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Identification of a novel *B. gibsoni* 27-kDa protein as a serodiagnostic antigen

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ABSTRACT

A novel gene encoding 27-kDa protein was identified by the screening of *Babesia gibsoni* cDNA library with acutely infected dog serum. The *BgP27* is a single copy gene with a predicted open reading frame of 762 bp and 254 amino acids. The phylogenic analysis of the deduced amino acid of *BgP27* demonstrated considerable identities with members of *Plasmodium berghei* circumsporozoite protein family that ranged between 18.4% and 22.8%. The BgP27 was expressed as a glutathione *S*-transferase fusion protein in *Escherichia coli*. The serum raised in mice against the recombinant protein specifically reacted with a 27-kDa protein in the extracts of *B. gibsoni* parasites. Confocal laser scanning microscopic observation showed high fluorescent reactivity with both extracellular and intracellular merozoite. Furthermore, recombinant BgP27 was used as an antigen in an enzyme-linked immunosorbent assay (ELISA). Thus, the kinetics of the anti-BgP27 antibody was detected in serial serum samples collected from a *B. gibsoni*-infected dog. IgG levels were high throughout the course of infection. In addition, the ELISA was able to differentiate between *B. gibsoni*-infected dog serum and *B. canis* subspecies-infected dog serum or normal dog serum. The diagnostic performance of BgP27-ELISA revealed the potential use of the antigen for detection of infection in dogs.

Key words: *Babesia gibsoni*, enzyme-linked immunosorbent assay, diagnostic performance.

INTRODUCTION

Babesia gibsoni is a tick-borne hemoprotozoan parasite, which causes piroplasmosis in dogs. *B. gibsoni* infection is documented to be prevalent worldwide, in Asia, Europe, Africa, and the Americas (Farwell *et al*., 1982; Jefferies *et al*., 2003; Lobetti, 1998). Recently, this disease has been found to occur frequently in companion animals and has become a big problem clinically (Macintire *et al.*, 2002; Muhlnickel *et al.*, 2002). The transmission occurs via *Haemaphysalis* spp, *Rhipicephalus sanguineus*, and *Dermacentor reticulatus* tick-vectors when sprozoites are released with saliva during their feeding (Levine, 1998; Boozer and Macintire, 2003). The pathogenicity begins when the merozoites invade and replicate intensively within host erythrocytes. Infected dogs typically suffer from acute form of babesiosis, which is generally characterized by pyrexia, weakness, pale mucous membrane, depression, lymphadenopathy, splenomegaly, malaise and rarely death (Boozer and Macintire, 2003). In chronically infected dogs, the disease recurs and causes anemia after an operation or while a dog is on immunosuppressive therapy (Farwell *et al.*, 1982; Adachi *et al.*, 1993).

Both specific and sensitive diagnostic test and protective vaccination are important for controlling the disease. Clinically, *B. gibsoni* infection is very difficult to differentiate from other diseases, which are characterized by hemolytic anemia. Giemsa-stained blood smears examination is considered to be a basic and standard method for diagnosis. However, in subclinical or chronic infections, this method is not reliable because of the low parasitemia in peripheral blood (Verdida *et al.*, 2004). Recently, PCR has been established

for direct parasites detection. Indeed, PCR offers the advantages of high degrees of sensitivity and specificity, but the disadvantage of the test is the requirement for specialized laboratory equipment and facilities as well as well-trained laboratory personnel. Alternatively, other researchers have used indirect parasites detection techniques that are based on the measurement of antibody levels against parasites in serum. The indirect fluorescent-antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) with whole parasite as the antigen have been utilized for serological diagnosis of *B. gibsoni* infection. Although, such techniques proved convenient tools for serological diagnosis, the poor quality of the antigens, and sometimes the cross-reactions with other *Babesia* species resulting in false positive reactions have limited their application (Bose *et al.*, 1995). Recombinant antigens provide better options because antigens are usually available in pure forms and offer higher specific activity than whole parasite (Tebele *et al.*, 2000; Boonchit *et al.*, 2006). In the same regard, several *B. gibsoni* merozoite antigens that previously identified, demonstrated promising results in ELISAs, such, rBgP50, rBgBSA1, rBgP32 and rBgTRAP (Fukumoto *et al.*, 2001a; Jia *et al.*, 2006; Aboge *et al.*, 2007; Goo *et al.*, 2008). However, their sensitivities have not yet achieved perfect result, therefore, further research on new antigens is extremely desirable.

