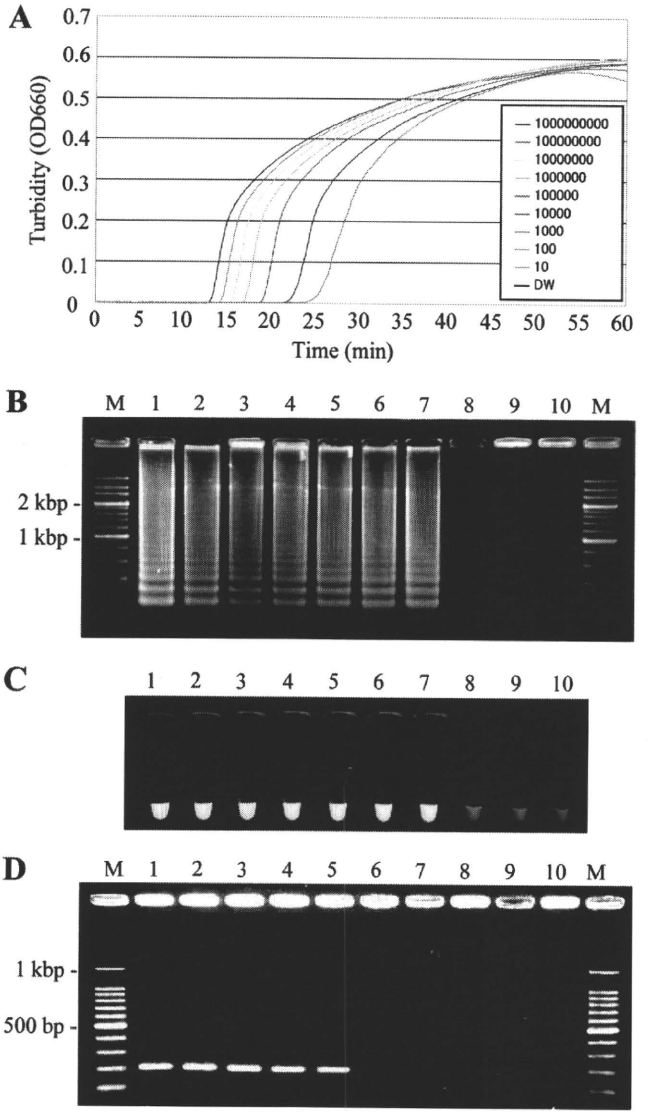

FIG. 3. Comparison of sensitivities of three methods of detection of *Pk*LAMP and conventional single PCR for the detection of the *P. knowlesi* β-tubulin gene. Template DNA was prepared on serial dilutions of plasmid DNA (10^8 copies to 1 copy per reaction) containing a β-tubulin gene for each assay. (A) Real-time LAMP assay monitored by real-time measurement of turbidity. OD_{660}, optical density at 660 nm. (B) Agarose gel electrophoresis of LAMP products. (C) Visual detection of LAMP products under UV light using the Loopamp fluorescent detection reagent. (D) Agarose gel electrophoresis of single-PCR products using the F3 and B3 primers. Lanes M, 200-bp ladder size markers (A) and 100-bp ladder size markers (B); lanes 1 to 9, 10^8 copies to 1 copy of plasmid; lanes 10, distilled water (B to D).

infection. *P. knowlesi* DNA could be detected by *Pk*LAMP throughout the course of infection, while the earliest detection of parasite DNA by the nPCR assay was on the third day after infection (Table 1).

We also compared the amplification efficiencies of *Pk*LAMP and nPCR using frozen whole blood as a template. As shown in Table 1, *Pk*LAMP could amplify the target from whole blood with an efficiency similar to that with DNA extracts throughout the course of infection. These results clearly indicate that *Pk*LAMP could detect even the target DNA from nonpurified whole blood. In contrast, nPCR assay using whole blood from J58 and J64 could amplify parasite DNA only on day 3 and day 9, respectively, when parasite densities were markedly increased in the blood (Table 1).

DISCUSSION

The diagnosis of malaria at regional clinics in areas of endemicity has been performed mainly by microscopic examination of blood smears because of its ease and rapid application. However, the morphology of the asexual stages of the zoonotic simian *Plasmodium* parasites substantially resembles that of human parasites, particularly on thick blood films, and laboratory technicians are trained to recognize only the four species of human parasites (16). In fact, numerous human cases of *P. knowlesi* infection have been misdiagnosed by microscopy as *P. malariae* due to their morphological similarities (4, 5, 16). The application of DNA amplification to the diagnosis of malaria can solve these problems. Amplification of parasite DNA using a specific PCR has been applied to various *Plasmodium* species, including four human malarial parasites and *P. knowlesi* (5, 15, 16). However, despite the excellent specificity and sensitivity of PCR and real-time PCR, these methods require complicated procedures and sophisticated instrumentation such as a thermal cycler, and they are often impractical under conditions requiring field diagnosis. In this regard, the LAMP method has the advantages of simplicity, specificity, and sensitivity compared to other molecular diagnostic methods. Thus, the LAMP method is a promising candidate for wide use in regional clinics and under field conditions.

In the present study, we successfully developed a LAMP method for detecting *P. knowlesi* infection, using a primer set that targets the β-tubulin genes of parasites. The specificity of the primers was evaluated using nine species of simian malaria parasites and four species of human malaria parasites. The results showed that the primer set for *Pk*LAMP amplified only the autologous DNA samples of *P. knowlesi* in typical ladder bands. In contrast, no ladder bands were obtained from any other control. These findings indicate that this primer set is specific for *P. knowlesi* and can be used to examine for *P. knowlesi* malaria as well as to distinguish between it and other types of malaria. The sensitivity of the test was evaluated, and the results showed that *Pk*LAMP was 100-fold more sensitive than single-PCR assay using the F3 and B3 primers. Moreover, the present study showed that an isothermal reaction time of 1 h was enough to amplify 10^9 copies of the target DNA in reaction tubes containing from 10^8 to 10^2 copies/μl of the DNA template and that results could be easily judged by visual inspection of the turbidity or fluorescence of the reaction mixture (10, 13). These results suggest that the *Pk*LAMP assay is reliable and useful for the diagnosis of *P. knowlesi* malaria.

To evaluate the feasibility of using *in vivo* materials, comparisons of *Pk*LAMP and the conventional nested PCR method and microscopic examination were made with blood samples from two infected monkeys. These studies validated *Pk*LAMP as an alternative molecular diagnostic tool, which can be used in the diagnosis of early and advanced infections of *P. knowlesi*. Early species identification in the diagnosis of malaria is very important in preventing disease progression. In particular, early

TABLE 1. Comparison of *Pk*LAMP with nPCR and microscopic examination for detection of *P. knowlesi* in two infected monkeys[a]

Day after infection	Monkey J58					Monkey J64				
	Parasitemia (%)	*Pk*LAMP result with:		nPCR result with:		Parasitemia (%)	*Pk*LAMP result with:		nPCR result with:	
		DNA extract	Whole blood	DNA extract	Whole blood		DNA extract	Whole blood	DNA extract	Whole blood
0	−	−	−	−	−	−	−	−	−	−
1	<0.01	+	+	+	−	−	+	+	−	−
2	0.2	+	+	+	−	−	+	+	−	−
3	10.8 (autopsy)	+	+	+	+	−	+	+	+	−
4						−	+	+	+	−
5						−	+	+	+	−
6						0.01	+	+	+	−
7						0.1	+	+	+	−
8						2.0	+	+	+	−
9						58.0 (autopsy)	+	+	+	+

[a] Monkeys J58 and J64 were infected with *P. knowlesi* strains H and Hackeri, respectively.

identification of *P. knowlesi* infection is essential, since the unique 24-h asexual replication cycle among human and simian malaria parasites can rapidly result in high levels of parasitemia with a fatal outcome in humans (4, 5). Although nPCR and sequencing have been applied to species identification for malaria diagnosis, a more rapid diagnostic test such as *Pk*LAMP would be a convenient and powerful tool for enabling the delivery of prompt and adequate medical treatment.

The present study also assessed the detection performance of *Pk*LAMP with different DNA template preparations, including frozen whole blood or genomic DNA extracts. The detection efficiency of *Pk*LAMP using whole blood was identical to that of *Pk*LAMP when gDNA extracts were used as the template. However, the detection performance of nPCR using the whole-blood templates was quite poor. It appears that this is due to blood components, such as myoglobin, hem-blood protein complexes, and immunoglobulin G, that inactivate the *Taq* DNA polymerase used in standard PCR (1). In contrast, such inhibitors do not affect the *Bst* polymerase used in LAMP (6). According to previous reports, the specificity and sensitivity of detection appear to be unaffected by LAMP processing conditions or sample type, including whole blood, filter paper- or card-processed blood, serum, sputum, and crudely processed tissue samples (8). Furthermore, Poon et al. have reported that *P. falciparum* DNA was detected by LAMP using a promising simple DNA template method of preparation from heat-treated blood (14). Further improvement of template production methods for *Pk*LAMP will be required to optimize and simplify template preparation.

In conclusion, *Pk*LAMP can be considered an efficient candidate for the molecular diagnosis of *P. knowlesi* infection in areas of endemicity. Thekisoe et al. reported that LAMP reagents are stable at ambient temperature for up to 2 weeks (16a). In addition, a recent study of the LAMP method showed that it is able to detect both *Plasmodium* oocysts and sporozoites from an "all-in-one" template using whole mosquito bodies (2). These observations further emphasize the potential usefulness of the LAMP method as a diagnostic and new epidemiological surveillance tool for malaria. Our studies will also provide a powerful method for the diagnosis and monitoring of *P. knowlesi* infection in the field.

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Short communication

Artesunate, a potential drug for treatment of *Babesia* infection

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ABSTRACT

The effects of artesunate, a water-soluble artemisinin derivative, against *Babesia* species, including *Babesia bovis*, *Babesia gibsoni* and *Babesia microti* were studied. Cultures of *B. bovis* and *B. gibsoni* were treated with 0.26, 2.6, 26 and 260 μM artesunate, showing inhibition of parasite growth at concentrations equal to and greater than 2.6 μM artesunate by days 3 post-treatment for *B. gibsoni* and *B. bovis* in a dose-dependent manner. Consistent with *in vitro* experiments, artesunate was effective in the treatment of mice infected with *B. microti* at doses equal to and greater than 10 mg/kg of body weight on days 8–10 post-infection. Taken together, these results suggest that artesunate could be a potential drug against *Babesia* infection.

Babesiosis is a parasitic disease caused by intraerythrocytic protozoa of the genus *Babesia* and transmitted by ticks to their vertebrate hosts. The disease is recognized to be of veterinary importance in cattle, horses, and dogs and is highlighted as an emerging zoonosis in humans. Symptoms can include a malaria-like syndrome, including fever, haemolytic anemia, and hemoglobinuria, and clinical cases appear suddenly and severe [1]. There are a number of babesiacides, but only a few drugs are currently available such as imidocarb dipropionate (Imizol®, Schering-Plough Animal Health) and diminazene aceturate (Berenil®, Intervet India Pvt. Ltd.) for animals, such as cattle, horses, and dogs, and quinine, clindamycin and atovaquone (Mepron®, Glaxo Welcome) for humans [2]. However, an increasing number of resistant parasites to commercial drugs are appearing, adverse effects of drugs are well documented, and the long term persistence of low level parasitemia after treatment still necessitate development of an effective treatment.

Artemisinin and its derivatives, such as artesunate, artemether, arteether, and dihydroartemisinin, are the most potent antimalarial drugs available throughout the world [3]. The artemisinin derivatives act rapidly on the parasites leading to their quick elimination thereby rendering these derivatives effective against severe malaria. Furthermore, parasites are slow to develop resistance and these derivatives exhibit high efficacy against all asexual stages of *Plasmodium falciparum* with rare adverse effects [4–7].

