J. Protozool. Res. 19, 62-71 (2009)

Expression analysis of a *Babesia bovis* BboP67 gene homologous to the *Theileria parva* p67 gene

Sivakumar, T., Khukhuu, A., Alhassan, A., Rezk Ali Aboulaila, M.,
Iseki, H., Ota, N., Lijia, Z., Yokoyama, N.* and Igarashi, I.
National Research Center of Protozoan Diseases,
Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan
*Correspondence author (Naoaki Yokoyama) National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
Tel.: +81-155-49-5649; Fax: +81-155-49-5643; E-mail address: yokoyama@obihiro.ac.jp

ABSTRACT

A fragment of a novel gene, designated as the BboP67 gene, of *Babesia bovis*, which had been recently reported as a homolog of the gene encoding a sporozoite-specific P67 antigen of *Theileria parva*, was isolated from the genomic DNA of *B. bovis* and expressed as a recombinant (rBboP67) in *E. coli*. The antigenicity of rBboP67 was evaluated using 102 and 68 field serum samples collected from cattle in Ghana and Mongolia, respectively. All of the *B. bovis*- and *B. bigemina*-infected sera, which had been identified by the species-specific antigens, rRAP-1/CTs, for *B. bovis* and *B. bigemina* in the standard ELISA, positively reacted with the rBboP67 in ELISA. While *B. bovis*-infected field serum samples recognized the rBboP67 in Western blot analysis, sera collected from the experimentally infected cattle with *B. bovis* merozoites failed to react to it. IFAT analysis conducted with polyclonal antisera collected from the immunized mice with rBboP67 did not show any positive reaction to the *B. bovis* merozoites. These results indicate that the p67 homolog is not expressed in merozoite stage but is likely expressed in sporozoites; they also suggest that the antigen might be relatively conserved among different isolates of *B. bovis* and, at least, several epitopes of the antigens might be shared between *B. bovis* and *B. bigemina* sporozoites.

Key words: BboP67, *Babesia bovis*, *Babesia bigemina*, sporozoite, *Theileria parva*

INTRODUCTION

Bovine babesiosis is caused by members of the genus *Babesia*, especially, *B. bovis* and *B. bigemina*, and more than one half billion cattle worldwide are at risk of infection (McCosker, 1981). The infectious disease is mainly found in tropical and sub-tropical regions where the intermediate hosts, *Ixodid* ticks, exist (Brown *et al.*, 2006). *Babesia* sporozoites, which are injected into the host by the ticks, directly infect red blood cells (RBC) and then develop into merozoites (Uilenberg, 2006). Multiplication of the merozoites results in the lysis of the infected RBC (Uilenberg, 2006). The clinical symptoms of bovine babesiosis vary, depending on the causative species of *Babesia*. The clinical signs of *B. bigemina* infection are fever, hemoglobinuria, and acute anemia, and parasitemia is often as high as 30% in the infected cattle. On the other hand, in addition to the above clinical signs, cerebral or nervous signs characterize the *B. bovis* infection with lower parasitemia (Shkap *et al.*, 2005). Early diagnosis and proper selection of effective drugs are essential for the successful prevention of the clinical disease, but success is less likely when the treatment is delayed (Vial and Gorenflot, 2006). Therefore, the new development of proper control measures is very important to prevent the disease

and minimize the resultant economic losses.

The control strategies can be divided into three, i.e., vector control, chemoprophylaxis, and vaccination (De Waal and Combrink, 2006). Tick control is becoming more difficult because of the increasing development of acaricide resistance and the global spreading of the infected ticks to new areas due to abnormal climate changes (De Waal and Combrink, 2006). Live attenuated vaccines have been used to control bovine babesiosis in many parts of world (Shkap *et al.*, 2007). However, vaccination failure is not uncommon because of the long use of region-specific strains, which possibly results in vaccine breakthroughs (De Vos and Bock, 2000). In addition, there is a risk of transmission of other blood-borne pathogens during vaccination (De Waal, 1996). Therefore, alternative vaccination strategies, such as the choice of recombinant vaccines, must be explored.