In the present study, we screened a cDNA expression library of *B. gibsoni* merozoites with *B. gibsoni*-infected sera in order to identify the genes coding the antigenic proteins that could be useful candidates for serodiagnosis. Furthermore, we identified and characterized a *B. gibsoni* 27-kDa protein (BgP27), which shares homology with *Plasmodium berghei* circumsporozoite protein and shown to be a promising serodiagnostic antigen.

MATERIALS AND METHODS

Parasites and experimental animals

The *B. gibsoni* NRCPD strain isolated from a hunting dog in Hyogo Prefecture, Japan (Ishimini *et al.*, 1978) was maintained in one-year-old splenoctomized beagle dogs by intravenous passage of infected erythrocytes (Fukumoto *et al.*, 2001). Cultured *B. gibsoni* Oita strain was maintained on fresh canine erythrocytes *in vitro* as previously described (Sunaga *et al.*, 2002).

Beagle dogs (Nihon Nosan, Japan), Japanese white rabbits (CLEA, Japan), and ddY (SLC, Japan) mice were used for the infection and immunization experiments. All animal experiments described in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals Promulgated by Obihiro University of Agriculture and Veterinary Medicine.

Immunoscreening of cDNA expression libraries

A cDNA library of *B. gibsoni* merozoites was previously constructed as earlier described (Fukumoto *et al.*, 2001). Briefly, the cDNA was synthesized by using a Zap-cDNA synthesis kit, as per manufacturer's instructions (Stratagene, USA). The cDNA expression library (10^7 PFU) was immunoscreened with the serum from acutely *B. gibsoni*-infected dog and the cDNA inserts of positive clones were sequenced using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, USA). Complete nucleotide sequences of identical cDNAs were analyzed using basic local alignment search tool (BLAST) accessed through the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). A cDNA clone encoding BgP27 of *B. gibsoni* was selected for further study. The phylogenic analysis was carried out using DNASTAR software (NetWell Corporation, Japan).

Southern blotting

Genomic DNA of *B. gibsoni* was extracted from the erythrocytic stages of the parasite and digested with *Eco*RI, *Xho*I, *Hin*dIII and *Pvu*II overnight. The DNA samples were separated on 0.8% (w/v) agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham-Buchler, Germany). The blots were pre-hybridized at 56°C for 4 hrs and probed overnight with either full or partial length of *BgP27* gene labeled with alkaline phosphatase (Amersham Pharmacia Biotech, UK).

Cloning, expression, and purification of recombinant BgP27

The entire fragment encoding BgP27 was amplified using primer set: 5`-CGAGAATTCATGGCTGGCATGACA-3` and 5`-ATTCTCGAGTTTGGCGTACA GGTTG-3`. The PCR product was inserted into the pGXE-4T vector (Amersham Pharmacia Biotech, USA) using the *Eco*RI and *Xho*I sites and expressed in *Escherichia coli* BL21 strain, according to the manufacturer's instructions. The recombinant proteins were then purified using glutathione-Sepharose 4B beads (Amersham Biosciences, USA).

Production of anti-rBgP27 antiserum and IgG purification

Six-week-old ddY mice (n = 5) were immunized intraperitoneally (i.p.) with 100 μg of the purified rBgP27 and rGST proteins emulsified with 100 μl Freund's complete adjuvant (Difco Laboratories, USA). Two boosters of 50 μg of the same antigens emulsified with Freund's incomplete adjuvant (Difco, USA) were given i.p. at 14-day intervals. Sera were collected two weeks after the last booster and checked for specific antibodies by IFAT. The anti-rBgP27 antibody was also raised in rabbit to prepare purified IgG. Following the same immunization regime mentioned above, 0.5 mg of the proteins (rBgP27 or rGST) emulsified with Freund's incomplete adjuvant were administered subcutaneously (s.c.) to Japanese white rabbits (2.5 kg) for priming and 0.25 mg emulsified in Freund's incomplete adjuvant for boosters. Polyclonal rabbit antibodies were checked using Western blotting and IFAT. Total IgGs were purified from rabbit sera through Protein A chromatography columns according to the manufacturer's instructions (Bio-Rad Laboratories, USA). The fractions containing IgG were pooled and run on SDS-PAGE to test the purity and concentration. An ELISA was performed to test the binding activity of the IgGs either against rBgP27 or GST.

