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Clinical Usefulness of Antibodies against *Babesia gibsoni* Detected by ELISA with Recombinant P50

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Running Head: BABESIA GIBSONI ELISA IN DOGS

ABSTRACT. The clinical usefulness of antibodies against *Babesia gibsoni* detected by ELISA with recombinant P50 was examined in dogs in an area where *B. gibsoni* infection was endemic. Only 8 among 14 dogs with acute type *B. gibsoni* infection without a previous history of infection were positive. This high percentage of false-negative results is thought to be a weak point of ELISA as a diagnostic tool. However, 14 other anemic dogs with a confirmed history of *B. gibsoni* infection were all positive, thus confirming the higher sensitivity of ELISA in detecting a history of infection.

KEY WORDS: *Babesia gibsoni*, diagnosis, ELISA

要約

*Babesia gibsoni*組換えP50-ELISAで検出される抗体の臨床的有用性（短報）--------

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Babesia gibsoni 感染流行地において組換えP50抗原ELISAで検出される犬抗体の臨床的有用性を検討した。過去に感染歴のない*B. gibsoni* 発症犬14頭中8頭のみが陽性を示し、本法は急性症診断のためには感度が不十分であると思われた。しかし感染歴の明らかな貧血症例14頭は全て陽性を示し、本法は感染歴検出には感度が高いことが示唆された。

Babesia gibsoni is a hemoprotozoan parasite reported to cause clinically important hemolytic anemia in dogs. The diagnosis of canine *B. gibsoni* infection is generally based on the demonstration of parasites in a blood smear under a microscope after Romanowsky staining [7]. As the degree of parasitemia in *B. gibsoni* infection is not related to clinical or laboratory findings, it is very difficult for clinical veterinarians to confirm *B. gibsoni* infection and differentiate it from immune-mediated hemolytic anemia (IMHA) if there is no evidence of *Babesia* parasites in blood smears, especially in endemic areas [5]. Recently, the polymerase chain reaction (PCR) has been used to diagnose *B. gibsoni* infection with a higher sensitivity and specificity [3, 8, 9]. After the introduction of PCR, animals with *B. gibsoni* infection showing low parasitemia have been easily diagnosed [4]. Thus, *B. gibsoni*-specific PCR is a very useful tool for diagnosing acute *B. gibsoni* infection in dogs. However, the limitation of PCR is that this method can detect only current infection in the peripheral blood, and not past infections. In anemic dogs with histories of *B. gibsoni* infection, the possibility of infection recurrence should always be considered, because *B. gibsoni* can not be eradicated completely from the dog's body after successful treatment. An enzyme-linked immunosorbent assay (ELISA) using recombinant proteins of *B. gibsoni* is an alternative method to detect evidence of infection with a high sensitivity and specificity. An improved ELISA specific for *B. gibsoni* using recombinant C-terminal hydrophobic regions of P50 expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST) has

been recently reported [10]. Although ELISA has been used for epidemiological studies of *B. gibsoni* infection in dogs [1, 2, 10], little information is available about the clinical application of this method. Thus, in the present study, the clinical usefulness of antibodies against *B. gibsoni* detected by ELISA with recombinant truncated P50 was examined in dogs with various types of anemia in an area in which *B. gibsoni* infection was endemic.

A total of 41 dogs with anemia were used in this study. All animals were treated at the Veterinary Teaching Hospital, Yamaguchi University between 1998 and 2004, with conditions related to anemia, including anorexia, pale mucosal membranes, hematuria or fever. All animals lived in an area where *B. gibsoni* infection was endemic: Yamaguchi Prefecture and the surrounding area in the western part of Japan. Physical and blood examination, Coom's test, and/or evaluation of bone marrow or lymph nodes aspiration were used for the differential diagnosis of anemia. *Babesia gibsoni*-specific PCR amplification was also performed using the primer set of Gib599F and Gib1270R [5]. Fourteen anemic dogs were diagnosed with the acute type of *B. gibsoni* infection: they showed typical symptoms of *B. gibsoni* infection such as anorexia, lethargy, fever, or splenomegaly. All of them were *B. gibsoni* PCR-positive, and without history of previous infection (group A). The other 27 anemic dogs were *B. gibsoni* PCR-negative and diagnosed with IMHA (n=15), lymphoma (n=4), aplastic pancytopenia (n=2), myelodysplastic syndrome (MDS) (n=1), or anemia of chronic diseases (ACD) (n=5). They were separated into 2 groups according to their history

of *B. gibsoni* infection. Seven dogs with IMHA, 2 dogs with lymphoma, and 5 dogs with ACD were categorized into group B. These dogs had confirmed histories of *B. gibsoni* infection at least 3 months before the examination by demonstrating *B. gibsoni* protozoa in their blood smears and they were treated for *B. gibsoni* infection. The remaining 13 dogs who were *B. gibsoni* PCR-negative without a history of previous infection were categorized into Group C. This group included 8 cases of IMHA, 2 of lymphoma, 2 of aplastic pancytopenia, and 1 of MSD. As a negative control, 1021 non-anemic dogs without an apparent history of previous infection of *B. gibsoni* were randomly selected (group D). Plasma or serum was obtained from all animals and kept at -20 degrees centigrade until ELISA was performed.