Studies on *Theileria parva*, which is known as a causative agent of the serious east coast fever, indicated that antibodies produced against the sporozoites have a neutralizing activity to prevent the parasite from the subsequent lymphocyte invasion, therefore suggesting that antigens derived from this stage might become vaccine candidates to induce protective immunity against *T. parva* infection (Dobbelaere *et al.*, 1985). Importantly, the P67 antigen, which is well-conserved among different isolates of *T. parva* (Nene *et al.*, 1996), is known to locate on the surface of the sporozoite, and P67 has been studied as a novel vaccine candidate because of its neutralizing activity (Dobbelaere *et al.*, 1984; Nene *et al.*, 1999). Recently, Musoke *et al.* (1992) reported that six of nine cattle immunized with the recombinant P67 antigen were protected against *T. parva* infection when challenged with the sporozoites. A P67 homologous protein of *T. annulata*, called sporozite antigen-1 (SPAG-1), has also been studied as a leading vaccine candidate against *T. annulata* infection (Boulter *et al.*, 1993).

A recent genome project indicated that the genomic sizes and structural features are very similar in *B. bovis* and *T. parva* (Brayton *et al.*, 2007). From the project, a partial fragment of the p67 homologous gene (Accession No XM_001610647) was identified in the genome of *B. bovis* (Brayton *et al.*, 2007). Brayton *et al.* pointed out the possibility that the *B. bovis* homolog might provide a similar vaccine potential against *B. bovis* infection. In our present study, we isolated a fragment of the p67 homologous gene (BboP67 gene) from the *B. bovis* genomic DNA and produced the recombinant protein (rBboP67) in order to evaluate its antigenicity.

MATERIALS AND METHODS

Parasite

The Texas strain of *B. bovis* (Hines *et al.*, 1992) was grown in bovine RBC using a previously established continuous micro-aerophilous stationary phase culture system (Igarashi *et al.*, 1998; Vega *et al.*, 1985). When the parasitemia exceeded 5%, RBC were washed three times with phosphate-buffered saline (PBS) and then stored at -80˚C until use.

Cloning of theBboP67 gene

The genomic DNA of *B. bovis* was extracted from the parasitized RBC as previously described (Yokoyama *et al.*, 2002). The DNA was suspended in a Tris-EDTA buffer and then used as a template for the subsequent PCR amplification. A forward primer, 5´-gggaattcATGGCATTTGCAAAGTTGTC-3´, and a reverse primer, 5´-ctcctcgagCTCCTTGCTTGCCTCGTAG-3´ (the lowercase letters form two restriction sites for *Eco*RI and *Xho*I, respectively), were used to amplify a 1,356-bp fragment of the BboP67 gene. The PCR product was purified by a QIAquick gel extraction kit (Qiagen, Tokyo, Japan) and ligated into a pCR

2.1 plasmid vector using a TA cloning kit (Invitrogen, Carlsbad, CA, USA). The ligated plasmids were transferred to *E. coli* competence cells (Top 10) and then plated on ampicillin-containing LB plates in the presence of X-gal (Wako, Osaka, Japan). After overnight incubation at 37˚C, white clones were collected and cultured in ampicillin-containing LB broths at 37˚C overnight. The presence of the inserted gene in each culture was confirmed by PCR. Finally, plasmids were extracted from the positive cultures by using a QIAprep spin miniprep kit (Qiagen), and an ABI PRISM 3100 Genetic Analyzer confirmed the sequence of the inserted fragment of the target gene (Földvári *et al.*, 2005).

Expression and purification of rBboP67

A 1,356-bp fragment of the BboP67 gene was purified from the cloned pCR 2.1 plasmid by digesting with two restriction endoneucleases, *Xho*I and *Eco*RI, and then ligated into a similarly pre-digested expression plasmid vector, pGEX-4T-1 (GE Healthcare, Uppsala, Sweden). After ligation, the plasmids were transferred to *E. coli* competence cells (BL 21), and PCR positive clones were obtained as described above. For gene expression, 6 ml of the positive culture was added with 400 ml of ampicillin-containing LB broth and incubated at 37˚C. When the optical density (OD) at 600 nm reached 0.5, the plasmid was induced by the addition of IPTG (Wako) in order to synthesize a GST fusion recombinant protein (rBboP67) in *E. coli* by subsequent incubation for 4 h. Finally, the recombinant protein was purified by using glutathione-sepharose 4B (GE Healthcare), as described previously (Smith and Johnson, 1988.).

