Expression of C-terminal truncated and full-length *Babesia bigemina* rhoptry-associated protein 1 and their potential use in enzyme-linked immunosorbent assay

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Abstract

Recombinant antigen-based enzyme-linked immunosorbent assay (ELISA) was developed for the serological diagnosis of *Babesia bigemina* infection by using a full-length *B. bigemina* rhoptry-associated protein 1 (rRAP-1) and the truncated C-terminal RAP-1 (rRAP-1/CT). While the rRAP-1 showed cross reactivity between *B. bigemina* - and *Babesia bovis*-infected bovine sera, the rRAP-1/CT was highly specific to *B. bigemina*-infected bovine sera and proved useful in the detection of sequential sera collected from an experimentally infected cow during the acute and latent infection. The high yield of soluble rRAP-1/CT and its diagnostic specificity demonstrate its potential in the diagnosis of *B. bigemina* infection. Its usefulness for epidemiological investigation is currently being evaluated.

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1. Introduction

*Babesia bigemina* is a tick-borne intraerythrocytic protozoan parasite that causes bovine babesiosis in tropical and sub-tropical areas and constitutes one of the most important diseases affecting cattle industry worldwide (McCosker, 1981; Kuttler, 1988). While the acute infection can readily be diagnosed by direct microscopic examination of Giemsa-stained blood smears, in sub-clinical cases, this may be impractical due to low levels of parasitemia (Bose et al., 1995). Serological tests such as the immunofluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) have
been used in the detection of sub-clinical cases and epidemiological surveys (Weiland and Reiter, 1988). Unlike ELISA, the interpretation of IFAT has disadvantages of low sample throughput and subjectivity, and is affected by cross reactivity with Babesia bovis (Fuginaga et al., 1980; Wright, 1990; Bose et al., 1995).

The detection of specific antibodies (Abs) by ELISA based on the native crude Babesia antigens requires mass production of the parasite either from experimentally infected animals or cultures, and is therefore laborious, expensive, and limited in terms of both specificity and assay-reproducibility (Bose et al., 1995). Thus, emphasis has shifted to the characterization of Babesia antigenic components and their use in diagnostics. Several Babesia recombinant antigens have been expressed in Escherichia coli and evaluated purportedly to replace native parasite antigens to improve the sensitivity and specificity of serological tests (Ikadai et al., 1999; Tebele et al., 2000; Hirata et al., 2002; Huang et al., 2003; Goff et al., 2003). The utilization of recombinant antigen(s) creates improved standardization of tests and reduces the production cost.

Among several proteins of B. bigemina merozoites, rhoptry-associated protein 1 (RAP-1) (McElwain et al., 1987; Machado et al., 1993) has been well characterized for its immunogenicity and conservation among different geographic isolates (Figueroa et al., 1990; McElwain et al., 1987, 1991; Suarez et al., 1994; Vidotto et al., 1995). Suarez et al. (1991) earlier described a relatively conserved N-terminal region of RAP-1 in B. bigemina and B. bovis. In a related study, using the full-length RAP-1 antigen, Boonchit et al. (2002) have noted cross reactivity between B. bigemina and B. bovis. In a related study, using the full-length RAP-1 antigen, Boonchit et al. (2002) have noted cross reactivity between B. bigemina and B. bovis. In a related study, using the full-length RAP-1 antigen, Boonchit et al. (2002) have noted cross reactivity between B. bigemina and B. bovis.

2. Materials and methods

2.1. Parasites

B. bigemina, Argentina strain that has been continuously cultured in vitro with bovine erythrocytes employing the microaerophilous stationary-phase culture system (Vega et al., 1985) in our laboratory was used. When the level of parasitemia reached 5–10%, the infected erythrocytes were washed three times with phosphate-buffered saline (PBS), and the pellets were stored at –80 °C until use.

