Molecular characterization of a novel 32-kDa merozoite antigen of *Babesia gibsoni* with a better diagnostic performance by enzyme-linked immunosorbent assay

G. O. ABOGE[1], H. JIA[1], K. KURIKI[2], J. ZHOU[1], Y. NISHIKAWA[1], I. IGARASHI[1], K. FUJISAKI[1], H. SUZUKI[1] *and* X. XUAN[1]*

[1] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*
[2] *Kyoritsu Seiyaku Corporation, Chiyoda-ku, Tokyo 102-0073, Japan*

(*Resubmitted 27 December 2006; revised 1 February 2007; accepted 2 February 2007; first published online 23 March 2007*)

SUMMARY

We cloned and expressed a novel gene encoding a 32-kDa merozoite protein of *Babesia gibsoni* (BgP32). The length of nucleotide sequence of the cDNA was 1464 bp with an open reading frame of 969 bp. The truncated recombinant BgP32 (rBgP32) without a signal peptide and C-terminal hydrophobic sequence was expressed in *Escherichia coli* as a soluble glutathione-*S*-transferase (GST) fusion protein. Western blotting demonstrated that the native protein was 32-kDa, consistent with molecular weight of the predicted mature polypeptide. Enzyme-linked immunosorbent assay (ELISA) using rBgP32 detected specific antibodies from 8 days to 541 days post-infection in the sequential sera from a dog experimentally infected with *B. gibsoni*. Moreover, the antigen did not cross-react with *B. canis* subspecies and closely related protozoan parasites, indicating that rBgP32 is a specific diagnostic antigen. Analysis of 47 sera taken from dogs with anaemic signs revealed that rBgP32 detected a higher proportion of *B. gibsoni* seropositive samples (77%) than its previously identified rBgP50 (68%) homologue. These results indicate that the BgP32 is a novel immunodominant antigen of *B. gibsoni*, and rBgP32 might be useful for diagnosis of *B. gibsoni* infection.

Key words: 32-kDa merozoite protein, *Babesia gibsoni*, diagnostic performance, ELISA.

INTRODUCTION

Babesia gibsoni is an intraerythrocytic apicomplexan parasite that infects dogs and is naturally tick-transmitted (Baneth *et al*. 1998). There are, however, reports of transmissions associated with blood transfusion (Stegeman *et al*. 2003) and via the transplacental route to the developing foetus (Fukumoto *et al*. 2005) and, in some instances, infection associated with dog bites has been documented (Birkenheuer *et al*. 2005). The parasite affects wild and domestic canids worldwide, in Africa, Asia, the United States and Europe (Kjemtrup *et al*. 2000). The acute form of *B. gibsoni* infection usually is clinically characterized by fever, anaemia, lethargy and splenomegally (Conrad *et al*. 1991). In contrast, the chronic form of the disease is clinically characterized poorly (Conrad *et al*. 1991), and infected animals may become chronic carriers without clinical manifestations thus complicating the diagnosis. The classical diagnosis of animals acutely infected with *B. gibsoni* is based on light microscopical demonstration of intraerythrocytic parasites in Giemsa-stained blood smears (Conrad *et al*. 1991). However, in subclinical or latent infections, this may be impractical due to low levels of parasitaemia.

Although the polymerase chain reaction (PCR) can provide an alternative test, with good sensitivity and specificity (Ano *et al*. 2001; Birkenheuer *et al*. 2003*b*), and is able to detect current, carrier infections, the test requires specialized laboratory equipment and highly trained personnel. Furthermore, cases of seropositive dogs, which are PCR and blood smear negative, have been reported (Birkenheuer *et al*. 2003*b*) especially when the parasite antibodies persist for a period even after the living pathogen has been eliminated. Serological tests using the immunofluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) have also proved useful in the detection of subclinical cases and field surveys (Birkenheuer *et al*. 2003*a*). However, unlike IFAT, the interpretation of ELISA results has the advantage of high sample throughput and less subjectivity since results are based on measurements of optical density (OD) values (Birkenheuer *et al*. 2003*b*). Furthermore, an

* Corresponding author: National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan. Tel: +81 155 49 5648. Fax: +81 155 49 5643. E-mail: gen@obihiro.ac.jp

doi:10.1017/S0031182007002594 Printed in the United Kingdom

ELISA analysis based on recombinant 50-kDa major surface antigen (rBgP50) of *B. gibsoni* was shown to be a promising diagnostic candidate (Verdida *et al.* 2004). Despite the promising findings, the sensitivity of ELISA using this antigen has yielded varying results, and therefore has not been fully characterized satisfactorily, necessitating further research on production of novel recombinant antigens. The initial strategies have focused on cloning and characterizations of novel genes encoding merozoite proteins of *B. gibsoni* that are believed to be antigenic (Fukumoto *et al.* 2001*a*, 2003*a*; Zhou *et al.* 2006).

In this regard, we immunoscreened a cDNA expression library constructed from *B. gibsoni* merozoites and isolated a novel cDNA encoding a 32-kDa protein (BgP32), which shares homology with the previously reported BgP50 (Fukumoto *et al.* 2001*a*). We evaluated whether the recombinant protein (rBgP32) is a potential diagnostic antigen, and determined whether ELISA using rBgP32 was more sensitive than the rBgP50, using field serum samples collected from anaemic dogs in Japan. In addition, we investigated whether or not there is an antigenic relationship between BgP32 and BgP50 merozoite proteins of *B. gibsoni*.

