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FULL PAPER *Public Health*

U.S.-Type *Babesia microti* Isolated from Small Wild Mammals in Eastern Hokkaido, Japan

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ABSTRACT. Our previous report demonstrated that small wild rodents in Japan harbored two types of novel *Babesia microti*-like parasites (Kobe and Hobetsu types), but not the type widely distributed throughout the temperate zones of North American and Eurasian Continents (U.S. type). In this study, we surveyed small wild mammals collected at various places in the northern part of Japan, seeking for U.S.-type *B. microti*. A total of 197 small mammals comprising 10 species, *Apodemus speciosus, A. argenteus, Clethrionomys rufocanus, C. rutilus, Eothenomys andersoni, Microtus montebelli, Tamias sibiricus, Sorex unguiculatus, S. caecutiens,* and *Urotrichus talpoides*, were examined. *Babesia* parasites were detected in *A. speciosus, C. rufocanus, C. rutilus, M. montebelli, S. unguiculatus,* and *S. caecutiens* by microscopy of blood smears and by PCR targeting babesial nuclear small-subunit rRNA (rDNA) and β-tubulin genes. Inoculation of their bloods into experimental animals gave rise to 23 parasite isolates, which included 16 from *A. speciosus*, 4 from *C. rufocanus*, and 1 each from *C. rutilus, M. montebelli* and *S. unguiculatus*. Sequencing analyses of their rDNA and β-tubulin genes revealed that, of the 23 isolates, 20 and 3 were of Hobetsu and U.S. types, respectively. The U.S.-type *B. microti* strains isolated in Japan, however, were distinguishable from the isolates in the United States when their β-tubulin gene sequences and antigen profiles in Western blots were compared. We conclude that U.S.-type *B. microti* exists in Japan although it has been genetically and antigenically diversified from that distributed in the United States. The results also suggest that not only rodents, but also some insectivores may serve as reservoirs for the agent of human babesiosis.

KEY WORDS: *Babesia microti*, Japan, reservoir, U.S. type, wild animal.

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Babesia microti is an erythroparasitic protozoon frequently seen in small wild rodents. This parasite is known to be the causative agent of human babesiosis, an emerging tick-borne zoonosis which has increasingly been recognized in the Northeastern and upper-Midwestern United States [8, 11, 20]. Parasitization of *B. microti* in various rodent species has been reported in North America [3, 7, 26], Europe [9, 10, 26], and East Asia [18, 24, 29]. Curiously, however, despite such worldwide distribution of *B. microti* throughout the northern temperate zone, symptomatic human infections have been reported almost exclusively from the United States [11, 20]. It is well established that white-footed mouse, *Peromyscus leucopus*, and deer tick, *Ixodes dammini* (also known as *I. scapularis*), serve as the rodent reservoir and the tick vector, respectively, for *B. microti* in the northeastern United States [7, 19]. The parasite was also found in short tailed shrews, *Blarina brevicauda*, indicating the role of Insectivora as additional reservoir hosts [21].

Recently, the first case of transfusion-acquired, symptomatic human babesiosis has been reported in Japan [13, 16]. In order to identify wild animals that serve as the reservoirs for the agents of human babesiosis, we conducted epizootiologic surveys at various places in the country [23]. The results reveal that there are two types of *B. microti*-like parasites (designated as the Hobetsu and Kobe types) in Japan, and that the large Japanese field mouse, *Apodemus speciosus*, serves as the major reservoir host for both of the two types. The nuclear small-subunit rRNA gene (rDNA) sequences of these two types (GenBank accession nos. AB032434 and AB050732 for the Kobe and Hobetsu types, respectively) clearly differed from that of the United States *B. microti* (the U.S. type; GenBank accession no. U09833). Therefore, although human babesiosis in Japan may be able to be categorized as an emerging disease because of its sudden appearance, it became apparent that *B. microti*-like parasites in the country were not imported from the regions in the United States where human babesiosis is endemic. The U.S.-type *B. microti* has not yet been found in any places surveyed in our previous study [23].

More recently, we have extended our epizootiologic surveys to regions surrounding Japan, including South Korea, Far East Russia, and the northwestern China [29]. Animals harboring *B. microti* were detected in these three regions, and all of the parasites were identified to be of U.S. type, based on their rDNA sequences being identical to that of the United States *B. microti* [29]. Several European strains of *B. microti* have also been reported to have the U.S.-type rDNA sequence [6, 28]. These lines of epidemiological evidence suggest that the U.S.-type *B. microti* is probably distributed throughout the temperate zones of not only North American but also Eurasian Continents, whereas that the Hobetsu and Kobe types may be the parasites unique to Japan [23, 29]. Phylogenetically, the Hobetsu-, Kobe-, and U.S.-type parasites are quite closely related to each other,

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but clearly distinguishable from each other antigenically [23].

In our previous study [29], *A. peninsulae*, *A. agrarius*, and *Clethrionomys rufocanus* are identified to be the rodent species harboring the U.S.-type *B. microti* in northeastern Eurasia. Both *A. peninsulae* and *C. rufocanus* are also known to exist on Hokkaido Island, the far northernmost part of Japan, because this island had been connected with Eurasian Continent via Sakhalin Island during the latest ice age until approximately ten thousand years ago. Hence, despite the current unavailableness of U.S.-type *B. microti* in Japan, it may be possible to find this type in that region of the country. In the present study, therefore, we conducted epizootiologic surveys at various places on Hokkaido Island and the northern part of Honshu Island (the mainland) of Japan, seeking for U.S.-type *B. microti* in small wild rodents.

MATERIALS AND METHODS

Field collections: Small wild mammals examined in the present survey were collected during 1999 and 2001 at various places on Hokkaido Island and the northern part of Honshu Island (the mainland) of Japan. The locations of the field collections are shown in Fig. 1. Identification of animal species was done according to the key characteristics described by Abe *et al.* [1]. We collected a total of 197 small mammals comprising 10 species, which included 104 of *A. speciosus*, 41 of *A. argenteus*, 25 of *C. rufocanus*, 12 of *C. rutilus*, 1 of *Eothenomys andersoni*, 1 of *Microtus montebelli*, 1 of *Tamias sibiricus*, 4 of *Sorex unguiculatus*, 5 of *S. caecutiens*, and 3 of *Urotrichus talpoides*. Their blood samples were processed according to the methods described previously [23] for preparations of blood smear and DNA, which were used for microscopic examination and for PCR targeting babesial rDNA and β-tubulin gene, respectively [27, 29].

Detection of Babesia parasites: Nested PCRs targeting the babesial rDNA and β-tubulin gene were carried out for detection of *Babesia* parasites in the blood specimens from the field collections according to previously published protocols [27, 29]. DNA samples were prepared from 100 μl of blood samples with a DNA Extractor WB kit (Wako Pure Chemical Industries, Osaka). Approximately 1/30 of the final DNA preparation was used for the first round of PCR, followed by the second round of PCR with 1 μl of the first-round PCR product. Type-specific PCRs based on the sequences of β-tubulin gene for genotypic classification were carried out as described previously [29].

Experimental animals: Syrian hamsters (Std:Syrian), Mongolian gerbils (MON/Jms/Gbs) were obtained from SLC Inc. (Shizuoka). House musk shrews, *Suncus murinus* (Jic:Sun-Her), were purchased from CLEA Japan, Inc. (Tokyo). NOD/shi-*scid* mice [12] had been provided from the Central Institute of Experimental Animals, Kawasaki, and have been maintained in the laboratory animal facility in Rakuno-Gakuen University. Animals were housed in

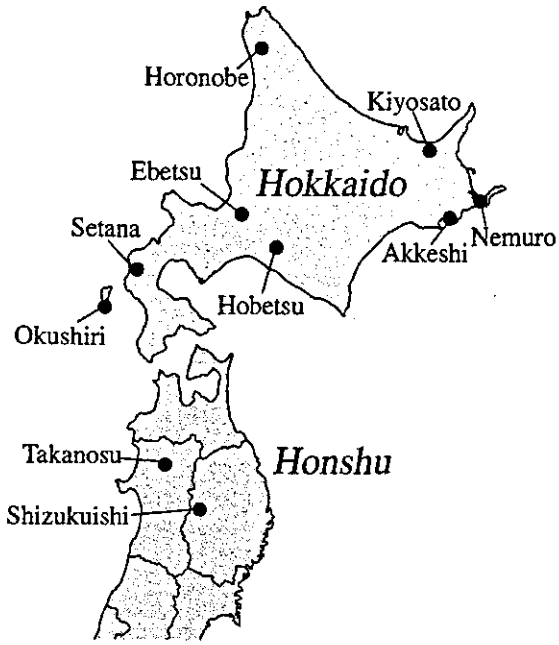

Fig. 1. Map of the northern part of Japan, showing the locations of field collections (●).

vinyl-film isolators at a temperature of between 22 and 25°C, and were provided with γ-ray-irradiated pellet diet and autoclaved tap water. All hamsters and gerbils were splenectomized, and were used for experiments after the surgical wounds had healed completely. Animal experimentation was carried out according to the Laboratory Animal Control Guidelines of Rakuno-Gakuen University.

