Molecular characterization and expression of a 47-kDa merozoite surface protein of *Babesia gibsoni* for serodiagnosis by enzyme-linked immunosorbent assay

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ABSTRACT

A novel gene encoding a 47-kDa surface protein was identified by bioinformatic analyses of Expressed Sequence Tags (EST) prepared from mRNA of *Babesia gibsoni*. The Bgp47 cDNA encodes a 47-kDa polypeptide having an-N terminal signal peptide, a central hydrophilic core and a C-terminal transmembrane domain. This protein shares homology with 50-kDa (Bgp50) and 32-kDa (Bgp32) proteins of *B. gibsoni*. The expressed recombinant protein without the signal peptide and the transmembrane domain (Bgp47t) was approximately 46-kDa after glutathione S-transferase (GST) cleavage, similar to an approximately 47-kDa mature native protein identified in the parasite merozoite after probing with mouse anti-Bgp47t serum. A 32-kDa band was also identified using the same antiserum suggesting that Bgp47 and Bgp32 might share common B cell epitopes. The Bgp47 was localized predominantly on the parasite cell surface. An enzyme-linked immunosorbent assay (ELISA) revealed that the rBgp47t did not cross-react with *B. canis* subspecies and other genetically related apicomplexans indicating that the antigen is specific. Out of 106 sera from dogs with *B. gibsoni*-like symptoms, 79.2% were seropositive by rBgp47t-ELISA. Therefore, the Bgp47t is a prospective serodiagnostic antigen.

**Key words: 47 kDa merozoite protein; B. Gibsoni; serodiagnosis ELISA**

INTRODUCTION

*Babesia gibsoni* is a tick-borne intraerythrocytic parasite that infects dogs and causes canine babesiosis. Although the disease is endemic in Asia including Japan, of late, the infection is increasingly becoming an important emerging disease worldwide with new infections being reported in many countries such as Brazil (Trapp et al., 2006), Germany (Hartelt et al., 2007), South Africa (Matjila et al., 2007), South Korea (Lee et al., 2009), Croatia (Beck et al., 2009), Italy (Trotta et al., 2009) and the United States (Yeagley et al., 2009). Some of these infections has been attributed to introduction of infected dogs from endemic country (Matjila et al., 2007). Subsequently, there is need to develop sensitive and specific high throughput diagnostic tools for screening potentially infected animals to be able to prevent spread of this disease.

The intraerythrocytic parasite can be confirmed by examination of Giemsa stained blood smears using a light microscope. Additionally, the parasite DNA can be detected by polymerase chain reaction
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(PCR) and sequencing of the genetic material (Beck et al., 2009). Alternatively, ELISA is relatively easier to perform, is specific and appropriate for testing large number of samples, especially in large scale epidemiological surveys. Previously, ELISAs have been evaluated for detection of antibodies against *Babesia* parasites using native antigens but cross-reactions between the *Babesia* species and with closely related apicomplexan parasites resulting in false positive results have limited their application (Bose et al., 1995). Nevertheless, the use of recombinant antigens has been shown to result in higher specificity and allow for better standardization of the antigens for use in serodiagnosis.

Therefore, recombinant proteins such as, rBgp50 (Fukamoto et al., 2001), rBgp51 (Jia et al., 2006), Bgp32 (Abose et al., 2007a), rBgp57 (Abose et al., 2007b) as well as rBgp5TrAP (Goo et al., 2008) have been generated using *B. gibsoni* merozoite. These proteins have been shown to possess promising antigenic properties for use in serodiagnosis by indirect ELISAs with BgpTRAP being the best candidate antigen. However, BgpTRAP is difficult to express, moreover, some seminested PCR positive samples were still shown to be negative for ELISA using this antigen. Consequently, these shortcomings necessitate further research into the development of more prospective antigens. Additionally, we argued that if an array of recombinant proteins can be generated and used in combination with an ELISA, then it would be possible to improve the sensitivity of ELISA for serodiagnosis of infection with *B. gibsoni*.

