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Evaluation of a novel multiplex PCR amplicon sequencing assay for detection of human pathogens in *Ixodes* ticks

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Abstract

Tickborne diseases are an increasing public health concern in the United States, where the majority of notifiable cases are caused by pathogens vectored by *Ixodes* ticks. To better monitor changes in acarological risk of human encounters with these ticks and their associated pathogens, the Centers for Disease Control and Prevention (CDC) recently established a national tick and tickborne pathogen surveillance program. Here, we describe and evaluate a new Multiplex PCR Amplicon Sequencing (MPAS) assay for potential use in surveillance programs targeting two common human-biting vector ticks, *Ixodes scapularis* and *Ixodes pacificus*. The ability of the MPAS assay to detect five *Ixodes*-associated human pathogens (*Borrelia burgdorferi* sensu stricto, *Borrelia mayonii*, *Borrelia miyamotoi*, *Anaplasma phagocytophilum* and *Babesia microti*) was compared to that of a previously published and routinely used probe-based (TaqMan) PCR testing algorithm for pathogen detection in *Ixodes* ticks. Assay performance comparisons included a set of 175 host-seeking *Ixodes* nymphs collected in Connecticut as well as DNA from our pathogen reference collection. The MPAS assay and the CDC standard TaqMan PCR pathogen testing algorithm were found to have equivalent detection sensitivity for *Ixodes*-associated human pathogens. However, the MPAS assay was able to detect a broader range of tick-associated microorganisms, more effectively detected co-infections of multiple pathogens in a single tick (including different species within the *Borrelia burgdorferi* sensu lato complex), and required a smaller volume of test sample (thus preserving more sample for future testing).

Keywords

Anaplasma ; *Babesia* ; *Borrelia* ; *Ixodes* ; Next generation sequencing

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Disclaimer

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

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101504.

1. Introduction

Tickborne diseases are an increasing public health concern in the United States, where they represent over 75 % of vector-borne disease cases reported to the Centers for Disease Control and Prevention (CDC) (Adams et al., 2016; Rosenberg et al., 2018). The majority of these cases are Lyme disease, primarily caused by *Borrelia burgdorferi* sensu stricto (s.s.) and less commonly and more focally by *Borrelia mayonii* (Pritt et al., 2016). In the eastern United States, the blacklegged tick, *Ixodes scapularis* is the primary vector to humans of these Lyme disease spirochetes as well as other disease agents including *Borrelia miyamotoi* (relapsing fever), *Anaplasma phagocytophilum* (anaplasmosis), *Babesia microti* (babesiosis), and Powassan virus (Eisen and Eisen, 2018). The number of counties in which *I. scapularis* is considered to be established has more than doubled over the past two decades and during that same time period, the geographic range over which Lyme disease cases occur has also expanded (Kugeler et al., 2015; Eisen et al., 2016). Together, these trends underscore that an increasing number of communities are at risk for exposure to *I. scapularis*-borne pathogens. To provide the public, health care providers and policy makers with current and accurate data on the distribution of medically important ticks and their associated pathogens, the CDC initiated a national surveillance program focused on *I. scapularis* and its close relative in the western United States, *Ixodes pacificus* (https://www.cdc.gov/ticks/resources/TickSurveillance_Iscapularis-P.pdf). This program includes routine testing of collected host-seeking *Ixodes* ticks for presence of the following bacterial and parasitic agents: *Bo. burgdorferi* s.s., *Bo. mayonii*, *Bo. miyamotoi*, *A. phagocytophilum*, and *Ba. microti*.

Here, we describe a new Multiplex PCR Amplicon Sequencing (MPAS) assay that, compared with the currently used CDC in-house standard probe-based (TaqMan) PCR assay for pathogen detection in *Ixodes* ticks (Graham et al., 2018), reduces the amount of nucleic acid used and improves specificity while still retaining the same level of sensitivity. The performance of the new MPAS assay was evaluated using 1) a set of host-seeking *Ixodes* nymphs collected in Connecticut, United States and 2) various pathogen DNA sources from our reference collection.

2. Materials and methods

2.1. Tick and pathogen samples

Archived nucleic acids from 175 *Ixodes* nymphs collected in 2018 by drag sampling from various locations in Connecticut were used to compare the CDC standard in-house TaqMan PCR pathogen testing algorithm, described by Graham et al. (2018), and the new MPAS assay. After collection, the ticks were stored at 4 °C in RNA/DNA Shield (Zymo Research, Irvine, CA, USA) and shipped to the CDC, Fort Collins, Colorado for nucleic acid extraction. Nucleic acid from pathogen culture samples and *Ixodes* ticks from our reference collection (Supplemental Table 1) were used to further evaluate the specificity and sensitivity of the MPAS assay. In addition, a multi-pathogen sample was generated by mixing equal amounts of DNA from *I. scapularis* and six different pathogens from our reference collection (*Bo. burgdorferi* s.s., *Bo. mayonii, Bo. miyamotoi, A. phagocytophilum, Ba. microti,* and *Ehrlichia muris eauclairensis*). This sample was used to assess the ability

of the new assay to detect human pathogens in ticks that are simultaneously infected with multiple pathogens. All *Borrelia* spp. DNA were extracted from cultures of isolates maintained at the CDC, Fort Collins (Graham et al., 2018) (Supplemental Tables 1–3). DNA from *A. phagocytophilum* (USG3), *E. muris eauclairensis*, and *Ba. microti* were provided by the CDC, Atlanta, GA. Additionally, DNA from a female *Ixodes angustus* harboring "*Candidatus* Ehrlichia khabarensis" was provided by the British Columbia Centre for Disease Control, Vancouver, Canada.

