Development of Serodiagnostic Methods for Bovine Babesiosis

（ウシバシア症の血清診断法の開発）

2009

KHUKHUU Altangerel

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

Contents

Abbreviations

B. bovis	:	*Babesia bovis*
B. bigemina	:	*Babesia bigemina*
BvP36	:	*Babesia bovis* protein 36
BvP29	:	*Babesia bovis* protein 29
CFT	:	Complement fixation test
DNA	:	Deoxyribonucleic acid
FITC	:	Fluorescein isothiocianate conjugate
ELISA	:	Enzyme linked immunosorbent assay
EDTA	:	Ethylenediaminetetraacetic acid
E. coli	:	*Escherichia coli*
GST	:	Glutathione S-transferase
IFAT	:	Immunofluoresence antibody test
IgG	:	Immunoglobin G
IPTG	:	Isopropyl-b-D-thiogalactopylanoside
IRBC	:	Infected red blood cells
i.p	:	Intraperitoneally
LB	:	Luria-Bertani medium
MW	:	Molecular weight
N. caninum	:	*Neospora caninum*
OD	:	Optical density
PBS	:	Phosphate-buffer saline
PBST	:	Phosphate-buffer saline containing 0.05% Tween 20
PCR	:	Polymerase chain reaction

P. berghei	:	*Plasmodium berghei*
RAP-1	:	Rhoptry-associated protein 1
RBC	:	Red blood cells
RT	:	Room temperature
SD	:	Standart deviation
SE	:	Standart error
SDS-PAGE	:	Sodium dodesyl sulfate polyacrylamide gel electrophoresis
ORF	:	Open-reading frame
TBDs	:	Tick- borne diseases
T. gondii	:	*Toxoplasma gondii*

Unit abbreviation

bp	:	base pair
kDa	:	kilo Dalton
h	:	hour
°C	:	degree celcius
M	:	moli/liter
Ml	:	mililitter
mM	:	milimol/litter
pM	:	picomol/litter
min	:	minute
mg	:	milligram
µg	:	microgram
ng	:	nanogram
sec	:	second
mm	:	millimeter
nm	:	nanometer

General introduction

1. Bovine babesiosis

Bovine babesiosis or redwater is a tick-born disease caused mainly by the intraerythrocytic protozoan parasites mainly *B. bovis* and *B. bigemina* (McCosker, 1981). The genus *Babesia* belongs to the phylum Apicomplexia, class Sporozoasida, order Piroplasmorida, and family Babesidae (Levine, 1985). The parasite was first described by Babes in 1888 in the blood of cattle showing haemoglobinuria (Babes, 1888), and the named *Babesia* was adopted in his honor (Ristic and levy, 1981). Ticks were first recognized as vectors of babesiosis in 1893 when Smith and Kilbourne described *Boophilus* ticks as the vector of *B. bigemina* (Smith and Kilbourne, 1893). It is now known worldwide that ticks are the main vectors of *Babesia* of domestic animals (Friedhoff and Smith, 1981).

Bovine babesiosis causes serious economic losses (Carson and Philips, 1981) in tropical and subtropical countries (McCosker, 1981). It has been estimated that bovine babesiosis endangers half a billion cattle throughout the world (Ristic and Levy, 1981). The diseases is endemic in many regions of world where the *Boophilus* ticks are present (Vial and Gorenflot, 2006). *B. bovis* and *B. bigemina* is present in many countries including North and South America, south Europe, Africa, Asia and Australia (Ristic, 1988). The acute cases of *B. bigemina* and *B. bovis* infections are characterized by fever, haemoglobinuria and anemia. (Shkap et al., 2005).

In addition to the clinical signs exhibited by the *B. bigemina* infected cattle, *B. bovis* infection is characterized by cerebral/nervous signs (Shkap et al., 2005) and the diseases is considered to be more severe than that of *B. bigemina* (Ristic, 1981). *B. bovis* infection usually occurs in the same areas as in case of *B. bigemina,* however, it has been identified in

some parts of Europe where the *Boophilus* ticks do not exist suggesting that there can be other vectors which may be involved in the transmission of *B. bovis* (Vial and Gorenflot, 2006).

2. Life cycle

Life cycle of the *Babesia* parasites involves two hosts, definitive vertebrate host and tick as intermediate host (Fig. 1). The life cycle in the vertebrate host begins with the injection of sprozoites by Ixodid ticks (Uilenberg, 2006), that can directly invade the red blood cells (RBC) (Brown et al., 2006), and differentiate into merozoites (Uilenberg, 2006). The process of erythrocyte invasion by merozoites can be divided into three steps, attachment, penetration and internalization. (Yokayama et al., 2006). Within the host RBC most of the merozoites transform into trophozoites and new merozoites will be produced by asexual binary fission (Homer et al., 2000). However, some of the trophozoites become gamatocytes that can not replicate in the erythrocytes (Rudzinska et al., 1979).

