

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 crysolais* 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

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 <i>B. afzelii</i>	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.

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 mm³) 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.

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 IIb 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.

We thank Masahito Fukunaga, University of Fukuyama, Hiroshima, Japan, for advice in classifying *Borrelia* species.

This study was supported by grant-in-aid for scientific research 05670218 from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

- Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J. C. Piffaretti, M. Assous, and P. A. Grimont. 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. *Int. J. Syst. Bacteriol.* **42**:378–383.
- Barbour, A. G. 1984. Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med.* **57**:521–525.
- Fikrig, E., S. W. Barthold, D. H. Persing, X. Sun, F. S. Kantor, and R. A. Flavell. 1992. *Borrelia burgdorferi* strain 25015: characterization of outer surface protein A and vaccination against infection. *J. Immunol.* **148**:2256–2260.
- Fukunaga, M., and I. Mifuchi. 1989. The number of large ribosomal RNA genes in *Leptospira interrogans* and *Leptospira biflexa*. *Microbiol. Immunol.* **33**:459–466.
- Fukunaga, M., M. Sohnaka, M. Nakao, and K. Miyamoto. 1993. Evaluation of genetic divergence of borrelial isolates from Lyme disease patients in Hokkaido, Japan, by rRNA gene probes. *J. Clin. Microbiol.* **31**:2044–2048.
- Fukunaga, M., M. Sohnaka, and Y. Yanagihara. 1993. Analysis of *Borrelia* species associated with Lyme disease by rRNA gene restriction fragment length polymorphism. *J. Gen. Microbiol.* **139**:1141–1146.
- Kawabata, H., T. Masuzawa, and Y. Yanagihara. 1993. Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. *Microbiol. Immunol.* **37**:843–848.
- Marin Canica, M., F. Nato, L. du Merle, J. C. Mazie, G. Baranton, and D. Postic. 1993. Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. *Scand. J. Infect. Dis.* **25**:441–448.
- Nakao, M., and K. Miyamoto. 1993. Reservoir competence of the wood mouse, *Apodemus speciosus ainu*, for the Lyme disease spirochete, *Borrelia burgdorferi*, in Hokkaido, Japan. *Jpn. J. Sanit. Zool.* **44**:69–84.
- Nakao, M., K. Miyamoto, and M. Fukunaga. 1994. Lyme disease spirochetes in Japan: enzootic transmission cycles in birds, rodents, and *Ixodes persulcatus* ticks. *J. Infect. Dis.* **170**:878–882.
- Nakao, M., K. Miyamoto, M. Fukunaga, Y. Hashimoto, and H. Takahashi. 1994. Comparative studies on *Borrelia afzelii* isolated from a patient of Lyme disease, *Ixodes persulcatus* ticks, and *Apodemus speciosus* rodents in Japan. *Microbiol. Immunol.* **38**:413–420.
- Nakao, M., K. Miyamoto, K. Uchikawa, and H. Fujita. 1992. Characterization of *Borrelia burgdorferi* isolated from *Ixodes persulcatus* and *Ixodes ovatus* ticks in Japan. *Am. J. Trop. Med. Hyg.* **47**:505–511.
- Schaible, U. E., R. Wallich, M. D. Kramer, L. Gern, J. F. Anderson, C. Museteanu, and M. M. Simon. 1993. Immune sera to individual *Borrelia burgdorferi* isolates or recombinant OspA thereof protect SCID mice against infection with homologous strains but only partially or not at all against those of different OspA/OspB genotype. *Vaccine* **11**:1049–1054.
- Schwartz, J. J., A. Gazumyan, and I. Schwartz. 1992. rRNA gene organization in the Lyme disease spirochete, *Borrelia burgdorferi*. *J. Bacteriol.* **174**:3757–3765.
- Sinsky, R. J., and J. Piesman. 1989. Ear punch biopsy method for detection and isolation of *Borrelia burgdorferi* from rodents. *J. Clin. Microbiol.* **27**:1723–1727.
- Takahashi, Y., M. Sohnaka, M. Nakao, K. Miyamoto, and M. Fukunaga. 1993. Characterization of *Borrelia* species isolated from ixodid ticks, *Ixodes ovatus*. *Microbiol. Immunol.* **37**:721–727.
- van Dam, A. P., H. Kuiper, K. Vos, A. Widjojokusumo, B. M. de Jongh, L. Spanjaard, A. C. Ramselaar, M. D. Kramer, and J. Dankert. 1993. Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. *Clin. Infect. Dis.* **17**:708–717.
- Wilske, B., V. Preac-Mursic, U. B. Göbel, B. Graf, S. Jauris, E. Soutschek, E. Schwab, and G. Zumstein. 1993. An OspA serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal antibodies and OspA sequence analysis. *J. Clin. Microbiol.* **31**:340–350.