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Ixodes persulcatus Ticks as Vectors for the *Babesia microti* U.S. Lineage in Japan

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ABSTRACT

The U.S. lineage, one of the major clades in the *Babesia microti* group, is known as a causal agent of human babesiosis mostly in the northeastern and upper midwestern United States. This lineage, however, also is distributed throughout the temperate zone of Eurasia with several reported human cases, although convincing evidence of the identity of the specific vector(s) in this area is lacking. Here, the goal was to demonstrate the presence of infectious parasites directly in salivary glands of *Ixodes persulcatus*, from which U.S. lineage genetic sequences have been detected in Asia, and to molecularly characterize the isolates. Five PCR-positive specimens were individually inoculated into hamsters, resulting in infections in four; consequently, four strains were newly established. Molecular characterization, including 18S rRNA, β-tubulin, and *CCT7* gene sequences, as well as Western blot analysis and indirect fluorescent antibody assay, revealed that all four strains were identical to each other and to the U.S. lineage strains isolated from rodents captured in Japan. The 18S rRNA gene sequence from the isolates was identical to those from *I. persulcatus* in Russia and China, but the genetic and antigenic profiles of the Japanese parasites differ from those in the United States and Europe. Together with previous epidemiological and transmission studies, we conclude that *I. persulcatus* is likely the principal vector for the *B. microti* U.S. lineage in Japan and presumably in northeastern Eurasia.

IMPORTANCE

The major cause of human babesiosis, the tick-borne blood parasite *Babesia microti*, U.S. lineage, is widely distributed in the temperate Northern Hemisphere. However, the specific tick vector(s) remains unidentified in Eurasia, where there are people with antibodies to the *B. microti* U.S. lineage and cases of human babesiosis. In this study, the first isolation of *B. microti* U.S. lineage from *Ixodes persulcatus* ticks, a principal vector for many tick-borne diseases, is described in Japan. Limited antigenic cross-reaction was found between the Japan and United States isolates. Thus, current serological tests based on U.S. isolates may underestimate *B. microti* occurrence outside the United States. This study and previous studies indicate that *I. persulcatus* is part of the *B. microti* U.S. lineage life cycle in Japan and, presumably, northeastern Eurasia. This report will be important for public health, especially since infection may occur through transfusion, and also to researchers in the field of parasitology.

*B*abesia is a protozoan transmitted by ixodid ticks that infects erythrocytes in the host animal. The *Babesia microti* group is a diverse group of worldwide-distributed parasites that includes various lineages, such as U.S., Kobe, Hobetsu, Munich, monkey/squirrel, and some still-unnamed groups (1–5). The parasites of this group have been detected from various animals, including mouse (U.S., Kobe, Hobetsu, and Munich), rat (U.S. and Kobe), vole (U.S., Hobetsu, and Munich), shrew (U.S., Hobetsu, and Munich), lemming (U.S.), squirrel (monkey/squirrel), and non-human primate (monkey/squirrel) (summarized in Zamoto-Niikura et al. [6]). To date, parasites belonging to the U.S. lineage around the world and the Kobe lineage from Japan have been isolated from patients and are apparently pathogenic to humans (5, 7, 8). However, a patient(s) infected with another parasite, such as Hobetsu lineage (9), may emerge as a consequence of improved detection techniques and recent increased attention to emerging tick-borne diseases, such as severe fever with thrombocytopenia syndrome (SFTS), relapsing fever, anaplasmosis, and neoehrlichiosis (10–13).

The largest lineage of the *B. microti* group, the U.S. lineage, contains *B. microti sensu stricto*, a causative agent of human babesiosis in the northeastern and upper midwestern United States, where most of the human cases worldwide have been reported.

Outside of the United States, a few autochthonous infections have been reported from Canada, Australia, and Germany (7, 8, 14, 15), and antibodies against the U.S. lineage in humans are evident widely in Germany (16), Austria (17), Belgium (18), Switzerland (19), and Mongolia (20). These findings indicate that the *B. microti* U.S. lineage parasites distributed worldwide are infectious to humans, but pathogenicity may vary among parasite populations. Recently the U.S. lineage parasites were demonstrated to be genetically diverse in β-tubulin and chaperonin containing TCP1 subunit eta (*CCT7*) gene sequences (21–23). Furthermore, parasites in the U.S. lineage phylogenetically grouped into distinct 3 sublineages, North America, Europe-Central Asia, and East Asia, each of which reflects the geographic origin of the para-
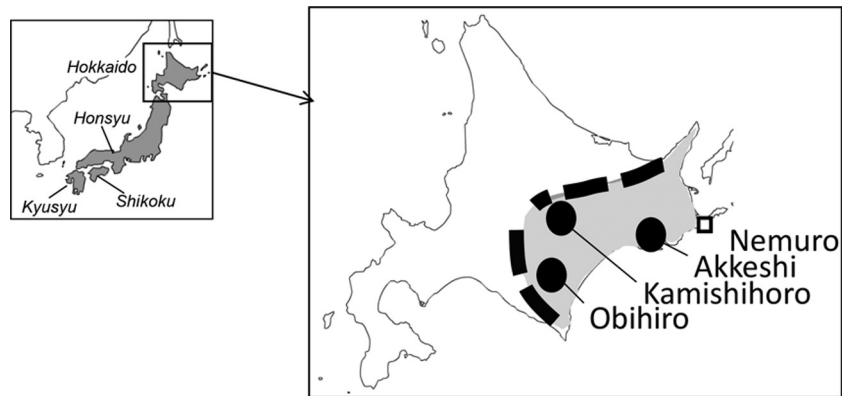
FIG 1 Map of Hokkaido, Japan, showing the locations of field collections (circles). The dashed line shows the eastern Hokkaido area and mountain ranges along the border of eastern Hokkaido. Nemuro (square) is the area in which the *B. microti* U.S. lineage was previously isolated from rodents.

sites. The relationship between the genetic classification (or the sublineage) and pathogenicity remains unclear.

