New Molecules in *Babesia gibsoni* and Their Application for Diagnosis, Vaccine Development, and Drug Discovery

Youn-Kyoung Goo1,2 and Xuenan Xuan1,*

1National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan; 2Department of Parasitology and Tropical Medicine, Kyungpook National University School of Medicine, Daegu, Korea

Abstract: *Babesia gibsoni* is an intraerythrocytic apicomplexan parasite that causes piroplasmosis in dogs. *B. gibsoni* infection is characterized clinically by fever, regenerative anemia, splenomegaly, and sometimes death. Since no vaccine is available, rapid and accurate diagnosis and prompt treatment of infected animals are required to control this disease. Over the past decade, several candidate molecules have been identified using biomolecular techniques in the authors’ laboratory for the development of a serodiagnostic method, vaccine, and drug for *B. gibsoni*. This review article describes newly identified candidate molecules and their applications for diagnosis, vaccine production, and drug development of *B. gibsoni*.

Key words: *Babesia gibsoni*, serodiagnosis, vaccine, drug

INTRODUCTION

*Babesia gibsoni* is an intraerythrocytic apicomplexan parasite that causes piroplasmosis in dogs. The organism was recognized in India in 1910, and since, has been reported in Asia, Northern and Eastern Africa, Brazil, Europe, and even Australia [1-3]. This disease is mainly transmitted naturally by ticks, but many reports have demonstrated transmission by dog bites and blood transfusions and transmission via the transplacental route to the developing fetus [4-7]. *B. gibsoni* infection occurs frequently in dogs, and recently has become a serious problem from a clinical viewpoint, because the acute form of the disease typically results in serious clinical problems, such as, fever, thrombocytopenia, regenerative anemia, splenomegaly, and sometimes death [8,9]. Furthermore, infected animals may become chronic carriers and transmit the disease via ticks to other animals. Therefore, in order to control *B. gibsoni* infection, rapid, accurate diagnosis followed by prompt effective treatment, and the prevention of chronic carriers are needed.

Apart from clinical signs, microscopic examination is the simplest and most accessible diagnostic test for most veterinarians, and is reasonably sensitive for detecting intraerythrocytic parasites in Giemsa-stained blood smears during acute infection [10]. Moreover, microscopic examination is the only viable option available to veterinarians in many developing countries where babesiosis is endemic. However, the diagnosis of babesiosis in chronically infected and carrier dogs remains a significant challenge due to very low, often intermittent parasitemia [9]. Indeed, failure to detect parasites in animals with hemolytic anemia or thrombocytopenia has led to incorrect diagnoses in several documented cases [5,11].

PCR provides an alternative diagnostic test with good sensitivity and specificity [12-15]. Since parasite morphology is a poor guide to speciation, modified PCR techniques have been utilized to differentiate piroplasm species rapidly. For example, PCR-restriction fragment length polymorphism (RFLP) and nested PCR have been reported to differentiate *B. canis* subspecies and *B. gibsoni* in Australia and in endemic regions [16,17]. In addition, loop-mediated isothermal amplification (LAMP) has been reported to have the advantages of speed and specificity over nested PCR for the detection of *B. gibsoni* infection [18], and real-time PCR has been shown to enable the quantification of pathogen levels in blood and tissue samples [13]. Furthermore, a number of these PCR methods have been applied to filter-paper technologies, such as, FTA (Whatman Bioscience) and IsoCode Stix (Schleicher and Scheull) cards to fa-
ciliate the transport of samples to distant laboratories. Although the sensitivities of PCR methods continue to improve, access to molecular techniques for routine clinical diagnosis is still restricted to relatively few clinics worldwide, and these methods will not detect target DNA when there are no organisms in a sample. In addition, “false negative” results may be encountered during the chronic disease stage, and it is critical that this limitation is appreciated during the screening of asymptomatic dogs as potential blood donors and of potential carriers [19]. The ability of PCR to detect parasite DNA in chronically infected dogs can be improved by testing on more than one occasion, but complementary serological testing is advisable [20,21].

Immunofluorescent antibody test (IFAT) is the most widely used serological diagnostic test used for babesiosis [22-24]. However, its poor specificity due to cross-reactions between B. gibsoni and other closely related parasites with B. gibsoni, operator subjectivity, and its unsuitability for large-scale screening limit its use. ELISA is a more sensitive technique than IFAT and is appropriate for testing large number of samples, especially in field surveys [25,26]. However, although ELISA tests are powerful tools for serological surveys, poor quality antigens and sometimes cross-reactions limit their applications [27].

After the accurate diagnosis of B. gibsoni infection, effective control and management measures, which include the treatment of infected animals and tick control, are essential. The treatment of B. gibsoni-infected dogs is challenging because it is difficult to eradicate the parasite completely. Various drug regimens improve clinical manifestations, but fail to achieve eradication as determined by follow-up. In particular, clindamycin reduces parasitemia and the clinical symptoms of Babesia infection [28], but does not eradicate the parasite. In an experimental trial of atovaquone, parasite clearance and concomitant clinical symptom resolution were observed in a B. gibsoni-infected dog after 2 days of atovaquone treatment, but the parasite reappeared at ~1 month after last treatment [29]. In one study, atovaquone and azithromycin were found to reduce parasitemia but not to eliminate the parasite; furthermore the development of drug resistance was suspected [30].

