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# Experimental infection of dogs with *Babesia microti*

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## ABSTRACT

To know the infectivity of *Babesia microti* parasites in dogs, two dogs were experimentally inoculated with *B. microti* and parasitemia levels were monitored. Number of erythrocytes gradually decreased until 32 days after the inoculation and then recovered to normal levels thereafter. Parasites in the erythrocytes were first observed at 35 days after the inoculation and parasitemia levels reached approximately 1% around 50 days after inoculation in 2 dogs. Infected dogs showed transiently decreased number of erythrocytes but no clinical signs during the experimental periods. Smears of the parasitized dog erythrocytes showed positive reaction with anti-*B. microti* serum. The PCR assay in parasites DNA from the dogs showed the amplification of *B. microti* marker gene and the sequence showed 99% homology with the 16S-like small subunits from *B. microti*. These findings show the replication of *B. microti* in dogs and suggest the possibility that *B. microti* is a source of canine babesiosis if the parasite can be transmitted by ticks.

**Keywords:** *Babesia microti*; dog; experimental infection; polymerase chain reaction; indirect immunofluorescence test.

## INTRODUCTION

*Babesia microti* is a tick-borne blood parasite that causes babesiosis in rodents (Chen *et al*., 2000; Gray *et al*., 2002; Igarashi *et al*., 1999; Ike *et al*., 2005; Ruebush and Hanson, 1979; Zamoto *et al*., 2004a) and humans (Kjemtrup and Conrad, 2000; Ruebush and Hanson, 1979). Cases of human babesiosis due to this parasite have been reported in numerous countries (Eskow *et al*., 1999; Hildebrandt *et al*., 2007; Kogut *et al*., 2005; Saito-Ito *et al*., 2000; Siński *et al*., 2006; Shih *et al*., 1997; Zamoto *et al*., 2004b).

In Japan, a case of human infection by *B. microti* was attributed to a blood transfusion administered during a gastric ulcer operation, and the etiological agent was determined to be an indigenous strain of *Babesia* as neither the patient nor the donor had any record of overseas travel (Saito-Ito *et al*., 2000). Furthermore, *B. microti* has been detected in domestic rodents and there is concern about the spread of the disease in Japan (Saito-Ito *et al*., 2004; Zamoto *et al*., 2004a). Within this context, *B. microti* infection is considered to be one of the most important zoonotic diseases in Japan.

Recently, cases of a *B. microti*-like parasites infection have been reported in dogs with clinical babesiosis characterized by hemolytic anemia (Camacho *et al*., 2001; Camacho *et al*., 2004; Zahler *et al*., 2000). The infected *B. microti*-like parasites had a distinct ring-shaped appearance in blood smears and a 99% homology with *B. microti*, as revealed by genotype analysis (Camacho *et al*., 2001). These natural cases in dogs suggested the importance of canine babesiosis as an additional causative agent of canine piroplasmoses that have previously been attributed to *Babesia gibsoni* or *Babesia canis*. However, there are currently no reports of experimental *B. microti* infection in dogs. Thus in the present report, we examined the possibility whether *B. microti* can replicate in dogs using standard strain of *B. microti*. Furthermore, if the parasites can replicate in dogs we compared the pathogenicity of *B. microti* to other canine babesia such as *B. gibsoni*.

## MATERIALS AND METHODS

Experimental animals and protozoa

Six-week-old, female, ddY mice (Japan SLC Inc, Shizuoka, Japan), and 2 one-year-old female beagles (Nisseiken Corporation, Tokyo, Japan) were used in this study. The standard strain of *B. microti* (Munich strain) was kindly supplied by the University of Tokyo and was maintained in our laboratory by serial passage of parasitized erythrocytes in mice.

Inoculation of *B. microti*-infected erythrocytes in dogs

Blood containing *B. microti*-infected erythrocytes were collected by cardiac puncture from mice infected with *B. microti*. After measuring the total erythrocyte count and infection rate, parasitized erythrocytes were adjusted to $1\times10^8$/mL using phosphate buffered saline (PBS), 10 mM sodium phosphate, pH 7.4. The two dogs were injected intravenously with $1\times10^8$ of the infected erythrocytes. Blood samples were then collected from the dogs twice a week for the first 12 weeks and once a week thereafter until 183 days after inoculation.

Calculation of total and infected erythrocytes

Total erythrocyte counts were performed using a hemocytometer (Erma, Tokyo, Japan). Parasitemia in the peripheral blood was examined by staining blood smears with Wright-Giemsa solution (Merk, Darmstadt, Germany) and counting parasites in erythrocytes under a light microscope.