Identification of tick vectors is important, since they bridge the animal reservoir and humans. The vector's preference for humans (questing behavior), seasonal and regional abundance, geographical distribution, and transmission efficiency as a vector directly or indirectly affect the emergence of human cases (24–27). The *B. microti* Munich lineage, sequences of which have been detected in various species of rodents from Europe to Russia (summarized in Zamoto-Niikura et al. [6]), is regarded as a nonzoonotic pathogen partly because a nidicolous (nest-dwelling) tick, *Ixodes trianguliceps*, is considered the principal vector for this lineage (28), and this tick does not infest humans.

Since the *B. microti* U.S. lineage is distributed widely over the temperate zones of the Northern Hemisphere, species of ticks transmitting parasites of this lineage would be expected to vary. In the U.S., an extensive survey in areas where human babesiosis is endemic demonstrated *Ixodes scapularis* (formerly *I. dammini*) as the principal tick vector. The ecology of this tick is well understood. (i) Both nymphal and adult *I. scapularis* ticks carry the parasites, but nymphs have a higher infection rate and number of developed sporozoites in their salivary glands (29). This is one reason why the infection is usually transmitted through the bite of infected nymphs, although adult ticks occasionally transmit *B. microti* to humans. (ii) The maximum seasonal activity of *I. scapularis* nymphs, in May through July, is followed by human infections, which are diagnosed mainly in June, July, or August (27). (iii) The geographical extension of human babesiosis from the northeastern coastal region to inner and southern areas of the U.S. has been partially attributed to geographic expansion of *I. scapularis* and its deer host (27, 30).

In the Eurasian region where *I. scapularis* is absent, PCR surveillance and DNA sequence analyses of field-collected ticks have revealed that *Ixodes persulcatus* (31–33) and *Ixodes ricinus* (1, 34, 35) in Asia and Europe, respectively, carry the *B. microti* U.S. lineage. Although experimental transmission of *B. microti* U.S. lineage to hamsters or gerbils has been shown (33, 36), there is still a lack of biological evidence demonstrating the live pathogen in field-derived specimens. In this study, we provide direct evidence that *I. persulcatus* ticks carry the infectious *B. microti* U.S. lineage in their salivary glands. We successfully isolated the *B. microti* U.S. lineage from field-collected *I. persulcatus* females in Japan and established 4 strains. We show here the genetic and antigenic features of these strains compared to those previously reported in the U.S. and Europe.

MATERIALS AND METHODS

Field collection. Unfed, host-seeking ticks were collected by flagging vegetation on the sides of forest paths, which were easily accessible by car and on foot. In Japan, *B. microti* U.S. lineage has been detected in *I. persulcatus* collected only in the eastern part of Hokkaido (6, 33); thus, 3 areas, including 7 sites, were selected for this study (Fig. 1). The duration times and number of tick collections were recorded at each site. The species identification of ticks was determined morphologically (body size, color, and shape of idiosoma and legs, shape of capitulum, and shape and size of internal spur of coxa I). Microscopically identified female ticks were kept in tightly sealed plastic tubes over a moistened filling of solidified plaster with activated charcoal at 4°C until infested on gerbils. Male ticks were frozen and kept at −30°C until DNAs were extracted.

Collection efficiency. Tick collection efficiency was used to postulate how often wild animals carrying the ticks appeared at the survey site. The collection efficiency was the number of ticks collected divided by duration time (ticks/minute) (Table 1).

Extraction of DNA from ticks. DNA extractions were performed as described previously (33). Briefly, individual ticks were homogenized in TNE buffer (10 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, pH 8.0) containing 0.1% sodium dodecyl sulfate (SDS) and digested with proteinase K. DNAs were purified by phenol extraction followed by ethanol precipitation, and the DNA pellet was resuspended in 100 μl of Tris-EDTA (TE) buffer. All DNAs were stored frozen at −30°C until use.

PCR. To detect *B. microti* U.S. lineage, lineage-specific PCR based on the β-tubulin gene sequence was performed as described previously (23), using 1 μl of DNA. Briefly, two sets of primers, BmTubu93F/BmTubu897R and Tubu-US5′/Tubu-US3′, were used for primary and nested PCR, respectively. Data collected by the lineage-specific PCR test were used to provide an estimate of infection prevalence. The infection rate (as a percentage) was calculated by comparing the number of PCR samples positive for *B. microti* group U.S. lineage to the total number of ticks examined.

Experimental animals. Specific-pathogen-free golden hamsters (slc: Syrian) and gerbils (MON/Jms/GbsSlc) 8 weeks of age were purchased from SLC Inc. (Shizuoka, Japan). Animal experimentation was carried out according to the laboratory animal control guidelines of the National Institute of Infectious Diseases (institutional permission no. 213126 and 114014).

TABLE 1 Ticks collected and screening of male and female adult *I. persulcatus* ticks for *B. microti* by nested PCR targeting β-tubulin gene

Location	Value(s) for:					
	Ticks collection (all species)			*I. persulcatus* screened for *B. microti* U.S. lineage[g]		
	No. collected	Time[a] (min)	No. of ticks/min	Total no. screened (no. male/no. female)	No. PCR positive (no. male/no. female[b])	% of total (% male/ % female)
Akkeshi 1	387	160	2.4	111 (111/ND)	1 (1/ND)	0.9 (0.9/ND)
Akkeshi 2	67	40	1.7	11 (9/2[c])	1 (1/0)	9.0 (11.1/0)
Kamishihoro 1	137	80	1.7	62 (47/16)	6 (3/3[d])	9.7 (6.4/18.8)
Kamishihoro 2	35	60	0.6	20 (20/ND)	0 (0/ND)	0 (0.0/ND)
Obihiro 1	35	40	0.9	5 (5/ND)	0 (0/ND)	0 (0.0/ND)
Obihiro 2	88	60	1.5	28 (28/ND)	0 (0/ND)	0 (0/ND)
Obihiro 3	111	50	2.2	56 (33/23)	9 (7/2[e])	15.3 (21.2/9.0)
Total	860[f]	490	1.8	294 (253/41)	17 (12/5)	5.8 (4.7/12.2)