MATERIALS AND METHODS

Experimental animals

Three 1-year-old female beagle dogs were confirmed to be free of natural *B. gibsoni* infection by detection of specific antibodies using IFAT and microscopical examination of Giemsa-stained blood smears. The dogs were fed once per day and given drinking water *ad libitum*. Five 6-week-old female ddY mice were fed on conventional food and given drinking water *ad libitum*. The experimental animals were housed in P2 and P3 level facilities, respectively. The experiments were performed in accordance with the Stipulated Principles for the Care and Use of Research Animals Promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Parasite

B. gibsoni NRCPD strain (Fukumoto *et al.* 2001*a*) was maintained in splenectomized beagle dogs. *B. gibsoni*-infected erythrocytes were collected from the experimentally infected dogs at peak parasitaemia (20%), and the blood samples were centrifuged at 2000 rpm (800 ***g***) for 30 min at 4 °C. The plasma layer was decanted and the remaining erythrocytes were washed 3 times with phosphate-buffered saline (PBS), and lysed with 0·83% ammonium chloride at 37 °C for 20–30 min. The lysate was centrifuged at 2500 rpm (1250 ***g***) for 20 min and the merozoite pellet was recovered and washed 3 times with PBS. The pellet was re-suspended in PBS and stored at −80 °C until use.

Immunoscreening of cDNA expression library

A cDNA expression library of *B. gibsoni* merozoites was previously constructed as described by Fukumoto *et al.* (2001*a*). Briefly, the cDNA was synthesized by using a Zap-cDNA synthesis kit, ligated to a λ Zap II phage expression vector and packaged by using a Gigapack III packaging system (Stratagene, San Diego, Calif.). The cDNA expression library (10^7 PFU) was screened with the serum from a *B. gibsoni*-infected dog. The cDNA inserts of positive clones were sequenced by an automated sequencer using M13 reverse primer and T-7 primer (ABI PRISM 3100 Genetic Analyzer, USA). Two identical cDNA sequences, which shared homology with the previously identified *B. gibsoni* P50 (*BgP50*) gene (Fukumoto *et al.* 2001*a*), were selected and their nucleotide sequences were determined by using a primer-walking strategy, moving downstream from the 5′ end and upstream from the 3′ end of the cDNA sequences. The nucleotide sequences of the cDNAs were analysed using the basic local alignment search tool (BLASTX) accessed through the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov). The hydropathic plots of the proteins were determined using the DNAstar software based on the Kyte-Doolittle approach (Kyte and Doolittle, 1982). The SignalP server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the presence and location of the putative N-terminal signal peptide in the BgP32 sequence.

Preparation of B. gibsoni *genomic DNA and Southern blotting*

The genomic DNA was prepared from purified merozoites by standard methods of proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation as described by Fukumoto *et al.* (2001*b*). For Southern blot analysis, the *B. gibsoni* genomic DNA was digested with *Bam*HI, *Stu*I and *Pvu*II restriction enzymes and then electrophoresed in 0·7% agarose gel. The subsequent procedures were performed using a method described by Ikadai *et al.* (1999) and Sambrook and Russell (2001).

Analysis of intron presence in genomic DNA

The genomic DNA of *B. gibsoni* was amplified by PCR using forward and reverse primers described below. The amplified fragments were electrophoresed in 1·0% agarose gel, purified using geneclean® kit and then ligated into pGEM®-T-vector

https://doi.org/10.1017/S0031182007002594 Published online by Cambridge University Press

(Promega Corporation Madison, USA). The genomic DNA fragment ligated to pGEM®-T-vector was sequenced in order to confirm the presence of introns.

Cloning of BgP32 gene into pGEX-4T-3 vector

The pGEX-4T-3 vector, containing an open reading frame (ORF) encoding a glutathione *S*-transferase (GST)-fusion protein and *Eco*RI *and Xho*I restriction enzyme sites was used to clone the cDNA of BgP32. One pair of oligonucleotide primers including the *Eco*RI and *Xho*I restriction enzyme sites (in italics) was designed and used to clone the truncated gene encoding rBgP32 without the predicted N-terminus signal peptide and C-terminal 18 amino acids (aa), (forward primer, 5′-TT*GAATTC*-TGGAGAAGGGCAAGCAGGAGGT-3′; reverse primer-1, 5′-TT*CTCGAG*TTAGTTCGCACCA-TCTTCACCTT-3′). In addition, another reverse primer including the *Xho*I site was designed in order to clone the non-truncated rBgP32 with nucleotides encoding C-terminus hydrophobic amino acids sequence (reverse primer-2, 5′-TG*CTCGAG*CT-TAAAATACAGCGACAGCCACA-3′). The open reading frames (ORFs) of both the truncated and non-truncated cDNA inserts were cloned into the *Eco*RI and *Xho*I restriction enzyme sites of the pGEX-4T-3 *E. coli* expression vector (Amersham Pharmacia Biotech, Piscataway, NJ). The constructs were designated as pGEX-4T3/rBgP32 (truncated) and pGEX-4T-3/nrBgP32 (non-truncated), and were checked for accurate insertion by restriction enzyme digestion and nucleotide sequencing by using a model ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Ca, USA).

Expression and purification of the rBgP32 in E. coli

The truncated BgP32 (rBgP32) and non-truncated BgP32 (nrBgP32) were expressed as a GST-fusion protein in the *E. coli* BL21 (DE3) strain according to the manufacturer's instructions (Amersham Pharmacia Biotech). The resulting *E. coli* cells were washed 3 times with PBS, lysed in 1% Triton X-100–PBS, sonicated, and then centrifuged at 12 000 rpm (8000 ***g***) for 10 min at 4 °C. Supernatants containing the soluble rBgP32 were purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Preparation of the rBgP50 antigen

The *rBgP50* gene was previously cloned and expressed in *E. coli* as a fusion protein with GST in our laboratory (Verdida *et al*. 2004).

Preparation of mouse anti-rBgP32 immune serum

Five 6-week-old female ddY mice were intraperitoneally immunized with 0·25 ml (250 μg) of the purified rBgP32 emulsified with an equal volume of complete Freund's adjuvant. Two additional boosters with the same amount of rBgP32 emulsified with 0·25 ml of incomplete Freund's adjuvant were intraperitoneally administered at 2-week intervals. The mice were bled 14 days after the last booster and serum samples were stored at −30 °C until use.

