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Title: Babesia gibsoni ribosomal phosphoprotein P0 induces cross-protective immunity against B. microti infection in mice

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Abstract: Babesia gibsoni ribosomal phosphoprotein P0 (BgP0) was identified as an immunodominant cross-reactive antigen with B. microti. The BgP0 gene is a single copy with a predicted open reading frame of 942 bp and 314 amino acids. The serum raised in mice with the recombinant BgP0 showed a specific band with a 34-kDa molecular mass on the extracts of B. gibsoni and B. microti merozoites. As other apicomplexan parasites, BgP0 seems to be located on the surface of merozoites and might be important for the erythrocyte invasion process. Furthermore, we have demonstrated that the recombinant BgP0 could induce cross-protective immunity against B. microti infection in mice. These data suggest that BgP0 is a potentially universal vaccine candidate for both B. gibsoni and B. microti infections.

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Cover Letter

Dear Sir:

We send the paper entitled "*Babesia gibsoni* ribosomal phosphoprotein P0 induces cross-protective immunity against *B. microti* infection in mice" that we would like to submit for publication in the *vaccine Journal*.

We, Alaa Terkawi, Honglin Jia, Yoshifumi Nishikawa, Kozo Fujisaki, and Xuenan Xuan, are the authors of this above manuscript.

In this report, we identified ribosomal phosphoprotein P0 as cross reactive antigen between *B. gibsoni* and *B. microti*, we also evaluated the cross-protective immunity induced by recombinant *B. gibsoni* P0 against challenge infection of *B. microti* in mice.

We, Alaa Terkawi, Honglin Jia, Yoshifumi Nishikawa, Kozo Fujisaki, and Xuenan Xuan, acknowledge that we have read the above manuscript and accept responsibility for its contents. We confirm this manuscript has not been published previously and if accepted in the *vaccine Journal* will not be published elsewhere without the approval of the Editor-in-Chief. We also confirm there are no financial or other relationships that might lead to a conflict of interest. Your consideration of this paper is greatly appreciated.

Our manuscript is contained all contents of regular paper published in *Vaccine journal* including abstract, material and methods, results, discussion, acknowledgment and references.

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Title

Babesia gibsoni ribosomal phosphoprotein P0 induces cross-protective immunity against *B. microti* infection in mice

Running title

Identification of *Babesia gibsoni* P0

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Abstract

Babesia gibsoni ribosomal phosphoprotein P0 (BgP0) was identified as an immunodominant cross-reactive antigen with *B. microti*. The *BgP0* gene is a single copy with a predicted open reading frame of 942 bp and 314 amino acids. The serum raised in mice with the recombinant BgP0 showed a specific band with a 34-kDa molecular mass on the extracts of *B. gibsoni* and *B. microti* merozoites. As other apicomplexan parasites, BgP0 seems to be located on the surface of merozoites and might be important for the erythrocyte invasion process. Furthermore, we have demonstrated that the recombinant BgP0 could induce cross-protective immunity against *B. microti* infection in mice. These data suggest that BgP0 is a potentially universal vaccine candidate for both *B. gibsoni* and *B. microti* infections.

Key words: *B. gibsoni*; *B. microti*; Ribosomal phosphoprotein P0; Cross-reactive antigen.

1. Introduction

Babesia organisms are tick-transmitted intraerythrocytic protozoa capable of infecting a wide spectrum of wild and domestic mammals [4]. The pathogenesis of *Babesia* species results from the asexual erythrocytic stage, during which the parasites invade the host and extensively replicate within the erythrocytes. The parasite normally causes clinical manifestations ranging from an asymptomatic carrier state to malaria-like episodes that could be life-threatening [20]. To date, chemotherapeutics have been reportedly unable to succeed in the elimination of babesial infection [5]. *B. gibsoni* is a canine form of *Babesia* identified to be epidemic in many areas throughout the world, such as Asia, Europe, Africa, and the Americas [5,22,23]. *B. microti* is assumed to be a major etiologic agent of rodent and human babesiosis [15]. Moreover, *B. microti* has been used in model studies with mice to learn more about the mechanisms of babesial infection and the requirements for effective vaccination [18]. Cross reactivity between these parasites has been observed [11]. Based on this observation, the identification of cross-reactive antigens might be important to develop a universal vaccine to control both of these parasites.

