NOTE *Parasitology*

Identification, Cloning and Characterization of BmP41, a Common Antigenic Protein of *Babesia microti*

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ABSTRACT. *Babesia microti* is a rodent tick-borne blood parasite and the major causative agent of emerging human babesiosis. Here, we identified a candidate of common antigenic protein BmP41 of *B. microti* by serological screening of cDNA library of human-pathogenic Gray strain with antisera against rodent Munich strain. Immunofluorescent antibody test using mouse anti-recombinant BmP41 (rBmP41) serum revealed that native BmP41 was expressed in each of the developmental stages of *B. microti* merozoites. An enzyme-linked immunosorbent assay (ELISA) using rBmP41 detected specific antibodies in sera from hamsters infected with *B. microti* Gray strain and mice infected with *B. microti* Munich strain. Taken together, BmP41 could be a promising universal serological marker for diagnosis of human babesiosis.
KEY WORDS: *Babesia microti*, BmP41, common antigen, ELISA, immunoscreening.

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Babesia microti is a rodent tick-transmitted intraerythrocytic protozoan of the genus *Babesia*. Recently, babesiosis caused by *B. microti* arouses interest as an emerging zoonosis in humans [2, 6]. Human babesiosis caused by *B. microti* was first recognized as an endemic disease in North America and has recently emerged in Europe and East Asia [2–4]. Infection usually causes asymptomatic to mild flu-like clinical manifestations including fever, sweat, chills and anemia [4]. However, severe and sometimes fatal outcomes generally occur in immunocompromised or elderly individuals [4, 8, 15].

Transmission of *B. microti* occurs through the bite of infected *Ixodes scapularis* ticks or via blood transfusion from infected donors [8, 17, 20]. Thus, asymptomatic carriers are potential threat to public health, and the Red Cross and other blood donation agencies prohibit people with a history of babesiosis from donating blood [7]. Therefore, diagnosis of the infection in carriers is very beneficial for the control of this disease. Enzyme-linked immunosorbent assay (ELISA) is a sensitive, specific and practical test for screening blood donors for *Babesia* infection prior to transfusion [8, 10, 14]. However, recent phylogenetic study indicates that the *B. microti*-group parasite branched mostly into four clades (US, Kobe, Munich and Hobetsu types) [12], and they are serologically different [18]. Therefore, discovery of serological marker that can detect multi-strains of *B. microti* is required.

To identify a universal antigen for the detection of different species of *B. microti*, we attempted to find a common antigen between *B. microti* Gray strain (US type, American Type Culture Collection, Catalog No. 30221) and rodent *B. microti* Munich strain [13, 14] because these types are genetically different [2], and we speculate that the serological common antigen between these strains could serve as an universal antigen for the diagnosis of babesiosis in rodent and humans. The cDNA library of *B. microti* Gray strain previously constructed [10] was placed on plates, and then, the plaques were transferred onto nitrocellulose membranes as described previously [10]. Thereafter, the membranes were probed by acutely *B. microti* Munich strain-infected mice sera, and screening was performed according to the instruction manual for the picoBlue immunoscreening kit (Stratagene, La Jolla, CA, U.S.A.) [13, 14]. A total of 58 positive clones were obtained, isolated and sequenced. A gene that has 95% identities with BMN1-3 of *B. microti* MN1 strain was the most frequently detected (18 clones). The high similarity of these genes suggested that they belong to the same BMN1 family [5, 9] or the identified gene is the homologue of BMN1-3 in *B. microti* Gray strain. A previous study suggested that the BMN1 proteins are orthologue of the mature erythrocyte surface antigens (MESAs) of *Plasmodium falciparum* [9], that are known to be associated with erythrocyte cytoskeleton protein 4.1 and expected to play a role in altering the membrane of the infected erythrocyte [1, 11, 19]. Bioinformatics analyses revealed that the gene contained single open reading frame of 1,107 nucleotides encoding 369 amino acids protein (Fig. 1A). This protein is likely to have a signal peptide (1–23 aa) (http://www.cbs.dtu.dk/services/SignalP/), eight hexapeptide SEAGGP repeats (34–39, 40–45, 46–51, 52–57, 58–63, 70–75, 82–87 and 88–93 aa) and predicted transmembrane domain at the carboxy terminus (352–368 aa). Since the theoretical molecular weight of the protein is 40.7 kDa, without signal peptide, the

