Studies on development of serological diagnostic methods and molecular targeted drugs against canine babesiosis

2013

CAO, SHINUO

Doctor's Course of Animal and Food Hygiene
Graduate School of
Obihiro University of Agriculture and Veterinary Medicine

犬バベシア感染症に対する血清学診断法及び分子標的治療薬の開発に関する研究

平成 25 年

(2013)

曹　世諾

帯広畜産大学大学院畜産学研究科

博士後期課程　畜産衛生学専攻

Contents

Abbreviations

B BccSA1: *Babesia canis canis* secreted antigen 1

BccP33: *Babesia canis canis* 33 kDa protein

BgIMPDH: *Babesia gibsoni* inosine 5'-monophosphate dehydrogenase

BSA: bovine serum albumin

C cDNA: complementary DNA

D das-ELISA: double antibody sandwich enzyme-linked immunosorbent assay

DNA: deoxyribonucleic acid

DPI: days post-infection

E ELISA: enzyme-linked immunosorbent assay

G GST: glutathione *S*-transferase

H HCT: hematocrit

I IMPDH: inosine 5'-monophosphate dehydrogenase

IMP: inosine 5'-monophosphate

i.p.: intraperitoneal

ICT: immunochromatographic test

IFAT: immunofluorescent antibody test

IgG: immunoglobulin G

K kDa: kilodalton

M MMF: mycophenolate mofetil

MPA: mycophenolic acid

MZB: mizoribine

O OD: optical density

ORF: open reading frame

P PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFU: plaque forming unit

PI: propidium iodide

R RBV: ribavirin

RBC: red blood cell

RNA: ribonucleic acid

S SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

SPF: specific pathogen free

X XMP: xanthosine 5'-monophosphate

7-NID: 7-nitroindole

General introduction

1. Babesiosis in general

Babesiosis is caused by intraerythrocytic protozoan parasites of the genus *Babesia.* The disease, which is transmitted by ticks, affects a wide range of domestic and wild animals and occasionally humans. While the major economic impact of babesiosis is on the cattle industry, infections in other domestic animals, including horses, sheep, goats, pigs, and dogs, assume varying degrees of importance throughout the world. In 1888, the *Babesia* protozoa was firstly identified by Romanian biologist Babes, who isolated the parasite from cattle with febrile hemoglobinuria (Babes, 1888). Since then, newly recognized *Babesia* species continue to emerge around the world and the significant economic influence on livestock and substantial public health impact on humans are ongoing (Collett, 2000; Kivaria *et al.*, 2007).

Babesia species are classified in the order Piroplasmida and family Babesiidae within the phylum Apicomplexa based on their exclusive invasion of erythrocytes, multiplication by budding rather than schizogony, and lack of hemozoin (Hunfeld *et al.*, 2008). All species of *Babesia* are naturally transmitted by the bite of infected ticks, in which sporozoites are injected into host together with the saliva of the vector tick and directly infect red blood cells (Battsetseg *et al.*, 2007). The sporozoites infected into red blood cells develop into piroplasms inside the infected erythrocyte resulting two or sometimes four divisions develop as the parasites split. While still in the blood cells, the parasites are ingested by ticks eating the host's blood. Parasites move into the tick’s gut cells, which transforms them into phagocytes

as the tick passes through its larval stage to the nymph stage over the winter. Still inside the ticks, they transform into vermicular sporozoites through binary fission. The tick releases the sporozoites, which travel to the new host, when the tick takes another meal (Fig. 1).

The disease manifestations of babesiosis are caused by the asexual reproductive stage of the organism in the erythrocytes of the host and the subsequent lysis of host cells. Consequently, there is a very broad clinical spectrum, which is probably directly reflective of the level of parasitemia in the blood (Homer *et al.*, 2000a). The extreme case is often described as a fulminating malaria-like infection; symptoms may include anemia, fatigue, chills, myalgia, and fever. Some cases also described nausea, emesis, weight loss, and hematuria. Traditionally, *Babesia* was mainly grouped based on their morphology, host/vector specificity, and susceptibility to drugs. Recently, molecular genetic analyses clarified the somewhat confusing phylogenetic situation, sometimes resulting in the emergence of new groups and 18S rDNA analysis added new information to the taxonomic position of many piroplasm species (Kjemtrup and Conrad, 2000).

2. Canine babesiosis

Canine babesiosis is a tick-borne disease caused by hemoprotozoan parasites of the genus *Babesia*. The parasites *B. canis* and *B. gibsoni* are responsible for canine babesiosis throughout the world. *B. canis* was previously described as a group of three biologically different subspecies, namely *B. canis canis*, *B. canis vogeli*, and *B. canis rossi*, which have been confirmed with polymerase chain reaction (PCR) using restriction fragment length

polymorphism analysis. *B. c. canis* was found in Europe, *B. c. vogeli* in Northern Africa, North America and South Africa and *B. c. rossi* in Southern Africa. The pear-shaped trophozoite of *B. canis* measures 4-5 μm long and is usually found in pairs within the erythrocyte, but up to eight or more may be present. In comparison, the small *Babesia* in dogs is generally considered to be *B. gibsoni* and using PCR, this group has been further subdivided into two subspecies: the North American and Asian subspecies.

The pathophysiology of infection with canine *Babesia*, appear to resemble that of *Plasmodium* species. Dogs are affected after an infected tick bites and feeds on a susceptible host for a minimum of three days. When the *Babesia* organism is introduced into the host, it attaches to erythrocyte membranes and is endocytosed. Hemolytic anemia and hypotensive shock are typical clinical syndromes of infection. Hemolytic anemia results from direct erythrocyte damage by the parasite, and both intravascular and extravascular immune-mediated destruction of red blood cells. Infection can produce thrombocytopenia, the mechanism of which consists of immune-mediated destruction and sequestration in the spleen. Physical examination reveals splenomegaly, lymphadenomegaly, fever and less frequently, lethargy, vomiting, hematuria, and icterus. Hypotensive shock results from the release and production of vasoactive amines and cytokines, which produce vasodilation. It most often occurs in puppies with the peracute form of the disease. Chronic infections, subclinical carrier states and atypical canine babesiosis may also occur.

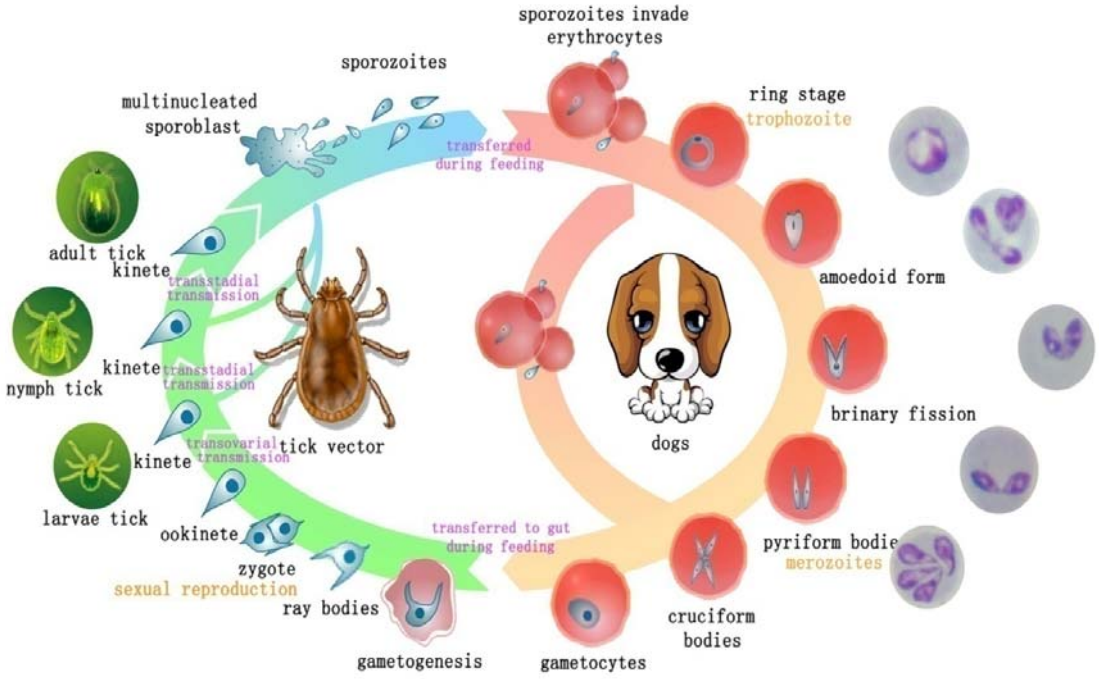


Fig. 1. Life cycle of *B. canis*

3. Diagnosis of canine babesiosis

Microscopic examination is the simplest and most accessible diagnostic test for the most veterinarians and reasonably sensitive for detecting intraerythrocytic parasites in Giemsa-stained blood smears during acute infections (Rosenblatt, 2009). Moreover, microscopic examination is still the only viable option available to veterinarians in many parts of the developing countries where babesiosis is endemic. However, the diagnosis of canine babesiosis in chronically infected and carrier animal remains a significant challenge due to very low, often intermittent parasitemias (Uilenberg, 2006; Hunfeld *et al.*, 2008). Indeed, failure to detect parasites in animals with hemolytic anemia or thrombocytopenia has led to an incorrect diagnosis in documented cases (Birkenheuer *et al.*, 2005; Verdida *et al.*, 2005;

Yeagley *et al.*, 2009).

PCR is an alternative test with good sensitivity and specificity (Ano *et al.*, 2001; Birkenheuer *et al.*, 2003; Song *et al.*, 2004; Matsuu *et al.*, 2005). However, access to molecular techniques for routine clinical diagnosis of canine babesiosis is still restricted to relatively few clinics worldwide. Immunofluorescent antibody test (IFAT) has been the most widely used serological diagnostic test for babesiosis (Anderson *et al.*, 1980; Levy *et al.*, 1987). However, poor specificity due to cross-reactions between *Babesia* species and with other apicomplexan parasites, operator subjectivity and its inadequacy for large-scale screening have all been limiting factor (Verdida *et al.*, 2005; Aboge *et al.*, 2007). Moreover, immunochromatographic test (ICT) was developed for the rapid diagnosis of canine *Babesia* species infection (Verdida *et al.*, 2005; Jia *et al.*, 2007). However, it required great amount of antigen and it is still developing. On the other hand, an enzyme-linked immunosorbent assay (ELISA) is quite sensitive and is appropriate for testing large number of samples especially in field surveys (Reiter and Weiland, 1989). Previously, ELISAs have been evaluated for detection of antibodies to canine *Babesia* species using native antigens. Although ELISA tests proved to be powerful tools for serological surveys, poor quality of antigens and sometimes cross-reactions between the *Babesia* species and closely related apicomplexan parasites have limited their application (Bose *et al.*, 1995). However, studies have shown that recombinant antigens provide better options because such antigens are usually available in pure forms and offer higher specific reaction than corresponding native antigen thus avoiding the problem of false positive results experienced with native antigens (Boonchit *et al.*, 2006; Jia *et al.*, 2006;

Aboge *et al.*, 2007). Therefore, ELISA with purified recombinant antigen could be the next selection for a promising diagnostic technique.

4. Treatment of canine babesiosis

For animal babesiosis, three babesiacides, quinuronium sulphate (Ludobal®, Bayer Ltd.), amicarbalide isothionate (Diampron®, May and Baker Ltd.) and diminazene aceturate (Berenil®, Hoechst Ltd.) were available in most European countries for the treatment of bovine babesiosis for long time (Rashid *et al.*, 2008; Schwint *et al.*, 2009). In the 1970s, a fourth, imidocarb dipropionate was introduced (Imizol; Schering-Plough), and it rapidly became the product of choice in those countries that licensed it because in addition to its therapeutic utility, it also proved to be an effective prophylactic at twice the therapeutic doses. This drug, imidocarb dipropionate, is currently available for the treatment of babesiosis in dogs (Vial and Gorenflot, 2006). Two injections of imidocarb diproprinate at 5.0-6.6 mg/kg given subcutaneously or intramuscularly at an interval of 2-3 weeks are reputed to be effective. In acute babesiosis, the therapeutic response is rapid, with increasing production of new red blood cells within 12-24 h. Another possible treatment is a single intramuscular injection of diminazene aceturate at a dose of 5 mg/kg (Taboada, 1998). In addition, atovaquone and azithromycin were tested for canine babesiosis (Matsuu *et al.*, 2004; Sakuma *et al.*, 2009). Supportive therapy such as intravenous fluids and blood transfusions should be employed when necessary (Vial and Gorenflot, 2006; Hunfeld *et al.*, 2008). Current chemotherapeutic agents used to treat canine babesiosis would be incapable of eliminating the

disease at the recommended dose; they only are capable of limiting mortality and the severity of clinical signs (Chaudhuri *et al.*, 2007; Subeki *et al.*, 2007; Aboge *et al.*, 2008). Dogs that have survived from babesiosis may remain subclinical infected and may suffer a relapse of disease in the future or serve as point sources for the further spread of disease in a given area (Imwong *et al.*, 2007). The failures of these antibabesials to clear parasites, together with the parasite resistance to these chemotherapeutics, and drug withdrawals from the market because of toxicity concerns have limited the range of drugs available for treatment of canine babesiosis (Miyama *et al.*, 2006; Suzuki *et al.*, 2007; Matsuu *et al.*, 2008). Subsequently, it is necessary to develop new drugs, which are effective and less toxic to the host, for the treatment of canine babesiosis.

5. Aims of the present study

Recently, the canine *Babesia* species infection has become an important emerging disease worldwide; thus, it is necessary to prevent its spread. The effective control and management of this infection relies first on accurate diagnosis, and then on subsequent treatment of the infected animals. This standard approach for prevention of the disease requires the development of a sensitive and specific diagnostic tool as well as drugs for treatment. Concerning serodiagnosis, the discovery and production of canine *Babesia* species antigens by recombinant DNA is particularly important because such a strategy produces pure and more specific recombinant antigens than the native proteins. Since recombinant antigens are standardized and have minimum batch-to-batch variation, they could be easily optimized for

use in ELISA and ICT system; hence, complementing the other established diagnostic methods, such as microscopy and IFAT, for the diagnosis of canine babesiosis. For drug development, although, the molecular drug targets of other apicomplexans including *Plasmodium* have been characterized, this has not been achieved in the canine babesiosis. Therefore, if I can identify and validate molecular drug target of canine *Babesia* species, then it would be possible to discover novel chemotherapeutic drugs for treatment and management of canine babesiosis. Therefore, the main objective of this study was to develop serological diagnostic methods of canine *Babesia* species and to characterize molecular drug target of the pathogen to facilitate drug discovery.

Chapter 1

Identification and characterization of a novel secreted antigen 1 of *Babesia canis canis* as a potential serodiagnostic antigen

1-1. Introduction

Babesia canis canis is a tick transmitted apicomplexan protozoa that causes piroplasmosis in dogs (Ano *et al.*, 2001). It is the most frequent causative agent of canine babesiosis in tropical and subtropical regions of Europe and west Asia (Cacciò *et al.*, 2002; Carret *et al.*, 1999b). Recently, many new endemic foci of this disease have been reported from European countries (Kubelová *et al.*, 2012). *B. canis canis* is a subspecies of *B. canis*, transmitted by *Dermacentor reticulatus* ticks, which causes a wide range of clinical signs such as lethargy, anorexia, fever, jaundice, anemia and lymphadenopathy (Schetters *et al.*, 1997). In general, *B. canis canis* infection is characterized by chronic infections. The treatment was limited in a few kinds of anti-*Babesia* drugs, necessitating the development of sensitive and specific diagnostic methods to prevent this disease.

The diagnosis of *Babesia* species infection is often based on intraerythrocytic piroplasm observation in peripheral blood smears. However, this method appropriates for the diagnosis of parasites infection in the acute stage (Jefferies, *et al.*, 2007). In addition, PCR represents a great advantage over microscopical detection due to its high sensitivity and specificity (Inokuma *et al.*, 2003), but the test requires skilled personnel and specialized laboratory. On

the other hand, serodiagnosis methods, such as the IFAT and ELISA with native antigens have been used to diagnose *B. canis canis* infection (Weiland and Reiter, 1988; Yamane *et al.*, 1993; Birkenheuer *et al.*, 2003). However, it is possible that the use of whole parasites or parasite-derived antigens could result in a false positive due to cross-reaction with other closely related parasites. Recent studies have shown that recombinant antigens are more acceptable than the corresponding native antigens, since they can usually be used in the pure form, contributing a high specific activity (Boonchit *et al.*, 2006). However, the recombinant antigen identified is still unavailable for serodiagnosis of *B. canis canis* infection. Thus, I focused on the development of a novel recombinant antigen for the serodiagnosis of *B. canis canis* infection.

In this context, I constructed a cDNA expression library of *B. canis canis* and serologically screened the cDNA expression library. A novel secret antigen of *B. canis canis* (BccSA1) was identified and characterized by Western blot analysis and IFAT. The recombinant BccSA1 (rBccSA1) was evaluated in ELISA, ICT, and double antibody sandwich ELISA (das-ELISA) methods as potential antigen. My data indicate that BccSA1 could be useful as an antigenic marker to detect *B. canis canis* infection.

1-2. Materials and methods

Parasites and experimental animals. *B. canis canis* isolate from a dog in Europe was stored in liquid nitrogen. The large scale of parasites were prepared for constructing the cDNA expression library according to intravenous injection on the two one-year-old

spleenectomized beagle dogs (Nihon Nosan, Japan) with 1×10^7 *B. canis canis*-infected erythrocytes, respectively. The blood was harvested with heparin when infected red blood cells reached near peak parasitemia (5-8%) and kept at -80℃. Two other nonspleenectomized beagle dogs were intravenously infected with 1×10^7 *B. canis canis*-infected erythrocytes for preparing the serial sera. Parasitemia was monitored daily by counting parasitized erythrocytes on Giemsa-stained thin blood smears. The blood samples were serially collected over a period of 222 days post challenge by using vacuum tubes with EDTA anticoagulant (NIPRO, Japan). The blood was centrifuged at 3,000 rpm for 15 min, and then the serum samples were kept at -30℃. Seven 6-week-old female ICR mice (CLEA, Japan) were used for the 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.

Construction of cDNA expression library. Total RNA was prepared from *B. canis canis*-infected splenectomized dog erythrocytes by acid guanidinium thiocyanate-phenol-chloroform extraction methods (Chomczynski *et al.*, 1987), and then polyadenylated RNA was purified with Oligotex-dT 30 (JSR and Nippon Roche, Japan). Next, cDNA was synthesized by using a ZapcDNA synthesis kit, ligated to a Uni-ZAP XR vector and packaged by using a Gigapack III packaging system (Stratagene, USA) according to the manufacturer's instructions.

Immunoscreening of cDNA expression library. The constructed library was plated on a total of 50 plates at a concentration of approximately 20,000 plaque-forming units (PFUs)

per plate to lift plaques. The plaques were transferred to nitrocellulose membranes and screened with the *B. canis canis*-infected dog serum according to the protocol of the picoBlue Immunoscreening Kit (Stratagene, USA). After an *in vivo* excision, the cDNA inserts in the positive clones were transferred into pBluescript phagemids and then sequenced with M13 forward, reverse, and internal DNA primers by using an automated sequencer (ABI PRISM 310 Genetic Analyzer, USA).

Cloning, expression, and purification of rBccSA1 in *E. coli*. A cDNA homology sequence of *B. gibosoni* secret antigen 1 (BgSA1) was found as the most frequent gene and was thus selected for further study. The gene was designated as BccSA1. The hydropathic plot and antigenic index of predicted amino acid were determined using the DNASTAR software (Netwell, Japan). One pair of oligonucleotide primers including *Eco*RI and *Sal*I restriction enzyme sites were designed and used to clone the truncated gene encoding BccSA1 without the N-terminus predicated signal peptide (forward primer: 5'-CGGAATTCCAATCAACAAGCAGCCAG -3'; reverse primer: 5'-CGGTCGACCTAGTTGATTCATTCTTA -3'). The PCR product was cloned into *E. coli* expression vector pGEX-4T-1 (Amersham Pharmacia Biotech, USA). The resulting plasmid was identified by sequencing and designated as pGEX-4T-1/BccSA1. The recombinant BccSA1 was expressed as a glutathione S-transferase (GST) fusion protein in the *E. coli* BL21 strain. The supernatant containing soluble rBccSA1 was purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, USA). Then the BccSA1 fused with GST was cleaved by thrombin protease according to the manufacturer's

instructions (Amersham Pharmacia Biotech, USA). The protein concentration was measured by using a modified Lowry protein assay kit (Thermo Scientific, USA).

Production of mouse and rabbit anti-BccSA1 serum. Six-week-old female ICR mice were immunized intraperitoneally (i.p.) with 100 μg of purified rBccSA1 without GST in an equal volume of Freund's complete adjuvant (Difco Laboratories, USA) for the first injection. Two boosters were given i.p. using 50 μg of the same protein emulsified in Freund's incomplete adjuvant at 2 weeks intervals. Serum was collected 10 days after the last booster and checked for specific antibodies by an indirect immunofluorescence assay (IFAT) and stored at -30℃. For the preparation of rabbit serum against BccSA1, one 2.5 kg Japanese white rabbit (CLEA, Japan) was immunized subcutaneously and was given boosters on days 14 and 28. The anti-rBccSA1 rabbit serum was collected 14 days after the last immunization.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. To identify the native BccSA1 on the parasite, the extracts of *B. canis canis* merozoite and normal dog erythrocytes lysates were sonicated, and then precipitated with acetone. Thereafter, mouse anti-BccSA1 serum was used to analyze the merozoite extract by SDS-PAGE and Western blot analysis as previously described (Zhou *et al.*, 2006). In addition, the rBccSA1 was analyzed by SDS-PAGE as previously described (Xuan *et al.*, 1996) and processed in the same way as for merozoite extract. The rBccSA1 was probed with *B. canis canis*-infected dog serum. Subsequent procedures were performed as described above for the native BccSA1. To determine of circulating BccSA1 in the blood, mouse anti-BccSA1 serum was used to analyze the plasma collected from a splenectomised

dog experimentally infected with *B. canis canis* and a health dog by Western blot analysis.

Indirect fluorescent antibody test (IFAT) and confocal laser microscopic observation. A thin blood smear film of *B. canis canis*-infected red blood cells was fixed with 100% methanol for 30 min at -30℃. Anti-rBccSA1 serum raised in a mouse was applied as the primary antibody on the fixed smear and incubated for 30 min at 37℃. After washing 3 times with phosphate-buffered saline Tween 20 (PBST), Alexa-Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) (Molecular Probes, USA) was subsequently applied as a secondary antibody and incubated for another 30 min at 37℃. The slide was washed 3 times with PBST and incubated with 6.25 mg/ml propidium iodide (PI) (Wako, Japan) containing 50 mg/ml RNase A (Qiagen, Germany) for 20 min at 37℃. After washing twice with PBS, the glass slides were mounted by adding 200 ml of a 50% glycerol-PBS (v/v) solution and covering with a glass cover-slip. The slides were examined under a confocal laser scanning microscope (TCSNT, Leica, Germany).

Indirect fluorescent antibody test (IFAT). *B. canis canis*-infected RBC were coated on indirect fluorescent antibody test (IFAT) slides, dried, and then fixed in absolute acetone for 20 min for standard IFAT observation. The 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℃ in a moist chamber. After washing with PBS three times, the fluorescein isothiocyanate (FITC)-conjugated goat anti-dog IgG antibody (Bethyl Laboratories, Montgomery, TX, USA) was applied as a secondary antibody (1:500) and then incubated for 1 h at 37℃. The slides were washed three times with PBS and then examined using a fluorescent microscope (Nikon,

Kawasaki, Japan).

Enzyme-linked immunosorbent assay (ELISA). Purified rBccSA1 without GST was diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6 to a final concentration of 4 μg/ml. Each well of 96-well microtiter plates (Nunc, Denmark) was coated with 50 μl of the protein overnight at 4℃. The ELISA was performed as described by Xuan et al (2001). Briefly, Blocking was performed with a blocking solution (3% skim milk in PBS, pH 7.2) at 37℃ for 2 h. The plates were incubated at 37℃ for 30 min with 50 μl of each of the serial sera samples from a dog experimentally infected with *B. gibsoni*. After washing 6 times with PBS-Tween 20, the plates were incubated with 50 μl per well of HRP-conjugated goat anti-mouse IgG (Bethyl Laboratories, USA) diluted in a blocking solution. The bound-antibody was visualized with 100 μl per well of a substrate solution (0.3 mg/ml 2,2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid), 0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2). The absorbance at 415 nm was measured by using an MTP-500 microplate reader (Corona Electric, Japan). The cut-off value was defined as the mean value plus 3 standard deviations of the mean optical density (OD) obtained from 28 SPF dog serum samples.

Immunochromatographic test (ICT). Purified rBccSA1without GST was diluted to an optimal concentration of 200 μg/ml as the test line or conjugated with gold colloid particles (British BioCell International, UK) as described previously (Huang *et al.*, 2004). Rabbit anti-rBccSA1 polyclonal IgG was purified as described above and rabbit anti-rBccSA1 IgG (1,500 μg/ml) were linearly jetted onto a nitrocellulose membrane (Schleicher & Schuell,

USA) as the control line using a BioDot Biojet 3050 quanti-dispenser (BioDot, Inc., USA). The NC membrane, absorbent pad, conjugate pad, and sample pad were assembled on an adhesive card (Schleicher& Schuell, USA) and cut into 2 mm wide strips using a BioDot cutter (BioDot, Inc., USA). Detection was performed by pipetting 40 µl of the diluted serum (1:2 in PBS) on the sample pad. For dog serum samples, 40 µl of the diluted serum (1:40 in PBS) was applied due to the limited amount of serum. The result was judged 15 min after the application of serum samples. The presence of a control band alone indicated a negative result, whereas the presence of two bands (control and test bands) indicated a positive result.