Recent advances in the design of a vaccine against babesial infection are based on molecularly defined antigens. One of these molecules is called the ribosomal phosphoprotein P0, a neutral protein related to the family of acidic ribosomal phosphoproteins. P0 has been documented as a multifunctional protein in many cellular processes [26,27], having a regulatory role in DNA repair, cell development, apoptosis, and carcinogenesis [6,12,21]. The multiple roles of the P0 protein in the ribosomes and nucleus and on the cell surface presumably occur through interactions with other protein syntheses [3]. In protozoan parasites, the presence of P0 on the surface of the sexual and asexual stages of the human malarial parasite *Plasmodium*

falciparum [8] induced immunological status against parasites, since the anti-PfP0 antibodies blocked the invasion of merozoites into red blood cells in an *in vitro* culture [8-14] and also conferred protection to mice against *P. yoelii* infection [9]. Interestingly, the anti-PfP0 antibodies reacted specifically with the surface of the *T. gondii* tachyzoite and blocked its invasion into human foreskin fibroblasts [28]. Moreover, the vaccination with recombinant P0 or DNA carrying the P0 gene induced a protective immunity against cutaneous leishmaniasis in mice [16,17].

In the present paper, we report the characterization of BgP0, identified as a cross-reactive antigen with *B. microti*. We also evaluate the cross-protective immunity induced by rBgP0 against a challenge infection with *B. microti* in BALB/c mice.

2. Materials and methods

2.1. Parasites

The *B. gibsoni* NRCPD strain isolated from a hunting dog in Hyogo Prefecture, Japan [19], was maintained in one-year-old splenoctomized beagle dogs [13]. The *B. microti* Munich strain was maintained in BALB/c mice by serial passages. Infections were initiated by an intraperitoneal (i.p.) injection of the infected erythrocytes [18].

2.2. Immunoscreening of cDNA expression libraries

A cDNA expression library constructed from *B. gibsoni* merozoite mRNA was used for immunoscreening [13]. The library was plated at a concentration of approximately 20,000 plaque-forming units (PFUs) per plate to lift plaques. The plaques were transferred to nitrocellulose membranes and then screened with the *B. microti*-infected serum according to the protocol of the picoBlue^{TM} Immunoscreening Kit (Stratagene, San Diego, CA). After an *in vivo* excision, the cDNA inserts in the positive clones were transferred into pBluescript phagemids and then sequenced with

M13 forward, reverse, and internal DNA primers using an automated sequencer (ABI PRISM 310 Genetic Analyzer, USA).

B. microti P0 was obtained from a cDNA library of the *B. microti* merozoite [24] by screening with a specific anti-rBgP0 antiserum, and the gene was then isolated by following the same procedure reported above.

2.3. Southern blotting

Genomic DNA of *B. gibsoni* was extracted from the total erythrocytic stages of the parasite and digested with *Eco*RI, *Xho*I, *Hin*dIII, *Pvu*I, and *Eco*RV overnight. The DNA samples were separated on 0.8% agarose gel and transferred to a nylon membrane (Hybond-N+, Amersham-Buchler, Munich, Germany). The blots were pre-hybridized at 56°C for 4 hrs and probed overnight with the full length of the *BgP0* gene labeled with alkaline phosphatase (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

2.4. Cloning, expression, and purification of recombinant BgP0 (rBgP0)

The entire fragment encoding BgP0 was amplified using a primer set: forward primer 5'-CGAGAATTCATGGCTGGCATGACA-3 and reverse primer 5'-ATTCTCGAGTTTGGCGTACAGGTTG-3. The PCR product was inserted into the pGXE-4T-3 vector (Amersham Pharmacia Biotech, Piscataway, NJ) using the *Eco*RI and *Xho*I sites and expressed in *E. coli* BL21 strain, according to the manufacturer's instructions. Bacterial protein was solubilized in a 2% sacosyl solution, and the protein was then purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Lastly, the GST tag was digested with thrombin (Amersham Biosciences) overnight according to the manufacturer's instructions.

2.5. Production of mice antiserum against rBgP0

Six-week-old DDY mice were immunized intraperitoneally with 100 μg of the purified rBgP0 or GST proteins emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). The mice were boosted later with the same antigens emulsified in Freund's incomplete adjuvant three times at 14-day intervals. Sera were collected two weeks after the last boosting and checked for specific antibodies with the indirect immunofluorescent test (IFAT).