Packed cell volume (PCV)

The hematocrit ratio was expressed as the packed cell volume (PCV) of a venous blood sample taken from the jugular vein. A hematocrit capillary tube filled with blood was centrifuged for 5 min at 12,000 rpm and the PCV was read using a hematocrit reader.

Polymerase chain reaction (PCR)

For PCR, erythrocytes from 500 μl blood were washed three times in 500 μl of PBS. Then, 40 μg proteinase K (Takara, Otsu, Japan) was added to 50 μl of PBS containing erythrocytes and incubated at 55°C for 2 hours. Sample preparation was the same as that described elsewhere (Persing *et al.*, 1992). Briefly, the extracted DNA was amplified using the Bab 1 (5'-CTT AGT ATA AGC TTT TAT ACA GC-3') and Bab 4 (5'-ATA GGT CAG AAA CTT GAA TGA TAC A-3') primers, which target the 16S-like small subunit gene of *B. microti*. Amplification conditions consisted of 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. An additional PCR using primers Bab 2 (5'-GTT ATA GTT TAT TTG ATG TTC GTT T-3') and Bab 3 (5'-AAG CCA TGC GAT TCG CTA AT-3') was also conducted to amplify the 154-bp internal fragment of *B. microti* (Persing *et al*., 1992). Amplification conditions consisted of 50 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Electrophoresis was performed on 2% agarose gels followed by ethidium bromide staining.

Indirect immunofluorescence test (IFAT)

The IFAT was performed as described elsewhere (Terkawi *et al*., 2007). Briefly, slide glasses were coated with erythrocytes and fixed in a mixture of methanol and acetone 1:4 (v/v) at -20°C for 20 min. The fixed erythrocytes were then incubated with sera from mice infected with *B. microti* that had been used for dog inoculation for 30 min at 37°C. After washing with PBS, the fixed erythrocytes were stained with FITC-conjugated anti-dog or anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories, PA, USA) for 30 min at 37°C. The slides were examined using a fluorescence microscope.

All animal experiments were conducted in accordance with the Guidelines for Laboratory Animal Welfare and Animal Experiment Control at Nippon Institute for Biological Science.

**RESULTS**

In the two inoculated dogs, numbers of erythrocytes started decreasing at 18 days in dog A and at 25 days in dog B, and became the lowest levels at 32 days after the inoculation and then recovered to normal levels thereafter (Fig. 1-A and 1-B).