IFAT and confocal laser microscopic observation

Thin blood smears of cultured *B. gibsoni*-infected erythrocytes was fixed with absolute methanol at -20°C for 30 min. Standard protocol of immonofluorescence assay was carried out to confirm the reactivity of antiserum with native protein. Briefly, the antiserum against the rBgP27 was applied as the first antibody on the fixed smears and incubated at 37°C for 1 hr in moist chamber. After washing with phosphate-buffered saline Tween 20 (PBST) three times, Alexa-Fluor® 488-conjugated goat anti-mouse immunoglobin G (IgG) (Molecular Probes, USA) was applied as a secondary antibody (1:200) and then incubated at 37°C for 30 min. The slides were washed three times with PBST and incubated with 2.5 μg/ml propidium iodide (PI) (Molecular Probes, USA) containing 50 μg/ml RNase (Qiagen, Germany) at 37°C for 10 min. After washing with PBS twice, the glass slides were mounted by adding 10 μl of a 50% glycerol-PBS (v/v) solution and covered with a glass cover slip. The slides were examined using a confocal laser scanning microscope (TCS NT, Leica, Germany).

Preparing the parasites lysate

B. gibsoni-infected erythrocytes were obtained from infected dogs at the peak of parasitemia, and then the mononuclear cells were removed by HISTOPAQUE®-1077 (Sigma-Aldrich, USA) according to the manufacturer's instructions, while the Buffy coat was removed by washing four times with PBS. The erythrocytes were later lysed with 0.25% saponin, the pellet was washed four times with cold PBS, and then the final pellet was resuspended in PBS, disrupted three times by a freeze-thaw cycle in liquid nitrogen, and then sonicated in ice slurry. The protein concentration of the lysates was determined by a BCA protein assay kit (PIERCE, USA), and finally stored at −80°C until use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Proteins in the merozoite extracts were size-separated under reducing conditions by electrophoresis in 12% SDS–PAGE and then electroblotted onto a nitrocellulose membrane (Immobilon™-P, Millipore, USA). The immunoblotting and bands detection was performed as previously described (Xuan *et al.*, 1996).

ELISA

The standard ELISA was performed as described (Terkawi *et al.*, 2007). Briefly, microtiter plates (Nunc, Denmark) were coated overnight at 4°C with 2 μg/ml of the rBgP27 and GST emulsified in a coating buffer (0.05 M carbonate buffer, pH 9.6). The plates were then blocked with a 3% (w/v) skim milk solution for 1 hr at 37ºC. After washing, the plates were incubated with serum samples. The bound antibody was detected by treatment with horseradish peroxidase HRP-conjugated (BETHYL, Labratories, USA) anti-dog IgG (1:6,000) and ABTS [2,2 -azinobis (3-ethylbenzthiazolinesulfonic acid)] (Sigma, USA). The color was allowed to develop at room temperature. Optical density (OD) was measured by MTP-500 microplate reader (Corona Electric, Japan) at 415 nm. The OD of GST-background values were subtracted from those of rBgP27.

***In vitro* inhibition assay of *B. gibsoni* growth**

The parasites culture was performed as described previously (Sunaga *et al.*, 2002). Briefly, *B. gibsoni*-infected canine RBCs with approximately 5% parasitemia were suspended in a 900 μl RPMI-1640 medium (GIBCO) supplemented with 25 mM HEPES, 2 mM L-glutamine, 1 mM pyruvic acid, 24 mM $NaHCO_3$, 10% dog serum free of *Babesia* infection, and then diluted with fresh canine RBCs to 1% parasitemia and 10% packed cell volume (PCV). Two hundred microliters of the culture mixture was dispensed into 96-well plates (Nunc, Denmark) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 4 days. The supernatant of culture was replaced daily with a fresh medium. The inhibition assay was performed using IgGs at final concentration of 1 mg/ml for anti-rBgP27 IgG, anti-rBgP50 and anti-rGST IgG. Percent parasitemia of Giemsa-stained smears was determined by observing microscopic fields covering approximately 1,000 erythrocytes.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the means parasitemia levels (GraphPad Prism 3, USA). The mean values were considered to be statistically different when $P<0.05$.

