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A molecular algorithm to detect and differentiate human pathogens infecting *Ixodes scapularis* and *Ixodes pacificus* (Acari: Ixodidae)

Christine B. Graham[*], **Sarah E. Maes**, **Andrias Hojgaard**, **Amy C. Fleshman**, **Sarah W. Sheldon**, and **Rebecca J. Eisen**

Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, 3156 Rampart Rd., Fort Collins, CO 80521, United States

Abstract

The incidence and geographic range of tick-borne illness associated with *Ixodes scapularis* and *Ixodes pacificus* have dramatically increased in recent decades. Anaplasmosis, babesiosis, and *Borrelia* spirochete infections, including Lyme borreliosis, account for tens of thousands of reported cases of tick-borne disease every year. Assays that reliably detect pathogens in ticks allow investigators and public health agencies to estimate the geographic distribution of human pathogens, assess geographic variation in their prevalence, and evaluate the effectiveness of prevention strategies. As investigators continue to describe new species within the *Borrelia burgdorferi* sensu lato complex and to recognize some *Ixodes*-borne *Borrelia* species as human pathogens, assays are needed to detect and differentiate these species. Here we describe an algorithm to detect and differentiate pathogens in unfed *I. scapularis* and *I. pacificus* nymphs including *Anaplasma phagocytophilum, Babesia microti, Borrelia burgdorferi* sensu stricto, *Borrelia mayonii*, and *Borrelia miyamotoi*. The algorithm comprises 5 TaqMan real-time polymerase chain reaction assays and 3 sequencing protocols. It employs multiple targets for each pathogen to optimize specificity, a gene target for *I. scapularis* and *I. pacificus* to verify tick-derived DNA quality, and a pan-*Borrelia* target to detect *Borrelia* species that may emerge as human disease agents in the future. We assess the algorithm's sensitivity, specificity, and performance on field-collected ticks.

Keywords

Borrelia; *Anaplasma phagocytophilum*; *Babesia microti*; Real-time PCR; *Ixodes*

1. Introduction

The incidence and geographic range of human tick-borne illnesses caused by pathogens transmitted by the blacklegged tick, *Ixodes scapularis*, in the eastern United States have dramatically increased in recent decades. The western blacklegged tick, *Ixodes pacificus*,

[*]Corresponding author. hyb4@cdc.gov (C.B. Graham).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ttbdis.2017.12.005.

transmits several of the same pathogens in the far western United States (Eisen et al., 2017). Moreover, new human pathogens associated with these and other tick species continue to emerge in the United States (Eisen et al., 2016, 2017). *Ixodes scapularis* is an experimentally-confirmed vector of, and is naturally infected with, at least 7 human pathogens: *Borrelia burgdorferi* sensu stricto (s.s.) and *Borrelia mayonii* (Lyme borreliosis spirochetes), *Borrelia miyamotoi* (agent of *Borrelia miyamotoi* disease, a relapsing fever-like illness), *Anaplasma phagocytophilum* (agent of anaplasmosis), *Babesia microti* (agent of babesiosis), *Ehrlichia muris* subsp. *eauclarensis* (agent of ehrlichiosis), and Powassan virus lineage 2 (formerly termed deer tick virus, agent of Powassan virus disease) (Bakken and Dumler, 2008; Dolan et al., 2016, 1998; Ebel, 2010; Eisen et al., 2016; Johnson et al., 2015; Karpathy et al., 2016; Krause et al., 2015; Merten and Durden, 2000; Pritt et al., 2017, 2016a, 2016b, 2011; Scoles et al., 2001; Spielman, 1976; Steere et al., 1983; Teglas and Foley, 2006; Vannier and Krause, 2012). *Ixodes pacificus* is a vector of a subset of the human pathogens that *I. scapularis* transmits, including *B. burgdorferi* s.s, *A. phagocytophilum*, and most likely also *B. miyamotoi* (Eisen et al., 2016; Krause et al., 2015; Lane et al., 1994; Merten and Durden, 2000; Teglas and Foley, 2006).

Lyme borreliosis is the most commonly reported vector-borne disease in the United States. In recent years, state and local health departments have reported more than 30,000 cases annually, which is 3 times the number of cases reported each year in the early 1990's (Mead, 2015). Anaplasmosis and babesiosis case counts have also increased over recent years, though these diseases are not as common as Lyme borreliosis (Adams et al., 2016; CDC, 2016a, 2016b, 2016c). In 2016, state and local health departments reported more than 4000 cases of anaplasmosis and 1910 cases of babesiosis (CDC, 2016b).

There is substantial overlap among the areas that report Lyme borreliosis, anaplasmosis, and babesiosis. Based on cases reported between 2008 and 2015, fourteen states in the Northeast, mid-Atlantic and upper Midwest regions have been classified as high Lyme borreliosis incidence states (Schwartz et al., 2017). In 2016, these 14 states reported more than 96% of Lyme disease, anaplasmosis and babesiosis cases (CDC, 2016b). Although it is not associated with a notifiable condition, *Borrelia miyamotoi* was recently recognized as another tick-borne human pathogen in the United States. The spirochete appears to have a broad geographic distribution, and clinicians have documented human cases in northeastern and midwestern states (Chowdri et al., 2013; Crowder et al., 2014; Gugliotta et al., 2013; Jobe et al., 2016; Krause et al., 2015, 2013; Molloy et al., 2015; Nelder et al., 2016). Given that the etiologic agents of Lyme disease, Borrelia miyamotoi disease, anaplasmosis, and babesiosis are sympatric in several regions, it is not surprising that researchers have observed evidence of co-infection with two or more of these agents in both ticks and humans (Barbour et al., 2009; Belongia et al., 1999; Fiorito et al., 2017; Johnson et al., 2017; Krause et al., 2002, 2014; Pritt et al., 2016b). Investigators and public health officials need assays that reliably detect a range of pathogens associated with *I. scapularis* and *I. pacificus* in field-collected ticks, including ticks infected with multiple pathogens, to estimate the geographic distribution of pathogens and to assess geographic variation in their prevalence.

Hojgaard et al. (2014b) described a testing algorithm employing paired TaqMan real-time polymerase chain reaction (PCR) assays to detect *B. burgdorferi* s.s., *A. phagocytophilum*,

and *Ba. microti* in *I. scapularis*. The algorithm incorporated 2 different targets per pathogen as well as an *I. scapularis* actin target. We subsequently determined that the actin primer-probe set could be used to verify DNA integrity in both *I. scapularis* and *I. pacificus*-derived samples (Graham et al., 2016). The algorithm included 2 *Borrelia* targets: a non-coding segment of the chromosome, "gB31", which is present in *Borrelia* species including *B. burgdorferi* s.s. and *B. miyamotoi*, and a segment of the flagellin gene (*fliD*), which is present in *B. burgdorferi* sensu lato (s.l.) species including *burgdorferi* s.s., but not in *B. miyamotoi*. Without a *B. miyamotoi*-specific target, however, identification of *B. miyamotoi* in ticks required additional amplification and sequencing. Moreover, the algorithm could not differentiate ticks co-infected with *B. burgdorferi* s.s. and *B. miyamotoi* from ticks infected with *B. burgdorferi* s.s. alone (both tested positive for the gB31 and *fliD* targets) (Hojgaard et al., 2014b).

Following the recent discovery of B. mayonii, a new member of the *B. burgdorferi* s.l. complex that causes Lyme borreliosis (Pritt et al., 2016a, 2016b), we found that the 2 multiplex assays were not adequately specific. That is, we could not differentiate *B. burgdorferi* s.s. from *B. mayonii* using the gB31 and *fliD* targets. Additional testing suggested that the algorithm also detected both the gB31 and *fliD* targets in some, but not all, other *B. burgdorferi* s.l. species. There are 9 named species within the *B. burgdorferi* s.l. complex that are present in the United States: *Borrelia americana*, *Borrelia andersonii*, *Borrelia bissettiae*, *B. burgdorferi* s.s., *Borrelia californiensis*, *Borrelia* carolinensis, *Borrelia kurtenbachii*, *Borrelia lanei* sp. nov., and *B. mayonii* (Margos et al., 2017; Pritt et al., 2016b; Schotthoefer and Frost, 2015). Investigators have detected *B. burgdorferi* s.s. and *B. mayonii* as well as B. andersonii and B. kurtenbachii in field-collected *I. scapularis* (Burgdorfer et al., 1982; Hamer et al., 2012; Johnson et al., 1984; Lin et al., 2001; Margos et al., 2010; Pritt et al., 2016a, 2016b). *Ixodes pacificus* is naturally associated with *B. burgdorferi* s.s. as well as *B. americana*, *B. bissettiae*, *B. californiensis*, and *B. lanei* sp. nov. (Fedorova et al., 2014; Lane et al., 2013; Margos et al., 2017, 2016; Postic et al., 2007, 1998; Rudenko et al., 2009). *Borrelia burgdorferi* s.s. and *B. mayonii* have been culture confirmed as human pathogens in the United States (Benach et al., 1983; Pritt et al., 2016a, 2016b; Steere et al., 1983). Golovchenko et al. (2016) recently isolated a *B. bissettiae* strain from a Florida resident, but they could not report specific clinical manifestations of the infection due to a lack of clinical evidence. *Borrelia bissettiae* and/or other species may emerge as human pathogens in the United States in the future. Therefore, it is advantageous to detect and differentiate the full range of *Borrelia* in field-collected ticks.

Despite the shortcomings of the 2 multiplex assays described by Hojgaard et al. (2014b), we found that combining sensitive, multiplex, real-time PCR assays incorporating a tick DNA control and multiple targets for each pathogen was an efficient and reliable approach for high-throughput testing of field-collected ticks (Feldman et al., 2015; Johnson et al., 2017; Morshed et al., 2015). We therefore sought to refine and expand our pathogen testing scheme in accordance with the ever-growing complexity of *ixodes*-borne *Borrelia*. Our goals included (1) incorporating targets to detect and differentiate the 3 *ixodes*-borne *Borrelia* species known to cause human disease in the United States (*B. burgdorferi* s.s., *B. mayonii*, and *B. miyamotoi*) from each other and from other *ixodes*-borne borreliae, (2) integrating a target that would allow us to detect other *Borrelia* species for putative identification by

sequencing and/or to bank for future testing if new pathogenic *Borrelia* species emerge or known species are shown to cause human illness, (3) incorporating at least 2 targets per pathogen, and (4) maintaining the ability to verify DNA integrity. While others have developed molecular testing schemes to detect and differentiate multiple pathogens in *Ixodes* ticks (e.g., Courtney et al., 2004; Dibernardo et al., 2014; Eshoo et al., 2015; Tokarz et al., 2009, 2017; Ullmann et al., 2005; Wroblewski et al., 2017), we know of no published algorithm to detect and differentiate the etiologic agents of Lyme borreliosis (both *B. burgdorferi* s.s. and *B. mayonii*), *Borrelia miyamotoi* disease, anaplasmosis, and babesiosis, and certainly not one that achieves all 4 of our goals.