2.6. IFAT and confocal laser microscopic observation

A thin blood smear prepared with *Babesia* parasite-infected red blood cells was fixed with a mixture of methanol and acetone (v:v / 1:4) at -20°C for 20 min. The antiserum against the rBgP0 was applied as the first antibody on the fixed smears and incubated for 30 min at 37°C. After washing with PBS three times, Alexa-Fluor® 488-conjugated goat anti-mouse immunoglobin G (IgG) (Molecular Probes Inc.) was applied as a secondary antibody and then incubated for 30 min at 37°C. The slides were washed three times with PBS and incubated with 2.5 μg/ml propidium iodide (PI) (Molecular Probes Inc.) containing 50 μg/ml RNase (Qiagen, Inc.) for 10 min at 37°C. 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 cover slip. The slides were examined using a confocal laser scanning microscope (TCS NT, Leica, Germany).

2.7. Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described previously [33].

2.8. ELISA

Microtiter plates (Nunc, Denmark) were coated overnight at 4°C with 2 μg/ml of the rBgP0 (removed GST) or BSA using a coating buffer (0.05 M carbonate buffer, pH 9.6). The plates were then blocked with a 3% skim milk solution for 1 hr at 37ºC. After washing, the plates were incubated with dog sera diluted at 1:100 in a 3% skim milk solution and detected with HRP-conjugated anti-dog (1:4,000) visualized with substrate (ABTS). The absorbance values were read at 415 nm using an MTP-500 microplate reader (Corona Electric, Tokyo, Japan).

2.9. Vaccination of rBgP0 and challenge infection with B. microti in BALB/c mice

A total of 24 female BALB/c mice that were approximately 6 weeks old were divided into four groups (n = 6). One hundred fifty μg purified rBgP0 fused with GST-emulsified in Freund's adjuvant was administered to the mice i.p. Subsequently, on days 14, 28, and 42, one hundred μg of the same protein in incomplete Freund's adjuvant (Difco) was injected via the same route. This group was designated as a test group. Following the same immunization regime, control groups received either GST protein or PBS emulsified in adjuvant. One group did not receive any immunization. Ten days after the last boosting, the mice were challenged with 10^7 *B. microti*-infected erythrocytes. Parasitemia was monitored daily for 30 days by examination of Giemsa-stained smears. The means were compared using one-way ANOVA and considered to be significantly different when the P values were $P< 0.05$. An automatic blood-cell counter was used for monitoring the changes of the homological blood values of mice (NIHON KOHDEN, Tokyo, Japan). The antibody responses were confirmed before the challenge infection by IFAT and monitored during the infection course of *B. microti* by ELISA using the rBgP0. The antibody titers were determined

by serial dilution of the sera and then defined as the inverse of the highest dilution factor giving an absorbance of 0.2.

2.10. Nucleotide sequence accession number

The complete gene sequences of *BgP0* and *BmP0* have been submitted to the GenBank with the Accession Nos. AB266721 and AB267377, respectively.

3. Results

3.1. Molecular characterization of a gene encoding BgP0

A total of 40 positive clones were obtained by screening of the *B. gibsoni* cDNA expression library with sera from *B. microti*-infected mice, and 12 clones were identified as the *BgP0* gene. The full length of *BgP0* contains a single open reading frame of 942 nucleotides encoding a polypeptide of 314 amino acid residues, with a calculated molecular mass of 34193.28 Da. The *BmP0* sequence was isolated from *B. microti* cDNA expression library screened with a specific anti-rBgP0 antiserum. The *BmP0* gene contains an ORF that is 936 bp long and encodes a protein with 312 amino acids and a calculated molecular mass of 34066.5Da.

BLAST analysis showed that this gene had very high identity with other apicomplexan parasites, such as *B. bovis* (80% identity), *T. annulata* (67%), *B. microti* (60%), *T. gondii* (60%), *P. falciparum* (58%), *P. berghei* (58%), and *Eimeria tenella* (53%). Accordingly, the homology of the BgP0 is strongly conserved among protozoan P0 proteins. Figure 1 shows the alignment of *BgP0* with those from other apicomplexan parasites.

