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Short communication

Transmission of the relapsing fever spirochete, *Borrelia miyamotoi*, by single transovarially-infected larval *Ixodes scapularis* ticks

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ABSTRACT

The relapsing fever spirochete, *Borrelia miyamotoi,* is increasingly recognized as a cause of human illness (hard tick-borne relapsing fever) in the United States. We previously demonstrated that single nymphs of the blacklegged tick, *Ixodes scapularis*, can transmit *B. miyamotoi* to experimental hosts. However, two recent epidemiological studies from the Northeastern United States indicate that human cases of hard tick-borne relapsing fever peak during late summer, after the spring peak for nymphal tick activity but coincident with the peak seasonal activity period of larval ticks in the Northeast. These epidemiological findings, together with evidence that *B. miyamotoi* can be passed from infected *I. scapularis* females to their offspring, suggest that bites by transovarially-infected larval ticks can be an important source of human infection. To demonstrate experimentally that transovarially-infected larval *I. scapularis* ticks can transmit *B. miyamotoi*, outbred *Mus musculus* CD1 mice were exposed to 1 or 2 potentially infected larvae. Individual fed larvae and mouse blood taken 10 d after larvae attached were tested for presence of *B. miyamotoi* DNA, and mice also were examined for seroreactivity to *B. miyamotoi* 8 wk after tick feeding. We documented *B. miyamotoi* DNA in blood from 13 (57%) of 23 mice exposed to a single transovarially-infected larva and in 5 (83%) of 6 mice exposed to two infected larvae feeding simultaneously. All 18 positive mice also demonstrated seroreactivity to *B. miyamotoi*. Of the 11 remaining mice without detectable *B. miyamotoi* DNA in their blood 10 d after infected larvae attached, 7 (64%) had evidence of spirochete exposure by serology 8 wk later. Because public health messaging for risk of exposure to Lyme disease spirochetes focuses on nymphal and female *I. scapularis* ticks, our finding that transovarially-infected larvae effectively transmit *B. miyamotoi* should lead to refined tick-bite prevention messages.

1. Introduction

The relapsing fever spirochete, *Borrelia miyamotoi,* is increasingly recognized as a cause of human illness (hard tick-borne relapsing fever) in the United States (Krause et al., 2013, 2014; Molloy et al., 2015; Fiorito et al., 2017). The primary vector of *B. miyamotoi* to humans in the eastern part of the United States is the blacklegged tick, *Ixodes scapularis*, which also transmits the Lyme disease spirochetes, *Borrelia burgdorferi* sensu stricto (s.s.) and *Borrelia mayonii* (Krause et al., 2015; Breuner et al., 2017; Eisen, 2018). For nymphal and adult *I. scapularis* ticks, local infection prevalence with the relapsing fever spirochete, *B. miyamotoi*, is commonly 10-fold lower than for the Lyme disease spirochete, *B. burgdorferi* s.s. (Tsao et al., 2004; Krause et al., 2015; Nelder et al., 2016). However, in contrast to *B. burgdorferi* s.s., female ticks infected with *B. miyamotoi* can pass spirochetes transovarially to their offspring, resulting in host-seeking infected larval ticks (Scoles et al., 2001; Rollend et al., 2013; Breuner et al., 2017).

Two recent epidemiological studies from the Northeastern United States indicate that human cases of hard tick-borne relapsing fever peak during late summer (Molloy et al., 2015; Fiorito et al., 2017). This peak in cases coincides with the peak seasonal activity period of larval ticks in the Northeast, but occurs later in the year than the peak for nymphal tick activity and earlier than the fall peak activity period for adult ticks (Sonenshine, 1993; Stafford, 2007). Support for involvement of larval *I. scapularis* ticks in the natural transmission of *B. miyamotoi* in the Northeast was provided by an earlier study showing rising infection prevalence of white-footed mice (*Peromyscus leucopus*) with this spirochete during the summer period of peak larval activity (Barbour et al., 2009)