2.2. Cloning of the full-length and the C-terminal truncated genes of RAP-1 (p58)

B. bigemina genomic DNA was extracted from B. bigemina-infected erythrocyte pellets with phenol–chloroform as previously described (Boonchit et al., 2002) and used as a template DNA in PCR. Oligonucleotide primers were designed based on the DNA sequence of B. bigemina RAP-1 (p58) gene (Gene Bank accession no. M60878) with restriction enzyme-compatible ends for the subsequent DNA cloning. The nucleotide sequences (nt 186–1625 and 1352–1625) coding the entire RAP-1 (p58) and the Carboxy Terminal Variant Type 1 (CT1) which is highly conserved among strains were obtained by PCR using a pair of primers, RAP-1-1 (5′-ACGCGGCCGCAATGTACAGCTAAATTGCTGT-TA-3′; the underlined sequence contains an NotI restriction site) and RAP-1-3 (5′-ACGTCGACAA-CATGAGGAGGAGCTTCTTGGGTGTGT-3′; the underlined sequence contains a SalI restriction site), and another pair of primers, RAP-1-2 (5′-ACGCGG-CCGCCCTGTTGTGCCGATAAAG-3′; the underlined sequence contains a SalI restriction site), and another pair of primers, RAP-1-2 (5′-ACGCGG-CCGCCCTGTTGTGCCGATAAAG-3′; the underlined sequence contains a SalI restriction site) and RAP-1-3. The PCR conditions were 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and then extension at 73 °C for 2 min. Each of the PCR-amplified DNA was digested with restriction enzymes, SalI and NotI, and then ligated to a similarly digested pGEX-4T expression plasmid (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England), resulting in the generation of pGEX-RAP-1 and pGEX-RAP-1/CT, which contain the full-length RAP-1 and the C-terminal RAP-1 fragments, respectively.

2.3. Expression and purification of the recombinant proteins

The plasmids, pGEX-RAP-1 and pGEX-RAP-1/CT were transformed in E. coli (strain DH5α). Each
transformed colony was cultured in LB medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl and 0.1% 5N NaCl) with ampicillin sodium (50 μg/ml) at 37 °C. When the optical density at 600 nm reached 0.3, the plasmids were induced to synthesize the recombinant fusion proteins by the addition of 0.5 mM IPTG (isopropyl-b-D-thiogalactopyranoside), and incubation for another 4 h. The bacterial cultures were harvested by low centrifugation, lysed with TNE buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl and 2 mM EDTA) containing 100 μg/ml lysozyme and 1% Triton X-100 with sonication. The supernatant containing the recombinant fusion proteins, GST-rRAP-1 and GST-rRAP-1/CT were removed by high centrifugation, and then purified with glutathione–sepharose 4B according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The expression and purification of recombinant proteins were confirmed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), that was performed using 12% acrylamide gel as described previously (Boonchit et al., 2002). The purified recombinant antigens were used in ELISA.

2.4. ELISA

ELISA was performed as previously described (Boonchit et al., 2002), with some modifications. Briefly, 96-well microtiter plates (Nalge Nunc International, Roskilde, Denmark) were coated overnight at 4 °C with 50 μl of the indicated recombinant antigens at a concentration of 0.1 μg per well in a coating buffer (50 mM carbonate–bicarbonate buffer, pH 9.6). The plates were washed once with 0.05% Tween 20-PBS (PBST), and then incubated with 100 μl of a blocking solution (3% skim milk in PBS) for 1 h at 37 °C. Serum samples were diluted to 1:200 with the blocking solution containing 5 μg/ml purified GST, and then incubated for 1 h at 37 °C in order to remove the anti-GST specific antibodies from the samples. After one wash with PBST, the coated plates were incubated with a 50 μl per well of the pre-treated serum samples for 1 h at 37 °C. The plates were washed six times with PBST, and then incubated with 50 μl of horse radish peroxidase (HRPO) conjugated sheep anti-bovine IgG (Bethyl Laboratories, USA) diluted to 1:5000 with the blocking solution for 1 h at 37 °C. The plates were washed as described above, and 50 μl of the substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H2O2 and 0.3 mg of 2,2'-azide-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical, St. Louis, MO) per ml] was then added to each well. After 1 h of incubation at room temperature (RT), the optical density (OD) was measured at wavelength 415 nm using an MTP-120 ELISA reader (Corona Electric, Ibaraki, Japan). The OD415 cut-off was set as the mean value of the 30 negative bovine sera plus three standard deviations (S.D.).

