IDENTIFICATION OF A NOVEL GENE ENCODING A SECRETED ANTIGEN 1 OF BABESIA GIBSONI AND EVALUATION OF ITS USE IN SERODIAGNOSIS

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Abstract. Serum from a dog immunized with blood plasma from a B. gibsoni-infected dog, putatively containing secreted antigens, was used to screen a cDNA expression library. A novel gene encoding BgSA1 was identified from the isolated clones. The serum raised in mice immunized with the recombinant BgSA1 expressed in Escherichia coli could recognize a native parasite protein with a molecular mass of 59 kDa. Comparing with the previously established ELISA with recombinant P50 as antigen, the ELISA with recombinant BgSA1 as the antigen was more sensitive when they were used to detect field samples. Moreover, a sandwich ELISA with anti-BgSA1 antibodies could detect the circulating BgSA1 in a serial blood plasma from a dog experimentally infected with B. gibsoni. These results indicated that BgSA1 could be a useful target for the development of a diagnostic test for the detection of specific antibodies and circulating antigens.

INTRODUCTION

Babesia gibsoni is a tick-transmitted, intraerythrocytic protozoan parasite. Some infected dogs develop severe hemolytic anemia, remittent fever, thrombocytopenia, splenomegaly, and hepatomegaly.1–5 B. gibsoni has been identified to distribute in many areas throughout the world, such as Asia, Europe, Middle East Africa, and Americas, since it first was recognized in India in 1910.1–4 6 This disease has been considered to be emerging in dogs in the United States and Europe because of the increasing numbers in new areas. In some epidemic regions, it has become a significant problem because of the lack of effective vaccines or control measures.7–9

For the elimination of this disease, it would be important to have a method for the sensitive and reliable diagnosis and detection of dogs that carry this parasite or that have a chronic infection. In this regard, many methods, including polymerase chain reaction (PCR), the indirect fluorescent antibody test (IFAT), and the enzyme-linked immunosorbent assay (ELISA) with native or recombinant proteins as antigens, have been recently developed.10–13 These methods are particularly useful for the identification of chronically infected dogs with significantly low parasitemia. For mass detection, the advantages of ELISA compared with other techniques are its sensitivity, specificity, and convenience. However, the antibody level can remain for a long time even when the infection has finished; therefore, antibody-positive results must be confirmed by the observation of a Giemsa-stained thin blood smear to determine the current infection status of dogs. The aim of this study is to screen circulating proteins that can be used as diagnostic targets in antigen detection tests from the blood plasma of infected dogs to avoid such problems.

To isolate and report available antigens, a cDNA expression library constructed from B. gibsoni merozoite mRNA was screened using a special method that we designed for the identification of secreted antigens. From the candidate cDNA sequences, a novel gene encoding a secreted antigen was identified. As shown in our report, a soluble protein with good antigenicity could be a useful antigenic marker of active B. gibsoni infection.

MATERIALS AND METHODS

Serum. A B. gibsoni NRCPD strain maintained in splenectomized and normal beagles was used.14 Parasitemia was monitored by daily observation of Giemsa-stained thin blood smears. Blood was harvested when infected red blood cells reached approximately 20%. Blood plasma was separated by centrifuging the harvested blood at 1,000 × g for 10 minutes, collected from the top of the cell pellet and debris, and then ultracentrifuged at 130,000 × g for 1 hour. Another healthy beagle was immunized subcutaneously with 10 mL of the plasma mixed with the same volume of Freund’s complete or incomplete adjuvant (Difco Laboratories, Detroit, MI) every 3 weeks for 7 times. The last immunization was performed by intravenous injection without an adjuvant. Serum was collected 10 days after the last immunization.

Immunoscreening of cDNA expression library. A cDNA expression library constructed from B. gibsoni merozoite mRNA was used for immunoscreening.15 The library was plated on a total of 15 plates at a concentration of approximately 20,000 plaque-forming units (PFUs) per plate to lift plaques. The plaques were transferred to nitrocellulose membranes and screened with the serum prepared above according to the protocol of the picobluetM Immunoscreening Kit (Stratagene, San Diego, CA). After an in vivo excision, the cDNA inserts in the positive clones were transferred into pBluescript phagemids and then sequenced with M13 forward, reverse, and internal DNA primers by using an automated sequencer (ABI PRISM 310 Genetic Analyzer, USA).