Here we describe an algorithm to detect and differentiate *A. phagocytophilum*, *Ba. microti*, *B. burgdorferi* s.s., *B. mayonii*, *B. miyamotoi*, and other *Borrelia* species in unfed *I. scapularis* and *I. pacificus*. The algorithm includes: (1) paired multiplex TaqMan real-time PCR assays for the detection of *A. phagocytophilum*, *Ba. microti*, and *Borrelia*, (2) a duplex TaqMan real-time PCR assay to detect and differentiate *B. burgdorferi* s.s. and *B. mayonii* in *Borrelia*-positive samples, (3) paired TaqMan real-time PCR assays to detect *B. miyamotoi* in *Borrelia*-positive samples, and (4) sequencing protocols to putatively identify other *Borrelia* species, verify *B. mayonii* positives, and resolve suspect realtime PCR results (Fig. 1). We assessed the algorithm's sensitivity to each target pathogen, its overall specificity, and its performance on field-collected *I. scapularis* nymphs from Minnesota.

2. Materials and methods

2.1. Real-time PCR

Our testing algorithm incorporated 5 real-time PCR assays (Fig. 1). All primer and probe sequences and per-reaction concentrations appear in Table 1. For the first step, in which we sought to detect *A. phagocytophilum*, *Ba. microti*, and *Borrelia*, we modified the paired multiplex TaqMan real-time PCR assays described by Hojgaard et al. (2014b). The modified assays incorporated a pan-*Borrelia* primer-probe set adapted from Parola et al. (2011) as well as adjustments to reaction volume, some primer concentrations, cycling conditions, and the quantitation cycle (Cq) determination mode. The first assay targeted segments of the genes encoding flagellin (*fliD*) in *B. burgdorferi* s.s. and *B. mayonii*, and the genes encoding *A. phagocytophilum* P44 outer membrane proteins (*p44*), and *Ba. microti* secreted antigen 1 (sa1). For simplicity, we hereafter refer to the *fliD* target as "*B. burgdorferi* s.l. *fliD*" because the assay detected this target in multiple *B. burgdorferi* s.l. species including both *B. burgdorferi* s.l. species known to cause human disease in the United States. The first assay also included a tick actin target to verify the integrity of DNA samples derived from *I. scapularis* and *I. pacificus*. Hereafter, we refer to this assay as "M1b", because it incorporates the same targets as the assay previously termed "M1" (Hojgaard et al., 2014b) with modified *fliD* primer concentrations. The second assay targeted *Borrelia* 16S rDNA, the genes encoding *A. phagocytophilum* major surface protein 4 (*msp4*), and *Ba. microti* 18S rDNA. We developed this assay to replace "M2" (Hojgaard et al., 2014b). As it incorporated a unique combination of targets, we named it "M3." For both M1b and M3, each 10-μl reaction included 1X iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA, USA), 200 nM each probe, 300–600 nM each primer (see Table 1), and 4.8 μl template. Real-time

cycling conditions comprised an initial 3-min activation step at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s.

To detect and differentiate *B. burgdorferi* s.s. and *B. mayonii* in *Borrelia*-positive samples (Fig. 1), we designed a duplex TaqMan realtime PCR assay targeting the oligopeptide permease periplasmic A2 gene (*oppA2*). This duplex assay, hereafter "M4," employed 2 species-specific forward primers (Bb-F and Bmayo-F), a single reverse primer designed to anneal to a conserved segment of the *oppA2* sequence (Bb/Bmayo-R), and species-specific probes (Bb-probe, Bmayo-probe). In addition to the 3 primers and 2 probes (Table 1), each 25-μl duplex reaction included 1X iQ Multiplex Powermix (Bio-Rad), and 5–10 μl template. Real-time cycling conditions comprised an initial 3-min activation step at 95 °C followed by 40 cycles of 95 °C for 15 s and 58 °C for 1 min.

To detect *B. miyamotoi* in *Borrelia*-positive samples, we incorporated a pair of real-time TaqMan PCR assays into the algorithm, hereafter "M5" and "M6". These assays are described in detail elsewhere (Graham et al., 2016). Briefly, M5 targeted the gene encoding *B. miyamotoi* glycerophosphodiester phosphodiesterase (*glpQ*). M6 targeted the adenylosuccinate lyase (purB) gene and the tick actin target included in M1b (Table 1). Each 10-μl reaction included 1X iQ Multiplex Powermix (Bio-Rad), 200 nM probe, 600 nM each primer, and 4.8 μl template. Real-time cycling conditions were identical to those for assays M1b and M3.

We used CFX Manager 3.1 software (Bio-Rad) with the Cq determination mode set to regression to analyze results for all real-time PCR assays.

2.2. Sequencing

We integrated a set of 3 amplification and sequencing protocols into the algorithm to (1) putatively identify *Borrelia*-positive samples that test negative for *B. burgdorferi* s.s., *B. mayonii*, and *B. miyamotoi*, (2) putatively identify *B. burgdorferi* s.l. *fliD*-positive samples that test negative for *B. burgdorferi* s.s. and *B. mayonii*, (3) verify *B. mayonii*-positive samples, and (4) confirm the presence of *B. burgdorferi* s.s. in samples with suspect real-time PCR results (Fig. 1). All 3 protocols employed (semi-)nested PCR to facilitate specific amplification of potentially scarce *Borrelia* DNA from tick material. Primer sequences and per-reaction concentrations appear in Table 1.

To identify borreliae in ticks infected with a single *B. burgdorferi* s.l. species, we amplified and sequenced segments of the housekeeping genes encoding Clp protease subunit A (*clpA*) or Dipeptidyl aminopeptidase (*pepX*) as described at pubMLST (http://pubmlst.org/borrelia/ ; Margos et al., 2015) and elsewhere (Margos et al., 2008; Wang et al., 2014) with minor modifications. Briefly, each 25-μl outer reaction included 1X HotStar Taq Master Mix (Qiagen, Valencia, CA, USA), primers clpAF1237 and clpAR2218 or pepXF449 and pepXR1172, 0.5 μl 25mM $MgCl_2$ to bring the final concentration to 2mM $MgCl_2$, and 5–10 μl template. Touchdown cycling conditions for outer reactions were as described at PubMLST, but with annealing temperatures of 60 °C-52 °C for the first set of *pepX* amplification cycles and 52 °C for the second set of *pepX* amplification cycles as suggested by Wang et al. (2014). Each 50-μl inner reaction included 1X HotStar Taq Master Mix

(Qiagen), inner primers clpAF1255 and clpAR2104 or pepX449 and pepXR1115, 1 μl 25mM $MgCl_2$ to bring the final concentration to 2mM $MgCl_2$, and 5–10 μl of the outer reaction product. Cycling conditions were as described at PubMLST, but with a 15 min initial activation at 95 °C and an annealing temperature of 52 °C for *pepX*.

Because the *clpA* and *pepX* primers generate amplicons from all *B. burgdorferi* s.l. species, they do not allow for verification of *B. mayonii* in samples that are co-infected with another *B. burgdorferi* s.l. species, e.g., *B. burgdorferi* s.s. We therefore developed an amplification and sequencing protocol targeting a *B. mayonii*-specific segment of circular plasmid 26 (cp26) between Bmayo_06250 and Bmayo_06255 (Kingry et al., 2016). Each 25-μl outer reaction and each 50-μl inner reaction included 1X HotStar Taq Master Mix (Qiagen), 900 nM each primer (outer reaction: Bm_cp26_OF, Bm_cp26_OR; inner reaction: Bm_cp26_IF, Bm_cp26_IR), 0.5 μl 25mM $MgCl_2$ to bring the final $MgCl_2$ concentration to 2mM per reaction, and 5–10 μl DNA (outer reaction) or 5–10 μl outer reaction product (inner reaction). Cycling conditions included a 15 min initial activation at 95 °C followed by 30 (outer reaction) or 40 (inner reaction) cycles of 94 °C for 30 s, 56 °C (outer reaction) or 55 °C (inner reaction) for 30 s and 72 °C for 1 min, and a final 10 min extension at 72 °C.

Before sequencing, we visualized inner products on a 1% agarose gel to verify the presence of an approximately 850-nucleotide (nt) (*clpA*), 668-nt (*pepX*) or 337-nt (cp26) amplicon. The remaining product was purified using the QIAquick PCR Purification Kit (Qiagen). We sequenced each product using the inner amplification primers and BigDye Terminator v3.1 Ready Reaction Mix, removed unincorporated dyes with the BigDye Xterminator Kit (ThermoFisher Scientific Inc., Waltman, MA, USA), and analyzed the samples on an ABI 3130XL genetic analyzer. Using Lasergene 12 software (DNASTAR, Madison, WI, USA), we aligned forward and reverse sequences to generate a consensus sequence with at least 2-fold coverage of every nt. We manually trimmed poor or ambiguous sequence and any primer sequence from either end of the consensus sequence. We then used the Basic Local Alignment Search Tool (BLAST) to identify similar sequences in GenBank. We also queried the PubMSLT database using the *clpA* and *pepX* consensus sequences to identify similar alleles.

2.3. Analytical sensitivity and specificity

2.3.1. Pathogen DNA—All *Borrelia* DNA used in this study came from reference collections maintained in the Bacterial Diseases Branch of the Division of Vector-Borne Diseases, Centers for Disease Control and Prevention (DVBD, CDC, Fort Collins, CO, USA), and had been extracted from cultured isolates (Supplemental Table 1). DNA from *A. phagocytophilum* USG3, cultured in HL-60 human promyeloblast cells, was provided by the Rickettsial Zoonoses Branch, DVBD, CDC (Atlanta, GA, USA). Babesia microti DNA, extracted from a human blood sample (reference number 1953), was provided by the Division of Parasitic Diseases and Malaria, CDC, in accordance with the CDC Human Subjects Research Protocol, “Use of residual diagnostic specimens from humans for laboratory methods research” (Atlanta, GA, USA).