2.2. Nucleic acid extraction

The archived samples were originally created by placing individual ticks in 350 μL of tissue lysis buffer (328 μL ATL, 20 μL Proteinase K, and 2 μL DX Reagent (Qiagen, Germantown, MD, USA) and homogenized using a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA) with 545 mg 2.0 mm Very High Density Yttria stabilized zirconium oxide beads (GlenMills, Clifton, NJ, USA). Nucleic acid was then extracted from tick lysates (300 μL) using the KingFisher DNA extraction system (Thermo Fisher Scientific, Waltham, MA, USA) and the MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific) according to manufacturer recommendations.

2.3. CDC in-house standard TaqMan PCR pathogen testing algorithm

The CDC standard in-house TaqMan PCR pathogen testing algorithm (Graham et al., 2018) uses five multiplex real time PCR assays to detect *Bo. burgdorferi* sensu lato (s.l.), *Bo. burgdorferi* s.s., *Bo. mayonii, Bo. miyamotoi, Ba. microti,* and *A. phagocytophilum* in *Ixodes* ticks. This pathogen detection algorithm was thoroughly tested for sensitivity and specificity as described previously (Graham et al., 2018): the limit of detection for *Bo. burgdorferi* s.s., *Bo. mayonii*, *Bo. miyamotoi*, *A. phagocytophilum*, and *Ba. microti* was found to be ≤ 6 genomes.

2.4. Description of multiplex PCR amplicon sequencing (MPAS) assay

The MPAS assay is comprised of six component parts, each described below.

2.4.1. Primary PCR reaction—The first step in the process is to perform a multiplex PCR reaction targeting tick-borne pathogens of interest, using genus level PCR primers (Supplemental Table 4). This is done to narrow the scope of targeted microorganisms to genera of interest to human health. That is, we optimized the assay for surveillance of *Ixodes*-borne human pathogens rather than broadly targeting all microorganisms present on the surface of or within the ticks. The primary multiplex PCR reactions were performed in 25 μL, which included 12.5 μL 2x Sso Advanced (BioRad, Hercules, CA, USA), 10 μL tick nucleic acids extract, PCR primers (Supplemental Table 4) and 2.5 μL PCR grade H_2O. Cycling conditions consisted of 98 °C for 3 min to denature DNA followed by 40 cycles of 98 °C for 20 s, 58 °C for 20 s and 68 °C for 1 min, ending with a 5 min incubation at 68 °C, using a C1000 Touch thermal cycler (BioRad).

2.4.2. PCR cleanup—Following the primary multiplex PCR reaction, the PCR amplicons are purified and prepared before barcodes/indexes can be attached. The PCR amplicons were purified and prepared for barcodes/indexes using Agencourt AMpure XP

magnetic beads (Beckman Coulter, Brea, CA, USA). A 1X volume of AMpure XP was added to each sample and incubated at room temperature for 10 min. Following incubation, the samples were placed on a magnet (96-well plate or individual tube) for 5 min to allow the magnetic particles to adhere to the side of the vessel. After the magnetic particles adhered to the vessel the liquid was removed, and the magnetic particles were washed twice using 180 µl of freshly made 80 % ethanol, while still on the magnet. Following the ethanol wash on the magnet, the magnetic beads were air-dried for 2 min. Thereafter nucleic acid was eluted from the magnetic beads by 1) adding 55 µl molecular grade H_2O to the sample; 2) taking the vessel off the magnet; 3) pipetting up and down 5 times to mix; 4) reapplying the vessel to the magnet; 5) allowing the magnetic beads to adhere to the side of the vessel; and 6) transferring 40 µl of the elution to a new tube/plate.

2.4.3. Index PCR—Following the primary PCR cleanup step, a unique index (barcode) was added to each primary multiplex PCR reaction, which facilitates tracking of unique PCR amplicons in a pooled sample. Indexing was performed using the Nextera XT index kit V2 (Illumina, San Diego, CA, USA) in a PCR reaction consisting of 25 µL 2x Sso Advanced (BioRad), 10 µL PCR grade H_2O, 5 µL forward index primer, 5 µL reverse index primer, and 5 µL of the cleaned-up PCR amplicon described above. PCR cycling conditions consisted of 98 °C for 3 min to denature DNA followed by 12 cycles of 98 °C for 20 s, 55 °C for 20 s, and 68 °C for 1 min, ending with a 5 min incubation at 68 °C, using a C1000 Touch thermal cycler (BioRad).