Southern blotting. Southern blot analysis was performed as described previously.16 Briefly, 10 µg of B. gibsoni genomic DNA extracted from the infected red blood cells, was digested with relative restriction enzymes, and then separated on a 0.8 agarose gel. The DNA fragments were transferred to a nylon membrane (Hybond-N*: Amersham-Buchler, Munich, Germany) and hybridized with a cDNA probe labeled with alkaline phosphate by an AlkPhos Direct kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

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Expression and purification of recombinant BgSA1. The cDNA fragment of the BgSA1 without a signal peptide was inserted into Escherichia coli expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting plasmid was designated as pGEX-4T-3/BgSA1 after it was identified by restriction enzyme analysis and sequencing. The recombinant protein fused with a glutathione S-transferase (GST) tag was expressed in the E. coli BL21 strain according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Purification of recombinant BgSA1 (rBgSA1) was performed with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Preparation of rabbit and mouse sera against BgSA1. Two Japanese white rabbits (6 weeks old) were immunized subcutaneously with 1 mg of purified rBgSA1 or rGST in Freund’s complete adjuvant (Difco Laboratories, Detroit, MI) for the first injection. Five hundred micrograms of the same antigen in Freund’s incomplete adjuvant (Difco) was subcutaneously injected into the rabbit on days 14 and 28. For the preparation of mice antiserum (DDY mice, 5 weeks old), 100 μg and 50 μg of rBgSA1 were used for the first immunization and for boosting on days 14 and 28, respectively. Sera were collected 14 days after the last immunization.

Indirect fluorescent antibody test and confocal laser microscopic observation. A thin blood smear prepared with B. gibsoni-infected red blood cells was fixed with a mixture of methanol and acetone (v:v / 1:4) at −20°C for 20 minutes as described previously. Briefly, The anti-rBgSA1-specific mouse serum was applied as the first antibody on the fixed smears and incubated for 30 minutes at 37°C. After 3 washings with PBS, Alexa-Fluor® 488 conjugated goat anti-mouse immunoglobulin G (IgG) ( Molecular Probes Inc.) was subsequently applied as a secondary antibody and incubated for another 30 minutes at 37°C. The slides were washed 3 times with PBS and incubated with 6.25 μg/ml propidium iodide (PI) ( Molecular Probes Inc.) containing 50 μg/ml RNase A (Qiagen, Inc.) for 10 minutes at 37°C. After 2 washings with PBS, the glass slides were mounted by adding 200 μL of 50% glycerol-PBS (V/V) solution and covering with a glass cover slip. The slides were examined under a confocal laser scanning microscope (TCS NT, Leica, Germany).

Western blotting. B. gibsoni-infected dog erythrocytes and normal dog erythrocytes were treated with a 0.83% NH4Cl solution and then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was electrically transferred to a nitrocellulose membrane, and Western blotting was carried out as described previously.

ELISA. For indirect ELISA, 50 μL purified rBgSA1 or control GST diluted in a coating buffer (a 0.05 M carbonate-bicarbonate buffer, pH 9.6) was used to coat the individual wells of 96-well microtiter plates. The ELISA was performed as described previously. The cut-off value of 0.12 was calculated from the results of the ELISA of 28 specific pathogen-free (SPF) dog sera as follows: 0.051 (mean value) + 3 × 0.022 (standard deviation). Sera used for ELISA were as follows: 14 sera from dogs experimentally infected with B. gibsoni, 5 sera from dogs experimentally infected with B. canis canis, 3 sera from dogs experimentally infected with B. canis rossi, 2 sera from dogs experimentally infected with B. canis vogelii, 5 sera from dogs experimentally infected with L. infantum, 4 sera from N. caninum, 28 sera from SPF dogs, and serial serum samples from a dog experimentally infected with B. gibsoni. In addition, 931 sera from domestic dogs collected from various areas of Japan were also used. The ELISA with recombinant truncated BgPS0 and IFAT were also performed for the detection of the antibody to B. gibsoni in the field samples.

Double-antibody sandwich ELISA. In the sandwich ELISA, rabbit anti-rBgSA1 polyclonal IgG was purified using Econo-Pac A Columns (Bio-RAD Laboratories) according to the manufacturer’s guidelines. One microgram of the IgG diluted in a 0.05 M carbonate buffer (pH 9.6) was used as the capture antibody to coat microtiter plates at 4°C overnight, and purified rabbit anti-GST IgG was used as the control antibody. Blocking was performed with a blocking solution (3% skim milk in PBS, pH 7.2) at 37°C for 2 hours. The plates were incubated at 37°C for 30 minutes with 50 μL of each of the serial plasma samples from a dog experimentally infected with B. gibsoni. After washing 6 times with PBS-Tween 20, mouse anti-rBgSA1 polyclonal serum diluted in a blocking solution was added in each well as a detection antibody. After washing 6 times again, the plates were incubated with 50 μL per well of HRPO-conjugated goat anti-mouse IgG (Bethyl Laboratories) diluted in a blocking solution. Binding was visualized with 100 μL per well of a substrate solution (0.3 mg/ml 2,2’-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid), 0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H2O2). The absorbance at 415 nm was measured by using an MTP-500 microplate reader (Corona Electric, Tokyo, Japan).

