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Molecular and Immunological Characterization of a Novel 32-kDa Secreted Protein of *Babesia microti*

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ABSTRACT: A cDNA encoding the *Babesia microti* 32-kDa protein was identified by serological immunoscreening of a cDNA expression library and designated as BmP32. The full length of BmP32 contains an open reading frame of 918 base pairs consisting of 306 amino acids having a significant homology with *B. microti* secreted antigen 1. Antiserum raised against recombinant protein (rBmP32) specifically reacted with a 32-kDa native protein of the parasite lysate using western blot analysis. The indirect immunofluorescent antibody test showed a preferable localization of BmP32 in the cytoplasm of the intra- and extracellular parasites. Moreover, BmP32 was secreted in the cytosol of infected erythrocytes, especially during the peak parasitemia and the recovery phase of the infection. Next, the antigenicity of rBmP32 was examined by an enzyme-linked immunosorbent assay (ELISA) and sera from mice experimentally infected with either *B. microti* or closely related parasites. ELISA was highly specific and sensitive when used for the detection of *B. microti* antibody in a mouse model. Furthermore, mice immunized with rBmP32 emulsified with Freund's adjuvant were not significantly protected against challenge infection with *B. microt*i. However, high antibody titer was detected just before the challenge infection. Our data suggest that rBmP32 may be a specific diagnostic antigen but not a subunit vaccine.

Babesia microti is a tick-borne intra-erythrocytic parasite that has recently emerged as a lethal opportunistic pathogen in immunocompromised patients. Human babesiosis, caused by *B. microti*, was first recognized as an endemic disease in North America and has recently surfaced in Europe and East Asia (Homer et al., 2000; Hildebrandt et al., 2007). The parasite normally causes clinical manifestations ranging from an asymptomatic carrier state to malaria-like episodes that could be life-threatening, especially in immunocompromised or elderly patients. Transmission occurs through the bite of infected *Ixodes scapularis* ticks or via blood transfusion from an infected donor (Leiby, 2006). Therefore, asymptomatic carriers are a potential threat to public health and, currently, the Red Cross and other blood donation agencies prohibit people with a history of babesiosis from donating blood (Krause et al., 2003; Krause et al., 2008). The increase in reported fatal cases of *B. microti* infection worldwide has spurred an interest in developing effective diagnostic tools and vaccine strategies that could lead to better management and control of infection (Homer et al., 2000; Krause et al., 2008).

The development of diagnostic tools and subunit vaccines against babesiosis has recently shifted to molecular-defined antigen technology that offers the choice of particular proteins interacting with immune effector cells (Homer et al., 2000; Brown et al., 2006; Terkawi et al., 2011). Although several recombinant proteins of *Babesia* have been identified and evaluated as potential diagnostic and vaccine antigens, results in experimental model have not been satisfactory and, therefore, further research on the discovery of new antigens is extremely desirable (Homer et al., 2000; Brown et al., 2006). In the present study, a novel gene encoding a 32-kDa protein of *B. microti* (BmP32) was identified by immunoscreening. The diagnostic potential of a recombinant BmP32 (rBmP32) protein was evaluated with ELISA technology. In addition, the protective effect of the rBmP32 against challenge infection with *B. microti* was evaluated using a mouse model.

