

## Serodiagnosis of *Babesia gibsoni* Infection in Dogs by an Improved Enzyme-Linked Immunosorbent Assay with Recombinant Truncated P50

Rodolfo A. VERDIDA<sup>1,6)\*</sup>, Olga A. HARA<sup>1,6)\*</sup>, Xuenan XUAN<sup>1)\*</sup>, Shinya FUKUMOTO<sup>1,2)</sup>, Ikuo IGARASHI<sup>1)</sup>, Shoufa ZHANG<sup>3)</sup>, Junyan DONG<sup>3)</sup>, Hisashi INOKUMA<sup>4)</sup>, Hidenori KABEYA<sup>5)</sup>, Yukita SATO<sup>5)</sup>, Tadaaki MORITOMO<sup>5)</sup>, Soichi MARUYAMA<sup>5)</sup>, Florencia CLAVERIA<sup>6)</sup> and Hideyuki NAGASAWA<sup>1)</sup>

<sup>1)</sup>National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555, <sup>2)</sup>The Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan, <sup>3)</sup>Department of Veterinary Medicine, Yanbian University, Longjing, Jilin Province 133400, China, <sup>4)</sup>Faculty of Agriculture, Yamaguchi University, Yamaguchi 753–8515, <sup>5)</sup>College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252–8510, Japan and <sup>6)</sup>Department of Biology, De La Salle University, 2401 Taft Avenue, Manila, Philippines

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**ABSTRACT.** The surface antigen P50 of *Babesia gibsoni* is an important candidate for the development of a diagnostic reagent for canine piroplasmiasis. In order to establish an effective diagnostic method for practical use, the gene encoding truncated P50 (P50t) lacking a signal peptide and C-terminal hydrophobic regions were cloned and expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST). More than 90% portion of the GST-P50t was expressed as a soluble form, in contrast with GST-P50f (full-length gth), which was completely expressed as an insoluble form. This result indicates that removal of the hydrophobic signal peptide and C-terminus had dramatically improved its hydrophilicity. The purified GST-P50t was tested in an enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to *B. gibsoni* in dogs. The ELISA with GST-P50t clearly differentiated between *B. gibsoni*-infected dog sera and uninfected dog sera. In addition, the ELISA detected no cross-reactivity with sera from dogs experimentally infected with the closely related parasites, *B. canis canis*, *B. canis vogeli*, and *B. canis rossii*. Field serum samples collected from dogs in Japan and China were examined for the diagnosis of *B. gibsoni* infection by using the ELISA. 14.5% (9/62), 5.8% (7/120), and 5.4% (2/37) of tested samples were positive for dogs from Okinawa, Yamaguchi, and Osaka prefectures, Japan, respectively. On the other hand, 4.8% (2/41) of tested samples were positive for dogs from Nanjing, China. These results suggest that the GST-P50t could be a reliable reagent for practical use in ELISA for the serodiagnosis of canine piroplasmiasis caused by *B. gibsoni*.

**KEY WORDS:** *Babesia gibsoni*, canine, ELISA, *Escherichia coli*, P50.

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*Babesia gibsoni* is a tick-borne hemoprotozoan parasite that causes piroplasmiasis in dogs. The most common clinical signs of acute cases are fever, marked anemia with icterus, inappetence, marked thirst, weakness, prostration, and often death [1, 17]. Both young and old dogs are susceptible to *B. gibsoni* infection; however, young dogs are more susceptible and frequently have more severe diseases [5].

The diagnosis of canine *B. gibsoni* infection is mainly based on the detection of small intraerythrocytic *Babesia* organisms by the microscopic examination of Giemsa-stained thin blood smear films. The microscopic examination is simple and suitable for the acute stage of canine babesiosis. However, the detection of the intraerythrocytic *Babesia* organisms is sometimes very difficult and not suitable for practical use in an inapparent or chronic infection, because of low levels of parasitemia [18]. Hence, alternatively, the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) with infected erythrocytes as antigen have been used for the serological

diagnosis of *B. gibsoni* infection [2, 18, 19]. These tests are particularly useful for identification of chronically infected dogs with significantly low parasitemia. However, it is feared that the use of whole parasites or parasite-derived antigens may result in false-positives due to cross-reaction with other closely related parasites, such as *B. canis* [19]. Therefore, it is necessary to develop a reliable, sensitive, and specific test using *B. gibsoni*-specific antigens.

