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Identification of an immunodominant *Babesia gibsoni* 47-kDa antigen

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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 (Baneth *et al*., 1998; Birkenheuer *et al*., 2005; Fukumoto *et al*., 2005; Stegeman *et al*., 2003). This organism has been reported in Asia, Northern and Eastern Africa, Brazil, Europe, and even Australia (Patton, 1910; Kjemtrup *et al*., 2000; Muhlnickel *et al*., 2002). 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 (Conrad *et al*., 1991; Boozer *et al*., 2003).

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 the potential for effective diagnosis and putative vaccine candidates in preliminary experiments (Goo *et al*., 2008; Jia *et al*., 2009; Terkawi *et al*., 2009), a successful antigen to diagnose and prevent this disease has not been found. Meanwhile, the results of earlier study showed that sera derived from the infected dogs inhibited the parasite growth in *B. gibsoni*-infected canine red blood cell-substituted severe combined immunodeficiency (Ca-RBC-SCID) mice (Fukumoto *et al*., 2000), indicating that the immunodominant antigens exposed to the protective serum should be promising as candidates for serological tests as well as vaccine. In this regard, continued search for new antigen is extremely desirable.

In this study, to identify an immunodominant antigen, pooled sera from dogs naturally infected with *B. gibsoni* were used to immunoscreen the cDNA expression library (10^7 PFU) of *B. gibsoni* merozoite constructed as described previously (Fukumoto *et al*., 2001), and the cDNA inserts of positive clones were sequenced using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, USA). A total of 98 clones were obtained and analyzed by the BLAST accessed through the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). Since 2 clones showed high values of homology with BgP50 (49%) and BgP32 (47%), identified previously as potential serodiagnostic antigens (Fukumoto *et al*., 2001; Aboge *et al*., 2007), these clones were selected for a molecular characterization and named as BgP47. The full length of the nucleotide sequence of the cDNA was 1,830 bp contained a single ORF with 1,332 bp predicted to encode a polypeptide consisting of 444 amino acid residues. The nucleotide sequence is available in the DDBJ/EMBL/GenBank nucleotide sequence database with the Accession No. GQ494996. The molecular mass of the mature protein was 47.4 kDa, as calculated by Statistical Analysis of Protein Sequences (SAPS, http://www.isrec.isb-sib.ch/software/SAPS_form.html). Analysis by the SignalP server (http://www.cbs.dtu.dk/services/SignalP/) of the putative N-terminal signal peptide showed that this sequence has a highly predicted signal peptide probability (0.999) and a maximum cleavage site probability (0.715) between amino acids in positions 19 and 20.

The truncated gene encoding rBgP47 lacking the predicted N-terminus signal peptide was cloned into *Escherichia coli* expression vector, pGEX-4T-1 (GE Healthcare, UK), with oligonucleotide primers, including *BamH*I and *Xho*I restriction enzyme sites (forward primer, 5'-GAA GGA TCC ACT GGA GAA GGG CAA GCA GGA-3'; reverse primer, 5'-CGC CTC GAG ACT TAA AAT ACA TCG ACA GCC-3') for further molecular characterization as previously described (Goo *et al*., 2009). The construct of the resulting plasmid was checked for accurate insertion by restriction enzymes and nucleotide sequencing. Then, rBgP47 was expressed as a GST-fusion protein of approximately 73 kDa in the *E. coli* BL21 (DE3) strain according to the manufacturer's instruction (GE Healthcare, UK). To confirm the antigenicity of this recombinant protein, Western blot analysis was performed as previously described (Goo *et al*., 2009). A serum from a dog experimentally infected with *B. gibsoni* was probed with rBgP47 and a specific 73-kDa band was observed (Fig. 1). Based on this result, this recombinant antigen was used to raise antibodies in mice as previously described (Goo *et al*, 2009) in order to further characterize the native BgP47. Furthermore, another Western blot analysis was done to test the efficacy of the raised antibodies and identify a native BgP47 in *B. gibsoni* parasites, an anti-rBgP47 mouse serum was reacted with *B. gibsoni*-infected erythrocyte lysate. However, an approximately 30-kDa band inconsistent with the band of a predicted molecular mass (47 kDa) was detected in *B. gibsoni*-infected erythrocyte lysate but not in normal dog erythrocyte lysate (Fig. 1). It is likely that a degradation of this protein occurred to be a mature form. In addition, to determine the cellular localization of BgP47 in parasites and the antibody's ability to detect native parasites, indirect fluorescence antibody test (IFAT) was performed (Goo *et al*., 2009). Figure 2 demonstrates that the immunofluorescent detection of BgP47 using the anti-rBgP47 mouse serum was strong in a membrane of the ring stage of trophozoite.