[a] Time during which ticks were collected at each location.
[b] Whole bodies and salivary glands of males and females, respectively, were used.
[c] Only 2 female ticks were collected.
[d] Two of three were successfully isolated in hamsters.
[e] Two were isolated in hamsters.
[f] *I. persulcatus* male ($n = 253$), female ($n = 193$), and nymph ($n = 22$); *I. ovatus* ($n = 315$) and *Hemaphysalis* spp. ($n = 77$).
[g] ND, not determined.

Isolation of *B. microti* from *I. persulcatus*. Isolation of *B. microti* was done by inoculation of salivary glands as previously described ([33]). *I. persulcatus* females collected at the selected site (Obihiro, Akkeshi, and Kamishihoro) ([Table 1]) were fed on naive gerbils for 3 or 4 days to activate quiescent *B. microti* sporozoites in the salivary glands into a state of readiness for infection, and then the partially engorged ticks were removed manually. The salivary glands were dissected from the ticks under a stereomicroscope, and the glands from each individual tick were soaked in 500 µl of cold phosphate-buffered saline (PBS) and homogenized by a glass tissue grinder (Radnoti). An aliquot of 100 µl was used for DNA extraction and PCR screening as described above to predict the presence of the *B. microti* parasites. The remaining 400 µl of the homogenate was inoculated intraperitoneally into individual Syrian hamsters. The hamsters were monitored every 2 or 3 days for 3 months by determining the parasitemia in Giemsa-stained thin blood smears. When the parasitemia in the hamsters reached at least 10%, whole blood was collected by cardiac puncture. The red blood cells (RBCs) were washed 3 times by centrifugation in cold PBS and used to prepare DNA for sequencing, antigen for indirect fluorescent antibody (IFA) test, and antigen for Western blot analysis, and to produce antiserum to *B. microti* U.S. lineage in hamsters. For the latter, a portion of the washed RBCs was injected intravenously into naive hamsters, and convalescent-phase serum was collected 8 weeks postinfection and stored at −30°C until it was used as described below. It is noted that the isolation was attempted by inoculation of the salivary glands, since we failed to isolate parasites from female ticks by direct infestation (data not shown).

Absence of parasites in the hamsters was determined at 3 months after inoculation by PCR. Nested PCR targeting the 18S rRNA gene was performed on the DNA extracted from erythrocytes as described below.

Western blot analysis. The antigens described above were separated by SDS-PAGE as described previously ([5]). The loading volume was compensated based on the percent parasitemia of the blood at harvest from hamsters to prepare erythrocyte antigens ([5]), except for isolate IpSG13-1-2. Since the parasitemia in the hamster infected with IpSG13-1-2 was 10%, which was the lowest level among the isolates, and since cross-reactivity was relatively low in preliminary experiments, the IpSG13-1-2 sample was loaded at twice the calculated volume. The separated proteins were blotted onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore), and the membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20. The membranes were incubated first with antisera diluted 1:200 and subsequently with goat poly-

clonal antibody to Syrian hamster IgG H&L (alkaline phosphatase) (Abcam). Immunoreactive antigens were detected using the 5 bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium alkaline phosphatase (NBT-BCIP) liquid substrate system (Sigma). IpSG13-10-2 antiserum was used as a representative of antisera raised against *B. microti* U.S. lineage isolated from *I. persulcatus* in this study. For negative controls, antigens were prepared from two noninfected hamsters, which had been inoculated with piroplasma-free salivary glands from *I. persulcatus* females.

IFA tests. Infected RBCs were mixed with an equal volume of fetal bovine serum, and thin blood smears were made on microscope slides. The slides were transferred into double-distilled water (DDW) to lyse the RBCs and air dried. Hydrophobic circles, used to form wells, were drawn on the slides using a PAP pen (Super PAP pen; Daido, Tokyo, Japan). The slides were placed in a humidified chamber, and 20 µl of serial 2-fold dilutions of serum was added to each well. After 1 h of incubation at 37°C, the slides were washed in PBS, and 20 µl of Alexa Fluor488-conjugated goat anti-hamster IgG (H+L) (Life Technologies) diluted 1:200 in Tris-buffered saline containing 5% Immunoblock (DS Pharma) and Tween 20 was added. The slides were incubated at room temperature for 1 h and washed in PBS. Fluorescent parasites in RBCs were observed with a fluorescence microscope (Olympus IX71) at a magnification of ×200.

Amplification and direct sequencing of 18S rRNA, β-tubulin, and *CCT7* gene sequences. PCR was performed using 1 µl of DNA and primer pairs Piro0F/6R ([4]), TubuATG5F/Tubu1538R ([2]), and UScct-1/UScct-4 ([22]) to amplify 18S rRNA, β-tubulin, and *CCT7* gene sequences, respectively. For *CCT7*, the PCR primer set UScct-2/UScct-3 ([22]) was used for nested PCR when the primary PCR amplicons were insufficient for direct sequence analysis.

Specific amplicons were subjected to cycle sequencing using the PCR primers and BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). For *CCT7*, additional primers UScct-sq1 (5′GCTGCTACGAT GCGCGGAAACGACAC3′), UScct-sq2 (5′GAATGCCCTAATACAAAG ACCGCAACUSCCT3′), USCCT-sq3R (5′CCTCAGATAATAATCAAAT ACTATAGGGAAGC3′), and USCCT-sq4R (5′GATGAAATTGATATTA AGCGAGTGGCCAAAG3′) were used for direct sequencing. Sequencing was performed using a 3130 genetic analyzer (Applied Biosystems). All sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ). The sequences have been released to the public and are available through GenBank.