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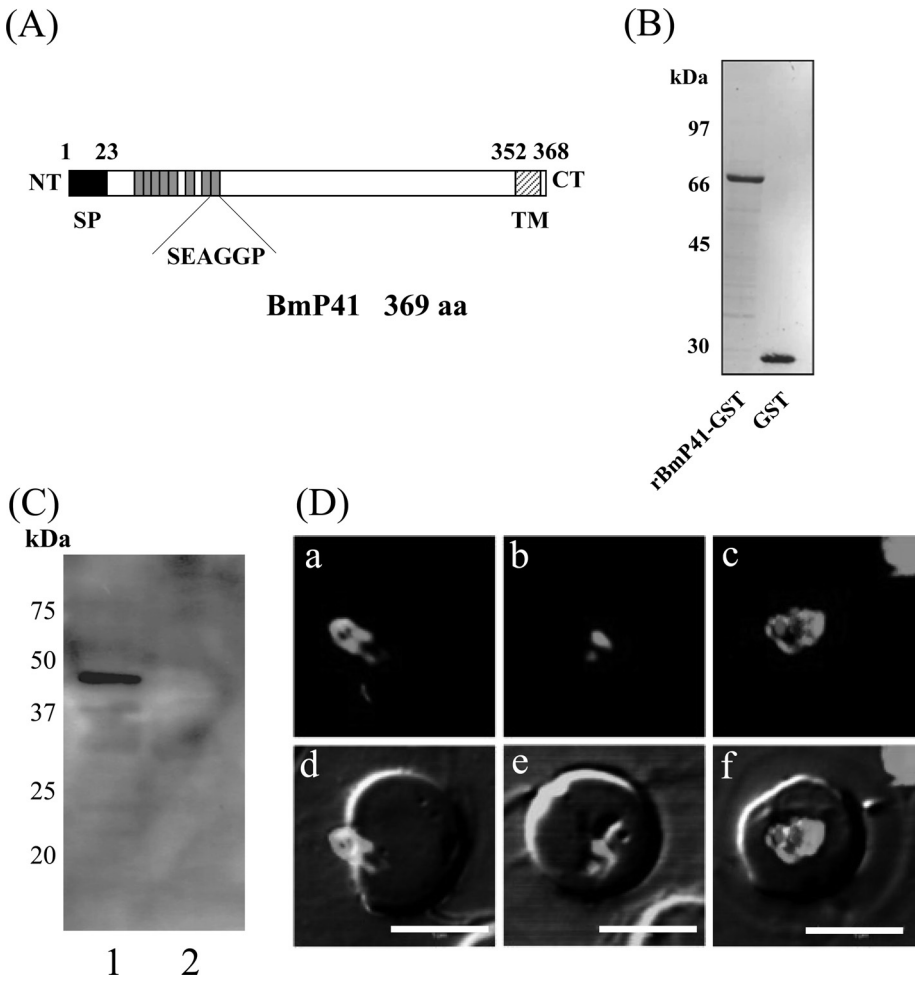