Among artemisinin derivatives, artesunate, a water-soluble half-ester succinate derivative, has been the most commonly used derivative for more than 15 years; many clinicians feel that parenteral administration of artesunate is the most effective treatment for severe malaria [8,9]. Since *Babesia* species share a similar life cycle, as well as clinical symptoms, with *Plasmodium* species, coupled with the previously observed growth-inhibitory effect of artesunate on *Babesia* (*Theileria*) *equi* and *B. caballi in vitro* and on *B. microti* in hamster [10,11], we tested whether artesunate inhibited the growth of other *Babesia* species. In previous studies, the significant growth-inhibitory effects of atovaquone and diminazene aceturate were shown on *B. microti* and *B. divergens* and on *B. rodhani*, *B. divergens* and *B. bovis*, respectively [12–18]. Therefore, atovaquone and diminazene aceturate were used to compare an efficacy of artesunate against *Babesia* parasites. With this in mind, we evaluated the efficacy of artesunate against *B. bovis* for cattle and *B. gibsoni* for dogs *in vitro*, and *B. microti* for mice and humans *in vivo* and compared these growth-inhibitory effects with those of currently available drugs, such as atovaquone and diminazene aceturate.

For *in vitro* assays, solutions of 156 mM artesunate (Guangxi, China), 26 mM atovaquone (Toronto Research Chemical Inc., Canada) and 260 mM diminazene aceturate (Kanto Chemical Co., Inc., Japan) added to growth media were prepared. The Texas T2B strain of *B. bovis* and NRCPD strain of *B. gibsoni* were maintained in bovine and canine RBC as previously established methods [19,20]. The *in vitro* growth-inhibitory assay was carried out in 48-well tissue culture plates by modified methods described previously [10]. Initial *Babesia* parasite cultures containing 1% infected erythrocytes were prepared from cultures that had reached 3 to 5% parasitemia by mixing with

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uninfected bovine and canine RBC. To each well, 50 μl of infected erythrocytes were added to 450 μl of growth medium containing either 0.26, 2.6, 26 and 260 μM of one of the drugs (artesunate, atovaquone or diminazene aceturate). Evaluation of growth-inhibitory effects of each drug and concentration was performed for each parasite species and monitored in triplicate and in three independent trials. Culture plates were kept in a humidified 5% CO_2 incubator at 37 °C. Per well, 250 μl of the culture medium with the indicated concentration of drug was replaced daily for 4 days. Thereafter, to demonstrate whether the inhibitory effect was maintained after withdrawal of treatment, parasite cultures were subcultured with uninfected bovine and canine RBC as described above and parasite re-growth was monitored for another two days. Parasitemia in Giemsa-stained culture smears was calculated from eight to ten microscopic fields covering approximately 2000 cells.

B. bovis and *B. gibsoni* were grown *in vitro* beginning at 1% parasitemia in the presence of the aforementioned concentrations of artesunate, atovaquone and diminazene aceturate, and parasitemia was compared to a control. Statistical significance between the mean parasitemia of a control group and that of each group treated with drugs was determined using one-way ANOVA and Tukey's tests using the JMP Version 8 Program (SAS Institute Inc., USA). Beginning at day 2 post-treatment significant growth inhibition ($P<0.05$) of *B. bovis* was observed in groups treated with equal to and greater than 2.6 μM artesunate (Fig. 1A). Moreover, this growth-inhibitory effect was maintained in equal to and greater than 2.6 μM artesunate even after withdrawal of the treatment at day 4. Upon comparison of the growth-inhibitory effect against three drugs on *B. bovis*, it appears that artesunate may be moderately effective than atovaquone but less than diminazene aceturate at day 4 post-treatment (Fig. 1A versus 1B and C, respectively). These results could be explained by their mechanisms acting to parasites. Diminazene aceturate binds to the AT-rich domains of the DNA double helices, which leads to an interference with the activities of the eukaryotic type II topoisomerase enzyme and finally causes death of parasites [21,22]. On the other hand, atovaquone suppresses electron flow in mitochondrion of parasites by inhibition of binding between ubiquinone and cytochrome bc_1 [23]. In addition, artesunate inhibits sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) responsible for the maintenance of calcium ion concentrations, which is related to the generation of calcium-mediated signaling and the invasion of parasites to erythrocytes, thereby inhibiting the parasite growth [24].

Artesunate was also found to be effective against *B. gibsoni*, where significant growth inhibition ($P<0.05$) was observed at 26 and 260 μM from day 1 post-treatment. Moreover, this significant difference was observed in all test concentrations at day 3 post-treatment (Fig. 2A). Furthermore, upon withdrawal of the treatment, reemergence of the parasite failed to occur in concentrations equal to and greater than 2.6 μM artesunate. As with *B. bovis*, artesunate was found to be less effective than diminazene aceturate in suppressing the growth of *B. gibsoni* (Fig. 2). Regarding the efficacy of artesunate and atovaquone, while 2.6 μM artesunate could rather effectively inhibit parasite growth than 2.6 μM atovaquone, 26 μM atovaquone was more effective than 26 μM artesunate on *B. gibsoni*. Therefore, it is difficult to conclude which drug is more effective in the growth inhibition of *B. gibsoni*.

The growth inhibition of both *B. bovis* and *B. gibsoni* exhibited a dose dependence and therefore the half maximal inhibitory concentration (IC_{50}) for each parasite was calculated as the concentration required for a 50% reduction in the mean parasitemia of drug-treated groups by a comparison with that of control groups at day 4 post-treatment. The IC_{50} was calculated using non-linear curve-fitting of the percent inhibitions against various concentrations of three drugs by a calculation software (Sigma Plot, Japan). Although the IC_{50} values for diminazene aceturate (*B. bovis*, 24.82 ± 2.37 nM; *B. gibsoni*, 41.93 ± 2.32 nM) suggested this drug to be more effective than artesunate (*B. bovis*, 372.2 ± 24.32 nM; *B. gibsoni*, 924.0 ± 97.26 nM) in treatment of both *B. bovis* and *B. gibsoni*, previous attempts at using diminazene aceturate for treatment of babesiosis failed due to its toxicity to kidney, brain, and liver which can result in serious side-effects such as weakness, paralysis, lack of responsiveness to stimuli in the central nervous system especially in dogs as well as humans [25–27]. Moreover, due to these side-effects, the diminazene aceturate was recently withdrawn from the market in Japan, and this drug is not approved by the Food and Drug Administration (FDA) in the U.S.A. [28]. In contrast to diminazene aceturate, few significant side-effects of artesunate have been reported in more than two million patients treated with artesunate [3,6]. Therefore, artesunate could be a preferential choice for the treatment of *B. bovis* and *B. gibsoni* based on these results.

In order to determine anti-babesial effects of artesunate against *B. microti*, female 6-week-old BALB/c mice (Japan CLEA, Japan) were used in an experimental infection study. Moreover, atovaquone was tested in parallel on mice infected with *B. microti* as a currently available anti-babesial drug in order to compare its anti-babesial effects with artesunate. Infection was initiated by intraperitoneal (i.p.) injection of 1×10^7 *B. microti* Munich strain infected erythrocytes isolated from a mouse with 40.7% parasitemia. Infected mice were divided into 8 groups as follows: control groups were either administered 5% sodium bicarbonate (SB) for artesunate or phosphate buffered saline (PBS) for atovaquone intramuscularly. Experimental groups were divided as 1, 10 or 50 mg/kg of body weight of artesunate (AR1, AR10 and AR50, respectively) or atovaquone (AT1, AT10 and AT50, respectively) and subsequently administrated 0.2 ml of the indicated doses of artesunate or atovaquone dissolved in 5% sodium bicarbonate or PBS, respectively, by intramuscular route. The doses of atovaquone for this study were decided based on previous studies in which 50–100 mg/kg of body weight of atovaquone inhibited the growth of *B. microti* in hamster and Mongolian gerbil [13,14]. Parasitemia was monitored by examination of Giemsa-stained, thin blood smears using a light microscope. The body weight of mice was measured every 2 days and each mouse was given the indicated doses of the drug once per day for 6 consecutive days beginning 2 days post-infection. The infected erythrocytes appeared in peripheral blood of all mice on day 2 post-infection and thus treatment was started from day 2 post-infection. In Fig. 3A, peak parasitemia (35.5%) was observed in SB on day 10 post-infection. In contrast, lower parasitemia was observed in AR10 as well as AR50. Statistical significance of mean parasitemia between SB or PBS and each experimental group was determined using ANOVA and Tukey's tests using the JMP Version 8 program (SAS Institute Inc., USA). Significant differences ($P<0.05$) between SB and experimental AR50 and AR10 groups were observed on days 8–10 post-infection. Although artesunate failed to eradicate parasites and parasitemia increased up to 19.6% and 24.7% (standard deviation: ±6.32 and ±9.34) for AR10 and AR50 after the cessation of the treatment, respectively, artesunate not only inhibited the growth of the parasites but also delayed the increase of parasitemia, indicating that artesunate could be used for controlling *B. microti* infection. Moreover, artesunate was able to suppress more effectively the increase in parasitemia compared to atovaquone which showed significant inhibition ($P<0.05$) between PBS group and AT50 only at day 11 post-infection. Atovaquone did not effectively inhibit parasite growth in BALB/c mice, which is different from previous studies in gerbils and hamsters. Although mice, gerbils and hamsters are closely related in aspect of taxonomy, species differences in pharmacokinetics might affect absorption, distribution, metabolism and excretion of drugs [29,30]. The efficacy of artesunate could be improved by a combination with another effective babesiacide. Indeed, the combination of artesunate with other drugs has been advocated to malaria patients to prevent drug resistant parasites and to improve its efficacy by using drugs which have the different mode of action [31].