Polyclonal antibody production

Six-week-old BALB/c mice were used to produce a polyclonal antibody against BboP67. One-hundred micrograms of rBboP67 was injected intraperitoneally with an equal volume of Freund's complete adjuvant (Sigma Aldrich, Saint Louis, MO, USA). A control group was injected with an equal amount of GST with the adjuvant. Subsequently, 50 μg of rBboP67 with an equal volume of Freund's incomplete adjuvant (Sigma Aldrich) was injected on the 14^{th} and 28^{th} day after the primary immunization. Blood was collected from the immunized mice on the 10^{th} day after the last immunization, and sera were obtained and stored at -20˚C until use. ELISA using the rBboP67-coated plates confirmed the full production of a specific antibody against rBboP67 in mice.

SDS-PAGE and Western blot analyses

SDS-PAGE analysis was performed as described previously (Laemmli, U.K., 1970). The purified recombinant protein and total *E. coli* cellular lysate were loaded to the SDS-PAGE. The method described by Towbin *et al.*, (1979) was adopted to transfer the protein to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) for Western blot analysis. Transferred membranes were treated with antibodies and chemicals according to the protocol described by Boonchit *et al.* (2002). Bovine serum samples collected from experimentally infected cattle with *B. bovis* merozoites (Boonchit *et al.*, 2006) and from naturally infected cattle of Ghana and Mongolia and murine serum samples collected from the mice immunized with rBboP67 as described above were used as the primary antibodies for Western blot analyses. The transferred nitrocellulose membranes were blocked overnight with a 3% skim milk solution at 4˚C and then incubated with the primary antibodies (1:100 dilution) for 1 h at 37˚C. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-bovine or murine antibody (Bethyl, Montgomery, TX, USA) (1:5,000 dilution). The reaction was detected by placing the membrane into a substrate solution containing 0.5 mg of diabinobenzidine per ml and 0.005% H_2O_2.

ELISA

For ELISA, 102 and 68 bovine serum samples from Ghana and Mongolia, respectively, (unpublished

samples) were used. Truncated forms of rhoptry-associated protein-1 (RAP-1/CTs) of *B. bovis* (Boonchit *et al.*, 2004) and *B. bigemina* (Boonchit *et al.*, 2006) were produced as GST fusion proteins and subjected to these standard serological diagnoses in ELISA. In addition, ELISA with the rBboP67 produced in the present study was also performed against all of the serum samples according to the same procedure as that for the standard ELISA. In brief, microtiter plates were coated with 5 μg/ml of each recombinant protein using a coating buffer (a 50 mM carbonate-bicarbonate buffer, pH 9.6) and then incubated overnight at 4°C. Subsequently, the plates were blocked with a 3% skim milk solution at 37°C for 1 h. Plates were washed six times with 0.05% Tween 20-PBS (PBST), and 50 μl of 1:100 diluted serum samples was added into each well. After incubation at 37°C for 1 h, plates were washed six times with PBST and added with HRP-conjugated rabbit anti-bovine antibody (Bethyl) diluted to 1:5,000. After incubation at 37°C for 1 h, plates were washed six times with PBST, and positive reactions were detected by adding a substrate solution [0.1M citric acid, 0.2M sodium phosphate, 0.003% H_2O_2, and 0.3 mg of 2,2′-azide-bis (3-etylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma Chemical, St. Louis, MO) per ml]. The OD values were measured at 415 nm using an MTP 500 microplate reader (Corona Electric, Katsuta, Japan). Positive samples were identified on the basis of the cut-off value set by a checkerboard assay using several negative serum samples (Goncalves *et al.*, 1999).

IFAT

In vitro culture-derived *B. bovis*-infected RBC were washed three times with PBS, and the smears prepared on glass slides were fixed in a 50% acetone-50% methanol solution at -20°C for 5 min (Yokoyama *et al.*, 2002). After blocking with a 10% skim milk solution for 30 min at room temperature, slides were incubated with anti-BboP67 murine immune serum diluted to 1:100 with the blocking solution for 30 min at 37°C. Subsequently, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL, USA) diluted to 1:200 was added and incubated at 37°C for 30 min. Between the indicated steps (blocking and the first and second antibody incubations), the slides were washed three times with PBS. Then, 25 μg of propidium iodide (Wako) and 50 μg of RNase A (Roche, Indianapolis, IN, USA) in 1 ml of PBS were added to the slides, incubated for 10 min at 37°C, and then washed with PBS. Slides were finally mounted in a fluorescent mounting medium (Dako, Carpinteria, CA, USA) and observed under a confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). As the positive control, anti-*B. bovis* merozoite surface antigen 1 (MAS-1) murine immune serum (unpublished sample) was used in the IFAT.