Fig. 1. Molecular characterizations of the *Babesia microti* 41-kDa protein (BmP41). (A) Schematic representation of the protein structure of BmP41. NT, N-terminus; CT, C-terminus; SP, signal peptide (1–23 amino acids); SEAGGP, hexapeptide SEAGGP repeats; TM, transmembrane region (352–368 amino acids). (B) SDS-PAGE analysis of recombinant BmP41 fused with GST (rBmP41-GST). The positions of molecular mass standards are indicated on the left. (C) Western blot analysis of native BmP41 using mouse anti-rBmP41 serum. Lysate of *B. microti* Gray strain-infected hamster erythrocytes (lane 1) and normal erythrocytes lysate (lane 2, negative control) were loaded. The positions of molecular mass standards are indicated on the left. (D) Localization of BmP41 in *B. microti* Gray strain-infected hamster erythrocytes by immunofluorescent staining and confocal microscopy. (a and d) Extracellular parasite; (b and e) Intracellular parasite; (c and f) Dividing form. Panels (a–c), the overlaid image of fluorescent reactivity (green) and propidium iodide (PI) staining of nuclei (red); Panels (d–f), the overlaid image of fluorescent green reactivity and red PI staining on phase-contrast images of the parasites. Bars, 5 μm.

protein was designated as BmP41.

To further characterize the BmP41, the BmP41 gene of *B. microti* Gray strain lacking N-terminal signal peptide sequence was amplified using primer sets: BmP41 w/o SP-F (5′-CAG AAT TCA GTG ATA CCG ATC CCG AAG CT-3′) and BmP41 w/o SP-R (5′-GGC TCG AGC ATC TGG TAT TAC ATC TAT TT G-3′) (the underlined sequences contain *Eco*RI site and *Xho*I site, respectively). The resulting PCR product was cloned into the prokaryotic expression vector pGEX-4T1 (GE Healthcare, Little Chalfont, UK) and expressed in *Escherichia coli* (strain BL21) as a soluble glutathione *S*-transferase (GST)-fusion protein (rBmP41-GST) as reported previously [14]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated a 67-kDa protein of rBmP41-GST, including the 26-kDa GST tag (Fig. 1B). Next, mouse anti-rBmP41 polyclonal serum was prepared as previously described [10] and used to identify the native BmP41 in the *B. microti* parasites. Preparation of antiserum and all animal experiences were performed under the guiding principles for the care and use of research ani-

mals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan. The lysate of *B. microti* Gray strain-infected and normal Syrian hamster erythrocytes was analyzed by Western blotting as previously reported [21]. As shown in Fig. 1C, an about 45-kDa band was detected in *B. microti*-infected erythrocytes (lane 1), but not in normal erythrocytes lysate (lane 2) when probed with anti-rBmP41 serum. The difference in the observed molecular weight and the predicted size (41-kDa) may be due to post-translational modification of eukaryotic cells. To determine the cellular localization of BmP41, *B. microti* Gray strain-infected hamster blood smear was used to perform immunofluorescent antibody test with the anti-rBmP41 mouse serum (Fig. 1D) as previously described [16]. Observation of extracellular and intracellular parasites by a confocal laser microscope demonstrated that BmP41 was expressed in each of the developmental stages of *B. microti* merozoites (Fig. 1D a–f). Notably, the fluorescence signal was consistently observed within the cytosol of parasites.

We evaluated the antigenicity of BmP41 by Western blotting (Fig. 2A). Serum from a hamster infected with experimentally infected with *B. microti* Gray strain recognized the rBmP41-GST in Western blotting, but there was no reaction with GST protein (Fig. 2A-a). Importantly, serum from a mouse infected with *B. microti* Munich strain also reacted with the rBmP41-GST (Fig. 2A-b). Normal hamster serum and normal mouse serum did not react with rBmP41-GST (Fig. 2A c and d, respectively). Next, the antigenicity of the BmP41 was evaluated using an ELISA with *B. microti*- and closely related parasites-infected sera (Fig. 2B) [10, 14]. Sera from *B. microti* Munich strain-infected mice showed a highly reaction as evidenced by high ODs, whereas sera from infected mice with *B. rodhaini*, *Plasmodium berghei* and *Toxoplasma gondii* showed a clear negative reaction (Fig. 2B, lanes 1–5). Notably, *B. microti* Gray strain-infected hamsters also showed highly reaction (Fig. 2B, lane 6). These results indicated that the rBmP41 from the *B. microti* Gray strain could be a common antigen and might be suitable for the detection of different strains of *B. microti*. Our further study is to evaluate the performance of this antigen with sera from rodents infected with other type of *B. microti*(Hobetsu type and Kobe type) and human sera collected from endemic areas.