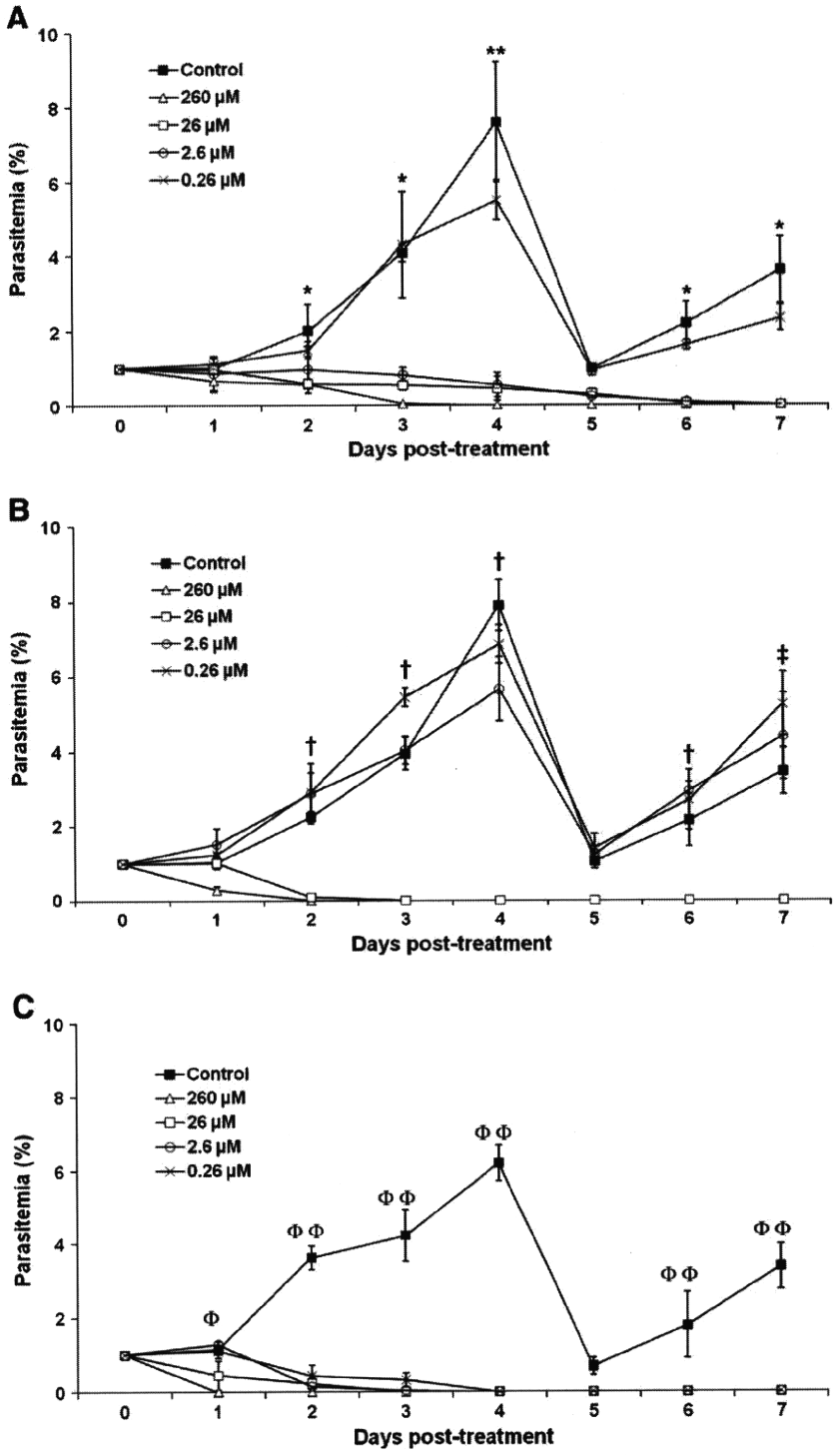

Fig. 1. Growth curves of *B. bovis in vitro* cultures treated with 0.26, 2.6, 26 and 260 μM of artesunate (A), atovaquone (B) and diminazene aceturate (C). Cultures started at 1% parasitemia and Giemsa-stained thin blood smears were prepared to determine daily parasitemia. (A) *, significant difference ($P<0.05$) between control group and groups tested with 2.6, 26 and 260 μM of artesunate; **, significant difference ($P<0.05$) between control group and all groups tested with artesunate. (B) †, significant difference ($P<0.05$) between control group and groups tested with 26 and 260 μM of atovaquone; ‡, significant difference ($P<0.05$) between control group and all groups tested with atovaquone. (C) Φ, significant difference ($P<0.05$) between control group and groups tested with 26 and 260 μM of diminazene aceturate; ΦΦ, significant difference ($P<0.05$) between control group and all groups tested with diminazene aceturate (the error bars, standard deviations).

This study has used a mouse model to evaluate the efficacy of artesunate against *B. microti* which causes rodent and human babesiosis. These results might help to discover a drug used for humans infected with *B. microti*. Therefore, an i.m. artesunate regimen was selected to treat *B. microti* infection since a prompt treatment is required to treat these infections that occur suddenly and severely in humans. While i.v. administration is also recommended for patients in severe condition, particularly those in comas, venous access may not be possible where only basic health care facilities exist. In addition, even when the drugs can be administered by i.v., patient discomfort and inconvenience, as well as risks such as overhydration and thrombophlebitis may make i.v. less attractive than i.m. [32].

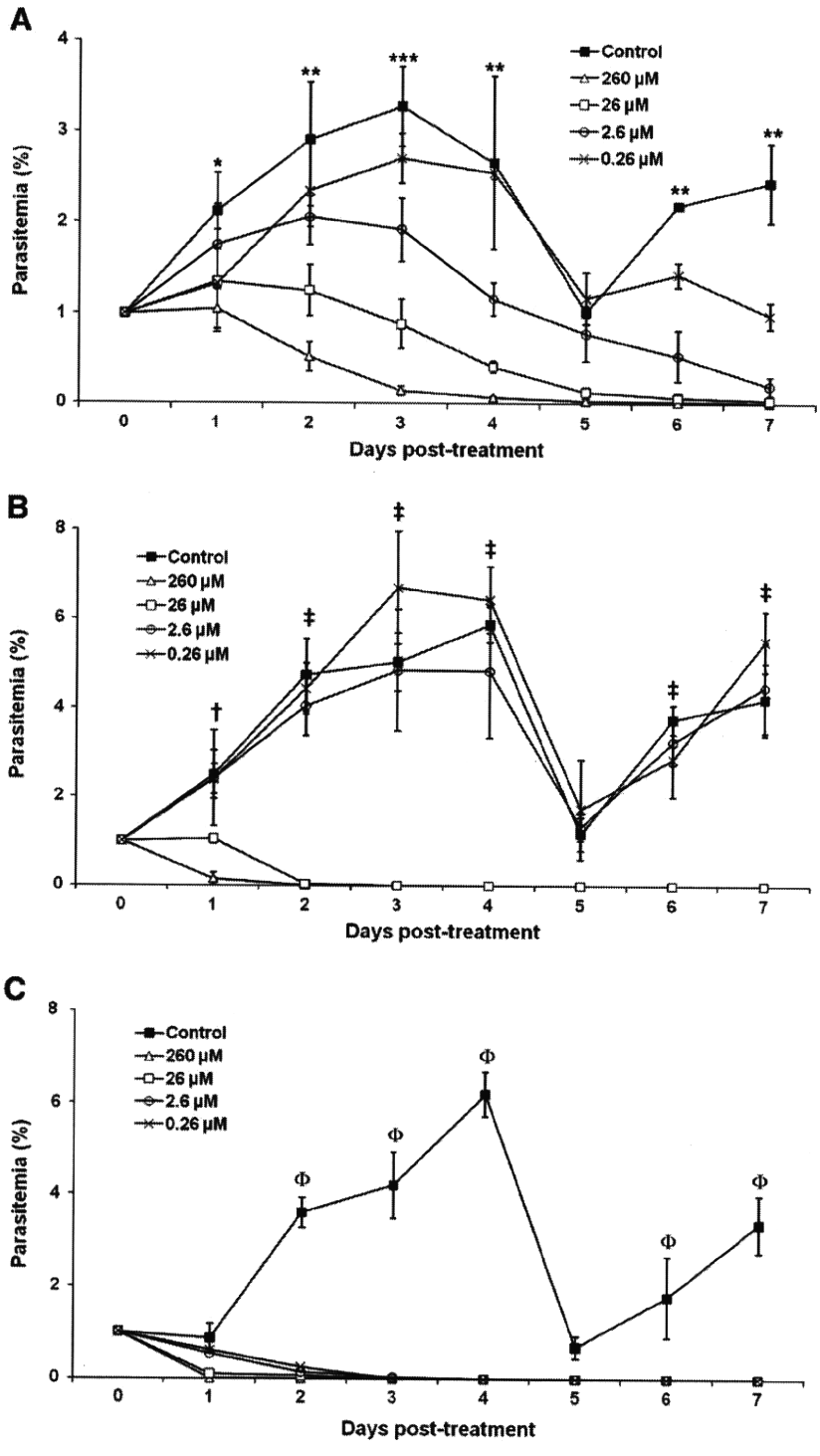

Fig. 2. Growth curves of *B. gibsoni in vitro* culture treated with 0.26, 2.6, 26 and 260 μM of artesunate (A), atovaquone (B) and diminazene aceturate (C). Cultures started at 1% parasitemia and Giemsa-stained thin blood smears were prepared to determine daily parasitemia. (A) *, significant difference ($P<0.05$) between control group and groups tested with 26 and 260 μM of artesunate; **, significant difference ($P<0.05$) between control group and groups tested with 2.6, 26 and 260 μM of artesunate; ***, significant difference ($P<0.05$) between control group and all tested groups with artesunate. (B) †, significant difference ($P<0.05$) between control group and groups tested with 260 μM of atovaquone; ‡, significant difference ($P<0.05$) between control group and groups tested with 26 and 260 μM of atovaquone. (C) Φ, significant difference ($P<0.05$) between control group and all groups tested with diminazene aceturate (the error bars, standard deviations).

Although i.m. administration of the oil-soluble antimalarial artemisinins could damage brain stem centers mainly involved in auditory processing and vestibular reflexes [33–35], artesunate, a water-soluble artemisinin derivative, has shown to cause less neurotoxic effects [36]. Mice treated with artesunate here displayed neither decrease in body weight on day 12 post-infection compared to that on day 1 post-infection (data not shown) nor clinical abnormalities such as gait and equilibrium disturbances, suggesting that doses between 1–50 mg/kg of body weight of artesunate were not responsible for neurotoxicity.

In conclusion, we have demonstrated that artesunate inhibits the growth of *B. bovis* and *B. gibsoni in vitro* and i.m. administration of

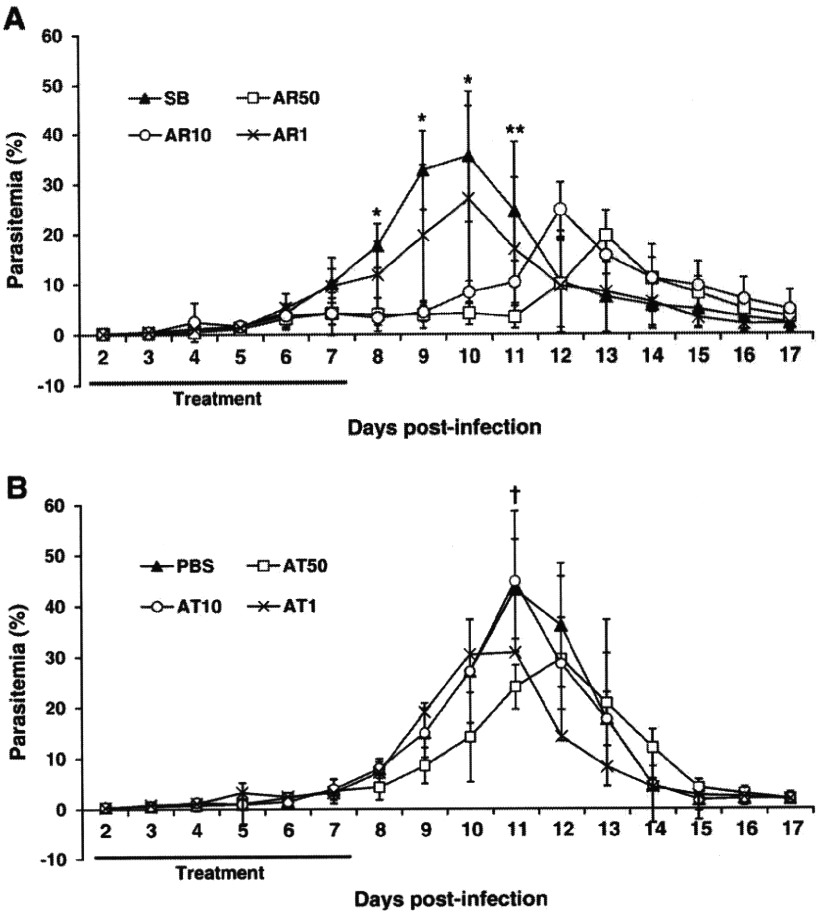

Fig. 3. Course of parasitemia in artesunate (A) and atovaquone (B) treated mice infected with *B. microti*. *B. microti* infected mice were treated with artesunate or atovaquone for 6 days, from day 2 to day 7 post-infection, and Giemsa-stained thin blood smears were prepared to determine daily parasitemia. (A) Artesunate treatment in mice infected with *B. microti*. SB, group for 5% sodium bicarbonate; AR50, group for 50 mg/kg of body weight of artesunate; AR10, group for 10 mg/kg of body weight of artesunate; AR1, group for 1 mg/kg of body weight of artesunate. *, significant difference ($P<0.05$) between SB and tested groups, AR50 and AR10; **, significant difference ($P<0.05$) between SB and AR50. (B) Atovaquone treatment in mice infected with *B. microti*. PBS, group for phosphate buffered saline; AT50, group for 50 mg/kg of body weight of atovaquone; AT10, group for 10 mg/kg of body weight of atovaquone; AT1, group for 1 mg/kg of body weight of atovaquone. †, significant difference ($P<0.05$) between PBS and AT50 (the error bars, standard deviations).

artesunate suppressed growth of *B. microti in vivo* without side-effects, suggesting that artesunate could be a potential drug for *Babesia* infection. However, *in vivo* experiments for *B. microti* indicated possible recrudescence of parasite growth after cessation of artesunate treatment.

