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Characterization of the *Babesia gibsoni* 12-kDa protein as a potential antigen for the serodiagnosis

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ABSTRACT

A novel gene, BgP12, encoding a 12-kDa protein was identified from *Babesia gibsoni*. The full-length cDNA of BgP12 contains an open reading frame of 378 bp, corresponding to 126 amino acid (aa) residues consisting of a putative 26 aa signal peptide and a 100 aa mature protein. The recombinant BgP12 (rBgP12) lacking the N-terminal signal peptide was expressed in *Escherichia coli* as a soluble glutathione *S*-transferase (GST) fusion protein (rBgP12) that produced an anti-rBgP12 serum in mice after immunization. Using this anti-rBgP12 serum, a native 12-kDa protein in *B. gibsoni* was recognized by Western blot analysis. Immunofluorescent antibody tests (IFAT) revealed that BgP12 was mainly seen during the ring stage of *B. gibsoni* trophozoite. An indirect enzyme-linked immunosorbent assay (ELISA) using the rBgP12 detected specific antibodies in the sequential sera of a dog experimentally infected with *B. gibsoni* beginning 10 days post-infection to 442 days post-infection, even when the dog became chronically infected and showed a low level of parasitemia. Moreover, the antigen did not show cross-reaction with antibodies to the closely related apicomplexan parasites, indicating that the rBgP12 might be an immunodominant antigen for *B. gibsoni* infection that could be used as a diagnostic antigen for *B. gibsoni* infection with high specificity and sensitivity.

1. Introduction

Babesia gibsoni is an intraerythrocytic apicomplexan parasite that causes piroplasmosis in dogs. This disease is mainly transmitted naturally by ticks; however, there are many reports demonstrating transmission via dog bites and blood transfusions as well as via the transplacental route to the developing foetus [1–4]. This organism was recognized in India in 1910 and, since then, has been reported in Asia, Northern and Eastern Africa, Brazil, Europe, and even Australia [5–7]. The disease has been found to occur frequently in dogs and recently has become a serious problem from a clinical viewpoint where the acute form of *B. gibsoni* infection typically results in serious clinical problems, such as fever, thrombocytopenia, regenerative anemia, splenomegaly, and sometimes death [8,9].

Considering these problems associated with *B. gibsoni* infections, it is therefore necessary to develop a reliable diagnostic method and vaccination for this disease. Although some antigens have shown potential for effective diagnosis and putative vaccine candidates in preliminary experiments, a successful antigen to diagnose and prevent this disease has not been found. Several factors have contributed to this lack of proper antigens including a complicated life cycle of the parasite, the wide variety of immune responses induced by the parasite, and insufficient information concerning the relationship between the parasite and its hosts [10,11]. Furthermore, the clinical diagnosis of animals acutely infected with *B. gibsoni* is still based on the light-microscopy to detect intraerythrocytic parasites in Giemsa-stained blood smears [8]. This is particularly problematic in subclinical or latent infections where low levels of parasitemia may be difficult to detect. Recent methods to diagnose this disease include polymerase chain reaction (PCR)-based tests, an immunofluorescent antibody test (IFAT), an enzyme-linked immunosorbent assay (ELISA), and the immunochromatographic test (ICT) [12–16]. Among these methods, the ELISA test with native or recombinant proteins used as antigens has been developed and proven useful for the determination of chronically infected dogs with significantly low levels of parasitemia. Moreover, previous studies have shown that the recombinant proteins used in the ELISA have advantages because they are available in pure forms and can prevent false-positive reactions better than native antigens [17,18]. However, their sensitivities have not achieved

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ideal results; thus, further research on the development of a novel candidate antigen is required.

In this regard, we constructed a library of expressed sequence tags (ESTs) from *B. gibsoni* in order to select candidates that might make potentially powerful antigens for future diagnostic methods. From this bank of ESTs, a novel cDNA encoding a 12-kDa protein was isolated. The successful expression of the recombinant protein (rBgP12) was performed in *Escherichia coli*, and the corresponding 12-kDa native protein of the parasite was identified after isolation of anti-sera. In addition, we demonstrated that rBgP12 is an immunodominant antigen that could induce a strong immune response in both the acute and chronic infectious stages thereby demonstrating the utility of this antigen even at low levels of parasitemia.

2. Materials and methods

2.1. Parasite

B. gibsoni was initially isolated from a hunting dog from Hyogo Prefecture in Japan and designated as the NRCPD strain and maintained in splenectomized beagles as described earlier [19]. The *B. gibsoni*-infected dog erythrocytes were collected from the experimentally infected dog at peak parasitemia (14%) and stored at −80 °C. Furthermore, *in vitro* culture of *B. gibsoni*-infected RBC was maintained as described by Sunaga et al. [20].

2.2. Construction of the B. gibsoni full-length cDNA library and cDNA sequencing

A full-length cDNA library was made using the vector-capping method [21]. Total RNA was prepared from *B. gibsoni*. The cDNA was synthesized with 5 μg total RNA by the G-capping method and ligated into the plasmid vector pGCAP1; the resulting plasmid was transformed into electrocompetent DH12S cells (Invitrogen, USA). ESTs were constructed by random partial sequencing of the 5′-terminus of 10,000 cDNA clones from the cDNA library, and similarities in the protein databases were then examined using the BLASTX program. The cDNA clones encoding a putative 12-kDa antigen were chosen for further analysis.

