

# Transmission of the Lyme Disease Spirochete *Borrelia mayonii* in Relation to Duration of Attachment by Nymphal *Ixodes scapularis* (Acari: Ixodidae)

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## Abstract

The recently recognized Lyme disease spirochete, *Borrelia mayonii*, has been detected in host-seeking *Ixodes scapularis* Say ticks and is associated with human disease in the Upper Midwest. Although experimentally shown to be vector competent, studies have been lacking to determine the duration of time from attachment of a single *B. mayonii*-infected *I. scapularis* nymph to transmission of spirochetes to a host. If *B. mayonii* spirochetes were found to be transmitted within the first 24 h after tick attachment, in contrast to *Borrelia burgdorferi* spirochetes (>24 h), then current recommendations for tick checks and prompt tick removal as a way to prevent transmission of Lyme disease spirochetes would need to be amended. We therefore conducted a study to determine the probability of transmission of *B. mayonii* spirochetes from single infected nymphal *I. scapularis* ticks to susceptible experimental mouse hosts at three time points postattachment (24, 48, and 72 h) and for a complete feed (>72–96 h). No evidence of infection with or exposure to *B. mayonii* occurred in mice that were fed upon by a single infected nymph for 24 or 48 h. The probability of transmission by a single infected nymphal tick was 31% after 72 h of attachment and 57% for a complete feed. In addition, due to unintended simultaneous feeding upon some mice by two *B. mayonii*-infected nymphs, we recorded a single occasion in which feeding for 48 h by two infected nymphs resulted in transmission and viable infection in the mouse. We conclude that the duration of attachment of a single infected nymphal *I. scapularis* tick required for transmission of *B. mayonii* appears to be similar to that for *B. burgdorferi*: transmission is minimal for the first 24 h of attachment, rare up to 48 h, but then increases distinctly by 72 h postattachment.

**Key words:** *Borrelia mayonii*, *Ixodes scapularis*, Lyme disease, time to transmission

The recently described Lyme disease spirochete, *Borrelia mayonii*, has been detected in naturally infected blacklegged ticks, *Ixodes scapularis* Say, collected in Wisconsin (Pritt et al. 2016a, b). Subsequent experimental studies confirm that *I. scapularis* originating from the Upper Midwest and Northeast are vector competent for *B. mayonii* (Dolan et al. 2016, Eisen et al. 2017). Dolan et al. (2016) also included preliminary data on the duration of attachment required for *I. scapularis* nymphs to transmit *B. mayonii*; however, this was based on exposure of white mouse hosts to simultaneous feeding by multiple infected nymphs. Within the first 24–48 h of nymphal attachment, *B. mayonii* transmission was recorded for one of the six outbred mice exposed to two to nine infected nymphs each, with the single transmission event occurring for a mouse exposed to the simultaneous feeding by six infected nymphal ticks. At  $\geq 72$  h postattachment, transmission of *B. mayonii* occurred in

9 of the 11 mice exposed to simultaneous feeding by multiple (range of 2–6) infected nymphs.

Humans are less likely than small mammal reservoir hosts to be simultaneously exposed to bites by more than a single *B. mayonii*-infected nymphal tick; therefore, these data may have inflated the probability of transmission at earlier time points ( $\leq 48$  h after attachment) when considering human bites by single infected ticks. Previous studies on the closely related Lyme disease spirochete, *Borrelia burgdorferi*, indicates that transmission to experimental hosts within the first 48 h of attachment by *I. scapularis* nymphs is more likely to occur during simultaneous feeding by multiple infected nymphs when compared with feeding by a single infected nymph (Piesman et al. 1987, Piesman 1993, Shih and Spielman 1993, des Vignes et al. 2001, Piesman and Dolan 2002, Hojgaard et al. 2008). Transmission by a single *B. burgdorferi*-infected

*I. scapularis* nymph has not been reported within the first 24 h of attachment and only occasionally by 48 h (Piesman et al. 1987, des Vignes et al. 2001, Piesman and Dolan 2002, Hojgaard et al. 2008). These studies report that transmission of *B. burgdorferi* occurs most frequently after  $\geq 60$  h of nymphal attachment. The aim of this study was to assess the probability of transmission of *B. mayonii* by single infected nymphal ticks to outbred mice at three time points after attachment and for a complete feed. To facilitate messaging to the public regarding the importance of prompt removal of attached ticks, we chose time points (24, 48, and 72 h) that are easy to understand in terms of checking for and removing attached nymphs daily or every 2 or 3 d.

