FULL PAPER *Parasitology*

Development of a Polymerase Chain Reaction Method for Diagnosing *Babesia gibsoni* Infection in Dogs

Shinya FUKUMOTO[1)], Xuenan XUAN[1)], Shinya SHIGENO[2)], Elikira KIMBITA[1)], Ikuo IGARASHI[1)], Hideyuki NAGASAWA[1)], Kozo FUJISAKI[1)] and Takeshi MIKAMI[1)]

[1)]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555 and [2)]Leo Animal Hospital, 9 Yoriai-cho, Wakayama 640–8214, Japan

(Received 16 January 2001/Accepted 17 May 2001)

ABSTRACT. A pair of oligonucleotide primers were designed according to the nucleotide sequence of the P18 gene of *Babesia gibsoni* (*B. gibsoni*), NRCPD strain, and were used to detect parasite DNA from blood samples of *B. gibsoni*-infected dogs by polymerase chain reaction (PCR). PCR was specific for *B. gibsoni* since no amplification was detected with DNA from *B. canis* or normal dog leucocytes. PCR was sensitive enough to detect parasite DNA from 2.5 *µl* of blood samples with a parasitemia of 0.000002%. PCR detected parasite DNA from 2 to 222 days post-infection in sequential blood samples derived from a dog experimentally infected with *B. gibsoni*. The detection of *B. gibsoni* DNA by PCR was much earlier than the detection of antibodies to *B. gibsoni* in blood samples by the indirect fluorescent antibody test (IFAT) or that of the parasite itself in Giemsa-stained thin blood smear film examined by microscopy. In addition, 28 field samples collected from dogs in Kansai area, Japan, were tested for *B. gibsoni* infection. Nine samples were positive in blood smears, 9 samples were positive by IFAT and 11 samples were positive for *B. gibsoni* DNA by PCR. The nucleotide sequences of PCR products from all 11 samples found positive by PCR were completely identical to that of the P18 gene of the *B. gibsoni*, NRCPD strain. These results suggest that PCR provides a useful diagnostic tool for the detection of *B. gibsoni* infection in dogs.
KEY WORDS: *Babesia canis*, *Babesia gibsoni*, IFAT, PCR, P18 gene.

J. Vet. Med. Sci. 63(9): 977–981, 2001

Babesia gibsoni (*B. gibsoni*), a tick-borne hemoprotozoan parasite, causes babesiosis in dogs, which results in remittent fever, progressive anemia, haemoglobinuria, marked splenomegaly and hepatomegaly, and sometimes death. *B. gibsoni* infection is prevalent in many regions of Asia, Africa, Europe and America [8, 16, 17]. Recently, this disease has frequently been found in companion dogs, creating a clinical problem [2, 9]. In dogs chronically infected with *B. gibsoni*, the disease recurs and causes progressive anemia postsurgically or under immunosuppressive therapy. Therefore, the diagnosis and detection of carrier or subclinical dogs are very important. Generally, diagnosis of the babesiosis infection is carried out by the detection of *Babesia* parasites in Giemsa-stained thin blood smear film examined by microscopy. However, the detection of *Babesia* parasites is very difficult in inapparent or chronic infections since the parasitemia level is very low.

B. gibsoni infection in dogs can be determined by the detection of antibodies using such serological tests as the indirect fluorescent antibody test (IFAT) [5, 18] or the enzyme-linked immunosorbent assay (ELISA) [15] using whole *B. gibsoni*-infected erythrocytes or the lysates, respectively, as antigens. These tests are useful for the diagnosis of chronically infected dogs with significantly low levels of parasitemia. In general, IFAT and ELISA for *B. gibsoni* parasites are highly sensitive, but only moderately specific, because of antigenic cross-reactions to *Babesia canis* (*B. canis*) [18] and normal dog erythrocytes [1, 3, 4 18]. Therefore, the development of a highly specific and sensitive system for the diagnosis of *B. gibsoni* infection is required.

Recently, the introduction of polymerase chain reaction (PCR) has allowed the development of simple, specific and sensitive diagnostic methods for the detection of many types of piroplasmosis [6, 7, 13, 14]. However, this method has not yet been evaluated for the diagnosis of *B. gibsoni* infection. In this study, we developed a PCR method for the detection of *B. gibsoni* infection in dogs, and evaluated it's sensitivity and specificity by microscopic examination, antibody determination and nucleotide sequencing.