RESULTS

Cloning and expression of the BboP67 gene

A 1,356-bp fragment of the BboP67 gene was successfully amplified from the *B. bovis* genomic DNA (Fig. 1A) and then cloned into a plasmid vector. The DNA sequence of the inserted fragment was completely identical to a previously registered one (Accession No. XM_001610647) (Brayton *et al.*, 2007). Next, the BboP67 gene was expressed as a GST fusion recombinant protein in *E. coli*, and approximately 80-kDa rBboP67 was detected in the clarified lysate of transformed *E. coli* cells (Fig. 1B, Lane 1). The SDS-PAGE results of the clarified and purified fractions also showed that the expressed protein was successfully obtained as a highly soluble protein (Fig. 1B, Lanes 1 and 2). This purified rBboP67 was immunized into mice, and the anti-BboP67 immune sera were also successfully obtained. The antisera raised in the mice were confirmed to effectively recognize the rBboP67 in Western blot analysis (Fig. 2, Panel A).

Antigenicity of BboP67

While *B. bovis*-infected field serum samples strongly reacted to rBboP67 in Western blot analysis (Fig. 2, Panel B), serum samples collected from experimentally infected cattle with *B. bovis* merozoites failed to recognize any visible bands (Panel C). On the other hand, *B. bigemina*-infected field serum samples also showed a comparatively lower, but certain reactivity to the rBboP67 in Western blot analysis (data not shown).

Next, rBboP67 was subjected to serological ELISA using a series of field bovine samples collected from cattle living in Ghana and Mongolia. First, all sera were diagnosed using the previously developed, species-specific standard ELISAs (Table 1). In the ELISA with *B. bovis* rRAP-1/CT, 35 of 102 serum samples from Ghana (34.3%) and 23 of 68 serum samples from Mongolia (33.8%) were determined to be positive for *B. bovis* infection. On the other hand, when *B. bigemina* rRAP-1CT was used as the diagnostic antigen for *B. bigemina* infection, 55 (53.9%) and 40 (58.8%) serum samples yielded positive results from Ghana and Mongolia, respectively. Furthermore, 11 Ghana (10.8%) and 5 Mongolian (7.4%) serum samples were positive for both *B. bovis* and *B. bigemina* infections. Next, these field serum samples were analyzed in the rBboP67 ELISA in order to evaluate its reactivities against both infections. Interestingly, the rBboP67-coated ELISA plates positively recognized all of the *B. bovis*- and *B. bigemina*-infected sera from both countries but never reacted with all of the remaining samples that had been negatively diagnosed in the standard ELISAs for both species (Table 1).

IFAT was carried out with the anti-BboP67 and MSA-1 immune sera. Although certain positive results were obtained in the parasite body of the control smear with the anti-MSA-1 immune serum (Fig. 3A), the anti-BboP67 immune serum failed to recognize any antigen in *B. bovis* merozoites (Fig. 3B).