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

To identify the native BgP32 in extract of merozoites of *B. gibsoni* purified *B. gibsoni* merozoite extract and normal dog erythrocytes lysates were sonicated, and then precipitated with acetone. Thereafter, mouse anti-rBgP32 serum was used to analyse the merozoite extract using SDS-PAGE and Western blotting as previously described (Zhou *et al*. 2006). In addition, the rBgP32 was analysed by SDS-PAGE as previously described (Zhou *et al*. 2006) and processed in the same way as for the purified merozoite extract. The rBgp32 was probed with the *B. gibsoni*-infected dog serum and non-infected dog serum, respectively. Subsequent procedures were performed as described above for the native BgP32.

Indirect fluorescent antibody test (IFAT)

B. gibsoni-infected dog erythrocytes, fixed on IFAT slides, were reacted with mouse anti-rBgP32 serum and observed under IFAT microscopy as previously described by Fukumoto *et al*. (2003*b*). Briefly, thin blood films from *B. gibsoni*-infected dog erythrocytes were made on individual wells of IFAT slides. The blood films were air-dried and fixed for 5 min in an oven at 80 °C. The IFAT slides were then covered with aluminium foil, and stored at −80 °C until use.

Serum samples

Canine serum samples used in this experiment were as follows: 30 sera from non-infected dogs; 30 sera from specific pathogen-free (SPF) dogs (Nihonnosan, Japan); sequential serum samples (0–541 days post-infection) from a dog experimentally infected with *B. gibsoni* NRCPD strain; 2 sera each from dogs experimentally infected with *B. canis canis*, *B. canis vogeli*, and *B. canis rossi*; 2 sera from dogs experimentally infected with *Leishmania infantum*; 2 sera from dogs infected with *Neospora caninum* and 2 sera from *Toxoplasma gondii*-infected mice. In addition, 47 field serum samples collected from anaemic dogs in Japan and 6 anti-rBgP50 rabbit specific sera were used in this study. The serum was produced in rabbit and purified as described by Fukumoto *et al*. (2003*b*).

https://doi.org/10.1017/S0031182007002594 Published online by Cambridge University Press

ELISA

The optimal concentrations or dilutions of the antigen coated to the plate, serum, enzyme–antibody conjugate, and substrate solution were determined through 'checkerboard' titrations of each reagent against all other reagents. Analytical sensitivity of the assay was determined by end-point dilution analysis of samples from known positive serum of *B. gibsoni*-infected dog, in order to define the penultimate dilution of sample in which the analyte (antibody) is no longer detectable. The analytical specificity was determined by testing cross-reactivity of rBgP32 with sera from dogs infected with *B. canis*, *canis*, *B. canis vogeli* and *B. canis rossi*. Cross-reactivity with other closely related protozoa such as *Neospora caninum*, *Leishmania infantum* and *Toxoplasma gondii* was also determined. The cut-off value was defined as the mean value plus 3-fold standard deviations of the optical density (OD) obtained from 30 specific pathogen-free (SPF) dog serum samples.

In order to calculate the above diagnostic parameters, the following definitions were used: true positive number (tp) of *B. gibsoni*-infected dog sera showing positive readings, false-negative number (fn) of *B. gibsoni*-infected dog sera showing negative reading, false-positive number (fp) of sera from healthy dogs showing a positive reading, true-negative number (tn) of sera from healthy dogs showing a negative reading, sensitivity = $tp \times 100/(tp + fn)$ and specificity = $tn \times 100/(tn + fp)$ (Gonzalez-Sapienza *et al*. 2000).

The rBgP32, rBgP50 and GST were diluted with a 0·05 M carbonate-bicarbonate buffer (pH 9·6) as ELISA antigens to a final concentration of 5 μg/ml. Each well of 96-well plates (Nunc-Immuno Plate; Nunc, Roskilde, Denmark) was coated with 50 μl of each of the proteins overnight at 4 °C. The subsequent protocols were performed as previously described by Boonchit *et al*. (2002). Horseradish peroxidase-conjugated to goat anti-dog IgG (Cappel, Durham, N.C.) antibody or peroxidase-conjugated to goat anti-rabbit IgG for specific rabbit antisera were used as secondary antibodies.

PCR analysis

In addition, respective blood samples collected from the anaemic dogs were analysed using PCR in order to detect DNA of *B. gibsoni* using a method previously described by Birkenheuer *et al*. (2003*b*).

RESULTS

Cloning of a cDNA encoding the BgP32

The cDNA expression library (10^7 PFU) of *B. gibsoni* was screened with the infected dog serum. The partial cDNA sequences of 200 positive clones were isolated and then subjected to blastx computer analysis. Two identical cDNA sequences, which had homology to the previously identified BgP50 merozoite protein that was shown to be a promising diagnostic antigen, were selected for molecular characterization. Analysis of the nucleotide of the cDNA revealed that it is composed of 1464 bp, with an ORF of 969 bp from bases 20 to 988. Starting from methionine at position 20, the ORF encoded a polypeptide of 322 amino acid residues with a predicted size of 32 kDa as calculated by computer. A TAA stop codon at position 986 and a poly (A) tail at positions 1451 to 1464 demonstrated that the 3′ end of BgP32 cDNA was complete (Fig. 1). The 5′ end of the sequence had no in-frame stop codon in the 19 bases before the ATG start codon of the nucleotide sequence suggesting that the 5′ end may not be complete. However, it is likely that the sequence is full-length gene.

Analysis of the putative N-terminal signal peptide in BgP32 sequence by using SignalP-server revealed that this part of the sequence had a high-predicted signal peptide probability (0·998) and a maximum cleavage site probability of 0·706 between amino acids in positions 19 and 20. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the Accession number AB282646. BLAST analysis (blastp) of the predicted polypeptide sequence of rBgP32 against all non-redundant databases accessed through NCBI revealed a highest score alignment with previously identified BgP50 of *B. gibsoni* (E-value = 2e-35; score = 152). These values were from amino acids 1 to 256 in the BgP32 sequence, and the amino acid identity with BgP50 for this region of the sequence was 115/271 (42%) and positives of 145/271 (53%). In addition, the same output of blastp results revealed an E-value of 2e-05, with score of 52·8 from amino acids 235 to 322 in the same BgP32 sequence. The amino acid identities with BgP50 for this part of the sequence was 44/88 (50%) and positives of 55/88 (62%). Computer based Kyte-Doolittle's plot of the predicted polypeptide revealed 3 distinct regions within the sequence: a hydrophobic N-terminal predicted signal peptide and C terminal hydrophobic region flanking a central hydrophilic core (data not shown).