Double-antibody sandwich enzyme-linked immunosorbent assay (das-ELISA). The sandwich ELISA was performed as previously described (Jia *et al.*, 2006). Briefly, rabbit anti-rBccSA1 polyclonal IgG was purified using Econo-Pac protein A kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's guidelines. One microgram of the IgG diluted in a 0.05 M carbonate buffer (pH 9.6) was used as the capture antibody to coat microtiter plates at 4℃ overnight and purified rabbit anti-GST IgG was used as the control antibody. Blocking was performed with a blocking solution (3% skim milk in PBS, pH 7.2) at 37℃ for 2 h. The plates were incubated at 37℃ for 30 min with 50 µl of each of the serial plasma samples from a dog experimentally infected with *B. canis canis*. After washing six times with PBST, mouse anti-rBccSA1 polyclonal serum diluted in a blocking solution was added in each well as a detection antibody. After washing six times again, the plates were incubated with 50 µl per well of horseradish peroxidase-conjugated goat anti-mouse IgG (Bethyl Laboratories, USA) diluted in a blocking solution. Binding was visualized with 100 µl

per well of a substrate solution [0.3 mg/ml of 2, 2'-azinobis-(3-ethylbenz-thiazoline-6-sulfonic acid), 0.1 Mcitric acid, 0.2 Msodium phosphate, and 0.003% H_2O_2]. The absorbance at 415 nm was measured using an MTP-500 microplate reader (Corona Electric, Japan). The cut-off value was defined as the mean value plus three standard deviations of the mean optical density (OD) obtained from 14 SPF canine serum samples. At the same time, 0.1 μg/ml to 8.0 μg/ml of rBccSA1 in diluents buffer containing normal canine sera were used to generate a standard curve for evaluating test samples quantitatively.

Serum samples. Dog sera used for the specificity detection were prepared as follows: 11 sera from dogs experimentally infected with *B. canis canis,* 3 sera from dogs experimentally infected with *B. canis vogeli*, 3 sera from dogs experimentally infected with *B. canis rossi*, 3 sera from dogs experimentally infected with *B. gibsoni*, 3 sera from dogs experimentally infected with *Neospora caninum*, and 30 sera from SPF dogs. In addition, 21 field serum samples collected in the epidemic areas of Europe were then investigated using the ELISA and compared with ICT based on the rBccSA1.

1-3. Results

Identification and characterization of the BccSA1 gene. The partial length of BccSA1 contains 1,255 nucleotides encoding a polypeptide of 417 amino acid residues (Fig. 2). The isoelectric point and molecular mass for the mature protein without signal peptide were 8.77 and 44.06 kDa, as calculated with software

(http://web.expasy.org/cgi-bin/compute_pi/pi_tool). Analysis of the putative N-terminal signal peptide in the BccSA1 sequence by the SignalP server (http://www.cbs.dtu.dk/services/SignalP/) showed that this sequence had a high predicted signal peptide probability (0.951) and a maximum cleavage site probability (0.512) between amino acids in positions 38 and 39 (Fig. 2A). The amino acid sequence shared homology with the *B. gibsoni* secreted antigen 1 (BgSA1) (Jia *et al.*, 2007). To improve the expression level of rBccSA1, the recombinant protein was truncated based on its hydropathy plot and antigenic index of predicted amino acid (Fig. 2B).

Expression of the rBccSA1 in *E. coli*. The BccSA1 lacking the N-terminal signal peptide sequence gene was cloned into the prokaryotic expression vector, pGEX-4T-1, and the resulting plasmid was transformed into an *E. coli* BL21 strain. The rBccSA1 was expressed in *E. coli* as a soluble GST-fusion protein with expected molecular mass. Sera from dogs experimentally infected with *B. canis canis* could recognize the GST-fused rBccSA1 by Western blot analysis. This result suggested that the rBccSA1 successfully expressed in *E. coli* maintained its antigenicity (Fig. 3).

Characterization of the native BccSA1 of *B. canis canis*. Mouse anti-rBccSA1 polyclonal serum was used to identify the native BccSA1 in the lysate of *B. canis canis* parasites. As shown in Fig. 4, specific band with size of 44 kDa was detected in *B. canis canis*-infected erythrocyte lysates by Western blot analysis but not in normal ones (Fig. 4A). In addition, specific band was also detected in blood plasma with 8% parasitemia from a splenectomized dog experimentally infected with *B. canis canis* using Western blot analysis

(Fig. 7A). In addtion, to determine the cellular localization of BccSA1, the merozoite stage of *B. canis canis* was assayed by IFAT. Confocal laser microscopic observations revealed that anti-rBccSA1 antibody specifically reacted with merozoite (Fig. 4B).

Evaluation of the diagnostic potential of rBccSA1 by ELISA. In order to determine the potential of rBccSA1 as diagnostic antigen, the cross-reactivity of rBccSA1 with closely related parasite-infected dog serum was illustrated by ELISA. The cut off value of rBccSA1 ELISA calculated using SPF canine serum was 0.093. Eleven serum samples from *B. canis canis* experimentally infected dogs were positive (OD > 0.093), whereas the serum samples from the dogs infected with *B. canis rossi*, *B. canis vogeli*, *B. gibsoni*, *N. caninum* and SPF dogs were negative (OD < 0.093) in the ELISA (Fig. 5A). The kinetics of the anti-rBccSA1 antibody was detected by ELISA in serial serum samples collected from *B. canis canis*-infected dog for determining the sensitivity of ELISA method. Specific antibody against rBccSA1 could be detected on the 21th day post-infection. The high antibody level was maintained until 222 days post-infection when the infection was in chronic stage, when no parasitemia was detectable (Fig. 5B).

Evaluation of the diagnostic potential of rBccSA1 by ICT. Serum samples from dogs experimentally infected with *B. canis rossi*, *B. canis vogeli*, *B. gibsoni*, *N. caninum* and SPF dogs were used to determine the specificity of ICT based on the rBccSA1. Only the *B. canis canis*-infected dog serum sample was positive in the ICT. The other serum samples from the dogs infected with closely related parasites and from SPF dogs were negative (Fig. 6A). In addition, the ICT was evaluated with sequential serum samples from a dog experimentally

infected with *B. canis canis*. The specific antibodies could be detected from as early as 21 days to 222 days after infection, which is quite consistent with the results of the ELISA (Fig. 6B).

Evaluation of das-ELISA. A standard curve generated using serial dilutions of rBccSA1 yielded a coefficient of determination (R^2) of 0.991 with a quantitation limit of 0.78 μg/ml of the rBccSA1 (Fig. 7B). Therefore, from this standard curve it might be possible to quantify the circulating BccSA1 in clinically infected host. The sensitivity of the assay was tested with sequential sera obtained from splenectomized and nonsplenectomized dogs infected with *B. canis canis*. The circulating BccSA1 was detectable in the infected plasma of the splenectomized dog with the peak of parasitemia (5%), and the das-ELISA titer of the samples was well associated with the parasitemia post infection. However, the circulating BccSA1 was not detectable in the plasma of the nonsplenectomized dog infected with *B. canis canis* with the peak of parasitemia (0.5%) (Fig. 7D).

The specificity of the assay was evaluated by using sera of splenectomized and nonsplenectomized dogs infected with *B. canis canis* and that of non-infected dogs. I found that the assay system detected circulating BccSA1 in sera of splenectomized dogs infected with *B. canis canis* (OD > 0.267). However, no circulating BccSA1 was detected in sera of nonsplenectomized dogs infected with *B. canis canis* and non-infected dogs (Fig. 7C).

Comparison of the IFAT, ELISA, and ICT with rBccSA1. A total of 21 dog serum samples from Europe were detected by IFAT, ELISA and ICT with rBccSA1. As shown in Table 1, 11 (52.4%) of the tested samples were positive when analyzed by the IFAT, 10

(47.6%) were positive on ELISA, and 9 (42.9%) were positive on ICT.

1-4. Discussion

This is the first report describing the recombinant antigen for serodiagnosis of *B. canis canis* infection. Canine babesiosis in Europe has always been a severe and frequent disease, attributed to infection with *B. canis canis* (Caccio *et al.*, 2002; Criado-Fornelio *et al.*, 2003). However, no recombinant antigen of *B. canis canis* had been identified for serodiagnosis so far. Therefore, the identification of sensitive and specific antigen is very important for the researchers in this field. The secreted antigens are soluble antigens released into the *in vitro* culture medium or blood plasma fraction of the infected animals during the parasite's asexual development. The secreted antigens can induce protective immunity against the infections of *Babesia* species and could be a suitable source of antigen for the detection of parasites infection (Valentin *et al.*, 1993; Patarroyo *et al.*, 1995; Schetters *et al.*, 2007). Therefore, identification of secreted antigens could be very useful in the development of effective serodiagnostic methods for the detection of circulating antigens and antibodies.

In answer to this need, a cDNA expression library prepared from *B. canis canis* merozoites mRNA was constructed and serologically screened with *B. canis canis*-infected serum. A novel secreted antigen gene of *B. canis canis* (BccSA1) was isolated and identified, which shared homology with the BgSA1 (Jia *et al.*, 2006). Moreover, analysis of predicted polypeptide of BccSA1 using SignalP-3.0 server revealed that this protein had a predicted signal peptide indicating that it might be a secretary protein. Moreover, the lysates of *B. canis*

canis-infected and non-infected canine erythrocytes were analyzed by Western blot analysis. A specific 44 kDa band was detected in the *B. canis canis*-lysate, which is consistent with the molecular mass of BccSA1 without signal peptide. Another unexpected 69 kDa band was observed, which might be a mature complex form of BccSA1 to give a band. To determine the cellular localization of rBccSA1, *B. canis canis* parasitized erythrocytes were analyzed by IFAT using a confocal laser microscopy. Specific fluorescence was observed within the cytoplasm of the parasites.

In this study, the BccSA1 was expressed as a soluble GST-fusion protein and purified, and the potential of rBccSA1 as a diagnostic antigen was evaluated by ELISA. There is no cross-reactivity of rBccSA1 with closely related parasite-infected dog sera and the specific antibody was detected as early as the 21th day post-infection in dog. Furthermore, analysis the serum samples from the Europe show a specific reaction in some of the sera. I can conclude from these results that rBccSA1 could be used as a potential antigen for the detection of *B. canis canis* infection at the chronic stage.

ICT has provided an analytical platform that permits one-step rapid analysis without any equipment or testing skills and the result can be determined in a few minutes with the naked eye; therefore, this test is more practical for clinical application than any other test (Huang *et al.*, 2004; Jia *et al.*, 2007). In this study, I try to develop an ICT for detection of *B. canis canis* infection, particularly for epidemiological survey. The ICT based on rBccSA1 showed that the specific antibodies could be detected from *B. canis canis*-infected dog, which was identical to that of ELISA. Furthermore, ELISA based on the rBccSA1 compared with the ICT based on

the rBccSA1 and IFAT by detected 21 serum samples collected from Europe. The positive detection rates were 47.6% on ELISA, 42.9% on ICT, and 52.4% on IFAT. Eleven serum samples were positive by the IFAT, but one of them was negative on ELISA. This serum may be collected during the acute stage of *B. canis canis*-infected dog. One-serum samples were positive when analyzed by the ELISA, but negative on ICT. The possible explanation was that ICT was less sensitive than ELISA. Therefore, it is necessary to optimize the ICT condition and to confirm using large number of field samples in future study.

The native BccSA1 was detected in plasma from a dog experimentally infected with *B. canis canis* by Western blot analysis. These results demonstrated that the BccSA1 was as a circulating antigen in plasma of *B. canis canis*-infected dogs. Therefore, BccSA1 was evaluated by das-ELISA as a diagnostic target for the detection of circulating antigen. The results shown that the native BccSA1 could be detected in the plasma of a splenectomised dog infected with *B. canis canis* when the parasitemia reached 5% after infection. However, when the parasitemia reached 0.5% in the nonsplenectomised dog, the native BccSA1 could not be detected by the das-ELISA. Moreover, I was able to quantify the circulating BccSA1 by das-ELISA. The quantitation limit of 0.78 μg/ml. This revealed that the sensitivity of double-antibody sandwich ELISA based the detection of native BccSA1 did not allowed it used in diagnosis of the infection with low parasitemia. Thus, the sensitivity of das-ELISA based on the BccSA1 should be improved in the future study.

In conclusion, I have identified and characterized a novel secreted antigen of *B. canis canis* by immunoscreening of the *B. canis canis* cDNA expression library and evaluated its

potentiality for serodiagnosis. The results demonstrated that the rBccSA1 was a promising chronic serodiagnostic target with a good diagnostic performance in ELISA and ICT to detect *B. canis canis* infection. In addition, the das-ELISA could detect circulating BccSA1 in the plasma of 5% parasitemia infection of *B. canis canis*, but could not detect BccSA1 in the plasma of the infection with low parasitemia. Overall, the BccSA1 is a promising serodiagnostic antigen for clinical detection and epidemiological survey.

1-5. Summary

In this report, a cDNA expression library prepared from *Babesia canis canis* merozoites mRNA was constructed and serologically screened with *B. canis canis*-infected serum. A cDNA encoding a novel secreted antigen was identified and designated as BccSA1. Antibodies raised in mice immunized with rBccSA1 recognized a native 44 kDa parasite protein. An ELISA of rBccSA1 detected specific antibodies in the sequential sera of a dog infected with *B. canis canis* beginning 21 days to 222 days post-infection, even when the dog became chronically infected and showed a low level of parasitemia. A rapid ICT using rBccSA1 was evaluated and detected the specificity and sensitivity, which was quite consistent with the results of the ELISA. Moreover, a das-ELISA assay with anti-rBccSA1 antibodies could detect the circulating BccSA1 in the plasma of splenectomized dog experimentally infected with *B. canis canis* at 5% parasitemia. Finally, 21 serum samples from Europe were analyzed by IFAT, ELISA and ICT, 52.4%, 47.6%, and 42.9% of the tested samples were positive by IFAT, ELISA, and ICT, respectively. Taken together, these results

indicated that the rBccSA1 might be a useful target to develop both ELISA and ICT for serodiagnosis of *B. canis canis* infection in dogs and for epidemiological survery.

A

```
GGCACGAGGCCTCGTGCCGAATTCGGCACGAGGGTTCTATCCATTAAACCTCCTGAAAACTCATCCACATAATTTTATTTTAATTTTTCAATTATTAGCAACAATGGGTAACGCTCAATCA
  A  R  G  L  V  P  N  S  A  R  G  F  Y  P  L  N  L  L  K  T  H  P  H  N  F  I  L  I  F  Q  L  L  A  T  M  G  N  A  Q  S
ACAAGCAGCCAGGAAAACTCACGGGATGGCTTACGGGAAGTCTTGGAATATACGAACCAGCTGCACAACAACTACGGTTCTGCAGTAAGGAAGGTTACAGACAAGCTTAAGAATGAGATT
 T  S  S  Q  E  N  S  R  D  G  L  R  E  V  L  E  Y  T  N  Q  L  H  N  N  Y  G  S  A  V  R  K  V  T  D  K  L  K  N  E  I
GACGTATACTGCAAATCCACTGATGATAAGGGTTACTACTTCGCCAATGGTTCTTTCGGATATTTCCGCAAGGCCCTCAACGATTCCTTCAACTTTAGGTTCCAGTTGCTCTCCAACTAC
 D  V  Y  C  K  S  T  D  D  K  G  Y  Y  F  A  N  G  S  F  G  Y  F  R  K  A  L  N  D  S  F  N  F  R  F  Q  L  L  S  N  Y
AACGATTACAGGAAGTATAAGACGCGCTTCCAGGACACCGATGATGAGGCCGAAAAGCACGTTAAGTACCTCAAAGAAAACCTTTTCGATCTATTCGGTACCTTGTCCTACATGTATTTC
 N  D  Y  R  K  Y  K  T  R  F  Q  D  T  D  D  E  A  E  K  H  V  K  Y  L  K  E  N  L  F  D  L  F  G  T  L  S  Y  M  Y  F
CAGTGCTCTCACAAATGCCAGAAATACAACGGAGGGAAATGGGAAGAACAAAGTATGAACCAAAGTGGTAGTGAGGTGAGCAAATGGCTTATGGGGAGTAATTCTGCTGCCACTGACTCC
 Q  C  S  H  K  C  Q  K  Y  N  G  G  K  W  E  E  Q  S  M  N  Q  S  G  S  E  V  S  K  W  L  M  G  S  N  S  A  A|T  D  S
GTTCATTTTCTGGGGAGGGACTTCTCAACTTCAGAGTTAACCAACATCAAAGGCAAAGAGCTGGCCGACAAGGATCGCGCTAGCCTATCTGATTTAATTAAATACAGTGGCAGAGGTAAC
 V  H  F  L  G  R  D  F  S  T  S  E  L  T  N  I  K  G  K  E  L  A  D  K  D  R  A  S  L  S  D  L  I  K  Y  S  G  R  G  N
CTTCAGCATGCCCTGTTCTGGATGCTGTTCATTGGACCATGGGTGGATGGAAAAACTGGTCATGCCATACTGTTCTTGATCCAATTTATCGAATTAACAGAATATGATTACTTCAAAGAG
 L  Q  H  A  L  F  W  M  L  F  I  G  P  W  V  D  G  K  T  G  H  A  I  L  F  L  I  Q  F  I  E  L  T  E  Y  D  Y  F  K  E
AATAGCAAGAAGAAGTACGATTACAAAAAGCTGGTTACCCCTTGCAAGAATCTGAAGACCCCTCTTGGTGACGTATCAAGGAAAGTAGTACCTGTAGGAAAGAACCCCAACGTTAAATAC
 N  S  K  K  K  Y  D  Y  K  K  L  V  T  P  C  K  N  L  K  T  P  L  G  D  V  S  R  K  V  V  P  V  G  K  N  P  N  V  K  Y
AGAGACCTCCTATCCGGCGAATTTTTCGATGCATATGTTGATTGGTTGGAGAAGAACATTGACAACCTTATTAAATCTTTGGAGGACATGTCTAAAGACTGCAACAATTGGACTTACGAT
 R  D  L  L  S  G  E  F  F  D  A  Y  V  D  W  L  E  K  N  I  D  N  L  I  K  S  L  E  D  M  S  K  D  C  N  N  W  T  Y  D
AAACTAAACAGCGCGTCTTCCTATGGTCCTTTCCCATGGGGATGTGTTTTCAAAGATGAGGATTGGCACACCAGTGGTAGGCTGCCAAAGTTGCCTGAAGATGTCAAGAAAGCCACTGAT
 K  L  N  S  A  S  S  Y  G  P  F  P  W  G  C  V  F  K  D  E  D  W  H  T  S  G  R  L  P  K  L  P  E  D  V  K  K  A  T  D
GCCTTAAAAAAACTTAAAGAGGCTATTGAAGAAATGAAGAAAGAGAAAAAGTAAGAATGAATCAACTAGTTTTAATTTGTGACTTGCCATCCTACTAACATGGAATTCGTTTAGAAAATG
 A  L  K  K  L  K  E  A  I  E  E  M  K  K  E  K  K  *
TTGCTTCTGGGTGGTGAAGACGCAACATTCATGTCAGCGTAAATTTCCGATGCGTATTTGTTGCCATGTAAAATACGCATGGTCACTCAACACATACTGATTTTAATCTTATTATAACAT

TTAAAAAAAAAAAAAAAAAA
```

B

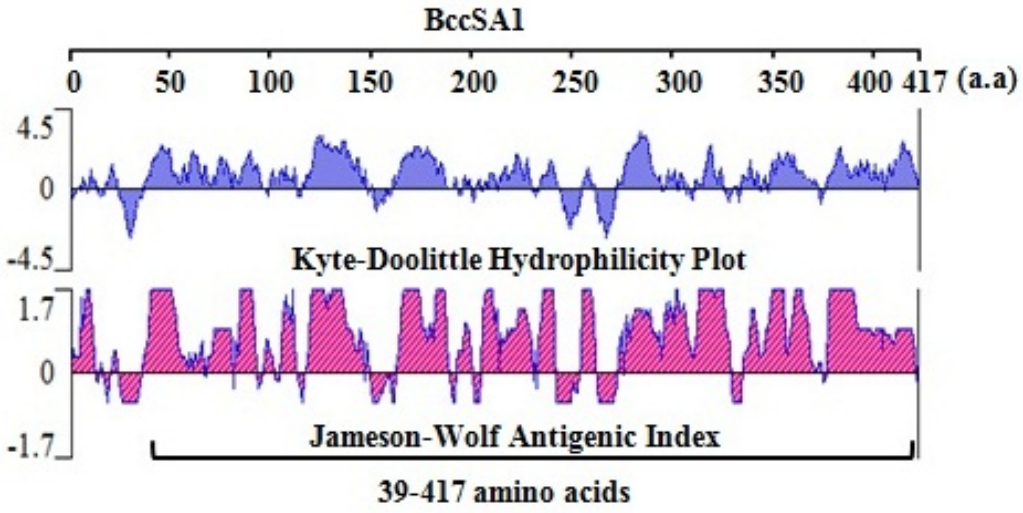


Fig. 2. (A) Nucleotide sequences and predicted amino acid sequences of cDNA coding for BccSA1.

(B) The software analysis of hydrophilicity and antigenicity of BccSA1.

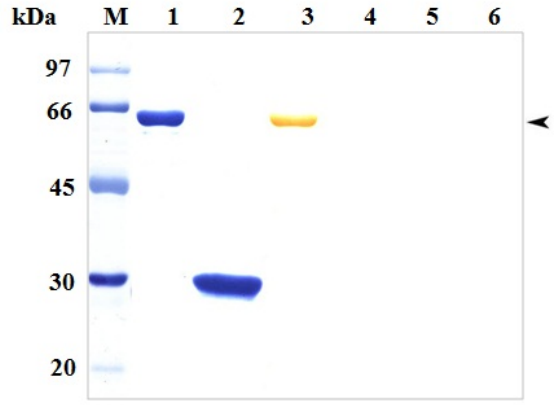


Fig. 3. SDS-PAGE and Western blot analysis of recombinant BccSA1. M, molecular size markers. Lanes 1 and 2, the rBccSA1 fused with GST and recombinant GST were stained by amide black. Lanes 3 and 4, rBccSA1 fused with GST and the purified recombinant GST were incubated with serum from a dog infected with *B. canis canis*. Lanes 5 and 6, rBccSA1 fused with GST and the purified recombinant GST were incubated with serum from a normal dog.

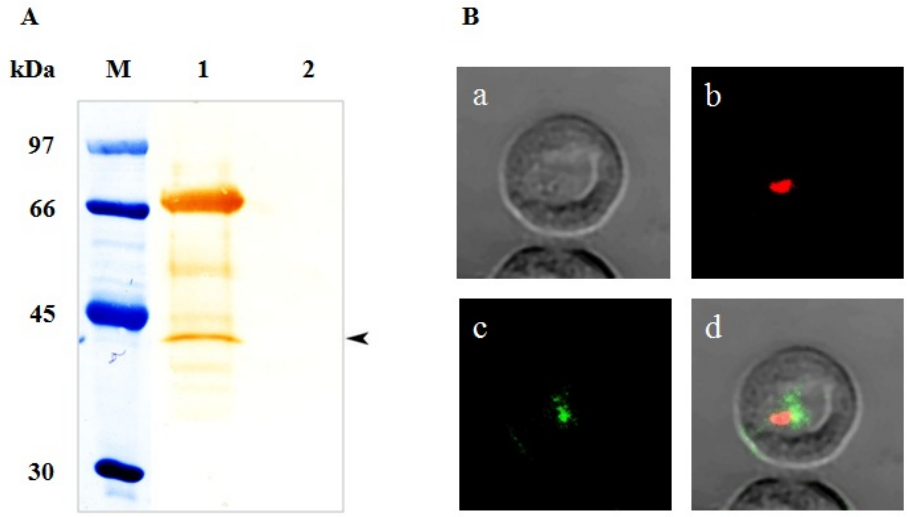
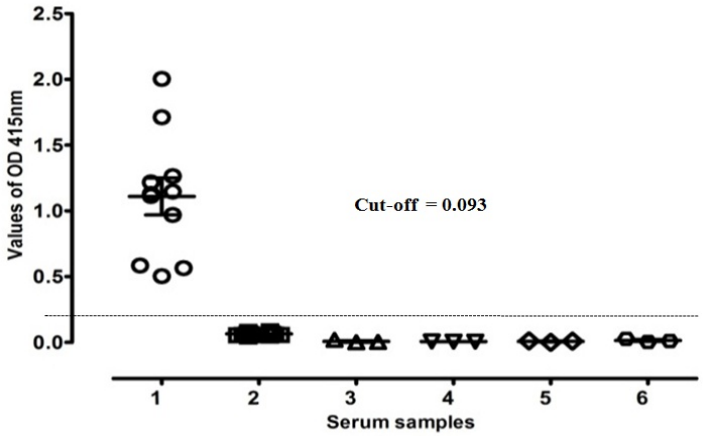


Fig. 4. Molecular characterization of the native BccSA1. (A) Lane M, molecular size marker. Lanes 1 and 2, Western blot analysis of the lysates of *B. canis canis* and non-parasitized canine erythrocytes reacted with anti-rBccSA1 serum raised in mice, respectively. (B) Observation of the native BccSA1 recognized by mice anti-rBccSA1 serum in confocal laser micrographs. (a) Phase-contrast images of *B. canis canis* merozoites. (b) Immunofluorescent staining of *B. canis canis* merozoites with mice anti-rBccSA1 serum. (c) Propidium iodide staining of *B. canis canis* merozoite nuclei. (d) Panels b and c are overlaid on panel a. The images were derived from a single section.

