HHS Public Access

Author manuscript

Ticks Tick Borne Dis. Author manuscript; available in PMC 2024 July 01.

Published in final edited form as:

Ticks Tick Borne Dis. 2024 July ; 15(4): 102343. doi:10.1016/j.ttbdis.2024.102343.

A next generation sequencing assay combining *Ixodes* species identification with pathogen detection to support tick surveillance efforts in the United States

Lynn M. Osikowicz,
Sarah E. Maes,
Rebecca J. Eisen,
Andrias Hojgaard*

Division of Vector-Borne Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, United States

Abstract

The burden of tick-borne diseases continues to increase in the United States. Tick surveillance has been implemented to monitor changes in the distribution and prevalence of human disease-causing pathogens in ticks that frequently bite humans. Such efforts require accurate identification of ticks to species and highly sensitive and specific assays that can detect and differentiate pathogens from genetically similar microbes in ticks that have not been demonstrated to be pathogenic in humans. We describe a modification to a next generation sequencing pathogen detection assay that includes a target that accurately identifies *Ixodes* ticks to species. We show that the replacement of internal control primers used to ensure assay performance with primers that also act as an internal control and can additionally differentiate tick species, retains high sensitivity and specificity, improves efficiency, and reduces costs by eliminating the need to run separate assays to screen for pathogens and for tick identification.

Keywords

Borrelia; Anaplasma; Babesia; Ehrlichia; Ixodes scapularis

*Corresponding author. fth3@cdc.gov (A. Hojgaard).

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.

CRediT authorship contribution statement

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

Declaration of competing interest

none.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2024.102343.

1. Introduction

Tick-borne diseases account for more than three-quarters of vector-borne disease cases reported annually to the US Centers for Disease Control and Prevention (CDC) (Rosenberg et al., 2018). Although *Amblyomma* and *Dermacentor* ticks play a significant role in transmitting bacterial and viral disease agents to humans (e.g., *Ehrlichia chaffeensis, Rickettsia rickettsii, Rickettsia parkeri*, Heartland virus, Colorado tick fever virus), the majority of tick-borne disease cases in the US are caused by pathogens spread by *Ixodes* ticks, primarily *Ixodes scapularis* in the eastern US (Eisen et al., 2017; Eisen and Eisen, 2018; NNDSS, 2023). The geographic distribution of *I. scapularis* and its associated pathogens has expanded in recent decades, putting an increased number of communities at risk for encounters with infected ticks (Eisen et al., 2016; Fleshman et al., 2022; Eisen and Eisen 2023). To monitor changes in the distribution and abundance of ticks and human pathogens within them, in 2018 the CDC initiated a national tick surveillance effort (CDC, 2018; Eisen and Paddock, 2021).

As part of this program, thousands of *Ixodes* ticks are collected annually throughout the US and are tested by CDC, state health departments, and universities for human disease-causing pathogens; testing results are submitted to a CDC database, ArboNET, for compilation and dissemination to the public (CDC, 2023). Initially, the majority of ticks were tested using TaqMan PCR assays (Graham et al., 2018) that were capable of detecting the most commonly encountered human pathogens in *I. scapularis: Borrelia burgdorferi* sensu stricto (s.s.) and *Borrelia mayonii* (Lyme disease), *Borrelia miyamotoi* (hard tick relapsing fever), *Anaplasma phagocytophilum* (anaplasmosis), and *Babesia microti* (babesiosis). In areas where morphologically similar tick species co-occurred, ticks were further tested to confirm species identification (Wright et al., 2014). With follow up sequencing of *Borrelia* infected ticks, other *Borrelia burgdorferi* sensu lato species could be identified (Graham et al., 2018). However, the assay was not optimized to detect *B. burgdorferi* sensu lato coinfections, could not differentiate among *A. phagocytophilum* variants known or not known to cause pathology in humans, and did not include targets to detect the newly described *Ehrlichia muris eauclairensis* (ehrlichiosis).

A more recent next generation sequencing (NGS) assay was designed to retain sensitivity of the TaqMan assay, improve specificity including differentiation of *A. phagocytophilum* variants, detect *E. muris eauclairensis*, identify *B. burgdorferi* sensu lato coinfections, and reduce the volume of nucleic acid consumed per reaction (Hojgaard et al., 2020, 2021, 2022). As the numbers of ticks tested per year increased and ticks were collected from a larger geographic coverage with expanded diversity in species sampled, we identified a need for molecular identification of tick species to complement morphological identification. This NGS target was described recently (Osikowicz et al., 2023a).

