Development of Serological Diagnostic Methods against Canine *Babesia gibsoni* Infection

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Contents

Abbreviations

AP: alkaline phosphatase

BgIRA: interspersed repeat antigen of *Babesia gibsoni*

BgSA1: secreted antigen 1 of *Babesia gibsoni*

BSA: bovine serum albumin

bp: base pair

cDNA: complementary DNA

DIC: differential interference contrast

DNA: deoxyribonucleic acid

DPI: days-post infection

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

GST: glutathione *S*-transferase

HCT: hematocrit

HRPO: horse radish peroxidase

ICT: immunochromatographic test

IFAT: indirect fluorescent antibody test

Ig: immunoglobulin

kDa: kilodalton

MAb: monoclonal antibody

mRNA: messenger RNA

NRS: normal rabbit serum

OD: optical density

ORF: open reading frame

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PCV: packed cell volume

PFU: plaque forming unit

pI: isoelectric point

PI: propidium iodide

RBC: red blood cell

RK: rabbit kidney

RNA: ribonucleic acid

SCID: severe combined immunodeficiency

SDS: sodium dodecyl sulfate

TK: thymidine kinase

General introduction

1. Canine babesiosis

Tick-transmitted hemoparasites of the genus *Babesia* (phylum Apicomplexa) are the second most common blood-borne parasites of mammals after the trypanosomes (Henfeld et al., 2008). Most significantly, molecular analysis of the implicated pathogens suggests that the host-range of many *Babesia* is less restricted than believed previously and also that hitherto unrecognised species can cause infections in a variety of animal hosts and in humans (Zahler et al., 2000; Cho et al., 2002; Herwaldt et al., 2003, 2004; Conrad et al., 2006; Kjemtrup et al., 2006; Haselbarth et al., 2007; Kim et al., 2007).

Babesia is classified as apicomplexan parasites of the suborder Piroplasmidea and family Babesiidae on the basis of their exclusive invasion of erythrocytes, multiplication by budding rather than schizogony, and lack of hemozoin (Hanfeld et al., 2008). The life cycles of the parasites are very similar. All species of *Babesia* are naturally transmitted by the bite of infected ticks (almost all ixodids rather than argasids) and the main difference in lifecycle amounts to the presence of transovarial transmission in some species (Bonnet et al., 2007; Howell et al., 2007; Ikadai et al., 2007) and not in others (Gray et al., 2002; Hanfeld et al., 2008). During the tick bite, sporozoites are injected into the host and directly infect red blood cells. This phenomenon separates *Babesia* spp. from *Theileria* spp., where sporozoites do not readily infect red blood cells but initially penetrate a lymphocyte or macrophage in which development into schizonts takes place (Uilenberg, 2006).

Traditionally, *Babesia* was mainly grouped on the basis of their morphology, host/vector specificity, and susceptibility to drugs. Pragmatically, they are divided into the small *Babesia* (trophozoites of 1.0-2.5 μm; including *B. gibsoni*, *B. microti*, and *B. rodhaini*) and large *Babesia* (2.5-5.0 μm; including *B. bovis*, *B. caballi*, and *B. canis*). These morphological classifications are generally consistent with the phylogenetic characterization based on the nuclear small subunit ribosomal RNA gene (18S rDNA) sequences, which shows that the large and small *Babesia* fall into two phylogenetic clusters, the small *Babesia* being more related to *Theileria* spp. than the large *Babesia* (with the exception of *B. divergens*, which appears small on blood smears, but is genetically related to large *Babesia*) (Homer et al., 2000). 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, 2006). Recently, a careful study by Criado-Fornelio et al. (2003) recently suggested classification of the piroplasms into five distinct clades: (1) *B. microti* group containing *B. rodhaini*, *B. felis*, *B. leo*, *B. microti*, and a *B. microti*-like canine isolate; (2) western US *Theileria*-like group, containing *B. conradae*; (3) *Theileria*-group, containing all *Theileria* spp. from bovines; (4) a first group of 'true' *Babesia* spp. including *B. canis* and *B. gibsoni* from canines together with *B. divergens* and *B. odocoilei*; and (5) a second *Babesia* spp. group composed mainly of *Babesia* spp. from ungulates: *B. caballi*, *B. bigemina*, *B. ovis*, *B. bovis*, and other *Babesia* spp. from cattle.

Canine babesiosis is mainly caused by *B. canis* and *B. gibsoni*. Both *B. canis* and *B. gibsoni* infections are prevalent in Asia, although the former is less common. Until recently, canine piroplasms were mainly identified based on their morphological

properties: all large piroplasms were thought to be *B. cannis*, whereas all small piroplasms were thought to be *B. gibsoni*. But molecular genetic analyses of 18S rDNA from canine piroplasms shows that there are at least three distinct subtypes or subspecies for small piroplasms: a classic Asian type *B. gibsoni*; a small organism recently identified in northern Spain called *T. annae*; and a small organism recently identified in California named as *B. conradae* (Kjemtrup et al., 2000; Camacho et al., 2001; Garcia et al., 2006).

Dogs infected with *B. gibsoni* develop severe hemolytic anemia, remittent fever, thrombocytopenia, splenomegaly, and hepatomegaly (Wozniak et al., 1997; Casapulla et al., 1998; Boozer and Macintire, 2003). *B. gibsoni* was first recognized in India in 1910, and, since this parasite was first reported in the United States in 1968, it has been identified to distribute in many areas throughout the world, such as Asia, Europe, Middle East, Africa, and Americas (Macintire et al., 2002; Muhlnickel et al., 2002; Boozer and Macintire, 2003; Inokuma et al., 2004; Miyama et al., 2005; Blaschitz et al., 2008; Oosthuizen et al., 2008). In some epidemic regions, it has become a significant problem because of the lack of effective preventive and control medicines, as well vaccines (Adachi et al., 1993; Inokuma et al., 2004).

2. Diagnosis of canine *B. gibsoni* infection

The diagnosis of canine *B. gibsoni* infection is mainly based on the detection of intraerythrocytic babesial organisms by the microscopic examination of Giemsa-stained thin blood smear films. Annular forms, including rings and ovals, are the most common morphologic form of *B. gibsoni* parasite in peripheral blood in dogs (Maronpot and

Guindy, 1970; Roher et al., 1985). Other morphologic forms include elongated, comma, band, ameboid, paired pyriform and quadruplet forms. The microscopic examination is simple and suitable for the acute stage of canine babesiosis.

Serological diagnostic methods, such as indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) with infected erythrocytes have been widely used for the serological diagnosis of *B. gibsoni* infection (Anderson et al., 1980; Yamane et al., 1993; Bose et al., 1995). Recently, recombinant antigens expressed in *Escherichia coli* or insect cells have been evaluated in the serological tests for the diagnosis of *B. gibsoni* infection (Fukumoto et al., 2004; Aboge et al., 2007; Goo et al., 2008). The application of specific antigens greatly improved the specificity of these kinds of tests. In addition, polymerase chain reaction (PCR) has also been developed for detection of *B. gibsoni* DNA. These tests are particularly useful for identification of chronically infected dogs with significantly low parasitemia (Birkenheuer et al., 2003).

3. Aims of the present study

For the control of canine *B. gibsoni* infection, it is very important to develop specific and sensitive diagnostic methods. A successful diagnostic method should possess characters, such as: (1) the method should be simple to perform; (2) the cost must be minimal; (3) the method should be suitable for handling large number of samples.

In the clinical hospital, examination of Giemsa-stained blood smears for the parasite is still considered as gold standard for diagnosis of this disease. However, in subclinical or latent infections, this may be impractical due to low levels of parasitemia. In addition, microscopy of parasites in blood smears without additional molecular analysis of the

pathogen is not reliable for species identification. PCR is a sensitive method for the diagnosis of *B. gibsoni* infection. However, this method requires highly trained personnel and costly equipments, thus limiting its application in the mass scale diagnosis of the disease. Serological tests, such as IFAT bassed on lysates of whole babesial parasites are highly sensitive, but only moderately specific, because of antigenic cross-reactions with other closely related *Babesia* spp. (Yamane et al., 1993). In these tests, antigenic cross-reactivity between the two canine *Babesia* spp. has been reported (Anderson et al., 1980; Yamane et al., 1993). Furthermore, dogs that were experimentally infected with *B. gibsoni* develop cross-reactive antibody to other canine protozoan parasites such as *B. canis*, *Toxoplasma gondii* and *Neospora canninum* (Yamane et al., 1993). In addition, when whole parasites are used as antigens, their quantities can vary from batch to batch. Also, the antigen production for these tests requires experimentally infected dogs, making production of such antigens time-consuming and expensive. Moreover, the serum from *B. gibsoni*-infected dogs sometimes shows a cross-reaction to normal dog erythrocytes (Adachi and Makimura, 1992; Adachi et al., 1994a, 1994b, 1995) or *B. canis* (Yamane et al., 1993) when native antigens are used. The accumulation of fundamental knowledge concerning the immunoserology of *B. gibsoni* infections has led to the discovery of a number of specific angtigens. Although these antigens have revealed promising results in ELISAs in previous studies, there is still need for further evaluation.

On the other hand, there are still no serological tests suitable for the detection of early infection. Therefore, the negative results do not indicate that the animal is free from infection. Moreover, the antibody titer can maintain at a high level for a long time even after terminating resolution of an infection by autosterilisation or chemotherapy. The

need for a serological test is obvious to be able to determine whether eradication programmes have been successful or whether transmission of babesiosis in endemic areas has been interrupted. In this regards, efforts must continue toward the preparation of more sensitive and specific antigens, which will be able to define the status of infected animals.

Based on these backgrounds, more sensitive and specific diagnostic methods need to be developed and the objectives in this thesis can be summarized as follows: (1) isolation and identification of novel immunodominant or secreted antigens; (2) development of more sensitive and specific diagnostic methods to detect antibodies for *B. gibsoni* infection; (3) development of sensitive and specific diagnostic methods to detect circulating antigens for *B. gibsoni* infection.

Chapter 1

Identification and characterization of an immunodominant repetitive antigen of *Babesia gibsoni*

1-1. Introduction

For understanding of the prevalence and the elimination of canine *Babesia gibsoni* infection, it would be important to have a method for the sensitive and reliable diagnosis of dogs in the chronic infection or that carry this parasite. In this regard, many methods, including polymerase chain reaction (PCR) (Birkenheuer et al., 2003), indirect fluorescence antibody test (IFAT) (Yamane et al. 1993), and enzyme-linked immunosorbent assay (ELISA) with native or recombinant proteins (Fukumoto et al. 2004; Verdida et al. 2004), have been developed. These methods are particularly useful for the identification of chronically infected dogs with significantly low parasitemia. For mass detection, the advantages of ELISA compared to other techniques are its sensitivity, specificity, and convenience. Isolation of parasite specific antigens is essential for the development of this kind of high-quality serodianostic system. However, several factors, such as the complexity of the life cycle, the wide variety of immune responses induced by the parasites, and incomplete knowledge of protective immunity have hindered the search for a promising antigen. The currently available antigens still need to be further evaluated. In this regard, continued search for new antigens is extremely desirable.

Recently, serum samples from several dogs experimentally infected with *B. gibsoni*, including a splenectomized dog and dogs in acute infection stages or chronic stages, were used to isolate immunodominant antigens from a cDNA library (Fukumoto et al., 2001). In this chapter, a gene encoding a highly repetitive antigen, which showed strong reaction and a dominant number in the immunoscreening, was identified and designated as *B. gibsoni* Interspersed Repeat Antigen (BgIRA). Studies on several protective antigens from *Plasmodium*, such as CSP and RESA (Cochrane et al., 1982; Cowman et al., 1984), indicated the important roles of individual repetitive regions in the immune response of malaria patients. The BgIRA described here is further repetitive at the amino acid level. Needless to say, a highly repetitive and immunodominant antigen exposed by the immune system of the dogs infected with *B. gibsoni* should be the first choice to be applied in serological tests.