**Fig.1-A**

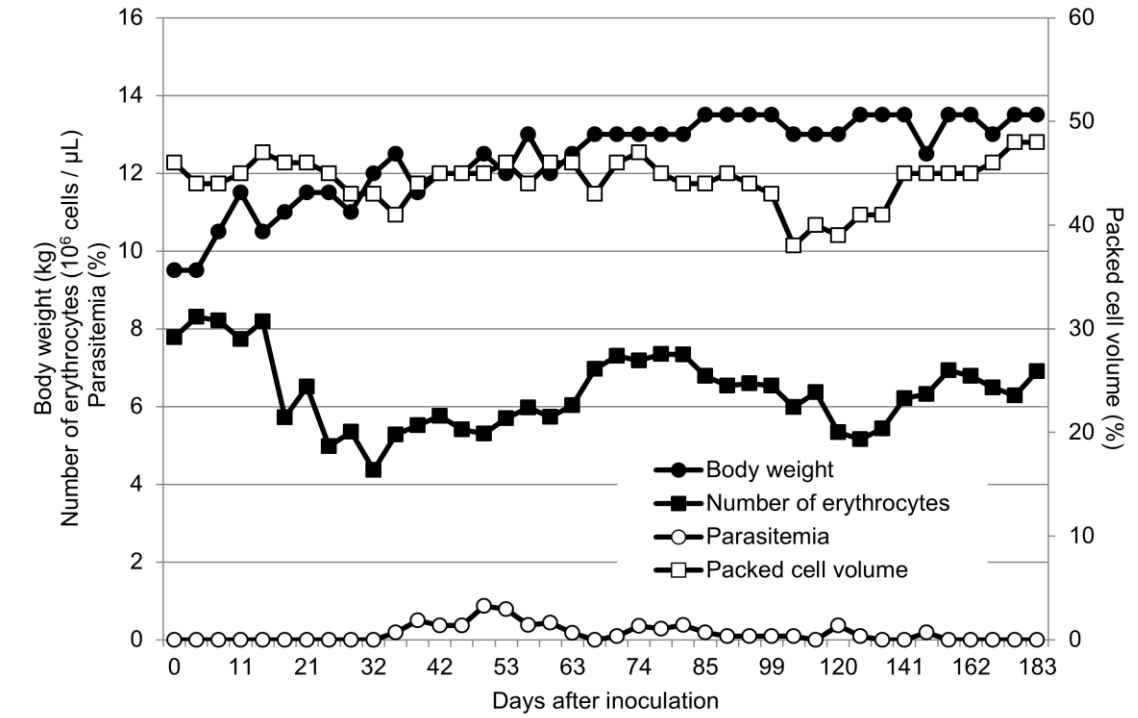


**Fig.1-B**

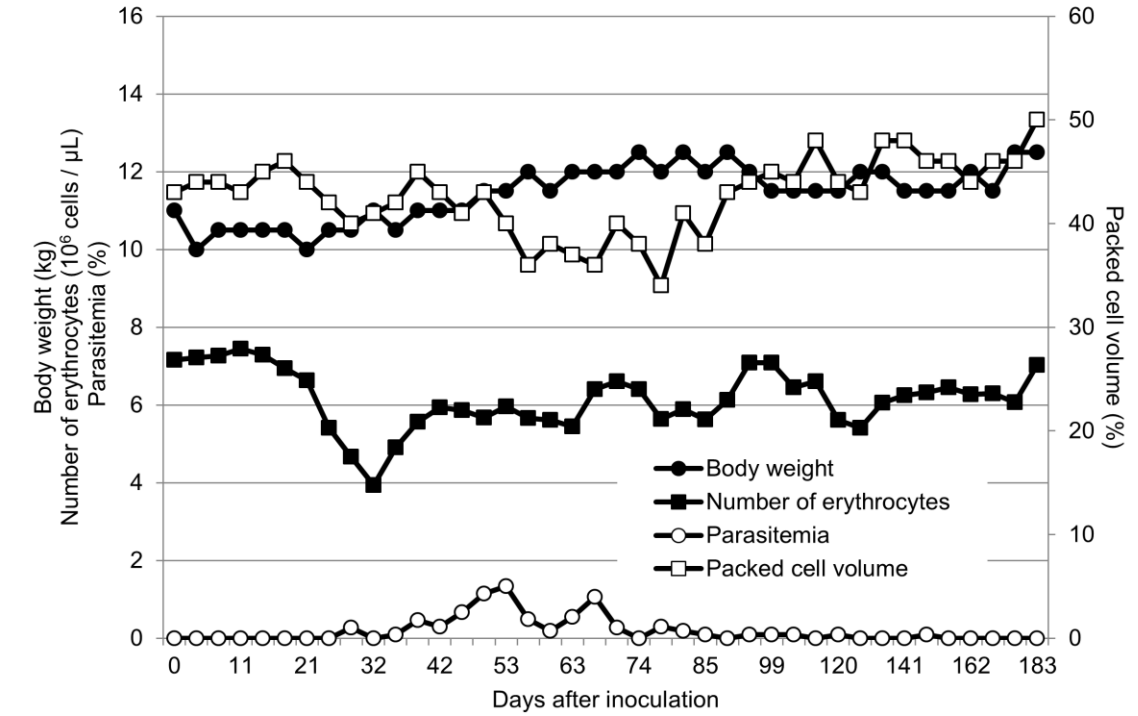


Figure 1. Changes in number of erythrocytes, parasitemia, body weight and packed cell volume (PCV) in dog A (Fig. 1-A) and dog B (Fig. 1-B). Solid circles indicate body weight, solid squares indicate number of erythrocytes, open circles indicate parasitemia and open squares indicate PCV.

Parasites were first observed in erythrocytes at 35 days after inoculation in 2 dogs with peak parasitemia levels reaching 0.87% at 49 days in dog A (Fig. 1-A) and 1.34% at 53 days in dog B (Fig. 1-B) after the inoculation. Blood smears prepared at the time of the peak parasitemia levels showed evidence of intraerythrocytic parasites, usually as small, ring-shaped babesias (Fig. 2-B). To know the antigenic properties of the parasites, fixed parasitized dog erythrocytes were examined by IFAT. Smears of the parasitized dog erythrocytes showed positive reaction with anti-*B. microti* mice sera (Fig 4-C). To more accurately characterize the parasite in dogs, specific *B. microti* marker gene analysis was performed by using PCR. The PCR assay revealed the amplification of bands in erythrocytes prepared from both inoculated dogs and mice infected with *B. microti* (Fig. 3). A comparison of these amplified dog and mouse parasite DNA gene sequences showed 99% homology with sequences of the 16s-like small subunits from *B. microti* (GenBank sequence accession number: M93660) (data not shown).

