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Lyme disease spirochetes in Japan: enzootic transmission cycles in birds, rodents, and Ixodes persulcatus ticks

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Lyme Disease Spirochetes in Japan: Enzootic Transmission Cycles in Birds, Rodents, and *Ixodes persulcatus* Ticks

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The ixodid tick, *Ixodes persulcatus*, serves as a vector of *Borrelia* species associated with Lyme disease in Hokkaido, Japan. The migratory birds of genera *Emberiza* and *Turdus* and the woodland rodents of genera *Apodemus* and *Clethrionomys* are the wildlife reservoirs. To systemize the enzootic transmission cycles, borreliae isolates were classified by ribosomal RNA gene restriction fragment length polymorphism (RFLP) analysis. Most (>60%) isolates from the bird-feeding *I. persulcatus* larvae belonged to *Borrelia garinii;* the rest were classified as RFLP ribotype group IV (unknown species). In contrast, no *B. garinii* were found among isolates from rodent-feeding *I. persulcatus* larvae. These isolates were classified as *Borrelia afzelii*, group IV, and group V (unknown species). These observations suggest that two enzootic cycles in nature (bird-tick and rodent-tick) maintain borreliae specifically. The group IV species, which was predominant among clinical isolates from Lyme disease patients in Hokkaido, appears to be the most important pathogen for humans.

Lyme disease is a tickborne zoonosis caused by the spirochetes of genus *Borrelia: B. burgdorferi*, *B. garinii*, and *B. afzelii* [1, 2]. Although *B. burgdorferi* is geographically restricted in North America and Europe, *B. garinii* and *B. afzelii* are widely distributed in Eurasia [1–3]. Ticks of the *Ixodes ricinus* species complex, including North American *Ixodes scapularis* and *Ixodes pacificus*, European *I. ricinus*, and East European and Siberian *Ixodes persulcatus*, are major vectors for transmitting the infection to humans [4, 5]. Lyme disease spirochetes are maintained in enzootic transmission cycles through vertebrate reservoirs and tick vectors [4]. In general, adult ticks parasitize large mammals, while nymphal and larval ticks exhibit a broader host range that includes small mammals, birds, and reptiles. The immature ticks play an important role in perpetuating spirochetal infections in wildlife reservoirs.

In Japan, most human cases of Lyme disease are concentrated in the Hokkaido and Nagano districts, where epidemiologic surveys have shown that questing adult ticks of both *I. persulcatus* and *Ixodes* (*Partipalpiger*) *ovatus* are highly infected with spirochetes [6]. Antigenic and genetic properties of the spirochetal isolates from *I. ovatus* are homogeneous and quite different from those from *I. persulcatus* [7–9]. On the basis of whole DNA-DNA hybridization, spirochetes of *I. ovatus* were named *Borrelia japonica* [10]. Vertebrate animals belonging to the order Insectivora have been incriminated as reservoirs of *B. japonica* [11], but no human cases of Lyme disease caused by *B. japonica* have been confirmed in Japan. In contrast, *B. garinii*, *B. afzelii*, and unknown species have been found on *I. persulcatus* [1–3]. Because all isolates from Lyme disease patients in Japan have been genetically identical to the spirochetes of *I. persulcatus* [12], the tick is thought to be the most important vector to humans. In Hokkaido, migratory birds of genera *Emberiza* and *Turdus* and the woodland rodents of genera *Apodemus* and *Clethrionomys* have been found to be reservoirs for spirochetes transmitted by *I. persulcatus* [13, 14]. However, the possibility that *Borrelia* specificity is associated with the vector and reservoir relationships has not been evaluated.

The present study was undertaken to systemize the enzootic transmission cycles involving birds, rodents, and *I. persulcatus* ticks. For this purpose, a large number of spirochetal isolates from various culture sources were classified by ribosomal RNA gene restriction fragment length polymorphism (RFLP) analysis.

