Short Report: Identification and Phylogenetic Analysis of Japanese Macaque *Babesia*-1 (JM-1) detected from a Japanese Macaque (*Macaca fuscata fuscata*)

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Abstract. We demonstrate here the identification and phylogenetic characterization of *Babesia microti* (*B. microti*)-like parasite detected from a splenectomized Japanese macaque (*Macaca fuscata fuscata*) at a facility for laboratory animal science. On Day 133 after splenectomy, intra-erythrocytic parasites were found on light microscopic examination, and the level of parasitemia reached 0.3% on blood smear. Molecular characterization of the parasite using nested-polymerization chain reactions targeting the *18S rRNA*, β-*tubulin*, and subunit 7 (eta) of the chaperonin-containing t-complex polypeptide 1 (*CCT7*) genes were identified as a *B. microti*-like parasite, designated the Japanese Macaque *Babesia*-1 (JM-1).

The genus *Babesia* belongs to the family Piroplasmida, closely related to *Plasmodium* and *Theileria* genera, and comprises over 70 species that parasitize mammals and birds.[1] *Babesia microti* (*B. microti*) is a rodent-infective *Babesia* species transmitted by ixodid ticks and is also a major etiological agent of human babesiosis.[2–5] Nonhuman primates in Africa and Asia are natural hosts for *Entopolypoides macaci*, of which is a piroplasm phylogenetically close to the *B. microti* parasite and similar in morphology.[6–9] *Babesia microti*-like parasites have recently been reported to infect various vertebrate hosts such as the raccoon, domestic dog, fox, squirrel, and rarely humans.[10–14] Natural infections with *B. microti*-like parasites have also been found in some species of nonhuman primates including the African baboon (*Papio cynocephalus*), cynomolgus macaque (*Macaca fascicularis*), rhesus macaque (*Macaca mulatta*), and cercopithecus monkey (*Cercopithecus pygerythrus*).[6,7,9,15,16] Moreover, the susceptibility of the squirrel monkey (*Saimiri sciureus*) and capuchin monkey (*Cebus paella*) to *B. microti* infection has been reported.[17,18]

The Japanese macaque (*Macaca fuscata*), as well as rhesus and cynomolgus macaques, is an Old World monkey species native to Japan. It is found only in Japan and classified into two subspecies, *Macaca fuscata fuscata*, which is widely distributed in three major islands of Japan, and *Macaca fuscata yakui,* found only in Yaku Islet.[19] We demonstrate here the identification and phylogenetic analysis of a *B. microti*-like parasite detected from *M. fuscata fuscata* at a primate center for biomedical research.

The monkey (animal no. J79) housed in a cage outdoors, an eight-year-old female, was a second-generation offspring bred in captivity, and was offered by a commercial animal facility after a 28-day quarantine period. No clinical problems or specific pathogens were found during the quarantine period. Monkey J79 was splenectomized for an experiment involving primate malaria infection, and was kept in an individual cage

in controlled Biosafety Level II condition at Tsukuba Primate Research Center, given commercial food pellets supplemented with fresh fruits, and maintained in accordance with the Guidelines for the Use of Experimental Animals authorized by the Japanese Association for Laboratory Animal Science. The protocol was approved by the Ethics Committee of Animal Experiments, Dokkyo University of School of Medicine (permit no.: 0536).

Although no marked clinical signs were observed in the monkey during the postoperative period after splenectomy, at 133 days after operation intra-erythrocytic parasites were found on light microscopic examination. Parasitemia reached 0.3% spontaneously, and dot forms (Figure 1A), ovoid forms measuring about 2 μm in diameter (Figure 1B), pyriforms (Figure 1C), and ring-forms (data not shown) were frequently detected on Giemsa-stained thin blood smears. In addition, multiply-infected erythrocytes were often observed (Figure 1D). The parasites were morphologically distinct from primate malarial parasites, but were very similar to *B. microti*.

Polymerase chain reaction (PCR) was performed using genomic DNA (gDNA) of the parasite from the peripheral blood. Heparinized blood obtained from J79 was centrifuged at $1,200 \times g$ for 10 min at 4°C. Erythrocytes were washed three times with phosphate-buffered saline (PBS) by centrifugation at $1,200 \times g$ for 10 min at 4°C, and the buffy coat was removed completely. The gDNA was extracted using a QIAamp DNA blood mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The gDNA was used as a template for nested PCR, which was carried out targeting the *18S rRNA*, β-*tubulin*, and subunit 7 (eta) of the chaperonin-containing t-complex polypeptide 1 (*CCT7*) genes of piroplasma belonging to *Babesia* and *Theileria*, as described previously with minor modifications.[20,21] The *18S rRNA* gene was amplified using Piro0F/Piro6R for the first-round PCR and Piro1F/Piro 5.5R for nested PCR (Table 1).[12] The β-*tubulin* gene was amplified using the primers TUBU-ATG5F/Tubu-1538R for the first-round PCR and Tubu-63F/Tubu-3R for nested PCR (Table 1). The *CCT7* gene was amplified using the primers TBCCT35F/TBCCTR0 for the first round, and TBCCT70F/TBCCT1519R-3 for nested PCR (Table 1).[20] The PCR reaction

