Prevalence of Lyme Borrelia in *Ixodes persulcatus* Ticks from an Area with a Confirmed Case of Lyme Disease

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**ABSTRACT.** In this study, the prevalence of *Borrelia* infections in *Ixodes* ticks from a site in Hokkaido, Japan, with confirmed cases of Lyme disease was determined by a PCR method capable of detecting and differentiating between strains of pathogenic *Borrelia*, with particular emphasis on *Borrelia garinii* (B. *garinii*) and *Borrelia afzelii* (B. *afzelii*), using tick-derived DNA extracts as template. A total of 338 ticks, inclusive of 284 *Ixodes persulcatus* (I. *persulcatus*), were collected by flagging vegetation in mid-spring. Ninety-eight (34.5%) of *I. persulcatus* tested positive for *Borrelia* species DNA, whereas the overall prevalence of *Borrelia* species in *Ixodes* ovatus and *Haemaphysalis longicornis* ticks was 19.5 and 7.7%, respectively. PCR-RFLP and sequence analysis of *Borrelia* *rrs* (5S-rrl(23S) intergenic spacer DNA amplicons indicated that they originated from three different *Borrelia* species namely, *B. garinii*, *B. afzelii* and *B. japonica*. Among the I. *persulcatus* species, which is a known vector of human borreliosis, 86 were mono-infected with *B. garinii*, 2 ticks were mono-infected with *B. afzelii* and whereas 12 ticks had dual infections. Most significant, 11 of the *I. persulcatus* ticks were coinfected with *Anaplasma phagocytophilum* and *B. garinii*. The difference between the number of obtained and expected co-infections was significant (χ²=4.32, *P*=0.038).

**KEY WORDS:** *B. afzelii*, *B. garinii*, I. *persulcatus*, Lyme borreliosis.

Lyne disease is a multi-systemic disorder caused by infection with at least three spirochete species including *Borrelia burgdorferi*, *B. garinii* and *B. afzelii*. Human Lyme borreliosis is vectored by *Ixodes* ticks, specifically *Ixodes scapularis*, *I. ricinus*, *I. pacificus* and *I. persulcatus* [24]. *I. persulcatus* is distributed in Russia and Far East Asia, and has been implicated as a vector of several human pathogens including Lyme disease [15]. In Japan, Lyme disease in humans is due to infection with *B. garinii* or *B. afzelii*, which are biologically and specifically transmitted by *I. persulcatus* [21]. In 1986, a human Lyme borreliosis case was reported for the first time in Japan [8] and since then, an increasing number of patients have been reported. There were 124 confirmed cases of Lyme disease in Japan between 1999 and 2010 (the data are cited from Infectious Agents Surveillance Report (IASR), http://idsc.nih.go.jp/iasr/index-j.html). Determining vector competence in a locality can help in estimating the prevalence of the pathogens transmitted by such vectors leading to effective and targeted interventions. This is of particular relevance in the case of Lyme borreliosis, a zoonotic infection with a wide range of wild reservoir hosts [1]. Host preference and hence reservoir capacity of such hosts, including rodents and birds, is determined by multiple factors, including geographical distribution and density of the host population, as well as the feeding behavior of tick species prevalent in the area, which in turn influences the prevalence of borrelia species in a particular region [15]. In the present study, we aimed to apply a PCR detection method to detect *Borrelia* species DNA from individual ticks that were captured on a farm with a known case of Lyme disease. Further, we performed PCR-restriction fragment length polymorphism and sequence analysis to differentiate between the detected *Borrelia* pathogens.

In 2008, a woman with erythema migrans resulting from a tick bite was clinically diagnosed with Lyme disease in the central part of Hokkaido, Japan. However, the species of both the tick and the infecting *Borrelia* were not identified. In May of 2009, to investigate the prevalence of *Borrelia* infections in *Ixodes* ticks, host-seeking adult ticks were collected by flagging with a white cotton flannel at the ranching farm where the woman diagnosed with Lyme disease had lived. Collected ticks were examined morphologically to determine their species [color of tick’s basal side (idiosoma), length of internal spur on cox 1] [9], and genomic DNA samples obtained by disrupting the ticks as previously described [10]. Tick identification was further confirmed by amplifying tick mitochondrial 16S DNA and sequencing the *Bataillonella* DNA. Among the 173 ticks collected, 117 were *I. persulcatus* (102 mono-infected, 15 coinfected with *Anaplasma phagocytophilum* and *B. garinii*), 9 were *I. scapularis*, 17 were *I. ovatus* and 2 were *I. ricinus*. Of these, 94 were positive for *Borrelia* DNA, with *B. garinii* detected in 60, *B. afzelii* in 14, *B. japonica* in 4, *B. rickettsii* in 1, and *B. afzelii* and *B. rickettsii* in 1. PCR-RFLP and sequence analysis of *Borrelia* DNA amplicons confirmed the presence of *B. garinii*, *B. afzelii* and *B. japonica*. The prevalence of *B. garinii* in *I. persulcatus* ticks was 19.5 and 7.7%, respectively. PCR-RFLP and sequence analysis of *Borrelia* DNA amplicons indicated that they originated from three different *Borrelia* species namely, *B. garinii*, *B. afzelii* and *B. japonica*. Among the *I. persulcatus* species, which is a known vector of human borreliosis, 86 were mono-infected with *B. garinii*, 2 ticks were mono-infected with *B. afzelii* and whereas 12 ticks had dual infections. Most significant, 11 of the *I. persulcatus* ticks were coinfected with *Anaplasma phagocytophilum* and *B. garinii*. The difference between the number of obtained and expected co-infections was significant (χ²=4.32, *P*=0.038).

