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Mixed Infection of Different *Borrelia* Species among *Apodemus speciosus* Mice in Hokkaido, Japan

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The wood mouse (*Apodemus speciosus*) serves as a wildlife reservoir for Lyme disease spirochetes in Hokkaido, Japan. To isolate *Borrelia* species, we captured 34 wood mice in an area where *Borrelia* species are endemic during October 1993. The earlobes (right and left), heart, spleen, and urinary bladder from each mouse were used as culture sources. As a result of culture 73 isolates from 21 mice were classified by rRNA gene restriction fragment length polymorphism analysis. Ribotype groups III (*Borrelia afzelii*) and IV (unknown species) were detected among those isolates. Thirty-one (77.5%) of 40 earlobe isolates were classified as group IV. In contrast, 6 (40.0%) of 15 heart isolates, 5 (50.0%) of 10 spleen isolates, and 7 (87.5%) of 8 urinary bladder isolates were *B. afzelii*. Seven mice showed mixed infection with *B. afzelii* and group IV. The data indicate that different *Borrelia* species can coexist in a reservoir host that is suitable for them.

Lyme disease is a multisystem disorder caused by tick-borne spirochetes. On the basis of DNA relatedness, the spirochetes have been divided into three genospecies: *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (1, 8). Although *B. burgdorferi* sensu stricto is the only species found in North America, all three species prevail in Europe. In the clinical spectrum, several investigators suggested that these species have different pathogenic potentials (8, 17, 18). The diversity of the microorganisms also indicates that their enzootic transmission cycles through tick vectors and vertebrate reservoirs should be investigated. Ticks of the *Ixodes ricinus* species complex are major vectors that transmit the infection to humans. In Japan, we found that the non-*I. ricinus* complex tick, *Ixodes* (*Partipalpiger*) *ovatus*, specifically harbors a characteristic spirochete (12, 16). The spirochete was named *Borrelia japonica* and was placed within the *B. burgdorferi* complex (7). No cases of Lyme disease

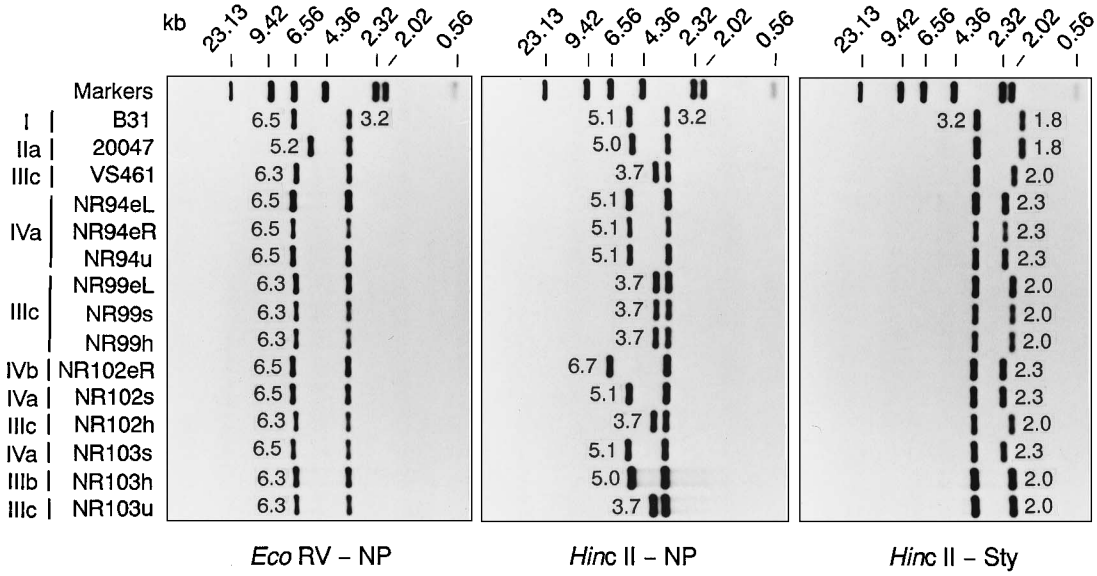

FIG. 1. Southern blot patterns of spirochete isolates from *A. speciosus* mice (mice NR94, NR99, NR102, and NR103). Genomic DNAs were digested with *Eco*RV or *Hinc*II and were hybridized with 23S rRNA gene probe (NP or Sty). The sizes of the fragments are in kilobase (kb) pairs. Markers, fluorescein-labeled bacteriophage lambda DNAs digested with *Hin*dIII; B31, type strain of *B. burgdorferi* sensu stricto; 20047, type strain of *B. garinii*; VS461, type strain of *B. afzelii*; NR94eL, isolate from left earlobe of NR94; NR94eR, isolate from right earlobe of NR94; NR94u, isolate from urinary bladder of NR94; NR99eL, isolate from left earlobe of NR99; NR99s, isolate from spleen of NR99; NR99h, isolate from heart of NR99; NR102eR, isolate from right earlobe of NR102; NR102s, isolate from spleen of NR102; NR102h, isolate from heart of NR102; NR103s, isolate from spleen of NR103; NR103h, isolate from heart of NR103; NR103u, isolate from urinary bladder of NR103. The RFLP ribotype subgroups are given on the left.

