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Paired real-time PCR assays for detection of *Borrelia miyamotoi* in North American *Ixodes scapularis* and *Ixodes pacificus* (Acari: Ixodidae)

Christine B. Graham[*], **Mark A. Pilgard**, **Sarah E. Maes**, **Andrias Hojgaard**, 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

Borrelia miyamotoi is an emerging, tick-borne human pathogen. In North America, it is primarily associated with *Ixodes scapularis* and *Ixodes pacificus*, two species known to bite humans. Here we describe the development and evaluation of a pair of real-time TaqMan PCR assays designed to detect *B. miyamotoi* in North American ticks. We sought to achieve sensitivity to *B. miyamotoi* strains associated with ticks throughout North America, the full genetic diversity of which is unknown, by targeting sequences that are largely conserved between *B. miyamotoi* strains from the eastern United States and genetically distinct *B. miyamotoi* strains from Japan. The two assays target different loci on the *B. miyamotoi* chromosome and can be run side by side under identical cycling conditions. One of the assays also includes a tick DNA target that can be used to verify the integrity of tick-derived samples. Using both recombinant plasmid controls and genomic DNA from North American and Japanese strains, we determined that both assays reliably detect as few as 5 copies of the *B. miyamotoi* genome. We verified that neither detects *B. burgdorferi*, *B. lonestari* or *B. turicatae*. This sensitive and specific pair of assays successfully detected *B. miyamotoi* in naturally-infected, colony-reared nymphs and in field-collected *I. scapularis* and *I. pacificus* from the Northeast and the Pacific Northwest respectively. These assays will be useful in screening field-collected *Ixodes* spp. from varied regions of North America to assess the risk of human exposure to this emerging pathogen.

Keywords

Borrelia miyamotoi; *Ixodes scapularis*; *Ixodes pacificus*; Tick-borne disease; Real-time PCR

1. Introduction

Borrelia miyamotoi is a tick-borne spirochete that was first incriminated as a cause of human illness in Russia in 2011 (Platonov et al., 2011). Human cases of *Borrelia miyamotoi* disease have since been reported in the Netherlands, Japan, and the United States (Chowdri et al., 2013; Gugliotta et al., 2013; Hovius et al., 2013; Krause et al., 2013; Molloy et al., 2015;

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

Sato et al., 2014). In North America, *B. miyamotoi* is primarily associated with *Ixodes scapularis* and *Ixodes pacificus* (Krause et al., 2015), although it has also been detected in *Ixodes dentatus*, a tick species usually associated with birds and rabbits that rarely bites humans (Hamer et al., 2012). Both *I. scapularis* and *I. pacificus* readily bite humans (Merten and Durden, 2000). To accurately ascertain the range of *B. miyamotoi*-infected ticks in North America and assess the risk of human exposure to this pathogen in different states or counties or at specific sites, assays are needed to detect *B. miyamotoi* in field-collected *Ixodes* spp.

Recent phylogenetic and comparative genomic analyses place *B. miyamotoi* firmly within the relapsing fever (RF) *Borrelia* group, which is distinct from the *Borrelia burgdorferi* sensu lato (sl) complex, or Lyme disease (LD) group (Adeolu and Gupta, 2014; Barbour, 2014; Hue et al., 2013). Soft ticks and lice vector most RF *Borrelia* (Cutler, 2015). To date, *B. miyamotoi* is the only RF *Borrelia* that has been associated with *Ixodes* spp., but there are at least four other RF *Borrelia* associated with other hard ticks (Barbour, 2014). Investigators first detected *B. miyamotoi* in *Ixodes* spp. and *B. lonestari* in *Amblyomma* spp. in the mid-1990s, and both the association of *B. turcica* with hard ticks and the association of another RF *Borrelia* sp. with an *Amblyomma* sp. in Japan were first reported within the last seven years (Barbour, 2014). It is possible that *I. scapularis* and *I. pacificus* harbor other, as-yet-unidentified RF *Borrelia* spp. Moreover, both *I. scapularis* and *I. pacificus* vector multiple *Borrelia burgdorferi* sl spp. (Schotthoefer and Frost, 2015). *Borrelia miyamotoi* and *B. burgdorferi* sl spp. are often sympatric, and they may even co-infect the same tick (Barbour et al., 2009; Dibernardo et al., 2014; Padgett et al., 2014; Wagemakers et al., 2015). Any effort to assess the prevalence of *B. miyamotoi* in North American ticks therefore requires an assay that is specific to RF *Borrelia*, and ideally one that is specific to this species.