2.3.2. Tick DNA—To extract DNA from individual *I. scapularis* nymphs, we homogenized individual ticks with ≈545 mg 2.0mm yttria-stabilized zirconium oxide beads (Glen Mills, Clifton, NJ, USA) in 470 μl lysis mix comprised of buffer ATL, 20 μl proteinase K, and 0.5% DX anti-foaming reagent (Qiagen). We disrupted the sample for a total of 2 min using a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA) as described in Graham et al. (2016), then incubated the homogenate for 10–12 min at 56 °C. After centrifuging the sample for 30 s at 1000 x g, we processed 200 μl using the QIAcube HT automated nucleic acid isolation system and the *cador* Pathogen 96 QIAcube HT Kit (Qiagen). Following lysis with buffer VXL and the addition of buffer ACB, the instrument loaded 650 μl sample into the capture plate and applied a 3 min vacuum at 35 kPa. The column was washed with 600 μl buffer AW1 and subjected to a 2 min vacuum at 35 kPa. Subsequent wash and dry steps followed the *cador* Pathogen 96 QIAcube HT Kit V3 program (Qiagen). At the final step, DNA was eluted by adding 100 μl buffer AVE to the column, incubating for 2 min, and applying a 55 kPa vacuum for 6 min.

To prepare tick DNA for use in spiking reactions, we homogenized pooled, colony-reared *I. scapularis* nymphs (DVBD, Fort Collins) in 180 μl buffer ATL with 0.5% DX anti-foaming reagent (Qiagen) for 2 min in a Mini-Beadbeater-96 (BioSpec) with ≈545 mg 2.0mm yttria-stabilized zirconium oxide beads (GlenMills). We then added 20 μl proteinase K and incubated the homogenate overnight at 56 °C before extracting DNA using the DNeasy Blood and Tissue Kit (Qiagen).

2.3.3. Recombinant plasmids—To assess the sensitivity of the paired assays (M1b and M3) that provide the foundation for our testing algorithm (Fig. 1), we constructed 6 recombinant plasmids. We used the M1b and M3 primers (Table 1) to amplify the *fliD* and 16S targets from *B. burgdorferi* B31, the *p44* and *msp4* targets from *A. phagocytophilum* USG3, and the *sa1* and 18S targets from *Ba. microti*. We purified each product from a 2% agarose gel using Freeze 'N Squeeze DNA extraction columns (Bio-Rad). Each purified product was then cloned it into a pCR4-TOPO plasmid vector and propagated in TOP10 chemically competent *E. coli* using the TOPO TA Cloning Kit for Sequencing (ThermoFisher). We verified that each recombinant plasmid contained the correct insert by preparing forward and reverse sequence reactions using BigDye Terminator v3.1 Ready Reaction Mix and pCR4-TOPO plasmid vector T3 and T7 primers (ThermoFisher). We removed unincorporated dyes and analyzed each sequence as in Section 2.2. We subsequently linearized plasmids containing each complete target sequence using restriction enzyme NotI and purified the linearized plasmid DNA using the QIAquick PCR Purification Kit (Qiagen). Samples with an absorption ratio (A260/A280) < 1.79 were re-purified by ethanol precipitation.

2.3.4. Limit of detection for each real-time PCR target—We determined the double-stranded DNA concentration of each plasmid or genomic DNA template using a Qubit 2.0 fluorometer and the Qubit dsDNA HS Assay Kit (ThermoFisher) immediately before preparing serial dilutions for limit of detection (LOD) experiments. We included 5 replicates at each concentration, and each reaction also contained 6 ng *I. scapularis* DNA, which is the average amount of double-stranded DNA in 5 μl extract prepared from a single

nymph as described in Section 2.3.2. For all LOD experiments, primer-probe sets were run in multiplex. Unless otherwise noted, we conducted LOD testing for each target on 2 different days, using fresh DNA dilutions each day, for a total of 10 replicates per concentration per target, and we defined the LOD for each target as the lowest concentration for which all 10 replicates yielded positive PCR results (target Cq value < 40).

To determine the copy-number LOD for each M1b and M3 target, we prepared serial dilutions of the linearized recombinant plasmids containing each target to achieve the equivalent of 3, 6, or 10 target copies per reaction. To ascertain whether the *Borrelia* targets in M1b and M3 showed similar sensitivity to *B. burgdorferi* s.s., *B. mayonii*, and *B. miyamotoi*, we also determined the LOD for both targets using genomic DNA from these 3 species. Specifically, we determined the *B. burgdorferi* s.l. *fliD* LOD using *B. burgdorferi* B31 and *B. mayonii* MN14-1539 genomic DNA, and the *Borrelia* 16S LOD using genomic DNA from these 2 strains as well as *B. miyamotoi* CT13-2396. We prepared serial dilutions to achieve the equivalent of 3, 6, 10, 20, 30, or 40 genomes per reaction. Each reaction also included 6 ng *I. scapularis* DNA. We based our genome copy-number calculations on estimated genome sizes for *B. burgdorferi* B31 (1.52 Mbp; NCBI Genome Database), *B. mayonii* MN14-1539 (1.30 Mbp; Kingry et al., 2016), and *B. miyamotoi* CT13-2396 (1.28 Mbp; Kingry et al., 2017b). Because our *A. phagocytophilum* and *Ba. microti* genomic DNA stocks contained host DNA, we determined genomic DNA LODs empirically for *A. phagocytophilum* and *Ba. microti* by preparing 2-fold serial dilutions to achieve the equivalent of 25y–400 fg/reaction and identifying the lowest concentration at which both multiplex panels (M1b and M3) detected 10 replicates. We defined the M1b/M3 paired-assay LOD for each pathogen as the LOD associated with the less sensitive target.

Using the same method we used to determine genomic LODs for the *Borrelia* targets in M1b and M3, we used genomic DNA from *B. burgdorferi* s.s. strains B31 and MN88-0003 and *B. mayonii* strain MN14-1539 to determine the LODs for the *B. burgdorferi* s.s. and *B. mayonii oppA2* targets in M4. We previously determined the LOD for each *B. miyamotoi* target in assays M5 and M6 using similar methods (Graham et al., 2016).

2.3.5. Impact of increased tick DNA or co-infection on assay sensitivity—To assess the impact of increased tick DNA concentrations (> 6 ng/reaction) on M1b/M3 sensitivity, we prepared reactions containing genomic DNA from *B. burgdorferi* s.s., *A. phagocytophilum*, or *Ba. microti* at the M1b/M3 paired-assay LOD and *I. scapularis* DNA at each of 4 concentrations between 12 and 96 ng/reaction (Table 4). To assess the impact of co-infecting pathogens on the M1b/M3 LOD for *B. burgdorferi* s.s., *A. phagocytophilum* and *Ba. microti*, we prepared reactions containing 6 ng *I. scapularis* DNA, genomic DNA from a single pathogen at the paired-assay LOD, and genomic DNA from a second pathogen at 10, 10^2 and 10^3 times its LOD.

To assess the sensitivity of assay M4 to *B. burgdorferi* s.s. and *B. mayonii* in co-infected samples, we prepared reactions containing *I. scapularis* DNA, genomic *B. mayonii* DNA at concentrations equivalent to 10, 10^2, 10^3, or 10^4 genomes per reaction, and *B. burgdorferi* s.s. DNA at concentrations equivalent to 10, 10^2, 10^3, or 10^4 genomes per reaction. We

included 5 replicates at each *B. mayonii* concentration in the presence of *B. burgdorferi* s.s. at each concentration (Table 5).

2.3.6. Algorithm specificity—We assessed the algorithm's ability to differentiate *Borrelia* species using a panel of 20 *Borrelia* strains comprising *B. burgdorferi* s.s., *B. mayonii*, *B. miyamotoi*, and 6 other *Borrelia* species (Supplemental Table 1). Using the M1b, M3, M4, M5, and M6 assays, we screened *I. scapularis* DNA-spiked stocks of each strain at concentrations of approximately 10 pg and 50 fg *Borrelia* DNA per reaction. We subsequently amplified and sequenced the *clpA* and *pepX* targets from all *Borrelia* species that tested negative for *B. burgdorferi* s.s., *B. mayonii*, and *B. miyamotoi* to determine if we could reliably identify other *B. burgdorferi* s.l. species.

2.4. Pathogen detection in field-collected ticks

To further assess assay performance, we tested DNA extracted as described in Section 2.3.2 from 192 individual *I. scapularis* nymphs collected by drag sampling on public lands in Clearwater County (n=115) and Hubbard County (n=77) in Minnesota in June 2015. Each set of extractions included at least 1 tick-free extraction control for every 18 field-collected samples. In addition to extraction controls, each M1b/M3 real-time PCR run included at least 1 no-template control and a positive control comprised of pooled recombinant plasmid DNA containing each pathogen target at a concentration equivalent to approximately 20 copies per reaction. To assess the integrity of each field-collected tick extract, we analyzed the distribution of the *I. scapularis* actin Cq values generated by all 192 samples following M1b/M3 testing. Specifically, we used JMP 11 statistical software (v. 11.1.1 SAS Institute, Inc., 2013) to construct a boxplot, and we identified outliers with an *I. scapularis* Cq value > the upper whisker value (3rd quartile +1.5[interquartile range]). We re-tested these outliers, as well as any samples that yielded an *I. scapularis* actin curve with an end relative fluorescent unit (RFU) value < 400. If the *I. scapularis* actin results still indicated insufficient or poor quality DNA upon repeat, we prepared a second extract from 200 µl of the remaining tick triturate. If the *I. scapularis* actin results for the second extract did not indicate adequate DNA quantity and quality, we excluded the tick from infection prevalence analyses. We also repeated and/or prepared a second extract from samples yielding inconsistent M1b/M3 results (e.g., *A. phagocytophilum p44*-positive/*msp4*-negative).

Each M4 real-time run included at least 1 negative control for every 18 *Borrelia*-positive samples and a positive control containing genomic *B. burgdorferi* s.s. and *B. mayonii* DNA at concentrations equivalent to approximately 20 genome copies each per reaction. Each M5/M6 realtime run included at least 1 negative control for every 18 *Borrelia*-positive test samples and a positive control containing the appropriate recombinant plasmid at a concentration equivalent to approximately 10 target copies per reaction.

We used PooledInfRate, Version 4.0 (Biggerstaff, 2009) to calculate 95% confidence intervals for infection rates with each pathogen.

3. Results and discussion

Here we describe the development and evaluation of an algorithm for high throughput testing of field-collected, host-seeking *I. scapularis* and *I. pacificus* for 5 tick-borne human pathogens.

3.1. Algorithm sensitivity

3.1.1. Algorithm limits of detection for Anaplasma phagocytophilum and Babesia microti—

The LOD associated with each pathogen target in the algorithm appears in Table 2. Because the M1b and M3 assays each included 1 of 2 targets for *A. phagocytophilum* and *Ba. microti*, and because a sample must test positive for both targets to be considered positive for the pathogen (Fig. 1), we identified the algorithm LOD for each of these pathogens as the LOD associated with the target that was less sensitive to genomic pathogen DNA. The algorithm LOD for *A. phagocytophilum* was 200 fg of our genomic *A. phagocytophilum* stock, which our results suggest was approximately equivalent to 3 copies of the *msp4* target (Table 2). The *A. phagocytophilum* genome contains a single copy of the *msp4* gene (Dunning Hotopp et al., 2006). Assuming a single genome per bacterium, we would thus expect the algorithm to reliably detect as few as 3 *A. phagocytophilum* organisms per reaction. Notably, the *A. phagocytophilum* genome contains as many as 80 full-length or reserve/silent *p44* genes, although the specific *p44* gene family complement varies between strains (Dunning Hotopp et al., 2006). This explains why the *p44* target was much more sensitive to genomic *A. phagocytophilum* DNA than the *msp4* target even though the *p44* target copy LOD was higher.