Characterization of the BgP32 gene

A probe derived from the cDNA clone encoding BgP32 was hybridized to *B. gibsoni* genomic DNA fragments using Southern blotting (Fig. 2). The cDNA probe hybridizes to a single band with *Bam*HI (lane 1) that does not cut within the probe sequence. On the other hand, the cDNA probe hybridizes to 2 bands with *Stu*I (lane 2) and *Pvu*II (lane 3) that cut once within the probe sequence. The result suggested that the *BgP32* gene occurs as a

https://doi.org/10.1017/S0031182007002594 Published online by Cambridge University Press

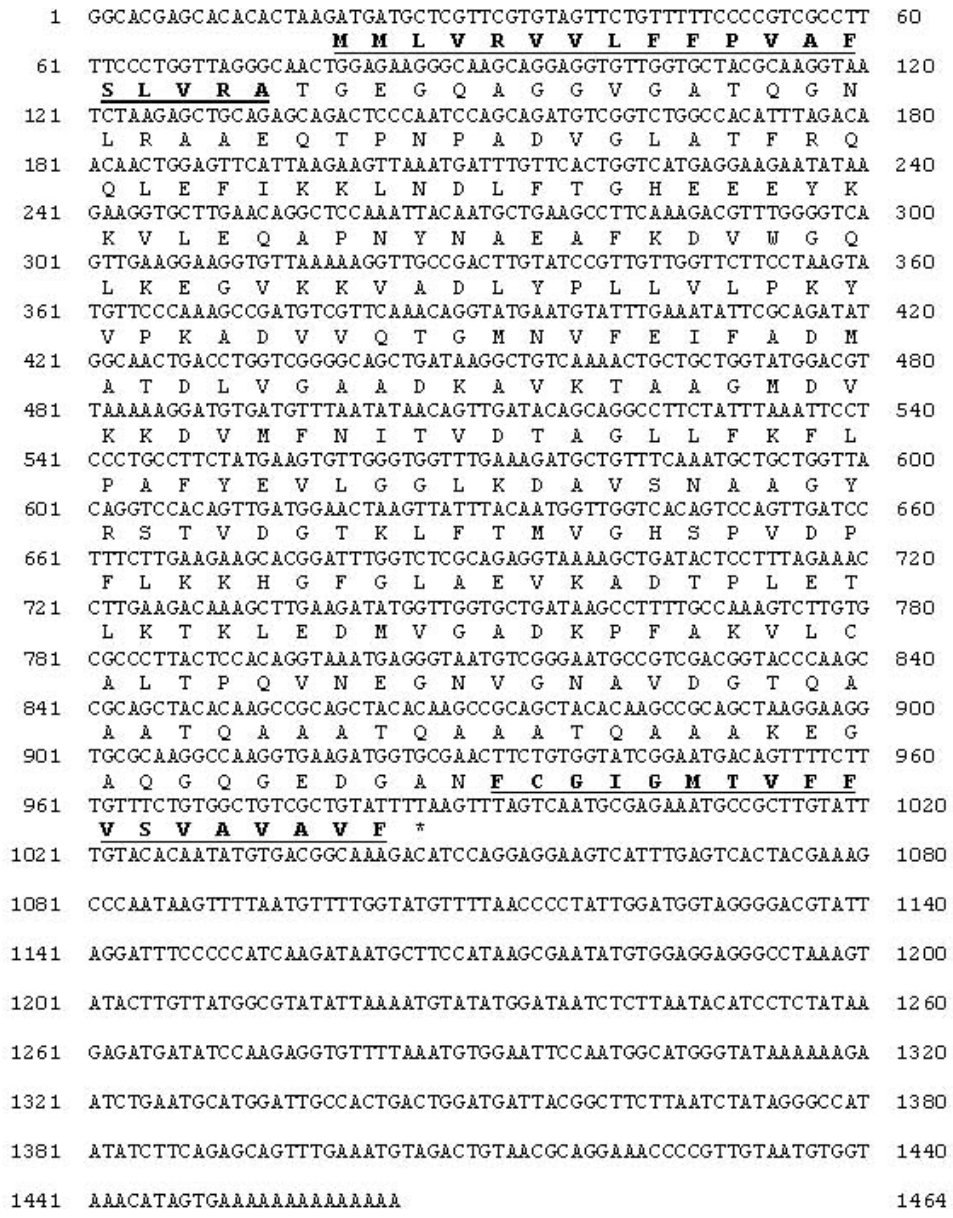

Fig. 1. Complete nucleotide sequence, including the 5′- and 3′-untranslated regions of the cDNA encoding BgP32. The predicted amino acid sequence translated from the ORF is shown below each codon. The amino acids in boldface and underlined at the 5′- and 3′-orientations of the sequence show the N-terminal signal peptide and C-terminal hydrophobic regions respectively.

single copy in the genome of *B. gibsoni*. In addition, sequence comparison of the genomic DNA fragment with the cDNA encoding BgP32 revealed that the genomic DNA consisted of 2 exons (354 bp at the 5′ end and 613 bp at the 3′ end) interrupted by a 37 bp (GTATGTTTAATATTCTATTTCTTC-AACCTTTTTCAG) intron between the 374th and 375th bases with the ORF.