Materials and Methods

Study site and field sampling. Migratory birds, rodents, and ticks were collected at a woodland on the shore of Lake Furen in Nemuro, Hokkaido. Field data have been described [13, 14]. In brief, we captured 1699 birds (20 species) with Japanese mist nets in October 1990 and October 1991 and examined them for ticks. The black-faced bunting, *Emberiza spodocephala*, and the red-bellied thrush, *Turdus chrysolaus*, were captured most frequently, 1565 and 63, respectively. Larval and nymphal ticks of *I. persulcatus* were predominantly found on birds. Sixty-nine rodents (3 species) were captured in Sherman box traps in May and October 1991 and examined for ticks. Of the rodents, 47

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Table 1. Categories of culture sources and numbers of spirochetal isolates studied.

Categories	Culture sources	No. of isolates used
Bird		
A	Larvae of *I. persulcatus* ticks feeding on 12 *Emberiza spodocephala*	12
B	Larvae of *I. persulcatus* ticks feeding on 5 *Turdus chrysolaus*	5
Rodent		
A1	Larvae of *I. persulcatus* ticks feeding on 19 *Apodemus speciosus*	33*
B1	Larvae of *I. persulcatus* ticks feeding on 3 *Clethrionomys rutilus*	4
A2	17 *A. speciosus* spleens	17
B2	2 *C. rutilus* spleens	2
Adult tick	68 *I. persulcatus*	68
Human	Erythema lesions, 12 patients	12

* Because many isolates were obtained from *I. persulcatus* larvae feeding on 19 *A. speciosus*, 1 or 2 per rodent were selected at random for testing.

(68.1%) were wood mice, *Apodemus speciosus*, and 21 (30.4%) were red-backed voles, *Clethrionomys rutilus*. Larval and nymphal *I. persulcatus* were highly prevalent on *A. speciosus*. Rodents were sacrificed, and spleen samples were used for spirochetal culture. Host-seeking adult ticks of *I. persulcatus* were collected by flagging vegetation in May 1993. Bird- and rodent-feeding ticks and unfed adult ticks were used for spirochetal culture.

Bacterial isolates. Spirochetes were isolated by culturing tick midguts, rodent spleens, and human cutaneous tissues in BSK II medium [15] as described [7, 12, 13]. Table 1 summarizes information about isolates. Clinical isolates originated from erythema migrans lesions of 12 Lyme disease patients who lived in various locales in Hokkaido. Eight isolates have been described [12]. In addition, isolates HT17, HT57, HT64, HT10, HT25, HT61, HT22, HT7, HT37, HT19, and HT55, which were selected from our culture collection of spirochetes isolated from *I. persulcatus* in Hokkaido [7], served as representatives for genomic analysis. The type strains, *B. burgdorferi* sensu stricto B31 (ATCC 35210), *B. garinii* 20047 (CIP 103362), and *B. afzelii* VS461 (CIP 103469), were used as controls.

Southern blot hybridization. Spirochetal DNAs were prepared as described [3, 16] and digested with *Eco*RV or *Hin*cII (Takara Biochemicals, Kyoto, Japan). Resulting DNA fragments were electrophoresed in 0.7% agarose gel with TAE buffer (40 m*M* TRIS, 20 m*M* sodium acetate, 30 m*M* acetic acid, 2 m*M* EDTA [pH 7.8]) and transferred to a nylon membrane (Hybond-N+; Amersham International, Tokyo) by the method of Southern [17]. The blotted DNAs were bound to the membrane by UV cross-linking. An enhanced chemoluminescence kit (ECL random prime labeling and detection systems; Amersham) was used for genomic hybridization. The 23S rRNA gene fragments of *B. burgdorferi* B31 designated NP and Sty [3] were labeled with fluorescein-11-dUTP and used as probes. Hybridization, subsequent washings, and signal detection on autoradiographic films were done as recommended by the manufacturer.

Cloning of isolate. One spirochetal isolate from an unfed adult *I. persulcatus* was cloned by limiting dilution. Spirochetes were counted by Thoma hemocytometer (Nitirin, Tokyo) under darkfield microscopy. Twenty tubes containing 6 mL of BSK II medium were used for inoculation of the single cell. Resulting clones were analyzed by Southern blot hybridization.