Isolation of parasites: Red blood cell (RBC) samples from the field collections were inoculated into splenectomized hamsters for isolation of parasites. Other experimental animals, including splenectomized gerbils, house musk shrews, and splenectomized NOD/shi-*scid* mice, were also used in attempts to isolate *Babesia* parasites from shrews (*Sorex unguiculatus*, and *S. caecutiens*). Blood samples were collected periodically from the tail vein of the inoculated animals, and Giemsa stained thin-smeared blood films were prepared for microscopic detection of parasitemia. When the level of parasitemia reached 20 to 40%, blood was harvested by cardiocentesis from anesthetized animals, washed in phosphate buffered saline (PBS), resuspended in a cell freezing solution (Cell Banker; Nippon Zenyaku Co., Ltd., Fukushima), and cryopreserved in liquid nitrogen. The isolates were further propagated by subpassage into new splenectomized hamsters, and their RBCs (parasitemia level, 30 to 50%) were washed in PBS and stored at –80°C without cryopreservatives for subsequent use to prepare parasite DNAs and antigens for Western blot analysis. For production of antibodies, hamsters with intact spleens were infected with parasites, and serum samples were collected from the animals when they had high antibody titers.

Sequencing analyses: For each parasite isolate, genomic

DNA was prepared from the frozen parasitized RBC stock described above using a Whole-blood DNA extraction kit (GenTLE; TaKaRa Biochemical, Shiga). Sequences of the rDNA and β-tubulin gene were amplified from the DNA samples by PCR with the primer sets described in previous studies [14, 29]. Specifically amplified products were cloned in a plasmid vector and sequenced as described elsewhere [16].

Phylogenetic analysis: Phylogenetic relationship of β-tubulin gene sequences was analyzed by using the MacVector software package, version 7.0 (Genetic Computer Group Inc., Madison, Wis, U.S.A.). The β-tubulin gene sequences (GenBank accession numbers are given in parentheses) included in phylogenetic analysis were of U.S.-type *B. microti* collected from Vladivostok in Russia (AB083379), from South Korea (AB083380), and from Xinjiang in China (AB083378), of the Hobetsu- and Kobe-type *B. microti*-like parasites (AB083441 and AB083440 for the strains Ho234 and Ko524, respectively), and of *B. rodhaini* (AB083442). The sequences were aligned with the program CLUSTAL W Alignment [22], and a phylogenetic tree was constructed by neighbor-joining method [17] from the aligned sequences with the program Phylogenetic Analysis in the MacVector software. Support for tree nodes was calculated with 1,000 bootstrap replicates by use of the bootstrap tree algorithm.

Antigenic analyses: The indirect immunofluorescent antibody test (IFAT) was carried out by the method described previously [16]. A mixture of fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (IgG) plus IgM, anti-rat IgG plus IgM and anti-Syrian hamster IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa, U.S.A.) was used for the detection of antibodies in the wild animals collected from the fields. Western blot analysis was performed as described previously [27]. Frozen stocks of *Babesia*-infected RBCs were thawed and washed five times at 4°C in TE (10 mM Tris-HCl and 10 mM EDTA, pH 7.5) by centrifugation at 10,000 × *g* for 10 min. The resulting pellets were dissolved in 125 mM Tris-HCl (pH 6.5) containing 5% β-mercaptoethanol, 2% sodium dodecylsulfate, 10% glycerol, and 0.1% bromophenol blue, heated at 98°C for 5 min, and vigorously vortexed. The samples were diluted such that each contained material from equivalent numbers of parasitized RBCs and were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis, followed by blotting onto Fluorotrans membranes (Pall Bio Support, Port Washington, N.Y., U.S.A.). After blocking was done with PBS containing 0.5% casein, the membranes were reacted with appropriately diluted antisera and subsequently with secondary antibodies (alkaline phosphatase-conjugated affinity purified goat anti mouse IgG heavy and light chains or anti-Syrian hamster IgG heavy and light chains; Jackson ImmunoResearch Laboratories). Immunoreactive antigens were detected with 5 bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium alkaline phosphatase substrates kit IV (Vector Laboratories, Inc., Burlingame, Calif., U.S.A.).

Reference parasite strains: The Gray and GI strains have been described in our previous study [16], and were used as the *B. microti* isolates obtained in the Northeastern United States. The strains Ho234 [23] and Ko524 [27] were used as the type strains of the Hobetsu- and Kobe-type *B. microti*-like parasites, respectively. The Australian strain of *B. rodhaini* was kindly provided from the National Institute of Animal Health, Tsukuba. These parasites were propagated in hamsters or NOD/shi-*scid* mice as described previously [23].

Nucleotide sequence accession number: All three U.S.-type *B. microti* isolates obtained in Japan had identical β-tubulin gene sequences. The sequence from the strain NM69, as a representative, was submitted to DNA Data Bank of Japan (DDBJ) and has been given the accession number AB085813.

RESULTS

Field surveys: Small wild mammals examined in the present survey were collected at various places on Hokkaido Island and the northern part of Honshu Island (the mainland) of Japan (Fig. 1). A total of 197 small mammals comprising 10 species were collected. Their blood samples were examined by microscopy of blood smears and by PCR targeting babesial rDNA and β-tubulin gene. The results of survey are summarized in Table 1. *Babesia* parasites were detected in 24 animals, consisting of 16 from *A. speciosus*, 4 from *C. rufocanus*, and 1 each from *C. rutilus, M. montebelli, S. unguiculatus* and *S. caecutiens*.

Parasite isolation: The 24 blood specimens, in which, *Babesia* parasites had been detected, were inoculated into experimental animals for isolation of parasites. A single splenectomized hamster (*Mesocricetus auratus*) was used for each of the 24 samples. In addition, *Suncus murinus* and NOD/shi-*scid* mice were used for the sample from *Sorex caecutiens* collected at Nemuro, and Mongolian gerbils (*Meriones unguiculatus*) for the samples from *Sorex unguiculatus* and *Microtus montebelli* collected at Kiyosato and Shizukuishi, respectively. We were able to finally obtain a total of 23 parasite isolates, which included 16 from *A. speciosus*, 4 from *C. rufocanus*, and 1 each from *C. rutilus, M. montebelli* and *S. unguiculatus* (Table 2). However, we failed to isolate a parasite from the sample of *S. caecutiens*, in which Hobetsu-type *B. microti*-like parasites had been detected by PCR.

Genotypic classification: On the 24 DNA samples from the field collections, in which the rDNA sequence of *B. microti* had been detected by PCR, we carried out the type-specific PCR based on the β-tubulin gene sequence [29] for genotypic classification. Of the 24 samples, 21 and 2 were identified to be of Hobetsu and U.S. types, respectively (Table 1), and another one from *C. rutilus* trapped in Nemuro were positive for both Hobetsu and U.S. types. We also carried out sequencing analysis for the rDNA of the 23 parasite isolates to determine their genotype (Table 2). Of the 23 isolates, 20 and 3 had the rDNA sequences which were

Table 1. Summary of field surveys of *B. microti*-like parasites among small wild mammals in the nothern part of Japan

Site	Species	No. of animals captured	No. of postive/no. of tested by:						No. of isolates[d]
				Nested PCR targeting the following genes:					
			Microscopy[a]	rDNA[b]	β-tubulin[c]				
					Universal	U.S.	Hobetsu	Kobe	
Nemuro	*Clethrionomys rufocanus*	4	2/4	2/4	2/4	1/2	1/2	0/2	2/2
	Clethrionomys rutilus	10	1/10	1/10	1/10	1/1	1/1	0/1	1/1
	Sorex caecutiens	3	1/3	1/3	1/3	0/1	1/1	0/1	0/1
Akkeshi	*Apodemus speciosus*	4	0/4	1/4	1/4	1/1	0/1	0/1	1/1
	Clethrionomys rufocanus	7	0/7	1/7	1/7	0/1	1/1	0/1	1/1
	Clethrionomys rutilus	2	0/2	0/2	0/2	–	–	–	–
	Tamias sibiricus	1	0/1	0/1	0/1	–	–	–	–
Horonobe	*Apodemus speciosus*	2	0/2	0/2	0/2	–	–	–	–
	Apodemus argenteus	3	0/3	0/3	0/3	–	–	–	–
	Clethrionomys rufocanus	1	0/1	0/1	0/1	–	–	–	–
	Sorex unguiculatus	2	0/2	0/2	0/2	–	–	–	–
Kiyosato	*Apodemus speciosus*	1	1/1	1/1	1/1	0/1	1/1	0/1	1/1
	Apodemus argenteus	2	0/2	0/2	0/2	–	–	–	–
	Clethrionomys rufocanus	2	0/2	0/2	0/2	–	–	–	–
	Sorex unguiculatus	1	1/1	1/1	1/1	0/1	1/1	0/1	1/1
Hobetsu[e]	*Apodemus speciosus*	10	7/10	7/10	7/10	0/7	7/7	0/7	7/7
	Apodemus argenteus	2	0/2	0/2	0/2	–	–	–	–
	Clethrionomys rufocanus	3	0/3	1/3	1/3	0/1	1/1	0/1	1/1
Ebetsu[e]	*Apodemus speciosus*	22	0/22	0/22	0/22	–	–	–	–
	Apodemus argenteus	15	0/15	0/15	0/15	–	–	–	–
	Clethrionomys rufocanus	7	0/7	0/7	0/7	–	–	–	–
	Sorex unguiculatus	1	0/1	0/1	0/1	–	–	–	–
Setana	*Apodemus speciosus*	11	0/11	0/11	0/11	–	–	–	–
	Apodemus argenteus	8	0/8	0/8	0/8	–	–	–	–
	Clethrionomys rufocanus	1	0/1	0/1	0/1	–	–	–	–
	Sorex caecutiens	2	0/2	0/2	0/2	–	–	–	–
Okushiri	*Apodemus speciosus*	14	0/14	0/14	0/14	–	–	–	–
Shizukuishi	*Apodemus speciosus*	23	0/23	0/23	0/23	–	–	–	–
	Apodemus argenteus	10	0/10	0/10	0/10	–	–	–	–
	Eothenomys andersoni	1	0/1	0/1	0/1	–	–	–	–
	Microtus montebelli	1	1/1	1/1	1/1	0/1	1/1	0/1	1/1
	Urotrichus talpoides	2	0/2	0/2	0/2	–	–	–	–
Takanosu	*Apodemus speciosus*	17	7/17	7/17	7/17	0/7	7/7	0/7	7/7
	Apodemus argenteus	1	0/1	0/1	0/1	–	–	–	–
	Urotrichus talpoides	1	0/1	0/1	0/1	–	–	–	–
Total		197	21/197	24/197	24/197	3/24	22/24	0/24	23/24

a) Detection of parasitezed RBCs by microscopy of thin-smear blood films.
b) Detection of rDNA sequence by nested PCR with primers specific for *B. microti* [27].
c) Detection of β-tubulin gene sequence by nested PCR with primers universal for three types of *B. microti*-like parasites, and genotypic classification with primers specific for the U. S., Hobetsu and Kobe types [29].
d) *Babesia* parasites were isolated by inoculation of the blood samples into experimental animals as shown in Table 2.
e) The samples of Hobetsu and Ebetsu were obtained in our previous study [27].