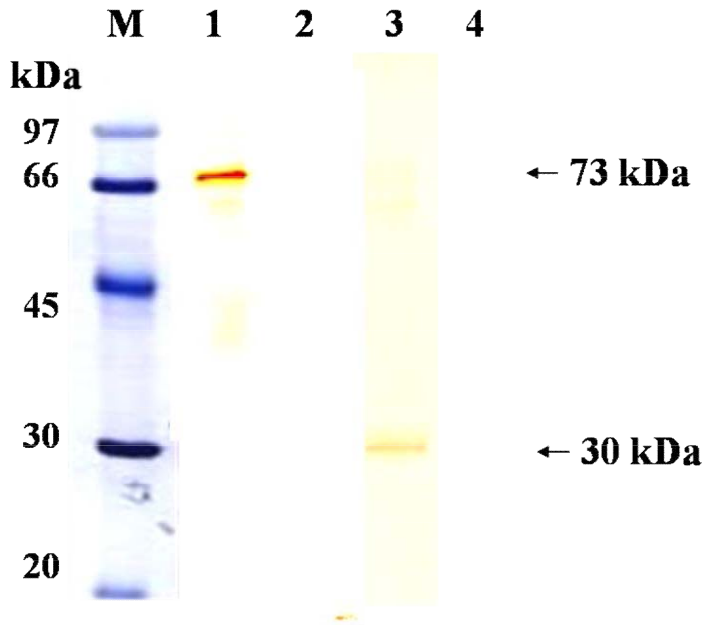

Fig. 1. Western blot analysis of recombinant and native BgP47 proteins (12% SDS-PAGE gel). Lane M, low molecular-mass marker; lane 1, rBgP47 probed with *B. gibsoni*-infected serum; lane 2, rBgP47 probed with uninfected dog serum; lane 3, *B. gibsoni*-infected erythrocyte lysate probed with anti-rBgP47 mouse serum; lane 4, the uninfected dog erythrocyte lysate probed with anti-rBgP47 mouse serum.

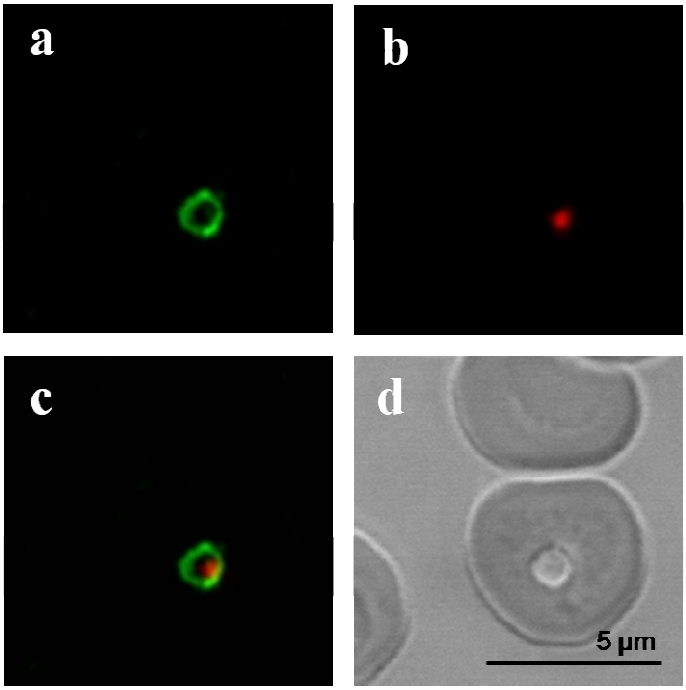

Fig. 2. Localization of rBgP47 in *B. gibsoni* parasite by immunofluorescence staining and confocal microscopy. (a) immunofluorescence staining; (b) propidium iodide staining; (c) merge; (d) Differential interference contrast.

Furthermore, to evaluate the pattern of the antibody development to rBgP47 in *B. gibsoni*-infected dog, serial sera (0-541 days post-infection) from a dog experimentally infected with *B. gibsoni* were tested by the enzyme-linked immunosorbent assay (ELISA) as described previously (Goo *et al*., 2008). A high antibody response to rBgP47 was observed from 4 days post-infection, and the high antibody level was maintained until 541 days post-infection even when the infection was in the chronic stage (Fig. 3a), indicating that BgP47 may play a dominant role in the immune response to *B. gibsoni* infection. Furthermore, the ELISA with sera from dogs infected with parasites closely related to *B. gibsoni* was performed to determine the specificity for BgP47 in *B. gibsoni* infection. The rBgP47 reacted specifically with *B. gibsoni* antibodies but not with the *B. canis* sub-species and *Neospora caninum* antibodies (Fig. 3b), indicating that BgP47 is specific to *B. gibsoni* parasites. These ELISA results indicated that this antigen is an immunodominant antigen stimulating an immune system of *B. gibsoni*-infected dog.

