

***Babesia gibsoni* rhoptry-associated protein 1 and its potential use as a diagnostic antigen**

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Abstract

A cDNA encoding the rhoptry-associated protein 1 (RAP-1) homologue was obtained by immunoscreening an expression library prepared from *Babesia gibsoni* merozoite mRNA. The complete nucleotide sequence of the gene was 1,740 bp. Computer analysis suggested that the sequence contains an open reading frame of 1,425 bp encoding an expected protein with a molecular weight of 52 kDa. Based on the sequence similarity, this putative protein was designated as the *B. gibsoni* RAP-1 (BgRAP-1). The BgRAP-1 gene was expressed in the *Escherichia coli* BL21 strain, and the recombinant BgRAP-1 was used as the antigen in the enzyme-linked immunosorbent assay (ELISA). The results can differentiate between the *B. gibsoni*-infected dog sera and the *Babesia. canis* infected dog sera or the normal dog sera. Furthermore, the antibody response against the recombinant protein was maintained during the chronic stage of infection, indicating that the recombinant BgRAP-1 protein might be a useful diagnostic antigen for the detection of antibodies to *B. gibsoni* infection in dogs.

Key words:

Babesia gibsoni; rhoptry-associated protein; enzyme-linked immunosorbent assay; diagnosis

1.Introduction

Babesia gibsoni is a tick-borne apicomplexan parasite that causes piroplasmosis in dogs. The disease is characterized by remittent fever, progressive anemia, hemoglobinuria, marked splenomegaly and hepatomegaly, and sometimes death. *B. gibsoni* infection is endemic in many regions of Asia, Africa, Europe, and the Americas (Casapulla et al. 1998; Zhou et al. 2006b). Recently, this disease has been found in companion animals and has become a significant clinical problem (Fukumoto et al. 2001). The identification of immunodominant antigens is an important way to find the molecules that have diagnostic potential. In our group, a merozoite surface antigen of *B. gibsoni*, the P50 protein, was identified as an immunodominant antigen and showed the diagnostic potential in the enzyme-linked immunosorbent assay (ELISA) (Fukumoto et al. 2001) and immunochromatographic test (ICT) (Verdida et al. 2005). Another immunodominant protein of *B. gibsoni*, the 29-kDa cytoplasmic protein, was also found to be candidate antigen for serodiagnosis (Fukumoto et al. 2003). To develop a diagnostic system based on a recombinant protein, we intend to obtain more immunodominant proteins as the candidates.

In the present study, we screened the cDNA expression library of *B. gibsoni* merozoites in order to identify a gene coding an immunodominant protein that would be useful as an antigen for

serodiagnosis. As a result, we identified a gene-coding protein that has sequence similarity to the rhoptry-associated protein 1 (RAP-1) from other *Babesia* parasites. Rhoptry proteins are prime candidates for the development of improved vaccines against bovine babesiosis (Brown and Palmer 1999). The RAP-1 gene family, initially described in *Babesia bovis* and *Babesia bigemina*, also occurs in other *Babesia* parasites, such as *Babesia canis*, *Babesia divergens*, *Babesia ovis*, and *Babesia caballi* parasites (Dalrymple et al. 1993; Kappmeyer et al. 1999; Skuce et al. 1996; Suarez et al. 1998). Immunization of cattle either with native *B. bigemina* RAP-1 (McElwain et al. 1991) or with a recombinant construct, including *B. bovis* RAP-1 (Dalrymple et al. 1993), results in protection. It has recently been reported that RAP-1 also is expressed in *B. bovis* sporozoites and that antibodies against RAP-1 are able to inhibit erythrocyte invasion by *B. bovis* sporozoites (Mosqueda et al. 2002). Taken together, these observations strongly suggest that RAP-1 proteins have an important functional role in parasite invasion and are targets of the protective immune response. Rhoptry proteins have also shown diagnostic potential for *Babesia* infections. The RAP-1 of *B. bovis* and *B. bigemina* has been used for the detection of specific antibodies (Boonchit et al. 2002; 2006). The 48-kDa rhoptry protein of *B. caballi* was also applied to the detection of *B. caballi*-infected horses in the field (Ikadai et al. 2000). Here, the gene encoding the *B. gibsoni* RAP-1 homologue was

expressed in *E. coli*. Then, the ELISA based on the recombinant antigen was developed, and its potential use for the detection of antibodies to *B. gibsoni* in dog was evaluated.