Here, we describe and evaluate an updated NGS assay intended for high throughput screening that combines the pathogen targets from previously described assays (Hojgaard et al. 2020, 2021, 2022) with the newly described target for molecular identification of *Ixodes* species (Osikowicz et al., 2023a).

2. Materials and methods

2.1. Nucleic acid extraction

Nucleic acid from archived *Ixodes* ticks used in the study was extracted following previously described protocols (Lehane et al., 2021). Briefly, individual ticks were placed in 470 µl of tissue lysis buffer (447 µl ATL, 20 µl Proteinase K, and 3 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 Yttrium stabilized zirconium oxide beads (GlenMills, Clifton, NJ, USA). Nucleic acid was then extracted from tick lysates (200 µl) using the KingFisher™ Flex DNA extraction system (Thermo Fisher Scientific, Waltham, MA, USA) and the MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific) according to manufacturer recommendations.

2.2. Description of pathogen and tick primers

The updated *Ixodes* tick surveillance NGS multiplex PCR amplicon sequencing (MPAS) assay (iPM-05, Illumina Primer Mix 05) and the former *Ixodes* tick surveillance NGS MPAS iPM-01 assay described by Hojgaard et al. (2020), contain the same PCR primers for detection of *Ixodes*-associated human pathogens (*Borrelia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp.), but the iPM-05 MPAS assay also has PCR primers for a target in the tick mitochondrial 16S rRNA region (tick 16S mt-rRNA) that can be used for molecular tick identification as described by Osikowicz et al. (2023a) (see Table 1 for primer sequences and concentrations). Additionally, this target can be used as an internal control as described in Section 2.4.

2.3. PCR, library preparation, and next generation sequencing

The DNA NGS sequencing procedure used in this study was derived from previously described assays (Hojgaard et al., 2020). Briefly, the primary PCR reactions for the NGS experiments were performed in 25 µl, which included 12.5 µl TEMPase 2x master mix (AMPLICON, Denmark), 10 µl tick nucleic acids extract and 2.5 µl PCR primers resuspended in PCR grade water (Table 1). Cycling conditions consisted of 95 °C for 15 min to denature DNA followed by 40 cycles of 95 °C for 20 s, 58 °C for 20 s and 72 °C for 1 min, ending with a 5 min incubation at 72 °C. Upon completion of the primary PCR reaction, the amplicons were incubated with 1X Agencourt AMpure XP magnetic beads (Beckman Coulter, Brea, CA, USA), washed twice in 200 µl of freshly made 80 % ethanol and eluted into 90 µl molecular grade H_2O using the KingFisher™ Flex DNA extraction system (Thermo Fisher Scientific). Nextera XT indexes (Illumina, San Diego, CA, USA) were added to the purified primary PCR amplicon during the index PCR reaction. The index PCR reaction was performed with 25 µl TEMPase 2x master mix (AMPLICON), 10 µl PCR grade H_2O, 5 µl forward index primer, 5 µl reverse index primer, and 5 µl from the primary PCR reaction. Index PCR cycling conditions consisted of 95 °C for 15 min to denature DNA followed by 12 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 1 min, ending with a 5 min incubation at 72 °C. Upon completion of the index PCR reaction, the amplicons were incubated with 2 µl MagSi-DNA allround magnetic beads (BOCA Scientific, Westwood, MA, USA), 5 µl sodium acetate (3 M) and 50 µl isopropanol, washed twice in 200 µl of freshly made 80 % ethanol and eluted into 90 µl molecular

grade H_2O using the KingFisher™ Flex DNA extraction system (Thermo Fisher Scientific). Pooling of purified indexed samples was done by mixing 50 µl of each sample. For the final library, 90 µl of the pooled sample, 10 µl sodium acetate (3 M) and 90 µl AMpure XP magnetic beads (Beckman Coulter) were combined and incubated in a 1.5 ml Eppendorf tube for 5 min, washed twice in 500 µl of freshly made 80 % ethanol and eluted into 30 µl molecular grade H_2O. Amplicon sequencing was performed on the Illumina MiSeq system using MiSeq Reagent Kits Nano 500V2 (Illumina) according to the manufacturer's protocol.