1-2. Materials and methods

Sera and parasite. A *B. gibsoni* NRCPD strain (Fukumoto et al., 2000) maintained in splenectomized and normal beagles was used. The sera used for immunoscreening include the serum samples collected from a dog infected with *B. gibsoni* at acute stage and chronic stage and serum from a splenetomized dog.

Sera used for ELISA were as follows: 14 sera from dogs experimentally infected with *B. gibsoni,* 2 sera from dogs experimentally infected with *B. canis canis*, 2 sera from dogs experimentally infected with *B. canis rossi*, 1 sera from dogs experimentally infected with *B. canis vogeli*, 2 sera from dogs experimentally infected with *Leishmania infantum*, 2 sera from *Neospora caninum*, 28 sera from SPF dogs, and serial serum

samples from a dog experimentally infected with *B. gibsoni*. In addition, 33 field serum samples collected from the *B. gibsoni*-infected dogs in the epidemic areas of Japan. These dogs were diagnosed to be infected with *B. gibsoni* by using a semi-nested PCR.

Immunoscreening of cDNA expression library. A cDNA expression library constructed from *B. gibsoni* merozoite mRNA (Fukumoto et al., 2001a) was used for immunoscreening. The library was plated on a total of 15 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 serum prepared above 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).

Southern blotting. Southern blot analysis was performed as described by Bork et al (2004). Briefly, 10 μg of *B. gibsoni* genomic DNA, which was extracted from the infected RBC, was digested with *Acc*I, *Hin*dII, *Xba*I, and *Kpn*I, and then separated on a 0.1% agarose gel. The separated DNAs were transferred to a nylon membrane (Hybond-N; Amersham-Buchler, Germany) and hybridized with a DNA fragment containing an entire ORF of the BgIRA gene. The fragment had been extracted from the pBS/BbLDH as described below and then labeled with alkaline phosphatase by using an AlkPhos Direct kit (Amersham Pharmacia Biotech, UK).

Expression and purification of recombinant BgIRA. The cDNA fragment of the BgIRA without a signal peptide was inserted into *Escherichia coli* expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, USA). The resulting plasmid was

designated as pGEX-4T-3/BgIRA after it was identified by restriction enzyme analysis and sequencing. The recombinant protein fused with a glutathione *S*-transferase (GST) tag was expressed in the *E. coli* BL21 strain according to the manufacturer's instructions (Amersham Pharmacia Biotech, USA). Purification of recombinant BgIRA (rBgIRA) was performed with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, USA) according to the manufacturer's instructions.

Preparation of rabbit and mouse sera against BgIRA. Two Japanese white rabbits (3 kg, SCL, Japan) were immunized subcutaneously with 1 mg of purified rBgIRA or rGST in Freund's complete adjuvant (Difco, USA) for the first injection. Five hundred micrograms of the same antigen in Freund's incomplete adjuvant (Difco, USA) was subcutaneously injected into the rabbit on days 14 and 28. Sera were collected 14 days after the last immunization.

IFAT and confocal laser microscopic observation. A thin blood smear prepared with *B. gibsoni*-infected red blood cells was fixed with a mixture of methanol and acetone (v:v / 1:4) at 20°C for 20 min as described previously (Fukumoto et al., 2001a). Briefly, the anti-rBgIRA-specific rabbit serum was applied as the first antibody on the fixed smears and incubated for 30 min at 37°C. After washings three times with PBS, Alexa-Fluor® 488 conjugated goat anti-rabbit immunoglobin G (IgG) (Molecular Probes, USA) was subsequently applied as a secondary antibody and incubated for another 30 min at 37°C. The slides were washed three times with PBS and incubated with 6.25 μg/ml propidium iodide (PI) (Molecular Probes, USA) containing 50 μg/ml RNase A (Qiagen, Germany) for 10 min at 37°C. After washings two times with PBS, the glass slides were mounted by adding 200 μl of a 50% glycerol-PBS (V/V) solution and covered with a glass cover slip. The slides were examined under a confocal laser

scanning microscope (TCS NT, Leica, Germany).

Western blotting. *B. gibsoni*-infected dog erythrocytes and normal dog erythrocytes were treated with a 0.83% NH_4Cl solution and then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was electrically transferred to a nitrocellulose membrane. The membrane was blocked with PBS containing 5% skim milk and then incubated with anti-rBgIRA or anti-GST polyclonal serum diluted in 5% skim milk at 37°C for 60 min. Then the membrane was washed three times with PBS and then incubated with peroxidase-conjugated goat anti-rabbit IgG in 5% skim milk. After washing three times with PBS, the bands on the membrane were visualized by incubation with diaminobenzine.

ELISA. Fifty microliters of purified rBgIRA or control GST diluted in a coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) was used to coat the individual wells of 96-well microtiter plates. 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°C for 2 h. The plates were incubated at 37°C 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 HRPO-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 of 0.11 was calculated from the results of the ELISA of 28 specific pathogen-free (SPF) dog sera as follows: 0.028 (mean value) + 3 × 0.027 (standard

deviation).

Semi-nested PCR. The semi-nested PCR was performed as reported by Birkenheuer et al (2003). The optimal conditions were used with a 50 μl reaction volume containing 1.25 U of AmpliTaq Gold (Perkin-Elmer, USA)/reaction, 25 pmol of each primer, 200 μM concentrations of each deoxynucleoside triphosphate, and a 1 × concentration of PCR buffer. DNA amplification with the outer primer pair was performed in a thermal cycler (PCR ExPress, UK) at the following temperatures: initial denaturation at 95°C for 5 min, followed by 50 amplification cycles (95°C for 45 s, 58°C for 45 s, and 72°C for 45 s), and a final extension step at 72°C for 5 min.

Semi-nested PCR (specific forward primers paired with the outer reverse primer) was each carried out in separate tubes under the same conditions as the outer primer pair, except for the following: 0.5 μl from the initial reaction was used as a DNA template, and the reactions were amplified for 30 cycles.

1-3. Results

Identification and characterization of the *BgIRA* gene. The full length of the *BgIRA* cDNA sequence had a single open reading frame of 2,058 nucleotides encoding a polypeptide of 686 amino acid residues. The nucleotide sequence was available in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number EF452231. The molecular weight of the mature protein was 76.2 kDa, as calculated with software (MacVector, version 7.0, Oxford Molecular Group, USA). The amino acid sequence shared low homology with the *P. falciparum* interspersed repeat antigen (FIRA) (Stahl et al., 1985). There were three distinct blocks of repeats, and the sequence

of each block consisted largely of inexact repeats of short units of amino acid residues (Fig. 1A). The sequence of the first block (93-147 aa) was followed continuously by the second block (148-284 aa). Next was a region of 44 amino acid residues, followed by a third block (328-491 aa) of repeats, which were closely related to those of the first block, but the function of these remarkable repeats was still unknown. Southern blotting and genomic analyses indicated that the *BgIRA* gene exist as a single copy in the genome of *B. gibsoni* (Fig. 2A) and contained 10 introns (Fig. 2B).

Expression of BgIRA in *E. coli.* A recombinant BgIRA was expressed as a fusion protein with GST, but it was approximately twice larger than the expected size. I suspected that the protein dimer might have been formed by two molecules of rBgIRA after it was expressed in *E. coli*. This was also supported by the results of native protein analysis discussed below. The rBgIRA expressed in BL21 strain of *E. coli* maintained its antigenicity and could be recognized by a serum from a dog experimentally infected with *B. gibsoni* in Western blotting (Fig. 3).

Identification of the native BgIRA of *B. gibsoni.* A rabbit anti-rBgIRA polyclonal serum was used to identify the native BgIRA in the lysate of *B. gibsoni* parasites. A clear band with twice the expected size was detected in *B. gibsoni*-infected red blood cells but not in normal ones in Western blot analysis (Fig. 3). These results indicated that the native dimer formation of BgIRA also existed in *B. gibsoni* parasites. In addition, the native BgIRA was recognized by the anti-rBgIRA antibody under a confocal laser scanning microscope (Fig. 4).

Application of recombinant BgIRA in an ELISA for the detection of a specific antibody. The immune response against BgIRA in dogs was determined by an ELISA with rBgIRA expressed in *E. coli*.

The specific antibody against BgIRA could be detected as early as the 8th day post-infection, and the antibody level was maintained until 541 days post-infection even when the infection was in the chronic stage, which was characterized by a recovered hematocrit value (data not shown) and a significantly low level of parasitemia (Fig. 5). The dominant immune response against BgIRA was also observed in the field samples. Up to 32 out of 33 field samples, which are positive in a semi-nested PCR, showed a positive reaction in the ELISA based on the rBgIRA (data not shown). Furthermore, no cross-reactions were observed in the ELISA with the serum samples from the dogs experimentally infected with closely related protozoan parasites (Fig. 6).

1-4. Discussion

In this chapter, a novel gene encoding a highly repetitive antigen was isolated and named as BgIRA from a cDNA library. The genomic analyses indicated that the BgSA1 gene exists as single copy in the genome and contains 10 introns. This might indicate that the *BgIRA* gene had various alternative splicing.

The dominant immune response against BgIRA was observed in the field samples. Up to 32 samples showed a positive reaction with the rBgIRA expressed in *E. coli*. The high level of antibody response suggested that BgIRA played a dominant role in the immune response of *B. gibsoni* infection. Unlike all other antigens of *B. gibsoni* studied in detail so far, it contains a structural unit bearing repeats of a short unit. Moreover, this entire structural unit is itself repeated within the antigen.

The variation among the repeats of BgIRA is considerably similar with FIRA of *P. falciparum*. The repeats of BgIRA varied extensively along the molecule in all three positions of several codons. Natural antibody responses to the circumsporozoite (CS)

protein, ring-infected erythrocyte surface antigen (RESA) of malaria parasites appear to be largely directed against epitopes encoded by the repeat units (Brown et al., 1989; Astagneau et al., 1994). The strength of signals given by dog sera in colony immunoassays (on BgIRA clones) suggested that this was also the case for the antibody response to BgIRA. High antibody levels to BgIRA in the great majority of dogs living in an area, in which disease is endemic, appears consistent with BgIRA being the diagnostic reagent.

But, given the extreme diversity of the BgIRA repeats, the antibody response to this antigen may represent much different specificity, including specificities directed against nonrepeat regions of the molecule, a subset of which might be specific. In light of the rapidly growing number of antigens containing repetitive sequences that have already been identified, the number of variant alleles of these molecules, and, at least in the case of BgIRA, the variation within and among different blocks of repeats in the one molecule, it is not surprising that infection with *B. gibsoni* is associated with a high-titer antibody response.

The recombinant BgIRA expressed in *E. coli* was evaluated in an ELISA as an antigen for the detection of a specific antibody to *B. gibsoni* in dogs. The ELISA could differentiate the sera of *B. gibsoni*-infected dogs from non-infected dogs and *B. caninis*-infected dogs. In addition, the specific antibody was detectable as early as 8 days post-infection, and the antibody titer was maintained at a high level until 541 days post-infection. These results indicated that the ELISA with rBgIRA might be a useful method for the detection of the antibody to *B. gibsoni* in both acutely and chronically infected dogs. Both the specificity and sensitivity of BgIRA shown in my data supported it as a good diagnostic antigen.

In summary, the full length of the BgIRA sequence was isolated from a cDNA expression library and characterized it as an immunodominant antigen in this chapter. The full length of the BgIRA sequence contains three distinct blocks of repeats, and each of them consisted largely of inexact repeats of short units of amino acid residues. The specificity and sensitivity of the rBgIRA in an ELISA indicated that this antigen might be useful in a diagnostic test. The functions of BgIRA and whether the repetitive sequences in the antigen play crucial roles in the biological activity of *B. gibsoni* remained unknown. The next step will be to evaluate BgIRA as a vaccine target for *B. gibsoni* infection in dogs. What remains to be resolved includes the protective effect of BgIRA and the extent to which immune responses against these repeating antigenic epitopes are host-protective or, conversely, counterproductive.