Phylogenetic analyses have also revealed pronounced genetic variation between geographically distinct *B. miyamotoi* isolates (Barbour, 2014; Bunikis et al., 2004; Cosson et al., 2014; Crowder et al., 2014; Takano et al., 2014). Isolates typically cluster into three types, designated “Siberian,” “American” and “European” by Takano et al. (2014); others have employed similar designations (Cosson et al., 2014; Geller et al., 2012). Studies have detected limited genetic variation, however, between *B. miyamotoi* isolates within the same geographic group (Bunikis et al., 2004; Cosson et al., 2014; Crowder et al., 2014; Takano et al., 2014). Bunikis et al. (2004) found only one 16S–23S intergenic spacer genotype among 22 *B. miyamotoi*-positive *I. scapularis* collected in Connecticut, while *I. scapularis* collected at the same time from the same site were infected with *B. burgdorferi* comprising eight different genotypes. Based on an analysis of basecount signatures at five loci, Crowder et al. (2014) identified a single genotype in *B. miyamotoi*-positive ticks from Connecticut (n = 16), New York (n = 7), Pennsylvania (n = 2), Indiana (n = 10), and California (n = 20). There is, however, at least some variation between *B. miyamotoi* strains within North America, including variation between strains associated with *I. scapularis* in the eastern United States and some strains detected in *I. pacificus* from California (Mun et al., 2006; Padgett et al., 2014; Salkeld et al., 2014). Padgett et al. (2014) reported that a 614-nt (nucleotide) segment of the flagellin gene amplified from *B. miyamotoi*-positive *I. pacificus* showed 96.9% alignment with eastern United States *B. miyamotoi* strain LB-2001. Not surprisingly,

researchers have also observed that variation between different North American *B. miyamotoi* strains is less pronounced than the variation between North American *B. miyamotoi* strains and type strain HT31, which was isolated from a Japanese *Ixodes persulcatus* and falls within the "Siberian" cluster (Fukunaga et al., 1995; Takano et al., 2014). Mun et al. (2006) found that a 516-nt segment of the 16S rRNA gene from an *I. pacificus* collected in Mendocino County, California was 99.8% similar to eastern United States strain MP2000, and 99.4% similar to strain HT31. A 534-nt segment of the flagellin gene showed 99.1% homology to strain MP2000 and 97.8% homology to strain HT31.

Here we describe the development of a pair of species-specific real-time Taqman polymerase chain reaction (PCR) assays designed to detect *B. miyamotoi* in North American *I. scapularis* and *I. pacificus*. Because we sought to achieve sensitivity to *B. miyamotoi* strains associated with ticks throughout North America, the full genetic diversity of which is unknown, we targeted sequences that are largely conserved between *B. miyamotoi* strains from the eastern United States and the distinct *B. miyamotoi* cluster comprising HT31 and other closely-related Asian strains. Other goals included (1) developing assays targeting different *B. miyamotoi* genes that can be run side-by side, ideally under identical cycling conditions, so that the results of the second assay confirm the results of the first, and (2) integration of a tick DNA target than can be used to verify the integrity of individual tick-derived DNA samples. We routinely use paired real-time PCR panels with an integrated tick DNA control target to detect three other pathogens in field-collected *I. scapularis* (Hojgaard et al., 2014), and we have found this to be an efficient and reliable approach.