One previous study from Europe demonstrated experimentally that transovarially-infected larval *Ixodes ricinus* ticks are capable of transmitting *B. miyamotoi* (van Duijvendijk et al., 2016). However, all

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instances where transmission to an individual experimental host was demonstrated resulted from mass feeding by mixed infected and non-infected larvae. This scenario most likely better represents natural enzootic transmission than transmission to a human host bitten by a single or a few larvae. We previously demonstrated that single infected nymphal *I. scapularis* ticks effectively transmit *B. miyamotoi* to experimental hosts (Breuner et al., 2017). The objective of this study was to similarly demonstrate that single transovarially-infected larval *I. scapularis* ticks can transmit *B. miyamotoi* to experimental hosts.

2. Materials and methods

2.1. Source of B. miyamotoi-infected larval I. scapularis ticks

The larval *I. scapularis* ticks used in this transmission experiment were first generation transovarially *B. miyamotoi*-infected larvae originating from two female ticks (MN17-30 and MN17-52) collected by drag sampling at the Camp Ripley Training Center, Morrison County, Minnesota, in May 2017. The field-collected females were fed on a New Zealand white rabbit (Charles River Laboratories, Wilmington, MA, USA) in the laboratory and allowed to lay eggs as described previously (Piesman et al., 1991). Infection of the spent females and their larval offspring with *B. miyamotoi* was confirmed by TaqMan polymerase chain reaction (PCR) as described in Section 2.3. The two *B. miyamotoi*-infected females (MN17-30 and MN17-52) produced larval batches with high prevalence of infection with *B. miyamotoi*: 91% (29/32) of tested resulting larvae were infected for female MN17-52 and 62% (15/24) for female MN17-30. Additional larvae originating from these two females were used in the transmission experiment described in Section 2.2. Larvae originating from a third infected female (MN17-43) were not used due to their low infection prevalence (12%; 3/26).

2.2. Challenge of experimental hosts with transovarially B. miyamotoi-infected I. scapularis larvae and assessment of hosts for spirochete infection and exposure

Following our previous study (Breuner et al., 2017), 1–3 mo old female CD-1 outbred *Mus musculus* mice (Charles River Laboratories) were used as the experimental hosts to confirm transmission of *B. miyamotoi* by feeding ticks. Depending on the likelihood of a larval tick being infected (see Section 2.1), mice were exposed to either a single larva (from the MN17-52 female) or two larvae (from the MN17-30 female). Larval feeding was accomplished within capsules attached to the shaved backs of mice and sealed at the top with mesh to prevent the ticks from escaping (Mbow et al., 1994; Soares et al., 2006; Breuner et al., 2017).

A first set of 20 mice were each challenged with a single larva from female tick MN17-52. These larvae fed poorly: only 8 attached and took some blood, and none ingested a complete blood meal. A second set of 29 mice were each challenged with 2 larvae from female tick MN17-30. At least one fed larva was recovered from 26 of these mice, and most larvae had fed to completion. All larvae recovered from the mice were scored for feeding status (no visible blood ingested; minimal blood meal; more than minimal but still only partial blood meal; or complete blood meal) using a stereo microscope. Ticks that ingested at least a minimal amount of blood were tested for presence of *B. miyamotoi* DNA by TaqMan PCR as described in Section 2.3. Of the 34 mice from which larval ticks took at least some blood, 5 mice were found to have been exposed only to non-infected larvae. Those exposed only to non-infected larvae included one mouse with a partially fed tick, one mouse with a fully fed tick, and one mouse with two fully fed ticks.

Baseline blood samples were collected from all 49 experimental mice prior to tick challenge. For the 34 mice with recovered ticks that took at least a minimal amount of blood, this was followed by (i) a second blood sample 10 d after larvae attached (to test for the presence of *B. miyamotoi* DNA in the mouse blood, as described in Section 2.3) and (ii) a third blood sample at 8 wk post-tick infestation (to test for mouse seroreactivity to *B. miyamotoi*; as described in Section 2.4). This timeline for taking mouse blood samples to document spirochete infection and exposure followed Breuner et al. (2017). For selected mice, an attempt was made to culture *B. miyamotoi* from blood taken 10 d after larvae attached, as described in Section 2.5.