## Materials and Methods

### *Borrelia mayonii* Source, *I. scapularis* Ticks, and Experimental Mouse Hosts

The original source of spirochete infection to initiate the mouse–tick transmission chain maintained in our laboratory tick colony was *B. mayonii* strain MN14-1420, originally isolated from human blood (Pritt et al. 2016a, b). Nymphal ticks used in the study were of the first or second generation from adults collected in Fairfield County, CT, or in Anoka or Washington County, MN. Females producing egg batches were confirmed to be free of *Borrelia* infection as described previously (Dolan et al. 2016, 2017; Eisen et al. 2017). The infected nymphs used for the time point transmission experiment described here originated from previously noninfected larvae that were allowed to feed on three different outbred mice infected with *B. mayonii* via tick-bite. Two of these mice (2084 and 2114) were described in a previous publication and the third mouse (A61) was infected via nymphs fed as larvae on another previously described *B. mayonii*-infected mouse, 2063 (Dolan et al. 2017). Mice used as experimental hosts to assess transmission by *B. mayonii*-infected nymphs were 1–3-mo-old female CD-1 *Mus musculus* outbred mice (Charles River Laboratories, Wilmington, MA).

### Feeding of *B. mayonii*-Infected Nymphs on Experimental Mouse Hosts

To determine the probability of transmission by a single *B. mayonii*-infected nymph, and based on expected nymphal tick infection rates of 25–50% (Dolan et al. 2016, 2017), each mouse was exposed to a total of two nymphs. To facilitate removal or recovery of partially or fully fed nymphs at predetermined time points following

attachment—24, 48, and 72 h after nymphs were introduced onto the mice or a complete feed—nymphs were confined within tick-feeding capsules attached to the shaved back of individual mice as described previously (Mbow et al. 1994, Soares et al. 2006).

All partially or fully fed nymphs recovered from mice were examined by polymerase chain reaction (PCR) for presence of *B. mayonii*. Nucleic acid was isolated from ticks using a Mini-Beadbeater-96 (BioSpec Products, Inc., Bartlesville, OK) and a QIAcube HT robot (Qiagen, Valencia, CA) as described previously (Dolan et al. 2016). The in-house multiplex PCR master mix (designated M59; see Table 1 for primer and probe sequences) included primers and probes for the following targets: an in-house 23S *rDNA* pan-*Borrelia* target (Johnson et al. 2017); the flagellar filament cap (*fliD*) of *B. burgdorferi* which is present also in *B. mayonii* (Hojgaard et al. 2014, Dolan et al. 2016); the intergenic spacer (*IGS*) target of *B. mayonii* (Johnson et al. 2017); and the *I. scapularis* actin target (Hojgaard et al. 2014) which serves as a control for both the DNA purification and the PCR. The multiplex PCR reactions were performed in 10  $\mu$ l solutions with 5  $\mu$ l iQ Multiplex Powermix (BioRad, Hercules, CA), 4.8  $\mu$ l DNA extract with forward and reverse primers (0.2  $\mu$ l) in a final concentration of 300 nM each, and probes in a final concentration of 200 nM each. The PCR cycling conditions were as follows: 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 (BioRad). Based on the PCR results, each mouse was then classified as having been fed upon by 0, 1, or 2 *B. mayonii*-infected nymphs.

Salivary glands were dissected from a total of 40 unfed and subsets of partially fed nymphal ticks removed from mouse hosts at 24, 48, and 72 h after attachment. Salivary glands were not dissected from fully fed ticks due to difficulty in excising them intact as they become increasingly distended and fragile during the course of feeding. Salivary glands were double-washed in sterile phosphate-buffered saline to minimize the risk of spirochetes remaining on their surface. Salivary glands and paired tick bodies were then examined separately for presence of tick actin and spirochete *fliD* targets by PCR as described above.