2. Materials and methods

2.1 Parasite and dog. *B. gibsoni* isolated from a hunting dog of Hyogo Prefecture, Japan, designated as the NRCPD strain. *B. gibsoni*-infected dog erythrocytes were collected from the experimentally infected dogs at peak parasitemia (14%) and stored at -80°C (Zhou et al. 2006a). One-year-old beagle dogs were used and confirmed to be free of *B. gibsoni* infection by the inability to detect specific P50 antibody prior to experiments (Fukumoto et al. 2001).

2.2. Cloning and recombinant expression of the BgRAP -1

A cDNA expression library of *B. gibsoni* merozoites was constructed previously (Zhou et al. 2006a). The cDNA library (10^5 PFU) was screened with the serum from a *B. gibsoni*-infected dog at 70 days post-infection. The positive clones were chosen for further analysis. The nucleotide sequences were determined using an automated sequencer (ABI PRISM 310 Genetic Analyzer, USA). The open reading frame (ORF) of the BgRAP-1 gene was subcloned into a pGEX-4T-3 *E. coli* expression vector (Amersham Pharmacia Biotech, Piscataway, NJ) and designated as the pGEX-4T-3/BgRAP-1 plasmid. The BgRAP-1 gene was expressed as a glutathione *S*-transferase (GST)-fusion protein in the

E. coli BL21 (DE3) and supernatants of cell lysates containing the soluble GST fusion protein were purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The recombinant protein was checked by standard SDS-PAGE protocol.

2.3. Indirect fluorescent antibody test (IFAT).

Thin blood smear films of *B. gibsoni*-infected blood samples collected from a *B. gibsoni*-infected dog were fixed with methanol containing 2.5% acetone for 20 min. The anti-rBgRAP-1 mouse serum was made with purified rBgRAP-1 according to the standard protocol. The diluted (appropriate dilutions were made in a 10% fetal calf serum in PBS (FCS-PBS)) anti-rBgRAP-1 mouse serum was applied as the first antibody on the fixed smears and incubated for 30 min at 37°C. After three washings with PBS, Alexa-Fluor® 488-conjugated goat anti-Mouse immunoglobulin G (IgG) (Molecular Probes, Inc.) was subsequently applied (1:500 dilution in FCS-PBS) as a secondary antibody and incubated for another 30 min at 37°C. The slides were washed three times with PBS, and the glass slides were mounted by adding 200 µl of a 50% glycerol-PBS (v/v) solution and covered with a glass cover slip. The slides were examined under a fluorescent microscope.

2.4. ELISA.

Individual wells of a microtiter plate (Nunc) were coated with the purified

GST-BgRAP-1 protein (0.05 µg/well) or the control GST protein (0.05 µg/well) in an antigen coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6) for overnight at 4°C. The ELISA was performed as described previously (Zhou et al. 2006c). Absorbance was shown as the different between the GST-rBgRAP-1 protein and the control GST protein. The canine sera used for ELISA were as follows: 16 sera from healthy dogs, 12 sera from dogs experimentally infected with *B. gibsoni* (NRCPD strain), 3 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 vogeli*, and sequential serum samples from a dog experimentally infected with *B. gibsoni* (NRCPD strain).

3. Results

3.1. Cloning and characterization of theBgRAP-1 gene.

A total of 46 positive clones were obtained from a cDNA expression library by immunoscreening with a *B. gibsoni*-infected dog serum 70 days post-infection, in which 4 repeat clones represented the same cDNA insert. The complete nucleotide sequence of the gene was 1,740 bp (accession number DQ386864) containing an open reading frame of 1,425 bp encoding an expected protein with a molecular weight of 52 kDa. BLASTP analysis of the predicted polypeptide sequence revealed

significant scores with the sequence to be the members of the RAP-1 family of *Babesia* parasites. The modular composition of the protein is the most similar to the RAP-1 of *B. bovis*. Based on the similarity analysis, this putative protein was designated as the *B. gibsoni* RAP-1 (BgRAP-1). The *B. gibsoni* RAP-1 showed 39%, 34%, and 33% homology with the RAP-1 of *B. canis*, *B. caballi*, and *B. bovis*, respectively (Fig. 1). The BgRAP-1 showed the defining molecular features of the babesial RAP-1 family, including the presence of a signal peptide and strict conservation of four cysteines, a 14 amino acid motif [PLS(T/V)LPN(D)PYQLD(E)AAF], and several shorter motifs in the first 300 amino acids of the molecules (Brown and Palmer 1999; Suarez et al. 1991; Suarez et al. 1994). Different from other RAP-1, BgRAP-1 has five repeat motifs [EA(D/V)TKAT(G/S)] in the C-terminal.