The molecular search for diagnostic antigens for canine *B. gibsoni* infection has been focused on the identification of immunodominant antigens that are recognized by sera from dogs infected with geographically distant isolates and from both acutely and chronically infected dogs. Recently, Fukumoto *et al.* have identified a novel gene encoding an immunodominant antigen, P50 of *B. gibsoni*, and evaluated its diagnostic potential in ELISA [6–8]. The ELISA with recombinant full-length P50 (P50f) expressed in *Escherichia coli* was able to differentiate clearly between *B. gibsoni*-infected dogs and *B. canis*-infected dogs or noninfected dogs [8]. However, the usefulness of the ELISA is hindered by a limited antigen supply, because the recombinant P50f was completely expressed as an insoluble form. In the present study, to solve this problem, a perfectly designed soluble P50 was expressed in *E. coli* by removal of

\* Equally contributed to this work

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

highly hydrophobic signal peptide and transmembrane regions and evaluated its diagnostic potential in an ELISA.

## MATERIALS AND METHODS

**Parasite:** The NRCPD strain of *B. gibsoni* was experimentally infected and maintained in Beagles as described previously [6, 12].

**Cloning of a truncated P50 gene:** The truncated P50 (P50t) gene without sequences encoding highly hydrophobic signal peptide (aa 1–19) and C-terminus (aa 447–466) using a plasmid containing entire P50 gene [6] and following primers: 5'-ACG AAT TCT TAC CAC TCC CCT TCT -3' and 5'-ACG AAT TCC ACC ATC CTC ACC TTG -3', which both contains introduced *EcoRI* sites to facilitate cloning. The PCR product was cut with *EcoRI* and cloned into the *EcoRI* site of a bacterial expression vector, pGEX-4T-3 (Amersham Pharmacia Biotech, U.S.A.). The resulting plasmid was designated as pGEX/P50t.

**Expression of the P50t gene in *E. coli*:** The P50t gene was expressed as a glutathione *S*-transferase (GST) fusion protein (GST-P50t) in *E. coli* (DH5 $\alpha$  strain). *E. coli* transformed with pGEX/P50t was cultured at 37°C until the OD<sub>600nm</sub> level reached between 0.3 and 0.5. Expression of GST-P50t was induced with 0.5 mM isopropylthio- $\beta$ -galactoside (IPTG) at 37°C for 4 hr. The resulting *E. coli* was harvested by centrifugation and treated by sonication in a TNE buffer (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 2 mM EDTA) containing lysozyme (100  $\mu$ g/ml) and 1% Triton X-100. After centrifugation, the supernatant was harvested, and the GST-P50t was purified with Glutathione Sepharose 4B, according to the manufacturer's instructions (Amersham Pharmacia Biotech, U.S.A.).

**ELISA:** ELISA was essentially carried out according to the method as described previously [8]. The antigens, GST-P50t and GST (negative control) were coated to 96-wells

microplates at concentration of 100 ng per well. The ELISA titer was expressed as the reciprocal of the maximum dilution that showed an ELISA value equal to or greater than 0.1, which is the difference in absorbance between that for the antigen (GST-P50t) well and that for the control antigen (GST) well.

**SDS-PAGE and Western blot analyses:** SDS-PAGE and Western blot analyses were performed as described previously [7].

**Sera:** Canine serum samples used for evaluation of the ELISA with GST-P50t were as follows: 22 sera from dogs experimentally infected with *B. gibsoni*; 3 sera from dogs experimentally infected with *B. canis canis*; 2 sera from dogs experimentally infected with *B. canis vogeli*; 3 sera from dogs experimentally infected with *B. canis rossi*; 4 sera from dogs experimentally infected with *Neospora caninum*; 5 sera from dogs experimentally infected with *Leishmania infantum*; 30 sera from specific pathogen free dogs (Nihonnosan, Co., Japan); 120 sera from domestic dogs in Okinawa prefecture, Japan; 62 sera from domestic dogs in Yamaguchi prefecture, Japan; 37 sera from domestic dogs in Osaka prefecture, Japan; 41 sera from domestic dogs in Nanjing, China.