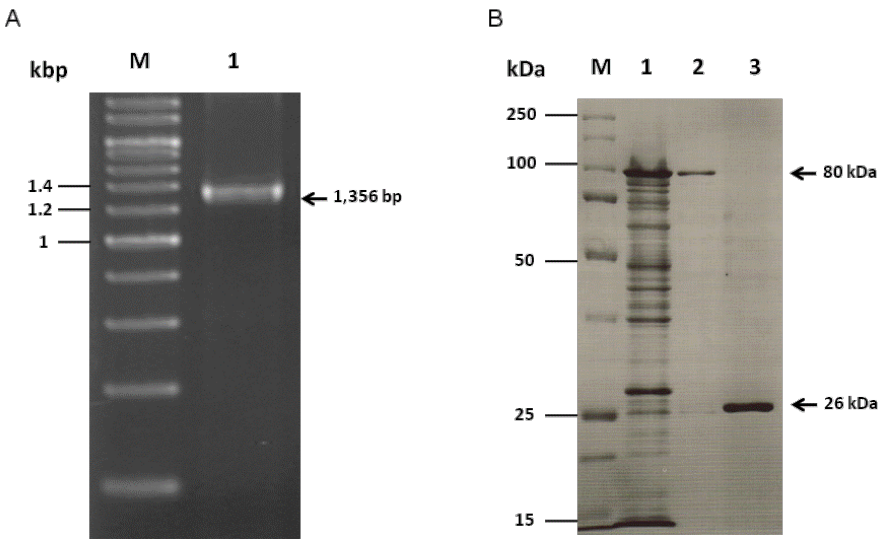

Fig. 1. PCR amplification and gene expression. Panel **A**: PCR amplification of a fragment of the BboP67 gene from *B. bovis* genomic DNA. M, Molecular DNA ladder maker; 1, PCR product derived from the BbpP67 gene. Panel **B**: Expression and purification of a recombinant GST fusion protein (rBboP67) in *E. coli* cells. M, Molecular protein size marker; Lane 1, Protein in soluble clarified fraction; Lane 2, Purified rBboP67; Lane 3, GST.

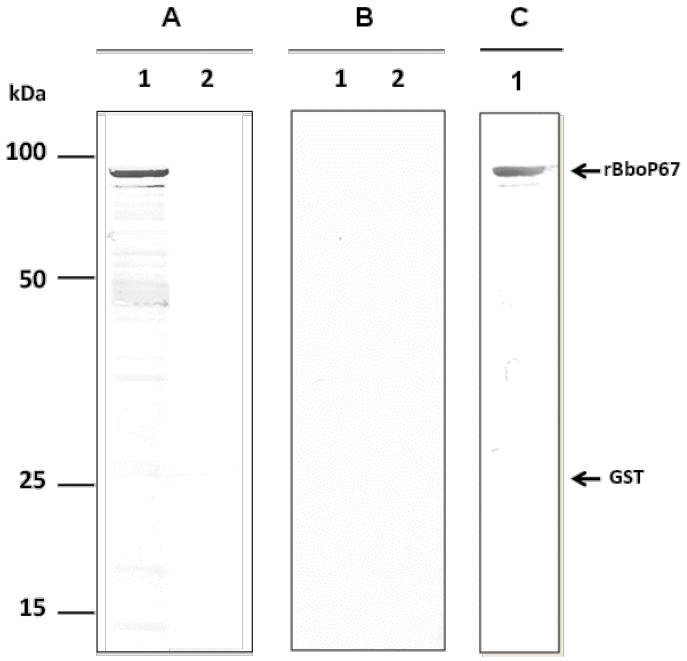

Fig. 2. Western blot analysis of rBboP67. Panel **A**, Treated with immune serum collected from the immunized mice with rBboP67; Panel **B**, Treated with *B. bovis*-infected field bovine serum; Panel **C**, Treated with bovine serum collected from the experimentally infected cattle with *B. bovis* merozoites. Lane 1, rBboP67; Lane 2, GST.

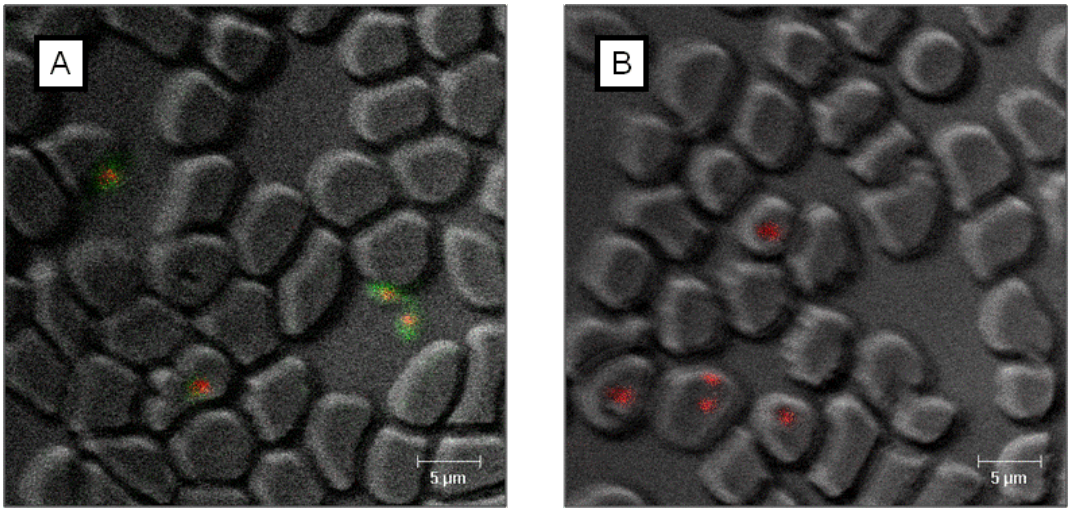

Fig. 3. IFAT. Treated with immune serum collected from the immunized mice with rMSA-1 (Panel **A**) and rBboP67 (Panel **B**). Note the positive reaction with anti-rMSA-1 but not with anti-rBboP67.