2. Materials and methods

2.1. Real-time PCR

To detect *B. miyamotoi* in North American ticks, we developed a singleplex real-time PCR assay targeting the *B. miyamotoi* glycerophosphodiester phosphodiesterase (*glpQ*) gene, and a duplex assay targeting both the *B. miyamotoi* adenylosuccinate lyase (*purB*) gene and the *I. scapularis* actin gene. For the singleplex assay, we modified the primers and probe from a real-time PCR assay previously used to detect *glpQ* in *B. lonestari* (Bacon et al., 2005) to target a 108-nt segment of the North American *B. miyamotoi glpQ* gene. The duplex reaction targets a 77-nt segment of the *I. scapularis* actin gene as previously described (Hojgaard et al., 2014), and a 121-nt segment of the adenylosuccinate lyase (*purB*) gene. See Table 1 for all primer and probe sequences. The Biotechnology Core Facility Branch at the Centers for Disease Control and Prevention (Atlanta, GA) synthesized all oligonucleotides.

Each 10-μl singleplex reaction contained 1X iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA), 600 nM of each *glpQ* primer, and 200 nM *glpQ* probe. Each 10-μl duplex reaction contained 1X iQ Multiplex Powermix, 100 nM of each *purB* primer, 300 nM of each actin primer and 200 nM each of the *purB* and actin probes. Real-time cycling conditions for both assays included a 3-min denaturation step at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. We analyzed samples using CFX Manager 3.1 software (Bio-Rad) with the quantitation cycle (Cq) determination mode set to regression.

2.2. DNA: borreliae and uninfected ticks

Except where otherwise noted, we determined the limit of detection (LOD), linear range of detection (LRD), and efficiency of each assay using DNA from cultured *B. miyamotoi* HT31, and *B. miyamotoi* US178-8(1-1), a North American strain that was originally isolated from a Rhode Island *I. scapularis*. We tested the specificity of each assay using DNA from cultured *B. burgdorferi* B31, *B. turicatae* FCB-1 (Schwan et al., 2005), and *B. lonestari* LS-1 (Varela et al., 2004). DNA from *B. lonestari*, cultured in ISE6 cells, was provided by the Microbiology and Pathogenesis Activity, and DNA from all other strains was provided by the Diagnostic and Reference Activity of the Bacterial Diseases Branch at the Division of Vector-Borne Diseases (DVBD), Centers for Disease Control and Prevention (Fort Collins, CO).

We extracted genomic DNA from uninfected, colony-reared *I. scapularis* nymphs (DVBD, Centers for Disease Control and Prevention, Fort Collins, CO) for use in spiking reactions. Using a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK), we homogenized up to 200 nymphs for 2 min in 0.5 ml tubes containing 159 µl ATL buffer, 20 µl Proteinase K and 1 µl DX antifoaming reagent (Qiagen, Valencia, CA), 410 mg 2.3 mm chrome steel beads (BioSpec), and 260 mg 1.3 mm chrome steel beads (BioSpec). Homogenates were incubated at 56 °C for 1 h, and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen).

2.3. Recombinant plasmids

We constructed recombinant plasmids to determine the LOD for each assay. We used our assay primers to amplify the *purB* and *glpQ* targets from *B. miyamotoi* US178-8(1-1) and extracted each amplicon from a 2% agarose gel using Freeze ‘N Squeeze DNA extraction columns (Bio-Rad). Each amplicon was cloned into a pCR4-TOPO plasmid vector, and we transformed the cloning reaction into TOP10 chemically competent *E. coli* using the TOPO TA Cloning Kit for Sequencing (ThermoFisher Scientific Inc., Waltham, MA). We used the PureLink Quick Plasmid Miniprep Kit (ThermoFisher) to isolate plasmid DNA from selected transformants and sequenced the insert using M13 forward (−20) and reverse primers and BigDye Terminator v3.1 Ready Reaction Mix (ThermoFisher). The BigDye Xterminator Kit (ThermoFisher) was used to remove unincorporated dyes before analyzing the samples on an ABI 3130XL genetic analyzer. We linearized plasmid DNA containing the correct insert with restriction enzyme NotI and purified the linearized plasmid using the QIAquick PCR Purification Kit (Qiagen). We determined the purity of each linearized plasmid stock on a NanoDrop2000 spectrophotometer (ThermoScientific). Samples with suboptimal absorption ratios (A260/A280 < 1.80 or A260/A230 < 1.90) were re-purified by ethanol precipitation. We used the Qubit dsDNA HS Assay Kit and a Qubit 2.0 fluorometer (ThermoFisher) to measure the double-stranded DNA concentration immediately before preparing serial dilutions for use as templates in LOD experiments.