MATERIALS AND METHODS

Parasites: *B. gibsoni*, NRCPD strain [12], was experimentally infected and maintained in splenectomized dogs or canine red blood cell (RBC)-substituted severe combined immune deficiency (SCID) mice (Ca-RBC-SCID mice) as described earlier [10]. *B. canis vogeli* isolated from a dog in Okinawa Prefecture, Japan [11], was maintained and infecting in Ca-RBC-SCID mice.

Dogs: One-year-old Beagles were used. The dogs were confirmed to be free of natural *B. gibsoni* infection by specific antibody examination prior to the experiments.

Experimental infection of B. gibsoni in a dog: One-half milliliter of *B. gibsoni*-infected dog erythrocytes with 10% parasitemia was injected to the dogs. The *B. gibsoni*-infected blood samples were collected at 2-to-7 day intervals and stored at –20°C until use.

* CORRESPONDENCE TO: PROF. DR. MIKAMI, T., National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555, Japan.

Field blood samples: Twenty-eight blood samples were collected from field dogs, which showed clinical symptoms such as anemia and suspected of the babesiosis in Wakayama and Osaka prefecture.

IFAT: A thin blood smear of a *B. gibsoni*-infected blood sample collected from a *B. gibsoni*-infected Ca-RBC-SCID mouse was fixed with acetone for 10 min and incubated with dog serum at 37°C for 1 hr. The slide was washed with phosphate-buffered saline (137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) (PBS) for 10 min and incubated with FITC-conjugated goat anti-dog IgG antibody (Bethyl Lab, TX) at 37°C for 1 hr. The slide was washed with PBS for 10 min, and then the stained cells were observed by fluorescent microscopy.

Light microscopic examination: A thin blood smear of blood samples was fixed with methanol for 1 min and stained with Giemsa solution for 30 min. Then, the parasitemia was determined with light microscopic examination.

Extraction of DNA: The blood samples collected from the dogs were centrifuged at 5,000 × g for 5 min and the blood plasma and leucocytes were discarded. The separated 25 μl of erythrocytes were washed 3 times with PBS and lysed in 0.1 M Tris-HCl (pH 8.0) containing 1% SDS, 0.1 M NaCl and 10 mM EDTA. The samples were then treated with proteinase K (100 μg/m*l*) for 2 hr at 55°C. The DNA was extracted with phenol/chloroform, precipitated by ethanol, and then dissolved in 50 μl of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). The DNA samples were stored at 4°C.

PCR: A cDNA clone capable of encoding an 18 kDa protein of *B. gibsoni* was identified and designated as a P18 gene. The nucleotide sequence of the P18 gene of *B. gibsoni*, NRCPD strain, has been submitted to the DDBJ database under accession no. AB053292. Two oligonucleotide primer targets for the 182 bp fragment (Fig. 1) of the *B. gibsoni* P18 gene were designed. PCR was performed in 50 μl of a mixture containing about 1 μg of template DNA, 50 pmol of each primer, 200 μM of dNTPs and 1.25 U of Taq Gold DNA polymerase (ABI, CA) in 1 × buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.001% gelatin) (ABI). PCR was performed for 10 min at 95°C to activate the Taq Gold DNA polymerase, and then the reaction was repeated for 40 cycles under the following conditions: 30 sec of denaturation at 94°C, 1 min of annealing at 54°C and 1 min of extension at 72°C. Five microliters of the PCR products were analyzed by 1.5% agarose gel electrophoresis followed by ethidium bromide staining and photography.

Sequencing of PCR products: Five microliters of each PCR product was treated with a PCR product pre-sequencing reagent pack (Amersham Pharmacia Biotech, NJ), and then used as the templates for sequencing. Sequencing of the PCR products was performed on both strands using the Dye terminator cycle sequencing kit supplied by ABI with the two primers d3 and d4. Analysis was done with a Model 377A ABI sequencer. The sequencing analysis was performed by computer program, MacVector Ver. 6. 5. 3. (Oxford Molecular, CA).