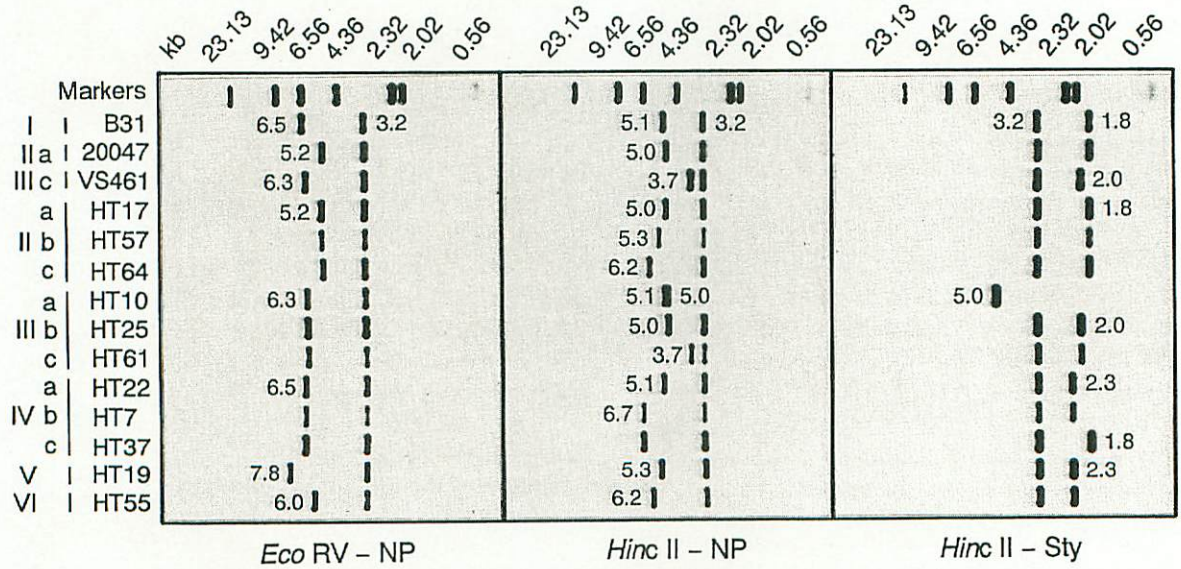

Figure 1. Southern blot patterns of *Borrelia* strains B31, 20047, and VS461 and representative Japanese isolates (HT17, HT57, HT64, HT10, HT25, HT61, HT22, HT7, HT37, HT19, and HT55). Genomic DNAs were digested with *Eco*RV or *Hin*cII and hybridized with 23S rRNA gene probe (NP or Sty). Sizes of fragments are in kilobase (kb) pairs. Markers, fluorescein–labeled λ DNAs digested with *Hin*dIII. Restriction fragment length polymorphism ribotype groups are at left.

Table 2. Frequency of restriction fragment length polymorphism (RFLP) ribotype groups among spirochetal isolates from various culture sources.

Genospecies, ribotype group	Bird		Rodent				Adult tick	Human
	A	B	A1	B1	A2	B2		
Borrelia burgdorferi, I	0	0	0	0	0	0	0	0
Borrelia garinii								
IIa	8 (66.7)	3 (60.0)	0	0	0	0	16 (23.5)	1 (8.3)
IIb	1 (8.3)	0	0	0	0	0	1 (1.5)	1 (8.3)
IIc	0	0	0	0	0	0	1 (1.5)	1 (8.3)
Borrelia afzelii								
IIIa	0	0	0	0	0	0	3 (4.4)	0
IIIb	0	0	0	0	1 (5.9)	0	0	0
IIIc	0	0	4 (12.1)	0	4 (23.5)	0	2 (2.9)	1 (8.3)
Unknown								
IVa	3 (25.0)	2 (40.0)	5 (15.2)	4 (100)	5 (29.4)	1 (50.0)	21 (30.9)	6 (50.0)
IVb	0	0	14 (42.4)	0	4 (23.5)	1 (50.0)	10 (14.7)	1 (8.3)
IVc	0	0	2 (6.1)	0	1 (5.9)	0	0	0
V	0	0	6 (18.2)	0	0	0	5 (7.4)	1 (8.3)
VI	0	0	0	0	0	0	3 (4.4)	0
Other*	0	0	2 (6.1)	0	2 (11.8)	0	6 (8.8)	0
Total	12	5	33	4	17	2	68	12

NOTE. Data are no. (%).
* Isolates with mixed RFLP patterns (2 ribotypes).