Nucleotide sequence accession numbers

The complete sequence of *BgP27* gene has been submitted to the GenBank with the accession number (AB454055).

RESULTS AND DISCUSSION

The cDNA library of *B. gibsoni* was screened with serum from an acutely *B. gibsoni*-infected dog. A total of 124 positive clones were obtained, isolated, sequenced, and then subjected to BLAST analysis. Seventy two clones that showed high reactivity with the sera were categorized in high antigenicity candidates group, while fifty two clones that showed low reactivity were categorized in low antigenicity candidates group (data not shown). Five clones from the first category, which showed low homology with members of *P. berghei* circumsporozoite proteins family, were selected for further molecular characterization. The identical cDNA sequence was designated as *BgP27* gene. The full length of *BgP27* contained a single open reading frame of 762 nucleotides encoding a polypeptide of 254 amino acid residues with a calculated molecular mass of 26.7-kDa. Analysis of putative N-terminal signal peptide in BgP27 sequence by using SignalP-server revealed a low probability of presence of signal peptide. Phylogenic analysis of the deduced amino acid of BgP27 demonstrated considerable identities with different *P. berghei* circumsporozoite proteins, which were ranged between 18.4% and 22.8 (Fig. 1B). Southern blot analysis of the genomic DNA probed with the *BgP27* gene demonstrated single hybridizing band after DNA digestion with *Eco*RI (13.3 kbp), *Hin*dIII (12.9 kbp) and *Acc*I (4.3 kbp) that cut a single site within the *BgP27* gene (Fig. 1D, lanes1, 2 and 3). On the other hand, *Pvu*II, which is supposed to cut outside gene yielded two hybridizing bands (2.6 and 10 kbp) as shown in Figure 1C, lane 4. These results revealed that the genomic DNA of *B. gibsoni* contains one copy of the *BgP27* gene.

(A)

