Experimental Parasitology 133 (2013) 346–352

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Identification and characterization of an interspersed repeat antigen of *Babesia microti* (BmIRA)

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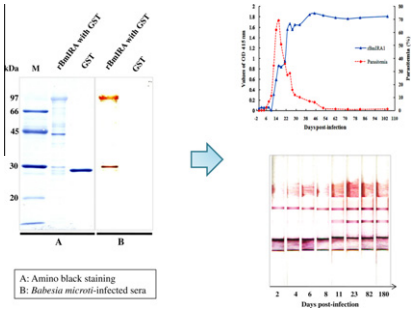
HIGHLIGHTS

- A novel interspersed repeat antigen (BmIRA) of *Babesia microti* was identified.
- The rBmIRA showed a good performance in an ELISA.
- An immunochromatographic test (ICT) was developed for detection of *B. microti* infection.
- BmIRA could be a potential marker for the serodiagnosis of *B. microti* infection.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 19 November 2012
Received in revised form 24 December 2012
Accepted 26 December 2012
Available online 3 January 2013

Keywords:
Babesia microti
BmIRA
ELISA
Immunochromatographic test

ABSTRACT

In this report, a novel gene encoding an interspersed repeat antigen from *Babesia microti* (BmIRA) was identified and described. The full-length cDNA containing an open reading frame of 1,947 bp was obtained by immunoscreening a *B. microti* cDNA expression library. The full-length of BmIRA gene was expressed as a GST fusion recombinant BmIRA (rBmIRA) in *Escherichia coli*. Sera of mice immunized with the rBmIRA detected a native parasite protein with a molecular mass of 76 kDa on Western blot analysis. The same protein was detected in the parasites by immunofluorescent antibody test (IFAT). An enzyme-linked immunosorbent assay (ELISA) using rBmIRA detected specific antibodies as early as 11 days post-infection in sera from a hamster experimentally infected with *B. microti* Gray stain (US type). Furthermore, a rapid immunochromatographic test (ICT) using rBmIRA detected specific antibodies in a hamster experimentally infected with *B. microti* from day 11 to at least day 180 post-infection. The results indicate the antibody response against the rBmIRA was maintained during the chronic stage of infection. On the other hand, an immunoprotective property of rBmIRA as a subunit vaccine was evaluated in hamsters against *B. microti* challenge, but no significant protection was observed. Our data suggest that the immunodominant antigen BmIRA could be a useful serodiagnostic antigen for screening of *B. microti* infection.

1. Introduction

Babesia microti is a tick-borne intraerythrocytic protozoan belonging to the genus *Babesia*. *B. microti* is considered to be a major etiological agent of emerging human babesiosis (Homer et al., 2000; Vannier and Krause, 2009). Human babesiosis is a malaria-like illness caused by *B. microti* and was initially recognized as an emerging endemic disease in the eastern and upper Midwestern United States (Scholtens et al., 1968; Krause et al., 2003) and recently in Europe (Homer et al., 2000; Hildebrandt et al., 2007) as well as East Asia (Shih et al., 1997; Wei et al., 2001). The clinical manifestations in patients with *Babesia* infection vary substantially

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http://dx.doi.org/10.1016/j.exppara.2012.12.015

from asymptomatic presentation to severe and occasionally fatal infections. Asymptomatic carriers can harbor the circulating parasites for months or years, thus complicating the diagnosis and causing a potential threat to blood donors. The bite by an infected hard tick, *Ixodes scapularis*, is considered to be the primary mode of transmission. However, many transfusion-acquired infections have been documented, and such cases appear to be persistently increasing (Leiby, 2006; Gubernot et al., 2009). Therefore, it is important to establish a reliable diagnosis and vaccine to control and eliminate this disease.

Examination of Giemsa-stained blood smears for the parasite is still considered as gold standard for diagnosis of *Babesia* infection. However, in subclinical or latent infections, this may be impractical due to low levels of parasitemia. Many methods, including polymerase chain reaction (PCR), indirect fluorescent antibody test (IFAT), immunochromatographic test (ICT), and enzyme-linked immunosorbent assay (ELISA) based on either native or recombinant proteins as antigens, have been recently developed. These tests have proved to be particularly useful for the identification of chronically infected hamsters with markedly low parasitemia (Luo et al., 2012). Among these methods, the advantages of ELISA compared to other techniques are its sensitivity, specificity, and convenience, especially for mass detections. In addition, the ICT has provided an analytical platform that permits one-step, rapid analysis independent of any instrument (Chandler et al., 2000; Richardson et al., 2002). Hence, ICT would be extremely valuable for use in clinical and field applications for the diagnosis of this disease. However, the *B. microti* antigens identified so far have not yielded satisfactory results. In this regard, there is a need to identify novel *B. microti* antigens to develop reliable, rapid, and sensitive diagnostic tests. Therefore, the identification of *B. microti*-novel antigens would be very useful for the development of novel diagnostic methods for the detection of human babesiosis.