2.3. Detection of B. miyamotoi DNA from ticks and mouse blood

Nucleic acids were isolated from ticks as follows. Fed larval ticks were homogenized in 350 µl of lysis buffer using a Mini-Beadbeater-96 (BioSpec Products, Inc., Bartlesville, OK, USA) as previously described (Graham et al., 2016, 2018). Spent female ticks were homogenized by hand in 500 µl of lysis buffer using a 1.5 ml microcentrifuge tube and a pestle. Thereafter, the tick lysates (300 µl for fed larvae and 30 µl for spent females) were processed using the KingFisher DNA extraction system (Thermo Fisher Scientific, Waltham, MA, USA) and the MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific).

Nucleic acids were isolated from mouse blood as follows. First, 200 µl of blood was mixed with 450 µl of lysis buffer (426 µl ATL; 20 µl proteinase K; 2 µl Reagent DX; and 2 µl Carrier RNA, 1 µg/µl) (Qiagen, Valencia, CA, USA). Two aliquots of 300 µl from each blood lysate was then processed using the KingFisher DNA extraction system and the MagMAX™ Pathogen RNA/DNA Kit. Each blood lysis sample was eluted in 90 µl. Finally, DNA elutes from the same original blood sample were combined after processing to generate one elute from each mouse blood sample.

The subsequent real-time TaqMan PCR reactions included primers and probes for the following DNA targets: a pan *Borrelia* 16S rDNA target (Kingry et al., 2018) and the adenylosuccinate lyase (*purB*) target for *B. miyamotoi* (Graham et al., 2016). PCR and DNA purification controls included the *I. scapularis* actin target (Hojgaard et al., 2014) for tick samples and the rodent GAPDH target (Applied Biosystems® TaqMan® Rodent GAPDH ControlReagents kit; ThermoFisher) for mouse blood samples.

PCR reactions were performed in 10 µl solutions with 5 µl iQ Multiplex Powermix (Bio-Rad, Hercules, CA, USA) and 4.8 µl DNA extract, with forward and reverse primers (0.2 µl) at a final concentration of 300 nM each, and probes at a final concentration of 200 nM each. The real-time TaqMan PCR cycling conditions consisted of: denature DNA 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). We analyzed samples using CFX Manager 3.1 software (Bio-Rad) with the quantitation cycle (Cq) determination mode set to regression. Based on Graham et al. (2018), only Cq values < 40 were considered indicative of the *B. miyamotoi purB* target being present in the tested sample.

2.4. Serological reactivity of mouse blood to B. miyamotoi

Antibody development to *B. miyamotoi* was assessed in mouse sera collected 8 wk after tick exposure for the 29 mice exposed to at least one infected larval tick, regardless of whether or not the blood of a given mouse tested positive for *B. miyamotoi* DNA 10 d after larvae attached. An additional 5 mice from which only non-infected fed ticks were recovered were included as negative controls. Serologic testing was as follows: whole cells from North American *B. miyamotoi* (strain CT13-2396; National Center for Biotechnology Information accession number: PRJNA310783) were cultivated in modified Barbour-Stoenner-Kelly (BSK) medium (in-house BSK-R medium) and harvested by centrifuging at 10,000 g for 10 min at 4 °C. The resulting cell pellet was frozen, thawed, and re-suspended in TE buffer, then sonicated, and finally diluted in TE buffer to a final protein concentration of 2.0 mg/ml. The sonicate was mixed with an equal volume of 2X Laemmli sample buffer with DTT (Bio-Rad), placed in a 95 °C heat block for 10 min, and run with 246 µl sonicate and sample buffer mixture on in-house made