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1    AATTTTAATATGGATCACAAAAGTTTTCGCTATCTCTTATTAAAGAGCCCCTCCTCTGAAGGAGTTGCTGGCAGACAGGCCATGACCAGAGCTATCGCCG  100
          M  D  H  K  S  F  R  Y  L  L  L  K  S  P  S  S  E  G  V  A  G  R  Q  A  M  T  R  A  I  A  G
101  GAATTGGTGGAGCTATTGTCAAGGTCCCCGATGTGATCCCTGGGCAGGGTGAAGTTCTGATCAAGGTAGCAATGGACACCGATATTTCTAACGTATTAGC  200
       I  G  G  A  I  V  K  V  P  D  V  I  P  G  Q  G  E  V  L  I  K  V  A  M  D  T  D  I  S  N  V  L  A
201  ATATTCATGTAGGGAGGTTAACGCTGATGGAACGAGGACTGACCTTAGCGGGAAAAGCGATGGTTCATCTGGAAGACGCGATTCTGATGGGAAGAAAGTT  300
       Y  S  C  R  E  V  N  A  D  G  T  R  T  D  L  S  G  K  S  D  G  S  S  G  R  R  D  S  D  G  K  K  V
301  AAGGATAGCCCAAAGCAGGACCTTCCAAGAAAGCCACCTGCCAACGATACCAAGCCCCCCGCCCCTAAGGTTGACAGGCCTCCTGTCCCTAAAGATGACA  400
     K  D  S  P  K  Q  D  L  P  R  K  P  P  A  N  D  T  K  P  P  A  P  K  V  D  R  P  P  V  P  K  D  D  K
401  AAGCTCCCACCGCCAATGACTCAAAGCCTCCTGCTGCAAATGACACAAAGCCCCCTGCCCCTAAGGTTGACAGGCCTCCTGTAGCAAATGACACCAAGCC  500
       A  P  T  A  N  D  S  K  P  P  A  A  N  D  T  K  P  P  A  P  K  V  D  R  P  P  V  A  N  D  T  K  P
501  CCCCGCCCCTAAGGTTGACAGGCCTCCTGTTCCTAAAGATGACAAAGCTCCCACCGCCAATGACTCAAAGCCTCCTGCTGCAAAGGATGACAAGGTGCCT  600
       P  A  P  K  V  D  R  P  P  V  P  K  D  D  K  A  P  T  A  N  D  S  K  P  P  A  A  K  D  D  K  V  P
601  GGAGCCAAGGAAGATAAGGCCCCCACCGTTAAGGATGACAAGGTTCCTGGTGCTGAGAAGAAGGTTGATGGCAAAGCAGCTGGCCAAGCTAAGGATGGCA  700
       G  A  K  E  D  K  A  P  T  V  K  D  D  K  V  P  G  A  E  K  K  V  D  G  K  A  A  G  Q  A  K  D  G  K
701  AGAAGGGTGATGCACTAGCGAAAGAAGCTGAAAAATCAACTTCCCATTTGGTGCCACAGGTATGTGAGATGTAAAAACTTTTAATTCGTATCAGCTCGCG  800
       K  G  D  A  L  A  K  E  A  E  K  S  T  S  H  L  V  P  Q  V  C  E  M  *
801  AACAAACTGAAAAACTTGAAAAGAATGAAACCTATAAGAGGAGGCATCGTCGCATTGCTTAAGCAGCATACATTATGCCACGTTCTTTTCTACGGACTAC  900
901  TTACATGTAGTGTGCTACTGGAGTTGCTTTGTTAATAGTGGTGCGTTCGGATTTAAACAGTGAAGGTTTCATACTAAAGGCTATCCATCCAAAACAGCGT  1000
1001 ATTATAGTTATCCACTCCAAACAACAATGAGAAATCATGTTAATATTCGTATCGCTAAAAAAAAAAAAAAAAA
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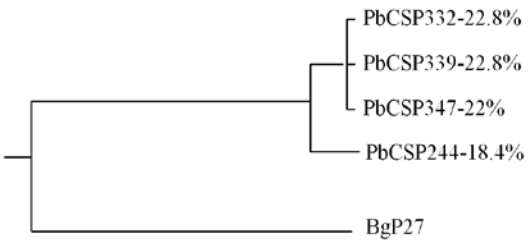
(B)



(C)

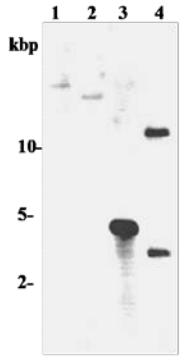


Fig. 1. (A) Complete nucleotide and predicted amino acid sequences of the cDNA coding for BgP27. **(B)** The phylogenic analysis of the deduced amino acid of BgP27 with different circumsporozoite proteins from *P. berghei*. The deduced amino acids of BgP27 were aligned with *P. berghei* circumsporozoite proteins PbCSP-332 (AAA29541), PbCSP-339 (P06915), PbCSP-347 (P23093) and PbCSP-244 (Q4Y9F2). The identities of each sequence with BgP27 were calculated by DNASTAR software. **(C)** Southern blot analyses. *B. gibsoni* genomic DNA was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2)and *Acc*I (lane 3) that cut a single site within the *BgP27* gene, and *Pvu*II (lane 4) that cut outside gene.

Next, expression of rBgP27 in *E. coli* yielded a soluble GST-fusion protein having molecular mass of 53-kDa. Sera from dogs experimentally infected with *B. gibsoni* reacted with the rBgP27 by Western blotting (Fig. 2A) indicating that the rBgP27 expressed in *E. coli* maintained its antigenicity. Moreover, the anti-rBgP27 serum specifically reacted with 27-kDa protein in *B. gibsoni* lysates by Western blot analysis (Fig. 2B, lane 1). The observed molecular size of native BgP27 was consistent with the expected molecular weight. The antiserum raised against rBgP27 was tested with *B. gibsoni* merozoites by IFAT. Highly specific fluorescence was observed in different developing stages of both intracellular and extracellular parasites (Fig. 2).