On the other hand, vaccination as a preferable way has been used to control babesiosis; however, at present there is no vaccine for human *Babesia* infection. Recently, the development of subunit vaccine has moved towards recombinant proteins (Shkap et al., 2007), and from an immunological point of view, the immunodominant antigens identified by protective serum could be candidates for a subunit vaccine (Jia et al., 2008). In this context, we serologically screened a cDNA library of *B. microti* Gray strain in order to select candidates for evaluation of the serodiagnostic potentiality and protective efficacy. A novel gene encoding an interspersed repeat antigen of *B. microti* (BmIRA) was isolated. The recombinant BmIRA (rBmIRA) was expressed in *Escherichia coli*. Then, the ELISA and ICT based on the rBmIRA were developed, and its potential use for the vaccination to protect *B. microti* challenged was evaluated.

2. Materials and methods

2.1. Parasites and experimental animals

The human isolate *B. microti* Gray strain (US type, American Type Culture Collection, Catalog No. 30221) was maintained in Golden Syrian hamsters by intraperitoneal injection with 1×10^7 *B. microti*-infected erythrocytes. The specific pathogen-free (SPF) female Golden Syrian hamsters were purchased from Clea (Japan). Parasitemia was monitored by counting parasitized erythrocytes on Giemsa-stained thin blood smears. The blood was harvested with heparin when infected red blood cells reached at least 50% parasitemia or more. Five 6-weeks-old female ICR mice (Clea, Japan) were used for the immunization experiments. All animal experiments described in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

2.2. Immunoscreening of the cDNA expression library

The cDNA library of *B. microti* Gray strain previously constructed (Luo et al., 2011) was placed on a total of four plates at a concentration of approximately 20,000 plaque-forming units (PFUs) per plate, and then the plaques were transferred onto nitrocellulose membranes. Thereafter, the membranes were screened with the sera of hamsters having acute infections with *B. microti* according to the instruction manual for the picoBlue™ immunoscreening kit (Stratagene, USA). The positive clones were picked and after an *in vivo* excision, the cDNA inserts in the lambda phage system were obtained as pBluescript phagemids (Strategene, USA). The plasmid DNA was further purified using the QIAGEN miniprep kit (Qiagen, Germany), and the nucleotide sequences were determined using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, USA) using M13 forward, reverse and internal DNA primers.

2.3. Cloning of the BmIRA gene into pGEX-4T-3 vector

The cDNA fragment of BmIRA was amplified by PCR using primers with the introduced *Bam*HI and *Xho*I sites (underlined), P1 (5′-ATGGATCCATGATGAAAATTAACATAG-3′) and P2 (5′-ATCTCGAGC TTCGTTAAATTATTTCAC-3′). Amplification was performed in a total volume of 50 μl of PCR mixture with buffer containing 2.5 U of *Taq* polymerase (Roche, Switzerland), 10 pmol of each primer, 2 mM of each deoxynucleoside triphosphate, and 2 μl of DNA template. The PCR consisted of an initial denaturation at 95 °C for 5 min, followed by 30 amplification cycles (95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min), and a final extension step at 72 °C for 7 min. The PCR product was cloned into *E. coli* expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, USA). The resulting plasmid was identified by sequencing and designated as pGEX-4T-3/BmIRA.

2.4. Expression and purification of rBmIRA in E. coli

The rBmIRA was expressed as a glutathione S-transferase (GST) fusion protein in the *E. coli* BL21 strain according to the manufacturer's instructions (Amersham Pharmacia Biotech, USA). The resulting *E. coli* cells were washed with cold phosphate-buffered saline (PBS), lysed in TNE buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 0.5% triton X-100, and 1 mg/ml lysozyme, sonicated, and then centrifuged at 15,000 *g* for 10 min at 4 °C. The supernatant containing soluble rBmIRA was purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, USA).

2.5. Production of anti-rBmIRA sera

Five six-week-old ICR mice (Clea, Japan) were immunized intraperitoneally with 100 μg of purified rBmIRA without GST in an equal volume of Freund's complete adjuvant (Sigma, USA) for the first injection. One hundred micrograms of the same antigen in Freund's incomplete adjuvant (Sigma, USA) was intraperitoneally injected into the mice on days 14 and 28 post-primary injections.

2.6. Indirect fluorescent antibody test and confocal laser microscopic observation

A thin smear of hamster erythrocytes infected with *B. microti* was made and then fixed with 100% methanol for 30 min at −30 °C. Anti-rBmIRA-specific mouse serum 1:200 diluted with PBS containing 3% bovine serum albumin (BSA) was applied as the first antibody on the fixed smears and incubated for 1 h at 37 °C. After four washes with PBST, Alexa-Fluor® 488 conjugated goat anti-mouse IgG (Molecular Probes, USA) 1:600 diluted in