2.4. Bioinformatics

The demultiplexed FASTQ files were analyzed with a custom bioinformatics pipeline described by Osikowicz et al. (2023b), and publicly available from GitHub (https://github.com/CDCgov/tick_surveillance). In short, after read clean-up and filtering, all amplicon sequence variants (ASVs) in a dataset were identified (Osikowicz et al., 2023b). The ASVs were then aligned to the input reference sequences (Supplement A) using a minimum percent identity and minimum percent aligned of 95 % and 99 %, respectively, for the *Borrelia-flaB, Babesia-18S, Anaplasma--groEL,* and tick 16S mt-rRNA targets. The tick-actin amplicons produced with iPM-01 were aligned to the reference sequences using a 90 % minimum percent identity and 95 % minimum percent alignment. A sample was considered positive if it contained a minimum of 50 reads for the given pathogen reference sequence and sufficient tick internal control reads (Tick-actin for iPM-01 and tick 16S mt-rRNA for iPM-05) as described by Osikowicz et al. (2023b).

3. Nucleic acids used to evaluate sensitivity (limit of detection) and specificity

We previously showed that iPM-01 retained the sensitivity but improved specificity compared with a previously described TaqMan testing algorithm (Hojgaard et al., 2020; Graham et al., 2018). In this assay, we exchanged the tick-actin target that was used as an assay internal control with a 16S mt-rRNA primer that also acts an internal control and can serve as a molecular confirmation of tick identification.

A plasmid with DNA for the *Borrelia-flaB, Babesia-18S*, and *Anaplasma-groEL* targets was used to assess and compare the limit of detection (LOD) of the two assays. Performance of the two assays, iPM-01 and iPM-05, was assessed across 7 plasmid dilutions (60 copy, 40 copy, 20 copy, 10 copy, 5 copy, 1 copy, 0 copy) with each concentration performed in triplicate.

Nucleic acids from an additional 14 field collected, host-seeking *Ixodes* ticks were used to assess the specificity of iPM-05. These 14 *Ixodes* tick samples consisted of 4 different *Ixodes* species (*I. scapularis, Ixodes pacificus, Ixodes dentatus*, and *Ixodes angustus*) harboring a total of 16 different previously identified organisms (*B. burgdorferi* s.s., *B. mayonii, B. miyamotoi* Am-East and *B. miyamotoi* Am-West (*B. miyamotoi* clades described by Hojgaard et al., 2021), *Borrelia andersonii, Borrelia bissettiae, Borrelia lanei, Borrelia carolinensis, A. phagocytophilum* human active variant (Ha), *A. phagocytophilum* non-human variant (non-Ha), *E. muris eauclairensis, Ehrlichia khabarensis, Ba. microti,*

Babesia odocoilei, Ba. microti-Clethrionomys (strain 400, GenBank Accession AY144687, detected in *Clethrionomys* spp.) and *Ba. microti-Sorex* (strain AF41002, GenBank Accession AY918952, detected in *Sorex* spp.). It should be noted that the genus name for most species in the genus *Clethrionomys* has been changed to *Myodes,* except for *Clethrionomys gapperi* (Schoch et al., 2020). Ticks were identified to species using morphological keys (Keirans and Clifford, 1978; Durden and Keirans, 1996); pathogens were identified previously using the iPM-01 assay.

Nucleic acids from 247 *Ixodes* ticks collected in 2022 by drag sampling from various locations in Vermont and Rhode Island were used to compare the presence of *Ixodes*-associated human pathogens between CDC's current *Ixodes* tick surveillance assay (iPM-01), with the updated MPAS assay (iPM-05).

4. Results

4.1. Limit of detection

DNA from plasmid dilutions was used to assess and compare the limit of detection (LOD) of the two assays, iPM-01 and iPM-05. Plasmids diluted from 60 target copies per reaction to 0 target copies per reaction showed equivalent sensitivity, with a LOD of five target copies per PCR reaction (Table 2).

4.2. Specificity comparison between iPM-01 and iPM-05

Molecular identification was confirmed for all 14 ticks included in the specificity panel when analyzed with iPM-05 providing 100 % concurrence between the morphological and molecular tick identification. All 16 different organisms previously identified using iPM-01 were correctly identified in the 14 *Ixodes* ticks using both iPM-01 and iPM-05. Six of the ticks had a single organism, another six ticks were co-infected with two organisms, and two were co-infected with three organism (Table 3).