Table1 Comparison of the reactivity of rBboP67 ELISA with those of standard serological ELISAs with species-specific rRAP-1CTs for *B. bovis* and *B. bigemina*, by using field bovine serum samples from Ghana and Mongolia.

Country	No of serum samples	No of samples positive for *B. bovis**	No of samples positive for *B. bigemina**	No of samples positive for both species*	No of samples negative for both species*
Ghana	102	35 (34.3%)	55 (53.9%)	11 (10.8%)	23 (22.5%)
BboP67-positive	79 (77.5%)	35 (34.3%)	55 (53.9%)	11 (10.8%)	0 (0.0%)
BboP67-negative	23 (22.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	23 (22.5%)
Mongolia	68	23 (33.8%)	40 (58.8%)	5 (7.4%)	9(13.2%)
BboP67-positive	59 (86.8%)	23 (33.8%)	40 (58.8%)	5 (7.4%)	0 (0.0%)
BboP67-negative	9 (13.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	9 (13.2%)

*rRAP-1CTs of *B. bovis* and *B. bigemina* were used as species-specific antigens for the standard serological ELISAs.

Note: The ELISA with rBboP67 recognized only all of the positive samples for *B. bovis* and *B. bigenima*.

DISCUSSION

The lifecycle of *Babesia* parasites in the vertebrate host begins with the injection of sporozoites by the ticks (Uilenberg, 2006). Sporozoites invade RBC, multiply, and then release the merozoites (Homer *et al.*, 2000). These extracellular merozoites invade RBC again and undergo an asexual reproduction, which results in clinical hemolysis (Vial and Gorenflot, 2006). The invasion process of merozoites is mediated by ligands, such as the rhoptry-associated antigen (RAP-1) (Yokoyama *et al.*, 2002) and merozoite surface antigen-1 (MSA-1) (LeRoith *et al.*, 2006). Therefore, immunization with such ligand antigens was expected to protect the animals from clinical disease. However, cattle immunized with recombinant MSA-1 were not fully protected against the challenge infection with virulent *B. bovis* despite the fact that the produced antibody could neutralize the merozoite infectivity *in vitro* (Hines *et al.*, 1995). In the study involving RAP-1, the monoclonal antibody against RAP-1 reduced the parasitemia only by 20% in an *in vitro* culture of *B. bovis* (Yokoyama *et al.*, 2002). These results suggest the possibility that the inhibition of sporozoite invasion, rather than that of merozoite invasion, might become an alternative vaccination strategy (Yokoyama *et al.*, 2006). This became more evident with the studies conducted with the recombinant P67 antigen of *T. parva*.

In the present study, we cloned a “BBOV_IV007750” gene, which is a homologous gene of *B. bovis* to the p67 gene of *T. parva*. Using an *E. coli* expression system, an approximately 80-kDa GST-fusion recombinant protein, designated as rBboP67, was successfully produced. Although the positive results were obtained in both Western blot and ELISA analyses using *B. bovis*- and *B. bigemina*-infected field bovine

serum samples, the sera collected from the experimentally infected cattle with *B. bovis* merozoites did not show any reactivity in the Western blot analysis. Furthermore, the results of IFAT showed that the anti-BboP67 murine immune serum did not recognize any antigen of the merozoites. RT-PCR analysis was also carried out with the extracted RNA from *B. bovis* merozoites but failed to amplify the target gene (data not shown). These observations strongly suggest that the BboP67 gene of *B. bovis* is not expressed in the merozoite stage of their life cycle, as in the case of the *T. parva* P67 gene (Nene *et al.*, 1996). We presume that the BboP67 gene must be expressed in the sporozoite stage of *B. bovis* in the tick vector and injected cattle. Further studies using *B. bovis* sporozoites collected from the infected ticks are essential to confirm our suggestion.