To identify a promising antigen for development of a diagnostic marker or vaccine candidate, a cDNA library of *B. microti* (Munich strain) merozoites (Nishisaka et al., 2001) was screened with pooled sera of mice having an acute *B. microti*-infection; a total of 164 positive clones was obtained, isolated, sequenced, and then subjected to BLAST analysis (Ooka et al., 2011). Two clones that showed 34% identities with BmSA1 of *B. microti* (Luo et al., 2011) were selected for testing; these cDNAs were designated as the BmP32 gene (AB672865). The full length of BmP32 contained an open reading frame consisting of 918 nucleotides, encoding a polypeptide with 306 amino acid residues, having a calculated molecular mass of 34.4 kDa and an isoelectric point of 5.5. The hydrophobic region at the N-terminus of BmP32 had a signal peptide, and the most likely cleavage site was predicted to be between 23 and 24 amino acids. The expected molecular weight of authentic protein without the signal peptide is 32 kDa. A computer analysis of potential subsequence motifs in the BmP32 amino acid sequence revealed the presence of 3 bipartite nuclear localization signals (NLS-BP) distributed across the entire length of the protein (Lys27–Thr44, Lys203–Glu217, and Lys249–Glu263), a lysine rich region (LYS-RICH) extending over amino acid residues Lys27–Lys108, and a remorin C-terminal extending over amino acid residues Lys178–Leu294 (Fig. 1A). The LYS-RICH that was found in BmP32 is also present in a large number of variant erythrocyte surface antigens (VESAs) of *Babesia bovis*. The proteins of the VESAs family are believed to have an important role in the survival of the parasites, discharging into their host cells and mediating the cytoadherence and sequestration of iRBCs (Allred, 2001). Remorins are plant-specific plasma membrane-associated proteins that may have regulatory functions during the pathogen infection due their ability to bind the extracellular matrix component and, possibly, to restrict movement of the pathogen in the plants (Jarsch and Ott, 2011). Further study investigating the function of remorin-protein in *Babesia* should be beneficial in understanding its role in the survival of the parasites. Subsequently, southern blot analysis (Ooka et al., 2011) has revealed that the genomic DNA of *B. microti* contains a single copy of the BmP32 gene (Fig. 1B).

To further characterize BmP32, the cDNA encoding BmP32 but lacking an N-terminus signal peptide was amplified using primer sets: 5′-CCGGATC-CAACGGTGATAAAAAAGA-3′ and 5′-AGCTCGAGTTGTGCTTA-GATTACAC-3′; restriction enzyme sites are underlined (*Eco*RI and *Xho*I, respectively). The resulting PCR product was cloned into the pGEX-4T1 plasmid and then expressed in *Escherichia coli* BL21. Upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a rBmP32 protein fused with glutathione *S* transferase (GST) and having a molecular mass of 58 kDa was detected (Fig. 2, lane 1). Upon western blotting, this recombinant protein reacted with sera of mice experimentally infected with *B. microti* to give a specific band (Fig. 2, lane 3), indicating that the protein is antigenic.

Mouse anti-rBmP32 serum reacted with endogenous protein in the *B. microti* lysate to yield a 32-kDa specific band, a molecular mass consistent with that of authentic or endogenous protein (Fig. 2, lane 5). In contrast, no specific band was observed when the parasites were probed by anti-GST serum used as a negative control in both western blot analysis and indirect immunofluorescent antibody test (IFAT; data not shown). The anti-rBmP32 serum was further used to determine cellular localization of BmP32 in the *B. microti* by IFAT (Fig. 3). Observation of extracellular and intracellular parasites by confocal microscopy demonstrated that BmP32 was expressed in each of the developmental stages of *B. microti* merozoites (Fig. 3A–E). Specific green fluorescence was observed within the cytoplasm of extra-erythrocytic and intra-erythrocytic merozoites for both single and dividing forms (Fig. 3A–C). Moreover, BmP32 was secreted from the parasite cell into the cytoplasm of infected erythrocytes as evidenced by the specific green fluorescence seen within the erythrocyte cytoplasm. This green fluorescence was observed in 5.4–7.8% of infected

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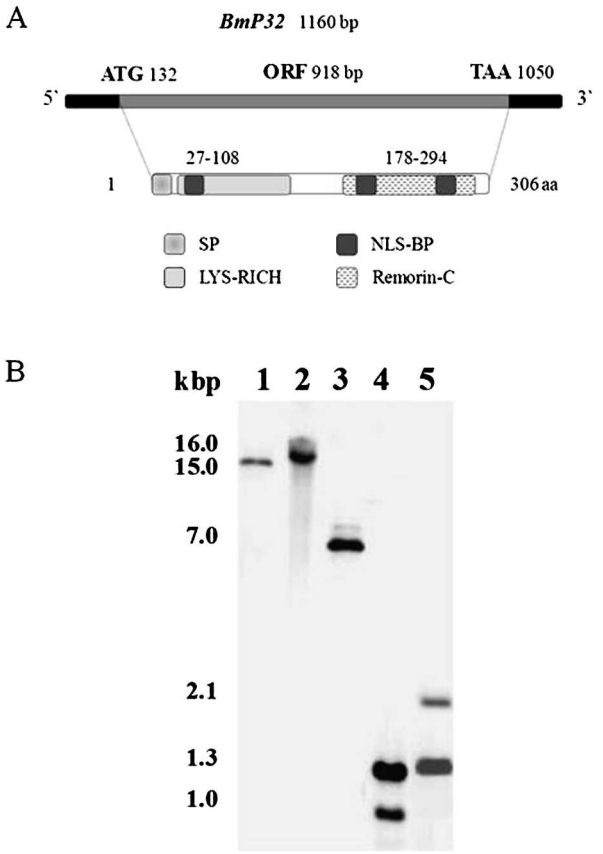