In this regard, we have isolated a novel cDNA encoding a 47 kDa protein (Bgp47), which shares homology with the previously identified Bgp50 (Fukamoto et al., 2001) and Bgp32 (Abose et al., 2007a) proteins. The truncated 47 kDa recombinant merozoite protein (rBgp47t), without N-terminal signal peptide and C-terminal transmembrane domain was expressed in *E. coli*. Next, this recombinant protein was used to generate antibodies that can be used to identify and localise the native protein from the parasite. Thereafter, the suitability of rBgp47t for serodiagnosis by ELISA was evaluated using serum samples obtained from a dog experimentally infected with *B. gibsoni*. This recombinant protein was used further to screen 106 samples collected from dogs that were admitted to veterinary clinics in Japan and manifested *B. gibsoni*-like symptoms. Finally, we have evaluated whether or not these homologous proteins could cross react with each other.

**MATERIALS AND METHODS**

**Cultivation of *B. gibsoni* stock**

*B. gibsoni*-Oita strain was obtained from the Department of Infectious Diseases, School of Veterinary Medicine, Azabu University, Japan. The parasite stock was cultured in RPMI 1640 medium (2.05 mM L-glutamine, 25 mM Heps buffer, 23.8 mM NaHCO₃, 0.91 mM sodium pyruvate, penicillin G at 100 units/ml, streptomycin at 100 µg/ml) having 20% dog serum in a humidified carbon dioxide incubator (Brand Bio-Lab) at 37°C in the presence of 5% carbon dioxide. The old parasite cultures were sub-cultured in fresh dog erythrocytes containing fresh complete medium at 5% hematocrit.

**Experimental animals**

Six adult-beagles dogs, which included 1 male and 5 female dogs (Nihonnosan, Japan), were fed once per day, and given water ad libitum. The dogs were housed in a level P2 facility, and the experiments were conducted in accordance with the Stipulated Regulations for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

**Construction and bioinformatic analyses of a *B. gibsoni* EST database**

An EST database of *B. gibsoni* was previously constructed in our laboratory using mRNA derived from the wild type *B. gibsoni*-NRCPD strain (Abose et al., 2008). From this EST database, having about
10,000 cDNA inserts, we analyzed all translated polypeptides of the partial cDNA sequences against all nonredundant databases accessed through NCBI GenBank. Thereafter, we selected 4 identical cDNA clones that showed consensus nucleotide sequences and shared homology with a previously identified 50-kDa merozoite protein of *B. gibsoni* (Fukumoto et al., 2001).

**Determination of full-length sequences of the selected cDNA clones**

To determine the full-length of the partial cDNA sequences, the 5' ends of 2 consensus cDNA clones were sequenced using the pGCAp1-2 (5'-ACTGCTCTTCCAGTGGATTT-3') vector primer. Then, 5 nucleotide primers, namely, BgH1, BgH2, BgH3, BgH4 and BgH5 (Table 1) were designed, and then used to sequence the partial cDNA sequences moving downstream from the 5' to the 3' ends. The resulting 6 sets of overlapping cDNA sequence fragments were assembled into consensus full length sequences by using Genetyx® software (Genetyx Corporation, Japan). The predicted polypeptide translated from open reading frames (ORF) of the full-length cDNAs were analyzed for signal peptide and other functional domains using SignalP 3.0 Server, Gene Runner (Hastings Software, USA) for windows, and the ProfileScan server (http://www.hits.isb-sib.ch/cgi-bin/PFSCAN), respectively. The gene encoding the mature protein of *B. gibsoni* without signal peptide was named as *BgP47* gene and the corresponding protein as BgP47 protein.

Table 1. Oligonucleotide primers designed to determine the full-length sequence of the BgP47 cDNA and primers designed for cloning and expression of ORF of the BgP47 cDNA.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequences (5' =&gt;3')</th>
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<tr>
<td>BgH1</td>
<td>GCCGCAGATGGAAGAGAAAG</td>
</tr>
<tr>
<td>BgH2</td>
<td>CCGTTGAACCTACTTGAAG</td>
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<tr>
<td>BgH3</td>
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<td>BgH5</td>
<td>TGGCCATGTTCTGTGTTTCTG</td>
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<tr>
<td>BgP47RI</td>
<td>GACTCGAGCACCAGTAAATTGTGCACCATC</td>
</tr>
<tr>
<td>BgP49Ro</td>
<td>GACTCGAGCTTAAATACAGCGACAGCCAC</td>
</tr>
</tbody>
</table>