2.4. Limit of detection, linear range of detection, efficiency

We prepared dilutions of *purB* and *glpQ* recombinant plasmids to attain 40, 20, 10, 5, and 1 copy per reaction and ran six replicates of each dilution. Each reaction also included 2.5 ng genomic *I. scapularis* DNA, which we had found yielded *I. scapularis* actin Cq values typical of those we observe in DNA prepared from individual nymphs (data not shown). We defined

the LOD as the lowest concentration at which all six replicates returned *purB* or *glpQ* Cq values within 40 PCR cycles.

We used genomic DNA from *B. miyamotoi* US178-8(1-1) to determine the LRD and efficiency of each assay. We verified genomic DNA purity (A260/A280 ≥ 1.80) and determined the double-stranded DNA concentration as described in Section 2.3 immediately before preparing 10-fold serial dilutions to attain 7 fg to 7×10^5 fg per reaction. We ran six replicates of each dilution, and each reaction also included approximately 2.5 ng *I. scapularis* DNA. To verify that both assays showed similar sensitivity to genetically variable *B. miyamotoi*, we also ran six replicates containing 7 fg genomic DNA from *B. miyamotoi* HT31 and 2.5 ng *I. scapularis* DNA.

2.5. Specificity

To determine if the *purB* or the *glpQ* assay detected *Borrelia burgdorferi* ss or RF *Borrelia* spp. other than *B. miyamotoi*, we prepared dilutions of *B. burgdorferi* B31 and *B. turicatae* FCB-1 DNA to achieve approximately 200 pg per reaction. Based on quantitative real-time PCR analysis (Bacon et al., 2005), we diluted *B. lonestari* LS-1 DNA to attain approximately 10^5 copies per reaction. All RF *Borrelia* spp. were run in duplicate.

2.6. Borrelia miyamotoi *detection in naturally-infected and field-collected ticks*

To verify that the paired real-time PCR assays could reliably detect *B. miyamotoi* DNA in naturally-infected ticks, we tested ten nymphs that had been reared in the lab from an egg batch laid by a *B. miyamotoi*-infected *I. scapularis* female collected in Connecticut. The nymphs were generated under protocols approved by the Animal Care and Use Committee at the DVBD, Centers for Disease Control and Prevention (Fort Collins, CO). To determine how the assays performed on field-collected ticks, we tested DNA in our reference collection from 299 questing *I. scapularis* nymphs collected by drag sampling in Suffolk County, NY in 2014 and 2015, and from 117 questing *I. pacificus* (114 adults, 3 nymphs) collected by drag sampling in multiple Washington State counties between February and May, 2015. In testing the Washington State samples, we sought both to detect *B. miyamotoi*—if present—in ticks from this region, and to verify that the *I. scapularis* actin primer-probe set could be used to detect amplifiable tick DNA in extracts from *I. pacificus*.

DNA was extracted from naturally-infected and field-collected ticks using a modification of the procedure described in Hojgaard et al. (2014). Individual ticks were homogenized in 96-well plates. Each 1.1 ml well contained 410 mg 2.3 mm chrome steel beads (BioSpec), 260 mg 1.3 mm chrome steel beads (BioSpec), and 375 µl lysis buffer composed of buffer ATL, 20 µl proteinase K, 1 µg carrier RNA, and 0.5% reagent DX (Qiagen, Valencia, CA). We disrupted the sample for 2 min using a Mini-Beadbeater-96 (BioSpec), allowing the sample to cool for at least 3 min at ambient temperature and 1 min on ice after both the first and second min of shaking. We then incubated the homogenate for ≥10 min at 56 °C. The homogenate was subsequently centrifuged for 30 s at 1,000 × *g*, and 150 µl was mixed with 150 µl AL buffer (Qiagen) and incubated for 10 min at 70 °C. We then centrifuged the sample briefly and processed the lysate using a QIAcube HT automated nucleic acid isolation system and the *cador* Pathogen 96 QIAcube HT Kit (Qiagen). 350 µl buffer ACB