FIGURE 1. Genetic and molecular characterizations of the *Babesia microti* 32-kDa protein (BmP32). (**A**) Graphic depiction of BmP32 (AB672865). The *B. microti* Munich cDNA BmP32 contains a complete open reading frame encoding the predicted protein shown in the bar below, with characteristic structures: a signal peptide (1–23 aa) at the N-terminal, three bipartite nuclear localization signals (NLS-BP), lysine rich region (LYS-RICH), and remorin C at the C-terminal region. (**B**) Southern blot analysis of genomic DNA of *B. microti* digested with different restriction enzymes, *Spe*I (lane 1), *Apa*I (lane 2), *Bam*HI (lane 3), *Acc*I (lane 4), and HindIII (lane 5) and hybridized with the specific probe of BmP32 cDNA.

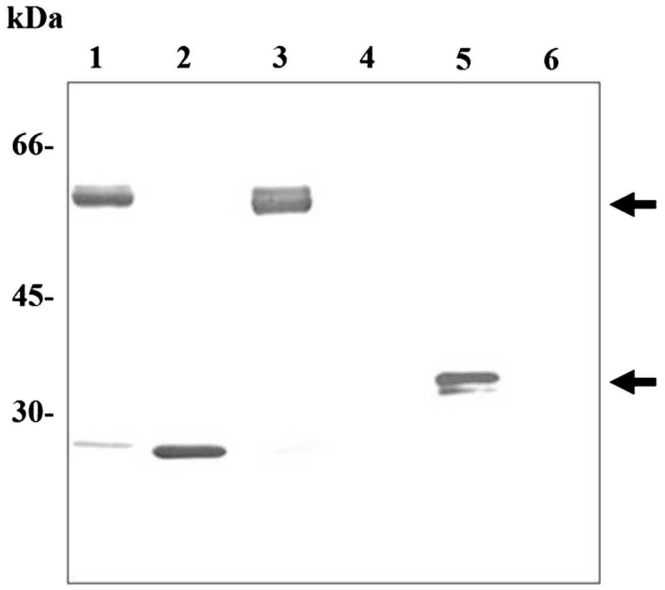

FIGURE 2. SDS-PAGE and western blot analysis of recombinant and native BmP32. The 12% SDS-PAGE stained with Coomassie blue: recombinant BmP32 (lane 1) and GST (lane 2). Western blot of recombinant protein: rBmP32 (lane 3) and GST (lane 4) probed with mouse *B. microti*-infected serum. Western blotting of native BmP32: *B. microti*-infected RBC lysate (lane 5) and normal murine RBC (lane 6) probed with anti-rBmP32 serum. Arrows at the right indicate the recombinant and native proteins.

erythrocytes, particularly at peak-parasitemia and when parasitemia was resolving (Fig. 3D, E). The secreted form of BmP32 was not observed when the blood smears were made from mice at an early stage of the infection (data not shown). The presence of BmP32 within the cytoplasm of infected RBCs was puzzling as to whether this protein can be released into the blood circulation of infected mice. Therefore, western blotting was performed using the plasma obtained from *B. microti*-infected mice with the anti-rBmP32 serum; an apparent band of the 32-kDa protein corresponding to the native BmP32 was observed (data not shown).

In general, the release of *B. microti* proteins into the cytosol and membrane is associated with alteration in the biochemical and morphological characteristics of iRBC, including membrane permeability, rigidity, and deformability (Hutchings et al., 2007). These dramatic changes may maintain the mechanical integrity and stability of the host cells during intracellular parasite replication, preventing RBC lysis and consequent parasite release. The other possible functions of these proteins may be based on the fact that *B. microti* parasites have a high nutritional demand and require an extensive network within the host cells to facilitate protein trafficking, nutrient uptake, and delivery between the parasites and the RBC membrane compartment (Yokoyama et al., 2006). In addition, these proteins may serve as RBC ligands for cytoadhesion in organ capillaries, allowing the iRBC to adhere to endothelial cells of the host's microcapillaries and, subsequently, escape from destruction in the spleen (O'Connor et al., 1999; Hutchings et al., 2007). Therefore, the presence of BmP32 shed within iRBCs may suggest its role in the survival of the parasites, i.e., the acquisition of nutrient from the cytosol of host cells or from extracellular milieu. However, further study, including the elucidation of the precise function of BmP32, could be beneficial for a better understanding of the pathogenesis leading to the development of new therapeutic strategies.

