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オオアシトガリネズミが北海道におけるヤマトマダニ媒介性ボレリアの保菌動物である可能性

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Long-tailed shrew, *Sorex unguiculatus*, as a potential reservoir of the spirochetes transmitted by *Ixodes ovatus* in Hokkaido, Japan

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Abstract: To determine the vertebrate reservoirs for the spirochetes transmitted by *Ixodes ovatus*, the wood mice, *Apodemus speciosus ainu*, and the long-tailed shrews, *Sorex unguiculatus*, were captured in Furano, Hokkaido during October 1992. Larval and nymphal ticks of *I. ovatus* were more abundant on the shrews than on the mice. The spirochetes were isolated repeatedly from earlobe tissues of both the mice and the shrews. The spirochetal isolates derived from the shrews contained the 30 kDa homogeneous OspA protein, and were similar to the control strain of *I. ovatus* adult origin in their SDS-PAGE protein profile. In contrast, the isolates from the mice were polymorphic in their OspA proteins, and distinguishable from the shrew-derived isolates. All the spirochetal isolates from *I. ovatus* larvae feeding on the shrews were also identical with the control strain of *I. ovatus* adult origin. These data strongly suggest that *S. unguiculatus* is a reservoir for the spirochetes transmitted by *I. ovatus*. Similar enzootic cycles involving insectivores should be sought in the mainland of Honshu, Japan.

Since Baranton *et al.* (1992) delineated three genomic species of *Borrelia* associated with Lyme disease, *B. burgdorferi* sensu stricto, *B. garinii*, and group VS461, the spirochetal isolates derived from humans and ixodid ticks in the world have been reinvestigated and divided into several species groups by various techniques of molecular biology (Welsh *et al.*, 1992; Marconi and Garon, 1992; Boerlin *et al.*, 1992; Wilske *et al.*, 1993). In Japan, many spirochetal isolates were obtained mainly from the unfed adults of ixodid ticks, *Ixodes persulcatus* Schulze and *Ixodes ovatus* Neumann (Miyamoto *et al.*, 1992). These isolates have tentatively been identified as *B. burgdorferi* according to their reactivity to the monoclonal antibody H5332 and constituent protein profile (Nakao *et al.*, 1992b); however, further studies are required to determine their taxonomic status and pathogenicity. Previous report demonstrated that the isolates from *I. ovatus* adults collected at various localities in Japan were homogeneous and distinguishable from the heterogeneous isolates from *I. persulcatus* adults in their protein composition (Nakao *et al.*, 1992b). This phenomenon strongly suggests that the spirochetes of *I. ovatus* are a distinct species from those of *I. persulcatus*, and that each vector tick independently supports the enzootic transmission cycle together with vertebrate reservoirs. The prin-

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cipal reservoir host for the spirochetes transmitted by *I. persulcatus* in Hokkaido is the wood mouse, *Apodemus speciosus ainu* Thomas (Nakao and Miyamoto, 1993b). However, the reservoir hosts for the spirochetes transmitted by *I. ovatus* still remain to be determined. We report herein the repeated isolation of spirochetes from the long-tailed shrews, *Sorex unguiculatus* Dobson, and from *I. ovatus* larvae feeding on these hosts in an area of Hokkaido. These spirochetal isolates were characterized to be identical with the spirochetes isolated from unfed adults of *I. ovatus*.

Materials and Methods

During October 1992, 45 mice (*A. speciosus ainu*) and 13 shrews (12 *S. unguiculatus* and 1 *Sorex caecutiens saevus* Thomas) were captured in Sherman box traps (7×9× 29 cm) baited with raw peanuts at a forest in Furano (43°14′N, 142°24′E), Hokkaido. The traps were set in woodland-grass in the afternoon and recovered in the following morning. Although the mice were trapped alive, all the shrews died within the traps. These carcasses were held individually in sealed plastic bags. In the laboratory, all ticks attached on the mice were removed upon visual inspection under a magnifying glass. Ticks detached from the carcasses of shrews were recovered from the plastic bags, and the ticks still remaining on their bodies were also removed. The collected ticks were counted and identified by species and stages.