Because eradication of the parasite is not an option using available drugs, a vaccine against B. gibsoni provides another possible control strategy. Unfortunately, no commercially available vaccine is available for B. gibsoni, despite the efforts of several researchers. In a study by Sunaga et al., no detectable parasitemia was observed after B. gibsoni challenge in dogs inoculated with parasites attenuated by irradiation and serially passage in vitro [31,32]. However, the instability in vitro culture systems and the low parasitemia of B. gibsoni hamper the commercialization of such vaccines.

Therefore, in this review, we introduce some of the candidate molecules produced in the authors’ laboratory for 1) diagnosis of B. gibsoni infection, 2) development of vaccines against the parasite, and 3) identification of potential drug targets.

**IDENTIFICATION OF GENES ENCODING NEW PROTEINS**

Since B. gibsoni is a eukaryotic organism, its genome contains untranslated introns. Furthermore, the complete genome sequence of B. gibsoni has not been determined, and thus, it is not possible to predict the locations of introns and exons, to amplify specific genes without introns by PCR, or to clone and express recombinant proteins for diagnostic, antigenic, or developmental purposes. Alternatively, the generation of complementary DNA (cDNA) from mRNA transcripts lacking introns provides a means of obtaining genes encoding novel candidate proteins in B. gibsoni for a development of diagnostic method, vaccine, and drug. In this regard, a full-length cDNA library was prepared from canine erythrocytes infected with the NRCPD strain of B. gibsoni using the vector-capping method [33,34].

In order to isolate high antigenic proteins or proteins with a conserved domain, a cDNA expression library constructed from B. gibsoni mRNA was used for immunoscreening. A number of potent antigens have been isolated using this immunoscreening technique, and their diagnostic and vaccine potentials subsequently demonstrated (Table 1) [34-57]. In parallel to the immunoscreening strategy, expressed sequence tag (EST) databases can be used to isolate and identify novel genes for developmental purposes. In our laboratory, the ESTs were mainly used to isolate drug targets for B. gibsoni infection after bioinformatics analysis to determine functional motifs, and experimental studies on parasites demonstrated their potencies as drug targets. Recently, several drug candidates of B. gibsoni, 2-Cys peroxiredoxin, leucine aminopeptidase, inosine 5'-monophosphate dehydrogenase, and dihydrololate reductase-thymidylate synthase have been reported (Table 1) [58-61]. On the other hand, ESTs can be used to mine antigenic proteins by using bioinformatics software to predict the antigenicity (T- or B-cell epitope) and solubility of protein candidates for vaccine and serodiagnostic method development.
After selecting a target gene by immunoscreening or bioinformatics analysis of ESTs, genes were amplified from the cDNA library or total mRNA of *B. gibsoni* by PCR or RT-PCR, respectively, using specifically designed primers with restriction enzyme sites in our laboratory. However, to improve the solubility and expression efficacy of recombinant proteins in the expression system, sequences with hydrophobic characteristic or low antigenicity were truncated. In the event, this did increase the solubility and expression of certain recombinant proteins [65,66].

### APPLICATION OF RECOMBINANT PROTEINS

#### Serodiagnostic methods

Prior to using purified recombinant proteins for serodiagnosis, their antigenicities were evaluated by Western blotting using a canine serum infected with *B. gibsoni*. Proteins that reacted with *B. gibsoni*-infected serum were selected and then applied to develop serodiagnostic methods, such as, ELISA or ICT, with the sera from dogs experimentally and naturally infected with *B. gibsoni*.

**ELISA:** We performed indirect ELISA or double-antibody sandwich ELISA with the recombinant antigen of *B. gibsoni* to detect antibodies against the parasite. Among ELISAs using the identified antigens in our laboratory so far, ELISA with recombinant BgTRAP showed best performance at detecting antibody against *B. gibsoni* with high specificity and sensitivity [20,67]. However, recombinant BgTRAP was difficult to express in large amounts in the *E. coli* system. Thus, in order to increase expression efficacy, full-length BgTRAP was truncated and expression was optimized by adding ethanol, reducing the concentration of IPTG concentration, and culturing at lower temperature [65]. For sandwich ELISA, BgSA1 was selected and the sandwich ELISA could detect circulating BgSA1 in the plasma of dogs experimentally infected with *B. gibsoni* when parasitemia is 0.2% [40].