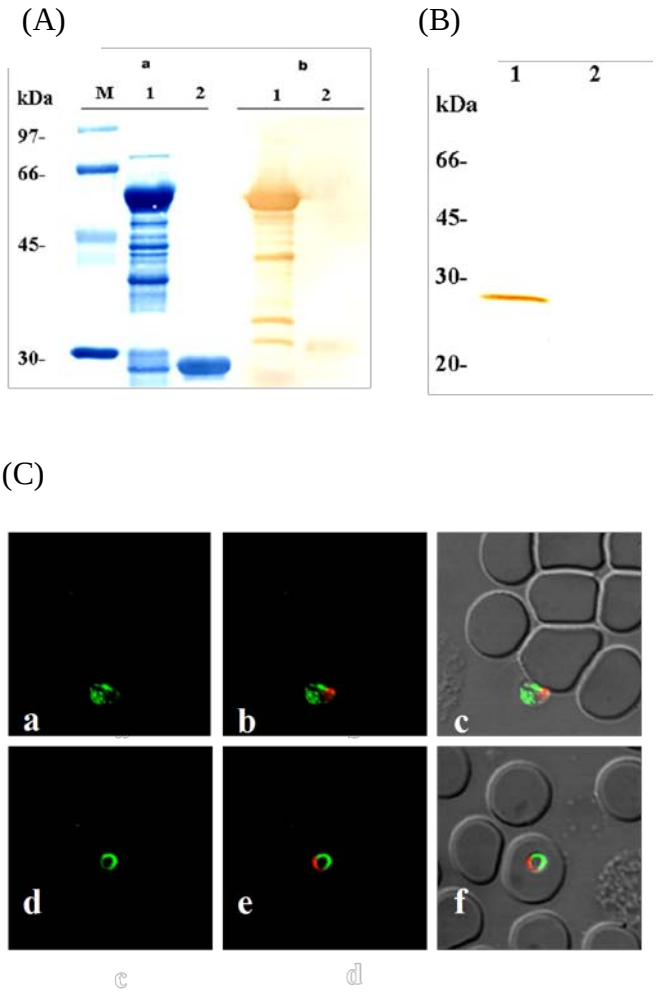


Fig. 2. (A) Western blot analysis of recombinant and native BgP27. (a) Recombinant rBgP27 and rGST proteins separated on a 12% SDS-polyacrylamide gel and stained with Coomassie blue. (b) Western blotting membrane of recombinant proteins probed with *B. gibsoni*-infected dog serum. Lanes 1, rBgP27 and 2, rGST. **(B)** Native BgP27 reacted with mouse anti-BgP27 serum. Lane 1, *B. gibson*i-infected erythrocytes; lane 2, erythrocytes from healthy dogs. **(C)** Immunofluorescence microscopy analysis. Methanol-fixed smears of *B. gibsoni*-infected RBC were preincubated with the anti-rBgP27 serum, visualized with Alexa-Fluor® 488-conjugated IgG secondary antibody and propidium iodide (PI) staining, and finally examined by confocal laser microscopy. The specific reaction of the antigen and anti-rBgP27 serum is green and the nucleus is red. The reactivity of antiserum with extracellular (c) and intracellular merozoites (f). Panels (a, d), immunofluorescent staining; (b, e), overlaid image of PI staining merozoite nuclei and immunofluorescent staining; (c, f), overlaid image of PI staining phase and green fluorescence on phase-contrast images of the parasites.

The *Plasmodium* circumsporozoite proteins are considered to be the principal antigens on the surface of sporozoites. Epitopes found on this antigen react with antibodies that inhibit the invasion of hepatocytes by sporozoites and induce cellular responses that kill sporozoite-infected liver cells. Monoclonal antibodies to the circumsporozoite (CS) proteins *P. falciparum* and *P. vivax* sporozoites blocked invasion into cultured human hepatoma cells (Hollingdale *et al.*, 1984; Rieckmann *et al.*, 1974). Therefore, we investigate the effects of purified anti-rBgP27 IgG on the growth of *B. gibsoni in vitro*. *B. gibsoni* parasites were cultured in RPMI medium in the presence and absence of IgG. Test cultures contained 1 mg/ml of anti-rBgP27 IgG, while control cultures were prepared using either 1 mg/ml anti-GST IgG or without IgG. Anti-rBgP50 IgG that previously showed protective effects in SCID mice (*in vivo*) was used as positive control (Fukumoto *et al.*, 2004). However, there was no inhibition of the parasites growth in both test and control cultures. In contrast, significant inhibition was only observed with anti-BgP50 IgG after 4 days of

culturing (data not shown).