2.4.4. Index PCR amplicon purification—The PCR reactions for the MPAS assay were performed in a 96-well format, resulting in 96 unique indexes for 96 individual samples. Following the index PCR reaction, the PCR amplicons were again purified and prepared before all the individual samples were mixed together creating one pooled sample, representing all the unique samples with unique traceable indexes. During this process DNA concentrations are normalized among all the samples before a pooled sample is generated (step 6 in this section). If there are more PCR amplicons in the sample than needed to saturate the magnetic beads, the excess PCR amplicons will be washed away and will therefore not be part of the final library. Without magnetic bead-based normalization, samples with high pathogen concentrations will be overrepresented making it harder to detect samples with low pathogen concentrations, and thereby making the assay less sensitive. A total of 43 µL from the Index PCR reaction described above was mixed with 2 µL MagSi-DNA allround magnetic beads (BOCA Scientific, Westwood, MA, USA), 5 µL sodium acetate (3 M) and 50 µL isopropanol. The reaction was mixed by pipetting up and down 10 times and incubated at room temperature for 10 min. Following incubation, the samples were placed on a magnet (96-well plate or individual tube) for 5 min allowing the magnetic particles to adhere to the side of the vessel. After the magnetic particles adhered to the vessel the liquid was removed, and the magnetic particle were washed twice using 180 µl freshly made 80 % ethanol, while still on the magnet. Following the ethanol wash, the magnetic beads were air-dried for 2 min. Subsequently, nucleic acid was eluted from the magnetic beads by 1) adding 40 µl molecular grade H_2O to the sample vessel; 2) taking the vessel off the magnet; 3) pipetting up and down 5 times to mix; 4) reapplying the vessel

to the magnet; 5) allowing the magnetic beads to adhere to the side of the vessel; and 6) transferring 20 µl of elution to a new vessel.

2.4.5. Purification of pooled samples with unique Indexes—After indexing individual samples, a new (pooled) sample was generated containing DNA (amplicons) representing all samples to be analyzed in the same sequencing experiment. Here, 90 µl of the pooled sample, 10 µL sodium acetate (3 M) and 90 µL AMpure XP magnetic beads were combined and then mixed by pipetting up and down 10 times and incubated at room temperature for 10 min. Following incubation, the samples were placed on a magnet allowing the magnetic particles to adhere to the side of the vessel for 5 min. After the magnetic particles adhered to the vessel the liquid was removed, and the magnetic particles were washed twice using 500 µl of 80 % freshly made ethanol, while still on the magnet. Following the ethanol wash the magnetic beads were air-dried for 2 min. Subsequently nucleic acid was eluted from the magnetic beads by 1) adding 32 µl molecular grade H_2O to the sample; 2) removing the vessel from the magnet; 3) pipetting up and down 10 times to mix; 4) reapplying the vessel to the magnet; 5) allowing the magnetic beads to adhere to the side of the vessel; and 6) transferring 30 µl of elution to a new tube. The DNA concentration of the pooled library was determined using the Qubit dsDNA HS Assay kit (Life Technologies, Carlsbad, CA, USA), and sequencing was performed on the MiSeq system (Illumina) using the V3 600 cycle reagent kit according to the manufacturer's protocol (Illumina).

2.4.6. Bioinformatics pipeline creation and sequencing analysis—The CLC Genomic Workbench (Qiagen) software was used to create a workflow pipeline and perform sequence assembly and read mapping. First, reads were merged with overlapping pairs and a quality trim was performed using a quality limit of 0.05, then adapters were trimmed and a minimum number and maximum number of nucleotides in reads was set to 100 and 1,000, respectively. The reads were then mapped to a reference library. The sequences used in the reference sequence library are listed in Supplemental Table 2. All reference sequences that were obtained from cultures were acquired after performing the MPAS assay, including analyzing the PCR amplicons using the bioinformatic pipeline described in this section. We performed a *de novo* assembly on all unmapped reads. Reports were generated for the quality control of sequencing reads, merge of overlapping pairs, read trimming, mapping, and *de novo* assembly. It is important to note that the MPAS assay is not a quantitative assay, since a magnetic bead normalization step is included in the process as described in Section 2.4.4. This was done to increase the sensitivity of the assay when processing samples with low pathogen concentrations. To determine if a sample was positive for any of the targeted *Ixodes*- associated pathogens, a negative water control sample was used to create the average coverage cutoff for classifying the test sample as pathogen positive or negative. Infection status was determined for each 96 well plate processed and each tick sample was compared with the negative water control. One negative control sample was included for every 15 tested tick samples and, any pathogen target with a 10-fold read increase above the negative control sample was deemed positive. Several cut-off thresholds were evaluated, and the 10-fold read increase yielded the greatest accuracy for correctly scoring samples as positive or negative.