## RESULTS

**Expression of GST-P50 in *E. coli*:** The P50t gene (Fig. 1) without sequences encoding highly hydrophobic signal peptide (aa 1–19) and C-terminus (aa 447–466) was amplified by PCR, inserted into the bacterial expression vector pGEX-4T-3, and then expressed as a GST fusion protein in *E. coli*. As shown in Fig. 2, more than 90% portion of the recombinant GST-P50t was expressed as a soluble form, in contrast with GST-P50f, which was completely expressed as an insoluble form. This result indicates that removal of the hydrophobic signal peptide and C-terminus had dramati-

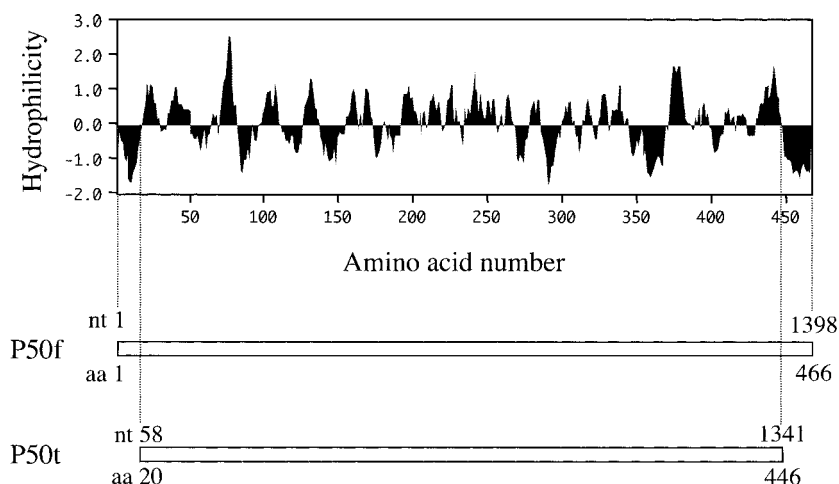


Fig. 1. Hydrophilicity plots of P50f and P50t. The plots shown were derived from the amino acid sequence of the open reading frame of the P50 gene by using computer analysis programs developed by Hopp and Woods [9]. nt, nucleotide; aa, amino acid.

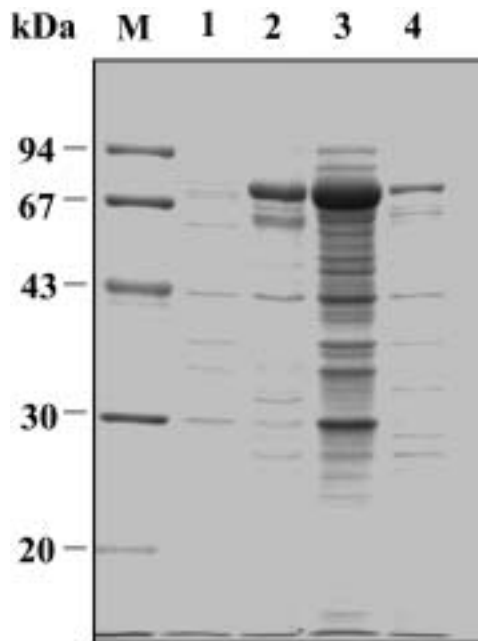


Fig. 2. SDS-PAGE analysis of the recombinant P50f and P50t. Lanes 1 and 2, soluble and insoluble fractions extracted from *E. coli* expressing GST-P50f, respectively; lanes 3 and 4, soluble and insoluble fractions extracted from *E. coli* expressing of GST-P50t, respectively.

cally improved its hydrophilicity. The molecular masses of control GST and GST-P50t were estimated as 26 and 71 kDa, respectively, as expected (Fig. 2). Western blot analysis shows that the GST-P50t reacted strongly with sera from *B. gibsoni*-infected dog but not with sera from uninfected dog (Fig. 3).