Furthermore, the specificity and sensitivity of rBmP32 was evaluated in a standard ELISA with a variety of experimentally infected and negative-control murine sera (Ooka et al., 2011). The cutoff optical density (OD) value was initially determined based on the OD values of the 20 negative-control sera to be 0.1 (Fig. 4A). Notably, the rBmP32–ELISA succeeded in clearly differentiating between *B. microti*-infected sera (Munich strain) and either the negative-control sera or *Babesia rodhaini*-infected and *Plasmodium berghei*-infected sera (Ooka et al., 2011). Thus, sera from *B. microti*-infected mice exhibited a highly specific reaction, whereas sera from infected mice with closely related parasites revealed a clear negative reaction (OD <0.1). Strikingly, there was no reaction with serum samples from either hamsters infected with the *B. microti* Gray strain or humans infected with the Kobe strain (data not shown). Moreover, rBmP3–ELISA detected a specific antibody response in sequential sera from mice experimentally infected with *B. microti* beginning from 4 days post-infection (PI) until 116 days PI (Ooka et al., 2011), even when the parasites were difficult to detect in stained blood smears (Fig. 3B). The specificity and sensitivity of the current ELISA suggest that rBmP32 can be used as specific serological marker for diagnosis of the *B. microti* Munich strain, which has the potential to infect humans. On the contrary, antigens previously identified from either Gray or Munich strains showed good sero-diagnostic performance with multiple stains of *B. microti* (Luo et al., 2011; Ooka et al., 2011). In addition, an attempt to identify BmP32 from *B. microti* (Gray strain) was made by screening the parasites with specific anti-BmP32 serum. Neither the immunoscreening of the cDNA library nor the IFAT with *B. microti* (Luo et al., 2011) produced a positive reaction (data not shown). These results indicate that BmP32 can be used as a specific serological marker for diagnosis of the *B. microti* Munich strain, which has the potential to infect humans. Further study to investigate the molecular differences among the strains of same species must be made to understand the pathogenesis of the infection.

To evaluate the protective effects of rBmP32 against *B. microti* infection, mice were immunized with antigen followed by 3 consecutive