Southern blot analysis of the genomic DNA probed with the full-length *BgP0* gene demonstrated two hybridizing bands following DNA digestion with *Hin*dIII or with *Eco*RV (0.9 kbp, 2.8 kbp) that cut a single site within *BgP0* (Fig. 2, lanes 5 and

6). On the other hand, the enzymes that cut outside of *BgP0*, *Xho*I, *Eco*RI, *Pvu*I, and *Dra*I yield single hybridizing band (Fig. 2, lanes 1, 2, 3, and 4). These results revealed that the genomic DNA of *B. gibsoni* contains a single copy of the *P0* gene.

3.2. Characterization of BgP0

Recombinant BgP0 was expressed in *E. coli* as an insoluble form, which was solubilized and purified by affinity chromatography using sepharose 4B columns. The molecular mass of the rBgP0 was estimated to be 60 kDa, with 26 kDa of the GST tag. Sera from dogs experimentally infected with *B. gibsoni* could recognize the GST-fused BgP0 in Western blotting and ELISA (data not shown). This result suggested that the rBgP0 expressed in *E. coli* maintained its antigenicity. In addition, anti-rBgP0 antiserum recognized the native BgP0 on the *B. gibsoni* lysate by Western blotting. As shown in figure 3, a specific single band of the 34-kDa protein was detected on the lysates of dog erythrocytes infected with *B. gibsoni* erythrocytes but not in erythrocyte lysates from healthy dogs. The observed molecular size of the native BgP0 is consistent with the expected molecular weight.

To determine the cellular localization of BgP0, the merozoite stage of *B. gibsoni* was assayed by immunofluorescence. Confocal laser microscopic observations revealed that BgP0 might be localized on the surface of the merozoite, as evidenced by the specific fluorescent reactivity observed in the overlaid images and shown in figure 4.

Furthermore, the kinetics of the anti-rBgP0 antibody was detected by ELISA in sera collected from *B. gibsoni*-infected dogs at different stages of infection. As shown in figure 5, the anti-BgP0 antibodies were detectable as early as day 6 post-infection and maintained a high level until 441 days post-infection, when no parasitemia was detectable.

3.3. Cross reactivity of P0 between B. gibsoni and B. microti

The ribosomal phosphoprotein P0 was identified as a cross-reactive antigen between *B. gibsoni* and *B. microti* by immunoscreening. The cross reactivity of P0 between these parasites was illustrated by gene sequence analysis that showed high similarity. The anti-rBgP0 antiserum specifically reacted with the native protein of BmP0 by both IFAT (data not shown) and Western blot analysis. As shown in figure 3, a specific single band of the 34-kDa protein was observed on the *B. microti* lysate. Moreover, the rBgP0 reacted with sera from *B. microti*-infected mice by both Western blotting and ELISA (data not shown).

3.4. Recombinant BgP0 induced cross-protective immunity against B. microti infection in mice

The antibody responses induced by rBgP0 immunization were detected by IFAT (*B. microti* used as an antigen) before the challenge; high IgG antibody titers of 1/1000 to 1/2000 were found in the test mice, but no positive reactions were observed in the control mice. Likewise, the antibody titers and isotypes against rBgP0 were determined by ELISA on days 0, 8, 14, and 20 post-challenge. No specific antibodies were observed in the control groups before challenge, while the total IgG antibody titers showed a gradual increase associated with the progress of infection. The isotypes IgG1 and IgG2a were observed later in the descending and resolution phase of parasitemia (Fig. 6). In contrast, following immunization with rBgP0, the mice showed extremely high titers of all isotypes tested, which continued to gradually increase after challenge with the parasites. No significant differences in the titers of IgG1 and IgG2a isotypes were observed either before or after the challenge. The statistical analyses of the antibody responses revealed that the titers of mice vaccinated with rBgP0 were significantly higher than the titers in the controls either

before or after the challenge. On the other hand, control mice challenged with *B. microti* developed parasitemia as early as 2 days post-challenge and showed a peaks on day 9 post-infection. The averages of the peaked parasitemia of GST-immunized, PBS-immunized, and non-immunized mice were 28.7%, 30.1%, and 39.2%, respectively, as shown in figure 7. On the other hand, the rBgP0-immunized mice had 2-days average delaying of the parasitemia development as compared to controls. Moreover, parasitemia was only observed during the second week post-challenge in 2 test mice. Of note, the rBgP0-immunized mice developed 2 faint peaks of parasitemia on day 9 (4%) and day 18 (3.5%) post-challenge. Statistical analysis revealed that significant differences between test and control mice in the mean of the parasitemia levels ($P<0.05$) were observed. Additionally, the hematological parameters correlated with the parasitemia; thus, the hematocrits (Hct) of controls tended to be lower than those of test mice at the peak of parasitemia (data not shown).