Positive results obtained in ELISA with the sera collected from two different countries, Ghana and Mongolia, suggest that the BboP67 antigen might be relatively conserved among geographical different isolates of *B. bovis*. Furthermore, the recognition of rBboP67 with *B. bigemina*-infected bovine sera is an interesting finding since it strongly suggests that several immune epitopes of the antigen must be shared between *B. bovis* and *B. bigemina* sporozoites. This suggestion could further strengthen the consideration of BboP67 as a novel vaccine, since immunization with BboP67 might protect cattle from the disease caused by *B. bigemina* as well as *B. bovis*. However, it will be of paramount importance to conduct further studies on the sporozoite expression in different isolates of both *B. bovis* and *B. bigemina* before any conclusion is reached.

ACKNOWLEDGEMENTS

This study was supported by the Program for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), Grants-in-Aid for Scientific Research from the Japan Society for Promotion of Science (JSPS), and the Japan International Cooperation Agency (JICA), Japan.

REFERENCES

Brayton, K.A., Lau, A.O., Herndon, D.R., Hannick, L., Kappmeyer, L.S., Berens, S.J., Bidwell, S.L., Brown, W.C., Crabtree, J., Fadrosh, D., Feldblum, T., Forberger, H.A., Haas, B.J., Howell, J.M., Khouri, H., Koo, H., Mann, D.J., Norimine, J., Paulsen, I.T., Radune, D., Ren, Q., Smith, R.K.Jr., Suarez, C.E., White, O., Wortman, J.R., Knowles, D.P.Jr., McElwain, T.F. and Nene, V.M. 2007. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS. Pathog. 3:1401-1413.

Boonchit, S., Alhassan, A., Chan, B., Xuan, X., Yokoyama, N., Ooshiro, M., Goff, W.L., Waghela, S.D., Wagner, G. and Igarashi, I. 2006. Expression of C-terminal-truncated and full-length *Babesia bigemina* rhoptry-associated protein 1 and their potential use in enzyme-linked immunosorbent assay. Vet. Parasitol. 137:28-35.

Boonchit, S., Xuan, X., Yokoyama, N., Goff, W.L., Waghela, S.D., Wagner, G. and Igarashi, I. 2004. An improved enzyme-linked immunosorbent assay using a C-terminal truncated recombinant antigen of *Babesia bovis* rhoptry-associated protein-1 for the detection of specific antibodies. J. Clin. Microbiol. 42:1601-1604.

Boonchit, S., Xuan, X., Yokoyama, N., Goff, W.L., Wagner, G. and Igarashi, I. 2002. Evaluation of an enzyme-linked immunosorbent assay with recombinant rhoptry-associated protein 1 antigen against *Babesia bovis* for the detection of specific antibodies in cattle. J. Clin. Microbiol. 40:3771-3775.

Boulter, N., Knight, P.A., Hunt, P.D., Hennessey, E.S., Katzer, F., Tait, A., Williamson, S., Brown, D., Baylis, H.A. and Hall, R. 1993. *Theileria annulata* sporozoite surface antigen (SPAG-1) contains neutralizing determinants in the C terminus. Parasite Immunol. 16:97-104.

Brown, W.C., Norimine, J., Knowels, D.P. and Goff, W.L. 2006. Immune control of *Babesia bovis* infection. Vet. Parasitol. 138:75-87.

De Vos, A., and Bock, R.E. 2000. Vaccination against bovine babesiosis. Ann. N. Y. Acad. Sci. 916:540–545.

De Waal, D.T. 1996. Vaccination against babesiosis. Acta. Parasitol. Turcica. 20:487-499.

De Waal, D.T., and Combrink., M.P. 2006. Live vaccines against bovine babesiosis. Vet. Parasitol. 138:88-96.

Dobbelaere, D.A., Shapiro, S.Z. and Webster, P. 1985. Identification of a surface antigen on *Theileria parva* sporozoites by monoclonal antibody. Proc. Natl. Acad. Sci. U. S. A. 82:1771-1775.

Dobbelaere, D.A., Spooner, P.R., Barry, W.C. and Irvin, A.D. 1984. Monoclonal antibody neutralizes the sporozoite stage of different *Theileria parva* stocks. Parasite. Immunol. 6:361-370.