Characterization of BgP32 on the parasite

The truncated rBgP32 was composed of a central hydrophilic core but the lacking N-terminal signal peptide sequence and C-terminal hydrophobic sequence were expressed in *E. coli* as a soluble GST fusion protein and the amount was about 7·5 mg per l of the culture. Moreover, *B. gibsoni*-infected dog serum reacted with the rBgP32-GST on Western blot analysis resulting in a specific band of 58-kDa (data not shown). However, the expression of non-truncated rBgP32, consisting of C-terminal hydrophobic sequence in *E. coli* was unsuccessful even with several attempts to optimize the expression conditions. Consequently, we used the soluble truncated rBgP32 for our subsequent studies. The merozoite extract of *B. gibsoni* was analysed by Western blotting using mouse antiserum against the rBgP32. As shown in Fig. 3, a specific 32-kDa band was detected in the merozoite extract of *B. gibsoni* and the molecular weight of the native protein was

https://doi.org/10.1017/S0031182007002594 Published online by Cambridge University Press

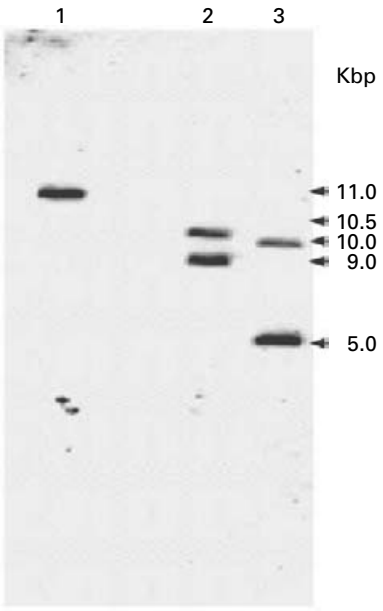

Fig. 2. Southern blot analysis of the *BgP32* gene of *Babesia gibsoni*. The genomic DNA (10 μg per lane) from *B. gibsoni* merozoites was digested with *Bam*HI (lane 1), *Stu*I (lane 2) and *Pvu*II (lane 3), and then probed with *BgP32* cDNA. The molecular sizes (in kilobase pairs) of the specific DNA bands that hybridized with the cDNA are shown on the right-hand side by the arrowheads.

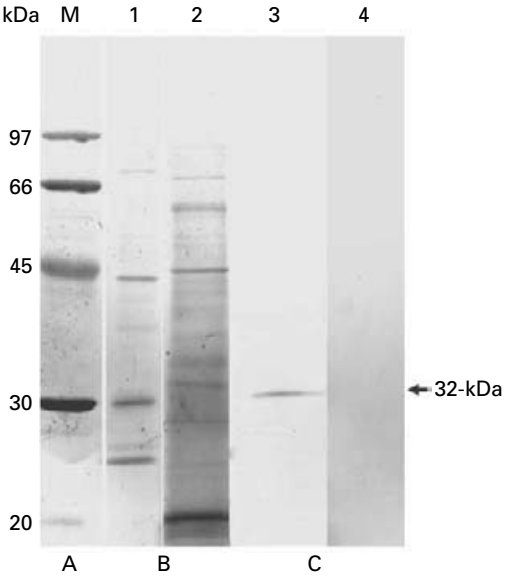

Fig. 3. SDS-PAGE and Western blot analysis of native protein of rBgP32 in *Babesia gibsoni* merozoites. (A) Lane M, low molecular weight marker; (B) lanes 1 and 2, normal RBC lysate and *B. gibsoni* merozoite lysate, respectively, on CBB staining; (C) lane 3 shows a 32-kDa specific band due to the reactions of anti-rBgP32 mouse antiserum with *B. gibsoni* purified merozoite, and lane 4 reveals no reaction between the antiserum and normal dog erythrocyte lysate.

consistent with the predicted molecular weight of mature polypeptide. The mouse anti-rBgP32 serum did not react with erythrocyte lysates from the *B. gibsoni* non-infected dog.

Table 1. The mean optical density (OD at 415 nm) of ELISA reactions with the rabbit anti-BgP50 and mouse anti-BgP32 sera with either of the two recombinant antigens

Antigen	Antiserum	Mean OD-value ± S.E.M.* ($n=6$)
rBgP50	Rabbit anti-BgP50	1·4808 ± 0·01567
rBgP50	Mouse anti-BgP32	1·3657 ± 0·02579
rBgP32	Mouse anti-BgP32	1·2051 ± 0·03446
rBgP32	Rabbit anti-BgP50	−0·0488 ± 0·03968

* S.E.M., Standard error of the mean.

B. gibsoni-infected dog erythrocytes fixed on IFAT slides were reacted with mouse anti-rBgP32 serum and then observed under IFAT microscopy. The mouse anti-rBgP32 serum strongly reacted with *B. gibsoni* parasites in infected dog erythrocytes and the antibody titre was 1/20 000 when observed under IFAT microscopy. In contrast, mouse anti-GST serum did not react with the parasite-infected dog erythrocytes fixed on the IFAT slide (data not shown).

Cross-reactivity between rBgP32 and rBgP50

We evaluated whether species-specific antibodies generated against rBgP32 and rBgP50 cross-react with the antigens by ELISA. Specific rabbit anti-rBgP50 antibodies only reacted with rBgP50 but not with rBgP32. On the other hand, specific mouse anti-rBgP32 antibodies cross-reacted with both rBgP32 and rBgP50 proteins (Table 1). As expected, the recombinant proteins reacted with *B. gibsoni*-infected dog serum but not with non-infected dog serum.

Diagnosis of B. gibsoni *infection by ELISA with rBgP32*

In order to develop and validate the ELISA, we determined the optimal concentrations and dilutions of the antigens and antibodies to be used in this assay using a checkerboard titration. The amount of antigen coated into each well of the 96-well plate was 0·25 μg for both recombinant antigens. The optimal dilutions of serum samples and enzyme antibody conjugate used in this assay were 1/100 and 1/2500 respectively. The cut-off value for ELISA based on both recombinant antigens was 0·100.