After collecting ticks, earlobe tissues of the mice and the shrews were cultured to detect spirochetal infections. The method for culturing the earlobe tissues described by Sinsky and Piesman (1989) was slightly modified as follows: The surface of earlobes was wetted with 10% povidone iodine solution (Isodine, Meiji Seika Co., Ltd., Tokyo, Japan) for 1 min, then cleaned with 70% ethanol. Subsequently, the skin samples (2 mm in diameter) were obtained from each ear with a rodent ear-notching punch (Natsume Seisakusho Co., Ltd., Tokyo, Japan) and inoculated individually into 6 ml of BSK II medium (Barbour, 1984) containing rifampin (50 μg/ml) in a culture tube. The single punch was taken from right earlobe of each shrew and the duplicate punches were taken from right and left earlobes of each mouse. Spirochetal culture was also conducted in the collected ticks. According to the methods of Miyamoto *et al.* (1992), the internal tissues of each tick (mainly midgut) were inoculated into 6 ml of BSK II medium. All the cultures were kept at 31°C and examined weekly for spirochetes by dark-field microscopy for 4 weeks. The positive cultures were passaged and used for immunochemical analysis.

The spirochetal isolates established in culture were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blots according to the methods described previously (Nakao *et al.*, 1992b). The North American B31 strain of *B. burgdorferi* (ATCC 35210) and the Japanese HT103 strain isolated from an unfed adult of *I. ovatus* in Furano were used as controls. Monoclonal antibodies H9724 and H5332 were used as probes for western blots. H9724 reacts with a protein of the periplasmic flagella of the genus *Borrelia* (Barbour *et al.*, 1986), and H5332 is specific for the outer surface protein A (OspA) of *B. burgdorferi* (Barbour *et al.*, 1983). The isolates that possessed the major 30 kDa (OspA) and 34.5 kDa (OspB-like) proteins were considered to be identical with the spirochetes transmitted by *I. ovatus*, because the unfed adults of *I. ovatus* in Japan harbored this characteristic strain (Nakao *et al.*, 1992b).

Abundance of ticks on host animals was statistically analyzed between the species of hosts by nonparametric Mann-Whitney *U*-test.

Results

Tick data

Two species of immature ticks, *I. ovatus* and *I. persulcatus*, were found on the shrews and the mice examined. A total of 33 larvae and 7 nymphs of *I. ovatus* and 10 larvae of

Table 1 Density of *Ixodes ovatus* and *Ixodes persulcatus* naturally infesting the long-tailed shrews and the wood mice collected in Furano, Hokkaido during October 1992.

Hosts*	No. examined	Mean no. of ticks±SD			
		I. ovatus		*I. persulcatus*	
		Larvae (range)	Nymphs (range)	Larvae (range)	Nymphs (range)
Shrews (Su)	12	2.75±3.96 (0–14)	0.58±0.51 (0–1)	0.83±0.83 (0–2)	0
Mice (Asa)	45	0.56±1.06 (0–4)	0.13±0.55 (0–3)	1.80±2.14 (0–8)	0.09±0.29 (0–1)

* Su, *Sorex unguiculatus*; Asa, *Apodemus speciosus ainu*.

Table 2 Detection of spirochetes from the shrews and the mice collected in Furano by culturing their earlobe tissues in BSK medium.

Hosts*	No. hosts examined	Earlobe cultures		No. hosts infected (%)
		No. examined	No. (%) positives	
Shrews (Su)	12	12	9 (75.0)	9 (75.0)
(Scs)	1	1	1 (100)	1 (100)
Mice (Asa)	45	90**	22 (24.4)	13 (28.9)

* Su, *Sorex unguiculatus*; Scs, *Sorex caecutiens saevus*; Asa, *Apodemus speciosus ainu*.
** The samples from mice were taken in duplicate.