A



B

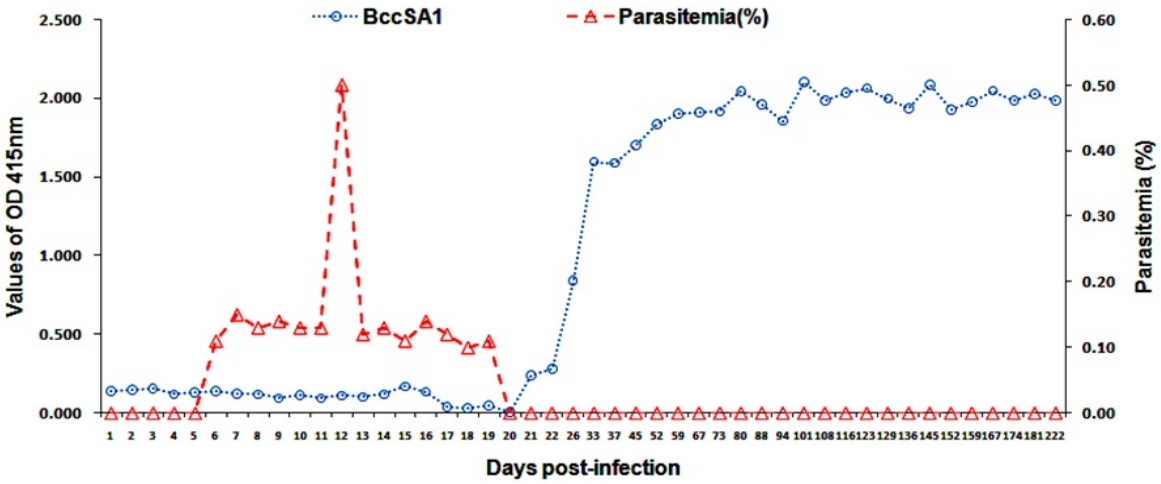


Fig. 5. Evaluation of BccSA1 as a diagnostic antigen by ELISA system. (A) Values of ELISA with experimentally infected canine serum. Lane 1, *B. canis canis*-infected dogs sera (n=11); lane 2, SPF dog sera (n=30); lane 3, *B. canis rossi*-infected dogs sera (n=3); lane 4, *B. canis vogeli*-infected dogs sera (n=3); lane 5, *B. gibsoni*-infected dogs sera (n=3); lane 6, *N. caninum*-infected dogs sera (n=3) (B) Detection of sequential serum samples against BccSA1 from a nonsplenectomized dog experimentally infected with *B. canis canis* by ELISA.

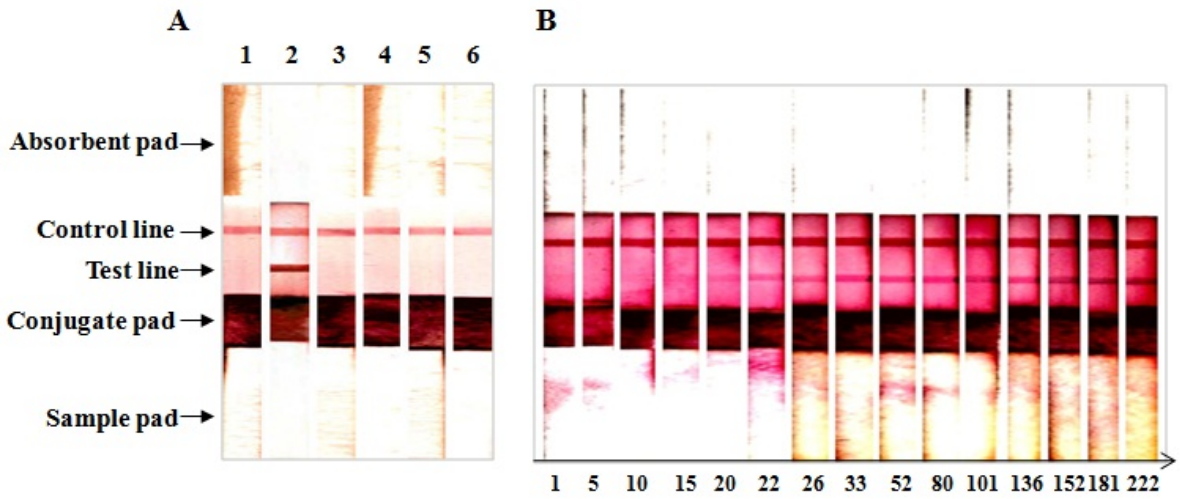


Fig. 6. Evaluation of rBccSA1 by ICT. (A) Cross-reactivity of rBccSA1 with closely related parasite-infected canine sera. Lane 1, SPF dog serum; lane 2, a *B. canis canis* fected dog serum; lane 3, *B. canis rossi*-infected dog serum; lane 4, *B. canis vogeli*-infected dog serum; lane 5, *B. gibsoni*-infected dog serum; lane 6, *L. infantum*-infected dog serum. (B) Specific antibody responses to BccSA1 in sequential serum samples from a nonsplenectomized dog experimentally infected with *B. canis canis*.

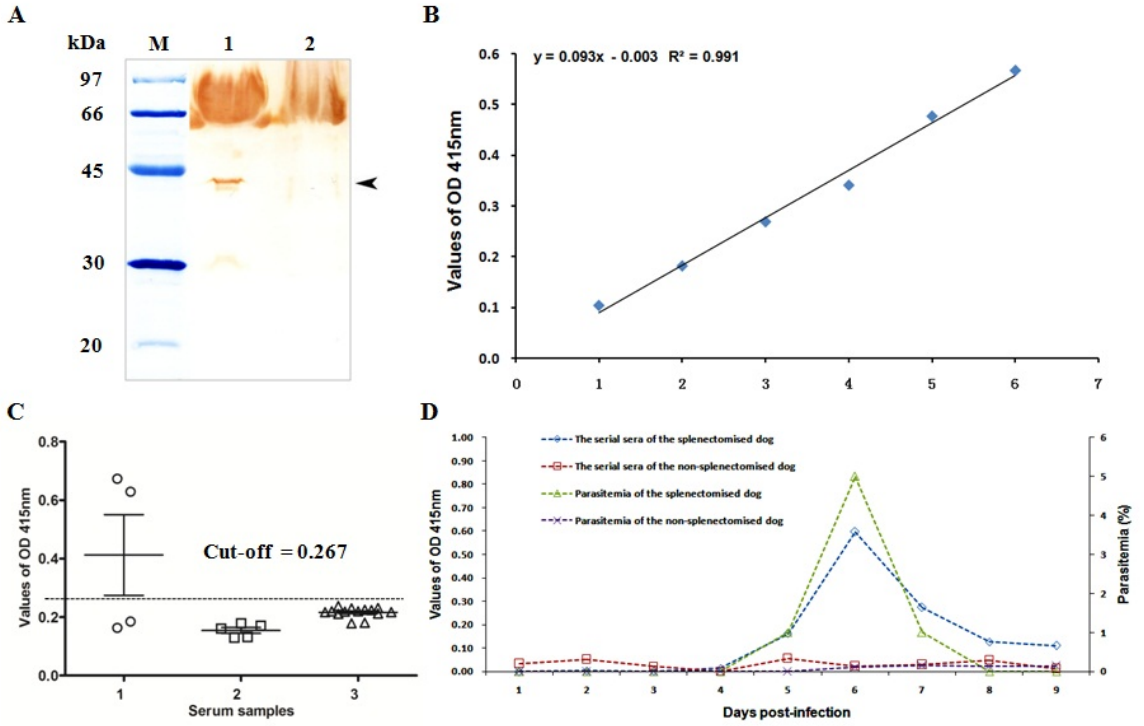


Fig. 7. Western blot analysis of circulating BccSA1 and evaluation of rBccSA1 by das-ELISA (A) Lane M, low molecular weight marker; lanes 1 and 2, detection of circulating BccSA1 in plasma of *B. canis canis*-infected splenectomized dog and SPF dog, respectively. (B) Generation of a standard curve based on serially diluted rBccSA1 added in SPF hamster sera in das-ELISA. (C) Cross-reactivity of das-ELISA with closely related parasite infections. Lane 1, Plasma from splenectomized and nonsplenectomized dogs infected with *B. canis canis* (n = 4); lane 2, *B. gibsnoni*-infected canine sera (n=5); lane 3, SPF dog sera (n=28) (D) BccSA1 and parasitemia levels in the serial sera samples from both splenectomized and nonsplenectomized dogs infected with *B. canis canis* detected by das-ELISA and microscopic examination of Giemsa-stained blood smears, respectively.

Table 1. Comparison of IFAT, ELISA, and ICT with the rBccSA1 for the detection of field samples from Europe.

No. (%) with BccSA1-ICT		No. (%) with IFAT		No. (%) with BccSA1-ELISA		No. (%) with BccSA1- ICT/ BccSA1-ELISA
		No. (%) positive	No. (%) negative	No. (%) positive	No. (%) negative	
No. (%) positive	9 (42.9)	9 (42.9)	0 (0)	9 (42.9)	0 (0)	10 (47.6)
No. (%) negative	12 (57.1)	2(9.5)	10 (47.6)	1 (4.7)	11 (52.4)	11 (52.4)
Total no. (%)	21 (100)	11 (52.4)	10 (47.6)	10 (47.6)	11 (52.4)	21 (100)

Chapter 2

Identification and characterization of a novel *Babesia canis canis* 33 kDa protein as a protential serodiagnostic antigen

2-1. Introduction

In chapter 1, my studies have shown that rBccSA1 provide better options because rBccSA1 is usually available in pure forms and offer higher specific activity than corresponding native antigens thus avoiding the problem of false positive results experienced with native antigens. However, its sensitivities have not achieved perfect result necessitating further research on development of novel candidate diagnostic antigens to detect antibody response during acute stage.

In this context, a cDNA expression library prepared from *B. canis canis* merozoites mRNA was serologically screened with *B. canis canis*-infected serum, again. A novel cDNA encoding 33 kDa proteins (BccP33) was isolated and identified. The recombinant protein of BccP33 (rBccP33) with GST was successfully expressed in *E. coli*, and evaluated its diagnostic potentiality by ELISA and ICT methods. In addition, I compared the results of the ELISA and ICT with IFAT using 21 sera samples collected from Europe. The results of ELISA and ICT indicated the rBccP33 detected specific antibodies as early as 9 days post-infection in the acute stage, which was more sensitive than rBccSA1.

2-2. Materials and methods

Parasites and experimental animals. Parasites and experimental animals using for rBccP33 was performed as described in chapter 1.

Construction and immunoscreening of cDNA expression library. The immunoscreening of a *B. canis canis* cDNA expression library was performed as described in chapter 1.

Cloning, expression and purification of recombinant BccP33. One third of the cDNA sequences isolated shared the same sequence encoding a novel antigen, which was designated as BccP33**.** The cDNA fragment of the BccP33 without a signal peptide was amplified by PCR using primers with the introduced *Bam*HI and *Sal*I sites (underlined), P1 (5'- CGGGATCCGAAAACACTATACTTTTATCC -3') and P2 (5'- CGGTCGAC TTATTAAAGTTTAGGAGAAGCAGCAGT -3') and inserted into *Escherichia coli* expression vector pGEX-4T-1 (Amersham Pharmacia Biotech, USA) using the *Bam*HI and *Sal*I sites and expressed in *E. coli* BL21 strain, according to the manufacturer's instructions. Bacterial protein was solubilized in a 2% (w/v) *N*-Lauroylsarcosine sodium (Sigma, USA), and the protein was then purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, USA). Lastly, the GST tag was digested with thrombin (Amersham Biosciences, USA) overnight according to the manufacturer's instructions. The protein concentration was measured by using a modified Lowry protein assay kit (Thermo Scientific, USA).

Production of mouse anti-rBccP33 sera. Six-week-old female ICR mice (CLEA,

Japan, n=5) were immunized intraperitoneally with 100 μg of the purified rBccP33 emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, USA). Two additional boosters with 50 μg of the rBccP33 antigen with incomplete Freund's adjuvant (Difco Laboratories, USA) were given intraperitoneally at 2-week intervals. The mice were bled 14 days after the last booster and serum samples were stored at -30℃.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. To identify the native BccP33 in the parasite extract of *B. canis canis*, the *B. canis canis*-infected and normal dog erythrocytes lysates were analyzed by SDS-PAGE and Western blot analysis as described in chapter 1.

Immunofluorescence analysis and confocal laser microscopic observation. For confocal microscopic observation, thin blood smears of *B. canis canis*-infected erythrocytes were fixed in absolute methanol at -30℃ for 30 min. Immunofluorescence assays were carried out as described in chapter 1.

Indirect fluorescent antibody test (IFAT). The IFAT was performed as described in chapter 1.

Enzyme-linked immunosorbent assay (ELISA). The ELISA based on rBccP33 was performed as described in chapter 1.

Immunochromatographic test (ICT). The ICT based on rBccP33 was performed as described in chapter 1.

Serum samples. Serum samples used for detection were performed as described in chapter 1.

2-3. Results

Identification and characterization of the BccP33 gene. The cDNA expression library of *B. canis canis* was screened with infected dog serum. One third of cDNA sequences of two hundred positive clones were isolated and then subjected to BLAST analysis. One identical sequence, which shows no match with the other gene, was selected for molecular characterization (Fig. 8A). The full length of the BccP33 cDNA sequence had a single open reading frame of 966 nucleotides encoding a polypeptide of 321 amino acid residues. Theoretical isoelectric point and molecular mass for the mature protein were 4.93 and 33.599 kDa, as calculated with software (http://web.expasy.org/cgi-bin/compute_pi/pi_tool). Analysis of the putative N-terminal signal peptide in the BccP33 sequence by the SignalP server (http://www.cbs.dtu.dk/services/SignalP/) showed that this sequence had a high predicted signal peptide probability (0.999) and a maximum cleavage site probability (0.741) between amino acids in positions 18 and 19 (Fig. 8A). Genomic analyses indicated that BccP33 contained 2 introns and 3 extrons (Fig. 8C). To improve the expression level of rBccP33, the recombinant protein was truncated based on its hydropathy plot and antigenic index of predicted amino acid (Fig. 8B).

Expression of the rBccP33 in *E. coli*. The BccP33 gene was ligated into the bacterial expression vector pGEX-4T-1 and the BccP33 was then expressed as about 60.6 kDa GST-fusion protein in *E. coli* BL21 strain. The rBccP33-GST was expressed as an insoluble form and then purified by affinity chromatography using Sepharose 4B Columns. As shown in

Fig. 9, the rBccP33 was recognized by the infected dog serum with *B. canis canis* on Western blot analysis (Fig. 9). This result suggested that the rBccP33 successfully expressed in *E. coli* and maintained its antigenicity.

Characterization of the native BccP33 of *B. canis canis*. The polyclonal anti-rBccP33 serum was prepared from mice and used to test for the presence of BccP33 in *B. canis canis* parasites. The lysate of *B. canis canis*-infected and normal canine erythrocytes were analyzed by Western blot analysis using mice serum against the rBccP33. As shown in Fig. 10A, a 39 kDa major band was detected in *B. canis canis*-infected erythrocyte lysates, but no band was detected in normal canine erythrocyte lysates. Moreover, to determine the localization of the BccP33 in parasites, *B. canis canis* merozoites collect from *B. canis canis*-infected dog were examined by IFAT by using anti-rBccP33 serum. Confocal laser microscopic observations revealed that anti-BccP33 antibody specifically reacted with merozoite (Fig. 10B).

Evaluation of the serodiagnostic potential of rBccP33 by ELISA. To evaluate whether the rBccP33 expressed in *E. coli* can be a suitable antigen for the diagnosis of *B. canis canis* infection in dogs, the purified rBccP33 was tested in ELISA. The cut off value of rBccP33 ELISA calculated using SPF canine sera was 0.111. As shown in Fig. 11A, all 12 sera from dogs infected with *B. canis canis* were positive (lane 1, OD > 0.111), whereas, all 30 sera from SPF dogs were negative (lane 2, OD < 0.111). Three sera from *B. canis rossi*-infected dogs (lane 3), 3 sera from *B. canis vogeli*-infected dogs (lane 4), 3 sera from *B. gibsoni*-infected dogs (lane 5) and *N. caninum*-infected dogs (lane 6) were negative (OD <

0.024). At the same time, the specific antibodies against BccP33 were detectable as early as 9th days post-infection in *B. canis canis*-infected canine serum (Fig. 11B). The antibody titers were maintained at a high level until 222 days post-infection, up to the chronic stage of infection in both strains, as evidenced by a recovering hematocrit value (data not shown) and a significantly low level of parasitemia.

Evaluation of the serodiagnostic potentiality of rBccP33 by ICT. To evaluate the performance of rBccP33 as a diagnostic antigen using a more rapid and simple method, ICT was performed to detect specific antibodies. As shown in Fig. 12A, the specific antibodies could be detected from day 9 to at least day 222 post-infection, which was quite consistent with the results of the ELISA. In addition, serum samples from dogs experimentally infected with closely related parasite, *B. canis rossi*, *B. canis vogeli*, *B. gibsoni* and *N. caninum*, were used to determine the specificity of ICT based on BccP33. Only the serum from the *B. canis canis*-infected dogs were positive in the ICT, other serum and the serum from SPF dog were negative (Fig. 12B).

Comparison of the IFAT, ELISA, and ICT with rBccP33. A total of 21 dog serum samples from Europe were detected by IFAT, ELISA and ICT with rBccP33. As shown in Table 2, 11 (52.4%) of the tested samples were positive when analyzed by the IFAT, 13 (61.9%) were positive on ELISA, and 9 (42.9%) were positive on ICT. The result showed that the ELISA was more sensitive than the other methods in detection the BccP33 specific antibody in field samples.

2-4. Discussion

In order to control canine babesiosis, it is important to develop sensitive and reliable tests to screen the parasite positive population or identify *Babesia canis canis* infection at acute and chronic stages. In this regard, a novel gene encoding 33 kDa protein was isolated from the cDNA expression library of *B. canis canis*, which shared no homology with any of the apicomplexan parasites that was important to confer a degree of specificity when using it as a diagnostic probe. The genomic analysis indicated that the BccP33 gene exist two introns in the genome of *B. canis canis*. Software based Kyte–Doolittle's hydropathy analysis of the predicted polypeptide of BccP33 demonstrated that the amino acid sequence had a hydrophilic core region with a good antigenic index suggesting that the antigen could be a good candidate for detection of *B. canis canis* antibodies. A 39 kDa protein of *B. canis canis* merozoites was recognized by anti-rBccP33 mice sera using Western blot analysis. The molecular mass of 39 kDa protein was higher than the predicted amino acides sequences of BccP33. Whilst two predicted N-glycosylation sites of BccP33 was identififed by the NetNGlyc predictor or the PROSITE pattern PS00001, indicading that this variant molecular mass was probably induced by glycosylation modification.

To evaluate whether the rBccP33 can be a suitable antigen for diagnosis of *B. canis canis* infection of dog, the ELISA was conducted for testing the antibody against BccP33 in the serum of *B. canis canis*-infected dog (Ikadai *et al.*, 1999; Xuan *et al.*, 2001). The ELISA with rBccP33 clearly differentiated between *B. canis canis*-infected dog sera and SPF dog sera. In addition, the ELISA detected no cross-reactivity with sera from dogs experimentally infected

with closely related parasites, such as *B. canis vogeli*, *B. canis rossi*, *B. gibsoni* and *N. caninum*. This result indicated that the BccP33 is a *B. canis canis* specific antigen. On the other hand, a dog experimentally infected with *B. canis canis* developed a significant level of antibody titer from nine days post-infection. The significant antibody titer was maintained until 222 days post-infection, even when the dog became chronically infected with a significantly low level of parasitemia. In chapter I, I developed an ELISA test based on BccSA1, which can detect the antibody starting from 22 days up to 222 days post-infection. Therefore, the BccP33 was determined as a specific antigen for early diagnosis of *B. canis canis* infection in clinical diagnosis. This result indicated that the BccP33 could induce strong humoral immunity in both the acute and the chronic stages of *B. canis canis* infections in dogs, thus further study need to be conducted in developing the subunit vaccine using the rBccP33 as a candidate antigen.

In order to develop a simple and rapid diagnosis method for the detection of *B. canis canis* infection in dogs, I try to develop an immunochromatographic test method, particularly for epidemiological survey according to previously study (Huang *et al.*, 2004; Hujakka *et al.*, 2001). The ICT based on the rBccP33 showed that the specific antibodies could be detected from *B. canis canis*-infected dog. The ICT based on the rBccP33 can detect the *B. canis canis* specific antibodies in serial serum samples of the dog as early as 9 DPI, which further indicating that rBccP33 owned the superiority in early diagnosis and could be used as a universal antigen for the development of both ELISA and ICT to detect *B. canis canis* infection at chronic and acute stages.

IFAT has been used as a gold standard for the diagnosis of *Babesia* infection together with the microscopic examination of the parasite (Anderson *et al.*, 1980). However, the disadvantages include low samples throughput and operator fatigue. Test results are influenced by subjective judgment of the operator, which makes standardization difficult. The ICT is a rapid and convenient method for the clinical diagnosis, whereas the sensitivity and specificity need to evaluate in the furtherlarge field samples. In this study, ELISA based on the rBccP33 compared with the ICT based on the rBccP33 and IFAT by detected 21 serum samples collected from Europe. The positive detection rates were 61.9% on ELISA, 42.9% on ICT, and 52.9% on IFAT. Thirteen serum samples were positive by the ELISA, but two of them were negative on IFAT and four were negative on ICT. The results could imply the ELISA with rBccP33 was more sensitive than IFAT and ICT with rBccP33. Taken together, the ELISA using rBccP33 could be useful in detecting dogs, which were exposed to *B. canis canis* infection by monitoring their antibody levels. On the other hand, the ICT was more simple and rapid and appropriate for the detection of large number of field samples, therefore, further research on the ICT need to carried out for improving epidemiological surveys and clinical diagnosis of *B. canis canis* infection.

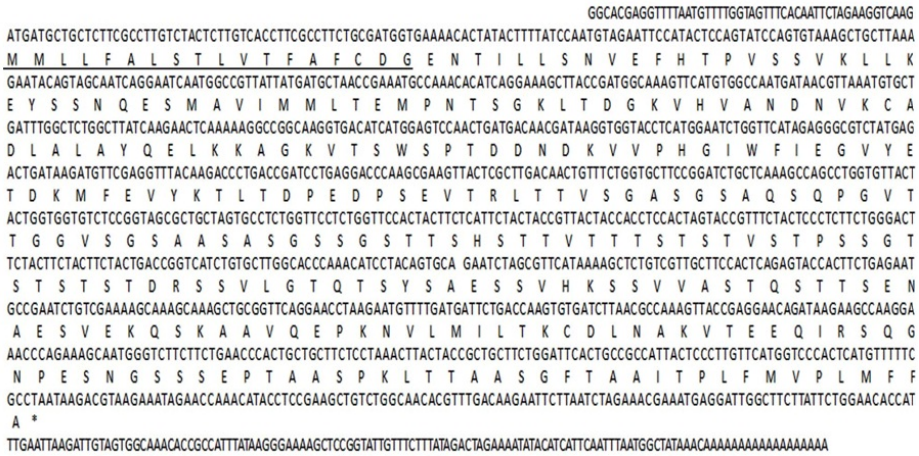
Consequently, a novel gene encoding 33 kDa proteins from *B. canis canis* cDNA expression library was characterized and determined. I demonstrated that ELISA and ICT based on rBccP33 have reliable specificity and higher sensitivity than the ELISA and ICT based on rBccSA1. This suggests that ELISA and ICT based on rBccP33 could be a promising serodiagnostic antigen for epidemiological survey and clinical diagnosis of *B. canis canis*

infection.

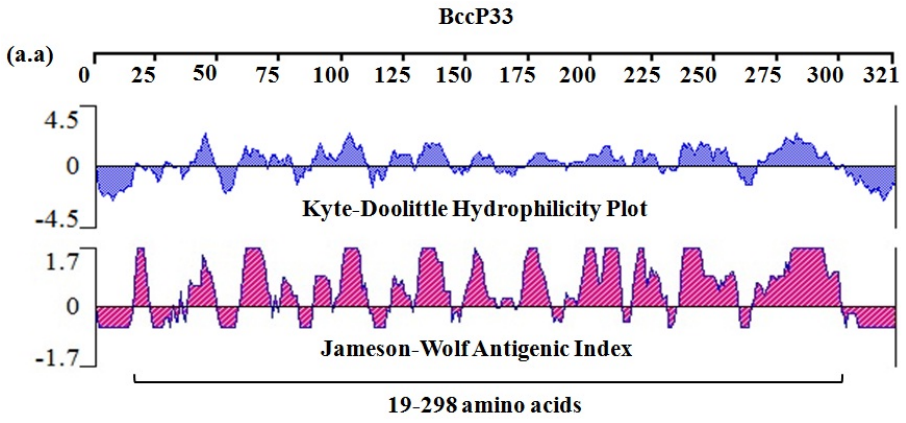
2-5. Summary

I identified a novel gene encoding 33 kDa protein by immunoscreening a *Babesia canis canis* cDNA expression library. The genomic analysis indicated that the BccP33 gene exists two introns in the genome of *B. canis canis*. The rBccP33 was expressed in *E. coli* as GST fusion protein that produced an anti-rBccP33 serum in mice. Using this anti- rBccP33 serum, a native 39 kDa protein in *B. canis canis* was recognized by Western blot analysis. An ELISA using the rBccP33 could detect specific antibodies as early as 9 DPI in serial sera from a dog experimentally infected with *B. canis canis*. It did not show a cross reaction with the closely related parasites. Moreover, a rapid ICT using the rBccP33 was developed and evaluated for detection of antibodies against *B. canis canis*, and it demonstrated high sensitivity and specificity, which was quite consistent with the results of ELISA and closely related parasites. In addition, 21 serum samples from Europe were analyzed by IFAT, ELISA and ICT based on the rBccP33. The result indicated that the ELISA of rBccP33 was more sensitive than IFAT and ICT of rBccP33. My data suggests that BccP33 is an immunodominant antigen. The rBccP33 could be a promising diagnostic antigen for a detection of both acute and chronic stages of *B. canis canis* infection to control canine babesiosis.

A



B



C

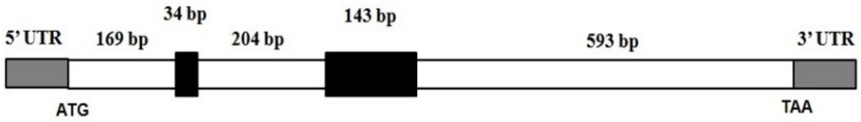


Fig. 8. (A) Complete nucleotide sequences and predicted amino acid sequences of cDNA coding for BccP33, including the 5'- and 3'- untranslated regions of the cDNA. The amino acids underlined at the 5'-orientation of the sequence are the N-terminal signal peptides (* stop codon). (B) The software analysis of hydrophilicity and antigenicity of BccP33 (C) Structure of the genomic BccP33 gene. White bold lines indicate exons and black rectangles indicate introns. The numeric numbers indicate the number of nucleotides.