Phylogenetic analysis. The sequences for each gene were aligned with the program CLUSTAL W 2.1 Alignment (http://clustalw.ddbj.nig.ac.jp/)

TABLE 2 Incubation of *I. persulcatus* and isolation of *B. microti*

| Salivary gland | *I. persulcatus* | | Day of isolation from hamster[b] |
	Origin of tick	Storage[a] (wk)	
IpSG13-1-2	Obihiro 3	3	18
IpSG13-10-2	Obihiro 3	7	26
IpSG13-16-3	Kamishihoro 1	11	46
IpSG13-18-1	Kamishihoro 1	19	52
IpSG13-19-1	Kamishihoro 1	19	Failed

[a] Period during which the ticks were stored at 4°C until used for inoculation.
[b] Number of days until detection of parasites in hamster erythrocytes after salivary gland inoculation.

with default settings. Phylogenetic trees were constructed by the neighbor-joining method in the program with 1,000 bootstrap replicates.

Reference parasite strains. *B. microti* Gray (37), GI (PRA-398; ATCC), and Nan-H5-2011 (PRA-399; ATCC) strains were used as the northeastern United States *B. microti* representative. NM69 and AK2273 are strains isolated from *Myodes rufocanus* and *Apodemus speciosus*, captured in Nemuro and Akkeshi, Hokkaido, Japan, respectively (Fig. 1) (38).

Accession number(s). All sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC127369 (IpSG13-1-2), LC127370 (IpSG13-10-2), LC127371 (IpSG13-16-2), and LC127372 (IpSG13-18-1) for the 18S rRNA gene; LC127373 (IpSG13-1-2), LC127374 (IpSG13-10-2), LC127375 (IpSG13-16-2), and LC127376 (IpSG13-18-1) for the β-tubulin gene; and LC127377 (IpSG13-1-2), LC127378 (IpSG13-10-2), LC127379 (IpSG13-16-2), and LC127380 (IpSG13-18-1) for *CCT7*.

RESULTS

Tick collection and PCR screening. In total, 860 ticks, including *Ixodes* and *Haemaphysalis* spp., were collected at 7 sites in 3 areas (Fig. 1 and Table 1). The numbers collected and the amount of time at each survey site are summarized in Table 1. Among all ticks collected, 315 were *I. ovatus* adults, 253 were *I. persulcatus* males, and 193 were *I. persulcatus* females.

Collection efficiency (ticks/minute) varied among the sites (Table 1). Ticks were efficiently collected at Akkeshi 1, Obihiro 3, Akkeshi 2, and Kamishihoro 1 (2.4, 2.2, 1.7 and 1.7 ticks/min, respectively). Collection efficiency at Akkeshi 1 (2.4 ticks/min) was 4 times greater than the lowest efficiency (0.6 ticks/min) at site Kamishihoro 2.

I. persulcatus adult males and females were screened by β-tubulin gene nested PCR for *B. microti* (Table 1), and infection rates were calculated for *I. persulcatus* males based on the PCR results. *I. persulcatus* males positive for *B. microti* U.S. lineage were found in 4 sites, Akkeshi 1 and 2, Kamishihoro 1, and Obihiro 3, and infection rates ranged from 0.9% at Akkeshi 1 to 21.2% at Obihiro 3 (Table 1). Judging from the infection rate in male ticks and the collection efficiency at each site, females collected in Akkeshi 2, Kamishihoro 1, and Obihiro 3 were selected for parasite isolation. Although Akkeshi 1 had the highest collection efficiency, the infection rate was low (0.9%). Thus, female ticks collected at Akkeshi 1 were not used for parasite isolation. *B. microti* U.S. lineage was not detected in any of the 315 *I. ovatus* ticks examined.

Isolation of *B. microti* U.S. lineage from *I. persulcatus*. *I. persulcatus* females collected at Obihiro 3 were kept at 4°C for 3 to 7 weeks and then fed on gerbils. Out of 23 ticks examined, the salivary glands from 2 ticks, designated IpSG13-1-2 and IpSG13-10-2, were PCR positive (Tables 1 and 2). Two hamsters were inoculated individually with the salivary glands IpSG13-1-2 or

IpSG13-10-2 and developed parasitemia 18 and 26 days after inoculation, respectively (Table 2). Hamsters ($n = 21$) inoculated individually with PCR-negative salivary glands from the remaining 21 ticks remained uninfected for 3 months.

I. persulcatus females collected at Kamishihoro 1 were kept at 4°C for 11 to 19 weeks (Table 2). Out of 15 examined, the salivary glands from 3 ticks, designated IpSG13-16-3, IpSG13-18-1, and IpSG13-19-1, were PCR positive (Tables 1 and 2). Two hamsters individually inoculated with IpSG13-16-3 and IpSG13-18-1 developed parasitemia 46 and 52 days after inoculation, respectively (Table 2). One hamster inoculated with PCR-positive IpSG13-19-1 salivary glands and 12 hamsters individually inoculated with PCR-negative salivary glands did not develop parasitemia for 3 months after inoculation. Lack of infection in the 13 hamsters was confirmed by nested PCR on peripheral blood samples.

At Akkeshi 2, two *I. persulcatus* females were collected and examined (Table 2). The salivary glands from 2 ticks were PCR negative, and hamsters individually inoculated with the salivary glands did not develop parasitemia for 3 months after inoculation.

In summary, 4 strains of *B. microti* U.S. lineage, designated IpSG13-1-2, IpSG13-10-2, IpSG13-16-2, and IpSG13-18-1, were isolated from hamsters by inoculation of infected salivary glands from *I. persulcatus* ticks (Tables 1 and 2; see also Fig. 6).