In summary, we isolated the full length of the BgP47 from a cDNA expression library and characterized it as an immunodominant antigen. The ELISA results indicated that this antigen stimulates the immune system of the host infected with *B. gibsoni*, which might be useful in a diagnostic test or a vaccine for *B. gibsoni* infection, and further research is needed to ascertain the efficacy of this antigen as the diagnostic and vaccine candidate.

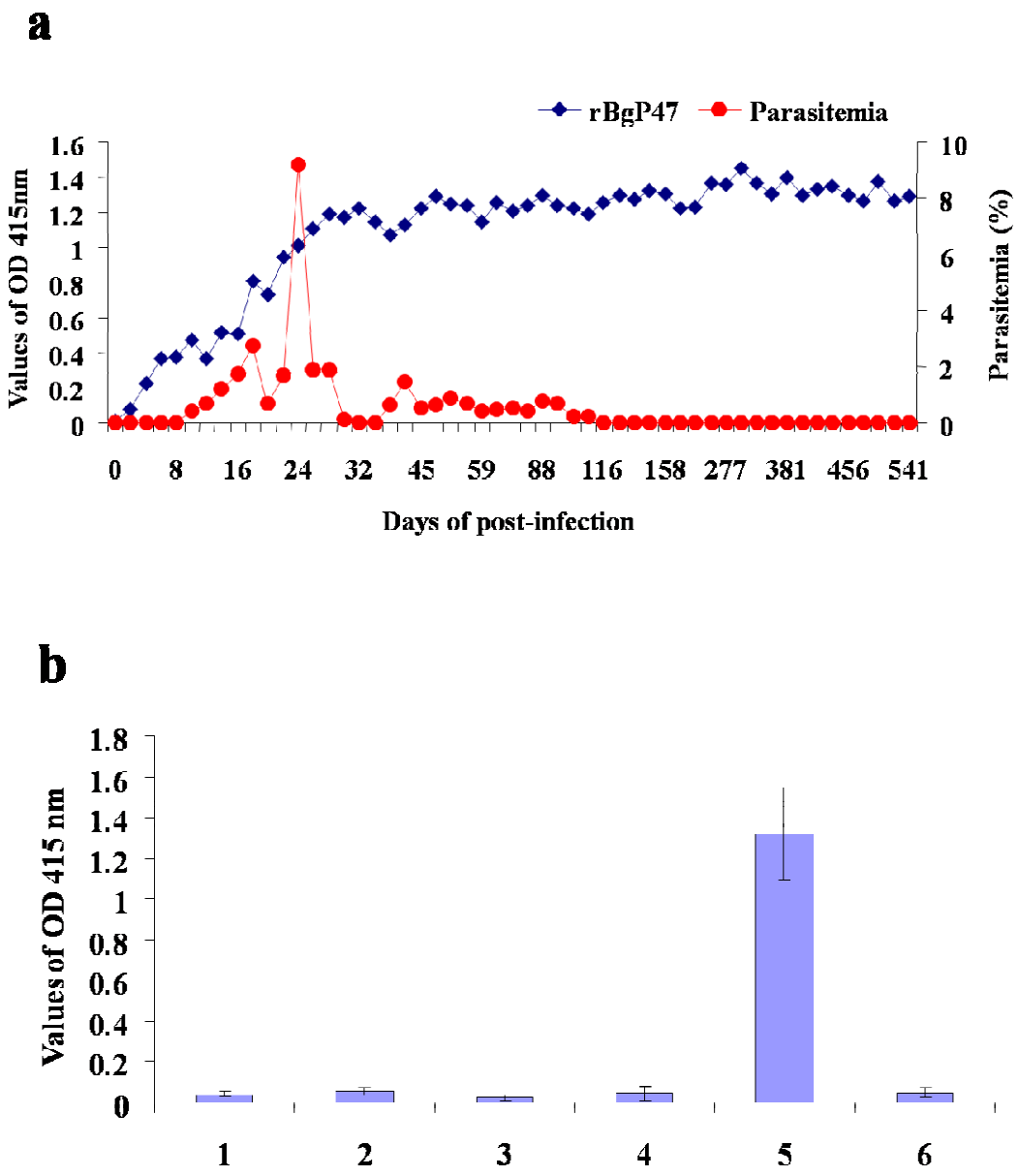

Fig. 3. Evaluation of BgP47 as an immunodominant antigen using an ELISA. (a) Detection of the antibody against BgP47 in a dog experimentally infected with *B. gibsoni* by using ELISA with BgP47. (b) Values of the ELISA with experimentally infected dog sera. Lane 1, sera from *B. canis rossi*-infected dogs (n = 3); lane 2, sera from *B. canis canis*-infected dogs (n = 3); lane 3, sera from *B. canis vogeli*-infected dogs (n = 3); lane 4, sera from *N. caninum*-infected dogs (n = 3); lane 5, sera from *B. gibsoni*-infected dogs (n = 20); lane 6, SPF dog sera (n = 20).

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