All reads in the *de novo* assembly were manually checked to confirm that the read mapping did not miss any sequence contigs of interest. A BLAST search was performed on all the *de novo* assembled contigs in order to determine their identity. If a contig for which the following BLAST search indicated the presence of a new pathogen was created during the *de novo* assembly, then a new reference sequence was generated and added to the CLC reference library. All samples were then reanalyzed using the updated bioinformatics pipeline. Any reads that mapped to a reference sequence were further analyzed using MEGA7 (Kumar et al., 2016). The sequences were aligned using CLUSTAL W function and a maximum likelihood phylogenetic tree was created. Reference sequences used in the phylogenetic analysis were either obtained from GenBank or generated from the culture isolates used for specificity testing (Supplemental Table 3).

2.5. Evaluation of the MPAS assay

In order to directly compare results from the MPAS assay with the CDC standard in-house pathogen testing algorithm, we performed each of these assays using the field-collected *Ixodes* nymphs from CT as described here (Section 2.4) or following Graham et al. (2018), respectively, on the same day using the same set of test samples. Results from each test sample and for each assay were scored independently as positive or negative for the pathogen target of interest (Table 1) and results were compared between assays for paired samples using Bowker's test in JMP 13 (SAS Institute, Cary, NC, USA). To further compare the performance of the MPAS assay and the CDC standard in-house pathogen testing algorithm, we evaluated assay sensitivity using 5-fold dilutions of DNA derived from cultures of five different human pathogens: *Bo. burgdorferi* s.s.*, Bo. mayonii, Bo. miyamotoi, A. phagocytophilum,* and *Ba. microti* (Supplemental Table 1). Each of the purified DNA samples were normalized, using the M1b and M3 PCR assays from the CDC standard in-house pathogen testing algorithm (Graham et al., 2018), in order to create stock samples with PCR *Cq* values close to 33 cycles. For each pathogen, samples were paired and run using each assay across 4 dilutions (1:1; 1:5; 1:25; 1:125). Each result was scored independently as positive or negative for the pathogen target of interest (Table 2).

The specificity of the MPAS assay was further assessed using DNA from pathogen sources from our internal reference collection (Supplemental Table 1). A 10 μl DNA sample from each of the pathogens in the internal reference collection were individually processed and the MPAS assay acquired DNA sequences were compared with GenBank sequences database following a BLAST search (Table 3). Finally, to assess the ability of the MPAS assay to detect human pathogens in ticks that are co-infected with multiple pathogens, we generated a multi-pathogen sample, as described in Section 2.1, by mixing equal amounts of DNA from *Bo. burgdorferi* s.s.*, Bo. mayonii, Bo. miyamotoi, A. phagocytophilum, Ba. microti, E. muris eauclairensis*, and *I. scapularis* from our reference DNA collection (Supplemental Table 1). DNA (10 μl) from the multi-pathogen sample was processed in the MPAS assay and all acquired DNA sequences were compared with GenBank sequences database following a BLAST search (Table 4).

3. Results

3.1. Comparison of assay performance using field-collected ticks and DNA from pathogen culture

Ticks tested using the MPAS assay were scored as either negative or positive based on the number of normalized reads relative to the negative controls. Among all negative controls (n = 40), the median number of reads was 0 (range: 0–4 reads). Similarly, the median number of reads for samples scored as negative (n = 642) was 0 (range: 0–6 reads). Positive samples (n = 58) had a median of 2,649.5 reads (range: 200−7,975 reads) (Supplemental Table 5).

The MPAS assay and the CDC standard in-house pathogen testing algorithm each identified several different human pathogens from the 175 tested field-collected *Ixodes* nymphs (Table 1). The same individual nymphs were identified by both assays as infected with *Bo. burgdorferi* s.s. (n = 29; 16.6 %), *Bo. miyamotoi* (n = 4; 2.3 %), and *A. phagocytophilum* (n = 5; 2.9 %). For *Ba. microti*, 16 (9.1 %) of the nymphs were identified as infected using the MPAS assay, compared with 15 (8.6 %) of the nymphs for the CDC standard in-house pathogen testing algorithm. No ticks infected with *Bo. mayonii* were detected with either assay. There were no statistically significant differences between the results of the two assays for the proportion of ticks infected with any of the above-mentioned pathogens (Bowker's test; $P > 0.05$ in all cases). One important difference between the two assays is that when performing the MPAS assay, only a single 10 μl DNA sample is required, whereas when performing the TaqMan PCR algorithm several reactions are performed, requiring between 30 and 50 μl of the DNA sample for complete analysis. Therefore, the MPAS assay preserves more specimen nucleic acids for archival samples and later additional use.

To further compare the performance of the MPAS assay and the CDC standard in-house pathogen testing algorithm, we evaluated assay sensitivity using 5-fold dilutions of DNA from 5 different human pathogens: *Bo. burgdorferi* s.s., *Bo. mayonii, Bo. miyamotoi, A. phagocytophilum,* and *Ba. microti*. As shown in Table 2, the MPAS assay and the CDC standard in-house pathogen testing algorithm performed equivalently across serial 5-fold dilutions for this suite of *Ixodes*-associated pathogens.