RESULTS

Specificity of the PCR method: The specificity of the PCR method was examined with DNA extracted from *B. gibsoni*-infected dog erythrocytes with 14% parasitemia, *B. canis*-infected erythrocytes with 10% parasitemia collected from *B. canis*-infected Ca-RBC-SCID mouse and normal dog leucocytes. As shown in Fig. 2, the expected 182 bp fragment was amplified only from *B. gibsoni* DNA but not from *B. canis* or normal dog leucocyte DNA. To further confirm the specificity of the PCR method, the PCR product was sequenced. The nucleotide sequence of the PCR product was found to be completely identical to that of the cDNA clone of the P18 gene.

Sensitivity of the PCR method: *B. gibsoni*-infected erythrocytes with 20% parasitemia were subjected to 10-fold serial dilutions using normal dog erythrocytes, and DNA was extracted from each diluted sample for testing the sensitivity of the PCR method. As shown in Fig. 3, PCR was sensitive enough to detect parasite DNA from 2.5 μl of blood samples with a calculated parasitemia of 0.000002%, equivalent to about 0.3 to 3 parasites.

Detection of B. gibsoni in sequential blood samples from an experimentally infected dog: DNA was extracted from sequential blood samples collected from a dog experimentally infected with *B. gibsoni*. Thereafter, it was tested for the presence of *B. gibsoni* DNA by PCR. As shown in Fig. 4, the parasite DNA was detected in all the blood samples

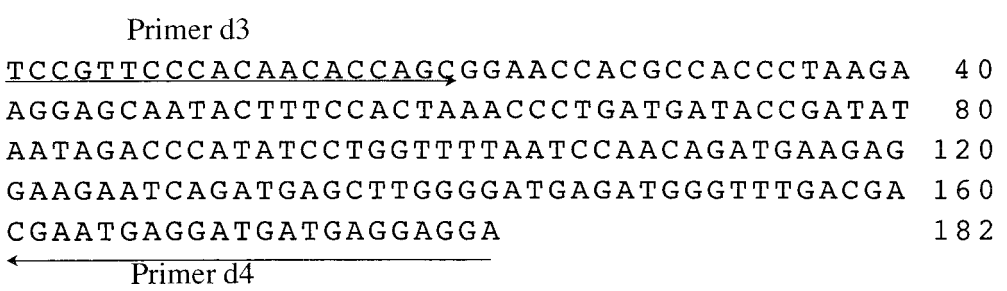

Fig. 1. Nucleotide sequence of the PCR target region and the location of two primers, d3 and d4, in the P18 gene of *B. gibsoni*.

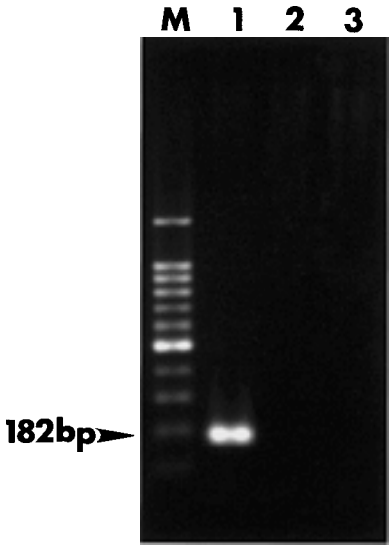

Fig. 2. Specificity of the PCR method. Ethidium bromide stained agarose gel electrophoresis of PCR products from *B. gibsoni* (lane 1), *B. canis* (lane 2) and normal dog leucocytes (lane 3). Lane M, 100 bp DNA ladder marker.

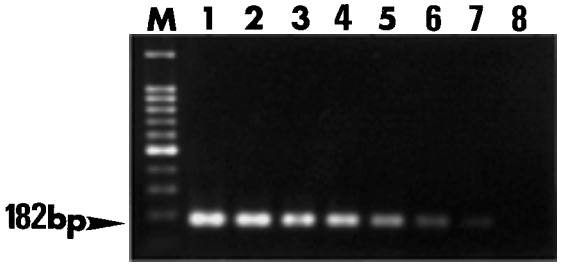

Fig. 3. Sensitivity of the PCR method. Ethidium bromide stained agarose gel electrophoresis of PCR products from 10-fold serial diluted samples. Lane 1, dilution of 10^{-1} with 2% parasitemia; lane 2, dilution of 10^{-2} with 0.2% parasitemia; lane 3, dilution of 10^{-3} with 0.02% parasitemia; lane 4, dilution of 10^{-4} with 0.002% parasitemia; lane 5, dilution of 10^{-5} with 0.0002% parasitemia; lane 6, dilution of 10^{-6} with 0.00002% parasitemia; lane 7, dilution of 10^{-7} with 0.000002% parasitemia; lane 8, dilution of 10^{-8} with 0.0000002% parasitemia. Lane M, 100 bp DNA ladder marker.