### Confirmation of Transmission of *B. mayonii* From Infected Nymphs to Experimental Mouse Hosts

Mice that were confirmed to have been exposed to at least one infected nymph were ear-biopsied at 3 to 4 wk after the tick feed

**Table 1.** Primers and probes used for PCR targets in the in-house M59 multiplex master mix

Primers and probes	Sequence (5'–3') <sup>a</sup>	Reference
<i>fliD</i> -F	TGGTGACAGAGTGATGATAATGGAA	Hojgaard et al. 2014
<i>fliD</i> -R	ACTCCTCCGGAAGCCACAA	Hojgaard et al. 2014
<i>fliD</i> -probe	FAM-TGCTAAAATGCTAGGAGATTGTCTGTCGCC-BHQ1	Hojgaard et al. 2014
23S-F	TCGGTGAAATGAAAGTATC	Johnson et al. 2017
23S-R	CARGCTATAGTAAAGTTCA	Johnson et al. 2017
23S-probe	HEX-CGTCTAACCAAGTAATCGGCATC-BHQ1	Johnson et al. 2017
<i>mIGS</i> -F	TGTCGTTATCGGTATGTG	Johnson et al. 2017
<i>mIGS</i> -R	AAGGGCCATGATGATTTG	Johnson et al. 2017
<i>mIGS</i> -probe	Quas705-CCTTCCTACGACTTATCACCGACAG-BHQ3	Johnson et al. 2017
<i>actin</i> -F	GCCCTGGACTCCGAGCAG	Hojgaard et al. 2014
<i>actin</i> -R	CCGTCGGGAAGCTCGTAGG	Hojgaard et al. 2014
<i>actin</i> -probe	Q670-CCACCGCCGCTCCTCTTCTCC -BHQ3	Hojgaard et al. 2014

<sup>a</sup> BHQ1, BHQ3: Black Hole Quencher 1 and 3, respectively; FAM: 6-carboxyfluorescein; HEX: hexachlorofluorescein phosphamidite; Q670: Quasar 670; Q705: Quasar 705.

(Sinsky and Piesman 1989). Ear biopsies were cultured in modified Barbour-Stoenner-Kelly (BSK) medium (in-house BSK-R medium) with antibiotics to detect live spirochetes as described previously (Dolan et al. 2016). Cultures were examined by dark-field microscopy (400× magnification) at 10 and 21 d. Serum samples were collected from mice that were determined to have been exposed to at least one infected nymph but produced spirochete-negative ear biopsy cultures. Serum was collected from these mice at 8–10 wk after the nymphal tick feed and examined for serological reactivity to *B. mayonii* using the MarDx *B. burgdorferi* (IgG) Marblot Strip Test System (MarDX Diagnostic Inc., Carlsbad, CA) with previously described modifications for testing of mouse serum (Eisen et al. 2017). Marblot strip banding patterns were analyzed and scored as positive, according to the manufacturer's recommendations, when ≥5 distinct bands were evident.

### Regulatory Compliance

Animal use and experimental procedures were in accordance with an approved protocol on file with the Centers for Disease Control and Prevention Division of Vector-Borne Diseases Animal Care and Use Committee.

## Results

### Transmission of *B. mayonii* by Infected Nymphal *I. scapularis* Ticks to Experimental Mouse Hosts at Different Time Points After Attachment

A total of 160 mice were challenged with potentially *B. mayonii*-infected nymphs during these time-to-transmission trials. It was determined that 69 (43%) mice were fed upon only by noninfected nymphs, 71 mice by a single infected nymph, and 20 mice by two infected nymphs (Table 2).

We found no evidence of infection with or exposure to *B. mayonii*, as determined by culture and serology, in mice that were fed

upon by a single infected nymph for 24 h (24 mice total) or 48 h (17 mice total; Table 2). The probability of transmission occurring during feeding by a single infected nymph was 31% following 72 h of attachment (evidence of transmission in 5/16 mice) and 57% for a complete feed (evidence of transmission in 8/14 mice; Table 2). Based on the expectation that the probability of transmission increases with duration of nymphal attachment, the observed increase in probability of transmission occurring from 48 to 72 h was statistically significant (Fisher's exact one-tailed test,  $P=0.018$ ). The observed 26% increase in probability of transmission that occurred between 72 h (31%) and a complete feed (57%) was not statistically significant ( $P=0.14$ ). None of the mice fed upon by a single infected nymph and failing to produce a spirochete-positive ear biopsy culture were serologically reactive to *B. mayonii*.

Data across time-points are based on very small sample sizes for the 20 mice unintentionally exposed to simultaneous feeding by two *B. mayonii*-infected nymphs (Table 2). Nevertheless, it is notable that we recorded a single instance of transmission in which simultaneous feeding by two *B. mayonii*-infected nymphs for 48 h resulted in transmission and viable infection for an individual mouse. As expected from the data reported here for feeding by single infected nymphs, transmission during feeding by two infected nymphs tended to increase from 24 h (0%) to 48 h (25%), and to 72 h or a complete feed (>70%; Table 2). None of the mice fed upon by two infected nymphs and failing to yield a spirochete-positive ear biopsy culture were serologically reactive to *B. mayonii*.