was added to the 300 µl lysate and mixed, and the 650 µl sample was then transferred to the capture plate and subjected to a 3 min vacuum at 35 kPa. Subsequent wash and dry steps were identical to those in the *cador* Pathogen 96 QIAcube HT V3 program (Qiagen). At the final step, DNA was eluted by incubating 100 µl buffer AVE on the column for 2 min, then applying a 50 kPa vacuum for 6 min. Each set of extractions included at least one tick-free extraction control for every 18 test samples. Extracts were stored at 4 °C or −80 °C. We used 4.8 µl eluate as the template in each 10-µl real-time PCR. In addition to DNA extraction controls, each real-time PCR run included at least one no-template control and a positive control comprised of the appropriate recombinant plasmid at a concentration equivalent to approximately 10 target copies per reaction.

2.7. Verification by sequencing

Borrelia miyamotoi-positive ticks were subjected to amplification and sequencing of the *B. miyamotoi* gene encoding ATP-dependent Clp protease subunit A (*clpA*) using the protocol described on pubMLST (http://pubmlst.org/borrelia/; Margos et al., 2015) with modifications. Each 50-µL PCR contained 5–15 µl template and 25 µl HotStar Taq Master Mix, 500 nM each primer (BmclpAF1268 and BmclpAR2051; Table 1), and $MgCl_2$ at a final concentration of 2.0 mM. Cycling conditions included a 15-min activation and denaturation at 95 °C followed by 9 cycles of 30 s at 94 °C, 30 s at 58 °C to 50 °C, decreasing 1 °C each cycle, and 1 min at 72 °C. This was followed by an additional 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C, and a final 5 min extension at 72 °C. We visualized 10 µl of the product on a 1% agarose gel to verify the presence of an approximately 826-nt amplicon. We purified the remaining 40 µl product using the QIAquick PCR Purification Kit (Qiagen) and eluted with 40 µl elution buffer. We used the amplification primers to sequence approximately 5 ng of purified amplicon using the sequencing procedure described in Section 2.3. We assembled and manually edited at least two forward and two reverse sequences from each sample using Lasergene 12 software (DNASTAR, Madison, WI). Each consensus sequence was based on at least double coverage of every nt and was manually trimmed to remove segments containing ambiguous base calls from either end. We then used BLAST to identify similar sequences in the GenBank database. We also queried pubMLST to identify similar *clpA* alleles.

3. Results and discussion

Here we describe the development and evaluation of paired real-time PCR assays designed to detect *B. miyamotoi* in North American ticks. A singleplex real-time PCR assay targets the *B. miyamotoi glpQ* gene, and a duplex assay targets both the *B. miyamotoi purB* gene and the *I. scapularis* actin gene. As in all RF *Borrelia*, the *glpQ* and *purB* genes are present on the *B. miyamotoi* chromosome, with the *purB* gene located in the 16S–23S intergenic spacer region (Barbour, 2014). Neither gene is present in LD *Borrelia* (Pettersson et al., 2007; Schwan et al., 2003). This makes both targets ideal for detection and differentiation of North American *B. miyamotoi* in ticks that may be infected with *Borrelia burgdorferi* sl spp., *B. miyamotoi*, or both.

Except for a single nt difference 10 bases from the 3′ end of the probe-annealing sequence, the *B. miyamotoi glpQ* primer-and probe-annealing sequences are identical in North American strain LB-2001 and Japanese strain FR64b (Fukunaga et al., 1995; Table 1). The *purB* primer and probe-annealing sequences in *B. miyamotoi* LB-2001 are identical to the corresponding sequences in strain FR64b (Table 1). As of April 1, 2016, the FR64b *glpQ* target sequence was identical to all homologous sequences available in GenBank from Japanese and Russian isolates, and FR64b was the only non-North American strain for which the *purB* target sequence was available. The LB-2001 *glpQ* and *purB* targets sequences were identical to all homologous sequences available in GenBank from North American isolates. While neither real-time PCR assay was designed to detect Western European strains of *B. miyamotoi*, alignment with the homologous *glpQ* sequence segments available in GenBank from European *B. miyamotoi* isolates (e.g., French strain tik371 (KJ425356.1:113-220)), revealed a single mismatch in the reverse primer-annealing region and a single mismatch in the probe-annealing region, neither of which would be expected to impact annealing. The *glpQ* assay is therefore likely to detect European strains of *B. miyamotoi*. There was no sequence data available from European isolates for the region corresponding to our *purB* target.