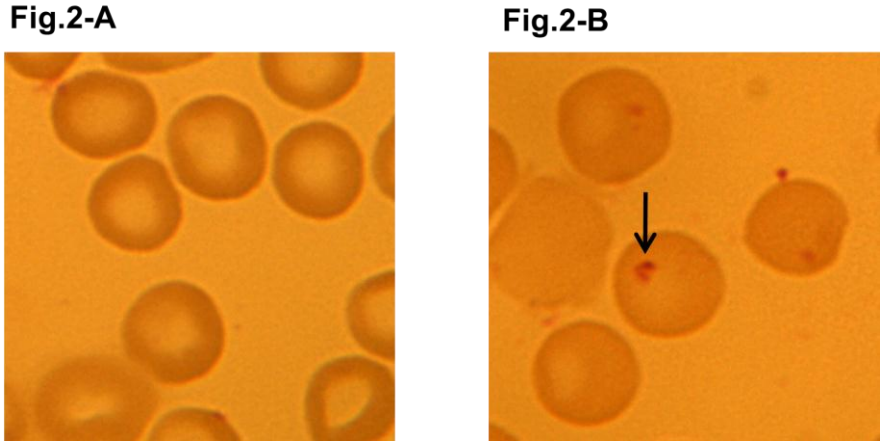


Figure 2. Dog erythrocytes infected by *B. microti*. Dog blood smears before inoculation (Fig. 2-A) and after inoculation (Fig. 2-B).

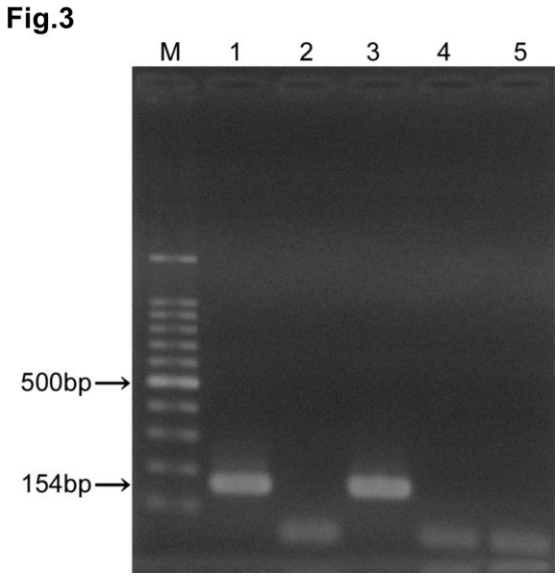


Figure 3. Polymerase chain reaction (PCR); (Lane M) 100 bp ladder, (Lane 1) erythrocytes from *B. microti*-infected mouse, (Lane 2) erythrocytes from uninfected-mouse, (Lane 3) erythrocytes from *B. microti*-inoculated dog, (Lane 4) erythrocytes from before inoculation, (Lane 5) negative control. Amplification product of *B. microti*-specfic marker was observed at 154 kb.