Nucleotide sequence accession number. The nucleotide sequence data reported in this article will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB246895.

All 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.

RESULTS

Identification of the BgSA1 gene. A total of 96 clones were isolated from the cDNA expression library with serum from the dog immunized with plasma from a B. gibsoni-infected dog. Alignment of the sequences indicated that the clones represent partial or complete ORFs of 11 genes or gene families. Table 1 shows a summary of the isolated cDNA sequences, along with some features of genes or putative encoded polypeptides. A novel gene named BgSA1 was identified from 84 clones. The cDNA sequence of BgSA1 was completely sequenced and is shown in Figure 1. The full length of BgSA1 contains a single open reading frame of 1,632 nucleotides encoding a polypeptide of 544 amino acid residues. The hydrophobic region at the N-terminus of BgSA1 clearly shows the characteristics of a signal peptide, and the most likely cleavage site was predicted between 23 and 24 amino acids. The molecular weight of the mature protein with 521 amino acid residues is 59 kDa, as calculated with MacVector, version 7.0, software (Oxford Molecular Group, Inc).

Four clones (Bg07, Bg21, Bg27, and Bg68) show identity with Plasmodium species, but all of them contain only partial ORFs. The remaining 7 genes or gene families, including BgSA1, appear to be novel sequences. No significant homologues were found in the GenBank databases by BLASTP.
analysis of the predicted polypeptide sequence. Among them, BgSA2 is another novel gene or gene family identified from 3 clones. Protein analysis programs predicted that BgSA2 encoding 283 aa also had a cleavable N-terminal signal peptide. However, the sera from dogs infected with B. gibsoni could not recognize the recombinant BgSA2 expressed in E. coli (data not shown). None of the other sequences was predicted to have an N-terminal signal sequence, except BgSA1 and

<table>
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<th>Name</th>
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<th>E-value</th>
<th>Number of isolated clones</th>
<th>Signal peptide</th>
<th>Prediction</th>
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<td>Bg18</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>1</td>
<td>Unknown</td>
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<td>Partial</td>
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</table>

* BlastX performed with NCBI BlastX program and an E-value cut-off of 0.05 against the protein database of NCBI.
† Indicates the significance of the match to sequences of the previous column.
‡ Results from all sequences after submission of the best open reading frame to the SignalP server.

**Figure 1.** Nucleotide and predicted amino acid sequences of the cDNA coding for BgSA1 from B. gibsoni. The predicted signal peptide is underlined.
BgSA2; however, because the ORFs start with the beginning of the nucleotide sequence, it is possible that the N terminals were not contained in the clones.

**Characterization of the BgSA1 gene.** A probe amplified from a BgSA1 cDNA clone was hybridized to the genomic DNA fragments of *B. gibsoni* digested with restriction enzymes. The result indicated that there were at least 4 copies of the BgSA1 gene in the genome of *B. gibsoni* (Figure 2). The genomic DNA of the BgSA1 gene was cloned into a pGEM-T vector (Promega, USA) and subjected to DNA sequencing analyses. The sequence was completely identical to the BgSA1 cDNA without any introns (data not shown).

**Expression of BgSA1 in E. coli.** The BgSA1 gene was cloned into the prokaryotic expression vector pGEX-4T-3, and the resulting plasmid was transformed into an *E. coli* BL21 strain. An rBgSA1 with a molecular weight of 85 kDa was expressed as a fusion protein with GST, as expected (data not shown). Sera from dogs experimentally infected with *B. gibsoni* could recognize the GST-fused BgSA1 in Western blotting (data not shown). This result suggested that the recombinant BgSA1 expressed in *E. coli* maintained its antigenicity. In addition, specific antibodies to *B. gibsoni* were induced in both mice and rabbits with immunization of rBgSA1 expressed in *E. coli* (data not shown).

**Identification of the native BgSA1 of B. gibsoni.** A mouse anti-rBgSA1 polyclonal serum was used to identify the native BgSA1 in the lysate of *B. gibsoni* parasites. As shown in Figure 3, a specific band with a size of 59 kDa was detected in *B. gibsoni*-infected red blood cells by Western blotting but not in normal ones. To determine the cellular localization of BgSA1, a thin blood smear was used to perform IFAT with the mouse anti-rBgSA1 serum and observed under a confocal laser microscope; the specific fluorescence was localized in the cytoplasm of *B. gibsoni* merozoites (Figure 4).