3.2. The anti-rBgRAP-1 mouse serum recognizes *B. gibsoni* parasites by IFAT.

The molecular mass of the recombinant BgRAP-1 was estimated to be 78 kDa as expected, including the GST tag with 26 kDa. The anti-recombinant BgRAP-1 mouse serum strongly reacted with the parasites but not the host cells in thin blood smear films by IFAT. However, the control mouse anti-rGST serum did not react with *B. gibsoni* parasites.

3.3. Evaluation of the diagnostic potential of rBgRAP as an antigen in

an ELISA.

The reactivity of the recombinant BgTRAP with the sera from infected dogs was checked by ELISA. As shown in Fig. 2, all 12 serum samples from dogs infected with *B. gibsoni* were positive (optical density >0.5), whereas 16 serum samples from uninfected dogs and 8 serum samples from *B. canis* infected dogs were negative (optical density < 0.2). A dog experimentally infected with *B. gibsoni* developed a significant antibody response to BgRAP within 8 days post-infection, as determined by ELISA (Fig. 3). The antibody titer was maintained for at least 207 days post-infection, even when the dog became chronically infected, a stage characterized by a recovering hematocrit rate (data not shown) and a significantly low level of parasitemia (Fig. 3).

4. Discussion

In the present study, the cDNA gene encoding a rhoptry-associated protein 1 of *B. gibsoni* merozoite was cloned, and its complete nucleotide sequence was determined. The genes encoding RAP-1 have been cloned and sequenced from a number of *Babesia* spp. (Dalrymple et al. 1993; Kappmeyer et al. 1999; Skuce et al. 1996; Suarez et al. 1998). Although the identities of the amino acid sequence are not very high, the structures of the protein are highly conserved. The N-terminal region contains four cysteine residues and additional amino acid motifs that are highly conserved among RAP-1 orthologs from the different species of *Babesia*.

The presence of such conserved amino acid motifs in the N-terminal region of RAP-1 suggests that the region is functionally important (Norimine et al. 2003). The C-terminal region of RAP-1 usually contains a repetitive region (Norimine et al. 2003; Ikadai et al. 1999; Skuce et al. 1996); this characteristic was also found in BgRAP-1.

Results from ELISA support the notion that BgRAP-1 is the immunodominant antigen recognized during infection in the dog, suggesting that it may play important roles in the development of protective immunity. Antibodies against BgRAP-1 were detected in both acute and chronic *B. gibsoni* infections in dogs and clearly differentiated sera from *B. gibsoni*-infected dogs, *B. canis*-infected dogs and normal dogs. Therefore, because of its sensitivity and specificity, rBgRAP-1 warrants further analysis on a larger sample size as a putative diagnostic agent for *B. gibsoni* in dogs.

Figure legends

Fig. 1. Alignment of the putative amino acid of *B. gibsoni* RAP-1 (BgRAP-1) with the RAP-1 of *B. bovis*, *B. caballi*, and *B. canis*. Identical residues are shaded black, and 4 conserved cysteine residues are indicated by arrowheads. The signal peptide sequence of BgRAP-1 is indicated by a solid line. Five repeat motifs [EA(D/V)TKAT(G/S)] are indicated by the dotted line. The nucleotide sequence accession numbers for *B. gibsoni*,

B. bovis, *B. caballi*, and *B. canis* are DQ386864, AAB84270, AAD4098, and AAA27807, respectively.

Fig. 2. Values from ELISA with rBgRAP-1 and experimentally infected dog sera. Lane 1, sera from *B. canis vogeli*-infected dogs; lane 2, sera from *B. canis canis*-infected dogs; lane 3, sera from *B. canis rossi*-infected dogs; lane 4, sera from healthy dogs; lane 5, sera from *B. gibsoni*-infected dogs; OD_{415nm}, Optical density at 415 nm.

Fig. 3. Antibody response to BgRAP-1 in a dog experimentally infected with *B. gibsoni*. The antibody response was detected using the enzyme-linked immunosorbent assay (ELISA). The antibody titer is indicated by the optical density at 415 nm (♦). The parasitemia of the infected dog was checked by microscopic observation (■).