ELISA was carried out according to the method described previously [10]. The antigens, GST-P50t or GST (negative control), were used to coat 96-well microplates at 250 ng per well. The ELISA titer was expressed as the reciprocal of the maximum dilution that showed an ELISA value, which is the difference between the absorbance for the antigen (GST-P50t) well and that for the control antigen (GST) well. An ELISA value equal to or greater than 0.1 was judged to be positive. The ELISA with GST-P50t clearly differentiated between *B. gibsoni*-infected dog sera and uninfected dog sera, and the ELISA showed no cross-reactivity with sera from dogs experimentally infected with the closely related parasites *B. canis canis*, *B. canis vogeli*, and *B. canis rossi* [10]. To compare ELISA values of each

group, one-factor ANOVA was performed using StatView Ver. 5.0 (Hulinks).

The results are summarized in Table 1. In group A, the parasites were detected by both PCR and blood smear observation in all 14 dogs at the time of diagnosis; however, only 8 among 14 dogs were ELISA-positive. The mean ELISA value in group A was 0.838. A period when dogs are ELISA-negative with parasites appearing in the peripheral blood may occur in the early stage of *B. gibsoni* infection before detectable antibody production. In experimental infections using a limited number of dogs, significant levels of antibody response to the P50 antigen measured by ELISA had developed by Day 8 or Day 14 post-infection [2, 3], which is just the beginning of the acute phase. However, as the antibody response in naturally infected dogs may be different from that in experimental infection, more clinical data should be accumulated to reach a conclusion. The higher percentage of false-negative results found in the present study is apparently a weak point of this ELISA as a diagnostic method for acute-type *B. gibsoni* infection in dogs.

All 14 dogs in group B showed positive ELISA results. The mean ELISA value in group B was 0.891. Although PCR failed to detect parasites in the peripheral blood of these patients, it is possible that *B. gibsoni* infection might have been related to the development of IMHA in these patients. This result supports the higher sensitivity of this ELISA for detecting a history of *B. gibsoni* infection. When immune-suppressive agents such as predonisone or azathioprine are used to treat ELISA-positive IMHA patients, veterinarians

should be aware that recurrence of the *B. gibsoni* infection may occur.

In group C, only one dog among 13 was positive for ELISA, with a value of 0.545. This dog was diagnosed with IMHA without an apparent history of *B. gibsoni* infection. However, the ELISA result suggests a previous exposure to *B. gibsoni*. The mean ELISA value in group C was 0.048, which was slightly lower than the value for groups A and B; however, the differences were not significant. Thirty four of 1021 dogs (3.3 %) without anemia in group D showed positive ELISA results. These dogs did not have an apparent history of previous infection with *B. gibsoni*; however, these results suggest that there are dogs with unapparent infection of *B. gibsoni* or dogs in a chronic phase of *B. gibsoni* infection without apparent symptoms or a history of infection in areas where *B. gibsoni* is endemic. Although the specificity of this ELISA was examined using *B. canis canis*, *B. canis vogeli*, and *B. canis rossi* [10], it is still possible that the ELISA-positive dogs might have been infected with other *Babesia* species such as an emerging *Babesia* sp. closely related to *B. divergens* detected from *Ixodes ovatus* in Japan recently [6]. The antibody response on experimental infection was maintained at high levels for at least 360 days post infection, even at a chronic stage of infection that was characterized by a significantly low level of parasitemia [1, 2]. Thus, when dogs are treated with immune-suppressive agents, or when dogs become candidate donors for blood transfusion, their close monitoring is required in areas where *B. gibsoni* is endemic.

In conclusion, ELISA has a high sensitivity for detecting a history of past infection of *B. gibsoni*. Positive results on ELISA provide useful information for veterinarians in order for them to choose the best treatment strategies in endemic areas.

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Table 1. Results of ELISA in dogs with anemia in an area where *B. gibsoni* is endemic

Group	Diagnosis	Numbers of dogs examined	Numbers of dogs positive
A	*B. gibsoni* infection (Acute type)	14	8
B	Immune-mediated hemolytic anemia	7	7
	Lymphoma	2	2
	Anemia of chronic diseases	5	5
C	Immune-mediated hemolytic anemia	8	1
	Lymphoma	2	0
	Aplastic pancytopenia	2	0
	Myelodysplastic syndrome	1	0
D	Not anemic	1021	34

Group A: PCR positive, without apparent history of *B. gibsoni* infection

Group B: PCR negative, with apparent history of *B. gibsoni* infection

Group C: PCR negative, without apparent history of *B. gibsoni* infection

Group D: Without anemia, without history of *B. gibsoni* infection or history unknown