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Research Brief

Babesia microti: Molecular and antigenic characterizations of a novel 94-kDa protein (BmP94)

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ABSTRACT

A novel gene, *BmP94*, encoding 94-kDa protein of *Babesia microti* was identified by immunoscreening of the cDNA expression library. The full-length of *BmP94* was expressed in *Escherichia coli* (rBmP94), which resulted in insoluble form with low yield, and the truncated hydrophilic C-terminus region of the gene was expressed as a soluble protein (rBmP94/CT) with improved productivity. Antiserum raised against rBmP94/CT recognized the 94-kDa native protein in the parasite extract by Western blot analysis. Next, an ELISA using rBmP94/CT was evaluated for diagnostic use, and it demonstrated high sensitivity and specificity when tested with the sera from mice experimentally infected with *B. microti* and closely related parasites. Moreover, the immunoprotective property of rBmP94/CT as a subunit vaccine was evaluated in BALB/c mice against a *B. microti* challenge, but no significant protection was observed. Our data suggest that the immunodominant antigen BmP94 could be a promising candidate for diagnostic use for human babesiosis.

1. Introduction

Babesia microti is a tick-transmitted intraerythrocytic parasite considered to be the primary etiological agent of human babesiosis. 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 (Homer et al., 2000; Saito-Ito et al., 2000; Hildebrandt et al., 2007). Infection usually causes asymptomatic to mild flu-like clinical manifestations including fever, sweat, chills, malaise, myalgia, and anemia (Homer et al., 2000). Severe symptoms, however, have been seen among patients who are immune-compromised, splenectomised, infants, or elderly (Homer et al., 2000; Leiby, 2006). The bite of an infected hard-bodied tick, *Ixodes scapularis*, is known to be the primary mode of transmission. Some cases, however, have occurred via transfusion of infected blood (Saito-Ito et al., 2000; Leiby, 2006; Tonnetti et al., 2009). Currently, the Red Cross and other blood donation agencies prohibit people with a history of babesiosis from donating blood (Vannier and Krause, 2009). Accordingly, there is an urgent and rational need to develop an effective means for the control including diagnosis and prevention of babesiosis.

Microscopic identification using a Giemsa-stained thin blood smear has been considered the standard technique for *Babesia* diagnosis, but the method has some limitations in the chronic stage of infection where the parasitemia is low. Polymerase chain reaction (PCR) has recently been used for detecting *B. microti* infection with high sensitivity and specificity (Persing et al., 1992). PCR, however, requires specialized laboratory equipment and facilities in addition to well-trained laboratory personnel. Alternatively, the indirect fluorescent-antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) with whole parasite have been utilized for serological diagnosis of *Babesia* infection. However, the poor quality of the antigens and occasional cross-reactions with other *Babesia* species resulting in false-positive reactions have limited their application (Bose et al., 1995). Recombinant proteins derived from parasites provide better options because they are usually available in pure forms and offer higher specificity than whole parasite (Tebele et al., 2000; Boonchit et al., 2006). Several antigens have been isolated from *B. microti* and evaluated as candidates for serodiagnosis in ELISA (Lodes et al., 2000; Homer et al., 2003). Thus far, however, no reliable and appropriately sensitive antigen for human use has been reported. Using ELISA along with recombinant protein needs to be considered as a measure for screening blood donors for silent *Babesia* infection prior to transfusion (Leiby, 2006). Therefore, further studies are desired to establish an effective ELISA method with a suitable recombinant antigen that can detect the infection with different *B. microti* strains.

On the other hand, over the past decade, several attempts have been undertaken to develop an anti-*Babesia* vaccine. However, no vaccine has been proven yet to be completely effective

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for preventing the infection (Brown and Palmer, 1999; Brown et al., 2006a). Recently, the development of subunit vaccines has moved towards recombinant proteins (Brown et al., 2006a; Shkap et al., 2007), but from an immunological point of view, the immunodominant antigens identified by protective serum could be candidates for a subunit vaccine (Jia et al., 2008). With this in mind, the present paper demonstrates the molecular and immunogenic characterizations of an immunodominant antigen of *B. microti* identified by immunoscreening of the cDNA expression library with acutely *B. microti*-infected sera. Results indicate promise of recombinant *Escherichia coli*-expressed BmP94 as a potential serodiagnostic antigen, but not a vaccine candidate for *B. microti* infection in mouse model.

2. Materials and methods

2.1. Parasites and experimental animals

The *B. microti* Munich strain, Australian strain of *Babesia rodhaini* (kindly provided by the Kyusyu Branch of the National Institute of Animal Health, Japan) and *Plasmodium berghei* ANKA strain were maintained in BALB/c mice (Clea, Tokyo, Japan) by serial passaging. Infections were initiated by an intraperitoneal (i.p.) injection of infected erythrocytes (Igarashi et al., 1993; Inoue et al., 2006). Blood was harvested during the acute stage for either sera or infected erythrocytes preparation. BALB/c and ICR mice (Clea, Tokyo, Japan) were used for the infection and immunization experiments. All animal experiments described in this article 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. Immunoscreening of the cDNA expression library

The cDNA library of *B. microti* merozoites (10^7 PFU) previously constructed (Nishisaka et al., 2001) was immunoscreened with the serum from acutely *B. microti*-infected BALB/c mice, and the cDNA inserts of positive clones were sequenced using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Complete nucleotide sequences of identical cDNAs were analyzed using a basic local alignment search tool (BLAST) accessed through the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). A cDNA clone encoding the 94-kDa protein (BmP94) was found as the most frequent gene and was thus selected for further study. The presence and location of a putative N-terminal signal peptide in the *BmP94* sequence were predicted using the SignalP server (http://www.cbs.dtu.dk/services/SignalP/). The presence of functional motifs and domains was predicted using the MyHits domain search database (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

2.3. Southern blot analysis

Genomic DNA of *B. microti* was extracted from the erythrocytic stages of the parasite and digested overnight with *Acc*I, *Bam*HI, *Sac*I, and *Sca*I. The DNA samples were separated on 0.8% (w/v) agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham-Buchler, Munich, Germany). The blots were pre-hybridized at 56 °C for 6 h and probed overnight with a full-length of *BmP94* cDNA labeled with alkaline phosphatase (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

2.4. Cloning, expression, and purification of recombinant BmP94 and BmP94/CT

The entire fragments encoding BmP94 without a signal peptide and its hydrophilic C-terminal region (618–863 amino acids, BmP94/CT), determined by software analysis (DNASTAR; NetWell Corporation, Tokyo, Japan), were amplified using primer sets: 5′-CAGGATCCGAATTTGTTGACCTACA-3′ and 5′-GACTCGAGATCTTAA ACAGATTGTTGCGG-3′; 5′-ATGGATCCAGCGATAATGAAACGCCCG CA-3′ and 5′-GACTCGAGATCTTAAACAGATTGTTGCGG-3′, respectively. According to the manufacturer's instructions, the PCR products were inserted into the pGEX-4T-3 vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using the *Bam*HI and *Xho*I sites and expressed in an *E. coli* DH5α strain. A fresh 10 ml overnight culture of transformed *E. coli* was grown in 1 l of LB base broth containing 50 μg/ml of ampicillin at 37 °C with shaking at 250 rpm until the optical density (OD) at 600 nm reached to 0.5. The expression of the proteins were induced by 1–5 mM-beta-galactoside (IPTG) followed by incubation at 27 °C overnight. The *E. coli* culture was centrifuged at 8000*g* for 15 min, the cell pellet was then suspended in TNE buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing 50 mg/ml lysozyme, 1% (w/v) *N*-Lauroylsarcosine sodium and protease inhibitors. The recombinant proteins were purified from the soluble fractions using glutathione-Sepharose 4B beads, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.5. Production of anti-rBmP94/CT serum

Six-week-old ICR mice were immunized i.p. with 200 μg of the purified rBmP94/CT protein emulsified in 200 μl of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA). Two boosters were given i.p. at 14 day intervals using the same amount of antigen emulsified in Freund's incomplete adjuvant (Difco, Detroit, MI, USA). Sera were collected two weeks after the last booster and checked for specific antibodies by IFAT.

2.6. IFAT and confocal laser microscopic observation

B. microti-parasitized erythrocytes were coated on IFAT slides, dried, and fixed in absolute acetone for 10 min. For confocal microscopic observation, thin blood smears of *B. microti*-infected erythrocytes were fixed with absolute methanol at −20 °C for 30 min. Standard IFAT protocol was employed to localize the protein (Terkawi et al., 2007). Briefly, the anti-rBmP94/CT serum was applied as the first antibody on the fixed smears and incubated in a moist chamber at 37 °C for 1 h. After washing four times with phosphate-buffered saline containing 0.05% Tween 20 (PBST), Alexa-Fluor® 488-conjugated goat anti-mouse immunoglobin G (IgG) (Molecular Probes, Eugene, OR, USA) was applied as a secondary antibody (1:200) and then incubated at 37 °C for 1 h. The slides were washed four times with PBST and incubated with 2.5 μg/ml propidium iodide (PI) (Molecular Probes, Eugene, OR, USA) 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 cover slip. The slides were examined using a confocal laser scanning microscope (TCSNT, Leica, Heidelberg, Germany).

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

Protein expressions were verified by SDS–PAGE stained with Coomassie blue, and their antigenicities were confirmed by Western blot analysis (Xuan et al., 1996). *B. microti*-infected erythro-

cytes obtained from infected mice at the peak of parasitemia were haemolysed with 0.15% saponin. After centrifugation, the pellet was washed four times with cold PBS, resuspended in PBS, sonicated and lastly precipitated with acetone (Terkawi et al., 2009). Native BmP94 was identified in the parasites extract by Western blot analysis. Proteins in the extract were size-separated by electrophoresis in 10% SDS–PAGE and electroblotted onto a nitrocellulose membrane. The membrane was blocked with phosphate-buffered saline containing 0.05% Tween 20 (PBST) plus 5% skim milk and probed with anti-rBmP94/CT serum as a primary antibody. After washing with phosphate-buffered saline containing 0.05% Tween 20 (PBST), a secondary antibody, horseradish peroxidase-conjugated to anti-mouse IgG (Bethyl, Montgomery, TX, USA), was applied. Finally, bands were visualized by using 3,3′-diaminobenzidine tetrahydrochloride (Nacalai Tesque, Inc., Kyoto, Japan) and H_2O_2.

2.8. ELISA

The standard ELISA was performed as previously described (Terkawi et al., 2007). Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 1 μg/ml of the rBmP94, 0.45 μg/ml of the rBmP94/CT, and 0.22 μg/ml of the control GST emulsified in a coating buffer (0.05 M carbonate buffer, pH 9.6). The plates were then blocked with PBS containing 3% (w/v) skim milk for 1 h at 37 °C. After washing, the plates were incubated with serum samples. The bound antibody was detected by treatment with horseradish peroxidase (HRP)-conjugated (Bethyl, Montgomery, TX, USA) to anti-mouse IgG (1:6000) and ABTS [2,2′-azinobis (3-ethylbenzthiazolinesulfonic acid)] (Sigma, St. Louis, MO, USA). The color was allowed to develop at room temperature. Optical density (OD) was measured by an MTP-500 microplate reader (Corona Electric, Hitachinaka-shi, Japan) at 415 nm. The positive cut-off value was calculated as the mean OD value of the serum samples from specific pathogen-free mice plus threefold of their standard deviation.