We found that M3 was slightly more sensitive to genomic *Ba. microti* DNA than M1b (Table 2). This is likely because there are 2 copies of the 18S target in the *Ba. microti* genome (Cornillot et al., 2012), and there is a single copy of the *sa1* target in the *Ba. microti* genome, as indicated by BLAST analysis of the *sa1* target site (JX112361.1:714-828) against the GenBank database, which contains the complete *Ba. microti* strain RI genome (Cornillot et al., 2012). The algorithm LOD for *Ba. microti* was 200 fg of our *Ba. microti* genomic stock, which our results suggest was equivalent to approximately 3 copies of the *sa1* target, or 3 *Ba. microti* genomes (Table 2). Therefore, assuming haploid form, we expect the algorithm to reliably detect 3 *Ba. microti* organisms per reaction. Souza et al. (2016) reported that, used in singleplex, the *Ba. microti sa1* and 18S primer-probe sets consistently detected the equivalent of 14 and 12 parasites per reaction, although they observed detection down to the equivalent of 2.4 and 0.5 parasites, respectively, in DNA extracted from parasitic hamster blood. *Babesia microti* is haploid in mammalian hosts (Goethert and Telford, 2014), so these results suggest that in a diagnostic context, a singleplex assay targeting *sa1* (1 copy/genome) would reliably detect as few as 14 genomes, and a singleplex assay targeting 18S (2 copies/genome) would reliably detect as few as 24 genomes. It is possible that these singleplex real-time PCR assays are in fact less sensitive to *Ba. microti* in DNA extracted from blood than M1b and M3 are to *Ba. microti* DNA in a tick extract. We acknowledge, however, that the true LOD for each target in the algorithm, in terms of actual organisms per reaction, is likely slightly higher than reported here, as the DNA extraction process is unlikely to yield 100% of the pathogen DNA present in a sample, regardless of the matrix.

3.1.2. M1b/M3 limit of detection for *B. burgdorferi* s.s., *B. mayonii*, and *B. miyamotoi*—

The pan-*Borrelia* 16S target LOD was between 3 and 6 genomes in representative strains of 3 human-disease causing *Borrelia* (Table 2). Assuming a single genome per spirochete, we would therefore expect M3 to reliably detect 6 or fewer *B. burgdorferi* s.s., *B. mayonii* or *B. miyamotoi* spirochetes per reaction. Our results for the *fliD* target, however, indicate that M1b has an LOD of 6 *B. burgdorferi* s.s. spirochetes and 30 *B. mayonii* spirochetes (Table 2). This may be due to flagellin gene sequence variations between these species. The *fliD* target sequence is well-conserved among *B. burgdorferi* s.s. strains; BLAST analysis of the 78-nt sequence from strain B31 (CP017201.1:149547-149704), the strain from which the *fliD* primer and probe sequences were derived, indicated 98.7% identity (1 nt difference) with *B. burgdorferi* s.s. strain JD1, and 100% identity with all other *B. burgdorferi* s.s. strains for which homologous sequence was available in the GenBank database (strains PAbe, PAli, CA382, N40, and ZS7). The *fliD* target sequence in *B. mayonii* (CP015780.1: 150092-150169) differed from the target sequence in *B. burgdorferi* B31 by 4 nt. Notably, however, mismatches between the *fliD* primer sequences and the primer-annealing sequences in *B. mayonii* fell at least 10 nt from the 3′ end of each primer. Because M1b might fail to detect *B. mayonii* at very low concentrations, we determined that all *Borrelia* 16S-positive samples – whether they are positive or negative for *fliD* – should undergo additional testing for *B. mayonii*, *B. burgdorferi* s.s., and *B. miyamotoi* (Fig. 1). Given this caveat, we concluded that the M1b/M3 assays had a combined LOD of ≤ 6 spirochetes for all 3 target *Borrelia* species. We treated 10 fg *B. burgdorferi* B31 DNA (equivalent to approximately 6 genomes) as the M1b/M3 *Borrelia* species LOD for all subsequent testing.

While the *Borrelia* 16S results determine the need for further testing with assays M4, M5 and M6, we kept the *B. burgdorferi* s.l. *fliD* target in M1b so that the first phase of testing would include two targets for *B. burgdorferi* s.s., the primary agent of the most commonly-reported vector-borne disease in the United States. In most cases, *fliD* also serves as a second target for *B. mayonii*. In addition, the *B. burgdorferi* s.l. *fliD* results are used to determine if additional testing is warranted for *Borrelia* 16S-positives samples that test negative for both *B. burgdorferi* s.s. and *B. mayonii* (Fig. 1).

3.1.3. Impact of co-infection or increased tick DNA on the limit of detection for each M1b/M3 pathogen target in multiplex—

All M1b and M3 targets reliably detected genomic DNA over at least 5 logs (LOD–10^4 x LOD). Preferential amplification of one target over another can impact pathogen detection using multiplex PCR, particularly when one target is highly abundant and a second target is at relatively low abundance (Bustin et al., 2009; Elnifro et al., 2000). To assess the impact of co-infecting pathogens on M1b and M3 sensitivity, we evaluated detection of genomic DNA from each pathogen at its M1b/M3 LOD in the presence of genomic DNA from a second pathogen at 10–10^3 times its LOD. The results are summarized in Table 3. *Borrelia burgdorferi* s.l. *fliD* and *Ba. microti sa1* were the only 2 targets for which we observed decreased sensitivity in the presence of other pathogen DNA.

Since we determined the analytical sensitivity of each assay using reactions spiked with the amount of tick DNA we would expect to have in 5 μl extract prepared from a single nymph

using our standard DNA extraction protocol (6 ng), we also assessed the impact of increasing the amount of tick DNA on sensitivity. Such a scenario could occur in extracts from adult ticks, extracts prepared using an alternative extraction method that produces a more concentrated eluate, or extracts from pooled ticks. The addition of 12 to 96 ng genomic tick DNA simulated the use of DNA extracted as in Section 2.3.2 from pools of 2 to 16 nymphs. The results are summarized in Table 4. Increasing the amount of tick DNA did not appear to impact detection using any of the M3 panel targets. Two of the M1b targets, however, *Ba. microti sa1*, and – more dramatically – *B. burgdorferi* s.l. *fliD*, lost some sensitivity as the tick DNA concentration increased above 12 ng/reaction. The *sa1* and *fliD* targets were likely less sensitive because the M1b assay, unlike the M3 assay, included a tick actin target; amplification of the actin target from increasingly abundant tick DNA may have impacted detection of the *sa1* and *fliD* targets in scarce pathogen DNA. It may also be that both co-infection and additional tick DNA impacted detection of *B. burgdorferi* s.l. *fliD* and *Ba. microti sa1* in multiplex because we were testing for detection at the combined assay LOD, which was equivalent to the target LOD for both *fliD* and *sa1*, whereas targets for the same pathogens in M3, (*Borrelia* 16S and *Ba. microti* 18S respectively), were slightly more sensitive (Table 2).

We previously observed similar amplification of the tick actin target from *I. scapularis* and *I. pacificus* DNA (Graham et al., 2016). Given that *I. scapularis* appeared to impact assay sensitivity to some pathogen targets multiplexed with the tick actin target in M1b, likely because preferential amplification of the tick target hindered amplification of scarce pathogen DNA, we would expect *I. pacificus* DNA to similarly impact M1b sensitivity to *B. burgdorferi* s.l. and *Ba. microti*. We would not expect *I. pacificus* DNA to have any more impact than *I. scapularis* DNA on the sensitivity of assays that do not include a tick target. Therefore, though we conducted all testing using *I. scapularis* DNA, we would expect the algorithm to yield similar results and to be subject to similar limitations for *I. pacificus*-derived DNA.

3.1.4. Re-testing samples that yield ambiguous M1b/M3 results using *B. burgdorferi* s.l *fliD* and *Ba. microti sa1* in singleplex—

One advantage of using paired real-time assays for pathogen detection is that if host material and/or co-infecting pathogen DNA in-hibits detection of a scarce pathogen target in one panel, we may still pick it up with the other panel. Based on the results of our LOD experiments, the algorithm specifies that a sample that tests negative for *B. burgdorferi* s.l. *fliD* and positive for *Borrelia* 16S, or negative for *Ba. microti sa1* and positive for 18S, should be re-tested using M3 along with *B. burgdorferi* s.l. *fliD* or *Ba. microti sa1* in singleplex.

Run in singleplex, we detected *B. burgdorferi* s.l. *fliD* in 10 fg *B. burgdorferi* s.s. DNA in at least 9 of 10 replicates in the presence of up to 96 ng *I. scapularis* DNA, up to 200 pg *A. phagocytophilum* DNA, and up to 200 pg *Ba. microti* DNA. While it is true that a *Borrelia* 16S-positive sample should undergo additional testing for *B. burgdorferi* s.s., *B. mayonii* and *B. miyamotoi* whether it tests positive for *B. burgdorferi* s.l. *fliD* or not (Fig. 1), it is helpful to determine if a sample is *B. burgdorferi* s.l. *fliD* positive or negative with as much confidence as possible. As noted in Section 3.1.2, these results are used to determine if

additional testing is needed for *Borrelia*-positives samples that test negative for both *B. burgdorferi* s.s. and *B. mayonii* (Fig. 1).

Run in singleplex, we consistently detected *Ba. microti sa1* in 200 fg *Ba. microti* DNA in at least 9 of 10 replicates in the presence of up to 48 ng *I. scapularis* DNA, and in 8 of 10 replicates in the presence of 96 ng *I. scapularis* DNA. Experiments did not indicate that *sa1* run in singleplex detected 200 fg *Ba. microti* in the presence of co-infecting pathogens more consistently than *sa1* run in M1b multiplex. We therefore acknowledge some loss of algorithm sensitivity to *Ba. microti* in co-infected samples.