3.2. MPAS assay specificity for samples containing DNA from single or multiple pathogen species

MPAS assay specificity was evaluated across nine different bacterial or protozoan species (or candidate species) within four genera (*Anaplasma*, *Babesia*, *Borrelia*, and *Ehrlichia*); all nine microorganisms (Table 3, Supplemental Table 1) occur naturally in *Ixodes* ticks and, with the exception of *Candidatus* Ehrlichia khabarensis, represent known human pathogens. DNA sequences generated by the MPAS assay for samples representing cultured pathogens (or in the case of *Candidatus* Ehrlichia khabarensis DNA from an infected tick) were evaluated against GenBank DNA sequences for the same microorganism species. As shown in Table 3, all tested microorganisms had ≥ 98.9 % identity with a strain from the same species in the GenBank database, thereby validating the high specificity of the MPAS assay for the tested suite of human pathogens associated with *Ixodes* ticks.

Evaluation of the ability of the MPAS assay to detect these pathogens in mixed samples (mimicking multi-pathogen infections in ticks) was done using a single pooled sample containing DNA from *I. scapularis*, *Bo. burgdorferi* s.s., *Bo. mayonii, Bo. miyamotoi, A. phagocytophilum, Ba. microti,* and *E. muris eauclairensis*. As shown in Table 4, the MPAS assay successfully identified all pathogens included in the generated multi-pathogen sample, thereby not only identifying pathogens across genera but also within the same genus (*Bo. burgdorferi* s.s., *Bo. mayonii*, and *Bo. miyamotoi*). To further increase confidence in the results from the multi-pathogen sample we also compared the DNA sequences that were acquired for single pathogen samples (Table 3) with the DNA sequences acquired for the multi-pathogen sample (Table 4). All DNA amplicons for a given pathogen species had ≥99.7 % identity between sequences from the individual pathogen sample and the multi-pathogen sample.

3.3. Additional findings from MPAS assay testing of field-collected Ixodes nymphs

As shown in Table 1 and Fig. 1, testing of 175 field-collected *Ixodes* nymphs with the MPAS assay revealed infections with two different species of Lyme disease group spirochetes, *Bo. burgdorferi* s.s. and *Bo. andersonii*, and the relapsing fever spirochete, *Bo. miyamotoi*. The infections with *Bo. andersonii* (n = 4 nymphs) would not have been routinely identified beyond *Bo. burgdorferi* s.l. using the CDC standard in-house pathogen testing algorithm and would have required additional testing (and use of more sample) in order to be identified to species. The MPAS assay acquired DNA sequences for the *flaB* locus were further used to generate a maximum likelihood phylogenetic tree for *Borrelia* spp. (Fig. 1), which provided additional information for genetic variability within *Bo. burgdorferi* s.s. across infected nymphs as well as for co-infections of single nymphs with multiple stains of *Bo. burgdorferi* s.s. (n = 2 nymphs) or with *Bo. burgdorferi* s.s. and *Bo. miyamotoi* (n = 1 nymph). The *Bo. burgdorferi* s.s. clade, comprising a total of 29 infected nymphs, included 3 different strains which aligned most closely with *Bo. burgdorferi* B31, *Bo. burgdorferi* N40, or *Bo. burgdorferi* MM1 (Fig. 1). In the case of *Bo. miyamotoi*, all four infected nymphs produced DNA sequences with 100 % identity to our experimental reference sample, *Bo. miyamotoi* RI13–2395, and the GenBank sequence for Connecticut isolate *B. miyamotoi* CT13–2396 (Fig. 1 and Supplemental Table 3).

Five nymphs were found to be infected with *A. phagocytophilum* in the MPAS assay when analyzing DNA sequences for the *groEL* locus. Three of the sequences had 100 % identity to each other and the other two sequences had 99.7 % and 99.4 % identity to the three identical ones. All five sequences from the ticks had > 99 % identity to the same *groEL* region of the human *A. phagocytophilum* isolate HZ-CA (GenBank: JF494839).

All 16 *Ixodes* nymphs found infected with *Ba. microti* yielded identical *18S rDNA* locus DNA sequences that were 270 bp in length and all were 100 % identical to the *Ba. microti* reference sequence (GenBank: MH523097) used in the CLC Genomic Workbench mapping library (Supplemental Table 2). Because the MPAS assay makes use of genus-specific primers that generate a PCR product also for other *Babesia* species, this assay additionally identified 21 nymphs infected with *Babesia odocoilei*, which is not known to be a human pathogen but can have negative impacts for cervids (Milnes et al., 2019).

As shown in Table 1, co-infections were detected in 20 (11.4 %) of the *Ixodes* nymphs using the MPAS assay. This included dual infections where *B. burgdorferi* s.s.-infected nymphs also carried *Ba. microti* (n = 11 nymphs), *Ba. odocoilei* (n = 2), *A. phagocytophilum* (n = 2), or *Bo. miyamotoi* (n = 1). Two additional nymphs were dually infected with *Ba. microti* and *A. phagocytophilum*. Finally, one nymph each was infected with three microorganisms (*B. burgdorferi* s.s., *Ba. microti*, and *A. phagocytophilum*) or four microorganisms (*B. burgdorferi* s.s., *Ba. microti*, *Ba. odocoilei*, and *A. phagocytophilum*).