1-5. Summary

In this chapter, an immunodominant antigen called BgIRA from *B. gibsoni* is identified and described. A highly repetitive antigen was screened from a cDNA library. The genomic BgIRA gene exists as single cope gene and contains 10 introns. The rBgIRA expressed in BL21 strain of *E. coli* maintained its antigenicity and could be recognized by a serum from a dog experimentally infected with *B. gibsoni*. The native BgIRA was recognized by the anti-rBgIRA antibody. BgIRA plays a dominant role in the immune response in dogs infected with *B. gibsoni*. The specificity and sensitivity of the rBgIRA in an ELISA indicated that this antigen might be useful in a diagnostic test.

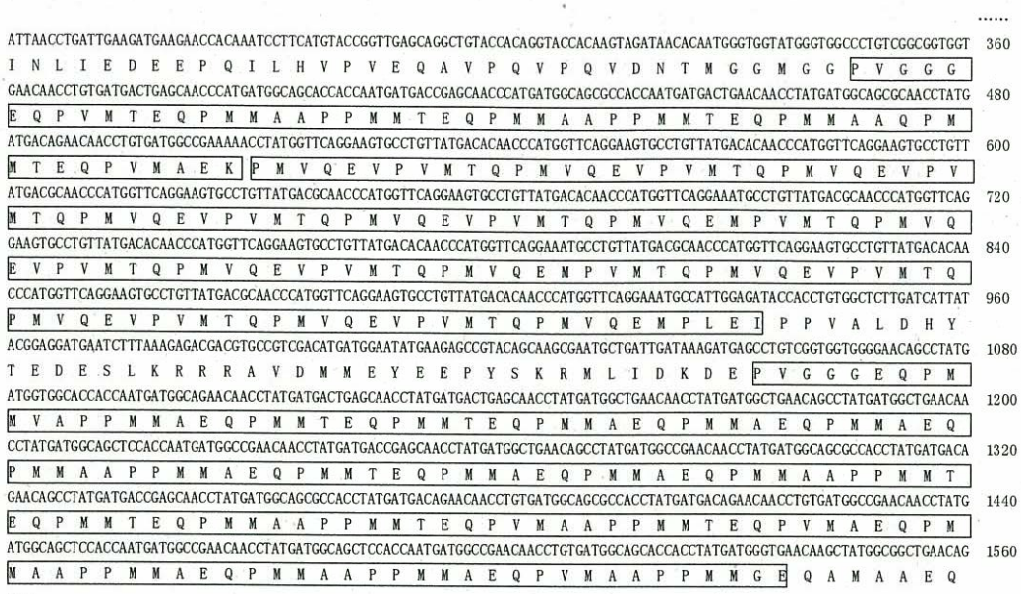

Fig. 1. Nucleotide and predicted amino acid sequences of the cDNA coding for BgIRA. The three repetitive blocks are indicated by boxed in areas.

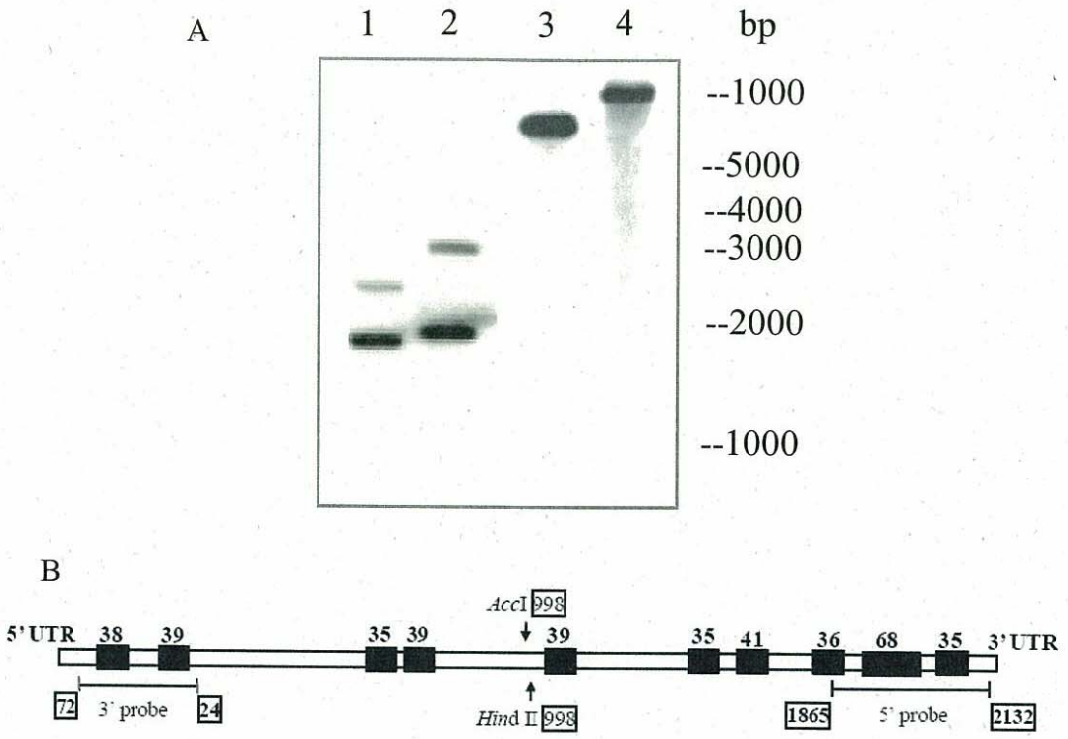

Fig. 2. A, southern blot analysis. The locations of the probes were indicated in panel B. The blots from lane 1 to lane 2 were hybridized with a mixture of the 3' probe and the 5' probe; the ones from lane 3 to lane 4 were hybridized with the 3' probe alone. There are one *Acc*I and one *Hin*dII cleavage sites within the genomic DNA sequence between the two probes, and no *Xba*I, and *Kpn*I cleavage sites within the genomic DNA sequence of the 3' probe. B, structure of the genomic *BgIRA* gene. White bold lines indicate exons, and black rectangles indicate introns. The numeric numbers indicate the number of nucleotides.

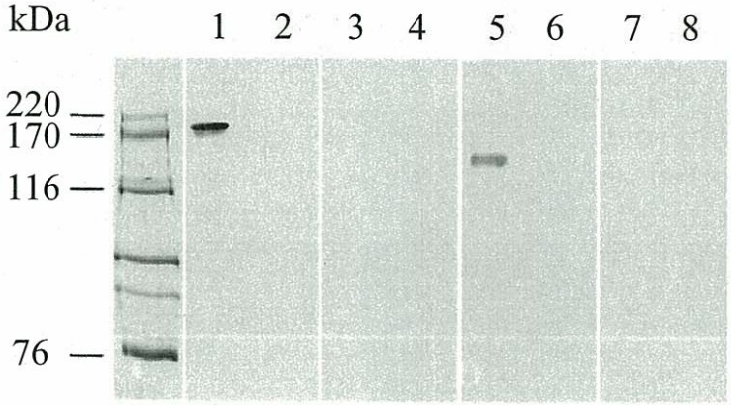

Fig. 3. Comparison of the expression profile of native BgIRA and rBgIRA by Western blot analysis. Lanes 1and 2, the purified rBgIRA and rGST were incubated with a serum from a dog infected with *B. gibsoni*; lanes 3 and 4, the purified rBgIRA fused with GST and rGST were incubated with a serum from a healthy dog; lanes 5 and 6, *B. gibsoni*-infected dog erythrocyte lysate and normal dog erythrocyte lysate were incubated with a rabbit polyclonal antibody against rBgIRA; lanes 7 and 8, *B. gibsoni*-infected dog erythrocyte lysate and normal dog erythrocyte lysate were incubated with a normal rabbit serum.

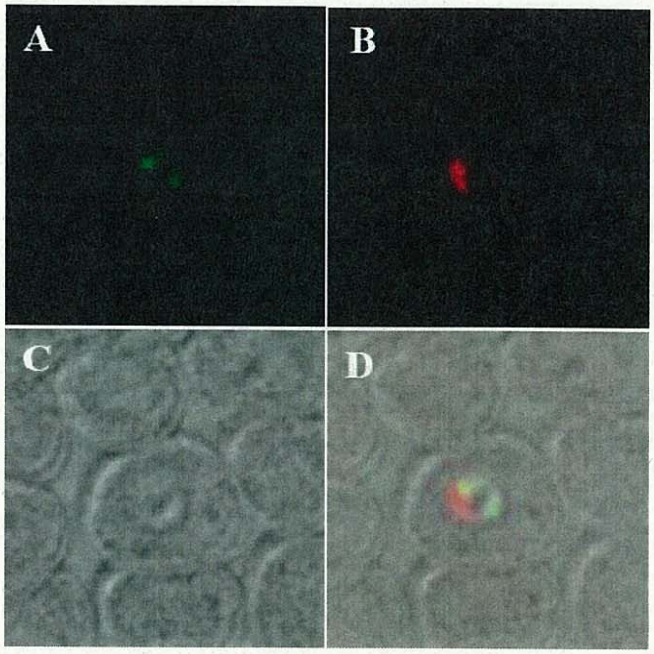

Fig. 4. Observation of the native BgIRA recognized by a rabbit anti-rBgIRA serum in confocal laser micrographs. A, Immunofluorescent staining of *B. gibsoni* merozoites with rabbit anti-rBgIRA serum; B, propidium iodide staining of *B. gibsoni* merozoite nuclei; C, phase-contrast images of *B. gibsoni* merozoites; D, panels A and B are overlaid on panel C. The images were derived from a single section.

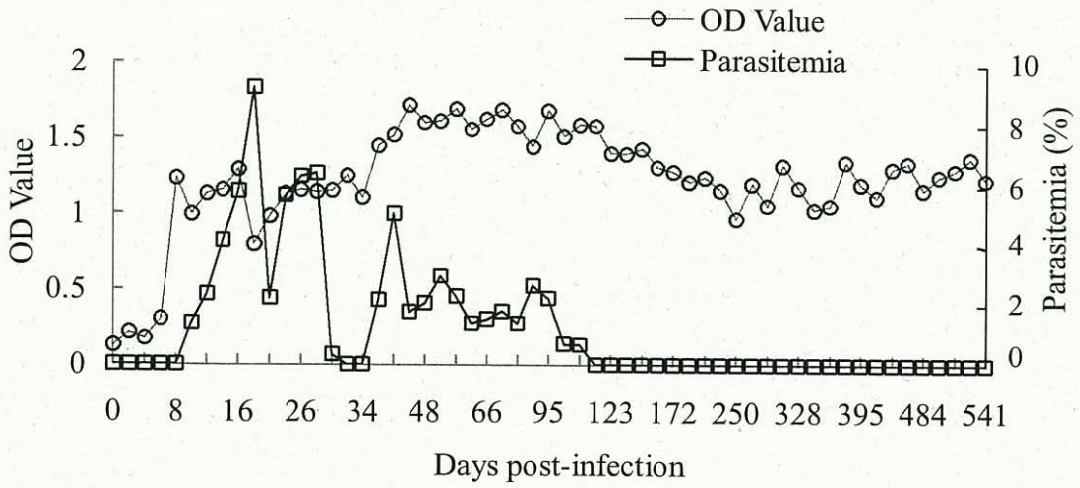

Fig. 5. Detection of the antibody against BgIRA in a dog experimentally infected with *B. gibsoni*.