In summary, we have identified and characterized an antigenic protein BmP41 of *B. microti* by serological screening of cDNA library of *B. microti* Gray strain with sera from acutely infected mice with *B. microti* Munich strain. The results demonstrated that the rBmP41 could be a potential universal diagnostic antigen with a satisfactory diagnostic performance in ELISA. These findings should hopefully provide a basis for future work that could exploit this protein as antigen for the detection of human babesiosis.

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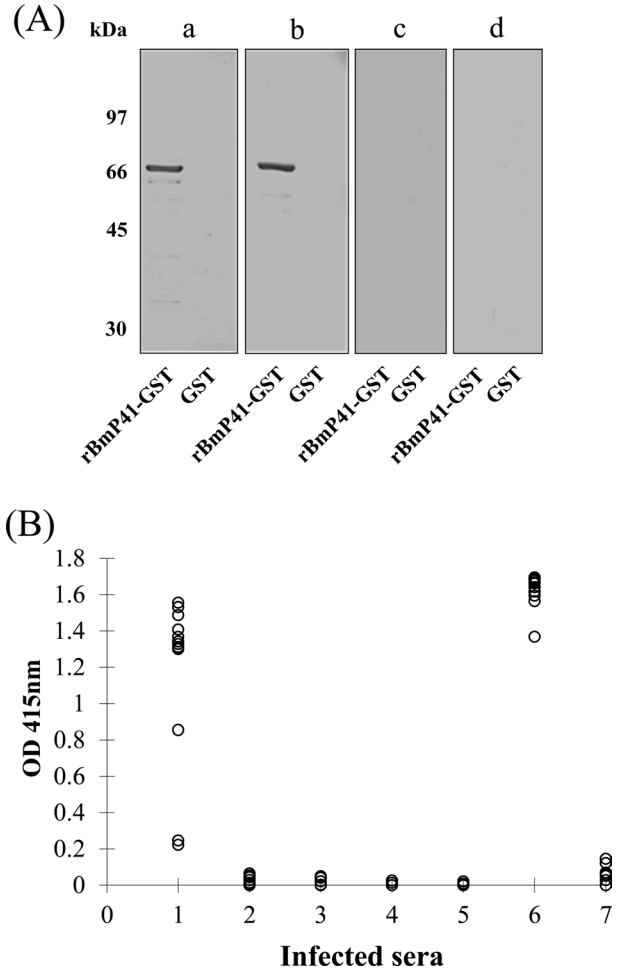

Fig. 2. Evaluation of BmP41 as universal antigen for the detection of deferent *B. microti* strains. (A) Western blot analysis of rBmP41. (a and c) The rBmP41-GST and purified GST were probed with sera from hamster infected with *B. microti* Gray strain (a) and normal hamster (c); (b and d) The rBmP41-GST and purified GST were probed with sera from mouse infected with *B. microti* Munich strain (b) and normal mouse (d). The positions of molecular mass standards are indicated on the left. (B) Values of the ELISA with experimentally infected hamster and mouse sera. Lane 1, sera from *B. microti* Munich strain-infected mice (n=14); lane 2, sera from *B. rodhaini*-infected mice (n=13); lane 3, sera from *P. berghei*-infected mice (n=6); lane 4, sera from *T. gondii*-infected mice (n=5); lane 5, sera from SPF mice (n=10); lane 6, sera from *B. microti* Gray strain-infected hamsters (n=11); lane 7, sera from SPF hamsters (n=7).

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