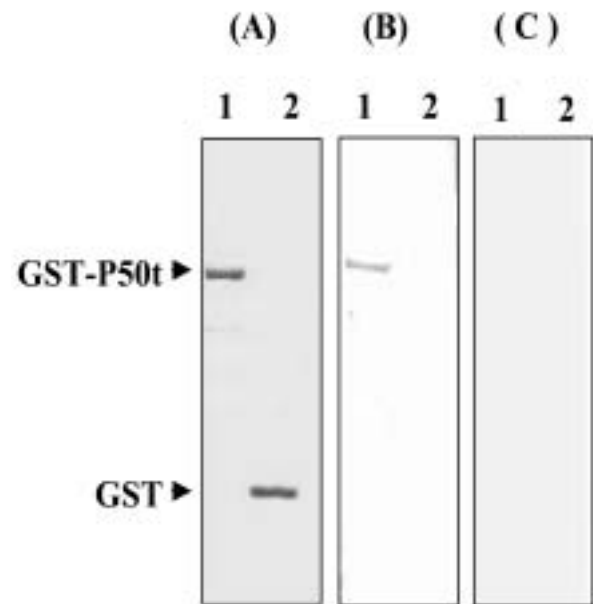


Fig. 3. SDS-PAGE and Western blot analyses of recombinant P50t. (A) Antigens stained with amido black 10B; (B) antigens reacted with *B. gibsoni* infected dog serum; (C) antigens reacted with SPF dog serum. Lane 1, GST-P50t; lane 2, GST.

**Evaluation of the ELISA with GST-P50t:** To evaluate whether the GST-P50t expressed in *E. coli* can be used as a suitable antigen for the diagnosis of *B. gibsoni* infection, the purified GST-P50t was tested in an ELISA using sera from dogs experimentally infected with *B. gibsoni*, or other closely related protozoan parasites. As shown in Fig. 4, all sera from dogs infected with *B. gibsoni* were positive (lane 1, optical density, > 0.1), whereas all sera from uninfected dogs (lane 2) or from dogs infected with *B. canis canis* (lane

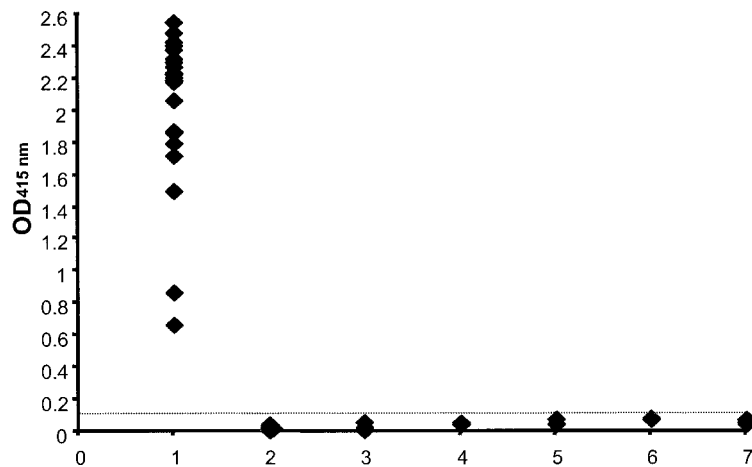


Fig. 4. ELISA with GST-P50t. Lane 1, *B. gibsoni*-infected dog sera (n=22); lane 2, SPF dog sera (n=30); lane 3, *B. canis canis*-infected dog sera (n=3); lane 4, *B. canis vogeli*-infected dog sera (n=2); lane 5, *B. canis rossi*-infected dog sera (n=3); lane 6, *N. caninum*-infected dog sera (n=4); lane 7, *L. infantum*-infected canine sera (n=5).

Table 1. Prevalence of canine *B. gibsoni* infection in Okinawa, Yamaguchi, Osaka prefectures, Japan and Nanjing, China

Samples from	No. examined	No. seropositive (%)
Okinawa	62	9 (14.5)
Yamaguchi	120	7 (5.8)
Osaka	37	2 (5.4)
Nanjing	41	2 (4.8)

3), *B. canis vogeli* (lane 4), *B. canis rossi* (lane 5), *N. caninum* (lane 6), *L. infantum* (lane 7) were negative (optical density, < 0.1).

**Diagnosis of *B. gibsoni* infection in dogs by the ELISA with GST-P50t:** Serum samples randomly collected from dogs in Okinawa, Yamaguchi, Osaka prefectures, Japan, and Nanjing, China were tested for detection of antibodies to *B. gibsoni* by the ELISA with GST-P50t. As shown in Table 1, 14.5%, 5.8%, and 5.4% of tested samples were positive for dogs from Okinawa, Yamaguchi, and Osaka prefectures, Japan, respectively. On the other hand, 4.8% of tested samples were positive for dogs from Nanjing, China.