identical to those of the Hobetsu type (GenBank accession no. AB050732) and the U.S. type (GenBank accession no. U09833), respectively. All of the three *B. microti* isolates identified to be of U.S. type were derived from animals trapped in an eastern area of Hokkaido Island (Nemuro and Akkeshi). The results of genotypic classification determined by the type-specific PCR targeting the β-tubulin gene and by sequencing of rDNA were in good agreement, except for the sample from *C. rutilus* trapped in Nemuro. Whereas the blood from this vole appeared to have contained both Hobetsu- and U.S.-type parasites, the parasites isolated after inoculation into a hamster were of U.S. type. To dissolve this discrepancy, we attempted to pass the isolated parasites through NOD/shi-*scid* mice three times. Since U.S.-type *B. microti* generally grew only very poorly in mice, this passage gave rise to isolation of Hobetsu-type parasite, con-

Table 2. Isolation of *Babesia* parasites from field collections

Animal species	No. of field animals tested	Experimental animals used for isolation	No. of isolated/No. of innoculated	No. of isolates with the following rDNA genotype[a)]		
				U.S.	Hobetsu	Kobe
Apodemus speciosus	16	Hamster	16/16	1	15	0
Clethrionomys rufocanus	4	Hamster	4/4	1	3	0
Clethrionomys rutilus	1	Hamster	1/1	1[b)]	0	0
Microtus montebelli	1	Hamster	1/1	0	1	0
		Girbil	1/1	0	1	0
Sorex unguiculatus	1	Hamster	0/1	–	–	–
		Girbil	1/1	0	1	0
Sorex caecutiens	1	Hamster	0/1	–	–	–
		Home musk shrew	0/2	–	–	–
		NOD/shi-*scid*	0/2	–	–	–
Total	24		24/30	3	20	0

a) Genotype was determined by sequencing analysis of near full-size rDNA gene.
b) The primaly isolate predominantly consisted of U.S.-type parasite, but the presence of Hobetsu-type parasite was demonstrated by passage in mice.

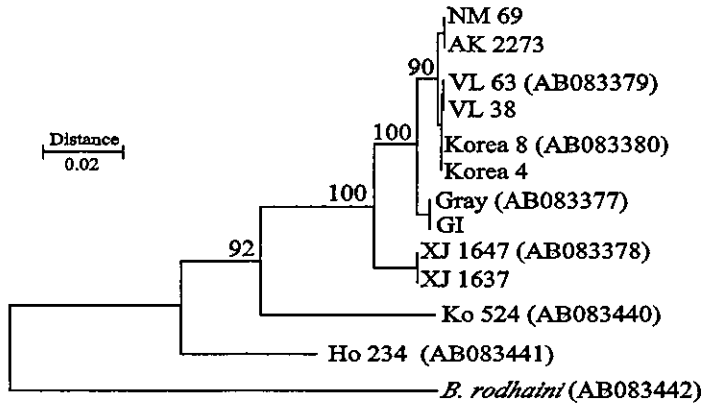

Fig. 2. Phylogenetic tree constructed with sequences of the β-tubulin genes of the U.S.-type *B. microti* and other closely related parasites. The U.S.-type parasites were collected from various places; NM69 and AK2273 from Hokkaido in Japan; VL63 and VL38 from Vladivostok in Russia; Gray and GI from the northeastern Unites States; XJ1647 and XJ1637 from Xinjiang in China. The strains Ko524 (Kobe-type) and Ho234 (Hobetsu-type) were also from Japan. GenBank accession numbers are given in parenthesis. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates.

firming mixed infections by both U.S.- and Hobetsu-type parasites.

Sequencing analysis of the β-tubulin gene: Sequencing analysis was also carried out for the β-tubulin gene of the 23 parasite isolates. Of the 23 β-tubulin gene sequences, 20 were identical to that of strain Ho234 (the type strain of Hobetsu type; GenBank accession no. AB083441), and the other 3 were very close, but not exactly identical, to that of the Gray strain (GenBank accession no. AB083377), the isolate from the United States index case patient [5]. With the β-tubulin gene sequences from the U.S.-type *B. microti* strains (NM69 and AK2273) isolated in Japan, together with those previously reported for the other U.S.-type *B. microti* parasites [29], we constructed a phylogenetic tree to analyze their interrelationship (Fig. 2). The U.S.-type *B. microti* isolated in Japan (NM69 and AK2273) was found to be quite closely related to those found in South Korea (Korea4 and 8) and Far East Russia (VL38 and 63), but relatively distantly related to those found in the United States (Gray and GI) or the northwestern China (XJ1637 and 1647).

Antigenic analysis: To examine the antigenic relationship between the U.S.-type parasites isolated from Japan (NM69 and AK2273) and those from the United States (Gray and GI), IFATs were carried out for pair wise comparison. Both

Table 3. Results of IFATs with *B. microti* parasites isolated from U.S. and Japan

Sera	Reciprocal titer against the following parasite:			
	Gray	GI	NM69	AK2273
anti-Gray	25,600	12,800	1,600	800
anti-GI	12,800	12,800	1,600	800
anti-NM69	<100	100	12,800	6,400
anti-AK2273	400	200	25,600	25,600

the Japanese and the Unites States parasites showed higher titers against the homologous than against the heterologous antiserum (Table 3). Similar results were also obtained in Western blot analysis (Fig. 3), in which we found that many of immunodominant antigens recognized by the homologous antiserum were poorly cross-reactive against the heterologous antiserum.

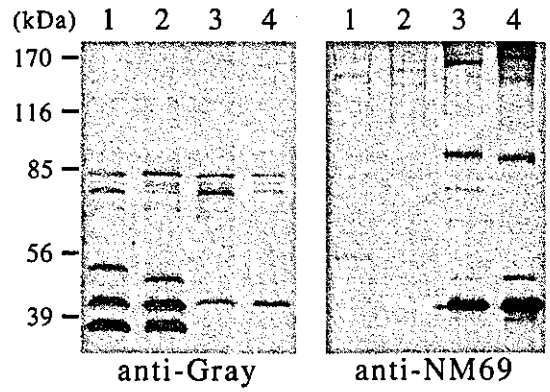

Fig. 3. Western blot analysis with the U.S.-type *B. microti* isolated in the United States (Gray and GI; lanes 1 and 2, respectively) and Japan (AK2273 and NM69; lanes 3 and 4, respectively). Parasite antigens were detected with anti-Gray mouse serum (left panel) and with anti-NM69 hamster serum (right panel).

DISCUSSION

This study represents the first isolation of U.S.-type *B. microti* in Japan. Previously, this type of *B. microti* was presumed not to exist in the country, because of its apparent absence in our field survey [23]. Recently, however, we found the presence of U.S.-type parasites in Northeastern Eurasia [29], which gave us motivation to seek this type of parasite in Japan. We focused on Hokkaido Island because this island had been connected with Eurasian Continent until approximately ten thousand years ago, and some species of small wild rodent seen in Northeastern Eurasia also exist on Hokkaido Island. The presence of U.S.-type *B. microti* in Japan appeared to be confined to a narrow area in the eastern part of Hokkaido Island. Although the exact reason for this is currently unclear, it may be related to the fact that natural environment in this area is so well preserved as to accommodate a large number of wild-life animals.

Finding of the U.S. type in this study, in addition to the Hobetsu and Kobe types in previous studies [23], demonstrated that there are three types of *B. microti*-like parasites in Japan. While the Hobetsu type is the major parasite that is widely distributed in Japan, the U.S. type appeared to be the predominant parasite that is distributed throughout the temperate zones of the North American and Eurasian Continents [4, 5, 28]. A major factor contributing to this difference is probably the geographic isolation, owing to which many mammals distributed in Japanese Islands are unique species, including *A. speciosus*, the major reservoir host for the Hobetsu type *B. microti*-like parasites.

