Tick-host-Borrelia interaction

Implications for host immunity and vaccination strategies

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*Borrelia miyamotoi* in ticks, reservoir hosts and patients referred to a tertiary multidisciplinary Lyme center in The Netherlands.

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Submitted

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ABSTRACT

Background
Ixodes ticks transmit Borrelia burgdorferi sensu lato (s.l.), the causative agent of Lyme borreliosis (LB). These tick species also transmit Borrelia miyamotoi, which was recently found to cause hard tick-borne relapsing fever (HTBRF) in humans. We were interested in the prevalence of B. miyamotoi infection in ticks and natural hosts in the Netherlands, and to what extent ticks are co-infected with B. burgdorferi. In addition, erythema migrans has been sporadically described in HTBRF patients, but these skin lesions might as well represent co-infections with B. burgdorferi s.l. We therefore investigated whether B. miyamotoi was present in LB-suspected skin lesions of patients referred to our tertiary Lyme disease clinic.

Methods
3360 questing Ixodes ricinus nymphs as well as spleen tissue of 74 rodents, 26 birds and 10 deer were tested by PCR for the presence of B. miyamotoi. Tick lysates were also tested for the presence of B. burgdorferi s.l.. Next, we performed a PCR for B. miyamotoi in 31 biopsies from LB-suspected skin lesions in patients visiting our tertiary Lyme center. These biopsies had been initially tested for B. burgdorferi sensu lato by PCR, and the skin lesions had been investigated by specialized dermatologists.

Results
Out of 3360 nymphs, 313 (9.3%) were infected with B. burgdorferi s.l., 70 (2.1%) were infected with B. miyamotoi, and 14 (0.4%) were co-infected with B. burgdorferi s.l. and B. miyamotoi. Co-infection of B. burgdorferi s.l. with B. miyamotoi occurred more often than expected from single infection prevalences (p=0.03). Both rodents (9%) and birds (8%) were found positive for B. miyamotoi by PCR, whereas the roe deer samples were negative. Out of 31 LB-suspected skin biopsies, 10 (32%) were positive for B. burgdorferi s.l. while none were positive for B. miyamotoi.

Conclusion
The significant association of B. burgdorferi with B. miyamotoi in nymphs implies the existence of mutual reservoir hosts. Indeed, the presence of B. miyamotoi DNA indicates systemic infections in birds as well as rodents. However, their relative contributions to the enzootic cycle of B. miyamotoi requires further investigation. We could not retrospectively diagnose HTBRF using biopsies of LB-suspected skin lesions, supporting the hypothesis that B. miyamotoi is not associated with LB-associated skin manifestations. However, this warrants further studies in larger sets
of skin biopsies. A prospective study focused on acute febrile illness after a tick bite could provide insight into the incidence and clinical manifestations of HTBRF in the Netherlands.
Borrelia miyamotoi is a tick-borne relapsing fever (TBRF) spirochete in Ixodes ticks, the same vector that transmits Borrelia burgdorferi s.l. and other tick-borne pathogens. The prevalence of B. miyamotoi in reservoir hosts, as well as the reservoir host range in nature, is yet scarcely described, but points towards small rodents and certain bird species being infected (88-93). Interestingly, B. miyamotoi can cause short-term systemic infection in rodents, and it is experimentally shown that ticks can acquire this pathogen from rodents (Burri et al., 2014). While B. burgdorferi s.l. causes Lyme borreliosis, B. miyamotoi was recently found to induce hard tick-borne relapsing fever (HTBRF) in humans (21). The incidence of HTBRF in humans is yet unknown. Two studies have described the prevalence of HTBRF in febrile patients suspected of a tick-borne infection, which found 97/11515 (0.84%) of patients in north-eastern United States and 51/302 (16.9%) of hospital-admitted Russian patients to be PCR positive on blood (21, 94). HTBRF presents around two weeks after a tick bite with a high fever and viral-like symptoms such as headache, myalgia, arthralgia and malaise (21, 94, 95). In two patients with severe immunodeficiency, including one case in our tertiary Lyme clinic, B. miyamotoi infection was found to cause a chronic meningoencephalitis (7, 96). Several studies have described HTBRF patients presenting with erythema migrans (EM), a classical symptom of early Lyme borreliosis; 9% of Russian HTBRF patients presented with EM, and it was hypothesized that EM was a sign of co-infection with B. burgdorferi s.l. rather than a true manifestation of HTBRF (21). A study in North America revealed several HTBRF patients to be co-infected with B. burgdorferi s.l., one of which presenting with an EM (94). Furthermore, a study in Japan described two febrile HTBRF patients that presented with EM and were shown to have antibodies against B. burgdorferi s.l. antigens, suggesting co-infections with both B. burgdorferi s.l. and B. miyamotoi (97).