Results

RFLP profiles of Borrelia rRNA genes. Lyme disease spirochetes have a unique organization of the rRNA gene. Two copies of the 23S and 5S genes are repeated in the order of 23S-5S-23S-5S and are not linked to the single 16S gene [18–21]. In our RFLP ribotyping system using the 23S gene probes (NP and Sty), hybridized signals are usually detected on two fragments: a common 3.2-kb fragment within the 23S–5S repetition and a distinct fragment that includes the flanking region. With *Eco*RV or *Hin*cII digestion, the NP probe detects a second band consisting of the rest of the first 23S gene and the upstream region to the next enzyme cleavage site. With *Hin*cII digestion, the Sty probe detects a second band consisting of the remainder of the second 23S gene and the downstream region to the next enzyme cleavage site.

To assess the genetic variability of Japanese *Borrelia* species, 67 isolates from unfed adult *I. persulcatus* from Hokkaido that we had previously characterized [7] were screened by Southern blot analysis. From those samples, we selected 11 representative isolates (HT17, HT57, HT64, HT10, HT25, HT61, HT22, HT7, HT37, HT19, and HT55). RFLP ribotype groups among the isolates and *Borrelia* type strains (B31, 20047, and VS461) are shown in figure 1. The grouping scheme was modified from that described by Fukunaga et al. [3]. Groups I (B31), II (20047, HT17, HT57, and HT64), and III (VS461, HT10, HT25, and HT61) correspond with *B. burgdorferi*, *B. garinii*, and *B. afzelii*, respectively. In addition, we designated groups IV (HT22, HT7, and HT37), V (HT19), and VI (HT55) for unknown species with unique RFLP patterns. On the basis of heterogeneity of the *Hin*cII cleavage sites, we classified groups II–IV into three subgroups each (designated a–c).

Analysis of field-sampled and clinical isolates. To determine if *Borrelia* species are related to specific reservoirs, field-sampled isolates (n = 141) were classified into RFLP ribotype groups. Likewise, clinical isolates (n = 12) were examined. As shown in table 2, none of the 153 isolates were classified as group I (*B. burgdorferi*). Most isolates (>60%) from bird-feeding *I. persulcatus* larvae belonged to group II (*B. garinii*; the rest were classified as group IV [unknown species]). In contrast, group II was not found among isolates from rodent-feeding *I. persulcatus* larvae or from rodent spleens. The rodent-derived isolates included groups III (*B. afzelii*) and IV and V (unknown species). Various ribotype groups were observed among isolates from unfed *I. persulcatus* adults and from humans. Group IV was predominant in rodents, adult ticks, and humans.

Demonstration of mixed infection. While conducting the RFLP analysis, we noticed that some isolates showed mixed patterns of two ribotype groups. In table 2, they are shown as "others." Of those, 1 isolate from an unfed *I. persulcatus* adult was cloned by limiting dilution technique. A total of 11 clones were obtained and analyzed by Southern blot hybridization. As shown in figure 2, these were separated into subgroup IVa (n = 10) and group VI (n = 1).

Discussion

Our RFLP ribotyping scheme was previously evaluated by using representative *Borrelia* isolates from North America

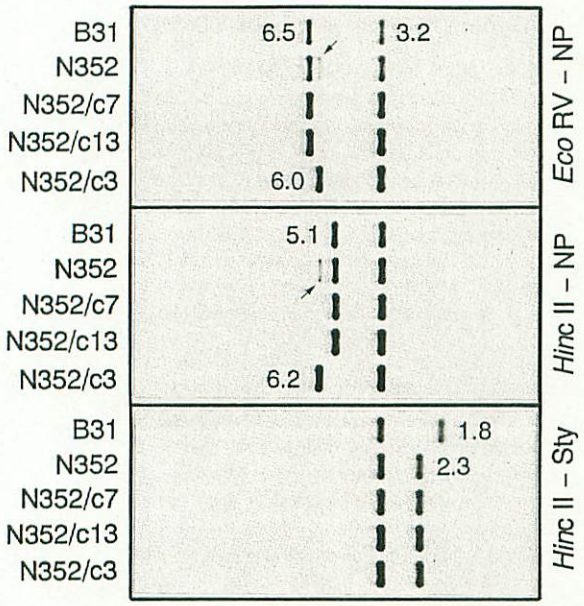

Figure 2. Southern blot analysis of mixed *Borrelia* infection. B31, type strain of *Borrelia burgdorferi*; N352, isolate from unfed adult *I. persulcatus;* N352/c7, N352/c13, and N352/c3, clones from N352. Genomic DNAs were digested with *Eco*RV or *Hinc*II and hybridized with 23S rRNA gene probe (NP or Sty). Sizes of fragments are in kilobase (kb) pairs. Arrows, mixed fragments. N352/c7 and N352/c13 were in subgroup IVa; however, N352/c3 was classified as group VI.

