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Identification and characterization of a novel secreted antigen 1 of *Babesia microti* and evaluation of its potential use in enzyme-linked immunosorbent assay and immunochromatographic test

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ABSTRACT

Here, we identified a novel secreted antigen designated as *Babesia microti* secreted antigen 1 (*Bm*SA1) by immunoscreening a *B. microti* cDNA expression library using the sera from hamsters immunized with plasma, putatively containing secreted antigens, from *B. microti*-infected hamsters. Antibodies raised in mice immunized with recombinant *Bm*SA1 (r*Bm*SA1) recognized a native 33-kDa parasite protein. An enzyme-linked immunosorbent assay (ELISA) of r*Bm*SA1 detected specific antibodies as early as 6 and 4 days post-infection in sera from a hamster experimentally infected with *B. microti* Gray strain (US type) and a mouse experimentally infected with *B. microti* Munich strain (rodent isolate), respectively. Moreover, a rapid immunochromatographic test (ICT) using r*Bm*SA1 detected specific antibodies in a hamster experimentally infected with *B. microti* from day 6 to at least day 270 post-infection, which was quite consistent with the results of the ELISA. In addition, analysis of the sera involved in the first case of human babesiosis in Japan (Kobe type) showed that specific antibodies were detectable in the patient and the positive donor by ELISA using r*Bm*SA1, and the ICT result was identical to the ELISA data. Taken together, these results indicated that *Bm*SA1 could be a promising and universal target for developing both ELISA and ICT for the serodiagnosis of human babesiosis and for an epidemiological survey of its rodent reservoir.

1. Introduction

Babesia microti, an intraerythrocytic protozoan of the genus *Babesia*, is a common rodent parasite and the major cause of emerging human babesiosis, an asymptomatic to fatal, malaria-like disease [1,2] which is mainly transmitted by the same tick vector (*Ixodes scapularis*) that transmits the aetiological agents of Lyme disease and human granulocytic ehrlichiosis [3]. *B. microti* in humans, first identified in 1968, can be locally prevalent in diverse regions of the United States [4,5] and recently emerged in Europe [6,7] and Asia [8,9]. The clinical manifestations in patients with *Babesia* infection vary substantially 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 donees. Indeed, many transfusion-acquired infections have been documented, and such cases appear to be persistently increasing [10]. Severe and sometimes fatal outcomes generally occur in immunocompromised or elderly individuals [11,12].

To control and eliminate this disease, it is important to establish a sensitive and reliable test to diagnose the parasite carriers or chronic infection in humans. Some methods have been developed for human babesiosis, including a blood smear test for the direct observation of intraerythrocytic parasites, inoculation of susceptible animals with blood from a suspected case, polymerase chain reaction (PCR), and immunofluorescent antibody test (IFAT) [13]. These tests are very useful but have some disadvantages. They insufficiently detect subclinical infections, yield nonspecific results, and are unfeasible for large-scale testing. Moreover, they are both expensive and time-consuming. Alternatively, an enzyme-linked immunosorbent assay (ELISA) is more sensitive, specific, and suitable for mass detection, and an immunochromatographic test (ICT) has provided an analytical platform that permits one-step, rapid analysis. Some antigens, including native or recombinant antigens, have been identified and evaluated by ELISA

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[2,14]; however, the antigens identified so far have not yielded satisfactory results. Several ICT methods for detecting antigens or antibodies in protozoan parasites have been developed [15–17], but there has been no report of the diagnosis of human *Babesia* infection. Clearly, there is a need to identify novel *B. microti* antigens to develop reliable, rapid, and sensitive diagnostic tests. Among candidate antigens, secreted antigens released into plasma by parasites, which have been successfully used to develop a serological test to detect antigens in malaria [18], could be used to determine the parasite burden of the host and thereafter accurately define the host infectious status. Therefore, the identification of secreted antigens of *B. microti* would be very useful for the development of novel diagnostic methods for the detection of circulating antigens and specific antibodies in human babesiosis.

In this context, we constructed a cDNA expression library from *B. microti* Gray strain and serologically screened the cDNA expression library for the identification of secreted antigens. A novel 33-kDa-secreted antigen was identified, and its potential as a candidate for serodiagnosis by both ELISA and ICT was evaluated.