3.1.5. Algorithm limit of detection for Borrelia miyamotoi—The algorithm specifies that samples that test positive for *Borrelia* 16S using the paired M1b and M3 assays should subsequently undergo testing for *B. burgdorferi* s.s., *B. mayonii*, and *B. miyamotoi* (Fig. 1). We incorporated paired TaqMan real-time PCR assays M5 and M6 to test for *B. miyamotoi* in all *Borrelia*-positive samples. Previous characterization of this assay revealed that it reliably detected ≤ 5 genomes, or 5 spirochetes, per reaction, did not detect *B. burgdorferi* s.s., *B. turicatae* or *B. lonestari*, and was unlikely to detect other relapsing fever *Borrelia* (Graham et al., 2016). Since we determined that *Borrelia* 16S had an LOD of 3 *B. miyamotoi* genomes (Table 2) in this study, we conclude that the algorithm provides specific detection of *B. miyamotoi* down to at least 5 spirochetes per reaction.

3.1.6. Algorithm limit of detection for *Borrelia burgdorferi* s.s. and *B. mayonii* in ticks infected with one or both pathogens—A tick that tests positive for *Borrelia* 16S might contain DNA from *B. burgdorferi* s.s., *B. mayonii*, or both; co-infected ticks accounted for more than 30% of the *B. mayonii*-positive ticks identified by Pritt et al. (2016b). Therefore, we developed an assay, M4, that could detect and distinguish *B. burgdorferi* s.s. and *B. mayonii* in ticks carrying one or both species. Using genomic DNA, we determined that this duplex assay had a *B. burgdorferi* s.s. LOD of ≤ 6 genomes (Table 2). M4 was slightly less sensitive to *B. mayonii*, reliably detecting DNA down to the equivalent of 10 genomes. Thus, a sample yielding between 6 and 10 *B. mayonii* genomes per reaction, the equivalent of between 6 and 10 spirochetes per reaction, would be expected to test positive for *Borrelia* 16S, and it might test negative for *B. burgdorferi* s.l. *fliD* and *B. mayonii oppA2* (Table 2). If it also tested negative for both *B. burgdorferi* s.s. and *B. miyamotoi*, 2X sample volume would be re-tested using M4 (Fig. 1), which should reliably detect *B. mayonii* at concentrations of ≤ 5 genomes, or 5 spirochetes, per 1X sample volume. Given this caveat, and given that the *Borrelia* 16S LOD was 3 genomes for *B. burgdorferi* s.s. and 6 genomes for *B. mayonii* (Table 2), we conclude that the algorithm has an overall LOD of ≤ 6 spirochetes for both *B. burgdorferi* s.s. and *B. mayonii*.

In reactions spiked with tick DNA and both *B. burgdorferi* s.s. and *B. mayonii*, M4 reliably detected *B. burgdorferi* s.s. at concentrations as low as 10 genomes per reactions in the presence of up to 10^4 *B. mayonii* genomes. It also reliably detected the equivalent of 10 *B. mayonii* genomes in the presence of up to 100 *B. burgdorferi* s.s. genomes (Table 5). Although M4 failed to detect 10 genomes *B. mayonii* in the presence of 10^3–10^4 genomes *B. burgdorferi* s.s. DNA, the assay consistently detected the *B. mayonii oppA2* target when *B. mayonii* was present at concentrations ≥ 100 genomes per reaction (Table 5).

Nonetheless, we acknowledge that the algorithm may fail to detect *B. mayonii* at very low concentrations in the presence of relatively abundant *B. burgdorferi* s.s. DNA.

3.2. Algorithm specificity

3.2.1. Real-time PCR specificity for Borrelia species—To assess the algorithm's specificity for *B. burgdorferi* s.s., *B. mayonii*, and *B. miyamotoi*, specifically its ability to differentiate these 3 species from each other and from other tick-borne borreliae, we tested high and low concentrations (10 pg and 50 fg per reaction) of the 20 *Borrelia* strains in Supplemental Table 1. Table 6 shows the real-time PCR results for each strain at both concentrations. The algorithm detected and correctly identified the 5 *B. burgdorferi* s.s., 2 *B. mayonii* and 4 *B. miyamotoi* strains we tested. As expected, the M3 assay detected the *Borrelia* 16S target in all *Borrelia* with similar Cq values for all 10^4 fg replicates (range: 24.15–25.18) and all 50 fg replicates (range: 31.16–32.49), suggesting similar sensitivity across species and strains. As expected, all *B. miyamotoi* strains were negative for *B. burgdorferi* s.l. *fliD*. M1b detected the *fliD* target in all *B. burgdorferi* s.s. and *B. mayonii* strains, with Cq values suggesting similar sensitivity across all *B. burgdorferi* s.s. strains, and slightly less sensitivity to *B. mayonii*, consistent with the findings from our LOD experiments (Table 2). The M1b assay showed a wide range of sensitivity, however, to all other *B. burgdorferi* s.l. strains. M1b detection of the *fliD* target in *B. bissettiae*, *B. californiensis*, and *B. kurtenbachii* was comparable to detection of the *fliD* target in the *B. burgdorferi* s.s. strains. The assay detected the *fliD* target only in the high concentration replicates of *B. americana* and 1 *B. andersonii* strain, and the *fliD* Cq values were well above the *Borrelia fliD* Cq values associated with *B. burgdorferi* s.s. and *B. mayonii* strains at the same concentration. Three strains tested negative for *fliD* at both the high and low concentrations. These findings confirmed the need for additional testing to differentiate *Borrelia*-positive samples.

Given that the *B. miyamotoi purB* and *glpQ* genes are not present in *B. burgdorferi* s.l. spirochetes (Pettersson et al., 2007; Schwan et al., 2003), it was not surprising that M5 and M6 clearly differentiated the 4 *B. miyamotoi* strains from all *B. burgdorferi* s.l. strains (Table 6). M4 correctly identified all *B. burgdorferi* s.s. and *B. mayonii* strains. We also observed poor detection of the *B. burgdorferi* s.s. *oppA2* target, however, in *B. americana* and *B. kurtenbachii* at high concentrations. The false *B. burgdorferi* s.s. *oppA2* positive Cq values were more than 6 cycles higher than the *Borrelia* 16S Cq values associated with the same strains at the same concentration, while the difference between *B. burgdorferi* s.s. *oppA2* and *Borrelia* 16S Cq values for the 5 *B. burgdorferi* s.s. strains was consistently less than 4 at both high and low concentrations (Table 6). The algorithm therefore specifies that a sample testing positive for *B. burgdorferi* s.s. using the M4 assay but with a *B. burgdorferi* s.s. *oppA2* Cq value more than 4 cycles above the sample's *Borrelia* 16S Cq value should be considered suspect, and the *Borrelia* species identification should be verified by sequencing (Fig. 1). While we recognize that this complicates the algorithm, we note that false *B. burgdorferi* s.s. *oppA2* positives are likely to be very rare. Assuming spirochete loads per nymph are similar across *B. burgdorferi* s.l. species, we would expect the spirochete load in the vast majority of naturally-infected nymphs to be less than 300,000 spirochetes (Barbour et al., 2009). We use approximately 2% of a nymph per reaction, so a single reaction would

very rarely contain 6000 *B. burgdorferi* s.l. genomes, which is roughly equivalent to 10 pg *B. burgdorferi* s.l. DNA, the high concentration in our specificity experiment.

3.2.2. Using clpA and pepX sequences to resolve Borrelia burgdorferi s.l species—In keeping with the algorithm, we amplified and sequenced the *clpA* and *pepX* targets from 9 *B. burgdorferi* s.l. strains: the 6 that tested negative for *B. miyamotoi*, *B. mayonii* and *B. burgdorferi* s.s., and the 3 that would be considered suspect based on their discordant *B. burgdorferi* s.s. *oppA2* and *Borrelia* 16S Cq values. We have deposited *clpA* and *pepX* sequences for strains *B. americana* SCW-41, *B. andersonii* 21038 and SI-10, *B. californiensis* CA20, and *B. carolinensis* SCW-22 in GenBank (accession numbers MF582579–MF582588). Target sequences for all other strains were already available. We were able to correctly identify all 9 strains from our test panel based on the *clpA* and *pepX* sequences. Our results indicated that *clpA* and *pepX* sequence analysis, using both the GenBank and MLST databases, should allow us to assign a sample to a particular species with reasonable confidence when both sequences show > 97% similarity to strains from a single species, and when one or both sequences show ≤ 97% identity with all other species. Analysis of the *clpA* and *pepX* sequences was also sufficient to verify *B. mayonii* positives, as indicated by the results from our field-collected tick testing (Section 3.3).

Overall, the algorithm detected and correctly identified all of the human disease causing *Borrelia* strains in our test panel, and it did not mistake any of the *B. burdorferi* s.l. species not known to be associated with human disease in the United States for *B. burgdorferi* s.s. or *B. mayonii*.

3.3. Pathogen detection in field-collected ticks

To assess the algorithm's performance on field-collected samples, we tested 192 host-seeking *I. scapularis* nymphs collected in Clearwater and Hubbard Counties in Minnesota in June 2015. Based on the *I. scapularis* actin Cq values associated with repeated testing of 1 nymph from Hubbard County, we determined that we could not reliably detect pathogen DNA in this specimen. This nymph was therefore excluded from all further analyses. Table 7 shows the infection prevalence with our 5 target pathogens: *B. burgdorferi* s.s., *B. mayonii*, *B. miyamotoi*, *A. phagocytophilum*, and *Ba. microti*.

Hubbard and Clearwater are adjacent counties in north-central Minnesota. We chose to use *I. scapularis* nymphs from these counties to assess algorithm performance on field-collected samples because Clearwater is one of the few counties in which *B. mayonii*-infected *I. scapularis* nymphs had been previously documented (Pritt et al., 2016b), and because we also expected to detect *B. burgdorferi* s.s., *A. phagocytophilum* and *Ba. microti* in nymphs from this region. In the period between 2008 and 2011, both counties reported human cases of Lyme borreliosis and human anaplasmosis (> 50 cases/100,000 residents), and between 2004 and 2011, both counties also reported a small number of babesiosis cases (Robinson et al., 2015). Though these data reflect the county of residence associated with each case and not necessarily the county of exposure, Robinson et al. (2015) found that residents of these and other rural counties were more likely to have been exposed in their county of residence than residents of more urban counties.