**Evaluation of recombinant BgSA1 in an ELISA for the detection of a specific antibody.** The potential of recombinant BgSA1 as a diagnostic antigen was evaluated in an indirect ELISA (BgSA1-ELISA). All 11 serum samples from *B. gibsoni*-infected dogs were positive (optical density > 0.12), whereas the serum samples from the dogs infected with *B. f.
canis (lane 2), B. canis vogeli (lane 3), B. canis rossi (lane 4), N. caninum (lane 5), L. infantum (lane 6), and uninfected dogs (lane 7) were negative (optical density < 0.12) (Figure 5). A specific antibody against BgSA1 could be detected on the eighth day post infection. The antibody level was maintained until 541 days post infection even when the infection was in chronic stage, which is characterized by a recovering hematocrit rate (data not shown) and a significantly low level of parasitemia (Figure 6).

A total of 931 dog serum samples from different regions of Japan were detected with BgSA1-ELISA. As shown in Tables 2 and 3, 72 (7.7%) of the tested serum samples were positive in BgSA1-ELISA (optical density > 0.12), 41 (4.4%) of the tested samples were positive in BgP50-ELISA (optical density > 0.1), and 53 (5.6%) were positive in PCR. Moreover, all of the positive samples, except 3 in BgP50-ELISA, were also positive in BgSA1-ELISA. It is notable that 20 positive samples in PCR (18 of them are positive in IFAT, data not shown) showed a negative reaction in BgP50-ELISA, but only 5 positive samples in PCR (3 of them are also negative in IFAT, data not shown) were negative in BgSA1-ELISA, and 4 of the 5 samples were also negative in BgP50-ELISA. The low sensitivity of P50 might have been caused by the antigenic variation of this antigen because it is exposed on the surface of the merozoite.

Evaluation of double-antibody sandwich ELISA for the detection of circulating BgSA1. Serial blood plasma samples from a dog experimentally infected with B. gibsoni were detected by using a double-antibody sandwich ELISA. As shown in Figure 7, the circulating BgSA1 was detectable in the infected plasma with a parasitemia of 0.2%, as determined by the sandwich ELISA. In addition, the infected dog developed a significant antibody response against BgSA1 in the same period (data not shown).

DISCUSSION

The aim of this study was to screen and identify circulating antigen candidates for their development and use in diagnostic methods to detect antigens of B. gibsoni. In a previous report, the authors described a method to screen soluble antigens from B. microti, and several of them showed encouraging results.20 We used the same technique to isolate the genes encoding soluble antigens from a cDNA library constructed from B. gibsoni merozoite mRNA by immunoscreening. The serum collected from a healthy dog immunized with an infected dog’s plasma containing secreted antigens putatively was used for the screening. Several sequences were isolated from the screening. The analysis of the selected sequences indicated that the method used in this study is effec-
tive to screen immunodominant antigens secreted or excreted into plasma by parasites.

From the isolated clones, a novel gene encoding a polypeptide of 544 amino acid residues was chosen and called BgSA1. The genomic analyses indicated that the BgSA1 gene exists as multi-copies in the genome and contains no introns. The serum raised in mice immunized with the recombinant BgSA1 expressed in \textit{E. coli} could recognize a native parasite protein with a molecular mass of 59 kDa. The native BgSA1 was also confirmed in blood plasma from a dog experimentally infected with \textit{B. gibsoni}. The procedure used in this study is different from those used in previous ones because it was designed, by the nature of the screening itself, for the isolation of secreted antigens. In addition, the antigen captured by the rabbit polyclonal anti-rBgSA1 IgG in the sandwich ELISA provided further important evidence that the novel secreted parasite antigen was released into the bloodstream during the merozoite stage. These results suggested that the BgSA1 is a \textit{B. gibsoni}-derived circulating antigen.