2.5. Preparation of anti-rRAP-1/CT specific mouse immune serum

Eight-weeks old female BALB/c mice were intraperitoneally (i.p.) immunized with 200 μg of rRap-1/CT antigen premixed with an equal volume of complete Freund’s adjuvant (Difco, Detroit, MI, USA). On days 14 and 28 post-initial immunization, the mice were i.p. injected with the same amount of the antigen in incomplete Freund’s adjuvant. Sera from the immunized mice were collected 10 days after the last booster and stored at –30 °C until use.

2.6. Indirect fluorescent antibody test (IFAT)

The indirect immunofluorescence antibody test (IFAT) was performed as follows. Smears of infected B. bigemina RBC were prepared on slides, dried and fixed in 50% acetone–50% methanol solution for 5 min at –20 °C. Anti-rRAP-1/CT specific mouse serum (1:100 dilutions) in 10% skim milk-PBS was applied as the first antibody on the fixed RBC and incubated for 30 min at 37 °C. After three washings with PBS, Alexa-Fluor® 488 conjugated goat anti-mouse immunoglobin G (IgG) (Molecular Probes Inc.) was subsequently applied (1:200 dilution in 10% skim milk–PBS) as a secondary antibody and incubated for another 30 min at 37 °C. The slides were washed three times with PBS, incubated with 25 μg of propidium iodide (PI) per ml (Molecular Probes) and 50 μg of RNase A (Qiagen Inc.) per ml for 10 min at 37 °C, and then mounted on a Dako cytometry fluorescent mounting medium (Dako cytometry 6392 via real carpenteria, CA 93013, USA). The slides were then examined with a confocal laser-scanning microscope (TCS NT, Leica, Germany).
Fixed smears of *B. bovis*-infected erythrocytes as described above were also incubated with anti-rRAP-1/CT specific mouse serum (1:100 dilution in 10% skim milk–PBS) at 37 °C for 30 min, followed by washing with PBS and subsequent incubation with Alexa-Fluor® 488 conjugated goat anti-mouse immunoglobulin G (IgG) at the same dilution as above. After two washings with PBS, the glass slides were mounted and then examined for reactivity using confocal microscopy.

2.7. Bovine sera

Serum samples from *B. bovis* (*n* = 14) and *B. bigemina* (*n* = 13)-infected and healthy (*n* = 14) cattle were kindly provided by the Washington State University (Pullman, WA) and Texas A&M University (College Station, TX, USA). Twenty-four sequential sera from a cow experimentally infected with *B. bigemina* (Kochinda strain) in Okinawa prefecture, Japan, were used. The serum samples were obtained prior to inoculation and daily until day 16 post-infection (p.i.), and thereafter on a weekly basis until 70 days p.i.