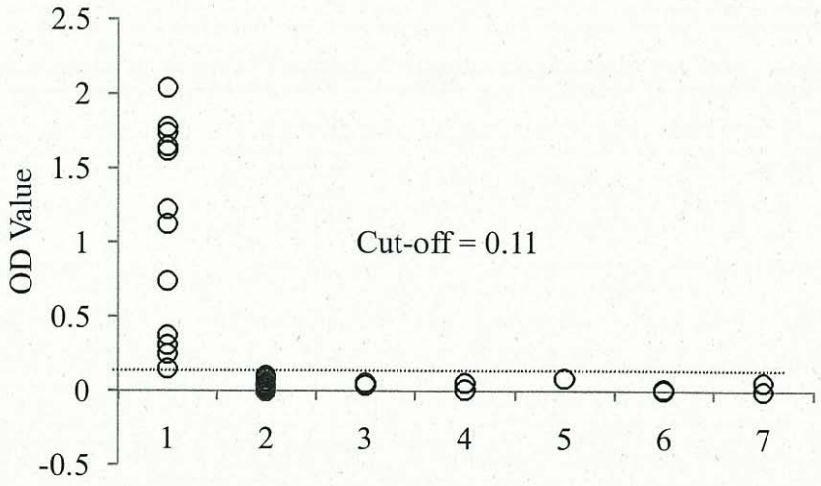

Fig. 6. Evaluation of the ELISA with experimentally infected dog sera. Lane 1, sera from *B. gibsoni*-infected dogs (n=14); lane 2, SPF dog sera (n=30); lane 3, sera from *B. canis canis*-infected dogs (n=2); lane 4, sera from *B. canis rossi*-infected dogs (n=2); lane 5, sera from *B. canis vogeli*-infected dogs (n=1); lane 6, sera from *N. caninum*-infected dogs (n=2); lane 7, sera from *L. infantum*-infected dogs (n=2).

Chapter 2

Identification and characterization of novel genes encoding secreted antigens of *Babesia gibsoni*

2-1. Introduction

Serological diagnostic tests are considered to be necessary for epidemic investigation and controlling of *Babesia gibsoni* infection, considering their advantages of sensitivity and convenience. All of currently available serological diagnostic tests of *B. gibsoni* are designed to detect antibodies. However, the antibody level can remain for a long time even when the infection has finished. Therefore, none of the available tests will revert to negative within a relatively short time after an infection had terminated by autosterilisation or chemotherapy. The need for development of a serological test is obvious to determine whether eradication programmes have been successful or whether transmission of babesiosis in endemic areas has been interrupted. In this regard, efforts must continue toward the preparation of more sensitive and specific *Babesia* spp. antigens which will be able to be used to define the status of infected animals.

Secreted antigens in the blood plasma of infected dogs derived from the parasites could reflect the real infection status or parasite burden in a host. In malaria, circulating antigens have been successfully used to develop serological tests to detect antigens. In this chapter, a special experiment was designed in order to isolate these specific secreted antigens.

In a previous report, a method was described to screen soluble antigens from *B. microti*, and several of them showed encouraging results (Homer et al., 2003). I used the same method to isolate the genes encoding soluble antigens from a cDNA library constructed from *B. gibsoni* merozoite mRNA by immunoscreening. From the candidate cDNA sequences, a novel gene encoding a secreted antigen was identified.

2-2. Materials and methods

Serum for immunoscreening. A beagle infected with *B. gibsoni* experimentally was splenectomized and parasitemia was monitored by daily observation of Giemsa-stained thin blood smears. Blood was harvested when infected red blood cells reached approximately 20%. Blood plasma was separated by centrifuging the harvested blood at 1,000 × *g* for 10 min, collected from the top of the cell pellet and debris, and then ultracentrifuged at 130,000 × *g* for 1 h. Another healthy beagle was immunized subcutaneously with 10 ml of the plasma mixed with the same volume of Freund's complete or incomplete adjuvant (Difco, USA) every three weeks for seven times. The last immunization was performed by intravenous injection without an adjuvant. Serum was collected 10 days after the last immunization.

Sera for ELISA. Sera used for ELISA were as follows: 14 sera from dogs experimentally infected with *B. gibsoni*, 5 sera from dogs experimentally infected with *B. canis canis*, 3 sera from dogs experimentally infected with *B. canis rossi*, 2 sera from dogs experimentally infected with *B. canis vogeli*, 5 sera from dogs experimentally infected with *L. infantum*, 4 sera from *N. caninum*, 28 sera from SPF dogs, and serial serum samples from a dog experimentally infected with *B. gibsoni*. In addition, 931 sera

from domestic dogs collected from various areas of Japan (Ikadai et al., 2004) were also used. The ELISA with recombinant truncated BgP50 (Verdida et al., 2004) and IFAT (Yamane et al., 1993) were also performed for the detection of the antibody to *B. gibsoni* in the field serum samples.

Immunoscreening of cDNA expression library. A cDNA expression library constructed from *B. gibsoni* merozoite mRNA (Fukumoto et al., 2001a) was immunoscreened by using serum described above. The procedure was described in the Chapter 1.

Southern blotting. Ten micrograms of *B. gibsoni* genomic DNA, which was extracted from the infected RBC, was digested with *Acc*I, *Hin*dII, *Xba*I, and *Kpn*I, and then separated on a 0.1% agarose gel. The separated DNAs were transferred to a nylon membrane (Hybond-N; Amersham-Buchler, Germany). Southern blot analysis was performed as described in Chapter 1.

Expression and purification of recombinant BgSA1. The cDNA fragment of *BgSA1* gene without a signal peptide was inserted into *E. coli* expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, USA). The recombinant proteins fused with a glutathione *S*-transferase (GST) tag was expressed in the *E. coli* BL21 strain according to the manufacturer's instructions (Amersham Pharmacia Biotech, USA). Purification of recombinant BgSA1 (rBgSA1) was performed with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, USA) according to the manufacturer's instructions.

Preparation of rabbit and mouse sera against BgSA1. Two Japanese white rabbits (3 kg, SCL, Japan) were immunized subcutaneously with 1 mg of each purified rBgSA1 in Freund's complete adjuvant (Difco, USA) for the first injection. Five

hundred micrograms of the same antigens in Freund's incomplete adjuvant (Difco) were subcutaneously injected into the rabbit on days 14 and 28. For the preparation of mice antiserum (ddY mice, 5 weeks old, SCL, Japan), 100 μg and 50 μg of antigens were used for the first immunization and for boosting on days 14 and 28, respectively. Sera were collected 14 days after the last immunization.

IFAT and confocal laser microscopic observation. A thin blood smear prepared with *B. gibsoni*-infected red blood cells was fixed with a mixture of methanol and acetone (v:v / 1:4) at 20°C for 20 min as described in Chapter 1.

Western blotting. *B. gibsoni*-infected dog erythrocytes and normal dog erythrocytes were treated with a 0.83% NH_4Cl solution and then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was electrically transferred to a nitrocellulose membrane, and Western blot analysis was performed as described in Chapter 1.

ELISA. Fifty microlitters of purified protein or control GST diluted in a coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) was used to coat the individual wells of 96-well microtiter plates. The ELISA was performed as described in Chapter 1. The cut-off value of 0.12 was calculated from the results of the ELISA of 28 specific pathogen-free (SPF) dog sera as follows: 0.051 (mean value) + 3 × 0.022 (standard deviation).

Double-antibody sandwich ELISA. In the sandwich ELISA, rabbit anti-rBgSA1 polyclonal IgG was purified using Econo-Pac A Columns (Bio-RAD, USA) 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°C 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°C for 2 h. The plates were incubated at 37°C for 30 min with 50 µl of each of the serial plasma samples from a dog experimentally infected with *B. gibsoni*. After washing 6 times with PBS-Tween 20, each mouse polyclonal serum diluted in a blocking solution was added in each well as a detection antibody. After washing 6 times again, the plates were incubated with 50 µl per well of HRPO-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).

2-3. Results

Identification of the genes encoding putative secreted antigens. A total of 96 clones were isolated from the cDNA expression library with serum from a dog immunized with plasma from a *B. gibsoni*-infected dog. Alignment of the sequences indicated that the clones represent partial or complete ORFs of eleven genes or gene families. Table 1 shows a summary of the isolated cDNA sequences, along with some features of genes or putative encoded polypeptides. A novel gene named BgSA1 was identified from 84 clones.

Seven genes or gene families, including BgSA1, appear to be novel sequences. No significant homologues were found in the GenBank databases by BLASTP analysis of the predicted polypeptide sequence. Among them, BgSA2 is another novel gene or gene

family identified from three clones. Protein analysis programs predicted that BgSA2 encoding 283 aa with a cleavable N-terminal signal peptide. However, the sera from dogs infected with *B. gibsoni* could not recognize the recombinant BgSA2 expressed in *E. coli* (data not shown). The remaining four clones (Bg07, Bg21, Bg27, and Bg68) show identity with *Plasmodium* species, but all of them contain only partial ORFs. None of the other sequences was predicted to have an N-terminal signal sequence, except BgSA1 and BgSA2; however, because the ORFs had no start codons at the beginning of the nucleotide sequence, it is possible that the N-terminals were not contained in these clones.

Characterization of the *BgSA1* gene. The cDNA sequence of BgSA1 was completely sequenced and is shown in Fig. 7. The full length of BgSA1 contains a single open reading frame of 1,632 nucleotides encoding a polypeptide of 544 amino acid residues. The hydrophobic region at the N-terminus of BgSA1 clearly shows the characteristics of a signal peptide, and the most likely cleavage site was predicted between 23 and 24 amino acids. The molecular weight of the mature protein with 521 amino acid residues is 59 kDa, as calculated with MacVector, version 7.0, software (Oxford Molecular Group, UK).

Southern blot analyses indicated that there were at least four copies of the BgSA1 gene in the genome of *B. gibsoni* (Fig. 8). The genomic DNA of the *BgSA1* gene was cloned into a pGEM-T vector (Promega, USA) and subjected to DNA sequencing analyses. The sequence was completely identical to the *BgSA1* cDNA without any introns (data not shown).

Expression of BgSA1 in *Escherichia coli.* The *BgSA1* gene was cloned into the prokaryotic expression vector pGEX-4T-3, and the resulting plasmid was transformed

into an *E. coli* BL21 strain. The rBgSA1 with a molecular weight of 85 kDa was expressed as a fusion protein with GST with expected molecular weight (data not shown). Sera from dogs experimentally infected with *B. gibsoni* could recognize the GST-fused rBgSA1 in Western blotting (data not shown). This result suggested that the rBgSA1 expressed in *E. coli* maintained theirs antigenicity. In addition, specific antibodies against *B. gibsoni* were induced in both mice and rabbits with immunization of rBgSA1 expressed in *E. coli* (data not shown).

Identification of the native BgSA1 of *B. gibsoni*. Mouse anti-rBgSA1 polyclonal serum was used to identify the native BgSA1 in the lysate of *B. gibsoni* parasites. As shown in Fig. 9, specific band with size of 59 kDa was detected in *B. gibsoni*-infected red blood cells by Western blotting but not in normal ones. In order to determine the cellular localization of BgSA1, a thin blood smear was used to perform IFAT with the mouse antiserum and observed under a confocal laser microscope; the specific fluorescence was observed on the *B. gibsoni* merozoites (Fig. 10).

Application of rBgSA1 in ELISA for the detection of specific antibody. The potential of rBgSA1 as diagnostic antigen was evaluated in an indirect ELISA. All 11 serum samples from *B. gibsoni*-infected dogs were positive (optical density > 0.12), whereas the serum samples from the dogs infected with *B. canis canis*, *B. canis vogeli*, *B. canis rossi*, *N. caninum*, *L. infantum*, and uninfected dogs were negative in the ELISA (Fig. 11). Specific antibody against BgSA1 could be detected on the 8th day post-infection. The antibody level was maintained until 541 days post-infection even when the infection was in chronic stage, which is characterized by a recovering hematocrit rate (data not shown) and a significantly low level of parasitemia (Fig. 12).

