Detection of *Babesia microti*-like Parasite in Filter Paper-Absorbed Blood of Wild Rodents

Tamaki OKABAYASHI1, Junko HAGIYA1, Masayoshi TSUJI1, Chiaki ISHIHARA1, Hiroshi SATOH1 and Chiharu MORITA1

1School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

(Received 3 April 2001/Accepted 2 October 2001)

**ABSTRACT.** The first case of human babesiosis was reported in Japan. The epidemiology of this disease in Japanese nature remains unclear. In this study, 97 common field mice captured in Hokkaido, Japan, were examined. Blood specimens absorbed onto filter papers were eluted and tested by nested PCR using specific primers for the *B. microti* nuclear small subunit rRNA genome. Twenty-three percent (11/47) of *Apodemus speciosus* and four percent (2/50) of *Clethrionomys rufocanus* were positive. The 159-bp primary sequences of PCR products tested exhibited 97.5% and 96.8% homology with those of the human isolate in Japan and of U.S. strains of *B. microti*, respectively.

**KEY WORDS:** *Babesia microti*, filter paper, rodent.

Human babesiosis is a malaria-like illness that is frequently caused by tick-borne intraerythrocytic protozoan parasites of the genus *Babesia* [17]. Human cases have mostly been reported in the United States and occasionally in Europe. Clinically, the disease ranges from asymptomatic to severe and occasionally fatal. Two species of *Babesia*, *B. microti* and *B. divergens*, are the most common zoonotic agents in the United States, especially in the eastern and the upper Midwestern areas, and in Europe, respectively. Other species of *Babesia*, referred to as WA1 [10], CA1 [8] and MO1 [1], the newly emerging species, are dominant in areas of the United States where *B. microti* is not endemic. The common reservoir for these organisms in the United States is the white-footed deer mouse (*Peromyscus leucopus*), which is the preferred host for the larval tick *Ixodes scapularis*, via which the disease is transmitted to humans [15]. Only a very few human cases have been reported in countries other than the United States and Europe, such as Taiwan, China, Egypt, South Africa and Mexico [2]. The first case of human babesiosis in Japan was a transfusion-infection case found in Hyogo Prefecture, and the agent isolated, referred to as Kobe strain, has been identified as an indigenous Japanese type of *B. microti* by antigenic and sequence analysis of the nuclear small subunit ribosomal DNA (rDNA) [11, 12]. The epidemiology of this organism in the Japanese nature, such as the species and types of zoonotic *B. microti*, reservoir rodents, vector ticks and regions of endemicity and prevalences of infection, are remained almost unclear, though Shiota et al. [13] reported in 1984 the presence of *B. microti* like-parasites in the most common Japanese field mice, *Apodemus speciosus*, captured in Shiga Prefecture.

The polymerase chain reaction (PCR) is now becoming a standard technique for rapid, sensitive and specific detection of *B. microti* infection in man [5]. This method is also useful for epidemiological studies in which ease of blood collection and transportation of blood samples is required. To try to determine the reservoir rodents in Japanese nature, we performed PCR amplification and sequence determination analysis of *Babesia* using filter paper-absorbed blood from wild rodents that has been captured in Hokkaido. A total of 97 field mice (*Apodemus speciosus*, 47; *Clethrionomys rufocanus*, 50) were captured in Hobetsu, Hokkaido from June to September, 1998. Each of the entrapped animals was anesthetized, bled and 100 µl of blood was collected using blood-sampling filter paper (Toyo Roshi, Tokyo, Japan). The filter paper was then dried in air and stored at room temperature [7]. For PCR analysis, the paper-absorbed blood was eluted in 600 µl of phosphate-buffered saline (pH 7.5; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan).