4. Discussion

In the present study, a gene encoding ribosomal phosphoprotein P0 from a cDNA expression library of *B. gibsoni* merozoites was identified using sera from *B. microti*-infected mice. Thus, P0 was characterized as a cross-reactive antigen between both parasites. The *BgP0* gene was present as a single copy in the genome of *B. gibsoni*, with a 942-bp ORF encoding 314 amino acids. The anti-rBgP0 antiserum specifically recognized the 34-kDa native BgP0 by Western blotting.

The florescent reactivity observed by confocal laser scanning microscopy revealed that BgP0 might be localized on the surface of the asexual stage of *B. gibsoni* and could be important for the erythrocyte invasion process of the parasite [8,14]. Although the P0 protein sequence has no signal sequence or transmembrane domain,

the protein can get transported on the surface via an unknown mechanism. Likewise, the P0 protein has been shown to be located on the surface in the asexual stage of *P. falciparum*, *P. yoelii*, and *T. gondii* tachyzoite [9,25,28]. The surface localization of P0 is not limited to parasite cells but includes yeast and mammalian cells [28,30].

The immunogenicity of the rBgP0 was observed through ELISA reactions using *B. gibsoni*-infected sera. The antibody responses against rBgP0 were detectable in the acute and chronic stages of infection. Similarly, P0 was detected as an immunodominant antigen recognized by the serum from animals infected with *Leishmania* [31,33]. Moreover, antibodies against the P0 protein existed in patients suffering from Chagas disease as well as in malaria-immune persons [8,32]. Accordingly, the immunogenic surface BgP0 might be a target for an anti-babesial invasion vaccine.

The *B. gibsoni* ribosomal phosphoprotein has all necessary characteristics for consideration as a cross-reactive antigen with *B. microti.* Therefore, we analyzed the immunogenic properties of BgP0 against *B. microti* infection as a model of study. Following immunization with rBgP0, the mice induced a strong antibody responses consisting of IgG1 and IgG2a isotypes. These mice had delayed the onset of parasitemia and showed significant reductions in its levels as compared with controls. Interestingly, the control mice showed antibody responses against the rBgP0 in the descending phase of parasitemia, at which time there was no predominance of IgG1 or IgG2a. Analysis of the immune responses against rBgP0 immunization showed that test mice developed mixed Th1 and Th2 responses, as deduced from the presence of similarly high levels of IgG1 and IgG2a in the sera [10].

In the *Babesia*-infection course, both humoral and cell-mediated immunities are required for protection [7,15]. The degeneration of parasites inside the erythrocyte

is due to the interaction with the soluble mediators of cell-mediated immunity [1,7,29]. On the other hand, the circulating antibodies can neutralize extracellular merozoites as well as the infected erythrocyte surface antigens, such as VESA1 [2]. In our study, the mixed immune responses induced by a combination of rBgP0 and an adjuvant inhibited the growth of *B. microti* in BALB/c mice. This finding is in agreement with previous studies that demonstrated that the immunization with *Leishmania* P0 induced mixed Th1/Th2 responses and stimulated the production of INFγ, which can be protective against cutaneous leishmaniasis [16,17]. In addition, *P. falciparum* P0 (PfP0) protected the mice against challenge with malaria parasites [25]. The specific anti-PfP0 antiserum could block the growth of parasites in an *in vitro* culture [8,14] and significantly reduced the initial levels of parasitemia and lengthened the survival period against challenge with *P. yoelii* [9].

Our present report might be valuable for developing a potential application for preventing protozoan infection. It is very interesting that molecules identified from *B. gibsoni* based on cross reactivity induced considerable cross-protective immunity against challenge infection with *B. microti* in mice. The unique attributes of such molecules, which stimulate an anamnestic response upon exposure to the parasite itself, might contribute to the realization of an effective vaccine against babesiosis

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Figure legends

Fig. 1. Multiple sequence alignments of the deduced amino acid sequence of the *B. gibsoni* ribosomal P0 protein with those of other apicomplexan parasites.

