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CHARACTERIZATION OF *BORRELIA* SPP. ISOLATED FROM THE TICK, *IXODES TANUKI* AND SMALL RODENTS IN JAPAN

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ABSTRACT: Spirochetes were isolated from the tick, *Ixodes tanuki,* as well as wood mice (*Apodemus speciosus*) and voles (*Clethrionomys rufocanus* and *Eothenomys smithii*), caught in Fukui, Tokushima, and Hokkaido, Japan, from 1991 to 1993. Spirochetes were characterized on the basis of protein profiles, reactivities with monoclonal antibodies (mAbs), Outer surface protein A gene (*ospA*) and Outer surface protein B gene (*ospB*) amplification analysis, rRNA gene and flagellin gene restriction fragment length polymorphism (RFLP) analysis, and DNA homology values. Protein profiles of all isolates were homologous and reacted with mAb to OspA, OspB, OspC, flagellin, and heat shock protein 60. The primer reactivity to *ospA* and *ospB* were different from those of *Borrelia burgdorferi* sensu stricto, *B. afzelii, B. japonica,* and *B. garinii.* Based on the DNA/DNA homology value and RFLP analysis of rRNA and flagellin gene, these *Borrelia* sp. isolates are a new group of *B. burgdorferi* sensu lato. The isolates from ticks and the host rodents were identical in these assays. We propose that these *Borrelia* sp. are adapted to *I. tanuki* and are maintained in these field rodents.

Key words: *Borrelia burgdorferi* sensu lato, Lyme disease, *Ixodes tanuki, Apodemus* sp., *Eothenomys* sp., *Clethrionomys* sp.

INTRODUCTION

Many *Borrelia* spp. strains have been isolated from ixodid ticks, reservoir animals, and Lyme disease patients in the world. Based on genomic DNA homology-values, *Borrelia burgdorferi* sensu lato, the cause of Lyme disease, is classified into four genospecies, *B. burgdorferi* sensu stricto (Johnson et al., 1984) isolated in North America and Europe, *Borrelia garinii* (Baranton et al., 1992) and *Borrelia afzelii* (Canica et al., 1993) isolated in Europe, and *Borrelia japonica* (Kawabata et al., 1993) isolated from *Ixodes ovatus* in Japan. Furthermore, Postic et al. (1994) recognized four new genomic groups among isolates in North America (Group DN127 and Group 21123) and Europe (Group VS116 and Group PotiB2). Casjens et al. (1995) found three genetic types (Types 21038, 25015, and CA55) in Group 21123. Types 21038, isolated from *I. dentatus* and cotton tail rabbit, has been renamed *B. andersonii* (Marconi et al., 1995). Spirochetes in Groups DN127 and 21123 had vectors with enzootic cycles quite different from those of *B. burgdorferi* sensu stricto (Postic et al., 1994).

Borrelia spp. was isolated from ixodid ticks, *Ixodes persulcatus* (Masuzawa et al., 1991b), *I. ovatus* (Masuzawa et al., 1991a), *Ixodes tanuki, Ixodes turdus,* and *Ixodes columnae* (Nakao and Miyamoto, 1993a) in Japan. Among these species, *I. persulcatus* is a member of the *Ixodes ricinus* complex of ticks which appear to be important as vectors of Lyme disease in Asia (Anderson, 1989). *Ixodes persulcatus* and *I. ovatus* are the dominant species in the northern part of Japan, and their prevalences of infection by spirochetes are similar to those of *Ixodes scapularis* and *I. ricinus* (Uchikawa et al., 1991).

Interestingly, isolates from *I. ovatus* are homogeneous in phenotypic, and genetic characteristics and reactivities with various monoclonal antibodies, and were classified as *B. japonica* (Kawabata et al., 1993). Reservoir hosts for *B. japonica* include a shrew (*Sorex unguiculatus*) and a vole (*Clethrionomys rufocanus*) in Hokkaido, the northernmost island of Japan, and

mice (*Apodemus specious, Apodemus argenteus*) and a vole (*Eothenomys smithii*) on Honshu Island, the main island of Japan (Nakao and Miyamoto, 1993b; Nakao et al., 1994a). Yet, isolates of *B. burgdorferi* from *I. persulcatus* and patients were heterogeneous and distinguishable from *B. japonica* (Nakao et al., 1992; Fukunaga et al., 1993b) and some of them were identified as *B. garinii* and *B. afzelii* (Fukunaga et al., 1993a).