A total of 931 dog serum samples from different regions of Japan were detected by

ELISA with rBgSA1. As shown in Tables 2 and 3, 72 (7.7%) of the tested serum samples were positive in ELISA with rBgSA1, 41 (4.4%) of the tested samples were positive in ELISA with rBgP50 (optical density > 0.1), and 53 (5.6%) were positive in PCR. Moreover, all of the positive samples, except three in ELISA with rBgP50, were also positive in ELISA with rBgSA1. It is notable that 20 positive samples in PCR (18 of them are positive in IFAT, data not shown) showed a negative reaction in ELISA with rBgP50, but only five positive samples in PCR (3 of them are also negative in IFAT, data not shown) were negative in ELISA with rBgSA1, and four of the five samples were also negative in ELISA with rBgP50. The low sensitivity of rBgP50 might have been caused by the antigenic variation of this antigen because it is exposed on the surface of the merozoite.

Evaluation of double-antibody sandwich ELISA for the detection of circulating BgSA1. Serial blood plasma samples from a dog experimentally infected with *B. gibsoni* were detected by using double-antibody sandwich ELISA. As shown in Fig. 13, the circulating BgSA1 was detectable in the infected plasma with a parasitemia of 0.2%, as determined by the sandwich ELISA. In addition, the infected dog developed significant antibody responses against BgSA1 in the same period.

2-4. Discussion

The aim of this chapter was to screen and identify circulating antigen candidates for their development and use in diagnostic methods to detect antigens of *B. gibsoni*. In a previous report, the authors described a method to screen soluble antigens from *B. microti*, and several of them showed encouraging results (Homer et al., 2003). I used the

same method to isolate the genes encoding soluble antigens from a cDNA library constructed from *B. gibsoni* merozoite mRNA by immunoscreening. The serum collected from a healthy dog immunized with an infected dog's plasma containing secreted antigens putatively was used for the screening. Several cDNA sequences were isolated from the library.

The procedure used in this chapter is different from those used in previous ones because it was designed, by the nature of the screening itself, for the isolation of secreted antigens. Moreover, the antigen captured by the rabbit polyclonal anti-rBgSA1 IgG in the sandwich ELISA provided further important evidence that the novel secreted parasite antigen was released into the bloodstream during the merozoite stage. These results indicated that the method used in this chapter is effective to screen immunodominant antigens secreted or excreted into plasma by parasites.

The rBgSA1 expressed in *E. coli* was evaluated in ELISA as antigens for the detection of specific antibody against *B. gibsoni* in dogs. The ELISA could differentiate the sera of *B. gibsoni*-infected dogs from non-infected dogs and *B. caninis*-infected dogs. In addition, the specific antibody was detectable as early as 8 days post-infection, and the antibody titers were maintained at a high level until 541 days post-infection. These results indicated that the ELISA with rBgSA1 might be useful method for the detection of the antibody against *B. gibsoni* in both acutely and chronically infected dogs. The ELISA with rBgSA1 was comparatively evaluated with previously established ELISA with rBgP50 (Verdida et al., 2004) and PCR using a large number of samples collected from dogs in Japan (Ikadai et al., 2004). Of the 931 samples analyzed, 72 (7.7%) samples were positive by ELISA with rBgSA1, whereas 41 (4.4%) and 53 (5.6%) samples were positive by ELISA with rBgP50 and PCR, respectively. In addition, of 53

PCR-positive samples, 48 (90.6%) samples were positive by ELISA with rBgSA1, whereas 33 (62.3%) samples were positive by ELISA with rBgP50. These results indicated that the sensitivity of ELISA with rBgSA1 was much higher than that of ELISA with rBgP50 and PCR. However, the reason that the 5 PCR-positive samples were ELISA-negative remains unclear (Tables 2 and 3). A possible reason for the result might be the antigenic variation of BgSA1 between field and NRCPD strains. In contrast, the 22 PCR-negative samples were positive in the ELISA with rBgSA1. These samples might have been taken from the dogs with a chronic infection without circulating parasites in peripheral blood. These results indicated that rBgSA1 is more reliable in serological diagnosis as a diagnostic antigen target.

Sometimes the detection of antibodies is unreliable as a method to determine the infection status of dogs because the titer of the antibodies against the parasites can remain very high for a long time even when the parasites have been completely eliminated. On the other hand, it is also necessary to determine the total parasite burden that accurately defines disease severity as a criterion of infection status that cannot be reflected by peripheral blood parasitemia. For example, it is important to decide when to stop the treatment for infected individuals or population groups. Circulating antigens secreted into plasma by parasites might be employed as diagnostic targets to be detected in serological tests to predict the total parasite biomass of infected dogs. In malaria, circulating antigens have been successfully used to develop serological tests to detect antigens (Noedl et al., 2002; Alifrangis et al., 2004; De Arruda et al., 2004), and several of them have already been used in the development of a model to estimate the total parasite biomass (Dondorp et al., 2005) or used in a commercial ELISA kit to test the sensitivity of a drug against *Plasmodium* in sandwich ELISA (Noedl et al., 2002). In

this chapter, I designed a double-antibody sandwich ELISA to detect BgSA1 in the plasma of dogs infected with *B. gibsoni.* The native BgSA1 could be detectable in the plasma of dogs infected with *B. gibsoni* when the parasitemia reached 0.2% after infection. The native antigen in the plasma of infected dogs, therefore, suggests the existence of *B. gibsoni* parasites in the peripheral blood. The sandwich ELISA titer is well associated with the parasitemia of *B. gibsoni*-infected blood post-infection. These results indicated that assays based on the secreted BgSA1 were suitable for the detection of parasite antigens in plasma. The sensitivity might be improved by using monoclonal antibodies instead of polyclonal antibodies or combining this system with an avidin/biotin system (Khusmith et al., 1992; Benito et al., 2005). Unfortunately, I only detected samples in the acute phase of infection in this experiment because all of the collected samples were serum samples, but it was demonstrated that plasma samples seemed to be more sensitive for the detection of native BgSA1. Therefore, further study on the detection of more samples in an acute or chronic stage of infection is still necessary to determine whether the sandwich ELISA to detect the antigen in plasma can reflect the infection status or parasite burden in infected dogs.

In short, the screening descried here was to identify immunodominant proteins that are exposed to the host immune system as a natural process of infection. As discussed above, the sensitivity and specificity of the secreted BgSA1 indicated its advantages for use as a target in a serodiagnostic test for the detection of both antibody and antigens. Moreover, the characterization of the novel molecules will increase the understanding of the mechanism involved in the parasite life cycle. More important is the possibility that the secreted antigens are associated the erythrocyte invasion of parasite. Therefore, secreted antigens are considered to be potential candidate molecules for the

development of new innovation strategies. It has been reported that the culture-derived soluble parasite antigens of several *Babesia* spp. could provide protective immunity against parasite infection (Valentin et al., 1993; Schettes et al., 1994; Lewis et al., 1995; Beniwal et al., 1997). The secreted BgSA1 is, therefore, promising potential vaccine against *B. gibsoni* infection. In addition, the detection of field serum samples suggested that *B. gibsoni* infection is a serious disease that should be reckoned with, especially in endemic areas. My next step is to determine the potential of the rBgSA1 as subunit vaccines to control canine *B. gibsoni* infection.

2-5. Summary

Serum from a dog immunized with blood plasma from a *B. gibsoni*-infected dog, putatively containing secreted antigens, was used to screen a cDNA expression library. A novel gene encoding BgSA1 was identified from the isolated clones. The BgSA1 was identified and characterized in this chapter. The serum raised in mice immunized with the recombinant BgSA1 expressed in *E. coli* could recognize a native parasite protein with a molecular mass of 59 kDa. Comparing with the previously established ELISA with rBgP50 as antigen, the ELISA with recombinant BgSA1 as the antigen is more sensitive when they were used to detect field samples. Moreover, the sandwich ELISA with anti-rBgSA1 antibody could detect the circulating BgSA1 in a serial blood plasma from a dog experimentally infected with *B. gibsoni*. These results indicated that BgSA1 could be useful target for the development of a diagnostic test for the detection of specific antibody and circulating antigen.

Table 1. Summary of clones isolated through immunoscreening.

Name	Best BlastX match to the database of NCBI[1]	E-value[2]	Number of isolated clones	Signal peptide[3]	Prediction
BgSA1	Unknown		84	Yes	Full-length
BgSA2	Unknown		3	Yes	Full-length
Bg07	Eukaryotic translation initiation factor 3 submit 10, putative [*P. falciparum* 3D7]	3e-15	1	Unknown	Partial
Bg18	Unknown		1	Unknown	Partial
Bg21	Protein serine/threonine phosphatase, putative [*P. chabaudi*]	2e-61	1	Unknown	Partial
Bg22	Unknown		1	Unknown	Partial
Bg26	Unknown		1	Unknown	Partial
Bg27	Sortilin, putative [*P. falciparum* 3D7]	2e-11	1	Unknown	Partial
Bg36	Unknown		1	Unknown	Partial
Bg50	Unknown		1	Unknown	Partial
Bg68	Conserved hypothetical protein [*P. berghei*]	2e-08	1	Unknown	Partial

[1]BlastX performed with NCBI BlastX program and an E-value cut-off of 0.05 against the protein database of NCBI.
[2]Indicates the significance of the match to sequences of the previous column.
[3]Results from all sequences after submission of the best open reading frame to the SignalP server.

Table 2. Comparison of BgSA1-ELISA with BgP50-ELISA for the detection of field samples.

No. (%) with BgSA1-ELISA	No. (%) with BgP50-ELISA		Total no. (%)
	+	-	
+	38 (4.1)	34 (3.7)	72 (7.7)
-	3 (0.3)	856 (91.9)	859 (92.3)
Total no. (%)	41 (4.4)	890 (95.6)	931 (100)

Table 3. Comparison of BgSA1-ELISA with PCR for the detection of field samples.

No. (%) with BgSA1-ELISA	No. (%) with PCR		Total no. (%)
	+	-	
+	48 (5.3)	22 (2.4)	70 (7.7)
-	5 (0.5)	838 (91.8)	843 (92.3)
Total no. (%)	53 (5.8)	860 (94.2)	913 (100)