Our *B. mayonii* results (Table 7) are consistent with the only previously published data for *B. mayonii* infection in *I. scapularis* nymphs from Minnesota. Pritt et al. (2016b) detected *B. mayonii* in 1 of 59 nymphs (1.7%; 95% CI: 0.1–7.9%) collected in Clearwater County between April and July 2015, the period within which our ticks were also collected. The algorithm also yielded *B. burgdorferi* s.s., *B. miyamotoi*, and *A. phagocytophilum* infection prevalence rates (Table 7) within the ranges reported for the prevalence of each pathogen in *I. scapularis* nymphs from the upper Midwest. Barbour et al. (2009) detected *B. burgdorferi* s.s. in 6.4–41.0% and *B. miyamotoi* in 0–8.2% of *I. scapularis* nymphs collected between mid-May and late August in the years 2004 through 2007 from each of 21 different sites in Minnesota, Michigan and Wisconsin. Murphy et al. (2017) detected *A. phagocytophilum* in 0.0–17.7% of questing *I. scapularis* nymphs collected by drag sampling at each of 24 sites is Wisconsin in 2015. There is limited data available on *Ba. microti* prevalence in questing *I. scapularis* nymphs collected in the Midwest. Stromdahl et al. (2014) detected *Ba. microti* in 3.3% of 215 *I. scapularis* nymphs submitted between 2002 and 2012 from soldiers at a Minnesota military installation. The algorithm yielded a higher *Ba. microti* infection prevalence in the nymphs we tested (8.4%), but the lower end of the 95% confidence interval (5.0%) falls within the 95% confidence interval associated with the Stromdahl et al. (2014) data (1.5–6.3%).

The algorithm detected co-infections in 24 ticks. Nine (38%) tested positive for *B. burgdorferi* s.s. and *A. phagocytophilum*, and 7 (29%) tested positive for *B. burgdorferi* s.s. and *Ba. microti*. Four ticks were infected with *B. burgdorferi* s.s., *A. phagocytophilum* and *Ba. microti*, and 1 tick was infected with *B. burgdorferi* s.s., *Ba. microti*, and *B. mayonii*. We also detected co-infections with *B. mayonii* and *B. miyamotoi*, *B. burgdorferi* s.s. and *B. miyamotoi*, and *B. burgdorferi* s.s. and *B. mayonii* (1 each).

Notably, though the algorithm includes a number of steps to clarify ambiguous real-time PCR results (Fig. 1), we determined the pathogen infection status for 185 of the 192 field-collected ticks (96%) based on a single round of real-time PCR testing using assays M1b and M3 followed by a single round of testing using assays M4, M5 and M6 to identify *Borrelia*-positive samples to species. All samples that tested positive for the *Borrelia* 16S target also tested positive for the *B. burgdorferi* s.l. *fliD* target, including the 2 *B. miyamotoi*-positive ticks, because 1 was coinfected with *B. burgdorferi* s.s. and the other with *B. mayonii*. All samples that tested positive for *Ba. microti* 18S also tested positive for *Ba. microti sa1*, so we did not need to conduct any follow-up testing using *B. burgdorferi* s.l. *fliD* or *Ba. microti sa1* in singleplex. Two samples initially yielded inconsistent results for the 2 *A. phagocytophilum* targets. One tested negative for both targets upon repeat and was therefore negative for *A. phagocytophilum* by the algorithm. The second sample yielded inconsistent *A. phagocytophilum* results again upon repeat, so we prepared a second DNA extract from the leftover triturate. We detected both *A. phagocytophilum* targets in the second extract and classified the sample as *A. phagocytophilum* positive.

The *B. burgdorferi* s.s. *oppA2* Cq values were within 4 of the *Borrelia* 16S Cq values for all samples that tested positive for *B. burgdorferi* s.s. We verified the presence of *B. mayonii* DNA in 2 samples by sequencing the *clpA* and *pepX* targets. The other 2 *B. mayonii*-positive samples were co-infected with *B. burgdorferi* s.s. We verified the presence of *B.*

mayonii in these samples by amplifying and sequencing the cp26 target. BLAST analysis confirmed that all sequences were identical to the homologous regions in *B. mayonii* MN14-1420 and MN14-1539.

Two *Borrelia*-positive samples that tested positive for both the *B. burgdorferi* s.l. *fliD* target and the *Borrelia* 16S target subsequently tested negative for *B. miyamotoi*, *B. burgdorferi* s.s., and *B. mayonii*. We repeated M4 testing using 10 μl of each sample, which revealed that 1 of the 2 samples was positive for *B. burgdorferi* s.s. We note that though M4 initially failed to detect *B. burgdorferi* s.s. in this sample, the algorithm prompted additional testing that ultimately allowed us to identify this human disease causing *Borrelia*. The second *Borrelia-positive* sample continued to test negative for *B. burgdorferi* s.s. and *B. mayonii*. BLAST analysis of the *clpA* sequence from this sample revealed 99% identity with *B. kurtenbachii* strains, 99% identity with a single *B. bissettiae* strain, and ≤ 96% identity with all other strains and species. The most similar allele in the pubMLST database was *clpA* 127, with which it shared 576/579 nts (99% identity). The *pepX* sequence was identical to the homologous region in *B. kurtenbachii* strain IL96-255, 99% similar to other *B. kurtenbachii* strains in GenBank, and ≤ 96% similar to available sequences for all other species. It contained *pepX* allele 107. The *clpA* 127 and *pepX* 107 alleles were associated exclusively with *B. kurtenbachii* isolates in pubMLST. We therefore concluded that this nymph was not infected with *B. burgdorferi* s.s., *B. mayonii*, or *B. miyamotoi*, and that it was most likely positive for *B. kurtenbachii*.

4. Conclusion

We conclude that the algorithm described here can reliably detect and distinguish 5 known human pathogens associated with *I. scapularis*. A 2-panel multiplex Taqman assay (M1b/M3) provides the foundation for the algorithm and detects *A. phagocytophilum*, *Ba. microti*, *B. burgdorferi* s.s., *B. miyamotoi* and *B. mayonii* at concentrations as low as 3–6 genomes per reaction. Subsequent testing with assays M4, M5, and M6 allows detection and differentiation of human disease causing *Borrelia* in *Borrelia*-positive samples, while amplification and sequencing protocols enable further verification and identification of other *Borrelia* species (Fig. 1). The algorithm integrates multiple targets for each pathogen with a tick actin target that can be used to verify the integrity of *I. scapularis* and *I. pacificus* DNA extracts. The inclusion of a pan-*Borrelia* target means that DNA samples testing positive for *Borrelia* but negative for *B. miyamotoi*, *B. burgdorferi* s.s. and *B. mayonii* can be putatively identified by sequencing and/or banked to allow for future screening for *Borrelia* species that have not yet been identified as human disease agents. We validated the algorithm using nymphal tick DNA because most Lyme borreliosis, anaplasmosis, and babesiosis cases occur during peak nymphal host-seeking activity, indicating that the host-seeking nymphal stage poses a particular risk to human health (Eisen et al., 2017). Based on our assessment of algorithm sensitivity in the presence of increased tick DNA, however, we believe that this testing scheme would also be useful for testing individual or pooled larvae or adult ticks. This scheme could also be used to test blood-fed *I. scapularis* and *I. pacificus*, although we do not know how the presence of blood in field-collected specimens might impact assay sensitivity. The algorithm will be useful for testing *I. scapularis* and *I. pacificus* collected throughout the United States in support of surveillance and research programs that assess the

risk of human exposure to the agents of Lyme borreliosis, *Borrelia miyamotoi* disease, anaplasmosis, and babesiosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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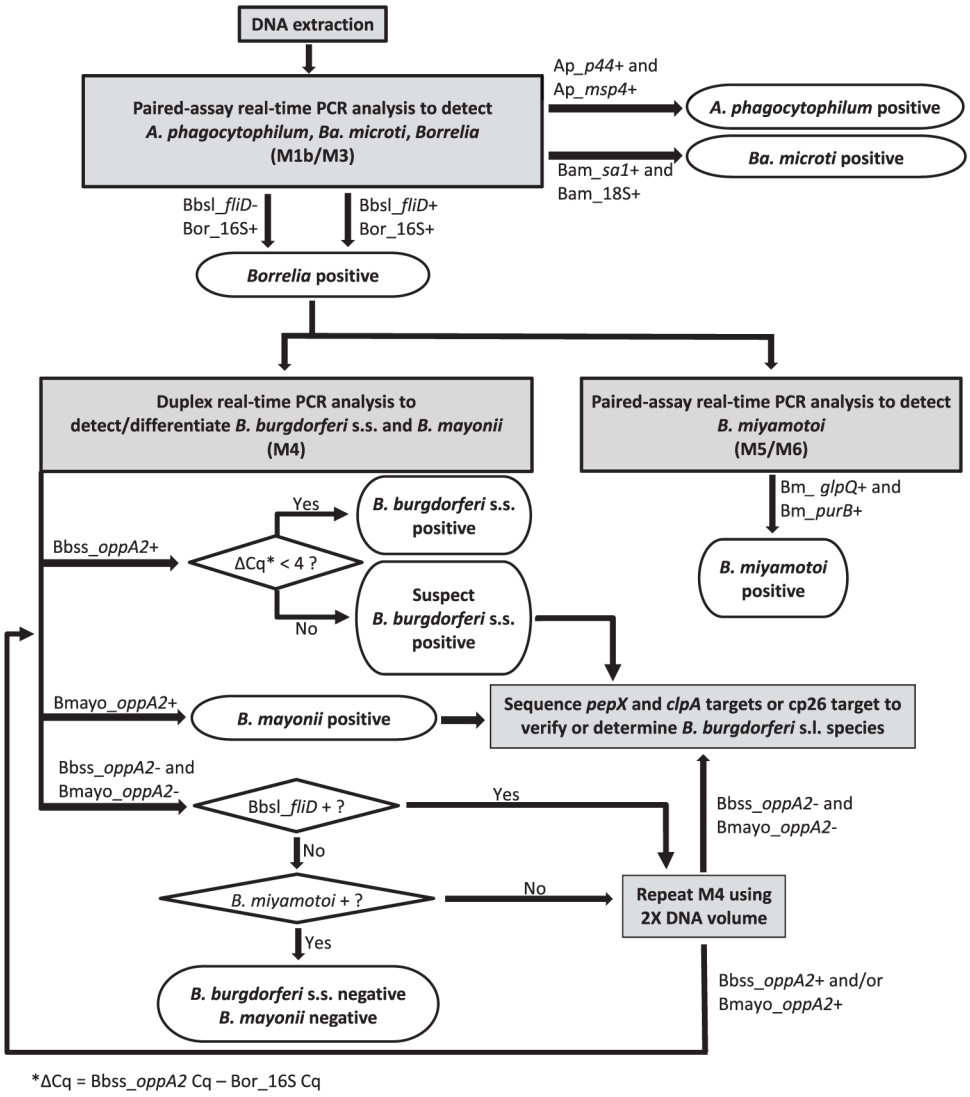

Fig. 1.