4. Discussion

The MPAS assay and the CDC standard in-house TaqMan PCR pathogen testing algorithm were found to have equivalent detection sensitivity for key *Ixodes*-associated human pathogens in ticks, but the MPAS assay had the further advantages of being able to detect a broader range of tick-associated microorganisms and more effectively detect co-infections of multiple pathogens in a single tick (including different species within the *Bo. burgdorferi* s.l. complex). When applied to field-collected nymphs, the MPAS assay was able to directly identify *Bo. andersonii*, which would have required additional Sanger sequencing reactions in the CDC standard in-house pathogen testing algorithm, as well as the deer-associated *Ba. odocoilei*, which is not targeted in the CDC standard in-house pathogen testing algorithm because it is not known to cause illness in humans. The ability of the sequencing-based MPAS assay approach to directly distinguish between *Bo. burgdorferi* s.l. species is useful because this complex comprises both species known to be human pathogens, for example *Bo. burgdorferi* s.s. and *Bo. mayonii* in North America, and species with unknown pathogenicity to humans, for example *Borrelia americana, Bo. andersonii*, *Borrelia californiensis, Borrelia carolinensis*, and *Borrelia lanei* in North America (Rudenko et al., 2011; Margos et al., 2016, 2017; Madison-Antenucci et al., 2020). Lack of specificity of detection assays for species within the *Bo. burgdorferi* s.l. complex could lead to artificial inflation in the prevalence of ticks considered to be infected with a human-pathogenic *Bo. burgdorferi* s.l. species and therefore provide an inaccurate acarological risk estimate.

Borrelia miyamotoi is genetically divided into three genospecies types: Asian, European, and North American. There is a high degree of DNA sequence homology between and within genospecies types, but some minor sequence variations do occur (Takano et al., 2014; Crowder et al., 2014; Mukhacheva et al., 2015; Cook et al., 2016; Iwabu-Itoh et al., 2017). Considering the origin (Connecticut) of the nymphs tested in this study, it is not surprising that all four *Bo. miyamotoi*-infected nymphs from the MPAS assay produced DNA sequences with 100 % identity to our experimental reference sample, *Bo. miyamotoi* RI13–2395 (Rhode Island), and the GenBank sequence for a Connecticut isolate *Bo. miyamotoi* CT13–2396 (Fig. 1). When comparing the *fla*B sequences from the nymphs infected with *Bo. miyamotoi* with sequences found in GenBank for a Californian isolate (CA17–2241) or with the sequence we obtained from analyzing a Japanese isolate (HT31) it is encouraging that we saw not only differences between samples from different continents (HT31 vs CT-infected nymphs) but also variation within the North American samples obtained from geographically distinct regions from the east and west coasts of the United States (CA17–2241 vs CT-infected nymphs) (Fig. 1). The ability to differentiate

Bo. miyamotoi at the genospecies type level when using the MPAS assay exemplifies the increased information that can be obtained compared with the TaqMan assay.

Additionally, compared with the TaqMan algorithm, the MPAS assay consumes a 3-fold lower volume of nucleic acids, thus preserving more sample for future testing. This is important because the amount of nucleic acid derived from field-collected ticks is limited. By reducing the volume of nucleic acids consumed to identify currently known human pathogens, we are better able to preserve the remaining sample for future use, such as testing of archived samples for newly recognized pathogens. *Ixodes scapularis* is currently known to harbor seven human pathogens and three of these were described only in the past decade (Eisen and Eisen, 2018). It therefore seems likely that additional microorganisms associated with this tick, particularly those occurring at low prevalence in the ticks or with highly focal geographic distributions, may be recognized as human pathogens in the future. Re-testing of archival samples from ticks collected across broad geographic areas may aid in rapidly assessing the distribution and prevalence of newly discovered human pathogens (Eisen and Paddock, 2020). Moreover, because the MPAS assay has the ability to recognize a wide array of tick-associated microorganisms that are taxonomically similar to known human pathogens, use of the assay could aid in the “reverse discovery” (recognition of organisms in ticks prior to their characterization as human pathogens) of novel tick-borne pathogens (Tijsse-Klasen et al., 2014).

Assay costs vary among laboratories and institutions, but within our laboratory setting, the cost per sample of the MPAS was comparable to the probe-based TaqMan assay. Moreover, using the Illumina MPAS assay, there are indexes (barcodes) to process 384 unique samples per analysis cycle, which will decrease the cost associated with each sample compared with the 96-well plate format described here. Drawbacks to the MPAS assay compared to the current TaqMan based testing algorithm include the more laborious laboratory work when preparing a sequencing library and the complexity of the current sequence analysis process. The current workflow performs well for developing new assays and working with a limited number of samples (< 500/month) but needs streamlining and optimization before it can be implemented as a part of the national tick and tickborne pathogen surveillance program that processes large volumes of ticks. We are therefore currently optimizing the library preparation by building automated workflows and building a custom bioinformatic workflow to be used for the national tick and tickborne pathogen surveillance program.