I. persulcatus were collected from 12 shrews of *S. unguiculatus*. A total of 25 larvae and 6 nymphs of *I. ovatus* and 81 larvae and 4 nymphs of *I. persulcatus* were collected from 45 mice of *A. speciosus ainu*. No ticks were recovered from a shrew of *S. caecutiens saevus*. Mean numbers of ticks/host animal are shown in Table 1. Mann-Whitney *U*-test indicated that *I. ovatus* larvae and nymphs were more abundant on *S. unguiculatus* than on *A. speciosus ainu* ($p<0.01$). Immature *I. persulcatus* were less abundant on *S. unguiculatus* than on *A. speciosus ainu*; however, these values were not statistically significant. Of the ticks collected, 27 larvae and 7 nymphs of *I. ovatus* from the shrews, 10 larvae of *I. persulcatus* from the shrews, 17 larvae and 6 nymphs of *I. ovatus* from the mice, and 57 larvae and 4 nymphs of *I. persulcatus* from the mice were used for spirochetal cultures.

Spirochetal culture

As shown in Table 2, spirochetal cultures were attempted from 103 earlobe samples of 58 small mammals representing 3 species, and 32 positive cultures were obtained. Of these mammals, 9 *S. unguiculatus*, 1 *S. caecutiens saevus*, and 13 *A. speciosus ainu* were found to be infected with spirochetes. Spirochetal cultures were also successful in both the immature ticks of *I. ovatus* and *I. persulcatus* collected from these mammals (Table 3). Spirochetes were isolated from 14 (51.9%) of 27 *I. ovatus* larvae feeding on 8 *S. unguiculatus* and from 5 (29.4%) of 17 *I. ovatus* larvae feeding on 11 *A. speciosus ainu*. From all the ticks examined, 35 positive cultures were obtained.

Table 3 Detection of spirochetes from immature ticks infesting the shrews and the mice collected in Furano by culturing their midgut tissues in BSK medium.

Hosts*	Tick species	Tick stages	No. examined	No. (%) positives
Shrews (Su)	*I. ovatus*	Larva	27	14 (51.9)
		Nymph	7	2 (28.6)
	I. persulcatus	Larva	10	0 (0)
Mice (Asa)	*I. ovatus*	Larva	17	5 (29.4)
		Nymph	6	2 (33.3)
	I. persulcatus	Larva	57	10 (17.5)
		Nymph	4	2 (50.0)

* Su, *Sorex unguiculatus*; Asa, *Apodemus speciosus ainu.*

Table 4 Reactivity of monoclonal antibodies H9724 and H5332 against the isolates from shrews, mice, and immature ticks by western blots.

Culture sources*	No. isolates examined	No. reactive isolates	
		H9724	H5332
Earlobes of shrews (Su)	9	9 (41 kDa)**	9 (30 kDa)
Earlobe of shrew (Scs)	1	1 (41 kDa)	1 (30 kDa)
Earlobes of mice (Asa)	22	22 (41 kDa)	18 (30–32 kDa)
I. ovatus larvae on shrews (Su)	14	14 (41 kDa)	14 (30 kDa)
I. ovatus nymphs on shrews (Su)	2	2 (41 kDa)	2 (30 kDa)
I. persulcatus larvae on mice (Asa)	10	10 (41 kDa)	10 (30–31.5 kDa)
I. persulcatus nymphs on mice (Asa)	2	2 (41 kDa)	2 (30–33 kDa)
I. ovatus larvae on mice (Asa)	5	5 (41 kDa)	4 (30–31 kDa)
I. ovatus nymphs on mice (Asa)	2	2 (41 kDa)	2*** (30–31 kDa)

* Su, *Sorex unguiculatus*; Scs, *Sorex caecutiens saevus*; Asa, *Apodemus speciosus ainu.* ** Molecular weights of proteins that reacted with monoclonal antibodies. *** One isolate showed the protein profile identical with the control strain of *I. ovatus* adult origin.