**NOTE** Parasitology

rRNA gene as described previously [26] by the primer pair
Tick mt-rss F (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') and Tick mt-rss R (5'-CCG GTC TGA ACT CAG ATC AAG TA-3'). The purified PCR products were
confirmed by direct sequencing using the CEQ 2000 Dye
CAG ATC AAG TA-3'). The purified PCR products were
targeting CEQ 2000 DNA analysis system (Beckman Coulter, Inc.)
kit (Beckman Coulter, Inc., Fullerton, CA, U.S.A.) and the
Terminator Cycle Sequencing method with the Quick Start
confirmed mixture after the second round of PCR were digested
for restriction enzyme digestion. Ten microliters of the puri-
fied PCR products were also
polyacrylamide gel. The purified PCR products were also
I. persulcatus
I. ovatus
H. longicornis

Table 1. Detection of Borrelia spp. and A. phagocytophilum in field-collected tick

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of tested</th>
<th>Detection and identification of borrelia species</th>
<th>Co-infection with A. phagocytophilum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. persulcatus</td>
<td></td>
<td>B. garinii (%)</td>
<td>B. afzelii (%)</td>
</tr>
<tr>
<td>Male</td>
<td>155</td>
<td>44 (28.4)</td>
<td>7 (4.5)</td>
</tr>
<tr>
<td>Female</td>
<td>129</td>
<td>52 (40.3)</td>
<td>7 (5.4)</td>
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<tr>
<td>(Total)</td>
<td>284</td>
<td>96 (33.8)</td>
<td>14 (4.9)</td>
</tr>
<tr>
<td>I. ovatus</td>
<td></td>
<td>7</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Female</td>
<td>34</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>(Total)</td>
<td>41</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>H. longicornis</td>
<td></td>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>0 (0)</td>
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a) All the 338 ticks analyzed tested positive for tick actin, which was used as control for template DNA loading. b) Results of detection of A. phagocytophilum were cited from the our report by Murase et al. (2011) [18].

In total, 338 adult ticks were collected (284 I. persulcatus, 41 I. ovatus and 13 H. longicornis), and DNA extraction and PCR assays were performed for detection of Borrelia species (Table 1). Overall, 31.7% (107/338) of the ticks were infected with Borrelia species. To identify the Borrelia sub-
species, PCR-RFLP was performed using two restriction en-
zymes that identified Lyme borrelia, B. garinii and B. afzelii, specifically from I. persulcatus ticks. The infection rates
of B. garinii and B. afzelii were 33.8% (96/284) and 4.9% (14/284), respectively. Of the I. persulcatus ticks from the field, 12 ticks (4.2%) had mixed infection with B. garinii and B. afzelii. Recently, we reported an epidemiological surveil-
ance of Anaplasma phagocytophilum in I. persulcatus ticks in Hokkaido [18]. As in the present study, DNA samples extracted from I. persulcatus had been used to detect A. phagocytophilum by PCR with primers MSP2-3F (5'-CCA GGC TTT AGC AGA ATA AGA G-3') and MSP2-3R (5'-GCC CAG TAA CAA CAT CAT AAG C-3') as described previously [13, 18, 27], which can specifically amplify a 334 bp portion of the p44 gene of A. phagocytophilum. Out of the 284 I. persulcatus ticks, 20 (7.0%) were found to harbor A. phagocytophilum DNA. The rate of co-infection with B. ga-inii and A. phagocytophilum in the I. persulcatus ticks was 3.9% (11/284) (Table 1), a statistically significant outcome (χ²=4.32, P=0.038) with an odds ratio of 2.6 (95% confi-
dence interval [CI]: 1.1–6.3). Similarly, a total of 41 I. ovatus from the same field were individually tested for the presence of Borrelial DNA. Out of 41 I. ovatus, 8 (19.5%) were found to be infected with Borrelial species (Table 1), while only one H. longicornis sample (7.7%) was positive, for Borrelial DNA by conventional PCR. However, Lyme borrelia could not be detected in both of the ticks. The tick actin gene was amplified to check the integrity of the template DNA and could be detected among all the 338 ticks sampled (data not shown). Genetic analysis was constructed from represen-
tative positive samples from each tick species, with the results indicating that these sequences were very close or identical with previously reported isolates of B. garinii, B. afzelii and B. japonica (data not shown). Furthermore, the results indicated that the Borrelia species identified from the I. persulcatus ticks were closely related to the Lyme borrelia derived from the human.