To our knowledge, this is the first assay combining multiplex PCR using genus-specific primers and Next Generation Sequencing (NGS) for detecting human pathogens in ticks. Several other NGS assays have been developed and used to analyze the microbiome of ticks and thereby also identifying human pathogens (Vayssier-Taussat et al., 2013; Estrada-Peña et al., 2018; Ravi et al., 2019; Tokarz et al., 2019; Egan et al., 2020; Chauhan et al., 2019), but common to them all is the need for secondary sequencing experiments to identify human pathogens beyond genus level. This is also, to our knowledge, the first time that the sensitivity and specificity of a novel NGS assay to detect pathogens in tick samples has been compared to that of an already verified tick testing algorithm (Graham et al., 2018).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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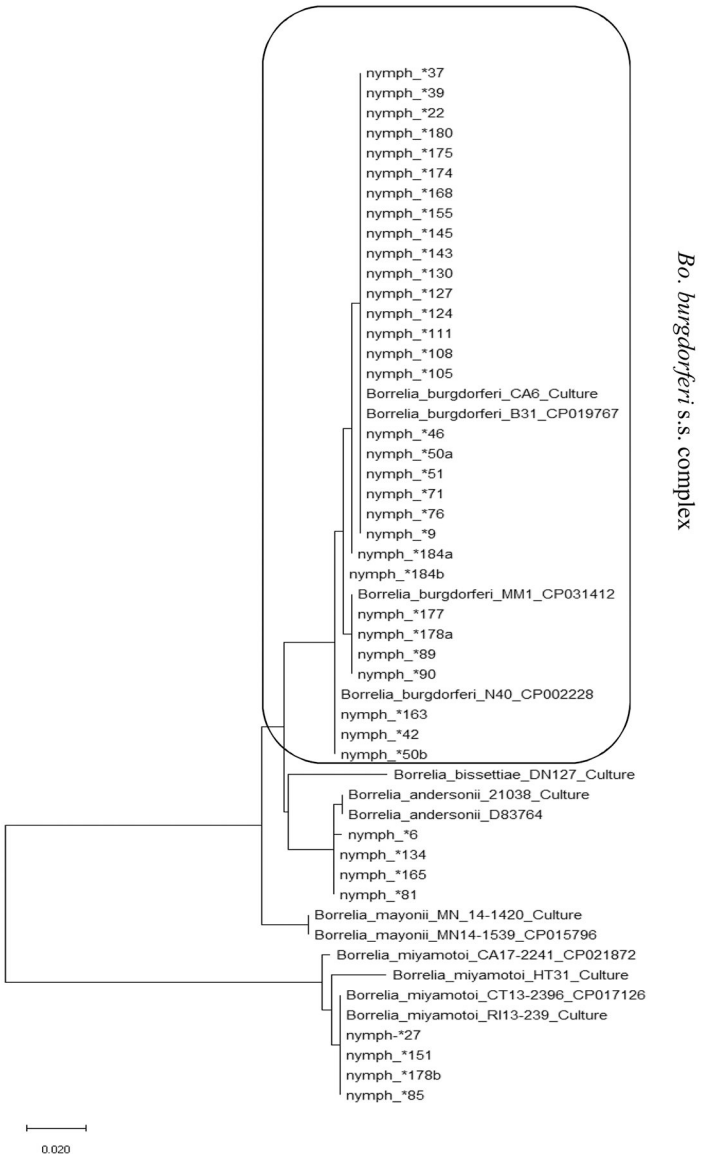

Fig. 1.

Maximum likelihood phylogenetic tree for the *Borrelia* spp. *flaB* target for the 37 *Ixodes* nymphs that were positive for *Borrelia* spp. Three of these nymphscarried either multiple strains of *Borrelia burgdorferi* sensu stricto (#50, black triangles; #184, black diamonds) or different *Borrelia* species (#178, black squares). Reference DNA sequences are described in Supplemental Table 4.

Table 1:

Performance comparison between the CDC standard in-house pathogen testing algorithm and the new MPAS assay based on testing of 175 field-collected *Ixodes* nymphs for presence of *Borrelia* (*Bo.*), *Anaplasma* (*A.*), and *Babesia* (*Ba.*) species.