4.3. Comparison of pathogen detection results in field collected ticks using iPM-01 and iPM-05

All field collected tick specimens were morphologically identified as *I. scapularis*. One of the ticks previously tested with iPM-01 did not pass the minimal read count, as described in Section 2.4, when tested with iPM-05. The iPM-05 MPAS assay confirmed the morphological tick identification in 245 of the 246 remaining samples. The tick 16S mt-rRNA target sequences identified in these samples were 99–100 % identical to the *I. scapularis* reference sequences used for this analysis (Supplement A). One sample did not match the original morphological identification (*I. scapularis*) but was instead molecularly identified as *Ixodes muris*. This sample produced a tick 16S mt-rRNA sequence that was 100 % identical an *I. muris* GenBank sequence (Accession U95896).

The iPM-05 assay produced comparable microorganism calls to the iPM-01 assay (Table 4 and Table 5). The iPM-05 assay identified an additional sample that was positive for *B. burgdorferi* s.s., one additional sample that was positive for *A. phagocytophilum* (Ha), and one sample that was positive for both *B. burgdorferi* s.s. and *A. phagocytophilum* (Ha).

The iPM-05 assay also contained an additional two samples with acceptable internal control reads (tick 16S mt-rRNA) compared to the iPM-01 assay (tick-actin).

5. Discussion

The new MPAS assay, iPM-05, and the currently used CDC *Ixodes* spp. tick surveillance assay, iPM-01, perform similarly for detecting *Ixodes*-associated human pathogens in *Ixodes* ticks. Both iPM-01 and iPM-05 detected and differentiated each of the 16 microbes included in the specificity panel, including the occurrence of coinfection, and both assays reliably detected plasmid DNA down to five copies, indicating similar analytic sensitivity. However, among the 247 field collected ticks tested, we observed three samples that were negative for human pathogens using the iPM-01 assay that were found to be positive for human pathogens using the iPM-05: one was positive for *B. burgdorferi* s.s., one was positive for *A. phagocytophilum* (Ha), and one was co-infected with *B. burgdorferi* s.s. and *A. phagocytophilum* (Ha). These results could be an indication that the new iPM-05 assay is slightly more sensitive than iPM-01, and this sensitivity increase could be due to the change of PCR primers (tick-actin vs. tick 16S mt-rRNA). Most importantly, no *Ixodes*-associated human pathogens were detected in iPM-01 that were not also detected in the new assay iPM-05.

Recognizing that field-collected ticks or DNA from ticks submitted to CDC's national tick surveillance program are of variable quality, it is essential to retain an internal tick control in the assay. This allows us to differentiate between inconclusive results, where the pathogen is not detected, possibly due to poor DNA quality or insufficient assay performance, and a negative sample where pathogens were not detected but the sample produced sufficient reads and was deemed acceptable. Such a differentiation provides more accurate estimates of pathogen prevalence by excluding inconclusive results from prevalence estimates. Sometimes tick samples are acquired from public health partners where a morphological tick identification is not possible. For example, in some cases specimens are damaged and key morphological characteristics are obscured. In other instances, ticks are destroyed entirely, the nucleic acids have already been purified, and a molecular identification of the tick is the only option. We showed that the standard assay internal control, tick-actin, could be replaced with a more informative target (tick 16S mt-rRNA) that verifies the identification of the *Ixodes* tick species and retains sensitivity and specificity for pathogen detection and differentiation. Further exploration of the target's ability to differentiate among *Ixodes* species ticks was described by Osikowicz et al. (2023a). As pathogen detection assays become increasingly more specific, it is critical to accurately identify infected ticks to species to improve our understanding of pathogen-tick associations and how tick-borne microbes are maintained in nature. Combining the molecular tick identification target with the existing pathogen detection assay increases efficiency and reduces cost through eliminating the need to retest the same samples using a secondary assay to differentiate morphologically similar species (Wright et al., 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank our academic and public health partners who collected and submitted ticks for testing and CDC's Advanced Molecular Detection program for equipment and reagent support.

Funding Source

This work was made possible through support from the Advanced Molecular Detection (AMD) program at CDC.

Data availability

Data will be made available on request.

References

CDC, 2018. Surveillance for *Ixodes scapularis* and pathogens found in this tick species in the United States. https://cdc.gov/ticks/resources/TickSurveillance_Iscapularis-P.pdf. (Accessed 29 April 2023).

CDC, 2023. ArboNET tick surveillance data sets. https://cdc.gov/ticks/surveillance/TickSurveillanceData.html. (Accessed 29 April 2023).