In the Netherlands, exposure levels to B. miyamotoi as measured by serology showed that risk groups such as forestry workers (10%) had higher seroprevalence levels than the control group. In the same study, B. miyamotoi antibodies were also found in patients suspected of human granulocytic anaplasmosis (HGA) (14.6%), suggesting that B. miyamotoi infections occur in Dutch high-risk populations and that they could be misdiagnosed (98). However, thus far only one PCR-confirmed patient has been described in the Netherlands, suggesting under-diagnosis due to a lack of awareness, lack of severe symptoms, lack of widely available diagnostic tools and/or misdiagnosis. A Russian study recently revealed a relatively high transmission efficiency of B. miyamotoi by adult Ixodes persulcatus ticks to humans (~8%), and because B. miyamotoi was recently demonstrated to be transmitted by Ixodes ricinus larvae patentis with HTBRF might not present with a history of known tick bites due
to the small size of larvae (27, 99). Indeed, a Russian study described *B. miyamotoi* to be under-diagnosed, leading to relapses in the absence of adequate antibiotic treatment (100). Because no routine diagnostics are currently performed for *B. miyamotoi* in our (tertiary) multidisciplinary Lyme disease center, we were interested whether we had missed (co-) infections with *B. miyamotoi* over the past years. Thus, in the current study, we performed *B. miyamotoi*-specific real-time PCR on ticks and potential reservoir hosts in nature and in LB-suspected human skin biopsies, which were previously tested for *B. burgdorferi* by PCR.

**METHODS**

**Collection of ticks and vertebrate hosts**

Questing ticks were collected by blanket dragging at 11 different forested areas throughout the Netherlands in 2014-2015. Spleen tissues were collected from different species of rodents, roe deer and birds (tables 1 and 2) at several sites in the Netherlands. The obtained spleen samples of roe deer, birds as well as the capturing of wild rodents have been described elsewhere (101, 102). Spleen samples were kept frozen (-80 °C) until testing. DNA from tissue samples was extracted using the Qiagen DNeasy Blood & Tissue Kit according to manufacturer’s protocol. Based on morphological criteria, tick species and stages were identified, and DNA from *I. ricinus* nymphs was extracted by alkaline lysis (103).

**Polymerase chain reactions and sequencing of ticks and vertebrate hosts**

All samples were screened for the presence of *B. miyamotoi* DNA with a real-time polymerase chain reaction (qPCR) targeting portion of the flagellin gene. The primers used were 200 nM forward (5’-AGA AGG TGC TCA AGC AG-3’) and reverse (5’-TCG ATC TTT GAA AGT GAC ATA T-3’) primers each, 200 nM probe (5’-Atto647N-AGC ACA ACA GGA GGG AGT TCA AGC-BHQ2-3’), and 3 to 8 µl of template DNA (Hovius et al., 2014). qPCR-positive samples were analysed further with primers targeting a fragment of a 700bp fragment of the glycerophosphodiester phosphodiesterase (*glpQ*) gene. The PCR was performed with the HotStarTaq master mix (Qiagen, Venlo, the Netherlands) using forward 5’-ATG GGT TCA AAC AAA AAG TCA CC-3’ and reverse primers 5’-CCA GGG TCC AAT TCC ATC AGA ATA TTG TGC AAC-3’ under the following conditions: 15 min 94°C, then 40 cycles of 30 sec 94°C, 30 sec 53°C, 90 sec 72°C and finishing with 10 minutes at 72°C. The sequences were stored and analysed in Bionumerics (Version 7.1, Applied Math, Belgium), after subtraction of the primer sequences.
Lyme borreliosis-suspected skin lesions