**Cloning of the BgP47 cDNA**

The ORF of the cDNA encoding the mature BgP47 merozoite protein, without nucleotide sequences encoding the signal peptide, was PCR amplified using oligonucleotide primers, namely, 5’ BgP47F and 3’ BgP47R, having *Eco*RI and *Xho*I restriction sites, respectively, as underlined (Table 1). To clone a truncated *BgP47* cDNA (*BgP47t*) without the nucleotide sequences that encode a C-terminal transmembrane region, the same forward primer (5’*BgP47F*) having *Eco*RI site as above and a second inner reverse primer (BgP47RI) containing *Xho*I restriction sites as underlined were used to amplify the cDNA fragment. After ligation to the vector-pGEX-4T-3 as previously described (Aboge et al., 2008), these products yielded pGEX-4T-3/BgP47 and pGEX-4T-3/BgP47t clones that would express a glutathione S-transferase (GST)-BgP47 fusion protein in bacteria.

**Expression and purification of BgP47 protein**

The *E. coli* BL21 (DE3), transformed with pGEX-4T-3/BgP47 and pGEX-4T-3/BgP47t clones, were each grown in 1.5 litres of LB base broth containing 50 µg/ml of ampicillin at 37°C with shaking at 250
rpm until the optical density (OD) at 600 nm reached 0.4. Subsequent purification of the expressed proteins was performed as previously outlined (Aboge et al., 2008). For the N-terminal signal peptide and C terminal truncated antigen (rBgP47t), GST was cleaved from the protein by thrombin protease at room temperature for at least 2 hours. The concentration of the rBgP47t protein was determined by using a bicinchoninic acid protein assay kit (Pierce, USA), applying bovine serum albumin (BSA) as standard. The specific bands corresponding to the GST/rBgP47t and rBgP47t proteins were confirmed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Generation of anti-rBgP47t serum in mice**

Anti-rBgP47t antibodies were generated in 6-weeks old ICR mice (CLEA Co., Japan) using previously established protocols (Aboge et al., 2007b). At first, a pre-immunization bleeding was done for each mouse, thereafter, the mice were intraperitoneally (i.p.) immunized with 250 μg of rBgP47t emulsified with an equal volume of complete Freund’s adjuvant (Sigma Chemicals., USA). After 2 weeks, additional booster of the same protein in Freund’s incomplete adjuvant was administered via the same route as indicated above. A final booster was administered, i.p., after 2-weeks and sera was collected 14 days after the last booster and then kept at -30°C pending analysis.

**Identification and cellular localization of native BgP47 protein**

* B. gibsoni* lysate was prepared using in vitro stock cultures as described earlier (Aboge et al., 2007a) and separated by 12% SDS-PAGE. The separated merozoite protein was electroblotted on nitrocellulose membrane and then probed with mouse anti-rBgP47t serum as highlighted previously (Aboge et al., 2007b). For control, pre-immune sera were reacted with the lysate as documented above. Cellular localization was performed by using confocal laser microscopy as described earlier (Fukumoto et al., 2001). Mouse anti-rBgP47t serum was used as primary antibody, whereas goat anti-mouse IgG-Alexa-Flour 488 was used as secondary antibody conjugate.

**Indirect ELISA using rBgP47t**

Optimization of assay reagents was done by performing a checkerboard titration as previously described (Aboge et al., 2007a). Each well of 96-well plate (Nunc-Immuno Plate; Nunc, Roskilde, Denmark) was coated, overnight at 4°C, with 0.150 μg of the rBgP47t protein diluted in a 0.05M carbonate-bicarbonate buffer (pH 9.6). Subsequent procedures were performed using previously established procedures. The cut off value of the rBgP47t-ELISA was determined as previously described (Aboge et al., 2007a). The cross-reactivity of the BgP47 protein with antibodies against genetically related apicomplexans was evaluated with sera of dogs infected with *B. canis canis, B canis rossi, B. canis vogeli, Neospora caninum* and *Leishmania infantum*. Sera from mice infected with *Toxoplasma gondii* was used to evaluate cross-reactivity of the recombinant antigen with *Toxoplasma*. Fifty six sequential serum samples (0–521 days post infection) of a dog experimentally infected with *B. gibsoni* were tested for the parasite’s antibodies using the rBgP47t-ELISA. The rBgP47t antigen was used to screen 106 reference sera taken from dogs admitted to veterinary clinics in Japan and manifested *B. gibsoni*-like symptoms. The calculations of sensitivity and specificity for the indirect rBgP47-ELISA were done as previously documented (Aboge et al., 2007a). The cross reactivity of mouse anti- BgP47t serum with rBgP32 and rBgP50 protein was also determined by indirect ELISA.