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Figure 1
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B.bovis	1	-MR-IISGVVGCTFLVFSN-MVSAFENHQVGS LAPAEVVGDLT-STL-ETAD-TL-MTLRDGHHNITKD-KHVLSNGRE	73
B.caballi	1	-MRCASSLFGALLV-ARGALAVC-NHSRVSIMAPSDSVGDVT-KTLAASE-SVDSAANAYM--INSD-SDYLSAVSD	73
B.canis	1	-MR-LVKQAIGTFLAFTT-SAFATKVPQFGGILSKSDGA-EKTLSTLLNV-DASTRAAL-E-GYRHHAAIANFSNGRRE	73
B.gibsoni	1	MVA-PIK-FISTELIA-TAGSAHALRLSN-NS-LAMTAGGNDP AEGV-AVAEEAMKA-LTQED-HMQFE-QAYAI VAP S	72
B.bovis	74	QIVHDVCSNAPEDSNCR-EVVMNADRCEMYGCTIDHVKTPLYQE-YQPLSLPDPYQLDAARLFKESASNPAKNSVKR	151
B.caballi	74	NFAERISQVPKGSNCS-ASVSASMSRCAKQDCLTLSLKYPL-EAKYQPLILPDPYQLEAAHILFKESDAMPANSTEKR	151
B.canis	74	EEEEAVCGNIAEETECC-KSVARIVESCVRIDCFSEIENQKYPQKEKE-YQPLILPDPYQLEAAHYVFRHSESNPIKH-PT	150
B.gibsoni	73	EITDAVGTQAPEPEECRLK-VGATVNRCCGDFDCLTLDVVNTPKERE-YQPLVLPDPYQLEAAHYILNCDSNPLKQEA	150
B.bovis	152	-EWLRFNGBANH-GDY-NYFVTGL-L-NHNVVNE-EGTT-DVE-YLVNKKVLIMATGHYKNTLVNSHNAKFFNRFSFTTK	223
B.caballi	152	-FWMRFRGKHH-S-YFHDLVFHL-L-EKHVTRD-ADAT-DIEHF-ASRYLMATLYYKNTLVNDFGASFFNKLSTTG	223
B.canis	151	AFVSRFRNG-GRYGAT-NHFLVNI-L-YKHLSDSMVDD--NLEGF-VKRYATMATHYKNTALDVVNRRIINKIAFSRH	223
B.gibsoni	151	QX-AAFR-SGGKYAEY-RTFLNLTLANKQFYQ-FTEHVYDLSF-VRSYVMATLYFKNTALDVVKQRIIMKVALAHN	225
B.bovis	224	IFSRRIRKQTLSDIRWVVEDE-ERSIEMITQLTSSDEDMLTQIPFLSKFAADRYADMT-KKVLGSLTSYVEAPWYKR	301
B.caballi	224	LFQWGRKRALQIIRSMLNLDIGTEHSVSLQNHITSSYKDMOTQIPALFKPAKRFSIMVQR-LLATVAGYVDTWYKK	302
B.canis	224	LFGRQIRHALTHIIRSMLNEDFG-KYNVDLDHVMGGDEEMHKKVPSLPMFAKKYAGMVKS-LIKHVAGYQKQWFKK	301
B.gibsoni	226	LFSGKIRKALVTYIKKMLNEDFG-KYNVADIKHIMDGYADMETQVPALFAFAKFAGLVTKT-LVKS VTD FQKLPWYRR	303
B.bovis	302	WIKKFRDFFSKN-VTQPTKKFIE-DTNEVTNMY-L--KANVA---EPTEK-FHQDTHEK-ITKG-YL--ENVAEPTKITFK	369
B.caballi	303	WNKLNHFMVNR-VFIPKKKFNKEIREPSMA--L--KEKVS--TDYDLY-ENKIGQGTVDFEKE-IRDPKAL-K	370
B.canis	302	LNNQIRNPFVHK-INEPTKEPFFVKINEPTDEF-F-V-NKIH--EPTEFFVN-KINEPTKEFFVHK--LNEPTKEPTV	371
B.gibsoni	304	LYRLLEADY-KKHVHYFAEPTEKTIGTPIAGPATOTKMRFRKAGEATKATY-Q-KFGEDKAGF-Q--FGTATADKF-	376
B.bovis	370	E-APQVTNHTDDNIGQPTKE-FR-EAPQATNIFLDENIGQPTKEFFREA-PQATKHFLDENIAQPTKEFFKDY-PQVT	444
B.caballi	371	EKVSNDADOLFENKIGQGTVD-FINNEIRDPSKALIR-KVSTGAEDLFENKIGQGTVDFINNEIRDPSKALIRKYTE-A	447
B.canis	372	NKLHEPTKE-F--FSNHVPGAT-Q-KISE--KAG-R-ML-RSSKTVPFE--DEPSSSLENEAVE-DGQLTNGDY-TDPE	436
B.gibsoni	377	QKNHEATKSGTQ-RFGKATKAGT-Q-KFGCVTKAGFH-KLGHVVGKDGKQG-LGD-NEG-DAQEVEGEGEAAEGEV-PD-A	447
B.bovis	445	KKVITENIAQPTKEFL-REVFNATHKVLNENIAQPAKEIINEFGTGAKNFI SAANEGTKQFLNETVGQPTKEFLNGALET	523
B.caballi	448	DDLFFENKIGQGTVDFINKEIRDPS-KALIRKVSTEADHLEK-----	488
B.canis	437	MATPTYEQGSQESLNEVGNE-----	456
B.gibsoni	448	-Q-PA-EAEAGPAEAEAPPAAEAEPEGATE-----	474
B.bovis	524	TKDALMHLGKSSSEANPYDATENTIQANDSTTSNGEDTAGYL	565
B.caballi	488	-----	488
B.canis	456	-----	456
B.gibsoni	474	-----	474