In our tertiary Lyme center, we offer more extensive diagnostic service than primary care centers will perform based on guidelines. Occasionally, biopsies of skin lesions are taken to confirm or rule out LB, which enabled us to retrospectively investigate the presence of *B. miyamotoi* in these lesions. Our dermatology department gathered 4 mm skin biopsies under local lidocaine anesthesia from 34 patients between 2009 and 2013, which were taken from the edge of suspected (atypical) EM lesions or central in suspected acrodermatitis chronica atrophicans (ACA) lesions. Biopsies were used for PCR and sent for pathologic examination when clinically indicated according to the treating dermatologist. A *B. burgdorferi*-specific PCR was initially performed, and 31/34 extracted DNA samples were included, while three DNA samples and corresponding patient cases were excluded from analysis due to qPCR inhibition. Patient records and PCR results were retrospectively reviewed to obtain a final diagnosis and to categorize the skin lesions into: 1. EM; 2. Multiple EM; 3. ACA; 4. LB-suspected skin lesion after previous treatment for LB, but active LB excluded 5. LB-suspected skin lesion without previous treatment for LB, but active LB excluded. As part of the current study a qPCR - see above - was performed to detect *B. miyamotoi*. C6 EIAs and qPCRs for *B. burgdorferi* s.l. and *B. miyamotoi* were performed as previously described (7, 104).

Statistical analysis

Confidence intervals (95%) were calculated using a Fisher’s exact test. The Chi-square test was used to assess the correlation between *B. miyamotoi* infections and *B. burgdorferi* s.l. infection in ticks based on a 2x2 contingency table with *B. miyamotoi* infection and *B. burgdorferi* infection as determining binary conditions.
RESULTS

Ticks
Questing *I. ricinus* nymphs (n=3360) were collected from 11 different areas in the Netherlands between 2014 and 2015. Of these nymphs 313 (9.3%) were positive for *B. burgdorferi* s.l. only and 70 (2.1%) were positive for *B. miyamotoi* only, while 14 nymphs (0.4%) were co-infected with both pathogens (Table 1). Thus, 14/327 (4.3%) *B. burgdorferi* s.l. positive nymphs were also positive for *B. miyamotoi*, and co-infection of *B. burgdorferi* with *B. miyamotoi* occurred significantly (p=0.03) more often than expected, suggesting the existence of mutual reservoir hosts.

Vertebrate hosts
Seven out of 74 (9%) examined rodent spleens were qPCR positive for *B. miyamotoi*. Three out of 21 (14%) wood mice (*Apodemus sylvaticus*), one out of 8 (13%) common voles (*Myodes arvalis*) and 3 out of 34 (9%) bank voles (*M. glareolus*) (Table 2) were found to be infected with *B. miyamotoi*. All 10 roe deer spleen samples were negative for *B. miyamotoi* DNA (Table 2). Two out of 26 studied birds (8%) were found qPCR positive for *B. miyamotoi*, namely a great tit (*Parus major*) and a European greenfinch (*Carduelis chloris*) (Table 3).

Table 1. Questing *Ixodes ricinus* nymphs (n=3360) tested for *Borrelia burgdorferi* s.l. and *Borrelia miyamotoi* by multiplex qPCR.

<table>
<thead>
<tr>
<th><em>Ixodes ricinus</em></th>
<th>Positive (n)</th>
<th>Percentage (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. burgdorferi</em> s.l.</td>
<td>327</td>
<td>10% (9-11%)</td>
</tr>
<tr>
<td><em>B. miyamotoi</em></td>
<td>84</td>
<td>2.5% (2-3%)</td>
</tr>
<tr>
<td><em>B. burgdorferi</em> and <em>B. miyamotoi</em> co-infection</td>
<td>14</td>
<td>0.4% (0.2-0.7%)</td>
</tr>
</tbody>
</table>

Table 2. Spleen tissue of different rodent species and roe deer tested for *Borrelia miyamotoi* by qPCR

<table>
<thead>
<tr>
<th>Rodent species</th>
<th>Total (n)</th>
<th>Positive (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apodemus flavicollis</em></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>Apodemus sylvaticus</em></td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td><em>Cociudra russula</em></td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td><em>Myodes arvalis</em></td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td><em>Myodes glareolus</em></td>
<td>34</td>
<td>3</td>
</tr>
<tr>
<td><em>Sorex araneus</em></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td><em>Capreolus capreolus</em></td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3. Spleen tissue of different bird species tested for *Borrelia miyamotoi* by qPCR

<table>
<thead>
<tr>
<th>Bird species</th>
<th>Total (n)</th>
<th>Positive (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Carduelis chloris</em></td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><em>Coccothraustes coccothraustes</em></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>Fringilla coelebs</em></td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><em>Parus major</em></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>Phylloscopus trochilus</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Pyrhula Pyrrhula</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Turdus iliacus</em></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td><em>Turdus merula</em></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>Turdus philomelos</em></td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