from 2 to 222 days post-infection but not from the pre-infection blood sample. On the other hand, the antibodies to *B. gibsoni* were detected from 8 days post-infection by IFAT using *B. gibsoni*-infected dog erythrocytes as an antigen, and the intraerythrocytic *B. gibsoni* organisms were detected 4 days post-infection by microscopy of the Giemsa-stained thin blood smear (Fig. 5). Using PCR, the parasite DNA was detected during the chronic stages of infection, when parasitemia was significantly low or no apparent parasite could be detected by light microscopy.

Table 1. Comparsion of the PCR method, microscopic examination, and IFAT to detection of *B. gibsoni* infection in field dogs from Kansai area, Japan

dog no.	Microscopy[a)]	IFAT[b)]	PCR[c)]
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	–	+
9	+	–	+
10	–	–	+
11	–	+	+
12	–	+	–
13	–	–	–
14	–	–	–
15	–	–	–
16	–	–	–
17	–	–	–
18	–	–	–
19	–	–	–
20	–	–	–
21	–	–	–
22	–	–	–
23	–	–	–
24	–	–	–
25	–	–	–
26	–	–	–
27	–	–	–
28	–	–	–

a) Microscopy was considered positive when *Babesia* organisms was detected in thin blood smear film.
b) IFAT was considered positive when fluorescence was observed at dilution of 1:100 and above.
c) PCR was considered positive when specific PCR product was detected in agarose gel electrophoresis.

Detection of B. gibsoni from the field samples: A comparison of PCR, microscopic examination and IFAT for detecting infection by *B. gibsoni* in samples from the field dogs in Kansai area, Japan, is shown in Table 1. Of the 28 samples, 9 samples were positive by light microscopic examination, 9 samples by IFAT and 11 by PCR. All samples found to be positive by microscopic examination were also found positive by PCR. In addition, the nucleotide sequences of the PCR products were analyzed. The sequences of all PCR products were completely identical to that of the P18 gene of the NRCPD strain of *B. gibsoni* (data not shown).

DISCUSSION

The diagnosis of *B. gibsoni* infection in dogs has been mainly carried out by microscopic examination of Giemsa-stained thin blood smear. However, the method requires expertise because parasites are often missed when parasitemia are significantly low. Serodiagnosis has also been used, but there are some problems with specificity because

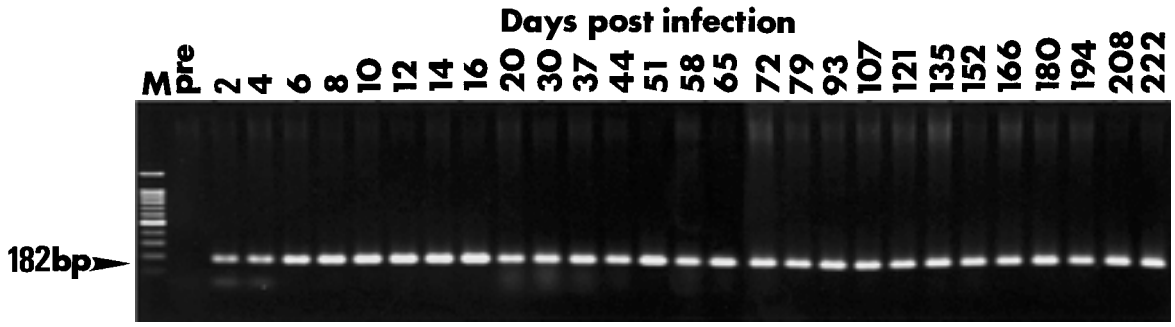

Fig. 4. Detection of the parasite DNA in a dog experimentally infected with *B. gibsoni* by PCR. Lane M, 100 bp DNA ladder marker.