3. Results

3.1. Cloning and expression of RAP-1 and C-terminal truncated RAP-1 genes

The DNA sequences encoding RAP-1 (aa 1–480) and C-terminal RAP-1 (RAP-1/CT) (aa 390–480) were successfully amplified by PCR and inserted into the plasmid pGEX-4T (Fig. 1). The recombinant proteins, GST-RAP-1 and GST-RAP-1/CT, which were expressed in *E. coli* as recombinant fusion proteins with GST showed molecular weights of approximately 84 and 36 kDa, respectively (Fig. 2). The high yield of soluble purified proteins was achieved at 1 and 5 mg/l of *E. coli* culture for GST-rRAP-1 and GST-rRAP-1/CT, respectively. The difference in yield is apparent in a heavier GST-rRAP-1/CT band (Fig. 2, lane 4), relative to that of GST-rRAP-1 (lane 3). The truncated recombinant antigen, rRAP-1/CT was used to immunize mice for raising specific anti-serum. In Western blot analysis both the rRAP-1 antigen and anti-rRAP-1/CT mouse immune serum specifically recognized the corresponding protein of 36 kDa in *B. bigemina* rRAP-1/CT antigen and merozoite lysate. In contrast, there was no reaction with *B. bigemina* rRAP-1/CT specific mouse sera in *B. bovis* merozoite lysate (data not shown).

To further confirm the specificity of rRAP-1/CT antigen, serum samples that were obtained from

![Fig. 1. Schematic representation of RAP-1 and C-terminal truncated RAP-1 (RAP-1/CT) fragments and hydrophilicity plot of a RAP-1 antigen sequence. The plot shown was derived from the amino acid (aa) sequence of the open reading frame of RAP-1 gene by using computer analysis programs (Hopp and Woods, 1981). nt, Nucleotide.](image1)

![Fig. 2. Expression and purification of the GST fusion with RAP-1 (lanes 1 and 2) and C-terminal truncated RAP-1 (lanes 3 and 4) protein expressed in *E. coli*. Proteins in soluble fractions (lanes 1 and 3), Purified proteins (lanes 2 and 4). Proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue.](image2)
rRAP-1/CT-immunized mice reacted with *B. bigemina* but not *B. bovis* in both IFAT (Fig. 3) and Western blot analysis (data not shown), clearly demonstrating the specificity of *B. bigemina* rRAP-1/CT antigen. Furthermore, IFAT revealed specific green fluorescence within the cytoplasm of *B. bigemina* merozoite-infected erythrocytes (Fig. 3).

### 3.2. Evaluation of ELISA with rRAP-1 and rRAP-1/CT

To evaluate the potential use of rRAP-1 and rRAP-1/CT as diagnostic antigens, a total of 13 *B. bigemina*-infected bovine sera, 14 *B. bovis*-infected bovine sera, and 30 negative bovine sera were tested in ELISA. The cut-off value for positive reactivity was determined at 0.12 and 0.11 for the rRAP-1 and rRAP-1/CT ELISA, respectively (Fig. 4). *Babesia bigemina*-infected bovine sera showed high absorbance above the cut-off in the range of 0.36–1.18 for rRAP-1 and 0.17–0.91 for rRAP-1/CT ELISA. The values for *B. bovis*-infected bovine sera were also above the cut-off in the range of 0.14–0.40 for the rRAP-1 ELISA, but below the cut-off in the rRAP-1/CT ELISA. The negative serum samples registered values below the cut-off in both rRAP-1 and rRAP-1/CT ELISA. As a result of the observed specificity of rRAP-1/CT as compared to that of rRAP-1, we then proceeded to use the rRAP-1/CT antigen to assay sequential sera obtained from a cow experimentally infected with *B. bigemina*. Reactivity to rRAP-1/CT antigen was detectable from 9 days p.i. until the termination of the study at 70 days p.i. (Fig. 5). Parasites were noted in blood smears beginning day 6 p.i., and with the RAP-1/CT-ELISA, the OD values were in the vicinity of the cut-off at 6–9 days p.i., and increased, thereafter.

### 4. Discussion

The rhoptry-associated protein 1 (RAP-1), present in the rhoptry and on the surface of live merozoites is one of the several proteins identified in *B. bigemina* merozoites (McElwain et al., 1987; Machado et al., 1993). *B. bigemina* RAP-1 is immunogenic and conserved among strains of *B. bigemina* isolated from different geographical regions (Figueroa et al., 1990; McElwain et al., 1991; Suarez et al., 1994; Vidotto et al., 1995). The sequence conservation of *B. bigemina* and *B. bovis* RAP-1 in the N-terminal region has been described (Suarez et al., 1991). Boonchit et al. (2002) have earlier reported cross reactivity of the conserved oligopeptide regions between *B. bovis* and *B. bigemina* in serodiagnosis. In order to develop a specific serological test for *B. bigemina* infection, we constructed both the recombinant C-terminal truncated and full-length RAP-1 and evaluated their diagnostic potential by ELISA.