## DISCUSSION

In the previous studies, we have identified and cloned a novel gene encoding a surface antigen with a molecular mass of 50 kDa (P50) by immunoscreening of cDNA library prepared from mRNA of *B. gibsoni* merozoites with canine infected serum, and demonstrated that P50 is an immunodominant antigen [6, 7]. The ELISA with GST-P50f expressed in *E. coli* was able to diagnose both acute and chronic *B. gibsoni* infections in dogs [8]. Unfortunately however, the GST-P50f was expressed as insoluble form, which makes the purification procedure complex, because the insoluble protein should be dissolved in an 8 M urea solution and then remove the urea by dialysis before the standard purification procedure [8]. The final yield of purified GST-P50f was still very low even after urea treatment. In general, for the expression of membrane proteins in *E. coli*, removal of their hydrophobic signal peptide and transmembrane regions can improve their hydrophilicity. Recently, we have succeeded in expressing several protozoan membrane associated antigens, such as SAG1 of *N. caninum* [4], SAG2 of *Toxoplasma gondii* [14], EMA-2 of *B. equi* [13] as soluble proteins in *E. coli*, by deleting the signal peptide and transmembrane regions. Our previous study shows that the N-terminus aa 1–19 and C-terminus aa 447–466 of P50 functions as signal peptide and transmembrane, respectively [7]. In this study, to obtain high amount of the soluble P50 for practical use in an ELISA, the gene encoding P50t without highly hydrophobic signal peptide and transmembrane regions was expressed in *E. coli*. The total expression level of GST-P50t was higher than that of GST-P50f, and more than 90% portion of the GST-P50t was expressed as a soluble form, in contrast with GST-P50f, which was completely expressed as an insoluble form as

mentioned above. This result indicated that removal of the hydrophobic signal peptide and C-terminus had dramatically improved its hydrophilicity. The yield of GST-P50t was as high as GST alone. The GST-P50t purified from one litter culture of *E. coli* is much enough to make approximately 1,000 microplates (96-wells) and is much enough to detect approximately 50,000 serum samples.

The GST-P50t purified from soluble fractions of *E. coli* extracts was recognized by the sera from dogs infected with *B. gibsoni* in Western blot analysis. This result indicated that the GST-P50t retained its immunoreactivity after deleting both ends of N- and C-terminuses.

The purified GST-P50t was tested in an ELISA for the detection of antibodies to *B. gibsoni* in dogs. The ELISA with GST-P50t clearly differentiated between *B. gibsoni*-infected dog sera and uninfected dog sera. In addition, the ELISA detected no cross-reactivity with sera from dogs experimentally infected with the closely related parasites, *B. canis canis*, *B. canis vogeli*, *B. canis rossi*, *N. caninum*, and *L. infantum*. Field serum samples collected from dogs in Japan and China were examined for the diagnosis of *B. gibsoni* infection by using the ELISA. 14.5%, 5.8%, and 5.4% of tested samples were positive for dogs from Okinawa, Yamaguchi, and Osaka prefectures, Japan, respectively. On the other hand, 4.8% of tested samples were positive for dogs from Nanjing province, China. All positive serum samples from domestic dogs both from Japan and China were IFAT-positive (data not shown). The present data of prevalence of canine *B. gibsoni* infection in western Japan is within the range reported previously [10, 11, 18]. In addition, to our knowledge, this is the first report describing a survey on canine piroplasmosis in Nanjing, China.

The recent knowledge based on genetic analyses of genes from canine piroplasms has shown that there are at least three distinct subtypes or subspecies for small piroplasms: a classic Asian type *B. gibsoni*; a small organism has been identified recently in northern Spain, which has been given the name *Theileria annae*; a small organism has been identified in California and remains unnamed [3, 15, 16, 20, 21]. Since the P50 gene is cloned from an Asian type *B. gibsoni*, an experiment is needed to evaluate whether the ELISA with GST-P50t can be used for detecting antibodies to *T. annae* or unnamed California type small piroplasm in dogs in Europe or North America.

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