We originally defined the three genotypes, namely, U.S., Hobetsu, and Kobe types, based on the sequences of their rDNA. All the U.S.-type *B. microti* isolated to date were reported to have virtually identical rDNA sequences [4, 5, 28, 29], regardless of the place of parasite isolation and also regardless of the host animal species. Sequencing analyses on the rDNA of the Hobetsu- and Kobe-type isolates also indicate that there are no sequence variations in their rDNA. Such high degree of sequence conservation, therefore, makes the rDNA sequence an ideal genotypic marker. However, since the number of nucleotides useful to distinguish between types is only a few, sequencing of the near full-size rDNA is needed for genotype determination. In this and previous studies [29], we included sequence analyses of the β-tubulin gene in addition to the rDNA. This enabled us to determine not only genotype but also intragenotypic variation, because sequence variation in the β-tubulin gene is much greater than that in the rDNA. Phylogenetic analysis of the β-tubulin gene sequences clearly demonstrated the presence of geographic diversity within the U.S.-type *B. microti* which could not previously be seen by analysis of rDNA.

Diversity of the U.S.-type *B. microti* was also observed in the parasite antigens recognized by the host animals. Western blot analysis clearly showed that the U.S.-type parasites isolated in Japan and those in the United States had several immunodominant antigens that were poorly cross-reactive against heterologous antiserum. This antigenic diversity indicates the necessity of a local isolate as an antigen for sensitive serodiagnosis of local infections. Indeed, in our previous study [29], we failed to detect specific antibodies in the serum samples from wild rodents collected in South Korea and Far East Russia, despite that their infection by U.S.-type *B. microti* was clearly detected by PCR. In that study, antibody detection was carried out by IFAT using the Gray strain of *B. microti* which was obtained from the United States. Recently, however, we were able to detect specific antibodies in the same serum samples by using the strain NM69, a U.S.-type parasite isolated in this study, as an antigen of IFAT (M. Tsuji, unpublished data). This also suggests that the antigenicities of the U.S.-type parasites distributed in Korea and Far East Russia are closely related with that in Japan, which is consistent with their close relationships in the phylogenetic tree (Fig. 2).

The results of the present survey reconfirmed our previ-

ous conclusion that *A. speciosus* serves as the major host for *B. microti*-like parasites in Japan. Besides *A. speciosus*, however, we also found *C. rufocanus, C. rutilus, M. montebelli, S. unguiculatus*, and *S. caecutiens* harboring *B. microti*-like parasites. These species of Rodentia and Insectivora may, therefore, serve as additional reservoir hosts for the agents of human babesiosis in the country. Interestingly, preference of experimantal hosts appeared to be somewhat different between the parasites infecting rodents and those infecting insectivores: all *B. microti*-like parasites derived from wild rodents were isolated by inoculation into hamsters which, however, appeared to be refractory to infection by the parasites derived from insectivores. Shrews have been suggested to play roles as reservoirs of not only babesiosis but also Lyme borreliosis in the Unites States [21] and Japan [15].

Previously, we speculated that the apparent absence of the U.S.-type *B. microti* in Japan might be relevant to the seemingly rare occurrence of symptomatic human babesiosis cases in the country [2, 23], which is in contrast to the relatively frequent clinical case reports from areas of endemicity in the Unites States [11, 20]. It was hypothesized for this speculation that the U.S.-type *B. microti* may be more pathogenic and virulent than the Hobetsu-type *B. microti*-like parasite, the major type widely distributing throughout Japan. Therefore, careful monitoring for the residents in the eastern area of Hokkaido Island, where the U.S.-type parasites have been found to be enzootic, may provide us with a good opportunity to test that hypothesis.

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FULL PAPER *Public Health*

Epizootiologic Survey for *Babesia microti* among Small Wild Mammals in Northeastern Eurasia and a Geographic Deversity in the β-Tubulin Gene Sequences

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ABSTRACT. We previously reported that small wild rodents in Japan harbor two types of novel *Babesia microti*-like parasites (designated as Hobetsu and Kobe types), but not the type commonly found in the northeastern United States (U.S. type) where human babesiosis is endemic. To determine whether these new types of parasites are distributed in places surrounding Japan, an epizootiologic survey was undertaken in three geographically distant areas in northeastern Eurasia; South Korea, Vladivostok in Russia, and Xinjiang in China. Blood samples were collected from a total of 387 animals comprising 24 species. DNAs extracted from the samples were tested by nested PCR targeting babesial nuclear small-subunit rRNA gene (rDNA), which revealed that small rodents harboring *B. microti* exist in all three survey areas. Sequence analysis showed that all PCR-positive samples had rDNA sequences virtually identical to that of U.S.-type *B. microti*. However, when β-tubulin gene sequences were compared, evident geographic variations were seen. By use of primers specific for each of the β-tubulin genes of Kobe-, Hobetsu-, and U.S.-type parasites, a type-specific PCR was developed. Parasite with Hobetsu- or Kobe-type sequence was not detected from any of the three survey areas. These findings suggest that U.S.-type *B. microti* is widely distributed among small wild mammals in temperate zones of not only North America, but also Eurasia, whereas that Hobetsu- and Kobe-type parasites may be uniquely distributed in Japan.

KEY WORDS: *Babesia microti*, β-tubulin, Epidemiology, Eurasia, wild rodent.

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Babesia microti is a tick-transmitted, intraerythrocytic protozoan parasite frequently seen in small wild rodents. This parasite is known to be the causative agent of human babesiosis, an emerging tick-bone zoonosis that is endemic mainly in the northeastern and upper midwestern United States [19, 29]. Extensive epidemiological studies have been conducted in the regions, revealing that the white-footed mouse (*Peromyscus leucopus*) and the black deer tick (*Ixodes scapularis*) serve as the rodent reservoir and the tick vector, respectively, for the agent of human babesiosis [19, 29].

In the literature, there are many reports that document the parasitization of *Babesia* among wide varieties of rodent species in various places in the world, including North America [3, 14, 33], Europe [15, 16, 18], Africa [8] and East Asia [28, 32]. The parasites reported in those studies are referred to as *B. microti*, suggesting that a single parasite species may widely be distributed throughout the world. However, because identification of the species in most studies was made primarily on the basis of parasite morphology and host species, and also because the taxonomy of rodent *Babesia* spp. has historically been quite complicated [20, 21], the possibility that several similar, but significantly distinct, parasite species might have been regarded as the same species can not be excluded. The possible presence of distinct "*B. microti*" in different places might give an easier explanation for the geographically biased occurrence of symptomatic human babesiosis cases, which have been reported frequently from the United States but only very rarely from other regions in the world.

Our previous investigations on the first Japanese case of human babesiosis [25, 34] proved that the patient was infected by blood transfusion from an asymptomatic carrier. A following epidemiological survey [31] further revealed that two types of *B. microti*-like parasites, namely Kobe and Hobetsu, are enzootic among Japanese small wild rodents, and that *Apodemus speciosus*, a species of Muridae unique to Japan, serves as the major reservoir of the agent of human babesiosis. Both the Kobe- and Hobetsu-type parasites were closely related phylogenetically to the U.S.-type parasite (=*B. microti* sensu stricto), but these three types were clearly distinguishable from one another antigenically and genotypically [31]. Parasites with a nuclear small-subunit rRNA gene (rDNA) sequence that is virtually identical to that of the United States *B. microti* have also been reported from Europe [7, 35], but to date not from Japan [31]. The objective of the present study was to conduct epizootiologic surveys in various places in northeast Eurasia for detection of *Babesia* parasites in small wild mammals.

MATERIALS AND METHODS

Field collections: Epizootiologic field surveys were car-

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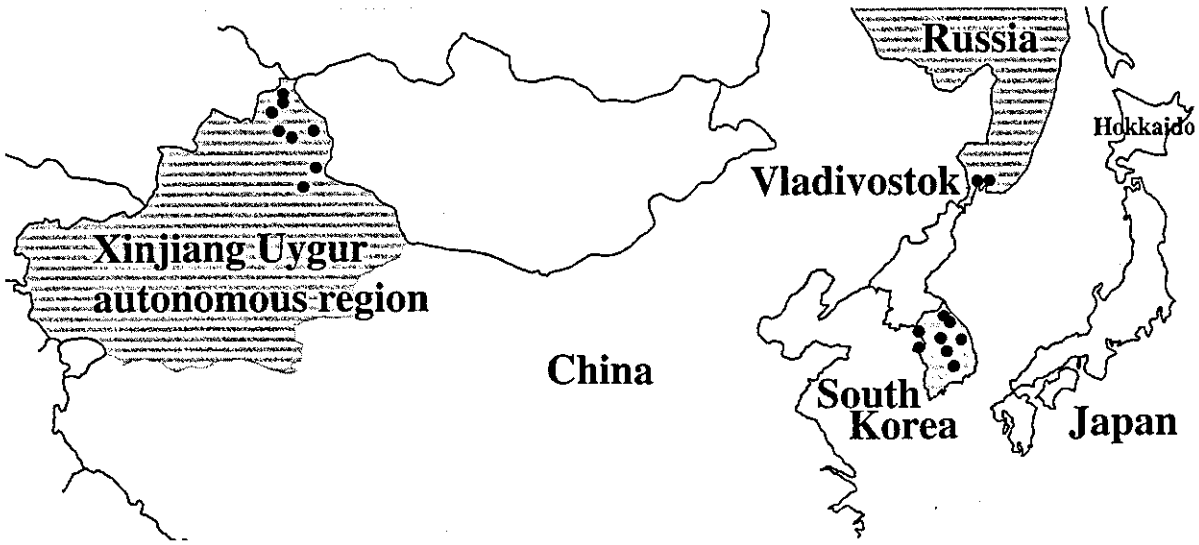

Fig. 1. Map of eastern Asia, showing locations of survey sites (●).

ried out in three geographically distant areas in the northeastern Eurasia: South Korea, Vladivostok in Russia, and Xinjiang Uygur autonomous region in China (Fig. 1). Samples from South Korea were collected in 2001 for this study, and obtained from 154 animals comprising three species of Rodentia and one of Insectivora. Field collections were carried out at eight locations; Odaesan National Park, Sobaeksan National Park, Songnisan National Park, Chirisan National Park, Soraksan National Park, Inchon in Kyodong, Tokyusan National Park, and Samsan in Inchon City. The blood specimens, which were stored in a dried form on blood sampling papers (Toyo Roshi, Tokyo), were brought to Japan and processed for subsequent analyses. Small wild mammals collected in these places are listed in Table 1. At two survey points near Vladivostok, 68 animals comprising five species of Rodentia were trapped in 1999. The serum samples collected were used for a previous study [22], and the remaining blood clots that had been stored at –80°C were made available for the present study. In Xinjiang, 165 small wild mammals comprising 19 species of Rodentia were captured during 1998 and 1999 at eight locations, namely Wuchai wan, Wulong-gu, Fuhai, Bulzin, Hanashi Lake, Jadenggu, Fuwen, and Kamushite [2]. The survey in Xinjiang was originally conducted for other purposes [2, 13], and the remaining stocks of dried bloods absorbed on filter papers were made available for this study.