The yield of GST-rRAP-1 in soluble form was much lower as compared to that of GST-rRAP-1/CT, which can be attributed to the hydrophobic characteristics of the RAP-1 N-terminal possibly causing antigen expression largely in the insoluble form. In the case of *Babesia equi* erythrocytic merozoites antigen-1
(EMA-1), the removal of the hydrophobic region enhanced the production of a soluble antigen (Huang et al., 2003). Thus, we think that the higher yield of GST-rRAP-1/CT may have been largely influenced by the removal of the sequence that encodes the hydrophobic region.

The cross reactivity of *B. bigemina* rRAP-1 antigen with *B. bovis* in the present study is consistent with earlier studies by Boonchit et al. (2002). Suarez et al. (1993) have reported highly specific immunogenic B cell epitopes in *B. bovis* RAP-1; while Boonchit et al. (2004), have demonstrated enhanced specificity of the N-terminal region of *B. bovis* RAP-1 in ELISA. The high degree of sequence identity in the first 300 amino acids between *B. bigemina* and *B. bovis* RAP-1 (Suarez et al., 1991), may explain the cross reactivity attributable to the conserved and non species-specific immunogenic epitopes shared by *B. bigemina* and *B. bovis* RAP-1 protein.

The rRAP-1/CT antigen in ELISA was able to differentiate *B. bigemina*-infected sera from *B. bovis*-infected and normal bovine sera. Using RAP-1 specific monoclonal antibodies and bovine CD4$^+$ T cell clones, Hötzel et al. (1996, 1997) have localized immunoreactive epitopes in both the C- and N-terminal regions of *B. bigemina* RAP-1 protein, and demonstrated the existence of isoforms or variants...
NT-1 and NT-2 (N-terminal) and the CT1, CT-2, CT-3 (C-terminal). Although these RAP-1 genes are generally and fairly conserved across several strains with the exception of CT-2 being absent in the SIA Argentina strain, they manifest dimorphic domains that are considered immunologically important in experimental vaccination (Hötzel et al., 1997). In the present work, the rRAP-1/CT fragment revealed a putative amino acid sequence that corresponds to the CT-1 earlier described by Hötzel et al. (1997) in nine South American strains of *B. bigemina*.

In IFAT and Western blot analysis, the specificity of rRAP-1/CT antigen with *B. bigemina* was clearly demonstrated. Interestingly, with IFAT and confocal laser scanning microscopy, specific green fluorescence was likewise demonstrable in the cytoplasm of infected erythrocytes (Fig. 3), suggestive of *B. bigemina* RAP-1 antigen shedding. A similar observation has been reported in *B. bovis* (Yokoyama et al., 2002) and *Babesia equi* (Kumar et al., 2004). The secretion of RAP-1 antigen inside red blood cells and its subsequent release into host circulation could be potential targeted in the development of antigen captured ELISA.

In summary, we have demonstrated the ability of *B. bigemina*-rRAP-1/CT to discriminate between *B. bigemina* and *B. bovis* infections in ELISA. The specificity of rRAP-1/CT antigen for *B. bigemina* infection points to the potential usefulness of rRAP-1/CT ELISA in the diagnosis of *B. bigemina* infection. Also, the detection of *B. bigemina* infection from days 9 to 70 p.i. in sera of an experimentally infected cow reflects its potential in the detection of acute and latent infections. We are currently carrying out additional work using other field serum samples, paired with whole blood collection for PCR analysis to further confirm the sensitivity and specificity of the rRAP-1/CT ELISA on a large scale.

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