Previously unknown *Borrelia* spp. isolated from *I. tanuki* collected in Honshu and *C. rufocanus* voles captured in Hokkaido had a homologous molecular mass of OspA- and OspB-ranging proteins (Nakao and Miyamoto, 1993a). The isolates from Honshu tentatively were named "It type *Borrelia*" (Ishiguro et al., 1994). The reservoir, *C. rufocanus,* is not distributed in Honshu island. We previously proposed that a wood mouse *A. speciosus* and a vole *E. smithii* were the initial reservoirs for *Borrelia* spp. in Honshu island (Ishiguro et al., 1995; Masuzawa et al., 1995).

In the present study, our objective was to characterize the *Borrelia* spp. isolates from field rodents and ticks which feed on mammals caught in Honshu, Hokkaido, and the southwestern island, Shikoku; we also determined the taxonomic position of these spirochetes.

MATERIALS AND METHODS

The small rodents were captured by Sherman live traps (H.B.Sherman Trap, Inc. Tallahassee, Florida, USA). Ticks feeding on small rodents and a raccoon dog (*Nyctereutes procyonoides albus*) were collected from 1991 to 1993 (Table 1). Spirochetes were isolated from earlobe and heart tissues of three species of rodents (*A. speciosus, E. smithii,* and *C. rufocanus*) and midgut of two species of ticks (*I. tanuki* and *I. ovatus*) by the method of Miyamoto et al. (1991) (Table 1). It type isolates, Hk501, OR1eR, OR2eL, and OR3eL were a gift from M. Nakao. They were cultivated at 32 C for less than seven passages in Barbour-Stoenner-Kelly II medium (Barbour, 1984). For controls, strains B31 (ATCC 35210), 20047 (CIP103362), VS461 (CIP103469), HO14 (JCM8951), and HS1 were used for the type strains of *B. burgdorferi* sensu stricto, *B. garinii, B. afzelii, B. japonica,* and *B. hermsii,* respectively. For the polymerase chain reaction (PCR) study, *B. garinii* strain PBi also was used.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses were carried out by the method of Masuzawa et al. (1991b). Samples were subjected to electrophoresis on 12.5% polyacrylamide (Wako Pure Chemical Co., Osaka, Japan) gels and stained with Coomassie brilliant blue (Laemmli, 1970). Antigens were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Richmond, California, USA) from the gels. Specific antigen bands were detected by immunostaining with monoclonal antibody (mAb) H9724 reactive to flagellin, mAb O1441b specific for *B. japonica* flagellin (Masuzawa et al., 1994), mAb H5332 reactive to outer surface protein (Osp) A, and mAbs P62 and 062 (Masuzawa et al., 1994) reactive to 62 kiloDalton (kDa) antigen which were a cross-reactive protein of heat shock protein 60 (Hsp 60). Monoclonal antibodies H9724 and H5332 were a gift from A.G. Barbour. Monoclonal antibodies P3134 reactive to OspB, and G7 reactive to OspC were prepared from mice immunized with strains NT24 and HP1 isolated from *I. persulcatus* in Nagano prefecture and Hokkaido, Japan, respectively (Masuzawa et al., 1994), by the cell fusion method (Masuzawa et al., 1988). This reactivity and specificity to OspB and OspC were confirmed using various Japanese isolates (T. Masuzawa, unpubl.).

The primers used in this analysis were OSP-A1, OSP-A2, OSP-A4, and B9, described by Persing et al. (1990) and Jonsson et al. (1992). We conduct a PCR targeting outer surface protein A and B gene (*ospAB*), and following restriction fragment length polymorphism (RFLP) analysis, by the method of Kawabata et al. (1994). Briefly, the PCR was set up with a final mixture containing each deoxynucleotide triphosphate at 200 μm, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 0.01% (wt/vol) gelatin (Sigma, St. Louis, Missouri, USA), and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut, USA) as well as 0.4 μM of each primer in a volume of 100 μl. Purified DNA (0.5 μg) was amplified for 30 cycles using a denaturing step (92 C for 30 sec), annealing (41 C for 30 sec) and extension step (72 C for 90 sec). After amplification, the sample was analyzed on 0.8% agarose-0.5 × tris borate ethylenediamine tetraacetate buffer gels. The DNA fragment amplified with OspA1 and B9 primer set was further characterized by subsequent endonuclease digestion using 15 U of *Hind* III, *Sty* I, *Pst* I and *Eco*R I according to the manufacturer's instructions.