DNA preparation: The samples collected near Vladivostok were stored as frozen clotted blood. DNAs from these samples were prepared with a whole blood DNA extraction kit (DNA Extractor WB Kit; Wako Pure Chemical Industries, Osaka) according to the instruction manual supplied by the manufacturer, except that clotted blood was mashed with a pestle (Handy Pestle; Toyobo, Osaka). The blood samples collected in Xinjiang and South Korea had been stored in a filter paper-absorbed, dried form. These filter papers were cut into small pieces and soaked in 500 μl of phosphate buffered saline (PBS) containing 0.05% NaN_3 for 2 days at 4°C. After centrifugation at 15,000 × g for 10 min, the supernatants were transferred to new tubes and used for IFA tests. The pellets were resuspended in 500 μl of TNE buffer (150 mM NaCl, 10 mM Tris-HCl, and 100 mM EDTA, pH 8.0) containing 1% sodium dodecyl sulfate and 100 $\mu g/ml$ of proteinase K, and incubated at 55°C overnight. DNA was purified by phenol-extraction followed by ethanol precipitation. DNA pellets were dissolved in 30 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5).

Detection of B. microti-specific DNA in blood samples: Nested PCR for detection of *Babesia* parasites in the blood specimens of the field collections was carried out according to the previously published protocol [31, 34]. All oligonucleotide primers used for various PCR are listed in Table 2. Approximately a 1/30 volume of the final DNA preparations described above was used for the first-round PCR, which was followed by the second-round PCR with 1 μl of the first-round product. Two kinds of primer sets were used to detect babesial rDNA: one set, Bab1A-4A (Bab1A and Bab4A for the first-round PCR, followed by Bab2A and Bab3A for the second round), was highly specific for rDNA of all three types of *B. microti*-like parasites (GenBank accession numbers U09833, AB050732, and AB032434 for U.S., Hobetsu, and Kobe types, respectively), and the other set, Bab5–8 (Bab5 and Bab8 for the first-round PCR, followed by Bab6 and Bab7 for the second round), was broadly specific for various piroplasms belonging to *Babesia* and *Theileria*. In order to minimize erroneous amplification during nested PCR, both DNA preparation described above and PCR detection were carried out in an isolated laboratory room with specialized equipment. Unused filter tips were always used for pipetting to avoid cross-contaminations.

Sequencing analysis of rDNA: In order to determine gen-

Table 1. Summary of the field surveys for *B. microti* among small wild mammals in Northestern Eurasia

Place	Animal species	No. of animals	No. of positives by nested PCR					Positive samples[d]
			rDNA		β-tubulin[c]			
			Bab5–8[a]	Bab1A-4A[b]	U.S.	Hobetsu	Kobe	
South Korea								
	Apodemus agrarius	146	30	16	16	0	0	Korea8 and others
	Apodemus peninsulae	2	2	1	1	0	0	Korea138
	Clethrionomys regulus	2	0	0				
	Crocidura lasiura	4	0	0				
	subtotal	154	32	17	17	0	0	
Vladivostok								
	Apodemus peninsulae	32	2	1	1	0	0	VL63
	Apodemus agrarius	28	0	0				
	Clethrionomys rufocanus	4	2	1	1	0	0	VL38
	Microtus fortis	2	0	0				
	Tamias sibiricus	2	0	0				
	subtotal	68	4	2	2	0	0	
Xinjiang								
	Citellus erythrogenys	1	1	1	0	0	0	XJ1648
	Citellus undulatus	7	7	0				
	Cricetulus migratorius	6	2	0				
	Cricetulus longicaudatus	9	4	0				
	Cricetulus eversmanni	10	7	0				
	Cricetus cricetus	1	1	0				
	Microtus arvalis	5	5	0				
	Lagurus luteus	3	3	2	2	0	0	XJ1637 and XJ1647
	Clethrionomys rufocanus	1	1	0				
	Clethrionomys rutilus	15	12	0				
	Meriones meridianus	28	12	0				
	Meriones tamariscinus	4	2	0				
	Apodemus uralensis	2	1	0				
	Apodemus peninsulae	14	5	0				
	Mus musculus	1	0	0				
	Alactagulus pumilio	1	1	0				
	Allactaga sibirica	20	14	0				
	Dipus sagitta	32	15	0				
	Euchoreutes naso	5	2	0				
	subtotal	165	53	3	2	0	0	
	total	387	131	22	21	0	0	

a) Nested PCR with primers Bab5 and Bab8 for the first round, and primers Bab6 and Bab7 for the second round.
b) Nested PCR with primers Bab1A and Bab4A for the first round, and primers Bab2A and Bab3A for the second round.
c) Genotyping of β-tubulin genes by type-specific PCR.
d) Identification numbers of the positive samples.

otypes of *B. microti*, near full-size rDNA sequences (approximately 1.6 kb) were amplified from DNA samples of field collections by nested PCR with the primer sets of Piro1F and rRNA-3', followed by Bab1A and Piro6R (Table 2). The procedures of DNA sequencing have been described previously [25].

Sequencing analysis of β-tubulin gene: The 1651 bp sequence encoding the full-size β-tubulin gene of the Gray strain was elaborated by combining three overlapping sequences. The first sequence contained a 1214 bp partial sequence of the β-tubulin gene, which was amplified from the parasite genomic DNA with a set of primers Tubu63F and Tubu-3' (Table 2). The second sequence was obtained by reverse transcription-PCR [27] from mRNA of Gray strain, which amplified the 551 bp 3'-end region of the β-tubulin mRNA. The third sequence contained both 5'- and 3'-end regions of the genomic β-tubulin gene, which were amplified by inverse PCR [27] from fragmented genomic DNAs of the Gray strain made by *Hinc* II-digestion followed by self-ligation. Amplification of β-tubulin gene sequences from various geographic samples was carried out by nested PCR with UStubu127F and UStubu1637R primers for the first-round PCR, and with another set of primers, UStubu161F and UStubu1609R, for the second-round PCR. The specific PCR products, approximately 1.5 kb in size, were cloned and sequenced as described previously [25]. Sequences of β-tubulin genes from genomic DNAs of Hobetsu- and Kobe-type parasites and of *Babesia rodhaini*

Table 2. Oligonucleotide primers used for amplification of babesial rDNA and β-tubulin genes

Primers	Oligonucleotide sequences (5'–3')
Primers for rDNA	
Piro1F	CCATGCATGTCTWAGTAYAARCTTTTA
Bab1A	GTCTTAGTATAAGCTTTTATACAGCG
Bab2A	CAGTTATAGTTTATTTGATGTTCGTTTTAC
Bab3A	CGGCAAAGCCATGCGATTCGCTAAT
Bab4A	GATAGGTCAGAAACTTGAATGATACATCG
Bab5	AATTACCCAATCCTGACACAGG
Bab6	GACACAGGGAGGTAGTGACAAGA
Bab7	CCCAACTGCTCCTATTAACCATTAC
Bab8	TTTCGCAGTAGTTCGTCTTTAACA
Piro6R	CTCCTTCCTYTAAGTGATAAGGTTCAC
rRNA-3'	ATCCTTCYGCAGGTTCACCTAC
Primers for β-tubulin	
Tubu63F	CAAATWGGYGCMAARTTYTGGGA
Tubu-3'	TCGTCCATACCTTCWCCSGTRTACCAGTG
UStubu127F	ACATTTTCCCCATAGAACTTTATATCG
UStubu161F	CTAACCATTTAAATTGTTTATAATGAG
UStubu1609R	AAACAACTGTGTTAATATGTGAGT
UStubu1637R	AATTAACAGTACAGGAACTAGGTTA
BmTubu 93F	GAYAGYCCCTTRCAACTAGAAAGAGC
BmTubu192F	ACHATGGATTCTGTTAGATCYGGC
BmTubu782R	GGGAADGGDATRAGATTCACAGC
BmTubu897R	CGRTCGAACATTTGTTGHGTCARTTC
Tubu-Ko5'[a]	CAAATGTTTTTTATAACCAGACGAGCG
Tubu-Ko3'[a]	GAAAGGAATAAGATTCACAGTGAGCT
Tubu-Ho5'[a]	AAGAGCTAACGTTTTTTACAATCTATCAAG
Tubu-Ho3'[a]	CGCAAATCCAATCATAAAAAAGTTTAGTC
Tubu-US5'[a]	GCAAAYGTTTTYTATAACCAGTTTAGTG
Tubu-US3'[a]	GAAATGCAATCTCGGGAAGGTAATGA

a) A single mismutch indicated by underline was introduced to enhance type specificity.

were amplified by PCR with a set of primers Tubu63F and Tubu-3' (Table 2). Analyses for DNA sequences and phylogenetic relationships were done by using the MacVector software package, version 7.0 (Genetic Computer Group Inc., Madison, Wis, U.S.A.). The sequences were aligned with the program Clustal W Alignment [30], and a phylogenetic tree was constructed by the neighbor-joining method [26] from the aligned sequences with the program Phylogenetic Analysis in the MacVector software. Support for tree nodes was calculated with 1,000 bootstrap replicates by use of the bootstrap tree algorithm.