The recombinant BgSA1 expressed in \textit{E. coli} was evaluated in an ELISA as an antigen for the detection of a specific antibody to \textit{B. gibsoni} in dogs. The ELISA could differentiate the sera of \textit{B. gibsoni}-infected dogs from non-infected dogs and \textit{B. canis}-infected dogs. In addition, the specific antibody was detectable as early as 8 days post infection, and the antibody titer was maintained at a high level until 541 days post infection. These results indicated that the ELISA with recombinant BgSA1 might be a useful method for the detection of the antibody to \textit{B. gibsoni} in both acutely and chronically infected dogs. The ELISA with recombinant BgSA1 was comparatively evaluated with previously established ELISAs with recombinant BgP50 and PCR using a large number of samples collected from dogs in Japan. Of the 931 samples analyzed, 72 (7.7\%) samples were positive by BgSA1-ELISA, whereas 41 (4.4\%) and 53 (5.6\%) samples were positive by BgP50-ELISA and PCR, respectively. In addition, of 53 PCR-positive samples, 48 (90.6\%) samples were positive by BgSA1-ELISA, whereas 33 (62.3\%) samples were positive by BgP50-ELISA. These results indicated that the sensitivity of BgSA1-ELISA was much higher than that of BgP50-ELISA. However, the reason that the 5 PCR-positive samples were BgSA1-ELISA-negative remains unclear (see Tables 2 and 3). A possible reason for the result might be the antigenic variation of BgSA1 between field and NRCPD strains. In contrast, the 22 PCR-negative samples were BgSA1-ELISA-positive. These samples might have been taken from the dogs with a chronic infection without circulating parasites in peripheral blood. These results indicated that BgSA1 is more reliable in serological diagnosis as a diagnostic antigen target.

Sometimes the detection of antibodies is unreliable as a method to determine the infection status of dogs because the titer of the antibodies against the parasites can remain very high for a long time even when the parasites have been completely eliminated. On the other hand, it is also necessary to determine the total parasite burden that accurately defines disease severity as a criterion of infection status that cannot be reflected by peripheral blood parasitemia. For example, it is important to decide when to stop the treatment of infected individuals or population groups. Circulating antigens secreted into plasma by parasites may be used as diagnostic targets to be detected in serological tests to predict the total parasite biomass of infected dogs. In malaria, circulating antigens have been successfully used to develop serological tests to detect antigens, and several of them have already been used in the development of a model to estimate the total parasite biomass or used in a commercial ELISA kit to test the sensitivity of a drug against \textit{Plasmodium} in sandwich ELISA. For the purposes of this study, we designed a double-antibody sandwich ELISA to detect BgSA1 in the plasma of dogs infected with \textit{B. gibsoni}. The native BgSA1 could be detectable in the plasma of dogs infected with \textit{B. gibsoni} when the parasitemia reached 0.2\% after infection. The native antigen in the plasma of infected dogs, therefore, suggests the existence of \textit{B. gibsoni} parasites in the peripheral blood.

### Table 3

Comparison of BgSA1-ELISA with PCR for the detection of field samples

<table>
<thead>
<tr>
<th>No. (%) with BgSA1-ELISA</th>
<th>No. (%) with PCR</th>
<th>Total no. (%)</th>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>48 (5.3)</td>
<td>22 (2.4)</td>
<td>70 (7.7)</td>
</tr>
<tr>
<td>−</td>
<td>5 (0.5)</td>
<td>838 (91.8)</td>
</tr>
<tr>
<td>Total no. (%)</td>
<td>53 (5.8)</td>
<td>860 (94.2)</td>
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</table>

**Figure 7.** Detection of circulating BgSA1 in plasma from a dog experimentally infected with \textit{B. gibsoni} by a double-antibody sandwich ELISA.
blood. The sandwich ELISA titer is well associated with the parasitemia of *B. gibsoni*-infected blood post infection. This result indicates that an assay based on the secreted BgSA1 is suitable for the detection of a parasite antigen in plasma. The sensitivity might be improved by using monoclonal antibodies instead of polyclonal antibodies or combining this system with an avidin/biotin system.

Unfortunately, we only detected samples in the acute phase of infection in this experiment because all of the collected samples were serum samples, but it was demonstrated that plasma samples seemed to be more sensitive for the detection of native BgSA1. Therefore, further study on the detection of more samples in an acute or chronic stage of infection is still necessary to determine whether the sandwich ELISA to detect the antigen in plasma can reflect the infection status or parasite burden in infected dogs.

In short, the screening described here was to identify immunodominant proteins that are exposed to the host immune system as a natural process of infection. As discussed previously in this article, the specificity and specificity of the secreted BgSA1 indicated its advantages for use as a target in a serodiagnostic test for the detection of both antibodies and antigens. Moreover, the characterization of the novel molecule will increase the understanding of the mechanism involved in the parasite life cycle. It has been reported that the culture-derived soluble parasite antigens of several *Babesia* species could provide protective immunity against parasite infection.

Therefore, secreted antigens are considered to be potential candidate molecules for the development of new intervention strategies. Our next step is to determine if the recombinant BgSA1 could be used as a potential vaccine to control canine *B. gibsoni* infection. In addition, the detection in field serum samples suggested that *B. gibsoni* infection is a serious disease that should be reckoned with, especially in endemic areas.

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