**Nucleotide sequence accession numbers**

The *BgP47* cDNA sequence reported in this paper is available in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB521673.
RESULTS AND DISCUSSIONS

To be able to identify, characterize, and express immunodiagnostic protein of *B. gibsoni* for subsequent use in ELISA, we needed to isolate a gene encoding putative antigenic protein. The strategy was to analyze cDNA sequences derived from mRNAs of the parasite, since it is these transcripts that are eventually translated to functional proteins. Therefore, we analysed ORF of about 10,000 partial cDNA sequences, derived from the previously constructed EST database of *B. gibsoni* (Aboge et al., 2008) and identified 73 cDNA clones that shared homology with the previously identified BgP50, which is known to be antigenic (Fukumoto et al., 2001). After further scrutiny, we realized that only 15 consensus cDNA sequences appear to encode BgP50 while the other 19 sequences encode the previously identified BgP32 protein also known to be antigenic (Aboge et al., 2007a). The remaining 11 and 28 consensus sequences appeared to encode 2 different unidentified merozoite proteins since neither BgP50 nor BgP32 shared 100% similarity with their translated partial amino acid sequences. Thus, we selected 4 cDNA clones from the latter 28 consensus sequences for further study.

Next, we were interested in getting the full-length consensus sequences of the 4 selected cDNAs to be able to translate them to polypeptides for bioinformatic analyses. Thus, we found that the translated full-length ORF encoded a polypeptide with 451 amino acid residues, including the N-terminal signal peptide, having a predicted molecular weight of 49-kDa. This finding led us to name the mature protein without signal peptide as a 47-kDa protein of *B. gibsoni* (BgP47). The polypeptide shared significant homology with the BgP50 having 56% primary amino acid sequence identity and similarity (E value = 1e-79; score = 301), and also with BgP32 having 56% amino acid residues identity and similarity (E value = 2e-43; score = 180). The 49-kDa polypeptide consists of an N-terminal hydrophobic signal peptide with 19 (1-19 aa) amino acid residues, a central hydrophilic core consisting of 414 (20-433 aa) residues and a C-terminal hydrophobic region having 18 (434-451-aa) residues predicted to be a transmembrane domain with an outer-inner orientation (Fig. 1). Prediction by computer based Kyte-Doolittle’s plot revealed that the BgP47 polypeptide has a good antigenic index and a high surface probability, especially at the central hydrophilic core. Therefore, these bioinformatic predictions indicated that this polypeptide could be a prospective serodiagnostic antigen.

Thereafter, we tried to express the rBgP47 protein having a C-terminal transmembrane region but the expression did not yield soluble GST fusion protein even with several attempts to optimize the expression conditions. Since truncation of C-terminal transmembrane have been reported to improve protein solubility (Aboge et al., 2007a), we truncated nucleotides encoding 15-aa residues situated at the extreme end of C-terminal transmembrane region and then expressed the truncated BgP47t cDNA in *E. coli*. This truncation resulted in expression of sufficient soluble rBgP47t protein having a concentration of 1.5 mg/ml, after GST cleavage, from 1.5 litres of *E. coli* culture. Moreover, analysis using 12% SDS-PAGE analysis revealed that the expressed protein was 46-kDa after GST cleavage, thus confirming the expression of pure rBgP47t (Fig. 2a). The C-terminal transmembrane region of BgP47 consists of phenylalanine (F), valine (V), alanine (A), cysteine (C), glycine (G) and methionine (M), which are hydrophobic amino acid residues, with serine (S) being the only hydrophilic amino acid (Fig 1). Therefore, it is possible that these hydrophobic amino acid residues led to the protein insolubility. Although, the truncation of some of the amino acid residues could potentially result in loss of some antigenic epitopes, the fact that this C-terminal transmembrane domain had low antigenic index in addition to being hydrophobic indicated that exclusion of this domain may not necessarily lead to loss of antigenic regions.
Figure 1. Amino acid sequence of the full-length BgP47 polypeptide. The N-terminal amino acid sequence in boldface shows the predicted sequence signal peptide domain (aa1 to 19). The C terminal amino acid sequence in boldface shows the truncated region of rBgP47t and the underlined amino acid residues shows the predicted transmembrane domain as analyzed by SOSUI by the N terminal amino acid sequence analysis of the peptide.