Type-specific PCR: Nested PCR for specific amplification of each of the β-tubulin genes from the U.S.-, Hobetsu-, and Kobe-type of *B. microti*-like parasites was developed. A set of primers used for the first-round PCR were BmTubu93F and BmTubu897R, which were designed to equally amplify all three types of β-tubulin gene sequences. The second-round PCR was carried out with four sets of primers (Table 2). One set, BmTubu192F and BmTubu782R, was designed to universally amplify most *B. microti* group parasites, and the other three sets were designed to specifically amplify each of the three types (Tubu-US5' and Tubu-US3', Tubu-Ho5' and Tubu-Ho3', and Tubu-Ko5' and Tubu-Ko3' for U.S., Hobetsu, and Kobe types, respectively). In each of these oligonucleotide primers (Table 2), an artificial single mismatch was introduced to a critical position near the 3' end in order to enhance the specificity of primers. The PCR mixtures contained 400 μM each deoxynucleoside triphosphate, 0.25 μM each primer, an appropriate amount of template DNA, and 1 U of La *Taq* DNA polymerase (Takara Biochemical, Otsu) in 20 μl of the PCR buffer supplied with the enzyme. Thermal cycling was carried out in a GeneAmp PCR system 9600 thermal cycler (Perkin Elmer, Norwalk, Conn, U.S.A.) with 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 60 s, and extension at 72°C for 90 s.

Serological analysis: The indirect immunofluorescent antibody test (IFAT) was carried out to detect specific antibodies against *B. microti* in PBS-eluates from blood specimens of field collections. The Gray strain was used as the antigen for IFAT. The method of IFAT has been described previously [25].

Reference strains of B. microti: The strains Gray and GI (U.S. type), Ho234 (Hobetsu type), and Ko524 (Kobe type) have been described in our previous study [25, 31], and were propagated in hamsters. The Australian strain of *B. rodhaini*, kindly provided by the National Institute of Animal Health, Tsukuba, Japan, was propagated in BALB/c mice. *Babesia canis vogeli*, Okinawa isolate, has been described in an earlier study [1].

Nucleotide sequence accession number: The sequence data generated in the present study were submitted to DNA Data Bank of Japan and were given accession numbers as listed in Table 3.

RESULTS

Epizootiologic survey: Small wild mammals examined in the present study are listed in Table 1. Blood samples were collected from 154 animals comprising four species in South Korea, from 68 animals comprising five species in Vladivostok, and from 165 animals comprising 19 species in Xinjiang. DNAs extracted from these samples were tested by nested PCR, for which two kinds of primer sets were used (Table 2): one set, Bab1A-4A, was highly specific for the rDNAs of three types of *B. microti*-like parasites (GenBank accession numbers U09833, AB050732, and AB032434 for U.S., Hobetsu, and Kobe types, respectively), the other set, Bab5–8, was broadly specific for various piroplasms belonging to *Babesia* and *Theileria*. The results of these PCR amplifications are summarized in Table 1, which revealed that small wild rodents harboring *B. microti* exist in all three places surveyed in the present study.

Sequencing analysis of rDNA: Twenty-two samples, which were PCR-positive with the primer set of Bab1A-4A, were subjected to another PCR for amplification of nearly full size rDNA sequences. Sequencing analysis revealed that both the 17 specimens from South Korea and the two specimens from Vladivostok had identical rDNA sequences which were exactly the same as that reported for a *B. microti*

Table 3. DNA sequence data generated in the present study

Accession no.	Gene	Size (bp)	Parasite (genotype)	Place[b)]
AB083374	rDNA[a)]	1708	*Babesia canis*	Okinawa, Japan
AB083375	rDNA	1665	*B. microti*, XJ1647 (U.S.)	Xinjiang, China
AB083376	rDNA	1598	*Babesia* sp., XJ1648	Xinjiang, China
AB083377	β-tubulin	1651	*B. microti*, Gray (U.S.)	U.S.A.
AB083378	β-tubulin	1476	*B. microti*, XJ1647 (U.S.)	Xnjiang, China
AB083379	β-tubulin	1448	*B. microti*, VL63 (U.S.)	Vladivostok, Russia
AB083380	β-tubulin	1482	*B. microti*, Korea8 (U.S.)	South Korea
AB083440	β-tubulin	1214	*B. microti*-like, Ko524 (Kobe)	Kobe, Japan
AB083441	β-tubulin	1214	*B. microti*-like, Ho234 (Hobetsu)	Hokkaido, Japan
AB083442	β-tubulin	1212	*B. rodhaini*	Uncertain

a) Nuclear small-subunit rRNA gene.
b) Place of parasite isolation or field sample collection.

isolate from United States (GenBank accession number U09833). The two specimens from *Lagurus luteus* in Xinjiang had rDNA sequences which were also identical to that of U.S.-type *B. microti*, except for two nucleotide substitutions at positions 605 and 1601 (GenBank accession number AB083375). Another sample from *Citellus erythrogenys* in Xinjiang, that was positive by *B. microti*-specific PCR, was found to have an rDNA sequence (GenBank accession number AB083376) with the highest similarity to that of *Babesia canis* (GenBank accession number AB083374). There were 131 specimens that were PCR-positive with the primer set of Bab5–8, but PCR-negative with the primer set of Bab1A-4A. Sequencing analysis of those rDNAs revealed that all of them were probably not derived from *B. microti* nor any closely related parasite, but may rather be derived from some other protozoan species belonging to Eimeriida, such as *Isospora*, *Sarcocystis*, and *Hepatozoon* spp.

Sequence analysis of β-tubulin gene: To further verify the presence of *B. microti* in the blood samples that were positive by rDNA-based PCR, babesial β-tubulin gene was also amplified from each of those samples and sequenced. Whereas the samples collected within a survey area had identical β-tubulin gene sequences, apparent sequence variations were seen among the samples obtained from distant geographic areas. We also amplified and sequenced partial β-tubulin gene sequences from two Japanese *B. microti*-like parasites (Kobe and Hobetsu types) and *B. rodhaini* (another rodent *Babesia*) for phylogenetic analysis. All of them contained two introns, the presence of which was experimentally confirmed by sequencing cDNA from β-tubulin mRNA. A neighbor joining tree constructed with those sequences was depicted in Fig. 2, showing that all the parasites detected in the present survey were closely related to *B. microti* from the United States.

Development of type-specific PCR: Designing primers specific for the β-tubulin genes of U.S.-, Hobetsu-, and Kobe-type parasites (Table 2), we developed type-specific nested PCR. The specificity of this PCR system is shown in Fig. 3a, demonstrating formation of a single positive signal which was specific for each type of the three *B. microti*-like parasites, namely Kobe- Hobetsu- and U.S.-type. The specificity of PCR products could further be verified without sequencing in aid of restriction enzyme digestion (Fig. 3b). The results obtained by this system were consistent with those obtained by rDNA-based PCR and sequencing (Table 1). A parasite with β-tubulin gene sequence of Hobetsu- or Kobe-type was not detected in any sample collected from the three survey areas.

Serological assay: The PBS-eluates from the blood samples, in which the presence of *B. microti* was detected by PCR, were tested by IFAT to detect specific antibodies against *B. microti*. Gray strain of *B. microti* [11] was used as the U.S.-type parasite antigen for IFAT. However, we were not able to find any sample which reacted with this parasite antigen (IFAT titers below 1:64).

DISCUSSION

In the present survey, we were able to demonstrate that U.S.-type *B. microti* is widely distributed among small wild rodents in northeastern Eurasia. Parasites with rDNA sequences that are virtually identical to that of *B. microti* from the United States (GenBank accession number U09833) have also been reported from Europe [7, 35]. Accordingly, it became increasingly clear that a single parasite species (*B. microti* sensu stricto, which is described as the U.S. type in this study) is distributed throughout the temperate zones of the Northern Hemisphere.

