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Original article

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

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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.

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## 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; 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.

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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