2.9. Vaccination and challenge infection

A total of 15 female BALB/c (six-week-old) mice were divided into three groups ($n = 5$). Two hundred micrograms of purified rBmP94/CT emulsified in 100 μl of Freund's complete adjuvant

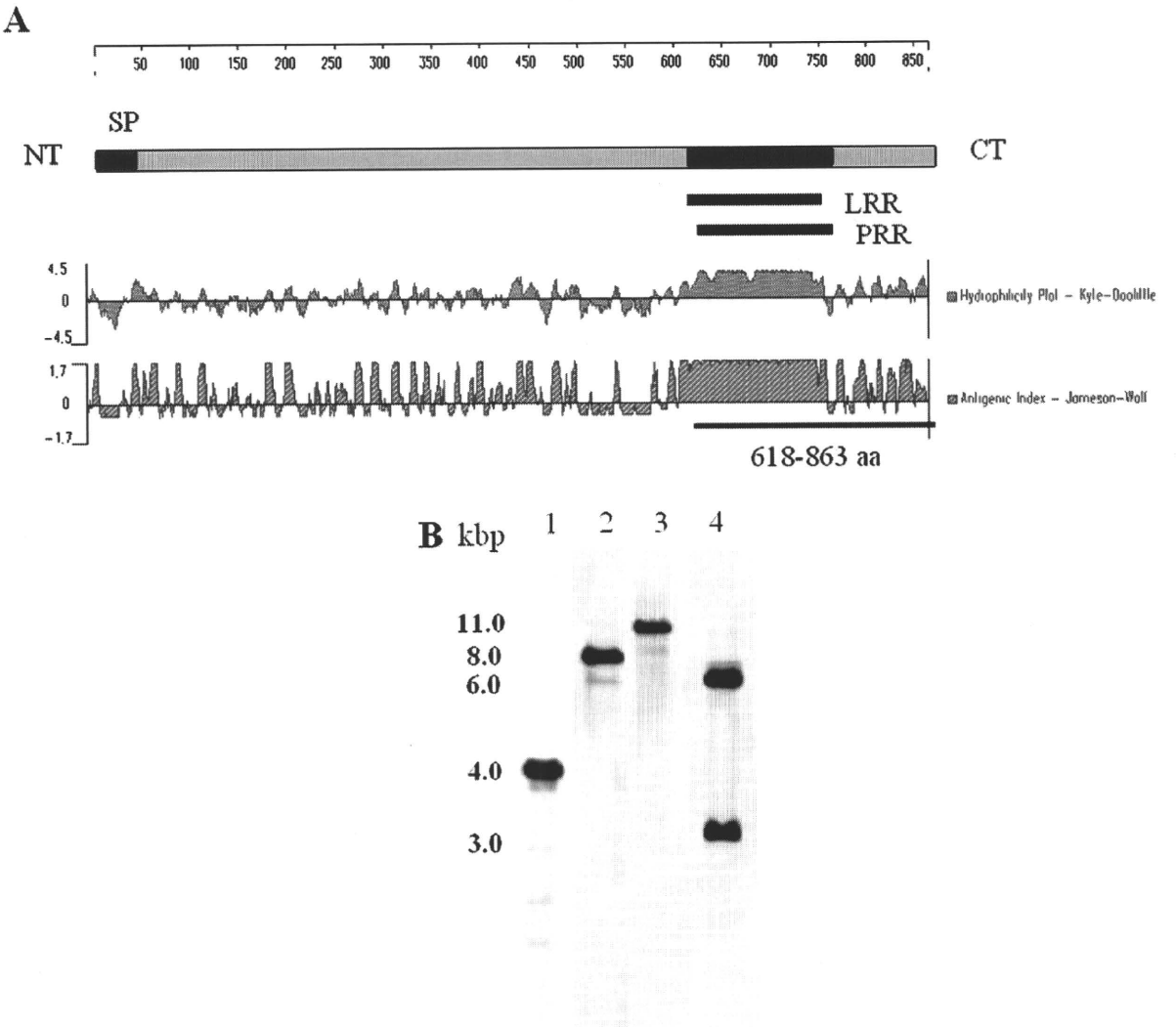

Fig. 1. (A) Schematic representation of the protein structure of BmP94 (upper), and software analysis of the hydrophilicity (middle) and antigenicity (lower) of BmP94 (DNASTAR; NetWell Corporation, Tokyo, Japan). The truncation region for rBmP94/CT is underlined. NT, N-terminus; CT, C-terminus; SP, signal peptide (1–39 amino acids); LRR, lysine-rich region (618–741 amino acids); PRR, proline-rich region (630–744 amino acids). (B) Southern blot analysis of genomic DNA of *Babesia microti* digested with different restriction enzymes, *Acc*I (lane 1), *Bam*HI (lane 2), *Sac*I (lane 3), and *Sca*I (lane 4), and hybridized with specific probe of the BmP94 gene.

was administered i.p. and followed by two additional boosters with the same amount of antigen in Freund's incomplete adjuvant at 14 day intervals. This group was designated as a test group. Control group mice received either GST protein or no immunization. Two weeks after the last boosting, the mice were challenged i.p. with 1×10^7 *B. microti*-infected erythrocytes. Parasitemias were monitored daily for 30 days by examination of Giemsa-stained smears. The antibody responses were determined before challenge. The blood samples were collected from tail bleeds using hematocrit capillary tubes (Hirschmann® laborgerate, Eberstadt, Germany).

2.10. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the means of all variables. The mean values were considered statistically different when $P < 0.05$.

2.11. Nucleotide sequence accession number

The complete sequence of the *BmP94* gene has been submitted to the GenBank with the accession number (AB540023).

3. Results and discussion

The emergence of human babesiosis caused by *B. microti* and the increase of immunocompromised patients' population in the world emphasize the need to search for effectively preventive means. Within this context, the present study demonstrates the identification of a novel *B. microti* antigen and the evaluation of its potential use for diagnosis and vaccination in rodent model. The cDNA library of *B. microti* was screened with sera from mice acutely infected with *B. microti*. A total of 164 positive clones were obtained, isolated, sequenced, and then subjected to BLAST analysis. Eighty-five clones that showed high reactivity with the sera were found to contain cDNA encoding novel amino acid sequences which were later designated as the *BmP94* gene. The full-length of *BmP94* contained an open reading frame of 2589 nucleotides encoding a polypeptide of 863 amino acid residues with a calculated molecular mass of 93.92-kDa and an isoelectric point of 6.1. The hydrophobic region at the N-terminus of *BmP94* showed the characteristics of a signal peptide, and the most likely cleavage site was predicted to be between 39 and 40 amino acids. In addition, the C-terminus of BmP94 contained a lysine-rich region (618–741 amino acids) and a proline-rich region (630–744 amino acids) that were predicted to be highly hydrophilic and antigenic (Fig. 1A) and thus determined to be the target region for further truncation (618–863 amino acids). Southern blot analysis of the genomic DNA probed with the *BmP94* gene demonstrated a single hybridizing band, after DNA digestion with *Acc*I, *Bam*HI, and *Sac*I, that cut outside the *BmP94* gene (Fig. 1B, lanes 1–3). On the other hand, the enzymes that cut a single site within the gene yielded two hybridizing bands (Fig. 1B, lane 4, *Sca*I). These results revealed that the genomic DNA of *B. microti* contains a single copy of the *BmP94* gene. Thereafter, the rBmP94 lacking an N-terminus signal peptide and truncated rBmP94/C-terminal region (618–863 amino acids) were expressed in *E. coli* as GST-fusion protein with a molecular mass of 120-kDa and 54-kDa, respectively, including a 26-kDa GST tag (Fig. 2). Sera from mice experimentally infected with *B. microti* reacted with the rBmP94 and rBmP94/CT by Western blot analysis (Fig. 2) indicating the antigenicity of the recombinant proteins. Notably, the expression of rBmP94/CT was increased in amount more than 10 times as compared to the expression of non-truncated rBmP94 (Fig. 2C), likely due to the hydrophobic nature of the BmP94′ N-termini that would lead to significant amounts of insoluble forms. The improvement in productivity enabled the immunization of mice and production of specific antiserum. Mouse-raised anti-rBmP94/CT serum specifically reacted with 94-kDa protein in *B. microti* extract by Western blot analysis (Fig. 3A, lane 1), and the observed molecular size of BmP94 was consistent with the expected molecular weight. The same serum was further used to know the localization of the protein in the *B. microti* merozoites by IFAT (Fig. 3B). Confocal microscopic observation of extracellular parasites and intracellular parasites demonstrated the expression of the BmP94 with various developmental stages of merozoites (Fig. 3B, a–h). However, weak or no reaction was sometimes observed with dividing form (Fig. 3B, e and f), suggesting that BmP94 probably expresses in a stage-dependent manner. Although, the fluorescence was consistently observed within the cytosol of parasites, the conclusive localization of the BmP94 needs further investigation using electron microscopic examination.

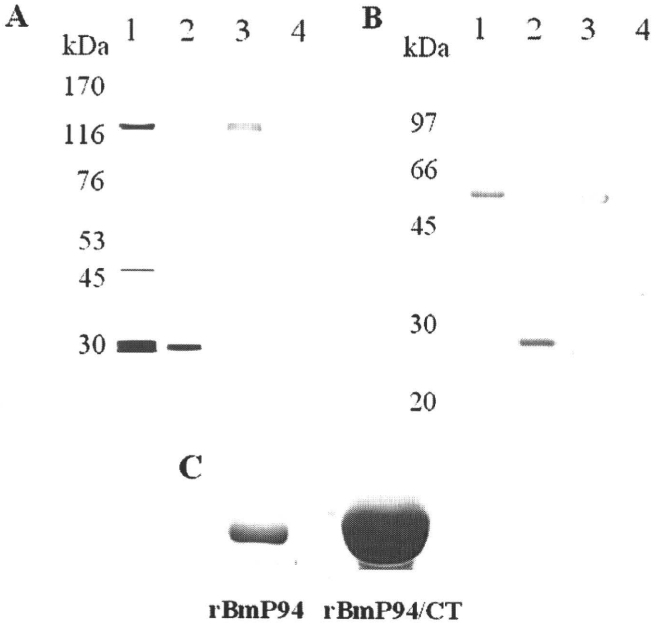

Fig. 2. SDS–PAGE and Western blot analysis of recombinant BmP94 (120-kDa), recombinant BmP94/CT (54-kDa), and GST (26-kDa). (A) Recombinant BmP94 (lanes 1 and 3) and GST (lanes 2 and 4), stained with amido black (lanes 1 and 2) and probed with *Babesia microti*-infected mouse serum (lanes 3 and 4), the bands around 45-kDa, 27-kDa, and 25-kDa appeared in SDS–PAGE might be degraded forms of the proteins due to stress response of the host cell. (B) Recombinant BmP94/CT (lanes 1 and 3) and GST (lanes 2 and 4), stained with amido black (lanes 1 and 2) and probed with *B. microti*-infected mouse serum (lanes 3 and 4); (C) Comparison of productivity between insoluble rBmP94 and soluble rBmP94/CT from the same amount of *Escherichia coli*.