The next objective was to authenticate whether the native BgP47 protein corresponding to the rBgP47 actually exist in the parasite. Thus, we inoculated mice with rBgP47t protein to generate the antibodies that could be used to identify the native protein in the parasite lysate and also to localize the endogenous protein in intact parasite cells. We found that mouse anti-rBgP47t serum reacted with total parasite lysate yielding 2 single bands, one corresponding to an approximately 47-kDa protein while the other one corresponded to a 32-kDa protein. Preimmune mouse serum, which was used as control did not react with the merozoite lysate (Fig. 2B). The weaker reactivity with the 47 kDa band than the 32 kDa band on Western blot could be either due to breakdown of the 47 kDa protein or cross reactivity with the 32 kDa protein. Additionally, the antibodies against the recombinant protein reacted with intact parasite cells resulting in the detection of green fluorescent signal associated with the merozoite membrane (Fig. 3A and B). For negative control, preimmune serum did not yield green fluorescence. Instead, only the parasite nucleus revealed red staining (Fig. 3C to D). A possible explanation for this finding is the presence of N-terminal signal sequence in this protein that is responsible for its targeting and secretion to the membrane of merozoite.
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Figure 2. SDS-PAGE and Western blot analysis of rBgP47 protein as well as identification of native Bg 47 protein by mouse anti-rBgP47t protein. (a) Lane M, low molecular weight marker; Lane 1, expressed rBgP47t without GST; (b) lane 2, *B. gibsoni* merozoite lysate; lane 3 shows 47-kDa and 32-kDa specific bands after reaction of mouse anti-rBgP47t serum with parasite lysate, and lane 4 shows that the parasite lysate did not react with preimmune serum of mouse.

Figure 3. IFAT and confocal laser microscopy of the native BgP47 protein in the merozoite. Panels a and b, overlaid images of the protein’s reaction with mouse anti-rBgP47t serum; panels c and d are images after reaction with mouse preimmune serum revealing only red staining of the parasite nucleus with propidium iodide.
When sequential sera taken from an experimentally infected dog over number of days (from days 0 to 521) were screened, antibodies to *B. gibsoni* were detected from about days 9-10 with high titer being maintained even during the chronic stage of the experimental infection (Fig 4A). Since it has been shown that it takes 8 to 10 days to develop antibodies to *B. gibsoni* infection (Aboge et al., 2007a; 2007b) it is possible that sera collected from the dog from day 0 to 8 did not have detectable antibodies. During natural infections of dogs with *B. gibsoni*, it is usually possible to have co-infections with genetically related apicomplexan parasites (Gotsch et al., 2009; Tabar et al., 2009). Therefore, from an epidemiological point of view, it is important to distinguish the more genetically closely related apicomplexans from *B. gibsoni* infection. In this regard, we evaluated the cross-reactivity of this antigen with genetically related apicomplexans and found that it reacted with *B. gibsoni* infected dog sera only. The antigen did not cross react with sera from dogs infected with *B. canis canis*, *B. canis vogeli*, *B. canis rossi*, *L. infantum* and *N. caninum* (Fig. 4B). This finding was consistent with the other previously identified rBgP50 (Verdida et al., 2005) and rBgP32 (Aboge et al., 2007a) proteins. Therefore, the use of recombinant BgP47t antigen could overcome the problem of cross-reactions between *B. gibsoni* and closely related apicomplexan parasites thus avoiding false positive results encountered with native proteins of *Babesia* (Bose et al., 1995).