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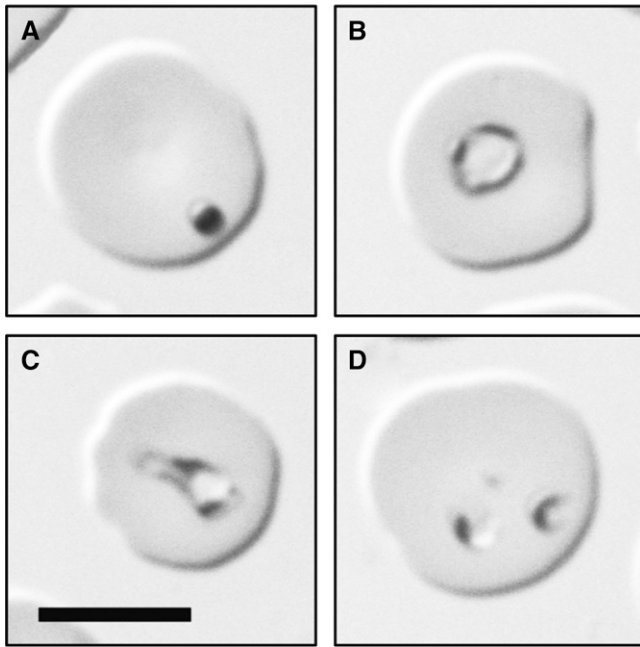
FIGURE 1. Light micrograph of a Giemsa-stained thin blood smear of peripheral blood showing various forms of intra-erythrocytic parasites. (**A**) dot form; (**B**) ovoid form; (**C**) pyriform; (**D**) a multiply-infected erythrocyte (bar = 5 μm).

mixture contained 0.1 μg of template DNA, 5 μL of 10× PCR buffer with 15 mM MgCl$_2$ (TaKaRa Bio Inc., Shiga, Japan), 5 μL of dNTP mix (2 mM of each dNTP) (TaKaRa), 2.5 U of *Takara LA Taq* DNA polymerase (TaKaRa), and 50 pmol of each primer set for the *18S rRNA*, β-*tubulin*, or *CCT7* gene-specific primers for PCR as described previously with minor modifications as reported.[21]

Nested PCR successfully amplified *18S rRNA*, β-*tubulin*, and *CCT7* genes from the gDNA of J79 (data not shown). The PCR products were isolated by 1.0% (w/v) agarose-gel electrophoresis in TAE buffer and purified with a GENECLEAN kit (BIO 101, Inc., Vista, CA). Nucleotide sequences of *18S rRNA*, β-*tubulin*, and *CCT7* genes were then determined using a CEQ8000 automated sequencer (Beckman Coulter, Inc., Brea, CA) with the DTCS DNA Sequence kit (Beckman Coulter). The *18S rRNA*, β-*tubulin*, and *CCT7* genes were

1,678, 1,300, and 1,720 bp in size, and the sequences were registered in GenBank under accession nos. of AB576641, AB576642, and AB576643, respectively.

A BLAST search with the *18S rRNA*, β-*tubulin*, and *CCT7* sequences detected no identical sequences in the public database. The most closely related sequences, with 98% sequence similarity, were from a *B. microti*-like parasite, which was isolated from a Hokkaido Squirrel.[13] We refer to the cognate sequence as Japanese Macaque *Babesia*-1 (JM-1).

Phylogenetic relationships among JM-1 and other *Babesia* and *Theileria* species were analyzed with the sequences for *18S rRNA*, β-*tubulin*, and *CCT7* genes using MacVector software version 8.0 (Genetic Computer Group Inc., Madison, WI). Published sequences of these genes were retrieved from the public database (Figure 2). These sequences were aligned using the CLUSTAL W Alignment program,[22] and a phylogenetic tree was constructed by the neighbor-joining (NJ) method from the aligned sequences with the Phylogenetic Analysis in the Mac Vector software package, version 8.0. Support for tree nodes was calculated with 1,000 bootstrap replicates using the bootstrap tree algorithm.[21,23]

The NJ phylogenetic tree constructed using the *18S rRNA* gene sequences separates the *B. microti* group into two closely related clades, one holding the rodent and human isolates including the Gray strain (*B. microti* sensu stricto), and one holding the nonhuman primate and squirrel isolates (Figure 2A). The trees constructed from β-*tubulin* and *CCT7* gene sequences, however, both place JM-1 within a single clade holding all corresponding sequences from *B. microti* sensu stricto and *B. microti*-like isolates, but more distant from *B. microti* sensu stricto (Figure 2B and C). JM-1 was most closely related to the *B. microti*-like parasite from a squirrel by all three analyses (Figure 2).

According to a previous survey in 1979, infections with *Babesia* sp. were found in 4 of 93 (4.3%) Japanese macaques (*M. fuscata yakui*) that had been reared in a monkey park in Japan.[19] Although the origin of the JM-1 infection in the Japanese macaque is unclear, it is likely that the infection was from colonies of Japanese macaques at the facility of animal science laboratory or the cage outdoors at the breeding facility. Our case is most likely an example of subclinical or opportunistic infection that manifested in the postoperative period after splenectomy in a previously immunocompetent host.