The antigenicity of the rBmP94 was evaluated using ELISA with variously *B. microti*-infected sera. Sera from *B. microti*-infected mice showed a highly specific reaction as evidenced by high ODs, whereas sera from infected mice with closely related parasites, *B. rodhaini* and *P. berghei*, showed a clear negative reaction (OD < 0.1). These revealed the specificity of rBmP94-ELISA in detection of the infection (Fig. 4A). Interestingly, rBmP94/CT-ELISA demonstrated a similar specificity when tested with the same sera (Fig. 4A). Next, the sensitivity of ELISA using rBmP94 and rBmP94/CT was examined by testing sera obtained sequentially after infection of mice infected with *B. microti*. Although the antibody responses to rBmP94 were detectable two days earlier than the antibody to rBmP94/CT, no significant differences in the OD values were observed during the acute and chronic stages (Fig. 4B). The slight difference might be due to the presence of antigenic regions found in the N-terminus (Fig. 1). However, the improvement in productivity together with similar diagnostic properties of the

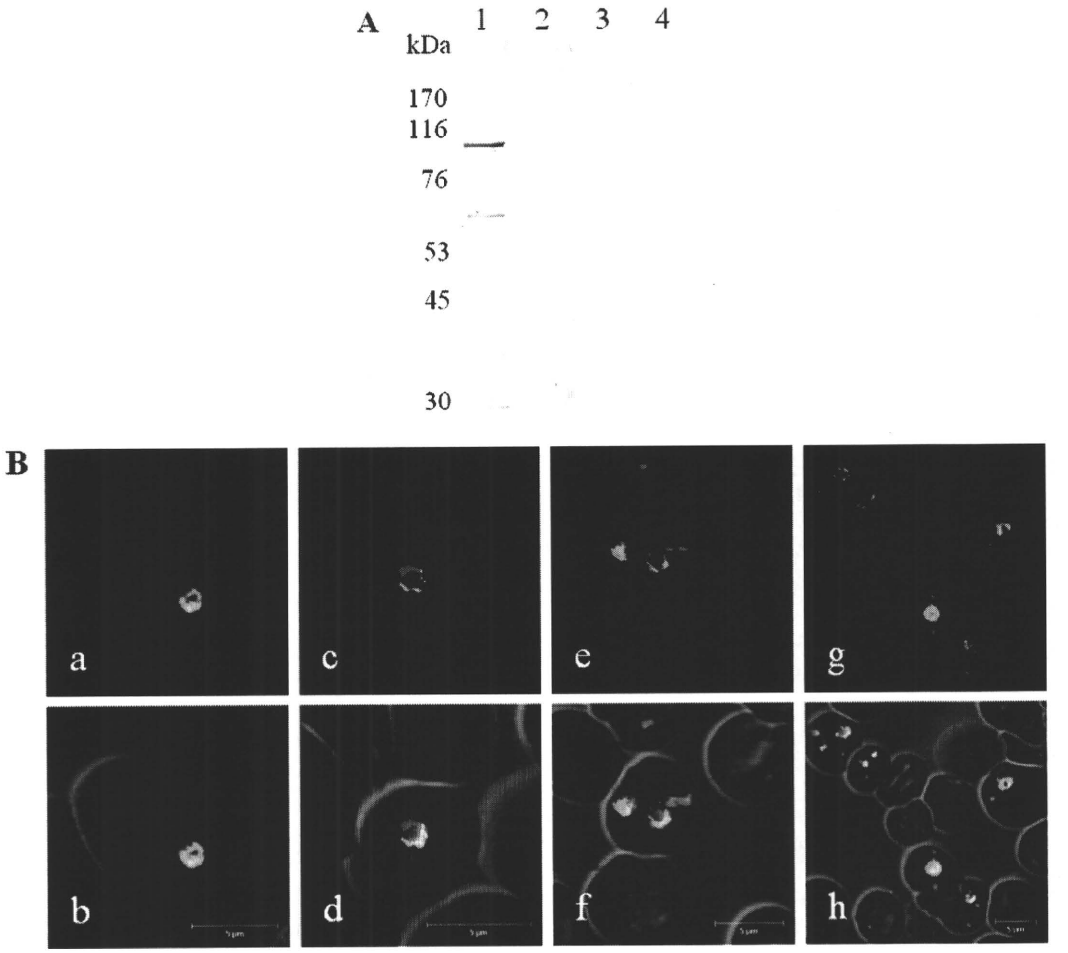

Fig. 3. Western blot and immunofluorescence microscopy analyses of native BmP94. (A) *Babesia microti*-infected erythrocytes (lanes 1 and 3) and erythrocytes from healthy mice (lanes 2 and 4) probed with anti-rBmP94/CT serum (lanes 1 and 2) or normal mouse serum (lanes 3 and 4). Apparent minor bands; 60-kDa and 26-kDa might be degraded forms of the native protein. (B) Immunofluorescence microscopy analysis. Methanol-fixed smears of *B. microti*-infected RBC were preincubated with the anti-rBmP94/CT serum, visualized with Alexa-Fluor® 488-conjugated IgG secondary antibody and propidium iodide (PI) staining, and finally examined with confocal laser microscopy. The specific reaction of the protein and anti-rBmP94/CT serum is green, and the nucleus is red. Panels a–h, the reactivity of antiserum with extracellular and intracellular *B. microti* merozoites. Panels a, c, e, and g, the overlaid image of fluorescent reactivity and red PI staining of nuclei; b, d, f, and h, the overlaid image of fluorescent green reactivity and red PI staining on phase-contrast images of the parasites. Bars, 5 μm.

full-length protein indicates the success of the truncation strategy of rBmP94/CT. Several recombinant merozoite antigens have been identified as immunodominant and have diagnostic significance in various *Babesia* species. Likewise, recombinant BmN1-17 derived from *B. microti* MN1 strain has been documented to be highly sensitive (98%) in detection the infection of human positive sera (Homer et al., 2003). However, none of these identified antigens were evaluated with *B. microti* Munich strain infected sera and our study is the first report about ELISA development based on Munich strain that considered potentially as agent for human babesiosis. Although our data did not show the performance of rBmP94 with human sera, the high sensitivity and specificity of the antigen in mouse model were promising enough to pursue development of a reliable serodiagnostic reagent for detection of *B. microti* infection. Further study including the examination of rBmP94-ELISA with different strains of *B. microti*-infected sera is required to obtain the conclusive remark of rBmP94 as antigen for serodiagnosic use.

The high antigenicity combined with high yield of rBmP94/CT was motivating to evaluate its potential as a vaccine candidate. The immunization regime induced highly specific antibodies (IFAT titers, 1:25,600) in mice that received rBmP94/CT but not in mice that received GST or the control mice (data not shown). The mice were then challenged and parasitemia was monitored for 30 days post-infection. All mice developed parasitemia as early as 2 days post-challenge, and peak levels were observed at day 8; no significant differences were observed between the test and control groups (data not shown). Although the vaccination was not protective, further study including different delivery schemes such as liposome vaccination, DNA vaccination, and virus vector vaccination might be necessary to attain the protective properties of this molecule. The failure in protection could support the concept that immunodominant antigens serologically identified are not necessarily protective (Brown et al., 2006b). However, several reports documented the usefulness of serological screening to identify subunit vaccine candidates. For example, immunization with ribosomal phosphoprotein P0 delayed the onset of *B. microti* infection and significantly reduced the peripheral parasitemia (Terkawi et al., 2007). In support of this, immunization with HSP-70 elicited significant protection against *B. microti* challenge infection (Terkawi et al., 2009).

In summary, we have identified and characterized a novel BmP94 antigen by immunoscreening of the *B. microti* cDNA library and evaluated its potential for serodiagnosis and as a subunit vaccine. The high specificity and sensitivity of ELISA-rBmP94/CT were promising enough to pursue development of a reliable serodiagnostic reagent for detection of infection in the mouse model.

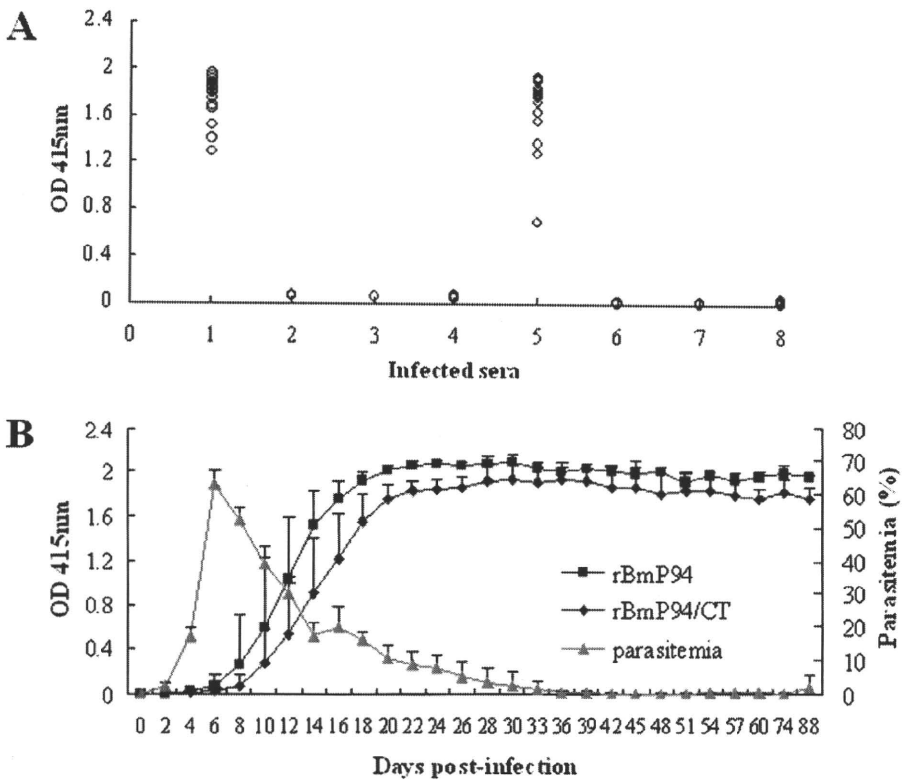

Fig. 4. Evaluation of rBmP94- and rBmP94/CT-ELISA. (A) The reactivity of rBmP94 (lanes 1–4) and rBmP94/CT (lanes 5–8) with B. microti - and closely related parasite-infected mice sera. Lanes 1 and 5: *Babesia microti*-infected mouse sera (n = 18); lanes 2 and 6: *Babesia rodhaini*-infected mouse sera (n = 6); lanes 3 and 7: *Plasmodium berghei*-infected mouse sera (n = 6); lanes 4 and 8: specific pathogen-free mouse sera (n = 12). (B) Sensitivity of rBmP94 (0.1 μg / well) and rBmP94/CT (0.045 μg / well) with serial serum samples from mice experimentally infected with *B. microti* (n = 5). The parasitemia was determined by microscopic examination. Each point represents the mean ± the standard deviation.

Immunization with rBmP94/CT combined with Freund's adjuvant was not significantly protective against challenge infection, although induced high titer of specific antibody. These findings should hopefully provide a basis for future work that could exploit this protein as antigen for diagnostic use.

Acknowledgments

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Spherical Body Protein 4 Is a New Serological Antigen for Global Detection of *Babesia bovis* Infection in Cattle▽

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Five *Babesia bovis* recombinant proteins, including merozoite surface antigen 2c (BbMSA-2c), C-terminal rhoptry-associated protein 1 (BbRAP-1/CT), truncated thrombospondin-related anonymous protein (BbTRAP-T), spherical body protein 1 (BbSBP-1), and spherical body protein 4 (BbSBP-4), were evaluated as diagnostic antigens to detect the infection in cattle. The recombinant proteins were highly antigenic when tested with experimentally *B. bovis*-infected bovine serum in Western blot analysis. Furthermore, five antisera that had been raised against each of the recombinant proteins reacted specifically with the corresponding authentic protein, as determined in Western blot analysis. Next, enzyme-linked immunosorbent assays (ELISAs) using these recombinant proteins were evaluated for diagnostic use, and the sensitivity and specificity of each protein were demonstrated with a series of serum samples from experimentally *B. bovis*-infected cattle. Furthermore, a total of 669 field serum samples collected from cattle in regions of *B. bovis* endemicity in seven countries were tested with the ELISAs, and the results were compared to those of an indirect fluorescent antibody test (IFAT), as a reference. Among five recombinant antigens, recombinant BbSBP-4 (rBbSBP-4) had the highest concordance rate (85.3%) and kappa value (0.705), indicating its reliability in the detection of specific antibodies to *B. bovis* in cattle, even in different geographical regions. Overall, we have successfully developed an ELISA based on rBbSBP-4 as a new serological antigen for a practical and sensitive test which will be applicable for epidemiologic survey and control programs in the future.