Fig. 2. Southern blot analysis of genomic DNA of *B. gibsoni* digested with different restriction enzymes, *Xho*I (lane 1), *Pvu*I (lane 2), *Dra*I (lane 3), *Eco*RI (lane 4), *Hin*dIII (lane 5), and *Eco*RV (lane 6), and hybridized with the full length of the *BgP0* gene.

Fig. 3. Western blot analysis of native P0 proteins reacted with mouse anti-BgP0 antiserum. Lane 1, *B. gibsoni*-infected erythrocytes; lane 2, erythrocytes from healthy dogs; lane 3, *B. microti*-infected erythrocytes; lane 4, erythrocytes from healthy mice.

Fig. 4. Localization of the BgP0 protein in *B. gibsoni*-infected RBC preincubated with the anti-rBgP0 antiserum, visualized with Alexa-Fluor® 488-conjugated IgG secondary antibody and PI staining, and finally examined with confocal laser micrographs. The specific reaction of the antigen and anti-rBgP0 antiserum is green and the nucleus is red. (A) Immunofluorescent staining of *B. gibsoni* merozoites. (B) Propidium iodide staining of merozoite nuclei. (C) Phase-contrast images of *B. gibsoni* merozoites. (D) Panels A and B overlaid on panel C.

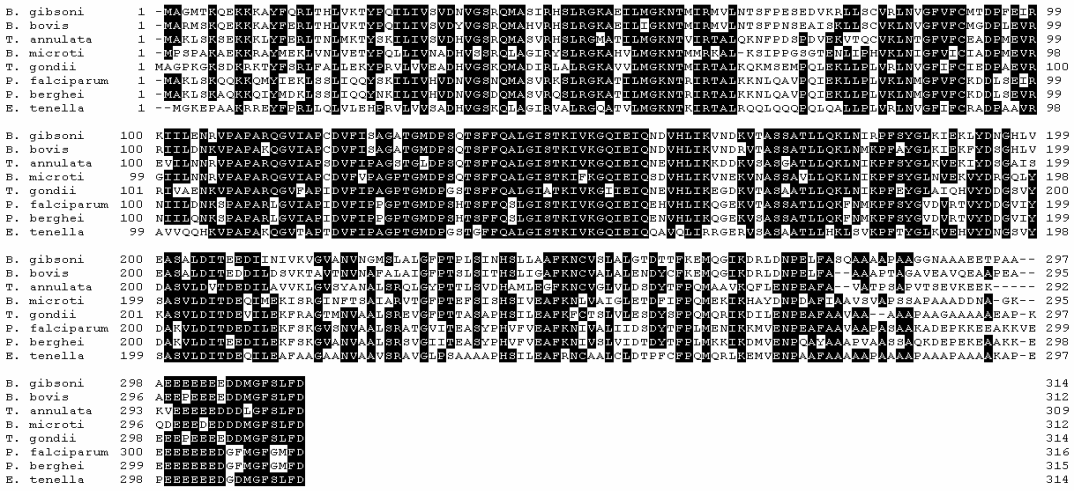
Fig. 5. OD values of IgG detected by ELISA: purified rBgP0 was used as the antigen, and the serial serum samples were obtained from *B. gibsoni*-infected dogs at different stages of infection.

Fig. 6. ELISA determination of antibody titers against the rBgP0 protein following immunization and challenge with *B. microti*. Test mice were immunized with rBgP0 emulsified in an adjuvant, while controls received either the emulsified GST protein or PBS in an adjuvant. One control group did not receive an injection (n= 6). Mice were challenged with 10^7 *B. microti*-infected RBC and monitored daily until 30 days post-challenge. The isotypes (IgG, IgG1, and IgG2a) were evaluated on days 0, 8, 14, and 20 post-challenge.

Fig. 7. Average parasitemia values over a period of 30 days of experimentally challenged mice with *B. microti.* Mice immunized with rBgP0 (A), the GST protein (B), and PBS (C) and non-immunized mice (D) were challenged with *B. microti.* Mice immunized with rBgP0 showed a significantly reduced average of parasitemia compared to the mice in the control groups. *, $P < 0.05$.

Figure(s)

Fig. 1. Terkawi et al., Vaccine.