Durden LA, Keirans JE, 1996. Nymphs of the Genus *Ixodes* (Acari: Ixodidae) of the United States: Taxonomy, Identification key, distribution, hosts, and Medical/Veterinary importance. Thomas Say Publications in Entomology: Mongraphs. Entomological Society of America, Lanham, MD, p. 95.

Eisen L, Eisen RJ, 2023. Changes in the geographic distribution of the blacklegged tick, *Ixodes scapularis*, in the United States. Ticks Tick Borne Dis. 14, 102233 10.1016/j.ttbdis.2023.102233. [PubMed: 37494882]

Eisen RJ, Eisen L, 2018. The blacklegged tick, *Ixodes scapularis*: an increasing public health concern. Trends Parasitol. 34, 295–309. 10.1016/j.pt.2017.12.006. [PubMed: 29336985]

Eisen RJ, Eisen L, Beard CB, 2016. County-scale distribution of *Ixodes* scapularis and *Ixodes* pacificus (Acari: ixodidae) in the continental United States. J. Med. Entomol 53, 349–386. 10.1093/jme/tjv237. [PubMed: 26783367]

Eisen RJ, Kugeler KJ, Eisen L, Beard CB, Paddock CD, 2017. Tick-borne zoonoses in the united states: persistent and emerging threats to human health. ILAR J. 58, 319–335. 10.1093/ilar/ilx005. [PubMed: 28369515]

Eisen RJ, Paddock CD, 2021. Tick and tick-borne pathogen surveillance as a public health tool in the United States. J. Med. Entomol 58 (4), 1490–1502. 10.1093/jme/tjaa087. [PubMed: 32440679]

Fleshman AC, Foster E, Maes SE, Eisen RJ, 2022. Reported county-level distribution of seven human pathogens detected in host-seeking *Ixodes* scapularis and *Ixodes* pacificus (Acari: ixodidae) in the contiguous United States. J. Med. Entomol 59, 1328–1335. 10.1093/jme/tjac049. [PubMed: 35583265]

Graham CB, Maes SE, Hojgaard A, Fleshman AC, Sheldon SW, Eisen RJ, 2018. A molecular algorithm to detect and differentiate human pathogens infecting *Ixodes scapularis* and *Ixodes pacificus* (Acari: ixodidae). Ticks Tick Borne Dis 9, 390–403. 10.1016/j.ttbdis.2017.12.005. [PubMed: 29258802]

Hojgaard A, Osikowicz LM, Eisen L, Eisen RJ, 2020. Evaluation of a novel multiplex PCR amplicon sequencing assay for detection of human pathogens in *Ixodes* ticks. Ticks Tick Borne Dis 11, 101504. 10.1016/j.ttbdis.2020.101504. [PubMed: 32993925]

Hojgaard A, Osikowicz LM, Maes S, Eisen L, Eisen RJ, 2021. Detection of genetic variability in *Borrelia miyamotoi* (Spirochaetales: spirochaetaceae) between and within the eastern and western United States. J. Med. Entomol 58, 2154–2160. 10.1093/jme/tjab075. [PubMed: 33956124]

Hojgaard A, Osikowicz LM, Rizzo MF, Ayres BN, Nicholson WL, Eisen RJ, 2022. Using next generation sequencing for molecular detection and differentiation of *Anaplasma phagocytophilum* variants from host seeking *Ixodes scapularis* ticks in the United States. Ticks Tick Borne Dis 13, 102041. 10.1016/j.ttbdis.2022.102041. [PubMed: 36257186]

Keirans JE, Clifford CM, 1978. The genus *Ixodes* in the United States: a scanning electron microscope study and key to the adults. J. Med. Entomol Suppl. 2, 1–149. 10.1093/jmedent/15.suppl2.1.

Lehane A, Maes SE, Graham CB, Jones E, Delorey M, Eisen RJ, 2021. Prevalence of single and coinfections of human pathogens in *Ixodes* ticks from five geographical regions in the United States, 2013–2019. Ticks Tick Borne Dis 12, 101637. 10.1016/j.ttbdis.2020.101637. [PubMed: 33360805]

NNDSS, 2023. Centers For Disease Control and Prevention. National Notifiable Diseases Surveillance System. CDC Division of Health Informatics and Surveillance, Atlanta, GA. Annual Tables of Infectious Disease Data. https://www.cdc.gov/nndss/data-statistics/infectious-tables/index.html.