### Table 1. List of genes identified for possible diagnostic, vaccine, or drug use for *Babesia gibsoni* infection

| Gene (Accession No.) | Origin of isolation | Purpose | Application | Reference |
|----------------------|--------------------|---------|-------------|-----------|
| 2-Cys peroxiredoxin (AB829722) | EST database | Drug | [61] |
| Dihydrololate reductase-thymidylate synthase (AB426521) | EST database | Drug | [58,72] |
| Inosine 5’-monophosphate dehydrogenase (JQ781073) | EST database | Drug | [60,74] |
| Leucine aminopeptidase (AB90782) | EST database | Drug | [59] |
| Thrombospondin-related adhesive protein (AB053292) | Immunoscreening | Serodiagnosis | ELISA, ICT | [20,41,52,65,67] |
| P32 (AB282646) | Immunoscreening | Serodiagnosis | ELISA | [44,69] |
| P47 (AB521673) | Immunoscreening | Serodiagnosis | ELISA | [53,69] |
| P45 (GQ494996) | Immunoscreening | Serodiagnosis | ELISA | [54,69] |
| P50 (AB051834) | Immunoscreening | Serodiagnosis | ELISA, ICT, Vaccinia virus vectored vaccine | [34,39,56,66,70,71] |
| P29 (AB085585) | Immunoscreening | Serodiagnosis | ELISA, Vaccinia virus vectored vaccine | [38,49] |
| P22 (FJ608703) | Immunoscreening | Serodiagnosis | ELISA | [51] |
| Rhoptry-associated protein 1 (a,b,c) (EU292825, AB480715, AB480716) | EST database | Serodiagnosis | ELISA | [46] |
| Heat shock protein-70 (AB440627) | Immunoscreening | Vaccine | [68] |
| Secreted antigen 3 (AB481149) | Immunoscreening | Serodiagnosis | ELISA | [50] |
| P12 (AB378695) | Immunoscreening | Serodiagnosis | ELISA | [21] |
| Glutamic acid-rich protein (JX964993) | EST database | Serodiagnosis | ELISA, Vaccinia virus vectored vaccine | [55,57] |
| Ribosomal phosphoriboprotein P0 (AB266721) | Immunoscreening | Vaccine | [35-37] |
| Interspersed repeat antigen (EF452231) | Immunoscreening | Serodiagnosis | ELISA | [48] |
| P57 (EF455059) | Immunoscreening | Serodiagnosis | ELISA | [47] |
| Secreted antigen 1 (AB246895) | Immunoscreening | Serodiagnosis | Sandwich ELISA | [40] |
| P38 (DQ368062) | Immunoscreening | Serodiagnosis | ELISA | [43] |
| Apical membrane antigen 1 (DQ368061) | Immunoscreening | Serodiagnosis | ELISA | [42] |
Immunochromatographic test (ICT): ICT is a rapid, simple, and easy method in which the diagnosis is completed within 15 min after dropping a serum sample to the test strip. Therefore, another serodiagnostic method used to evaluate the expressed recombinant antigens of *B. gibsoni* is the ICT, in which recombinant truncated BgP50 (BgP50t), BgSA1, or truncated BgTRAP (BgTRAPt) are used to detect antibodies to *B. gibsoni* [39,45,52]. These ICTs with respective recombinant antigen were found to be specific, and not to react with serum from specific pathogen-free dogs. Furthermore, these antigens did not cross-react with sera from dogs experimentally infected with closely related parasites, such as, *B. canis canis*, *B. canis vogeli*, and *B. canis rossi*, or with *N. canimun*, and *L. infantum*. In contrast, the test did identify dogs experimentally infected with *B. gibsoni* and clinically infected dogs. We found that ICT using these antigens to be rapid, simple, accurate, and suitable for use at clinical sites for the diagnosis of *B. gibsoni* infection in dogs. Favorable results, that is, a sensitivity of 100% and a specificity of 93.78%, were reported when the test was performed using BgTRAPt for the detection of antibodies against *B. gibsoni*.

Vaccine development

No effective commercial vaccine against *B. gibsoni* is available. Recent strategies used for vaccine development are based on the use of parasite recombinant antigens or specific DNA rather than crude parasite lysates. In studies of *B. gibsoni*, although in vitro culture was available, parasitemia did not usually reach 5%, and thus, vaccine development focused on identifying high antigenic vaccine candidates (usually recombinant antigens) [35-37,49,66,68].

A gene encoding a 50-kDa surface protein (BgP50) of *B. gibsoni* was identified by immunoscreening using a cDNA library of *B. gibsoni* and serum from a dog experimentally infected with the parasite [34,69]. Once the gene had been identified, it was expressed in insect cells using baculovirus, and the obtained recombinant antigen was evaluated for antigenicity [66,70]. Surface proteins of parasite are ideal candidates for vaccine development because these proteins are the main targets of host immune responses during the host-parasite interaction. Accordingly, the surface antigens of parasites are logical targets for subunit vaccines. We produced a truncated form of BgP50 (BgP50t) without its C-terminal hydrophobic region, and found that recombinant BgP50t (rBgP50t) was secreted into the supernatant of insect cells infected with recombinant baculoviruses. rBgP50t induced high antibody titers against the *B. gibsoni* merozoite when inoculated into mice, and antiserum to rBgP50t significantly inhibited *B. gibsoni* growth, indicating that rBgP50t might be a useful vaccine candidate. This inhibitory effect has been reported in a Severed Combined Immunodeficiency (SCID) mouse model transfused with canine erythrocytes [71]. Although the study focus of above-mentioned researches was on antibody-mediated immune response, it is vital to note that cellular immune responses could also play a role during *B. gibsoni* infection. Consequently, in future studies the antibody-mediated and cellular immune responses of the candidate vaccine antigens should be confirmed for full protection against *B. gibsoni* infection.

In another study, recombinant vaccinia virus-vectored plasmids were constructed to express rBgP50, rBgP29, or rBgGARP, respectively, and these vaccinia virus-vectored plasmids were used to immunize dogs [49,56,57]. The immunized dogs were shown to develop a high level of specific antibodies against the antigens as compared with non-immunized dogs. Furthermore, the antibody level was increased by booster immunization with a recombinant vaccinia virus. Additionally, the prime-boost immunization dose is known to provoke a specific IgG2 antibody response and IFN-γ production in dogs. However, when the immunized dogs were challenged with *B. gibsoni* parasites, only partial protection was afforded by this immunization regime.