As shown in Fig. 4, a dog experimentally infected with *B. gibsoni* developed a detectable level of antibody response to the rBgP32 by 8 days post-infection. The antibody titre was maintained until 541 days post-infection, even when the dog attained a chronic stage of infection as evidenced by a significantly low level of parasitaemia. In addition, the rBgP32 reacted specifically with antibodies to

https://doi.org/10.1017/S0031182007002594 Published online by Cambridge University Press

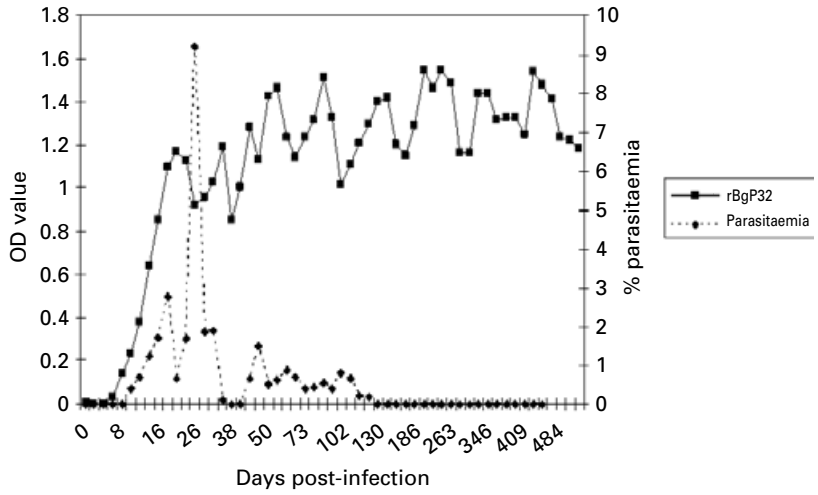

Fig. 4. Specific antibody responses in a dog experimentally infected with *Babesia gibsoni*. Detection of the antibody (IgG) against rBgP32 by ELISA and detection of the parasitaemia level (%) by microscopical examination of Giemsa-stained thin blood smear.

B. gibsoni but not with antibodies to *B. canis canis*, *B. canis vogeli* and *B. canis rossi*, suggesting that this recombinant protein could be a specific antigen for serological diagnosis of *B. gibsoni* in dogs. Moreover, there was no cross-reaction with sera from dogs experimentally infected with *Neospora caninum* and *Leishmania infantum* as well as mouse anti-*Toxoplasma gondii* serum. Serum from the non-infected dog did not react with rBgP32 on ELISA (data not shown). The sensitivities and specificities were determined using reference samples with known infection status for ELISAs based on the rBgP32 and rBgP50. The sensitivity of the ELISA using rBgP32 was 73% while that of the rBgP50 was 71%. On the other hand, the specificity of the rBgP32 ELISA was 100% while that of rBgP50-ELISA was 90%. It therefore appears that rBgP32 ELISA is more sensitive and specific than that based on rBgP50.

Thirty-six (77%) out of the 47 serum samples were positive for *B. gibsoni* antibodies using ELISA based on rBgP32 while 32 (68%) samples were positive for ELISA based on the rBgP50 antigen. PCR analysis revealed that 39 (83%) out of 47 samples were parasite positive. Moreover, 7 serum samples that tested negative for *B. gibsoni* antibodies on analysis using rBgP50, tested positive for the antibodies when rBgP32 was used, and 3 samples revealed strong reactions, as evidenced by high OD values (0·820, 0·859 and 1·055). On the other hand, only 3 sera that were negative for the antibodies on analysis using rBgP32, tested positive for the antibodies when rBgP50 was used. Furthermore, the positive reactions were weak as evidenced by the relatively low OD values (0·155, 0·260 and 0·262). Eight samples were negative on PCR test, 7 out of the 8 samples showed seropositive reaction to *B. gibsoni* antibodies when analysed by ELISA based on any of the 2 recombinant antigens, and some samples that were PCR negative revealed strong seropositive reactions (OD-values: 1·188, 1·220 for rBgP32, and 1·732 and 1·741 for rBgP50). Fourteen dog samples which were PCR-positive revealed negative results when analysed using ELISA based on either of the 2 antigens. Nine out of the 14 dog samples, tested negative when analysed using rBgP32 ELISA while 12 out of the 14 samples tested negative using rBgP50 ELISA. Furthermore, half (7 samples) of the PCR positive samples tested negative for ELISA based on both antigens.

DISCUSSION

In this study, a novel cDNA clone encoding *B. gibsoni* 32-kDa merozoite protein was isolated and was shown to share homology with previously reported *B. gibsoni* P50 protein (Fukumoto *et al.* 2001*a*). It has been demonstrated by using an ELISA system (Verdida *et al.* 2004) and immunochromatographic test (ICT) (Verdida *et al.* 2005) that the rBgP50 is a good diagnostic antigen. Therefore, we hypothesized that the rBgP32 could be as good or even a better diagnostic antigen since the predicted peptide shared homology with the previously identified rBgP50. Consequently, the BgP32 was molecularly characterized and its diagnostic performance was evaluated. Computer-based Kyte-Doolittle's hydropathy analysis of the predicted polypeptide of rBgP32 demonstrated that the sequence had 3 distinct regions, namely, a hydrophobic N-terminal predicted signal peptide, a central hydrophilic core and C terminal hydrophobic region. Similarly, computer analysis of the basic polypeptide structure of the previously reported BgP50 revealed similar distinct regions (Fukumoto *et al.* 2003*b*).

https://doi.org/10.1017/S0031182007002594 Published online by Cambridge University Press

The current study demonstrated that the *B. gibsoni* genomic DNA, which encodes BgP32, is a single copy gene and that a 37 bp intron interrupted the ORF between the 374th and 375th bases. Previous study revealed that the *BgP50* gene is also a single copy gene and that the gene encodes BgP50 on the merozoite surface (Fukumoto *et al.* 2001*a*). Furthermore, the ORF of the gene was interrupted by a 37 bp intron like that of the *BgP32* gene with which they encode proteins that share homology. Although the size of the intron in the *BgP50 gene* was the same as that of *BgP32 gene*, the position of the intron within the ORF of the *BgP50 gene* was different (between the 428th and 429th bases). Moreover, the nucleotide sequences of the 37 bp intron found in *BgP50* gene (GTTAGTTCAATATGTTATTGTCTCATTTCTGTCGTAG) was different from that of *BgP32* gene (GTATGTTTAATATTCTATTTCTTCAACCTTTTTTCAG). Predicted peptide analysis of BgP32 revealed that this protein had a predicted signal peptide suggesting that it might be a secretory protein or surface membrane protein.