2.3. Cloning of the BgP12 gene into the pGEX-4T-1 vector

The pGEX-4T-1 vector (GE healthcare, UK) containing an open reading frame (ORF) encoding a glutathione *S*-transferase (GST)-fusion protein. Oligonucleotide primers, including an EcoRI restriction enzyme site, were designed and used to clone the truncated gene encoding rBgP12 lacking the predicted N-terminus signal peptide (forward primer, 5′-AGAGAATTCGGGGTAACAGGAACTGGACTT-3′; reverse primer, 5′-GACGAATTCCGACAGGCACTCAAACAT-3′). Amplification was performed in a 50 μl of 1× PCR buffer (Roche, Switzerland) containing 2.5 U of Taq polymerase, 2 μl of DNA template, 10 pmol of each primer, and 2 mM concentration of each deoxynucleoside triphosphate. The PCR was performed as follows; initial denaturation at 96 °C for 5 min, followed by 30 amplification cycles (96 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and final extension step at 72 °C for 5 min. The PCR product was cloned into the EcoRI restriction enzyme site of the pGEM-4T-1. The construct of the resulting plasmid was checked for accurate insertion by restriction enzymes and nucleotide sequencing. The nucleotide sequence data is available in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB378695.

2.4. Expression and purification of rBgP12 in E. coli

The rBgP12 was expressed as a GST-fusion protein in the *E. coli* BL21 (DE3) strain according to the manufacturer's instructions (GE healthcare, UK). The resulting *E. coli* cells were washed three times with phosphate-buffered saline (PBS), lysed in 1% Triton X-100-PBS, sonicated, and then centrifuged at 10,000 ×*g* for 10 min at 4 °C. Supernatants containing the soluble rBgP12 were purified with glutathione-Sepharose 4B beads (GE healthcare, UK) according to the manufacturer's instructions.

2.5. Preparation of mouse anti-rBgP12 immune sera

Five 6-week-old female ddY mice (SLC, Japan) were intraperitoneally immunized with 100 μg of the purified rBgP12 emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, USA). Two additional boosters with 50 μg of the rBgP12 antigen with incomplete Freund's adjuvant (Difco) 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.

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

To identify the native BgP12 protein in the extract of *B. gibsoni*, the *B. gibsoni*-infected erythrocytes and normal erythrocytes were analyzed using SDS-PAGE and Western blot as previously described [11]. Briefly, the parasitized RBCs were harvested by low speed centrifugation (2000 rpm, 10 min) and washed three times with PBS. The pellets were suspended in PBS containing saponin at a final concentration of 0.075% and incubated at 37 °C in a water bath for 10 min to allow complete lysis of the RBC. The lysate was centrifuged at 8000 rpm for 10 min at 4 °C [22]. Normal erythrocytes were treated similarly. The lysates of the *B. gibsoni*-infected erythrocytes and normal erythrocytes were then sonicated and precipitated with acetone. The precipitated protein was dissolved in a sample buffer containing 5% 2-mercaptoethanol and boiled at 100 °C for 10 min. Samples were electrophoresed on a 15% polyacrylamide gel before transferring to an Immobilon-P Transfer membrane (Millipore, USA). After blocking with 5% skim milk in PBS, the membrane was incubated with anti-rBgP12 antibody (1:100) at 37 °C for 1 h. After washing with PBS containing 0.5% Tween 20, the membrane was soaked in peroxidase-conjugated goat anti-mouse immunoglobulin G (1:1000) and incubated at 37 °C for 1 h. Finally, the membrane was reacted with 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2 to detect BgP12 protein.

2.7. Southern blotting

The genomic DNA was prepared from *B. gibsoni* extracts by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation as described previously [23]. For Southern blot analysis, the *B. gibsoni* genomic DNA was digested with HinfI, BamHI, EcoRI, and HindIII restriction enzymes and electrophoresed on a 1.0% agarose gel. The DNA was transferred to a nylon membrane (Hybond-N+, GE healthcare, UK) using a method previously described [24]. Preparation of the labeled cDNA probe, hybridization, post-hybridization stringency washes, and signal generation and detection were performed using the AlkPhos Direct labeling kit (GE healthcare, UK).

2.8. Immunofluorescent antibody test (IFAT)

Thin blood smear films of *B. gibsoni*-infected blood samples collected from an *in vitro* culture were fixed with methanol for 30 min at −20 °C. The slides were incubated with anti-rBgP12-specific mouse serum appropriately diluted with PBS containing 10% fetal calf serum for 30 min at 37 °C. After washing with PBS, Alexa-Fluor® 488-conjugated goat anti-mouse immunoglobin G (IgG) (Molecular Probes, USA) was applied (1:1000 dilution in FCS-PBS) and the slides were incubated for another 30 min at 37 °C. After three washings with PBS, the slides were incubated with 6.25 μg/ml propidium iodide (PI) (Molecular Probes) containing 50 μg/ml RNase A (Qiagen, Germany) for 10 min at 37 °C. After two washings with PBS, the glass slides were mounted by adding 200 μl