and Europe [3]. The North American isolates were ribotype group I (*B. burgdorferi*), and European isolates clustered in groups I, II (*B. garinii*), and III (*B. afzelii*). Although the RFLP patterns of group I were uniform, the European isolates in groups II and III showed intraspecies variation of RFLP patterns. In the current study, we classified a large number of Japanese isolates by RFLP analysis and found no *B. burgdorferi* prevalent in Japan. Both *B. garinii* and *B. afzelii* were found from *I. persulcatus.* The Japanese *B. garinii* and *B. afzelii* also showed intraspecies variation of RFLP patterns. However, many of the tick-derived isolates showed unique RFLP patterns that were distinct from those of North American and European isolates. We tentatively classified these unknown species as groups IV–VI. Further studies on the levels of DNA relatedness by whole DNA-DNA hybridization will clarify their taxonomic positions.

To investigate the vector and reservoir relationships in an endemic area of Hokkaido, we captured *Emberiza* and *Turdus* birds and *Apodemus* and *Clethrionomys* rodents for sampling of *I. persulcatus* immature ticks. Spirochetes were isolated from the larval and nymphal ticks that fed on these animals. The origins of spirochetes isolated from the nymphs were uncertain because the nymphs (during their larval stages) had parasitized other animals. Therefore, we used larva-derived isolates for our investigation. Since transovarial transmission of spirochetes is negligible in *I. persulcatus* [22], spirochetes isolated from larvae were considered to have originated from the birds and rodents.

The results of the RFLP ribotype classification enable us to speculate that *Borrelia* species have host preferences. The data suggest that rodents have a broad potential to serve as reservoirs for various *Borrelia* species except for *B. garinii.* In contrast, the birds appeared to be specific reservoirs for *B. garinii,* although there was overlap of group IV ribotypes between birds and rodents. Reasons for the reservoir specificity are unclear; however, we believe that susceptibility of vertebrate reservoirs to various *Borrelia* species differs between birds and rodents. Experimental infections of various *Borrelia* species to birds and rodents will provide useful information on reservoir specificity.

We also classified many isolates from unfed adults of *I. persulcatus* collected at the same site and found various ribotype groups. Most of the groups were in common with those of isolates from bird- and rodent-feeding larvae. These results strongly suggest that various *Borrelia* species are transmitted transstadially from immature ticks, which had fed on birds or rodents, to adult ticks. Moreover, we demonstrated by a limiting dilution assay that 1 isolate from an adult tick was in 2 ribotype groups. This finding indicates that mixed infection of *Borrelia* species occurs in *I. persulcatus* adults. Since 1 tick has two chances for acquiring spirochetes (during larval and nymphal stages), 2 different *Borrelia* species may accumulate in the adult stage. If the mixed infection occurs in reservoirs, immature ticks will also acquire the mixed infection by feeding on the reservoirs.

In Hokkaido, people outdoors (e.g., hikers, hunters, and forest workers) are frequently bitten by female *I. persulcatus* ticks [23–25], but bites by nymphs are rarely documented [24]. In this study, we determined the ribotype groups of 12 human-derived isolates to confirm which *Borrelia* species are dominant in Lyme disease patients. Seven isolates (58%) were group IV, which was also dominant in isolates from *I. persulcatus* adults. Therefore, this group should be regarded as the most important Lyme disease pathogen for humans in Hokkaido.

In conclusion, on the basis of our findings in this study, we hypothesize that there are two enzootic transmission cycles (bird-tick and rodent-tick) in nature that maintain *Borrelia* species specifically. This hypothesis will be tested by further studies. From an evolutionary standpoint, transmission dynamics of *Borrelia* species should be investigated in various areas where Lyme disease is endemic.

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