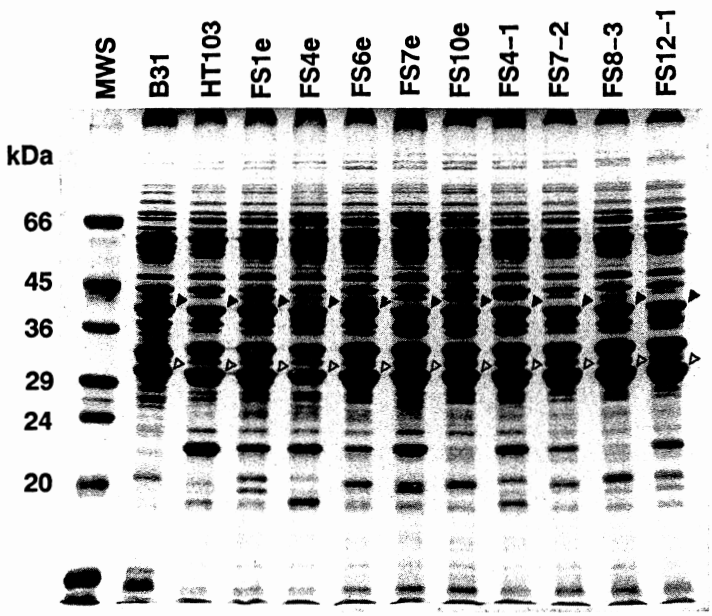

Fig. 1 Coomassie brilliant blue-stained proteins of the spirochetal isolates in a discontinuous SDS-PAGE gel (12.5%).

MWS, molecular weight standard; B31, the type strain of *B. burgdorferi*; HT103, the control strain of *I. ovatus* adult origin; FS1e, FS4e, FS6e, FS7e, and FS10e, the isolates from *S. unguiculatus*; FS4-1, FS7-2, FS8-3, and FS12-1, the isolates from *I. ovatus* larvae feeding on the shrews. Closed and open arrowheads indicate the 41 kDa flagellin and OspA proteins respectively.

Characterization of spirochetes

As summarized in Table 4, and Figs. 1 and 2, 67 isolates from shrews, mice, and immature ticks were analyzed by SDS-PAGE and western blots. None of the 67 isolates were identical to the North American B31 strain used in the comparison of protein composition. The epitope for H9724 was present in the 41 kDa flagellin protein of all isolates examined in this study. Ten isolates obtained from 9 *S. unguiculatus* and 1 *S. caecutiens saevus* reacted with H5332 and showed similar protein profile to each other. These isolates possessed the 30 kDa homogeneous OspA proteins and the 34.5 kDa homogeneous OspB-like proteins (Fig. 1). Moreover, 16 isolates obtained from 14 larvae and 2 nymphs of *I. ovatus* feeding on 8 *S. unguiculatus* also contained the 30 kDa homogeneous OspA proteins and the 34.5 kDa homogeneous OspB-like proteins. All isolates from the shrews and from immature *I. ovatus* feeding on these hosts were identical to the HT103 strain of *I. ovatus* adult origin. In contrast, 18 (81.8%) out of 22 isolates obtained from 13 *A. speciosus* ainu reacted with H5332, and these isolates possessed the 30–32 kDa heterogeneous OspA proteins (Fig. 2). These mouse-derived isolates were distinguishable from the shrew-derived isolates. Nineteen isolates obtained from 10 larvae and 2 nymphs of *I. persulcatus* feeding on 6 *A. speciosus ainu* and 5 larvae and 2 nymphs of *I. ovatus* feeding on 5 *A. speciosus ainu* were also polymorphic in their OspA proteins. Of these tick-derived isolates, one isolate from a nymph of *I. ovatus* feeding on *A. speciosus ainu* was identical to the HT103 strain.