BLAST analysis of the *B. miyamotoi glpQ* target revealed that species with very similar nt sequences at this locus included *B. lonestari*, *B. hermsii* (multiple strains), *B. anserina* (strain BA2), and *B. parkeri* (multiple strains). The *B. miyamotoi glpQ* primer- and probe-annealing sequences differed by a total of at least 9 nt from the corresponding sequences in each of these RF *Borrelia* strains. Species with very similar nt sequences at the *purB* locus included *B. anserina* (strain BA2), *B. crocidurae*, *B. hermsii* (multiple strains), *B. recurrentis* (strain A1), *B. duttoni*, and *B. turicatae* (strain 91E135). The *B. miyamotoi purB* primer- and probe-annealing sequences differed by a total of at least 11 nt from the corresponding sequences in each of these RF *Borrelia* strains.

The primers and probe targeting the *I. scapularis* actin gene were developed previously to serve as a positive DNA extraction control for tick-derived samples (Hojgaard et al., 2014). Although the full target sequence was not available in GenBank, available sequence for the *I. pacificus* actin gene (GU556973.1) differed by a single nt from the homologous region of the *I. scapularis* actin gene (AF426178.1:275-382). Results from field-collected *I. pacificus*, detailed below, confirmed that the *I. scapularis* actin primer-probe set also detects *I. pacificus* DNA.

Using recombinant plasmids containing the *purB* and *glpQ* target sequences, we determined that both assays detected as few as five copies of the target in six of six replicate reactions. Both assays detected genomic DNA from North American *B. miyamotoi* strain US178-8(1-1) over six logs (7 fg to 7×10^5 fg/reaction) with efficiencies between 90% and 100%, and R^2 values ≥0.994. The *glpQ* and *purB* assays also detected as little as 7 fg genomic DNA from *B. miyamotoi* HT31 in six of six replicates. All of these results held whether the *purB* assay was run in singleplex or duplexed with the *I. scapularis* actin primer-probe set. Given that genetic variation among North American *B. miyamotoi* strains is likely to be less than the variation between North American *B. miyamotoi* and Japanese *B. miyamotoi* strains (Crowder et al., 2014; Mun et al., 2006; Padgett et al., 2014), we conclude

that this assay is likely to show similar sensitivity to tick-borne strains of *B. miyamotoi* throughout North America. Based on an estimated *B. miyamotoi* genome size of 1.27 Mbp (Kingry et al., 2015), 7 fg genomic *B. miyamotoi* DNA would be expected to contain approximately 5 copies of the *B. miyamotoi* genome. We conclude that this pair of real-time PCR assays has an LOD ≤5 copies of the *purB* and *glpQ* targets, corresponding to an LOD ≤5 *B. miyamotoi* genomes. Assuming a single genome per spirochete, we would expect to be able to detect as few as 5 spirochetes per reaction.

Neither the *purB* nor the *glpQ* assay detected our *B. miyamotoi* targets in genomic DNA isolated from *B. burgdorferi* B31, or in RF strains *B. turicatae* FCB-1 or *B. lonestari* LS-1. While we cannot conclude with certainty that these assays would not detect any other *Borrelia* spp. or strains, BLAST analysis identified the *glpQ* target homolog in *B. lonestari*, which differed from the LB-2001 target sequence by nine nt, as the most similar non-*B. miyamotoi* homolog to either target, at least among those species with homologous sequences in the GenBank database. Given that neither assay detected any of the strains tested, including *B. lonestari* LS-1, we conclude that this pair of assays is likely to be specific to *B. miyamotoi*.