We previously reported that small wild rodents in Japan harbored two types of novel *B. microti*-like parasites, namely Hobetsu and Kobe types [31], whereas that U.S.-type *B. microti* has so far not been isolated in the country. Both the Hobetsu- and Kobe-type parasites have been isolated mainly from the large Japanese field mouse, *A. speciosus*, which is a major species of Muridae unique to Japanese Islands, indicating that these two types of *B. microti*-like parasites may also be unique to the country. Although some taxonomic criteria, such as morphology, pathogenicity, and ecological niche, place these Japanese parasites within the species of *B. microti*, our previous study clearly demonstrated that they differed from U.S.-type *B. microti* antigenically and genotypically [31]. If we are able to obtain further evidence for the geographic isolation of these two Japanese *B. microti*-like parasites, it may be reasonable to

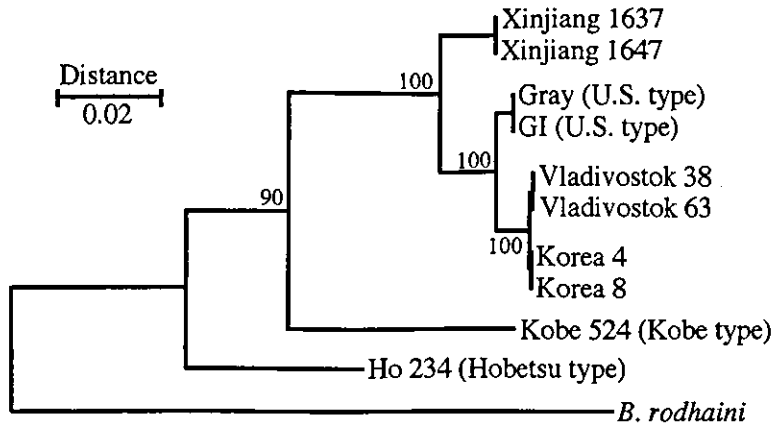

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method with β-tubulin gene sequences of several *B. microti* group parasites collected at various places. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates.

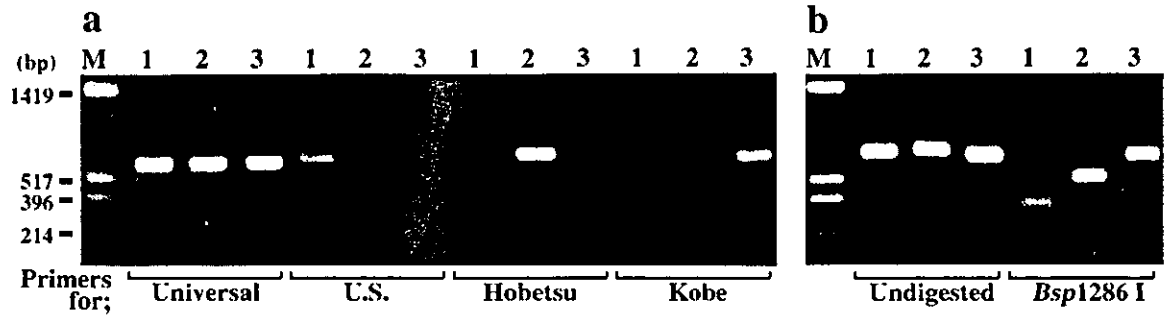

Fig. 3. Type-specific PCR targeting the β-tubulin genes of U.S.-, Hobetsu-, and Kobe-type parasites. Lanes; 1, Gray strain (U.S. type); 2, Ho234 (Hobetsu type); 3, Ko524 (Kobe type); M, DNA size makers. (a) Parasite DNA samples were amplified with either a set of the universal primers or each set of the three type-specific primers described in Materials and Methods. (b) Digestion of the specifically amplified PCR products by *Bsp*1286I, showing the presence of unique sites to each type.

propose separating them into new species or at least new subspecies.

The present survey showed that *Apodemus agrarius, A. penisulae, Clethrionomys rufocanus*, and *Lagurus luteus* were the rodent species harboring *B. microti* in northeastern Eurasia. *A. penisulae* and *C. rufocanus* are also known to exist on Hokkaido Island in Japan, because this island had been connected with Eurasian Continent via Sakhalin Island during the latest ice age until approximately ten thousand years ago. Hence, despite the apparent absence of U.S.-type *B. microti* in Japan, it may be possible to find this type in that region of the country. A sample from *Citellus erythrogenys* trapped in Xinjiang, China, contained a *Babesia* parasite whose rDNA was the most closely related to that of *B. canis vogeli* isolated from a dog in Okinawa Japan [1]. Whether this parasite is involved in any disease of dogs or humans is currently unknown, although a human case that was suggested to be due to *B. canis* has been reported [23].

The presence of *Babesia* parasites in the blood specimens was successfully determined by specific amplification of a short target sequence within the rDNA. However, sequencing near full length rDNA (approximately 1.6 kb) was needed for subsequent genotypic classification into Hobetsu, Kobe, and U.S. types, since the numbers of nucleotide sequences useful for type discrimination were only a very few; the three types of rDNAs showed more than 99.2% similarities [31]. Owing to such high degree of sequence conservation, the rDNA was not an appropriate target for further study on an intragenotypic, geographic variations. In fact, all the rDNA sequences amplified from the field collections in the present survey were virtually identical. Thus, we have analyzed β-tubulin gene sequences in addition to rDNA. All the β-tubulin gene sequences from the three survey areas exhibited high degrees of sequence similarity to that of *B. microti* isolated in the northeastern United States (GenBank accession number AB083377). Sequences obtained within a single survey area were identical, whereas significant numbers of base substitutions were seen among sequences obtained from distant areas, demonstrating intragenotypic, geographic variations. Most substitutions were found at the third nucleotide of the triplet genetic codon, therefore, resulted in little amino acid substi-

tution.

Sequencing analysis was carried out also for the β-tubulin genes of Hobetsu- and Kobe-type *B. microti*-like parasites and *B. rodhaini*. All of them contained two introns. While the position of the first intron is precisely conserved for most parasites in phylum Apicomplexa [4–6, 24], the presence and position of the second intron are probably unique to *B. microti* group (*B. microti* and closely related parasites, including *B. rodhaini*), because the other parasites belonging to genera *Babesia* and *Theileria* apparently lack it (M. Tsuji, unpublished data). The two introns were very small in size, 20 and 22 bp for the first and second ones, respectively, which are comparable to the smallest introns found in chloroplast nucleomorph of Chlorarachniophyte algae [10]. Since such introns may be too small to be recognized by the splicing mechanism known for general spliceisomal introns [12], *B. microti* may have a unique splicing mechanism. The introns in the β-tubulin genes of other *Babesia* and *Theileria* parasites are much longer than the first ones of *B. microti* group [4]. Gain and loss of introns have been regarded as a significant event in phylogenetic evolution [9]. Such features may be regarded as an additional line of evidence which supports the proposal that parasites in this group should belong to neither *Babesia* nor *Theileria*, and be re-classified as an independent genus [17, 35].

Phylogenetic analysis of the β-tubulin genes showed that sequence variations seen among the Kobe-, Hobetsu-, and U.S.-type parasites were much greater than those seen within various U.S. type *B. microti* from geographically distant areas. Whereas the former may be regarded as the interspecies variation, the latter may represent the intraspecies one. Evolutionary distances among these three types were comparable to those between some parasites in Piroplasmida that are well established to be distinct species, such as the distances between *Theileria parva* and *T. annulata* and between *Babesia bigemina* and *B. caballi* (M. Tsuji, unpublished data). This finding was in contrast to that reported in the previous studies based on rDNA sequences [25, 31], which showed that all the U.S.-, Hobetsu-, and Kobe-type parasites were quite closely related with each other. Because the degree of sequence conservation in the rDNA is exceptionally high, the phylogenetic analysis based on this gene may give rise to a significant underestimation. Further analyses with multiple genes will be needed to unequivocally address this issue.

We were able to develop type-specific nested PCR using primers that were specific for each β-tubulin gene of Kobe-, Hobetsu-, and U.S.-type parasites. This type-specific PCR easily and quickly generated results that were consistent with those obtained by rDNA-based PCR followed by sequencing analysis. Therefore, this system provided us with a much easier way of detection and typing *B. microti*-like parasites than that targeting their rDNA, and will become a powerful tool for facilitating epidemiological studies in much larger scale. With this type-specific PCR, we are currently conducting a field survey to identify vector ticks in Japan which transmit each of Hobetsu- and Kobe-type *B. microti*-like parasites.

Our serological assay failed to detect any specific antibody against *B. microti*. Because a local parasite isolate from any of the three survey areas has not been available in the present study, Gray strain, an isolate from the United State index case patient [11], was used as the U.S.-type parasite antigen for IFAT. However, variation in the β-tubulin gene sequences suggests that *B. microti* from different regions may significantly vary in their antigenicity. For more sensitive antibody detection, therefore, isolation of local parasites with homologous antigenicity may be needed.

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Sensitive and Specific Detection of *Yersinia pseudotuberculosis* by Loop-Mediated Isothermal Amplification

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We developed a loop-mediated isothermal amplification method able to detect *Yersinia pseudotuberculosis* strains in 30 min by using six primers designed by targeting the *inv* gene. This method is more sensitive than PCR and might be a useful tool for detecting and identifying *Y. pseudotuberculosis*.

Yersinia pseudotuberculosis is known to be an important causal agent of zoonosis. *Y. pseudotuberculosis* infection in humans causes several diseases, such as enteritis, mesenteric lymphadenitis, reactive arthritis, erythema nodosum, and septicemia (1, 14, 15). This bacterium has been isolated from many animals, including monkeys, dogs, pigs, rodents, rabbits, deer, and birds, and is sometimes fatal to them (1, 3, 4).

Of several molecular genetic methods, PCR is the most widely used for specific amplification of a target gene, and it has also been reported to be able to detect pathogenic *Yersinia* species from foods and environmental samples (7, 16, 17, 19). Recently, a novel nucleic acid amplification method, named loop-mediated isothermal amplification (LAMP), that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions has been developed (2, 9, 12). This method simply consists of incubating a mixture of the target gene, four different primers, DNA polymerase with strand displacement activity, and substrates at a constant temperature between 60 and 65°C. The target gene is detected by the increase in the turbidity of the reaction mixture that coincides with the production of precipitate correlated with the amount of target DNA synthesized, i.e., the amplicons. The aim of this study was to develop a *Y. pseudotuberculosis* detection method, more sensitive and specific than PCR, based on the LAMP method, and to evaluate the performance of this method for detection of *Y. pseudotuberculosis* in clinical samples.