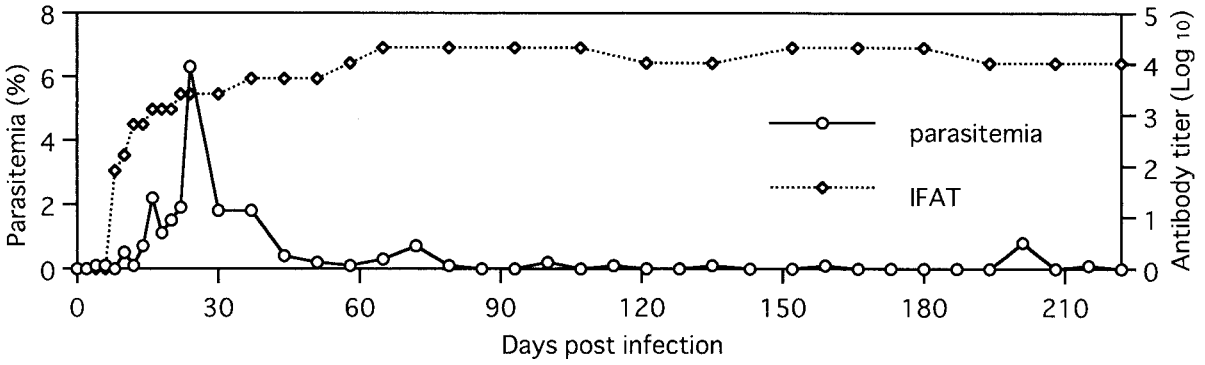

Fig. 5. Detection of *B. gibsoni* infection in an experimentally infected dog by IFAT and microscopic examination.

an antigenic cross-reaction to *B. canis* has been reported which may cause a negative result in the early stage of infection [18]. For these reasons, we developed a highly specific and sensitive diagnostic tool for *B. gibsoni* infection in dogs using a pair of oligonucleotide primers which targets the 182 bp fragment in the P18 gene of *B. gibsoni*. These primers appear to be highly specific for *B. gibsoni* since there was no detectable amplification from *B. canis* or canine leucocyte DNAs. PCR was sensitive enough to detect DNA from 2.5 μl of blood samples with a parasitemia of 0.000002%.

In sequential blood samples derived from a dog experimentally infected with *B. gibsoni*, the parasite DNA was detected from all samples taken from 2 to 222 days post-infection. PCR detected parasite DNA from earlier stages of infection than did IFAT and light microscopic examination. Moreover, PCR detected parasite DNA in the late stages of infection with a significantly low level of parasitemia or with no apparent parasite shown in Giemsa-stained thin blood smear. These results suggest that the PCR method has the potential to detect the parasite DNA in very early stages of infection, before the host animal shows clinical symptoms.

On examination of 28 field samples, PCR detected parasite DNA in 3 negative samples examined by IFAT and 2 negative samples examined microscopically, indicating that the sensitivity of PCR was higher than that of IFAT and microscopic examination for the diagnosis of *B. gibsoni* infection in dogs.

The nucleotide sequences of the PCR products were completely identical to that of the P18 gene of *B. gibsoni*, NRCPD strain, indicating that the target region of the P18 gene of *B. gibsoni* is probably well conserved in all isolates of *B. gibsoni* in Kansai area, Japan. Recently, it was reported that *B. gibsoni* isolated in North America and Asia probably belongs to a different species [19, 20]; therefore, experiments are needed in order to confirm the specificity of the PCR for canine Babesiosis in North America.

ACKNOWLEDGMENT. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Adachi, K. and Makimura, S. 1992. Changes in anti-erythrocyte membrane antibody level of dogs experimentally infected with *Babesia gibsoni*. *J. Vet. Med. Sci.* **54**: 1221–1223.
2. Adachi, K., Ueno, C. and Makimura, S. 1993. Immunosuppression in dogs naturally infected with *Babesia gibsoni*. *J. Vet. Med. Sci.* **55**: 503–505.