Ribosomal phosphoprotein P0 of *B. gibsoni* in its recombinant form (rBgP0) was evaluated as a universal vaccine candidate for *B. gibsoni*, *B. microti*, and *B. rodhaini* infections [36]. rBgP0 induced immunological response in mice against *B. microti* challenge [37]. In addition, the same rBgP0 showed cross-protective immunity against a lethal *B. rodhaini* infection in mice, and this cross-protection was mainly due to complement component 3 (C3) [35]. It is believed that antibody responses and cytokine production might play a role in this C3 mediated cross-protective immunity. In addition, heat shock protein-70 of *B. gibsoni* showed a cross-protective effect in mice infected with *B. microti*, and a partial protective effect was observed against *B. microti* infection [68].

Despite all these research attempts to produce candidate vaccines against *B. gibsoni* infection based on recombinant antigens, it has not been possible to identify an antigen that offers sufficient protection to justify its use as a commercial subunit vaccine. Nevertheless, studies on mechanisms of immune response during infection with *B. gibsoni* could provide new
insight and lead to the discovering of candidate vaccines.

Drug development

Currently available drugs, including clindamycin, atovaquone, and azithromycin, reduce parasitemia and the clinical symptoms of *B. gibsoni*-infection, but do not eradicate the parasite [28-30]. These drugs were discovered by random screening of the anti-babesial activity of chemical compounds against other pathogens, not by the rational identification of drug targets. Although some metabolic pathways of *B. bovis* have been predicted from the whole genome sequences of *B. bovis*, knowledge regarding the biochemistry of *Babesia* parasites, including *B. gibsoni*, is limited.

Given the situation that the whole genome sequence of *B. gibsoni* is not available, the EST strategy provides an alternative means of identifying drug target by aligning ESTs with genes of other apicomplexans using BLASTX. Using this strategy, 2-Cys peroxiredoxin, inosine 5'-monophosphate dehydrogenase, dihydrofolate reductase-thymidylate synthase, and leucine aminopeptidase of *B. gibsoni* were identified and studied as drug targets [58-61]. The proteins were used for biochemical characterization and antiserum preparation against the respective recombinant proteins to study endogenous native proteins in *B. gibsoni* parasites. Those 4 proteins were found in the cytoplasm in *B. gibsoni* in an IFAT confocal microscopy based study [58-61]. Subsequently, the enzyme activities of the recombinant enzymes were determined using in vitro enzymatic assays.

In the case of dihydrofolate reductase-thymidylate synthase of *B. gibsoni* (BgDHFR-TS), a well-validated antifolate drug target in certain pathogenic apicomplexans, the catalytic activity of the recombinant enzyme was confirmed, and its kinetic parameters and inhibition by novel or known compounds were assayed. GST fused and non-fused recombinant BgDHFR-TS enzyme displays catalytic activity and had Km values similar to corresponding recombinant DHFR-TS in *P. falciparum*. In the same study, it was demonstrated that the inhibitory effect of 3 antifolates, methotrexate, pyrimethamine, and trimethoprim, on the catalytically active recombinant DHFR-TS enzyme of *B. gibsoni* paralleled their inhibitions of parasite growth in vitro [58]. Furthermore, methotrexate, which is a more-potent inhibitor of the BgDHFR-TS enzyme activity than pyrimethamine or trimethoprim, is also a potent inhibitor of *B. gibsoni* proliferation in vitro. Because the chemical structures of these antifolates resemble that of the substrate, dihydrofolate, the compounds are believed to compete with the substrate for binding to amino acid residues in the hydrophobic pocket of active site of recombinant BgDHFR enzyme [58,72].

Inosine 5'-monophosphate dehydrogenase (IMPDH) is an attractive drug target in the purine pathway because parasites rely on this pathway to meet their purine demands for nucleic acid synthesis [73]. Its enzyme activity was confirmed by measuring *Km* and *IC*50 values. Subsequently, mycophenolic acid, a structural analog of the cofactor NAD+, showed an inhibitory effect on the enzyme activity of BgIMPDH and on the growth of *B. gibsoni*, which indicated mycophenolic acid might inhibit the replication of *B. gibsoni* by targeting IMPDH of the purine pathway [60]. In addition, several IMPDH inhibitors, including mycophenolate mofetil, mizoribine, ribavirin, 7-nitroindole, and mycophenolic acid, were tested in in vitro cultures of *B. gibsoni*, and mycophenolate mofetil showed the most potent inhibitory effect on parasite growth [74].

Two recombinant proteins, that is, 2-Cys peroxiredoxin and leucine aminopeptidase, of *B. gibsoni* were produced, and subsequently, respective native proteins were identified in the cytoplasm of *B. gibsoni*. 2-Cys peroxiredoxin is an antioxidant enzyme that controls redox balance and by so doing protects membrane lipids, nucleic acids, and proteins from damage by reactive oxygen species (ROS). Antioxidant activity was confirmed using recombinant 2-Cys peroxiredoxin of *B. gibsoni*, which suggests that 2-Cys peroxiredoxin controls redox balance in *B. gibsoni* [61]. In addition, the enzyme activity of recombinant leucine aminopeptidase of *B. gibsoni* (rBglAP) was confirmed using a leucine substrate. Although the substrate profile was slightly different from its homologue in *P. falciparum*, bestatin, an aminopeptidase inhibitor, showed similar inhibitory effect to enzyme activity of rBglAP and its homologues in *P. falciparum* [59]. Few studies have been conducted on identification of drug targets and on the metabolic pathways of *B. gibsoni* to date, and thus, undiscovered metabolic pathways could yield possible drug targets. For example, apicoplast metabolism was recently examined as drug target in *Plasmodium* species, but this has not been reported to be present in *B. gibsoni*.