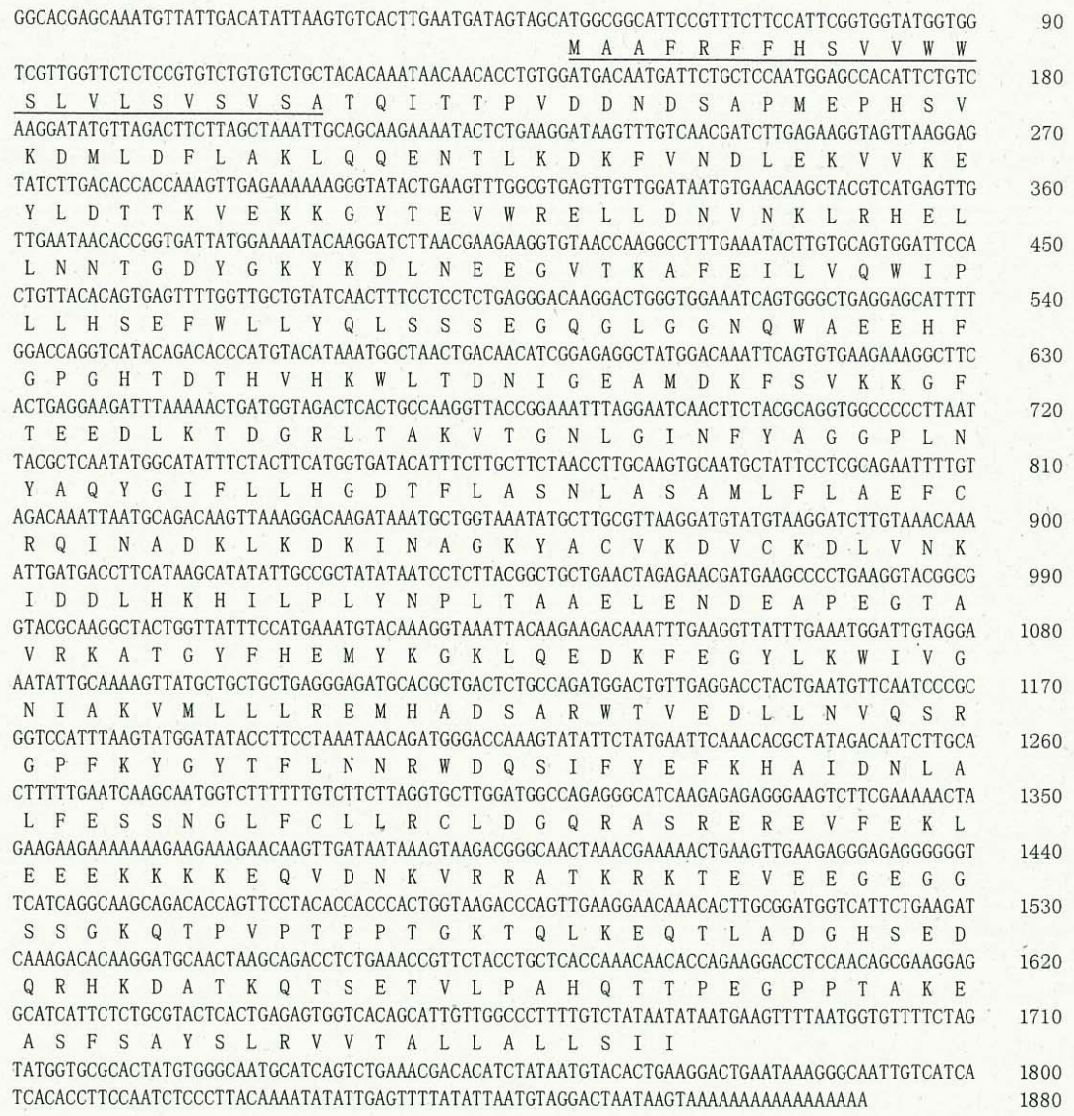

Fig. 7. Nucleotide and predicted amino acid sequences of the cDNA coding for BgSA1 from *B. gibsoni*. The predicted signal peptide is underlined.

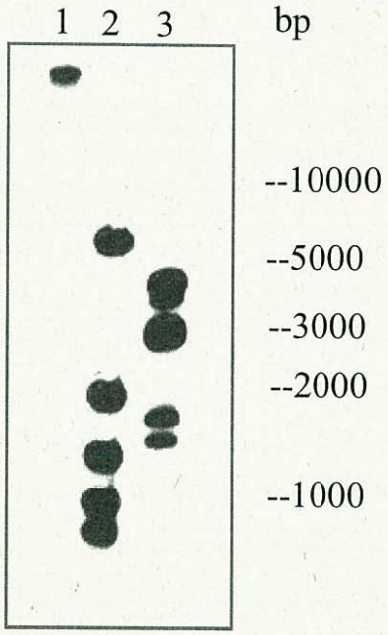

Fig. 8. Southern blot analysis. The *B. gibsoni* genomic DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), and *Taq*I (lane3). There is one *Eco*RI cleavage site and one *Taq* I cleavage site, and no *Bam*HI cleavage site in the *BgSA1* gene.

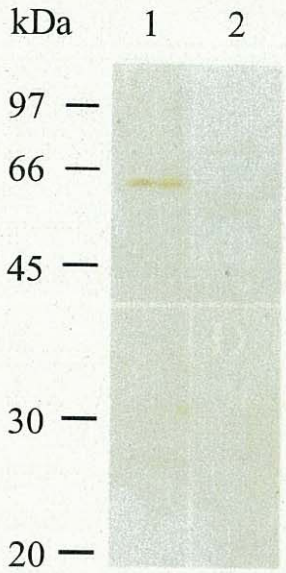

Fig. 9. Western blot analysis of native BgSA1 in merozoite lysate with a mice anti-rBgSA1 serum. The samples were separated on a 12% polyacrylamide gel and transferred onto a PVDF membrane. Lane 1, *B. gibsoni*-infected dog erythrocyte lysate; lane 2, normal dog erythrocyte lysate.

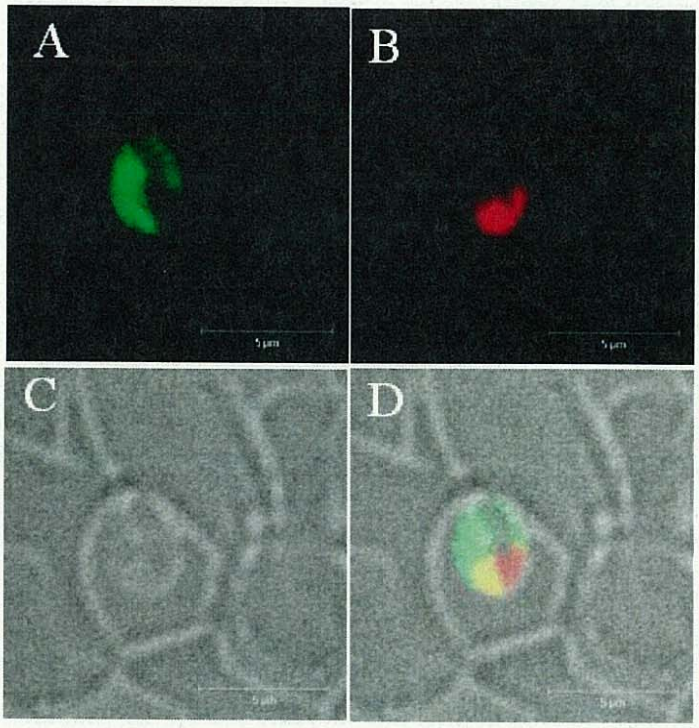

Fig. 10. Observation of an antigen recognized by a mouse anti-rBgSA1 serum in confocal laser micrographs. A, Immunofluorescent staining of *B. gibsoni* merozoites with mouse anti-rBgSA1 serum. B, Propidium iodide staining of *B. gibsoni* merozoite nuclei. C, Phase-contrast images of *B. gibsoni* merozoites. D, Panels A and B are overlaid on panel C. The images were derived from a single section.

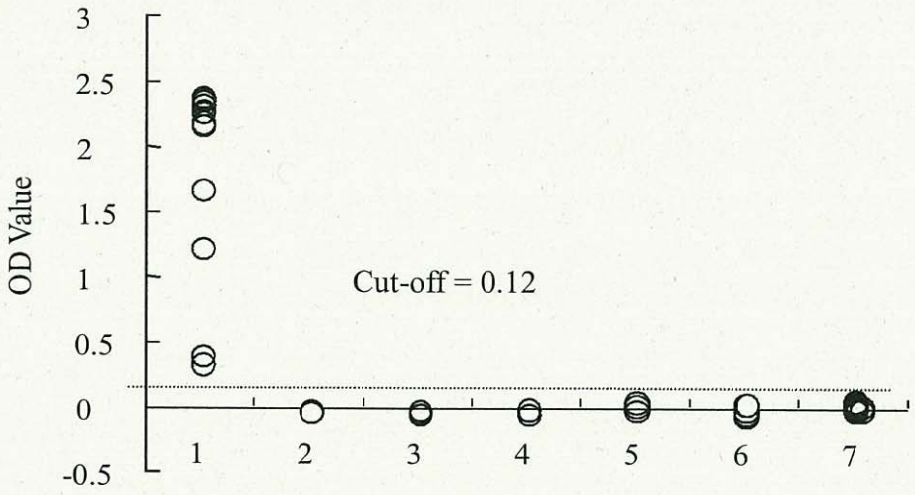

Fig. 11. Values of the ELISA with experimentally infected dog sera. Lane 1, sera from *B. gibsoni*-infected dogs (n=14); lane 2, sera from *B. canis canis*-infected dogs (n=5); lane 3, sera from *B. canis rossi*-infected dogs (n=3); lane 4, sera from *B. canis vogeli*-infected dogs (n=2); lane 5, sera from *N. caninum*-infected dogs (n=4); lane 6, sera from *L. infantum*-infected dogs (n=5); lane 7, SPF dog sera (n=28).

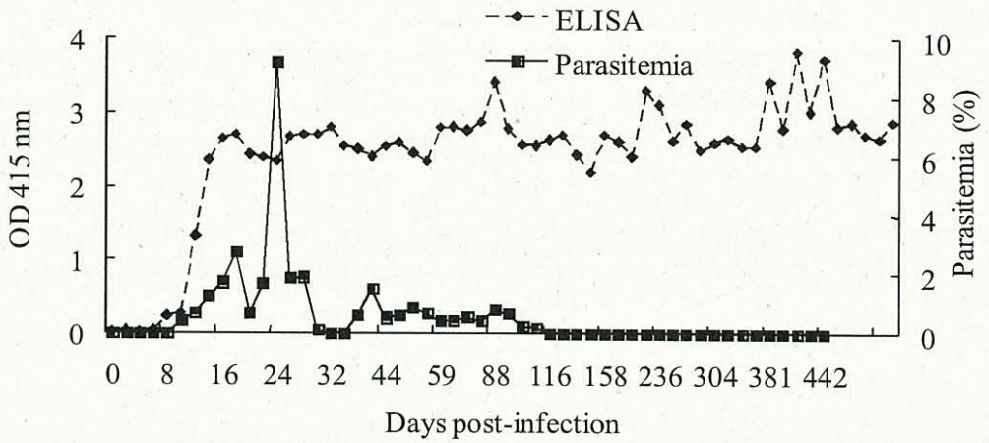

Fig. 12. Detection of antibody against BgSA1 in a dog experimentally infected with *B. gibsoni* by the ELISA with rBgSA1.

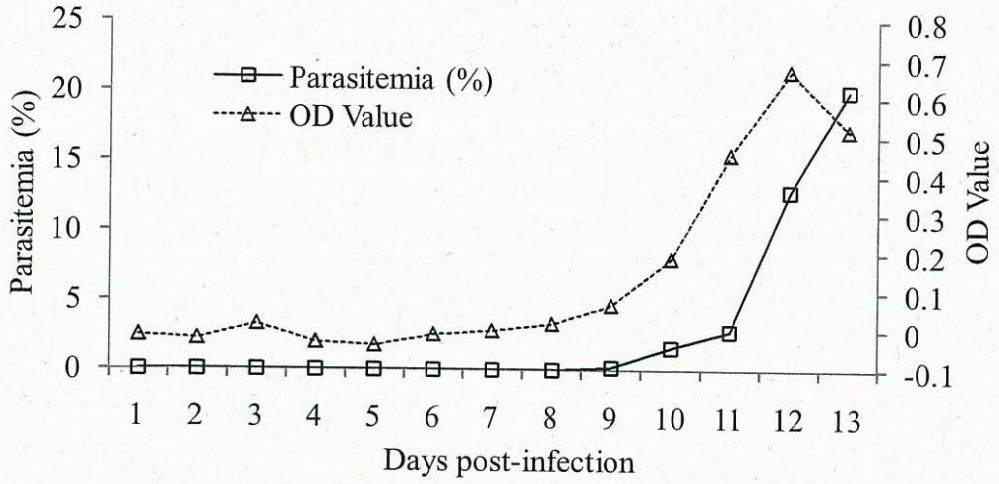

Fig. 13. Detection of circulating BgSA1 in plasma from a dog experimentally infected with *B. gibsoni* by a double-antibody sandwich ELISA.