3. Adachi, K., Tateishi, M., Horii, Y., Nagatomo, H., Shimizu, T. and Makimura, S. 1994. Reactivity of serum anti-erythrocyte membrane antibody in *Babesia gibsoni*-infected dogs. *J. Vet. Med. Sci.* **56**: 997–999.
4. Adachi, K., Tateishi, M., Horii, Y., Nagatomo, H., Shimizu, T. and Makimura, S. 1995. Immunologic characteristics of anti-erythrocyte membrane antibody produced in dogs during *Babesia gibsoni* infection. *J. Vet. Med. Sci.* **57**: 121–123.
5. Anderson, J. F., Magnarelli, L. A. and Sulzer, A. J. 1980. Canine babesiosis: indirect fluorescent antibody test for a North American isolate of *Babesia gibsoni*. *Am. J. Vet. Res.* **41**: 2102–2105.
6. Bose, R., Jorgensen, W. K., Dalgliesh, R. J., Friedhoff, K. T. and de Vos, A. J. 1995. Current state and future trends in the diagnosis of babesiosis. *Vet. Parasitol.* **57**: 61–74.
7. Calder, J. A., Reddy, G. R., Chieves, L., Courtney, C. H., Littell, R., Livengood, J. R., Norval, R. A., Smith, C. and Dame, J. B. 1996. Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. *J. Clin. Microbiol.* **34**: 2748–2755.
8. Casapulla, R., Baldi, L., Avallone, V., Sannino, R., Pazzanese, L. and Mizzoni, V. 1998. Canine piroplasmosis due to *Babesia gibsoni*: clinical and morphological aspects. *Vet. Rec.* **142**: 168–169.
9. Farwell, G. E., LeGrand, E. K. and Cobb, C. C. 1982. Clinical observations on *Babesia gibsoni* and *Babesia canis* infections in dogs. *J. Am. Vet. Med. Assoc.* **180**: 507–511.
10. Fukumoto, S., Xuan, X., Igarashi, I., Zhang, S., Mugisha, J., Ogata, T., Nagasawa, H., Fujisaki, K., Suzuki, N. and Mikami, T. 2000. Morphological changes of *Babesia gibsoni* proliferated in canine red blood cell-substituted severe combined immunodeficiency mice. *J. Parasitol.* **86**: 956–959.
11. Ishibashi, T., Sunaga, F., Namikawa, K., Kishikawa, S. and Kanno, Y. 1994. Isolation of *Babesia canis* from a dog in the Okinawa Mainland (in Japanese with English summary). *J. Jpn. Vet. Assoc.* **47**: 202–204.
12. Ishimine, T., Makimura, S., Kitazawa, S., Tamura, S. and Suzuki, N. 1978. Pathophysiological findings on blood of Beagles experimentally infected with *B. gibsoni*. *Jpn. J. Trop. Med. Hyg.* **6**: 15–26.
13. Persing, D. H., Mathiesen, D., Marshall, W. F., Telford, S. R., Spielman, A., Thomford, J. W. and Conrad, P. A. 1992. Detection of *Babesia microti* by polymerase chain reaction. *J. Clin. Microbiol.* **30**: 2097–2103.
14. Tanaka, M., Onoe, S., Matsuba, T., Katayama, S., Yamanaka, M., Yonemichi, H., Hiramatsu, K., Baek, B. K., Sugimoto, C. and Onuma, M. 1993. Detection of *Theileria sergenti* infection in cattle by polymerase chain reaction amplification of parasite-specific DNA. *J. Clin. Microbiol.* **31**: 2565–2569.
15. To, M. 1989. Basic studies on the screening test for the development of new drugs against canine babesiosis. Nippon Vet. Anim. Sci. Univ. Thesis.
16. Wozniak, E. J., Barr, B. C., Thomford, J. W., Yamane, I., McDonough, S. P., Moore, P. F., Naydan, D., Robinson, T. W. and Conrad, P. A. 1997. Clinical, anatomic and immunopathologic characterization of *Babesia gibsoni* infection in the domestic dog (Canis familiaris). *J. Parasitol.* **83**: 692–699.
17. Yamane, I., Conrad, P. A. and Gardner, I. 1993. *Babesia gibsoni* infections in dogs. *J. Protozool. Res.* **3**: 111–125.
18. Yamane, I., Thomford, J. W., Gardner, I. A., Dubey, J. P., Levy, M. and Conrad, P. A. 1993. Evaluation of the indirect fluorescent antibody test for diagnosis of *Babesia gibsoni* infections in dogs. *Am. J. Vet. Res.* **54**: 1579–1584.
19. Zahler, M., Rinder, H., Schein, E. and Gothe, R. 2000. Detection of a new pathogenic *Babesia microti*-like species in dogs. *Vet. Parasitol.* **89**: 241–248.
20. Zahler, M., Rinder, H., Zweygarth, E., Fukata, T., Maede, Y., Schein, E. and Gothe, R. 2000. *Babesia gibsoni* of dogs from North America and Asia belongs to different species. *Parasitology* **120**: 365–369.