Osikowicz LM, Rizzo MR, Hojgaard A, Maes SE, Eisen RJ, 2023a. Detection of *Borrelia burgdorferi* sensu lato species in host-seeking *Ixodes species* ticks in the United States. Ticks Tick Borne Dis. Tick Borne Dis 15, 102270. 10.1016/j.ttbdis.2023.102270.

Osikowicz LM, Hojgaard A, Maes S, Eisen RJ, Stenglein MD, 2023b. A bioinformatics pipeline for a tick pathogen surveillance multiplex amplicon sequencing assay. Ticks Tick Borne Dis 14, 102207. 10.1016/j.ttbdis.2023.102207. [PubMed: 37247570]

Rosenberg R, Lindsey NP, Fischer M, Gregory CJ, Hinckley AF, Mead PS, Paz-Bailey G, Waterman SH, Drexler NA, Kersh GJ, Hooks H, Partridge SK, Visser SN, Beard CB, Petersen LR, 2018. Vital Signs: trends in reported vector-borne disease cases - United States and Territories, 2004–2016. MMWR Morb. Mortal. Wkly. Rep 67, 496–501. 10.15585/mmwr.mm6717e1. [PubMed: 29723166]

Schoch CL, Ciufo S, Domrachev M, Hotton CL, Kannan S, Khovanskaya R, Leipe D, Mcveigh R, O'Neill K, Robbertse B, Sharma S, Soussov V, Sullivan JP, Sun L, Turner S, Karsch-Mizrachi I, 2020. NCBI Taxonomy: a comprehensive update on curation, resources and tools. Database : the journal of biological databases and curation 2020, baaa062. 10.1093/database/baaa062.

Wright CL, Hynes WL, White BT, Marshall MN, Gaff HD, Gauthier DT, 2014. Single-tube real-time PCR assay for differentiation of *Ixodes affinis* and *Ixodes scapularis*. Ticks Tick Borne Dis 5, 48–52. 10.1016/j.ttbdis.2013.08.003. [PubMed: 24192510]

Table 1

Primers used for PCR mix iPM-01 and iPM-05.

Target Organism	PCR Target	iPM Assay	Primer Name	Sequence	Concentration (nM)	Reference
Anaplasma/ Ehrlichia spp.	*groEL*	iPM-01, iPM-05	859_Esp-F 860_Esp-R	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTACTCAGAGTGCTTCTCAATGT GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCATACCATCAGTTTTTTCAAC	300	Hojgaard et al., 2020
Babesia spp.	*18S*	iPM-01, iPM-05	1960_panBabesia_F 1961_panBabesia_R	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAATTCCAGCTCCAATAGCGTA GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCTAAGAATTTCACCTCTGACAGT	300	Hojgaard et al., 2020
Borrelia spp.	*flaB*	iPM-01, iPM-05	1299_FlaF 1300 FlaR	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGCTTGGAATGCARCCTGC GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCAAGTCTATTTTGRAAAGCAC	300	Hojgaard et al., 2020
Ixodes spp.	Tick-Actin	iPM-01	2038_Isca_act_F 2039_Isca_act_R	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCCATGTACGTGGCCATCCA GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTCGGTGAGGATCTTCAT	150	Hojgaard et al., 2020
Ixodes spp.	Tick 16S mt-rRNA	iPM-05	2145_tickl 6S_mt-rRNA 2193_tickl 6S_mt-rRNA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTGCTCAATGATTTTTTAAATTGCTGTGG GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATTCWTAGGGTCTTCTTGT	150	Osikowicz et al., 2023

Table 2

Comparison of the assay performance of iPM-01 and iPM-05 using serially diluted plasmid DNA with inserts for the specific PCR targets. The assay performance was based on the number positive samples detected out of the total number samples tested. This testing was performed in triplicate.