Sequencing analysis of 18S rRNA, β-tubulin, and *CCT7* genes. The 18S rRNA, β-tubulin, and *CCT7* genes were successfully amplified from the 4 isolates designated IpSG13-1-2, IpSG13-10-2, IpSG13-16-2, and IpSG13-18-1. For all genes, nucleotide alignments revealed that the sequences from all 4 isolates were identical to each other and to those from *B. microti* NM69 and AK2273 strains isolated from rodent reservoirs, *Myodes rufocanus* and *Apodemus speciosus*, respectively, in Japan (38).

The phylogenetic tree based on the *B. microti* group 18S rRNA gene was constructed using sequences obtained in this study and sequences available in GenBank (Fig. 2). The *B. microti* 18S rRNA gene sequences from *I. persulcatus* were identical to those from humans in the United States (Gray and GI strains) and were placed in the U.S. lineage cluster with those from *I. ricinus* tick, human, and vole in Germany and from lemming in Xingiang, China (Fig. 2).

The phylogenetic trees based on the β-tubulin and *CCT7* genes were similarly constructed (Fig. 3 and 4) and showed similar topology to each other with high bootstrap values. The *B. microti* U.S. lineage sequences branched into 3 sublineages, namely, Europe-Central Asia, East Asia, and North America, as shown previously (6, 21, 22). The isolates from *I. persulcatus* all were placed in the East Asia sublineage, whereas isolates from *I. scapularis* and *I. ricinus* ticks belonged to North America and Europe-Central Asia sublineages, respectively (Fig. 3).

Antigenic analysis. To examine the antigenic relationship among the U.S. lineage parasites isolated from *I. persulcatus*, from rodents (NM69) in Japan, and from humans in the United States (Gray, GI, and Nan-H5-2011), IFA tests were carried out for pairwise comparison. All 4 strains isolated from *I. persulcatus* showed identical antigenicities; therefore, results for *I. persulcatus* IpSG13-10-2 are shown as representative (Table 3). The NM69 and IpSG13-10-2 antisera showed high titers of 12,800 against both the NM69 and IpSG13-10-2 parasite antigens (Table 3). Similarly, the United States Gray, GI, and Nan-H5-2011 antisera showed high titers of 12,800 against both homologous and heterologous U.S. parasite antigens for Gray, GI, and Nan-H5-2011 (Table 3).
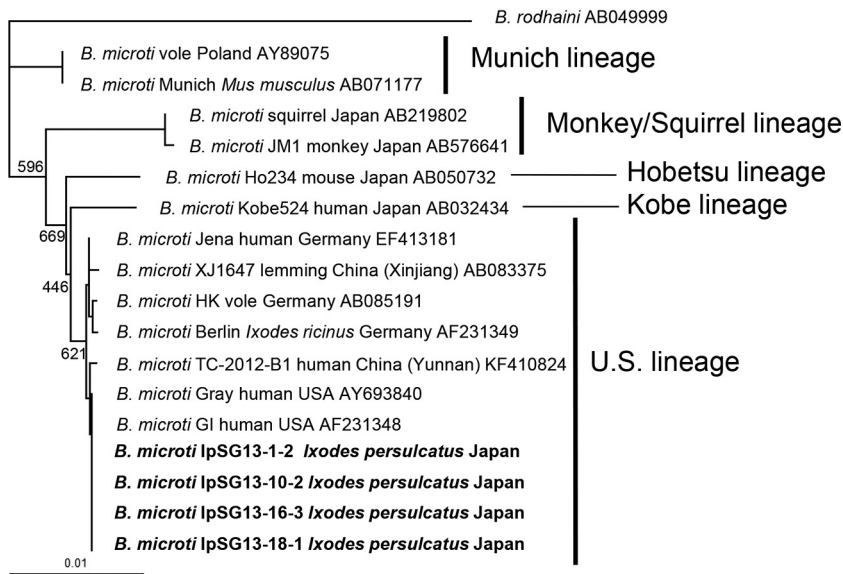
18S rRNA

FIG 2 Phylogenetic tree constructed with sequences of the 18S rRNA genes of the *B. microti* U.S. lineage and other closely related parasites. GenBank accession numbers are given for each strain. Strains for which sequences were determined in this study are shown in boldface. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates. Accession numbers of *B. microti* IpSG13-1-2, IpSG13-10-2, IpSG13-16-3, and IpSG13-18-1 are LC127369 to LC127372, respectively.

Lower titers resulted with the Japanese NM69 and IpSG 13-10-2 antisera and U.S. Gray, GI, and Nan-H5-2011 antigen and with the U.S. Gray, GI, and Nan-H5-2011 antisera reacted with the Japanese NM69 and IpSG13-10-2 antigen (Table 3). The IpSG 13-10-2 antiserum titers were 1:400 to 1:800 against the Gray, GI, and Nan-H5-2011 strains, which were 16 to 32 times lower than those of the U.S. strain homologous antisera (1: 12,800).
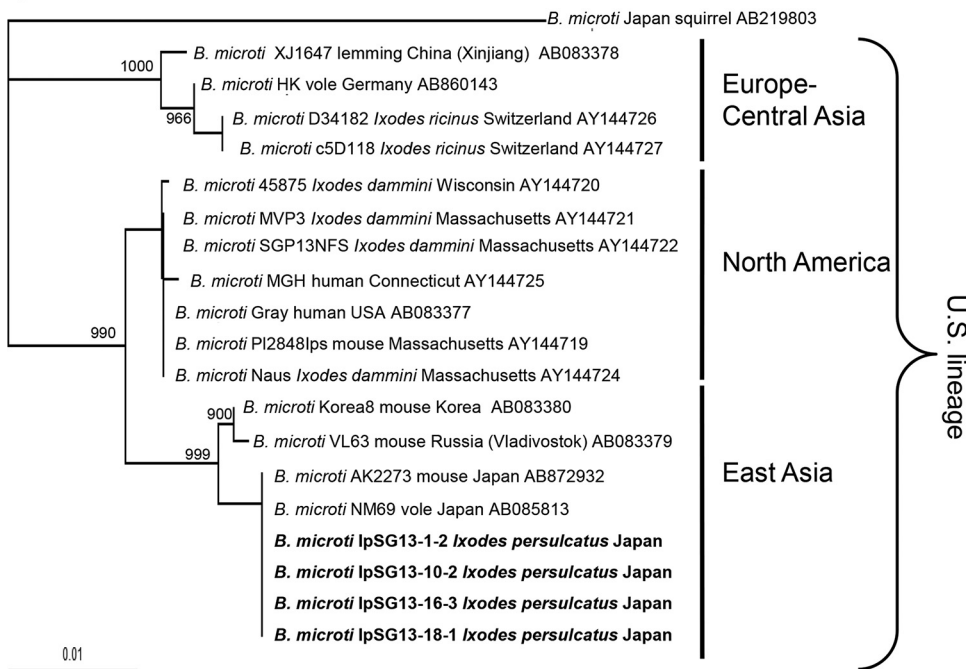
β-tubulin