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TABLE 1. Results of spirochete cultures in 34 *A. speciosus* mice captured in Nemuro, Hokkaido, Japan, and classification of the isolates by RFLP ribotyping

Mouse no.	Isolation from the following culture sources (RFLP ribotype subgroup):				
	Right earlobe	Left earlobe	Heart	Spleen	Urinary bladder
NR92	+ (IVb)	+ (IVa)	−	−	−
NR93	+ (IVa)	+ (IVa)	+ (IVa)	−	−
NR94	+ (IVa)	+ (IVa)	−	−	+ (IVa)
NR95	−	−	−	−	−
NR96	−	−	−	−	−
NR97	−	−	−	−	−
NR98	−	−	−	−	−
NR99	+ (IIIc)	+ (IIIc)	+ (IIIc)	+ (IIIc)	+ (IIIc)
NR100	−	−	−	−	−
NR101	+ (IVa)	+ (IVa)	+ (IIIc)	+ (IIIc)	+ (IIIc)
NR102	+ (IVb)	+ (IVb)	+ (IIIc)	+ (IVa)	+ (IIIc)
NR103	+ (Mix[a])	+ (Mix)	+ (IIIb)	+ (IVa)	+ (IIIc)
NR104	+ (IVa)	+ (IVa)	−	−	−
NR105	+ (IVc)	+ (Mix)	+ (IVc)	+ (IIIc)	+ (IIIc)
NR106	+ (IVb)	+ (IVb)	+ (IVb)	−	−
NR107	−	−	−	−	−
NR108	+ (IVa)	−	−	−	−
NR109	+ (IVa)	+ (IVa)	+ (IVa)	−	−
NR110	−	−	−	−	−
NR111	+ (IVb)	+ (IVb)	+ (Mix)	+ (IVb)	−
NR112	−	−	−	−	−
NR113	+ (IVa)	+ (Mix)	+ (IIIb)	+ (IIIc)	+ (IIIb)
NR114	−	−	−	−	−
NR115	−	−	−	−	−
NR116	−	−	−	−	−
NR117	+ (IVa)	+ (IVa)	+ (IVa)	+ (IVa)	−
NR118	−	−	−	−	−
NR119	−	−	−	−	−
NR120	+ (IVa)	+ (IVa)	−	−	−
NR121	+ (IVa)	+ (IVa)	+ (IIIc)	+ (IIIc)	−
NR122	+ (IVa)	−	−	−	−
NR123	+ (IVa)	+ (IIIc)	+ (Mix)	−	+ (IIIc)
NR124	+ (Mix)	+ (Mix)	+ (IVa)	+ (IVa)	−
NR125	+ (IVa)	+ (IVa)	+ (IVa)	−	−
No. (%) positive	21 (61.8)	19 (55.9)	15 (44.1)	10 (29.4)	8 (23.5)

[a] Mix, mixed RFLP patterns of two ribotype groups.

caused by *B. japonica* have been documented in Japan, suggesting that this bacterium is nonpathogenic to humans.

Ixodes persulcatus, which belongs to the *I. ricinus* complex of ticks, serves as a vector for several *Borrelia* species associated with Lyme disease in Hokkaido, Japan. We developed an hypothesis that there are two enzootic cycles (bird-tick and rodent-tick) in nature that specifically maintain *Borrelia* species (10). The migratory birds *Emberiza spodocephala* and *Turdus crysolaus* have been incriminated as reservoirs for *B. garinii*. In contrast, the wood mouse (*Apodemus speciosus*) has a broad potential to serve as a reservoir for *B. afzelii* and unknown *Borrelia* species. In the study described here we captured wood mice to isolate spirochetes from their various organs and then identified the isolates by rRNA gene restriction fragment length polymorphism (RFLP) analysis. The main purpose of the survey was to examine whether mixed infections with different *Borrelia* species occur in wood mice.

Field sampling was carried out at a woodland on the shore of Lake Furen in Nemuro, Hokkaido, Japan (43°15′N, 142°25′E). Epidemiologic surveys in this woodland had revealed that *A. speciosus* is a dominant species among wild rodents (9) and that *B. garinii*, *B. afzelii*, and unknown *Borrelia* species are prevalent in unfed *I. persulcatus* adults (10). During October 1993, 34 *A. speciosus* were captured in Sherman box traps. These mice were designated by the numbers NR92 to NR125. The earlobes (right and left), heart, spleen, and urinary bladder from each mouse were used as culture sources. Blood was not used because spirochetemia is rare in the mouse (9). An ear punch of 2 mm in diameter (15) and minced tissues of internal organs (3 to 5 mm^3) were inoculated individually into 6 ml of BSK II medium (2) containing rifampin (50 μg/ml) in a culture tube. The tubes were incubated at 31°C and were examined for spirochetes by dark-field microscopy weekly for 4 weeks. Spirochetes from positive cultures were passaged once and were used for subsequent genomic analysis.