**CONCLUSION**

In this review, we introduce molecules identified and characterized for serodiagnosis and vaccine development and molecules that are drug targets for *B. gibsoni* infection. Of the identified antigens, a truncated recombinant BgTRAP produced
promising results with high sensitivity and specificity for the detection of antibody specific to B. gibsoni by ELISA and ICT. For the next step, the BgTRAP could be used to develop a rapid diagnostic test of B. gibsoni infection using monoclonal antibodies specific to BgTRAP. The diagnostic test using the monoclonal antibodies would detect the antigen (BgTRAP) and by doing so differentiate dogs in current infection from past infection. In vaccine studies, parasitemia was only found to be partially reduced in studies of candidate vaccines, despite the induction of immune responses against B. gibsoni parasites in experimental animals. This could be improved by using effective adjuvants with the identified vaccine candidates based on better understanding of host immune responses induced by B. gibsoni. Regarding drug studies, few molecules have been identified to date, and the knowledge of metabolic pathways of B. gibsoni is still very limited. Therefore, further research is needed to identify the metabolic pathways that could be used to control this disease.

CONFLICT OF INTEREST

We have no conflict of interest related with this work.

REFERENCES

1. Groves MG, Yap LF. Babesia gibsoni (Patton, 1910) from a dog in Kuala Lumpur. The Med J Malay 1968; 22: 229.
2. Kjemtrup AM, Kocan AA, Whitworth I, Meinkoth J, Birkenheuer AJ, Cummings J, Boudreaux MK, Stockham SL, Irizarry-Rovira A, Conrad PA. There are at least three genetically distinct small piroplasms from dogs. J Parasitol 2000; 30: 1501-1505.
3. Muhlnickel CJ, Jefferies R, Morgan-Ryan UIM, Irwin PJ. Babesia gibsoni infection in three dogs in Victoria. Aust Vet J 2002; 80: 606-610.
4. Baneth G, Breitschwerdt EB, Hegarty BC, Pappalardo B, Ryan J. A survey of tick-borne bacteria and protozoa in naturally exposed dogs from Israel. Vet Parasitol 1998; 74: 133-142.
5. Birkenheuer AJ, Correa MF, Levy MG, Breitschwerdt EB. Geographic distribution of babesiosis among dogs in the United States and association with dog bites: 150 cases (2000-2003). J Am Vet Med Assoc 2005; 227: 942-947.
6. Stegeman JR, Birkenheuer AJ, Kruger JM, Breitschwerdt EB. Transfusion-associated Babesia gibsoni infection in a dog. J Am Vet Med Assoc 2003; 222: 959-963, 952.
7. Fukumoto S, Suzuki H, Igarashi I, Xuan X. Fatal experimental transplacental Babesia gibsoni infections in dogs. Int J Parasitol 2005; 35: 1031-1035.
8. Conrad P, Thomford J, Yamane I, Whiting J, Rosma I, Uno T, Holshuh HJ, Shelly S. Hemolytic anemia caused by Babesia gibsoni infection in dogs. J Am Vet Med Assoc 1991; 199: 601-605.
9. Boozer AL, Macintire DK. Canine babesiosis. Vet clin North Am Small Anim Pract 2003; 33: 885-904, viii.
10. Rosenblatt JE. Laboratory diagnosis of infections due to blood and tissue parasites. Clin Infect Dis 2009; 49: 1103-1108.
11. Yeagley TJ, Reichard MV, Hempstead JE, Allen KE, Parsons LM, White MA, Little SE, Meinkoth JH. Detection of Babesia gibsoni and the canine small Babesia ‘Spanish isolate’ in blood samples obtained from dogs confiscated from dog fighting operations. J Am Vet Med Assoc 2009; 235: 535-539.
12. Ano H, Makimura S, Harasawa R. Comparison of partial ribosomal DNA sequences of Babesia gibsoni occurring in Miyazaki prefecture, Japan. J Vet Med Sci 2001; 63: 561-562.
13. Matsuza A, Ono S, Ikadai H, Uchide T, Imamura S, Onuma M, Okano S, Higuchi S. Development of a SYBR green real-time polymerase chain reaction assay for quantitative detection of Babesia gibsoni (Asian genotype) DNA. J Vet Diagn Invest 2005; 17: 569-573.
14. Tani H, Tada Y, Sasai K, Baba E. Improvement of DNA extraction method for dried blood spots and comparison of four PCR methods for detection of Babesia gibsoni (Asian genotype) infection in canine blood samples. J Vet Med Sci 2008; 70: 461-467.
15. Fritz D. A PCR study of piroplasms in 166 dogs and 111 horses in France (March 2006 to March 2008). Parasitol Res 2010; 106: 1339-1342.
16. Zahler M, Schein E, Rinder H, Gothe R. Characteristic genotypes discriminate between Babesia canis isolates of differing vector specificity and pathogenicity to dogs. Parasitol Res 1998; 84: 544-548.
17. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminished PCR for detection and differentiation of Babesia gibsoni (Asian genotype) and B. canis DNA in canine blood samples. J Clin Microbiol 2003; 41: 4172-4177.
18. Ikadai H, Tanaka H, Shibahara N, Matsuza A, Uchi M, Itch N, Oshiro S, Kudo N, Igarashi I, Oymada T. Molecular evidence of infections with Babesia gibsoni parasites in Japan and evaluation of the diagnostic potential of a loop-mediated isothermal amplification method. J Clin Microbiol 2004; 42: 2465-2469.
19. Irwin PJ. Canine babesiosis: from molecular taxonomy to control. Parasit Vectors 2009; 2 Suppl 1: S4.
20. Goo YK, Jia H, Aboge GO, Terkawi MA, Kuriki K, Nakamura C, Kumagai A, Zhou J, Lee EG, Nishikawa Y, Igarashi I, Fujisaki K, Xuan X, White MA,Little SE,Meinkoth JH. Development of a SYBR green real-time polymerase chain reaction assay for quantitative detection of Babesia gibsoni (Asian genotype) DNA. J Vet Diagn Invest 2005; 17: 569-573.
21. Yeagley TJ, Reichard MV, Hempstead JE, Allen KE, Parsons LM, White MA, Little SE, Meinkoth JH. Detection of Babesia gibsoni and the canine small Babesia ‘Spanish isolate’ in blood samples obtained from dogs confiscated from dog fighting operations. J Am Vet Med Assoc 2009; 235: 535-539.
22. Anderson JE, Magnarelli LA, Sulzer AL. Canine babesiosis: indirect fluorescent antibody test for a North American isolate of Babesia gibsoni. Am J Vet Res 1980; 41: 2102-2105.
23. Levy MG, Breischwerdt EB, Moncol DJ. Antibody activity to *Babesia canis* in dogs in North Carolina. Am J Vet Res 1987; 48: 339-341.