Flow chart for a testing algorithm to detect and differentiate *A. phagocytophilum*, *Ba. microti*, *B. miyamotoi*, *B. burgdorferi* s.s., *B. mayonii*, and other *B. burdorferi* s.l. species in *I. scapularis* and *I. pacificus.* Ap, *A. phagocytophilum* target; Bam. *Ba. microti* target; Bbsl, *B. burgdorferi* s.l. target; Bor. pan-*Borreha* target; Bm, *B. miyamotoi* target; Bbss, *B. burgdorferi* s.s. target; Bmayo, *B. mayonii* target.

Table 1

Primers and probes used in a testing algorithm designed to detect and differentiate *B. burgdorferi* s.s., *B. mayonii*, *B. miyamotoi*, *A. phagocytophilum* and *Ba. microti* in *I. scapularis* and *I. pacificus*.

Target	Primer/Probe	Sequence (5′-3′)	Amplicon size (nt)	Reference	Concentration/Reaction (nM)
M1b Assay					
B. burgdorferi s.l. *fliD*[a]	fliD-F	TGG TGA CAG AGT GTA TGA TAA TGG AA	78	Zeidner et al., 2001	400
	fliD-R	ACT CCT CCG GAA GCC ACA A		Zeidner et al., 2001	400
	fliD-probe	FAM-TGC TAA AAT GCT AGG AGA TTG TCT GTC GCC-BHQ1		Zeidner et al., 2001	200
A. phagocytophilum p44	p44-F[b]	ATG GAA GGT AGT GTT GGT TAT GGT ATT	77	Courtney et al., 2004	300
	p44-R[b]	TTG GTC TTG AAG CGC TCG TA		Courtney et al., 2004	300
	p44-probe[b]	HEX-TGG TGC CAG GGT TGA GCT TGA GAT TG-BHQ1		Courtney et al., 2004	200
Ba. microti sa1	sa1-F	ACA GAA TGC AGT CGG TGA AG	115	Hojgaard et al., 2014b	300
	sa1-R	ATC AAG GAG AGT GGA TAG GTT TG		Hojgaard et al., 2014b	300
	sa1-probe	CalRd610-CCA TTG ACG CTG TTG TTG CTC ACA-BHQ2		Hojgaard et al., 2014b	200
I. scapularis and *I. pacificus* actin gene	actin-F	GCC CTG GAC TCC GAG CAG	77	Hojgaard et al., 2014b	300
	actin-R	CCG TCG GGA AGC TCG TAG G		Hojgaard et al., 2014b	300
	actin-probe	Quas705-CCA CCG CCG CCT CCT CTT CTT CC-BHQ3		Hojgaard et al., 2014b	200
M3 Assay					
Borrelia 16S rDNA	16S-F	AGC YTT TAA AGC TTC GCT TGT AG	148	Kingry et al., 2017a	600
	16S-R	GCC TCC CGT AGG AGT CTG G		Kingry et al., 2017a	600
	16S-probe	FAM-CCG GCC TGA GAG GGT GAW CGG-BHQ1		Kingry et al., 2017a	200
A. phagocytophilum msp4	msp4-F	TAT ATC CAA CTT CAA CTT CCA CTC	93	Hojgaard et al., 2014b	300
	msp4-R	CAT TCA AGT TCG CTA AGA GTT TAC		Hojgaard et al., 2014b	300
	msp4-probe	HEX-CTC CGC CAA TAG CAT AGC CAG TTG-BHQ1		Hojgaard et al., 2014a	200
Ba. microti 18S rDNA	18S-F	CGA CTA CGT CCC TGC CCT TTG	99	Hojgaard et al., 2014b	400
	18S-R	ACG AAG GAC GAA TCC ACG TTT C		Hojgaard et al., 2014b	400
	18S-probe	Quas705-AC ACC GCC CGT CGC TCC TAC CG-BHQ3		Hojgaard et al., 2014b	200

Target	Primer/Probe	Sequence (5′-3′)	Amplicon size (nt)	Reference	Concentration/Reaction (nM)
M4 Assay					
B. burgdorferi s.s. *oppA2*	Bb-F	AAT TTT TGG TTC CAT ACC C	162	This study	450
B. mayonii oppA2	Bmayo-F	GCC CGA TTT AAT CAA AGA	144	This study	450
B. burgdorferi s.s. *oppA2* and *B. mayonii oppA2*	Bb/Bmayo-R	CTG TCA ATA GCA AGA GTT AA		This study	900
B. burgdorferi s.s. *oppA2*	Bb-probe	HEX-CGT TCA ATA CAC ACA TCA AAC CAC T-BHQ1		This study[c]	200
B. mayonii oppA2	Bmayo-probe	FAM-ACA CGC ACA TTA AAC CGC TTG AT-BHQ1		This study[c]	200
M5 Assay					
B. miyamotoi glpQ	BmglpQ-F	GAC CCA GAA ATT GAC ACA ACC ACA A	108	Graham et al., 2016[d]	600
	BmglpQ-R	TGA TTT AAG TTC AGT TAG TGT GAA GTC AGT		Graham et al., 2016[d]	600
	BmglpQ-probe	CalRd610-CAA TCG AGC TAG AGA AAA CGG AAG ATA TTA CG-BHQ2		Graham et al., 2016[d]	200
M6 Assay					
B. miyamotoi purB	BmpurB-F	TCC TCA ATG ATG AAA GCT TTA	121	Graham et al., 2016	100
	BmpurB-R	GGA TCA ACT GTC TCT TTA ATA AAG		Graham et al., 2016	100
	BmpurB-probe	CalRd610-TCG ACT TGC AAT GAT GCA AAA CCT-BHQ2		Graham et al., 2016	200
I. scapularis and *I. pacificus* actin gene	actin-F, actin-R, and actin-probe as in panel M1b				
Amplification/Sequencing					
B. burgdorferi s.l. *clpA*	clpAF1237	AAA GAT AGA TTT CTT CCA GAC	982[e]	Wang et al., 2014	500
	clpAR2218	GAA TTT CAT CTA TTA AAA GCT TTC		Wang et al., 2014	500
	clpAF1255	GAC AAA GCT TTT GAT ATT TTA G	850[e]	Margos et al., 2008	500
	clpAR2104	CAA AAA AAA CAT CAA ATT TTC TAT CTC		Margos et al., 2008	500
B. burgdorferi s.l. *pepX*	pepXF449	TTA TTC CAA ACC TTG CAA TCC	724	Margos et al., 2008	500
	pepXR1172	GTT CCA ATG TCA ATA GTT TC		Margos et al., 2008	500
	pepXF449	as above	668	Margos et al., 2008	500
	pepXR1115	TGT GCC TGA AGG AAC ATT TG		Margos et al., 2008	500
B. mayonii	Bm_cp26_OF	CTC ATA TCC CTC TCC TTT GAT	749	This study	900
Bmayo_06250 –	Bm_cp26_OR	TCT GGG CAT ATT TCA GTG AT		This study	900

Target	Primer/Probe	Sequence (5′-3′)	Amplicon size (nt)	Reference	Concentration/Reaction (nM)
Bmayo_06255 (cp26)	Bm_cp26_IF	TTA CAG ACT AGT GAA CAT A	337	This study	900
	Bm_cp26_IR	CAA ATA CAT TAA CCA AGG AGC A		This study	900

nt, nucleotides; *fliD*, flagellin gene; *p44*, P44 outer membrane protein gene; *sa1*, secreted antigen 1 gene; *msp4*, major surface protein 4 gene; *oppA2*, oligopeptide permease periplasmic A2 gene; *glpQ*, glycerophosphodiester phosphodiesterase gene; *purB*, adenylosuccinate lyase gene; *clpA*, Clp protease subunit A gene; *pepX*, Dipeptidyl aminopeptidase gene; Bmayo_06250–Bmayo_06255 (cp26), segment of circular plasmid 26 between Bmayo_06250 and Bmayo_06255; CalRd610, CAL Fluor Red 610, FAM, 6-Carboxyfluorescein; HEX, Hexachloro-Fluorescein Phosphoramidite; Quas705, Quasar 705; BHQ1, BHQ2, BHQ3: Black Hole Quenchers 1–3.

[a]The *B. burgdorferi* s.l. *fliD* primer/probe set detects multiple *B. burgdorferi* s.l. species including *B. burgdorferi* s.s. and *B. mayonii*.

[b]*A. phagocytophilum* p44 primers and probe appear elsewhere as “msp2-F, msp2-R, and msp2-P” (Courtney et al., 2004; Hojgaard et al., 2014b). The *msp2* gene in *Anaplasma marginale* is in the same protein superfamily as the *p44* genes in *A. phagocytophilum*. There is, however, a distinct *msp2* gene in the *A. phagocytophilum* genome (Dunning Hotopp et al., 2006; Lin et al., 2004). The *A. phagocytophilum* primer/probe set in panel M1b detects a conserved *A. phagocytophilum p44* gene sequence. We have changed the primer and probe names accordingly.

[c]M4 panel probes were adapted from the TaqMan real-time PCR assay described in Pritt et al. (2016b) Supplemental Materials.

[d]*B. miyamotoi glpQ* primers and probe adapted from Bacon et al. (2005).

[e]Amplicon size for the *clpA* target varies slightly among *B. burgdorferi* s.l. species and strains. We’ve listed the amplicon sizes for *B. burgdorferi* B31.

Table 2

The limit of detection (LOD) for each pathogen target in the algorithm, defined as the lowest target copy or genome copy number consistently detected by real-time PCR. We determined each target copy number LOD using linearized recombinant DNA from a plasmid containing the target sequence. We determined each pathogen LOD using genomic DNA. *Borrelia* DNA was extracted from spirochete cultures and genome concentrations were estimated as described in Section 2.3.4. Because our *A. phagocytophilum* and *Ba. microti* genomic DNA stocks contained host DNA, we could not extrapolate a genome equivalent of the genomic DNA LOD.

Target (assay)	Target Copy LOD	Target copies per genome	Pathogen LOD (fg DNA or *Borrelia* spp. genomes)	
			species	LOD
A. phagocytophilum p44 (M1b)	20 copies	multiple	*A. phagocytophilum*	25 fg
A. phagocytophilum msp4 (M3)	3 copies	1	*A. phagocytophilum*	200 fg [*]
Ba. microti sa1 (M1b)	3 copies	1	*Ba. microti*	200 fg [*]
Ba. microti 18S (M3)	6 copies	2	*Ba. microti*	100 fg
B. burgdorferi s.l. *fliD* (M1b)	6 copies	1	*B. burgdorferi* s.s.	6 genomes
			B. mayonii	30 genomes
Borrelia 16S (M3)	3 copies	1	*B. burgdorferi* s.s.	3 genomes
			B. mayonii	6 genomes
			B. miyamotoi	3 genomes
B. burgdorferi s.s. *oppA2* (M4)	NT		*B. burgdorferi* s.s	3–6 genomes [*,a]
B. mayonii oppA2 (M4)	NT		*B. mayonii*	10 genomes [*,b]
B. miyamotoi glpQ (M5)	5 copies [c]	1	*B. miyamotoi*	5 genomes [c,*]
B. miyamotoi purB (M6)	5 copies [c]	1	*B. miyamotoi*	5 genomes [c]

NT, not tested.

