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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 (Macacafuscatafuscata) 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. Babesia microti (B. microti) is a rodent-infective Babesia species transmitted by ixodid ticks and is also a major etiological agent of human babesiosis. Nonhuman primates in species transmitted by ixodid ticks and is also a major etiological agent of human babesiosis. 1– 5 Nonhuman primates in Africa and Asia are natural hosts for Entopropodyoides 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). 6,7,15,16 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 (Macacafuscata) has been demonstrated as an Old World monkey species native to Japan. It is found only in Japan and classified into two subspecies, Macacafuscatafuscata, which is widely distributed in three major islands of Japan, and Macacafuscatayakutai, found only in Yakut Islet. 19 We demonstrate here the identification and phylogenetic analysis of a B. microti-like parasite detected from M.fuscatafuscata 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 breeder 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. 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), pyroforms (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 × g for 10 min at 4°C. Erythrocytes were washed three times with phosphate-buffered saline (PBS) by centrifugation at 1,200 × 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 piroplasms 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/Piro5.5R for nested PCR (Table 1). 22 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 TBCCCT35F/TBCCCTR0 for the first round, and TBCCCT70F/TBCCCT1519R-3 for nested PCR (Table 1). 22 The PCR reaction

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mixture contained 0.1 μg of template DNA, 5 μL of 10× PCR buffer with 15 mM MgCl₂ (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 GENE CLEAN 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, Brea, CA) using a CEQ8000 automated sequencer (Beckman Coulter, Brea, CA). Nucleotide sequences of 18S rRNA were then determined using a GENECLEAN buffer with 15 mM MgCl₂ (TaKaRa Bio Inc., Shiga, Japan), 18S rRNA genes were then determined from the gDNA of J79 (data not shown). The 18S rRNA, β-tubulin, or CCT7 genes were then amplified from a Hokkaido Squirrel. 13 We refer to the cognate similarity, were from a 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**

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primers</th>
<th>Oligonucleotide sequences (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>Piro 0F</td>
<td>GCCAGTAGTGCTATACTGGTGTAT</td>
</tr>
<tr>
<td></td>
<td>Piro 6R</td>
<td>CTCCTCTTCTAAGTGGAAAGTTCAC</td>
</tr>
<tr>
<td></td>
<td>Piro 1F</td>
<td>CCATGTCATGTCTWAGTAYAACCTTTTA</td>
</tr>
<tr>
<td></td>
<td>Piro 5.5R</td>
<td>CTTYTAAGTGAAAGGTCACAAGAATTCT</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>TUBU-ATG5F</td>
<td>ATGAGAGARATYTGATACATYCAAGC</td>
</tr>
<tr>
<td></td>
<td>Tubu-1538R</td>
<td>TAYTGTGTTGATCTCCTACRA</td>
</tr>
<tr>
<td></td>
<td>Tubu-63F</td>
<td>CAAATWGGYGCMAARTTYTGGGA</td>
</tr>
<tr>
<td></td>
<td>Tubu-3R</td>
<td>TCGTCCATACCTTTCWCCSGTRATACGAGT</td>
</tr>
<tr>
<td>CCT7</td>
<td>TBCCT35F</td>
<td>TGAAGGGARGGNACNGAYACWCTCAYCARG</td>
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<td></td>
<td>TBCCTR0</td>
<td>GYTCTRCATDSWNAGNACHWGGCANGNCYGTCGTVNC</td>
</tr>
<tr>
<td></td>
<td>TBCCT70F</td>
<td>CAAATYATYAGYAAAYFAWAAYGCTGYCA</td>
</tr>
<tr>
<td></td>
<td>TBCCT1519R-3</td>
<td>KTYYTTYTACMANNBBHDGYTCCCADATRCA</td>
</tr>
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</table>

**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).
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 Babesia microti strain, Babesia 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.