Bovine babesiosis is an economically important tick-borne disease in tropical and subtropical areas of the world (3). The disease is caused by hemoprotozoan parasites of the genus *Babesia*, namely, *B. bovis*, *B. bigemina*, *B. beliceri*, *B. divergens*, *B. major*, *B. ovata*, *B. occultans*, and *B. jakimovi* (28). Among them, *B. bovis* and *B. bigemina* are the most important species, and they are usually found together in most areas of endemicity (22, 31). Although *B. bovis* and *B. bigemina* are phylogenetically related and transmitted by *Rhipicephalus* (*Boophilus*) *microplus*, they cause remarkably different diseases in cattle (3). Infection by *B. bovis* is more severe than that of *B. bigemina*, due to the sequestration of infected erythrocytes in the microcapillaries of the kidneys, lungs, and brain, resulting in organ failure and systemic shock that leads to death. Cattle that survive *B. bovis* infection generally become carriers of the parasite and serve as reservoirs for transmission to other animals (3). Thus, highly sensitive and specific diagnostic tools are required to detect the carrier animals and to differentiate this infection from other closely related ones. Such diagnostic tests must lead to a better understanding of the protozoan epidemiology, providing useful information for disease management and control strategies (10).

A large number of serological tests have been developed for the detection of specific antibodies to bovine *Babesia* parasites for epidemiological surveys as well as for the identification of carrier animals (2, 7, 30). Among these assays, the indirect immunofluorescent antibody test (IFAT) is the most sensitive, but cross-reactivity with other *Babesia* spp., subjective interpretation, and low throughput have limited its usefulness (8). In contrast, enzyme-linked immunosorbent assays (ELISAs) can be used as a routine diagnostic test or as a screening test for epidemiologic studies. Although a number of diagnostic ELISAs have been developed, several problems regarding the sensitivity and specificity remain, related mostly to the characteristics and preparation of the antigens. For example, crude antigens have been used to detect the antibodies to *Babesia* parasites, but the poor purification quality of the antigen and the potential cross-reactivity with other protozoan parasites have impeded their application. Such crude antigens frequently contain host cell components, which may affect the accuracy of test results by increasing the nonspecific background (7–9, 21, 29). On the other hand, recombinant proteins derived from the parasites could become alternative sources of antigens, allowing a better standardization of the tests with high specificity and sensitivity (7, 8). Despite the potential advantages of using recombinant antigens in serological tests, their sensitivity needs to be significantly improved (29). Therefore, further research to identify new antigen makers is extremely desirable.

Babesia parasites are defined by the common characteristic structures of the apical complex, which consists of rhoptries, micronemes, and spherical body organelles. Proteins derived from these organelles, coupled with the membrane component of parasites, are believed to have critical functions in parasite

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survival and growth (34). These proteins include (i) variable merozoite surface antigens (VMSAs) (4), which are believed to play key roles in the initial attachment of merozoites and sporozoites during their invasion (34), (ii) microneme and rhoptry proteins, including apical membrane antigen 1 (AMA-1), thrombospondin-related anonymous protein (TRAP), and rhoptry-associated protein 1 (RAP-1), which seem to be involved in the formation of a tight junction between merozoites and erythrocytes (RBC) (11), and (iii) spherical body proteins (SBPs), which have roles in stabilizing the environment after invasion and in aiding parasite growth (14, 26). Importantly, they have been suggested as promising candidates for developing subunit vaccines or diagnostic antigens (34).

With this in mind, we validated five ELISAs with different recombinant proteins, including merozoite surface antigen 2c (BbMSA-2c) (19), C-terminal rhoptry-associated protein 1 (BbRAP-1/CT) (6), thrombospondin-related anonymous protein (BbTRAP) (11), spherical body protein 1 (BbSBP-1) (16), and spherical body protein 4 (BbSBP-4) (M. A. Terkawi, F. J. Seuseu, P. E. Wibowo, N. X. Huyen, M. Aboulaila, N. Yokoyama, X. Xuan, and I. Igarashi, submitted for publication), and then evaluated their potential application for the diagnostic detection of specific antibodies to *B. bovis*. The results indicate the promising use of an ELISA with rBbSBP-4 antigen as a global diagnostic marker for the detection of *B. bovis* infection.

MATERIALS AND METHODS

Parasites. The Texas strain of *B. bovis* and the Argentina strain of *B. bigemina* were continuously cultured with bovine erythrocytes (RBC) by using a microaerophilous stationary-phase culturing system (1, 15, 20). The cultured parasite was harvested when the parasitemia reached 8 to 10%.

Expression and purification of recombinant proteins and production of polyclonal antibodies. The DNA fragments encoding *B. bovis* merozoite surface antigen 2c (BbMSA-2c; GenBank accession number AY052542) (19), the C-terminal region of rhoptry-associated protein 1 (BbRAP-1/CT [388 to 490 amino acids {aa}]; AF030062) (5, 6), full-length or truncated thrombospondin-related anonymous protein (BbTRAP or BbTRAP-T [321 to 561 aa]; AY486102) (11), spherical body protein 1 (BbSBP-1; AAC37226) (16), and spherical body protein 4 (BbSBP-4; AB594813) (Terkawi et al., submitted) were amplified from a *B. bovis* cDNA phage expression library by standard PCRs using gene-specific primers (Table 1). The amplified DNA fragments were cloned into a pGEX-4T1 plasmid vector (Amersham Pharmacia Biotech, Madison, CA) using the suitable restriction enzyme sites and then expressed as glutathione *S*-transferase (GST) fusion genes in the *Escherichia coli* DH-5α strain (Amersham Pharmacia Biotech). The recombinant proteins were purified from the soluble fractions of *E. coli* lysates using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) and then analyzed by SDS-PAGE and Western blot analysis, as described previously (26).

Thereafter, each of the antisera for recombinant proteins was prepared in 6-week-old BALB/c mice ($n = 5$) according to the standard protocol (25). Briefly, mice were intraperitoneally (i.p.) immunized with 100 μg of each recombinant protein emulsified in Freund's complete adjuvant (Sigma, St. Louis, MO). Two boosters were given i.p. using 50 μg of the same proteins emulsified in Freund's incomplete adjuvant (Sigma) at 14-day intervals. Sera were collected 2 weeks after the last booster and then checked for the production of specific antibodies using the IFAT and Western blotting.

SDS-PAGE and Western blotting. The expressed recombinant proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent Coomassie blue staining, while their antigenicities were confirmed with Western blot analysis. To identify the authentic parasite proteins, the *B. bovis*-infected RBC were obtained from the *in vitro* culture, washed three times with cold phosphate-buffered saline (PBS), and then lysed with 0.25% saponin. The pellet was washed four times with cold PBS, resuspended in PBS, disrupted three times by a freeze-thaw cycle in liquid nitrogen, and then sonicated in an ice slurry (Terkawi et al., submitted). The protein concentrations of lysates were determined by a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL) and finally stored at −80°C until use. The extracted proteins were separated in 12% SDS-PAGE and then electroblotted onto a nitrocellulose membrane (32). The membrane was blocked with 0.05% Tween 20 in PBS (PBS-T) plus 5% skimmed milk and probed with the indicated protein-specific primary antibodies. After the membrane was washed with PBS-T, a secondary antibody, horseradish peroxidase (HRP)-conjugated anti-bovine or -mouse immunoglobulin G (IgG) antibody (Bethyl Laboratories, Montgomery, TX), was applied. Finally, reacted bands were visualized using a solution containing 3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 (Dojindo, Tokyo, Japan).

TABLE 1. Gene-specific primers for amplifying *BbMSA-2c*, *BbRAP-1/CT*, *BbTRAP-T*, *BbSBP-1*, and *BbSBP-4*

Gene	Oligonucleotide primer[a]
BbMSA-2c	5′-CGGAATTCATGGTGTCTTTAACATAATAACC-3′
	5′-TAGCGGCCGCGAATGCAGAGAGAACGAAGTAGCAG-3′
BbRAP-1/CT	5′-ACGGATCCGAGTTTTTCAGGGAA-3′
	5′-ACCTCGAGAAACTCATGTATGAT-3′
BbTRAP-T	5′-AGAATTCGAACCAAGCCGTGCTACACCG-3′
	5′-ACTCGAGCTATTGTTTTTCGCCCTCGTAG-3′
BbSBP-1	5′-GCGAATTCAACGAAGCTGAGGTATCTCAG-3′
	5′-GGCTCGAGTTAGTCTAGCATCTGTATTTT-3′
BbSBP-4	5′-AGGAATTCGAGGAGGAGGAAACTGATGAG-3′
	5′-GCCTCGAGTTATTCCTCAATGTCGGCTGT-3′

[a] Each oligonucleotide primer includes restriction enzyme sites at the 5′ end (underlined), which are EcoRI and NotI for *BbMSA-2c*, BamHI and XhoI for *BbRAP-1/CT*, and EcoRI and XhoI for *BbTRAP-T*, *BbSBP-1*, and *BbSBP-4*.

Bovine sera. Positive serum samples were collected from cattle experimentally infected with *B. bovis* ($n = 25$) or *B. bigemina* ($n = 30$) (National Institute of Animal Health, Tsukuba, Ibaraki, Japan) or with *Theileria orientalis* ($n = 6$) (Obihiro University of Agriculture and Veterinary Medicine, Japan), while non-*Babesia*-infected control sera ($n = 50$) were obtained from healthy cattle that had been bred at Obihiro University of Agriculture and Veterinary Medicine, Washington State University (Pullman, WA), and Texas A&M University (College Station, TX) (19). Field bovine sera were collected from Brazil ($n = 108$), Ghana ($n = 80$), China ($n = 100$), Thailand ($n = 100$), Mongolia ($n = 81$), South Korea ($n = 100$), and Hokkaido, Japan ($n = 100$) (1, 5, 18).

ELISAs. Standard enzyme-linked immunosorbent assays (ELISAs) were performed in the present study as described previously (25). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of each recombinant protein at a concentration of 2 μg/ml per well in a coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6). The plates were washed once with 0.05% Tween 20-PBS (PBS-T) and then incubated with 100 μl of a blocking solution (3% skim milk in PBS) for 1 h at 37°C. After the antigen-coated wells were washed once with PBS-T, they were incubated with 50 μl of the serum samples diluted 1:100 with the blocking solution for 1 h at 37°C. The plates were washed six times with PBS-T and then incubated with 50 μl of HRP-conjugated sheep anti-bovine IgG antibody (Bethyl) diluted 1:4,000 with the blocking solution for 1 h at 37°C as a secondary antibody. The plates were washed six times as described above, and 100 μl of a substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.3 mg/ml of 2,2′-azide-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma), and 0.01% of 30% H_2O_2] was then added to each well. After incubation for 1 h at room temperature (RT), the optical density (OD) was measured with an MTP-500 microplate reader (Corona Electric, Tokyo, Japan) at a wavelength of 415 nm. The cutoff points were calculated by the receiver operating characteristic (ROC) analysis with MedCalc statistical software (version 11.4; http://www.medcalc.be) for each recombinant protein with 50 non-*Babesia*-infected bovine sera (12, 13).

