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Original article

Transmission of *Borrelia miyamotoi* sensu lato relapsing fever group spirochetes in relation to duration of attachment by *Ixodes scapularis* nymphs

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ABSTRACT

Borrelia miyamotoi sensu lato relapsing fever group spirochetes are emerging as causative agents of human illness (*Borrelia miyamotoi* disease) in the United States. Host-seeking *Ixodes scapularis* ticks are naturally infected with these spirochetes in the eastern United States and experimentally capable of transmitting *B. miyamotoi*. However, the duration of time required from tick attachment to spirochete transmission has yet to be determined. We therefore conducted a study to assess spirochete transmission by single transovarially infected *I. scapularis* nymphs to outbred white mice at three time points post-attachment (24, 48, and 72 h) and for a complete feed (> 72–96 h). Based on detection of *B. miyamotoi* DNA from the blood of mice fed on by an infected nymph, the probability of spirochete transmission increased from 10% by 24 h of attachment (evidence of infection in 3/30 mice) to 31% by 48 h (11/35 mice), 63% by 72 h (22/35 mice), and 73% for a complete feed (22/30 mice). We conclude that (i) single *I. scapularis* nymphs effectively transmit *B. miyamotoi* relapsing fever group spirochetes while feeding, (ii) transmission can occur within the first 24 h of nymphal attachment, and (iii) the probability of transmission increases with the duration of nymphal attachment.

1. Introduction

The relapsing fever group spirochete, *Borrelia miyamotoi*, was first described from *Ixodes persulcatus* and the rodent *Apodemus argenteus* in Japan (Fukunaga et al., 1995), with subsequent records from *Ixodes scapularis* in the eastern United States (Scoles et al., 2001), *Ixodes ricinus* in Europe (Fraenkel et al., 2002), and *Ixodes pacificus* in the far western United States (Mun et al., 2006). Naturally infected *I. scapularis* have since been documented throughout most of the tick's geographic range in the eastern United States (Barbour et al., 2009; Crowder et al., 2014; Hamer et al., 2014; Nelder et al., 2016). Associations with human illness – named *Borrelia miyamotoi* disease, which typically manifests with symptoms including fever, headache, chills, and myalgia – were first reported from Russia in 2011 (Platonov et al., 2011) and soon thereafter from the eastern United States (Chowdri et al., 2013; Gugliotta et al., 2013; Krause et al., 2013) and Europe (Hovius et al., 2013; Jahfari et al., 2014). Although it remains unclear how many human cases of *Borrelia miyamotoi* disease occur worldwide, this illness may be more common than previously recognized in both North America and Eurasia (Krause et al., 2014; Molloy et al., 2015; Jobe et al., 2016; Siński et al., 2016).

A recent phylogenetic study (Barbour, 2014) indicated sufficient genetic variability among spirochete isolates to justify the provisional use of *B. miyamotoi* sensu lato for a putative species complex comprised of the originally described *B. miyamotoi* sensu stricto from Japan (Fukunaga et al., 1995) and other species complex members yet to be formally characterized and named. The latter potentially includes the North American LB-2001 *B. miyamotoi* sensu lato isolate which originated from an *I. scapularis* tick (Barbour, 2014). The infected *I. scapularis* nymphs used in this study originated from a naturally infected North American female tick which passed spirochetes to her offspring. Because we have not yet characterized the spirochetes used in this study genetically, they are most appropriately classified as *B. miyamotoi* sensu lato but hereafter referred to as *B. miyamotoi* for simplicity.

Transmission of *B. miyamotoi* spirochetes via the bite of infected ticks was demonstrated previously for *I. scapularis* (Scoles et al., 2001) and *I. ricinus* (van Duijvendijk et al., 2016). This included transmission by transovarially infected *I. scapularis* nymphs (Scoles et al., 2001) and transovarially infected *I. ricinus* larvae (van Duijvendijk et al., 2016). However, studies have yet to determine the duration of time required from tick attachment to spirochete transmission. *B. miyamotoi* spir-

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Table 1
Primers and probes included in the in-house M55 multiplex PCR master mix.

Primers and probes	Sequence (5′–3′)[a]	Refs.
fliD-F	TGGTGACAGAGTGTATGATAATGGAA	Hojgaard et al. (2014)
fliD-R	ACTCCTCCGGAAGCCACAA	Hojgaard et al. (2014)
fliD-probe	FAM-TGCTAAAATGCTAGGAGATTGTCTGTCGCC-BHQ1	Hojgaard et al. (2014)
23S-F	TCGGTGAAATTGAAGTATC	Johnson et al. (2017)
23S-R	CARGCTATAGTAAAGGTTCA	Johnson et al. (2017)
23S-probe	HEX-CGTCTAACCACAAGTAATCGGCATC-BHQ1	Johnson et al. (2017)
purB-F	TCCTCAATGATGAAAGCTTTA	Graham et al. (2016)
purB-R	GGATCAACTGTCTCTTTAATAAAG	Graham et al. (2016)
purB-probe	CalRd610-TCGACTTGCAATGATGCAAAACCT-BHQ2	Graham et al. (2016)
actin-F	GCCCTGGACTCCGAGCAG	Hojgaard et al. (2014)
actin-R	CCGTCGGGAAGCTCGTAGG	Hojgaard et al. (2014)
actin-probe	Q670-CCACCGCCGCCTCCTCTTCTTCC −BHQ3	Hojgaard et al. (2014)

[a] BHQ1, BHQ2, BHQ3: Black Hole Quencher 1, 2 and 3 respectively; CalRd610: CalFluor Red 610; FAM: 6-carboxyfluorescein; HEX: hexachlorofluorescein phosphramidite; Q670: Quasar 670.

ochetes are transmitted from infected *Ixodes* females to their offspring (Scoles et al., 2001; Richter et al., 2012; Rollend et al., 2013). We speculated that transovarially infected nymphs harbor disseminated spirochetes in their salivary glands prior to tick-attachment, and could therefore be capable of transmitting spirochetes shortly after attachment.