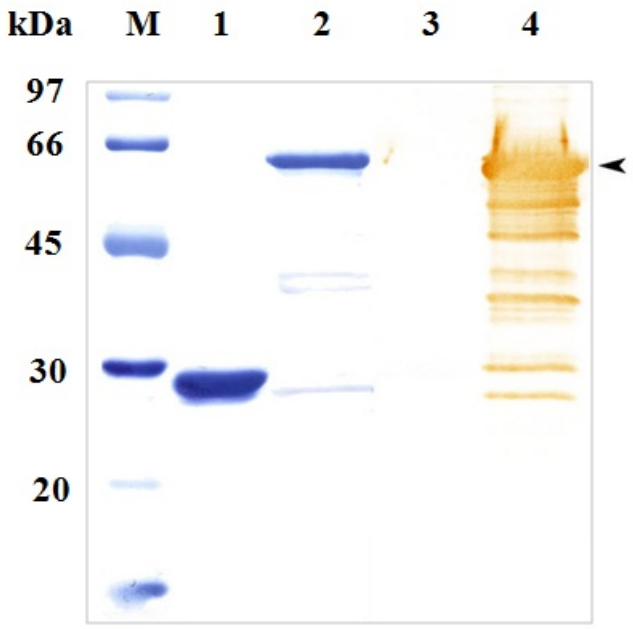


Fig. 9. SDS-PAGE and Western blot analysis of recombinant BccP33. M, molecular size markers. Lanes 1 and 2, the recombinant GST and rBccP33 fused with GST were stained by amide black. Lanes 3 and 4, the recombinant GST and rBcc P33 fused with GST were incubated with serum from a dog infected with *B. canis canis*.

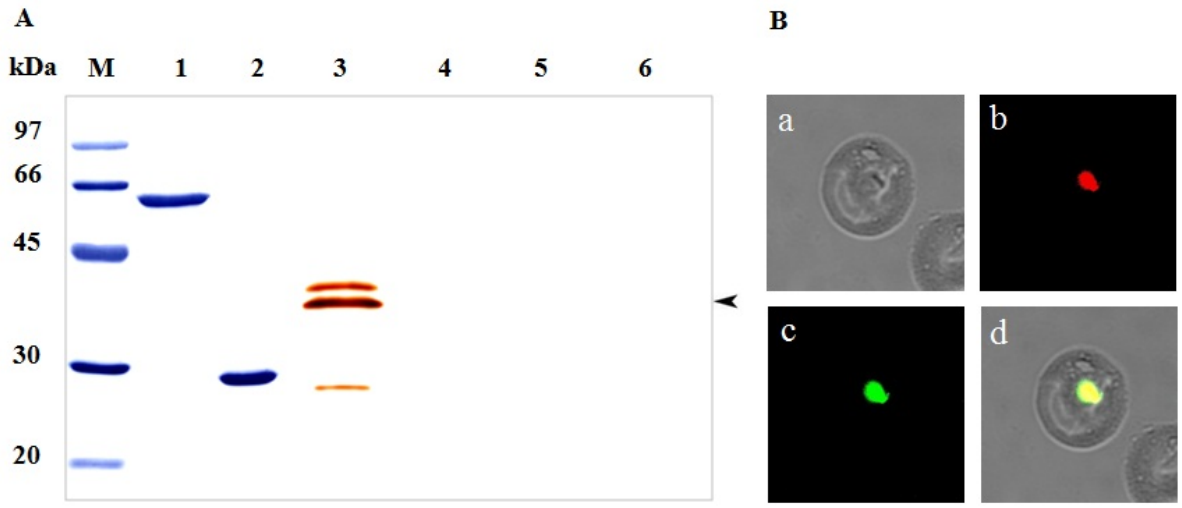
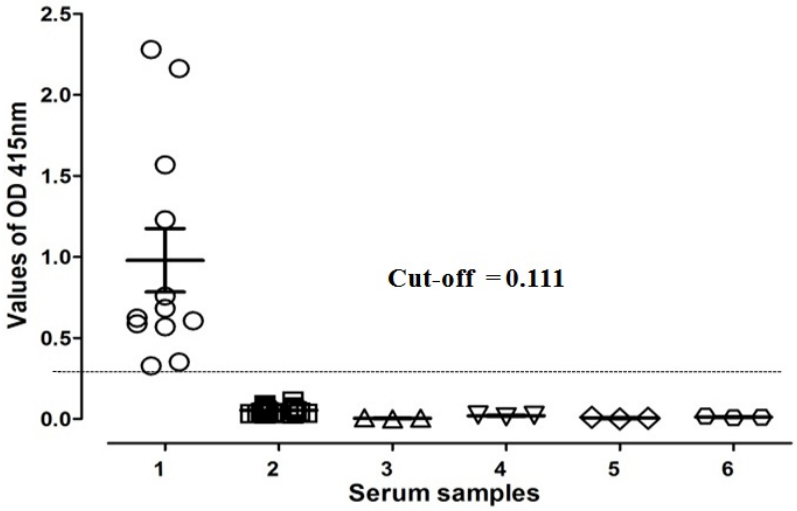


Fig. 10. Molecular characterization of native BccP33 enzyme (A) Lane M, molecular size marker. Lanes 1 and 2, rGST and rBccP33 fused with GST were stained by amide black. Lanes 3 and 4, detection of the lysates of *B. canis canis* and non-parasitized canine erythrocytes reacted with anti-rBccP33 serum raised in mice. Lanes 5 and 6, detection of the lysates of *B. canis canis* and non-parasitized canine erythrocytes reacted with a normal serum. (B) Observation of the native BccP33 recognized by mice anti-rBccP33 serum in confocal laser micrographs. (a) Phase-contrast images of *B. canis canis* merozoites. (b) Immunofluorescent staining of *B. canis canis* merozoites with mice anti-rBccP33 serum. (c) Propidium iodide staining of *B. canis canis* merozoite nuclei. (d) Panels b and c are overlaid on panel a. The images were derived from a single section.

A



B

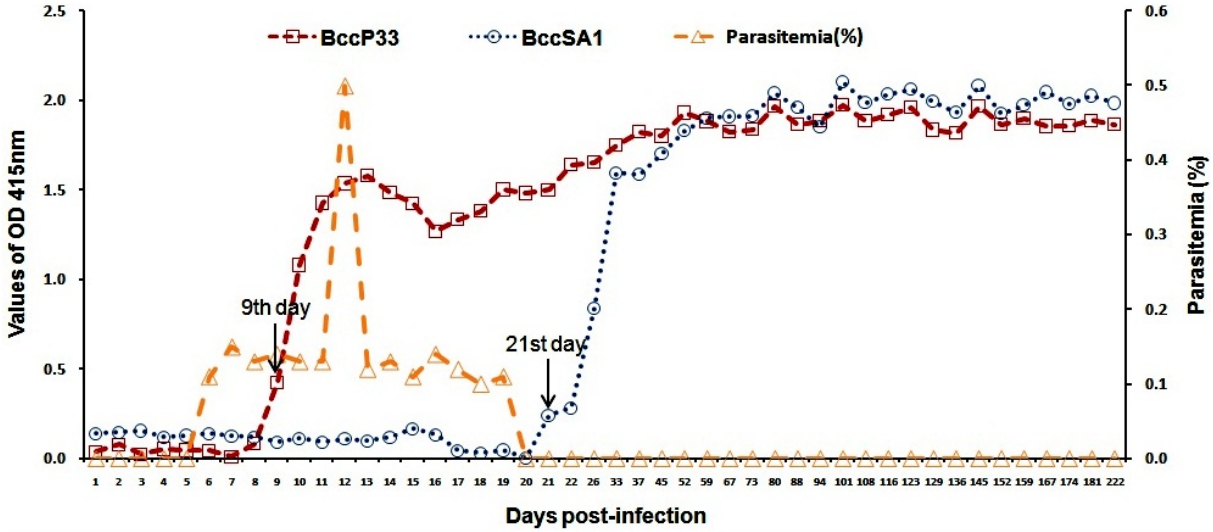


Fig. 11. Evaluation of BccP33 as a diagnostic antigen by ELISA system. (A) Values of ELISA with experimentally infected canine sera. Lane 1, *B. canis canis*-infected dogs sera (n=12); lane 2, SPF dog sera (n=30); lane 3, *B. canis rossi*-infected dogs sera (n=3); lane 4, *B. canis vogeli*-infected dogs sera (n=3); lane 5, *B. gibsoni*-infected dogs sera (n=3); lane 6, *N. caninum*-infected dogs sera (n=3) (B) Detection of sequential sera samples against BccP33 from a nonsplenectomized dog experimentally infected with *B. canis canis* by ELISA.

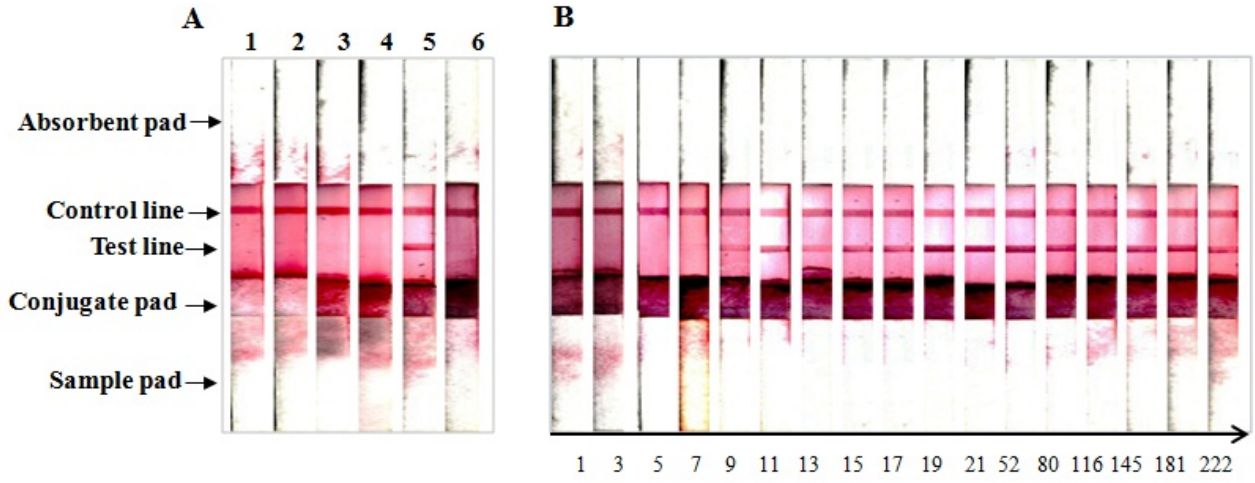


Fig. 12. Evaluation of rBccP33 by ICT. (A) Cross-reactivity of rBccP33 with closely related parasite-infected canine sera. Lane 1, *B. canis rossi*-infected dog serum; lane 2, *B. canis vogeli*-infected dog serum; lane 3, *B. gibsoni*-infected dog serum; lane 4, *L. infantum*-infected dog serum; lane 5, *B. canis canis*-infected dog serum; lane 6, a SPF dog serum. (B) Specific antibody responses to BccP33 in sequential serum samples from a nonsplenectomized dog experimentally infected with *B. canis canis*.

Table 2. Comparison of the IFAT, ELISA, and ICT based on the rBccP33 for the detection of sera samples from Europe.

No. (%) with BccP33-ICT		No. (%) with IFAT		No. (%) with BccP33-ELISA		No. (%) with BccP33- ICT/ BccP33-ELISA
		No. (%) positive	No. (%) negative	No. (%) positive	No. (%) negative	
No. (%) positive	9 (42.9)	8 (38.1)	1 (4.8)	8 (38.1)	1 (4.8)	14 (66.7)
No. (%) negative	12 (57.1)	3 (14.3)	9 (42.8)	5 (23.8)	7 (33.3)	7 (33.3)
Total no. (%)	21 (100)	11 (52.4)	10 (47.6)	13 (61.9)	8 (38.1)	21 (100)

Chapter 3

Cloning, characterization and validation of inosine 5'-monophosphate dehydrogenase of *Babesia gibsoni* as molecular drug target

3-1. Introduction

The *Babesia* parasites are protozoan pathogens transmitted by ticks and cause babesiosis in mammals including human (Rodríguez *et al.*, 1994; Homer *et al.*, 2000b; Vannier *et al.*, 2008). *B. bovis* infects cattle causing massive economic losses in livestock industry worldwide (Bock *et al.* 2004) while *B. gibsoni* causes canine babesiosis worldwide, sometimes leading to deaths (Birkenheuer *et al.*, 2005). Babesiosis is one of the most widely distributed, serious and poorly controlled infection globally. Chemotherapy is the only practical way of saving infected animals, and thus reducing economic losses as well as safeguarding animal welfare (Vial and Gorenflot, 2006; Ramharter *et al.*, 2010). However, only few drugs are available for babesiosis treatment. Therefore, there is need to develop novel anti-*Babesia* compounds to treat babesiosis.

The purine pathway of parasitic protozoa is an attractive chemotherapeutic target because these parasites rely entirely on the pathway to meet their purine demands for nucleic acid synthesis (Webster *et al.*, 1982; Striepen *et al.*, 2004). In this pathway, inosine 5’-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) is a rate limiting step catalyzing

the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP) with concomitant reduction of NAD^+ to NADH (Hedstrom, 2009). The XMP, which is formed, is a precursor of guanine nucleotides required for biosynthesis of DNA, crucial for the survival of parasites (Hedstrom, 2009). IMPDH enzyme is susceptible to inhibition by mycophenolic acid (MPA), which is a structural analogue of the cofactor NAD^+. MPA is an immunosuppressive drug (Ratcliffe, 2006) and has anticryptosporidial activity in cell cultures (Striepen *et al.*, 2004). The IMPDHs have been studied as molecular drug target in other apicomplexans, especially *Plasmodium*, *Cryptosporidium* and *Toxoplasma* spp (Hedstrom, 2009; Sullivan *et al.*, 2005; Umejiego *et al.*, 2004), but not in *Babesia* species. I think that MPA targets *Babesia* IMPDH enzyme, thereby disrupting the enzyme in *Babesia* cell and thus inhibit the growth of the parasite *in vitro*.

Therefore, I have cloned and expressed a *B. gibsoni* (BgIMPDH) cDNA in *Escherichia coli* BL21 (DE3) and validated the BgIMPDH enzyme as a molecular drug target. I then use the recombinant BgIMPDH (rBgIMPDH) to generate antibodies in mouse for characterization of a corresponding native enzyme in the parasite. The kinetic parameters of the rBgIMPDH enzyme and the enzyme inhibition constant of MPA are also reported. Finally, I have demonstrated that MPA inhibits the growths of both *B. gibsoni* and *B. bovis in vitro*. Therefore, my data suggest that MPA targets IMPDH of *Babesia* parasites.

3-2. Materials and methods

Reagents and experimental animals. Dimethyl sulfoxide (DMSO), isopropyl-β-D-

thiogalactopyranoside (IPTG), sodium pyruvate and sodium bicarbonate were purchased from Wako Pure Chemical Industries, Japan. MPA and IMP were purchased from Sigma Aldrich (USA). The other materials used in this study included Luria broth (LB) base (Invitrogen, USA), 0.45 μM Millex filter units (Corrigtwohill Co., Ireland), 48–well flat bottom tissue culture plates (Becton Dickinson and Co., USA), and 24-well multidish plates (Nunc, Denmark). For experimental animals, female 6-week-old ICR mice (CLEA, Japan) were used in addition to 10 Beagle dogs (Chugai, Japan). The animals used in this study were housed in a P2 facility. Mice were given food and water ad libitum while the dogs were fed every day and given water ad libitum. All experiments were done in line with the guidelines for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

Maintenance and culture of *Babesia* parasites. The parasites stocks used in this study were *B. gibsoni* Oita and *B. bovis Texas* strains. These parasites were stored in liquid nitrogen. The *in vitro* cultures of *B. gibsoni* and *B. bovis* were grown at 37^{o}C in humidified CO_2 (5%) and O_2 (5%) incubator (BIO-LABO, Japan) using canine and bovine erythrocytes. Briefly, *B. gibsoni* was cultured in canine erythrocytes suspended in RPMI 1640 supplemented with 20% dog serum while *B. bovis* was cultured in bovine erythrocytes suspended in GIT medium.

Isolation and bioinformatic analysis of BgIMPDH cDNA. An expressed sequence tag (EST) database of *B. gibsoni* constructed previously in my laboratory was analyzed to determine cDNAs that encode enzymes of the purine pathway. From these cDNAs, a *B. gibsoni* IMPDH (BgIMPDH) gene was isolated and its full-length was determined by

sequencing with an automated sequencer (ABI PRISM 3100 Genetic Analyzer, USA). The full-length BgIMPDH gene was translated and its polypeptide analyzed by BLASTp method. This polypeptide was compared with those of other apicomplexan parasites and mammals by amino acid alignment and phylogenetic analysis. The IMPDH-amino acid sequences of the other apicomplexan parasites and mammals were obtained from the GenBank database. Alignment of all the homologus peptides was done using Genetyx software (Genetyx Corporation, Japan) and CLUSTAL X software. A phylogenetic tree was generated by using the amino acid sequences of IMPDH using the neighbor-joining method. Functional domains, enzyme active sites and secondary structures of the deduced proteins were analyzed by the BLAST search tool and the DNAstar software (Netwell, Japan), respectively.

Cloning and expression of BgIMPDH gene. The cDNA fragments of the BgIMPDH gene was amplified by PCR using primers with *Sal*I and *Xho*I sites (underlined), P1 (5'-CGGTCGACATGGCTGACGGTTCCACT-3') and P2 (5'-CGCTCGAGTTAACTCGGCATAATAAC -3'). The PCR product was digested with *Sal*I and *Xho*I, and then inserted into *Sal*I and *Xho*I sites of the expression vector pET-28a (Amersham Pharmacia Biotech, USA). The cloned BgIMPDH cDNA was expressed in *E. coli* BL21 (DE3) as a His-tag fusion protein. Thereafter, the rBgIMPDH was purified by a Ni-NTA affinity chromatography according to the manufacturer's instructions (Amersham Pharmacia Biotech, USA). The recombinant protein concentration was measured by using a modified Lowry protein assay kit (Thermo Scientific, USA).

Production of mouse anti rBgIMPDH sera and characterization of the native

enzyme. Five 6-week-old female ICR mice were immunized with 0.5 mg of purified rBgIMPDH protein emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemicals, USA). Thereafter, two boosters consisting of 0.25 mg of the same antigen emulsified with Freund's incomplete adjuvant (Sigma Chemicals, USA) were administered to each mouse via the same route at days-14 and 28. Then, antiserum was collected from each mouse on the 14th day after the last booster. To identify the native BgIMPDH enzyme, *B. gibsoni* lysate were separated by a 12% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and then probed with the antiserum by Western blot analysis. Additionally, an indirect fluorescent-antibody test (IFAT) and confocal laser microscopy were performed by probing whole parasite cells with the same antiserum as reported earlier (Fukumoto *et al.* 2001).

Assay and inhibition of rBgIMPDH enzyme-activity. A standard buffer for rBgIMPDH assay consisted of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM DTT, and various concentrations of IMP and NAD^+. Enzyme activity was determined by measuring an increase in absorbance at 340 nm at 25°C by using a Beckman UV-800 visible spectrophotometer. The measurement of the increase in absorbance was based on conversion of NAD^+ to NADH. Data of the initial velocity were fitted to the Michaelis-Menten equation and lineweaver plots. The inhibition constant (*Ki*) of MPA was determined at 50 μM IMP and 1000 μM NAD^+. Assays were started by addition of IMP after pre-incubation of the enzyme with MPA dissolved in the assay buffer. To determine $K_{i,}$ a reciprocal of enzyme velocity was plotted against that of NAD^+ concentration for various inhibitor concentrations by using

Prism ver5.02 (GraphPad, USA).

Inhibition of growths of *Babesia* parasites *in vitro* by MPA. For inhibition assay of *in vitro* growths of *B. gibsoni* and *B. bovis*, the parasites were cultured in individual wells of a 48-well plate in the presence of MPA in a humidified incubator (BIO-LABO, Japan) set at 37^{o}C with 5% carbon dioxide and O_2 (5%). MPA was dissolved in DMSO and then added to the parasites cultures at concentrations ranging from 14.29 nM to 93750 nM for *B. gibsoni* and 128.6 nM to 281250 nM for *B. bovis*. Thereafter, the cultures of the *Babesia* parasites were grown for 3 days and then sub-cultured in non-treated medium for another 3 days. The cultures of the parasites treated with DMSO were used as negative control. Percent of parasitemias for both *Babesia* species were determined by microscopic examination of Giemsa stained blood smears. The dynamics of cellular morphology of erythrocytes was also examined.

Nucleotide sequence accession number. The nucleotide sequence of full-length *B. gibsoni* IMPDH genes are available in the DDBJ/EMBL/GenBank database with the accession number JQ781073. The library Genbank accession number of *B. gibsoni* EST is LIBEST_027758. The complete list of assigned Genbank accession numbers are in Table 3.

3-3. Results

Identification and bioinformatic analysis of BgIMPDH cDNA. I analyzed the *B. gibsoni* EST database constructed in my laboratory and found 10 different cDNAs that encode enzymes of the purine pathway (Table 3). Of these enzymes I focused on IMPDH, which

catalyzes a crucial step of the purine pathway making this enzyme a putative drug target (Terkawi and Igarashi, 2011). Therefore, I performed BLASTp analysis of translated BgIMPDH polypeptide and found that it shared 74% amino acid identity to the *B. bovis* IMPDH, 57% to the *Theileria parva* counterpart, and 56% to the *T. annulata* IMPDH as well as 52% identity to both *T. gondii* and *Plasmodium* spp enzymes. Some similarities to mammalian IMPDH were noted albeit at a relatively lower score values as compared to the apicomplexan enzymes. The predicted secondary structure of BgIMPDH consisted of TIM_phosphate_binding super family and two tandem repeats of the cystathionine beta-synthase (CBS pair) domains. This TIM_phosphate_binding super family domain contained a catalytic site with active site loop and flap as well as NAD^+ binding site (Fig. 13A). Thirteen amino acid residues responsible for IMP binding were found in the catalytic domains of BgIMPDH. The residues included Ser313, Ile314, Cys315, Asp350, Gly352, Gly373, Gly374, Tyr397 Gly399, Met400, Gly401, Gln431 and Gly432. The Gly374 in IMPDH of *Babesia* parasites was replaced by Ser388 in the mammalian IMPDH and this amino acid was conserved in mammals (Fig. 13B). Phylogenetic analysis revealed a wide divergence between *Babesia* IMPDH enzymes and the counterpart enzymes in mammalian host (Fig. 14). Taken together these observations suggest that *Babesia* IMPDH may have a distinct biochemical and catalytic properties different from their mammalian counterpart and thus could be selectively targeted by novel inhibitors.

Expression and characterization of BgIMPDH enzyme. The next objective was to express the BgIMPDH cDNA in bacteria for biochemical analysis and then generate

antibodies in mice for characterizing the native BgIMPDH enzyme. Purified rBgIMPDH revealed 57 kDa band on 12% SDS-PAGE (Fig. 15A), including an additional 3 kDa of the His-tag sequence. Furthermore, mouse anti-rBgIMPDH enzyme reacted with *B. gibsoni* lysate yielding a specific band of approximately 54 kDa. However, serum of non-immunized mice did not react with the *Babesia* lysate (Fig. 15A). On further analysis by IFAT and confocal laser microscopy, the mouse antiserum reacted with endogenous IMPDH in *B. gibsoni* yielding a specific green fluorescence mainly in the parasite cytosol. This green fluorescence was seen in both extracellular and intracellular parasites of single and dividing forms (Fig. 15B). It seems that the cDNA encodes an authentic IMPDH enzyme existing in a native form in *B. gibsoni* and possibly in other *Babesia* species.

Assay and inhibition of rBgIMPDH enzyme-activity. Since BgIMPDH cDNA encoded an authentic protein I theorized that the expressed rBgIMPDH is likely to be an active enzyme. Therefore, I determined the rBgIMPDH activity and found that the enzyme was catalytically active in a pH dependent manner revealing maximum activity at pH 8.0 (Fig. 16A). The steady-state kinetic parameters for this enzyme revealed K_m values of 8.18 ± 0.878 and 360.80 ± 43.41 μM for IMP and NAD^+, respectively (Fig. 16B and C). Since MPA have been widely used an inhibitor for determining the inhibition of IMPDH in other organism as well as mammals I selected it as a candidate inhibitor for my study. This compound inhibited the activity of rBgIMPDH enzyme with a K_i value of 20.93 ± 1.83 μM with respect to NAD^+ (Fig. 16D). These findings indicate that MPA may target the IMPDH enzyme in *Babesia* parasites.

Inhibitory effect of MPA on growths of *Babesia* parasites. The inhibition of rBgIMPDH activity by MPA convinced us that this compound may also target the endogenous enzyme in live *Babesia* cells and thus inhibit the growth of the parasites. Indeed, I observed inhibitions of the growths of both *B. gibsoni* and *B. bovis in vitro* by MPA. The compound caused inhibitions in dose dependent manner revealing reductions in percent parasitemias (Fig. 17A and B). The IC_{50}s of MPA were 0.95 ± 0.21 and 2.88 ± 0.49 μMs for *B. gibsoni* and *B. bovis,* respectively (Fig. 17C and D). The treated parasite cultures had predominantly single *Babesia* cells as opposed to untreated cells that had both single and dividing forms as observed on day two (Fig 18A-D). At a higher concentration of MPA the existing parasite cells were mainly in single forms as opposed to dividing forms (Fig. 18E and F). Likewise, treated culture showed predominantly dot-like form on the 3rd day. As shown in the Fig. 18G and H, there was a positive correlation between the percentage of dot forms and the concentration of MPA during the inhibitions of *B. gisoni* (R^2 = 0.9669 ± 0.6770) and *B. bovi*s (R^2 = 0.9592 ± 0.0470). Therefore, I can say that MPA may target the endogenous enzyme and thus inhibits the growth of the *Babesia* parasites.