Földvári, G., Hell, É. and Farkas, R. 2005. *Babesia canis canis* in dogs from Hungary: Detection by PCR and sequencing. Vet. Parasitol. 127:221-226.

Goncalves, P.M., Passos, L.M.F. and Ribeiro, M.F.B. 1999. Detection of IgM antibodies against *Babesia bovis* in cattle. Vet. Parasitol. 82:11-17.

Hines, S.A., Palmer, G.H., Jasmer, D.P., McGuire, T.C. and McElwain, T.F. 1992. Neutralization-sensitive merozoite surface antigens of *Babesia bovis* encoded by members of a polymorphic gene family. Mol. Biochem. Parasitol. 55:85-94.

Hines, S.A., Palmer, G.H., Jasmer, D.P., Goff, W.L. and McElwain, T.F. 1995. Immunization of cattle with recombinant *Babesia bovis* merozoite surface antigen-1. Infect. Immun. 63:349-352.

Homer, M.J., Aguilar-Delfin, I., Telford, S.R.3rd., Krause, P.J. and Persing, D.H. 2000. Babesiosis. Clin. Microbiol. Rev. 13:451-469.

Igarashi, I., Njonge, F.K. Kaneko, Y. and Nakamura. Y. 1998. *Babesia bigemina*: *In vitro* and *in vivo* effects of curdlan sulfate on growth of parasites. Exp. Parasitol. 90:290-293.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

LeRoith, T., Berens, S.J., Brayton, K.A., Hines, S.A., Brown, W.C., Norimine, J. and McElwain, T.F. 2006. The *Babesia bovis* merozoite surface antigen 1 hypervariable region induces surface-reactive antibodies that block merozoite invasion. Infect. Immun. 74:3663-3667.

McCosker, P.J. 1981. The global importance of babesiosis. In Babesiosis, Ristic, M., Kreier, J.P. Eds. Academic Press, New York.

Musoke, A., Morzaria, S., Nkonge, C., Jones, E. and Nene, V. 1992. A recombinant sporozoite surface antigen of *Theileria parva* induces protection in cattle. Proc. Natl. Acad. Sci. U. S. A. 89:514-518.

Nene, V., Gobright, E., Bishop, R., Morzaria, S. and Musoke, A. 1999. Linear peptide specificity of bovine antibody responses to p67 of *Theileria parva* and sequence diversity of sporozoite-neutralizing epitopes: Implications for a vaccine. Infect. Immun. 67:1261-1266.

Nene, V., Musoke, A., Gobright, E. and Morzaria, S. 1996. Conservation of the sporozoite p67 vaccine antigen in cattle-derived *Theileria parva* stocks with different cross-immunity profiles. Infect. Immun. 64: 2056-2061.

Shkap, V., de Vos, A.J., Zweygarth, E. and Jongejan, F. 2007. Attenuated vaccines for tropical theileriosis, babesiosis, and heartwater: The continuing necessity. Trends. Parasitol. 23:420-426.

Shkap, V., Leibovitz, B., Krigel, Y., Hammerschlag, J., Marcovics, A., Fish, L., Molad, T., Savitsky, I. and Mazuz, M. 2005. Vaccination of older *Bos taurus* bulls against bovine babesiosis. Vet. Parasitol. 129:235-242.

Smith, D.B. and Johnson, K.S. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene 67:31-40.

Towbin, H., Staechelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 79:4350.

Uilenberg, G. 2006. Babesia – A historical overview. Vet. Parasitol. 138:3-10.

Vega, C.A., Buening, G.M., Rodriguez, S.D., Carson, C.A. and McLaughlin, K. 1985. Cryopreservation of *Babesia bigemina* for *in vitro* cultivation. Am. J. Vet. Res. 46:421-423.

Vial, H.J., and Gorenflot, A. 2006. Chemotherapy against babesiosis. 2006. Vet. Parasitol. 138:147-160.

Yokoyama, N., Suthisak, B., Hirata, H., Matsuo, T., Inoue, N., Sugimoto, C. And Igarashi, I. 2002. Cellular localization of *Babesia bovis* merozoite rhoptry-associated protein 1 and its erythrocyte-binding activity. Infect. Immun. 70:5822-5826.

Yokoyama, N., Okamura, M. and Igarashi, I. 2006. Erythrocyte invasion by *Babesia* parasites: Current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. Vet. Parasitol. 138:22-32.