Analysis of rBgP32 using an ELISA system demonstrated that the recombinant protein detected *B. gibsoni*-specific antibodies in serial serum samples of a dog experimentally infected with the parasite starting from 8 days up to 541 days post-infection. It has been reported that antibodies usually take 8 to 10 days to develop in a *B. gibsoni* infection (Boozer and Macintire, 2003), and this observation was consistent with the finding of this study, in which specific antibodies were first detected as from 8 days post-infection. Cross-reactivity resulting in *B. gibsoni*-infected animals showing low levels of positive titres for *B. canis* has been documented (Yamane *et al.* 1993, 1994). Therefore, from an epidemiological point of view, it is important to distinguish the more common closely related *B. canis* infections and even other closely related apicomplexans from *B. gibsoni* infection. In this study, the rBgP32 reacted specifically with *B. gibsoni* antibodies but not with either *B. canis* subspecies antibodies or *Neospora caninum* and *Leishmania infantum* antibodies thus avoiding the problem of cross-reactivity. A similar finding was reported in the case of rBgP50 (Fukumoto *et al.* 2003*b*), indicating that the novel recombinant antigen is a potential diagnostic antigen as in the case of the rBgP50 antigen.

Initially we thought that the novel BgP32 could be a similar protein or even antigenically related to the BgP50 because blastp analysis revealed that 2 proteins shared some homology. Consequently, we evaluated whether rBgP32 could cross-react with the previously identified rBgP50, with which it shares some homology. Specific rabbit anti-rBgP50 serum only reacted with rBgP50 but not with rBgP32 by ELISA. Interestingly, specific mouse anti-rBgP32 serum reacted with both rBgP50 and rBgP32. It appears that specific rabbit anti-rBgP50 serum could not recognize B-cell epitopes present in rBgP32 while specific mouse anti-rBgP32 serum could recognize B-cell epitopes present in both rBgP32 and rBgP50. Moreover, *B. gibsoni*-infected serum reacted with both rBgP32 and rBgP50. The parasitic infection appears to produce antibodies that could detect epitopes present in both the recombinant proteins like the rBgP32. This is contrary to rBgP50 that elicited production of specific antibodies in rabbit that could only recognize epitopes found in rBgP50 but not in rBgP32.

ELISA based on rBgP32 detected a higher proportion of positive field serum samples than that based on the rBgP50. Moreover, 7 field serum samples that tested negative when analysed using the rBgP50 were seropositive for *B. gibsoni* antibodies when tested using rBgP32 in ELISA. This finding suggests that mouse anti-rBgP32 antibodies could recognize B cell epitopes in the parasite that could not be detected by rabbit anti-rBgP50 antibodies and appear to correlate with the above observation, in which specific mouse anti-rBgP32 serum reacted with both rBgP50 and rBgP32 while specific rabbit anti-rBgP50 serum only reacted with rBgP50 but not with rBgP32. If the results of the 2 antigens were merged and analysed together, the proportion of seropositive samples was 83%, which is the same as that of PCR (83%). Our observation suggests that the sensitivity of the ELISA system can be improved when using rBgP32 and rBgP50 in combination.

In addition, it is worth noting that 7 samples that were PCR negative tested positive when analysed by the ELISA system based on any of the recombinant antigens. Cases where PCR results and microscopical findings are not in agreement with seropositive samples have been reported (Birkenheuer *et al.* 2003*b*). It was thought that this situation was due to persistent infection characterized by a very low parasitaemia level below the detection of PCR and microscopical examination. This might be the same situation with the observation in the current study. On the other hand, this could be due to persistence of the parasite antibodies for a period even after the living pathogen has been eliminated. In another instance, we observed PCR parasite-positive samples, which were negative for ELISA based on the rBgP32 and rBgP50. It appears that the ELISA-negative samples were from dogs with early parasitic infection characterized by undetectable antibody titres. Samples that are seropositive on serological tests but are negative on PCR and microscopical analysis may be clinically problematic and thus complicating field diagnosis of canine babesiosis. However, in most cases an ELISA would be more appropriate for epidemiological surveys because of its advantage of high sample output.

In conclusion, we have shown that the rBgP32 is a potential diagnostic antigen and that ELISA using

https://doi.org/10.1017/S0031182007002594 Published online by Cambridge University Press

the antigen appears to reveal a better diagnostic performance than the rBgP50 when using field serum samples collected from anaemic dogs. In addition, we demonstrated that BgP32 might be antigenically related to the BgP50 and might share some B-cell epitopes with the protein. We further showed that mice immunized with rBgP32, generated antibodies that strongly reacted with *B. gibsoni*-infected dog erythrocytes on IFAT. Next, we plan to study prospects of rBgP32 as a vaccine candidate against *B. gibsoni*-infection and probably evaluate this antigen with large-scale field samples from *B. gibsoni*-infected dogs.

This work was funded by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The authors thank Drs Y. Kitano, K. Ishimaru, N. Ide and N. Suzaki for providing canine serum samples.

REFERENCES

Ano, H., Makimura, S. and Harasawa, R. (2001). Detection of *Babesia* species from infected dog blood by polymerase chain reaction. *Journal of Veterinary Medical Science* **63**, 111–113.

Baneth, G., Breitschwerdt, E. B., Hegarty, B. C., Pappalardo, B. and Ryan, J. (1998). A survey of tick-borne bacteria and protozoa in naturally exposed dogs from Israel. *Veterinary Parasitology* **74**, 133–142.

Birkenheuer, A. J., Correa, M. T., Levy, M. G. and Breitschwerdt, E. B. (2005). Geographic distribution of babesiosis among dogs in the United States and association with dog bites: 150 cases (2000–2003). *Journal of the American Veterinary Medical Association* **227**, 942–947.