3-4. Discussion

The purine pathway has been studied as attractive chemotherapeutic target in other apicomplexan parasites but not in *Babesia* parasites (Hariharan *et al.*, 1999; Sullivan *et al.*, 2005; Umejiego *et al.*, 2004). In this study, I identified *B. gibsoni* genes that encode putative enzymes of purine pathway suggesting that the pathway is present in this parasite. It has been

reported that IMPDH of purine pathway is a promising drug target for antimicrobial chemotherapeutic intervention (Hariharan *et al.*, 1999; Sullivan *et al.*, 2005; Umejiego *et al.*, 2004). The IMPDH enzymes of human and some apicomplexan parasites have been validated as molecular drug target (Hedstrom, 2009). Therefore, to validate IMPDH of *B. gibsoni* as a drug target, I cloned and expressed rBgIMPDH. The deduced secondary structure of BgIMPDH is similar to those of other known enzymes (Sullivan *et al.*, 2005). I found that the BgIMPDH polypeptide has CBS domains, active site loop as well as active site flap consistent with the structures of the other enzymes already characterized (Hedstrom, 2009). Mammalian and microbial IMPDHs have been reported to show marked structural and functional differences that may be exploited to develop inhibitors that selectively bind the parasite IMPDH (Berg et al, 2010). This study found that amino acid residues in the catalytic domain of mammalian and *Babesia* IMPDHs are not conserved. The Gly374 in IMPDH of *Babesia* parasites was replaced by Ser388 in the mammalian IMPDH (Sullivan *et al.*, 2005). These observations appear to concur with a previously documented report that mammalian and microbial IMPDH are probably structurally different having distinct biochemical properties (Umejiego *et al.*, 2004).

Recombinant IMPDH enzymes expressed in *E. coli* are known to provide the best choice for characterizing IMPDHs of humans (Sintchak *et al.*, 1996) and parasitic pathogens (Umejiego *et al.*, 2004). Nevertheless, a previous expression of the *T. gondii* IMPDH (TgIMPDH) enzyme in a variety of bacterial systems failed to yield the desired results (Sullivan *et al.*, 2005). In this study, I successfully expressed a corresponding rBgIMPDH in

E. coli suggesting that rBgIMPDH enzyme is more soluble protein than TgIMPDH. In fact, the BgIMPDH peptide had more polar amino acid residues (49.7%) than the TgIMPDH (48.5%), a factor that may have an influence on the protein solubility and hence a different outcome on the expressions of the enzymes. But other factors such as toxicity of the TgIMPDH gene to *E. coli* and the relative AT% content of the genes could not be ruled out. Previously, antisera against recombinant enzymes have been used to identify and characterize other enzymes of apicomplexan parasites including *Babesia* species (Aboge et al, 2008). Similarly, this study revealed that mouse antisera against the rBgIMPDH could identify and localize the native BgIMPDH in *B. gibsoni*. This convinced us that the BgIMPDH cDNA encoded an authentic functional enzyme that exists in some of the parasite-stages.

The expression of the recombinant IMPDH enzyme enabled us to study the biochemical properties of the enzyme *in vitro* as well as the inhibitory effect of MPA. The K_m of NAD^+ (μM) for BgIMPDH was 360.80 ± 43.41 μM a value high than the previously biochemically characterized enzymes of *T. gondii* (Sullivan *et al.*, 2005), *C. parvum* (Umejiego *et al.*, 2004), *E. tenella* (Hupe *et al.* 1986) and the human IMPDH (Hager *et al.*, 1995). It is possible that other factors such as assay conditions or even the differences in biochemical properties among the parasite species and mammals could have resulted in this variation. Nevertheless, the K_m of IMP (μM) for BgIMPDH was comparable to that of type II human IMPDH but slightly lower than that of the type I human enzyme (Hager *et al.*, 1995).

Since IMPDH is an important target for the development of chemotherapeutic drugs (Hariharan *et al.*, 1999; O'Gara *et al.*, 1997; Sharling *et al.*, 2010; Sullivan *et al.*, 2005;

Umejiego *et al.*, 2004), the rBgIMPDH enzyme was used to validate the inhibition of its activity by MPA. The inhibition of rBgIMPDH by MPA in dose dependent manner was consistent with other inhibition studies the recombinant enzymes of TgIMPDH (Sullivan *et al.*, 2005). Furthermore, this inhibition was consistent with suggests of growth and multiplication of *Babesia* parasites *in vitro.* This observation indicates that MPA kill *Babesia* parasites by targeting the IMPDH. MPA is known to be an uncompetitive inhibitor with respect to both IMP and NAD^+ for most IMPDHs because it has strong preference for the enzyme-XMP intermediate. This preference has been used to reverse the reaction, forming the enzyme-XMP-MPA complex from XMP with the Chinese hamster IMPDH type 2-enzyme (Fleming *et al.*, 1996). Consequently, I speculate that this selective affinity of MPA for the enzyme-XMP-complex may have contributed to the inhibition of rBgIMPDH activity as well as the growths and multiplications of *Babesia* parasites. Nevertheless, the inhibition was weaker since the *Ki* reported in this study was on the higher side.

The *Ki* of MPA for inhibition of the rBgIMPDH activity was higher than the IC_{50} of *Babesia* growth-inhibitions *in vitro* suggesting that the MPA was a strong inhibitor of the *Babesia* growths than the rBgIMPDH activity. It appears that the MPA had lower affinity for the enzyme in the assay solution but the affinity of the inhibitor for the enzyme was higher in parasites cultures. The trapping of the enzyme-XMP-intermediate by MPA could have been more effective with the endogenous IMPDH in the parasites as compared with the recombinant enzyme. I plan to screen analogs of MPA to further determine how the inhibition of rBgIMPDH activity is related to those of *Babesia* growth inhibitions *in vitro*. Interestingly,

MPA had a lower IC_{50} for *B. gibsoni* than for *B. bovis*, which may indicate that *B. gibsoni* is more sensitive to this drug. However, this can also be explained based on the differences in the nature of the parasites with regard to species-specific metabolic requirements needed for survival of the parasite. Alternatively, it could be due to the differences in components of the culture needed for cultivation of these parasites. Further study might be needed to confirm the theories above.

MPA inhibits the growths of *C. parvum* (Striepen *et al.*, 2004), *T. gondii* (Sullivan *et al.*, 2005) and *P. falciparum* (Hariharan *et al.*, 1999) similarly to the inhibition of the growths of *Babesia* parasites seen in this study. My results suggested that MPA targets BgIMPDH enzyme indicating that the inhibitor may have penetrated the erythrocyte and the parasite membranes barriers resulting in inhibition of cytosolic IMPDH in *Babesia* parasites. Consequently, I would expect MPA to inhibit the formation of XMP disrupting the subsequent synthesis of GMP. GMP is the source of guanine nucleotides required for RNA and DNA synthesis. Therefore, this disruption may have resulted in inhibition of the synthesis and replication of DNA in the parasites eventually, arresting the parasite cell division. This means that *Babesia* cells can only grow and increase in size but fail to divide into daughter cells. Indeed, this argument is supported by my results in which treated parasite cultures showed predominantly single parasite cells as opposed to untreated cultures with had both single cells and dividing merozoites.

In conclusion, this study reports for the first time, the isolation, cloning and expression of the *B. gibsoni* IMPDH gene to get an active 57 kDa purified rBgIMPDH enzyme. The

presence of the native enzyme in *B. gibsoni* was shown by Western blot analysis and confocal laser microscopy. I have also reported that *B. gibson*i and most probably other *Babesia* species have catalytically active IMPDH enzymes. Additionally, the inhibitory effect of MPA on the activity of rBgIMPDH enzyme and the growths of both *B. bovis* and *B. gibsoni in vitro* were demonstrated. Therefore, this study has shown validate that BgIMPDH enzyme may be a molecular target of MPA and thus could be exploited for future drug design and development.

3-5. Summary

The IMPDH have been characterized and validated as molecular drug target in other apicomplexans but not in the genus *Babesia*. Subsequently, I cloned and expressed a *Babesia gibsoni* IMPDH (BgIMPDH) cDNA in *E. coli*. I also determined the inhibitory effect of MPA on rBgIMPDH activity and the *Babesia*-growths *in vitro*. The translated BgIMPDH peptide contained thirteen amino acid residues responsible for substrate and cofactor binding in its catalytic domain with Gly374 in BgIMPDH being replaced by Ser388 in mammalian IMPDH. The native BgIMPDH enzyme in the parasite was approximately 54 kDa a mass similar to His-tag rBgIMPDH protein. The K_m values of the rBgIMPDH were 8.18 ± 0.878 μM and 360.80 ± 43.41 μM for IMP and NAD^+, respectively. MPA inhibited the rBgIMPDH activity yielding a K_i value of 20.93 ± 1.83 μM with respect to NAD^+. For *Babesia* growths, the IC_{50}s were 0.95 ± 0.21 and 2.88 ± 0.49 μM for *B. gibsoni* and *B. bovis*, respectively. Therefore, my results suggest that MPA may kill *Babesia* parasites by targeting IMPDH enzyme of the

purine pathway.

Fig. 13. Bioinformatics analysis of translated BgIMPDH polypeptide. (A) The predicted functional domains of BgIMPDH is shown by BLASTp. (B) IMPDH amino acid sequences for *B. gibsoni*, *B. bovis*, and *Bos taurus* were aligned using the CLUSTAL X program. Gray blocks indicate the predicted substrate-binding site residues.

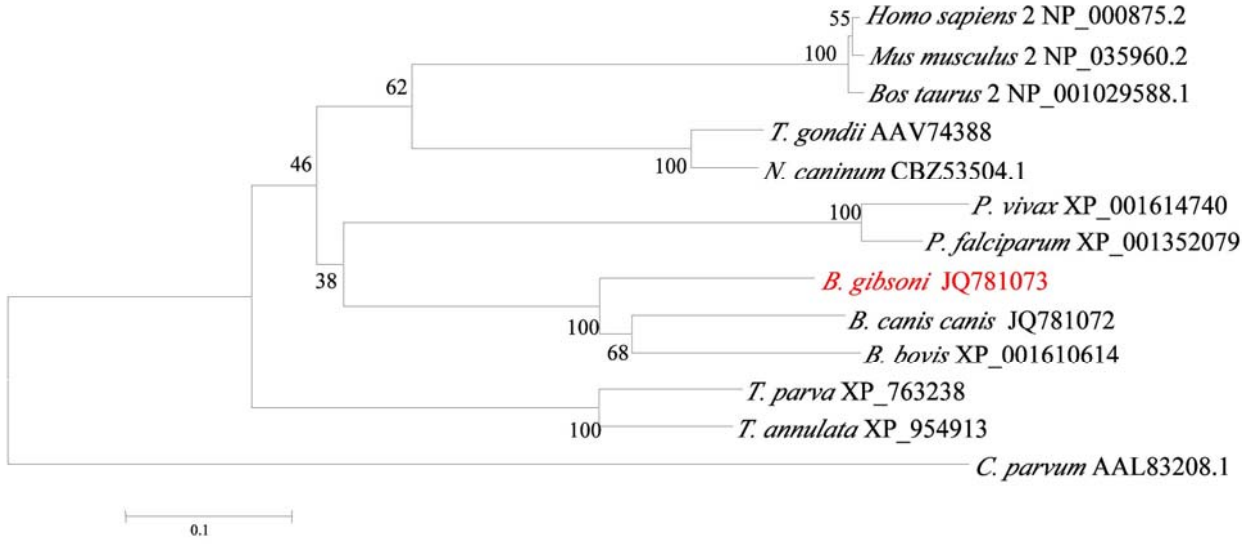


Fig. 14. A phylogenetic tree based on the IMPDHs amino acid sequences of *B. gibsoni*, *B. canis canis*, *B. bovis*, *T. parva*, *T. annulata*, *P. vivax*, *P. falciparum, T. gondii*, *N. caninum*, *C. parvum*, *Homo sapiens* 2, *Mus musculus* 2 and *Bos taurus* 2. The tree was generated using the neighbour-joining method incorporated into the MEGA 4 program.

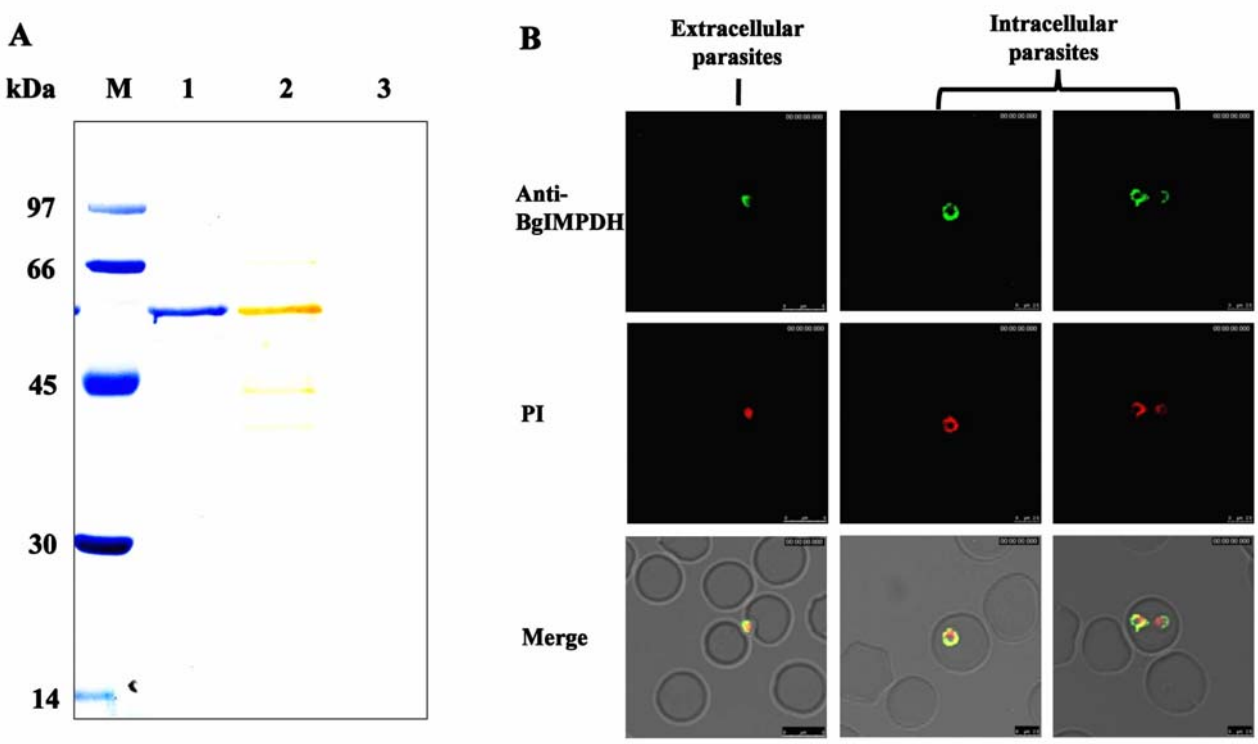


Fig. 15. Molecular characterization of native BgIMPDH enzyme. (A) Lane M, molecular size marker. Lane 1, purified recombinant BgIMPDH fused with his-tag after analysis by SDS-PAGE; Lane 2 and 3, Western blot analysis of the lysates of *B. gibsoni* and non-parasitized dog erythrocytes with anti-rBgIMPDH serum raised in a mouse, respectively. (B) The reactivity of anti-BgIMPDH serum with native enzyme of *B. gibsoni* on the IFAT and confocal laser microscopy. Specific green fluorescent was seen in extracellular (Left panel) and intracellular parasites in both single and dividing forms *of B. gibsoni*.

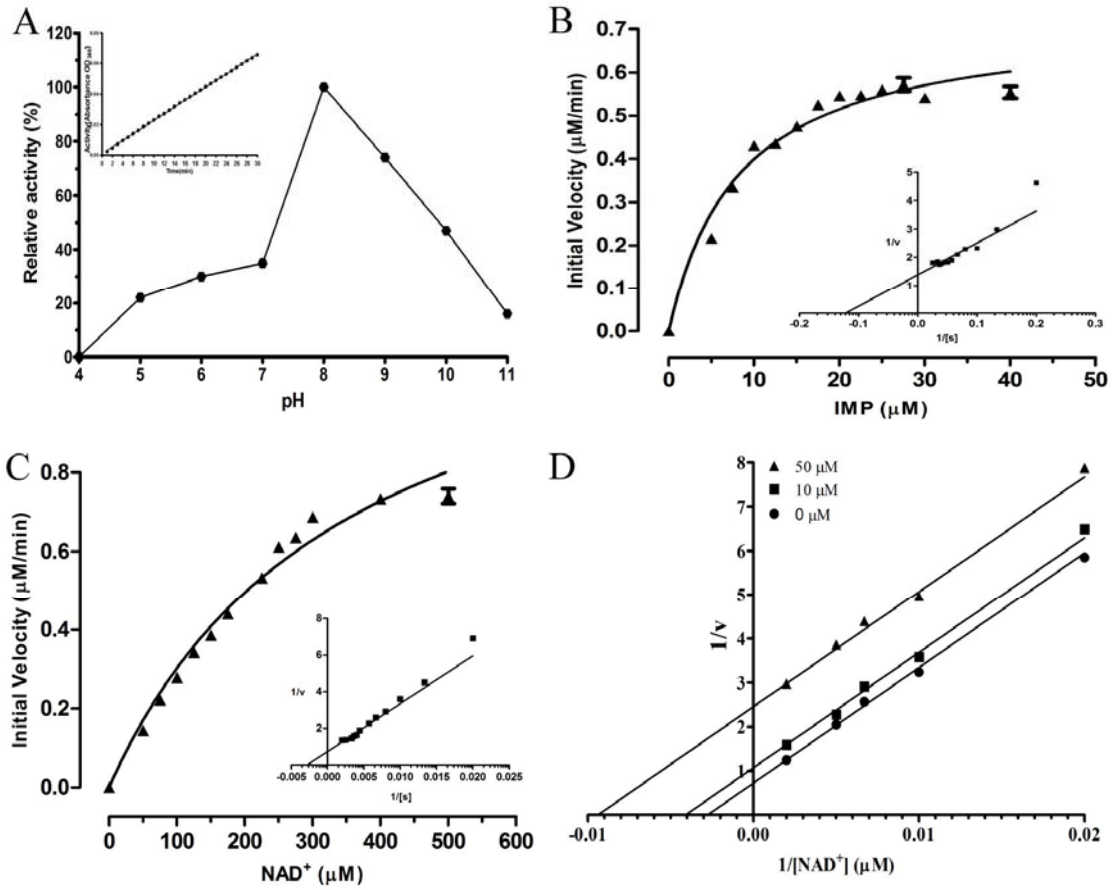


Fig. 16. Assay of enzymatic activity rBgIMPDH and its inhibition by MPA. (A) Effect of pH on catalytic activity. Inset is a graph showing the linear reaction. (B) Kinetic constants were determined by Michaelis-Menten equation. Various concentrations of IMP at a fixed NAD^+ concentration. (C) Various concentrations of NAD^+ concentrations at a fixed IMP concentration. (D) MPA inhibition of rBgIMPDH; 1/v versus 1/[NAD^+] plot. The concentration of IMP was 50 μM; NAD^+ concentration was varied. The mean ± standard error of the mean was calculated for each parameter.

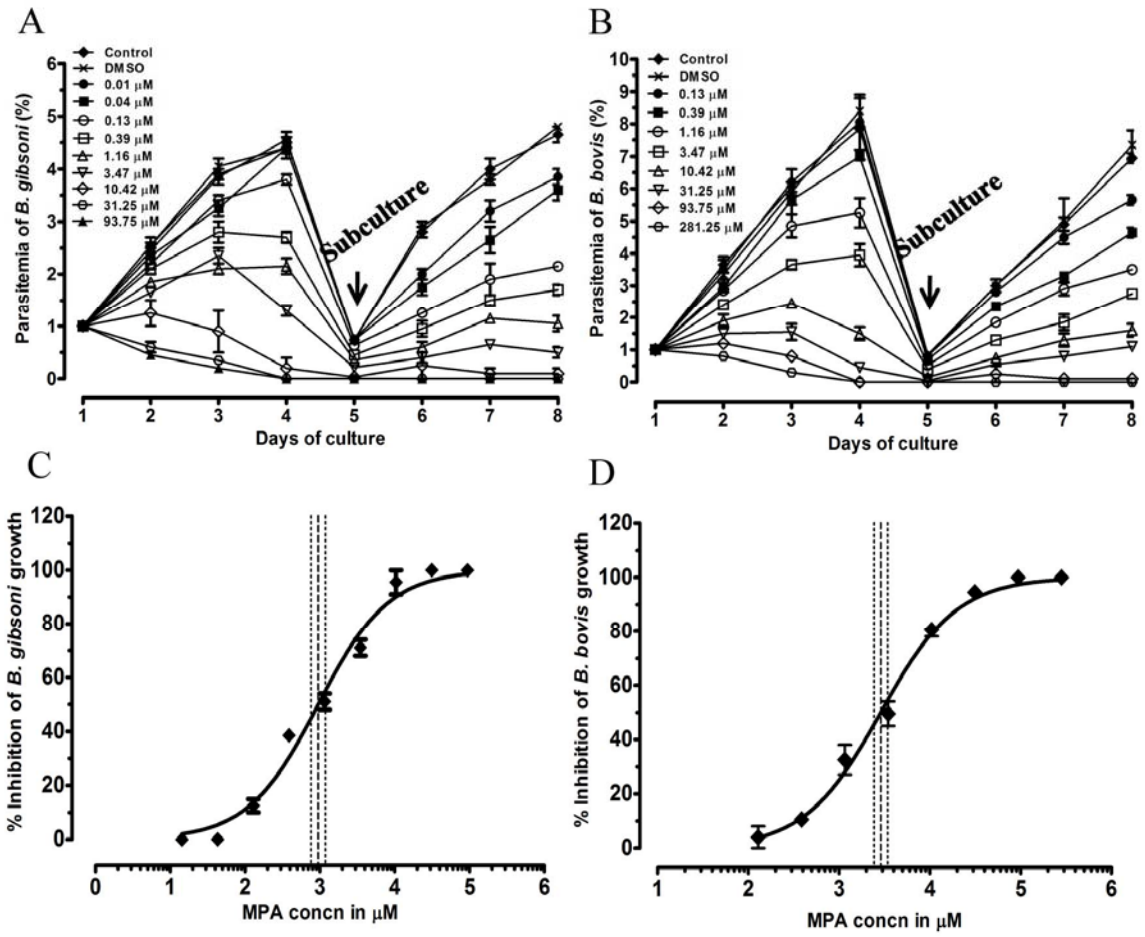


Fig. 17. Inhibition of *B. gibsoni* and *B. bovis* growths *in vitro*. (A) and (B) Inhibitions of *B. gibsoni* and *B. bovis* growths, respectively, *in vitro*. (C) and (D) represents IC_{50}s of MPA after inhibitions of *B. gibsoni* and *B. bovis* growths at day 5, respectively.

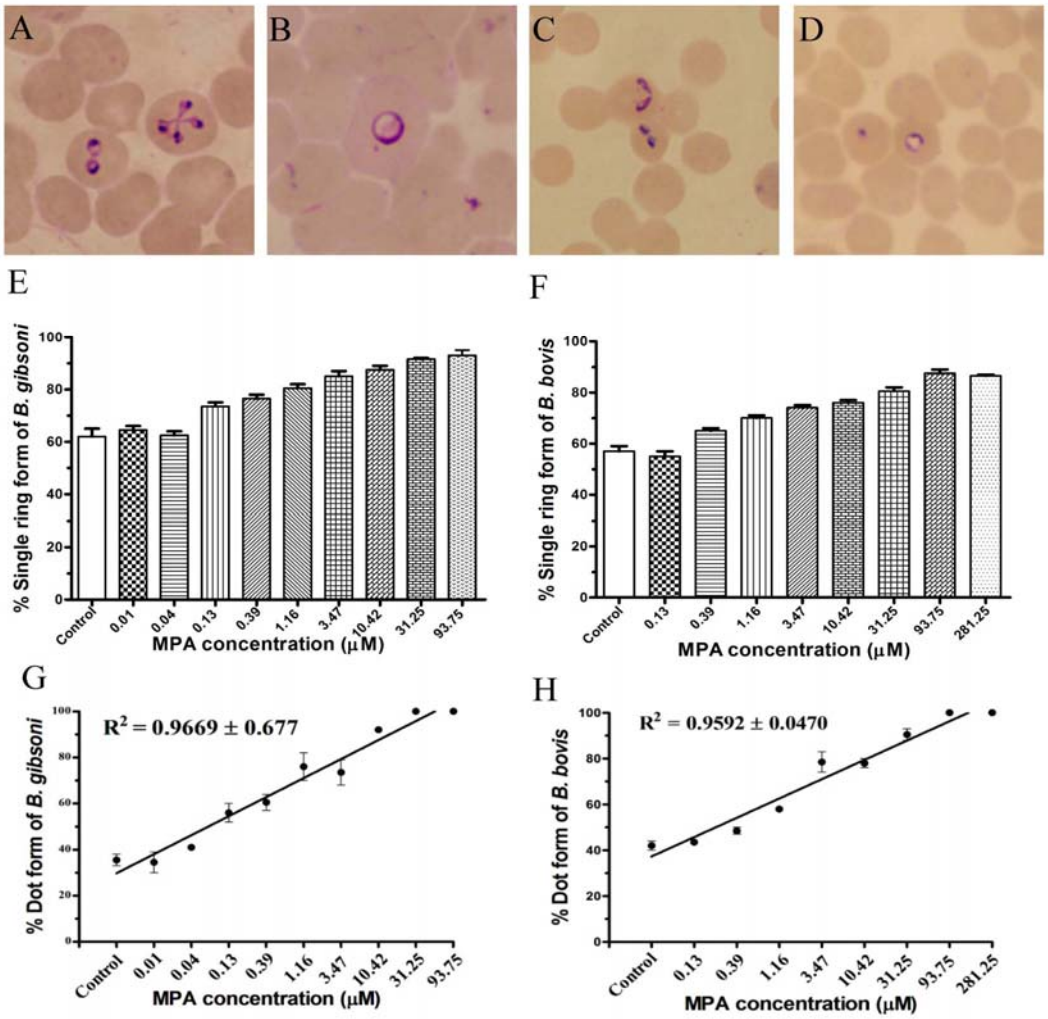


Fig. 18. Morphological changes of *Babesia* parasites after treatment with MPA. (A) Giemsa-stained thin blood smears of *B. gibsoni* in untreated and (B) treated *in vitro* cultures with 1.16 μM MPA. (C) Giemsa-stained thin blood smears of *B. bovis* in untreated and (D) treated *in vitro* cultures with 3.47 μM MPA. (E) Percentage of single ring form parasites of *B. gibsoni,* and *B. bovis* after the inhibition assay. The correlation between the percentage of dot forms parasites and concentration of MPA during inhibition for *B. gibsoni* (G) and *B. bovis* (H).