### Transmission Success in Relation to Spirochete Dissemination Within Infected Nymphal *I. scapularis* Ticks and Duration of Attachment

*Borrelia mayonii* infection in the salivary glands of nymphal ticks for which spirochetes were detected in the remainder of the tick exoskeleton or body increased from 13% prior to tick feeding to 30–40% for time-points of attachment from 24–72 h (Table 3). No similar data were generated for nymphs taking a complete

**Table 2.** Transmission outcomes for outbred mice fed upon for different durations of time by one or two *B. mayonii*-infected *I. scapularis* nymphs

Duration of nymphal feeding	Feeding by one <i>B. mayonii</i> -infected nymph			Feeding by two <i>B. mayonii</i> -infected nymphs		
	No. mice examined	No. mice with evidence of infection <sup>a</sup>	Percent of mice with evidence of infection	No. mice examined	No. mice with evidence of infection <sup>a</sup>	Percent of mice with evidence of infection
24 h	24	0	0	3	0	0
48 h	17	0	0	4	1	25.0
72 h	16	5	31.2	7	5	71.4
Complete feed	14	8	57.1	6	5	83.3

<sup>a</sup> As determined by culture of ear biopsies to detect live spirochetes and serological reactivity to *B. mayonii*.

**Table 3.** PCR-based evidence of *B. mayonii* infection in salivary glands of unfed versus partially fed *I. scapularis* nymphs with evidence of infection in the remaining tick body

Nymphal feeding status	No. <i>B. mayonii</i> -infected nymphs examined <sup>a</sup>	No. infected nymphs with <i>B. mayonii</i> DNA detected also in the salivary glands	Percent infected nymphs with <i>B. mayonii</i> DNA detected also in the salivary glands
Unfed	15	2	13.3
Fed—24 h	12	4	33.3
Fed—48 h	15	6	40.0
Fed—72 h	13	4	30.8

<sup>a</sup> Salivary glands were removed prior to testing of the remainder of the bodies.

**Table 4.** Transmission outcomes for mice fed upon for different durations of time by *I. scapularis* nymphs with PCR-based evidence of infection with *B. mayonii* in their bodies but not in their salivary glands versus mice fed upon by salivary-gland infected nymphs

Nymphal feeding status	Mice fed upon only by nymphs with <i>B. mayonii</i> -infection detected in their bodies but not in the salivary glands			Mice fed upon by nymphs with <i>B. mayonii</i> -infection detected in the salivary glands		
	No. mice examined	No. mice with evidence of infection <sup>a</sup>	Percent mice with evidence of infection <sup>a</sup>	No. mice examined	No. mice with evidence of infection <sup>a</sup>	Percent mice with evidence of infection <sup>a</sup>
Fed—24 h	6	0	0	4 <sup>b</sup>	0	0
Fed—48 h	8	0	0	5 <sup>c</sup>	1 <sup>d</sup>	20.0
Fed—72 h	7	0	0	4 <sup>b</sup>	3	75.0

Evidence of transmission to mice was determined by culture of live spirochetes from ear biopsies and serology.

<sup>a</sup> As determined by culture of ear biopsies to detect live spirochetes and testing of serum for serological reactivity to *B. mayonii*.

<sup>b</sup> Mice fed upon by a single nymph with infected salivary glands.

<sup>c</sup> Four mice fed upon by a single nymph with infected salivary glands and one mouse fed upon simultaneously by two nymphs with infected salivary glands.

<sup>d</sup> Mouse fed upon simultaneously by two nymphs with infected salivary glands.

bloodmeal. The probability of transmission of *B. mayonii* from salivary gland-infected nymphs to experimental mouse hosts increased distinctly over the course of nymphal attachment (Table 4). Following 24 h of nymphal attachment, none of the four mice fed upon by a single salivary gland-infected tick demonstrated evidence of spirochete exposure. After 48 h, none of the four mice fed upon by a single salivary gland-infected tick showed evidence of spirochete exposure, as compared with a single mouse fed upon simultaneously by two salivary gland-infected nymphs which developed a viable infection as determined by culture of spirochetes from ear tissue. In contrast, after 72 h of nymphal attachment, three of the four mice fed upon by a single salivary gland-infected tick developed viable infections with *B. mayonii*. As shown in Table 4, none of the mice that were fed upon only by nymphs with evidence of infection with *B. mayonii* in their bodies but not in their salivary glands showed evidence of spirochete exposure, regardless of whether the nymphs were attached for 24, 48, or 72 h.