Both assays detected *B. miyamotoi* in all of the naturally-infected, laboratory-reared nymphs we tested, and in 3.0% of 299 *I. scapularis* nymphs collected in Suffolk County, New York (Table 2). The latter is consistent with previous reports of *B. miyamotoi* infection prevalence in *I. scapularis* nymphs from this area. Scoles et al. (2001) detected *B. miyamotoi* in 4 of 160 (2.5%) nymphal *I. scapularis* collected in nearby Westchester County in 1999. Barbour et al. (2009) reported that 0–10.5% of *I. scapularis* nymphs collected from three sites in Suffolk County (sites 9, 10 and 11) were infected with *B. miyamotoi*, with an overall infection prevalence of 2.3% (n = 555 nymphs).

Both assays also detected *B. miyamotoi* in one *I. pacificus* adult from Washington State. The California Department of Public Health first detected *B. miyamotoi* in *I. pacificus* adults in 2000 (Padgett et al., 2014). Subsequent investigations have detected *B. miyamotoi* in *I. pacificus* nymphs and adults collected from 24 California counties, with the highest infection prevalence in the northern, coastal and foothills regions of the state (Eshoo et al., 2015; Mun et al., 2006; Padgett et al., 2014; Padgett and Bonilla, 2011; Salkeld et al., 2014). This is the second report of *B. miyamotoi* detection in a tick from Washington State (E. A. Dykstra, personal communication). In testing these samples using our paired PCR assays, we also verified that the *I. scapularis* actin primer-probe set reliably detects *I. pacificus* DNA. Actin Cq values associated with the three *I. pacificus* nymphs were well within the range of the *I. scapularis* actin Cq values yielded by the 299 New York *I. scapularis* nymph extracts (Table 2). Our paired real-time assays, including the built-in control for verifying the presence of amplifiable DNA in tick-derived samples, can thus be used to test for *B. miyamotoi* in both *I. scapularis* and *I. pacificus*.

Sequence analysis of the *clpA* gene segment confirmed that all field-collected ticks in which the paired real-time PCR assays detected the *purB* and *glpQ* targets were infected with *B. miyamotoi*. The nine consensus sequences generated from the *B. miyamotoi*-positive New York *I. scapularis* DNA extracts were identical to homologous sequences in GenBank for *B.*

miyamotoi CT14D4 (CP010308.1) and LB-2001 (CP006647.2), both of which originated in Connecticut. Each of the amplicons from the *B. miyamotoi*-positive New York *I. scapularis* contained a 570-nt segment identical to *clpA* allele 200 in pubMSLT. As of April 1, 2016, this allele was associated with only one isolate in the pubMLST database, *B. miyamotoi* M1029, which originated from a Connecticut *I. scapularis*. The 570-nt target segment of the *clpA* sequence from the *B. miyamotoi*-positive *I. pacificus* collected in Washington State differed from *clpA* allele 200 by two nt substitutions (205A > G, 340C > T). It differed from *clpA* allele 199, associated with Japanese *B. miyamotoi* isolates HT31 and MYK1, and from *clpA* allele 201, associated with German *B. miyamotoi* isolate EU T01, by 5 and 21 nt substitutions respectively. These findings are consistent with previous analyses indicating that there is very little genetic variation among *B. miyamotoi* strains from the eastern United States (Bunikis et al., 2004; Scott et al., 2010), but that there is some variation between eastern strains like LB-2001 and some *B. miyamotoi* strains detected in Californian *I. pacificus* (Mun et al., 2006; Padgett et al., 2014; Salkeld et al., 2014).

We conclude that the paired real-time PCR assays described here can be used to reliably detect *B. miyamotoi* in both North American *Ixodes* spp. known to harbor the spirochete and to bite humans. Others have developed PCR-based assays to detect *B. miyamotoi* in ticks (e.g., Dibernardo et al., 2014; Houck et al., 2011; Mukhacheva and Kovalev, 2014; Quarsten et al., 2015; Reiter et al., 2015; Tsao et al., 2004; Ullmann et al., 2005; Vayssier-Taussat et al., 2013; Venczel et al., 2016). The approach described here has the advantage of integrating two sensitive, species-specific targets designed to detect genetically diverse North American strains of *B. miyamotoi* with a tick DNA target that can be used to verify the integrity of DNA derived from *I. scapularis* or *I. pacificus*. Using this approach, we successfully detected at least two different *B. miyamotoi* strains in field-collected ticks from the Northeast and the Pacific Northwest. These paired assays will be useful in screening field-collected *I. scapularis* and *I. pacificus* from varied regions of North America as part of efforts to assess the risk of human exposure to this emerging pathogen.