Table 3. Expressed sequence tags associated with purine metabolism of *B. gibsoni*.

Enzyme	EC no.	GenBank accession number	Homologue (Reference)	E-value
Purine nucleoside phosphorylase	2.4.2.1	JK784394 JK784395	*T. annulata* (XP_952280.1)	3e-08
Adenylate kinase 2	2.7.4.3	JK784396 JK784397	*B. bovis* (XP_001609458.1)	2e-66
Adenylosuccinate lyase	4.3.2.2	JK784398	*B. bovis* (XP_001611211.1)	7e-60
Inosine 5'-monophosphate dehydrogenase	1.1.1.205	JK784399	*B. bovis* (XP_001610614.1)	8e-22
Guanylate kinase	2.7.4.8	JK784400	*B. bovis* (XP_001610134.1)	3e-67
Guanosine monophosphate reductase	1.7.1.7	JK784401 JK784402	*B. bovis* (XP_001610485.1)	2e-74
Guanosine monophosphate synthase	6.3.4.1	JK784403 JK784404	*B. bovis* (XP_001610833.1)	1e-56
DNA-directed RNA polymerase II	2.7.7.6	JK784405 JK784406	*B. bovis* (XP_001610446.1)	0.0
Nucleoside diphosphate kinase	2.7.4.6	JK784407 JK784408	*T. parva* (XP_764988.1)	2e-86
Nucleoside monophosphate kinase	2.7.4.4	JK784409	*B. bovis* (XP_001610732.1)	1e-34

Chapter 4

Evaluation of inosine 5'-monophosphate dehydrogenase-targeted drugs against *Babesia* parasites

4-1. Introduction

In chapter 3, my study has validated that the BgIMPDH enzyme may be a molecular drug target of *Babessia gibsoni* and could be exploited for further drugs screening and development. Therefore, the study of BgIMPDH enzyme supplied a platform to detect more effective and specific ihibitors for the treatment of *Babesia* infections.

In this context, the goal of this study was to determine whether the others IMPDH inhibitors inhibit catalytic activity of a recombinant *B. gibsoni* IMPDH (rBgIMPDH) as well as growths of parasites *in vitro* to deveplot the drugs of *Babesia* sepieces. Mycophenolate mofetil (MMF), which is a prodrug of MPA and mizoribine (MZB), an imidazole nucleoside, are known to inhibit guanine nucleoside synthesis and thus stop DNA synthesis (Goldstein *et al.*, 1999; Morath *et al.*, 2003). Ribavirin (RBV) a ribosyl purine analogue kills viruses by inhibiting IMPDH enzyme (Goldstein *et al.*, 1999) whereas indole fragments including 7-nitroindole (7-NID) are known to inhibit type II IMPDH enzyme (Beevers *et al.*, 2006). Here, I hypothesize that MMF, MZB, RBV and 7-NID may also target IMPDH enzyme in the parasites.

In this study, a 2D structure of the compounds while docked with a modeled IMPDH of *B.*

gibsoni (BgIMPDH) has been reported. The study also revealed that these compounds inhibit the activity of rBgIMPDH enzyme similarly to the growth inhibitions o f *B. bovis* and B. *gibsoni* replication *in vitro*. The inhibition of *B. microti* growth in mice by MPA was determined.

4-2. Materials and methods

Experimental animals. Thirty five female 6-week-old ICR mice (CLEA, Japan), and 2 Beagle dogs (Chugai, Japan) were housed in a P2 facility at the National Research Center for Protozoan Diseases (NRCPD) of Obihiro University of Agriculture and Veterinary Medicine. In addition, 2 healthy cows were used to provide bovine erythrocytes for the *B. bovis* culture *in vitro*.

Cultivation of *B. gibsoni*, *B. bovis*, and *B. microti*. *B. microti* Munich strain was maintained in mice by intraperitoneal (i.p.) passage as previously described (Igarashi *et al.*, 1999). Briefly, mice were infected with *B. microti* by i.p inoculations with 1×10^7 of parasitized erythrocytes (pRBCs). Parasitemia was monitored by microscopic examining of Giemsa-stained blood smears. To maintain the parasite, pRBCs were serially passed in non-infected mice. Stock cultures of *B. gibsoni* Oita and *B. bovis Texas strains* were grown in 24-well culture plates (Nunc, Denmark) in a humidified CO_2 incubator (BioLab) set at 37℃ in the presence of 5% CO_2. For *B. gibsoni*, the culture mixture contained dog erythrocytes at 5% packed cell volume (PCV), 20% dog serum and RPMI 1640 medium having 2.05 mM L-glutamine, 25 mM hepes buffer, 23.8 mM $NaHCO_3$, 0.91 mM sodium pyruvate, 100

units/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen, USA). The stock culture of *B. bovis* was grown in bovine erythrocytes at 5% PCV suspended in GIT medium (WAKO, Osaka, Japan) in the CO_2 incubator as described above. The culture media were changed daily and the parasites were sub-cultured in fresh erythrocytes at 72 h regular intervals. The parasites stocks were store were stored in liquid nitrogen when not in use.

Homology modeling and molecular docking of BgIMPDH. A BgIMPDH cDNA was isolated from an expressed sequence tag (EST) database available in my laboratory, sequenced with an automated sequencer (ABI Prism 3100 genetic analyzer) and then submitted to GenBank (Accession No. JQ781073). Homology modeling and docking was done with a translated BgIMPDH peptide using Accelrys Discovery Studio V2.5 (Accelrys Software Inc., USA). At first, the homologue search was done using the ExPASy website (http://swissmodel.expasy.org) and the template with the highest homology, 1jr1_a (inosine 5'-monophosphate dehydrogenase 2, 2.6 A˚, length 436 aa, 45% identity and 59% positive) was selected for modeling (Fig. 24). The final 2D model was generated by MODELER, the same program that was used to perform automated protein homology and loop modeling. IMP and NAD^+ were drawn in the ChemWindow 6.0 (Bio-Rad Laboratories, Sadtler Division, Grand Junction), converted to stereochemically correct configuration and then optimized by Dreiding-like force field in the Discovery Studio. The enzyme-inhibitors interaction was determined by a LigandFit/LigandScore, which is an automated tool for protein small molecule docking and scoring of protein with small molecule.

Cloning and expression of BgIMPDH gene. The cloning and expression of

rBgIMPDH was performed as described in chapter 3.

Assay of rBgIMPDH and inhibition of enzyme activity. Assay of rBgIMPDH enzymatic activity was done by measuring an absorbance at 340 nm at 25°C using Beckman UV-800 visible spectrophotometer. The assay buffer for IMPDH contained 50 mM Tris-HCL (pH 8.0), 100 mM KCl, 1 mM DTT, and various concentrations of IMP and NAD^+. For the inhibition assays, MMF, MZB, RBV, and 7-NID were pre-incubated with the enzyme dissolved in assay buffer and the assay started by addition of IMP while fixing the concentrations of IMP and NAD^+ at 50 and 500 μMs, respectively. The concentration ranges for rBgIMPDH inhibition assays were as follows; 0.01 μM to 30 μM for MMF, 0.05 μM to 99.9 μM for MZB, 0.5 μM to 666.7 μM for RBV, and 0.4 μM to 900 μM for 7-NID. To determine IC_{50}s, nonlinear curve-fitting plots of the percent inhibitions against the various inhibitor concentrations were fitted using Prism ver5.02 (GraphPad, USA).

Growth inhibition of *B. gibsoni* and *B. bovis in vitro*. *B. gibsoni* and *B. bovis* were cultured in individual wells of a 96-well plate in the presence of MMF (Roche, Japan), MZB, RBV, and 7-NID (Sigma Aldrich, USA) in a humidified incubator set at 37℃ with 5% carbon dioxide and 5% oxygen. MMF was dissolved in DMSO and then added to the parasites cultures at concentrations ranging from 0.016 μM to 25 μM for *B. gibsoni* and 0.2 μM to 300 μM for *B. bovis*. MZB was dissolved in the respective culture media and then added to the parasites cultures at concentrations ranging from 0.05 μM to 11.1 μM for *B. gibsoni* and 1.2 μM to 900 μM for *B. bovis*. RBV was also dissolved in the respective culture media and then used for the inhibition assays at concentrations ranging from 39 μM to 666.7 μM for *B.*

gibsoni and 263.4 μM to 2000 μM for *B. bovis*. 7-NID was dissolved in DMSO and inhibitory assay done at concentrations ranging from 0.4 μM to 900 μM for *B. gibsoni* and 1.2 μM to 900 for *B. bovis*. The compounds were each tested in parallel in an individual 96-well plate using DMSO, RPMI or GIT medium as negative internal control. The parasites were grown for 3 days and then sub-cultured in non-treated medium for another 3 days. The beginning parasitemias was 1%. Percent of parasitemia of both *Babesia* species were determined by microscopic examination of Giemsa stained culture smears. The dynamics of erythrocytes-morphology was also examined.

Growth inhibition of *B. microti* in mice. Thirty five female 6-week-old mice were inoculated with erythrocytes infected with *B. microti* Munich strain (at a dose of 1×10^7) initially obtained from a mouse infected with *B. microti* and having a parasitemia of 33.7%. The infected mice were divided into 11 groups consisting of 2 control groups, and 9 treatment groups with each group having 3 mice. On the 4^{th} day after infection with *B. microti*, mice in each of the 9 groups were injected i.p. with MPA (MMF metabolite) at dosage rates of 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 mg/kg body weight, respectively. For the control mice, DMSO was administered i.p. as indicated above. The parasitemia was monitored by microscopic examination of Giemsa-stained thin blood films at two days interval. Ten micro-liters of blood were collected from all the mice for hematocrit examination.

Statistical analysis. The 50% inhibition of the enzyme activity (IC_{50}) was determined by nonlinear curve-fitting plots of the percent inhibitions against the various inhibitor

concentrations using Prism ver5.02 (GraphPad, USA). The comparison of data obtained from cells treated with different drugs was analyzed by ANOVA (one way). Differences were considered statistically significant when $P < 0.05$.

4-3. Results

Homology modeling and molecular docking of BgIMPDH. The predicted BgIMPDH polypeptide consisted of 501 amino acid residues with IMP binding domains having Ser313, lle314, Cyc315, Asp350, Gly352, Gly373, Gly374, Tyr397, Gly399, Met400, Gly401, Gln431, and Gly432. These active site residues were docked with MMF, MZB, and RVB. The 2D structure modeling and docking showed that these inhibitors interact with the BgIMPDH catalytic sites by forming hydrogen bonds through some residues outside the active site also interacted with some inhibitors. The phenolic carboxyl group of MMF is an acceptor of H bond donated by the Arg400, and the Arg418 donates H-bond to the oxygen atom of morpholino moiety. Moreover, this inhibitor interacted with the enzyme-XMP intermediate to form a complex via covalent chemical bond or pi-bond (Fig. 20). For MZB, the imidazole ring forms a network of H-bonds with Ser313, Ile353, Gly373 and Tyr397 of the enzyme (Fig. 20B). RBV formed a network of H-bonds with Ile353, Gly352, Gly310, Gly373 and Asp 350 via hydroxyl and carbonyl groups (Fig. 20C). In this case, Asp350 was the only H-bond acceptor while the other residues were H-bond donors.

Inhibition of rBgIMPDH enzymatic activity. The inhibitory effect of MMF, MZB, RBV, and 7-NID on the rBgIMPDH catalytic activity was determinded. MMF was found to

the most potent inhibitor with an IC_{50} of 2.58 ± 1.32 μM while RBV was the least potent revealing an IC_{50} of 532.1 ± 142 μM. The IC_{50}s of MZB and 7-NID were 14.12 ± 1.25 and 75.42 ± 142 μMs, respectively (Fig. 21A-D). Negative control consisting of assay buffer only did not reveal inhibitory effect. The sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the recombinant enzyme is shown in Figure 25B.

Growth inhibition of *B. gibsoni* and *B. bovis in vitro*. The inhibition of rBgIMPDH activity by MMF, MZB, RBV, and 7-NID convinced us that these compounds could also inhibit the growth of the parasites. Therefore, I did growth inhibition assays of *B. gibsoni* and *B. bovis in vitro*. Indeed, these compounds inhibited the growths of both *B. gibsoni* and *B. bovis in vitro* with more potent inhibitions observed for *B. gibsoni* growth than for *B. bovis* (Table 4 and Fig. 25). In both cases, MMF was the most potent inhibitor with an IC_{50} of 0.13 ± 0.05 μM for *B. gibsoni* and 0.97 ± 0.49 μM for *B. bovis*. RBV was the least potent revealing IC_{50}s of 74.03 ± 25.42 and 486.30 ± 154.10 μMs for *B. gibsoni* and *B. bovis,* respectively. The IC_{50}s values of MZB and 7-NID were between those of RBV and MMF. The inhibition of growths of *Babesia* parasites *in vitro* by the compounds mirrored that of the rBgIMPDH activity. Smears made from parasite cultures with the inhibitor revealed predominantly single cells as opposed to the dividing and single parasites seen in nontreated parasites. The difference was obvious for *B. gibsoni* at inhibitor concentrations near the IC_{50}s and not obvious for *B. bovis*. However, at concentrations near IC_{90}s remarkable number of single parasites was seen for *B. bovis* (Fig. 22A and B).

Growth inhibition of *B. microti in vivo*. The inhibitory effect of MPA on the growth of

B. microti was determined in mice. MPA inhibited the growth and multiplication of *B. microti* in mice albeit at modest level. Blood smears made from mice treated with MPA revealed predominantly single *Babesia* cells as opposed to those from mice treated with DMSO only. The medium effective dose (ED_{50}) for this inhibition was 44.15 ± 12.53 mg/Kg. At relatively higher concentrations of MPA, the existing piroplasms were mainly in single forms as opposed to dividing forms. Generally, hematocrit values improved in mice treated with MPA as opposed to the infected mice treated with DMSO only. The hematocrit for non-treated infected mice was 17% while those of MPA treated-mice, at a maximum dose of 500 mg/Kg body weight, was 37%. Therefore, MPA appears to alleviate symptoms associated with anemia even though it did not completely inhibit multiplication of the parasite in mice (Fig. 23A-E).

4-4. Discussion

Babesiosis is caused by a protozoan that infects erythrocytes. Although several different species of *Babesia* infect humans, *B. microti* is the most common cause of infection in the United States. *B. bovis* infect cattle causing massive economic losses in livestock industry worldwide, while *B. gibsoni* causes canine babesiosis worldwide, sometimes leading to deaths (Homer *et al.*, 2000a; Krause *et al.*, 2008; Suarez *et al.*, 2011). A starting point for the development of antimetabolic chemotherapeutic agents involves drug target identification and validation to treat this disease. The IMPDHs have been studied as molecular drug target in other apicomplexans, especially *Plasmodium*, *Cryptosporidium* and *Toxoplasma* (Hariharan *et*

al., 1999; Sullivan *et al.*, 2005), but not in *Babesia* species The IMPDH enzyme of purine pathway is an important target for the development of chemotherapeutic agents (Braun-Sand *et al.*, 2010). Specific inhibitors targeting IMPDH are used clinically as antiviral, anticancer and immunosuppressive agents (Braun-Sand *et al.*, 2010), and experimentally as antiparasitic agents (Maurya *et al.*, 2009). In my case, I hypothesized that MMF, MZB, RBV and 7-NID could as well target *Babesia* IMPDH enzyme and inhibit the growths of the parasites. MMF was the most potent inhibitor of both rBgIMPDH activity and the *Babesia* growths *in vitro* suggesting that this compound inhibits the replication of *Babesia* parasites by targeting the enzyme.

MMF is a known inhibitor of IMPDH enzyme and this inhibition occurs after the compound is converted to MPA; the ultimate active moiety (Villarroel *et al.*, 2009). However, in this study, MMF inhibited both rBgIMPDH activity as well as growths of *B. gibsoni* and *B. bovis in vitro*. This was surprising because MMF is expected to be only active *in vivo,* where it is converted to the active MPA by undergoing esterase hydrolysis (Ransom *et al.*, 1995; Fujiyama *et al.*, 2010). I think that MMF may have inhibited *Babesia* IMPDH directly without being converted to MPA. In fact, an *in silico* model showed that the phenolic carbonyl group of MMF and the oxygen atom of morpholino moiety appeared to interact with some amino acid residues of the target enzyme. The inhibitor formed a complex with the enzyme-XMP intermediate via covalent chemical bond or pi-bond indicating an uncompetitive inhibition. Another theory for this inhibition by the prodrug is hydrolysis to its active moiety *in vitro*; however, this argument appears remote because the hydrolysis should

be rapid enough to release optimal concentration of the active compound and thus resulting in the potent inhibition observed *in vitro.*

MZB competitively inhibit activity of IMPDHs (Halloran, 1996) similarly to the inhibition of rBgIMPDH activity and *Babesia* growths *in vitro*. RBV, which was the least potent, also inhibited both rBgIMPDH activity and the parasites growths *in vitro* indicating the same mechanism of inhibition with MZB. RVB like MZB bind at the substrate site of IMPDH and thus competitively inhibit the enzyme activity (Reyes, 2001). The docking model suggests that Ser313, Gly373 and Tyr397, which are one of the amino acid residues that bind the substrate, also form H-bonding with MZB. For RBV, the Gly352, Asp350 and Gly373 that binds the substrate also interacted with the compound via H-bonding interacts. Therefore, it appears that MZB and RBV competitively inhibited the *Babesia* IMPDH activity and inhibited the growths of parasites.

It has been reported that MZB and RVB are activated to the 5'-monophosphate forms by adenosine kinase (AK) and then inhibit IMPDH enzyme (Willis *et al.*, 1978). But the enzyme assay solution did not contain adenosine kinase indicating that these compounds may have inhibited the recombinant enzyme without necessarily being converted to the 5'-monophosphate forms. However, it is possible that this AK enzyme mediated activation of MZB and RVB can occur in *Babesia* cultures *in vitro* since these parasites are known to have the AK enzyme (Carret *et al.*, 1999a; Goldstein *et al.*, 1999). 7-NID modestly inhibited rBgIMDH activity indicating that the low molecular mass indole fragment is an inhibitor of *Babesia* IMPDH. This indole fragment has been used to synthesize other indole derivates that

inhibit IMPDH (Beevers *et al.*, 2006). Generally, these compounds appeared to potently inhibit the growths of the *Babesia* parasites than the activity of the rBgIMPDH enzyme indicating a slight difference in the way IMPDH interacts with inhibitor in assay solution and in the parasites.

MMF and MZB, which are both immunosuppressant (Beevers *et al.*, 2006), also inhibited *Babesia* growth *in vitro* indicating that the drugs targeted native IMPDH in *Babesia* cells and thus deplete synthesis of guanine pool required for DNA synthesis and cell growth. It also appears that *Babesia* cells could not scavenge purines via a separate salvage pathway; otherwise this inhibition could have been circumvented. Therefore, I think that synthesis of purine in *Babesia* is crucial for supplying guanine nucleotides needed for DNA synthesis and cell multiplication. In this case, *Babesia* cells could only grow but fail to proliferate to daughter cells as seen in treated parasite cultures, which had predominantly single parasite and not in untreated ones with both single cells and dividing merozoites. RVB, an antiviral agent also showed some anti-*Babesia* activity *in vitro* albeit less potently than the other three compounds (Goldstein *et al.*, 1999). RBV inhibits RNA viruses by incorporating into RNA a base analogue and thus inducing lethal hypermutation (Crotty *et al.*, 2002). For DNA viruses, the RVB monophosphate form inhibits cellular IMPDH, thereby depleting intracellular pools of GTP. Perhaps the RVB killed *Babesia* cells by inhibiting the native IMPDH in the parasites. A similar mechanism of inhibition could as well be the reason for growth inhibition of *Babesia* by 7-NID since non-oxazole containing indole fragments has been shown to inhibit IMPDH (Beevers *et al.*, 2006).

In conclusion, this study shows for the first time that mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole inhibit the growths of *B. gibsoni* and *B. bovis in vitro*. The molecular mechanisms by which these chemical compounds may inhibit BgIMPDH activity is demonstrated *in silico*. The study also reveal that these compounds inhibit the activity of rBgIMPDH enzyme similarly to the growth inhibitions of *B. bovis* and *B. gibsoni* growths *in vitro*, thus making the compounds potential anti-*Babesia* drugs. The inhibition pattern suggests that purine pathway is functional in *Babesia* parasites studied. Therefore, IMPDH of *Babesia* species may be a molecular target of MMF, MZB, RBV and 7-NID, and thus could be exploited as a basis for antibabesia drug design and development. Nevertheless, more studies are needed to further confirm the molecular mechanisms of action of these compounds.

4-5. Summary

Babesiosis is a serious disease infecting both animals and humans, globally. Nevertheless, the development of new drugs for babesiosis treatment has been slow. Therefore, I validated IMPDH in *Babesia* parasite as a molecular target of mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole. A three-dimensional model of the BgIMPDH was generated, and validated using structure-checking programs and protein docking studies. Molecular docking of these compounds with BgIMPDH polypeptide suggests that they may interact with the enzymatic reactive sites by forming hydrogen bonds *in silico* model. Mycophenolate mofetil was the most potent inhibitor [50% inhibition concentration (IC_{50}) = 2.58 ± 1.32 μM] of the

recombinant BgIMPDH activity while ribavirin was the least potent [IC_{50} = 532.1 ± 142 μM]. The IC_{50}s of mizoribine and 7-nitroindole were 14.12 ± 1.25 μM and 75.42 ± 142 μM, respectively. Mycophenolate mofetil was the most potent inhibitor of the growths of *B. gibsoni* [IC_{50}s = 0.13 ± 0.05 μM] and *B. bovis* [IC_{50} = 0.97 ± 0.49 μM] *in vitro*. Ribavirin was the least potent with IC_{50}s of 74.03 ± 25.42 and 486.30 ± 154.10 μMs for *B. gibsoni* and *B. bovis*, respectively. Mycophenolic acid, a metabolite of MMF, caused a modest inhibition of *B. microti* growth in mice [ED_{50} = 44.15 ± 12.53 mg/kg] with noticeable improvement in hematological parameters of the infected mice. These results indicate that the inhibitions of recombinant BgIMPDH activity resembled those of the parasites growths *in vitro*. Therefore, I provide a basis for development of new anti-*Babesia* drugs targeting key metabolic pathways of the parasites.

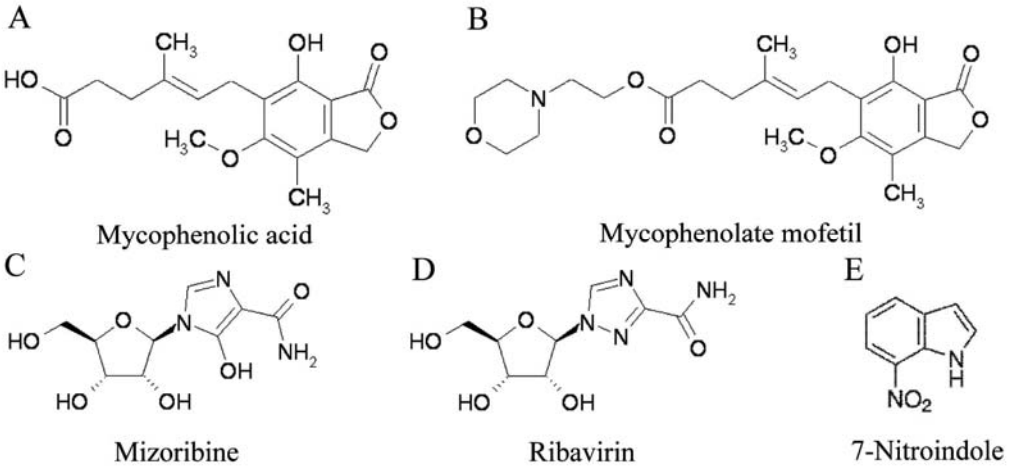


Fig. 19. Chemical structures of (A) MPA, (B) MMF, (C) MZB, (D) RBV, and (E) 7-NID.