FIG 3 Phylogenetic tree constructed with sequences of the β-tubulin gene of *B. microti* U.S. lineage. GenBank accession numbers are given for each strain. Strains for which sequences were determined in this study are shown in boldface. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates. Accession numbers of *B. microti* IpSG13-1-2, IpSG13-10-2, IpSG13-16-3, and IpSG13-18-1 are LC127373 to LC127376, respectively.

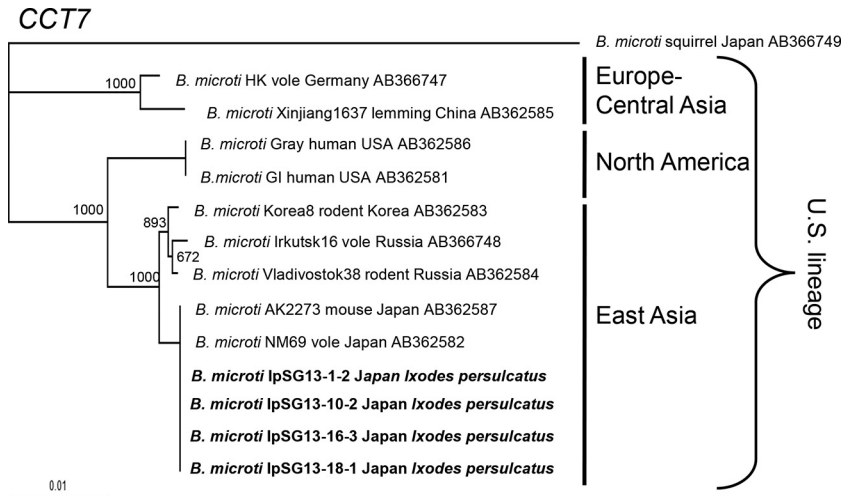Applied and Environmental Microbiology

CCT7

FIG 4 Phylogenetic tree constructed with sequences of the *CCT7* genes of *B. microti* U.S. lineage. GenBank accession numbers are given for each strain. Strains for which sequences were determined in this study are shown in boldface. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates. Accession numbers of *B. microti* IpSG13-1-2, IpSG13-10-2, IpSG13-16-3, and IpSG13-18-1 are LC127377 to LC127380, respectively.

Similar results were also obtained in the Western blot analysis (Fig. 5). As in the IFA, the antisera for the 4 strains from *I. persulcatus* (IpSG13-1-2, IpSG13-10-2, IpSG13-16-2, and IpSG13-18-1) all showed similar reactivity, so anti-*B. microti* IpSG 13-10-2 is shown as representative in Fig. 5. Among the isolates from *I. persulcatus* (IpSG13-1-2, IpSG13-10-2, IpSG13-16-2, and IpSG13-18-1) and rodent (NM69 and AK2273) in Japan, many immunodominant antigens were recognized by both *B. microti* IpSG 13-10-2 and *B. microti* NM69 antisera irrespective of the isolates. However, the heterologous antiserum was less cross-reactive between the Japanese and United States isolate antigens (Fig. 5).

In all three strains from the United States, three major antigens (25 to 50 kDa) were recognized by anti-*B. microti* Gray antisera, only one of which reacted strongly in the Japanese parasites (Fig. 5, lower left). The largest of the three major antigens migrated slower in the Gray strain than those from GI and Nan-H5-2011. This same result was observed with anti-*B. microti* Nan-H5-2011 (Fig. 5, lower right). The reactivity of anti-*B. microti* GI showed results similar to those of anti-*B. microti* Nan-H5-2011 (not shown).

DISCUSSION

In this study, we successfully isolated and established *B. microti* U.S. lineage strains from *I. persulcatus* ticks and characterized the parasites for the first time (Fig. 6). The isolation was achieved by selection of *I. persulcatus* females collected in areas where *B. microti* is highly endemic, allowing the salivary gland sporozoites to mature to the infective state, and then inoculating the infected

salivary glands into hamsters. A previous study demonstrated that *I. persulcatus* was a competent vector for *B. microti* U.S. lineage under experimental conditions where larval ticks were fed on infected hamsters and nymphs then transmitted *B. microti* to naive hamsters. The current study proves that *B. microti* sporozoites in the salivary glands of field-collected *I. persulcatus* ticks mature to the infective, transmissible stage, resulting in parasitemia in inoculated hamsters. Together with our previous studies (6, 33), the biological isolation and molecular characterization of the *B. microti* U.S. lineage in this study further supports that *I. persulcatus* is a competent vector for the *B. microti* U.S. lineage in Japan.