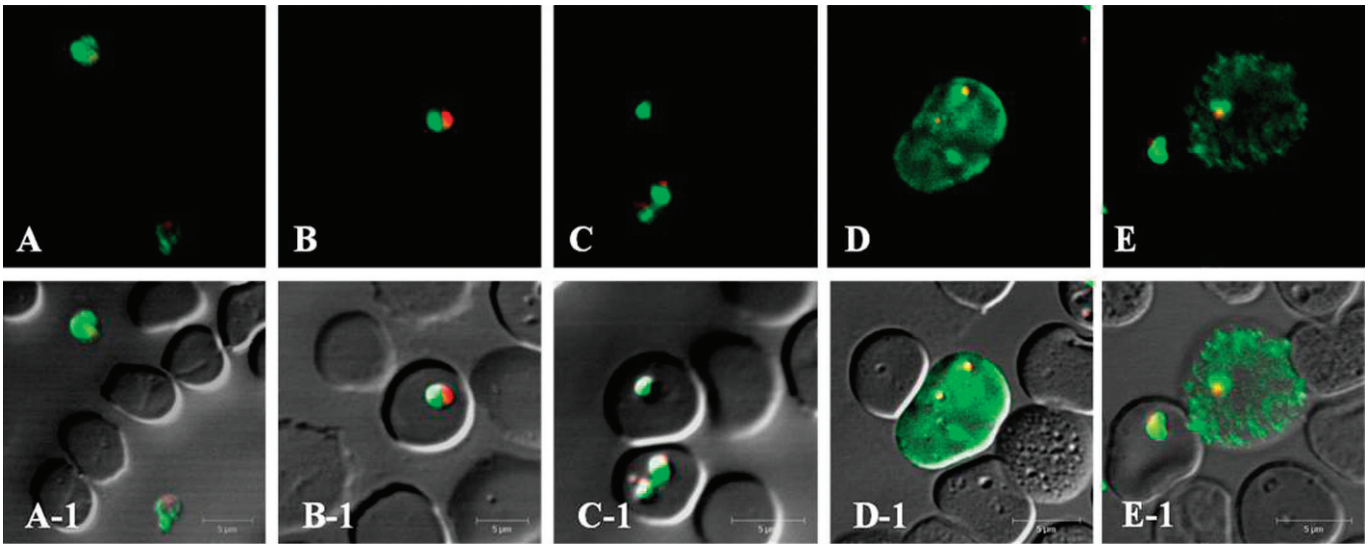

FIGURE 3. Cellular localization of BmP32. Confocal laser microscopic observation of BmP32 in thin blood smears of *B. microti*-infected RBC stained with specific antibody. Anti-rBmP32 (Wako Pure Chemical Industries, Osaka, Japan) was applied as the first antibody on the fixed smears and incubated at 37 C for 1 hr in a moist chamber. After washing with phosphate buffered saline Tween® 20 (PBST) 3 times, Alexa-Fluor® 488-conjugated goat anti-mouse immunoglobin G (IgG) (Molecular Probes, Provo, Utah) was applied as a secondary antibody (1:200) and then incubated at 37 C for 30 min. The slides were washed three times with PBST and incubated with 2.5 μg/ml propidium iodide (Molecular Probes) containing 50 μg/ml RNase (Qiagen, Hilden, Germany) at 37 C for 10 min. After washing with PBS twice, the glass slides were mounted by adding 10 μl of a 50% glycerol–PBS (v/v) solution and covered with a glass coverslip. The slides were examined using a confocal laser scanning microscope (TCS NT, Leica, Heidelberg, Germany). The specific reaction of the antigen is green and the nucleus is red. (**A**) the reactivity of antiserum with extracellular merozoites, and (**B**–**E**) with intracellular *B. microti* merozoites; single and dividing form. Panels (D, E) show the shed form of BmP32 in iRBC. Upper panels (A–E) are the overlaid image of fluorescent green reactivity and red propidium iodide staining of nuclei; lower panel (**A1–E1**) the overlaid image of fluorescent green reactivity and red propidium iodide staining on phase-contrast images of the parasites. Bars = 5 μm.