The primary aim of this study was to assess the probability of transmission of *B. miyamotoi* spirochetes by single infected *I. scapularis* nymphs occurring by different time points after attachment. To facilitate messaging to the public regarding the time of attachment required for spirochete transmission, we included a complete nymphal feed as well as three time points for a partial nymphal feed (24, 48, and 72 h) that are easy to understand in terms of checking for and removing attached ticks daily or every two or three days.

2. Materials and methods

2.1. Source of B. miyamotoi-infected I. scapularis ticks, and experimental mouse host

The *I. scapularis* ticks used in this transmission experiment were second generation transovarially *B. miyamotoi*-infected nymphs that originated from a single female (CT15-0840) collected in November 2014 from Fairfield County, CT and then allowed to feed to repletion on a New Zealand white rabbit (Charles River Laboratories, Wilmington, MA, USA). The spent female and her larval offspring were confirmed to be infected with *B. miyamotoi* by polymerase chain reaction (PCR) as described previously by Dolan et al. (2016). First generation transovarially infected larvae and nymphs were fed on naïve CD-1 outbred *Mus musculus* mice (Charles River Laboratories) and the resulting adults were fed on a white rabbit. Second generation transovarially infected larvae, originating from a single female, were then fed on two naïve mice to generate the infected nymphs used in this *B. miyamotoi* transmission experiment. PCR-based examination (see Section 2.3.) of a subset of the unfed nymphs showed 97% (37/38) to be infected with *B. miyamotoi*. Some of these unfed nymphs (n = 18) also had their salivary glands dissected out and tested for presence of *B. miyamotoi* separately from the remainder of their bodies. Prior to processing for PCR, excised salivary glands were double-washed in two separate drops of sterile phosphate-buffered saline (PBS) to minimize the risk of external spirochete contamination.

Female 1–3 month old CD-1 outbred mice were used as experimental hosts for the infected nymphs to confirm transmission of *B. miyamotoi*.

2.2. Challenge of naïve mice with single B. miyamotoi-infected I. scapularis nymphs and blood collection from experimental hosts

Single nymphal ticks were allowed to feed on naïve mice for either 24 h (n = 34 mice with successful recovery of the tick), 48 h (n = 37 mice), 72 h (n = 37 mice), or a complete nymphal feed lasting > 72–96 h (n = 35 mice). To facilitate removal or recovery of partially or fully fed ticks, the nymphs were contained within feeding capsules attached to the shaved dorsal-midline of the mice as described previously (Mbow et al., 1994; Soares et al., 2006). A single nymph was introduced into each capsule and sealed inside using a small mesh screen attached to the top of the capsule to prevent tick escape while allowing for air circulation. All recovered partially or fully fed nymphs were processed for detection of *B. miyamotoi* DNA by PCR as described in Section 2.3. Blood samples were collected from the mice before they were exposed to nymphal feeding (baseline sample), 9–11 days after nymphal feeding commenced (for detection of *B. miyamotoi* DNA in the blood by PCR as described in Section 2.4), and 8–10 weeks post-challenge (for examination of serological reactivity to *B. miyamotoi* as described in Section 2.5). Blood collections for detection of *B. miyamotoi* DNA were performed 9–11 days after nymphs first attached, based on preliminary studies (data not shown) indicating that this time period falls within a short optimal window of time when *B. miyamotoi* DNA can be detected in mouse blood before falling to undetectable levels thereafter.

2.3. PCR-based detection of B. miyamotoi DNA in nymphal ticks and salivary glands

Nucleic acids were isolated from ticks and tick salivary glands with a Mini-Beadbeater-96 (BioSpec Products, Inc., Bartlesville, OK, USA) and a QIAcube HT robot (Qiagen, Valencia, CA, USA) using the *cador* Pathogen Kit (Qiagen) as previously described (Dolan et al., 2016). The in-house multiplex PCR master mix (designated M55; see Table 1 for primer and probe sequences) included primers and probes for the following targets: the *I. scapularis actin* target (Hojgaard et al., 2014), which serves as a control for both the DNA purification and the PCR; an in-house *23S rDNA* pan-*Borrelia* target (Johnson et al., 2017); the adenylosuccinate lyase (*purB*) target for *B. miyamotoi* (Graham et al., 2016); and the flagellar filament cap (*fliD*) of *B. burgdorferi*, which is absent in *B. miyamotoi* (Hojgaard et al., 2014). The multiplex PCR was performed using 10 µl solutions containing 5 µl iQ Multiplex Powermix (Bio-Rad, Hercules, CA, USA) and 4.8 µl DNA extract, with forward and reverse primers (0.2 µl) in a final concentration of 300 nM each, and probes in a final concentration of 200 nM each. The PCR cycling conditions were: DNA denaturation at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 60 °C for 45 s on a C1000 Touch thermal cycler with a CFX96™ real time system (Bio-Rad).

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