TABLE 2. Frequencies of isolation of *B. afzelii* and group IV among *A. speciosus*-derived isolates

Culture source	No. of isolates examined	No. (%) of isolates classified as *B. afzelii*	No. (%) of isolates classified as group IV
Earlobe[a]	40	3 (10.0)	31 (77.5)
Heart	15	6 (40.0)	7 (46.7)
Spleen	10	5 (50.0)	5 (50.0)
Urinary bladder	8	7 (87.5)	1 (12.5)

[a] Data for isolates from the right and left earlobes were combined.

Southern blot hybridization for the examination of rRNA gene RFLPs is based on the tandem repeat of the 23S-5S rRNA gene unit (14). Extraction of spirochete DNAs, digestion with restriction endonucleases, agarose gel electrophoresis, and Southern transfer were performed as described previously (4, 6, 10, 11). An enhanced chemiluminescence kit (ECL random prime labeling and detection systems; Amersham International, Tokyo, Japan) was used for genomic hybridization. The 23S rRNA gene fragments of *B. burgdorferi* B31, designated NP and Sty (6), were used as probes. Hybridization, subsequent washing, and signal detection were done as recommended by the manufacturer. On the basis of the Southern blot patterns, the spirochetes were classified into ribotype groups as described previously (10). In brief, groups I, II, and III correspond to *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively. Additionally, we designated groups IV, V, and VI for Japanese isolates which showed RFLP patterns distinct from those of the three species and *B. japonica*. Groups II, III, and IV were also divided into three subgroups each (designated a, b, and c). Definitive identification of groups IV, V, and VI awaits the completion of ongoing phylogenetic studies such as whole DNA-DNA hybridization and 16S rRNA sequencing. Therefore, in the present study we regarded the groups as unknown species.

Seventy-three spirochete isolates were established from 170 murine samples (Table 1). As a result of culture we judged that 21 (61.8%) of 34 captured mice were infected with spirochetes. Earlobe and heart samples were superior as culture sources. Ribotype classifications are presented in Table 1 and Fig. 1. None of the 73 isolates were classified as *B. burgdorferi* sensu stricto or *B. garinii*. Those isolates were *B. afzelii* (subgroups IIIb and IIIc) or unknown species (subgroups IVa, IVb, and IVc). More than one subgroup was frequently observed among isolates that originated from the same mouse. Seven mice (NR101, NR102, NR103, NR105, NR113, NR121, and NR123) showed mixed infection with *B. afzelii* and group IV. The frequencies of isolation of *B. afzelii* and group IV are summarized by culture source in Table 2. Group IV was predominant in the earlobe. In contrast, *B. afzelii* was isolated repeatedly from the internal organs. The frequency of *B. afzelii* was highest in the urinary bladder.

In Hokkaido, Japan, *B. garinii*, *B. afzelii*, and group IV are the main species that prevail in unfed *I. persulcatus* adults (10). Among them, group IV has been isolated repeatedly from the erythema migrans lesions of patients with Lyme disease (5, 10). We also detected this group from replete *I. persulcatus* larvae which had fed on birds and rodents (10). However, *B. garinii* was not found among rodent-derived isolates. In the present study, we reconfirmed that no *B. garinii* was isolated from *A. speciosus* mice. Although the mice were captured in a woodland where *B. garinii*, *B. afzelii*, and group IV are distributed, they were infected only with *B. afzelii* or group IV. Some mice showed mixed infections with both species. The reasons why no *B. garinii* exists in the mice are unclear. However, this surprising phenomenon enables us to speculate that the wood mouse is not susceptible to *B. garinii*.

We have shown previously that *A. speciosus* serves as a suitable host for *I. persulcatus* larvae and nymphs (9). Repeated nymphal bites may cause mixed infections with different *Borrelia* species in the mice. In group IV species, the heterogeneity of outer surface proteins (Osps) has been reported (5). In contrast, *B. afzelii* showed antigenic homogeneity of OspA (8, 11, 18). Studies on Lyme disease vaccine showed that vaccination of laboratory mice with OspA did not provide protection from borreliae with other OspA genotypes (3, 13). Our field data also indicate that cross-protection against infection with different *Borrelia* species is lacking in the rodent reservoir.

In reservoir hosts, the organotropic potentials of *Borrelia* species are important for their transmission to tick vectors. The microorganisms in cutaneous sites may be easily acquired by ticks. In the present study, we noticed that the frequencies of infection with *B. afzelii* and group IV organisms were different in murine organs used as culture sources. Our observation suggests that the skin is a suitable environment in which group IV survives for a long time. Experimental infection of *B. afzelii* or group IV in *A. speciosus* mice is necessary to clarify the organotropisms of these organisms.

In conclusion, the data presented in this report indicate that different *Borrelia* species can coexist in a reservoir host that is suitable for them. In other Eurasian countries where several species of Lyme disease pathogens are distributed, each species may be associated with particular reservoirs.

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