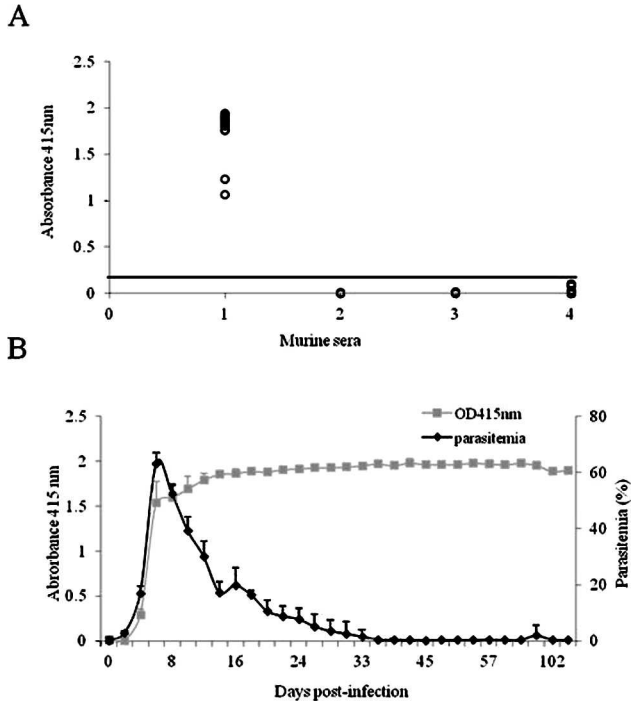

FIGURE 4. Reactivity of rBmP32-iELISA with varieties of murine sera. (**A**) The specificity of iELISA in detecting the infection. Lane 1, *B. microti*-infected mice sera (n = 18); lane 2, *B. rodhaini*-infected mice sera (n = 6); lane 3, *Plasmodium berghei*-infected mice sera (n = 6); lane 4, specific-pathogen-free mice sera (n = 20). (**B**) Sensitivity of rBmP32-ELISA in the detection of the infection in serial sera obtained from mice experimentally infected with *B. microti* (n = 5). Ninety-six microtiter plates (Nunc, Denmark) were coated overnight at 4 C with rBmP32 and GST at a concentration of 0.2 μg/well in a coating buffer (a 50 mM carbonate-bicarbonate buffer, pH 9.6). After blocking with 3% skim milk in PBS for 1 hr at 37 C, the plates were incubated with 50 μl of serum samples diluted 1:100 with the blocking solution for 1 hr at 37 C. The plates were washed 6 times with PBST and then incubated with 50 μl of horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (Bethyl Laboratories, Montgomery, Texas) diluted 1:4,000 with the blocking solution for 1 hr at 37 C as a secondary antibody. After washing, 100 μl of a substrate solution (0.1 M citric acid monohydrate, 0.2 M sodium phosphate, 0.3 mg/ml of 2,2′-azide-bis [3-ethylbenzthiazoline-6-sulfonic acid] [Sigma, St. Louis, Missouri] and 0.01% of 30% H_2O_2) was added and the plates were incubated for 1 hr at room temperature. The optical density (OD) was measured using an MTP-500 microplate reader (Corona Electric, Tokyo, Japan) at a wavelength of 415 nm. The OD values of the GST-background values were subtracted from those of rBmP32. The parasitemia was determined by microscopic examination. Each point represents the mean ± standard deviation.

boosters at 14-day intervals prior to challenge infection with *B. microti*. Notably, BmP32-immunized mice showed high specific antibody response detected by IFAT (titer ≥ 1:51,200) contrary to control mice immunized with GST that showed no reaction (data not shown). Two weeks after the last boost, mice were infected with *B. microti* and parasitemias were monitored for 30 days PI. All mice developed parasitemias as early as day 2 post-challenge infection, with peak-levels being observed at day 8. The mice that received rBmP32 demonstrated similar parasitemia profiles to control mice that received GST or no immunization, with no significant differences between test and control groups (data not shown). These results indicate that the rBmP32 was not protective, with immunization nonetheless inducing high antibody response in mice. Secreted antigens are considered to be potential candidates for vaccine development, as documented in other species of *Babesia* (Shkap et al., 2007). For instance, 4 exo-antigens identified in *B. bovis*, the spherical body protein-1 (SBP-1), cysteine-rich protein (12D3), rhoptry-associated protein-1 (RAP-1), and the high molecular weight antigen (11C5) confer

protection against experimental infection in vivo (Wright et al., 1992; Brown et al., 2006). Interestingly, all 4 antigens have the apical end localization and are secreted in the supernatant of in vitro culture. In these trials, the protection is associated with a rapid activation of memory and effector CD4+ T cells that secrete the IFN-γ needed to activate macrophages and mediate the production of protective antibody upon infection (Homer et al., 2000; Brown et al., 2006). Therefore, the inability of BmP32 to induce sufficient protection against *B. microti* infection is most probably due to the late exposure of protein to the immune cells, resulting in a delay in the activation of CD4+ T cells and macrophages on infection (Brown et al., 2006; Suarez and Noh, 2011). The secreted BmP32 detected in the blood may be derived from the rupture of immature iRBCs only during the peak-parasitemia and recovery phases of the infection. On the other hand, the cytoplasmic localization of BmP32 in the free merozoites may make the target protein inaccessible to immune cells. Further trials with different recombinant forms, immunization regime, and challenge infection time (1 or 2 mo after the last boost to allow the immune response to be completely developed) might be needed to re-evaluate the protective effects of BmP32 in vivo.

In summary, we have identified and characterized a 32-kDa protein that was detected in the cytoplasm of *B. microti* as well as in the cytoplasm of iRBC during the peak-parasitemia and recovery phases of infection. Recombinant BmP32 was a good antigen for serological diagnosis of only the *B. microti* Munich strain and no other. Immunization with the recombinant antigen in Freund's adjuvant showed no significant protective effects against *B. microti* challenge infection, although high specific antibody response was induced. The promising diagnostic performance of rBmP32 can make it a specific serological marker for diagnosis of the infection with *B. microti* Munich strain in rodents and humans.

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