Discussion

Borrelia burgdorferi sensu stricto may not be distributed in Japan because many spiro-

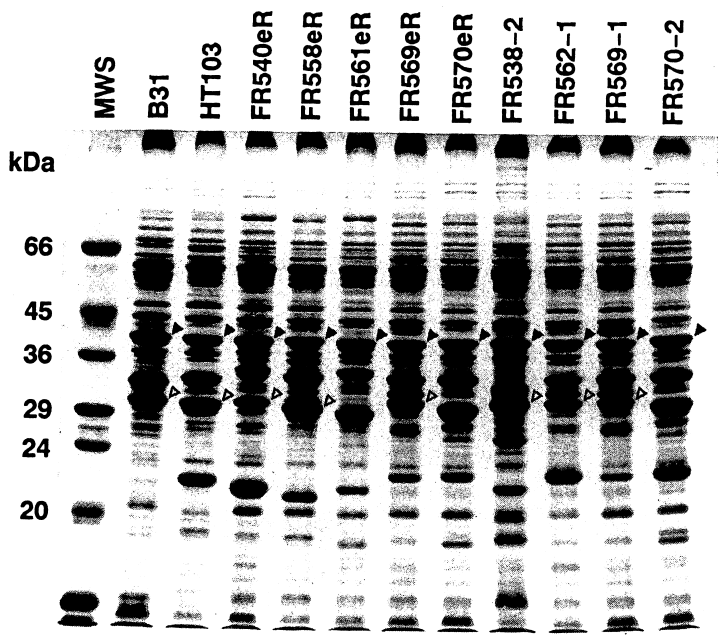

Fig. 2 Coomassie brilliant blue-stained proteins of the spirochetal isolates in a discontinuous SDS-PAGE gel (12.5%).

MWS, molecular weight standard; B31, the type strain of *B. burgdorferi*; HT103, the control strain of *I. ovatus* adult origin; FR540eR, FR558eR, FR561eR, FR569eR, and FR570eR, the isolates from *A. speciosus ainu*; FR538-2, FR562-1, and FR569-1, the isolates from *I. persulcatus* larvae feeding on the mice; FR570-2, the isolate from *I. ovatus* larva feeding on the mouse. Closed and open arrowheads indicate the 41 kDa flagellin and OspA proteins respectively.

chetal isolates obtained from various tick species, rodents, and humans are apparently different from the B31 strain of *B. burgdorferi* in their protein composition (Nakao *et al.*, 1992a, b; Nakao and Miyamoto, 1993a, b). The species of spirochetes transmitted by *I. persulcatus* in Japan include *B. garinii*, group VS461, and probably some new species (Baranton *et al.*, 1992; Fukunaga *et al.*, 1993b, c). The spirochetes transmitted by *I. ovatus* are suspected to be a new species that is distinct from the spirochetes of *I. persulcatus* (Takahashi *et al.*, 1993). Ecological data on the transmission dynamics of these spirochetes in nature will provide valuable suggestions on the taxonomic status of spirochetes, and also on the evolution of "vector-reservoir" relationship. The wood mouse, *Apodemus speciosus ainu*, has been implicated as a primary reservoir host of the spirochetes transmitted by *I. persulcatus* in Hokkaido (Miyamoto *et al.*, 1991; Nakao and Miyamoto, 1993b); however, no wild animals have been confirmed as the reservoirs for the spirochetes transmitted by *I. ovatus*. This study is the first reported demonstration of a potential reservoir for the spirochetes transmitted by *I. ovatus* in Hokkaido.

The host range of immature *I. ovatus* is limited to small mammals belonging to the orders of Rodentia and Insectivora (Hoogstraal *et al.*, 1973; Takada and Yamaguchi, 1974; Fujita *et al.*, 1981; Fujimoto *et al.*, 1986). Nevertheless, little information on the host preference of its immature ticks are available considering the spirochetal transmission in nature. The kind of animals on which immature *I. ovatus* most abundantly feed would be the reservoirs for the spirochetes transmitted by *I. ovatus*. In our study site, the larvae and nymphs were more

abundant on *S. unguiculatus* than on *A. speciosus ainu*. This host preference is probably related to the questing behavior of *I. ovatus*. The habitat of its immature ticks is thought to be the humid environment under litter layer. Since the shrews principally utilize the underground as living space, the questing larvae and nymphs may encounter the shrews more frequently than the mice. Further studies are required to determine the host preferential factors of immature *I. ovatus*.