24. Yamane I, Thomford JW, Gardner IA, Dubey JP, Levy M, Conrad PA. Evaluation of the indirect fluorescent antibody test for diagnosis of *Babesia gibsoni* infections in dogs. Am J Vet Res 1993; 54: 1579-1584.

25. Reiter I, Weiland G. Recently developed methods for the detection of babesial infections. Trans R Soc Trop Med Hyg 1989; 83 (suppl): 21-23.

26. Furuta PJ, Oliveira TM, Theixeira MC, Rocha AG, Machado RZ, Tinucci-Costa MG. Comparison between a soluble antigen-based ELISA and IFAT in detecting antibodies against *Babesia canis* in dogs. Rev Bras Parasitol Vet 2009; 18: 41-45.

27. Bose R, Jorgensen WK, Dalgleish RJ, Friedhoff KT, de Vos AJ. Current state and future trends in the diagnosis of babesiosis. Vet Parasitol 1995; 57: 61-74.

28. Wulansari R, Wijaya A, Ano H, Horii Y, Nasu T, Yamane S, Masunari N, Suzuki H. Identification and expression of a novel gene encoding a secreted antigen of *Babesia gibsoni* in vivo and in vitro. Vet Parasitol 2004; 124: 9-18.

29. Jerfieres R, Ryan UM, Jardine J, Robertson ID, Irwin PJ. *Babesia gibsoni*: detection during experimental infections and after combined atovaquone and azithromycin therapy. Exp Parasitol 2007; 117: 115-123.

30. Sunaga F, Nishikawa Y, Horii Y, Igarturi S, Fujisaki K, Suzuki H, Xuan X. A 38-kDa meront antigen of *Babesia gibsoni* infection in dogs. J Anim Prot Zoo 2003; 39: 558-562.

31. Matsuu A, Koshida Y, Kuriha Y, Nishikawa Y, Fujisaki K, Mikami T, Igarashi I. Efficacy of atovaquone against *Babesia gibsoni* infection. J Anim Protozooses 2002; 17: 15-18.

32. Zhou J, Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. Identification of a novel gene encoding a secreted antigen of *Babesia gibsoni* and evaluation of its use in serodiagnosis. Am J Trop Med Hyg 2006; 75: 843-850.

33. Zhou J, Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. Molecular characterization of a novel 29-kDa merozoite antigen of *Babesia gibsoni* with a better diagnostic performance by enzyme-linked immunosorbent assay. Parasitology 2007; 134: 1185-1194.

34. Zhou J, Jia H, Zhou J, Ikadai H, Matsuu A, Igarashi I, Fujisaki K, Xuan X. Development of an immunochromatographic test with recombinant P50 for the diagnosis of *Babesia gibsoni* infection in dogs. Exp Parasitol 2006; 114: 329-333.

35. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. Identification of a novel gene encoding a secreted antigen of *Babesia gibsoni* and its antibody response in an experimentally infected dog. Vet Parasitol 2006; 141: 345-348.

36. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. Molecular characterization of a novel 32-kDa merozoite antigen of *Babesia gibsoni* with a better diagnostic performance by enzyme-linked immunosorbent assay. Parasitology 2007; 134: 1185-1194.