Chapter 3

Development of an immunochromatographic test for the diagnosis of *Babesia gibsoni* infection

3-1. Introduction

An immunochromatographic test is also called lateral flow test or simply strip-test. It has been a popular platform for diagnostic test since its introduction in the late 1980s. ICTs are used for the specific qualitative or semi-quantitative detection of many analytes including antigens, antibodies, and even the products of nucleic acid amplification tests. ICTs are the simplest to use of all of the test formats, simply requiring the user to place the test strip in the specimen or added directly onto the strip itself and read the results after a specified amount of time. Results can usually be read in as little as 2 minutes, but around 15 minutes is more common.

Various serodiagnostic tests have been developed for diagnosing *Babesia gibsoni* infection, such as indirect fluorescence antibody test (IFAT) (Yamane et al. 1993), enzyme-linked immunosorbent assay (ELISA) (Fukumoto et al. 2004; Verdida et al. 2004), and immunochromatographic test (ICT) (Verdida et al. 2005) with native or recombinant proteins. Among these methods, the ELISA has many advantages, such as higher sensitivity, specificity, and greater objectivity in the determination of results. The ICT, however, is comparatively simple and easily handled, which makes it suitable for clinical or field applications.

Several ICT methods for detecting antigens or antibodies in protozoan parasites have

been recently developed (Farcas et al., 2003). An ICT based on the recombinant BgP50 (rBgP50) for detecting specific antibodies against *B. gibsoni* has also already established (Verdida et al., 2005). However, it has been shown that the rBgP50 is not sensitive enough as an antigen in the ELISA to diagnose *B. gibsoni* infection. The study in Chapter 2 indicated that the sensitivity of the ELISA could be improved by using the recombinant BgSA1 (rBgSA1) instead of rBgP50. BgSA1 is a *B. gibsoni*-derived circulating plasma antigen in infected dogs which could be used to detect specific antibody in an ELISA (Chapter 2). In this chapter, an ICT using the rBgSA1 expressed in *Escherichia coli* was described, and its potential in field serum samples was evaluated.

3-2. Materials and methods

Serum samples. The ICT with rBgSA1 was evaluated with sequential serum samples from a dog experimentally infected with *B. gibsoni.* In addition, serum samples from dogs experimentally infected with *B. cannis* subspecies, *N. caninum*, *L. infantum*, and healthy dogs were used to determine the specificity of ICT with rBgSA1. A total of 94 field serum samples collected in Okinawa, Japan were then investigated by using the ICT.

Expression and purification of rBgSA1 in *E. coli.* The rBgSA1 was expressed in *E. coli* as a fusion protein with glutathione *S*-tranferase (GST) as described in Chapter 2. Purification of the rBgSA1 was performed with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, USA), and the GST was cleaved by thrombin protease according to the manufacturer's instructions.

Assemblage of ICT strips. The preparation of rabbit IgG against the rBgSA1 and the assemblage of ICT strips were carried out as described previously with some modification (Verdida et al. 2005). Briefly, the purified rBgSA1 (200 μg/ml) and the anti-rBgSA1 IgG (1 mg/ml) were jetted linearly on a nitrocellulose membrane as the test and control lines, respectively. Ten milliliters of gold colloid (British BioCell International, UK) was conjugated with 1 ml of purified rBgSA1 (200 μg/ml) and then sprayed on a piece of glass fiber. The nitrocellulose membrane with the rBgSA1 and IgG, as well as the conjugated pad, sample pad, and absorbent pad, was assembled on an adhesive card and cut into 2 mm-wide strips (Fig. 14).

Detection of the specific antibody by ICT. Forty microliters of serum was loaded on the sample pad of an ICT strip as positive. If both the control and test lines were colored purple red, the sample was recorded as positive. Otherwise, if only the control line was colored, the sample was recorded as negative (Fig. 14). If no line was colored, the test was considered to have failed.

ELISA. ELISA was performed as described in Chapter 2. The sample was considered positive when the mean absorbance, a calculation of the mean absorbance from duplicated wells with rBgSA1-GST subtracted the mean absorbance from duplicated wells with the control antigen, GST, was equal to or greater than 0.12.

3-3. Results

Detection of the specific antigbodies in sera from experimentally infected dogs. The ICT was evaluated with sequential serum samples from a dog experimentally infected with *B. gibsoni.* The specific antibody could be detected from as early as 10

days till 222 days after infection, which is quite consistent with the results of the ELISA (Fig. 15). In addition, serum samples from dogs experimentally infected with *B. cannis* subspecies, *N. caninum*, *L. infantum*, and healthy dogs were used to determine the specificity of rBgSA1-ICT. Only the serum samples from the *B. gibsoni*-infected dogs were positive in the ICT, but the other serum samples from the dogs infected with closely related parasites and from healthy dogs were negative (Fig. 16).

Detection of the specific antibody in field samples by ICT. A total of 94 field serum samples collected in Okinawa, Japan were then investigated by using ICT; 66 of them were collected from free-roaming dogs, and 28 were obtained from domestic dogs. The results were compared with the previously established ELISA with rBgSA1 (Chapter 2). The two methods showed good agreement of 95.8%. As shown in Table 4, 22 (23.4%) of the 94 samples were positive in the ELISA, and 24 (25.5%) were positive in the ICT. Three ELISA-negative samples were ICT-positive.

3-4. Discussion

The diagnosis of canine babesiosis, particularly in remote areas lacking laboratory support, or for the economic consideration, frequently relies on the dog's symptoms. The first symptoms of *B. gibsoni* infection are not specific. Clinicians often misdiagnose *B. gibsoni* infection and misdiagnosis often leads to the unnecessary prescription of babesiosis medications. Thus, increasing the accuracy of clinical diagnosis is becoming more important. Currently available tools for the detection of *B. gibsoni* infection remain mostly laboratory based, requiring trained personnel and equipment. A simple rapid test that enables early detection at the point of care where laboratory facilities are

not readily accessible is, therefore, an unmet need for the management of *B. gibsoni*. ICT methods have apparent advantages over other routine diagnostic tests. For example, no special expertise or equipment is required when the strips are commercially available, and the test takes less than 15 minutes to complete. Furthermore, the ICT strip is quite stable during long storage under ordinary conditions.

In this chapter, an ICT with rBgSA1 was developed. The specific antibody could be detected as early as 10 days till 222 days after infection, which is quite consistent with the results of the ELISA with rBgSA1. Only the serum samples from the *B. gibsoni*-infected dogs were positive in the ICT, but the other serum samples from the dogs infected with closely related parasites and from healthy dogs were negative. Furthermore, the ICT showed good agreement with the ELISA with rBgSA1 established in Chapter 2. These results indicated that the ICT is a sensitive and specific method for the diagnosis of *B. gibsoni* infection.

In the field samples, three ELISA-negative samples were ICT-positive. This might be because the dogs were at a very early stage of *B. gibsoni* infection, at which only immunoglobulin M was detectable by ICT, or because much more concentrated samples were loaded for the ICT than for the ELISA (Table 4). In addition, all ELISA-positive samples were ICT-positive except one, with an optical density at 415 nm of 0.136, which had a weak positive reaction in the ELISA; however, this reaction is probably false-positive because the optical density was around the cut-off point of 0.12.

Collectively, the ICT with rBgSA1 is a rapid and simple diagnostic method. It is a more suitable diagnostic tool for the detection of the specific antibody in *B. gibsoni* infection of dogs under field conditions because it has higher sensitivity than the previously established method.

3-5. Summary

An ICT using rBgSA1 for the detection of antibody against *Babesia gibsoni* was developed and evaluated. Only the serum samples collected from dogs infected with *B. gibsoni* were positive in the ICT, but the serum samples from dogs infected with closely related parasites and from healthy dogs were negative. The specific antibody could be detected in a dog experimentally infected with *B. gibsoni* at both the acute and chronic infection stages by the ICT. To evaluate the clinical application of the ICT, a total of 94 serum samples collected from domestic dogs in Japan were tested with the ICT and the previously ELISA with rBgSA1. Twenty-one of the tested samples (22.3%) were positive in both the ICT and the ELISA. The concordance between the ELISA and the ICT was found to be 95.8%. These results suggested that the ICT using rBgSA1 is rapid, simple, accurate, and suitable for the diagnosis of *B. gibsoni* infection of dogs in the field.

Table 4. Comparison of the ELISA and the ICT both with rBgSA1 for the detection of *B. gibsoni* infection in dogs in Japan.

ELISA	ICT		
	No. (%) positive	No. (%) negative	Total no. (%)
No. (%) positive	21 (22.3)	1 (1.1)	22 (23.4)
No. (%) negative	3 (3.2)	69 (73.4)	72 (76.6)
Total no. (%)	24 (25.5)	70 (74.5)	94 (100)

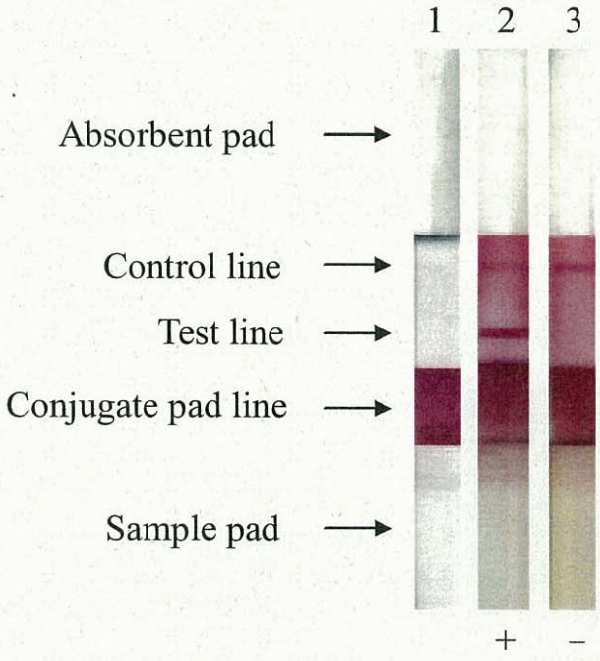

Fig. 14. Examples of ICT strips before (lane 1) and after (lanes 2 and 3) testing. Symbols: +, positive result; -, negative result.

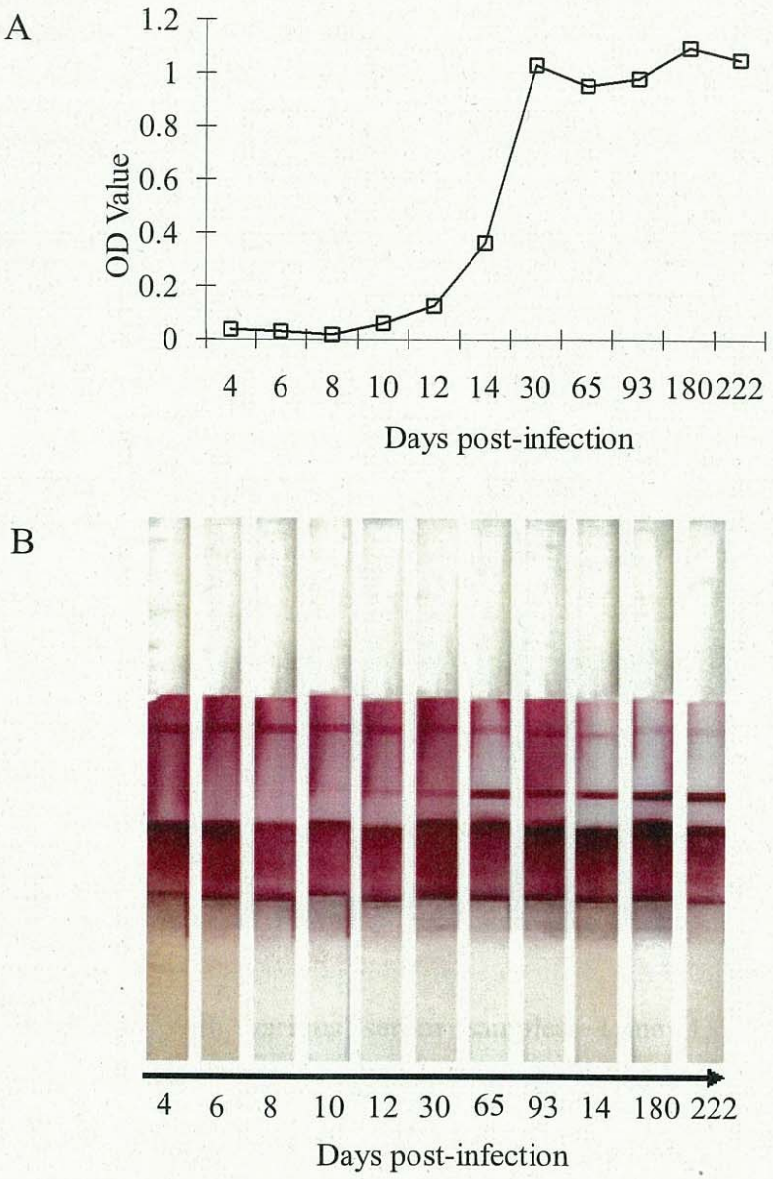

Fig. 15. Specific antibody responses in sequential serum samples from a dog experimentally infected with *B. gibsoni* detected by the ELISA and the ICT, both with rBgSA1. A, IgG examined by the ELISA; B, antibody responses examined by the ICT.