Target copy number in PCR reaction	***Anaplasma* spp. / *Ehrlichia* spp.**		***Babesia* spp.**		***Borrelia* spp.**	
	groEL		***18S***		***flaB***	
	iPM-01	**iPM-05**	**iPM-01**	**iPM-05**	**iPM-01**	**iPM-05**
60	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3
40	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3
20	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3
10	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3
5	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3	3 / 3
1	2 / 3	1 / 3	2 / 3	3 / 3	3 / 3	3 / 3
0	0 / 3	0 / 3	0 / 3	0 / 3	0 / 3	0 / 3

Table 3

iPM-01 and iPM-05 MPAS results for known positive North American, field collected, host-seeking *Ixodes* tick samples. *A. phagocytophilum* non-human active variant: non-Ha, *A. phagocytophilum* human active variant: Ha, *E. muris eauclarensis*: EME, *B. burgdorferi* sensu stricto: *B. burgdorferi* s.s.; *B. miyamotoi* (Am-East) and *B. miyamotoi* (Am-West) clades were described by Hojgaard et al., 2021; *Ba. microti (Clethrionomys*[*]*): Ba. microti* strain 400, GenBank Accession AY144687, detected in *Clethrionomys* spp.[*]; *Ba. microti (Sorex): Ba. microti* strain AF41002, GenBank Accession AY918952, detected in *Sorex* spp.

Expected microorganisms	Tick spp.	No. of known positive samples	No. positive samples	
			iPM-01	iPM-05
B. andersonii	*I. dentatus*	2	2	2
B. bissettiae	*I. pacificus*	1	1	1
B. lanei	*I. spinipalpis*	1	1	1
B. mayonii	*I. scapularis*	1	1	1
Ba. microti (Sorex)	*I. angustus*	1	1	1
A. phagocytophilum (non-Ha) + *Ba. odocoilei*	*I. scapularis*	1	1	1
A. phagocytophilum (Ha) + EME	*I. scapularis*	1	1	1
Bbss + *B. miyamotoi* (Am-West)	*I. pacificus*	1	1	1
B. carolinensis + *Ba. odocoilei*	*I. scapularis*	1	1	1
Ba. microti (Clethrionomys[*]*) + E. khabarensis*	*I. angustus*	2		
A. phagocytophilum human + *B. burgdorferi* s.s. + *B. miyamotoi* (Am-East)	*I. scapularis*	1	1	1
A. phagocytophilum (Ha) + *B. burgdorferi* s.s + *Ba. microti*	*I. scapularis*	1	1	1
	Total	14	14	14

[*]The genus name for most species in the genus *Clethrionomys* has been changed to *Myodes,* except for *Clethrionomys gapperi* Schoch et al., 2020.

Table 4

The number of positive tick samples identified with the iPM-01 and iPM-05 MPAS assays. A total of 247 North American, field collected, host-seeking *Ixodes* tick specimens from Vermont (n = 159) and Rhode Island(n = 88) were tested with each assay. Ha: *Anaplasma phagocytophilum* human active variant; non-Ha: *Anaplasma phagocytophilum* non-human active variant; *Borrelia burgdorferi* s.s.: *Borrelia burgdorferi* sensu stricto.

Identified microorganisms	**MPAS Assay**			
	iPM-01		**iPM-05**	
	No. positive	**% Positive**	**No. positive**	**% Positive**
Babesia microti	14	5.7	14	5.7
Babesia odocoilei	32	13.0	32	13.0
Borrelia burgdorferi s.s.	67	27.1	69	27.9
Borrelia kurtenbachii	2	0.8	2	0.8
Borrelia miyamotoi	1	0.4	1	0.4
Anaplasma phagocytophilum (Ha)	9	3.6	10	4.0
Anaplasma phagocytophilum (non-Ha)	3	1.2	3	1.2
Acceptable Tick-Actin reads	244	98.7	NA	NA
Acceptable Tick 16S reads	NA	NA	246	99.6

Table 5

The number of co-infected *Ixodes* spp. tick samples identified with the iPM-01 and iPM-05 MPAS assays. A total of 247 North American, field collected, host-seeking *Ixodes* tick specimens from Vermont (*n* = 159) and Rhode Island (*n* = 88) were tested with each assay. Ha: *Anaplasma phagocytophilum* human variant; non-Ha: *Anaplasma phagocytophilum* non-human variant; *B. burgdorferi* s.s.: *B. burgdorferi* sensu stricto.

Identified co-infections	**MPAS Assay**			
	iPM-01		**iPM-05**	
	No. positive	**% Positive**	**No. positive**	**% Positive**
B. burgdorferi s.s. + *A. phagocytophilum* (Ha)	6	2.4	7	2.8
B. burgdorferi s.s. + *Ba. odocoilei*	7	2.8	7	2.8
B. burgdorferi s.s. + *Ba. microti*	6	2.4	6	2.4
A. phagocytophilum (Ha) + *Ba. microti*	1	0.4	1	0.4
A. phagocytophilum (non-Ha) + *Ba. odocoilei*	1	0.4	1	0.4