DNA was extracted from the eluted blood using a QIAamp Tissue Kit (QIAGEN, Hiden, Germany) according to the manufacturer's instructions. A 5 µl of extracted DNA was used as template for amplification of *Babesia* gene. PCR was performed with nested PCR. Primer pairs of Bab1 and Bab4 and of Bab2 and Bab3 specific to the *B. microti* small subunit ribosomal RNA genome (rDNA) were used [9]. Amplification was carried out in a 50-µl volume containing 0.5 µM primers, 0.2 mM dNTP, 1.5 mM MgCl2 and 1.25 U of Taq polymerase and its PCR buffer) in 35 cycles (heating at 95°C for 5 min followed by denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min, with the last cycle extended to 10 min). The products amplified by secondary PCR were purified by SUPREC-02 (Takara Shuzo Co., Shiga, Japan) and sequenced utilizing a Big dye terminator cycle sequencing kit (Perkin-Elmer Co., Applied Biosystems, Tokyo, Japan) with a Perkin Elmer ABI Prism 310 Genetic Analyzer (Perkin-Elmer Co., Applied Biosystems, Chiba, Japan). Sequence analysis was carried out using a FASTA search on the GenBank database.
The use of blood specimens taken onto filter papers was demonstrated to be satisfactory for investigating *B. microti* infection in wild rodents by the nested PCR technique. Surprisingly, 13.4% (13/79) of the samples, including 23.4% (11/47) of samples from *A. speciosus* and 4.0% (2/50) of samples from *C. rufocanus*, were positive in amplification of the *B. microti*-rDNA (Table 1). The difference between the positive rates of *A. speciosus* and for *C. rufocanus* was significant (*P*<0.05). Sequence analysis of the amplified 16S rDNA fragments, 4 from *A. speciosus* and 2 from *C. rufocanus*, showed that they were all identical (100% homology) through the 159-bp primary sequences at positions 107 to 266. Moreover, this rDNA sequence showed 97.5% and 96.8% homology with the reported sequences of *B. microti* strains, which is known to be widely distributed in Hokkaido, Japan (Kobe strain) and of U.S. strains (Ruebush-Peabody and Gray) of *B. microti*, respectively (Table 2).

The present study demonstrated the prevalence of *B. microti* or *B. microti*-like organism in the wild rodents in Hokkaido, despite the fact that only two cases of infection from these organisms have been reported in Japan: a case of a transfusion-acquired human babesiosis in Hyogo in 1999 [11, 12] and a case of *A. speciosus* infection in Shiga in 1984 [13]. We also found that another field mouse, *C. rufocanus*, which is known to be widely distributed in Hokkaido, Japan and in northeastern Asia and Scandinavia [3] and was once shown to be free from *B. microti* infection by an examination of 13 animals from Atsuta, Hokkaido [13], could be an additional reservoir in Japanese nature (Table 1). *B. microti* (-like) rDNAs amplified from *A. speciosus* and *C. rufocanus* mice in this study are all classified into a single genotype and are distinctive in part from the Kobe strain and from the two U.S. reference strains with sequence homologies of 98.5% and 96.8%, respectively (Table 2). Moreover, it has been shown that the four U.S. reference *B. microti* strains, Ruebush-Peabody, Gray, GI and AJ, are all identical (100% homology) and are close to the Kobe strain with 99.2% homology [12]. Thus, it is possible that there exists in Japan another indigenous Japanese type of *B. microti* in addition to the Kobe strain, which has recently been recognized as an indigenous Japanese type [12]. Whether the *B. microti* (-like) organism detected in this study by PCR products is infectious and pathogenic in humans, however, remains unclear. An assay for the specific antibody in human sera is needed. Isolation of the type of parasites and experimental infections using a SCID mouse model whose erythrocytes has been exchanged with human’s may provide faithful results [12].

The nested PCR method using the filter paper absorbed blood seems to be effective for investigating *B. microti* infection in wild rodents. The 23.4% detection rate (11/47 *A. speciosus* mice; Table 1) is close to the highest previously reported rate in Japan of 30.2% (26/86, thin blood smears from *A. speciosus* mice from a spotted area in Shiga Prefecture; ref. 13) and is much higher than 18.7% (26/139) of the total tested rodents [13], though an infection rate up to 71% in field mice in Germany in early summer has been reported [6] and also in the United States [16]. The usefulness of the nested PCR technique in combination with the use of thin blood smears for detection of malaria and trypanosomiasis has recently been reported [4, 14]; however, method, which enable easy handling, transportation and storage of the test samples and is also very sensitive, may exclusively be very useful for remote and large-scale field surveys.

ACKNOWLEDGMENT. This work was supported in part by a research fellowship of the Japan Society for the Promotion of Science for Young Scientists (No. 04878).

**REFERENCES**

DETECTION OF *BABESIA MICROTI* IN RODENTS