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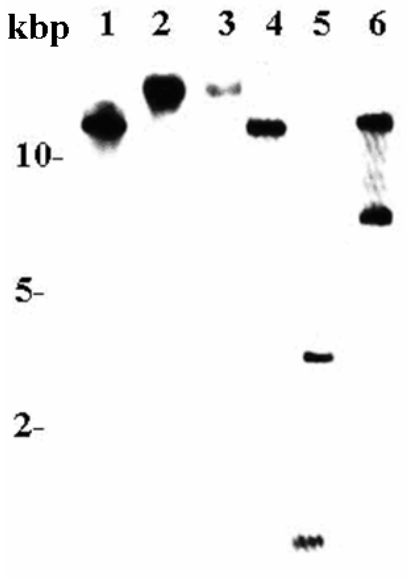

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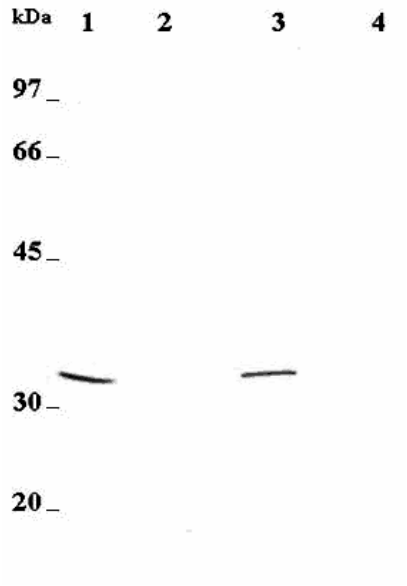

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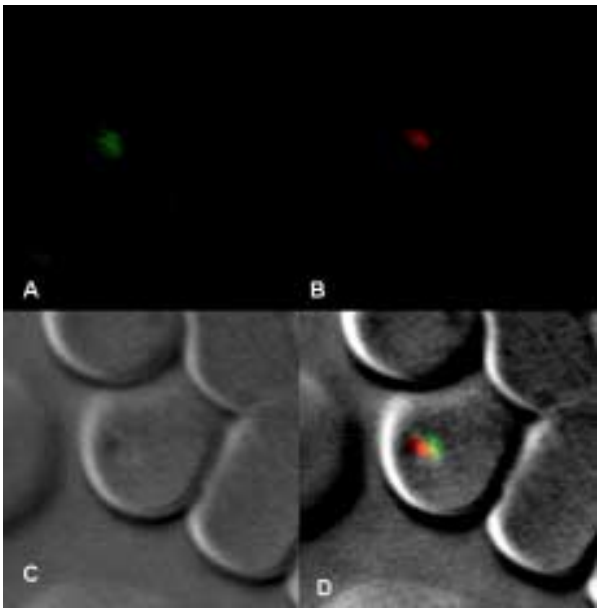

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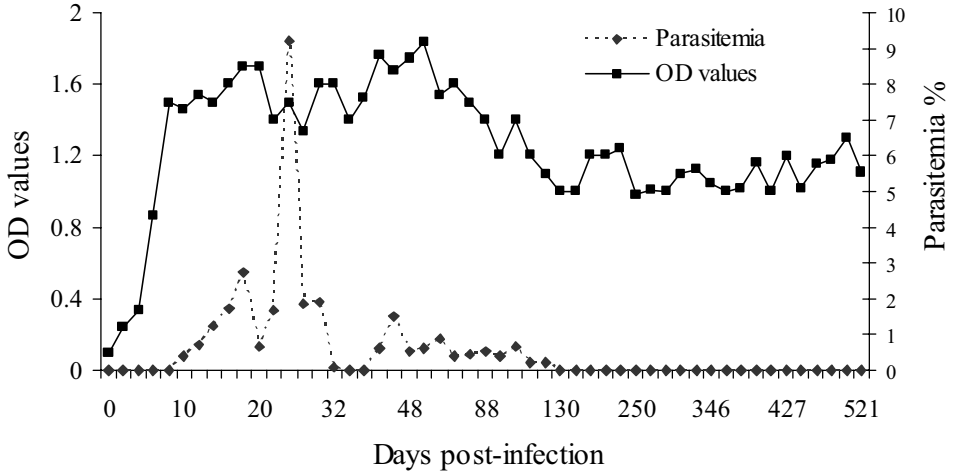