37. Jia H, Zhou J, Ikadai H, Matsuu A, Igarashi I, Fujisaki K, Xuan X. Development of an immunochromatographic test with recombinant BgS19 for the diagnosis of *Babesia gibsoni* infection in dogs. Parasitol Res 2007; 100: 1381-1384.

38. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. *Babesia gibsoni* rhoptry-associated protein 1 and its potential use as a diagnostic antigen. Vet Parasitol 2007; 145: 16-20.

39. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. A novel 57-kDa merozoite protein of *Babesia gibsoni* is a prospective antigen for diagnosis and serosurvey of canine babesiosis by enzyme-linked immunosorbent assay. Parasitol Res 2007; 100: 1401-1407.

40. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. *Babesia gibsoni* rhoptry-associated protein 1 and its potential use as a diagnostic antigen. Vet Parasitol 2007; 145: 16-20.

41. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. *Babesia gibsoni* rhoptry-associated protein 1 and its potential use as a diagnostic antigen. Vet Parasitol 2007; 145: 16-20.

42. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. *Babesia gibsoni* rhoptry-associated protein 1 and its potential use as a diagnostic antigen. Vet Parasitol 2007; 145: 16-20.

43. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. *Babesia gibsoni* rhoptry-associated protein 1 and its potential use as a diagnostic antigen. Vet Parasitol 2007; 145: 16-20.

44. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. *Babesia gibsoni* rhoptry-associated protein 1 and its potential use as a diagnostic antigen. Vet Parasitol 2007; 145: 16-20.

45. Jia H, Zhou J, Ikadai H, Matsuu A, Suzuki H, Igarashi I, Fujisaki K, Xuan X. *Babesia gibsoni* rhoptry-associated protein 1 and its potential use as a diagnostic antigen. Vet Parasitol 2007; 145: 16-20.
50. Jia H, Terkawi MA, Aboge GO, Goo YK, Ma I, Zhou J, Nishikawa Y, Igarashi I, Fujisaki K, Xuan X. Identification of secreted antigen 3 from Babesia gibsoni. Clin Vaccine Immunol 2009; 16: 944-948.

51. Goo YK, Jia H, Terkawi MA, Aboge GO, Yamagishi J, Nishikawa Y, Kim S, Jang HK, Fujisaki K, Xuan X. Babesia gibsoni: identification, expression, localization, and serological characterization of a Babesia gibsoni 22-kDa protein. Exp Parasitol 2009; 123: 273-276.

52. Goo YK, Lee N, Terkawi MA, Luo Y, Aboge GO, Nishikawa Y, Suzuki H, Kim S, Xuan X. Development of a rapid immunochromatographic test using a recombinant thrombospondin-related adhesive protein of Babesia gibsoni. Vet Parasitol 2012; 190: 595-598.

53. Aboge GO, Batbaatar V, Goo YK, Yamagishi J, Nishikawa Y, Sunaga F, Namikawa K, Igarashi I, Fujisaki K, Suzuki H, Xuan X. Molecular characterization and expression of a 47-kDa merozoite surface protein of Babesia gibsoni for serodiagnosis by enzyme-linked immunosorbent assay. J Protocool Res 2010; 20: 59-69.

54. Goo YK, Jia H, Aboge GO, Terkawi MA, Yamagishi J, Nishikawa Y, Igarashi I, Xuan X. Identification of an immunodominant Babesia gibsoni 47-kDa antigen. J Protocool Res 2009; 19: 16-21.

55. Moussa AA, Cao S, Aboge GO, Terkawi MA, Kirdasy AE, Salama A, Attia M, Aboulaila M, Zhou M, Kamykingik K, Moumouni PE, Masatani T, El Aziz SA, Moussa WM, Chahan B, Fukumoto S, Nishikawa Y, El Ballal SS, Luo Y, Li Y, Cao S, Yu L, Kamykingik K, Aboge GO, Nishikawa Y, Xuan X. Molecular characterization and antigenic properties of a novel Babesia gibsoni glutamic acid-rich protein (BgGARP). Exp Parasitol 2013; 135: 414-420.

56. Fukumoto S, Tamaki Y, Okamura M, Bannai H, Yokoyama N, Suzuki T, Igarashi I, Suzuki H, Xuan X. Prime-boost immunization with DNA followed by a recombinant vaccinia virus expressing BgGARP induced a partial protective immunity to inhibit Babesia gibsoni proliferation in dogs. Infect Immun 2007; 25: 1334-1341.

57. Cao S, Moussa AA, Aboge GO, Kamykingik K, Zhou M, Moumouni PFA, Terkawi MA, Masatani T, Nishikawa Y, Suzuki H, Fukumoto S, Xuan X. Prime-boost vaccination with plasmid DNA followed by recombinant vaccinia virus expressing BgGARP induced a partial protective immunity to inhibit Babesia gibsoni proliferation in dogs. Acta Parasitol 2013; 58: 619-623.

58. Aboge GO, Jia H, Terkawi MA, Goo YK, Nishikawa Y, Sunaga F, Namikawa K, Tsuji N, Igarashi I, Suzuki H, Fujisaki K, Xuan X. Cloning, expression, and characterization of Babesia gibsoni dihydrofolate reductase-thymidylate synthase: inhibitory effect of antifolates on its catalytic activity and parasite proliferation. Antimicrob Agents Chemother 2008; 52: 4072-4080.