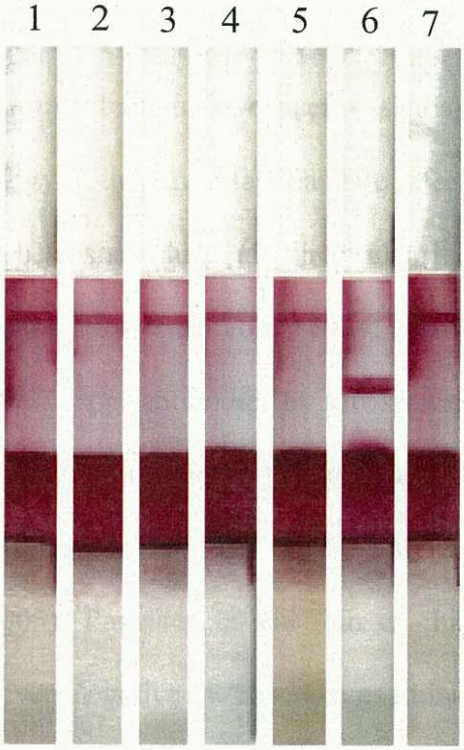

Fig. 16. ICT results with various serum samples. Lane 1, serum from a *B. gibsoni*-infected dog; lane 2, serum from a *B. canis canis*-infected dog; lane 3, serum from a *B. canis rossi*-infected dog; lane 4, serum from a *B. canis vogeli*-infected dog; lane 5, serum from a *N. caninum*-infected dog; lane 6, serum from a *L. infantum*-infected dog; lane 7, an SPF dog serum.

General discussion

Canine babesiosis caused by *B. gibsoni* is currently endemic in Japan and reemerging in many countries of the world. The development of specific, sensitive and inexpensive serological tests for *B. gibsoni* infection have been essential in the accumulation of knowledge on the epidemiology of this important parasite infection.

Diagnostic methods for babesiosis include: (1) direct identification of the *Babesia* spp. parasites by microscopic examination (Saal et al., 1964; Maronpot and Guindy, 1970), (2) indirect detection of *Babesia* spp. antibodies by serological tests (Ross et al., 1968; Yamane et al., 1993; Vercammen et al., 1995), (3) detection of *Babesia* DNA by using PCR or PCR-RFLP (Bashiruddin et al., 1999; Breitschwerdt et al., 2003; Oliveira-Sequeira et al., 2005). Parasites in the thin or thick blood films are true evidence of infection. However, negative microscopic examination does not exclude it. Actually, in the early phase of infection, in immune dog populations and in those which have been therapeutically treated, the detection of *Babesia* parasites in the stained blood films is uncommon. Direct identification of the *Babesia* parasites by microscopic examination of blood films is also time-consuming and tedious, especially when the vast majority of smears are negative. PCR method, however, requires highly trained personnel, as well as costly equipments, thus limiting its application for the mass scale diagnosis of many samples. The accumulation of the fundamental knowledge concerning the immunoserology of *Babesia* infections has led to the development of serological techniques which provide a means for studying the detection of animals with subclinical infections. Although the methods devised to date for serological diagnosis of

babesiosis are still obviously imperfect, the admittedly serological techniques now available will still be widely used for diagnosing *Babesia* spp. infections until better techniques are developed.

The ELISA principles are widely applied for the demonstration of antibodies against a variety of infectious agents, including *B. gibsoni.* But the currently available serological tests still need to be improved. In this study, an effort was made to isolate immunodominant antigens for development of more sensitive and specific ELISA. A novel gene encoding a highly repetitive antigen was isolated and named as BgIRA, which was identified as a major population among all positive cDNA clones. The high level of antibody response suggested that BgIRA plays a dominant role in the immune response of *B. gibsoni* infection. The ELISA with recombinant BgIRA might be a useful method for the detection of the antibody to *B. gibsoni* in both acutely and chronically infected dogs.

The ability of a test to distinguish between infected and non-infected individuals is often described by its diagnostic sensitivity and specificity. The sensitivity is defined as the proportion of infected individuals to be correctly identified by the test, and the specificity is the proportion of non-infected individuals to be correctly identified. In this regard, field samples and the serum samples from the dogs experimentally infected with closely related protozoan parasites, such as *B. canis canis*, *B. canis vogeli*, *B. canis rossi*, *N. caninum*, and *L. infantum* were used in the ELISA with the rBgIRA. No cross reaction was observed. For field samples, the ELISA with rBgIRA could detect all of the positive samples which were positive in a semi-nested PCR. Both the specificity and sensitivity of BgIRA shown in my data indicated that it is a good diagnostic antigen.

Immunity to babesiosis is based on so-called "premunition", or coinfectious

immunity. In the serology of babesiosis, there are still no tests suitable for the detection of early infection. In all tests reported, *Babesia* spp. parasitaemia appears in the blood before the rise of detectable *Babesia* spp. antibodies; therefore, the negative results do not indicate that the animal is free from infection. Moreover, none of the available tests will revert to negative within a relatively short time after an infection had terminated by autosterilisation or chemotherapy. There is need for a serological test to be able to determine whether eradication programmes have been successful or whether transmission of babesiosis in endemic areas has been interrupted. Therefore, efforts must continue toward the preparation of more sensitive and specific *Babesia* spp. antigens which will be able to define the status of infected animals.

In this context, an experiment was designed to screen and identify circulating candidate antigens for their production and use in diagnostic methods to detect antigens of *B. gibsoni*. The serum collected from a healthy dog immunized with an infected dog's plasma containing secreted antigens putatively was used for the screening. Several cDNA sequences were isolated by the immunoscreening. The analysis of the selected sequences indicated that the method used in this study is effective to screen immunodominant antigens secreted or excreted into plasma by the parasite. A gene encoding a secreted antigen named as BgSA1 was chosen and characterized.

In order to develop a test to predict the real infectious status of infected dogs, I designed a double-antibody sandwich ELISA to detect BgSA1 in the plasma of dogs infected with *B. gibsoni*. The native BgSA1 was detectable in the plasma of dogs infected with *B. gibsoni* when the parasitemia reached 0.2% after infection. Therefore, the presence of native antigen in the plasma of infected dogs suggests the existence of *B. gibsoni* parasites in the peripheral blood. The sandwich ELISA titer is associated with

the parasitemia of *B. gibsoni*-infected blood post-infection. These results indicated that assays based on the secreted BgSA1 were suitable for the detection of parasite antigens in plasma and therefore could be used as a method to define the current infectious status.

Soluble parasite antigens (SPA) have been used for a number of *Babesia* vaccines in order to stimulate protection without resulting in carrier status (Goodger et al., 1987; Goodger et al., 1992; Montenegro-James et al., 1992; Schetters et al., 1997; Schetters et al., 2001). The *B. canis* SPA vaccine, used in Europe under the name "Pirodog", is made up of exogenous antigens (Moreau et al., 1989). The vaccine limited the level of parasitemia, and the decline in packed cell volume (PCV), anemia, as well as splenomegaly associated with infection (Schetters et al., 1997). On the other hand, a culture-derived endogenous *B. bovis* or *B. bigemina* SPA vaccine was tested in Venezuela where bovine babesiosis is endemic and imported cattle suffer significant losses (Montenegro-James et al., 1992). The vaccine reduced the incidence of clinical disease and prevented death, however, the level of protection was not as high as with live vaccines. Alternatively, an endogenous (as opposed to exogenous) SPA vaccine against *B. bovis* was derived from a fraction of infected erythrocytes collected from infected cattle (Goodger et al., 1987; Goodger et al., 1992). The vaccinated cows had a lower mean daily parasitemia and survived homologous challenge, whereas the controls had a higher daily parasitemia and did not survive challenge. SPA vaccines are safer, more stable, and easier to develop than live vaccines. Needless to say, the BgSA1 in this study might be promising vaccine candidate. Moreover, the characterization of the novel molecule will increase the understanding of the mechanism involved in the parasite life cycle.

In addition to the sensitivity and specificity, a practical test for the diagnosis of babesiosis needs to be simple to perform; the test must be rapid; the cost should be minimal; and the test should be capable of performing reliable results in various laboratories and under different field conditions. The ICT was obviously the test which meets the criteria. This method has been a popular platform for rapid tests since its introduction in the late 1980s. In this study, an ICT with the rBgSA1 expressed in *E. coli* was described, and its potential in field serum samples was evaluated. These results indicated that the ICT with rBgSA1 is a rapid, reliable, and practical test for the diagnosis of *B. gibsoni* infection.

The parasite strain used in this study was the NRCPD strain, which was isolated from Japan (Fukumoto et al., 2000). Recent reports have indicated that there are at least three species of small piroplasms that infect dogs. Molecular characterization proved that they are genetically distinct (Kjemtrup et al. 2000; Kjemtrup et al. 2006; Garcia et al. 2006). Whether the serological tests established here could differentiate them or not is yet to be determined.

General summary

The aim of this study is to develop serological diagnostic method for *Babesia gibsoni* infection. Immunological screening of a cDNA library derived from *B. gibsoni* was performed with different sera to isolate immunodominant antigens. A novel gene encoding a highly repetitive antigen was identified and named as BgIRA. BgIRA plays a dominant role in the immune response in dogs infected with *B. gibsoni*. The specificity and sensitivity of the recombinant BgIRA in an ELISA indicated that this antigen might be useful in a diagnostic test.

A special experiment was also designed to isolate the circulating proteins in order to isolate the specific secreted antigens in this study. A novel gene encoding a secreted antigen was identified and named as BgSA1. Comparing with the previously established ELISA with recombinant BgP50 as antigen, the ELISA with recombinant BgSA1 as the antigen is more sensitive when they were used to detect field samples. Moreover, the sandwich ELISA with anti-rBgSA1 antibody could detect the circulating BgSA1 in a serial blood plasma from a dog experimentally infected with *B. gibsoni*. BgSA1 is *B. gibsoni*-specific antigen and could be useful target for the development of a diagnostic test for the detection of specific antibody and circulating antigen.

Finally, an ICT method was developed with rBgSA1. The concordance between the ELISA with rBgSA1 and the ICT was found to be 95.8%. My data indicated ICT using rBgSA1 is rapid, simple, accurate and suitable for the diagnosis of *B. gibsoni* infection of dogs in the field.

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