In the IFA test, the antiserum to strains of U.S. lineage isolated in Japan and the United States showed higher titers against the homologous than heterologous antigen. Furthermore, Western blot analysis showed antibodies raised against Japanese *B. microti* showed slight reactivity to all three *B. microti* strains from the United States. These results correspond to our previous findings (38). Serum from rodents captured in Korea and far eastern Russia detected Japanese *B. microti* strain NM69 in the IFA test but not the *B. microti* Gray strain from the United States (unpublished data), even though these rodents were genetically proven to be infected with the U.S. lineage (East Asian sublineage) (Fig. 3 and 4). Our results suggest that major antigenic cross-reactivities exist within the same sublineages only. In this regard, the commercially available *B. microti* IFA kits, which have been used worldwide for serological identification of *B. microti* infection (16, 18, 20), are

TABLE 3 Results of IFA test with *B. microti* U.S. lineage parasites

| Serum[a]	Reciprocal titer against parasite (sublineage):				
	IpSG13-10-2 (East Asia)	NM69 (East Asia)	Gray (North America)	Nan-H5-2011 (North America)	GI (North America)
Anti-IpSG13-10-2	12,800	12,800	800	800	400
Anti-NM69	12,800	12,800	3,200	800	400
Anti-Gray	800	800	12,800	12,800	12,800
Anti-Nan-H5-2011	3,200	1,600	12,800	12,800	12,800
Anti-GI	1,600	1,600	12,800	12,800	12,800

[a] Convalescent-phase sera from infected hamsters.
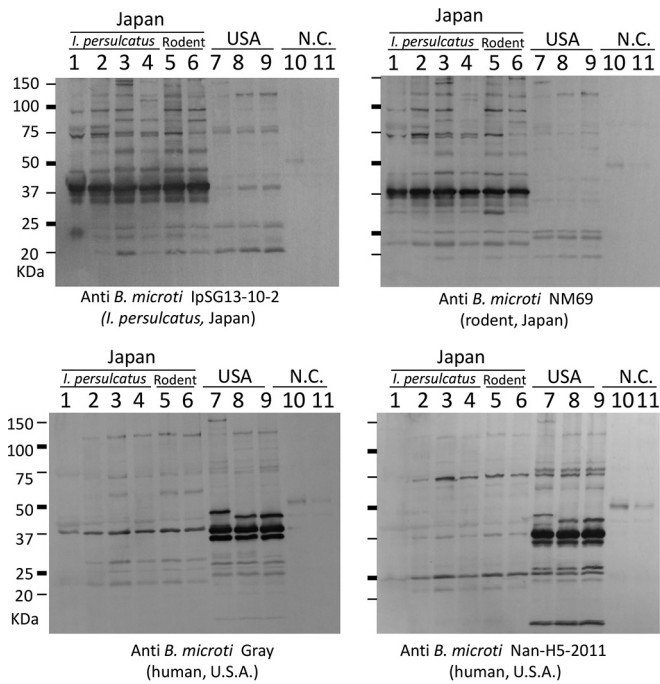
FIG 5 Western blot analysis with the *B. microti* U.S. lineage isolated from *I. persulcatus* (IpSG13-1-2, IpSG13-10-2, IpSG13-16-3, and IpSG13-18-1; lanes 1 to 4, respectively), rodents in Japan (NM69 and AK2273; lanes 5 and 6, respectively), and patients in the United States (Gray, GI, and Nan-H5-2011; lanes 7 to 9, respectively). Erythrocytes from noninfected hamsters were used as negative controls (N.C.; lanes 10 and 11). Parasite antigens were detected with anti-IpSG13-10-2 hamster serum (upper left), anti-NM69 hamster serum (upper right), anti-Gray hamster serum (lower left), and anti-Nan-H5-2011 serum (lower right).

FIG 6 Intraerythrocytic *B. microti* U.S. lineage parasites in hamster blood isolated from *I. persulcatus* in Japan. Giemsa stain was used. Bar, 10 μm.

based on antigen from parasites isolated in the United States. Thus, diagnostic serological testing outside the United States using these kits may underestimate or even fail to detect antibody titers in patients, and those who have low antibody titers may be disregarded. Therefore, it may be prudent to use parasites locally isolated or in the same sublineages as antigen for serological diagnostics or studies in different geographic locations.

At all survey sites where *I. persulcatus* ticks were collected, the sympatric tick *I. ovatus* was also collected. However, unlike *I. persulcatus*, in which PCR detected the *B. microti* U.S. lineage, all sympatric *I. ovatus* ticks tested were negative. This specific *I. persulcatus*-U.S. lineage relationship was observed in previous studies in Japan (33) and Russia (31). In the Novosibirsk region in Russia, where the *B. microti* U.S. lineage is distributed in voles, mice, and shrews, *I. persulcatus* ticks are found to carry U.S. lineages. However, in the Sverdlovsk region where the Munich lineage in the *B. microti* group (Fig. 2) is distributed in the same reservoir host species, *I. persulcatus* ticks do not carry the Munich lineage. These results indicate the *B. microti* U.S. lineage is transmitted specifically by *I. persulcatus* in Japan, Russia, and presumably other areas in northern Eurasia where *I. persulcatus* is dominantly distributed.