Birkenheuer, A. J., Levy, M. G., Stebbins, M., Poore, M. and Breitschwerdt, E. (2003*a*). Serosurvey of anti-*Babesia* antibodies in stray dogs and American Pit Bull Terriers and American Staffordshire Terriers from North Carolina. *Journal of the American Animal Hospital Association* **39**, 551–557.

Birkenheuer, A. J., Levy, M. G. and Breitschwerdt, E. B. (2003*b*). Development and evaluation of a semi-nested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. *Journal of Clinical Microbiology* **41**, 4172–4177.

Boonchit, S., Xuan, X., Yokoyama, N., Goff, W. L., Wagner, G. and Igarashi, I. (2002). Evaluation of an enzyme-linked immunosorbent assay with recombinant rhoptry-associated protein 1 antigen against *Babesia bovis* for the detection of specific antibodies in cattle. *Journal of Clinical Microbiology* **40**, 3771–3775.

Boozer, L. A. and Macintire, D. K. (2003). Canine babesiosis. *Veterinary Clinics Small Animal Practice* **33**, 885–904.

Corad, P., Thomford, J., Yamane, I., Whiting, J., Bosma, L., Uno, T., Holshuh, H. J. and Shelly, S. (1991). Haemolytic anaemia caused by *Babesia gibsoni* infection in dogs. *Journal of American Veterinary Medical Association* **199**, 601–605.

Fukumoto, S., Suzuki, H., Igarashi, I. and Xuan, X. (2005). Fatal experimental transplacental *Babesia gibsoni* infections in dogs. *International Journal for Parasitology* **35**, 1031–1035.

Fukumoto, S., Xuan, X., Inoue, N., Igarashi, I., Sugimoto, C., Fujisaki, K., Nagasawa, H., Mikami, T. and Suzuki, H. (2003*a*). Molecular characterization of a gene encoding a 29-kDa cytoplasmic protein of *Babesia gibsoni* and evaluation of its diagnostic potentiality. *Molecular and Biochemical Parasitology* **131**, 129–136.

Fukumoto, S., Xuan, X., Kadota, K., Igarashi, I., Sugimoto, C., Fujisaki, K., Nagasawa, H., Mikami, T. and Suzuki, H. (2003*b*). High-level expression of truncated surface antigen P50 of *Babesia gibsoni* in insect cells by baculovirus and evaluation of its immunogenicity and antigenicity. *Clinical and Diagnostic Laboratory Immunology* **10**, 596–601.

Fukumoto, S., Xuan, X., Nishikawa, Y., Inoue, N., Igarashi, I., Nagasawa, H., Fujisaki, K. and Mikami, T. (2001*a*). Identification and expression of a 50-kilodalton surface antigen of *Babesia gibsoni* and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* **39**, 2603–2609.

Fukumoto, S., Xuan, X., Shigeno, S., Kimbita, E., Igarashi, I., Nagasawa, H., Fujisaki, K. and Mikami, T. (2001*b*). Development of a polymerase chain reaction method for diagnosing Babesia infection in dogs. *Journal of Veterinary Medical Science* **63**, 97–981.

Gonzalez-Sapienza, G., Lorenzo, C. and Nieto, A. (2000). Improved immunodiagnosis of cystic hydatid disease by using a synthetic peptide with higher diagnostic value than that of the parent protein, *Echinococcus granulosus* antigen B. *Journal of Clinical Microbiology* **38**, 3979–3983.

Ikada, H., Xuan, X., Igarashi, I., Tanaka, S., Kanemaru, T., Nagasawa, H., Fujisaki, K., Suzuki, N. and Mikami, T. (1999). Cloning and expression of a 48-kiloDalton *Babesia caballi* merozoite rhoptry protein and potential use of the recombinant antigen in an enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* **37**, 3475–3480.

Kjemtrup, A. M., Kocan, A. A., Whitworth, L., Meinkoth, J., Birkenheuer, A. J., Cummings, J., Boudreaux, M. K., Stockman, S. L., Irizarry-Rovira, A. and Conrad, P. A. (2000). There are at least three genetically distinct small piroplasms from dogs. *International Journal for Parasitology* **30**, 1501–1505.

Kyte, J. and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105–132.

Sambrook, J. and Russell, D. W. (2001). *Molecular Cloning – A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Stegeman, J. R., Birkenheuer, A. J., Kruger, J. M. and Breitschwerdt, E. B. (2003). Transfusion-associated *Babesia gibsoni* infection in a dog. *Journal of the American Veterinary Medical Association* **222**, 959–963.

Verdida, R. A., Hara, O. A., Xuan, X., Fukumoto, S., Igarashi, I., Zhang, S., Dong, J., Inokuma, H., Kabeya, H., Sato, Y., Moritomo, T., Maruyama, S.,

Claveria, F. and Nagasawa, H. (2004). Serodiagnosis of *Babesia gibsoni* infection in dogs by an improved enzyme-linked immunosorbent assay with recombinant truncated P50. *Journal of Veterinary Medical Science* **66**, 1517–1521.

Verdida, R. A., Xuan, X., Fukumoto, S., Huang, X., Zhou, J., Igarashi, I., Claveria, F. G. and Nagasawa, H. (2005). Development of a practical immunochromatographic test with recombinant P50 for the diagnosis of *Babesia gibsoni* infection in dogs. *Parasitology* **131**, 769–774.

Yamane, I., Gardner, I. A., Ryan, C. P., Levy, M., Urrico, J. and Conrad, P. A. (1994). Serosurvey of *Babesia canis*, *Babesia gibsoni* and *Erhlichia canis* in pound dogs in California, USA. *Preventive Veterinary Medicine* **18**, 293–304.

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. *American Journal of Veterinary Research* **54**, 1579–1584.

Zhou, J., Fukumoto, S., Jia, H., Yokoyama, N., Zhang, G., Fujisaki, K., Lin, J. and Xuan, X. (2006). Characterization of the *Babesia gibsoni* P18 as homologue of thrombospondin related adhesive protein. *Molecular and Biochemical Parasitology* **148**, 190–198.

https://doi.org/10.1017/S0031182007002594 Published online by Cambridge University Press