## Discussion

Our data indicate that the duration of attachment of single infected nymphal *I. scapularis* ticks required for transmission of *B. mayonii* is similar to that for *B. burgdorferi*: transmission is minimal for the first 24 h of attachment, rare up to 48 h, but then increases distinctly by 72 h of attachment. Recommendations for regular tick checks and prompt tick removal as a way to prevent transmission of Lyme disease spirochetes to humans via the bites of infected ticks (CDC 2016a, b) therefore applies to the newly recognized *B. mayonii* as well as *B. burgdorferi*, for which these recommendations originally were developed. In contrast to feeding by single infected nymphs, *B. mayonii* transmission was observed already by 48 h after attachment in one instance where two infected *B. mayonii* nymphs fed simultaneously on a mouse (Table 2). This observation agrees with data from previous studies on *B. burgdorferi* showing transmission to occur more frequently within the first 48 h after attachment during feeding by multiple *B. burgdorferi*-infected *I. scapularis* nymphs (Piesman et al. 1987, Piesman 1993, Shih and Spielman 1993) compared to single infected nymphs (Piesman et al. 1987, des Vignes et al. 2001, Piesman and Dolan 2002, Hojgaard et al. 2008).

Piesman et al. (2001) used quantitative PCR to assess the number of *B. burgdorferi* spirochetes present in the salivary glands of unfed versus partially or fully fed *I. scapularis* nymphal ticks. They report a >17-fold increase in spirochetes in salivary glands from before feeding starts to 72 h postattachment, with the period of most rapid increase in number of spirochetes occurring from 48 to 60 h postattachment. Similarly, we report a 2–3-fold increase in the percent of nymphs found to contain *B.*

*mayonii* DNA in their salivary glands after they start to feed (Table 3) and, albeit based on a very small sample sizes precluding statistical analysis, an increase in transmission to mice from 48 to 72 h after attachment for salivary gland-infected nymphs (Table 4).

Data are accumulating to suggest that Lyme disease spirochetes are transmitted more effectively to a host when multiple infected nymphal ticks feed together (see Table 2 for *B. mayonii* and references provided above for *B. burgdorferi*). One caveat to the results presented here is that we cannot rule out the possibility that, in some cases, one of the two infected nymphs fed on a mouse was infected via cofeeding transmission rather than being infected prior to the start of the feed. A previous study on *I. scapularis* ticks infected with *B. burgdorferi* demonstrated cofeeding to occur, but only rarely (Piesman and Happ 2001). Although the underlying mechanism(s) for more effective transmission to a susceptible host when multiple infected nymphal ticks feed together remain to be revealed, *I. scapularis* saliva is known to facilitate the establishment of *B. burgdorferi* infection in mice (Zeidner et al. 2002) and feeding in proximity by multiple ticks should reasonably result in the passage of larger volumes of tick saliva to localized host tissues. We speculate that multiple infected nymphal ticks feeding in proximity may result not only in passage of higher numbers of spirochetes, as compared with a single infected nymph, but also in more effective suppression of the host immune response facilitating spirochete establishment. If the amount of injected tick saliva is a major factor facilitating establishment of viable spirochetal infection in the host, then transmission by a single infected nymph could, in the context of enzootic spirochete transmission, be facilitated by simultaneous cofeeding of non-infected ticks. This would seem a fruitful line of follow-up research.

We report here that transmission of *B. mayonii* appears to be associated with presence of spirochetes in the saliva of the feeding tick (Table 4). Although this finding is not surprising, it provides evidence to support the salivary route of transmission for this Lyme disease spirochete, similar to transmission of *B. burgdorferi* (Ribeiro et al. 1987, Ewing et al. 1994). Additional studies are needed to explore the dynamics of how *B. mayonii* spirochetes, as well as *B. burgdorferi* and the relapsing fever group spirochete *Borrelia miyamotoi*, disseminate within *I. scapularis* ticks and multiply within the salivary glands during attachment, resulting in subsequent passage to mammalian hosts via the saliva of a feeding tick.

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