In Europe, where *I. persulcatus* and *I. scapularis* are absent, *I. ricinus* is supported as a vector for the *B. microti* U.S. lineage based on PCR detection in field-collected ticks in Germany, Switzerland, and Slovenia (1, 34, 35) and transmission studies in gerbils (36). Thus, the parasites in the U.S. lineage worldwide exist in nature in
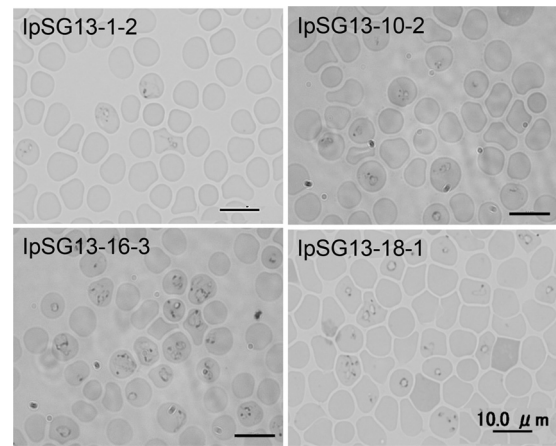
enzootic cycles involving ticks belonging to the *I. ricinus* species complex, and Europe-Central Asian, East Asian, and North American sublineages are specifically associated with *I. ricinus*, *I. persulcatus*, and *I. scapularis* (formerly *I. dammini*), respectively (Fig. 3). This corresponds with the overlapping distribution of the *B. microti* U.S. lineage parasites (Europe-Central Asia, East Asia, and North American sublineages) and the vector tick species (*I. ricinus*, *I. persulcatus*, and *I. scapularis*, respectively). Investigation in the countries where *I. ricinus* and *I. persulcatus* sympatry occur (39), such as Estonia (40), Latvia (41), Finland, Russia (42), and Poland (43), may elucidate the vector competency in nature for transmitting *B. microti* U.S. lineage parasites on the Eurasian continent.

Careful selection of *I. persulcatus* ticks, which were collected at rodent-enriched sites where *B. microti* is highly endemic, was crucial for efficient parasite isolation in this study (Tables 1 and 2). We timed the collection and counted the number of ticks collected at each site. Although sites in the same area were less than a few kilometers apart, they are different in habitat, such as streams and vegetation. Although the number of ticks collected varied among the sites, we could indirectly know how frequently wild animals dropped ticks at each site by calculating collection efficiency as the number of ticks collected per minute (tick density) (Table 1). We had speculated that *B. microti* could be maintained well in the area where the rodent reservoir and ticks were densely distributed. As we expected, *B. microti* PCR-positive *I. persulcatus* males were collected in sites with high collection efficiencies, Akkeshi 1 and 2, Kamishihoro 1, and Obihiro 3 (Table 1). Interestingly, at Akkeshi 1, which had the highest collection efficiency among all sites, only 1 out of 111 *I. persulcatus* males was PCR positive for *B. microti*. The *B. microti* parasite detected in the questing adult tick would have originated from the animal on which the tick fed as a nymph, the previous tick stage. Thus, the ticks collected at the Akkeshi 1 site might have fed on nonreservoir animals, such as deer, raccoon dog, fox, and mink, in their previous stage. Examination of the blood meal remnants in the collected ticks (44) may be useful to identify the source of the previous tick blood meal and to select specimens for parasite isolation in future endeavors.

Among five attempts at parasite isolation in hamsters using

tick salivary glands positive for *B. microti*, four were successful and one failed (Tables 1 and 2). The prepatent period until the parasites were detected at >0.05% parasitemia in the hamsters varied. Isolate IpSG 13-1-2 parasites emerged in the hamsters as early as 18 days after injection of *B. microti*-positive salivary glands, while IpSG 13-18-1 took 52 days. In our study, there is a direct correlation between the length of storage at 4°C and the length of time before intraerythrocytic parasites are observed in the hamsters. Ticks IpSG13-18-1 and IpSG13-19-1 were both kept at 4°C for 19 weeks before use, and the hamster injected with *B. microti*-positive salivary glands from IpSG13-19-1 never developed parasitemia throughout the period of this study. Thus, about 19 weeks of storage at 4°C was the threshold for isolating *B. microti* in hamsters using infected tick salivary glands in this study.

The refrigerated storage period of the ticks might influence parasite activity and survival in the salivary glands. If the infectious parasites in the salivary glands were gradually inactivated and/or reduced in number as *I. persulcatus* ticks were kept longer at 4°C, it likely would influence how long before the parasite emerged in the hamster RBC. It was reported that hamsters intraperitoneally infected with 25,000 salivarian parasites (sporozoites) from *I. scapularis* (formerly *I. dammini*) developed *B. microti* infection, but an inoculum of 10,000 was ineffective (45), which suggests that a minimum number of surviving parasites was important in the current study. In nature, a similar reduction of parasites in ticks may occur over time. In our studies, *B. microti*-positive ticks have been abundant in early spring rather than in late spring to early summer (unpublished data). A seasonal prevalence in the infection rate of *I. scapularis* by *B. microti* was also observed in ticks collected on Nantucket Island, Massachusetts (26).

I. persulcatus is a major tick species causing human tick bites and is an important vector of many tick-borne diseases, including human Lyme borreliosis (*Borrelia afzelii* and *B. garinii*), relapsing fever (*B. miyamotoi*), tick-borne encephalitis virus (TBEV; far eastern and Siberian subtypes), and neoehrlichiosis ("*Candidatus* Neoehrlichia mikurensis") in Russia and in northern regions of China and Japan where the tick is abundant (46–50). Human babesiosis caused by *B. microti* U.S. lineage has not been clearly documented in these areas, with the exception of China (51).

The study areas are at the feet of mountains and not far from residential areas. In fact, we sometimes found people collecting edible wild plants around our study sites during our field surveys. Inasmuch as *B. microti*-infected ticks were at these sites, we suspect such persons are at risk of acquiring *B. microti* infections through tick bites. Human babesiosis has been recognized occasionally by severe infection in asplenic patients and by infection through contaminated blood products from asymptomatic donors (37, 52). Large-scale surveys should be conducted for risk assessment, such as serological screenings for *B. microti* U.S. lineage in patients with febrile and/or undiagnosed illness as well as in healthy residents who live around *I. persulcatus* habitats.

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