TABLE 1
Polymerase chain reaction (PCR) primers used for amplification of the *18S rRNA*, β-*tubulin*, and *CCT7* genes

| Target genes	Primers	Oligonucleotide sequences (5′ to 3′)
18S rRNA	Piro 0F	GCCAGTAGTCATATGCTTGTGTTA
	Piro 6R	CTCCTTCCTYTAAGTGATAAGGTTCAC
	Piro 1F	CCATGCATGTCTWAGTAYAARCTTTTA
	Piro 5.5R	CCTYTAAGTGATAAGGTTCACAAAACTT
β-*tubulin*	TUBU-ATG5F	ATGAGAGARATYGTACACATYCAAGC
	Tubu-1538R	TAYTGYTGGTAYTCGCTRACYA
	Tubu-63F	CAAATWGGYGCMAARTTYTGGGA
	Tubu-3R	TCGTCCATACCTTCWCCSGTRTACCAGTG
CCT7	TBCCT35F	TGAAGGARGGNACNGAYACWTCYCARGG
	TBCCTR0	GTYTCRTCDATDSWNAGNACHWGGCANGCNGCYTCDGTNGC
	TBCCT70F	CAAATYATYAGYAAYATWAAYGCCTGYCA
	TBCCT1519R-3	KTYYTYTTNACMANNBHDGGYTCCCADATRCA
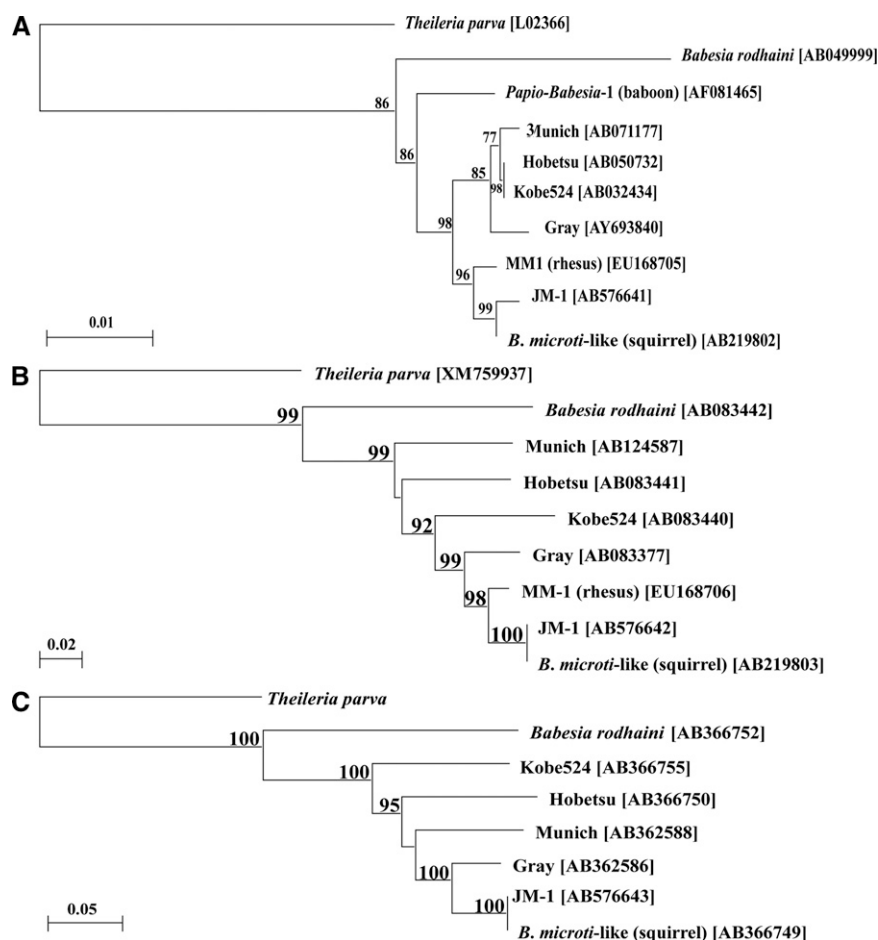
FIGURE 2. Neighbor-joining phylogenetic trees showing relationships between the (**A**) *18S rRNA*, (**B**) β-*tubulin*, and (**C**) *CCT7* gene sequences from the Japanese macaque J79 *Babesia microti*-like parasite (JM-1) and other *Babesia* isolates. GenBank accession numbers are shown in the trees for sequences from *B. microti* Gray strain, *B. microti*-like parasites (Kobe524, Hobetsu, and Munich strains; squirrel isolate, Japan), and *Babesia rodhaini*. The corresponding *Theileria parva* sequence served as the outgroup for each tree. The GenBank accession numbers are shown for *18S rRNA*, β-*tubulin*, and *CCT7* genes. The gene sequence was obtained from the Institute for Genomic Research (TIGR) website (http://www.tigr.org). Numbers at the nodes indicate bootstrap support form 1,000 repetitions.

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