Microorganisms identified	Number (%) nymphs infected out of 175 tested	
	CDC standard in-house pathogen testing algorithm	MPAS assay
Bo. burgdorferi s.s.	29 (16.6)	29 (16.6)
Bo. andersonii [a]	0[a]	4 (2.3)
Bo. miyamotoi	4 (2.3)	4 (2.3)
Ba. microti	15 (8.6)	16 (9.1)
Ba. odocoilei [b]	0[b]	21 (12.0)
A. phagocytophilum	5 (2.9)	5 (2.9)
Bo. burgdorferi s.s. and *Bo. miyamotoi*	1 (0.06)	1 (0.06)
Bo. burgdorferi s.s. and *Ba. microti*	11 (6.3)	11 (6.3)
Bo. burgdorferi s.s. and *Ba. odocoilei*	0[b]	2 (0.11)
Bo. burgdorferi s.s. and *A.phagocytophilum*	2 (0.11)	2 (0.11)
Ba. microti and *A. phagocytophilum*	1 (0.06)	2 (0.11)
Bo. burgdorferi s.s., *Ba. microti* and *A.phagocytophilum*	1 (0.06)	1 (0.06)
Bo. burgdorferi s.s., *Ba. microti, Ba.odocoilei*, and *A. phagocytophilum*	0[b]	1 (0.06)

[a] *Bo. andersonii* is not included as a primary species-level target for the CDC standard in-house pathogen testing algorithm but could have been identified with extended Sanger sequencing.

[b] *Ba. odocoilei* is not included as a target for the CDC standard in-house pathogen testing algorithm.

Table 2:

Performance comparison between the CDC standard in-house pathogen testing algorithm (Algorithm) and the new MPAS assay for serial dilutions of DNA from various *Ixodes*-associated human pathogens. Assay performance: number positive/number tested.

DNA stock dilution	*Anaplasma phagocytophilum*		*Babesia microti*		*Borrelia burgdorferi* s. s.		*Borrelia mayonii*		*Borrelia miyamotoi*	
	Algo-rithm	MPAS assay	Algo-rithm	MPAS assay	Algo-rithm	MPAS assay	Algo-rithm	MPAS assay	Algo-rithm	MPAS assay
1:1	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
1:5	4/4	4/4	4/4	4/4	3/4	3/4	4/4	4/4	4/4	4/4
1:25	3/4	2/4	0/4	4/4	2/4	3/4	1/4	2/4	2/4	4/4
1:125	1/4	1/4	0/4	2/4	0/4	0/4	1/4	0/4	0/4	2/4

Ticks Tick Borne Dis. Author manuscript; available in PMC 2024 August 28.

Table 3:

Specificity testing of MPAS assay acquired DNA sequences from internal reference collection against GenBank DNA sequences for *Ixodes*-associated microorganisms in samples containing DNA from a single microorganism. Further strain information for the tested microorganisms can be found in Supplemental Table 1.

Microorganism	Identity of DNA sequence	GenBank organism	GenBank ID
Borrelia mayonii MN14–1420	377/377 (100 %)	*Borrelia mayonii* strain MN14–1420	CP015780
Borrelia burgdorferi sensu stricto CA6	377/377 (100 %)	*Borrelia burgdorferi* strain B31_NRZ	CP019767
Borrelia andersonii 21038	373/377 (98.9 %)	*Bo. andersonii*	D83764
Borrelia miyamotoi HT31	329/329 (100 %)	*Borrelia miyamotoi* strain FR64b	CP004217
Borrelia miyamotoi RI13–2395	329/329 (100 %)	*Borrelia miyamotoi* strain CT13–2396	CP017126
Borrelia bissettiae DN-127	377/377 (100 %)	*Borreliella bissettii* DN127	CP002746
Anaplasma phagocytophilum USG3	357/359 (99.4 %)	*Anaplasma phagocytophilum* str. Dog2	CP006618
Babesia microti DPD1737	322/322 (100 %)	*Babesia microti* strain RI	LN871598
Ehrlichia muris eauclairensis #226	362/362 (100 %)	*Ehrlichia* sp. EMLA	KU214846
"*Candidatus* Ehrlichia khabarensis"	351/351 (100 %)	*Candidatus* Ehrlichia khabarensis isolate 13 MT101	MK956955

Table 4:

Specificity testing of MPAS assay acquired DNA sequences from the multi-pathogen sample against GenBank DNA sequences for *Ixodes*-associated microorganisms in a single sample containing DNA from multiple microorganisms. Further strain information for the tested microorganisms can be found in Supplemental Table 1.

Microorganisms included in the multi-pathogen sample	Identity of DNA sequence	GenBank organism	GenBank ID
Borrelia mayonii MN14–1420	377/377 (100 %)	*Borrelia mayonii* strain MN14–1420	CP015780
Borrelia burgdorferi sensu stricto CA6	377/377 (100 %)	*Borrelia burgdorferi* strain B31_NRZ	CP019767
Borrelia miyamotoi HT31	329/329 (100 %)	*Borrelia miyamotoi strain FR64b*	CP004217
Anaplasma phagocytophilum USG3	357/359 (99.4 %)	*Anaplasma phagocytophilum* str. Dog2	CP006618
Babesia microti DPD1737	322/322 (100 %)	*Babesia microti* strain RI	LN871598
Ehrlichia muris eauclairensis #226	362/362 (100 %)	*Ehrlichia* sp. EMLA	KU214846

Ticks Tick Borne Dis. Author manuscript; available in PMC 2024 August 28.