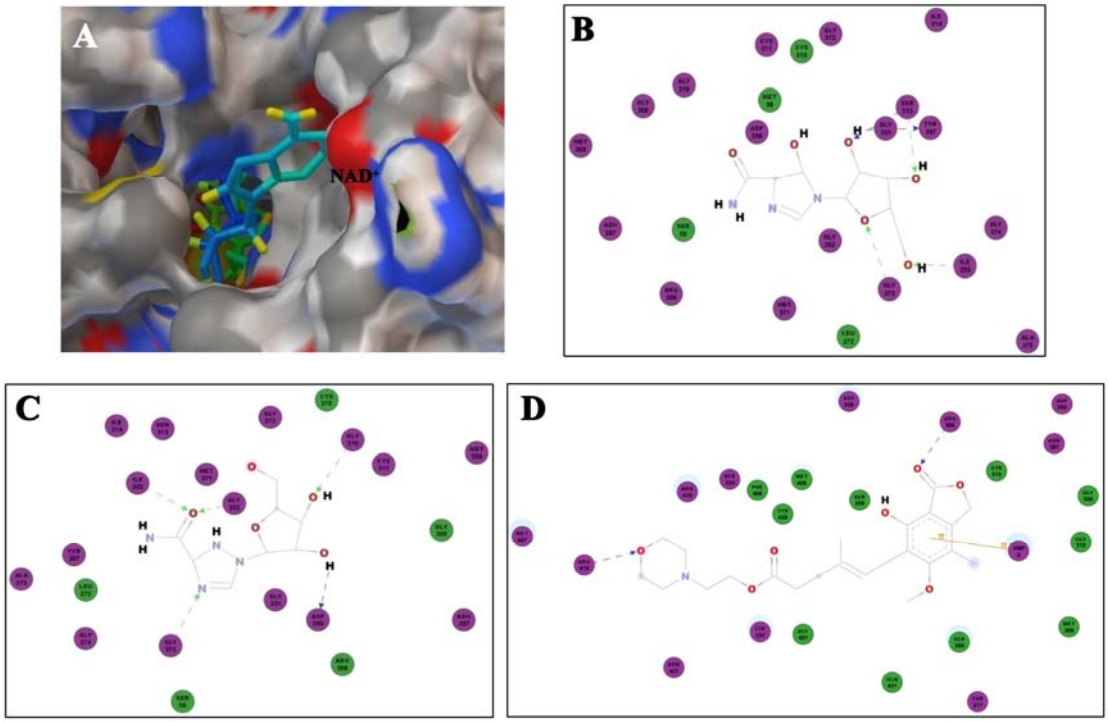


Fig. 20. The modelled structure and the predicted binding site of BgIMPDH. (A) Schematic representation of the binding mode of Mizoribine in the binding site. (B) Schematic representation of the binding mode of Ribavirin in the binding site. (C) Schematic representation of the binding mode of Mycophenolate mofetil in the binding site. The hydrophobic residues are in green and charged residues in purple. Hydrogen-bond interactions represented by a dashed line with an arrow head directed towards the electron donor atom. Cation-π interactions show as line with symbols indicating the interaction.

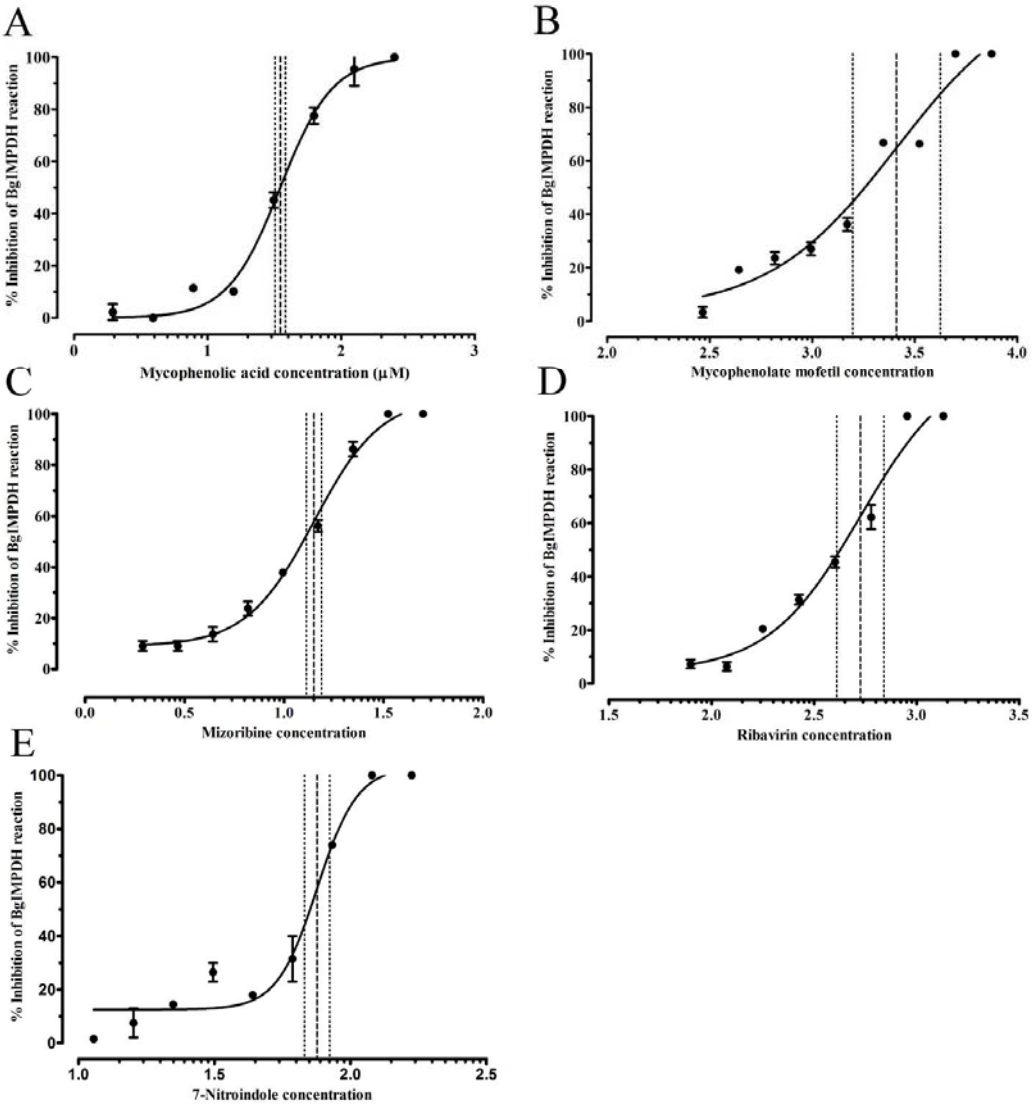


Fig. 21. Effect of five inhibitors on the enzymatic reaction of rBgIMPDH. The inhibition curve of (A) MPA, (B) MMF, (C) MZB, (D) RBV, and (E) 7-NID.

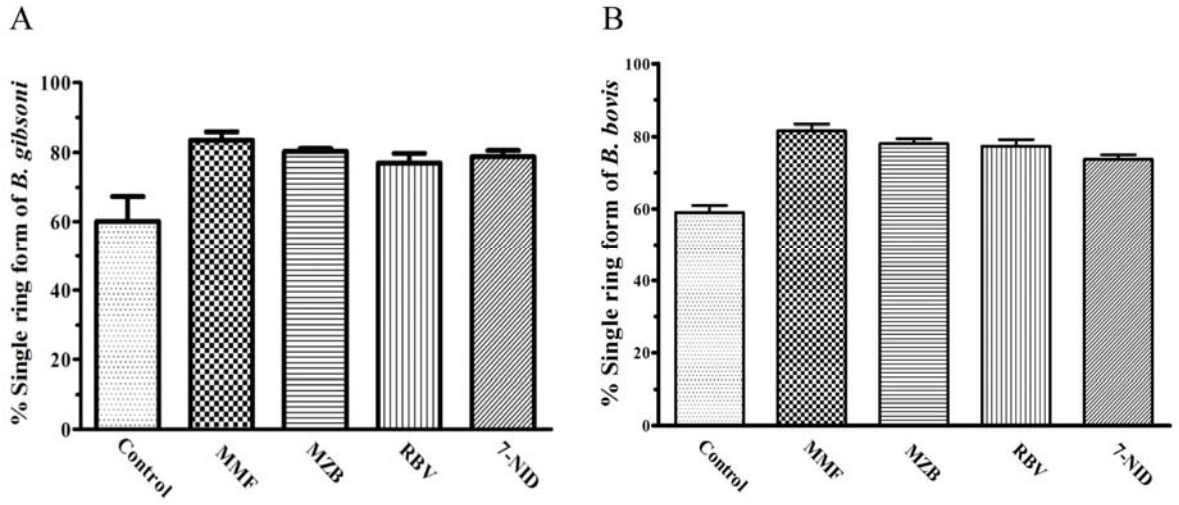


Fig. 22. Percentage of single or ring form parasites after *in vitro* treatments with the compounds. (A) *B. gibsoni* after 3 days of culture. The percentage of single ring form was determined at a dose close to the $IC_{50S.}$ (B) The percentage of single ring form of *B. bovis* was determined at a dose close to the $IC_{90S.}$ In boths control included untreated culture.

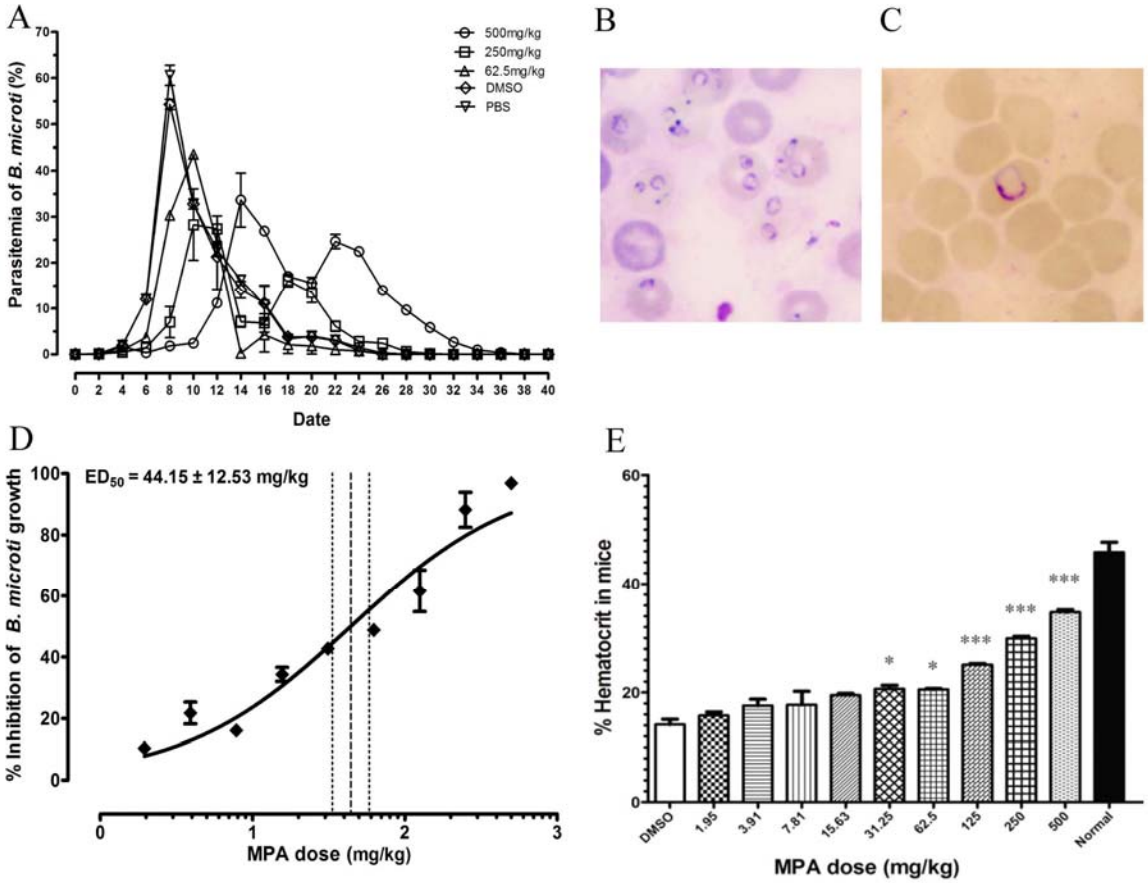


Fig. 23. Inhibition of *Babesia* microti growths *in vivo* and morphological changes after treatment with MPA. (A) Inhibition of *B. microti* growth *in vivo*. (B) Giemsa-stained thin blood smears of *B. microti* in untreated and (C) treated *in vivo*. (D) is the ED_{50} after inhibition of *B. microti* growth in mice by MPA. (E) Effect of different concentrations of MPA on hematocrit value in the infected *B. microti* mice.

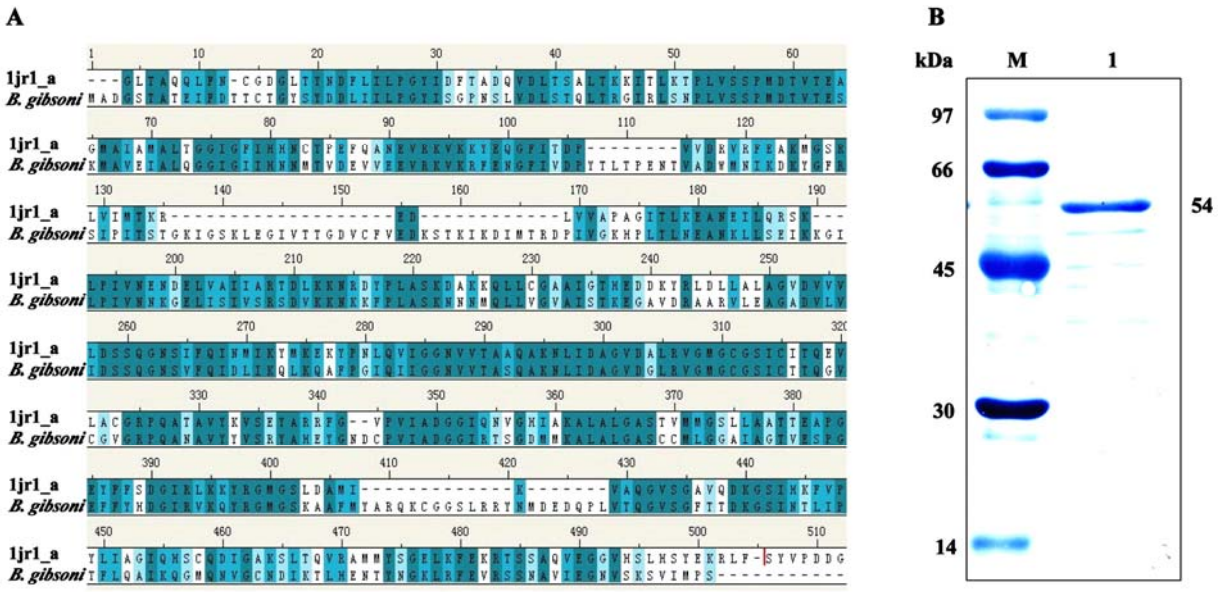


Fig. 24. (A) *B. gibsoni* IMPDH and the homology model 1jr1_a amino acid sequences were aligned using the Accelrys Discovery Studio V2.5 program. (B) 12% SDS-PAGE of the rBgIMPDH showing a specific band of 54 kDa.

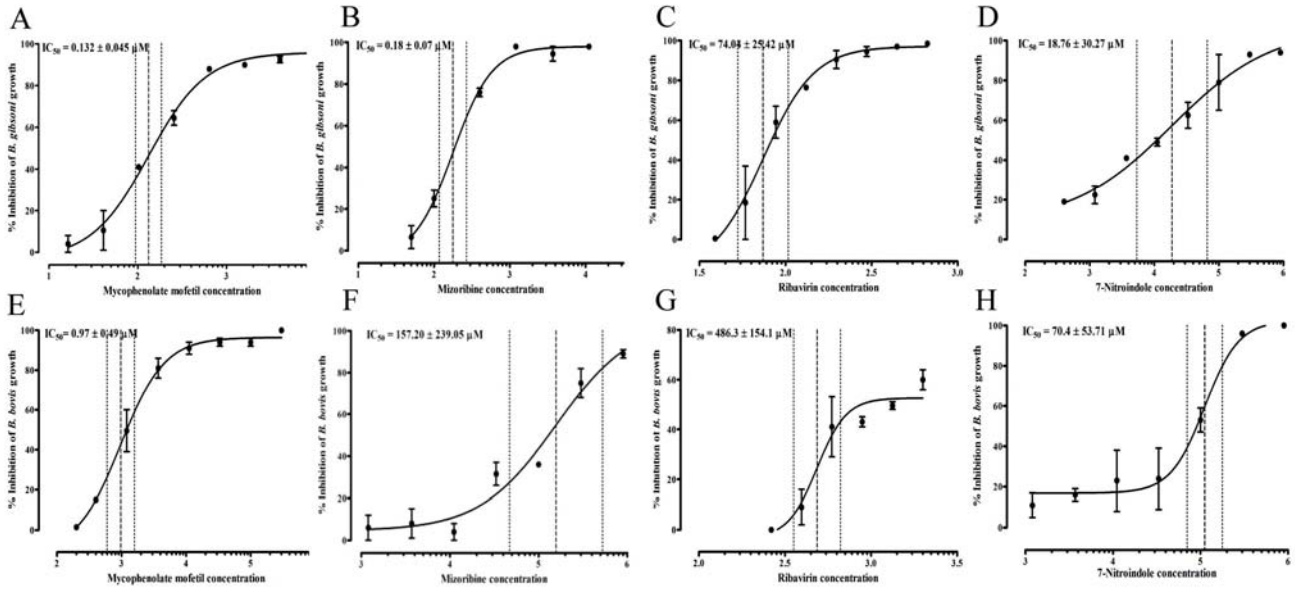


Fig. 25. Effect of four inhibitors for *B. gibsoni* and *B. bovis in vitro.* The inhibition curve of (A) mycophenolate mofetil, (B) mizoribine, (C) ribavirin, and (D) 7-nitroindole for *B. gibsoni in vitro.* The inhibition curve of (E) mycophenolate mofetil, (F) mizoribine, (G) ribavirin, and (H) 7-nitroindole for *B. bovis in vitro.* The mean ± standard error of the mean was calculated for each parameter.

Table 4. Effect of four inhibitors for *B. gibsoni* and *B. bovis in vitro*.

Compounds	IC_{50}s (μM)	
	B. gibsoni	*B. bovis*
Mycophenolate mofetil	0.13 ± 0.05	0.97 ± 0.49
Mizoribine	0.18 ± 0.07	157.20 ± 239.05
Ribavirin	74.03 ± 25.42	486.30 ± 154.10
7-Nitroindole	18.76 ± 30.27	70.40 ± 53.71

General discussion

Canine babesiosis caused by *B. canis* and *B. gibsoni* is a clinically significant emerging vector-borne disease in many countries of the world. Clinically, both species cause remittent fever, progressive anemia, hemoglobinuria, and marked splenomegaly and hepatomegaly in dogs and, in some cases, the death of infected animals. For control of canine babesiosis, it is important to block the transmission routes for controlling the spread of infection. Therefore, the development of reliable and sensitive serological tests and effective drugs for *Babesia* infection has been essential for the control of *Babesia* infection.

The ELISA principles are widely applied for the demonstration of antibodies against a variety of infectious agents, including *B. canis canis.* Currently, ELISA of *B. canis* was developed basis on the soluble antigen, which was made by infected blood. However, this method is not applicable to large-scale production. It is difficult to obtain the native protein highly enriched for specific antigen of intracellular parasites, particularly due to the presence of host contaminating components, such as red blood fragments. These contaminants can result in a nonspecific reaction on standardization of immunological assays. As well as the infected dogs may have antibodies, those are unpredictably cross-reactive. Consequently, the development of sensitive and specific ELISA tests basis on the recombinant protein is quit necessary.

The main objectives of this study were to identify the novel antigens for developing the serodiagnostic methods of *B. canis canis* and subsequently to discover the potent drug target

and drugs for *Babesia* infection. I constructed a cDNA expression library of *B. canis canis*. The sera derived from *B. canis canis*-infected dogs were used for immunoscreening. Several cDNA sequences were isolated by the immunoscreening. Two immunodominant antigens named as BccSA1 and BccP33 were chosen and characterized.

In order to characterize two candidates, BccSA1 and BccP33, I used the bioinformatics parameters such as antigenicity plot, signal peptide presence, and hydrophobicity plot of predicted peptide as benchmark for preliminary screeing of putative antigenic peptides. Subsequently, I showed that both the translated polypeptide of BccSA1 and BccP33 have signal peptides, good antigenic indices, and low hydrophobicity, suggesting that both recombinant proteins could be prospective diagnostic candidates. In order to confirm whether these recombinant proteins are indeed antigenic, Western blot analysis was done and both recombinant proteins reacted to sera from dogs infected with *B. canis canis*. Moreover, those proteins induced high antibody levels in mice immunized with respective antigen and each native protein was identified in erythrocyte lysates infected with *B. canis canis* by Western blot analysis with anti-BccSA1 and anti-BccP33 mice sera. Based on this data, I confirmed that BccSA1 and BccP33 are endogenous proteins of *B. canis canis* and may be able to detect the antibodies induced by those antigens when *B. canis canis* infection happens.

Subsequently, ELISA and ICT with rBccSA1 and rBccP33 identified previously as an immunodominant antigen were performed to ascertain whether those antigens could be used for the serodiagnosis of *B. canis canis* infection. The results revealed that all antigens are specific for *B. canis canis* antibodies only, indicating that they could be potential antigens for

detection of antibodies against *B. canis canis* as observations consistent with other findings (Aboge *et al.*, 2007; Jia *et al.*, 2008). Finally, these antigens were applied to ELISA with field canine sera from EU. It could be very important to differentiate the dogs in carrier stage, particularly among the dogs which do not have medical history, because these dogs would be needed certain control to prevent spreading of this disease.

In chapter 3, I identified and validated IMPDH of *B. gibsoni* as a molecular target of anti-IMPDH to facilitate discovery of novel drugs for treatment of canine babesiosis. The purine pathway of parasitic protozoa is an attractive chemotherapeutic target because these parasites rely entirely on the pathway to meet their purine demands for nucleic acid synthesis. In this pathway, IMPDH is a rate-limiting step catalyzing the conversion of IMP to XMP with concomitant reduction of NAD^+ to NADH. The XMP, which is formed, is a precursor of guanine nucleotides required for biosynthesis of DNA, crucial for the survival of parasites (Hedstrom, 2009). IMPDH enzyme is susceptible to inhibition by MPA, which is a structural analogue of the cofactor NAD^+. Although many drug targets have been validated in other apicomplexans related to *Babesia* parasites, only two molecular drug targets have been studied in the genus *Babesia.* Thus, to understand the mechanism of inhibition of *B. gibsoni* proliferation by purine pathway, I used the recombinant DNA technology to identify and validate BgIMPDH as a molecular drug target. The gene encoding the BgIMPDH enzyme was discovered from the EST database of *B. gibsoni*. The use of EST strategy ensures that only mRNA transcripts lacking introns are used to generate the cDNA libraries. This approach made it possible to identify the BgIMPDH cDNA, and to express it in bacteria to obtain the

enzyme for subsequent drug inhibition assays.

The inhibitory effects of MPA on the enzymatic activity parallel their inhibition of the parasite's growth *in vitro* indicating that the purified rBgIMPDH enzyme could be used for preliminary screening of the available anti-IMPDH libraries. This facilitates the initial selection of potent anti-IMPDH for subsequent evaluation of their inhibition of the parasite proliferation *in vitro*. MPA is a potent inhibitor of both the rBgIMPDH enzyme and the parasite growth *in vitro*; hence, I would expect it to be a prospective compound for treatment of canine babesiosis. However, the narrow therapeutics is a drawback for its use in clinical therapeutics. Nonetheless, the other anti-IMPDH inhibitors such as mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole are less toxic to mammalian host and have been shown to have antimalarial activity.

Therefore, I proposed in chapter 4 that these relatively safe inhibitors could inhibit *Babesia* parasite in a manner similar to that of *Plasmodium* species, and make them candidate antibabesial drugs. Subsequently, I found that mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole inhibit the BgIMPDH as well as the parasite proliferation with mycophenolate mofetil having a more-potent inhibitory effect than others in both *B. gibsoni* and *B. bovis in vitro*. From these results, I can confirm the previous hypothesis that these inhibitors directly target IMPDH enzyme of *Babesia* species, and inhibit the parasite growth *in vitro*. Therefore, IMPDH of *Babesia* species may be a molecular target of mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole, and thus could be exploited as a basis for anti-*Babesia* drug design and development. These results indicate that the inhibitions of

recombinant BgIMPDH activity resembled those of the parasites growths *in vitro*. Therefore, I provide a basis for development of new anti-*Babesia* drugs targeting key metabolic pathways of the parasites.

In conclusion, I identified two recombinant antigens, BccSA1 and BccP33, for developing serodiagnostic methods to detect *B. canis canis* infection and showed that two antigens are specific to *B. canis canis* infection. Interestingly, ELISA and ICT with rBccSA1 appeared that it could be a marker for the chronic stage of *B. canis canis* infection. Furthermore, ELISA and ICT with rBccP33 has showed higher sensitivity than ELISA and ICT with rBccSA1. Therefore, ELISA with rBccP33 could be a promising diagnostic method. Subsequently, the isolation, cloning and expression of the *B. gibsoni* IMPDH gene to get an active 57 kDa purified rBgIMPDH enzyme. The inhibitory effect of MPA on the activity of rBgIMPDH enzyme and the growths of both *B. bovis* and *B. gibsoni in vitro and* and *B. microti in vivo* were demonstrated. Additionally, growth inhibition effects of mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole were evaluated in *Babesia* species. The effective growth inhibitions of parasites were observed in *B. gibsoni* and *B. bovis in vitro*. Therefore, the IMPDH is a molecular drug target of anti-IMPDH drugs for *Babesia* parasites.

General summary

The main objective of this study was to develop serological diagnostic method of canine babesiosis, and to characterize molecular drug target of parasites to facilitate drug discovery.

In chapter 1, I have identified and characterized a novel secreted antigen of *Babesia canis canis* by immunoscreening of the *B. canis canis* cDNA expression library and evaluated its potentiality for serodiagnosis. The results demonstrated that the rBccSA1 was a promising chronic serodiagnostic target to detect *B. canis canis* infection. In addition, the das-ELISA could detect circulating BccSA1 in the plasma of 5% parasitemia infection of *B. canis canis*. Overall, the BccSA1 is a promising serodiagnostic antigen for clinical detection and epidemiological survey.

In chapter 2, a novel gene encoding 33 kDa proteins from *B. canis canis* cDNA expression library was characterized and determined. I demonstrated that ELISA and ICT based on rBccP33 have reliable specificity and higher sensitivity than ELISA and ICT based on rBccSA1. This suggests that ELISA and ICT based on rBccP33 could be a promising serodiagnostic antigen for epidemiological survey and clinical diagnosis of both acute and chronic stages of *B. canis canis* infection to control canine babesiosis.