2. Materials and methods

2.1. Parasites and sera

Human isolate *B. microti* Gray strain (US type, American Type Culture Collection, Catalog No. 30221) was maintained in Golden Syrian hamsters (Clea, Japan) by intraperitoneal injection with 10^7 *B. microti*-infected erythrocytes. Infection was monitored by making Giemsa-stained thin blood smears. Blood was harvested with heparin when infected red blood cells reached or were near peak parasitemia (50–70%). Blood plasma was separated by centrifuging the harvested blood at 3500 g for 15 min, collected from the top of the cell pellet and debris, further ultracentrifuged at 100,000 g for 1 h to ensure the removal of free merozoites, and then kept at −80 °C for immunization. Five 5-week-old specific pathogen-free (SPF) female Golden Syrian hamsters were purchased from Clea (Japan). The hamsters were immunized intraperitoneally with 300 μl of the plasma mixed with the same volume of Freund's complete adjuvant and boosted four times with the same amount of plasma with Freund's incomplete adjuvant (Difco Laboratories, USA) at 14-day intervals. Sera were collected 10 days after the final immunization. The experiments 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. Construction of cDNA expression library

Total RNA was prepared from *B. microti* Gray strain-infected hamster erythrocytes by the thiocyanate-phenol-chloroform extraction method [19]. Then the poly(A)$^+$ RNA was purified from the total RNA using oligotex-dT30 (JSR Co., Japan). Next, cDNA was synthesized by using a Zap-cDNA synthesis kit, ligated to a Uni-ZAP XR vector and packaged by using a Gigapack III packaging system according to the manufacturer's instructions (Stratagene, USA).

2.3. Immunoscreening of cDNA expression library

The constructed library was plated 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 prepared above according to the instruction manual for the picoBlue™ Immunoscreening Kit (Stratagene, USA), and positive clones were picked up. After an in vivo excision, the cDNA inserts in the lambda phage system were obtained as pBluescript phagemids (Strategene, USA). Plasmid DNA was further purified using the QIAGEN miniprep kit (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.

2.4. Expression and purification of recombinant BmSA1 (rBmSA1) in E. coli

The cDNA fragment of *Bm*SA1 without a signal peptide was amplified by PCR using primers with the introduced *Eco*RI and *Xho*I sites (underlined), P1 (5'-TGTGAATTCTGCTGGTGGTAGTGGTGGT-3') and P2 (5'-ACCTCGAGCTCCGTT TATTTAGAATAG-3'). Amplification was performed in a 50 μl of PCR buffer (Roche, Switzerland) containing 2.5 U of Taq polymerase, 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 96 °C for 5 min, followed by 30 amplification cycles (96 °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/*Bm*SA1. The recombinant *Bm*SA1 was expressed as a glutathione S-transferase (GST) fusion protein in the *E. coli* BL21 (DE3) 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 a TNE buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 2 mM EDTA) containing 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 r*Bm*SA1 was purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, USA). Then the leader protein, GST, was cleaved by thrombin protease according to the manufacturer's instructions. The protein concentration was measured by using a modified Lowry protein assay kit (Thermo Scientific, USA).

2.5. Production of mouse and rabbit anti-rBmSA1 sera

Six-week-old ICR mice (Clea, Japan) were immunized intraperitoneally with 100 μg of purified r*Bm*SA1 without GST in an equal volume of Freund's complete adjuvant (Difco Laboratories, USA) for the first injection. One hundred micrograms of the same antigen in Freund's incomplete adjuvant (Difco) was intraperitoneally injected into the mice on days 14 and 28 post-primary injections. For the preparation of rabbit antisera, one 2.5-kg Japanese white rabbit (Clea, Japan) was immunized subcutaneously with 1 mg of purified r*Bm*SA1 without GST for the first immunization and was given boosters on days 14 and 28. The anti-r*Bm*SA1 sera were collected 14 days after the last immunization.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

To identify the native *Bm*SA1 in the parasite extract of *B. microti*, the *B. microti*-infected hamster erythrocytes and normal hamster erythrocytes were analyzed by SDS-PAGE and Western blotting as described previously [20,21], with some modification. Briefly, the parasitized RBCs were harvested by centrifugation at 500 g for 10 min and washed three times with PBS. The pellets were treated with 0.075% of saponin in PBS (w/v) and incubated in a 37 °C water bath for 10 min to allow complete lysis of the RBCs. The lysate was centrifuged at 10,000 g for 10 min at 4 °C. Normal erythrocytes were treated similarly. The lysates were then sonicated and precipitated with cold acetone. The precipitated protein was dissolved in SDS-PAGE sample buffer (125 mM Tris–HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 3% (v/v) 2-mercaptoethanol, and 0.02% bromophenol blue) and was boiled at 100 °C for 5 min and then subjected to SDS-PAGE. After electrophoresis, the protein was transferred to an Immobilon-P transfer membrane (Millipore, USA). After being blocked with 5%