Furthermore, the antigenicity of the BgP27 was observed by ELISA reactions using serial *B. gibsoni*-infected sera. A dog experimentally infected with *B. gibsoni* developed detectable antibody responses to the BgP27 by 8 days, and maintained those levels until 541 days post-infection (Fig. 3A). From epidemiological point of view, it is important to differentiate the infection from closely related *B. canis* infections and other closely related apicomplexan parasite infections. Therefore, the specificity of rBgP27 was evaluated in dogs infected with different apicomplexan parasites. No cross-reaction with all sera from dogs experimentally infected with *B. canis canis*, *B. canis vogeli*, *B. canis rossi* or *N. caninum*, suggesting the specificity of antigen for detection of *B. gibsoni* antibodies in dogs (Fig. 3B). Previously reported antigens, such BgP50, BgTRAP, and BgSA1, showed similar performance in ELISA. Taken together, these results indicate that the novel rBgP27 could be a potentially specific antigen for detection of *B. gibsoni* infection and probably a candidate antigen for epidemiological investigation.

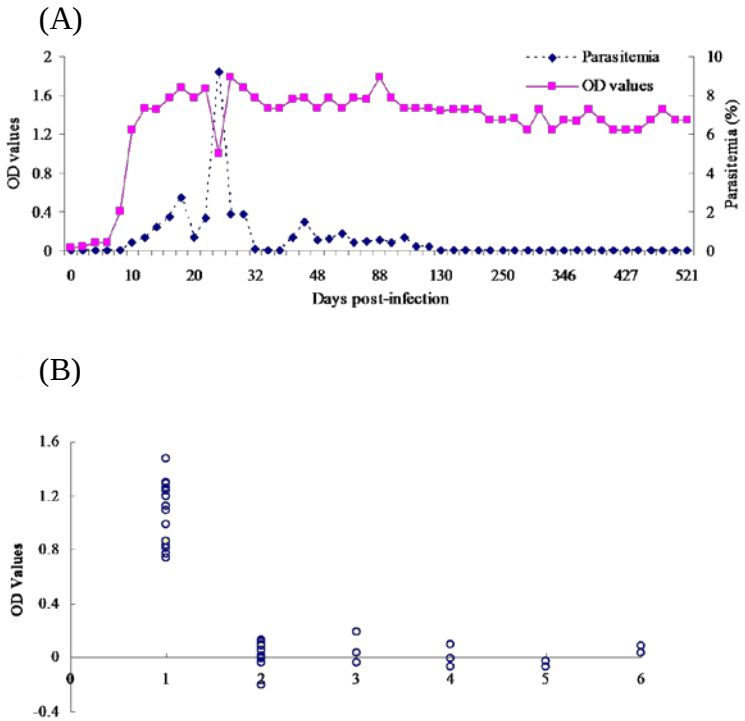


Fig. 3. Evaluation of BgP27 as a diagnostic antigen in an ELISA system. The cut-off value of 0.12 was calculated from the results of the ELISA of 28 specific pathogen-free (SPF). **(A)** Detection of the antibody against rBgP27 in a dog experimentally with *B. gibsoni* by using ELISA. **(B)** The ELISA values of experimentally infected dog sera. Lane 1, sera from *B. gibsoni*-infected dogs (n = 16); lane 2, SPF dog sera (n = 18); lane 3, sera from *B. canis canis*-infected dogs (n = 3); lane 4, sera from *B. canis rossi* infected dogs (n = 3); lane 5, sera from *B. canis vogeli*-infected dogs (n = 2); lane 6, sera from *N. caninum*-infected dogs (n = 2).

In summary, we isolated a full length of the BgP27 sequence from a cDNA expression library and characterized it as highly antigenic protein. Although, BgP27 appears to be highly antigenic, the anti-rBgP27 IgG was not protective against the parasites growth *in vitro*. On the other hand, the specificity and sensitivity of the ELISA-rBg27 indicated that this antigen might be useful in a diagnostic test.

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