In this study, both the shrews and the mice were found to be highly infected with spirochetes. All the spirochetal isolates derived from *S. unguiculatus* and from immature *I. ovatus* feeding on the shrews were homogeneous and identical with the control strain of *I. ovatus* adult origin in their protein profile and reactivity of monoclonal antibodies. In contrast, considerable heterogeneity existed among the spirochetal isolates derived from *A. speciosus ainu* and from immature *I. persulcatus* feeding on the mice, and these isolates were distinguishable from the control strain of *I. ovatus* adult origin. These data implicated *S. unguiculatus* as a likely enzootic reservoir of the spirochetes transmitted by *I. ovatus*. Xenodiagnostic method using the spirochete-free *I. ovatus* larvae is necessary for determination of the reservoir host. Definitive evidence of the reservoir competence of *S. unguiculatus* awaits completion of ongoing studies.

Although the host ranges of *I. ovatus* and *I. persulcatus* partially overlap each other in their immature stages, the types of spirochetes isolated from unfed adults of both species are clearly distinguishable (Nakao *et al.*, 1992b). This phenomenon strongly suggests that the spirochetes are strictly selected through the molting process of these ticks. In this study, all the isolates from *I. ovatus* larvae feeding on the mice were heterogeneous in their protein profile, and similar character was observed in the mice-derived isolates rather than the shrew-derived isolates. These data indicate the possibility that *I. ovatus* larvae accidentally ingest the spirochetes by feeding on the mice.

If *S. unguiculatus* proves to be a principal reservoir of the spirochetes transmitted by *I. ovatus* in Hokkaido, the dual enzootic cycles of spirochetal transmission, *i.e.*, "*I. persulcatus–A. speciosus ainu*" and "*I. ovatus–S. unguiculatus*" cycles, may exist in nature. These novel transmission cycles of the different spirochetes are maintained possibly by the following factors: (1) the difference of host preference between *I. persulcatus* and *I. ovatus*, (2) the susceptibility of reservoir animals to the spirochetes, (3) the selection of spirochetes through the molting process of vector ticks. Further studies will explain the formative processes of these complicated transmission cycles.

No reservoir animals have been found in the mainland of Honshu, Japan, although the spirochetes were isolated from unfed adults of *I. ovatus* collected in various localities (Miyamoto *et al.*, 1992; Nakao *et al.*, 1992b). The data presented in this report strongly suggest that the animals belonging to the Insectivora are candidates of the reservoirs for the spirochetes transmitted by *I. ovatus*. The enzootic cycles involving insectivores (*e.g.*, shrews, mole-shrews, and moles) should be sought in the Honshu regions.

The pathogenicity of the spirochetes transmitted by *I. ovatus* is unknown; however, we regard these spirochetes as a low-virulent agent to humans because no cases of Lyme disease caused by *I. ovatus* have been confirmed in Japan in spite of the abundance of human tick bites by this species. Recently, we isolated the spirochetes from skin lesions of 8 patients with erythema chronicum migrans in Hokkaido. These human-derived isolates were polymorphic in their OspA proteins and identical to the isolates of *I. persulcatus* adult origin (Nakao *et al.*, 1992a; Fukunaga *et al.*, 1993a).

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摘　要

オオアシトガリネズミが北海道における ヤマトマダニ媒介性ボレリアの 保菌動物である可能性

シュルツェマダニとヤマトマダニの未吸血成虫はおのおの異なったボレリアを保有している．北海道富良野市の森林において秋に小哺乳類を捕獲し，寄生マダニ相とボレリア感染状況を調査した．寄生マダニ相ではエゾアカネズミにシュルツェマダニ幼若虫，オオアシトガリネズミにヤマトマダニ幼若虫が優位であった．ボレリアはエゾアカネズミとオオアシトガリネズミから高率に分離された．オオアシトガリネズミ由来株はヤマトマダニ未吸血成虫由来株と同一で，エゾアカネズミ由来株はシュルツェマダニ未吸血成虫由来株と同一だった．また，オオアシトガリネズミ寄生ヤマトマダニ幼若虫から分離した株もヤマトマダニ未吸血成虫由来株と同一だった．以上の結果は食虫類がヤマトマダニ媒介性ボレリアの主要な保菌動物であることを強く示唆している．