Figure
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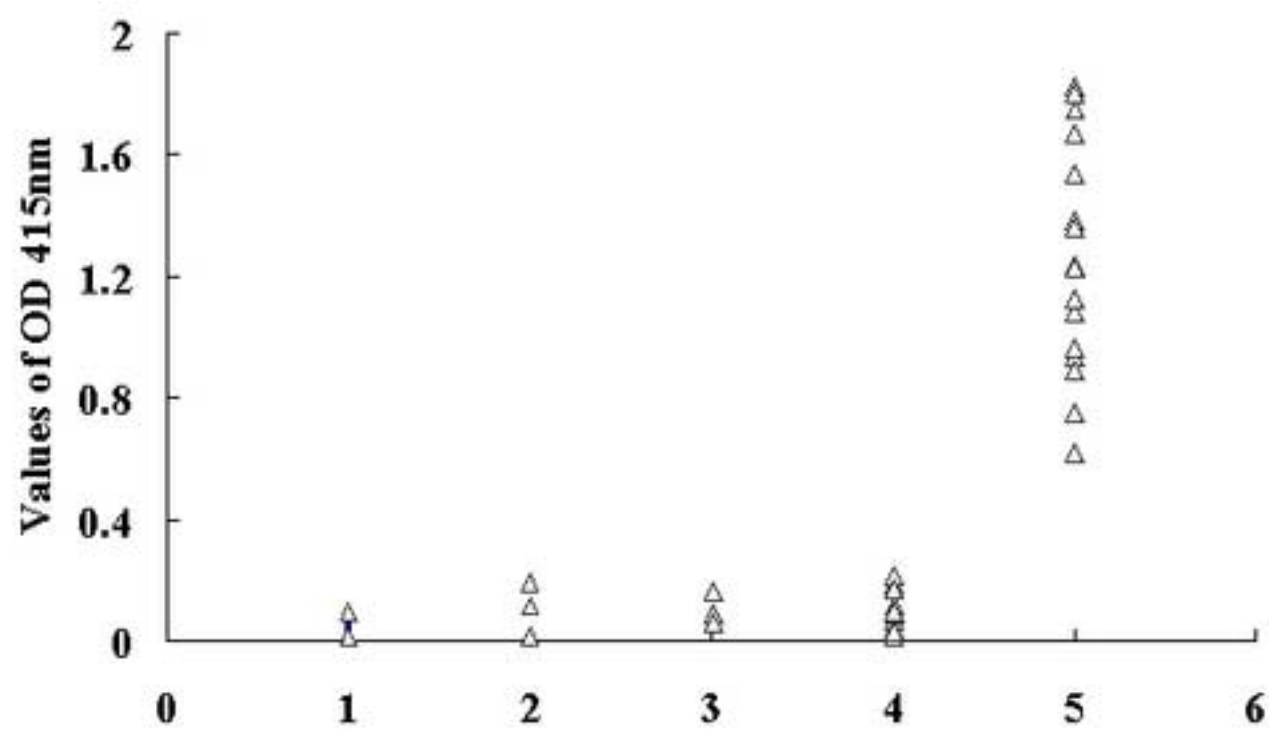


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