Figure 4. Evaluation of BgP47t as a diagnostic antigen in an ELISA system using sera from experimentally infected dogs. (A) Detection of the antibody against rBgP47t in a dog experimentally infected with *B. gibsoni* by using ELISA and the corresponding parasitemia. (B) The ELISA values of experimentally infected dog sera. Lane a, sera from *B. canis canis*-infected dogs (n = 3); lane b, sera from *B. canis vogeli*-infected dogs (n = 2); lane c, sera from *B. canis rossi* infected dogs (n = 3); lane d, sera from *L. infantum* infected dogs sera (n = 3); lane e, sera from *N. caninum*-infected dogs (n = 2). Lane f, sera from *T. gondii* -infected mice (n = 2); lane g, sera from *B. gibsoni*-infected dogs (n = 3); lane h, sera from non infected dogs (n = 3).
Based on a cut off value of 0.100, the rBgP47t-ELISA detected 84 (79.2%) as positive samples out of the 106 serum samples of dogs that manifested *B. gibsoni*-like symptoms and admitted at various veterinary hospitals in Japan. This proportion was higher than those of previously studied rBgP50 (68.2%) and rBgP32 (71%) proteins. The rBgP47t-ELISA identified 15, 10 and 7 seropositive samples that were previously shown to be negative for ELISAs with rBgP50 (Fukumoto *et al.*, 2001), rBgSA1 (Jia *et al.*, 2006) and BgP32 (Aboge *et al.*, 2007a), respectively. The advantage of this finding is that if the results of these recombinant proteins, which share homology (rBgP47, rBgP32, rBgP50) were merged and analyzed together, the proportion of the seropositive samples can be higher. Additionally, some 11 samples that were previously shown to be seminested PCR negative (Aboge *et al.*, 2007b), tested seropositive for the parasite antibodies on analysis by rBgP47t-ELISA. This finding could mean that the parasite may have been cleared from peripheral blood but antibodies still persisted. Alternatively, it may indicate that the PCR was not sensitive enough to detect the parasite DNA. In contrast, 14 samples that were previously seminested PCR positive (Aboge *et al.*, 2007b) tested seronegative for the parasite antibodies on analysis by rBgP47t-ELISA. It is likely that these samples were from dogs that had not produced detectable antibodies as is the case with early infections. These findings indicate that a PCR test could be appropriate for establishing early infections, while the rBgP47t-ELISA could be applied for chronic infections. Although parasite antibodies could persist for a period even after the parasite clearance, the detection of specific parasite antibodies is important because it could signal presence of underlying infection. In this regard, the ELISA with rBgP47t could complement other molecular methods such as PCR for establishing chronic infection status and screening potentially exposed dogs.

The rBgP47 protein shared homology with BgP50 and BgP32 proteins suggesting that these proteins may share common epitopes and thus could cross react with each other. Therefore, we evaluated whether anti-rBgP47t antibodies could react with these proteins. We found that mouse anti-rBgP47t antibodies reacted with both rBgP47t protein and rBgP32 but did not react with rBgP50 protein. Similarly, the mouse anti-rBgP32 antibodies reacted with both rBgP47t protein and BgP32 but not with rBgP50 (Table 2). These findings indicate that BgP47 shares some B cell epitopes with BgP32 but not with BgP50 protein. Thus BgP47 protein might be more closely antigenically related to BgP32 than to BgP50 protein of *B. gibsoni.*
Table 2. The mean optical density (at 415 nm) of ELISA results of the recombinant proteins and their corresponding mouse anti-recombinant sera.

<table>
<thead>
<tr>
<th>Recombinant antigens</th>
<th>Antiserum</th>
<th>OD-value</th>
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<tr>
<td>rBgP47t</td>
<td>Mouse anti-BgP47</td>
<td>(1.810) +</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-BgP32</td>
<td>(0.209) +</td>
</tr>
<tr>
<td></td>
<td>Rabbit anti-BgP50</td>
<td>(0.036) -</td>
</tr>
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<td></td>
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<td>Mouse anti-BgP47</td>
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</tr>
<tr>
<td></td>
<td>Rabbit anti-BgP50</td>
<td>(0.197) +</td>
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In conclusion, we have shown that the novel BgP47 gene identified from the EST database of B. gibsoni encodes BgP47 protein, which is likely to be a membrane associated protein and is prospective diagnostic antigen. Therefore, this study has contributed to the identification of a novel serodiagnostic antigen that could be used in combinations with array of the other recombinant proteins to improve the sensitivity of ELISA with recombinant antigen for serodiagnosis of infection by B. gibsoni.

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