TABLE 1. *Borrelia* spp. strains used in this study, 1991 to 1993.

Strains and isolates	Location	Biological source	Source
B. burgdorferi			
B31 (ATCC35210)	New York, USA	*Ixodes scapularis*	R. C. Johnson
B. garinii			
20047 (CIP103362)	Brittany, France	*Ixodes ricinus*	G. Baranton
PBi	Munich, Germany	Human cerebrospinal fluid	B. Wilske
B. afzelii			
VS461 (CIP103469)	Vouvry, Valais, Switzerland	*I. ricinus*	G. Baranton
B. japonica			
HO14 (JCM8951)	Hokkaido, Japan	*Ixodes ovatus*	N. Sato
It type isolates			
Fi81t	Fukui, Honshu, Japan	*Ixodes tanuki* nymph feeding on *Eothenomys smithii*	Our collection
Fi03o	Fukui, Honshu, Japan	*I. ovatus* larva feeding on *E. smithii*	Our collection
FiEE9	Fukui, Honshu, Japan	*E. smithii*	Our collection
FiAH1	Fukui, Honshu, Japan	*Apodemus speciosus*	Our collection
TkAE2	Tokushima, Shikoku, Japan	*A. speciosus*	Our collection
TkAE3	Tokushima, Shikoku, Japan	*A. speciosus*	Our collection
TkAE6	Tokushima, Shikoku, Japan	*A. speciosus*	Our collection
TkAE8	Tokushima, Shikoku, Japan	*A. speciosus*	Our collection
Hk501	Hokkaido, Japan	*I. tanuki* adult female feeding on *Nyctereutes procyonoides albus*	M. Nakao
OR1eR	Hokkaido, Japan	*Clethrionomys rufocanus*	M. Nakao
OR2eL	Hokkaido, Japan	*C. rufocanus*	M. Nakao
OR3eL	Hokkaido, Japan	*C. rufocanus*	M. Nakao
Other *Borrelia* spp.			
B. hermsii			
HS1	Washington, USA	*Ornithodoros hermsi*	R. C. Johnson

Purified *Borrelia* spp. DNAs digested with *Hind* III were electrophoresed in 1.0% agarose gel and transferred to nylon membranes (Hybond N+, Amersham, United Kingdom). The oligonucleotide probes used in this study were BBU30 (5′-AACTTCCTCTATCAGACTCTAGACATATAG-3′), complementary to region V4 of 16S rRNA gene and BBFN27 (5′-AGCTGATGTATTATGATTGATAATCAT-3′), and complementary to the 5′ end of the flagellin structural gene (Adam et al., 1991). The chemically synthesized probes were radio-labeled at the 5′ end with T4 polynucteotide kinase (Takara Shuzo Co., Otsu, Japan) and [γ^{32}P]-dATP. (NEN, 6,000Ci/mmol) according to the manufacturer's instruction. Hybridization was carried out by the method of Adam et al. (1991).

The DNA was extracted from a 50-ml culture and purified by the methods of Kawabata et al. (1993). Purified DNAs were used to determine the DNA-DNA homology value by the microplate hybridization method (Kawabata et al., 1993). The hybridization was performed with a hybridization solution containing 50% formamide (Wako Pure Chemical Co., Osaka, Japan) at 30 C.

RESULTS

Protein profiles of isolates tested were homogeneous (Fig. 1) and had a 41kDa flagellin protein that reacted with mAb H9724, but not with mAb 01441b. Also, these isolates had a 32 kDa Osp A, a 36 kDa Osp B, and a 61 kDa Hsp 60 (Table 2). The isolates had 22 to 23 kDa OspC which were reactive to mAb G7, but isolate Hk501 had a low expression or absence of OspC on this isolate.

In the RFLP analysis on the 16S rRNA

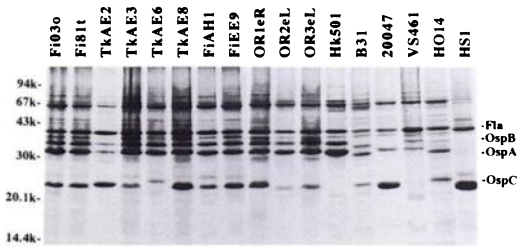

FIGURE 1. SDS-PAGE profiles of isolates used in this study. Molecular mass of standard proteins (kDa) is indicated at left. Location of flagellin (Fla), OspA, OspB, and OspC is indicated at right. Strains B31, 20047, VS461, HO14, and HS1 for the type strains of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. japonica*, and *B. hermsii*, respectively, were used as a standard.