Thirty-one *Yersinia* species comprising 21 strains of *Y. pseudotuberculosis*, 4 strains of pathogenic *Y. enterocolitica*, and 6 strains of nonpathogenic *Yersinia* species strains, as well as 10 other gram-negative bacilli, were tested (Table 1). Template DNAs used for LAMP were prepared as follows. Bacterial cells of each strain from colonies on trypticase soy agar (TSA; BBL) were suspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) to achieve a concentration of approximately 10^8 CFU/ml. In order to examine the detection limit for LAMP and PCR, a series of 10-fold dilutions of *Y. pseudotuberculosis* serovar 1b with TE buffer was made. The cells were heat treated in a boiling water bath for 10 min and were centrifuged for 10 min at 9,000 × *g*. The resulting supernatant was used as the template for LAMP and PCR. The LAMP reaction requires four oligonucleotide primers recognizing six distinct regions (F1, F2, F3, B1, B2, and B3) on the target DNA: the forward inner primer (FIP), back inner primer (BIP), and two outer primers (F3 and B3) (12). FIP consists of a complementary sequence of F1 and a sense sequence of F2. BIP consists of a sense sequence of B1 and a complementary sequence of B2. LAMP primers targeting the *inv* gene of *Y. pseudotuberculosis*, the chromosomal virulence gene (8), were designed based on the gene sequence of *inv* (accession no. M17448) obtained from the DNA Data Bank of Japan by using the software program Primer Explorer V2 (Fujitsu, Tokyo, Japan). The sequences of the designed primers are shown in Table 2. Those four primers are sufficient to carry out the amplification reaction; however, the LAMP reaction can be accelerated by using additional primers termed loop primers (10), so loop primers LF and LB targeting the *inv* gene of *Y. pseudotuberculosis* were designed (Table 2). These loop primers were used in the reactions through which the amplification data were collected. However, the designed loop primers react with the restriction site of restriction enzyme BssHII (New England BioLabs, Beverly, Mass.), which was used to digest the obtained amplicons so as to confirm that the amplicons are of the target genes. Therefore, the loop primers were not used in the reactions involving BssHII. The LAMP reaction was carried out with the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). A reaction mixture (25 μl) containing 1.6 μM each inner primer (FIP and BIP), 0.2 μM each outer primer (F3 and B3), 0.8 μM each loop primer (LF and LB), *Bst* DNA polymerase (0.5 μl), 2× reaction mix (12.5 μl), and template DNA (2 μl) was incubated at 63°C for 50 min and then heated at 80°C for 2 min to terminate the reaction. A DNA-omitted reaction mixture was used as a negative control. The amplification of the gene was confirmed by real-time monitoring of the increase of turbidity by using LA-200 (Teramecs, Kyoto, Japan), which sequentially measured the absorbance of the reaction mixture at 650 nm. To determine the detection

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TABLE 1. Bacterial strains subjected to LAMP and results

Species	Serotype	Strain	LAMP result[a]
Y. pseudotuberculosis	1a	3384	0.49
Y. pseudotuberculosis	1b	NYP95001	0.50
Y. pseudotuberculosis	1c	Kuratani	0.46
Y. pseudotuberculosis	2a	49	0.49
Y. pseudotuberculosis	2b	1799	0.49
Y. pseudotuberculosis	2c	274	0.49
Y. pseudotuberculosis	3	T-312	0.46
Y. pseudotuberculosis	4a	51	0.44
Y. pseudotuberculosis	4b	NYP01001	0.48
Y. pseudotuberculosis	5a	204	0.47
Y. pseudotuberculosis	5b	197	0.50
Y. pseudotuberculosis	6	#14	0.55
Y. pseudotuberculosis	7	141	0.50
Y. pseudotuberculosis	8	151	0.50
Y. pseudotuberculosis	9	R708Ly	0.49
Y. pseudotuberculosis	10	6088	0.48
Y. pseudotuberculosis	11	R80	0.43
Y. pseudotuberculosis	12	MW900-3	0.42
Y. pseudotuberculosis	13	N916	0.47
Y. pseudotuberculosis	14	CN7	0.45
Y. pseudotuberculosis	15	93422	0.47
Y. enterocolitica	O:3	8	0.00
Y. enterocolitica	O:5,27	S203	0.00
Y. enterocolitica	O:8	NY9306089	0.00
Y. enterocolitica	O:9	314-2	0.00
Y. enterocolitica	O:8,19	NY8904001	0.00
Y. aldovae		JCM 5892	0.00
Y. intermedia		JCM 7579	0.00
Y. cristensenii		JCM 7576	0.00
Y. bercovieri		NY8704001	0.00
Y. rohdei		JCM 7376	0.00
Campylobacter jejuni		ATCC33560	0.00
Campylobacter coli		JCM2529	0.00
Campylobacter lari		JCM2530	0.00
Citrobacter freundii		JCM1657	0.00
Enterobacter cloacae		JCM1232	0.00
Escherichia coli		JCM5431	0.00
Pasteurella haemolytica		NP8507001	0.00
Pseudomonas fluorescens		JCM 5963	0.00
Salmonella enterica serovar Typhimurium		NMJS1	0.00
Salmonella enterica serovar Enteritidis		NS9506003	0.00

[a] Turbidity after 30 min of incubation.

limit, 1 μl of the LAMP products was submitted to electrophoresis, and, to confirm the amplicon structure, the LAMP products were digested with restriction enzyme BssHII and submitted to electrophoresis. The electrophoresis was carried out in 2% Tris-acetic acid-EDTA (TAE) agarose gel, and staining was performed with ethidium bromide to confirm the presence of the expected DNA fragments. One kilobase of

TABLE 2. LAMP primers

Primer	Sequence (5'-3')
F3	CTCGTCGCGTGATTTCTCC
B3	GATCTACCCCGACAGTGAGT
FIP	CCAGTTGTGGGAGTGCAGGTAACTATAAAG AGCGCCCAGCC
BIP	CACCGGTGAGCGTGTTGCTTTGTGTAATTGA TCCCGGCAGT
LF	CATTCGCGCGCAAATCC
LB	GCAACGCAACCCTTATGC

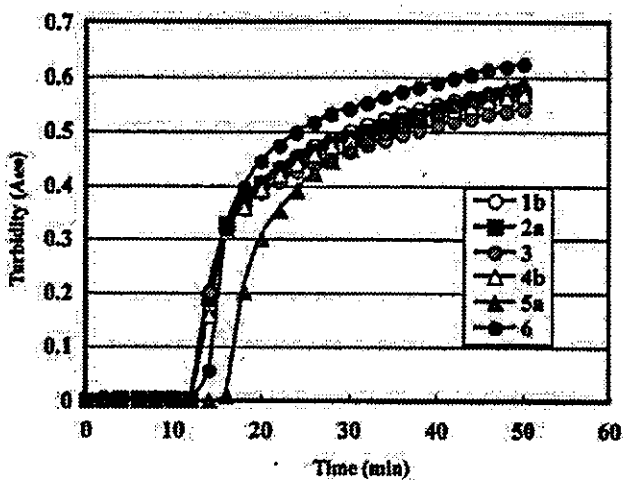

FIG. 1. Detection of the LAMP amplification signals. A total of 10^5 CFU of template DNA of *Y. pseudotuberculosis* 1b, 2a, 3, 4b, 5a, and 6 was used for the LAMP reaction.

Plus DNA ladder (Invitrogen) was used as a molecular weight standard. The PCR was carried out with the primers for the *inv* gene designed by Nakajima et al. (11). PCR was performed with a Program Temperature Control System PC-701 (ASTEC, Fukuoka, Japan) at 94°C for 1 min as an initial denaturation step and then was subjected to 30 cycles consisting of 30 s at 94°C, 1 min at 55°C, and 2 min at 70°C, followed by a single 5-min extension step at 70°C. The PCR mixture (50 μl) contained 4 μl of template DNA, 0.1 mM each of the four deoxynucleoside triphosphates, 5 μl of 10× PCR buffer (Applied Biosystems Japan Ltd., Tokyo, Japan), 0.1 μM each primer, and 0.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.). Ten microliters of the PCR amplification products was subjected to electrophoresis under the same protocol of the LAMP products in a 1.5% agarose gel.

The specificity of LAMP using the newly designed primers was examined by carrying out reactions with DNAs from the *Yersinia* species and other gram-negative bacilli. The results of turbidity measurements for the LAMP reaction for 30 min at 63°C are shown in Table 1, and the representative curves are shown in Fig. 1. Turbidities derived from the LAMP reaction of *Y. pseudotuberculosis* strains began to increase after approximately 15 min of incubation, and they continued to increase as the LAMP progressed. All *Y. pseudotuberculosis* strains examined showed turbidities above 0.4 at 650 nm after 30 min of incubation. In contrast, turbidities were not observed even after 50 min of incubation when template DNA from *Y. enterocolitica*, a nonpathogenic *Yersinia* species, and other gram-negative bacilli were tested. This result proved the specificity of the developed primers. The differences among the turbidities of *Y. pseudotuberculosis* and all of the other samples became evident after 20 to 25 min of incubation. The use of loop primers shortened the reaction time for amplification by about one-half compared to that of amplification performed without loop primers (data not shown). These results showed that the LAMP method using these newly designed primers is able to detect *Y. pseudotuberculosis* specifically.

The sensitivity of LAMP and PCR for *Y. pseudotuberculosis* was determined by determining the detection limit as de-