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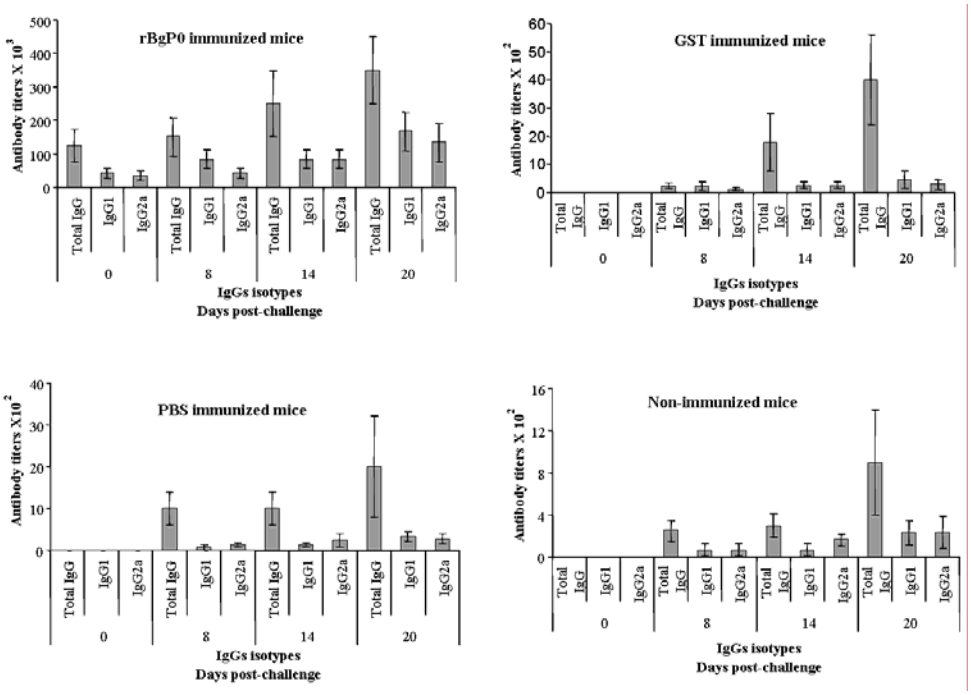

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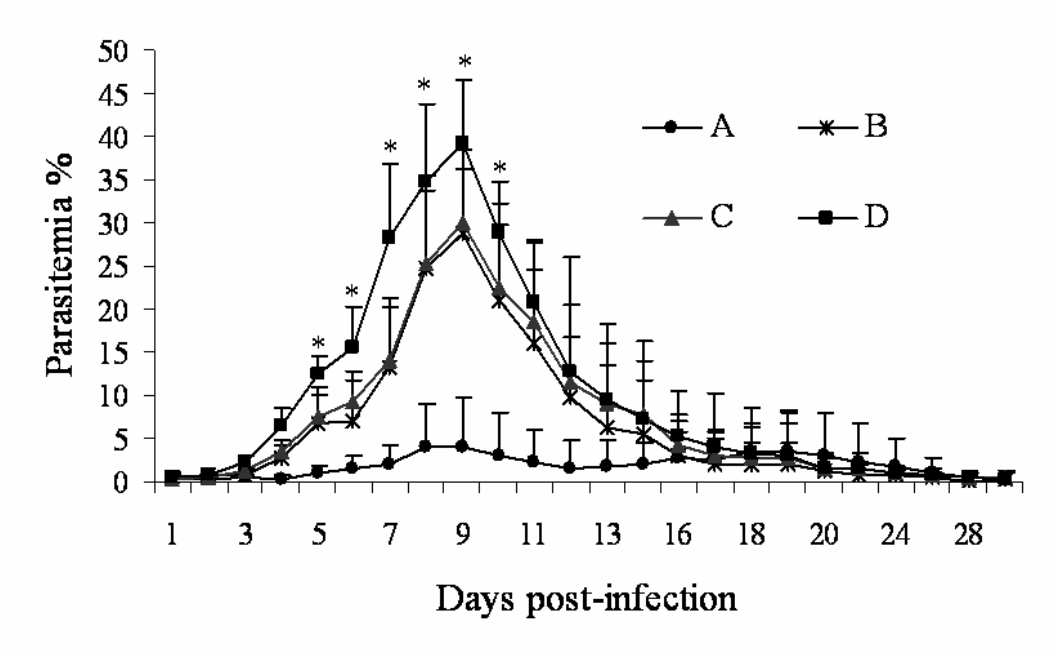

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