**LB-suspected skin biopsies**

Out of 31 patients with LB-suspected skin lesions who were referred to our tertiary Lyme borreliosis center, nine (29%) were PCR positive for *B. burgdorferi* in a skin biopsy: 3 patients were diagnosed as having ACA, 1 as multiple EM and 5 were diagnosed with definite EM (table 4). One patient was PCR-negative (possibly because the biopsy was taken after initiation of doxycycline treatment), but clinically diagnosed with EM by the dermatologist. None of these biopsies were taken from febrile patients, and none were concurrently positive for *B. miyamotoi*. In seven patients that had previously been diagnosed with LB, who were now presenting with persisting skin conditions, active LB was excluded by PCR and pathology. In three of these patients, another dermatological diagnosis could be made (progressive purpura pigmentosa, nummular eczema and morphea cutis). In none of these patients, *B. miyamotoi* could be identified as the cause of the symptoms. Finally, the remaining 14 patients with LB-suspected skin lesions tested negative for *B. burgdorferi*, but combined with pathology results, other dermatological diagnoses were made in 11 patients (angioma serpiginosum, contact dermatitis, toxicodermia, granuloma annulare, pityriasis lichenoides chronica, dermatomycosis corporis, eczematous dermatitis, chilblain lupus, erythema annulare centrifugum and hypersensitivity to a tick bite and a spider bite). These, including, the five patients without a definite diagnosis all retrospectively tested negative for *B. miyamotoi* (Table 4).
In this study we have evaluated the prevalence of *B. miyamotoi* in *I. ricinus* ticks, wild animals and LB-suspected skin lesions in the Netherlands. Previous studies in the Netherlands revealed that around 2-4% (Cochez et al., 2015, Hovius et al., 2013) of questing *I. ricinus* ticks were infected with *B. miyamotoi*, and around 4% (Fonville et al., 2014) of ticks that were collected from humans, which corresponds well with the incidence in other endemic areas (88). We found that *B. miyamotoi* infection in *I. ricinus* nymphs was present more often when ticks were infected with *B. burgdorferi* s.l. than in *B. burgdorferi* s.l. uninfected ticks (4.3% versus 2.3%), suggesting similar reservoir hosts. Indeed, we identified *B. miyamotoi* in rodents (9%) and birds (8%). The role of these animals in the transmission cycle is not clear; they could be amplifying hosts, a transitory or dead-end host for this spirochete, warranting further investigation.

Previously, several studies have shown wild rodents and small mammals to be infected with *B. miyamotoi* in up to 3.7% of animals (91-93). Although the enzootic cycle of *B. miyamotoi* is currently unknown, larvae are thought to play an important role in transmission (89). Indeed, we have recently described field-collected *I. ricinus* larvae to be able to transmit *B. miyamotoi* to laboratory-bred Naval Medical Research Institute (NMRI) mice (27). Since *B. miyamotoi* can be vertically transmitted, the relative contribution of mammalian reservoir hosts to the transmission cycle is yet unclear. Mice seem to clear *B. miyamotoi* from their blood by VMP-specific

DISCUSSION

Table 4. Retrospective qPCR analysis for *B. miyamotoi* in biopsies from Lyme borreliosis suspected skin lesions (positive/total tested).

<table>
<thead>
<tr>
<th>Patient category</th>
<th>PCR <em>B. burgdorferi</em> s.l.</th>
<th>C6 ELISA</th>
<th>Fever</th>
<th>Presenting May to September</th>
<th>PCR <em>B. miyamotoi</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>EM (n=6)</td>
<td>5/6</td>
<td>0/5*</td>
<td>0/6</td>
<td>5/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Multiple EM (n=1)</td>
<td>1/1</td>
<td>1/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>ACA (n=3)</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>LB-suspected skin lesion after previous treatment for LB; Active LB excluded (n=7)</td>
<td>0/7</td>
<td>4/7</td>
<td>0/7</td>
<td>1/7</td>
<td>0/7</td>
</tr>
<tr>
<td>LB-suspected skin lesion without previous treatment for LB; Active LB excluded (n=14)</td>
<td>0/14</td>
<td>1/12</td>
<td>1/14</td>
<td>8/14</td>
<td>0/14</td>
</tr>
<tr>
<td>Total</td>
<td>9/31</td>
<td>9/28</td>
<td>1/31</td>
<td>16/31</td>
<td>0/28</td>
</tr>
</tbody>
</table>