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Table 1

Primers and probes used in this study.

Primers and probes	Sequence (5′–3′)	Location in *B. miyamotoi* LB-2001, GenBank accession no. CP006647.2 (identity)	Location in *B. miyamotoi* FR64b, GenBank accession no. CP004217.1 (identity)	Reference
BmglpQ-F	GAC CCA GAA ATT GAC ACA ACC ACA A	248657–248681 (25/25)	657285–657261 (25/25)	This study[a]
BmglpQ-R	TGA TTT AAG TTC AGT TAG TGT GAA GTC AGT	248764–248735 (30/30)	657178–657207 (30/30)	This study[a]
BmglpQ-probe	CalRd610-CAA TCG AGC TAG AGA AAA CGG AAG ATA TTA CG-BHQ2	248701–248732 (32/32)	657241–657210 (31/32)	This study[a]
BmpurB-F	TCC TCA ATG ATG AAA GCT TTA	441938–441958 (21/21)	463965–463945 (21/21)	This study
BmpurB-R	GGA TCA ACT GTC TCT TTA ATA AAG	442058–442035 (24/24)	463845–463868 (24/24)	This study
BmpurB-probe	CalRd610-TCG ACT TGC AAT GAT GCA AAA CCT-BHQ2	442031–442008 (24/24)	463872–463895 (24/24)	This study
Actin-F	GCC CTG GAC TCC GAG CAG	n/a	n/a	Hojgaard et al., 2014
Actin-R	CCG TCG GGA AGC TCG TAG G	n/a	n/a	Hojgaard et al., 2014
Actin-P	Quas705-CCA CCG CCG CCT CCT CTT CTT CC-BHQ3	n/a	n/a	Hojgaard et al., 2014
BmclpAF1268	TTG ATC TCT TAG ATG ATC TTG G	378914–378893 (22/22)	526995–527016 (22/22)	PubMLST[b]
BmclpAR2051	CAA ACA TAA ACC TTT TCA GCC TTT AAT A	378089–378116 (28/28)	527820–527793 (28/28)	PubMLST[b]

CalRd610: CalFluor Red 610; Quas705: Quasar 705; BHQ2 and BHQ3: Black Hole Quencher 2 and 3 respectively.

[a] glpQ primers and probe adapted from Bacon et al. (2005).

[b] http://pubmlst.org/borrelia; Margos et al., 2015.

Table 2

Proportion of ticks that tested positive for *B. miyamotoi* and tick DNA (positive extraction control) using paired real-time PCR assays targeting the *B. miyamotoi purB* and *glpQ* genes and the *Ixodes* actin gene, with associated Cq values. Samples included laboratory-reared nymphs from a *Borrelia miyamotoi*-positive female, and field-collected *Ixodes* spp. from Suffolk County, New York and Washington State.

Sample source, species and life stage	***B. miyamotoi purB* No. positive/no. tested (Cq median, range)**	***B. miyamotoi glpQ* No. positive/no. tested (Cq median, range)**	***Ixodes* actin No. positive/no. tested (Cq median, range)**
Laboratory-reared			
I. scapularis nymphs	10/10 (24.82, 24.39–32.08)	10/10 (26.85, 26.57–34.68)	10/10 (26.14, 25.20–26.88)
New York			
I. scapularis nymphs	9/299 (27.06, 24.26–34.67)	9/299 (28.06, 26.56–37.60)	299/299 (26.88, 25.32–29.44)
Washington State			
I. pacificus nymphs	0/3	0/3	3/3 (27.47, 27.22–27.75)
I. pacificus adults	1/114 (21.94)	1/114 (24.73)	114/114 (24.75, 23.08–26.93)