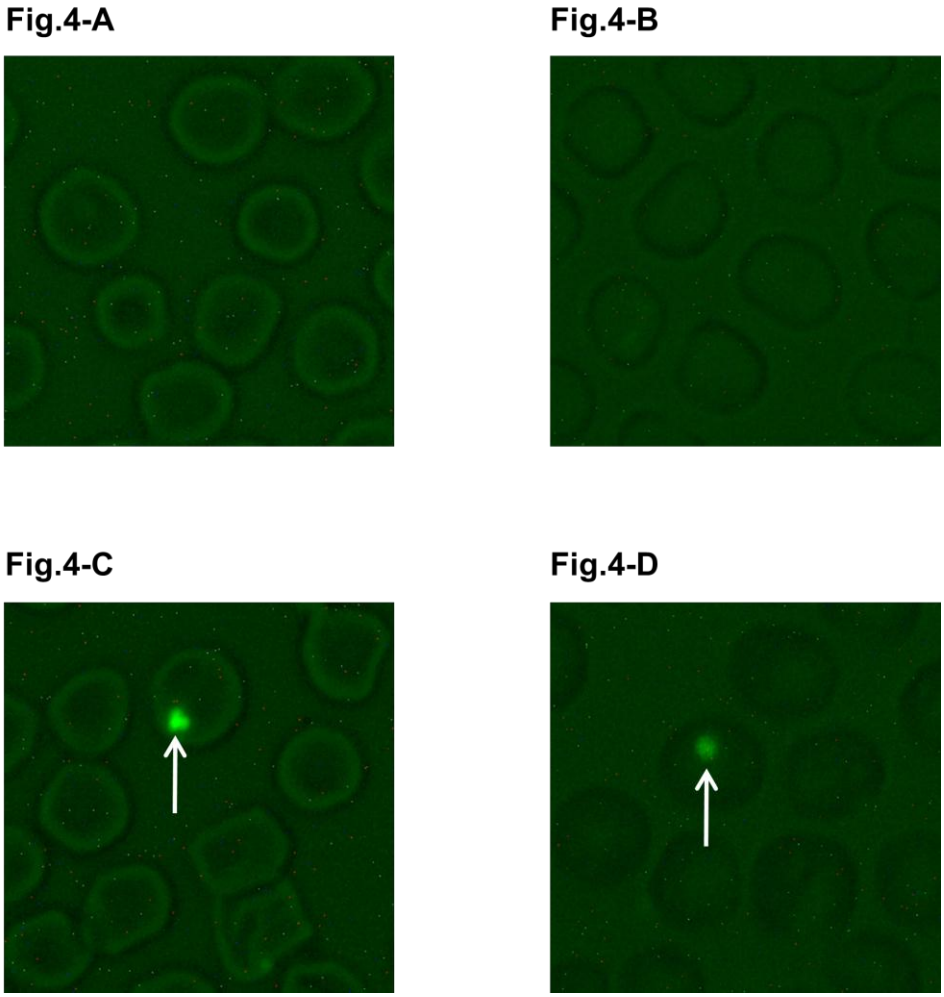


Figure 4. Indirect immunofluorescence antibody test (IFAT). Erythrocytes from a *B. microti*-inoculated dog against serum from (Fig. 4-A) non-infected mouse and (Fig. 4-B) dog serum before inoculation. Erythrocytes from a *B. microti*-inoculated dog against serum from (Fig. 4-C) *B. microti* infected mouse and (Fig. 4-D) inoculated dog.

Inoculated dogs showed lower parasitemia levels and no clinical signs of babesiosis, such as fever, apathy, or anemia in either of the dogs. In addition, no loss of body weight was observed throughout the experiment (Fig. 1-A and 1-B). No marked fluctuations were observed in the PCV, which remained within the normal range (Matijatko *et al*., 2007) throughout the experimental period (Fig. 1-A and 1-B).

**DISCUSSION**

*B. microti* has long been known to infect both humans and rodents (Ruebush and Hanson, 1979). Recent reports have suggested that *B. microti*-like parasites exhibit infectivity against dogs (Camacho *et al*., 2001; Camacho *et al*., 2004; Zahler *et al*., 2000). However the evidence whether *B. microti* can infect in dogs or not was not clear yet.

In the present experiment, we examined an inoculation of mouse-derived *B. microti* in two dogs. The following evidences strongly suggested that *B. microti* can replicate within dog erythrocytes. The parasitemia was first recognized at 35 days after inoculation when mouse origin erythrocytes are likely to have been destroyed by canine immune systems. Parasites derived from experimentally inoculated dogs were identified as *B. microti* by both serological and genetic analyses. This result suggests that *B. microti*

can infect and grow in dogs.

The parasitemia levels of *B. microti* in dogs indicated peak of parasitemia as 0.87% and 1.34%, respectively. Infected dogs showed the transient decrease of erythrocyte counts but PCV remained within normal range and no clinical signs throughout the experimental period. Field cases of a *B. microti*-like parasite infection showed clinical symptoms as typical babesiosis (Camacho *et al*., 2004; Zahler *et al*., 2000). The reasons why the dogs with lower parasitemia level and no clinical signs in the present experiment may be caused by an artificial intravenous injection but not through natural tick infestation, however the exact reason is not clear yet. An experimental *B. gibsoni*-infection in dogs (Matsuu *et al*., 2004) showed the typical anemia and thrombocytopenia despite the doses of *B. gibsoni* being lower than those of *B. microti* used in this study. It means that pathogenicity of *B. microti* against dog is lower than *B. gibsoni*, although it is not known whether inoculation with a higher dose of *B. microti* can induce clinical signs in dogs. Under natural condition, *B. microti* parasites are transmitted by tick infestation, however there are no reports concerning *B. microti* transmission to dog by ticks.

In conclusion, although further studies including experimental infection using ticks are necessary, it was suggested that *B. microti* is a source of canine babesiosis but the pathogenicity of this parasite is mild compared to that of *B. gibsoni*.

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