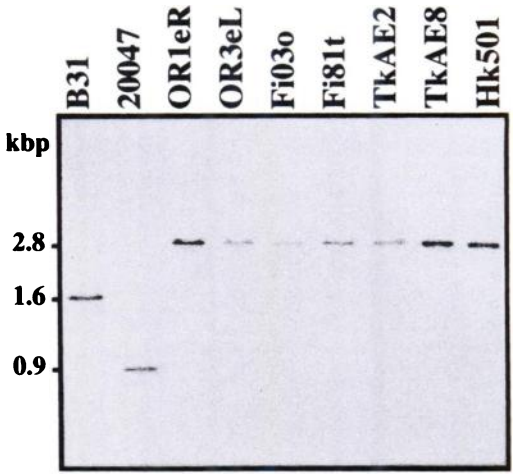

FIGURE 2. Genomic fingerprint of *Hin*dIII-digested DNA with ^{32}P-labeled oligonucleotide probe BBFN27 complementary to 5′ end of the flagellin structural gene. We used *B. burgdorferi* sensu stricto strain B31 and *B. garinii* strain 20047 as controls. Size standards are indicated to the left.

gene and flagellin gene, It type isolates generated 2.8 kilobase (kb) and 1.5 kb fragments on probing with BBFN27 (Fig. 2) and BBU30, respectively (Table 3). The 1.5 kb fragment hybridized with BBU30 was also observed in *B. burgdorferi* sensu stricto and *B. afzelii*. On the other hand, the 2.8 kb fragment detected with BBFN27 was not observed in *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, or *B. japonica*. Specific DNA amplification was observed on the spirochetes with OspA1/OspA2, and OspA1/B9 primer sets, but not with OspA4/B9 (Table 4). The amplified fragments with the OspA1/B9 primer set, with the exception of isolate OR1eR, were not digested by *Hin*d III, *Eco*R I, *Sty* I, and *Pst* I; in contrast, those of isolate OR1eR was digested by *Pst* I and generated 0.9 kb and 0.6 kb fragments. The resistance of amplified DNA against these restriction enzymes tested was the same as that of some *B. garinii* strains.

The DNA homology of the four representative isolates, Fi03o, Fi81t, TkAE2, and OR3eL, to strains B31 of *B. burgdorferi* sensu stricto, 20047 of *B. garinii*, VS461 of *B. afzelii* and HO14 of *B. japonica* ranged from 32% to 58% (Table 5). By contrast, the DNA homology of these isolates to isolate Fi03o, isolated from larval *I. ovatus* feeding on *E. smithii*, ranged from 79% to 99%.

DISCUSSION

Ixodes tanuki is distributed from Japan up to China and Nepal. The adult stage

TABLE 2. Reactivity of the isolates used in this study with monoclonal antibodies.

	Monoclonal antibodies						
	Hsp 60		Flagellin		OspB	OspA	OspC
Strains	O62	P62	H9724	O1441b	P3134	H5332	G7
It type isolates	+	+	+	−	+	+	+
B31 (*B. burgdorferi*)	+	+	+	−	−	+	+
20047 (*B. garinii*)	+	+	+	−	−	+	+
VS461 (*B. afzelii*)	+	+	+	−	−	−	+
HO14 (*B. japonica*)	+	−	+	+	−	+	+
HS1 (*B. hermsii*)	+	−	+	−	−	−	−

TABLE 3. Genomic fingerprint of *Hind* III-digested DNA with oligonucleotide probe BBU30 and BBFN 27[a].

