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

Jinlin Zhou [a,b], Honglin Jia [a], Yoshifumi Nishikawa [a], Kozo Fujisaki [a], Xuenan Xuan [a,*]

[a] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*

[b] *Shanghai Institute of Animal Parasitology, Chinese Academy of Agricultural Sciences, 3 Lane 345, Shilong Road, Shanghai 200232, China*

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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 1740 bp. Computer analysis suggested that the sequence contains an open reading frame of 1425 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.

Keywords: *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

* Corresponding author. Tel.: +81 155 49 5648; fax: +81 155 49 5643.
E-mail address: gen@obihiro.ac.jp (X. Xuan).

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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 immunoglobin 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 (0.05 M carbonate–bicarbonate buffer, pH 9.6) for overnight at 4 °C. The ELISA was performed as described previously (Zhou