* 1/5 dubious. 2/5 were re-tested at a later stage, both of which seroconverted

In this study we have evaluated the prevalence of *B. miyamotoi* in *I. ricinus* ticks, wild animals and LB-suspected skin lesions in the Netherlands. Previous studies in the Netherlands revealed that around 2-4% (Cochez et al., 2015, Hovius et al., 2013) of questing *I. ricinus* ticks were infected with *B. miyamotoi*, and around 4% (Fonville et al., 2014) of ticks that were collected from humans, which corresponds well with the incidence in other endemic areas (88). We found that *B. miyamotoi* infection in *I. ricinus* nymphs was present more often when ticks were infected with *B. burgdorferi* s.l. than in *B. burgdorferi* s.l. uninfected ticks (4.3% versus 2.3%), suggesting similar reservoir hosts. Indeed, we identified *B. miyamotoi* in rodents (9%) and birds (8%). The role of these animals in the transmission cycle is not clear; they could be amplifying hosts, a transitory or dead-end host for this spirochete, warranting further investigation.

Previously, several studies have shown wild rodents and small mammals to be infected with *B. miyamotoi* in up to 3.7% of animals (91-93). Although the enzootic cycle of *B. miyamotoi* is currently unknown, larvae are thought to play an important role in transmission (89). Indeed, we have recently described field-collected *I. ricinus* larvae to be able to transmit *B. miyamotoi* to laboratory-bred Naval Medical Research Institute (NMRI) mice (27). Since *B. miyamotoi* can be vertically transmitted, the relative contribution of mammalian reservoir hosts to the transmission cycle is yet unclear. Mice seem to clear *B. miyamotoi* from their blood by VMP-specific
antibodies, and only SCID mice have so far been demonstrated to experience persistent spirochetemia (105, 106). Therefore, further research should investigate 

*B. miyamotoi* infection dynamics in wild mammals and ticks in order to investigate the potential role of reservoir hosts to the *B. miyamotoi* life cycle. Interestingly, we identified two *B. miyamotoi* infected birds, namely a great tit and a European greenfinch. Previous studies revealed a very high prevalence of *B. miyamotoi* in 58% of wild Tennessee turkeys (*Meleagris gallopavo*), while experimental infection with field-collected *I. ricinus* nymphs did not succeed in common European songbirds (*Parus major*) (90, 107). Further studies should reveal which bird species are susceptible to *B. miyamotoi* infection, the relationship between species-specific complement sensitivity and the ecological implications of bird infections.

After establishing the presence of *B. miyamotoi* in ticks and wild animals from the Netherlands, we were interested whether we had missed *B. miyamotoi* in patients previously examined in our tertiary Lyme borreliosis clinic. *B. miyamotoi* was not found as a co-infection or primary explanation for 31 LB-suspected skin lesions examined in our clinic. These patients were mostly afebrile, while HTBRF is currently characterized by fever and generalized symptoms several weeks after a tick bite (21, 94, 97, 108, 109) or by chronic meningoencephalitis in immunocompromised patients (7, 96). Unfortunately, due to the retrospective nature of our study, no blood samples were available to establish serologic evidence or absence of infection with *B. miyamotoi*, and HTBRF in these patients, although unlikely, can therefore not be definitely excluded. Nonetheless, this is the first study investigating the presence of *B. miyamotoi* DNA in LB- suspected skin biopsies, and our findings support the hypothesis that *B. miyamotoi* is not associated with LB-related skin manifestations. However, more studies, with larger patient populations and with multiple body fluids and tissues, should be performed to corroborate our findings.

Considering the 2.5% *B. miyamotoi* prevalence in ticks that we found, combined with over a million tick bites per year in the Netherlands (110), an estimated 8% transmission efficiency to humans, elevated seroprevalences in Dutch high-risk populations and the incidence of HTBRF described in prospective studies in Russia and the U.S.A., we postulate that the diagnosis HTBRF is currently being missed in Dutch patients (21, 94, 98, 99). Therefore, we suggest that a prospective clinical study in Dutch patients presenting with fever after a tick bite is needed in order to assess the incidence of HTBRF and the occurrence of *B. miyamotoi* in blood and other tissues or body fluids of these patients.
ACKNOWLEDGEMENTS

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