Strains	Fragment size (kb) probed with	
	BBFN27	BBU30
It type isolates	2.8	1.5
B31 (*B. burgdorferi*)	1.6	1.5
20047 (*B. garinii*)	0.9	1.2
VS461 (*B. afzelii*)	0.9	1.5
HO14 (*B. japonica*)	0.9	2.1

[a] Oligonucleotide probes BBFN27 and BBU30, were complementary to the 5′ end of the flagellin structural gene and to region V4 of the 16S rRNA gene, respectively.

parasitizes raccoon dogs (*Nyctereutes procyonoides albus*) and occasionally humans, and wild animals; its larvae and nymphs have been found on field rodents (Takada, 1990). Previously, Nakao and Miyamoto (1993a) suggested that *C. rufocanus* was one reservoir host of It type *Borrelia* on Hokkaido Island. Furthermore, the antibody against strain Fi81t isolated from *I. tanuki* was most prevalent among *A. speciosus* and *E. smithii* in Honshu Island by indirect immunoperoxidase test (Ishiguro et al., 1995).

Since It type *Borrelia* from *I. tanuki* and small mammals have proteins corresponding to OspA, OspB and OspC, these isolates should be classified as *B. burgdorferi* sensu lato. Furthermore, It type *Borrelia* probably do not to belong to *B. japonica*, based on their reactivity with mAbs against Hsp60 and flagellin (Masuzawa et al., 1994). Based on the results from RFLP analysis on the 16S rRNA gene and flagellin gene, PCR of the *ospAB* amplicon, and DNA hybridization, It type *Borrelia* isolates should be classified as a new genomic group. From our findings, we believe that It type *Borrelia* is also maintained in the field rodent, *A. speciosus*, and *E. smithii* on the Honshu and Shikoku Islands. Furthermore, *I. ovatus* commonly feeds on these field rodents and maintains *B. japonica* (Masuzawa et al., 1995). One isolate, Fi03o from *I. ovatus* larva feeding on *E. smithii*, was also identified as the It type *Borrelia*. Isolation of It type *Borrelia* from *I. ovatus* adults collected by flagging or dragging vegetation has not been documented previously; furthermore, transovarial transmission of *Borrelia* spp. also has not been observed in *I. ovatus* (Nakao and Miyamoto, 1992). Therefore, we infer that the *I. ovatus* larva was infected with the spirochetes during feeding on *E. smithii* and the spirochetes originated from the animal. Based on these findings, we believe that each *Borrelia* sp. is maintained in the same species of field rodents and adapted independently to its own specific tick vector.

The pathogenicity of It type *Borrelia* to humans and animals is unknown and no human cases of Lyme disease from *I. ta-*

TABLE 4. OspA and OspB gene amplification and restriction endonuclease digestion of the amplified fragment.

Strains	Amplified fragment size (kb)			Fragment size generated by endonuclease digestion (kb)			
	OspA1/ OspA2	OspA4/ B9	OspA1/ B9	*Hind*III	*Eco*RI	*Sty*I	*Pst*I
OR1eR (It type isolate)	0.6	—[a]	1.5	1.5	1.5	1.5	0.9 + 0.6
It type isolates	0.6	—	1.5	1.5	1.5	1.5	1.5
B31 (*B. burgdorferi*)	0.6	1.0	1.5	1.4 + 0.1	1.0 + 0.5	1.5	0.9 + 0.5 + 0.1
20047 (*B. garinii*)	—	—	—	NT[b]	NT	NT	NT
P/Bi (*B. garinii*)	—	—	1.5	1.5	1.5	1.5	1.5
VS461 (*B. afzelii*)	—	1.0	1.5	1.0 + 0.5	1.5	1.5	1.5
HO14 (*B. japonica*)	—	—	1.5	1.0 + 0.5	1.2 + 0.3	1.1 + 0.4	1.5

[a] No amplified products were obtained.
[b] NT, not tested.

TABLE 5. Genomic DNA homology value of *Borrelia* spp. strains.

Labeled DNA	Relative fluorescent value (%)					
	B31	20047	VS461	HO14	*B. hermsii*	Fi03o
B31 (*Borrelia burgdorferi*)	100	56	55	56	NT[a]	NT
20047 (*Borrelia garinii*)	56	100	61	63	NT	NT
VS461 (*Borrelia afzelii*)	51	59	100	64	NT	NT
HO14 (*Borrelia japonica*)	53	63	64	100	NT	NT
It type isolates						
Fi03o	37	39	47	58	3	100
Fi81t	34	34	40	56	2	99
TkAE2	34	35	37	45	1	79
OR3eL	32	44	40	57	1	84

[a] NT, Not tested.

nuki bites have been reported in Japan. Further studies on the infectivity and virulence of It type *Borrelia* are needed to understand the etiology of this disease.

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