[*] Indicates algorithm LOD for each pathogen.

[a] The M4 assay consistently detected *B. burgdorferi* s.s. strain B31 down to the equivalent of 3 genomes, and *B. burgdorferi* s.s. strain MN88-0003 down to the equivalent of 6 genomes.

[b] *Borrelia*-positive samples that initially test negative for *B. mayonii oppA2* can be retested using 2X the standard DNA volume to bring the algorithm LOD for *B. mayonii* down to ≤ 5 genomes per 1X sample volume.

[c] Graham et al., 2016.

Table 3

Number of replicates in which assays M1b and M3 detected each target in genomic DNA at the paired-assay limit of detection (LOD; 10 fg *B. burgdorferi* s.s., 200 fg *A. phagocytophilum*, 200 fg *Ba. microti*) in the presence of DNA from a second pathogen at 10–10^3 x its LOD.

Second pathogen	***B. burgdorferi* s.s. (10 fg)**		***A. phagocytophilum* (200 fg)**		***Ba. microti* (200 fg)**	
	***fliD* (M1b)**	**16S (M3)**	***p44* (M1b)**	***msp4* (M3)**	***sa1* (M1b)**	**18S (M3)**
2 pg *A. phagocytophilum*	9/10	10/10	n/a	n/a	9/10	10/10
20 pg *A. phagocytophilum*	7/10	10/10	n/a	n/a	7/10	10/10
200 pg *A. phagocytophilum*	8/10	10/10	n/a	n/a	9/10	10/10
2 pg *Ba. microti*	9/10	10/10	10/10	10/10	n/a	n/a
20 pg *Ba. microti*	7/10	10/10	10/10	10/10	n/a	n/a
200 pg *Ba. microti*	8/10	10/10	10/10	10/10	n/a	n/a
100 fg *B. burgdorferi* s.s.	n/a	n/a	10/10	10/10	9/10	10/10
1 pg *B. burgdorferi* s.s.	n/a	n/a	10/10	9/10	8/10	10/10
10 pg *B. burgdorferi* s.s.	n/a	n/a	10/10	10/10	9/10	10/10

Table 4

Number of replicates in which assays M1b and M3 detected each target in genomic DNA at the paired-assay limit of detection (LOD; 10 fg *B. burgdorferi* s.s., 200 fg *A. phagocytophilum*, 200 fg *Ba. microti*) in the presence of increasing *I. scapularis* tick DNA.

I. scapularis **DNA (ng/reaction)**	**M1b**			**M3**		
	Bbsl_*fliD*	**Ap_*p44***	**Bam_*sa1***	**Bor_16S**	**Ap_*msp4***	**Bam_18S**
12	10/10	10/10	9/10	10/10	10/10	9/10
24	5/10	10/10	7/10	10/10	10/10	10/10
48	4/10	10/10	9/10	10/10	10/10	10/10
96	2/10	9/10	7/10	10/10	10/10	10/10

Bbsl, *Borrelia burgdorferi* s.l. target; Bor, pan-*Borrelia* target; Ap, *A. phagocytophilum* target; Bam, *Ba. microti* target.

Table 5

Number of replicates in which assay M4 detected the *B. burgdorferi* s.s. *oppA2* and *B. mayonii oppA2* targets in reactions spiked with genomic DNA from both pathogens at concentrations equivalent to 10–10^4 genomes per reaction.

***B. mayonii* genomes**	**10 *B. burgdorferi* s.s. genomes**		**10^2 *B. burgdorferi* s.s. genomes**		**10^3 *B. burgdorferi* s.s. genomes**		**10^4 *B. burgdorferi* s.s. genomes**	
	Bbss_*oppA2*	**Bmayo_*oppA2***	**Bbss_*oppA2***	**Bmayo_*oppA2***	**Bbss_*oppA2***	**Bmayo_*oppA2***	**Bbss_*oppA2***	**Bmayo_*oppA2***
10	5/5	5/5	5/5	5/5	5/5	3/5	5/5	0/5
10^2	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
10^3	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
10^4	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5

Table 6

Real-time PCR results (Cq values) for each *Borrelia* target included in our testing algorithm for *B. burgdorferi* s.l. and *B. miyamotoi* strains run at high (10^4 fg/reaction) and low (50 fg/ reaction) concentrations. Cq values<40 were considered positive. A target that failed to amplify within 40 cycles was considered negative (−). The difference between the *Borrelia* 16S (Bor_16S) and *B. burgdorferi* s.s. *oppA2* (Bbss_*oppA2*) Cq values was calculated for each Bbss_*oppA2*-positive replicate to allow for differentiation between true (ΔCq < 4) and false (ΔCq > 4) *B. burgdorferi* s.s. positives. .

Borrelia species	Strain	fg DNA per reaction	Bbsl_*fliD* Cq (M1b)	Bor_16S Cq (M3)	Bmiya_*purB* Cq (M5)	Bmiya_*qlpQ* Cq (M6)	Bmayo_*oppA2* Cq (M4)	Bbss_*oppA2* Cq (M4)	ΔCq (Bbss_*oppA2*–Bor_16S)
B. burgdorferi s.s.	B31^T	10^4	25.14	24.56	–	–	–	25.88	1.32
		50	32.29	31.49	–	–	–	33.27	1.78
B. burgdorferi s.s.	IRS	10^4	25.15	24.41	–	–	–	26.01	1.60
		50	32.61	31.61	–	–	–	33.94	2.33
B. burgdorferi s.s.	CA6	10^4	24.87	24.54	–	–	–	25.38	0.84
		50	32.72	31.19	–	–	–	32.51	1.32
B. burgdorferi s.s.	MN88-0003	10^4	24.86	24.89	–	–	–	26.16	1.27
		50	31.94	31.87	–	–	–	33.38	1.51
B. burgdorferi s.s.	SI1	10^4	25.01	24.77	–	–	–	26.23	1.46
		50	31.92	31.19	–	–	–	34.68	3.49
B. mayonii	MN14—1420^T	10^4	26.39	25.08	–	–	26.21	–	n/a
		50	32.80	31.16	–	–	33.55	–	n/a
B. mayonii	MN14-1539	10^4	26.64	25.18	–	–	26.20	–	n/a
		50	32.91	32.00	–	–	34.84	–	n/a
B. miyamotoi	HT31^T	10^4	–	24.59	22.88	25.69	–	–	n/a
		50	–	31.62	30.70	33.20	–	–	n/a
B. miyamotoi	HT24	10^4	–	24.99	23.59	26.27	–	–	n/a
		50	–	32.04	30.85	34.55	–	–	n/a
B. miyamotoi	CT13-2396	10^4	–	24.94	23.49	25.76	–	–	n/a
		50	–	31.55	30.61	34.50	–	–	n/a
B. miyamotoi	RI13-2395	10^4	–	25.00	23.60	26.22	–	–	n/a
		50	–	31.82	31.08	34.85	–	–	n/a
B. americana	SCW-41;	10^4	30.87	24.92	–	–	–	31.11	6.19

Borrelia species	Strain	fg DNA per reaction	Bbsl_*fliD* Cq (M1b)	Bor_16S Cq (M3)	Bmiya_*purB* Cq (M5)	Bmiya_*qlpQ* Cq (M6)	Bmayo_*oppA2* Cq (M4)	Bbss_*oppA2* Cq (M4)	ΔCq (Bbss_*oppA2*–Bor_16S)
	subgroup A^T	50		32.49					n/a
B. andersonii	21038	10^4	–	25.05	–	–	–	–	n/a
		50	–	31.89	–	–	–	–	n/a
B. andersonii	SI-10	10^4	32.76	24.15	–	–	–	–	n/a
		50	–	31.16	–	–	–	–	n/a
B. bissettiae	DN127^T	10^4	24.79	24.65	–	–	–	–	n/a
		50	32.20	31.40	–	–	–	–	n/a
B. bissettiae	CA389	10^4	24.81	24.53	–	–	–	–	n/a
		50	31.86	31.66	–	–	–	–	n/a
B. californiensis	CA20	10^4	25.53	24.92	–	–	–	–	n/a
		50	32.16	31.83	–	–	–	–	n/a
B. carolinensis	SCW-22	10^4	–	25.10	–	–	–	–	n/a
		50	–	31.93	–	–	–	–	n/a
B. kurtenbachii	25015^T	10^4	25.38	24.79	–	–	–	38.23	13.44
		50	32.71	32.00	–	–	–	–	n/a
B. kurtenbachii	IL96-255	10^4	25.10	24.72	–	–	–	35.64	10.92
		50	31.87	31.42	–	–	–	–	n/a

Bbsl, *Borrelia burgdorferi* s.l. target; Bbss, *Borrelia burgdorferi* s.s. target; Bor, pan-*Borrelia* target; Bmiya, *B. miyamotoi* target; Bmayo, *B. mayonii* target.

^{T}denotes Type Strain.

Table 7

Prevalence of each of the five pathogens targeted by our testing algorithm in *I. scapularis* nymphs collected in June 2015 on public lands in two north-central Minnesota counties, and prevalence of ticks testing positive for two or more of these pathogens.

Collection Site	Total no. nymphs	No. positive for each pathogen[a] (infection rate; 95% CI (%))					No. positive for multiple pathogens[a] (infection rate; 95% CI (%))
		Borrelia burgdorferi s.s.	*Borrelia mayonii*	*Borrelia miyamotoi*	*Anaplasma phagocytophilum*	*Babesia microti*	
Clearwater	115	36 (31.3; 23.4–40.2)	1 (0.9; 0.0–4.1)	0 (0.0; 0.0–3.2)	14 (12.2; 7.1–19.1)	13 (11.3; 6.5–18.1)	15 (13.0; 7.8–20.1)
Hubbard	76	31 (40.8; 30.2–52.0)	3 (3.9; 1.0–10.3)	2 (2.6; 0.5–8.3)	6 (7.9; 3.3–15.6)	3 (3.9; 1.0–10.3)	9 (11.8; 6.0–20.6)
Total	191	67 (35.1; 28.6–42.0)	4 (2.1; 0.7–4.9)	2 (1.0; 0.2–3.4)	20 (10.5; 6.7–15.4)	16 (8.4; 5.0–13.0)	24 (12.6; 8.4–17.8)

No., number; CI, confidence interval.

[a]If we detected two or more target pathogens in a tick, that tick is included in the “No. positive” for each pathogen for which it was positive. It is also included in the count of ticks positive for multiple pathogens.