In chapter 3, I cloned and expressed a BgIMPDH cDNA in *E. coli*. I also determined the inhibitory effect of mycophenolic acid (MPA) on rBgIMPDH activity and the parasites growths *in vitro*. The K_m values of the rBgIMPDH were 8.18 ± 0.878 μM and 360.80 ± 43.41 μM for IMP and NAD^+, respectively. MPA inhibited the rBgIMPDH activity yielding a K_i value of 20.93 ± 1.83 μM with respect to NAD^+. For *Babesia* growths, the IC_{50}s were 0.95 ±

0.21 and 2.88 ± 0.49 μM for *B. gibsoni* and *B. bovis*, respectively. Therefore, my results suggest that MPA may inhibite the growth of *Babesia* parasites by targeting IMPDH enzyme of the purine pathway.

In chapter 4, I validated IMPDH in *Babesia* parasite as a molecular target of mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole. A three-dimensional model of the BgIMPDH was generated, and validated using structure-checking programs and protein docking studies. Molecular docking of these compounds with BgIMPDH polypeptide suggests that they may interact with the enzymatic reactive sites by forming hydrogen bonds *in silico* model. The study also reveal that these compounds inhibit the activity of rBgIMPDH enzyme similarly to the growth inhibitions of *B. bovis* and *B. gibsoni* growths *in vitro*; thus making the compounds potential anti-*Babesia* drugs. The inhibition pattern suggests that purine pathway is functional in *Babesia* parasites studied. Therefore, IMPDH of *Babesia* species may be a molecular target of MMF, MZB, RBV and 7-NID, and thus could be exploited as a basis for anti-babesia drug design and development. These results indicate that the inhibitions of rBgIMPDH activity resembled those of the parasites growths *in vitro*. Therefore, I provide a basis for development of new anti-*Babesia* drugs targeting key metabolic pathways of the parasites.

In conclusion, the BccSA1 and BccP33 are novel immunodominant antigens that could be used for serodiagnosis of *B. canis canis* infection. On the other hand, the BgIMPDH enzyme is a molecular drug target of anti-*B. gibsoni* IMPDH drugs. Mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole target the BgIMPDH enzyme and inhibit growth of

the parasite.

Acknowledgments

This research work was carried out at National Research Center for Protozoan Diseases (NRCPD), Obihiro University of Agriculture and Veterinary Medicine (Obihiro Univ. Agric. Vet. Med.), and supported by a grant from the Global COE Program and a Grant-in-Aid for Scientific Research, both from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This research work was also supported by a doctoral fellowship from the Japanese Society for the Promotion of Science (JSPS).

I am indebted to my supervisor, Prof. Xuenan Xuan, for his intellectual guidance, constructive criticisms, invaluable suggestions, patiently revising this dissertation, and tireless supports during all the period of my study.

I sincerely thank Assoc. Prof. Yoshifumi Nishikawa, Prof. Ikuo Igarashi, Prof. Noboru Inoue, and Assoc. Prof. Makoto Igarashi, for their constructive criticisms, invaluable suggestions, and revising this dissertation.

I also would like to acknowledge Prof. Hiroshi Suzuki, Prof. Shin-ichiro Kawazu, Prof. Naoaki Yokoyama, and Assoc. Prof. Shinya Fukumoto, for their valuable advice, critical suggestions and comments.

I extend my gratitude to Dr. Yuzi Luo, Dr. Gabriel Oluga Aboge, and Dr. M. Alaa Terkawi, for their kind assistance, valuable suggestions and comments. I would like to thank all of the former and present staff and students of NRCPD for their kind assistance.

Finally, I would like to express my special gratitude to my family for their devoted supports.

References

Aboge, G.O., Jia, H., Kuriki, K., Zhou, J., Nishikawa, Y., Igarashi, I., Fujisaki, K., Suzuki, H., Xuan, X., 2007. Molecular characterization of a novel 32-kDa merozoite antigen of *Babesia gibsoni* with a better diagnostic performance by enzyme-liked immunosorbent assay. Parasitology 134, 1185-1194.

Aboge, G.O., Jia, H., Terkawi, M.A., Goo, Y.K., Nishikawa, Y., Sunaga, F., Namikawa, K., Tsuji, N., Igarashi, I., Suzuki, H., Fujisaki, K., Xuan, X., 2008. 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. 52, 4072-4080.

Anderson, J.F., Magnarelli, L.A., Sulzer, A.J., 1980. Canine babesiosis: indirect fluorescent antibody test for a North American isolate of *Babesia gibsoni*. Am. J. Vet. Res. 41, 2102–2105.

Ano, H., Makimura, S., Harasawa, R., 2001. Detection of *Babesia* species from infected dog blood by polymerase chain reaction. J. Vet. Med. Sci. 63, 111-113.

Babes, V., 1888. Sur l'hemoglobinurie bacterienne du boeuf. Compt. Rend. Acad. Sci. Ser. III Sci. Vie. 107, 692–694.

Battsetseg, B., Matsuo, T., Xuan, X., Boldbaatar, D., Chee, S.H., Umemiya, R., Sakaguchi, T., Hatta, T., Zhou, J., Verdida, A.R., Taylor, D., Fujisaki, K., 2007. *Babesia* parasites develop and are transmitted by the non-vector soft tick *Ornithodoros moubata*.

Parasitology 134; 1-8.

Beevers, R.E., Buckley, G.M., Davies, N., Fraser, J.L., Galvin, F.C., Hannah, D.R., Haughan, A.F., Jenkins, K., Mack, S.R., Pitt, W.R., Ratcliffe, A.J., Richard, M.D., Sabin, V., Sharpe, A., Williams, S.C., 2006. Low molecular weight indole fragments as IMPDH inhibitors. Bioorg. Med. Chem. Lett. 16, 2535-2538.

Berg, M., Van der Veken, P., Goeminne, A., Haemers, A., Augustyns, K., 2010. Inhibitors of the purine salvage pathway: a valuable approach for antiprotozoal chemotherapy? Curr. Med. Chem. 17, 2456-2481.

Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2003. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. J. Clin. Microbiol. 41, 4172–4177.

Birkenheuer, A.J., Correa, M.T., Levy, M.G., Breitschwerdt, E.B., 2005. Geographic distribution of babesiosis among dogs in the United States and association with dog bites: 150 cases (2000-2003). J. Am. Vet. Med. Assoc. 227, 942-947.

Bock, R., Jackson, L., de Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasitology 129, 247-269.

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

Bose, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T., de Vos, A.J., 1995. Current state

and future trends in the diagnosis of babesiosis. Vet. Parasitol. 57, 61-74.

Braun-Sand, S.B., Peetz, M., 2010. Inosine monophosphate dehydrogenase as a target for antiviral, anticancer, antimicrobial and immunosuppressive therapeutics. Future. Med. Chem. 2, 81-92.

Cacciò, S.M., Antunovic, B., Moretti, A., Mangili, V., Marinculic, A., Baric, R.R., Slemenda, S.B., Pieniazek, N.J., 2002. Molecular characterisation of *Babesia canis canis* and *Babesia canis vogeli* from naturally infected European dogs. Vet. Parasitol. 106, 285-292.

Carret, C., Delbecq, S., Labesse, G., Carcy, B., Precigout, E., Moubri, K., Schetters, T.P., Gorenflot, A., 1999a. Characterization and molecular cloning of an adenosine kinase from *Babesia canis rossi*. Eur. J. Biochem. 265, 1015-1021.

Carret, C., Walas, F., Carcy, B., Grande, N., Précigout, E., Moubri, K., Schetters, T.P., Gorenflot, A., 1999b. *Babesia canis canis*, *Babesia canis vogeli*, *Babesia canis rossi*: differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. J. Eukaryot. Microbiol. 46, 298-303.

Chaudhuri, S., Varshney, J.P., 2007. Clinical management of babesiosis in dogs with homeopathic *Crotalus horridus* 200C. Homeopathy 96, 90-94.

Chomczynski, P., and N. Sacci. 1987. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Collett, M. G., 2000. Survey of canine babesiosis in South Africa. J. S. Afr. Vet. Assoc. 71, 180–186.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Saraña, A., Barba-Carretero, J.C., 2003. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part I. Epizootiological aspects. Vet. Parasitol. 113, 189–201.

Crotty, S., Cameron, C., Andino, R., 2002. Ribavirin's antiviral mechanism of action: lethal mutagenesis? J. Mol. Med. (Berl). 80, 86-95.

Fleming, M.A., Chambers, S.P., Connelly, P.R., Nimmesgern, E., Fox, T., Bruzzese, F.J., Hoe, S.T., Fulghum, J.R., Livingston, D.J., Stuver, C.M., Sintchak, M.D., Wilson, K.P., Thomson, J.A., 1996. Inhibition of IMPDH by mycophenolic acid: dissection of forward and reverse pathways using capillary electrophoresis. Biochemistry 35, 6990-6997.

Fujiyama, N., Miura, M., Kato, S., Sone, T., Isobe, M., Satoh, S., 2010. Involvement of carboxylesterase 1 and 2 in the hydrolysis of mycophenolate mofetil. Drug Metab. Dispos. 38, 2210-2217.

Fukumoto, S., Xuan, X., Nishikawa, Y., Inoue, N., Igarashi, I., Nagasawa, H., Fujisaki, K., Mikami, T., 2001. Identification and expression of a 50-kilodalton surface antigen of *Babesia gibsoni* and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 39, 2603–2609.

Goldstein, B.M., Colby, T.D., 1999. IMP dehydrogenase: structural aspects of inhibitor binding. Curr. Med. Chem. 6, 519-536.

Hager, P.W., Collart, F.R., Huberman, E., Mitchell, B.S., 1995. Recombinant human inosine monophosphate dehydrogenase type I and type II proteins. Purification and characterization of inhibitor binding. Biochem. Pharmacol. 49, 1323-1329.

Halloran, P.F., 1996. Molecular mechanisms of new immunosuppressants. Clin. Transplant. 10, 118-123.

Hariharan, J., Rane, R., Ayyanathan, K., Philomena, Kumar, V.P., Prahlad, D., Datta, S., 1999. Mechanism-Based Inhibitors: Development of a high throughput coupled enzyme assay to screen for novel antimalarials. J. Biomol. Screen. 4, 187-192.

Hedstrom, L., 2009. IMP dehydrogenase: structure, mechanism, and inhibition. Chem. Rev. 109, 2903-2928.

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

Homer, M.J., Bruinsma, E.S., Lodes, M.J., Moro, M.H., Telford, S 3rd, Krause, P.J., Reynolds, L.D., Mohamath, R., Benson, D.R., Houghton, R.L., Reed, S.G., Persing, D.H., 2000b. A polymorphic multigene family encoding an immunodominant protein from *Babesia microti*. J. Clin. Microbiol. 38, 362-368.

Huang, X., Xuan, X., Hirata, H., Yokoyama, N., Xu, L., Suzuki, N., Igarashi, I., 2004. Rapid immunochromatographic test using recombinant SAG2 for detection of antibodies against *Toxoplasma gondii* in cats. J. Clin. Microbiol. 42, 351–353.

Hujakka, H., Koistinen, V., Eerikainen, P., Kuronen, I., Mononen, I., Parviainen, M., Lundkvist, A., Vaheri, A., Narvanen, A., Vapalahti,O., 2001. New immunochromatographic rapid test for diagnosis of acute *Puumala* virus infection. J. Clin. Microbiol. 39, 2146–2150.

Hunfeld, K. P., Hildebrandt, A. and Gray, J. S., 2008. Babesiosis: recent insights into an

ancient disease. Int. J. Parasitol. 38, 1219-1237.

Hupe, D.J., Azzolina, B.A, Behrens, N.D., 1986. IMP dehydrogenase from the intracellular parasitic protozoan *Eimeria tenella* and its inhibition by mycophenolic acid. J. Biol. Chem. 261, 8363-8369.

Igarashi, I., Suzuki, R., Waki, S., Tagawa, Y., Seng, S., Tum, S., Omata, Y., Saito, A., Nagasawa, H., Iwakura, Y., Suzuki, N., Mikami, T., Toyoda, Y., 1999. Roles of CD4 (+) T cells and gamma interferon in protective immunity against *Babesia microti* infection in mice. Infect. Immun. 67, 4143-4148.

Ikadai, H., Xuan, X., Igarashi, I., Tanaka, S., Kanemaru, T., Nagasawa, H., Fujisaki, K., Suzuki, N., Mikami, T., 1999. Cloning and expression of a 48-kilodalton *Babesia caballi* merozoite rhoptry protein and potential use ofthe recombinant antigen in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 37, 3475–3480.

Imwong, M., Snounou, G., Pukrittayakamee, S., Tanomsing, N., Kim, J.R., Nandy, A., Guthmann, J.P., Nosten, F., Carlton, J., Looareesuwan, S., Nair, S., Sudimack, D., Day, N.P., Anderson, T.J., White, N.J., 2007. Relapses of *Plasmodium vivax* infection usually result from activation of heterologous hypnozoites. J. Infect. Dis. 195, 927-933.

Inokuma, H., Yoshizaki, Y., Shimada, Y., Sakata, Y., Okuda, M., Onishi, T., 2003. Epidemiological survey of *Babesia* species in Japan performed with specimens from ticks collected from dogs and detection of new *Babesia* DNA closely related to *Babesia odocoilei* and *Babesia divergens* DNA. J. Clin. Microbiol. 41, 3494–3498.

Jia, H., J. Zhou, H. Ikadai, A. Matsuu, H. Suzuki, I. Igarashi, K. Fujisaki, Xuan, X., 2006.

Identification of a novel gene encoding a secreted antigen 1 of *Babesia gibsoni* and evaluation of its use in serodiagnosis. Am. J. Trop. Med. Hyg. 75, 843–850.

Jia, H., Liao, M., Lee, E., Nishikawa, Y., Inokuma, H., Ikadai, H., Matsuu, A., Igarashi, I., Xuan, X., 2007. Development of an immunochromatographic test with recombinant BgSA1 for the diagnosis of *Babesia gibsoni* infection in dogs. Parasitol. Res. 100, 1381-1384.

Jefferies, R., Ryan, U.M., Irwin, P.J., 2007. PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. Vet. Parasitol. 144, 20–27.

Kivaria, F. M., Ruheta, M. R., Mkonyi, P. A. and Malamsha, P. C., 2007. Epidemiological aspects and economic impact of bovine theileriosis (East Coast fever) and its control: a preliminary assessment with special reference to Kibaha district, Tanzania. Vet. J. 173, 384–390.

Kjemtrup, A. M. and Conrad, P. A., 2000. Human babesiosis: an emerging tick-borne disease. Int. J. Parasitol. 30, 1323-1337.

Krause, P.J., Gewurz, B.E., Hill, D., Marty, F.M., Vannier, E., Foppa, I.M., Furman, R.R., Neuhaus, E., Skowron, G., Gupta, S., McCalla, C., Pesanti, E.L., Young, M., Heiman, D., Hsue, G., Gelfand, J.A., Wormser, G.P., Dickason, J., Bia, F.J., Hartman, B., Telford, S.R. 3rd, Christianson, D., Dardick, K., Coleman, M., Girotto, J.E., Spielman, A., 2008. Persistent and relapsing babesiosis in immunocompromised patients. Clin. Infect. Dis. 46, 370-376.

Kubelová, M., Sedlák, K., Panev, A., Siroký, P., 2012. Conflicting results of serological, PCR and microscopic methods clarify the various risk levels of canine babesiosis in Slovakia: A complex approach to *Babesia canis* diagnostics. Vet. Parasitol. 191; 353-357.

Levy, M.G., Breitschwerdt, E. B., Moncol, D.J., 1987. Antibody activity to *Babesia canis* in dogs in North Carolina. Am. J. Vet. Res. 48, 339-341.

Matsuu. A., Koshida, Y., Kawahara, M., Inoue, K., Ikadai, H., Hikasa, Y., Okano, Higuchi, S., 2004. Efficacy of atovaquone against *Babesia gibsoni in vivo* and *in vitro*. Vet. Parasitol. 124; 9-18.

Matsuu, A., Ono, S., Ikadai, H., Uchide, T., Imamura, S., Onuma, M., Okano, S., Higuchi, S., 2005. Development of a SYBR green real-time polymerase chain reaction assay for quantitative detection of *Babesia gibsoni* (Asian genotype) DNA. J. Vet. Diagn. Invest. 17, 569-573.

Matsuu, A., Yamasaki, M., Xuan, X., Ikadai, H., Hikasa, Y., 2008. *In vitro* evaluation of the growth inhibitory activities of 15 drugs against *Babesia gibsoni* (Aomori strain). Vet. Parasitol. 157, 1-8

Maurya, S.K., Gollapalli, D.R., Kirubakaran, S., Zhang, M., Johnson, C.R., Benjamin, N.N., Hedstrom, L., Cuny, G.D., 2009. Triazole inhibitors of *Cryptosporidium parvum* inosine 5'-monophosphate dehydrogenase. J. Med. Chem. 52, 4623-4630.

Miyama, T., Inokuma, H., Itamoto, K., Okuda, M., Verdida, R.A., Xuan, X., 2006. Clinical usefulness of antibodies against *Babesia gibsoni* detected by ELISA with recombinant P50. J. Vet. Med. Sci. 68, 1371-1373.

Morath, C., Zeier, M., 2003. Review of the antiproliferative properties of mycophenolate mofetil in non-immune cells. Int. J. Clin. Pharmacol. Ther. 41, 465-469.

O'Gara, M.J., Lee, C.H., Weinberg, G.A., Nott, J.M., Queener, S.F., 1997. IMP dehydrogenase from *Pneumocystis carinii* as a potential drug target. Antimicrob. Agents Chemother. 41, 40-48.

Patarroyo, J.H., Prates, A.A., Tavares, C.A., Mafra, C.L., Vargas, M.I., 1995. Exoantigens of an attenuated strain of *Babesia bovis* used as a vaccine against bovine babesiosis. Vet. Parasitol. 59, 189–199.

Ramharter, M., Walochnik, J., Lagler, H., Winkler, S., Wernsdorfer, W.H., Stoiser, B., Graninger, W., 2010. Clinical and molecular characterization of a near fatal case of human babesiosis in Austria. J. Travel. Med. 17, 416-418.

Ransom, J.T., 1995. Mechanism of action of mycophenolate mofetil. Ther. Drug Monit. 17. 681-684.

Rashid, H.B., Chaudhry, M., Rashid, H., Pervez, K., Khan, M.A., Mahmood, A.K., 2008. Comparative efficacy of diminazene diaceturate and diminazene aceturate for the treatment of babesiosis in horses. Trop. Anim. Health. Prod. 40, 463-467.

Ratcliffe, A.J., 2006. Inosine 5'-monophosphate dehydrogenase inhibitors for the treatment of autoimmune diseases. Curr. Opin. Drug Discov. Devel. 9, 595-605.

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

Reyes, G.R., 2001. Ribavirin: recent insights into antiviral mechanisms of action. Curr. Opin.

Drug Discov. Devel. 4, 651-656.

Rodríguez, S.D., Aboytes, R., Figueroa, J.V., Vega, C., 1994. Bovine babesiosis: a review of recent advances. Arch. Med. Res. 25, 241-245.

Rosenblatt, J.E., 2009. Laboratory diagnosis of infections due to blood and tissue parasites. Clin. Infect. Dis. 49, 1103-1108.

Sakuma, M., Setoguchi, A., Endo, Y., 2009. Possible emergence of drug-resistant variants of *Babesia gibsoni* in clinical cases treated with atovaquone and azithromycin. J. Vet. Intern. Med. 23, 493-498.

Schetters, T.P., Moubri, K., Kleuskens, J., Scholtes, N.C., Gorenflot, A., 1997. Different *Babesia canis* isolates, different diseases. Parasitology 115, 485–493.

Schetters, T.P., Strydom, T., Crafford, D., Kleuskens, J.A., van de Crommert, J., Vermeulen, A.N., 2007. Immunity against *Babesia rossi* infection in dogs vaccinated with antigens from culture supernatants. Vet. Parasitol. 144, 10–9.

Schwint, O.N., Ueti, M.W., Palmer, G.H., Kappmeyer, L.S., Hines, M.T., Cordes, R.T., Knowles, D.P., Scoles, G.A., 2009. Imidocarb dipropionate clears persistent *Babesia caballi* infection with elimination of transmission potential. Antimicrob Agents Chemother 53, 4327-4332.

Song, K.H., Kim, D.H., Hayasaki, M., 2004. The PCR-based detection of *Babesia gibsoni* infection in dogs (German shepherds) reared in South Korea. Ann. Trop. Med. Parasitol. 98, 149-153.

Sharling, L., Liu, X., Gollapalli, D.R., Maurya, S.K., Hedstrom, L., Striepen, B., 2010. A screening pipeline for antiparasitic agents targeting *Cryptosporidium* inosine

monophosphate dehydrogenase. PLoS. Negl. Trop. Dis. 4, e794.

Sintchak, M.D., Fleming, M.A., Futer, O., Raybuck, S.A., Chambers, S.P., Caron, P.R., Murcko, M.A., Wilson, K.P., 1996. Structure and mechanism of inosine monophosphate dehydrogenase in complex with the immunosuppressant mycophenolic acid. Cell 85, 921-930.

Striepen, B., Pruijssers, A.J., Huang, J., Li, C., Gubbels, M.J., Umejiego, N.N., Hedstrom, L., Kissinger, J.C., 2004. Gene transfer in the evolution of parasite nucleotide biosynthesis. Proc. Natl. Acad. Sci. U. S. A. 101, 3154-3159.

Suarez, C.E., Noh, S., 2011. Emerging perspectives in the research of bovine babesiosis and anaplasmosis. Vet. Parasitol. 180, 109-125.

Subeki, Matsuura, H., Takahashi, K., Nabeta, K., Yamasaki, M., Maede, Y., Katakura K. 2007. Screening of Indonesian medicinal plant extracts for antibabesial activity and isolation of new quassinoids from *Brucea javanica*. J. Nat. Prod. 70, 1654-1657.

Sullivan, W.J. Jr, Dixon, S.E., Li, C., Striepen, B., Queener, S.F., 2005. IMP dehydrogenase from the protozoan parasite *Toxoplasma gondii*. Antimicrob Agents Chemother 49, 2172-2179.

Suzuki, K., Wakabayashi, H., Takahashi, M., Fukushima, K., Yabuki, A., Endo, Y., 2007. A possible treatment strategy and clinical factors to estimate the treatment response in *Bebesia gibsoni* infection. J. Vet. Med. Sci. 69, 563-568.

Taboada, J., 1998. Babesiosis. In: Greene, C. (Ed.), Infectious diseases of the dog and cat. WB Saunders, Philadelphia, 473-481.

Terkawi, M.A., Igarashi, I., 2011. Drug discovery against *Babesia* and *Toxoplasma*. In: Becker K, editors. Apicomplexan Parasites: Molecular Approaches toward Targeted Drug Development Drug Discovery in Infectious Diseases, Weinheim Germany: Wiley-VCH; p. 453-467.

Uilenberg, G., 2006. Chemotherapy against babesiosis. Vet. Parasitol. 138, 3-10.

Umejiego, N.N., Li, C., Riera, T., Hedstrom, L., Striepen, B., 2004. *Cryptosporidium parvum* IMP dehydrogenase: identification of functional, structural, and dynamic properties that can be exploited for drug design. J. Biol. Chem. 279, 40320-40327.

Valentin, A., Precigout, E., L'Hostis, M., Carcy, B., Gorenflot, A., Schrevel, J., 1993. Cellular and humoral immune responses induced in cattle by vaccination with *Babesia divergens* culture-derived exoantigens correlate with protection. Infect. Immun. 61, 734–741.

Vannier, E., Gewurz, B.E., Krause, P.J., 2008. Human Babesiosis. Infect. Dis. Clin. N. Am. 22, 469-488.

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

Verdida, R.A., Xuan, X., Fukumoto, S., Huang, X., Zhou, J., Igarashi, I., Claveria, F.G., Nagasawa, H., 2005. Development of a practical immunochromatographic test with recombinant P50 for the diagnosis of *Babesia gibsoni* infection in dogs. Parasitology 131, 769–774.

Villarroel, M.C., Hidalgo, M., Jimeno, A., 2009. Mycophenolate mofetil: An update. Drugs Today (Barc). 45, 521-532.

Webster, H.K., Whaun, J.M., 1982. Antimalarial properties of bredinin. Prediction based on identification of differences in human host-parasite purine metabolism. J. Clin. Invest. 70, 461-469.

Weiland, G., Reiter, I., 1988. Methods for serological response to *Babesia*. In: Ristic, M. (Ed.), Babesiosis of Domestic Animals and Man. CRC Press, Boca Raton, Florida, pp. 143–158.

Willis, R.C., Carson, D.A., 1978. Seegmiller, J.E., Adenosine kinase initiates the major route of ribavirin activation in a cultured human cell line. Proc. Natl. Acad. Sci. U. S. A. 75, 3042-3044.

Xuan, X., Maeda, K., Mikami, T., Otsuka, H., 1996. Characterization of canine herpesvirus glycoprotein C expressed by a recombinant baculovirus in insect cells. Virus Research 46, 57–64.

Xuan, X., Larsen, A., Ikadai, K., Tanaka, T., Igarashi, I., Nagasawa, H., 2001. Expression of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked Immunosorbent assay. J. Clin. Microbiol. 39, 705-709.

Yamane, I., Thomford, J.W., Gardner, I.A., Dubey, J.P., Levy, M., Conrad, P.A., 1993. Evaluation of the indirect fluorescent antibody test for diagnosis of *Babesia gibsoni* infections in dogs. Am. J. Vet. 54, 1579–1584.

Yeagley, T.J., Reichard, M.V., Hempstead, J.E., Allen, K.E., Parsons, L.M., White, M.A., Little, S.E., Meinkoth, J.H., 2009. Detection of *Babesia gibsoni* and the canine small

Babesia 'Spanish isolate' in blood samples obtained from dogs confiscated from dogfighting operations. J. Am. Vet. Assoc. 235, 535-539.

Zhou, J., Fukumoto, S., Jia, H., Yokoyama, N., Zhang, G., Fujisaki, K., Lin, J., Xuan, X., 2006. Characterization of the *Babesia gibsoni* P18 as homologue of thrombospondin related adhesive protein. Mol. Biochem. Parasitol. 148, 190–198.