Lyme disease is an emerging infectious disease and the most prevalent tick-borne zoonosis. Accordingly, epidemi-
ological studies for Lyme borrelia are conducted extensively
in endemic regions [24]. In Japan, previous surveys indicated high prevalence of Lyme borreli [16, 17, 20]. Lyme borreli infection rates among wildlife in Hokkaido have been reported to be 40.0–87.5% in wood mice [22], 0.2% in feral raccoon [6] and 69.0% in deer [7], respectively. In addition, the prevalence rates among ticks collected from some areas of Hokkaido have been reported to be 16.6% [20] and 6.7–15.5% [16]. In Hokkaido, a northern part of Japan, I. persulcatus is the main tick species due to the cool weather conditions that favor this tick, resulting into higher number of Lyme disease cases compared to other areas in Japan [the data were cited from Infectious Agents Surveillance Report (IASR), http://idsc.nih.go.jp/iasr/index-j.html]. There have been 49 confirmed cases of Lyme disease between 1999 and 2010 in Hokkaido (the data were cited from Historical Statistics of Hokkaido Infectious Disease Surveillance Center). A case of Lyme disease was found in a farming area located in the central part of Hokkaido in 2008. Thus, in this study, to evaluate the prevalence of Lyme borreli, host-seeking ticks were collected in the study area, and spirochete B. garinii and B. afzelii were detected by PCR targeting the 23S–5S rRNA intergenic spacer regions. I. persulcatus Schulze was found to be the predominant tick species (84.0%) in the area (Table 1). In a total of 284 I. persulcatus Schulze adult ticks collected at the farm, spirochetal DNA was detected in 34.5% on average of the ticks examined. The restriction patterns obtained after Dra I and Mse I digestion of the amplicons assigned 98 DNA samples to the following genomic groups: 96 (98.0%) to B. garinii, fourteen (14.3%) to B. afzelii, and twelve (12.2%) to mixed assigned B. garinii and B. afzelii. I. ovatus and H. longicornis ticks were negative for Lyme borreli, but B. japonica, a non-pathogenic spirochaete, was detected in both tick species. These results are consistent with previous findings indicating that the transmission of Lyme borreli in Japan is restricted to I. persulcatus [15, 21]. The rates of Lyme borreli infections observed in the present study are similar to findings from past studies that employed different diagnostic methods, thus confirming Hokkaido to be a disease-endemic zone. Lyme borreli and A. phagocytophilum, the agent of human granulocytic anaplasmosis, are both transmitted by Ixodes spp., including I. persulcatus and may occasionally co-infect a host. In our previous study [18], out of the 281 I. persulcatus ticks, twenty (7.1%) were found to harbor A. phagocytophilum DNA. Finally, the co-infection rate for ticks, twenty (7.1%) were found to harbor B. burgdorferi and A. phagocytophilum from a large cohort of I. persulcatus ticks in China, and nymph I. persulcatus ticks, indicating 33.8% were infected with B. burgdorferi, 4.6% with A. phagocytophilum, and 0.5% with both pathogens. These results from this study raise the possibility occurrence of Lyme disease patient with dual infection with B. burgdorferi and A. phagocytophilum in Asian countries as in North America and Europe. However, no human case of dual infection has been reported in Asia to the present. In addition, there is no documented case of human granulocytic anaplasmosis in Japan, including Hokkaido, the study area for this report. In North America, previous studies have led to the conclusion that A. phagocytophilum exists in a separate epizootic cycle within deer and I. scapularis [12], and that wild deer harbors a variant strain not associated with human infection [14]. It remains to be seen whether this pattern also holds true for co-infection with the agents of Lyme borreliosis and human granulocytic ehrlichiosis. Furthermore, it would be interesting to determine virulence of the Hokkaido isolates either singly or in case of dual infections. Studies are ongoing to identify the reservoir host for both pathogens and investigate their prevalence in the field.

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