59. Jia H, Terkawi MA, Aboge GO, Goo YK, Luo Y, Li Y, Yamagishi J, Nishikawa Y, Igarashi I, Sugimoto C, Fujisaki K, Xuan X. Characterization of a leucine aminopeptidase of Babesia gibsoni. Parasitology 2009; 136: 945-952.

60. Cao S, Aboge GO, Terkawi MA, Zhou M, Luo Y, Yu L, Goo Y, Kamykingik K, Masatani T, Suzuki H, Igarashi I, Nishikawa Y, Xuan X. Cloning, characterization and validation of a 5’-monophosphate dehydrogenase of Babesia gibsoni as molecular drug target. Parasitol Int 2013; 62: 87-94.

61. Masatani T, Asada M, Ichikawa-Seki M, Usui M, Terkawi MA, Hayashi K, Kawazu S, Xuan X. Cloning and Characterization of a 2-Cys Peroxiredoxin from Babesia gibsoni. J Vet Med Sci 2014; 76: 139-143.

62. Larsen MV, Lundegaard C, Lambeth K, Buis S, Lund O, Nielsen M. Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. BMC bioinformatics. 2007; 8: 424.

63. El-Manzalawy Y, Dobbs D, Honavar V. Predicting linear B-cell epitopes using string kernels. J Mol Recognit 2008; 21: 243-255.

64. El-Manzalawy Y, Dobbs D, Honavar V. Predicting MHC-II binding affinity using multiple instance regression. IEEE/ACM Trans Comput Biol Bioinform 2011; 8: 1067-1079.

65. Narantsartsral S, Goo YK, Battsetseg B, Myagmarsuren P, Terkawi MA, Soma T, Luo Y, Li Y, Cao S, Yu L, Kamykingik K, Aboge GO, Nishikawa Y, Xuan X. Expression of truncated Babesia gibsoni thrombospondin-related adhesive proteins in Escherichia coli and evaluation of their diagnostic potential by enzyme-linked immunosorbent assay. Exp Parasitol 2011; 129: 196-202.

66. Fukumoto S, Xuan X, Kodota K, Igarashi I, Sugimoto C, Fujisaki K, Nagasawa H, Mikami T, Suzuki H. High-level expression of truncated surface antigen P50 of Babesia gibsoni in insect cells by baculovirus and evaluation of its immunogenicity and antigenicity. Clin Diagn Lab Immunol 2003; 10: 596-601.

67. Konishi K, Sakata Y, Miyazaki N, Jia H, Goo YK, Luo Y, Xuan X, Inokuma H. Epidemiological survey of Babesia gibsoni infection in dogs in Japan by enzyme-linked immunosorbent assay using B. gibsoni thrombospondin-related adhesive protein antigen. Vet Parasitol 2008; 155: 204-208.

68. Terkawi MA, Aboge G, Jia H, Goo YK, Ooka H, Yamagishi J, Nishikawa Y, Yokoyama N, Igarashi I, Kawazu SI, Fujisaki K, Suzuki H. Molecular and immunological characterization of Babesia gibsoni and Babesia microti heat shock protein-70. Parasite Immunol 2009; 31: 328-340.

69. Goo YK, Aboge GO, Terkawi MA, Jia H, Yamagishi J, Sunaga F, Namikawa K, Cha SY, Jang HK, Kim S, Nishikawa Y, Xuan X. Four promising antigens, BgP32, BgP45, BgP47, and BgP50, for serodiagnosis of Babesia gibsoni and Babesia microti infection in dogs by enzyme-linked immunosorbent assay using B. gibsoni merozoite surface protein family. Parasitol Int 2012; 61: 364-368.

70. Fukumoto S, Tamaki Y, Shirafuji H, Harakawa S, Suzuki H, Xuan X. Immunization with recombinant surface antigen P50 of Babesia gibsoni expressed in insect cells induced parasite growth inhibition in dogs. Clin Diag Lab Immunol 2005; 12: 557-559.

71. Fukumoto S, Xuan X, Takabatake N, Igarashi I, Sugimoto C, Fujisaki K, Nagasawa H, Mikami T, Suzuki H. Inhibitory effect of antiserum to surface antigen P50 of Babesia gibsoni on growth of parasites in severe combined immunodeficiency mice given canine red blood cells. Infect Immun 2004; 72: 1795-1798.

72. Aboge GO, Jia H, Terkawi MA, Goo YK, Batbaatar V, Nishikawa Y, Sunaga F, Namikawa K, Fujisaki K, Suzuki H, Xuan X. Precursors of methotrexate target dihydrofolate reductase-thymidylate synthase of Babesia gibsoni and inhibit parasite proliferation. J Proto-
zool Res 2010; 20: 70-81.
73. Ginsburg H. Metabolism: Malaria parasite stands out. Nature 2010; 466: 702-703.
74. Cao S, Aboge GO, Terkawi MA, Zhou M, Kamilyingkird K, Mounouni PFA, Masatani T, Igarashi I, Nishikawa Y, Suzuki H, Xuan X. Mycophenolic acid, mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole inhibit propagation of Babesia parasites by targeting inosine 5’-monophosphate dehydrogenase. J Parasitol 2014 (in press). doi: http://dx.doi.org/10.1645/13-278.1.