IFAT. The *B. bovis*-infected RBC were coated on indirect fluorescent antibody test (IFAT) slides (Matsunami Glass Ind., Ltd., Osaka, Japan), dried, and then fixed in absolute acetone for 20 min for standard IFAT observation (4). Briefly,

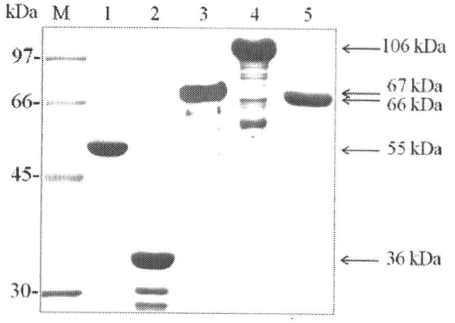

FIG. 1. Successful expression of recombinant proteins in *E. coli*. Twelve percent SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant protein stained with Coomassie blue. Lanes: M, molecular mass marker; 1, rBbMSA-2c; 2, rBbRAP-1/CT; 3, rBbTRAP-T; 4, rBbSBP-1; 5, rBbSBP-4. The size of each recombinant protein is indicated on the right.

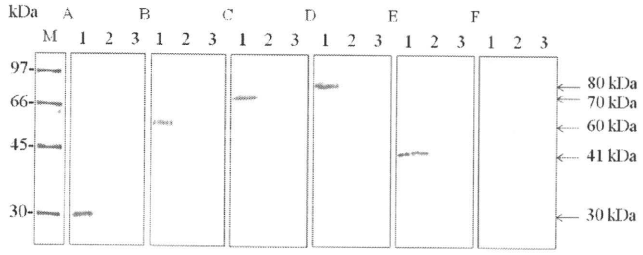

FIG. 3. Western blot analysis of authentic proteins with antisera against different recombinant proteins. BbMSA-2c (A), BbRAP-1 (B), BbTRAP (C), BbSBP-1 (D), and BbSBP-4 (E) were detected on the lysate of *B. bovis* parasites (lanes 1) but not on *B. bigemina* (lanes 2) and noninfected bovine RBC (lanes 3). The reactivity of the lysates was also examined with anti-GST serum (F) as a negative control.

a 10-μl field serum sample diluted in PBS (1:100) was applied as the first antibody on the fixed smears and then incubated for 1 h at 37°C in a moist chamber. After the slides were washed with PBS three times, fluorescein isothiocyanate (FITC)-conjugated sheep anti-bovine IgG antibody (Bethyl Laboratories, Montgomery, TX) was applied as a secondary antibody (1:250), and incubation proceeded for 1 h at 37°C. Propidium iodide (PI) (Molecular Probes) was used to stain the parasite's nuclei (26). After the glass slides were washed with PBS twice, they were mounted by adding 10 μl of a 50% (vol/vol) glycerol-PBS solution and covering them with glass coverslips and examined using a fluorescent microscope (E400 Eclipse; Nikon, Kawasaki, Japan).

Statistical analysis. The results of ELISAs were compared with those of the IFAT to calculate the percentages of agreement, the sensitivity and specificity (18), and the kappa values; thus, the strength of agreement between the ELISA and the IFAT was considered the kappa value: fair (0.21 to 0.40), moderate (0.41 to 0.60), and substantial (0.61 to 0.8) (http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTS

Production of recombinant proteins. The genes encoding BbMSA-2c, BbRAP-1/CT, BbTRAP-T, BbSBP-1, and BbSBP-4 were successfully expressed as soluble GST fusion proteins in *E. coli* with molecular masses of 55, 36, 66, 106, and 67 kDa, respectively (Fig. 1). Sera collected from cattle experimentally infected with *B. bovis* specifically reacted to all the recombinant proteins but not to the control GST protein in Western blot analysis (data not shown), suggesting their high antigenicity with the infected sera. Because full-length BbTRAP resulted in a low yield in the soluble fraction, only rBbTRAP-T was used for further validation (data not shown). Thereafter, five different antisera raised against each recombinant protein were used to identify the native proteins derived from *B. bovis*. Confocal microscopic observation of the IFAT demonstrated strong reactivity of each antiserum with both intra- and extraerythrocytic parasites of *B. bovis* (Fig. 2). Western blot analysis of *B. bovis* lysate probing these antisera revealed 30-, 60-, 70-, 80-, and 41-kDa proteins of BbMSA-2c, BbRAP-1, BbTRAP, BbSBP-1, and BbSBP-4, respectively (Fig. 3, lanes 1). All sizes of the authentic *B. bovis* proteins were consistent with the expected molecular weights for each mature protein. In contrast, these antisera did not cross-react with the lysate of *B. bigemina*-infected erythrocytes or normal bovine erythrocytes (Fig. 3, lanes 2 and 3, respectively).

Application of the recombinant proteins for serological diagnoses. The specificity and sensitivity of rBbMSA-2c, rBbRAP-1/CT, rBbTRAP-T, rBbSBP-1, and rBbSBP-4 were evaluated in a standard ELISA with experimentally infected and negative-control bovine sera. The cutoff OD values were determined to be 0.14, 0.20, 0.217, 0.173, and 0.11, respectively, for rBbMSA-2c, rBbRAP-1/CT, rBbTRAP-T, rBbSBP-1, and rBbSBP-4 using ROC analysis with 50 negative-control sera (Fig. 4). Notably, the ELISAs based on these recombinant proteins succeeded in clearly differentiating between *B. bovis*-infected sera and either the negative-control sera or B. *bigemina*-infected and *T. orientalis*-infected sera. All 25 *B. bovis*-

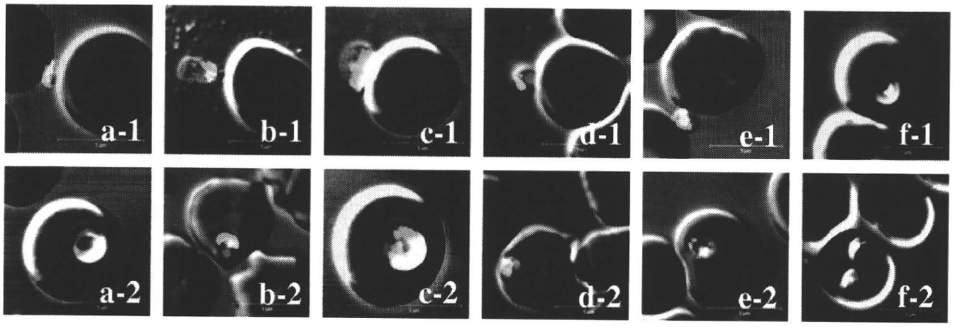

FIG. 2. Reactivity of each antiserum with *B. bovis* parasites by the IFAT. Confocal laser microscopic observation of extracellular (a-1 to e-1) and intracellular *B. bovis* parasites (a-2 to f-2). Thin blood smears of *B. bovis*-infected RBC fixed with absolute methanol were probed with each antiserum: a, anti-rBbMSA-2c; b, anti-rBbRAP-1/CT; c, anti-rBbTRAP-T; d, anti-rBbSBP-1; e, anti-rBbSBP-4; f, anti-GST. Specific immunofluorescent reaction (green) and nuclear staining (red) were observed.

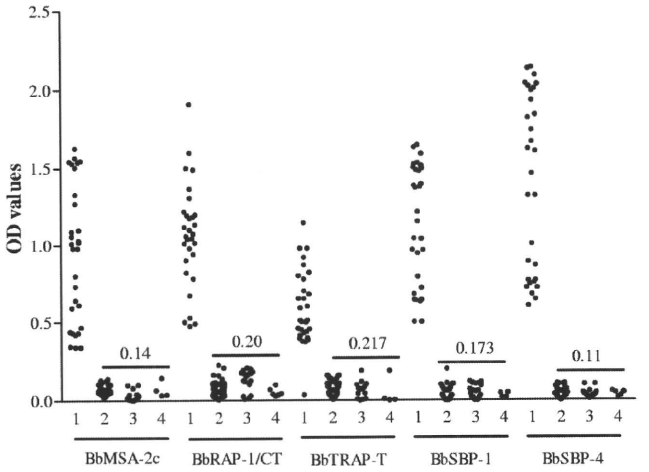

FIG. 4. Reactivity of ELISA using recombinant proteins with bovine sera. Lane 1, experimentally *B. bovis*-infected sera; lane 2, experimentally *B. bigemina*-infected sera; lane 3, noninfected bovine sera; lane 4, *Theileria orientalis*-infected sera. The cutoff of each recombinant protein is indicated by a bar.

infected serum samples tested had higher ODs than the cutoff value, contrary to the 30 *B. bigemina*-infected sera and 6 *Theileria orientalis*-infected sera, which had ODs lower than the cutoff values (Fig. 4). Although all recombinant proteins demonstrated good performance as ELISA antigens capable of identifying the experimental infection, rBbSBP-4 seemed to be the best due to its high OD values with *B. bovis*-infected sera and its lower OD values with normal bovine sera or *B. bigemina*- and *T. orientalis*-infected sera. Of note, rBbTRAP-T showed low OD values with *B. bovis*-infected sera. Furthermore, many samples were near the cutoff, and one sample was below, which indicates the low potential of rBbTRAP-T as a diagnostic antigen.

The diagnostic performances of these recombinant proteins were also evaluated with 669 field samples collected from areas of *B. bovis* endemicity, including Brazil (108 samples), Ghana (80 samples), China (100 samples), Thailand (100 samples), and Mongolia (81 samples), and areas where *B. bovis* is nonendemic, including South Korea (100 samples) and Hokkaido, Japan (100 samples). In addition, their results were compared to those of the IFAT, as a reference test (Table 2). None of the antigens showed a reaction, as tested using ELISAs and the IFAT with serum samples derived from areas where *B. bovis* is nonendemic (data not shown). On the other hand, the specificity and sensitivity of ELISAs with serum samples derived from the areas of endemicity ranged from 54.85% (rBbTRAP-T) to 96.01% (rBbSBP-4) and from 23.45% (rBbTRAP-T) to 96.43% (rBbSBP-4), respectively (Table 3). Next, the results were statistically compared to IFAT data. The agreement (con-

TABLE 2. Summary of ELISA results with recombinant proteins and IFAT results with field sera collected from different areas of *B. bovis* endemicity

Site or IFAT result	No. of IFAT samples	No. of ELISA samples									
		MSA-2c		RAP-1/CT		TRAP-T		SBP-1		SBP-4	
		+	−	+	−	+	−	+	−	+	−
Brazil											
+	80	52	28	35	45	10	70	74	6	78	2
−	28	2	26	0	28	1	27	8	20	4	24
Total	108	54	54	35	73	11	97	82	26	82	26
Ghana											
+	41	24	17	13	28	16	25	27	14	32	31
−	39	6	33	3	36	1	38	8	31	8	9
Total	80	30	50	16	64	17	63	35	45	40	40
China											
+	36	22	14	21	15	3	33	22	14	34	2
−	64	9	55	6	58	1	63	9	55	6	58
Total	100	31	69	27	73	4	96	31	69	40	60
Thailand											
+	57	45	12	34	23	10	47	38	19	51	6
−	43	16	27	16	27	2	41	14	29	11	32
Total	100	61	39	50	50	12	88	52	48	62	38
Mongolia											
+	29	17	12	18	11	9	20	16	13	18	11
−	52	11	41	10	42	4	48	6	46	10	42
Total	81	28	53	28	53	13	68	22	59	28	53
Overall											
+	243	160	83	121	122	48	195	177	66	213	30
−	226	44	182	35	191	9	217	45	181	39	187
Total	469	204	265	156	313	57	412	222	247	252	217