The Ixodid ticks ingest blood cells contained gamatocytes when they feed on an infected vertebrate host (Wenyon, 1926). Gametocytes then develop into male and female gametes in the tick gut and they fuse together to form zygotes, (Mehlhorn and Schein, 1984) that develop to vermicules which are capable of invading many tissues of ticks via the haemolymph (Rudzinska et al., 1983). In the infected female ticks, the vermicules reach the ovary and passes the infection to the next generation through eggs, larvae and nymph. When the progeny ticks attach to a new host, infectious sporozoites can be released from salivary glands during their feeding (Howell et al., 2007).

3. Diagnosis.

Three major methods, detection of parasites, detection of antibody and molecular identification, are currently used for the diagnosis of babesiosis.

3.1. Detection of parasites

The diagnosis of babesiosis should begin with the history of the clinical manifestation of the infected animals (Homer et al., 2000). The parasites could be detectable in circulting blood during the first few weeks of infection.

3.1.1. Microscopy: Examination of Giemsa-stained thin blood smears under the light microscopy is the most frequently used technique to detect the *Babesia* parasites (Etkind et al., 1980). Capillary blood smears are highly preferred over the venous blood smears since more parasites can be detected in the former (Tatchell, 1984). Thin blood smears or impression made from organs such as kidney, brain and heart muscle can also be examined under a microscope for the detection of parasites. However, sub-clinical infections and carrier stages cannot be detected by light microscopy alone (Calder et al., 1996).

3.1.2. *In vitro* culture: Many *Babesia* species such as *B. bovis* (Rodriguez et al., 1983), *B. bigemina* (Vega et al., 1985), *B. divergens* (Grande et al., 1997), *B. caballi* (Zweygarth et al., 1999, Avarzed et al., 1997) and *B. equi* (Holman et al., 1994, Avarzed et al., 1997) can be maintained in *in vitro* cultures. The continuous micro-aerophilous stationary phase culture system, an improved technique for the *in vitro* cultivation of *Babesia* parasites, allows culturing of the individual parasites (Vega et al., 1985, Igarashi et al., 1998). It could be used to detect the *Babesia* infected carrier animals (Holman et al., 1993). However, since this technique needs expensive culture facilities and skilled personnel, it can only be used for the disease certification of expensive animals for commercial purposes (Holman et al., 1993).

3.2. Detection of specific antibody

Serological diagnosis is the most reliable method of detecting the infection in carrier animals. Various serological diagnostic techniques have been used to demonstrate the presence of antibodies against *B. bigemina* and *B. bovis* infections in cattle, with varying levels of accuracy. The serological diagnostic techniques include examination of indirect immunofluorescent antibody test (IFAT), complement fixation test (CFT), enzyme linked immunosorbent assay (ELISA) and Immunochromatographic test (ICT).

3.2.1. Indirect fluorescent antibody test (IFAT): IFAT is also a commonly used technique for the detection of specific antibodies against the *Babesia* species (Araujo et al., 1998). Cross-reaction of the specific antibodies to *B. bovis* in the IFAT for *B. bigemina* is a particular problem in the areas where the two parasites coexist. Additionally, the IFAT has the disadvantages of low sample throughput and subjectivity.

3.2.2. Complement fixation test (CFT): CFT is mainly used for the detection of IgM antibodies which are produced in the early stage of the primary infections and therefore it has low sensitivity (Bose et al., 1995). This observation was confirmed by the studies conducted to compare the *in vitro* culture technique (Holman et al., 1993) and other sero-diagnostic assays (Tenter and Friedhoff, 1986) with CFT.

3.2.3. Enzyme Linked Immunosorbent Assay (ELISA): ELISA is widely used to detect the subclinical infection and for epidemiological surveys (Weiland and Reiter, 1988). Sensitive ELISA for *B. bovis* infection was developed with whole antigens derived from infected blood (Waltisbuhl et al., 1987). However, usage of these antigens for ELISA is severely constrained by the difficulties in obtaining large quantities and by the unfavorable contamination (Bose et al., 1995) what causes a cross reactivity. Several recombinant diagnostic antigens have also been developed for use in ELISA to detect both *B. bovis* and *B. bigemina* antibodies. Boonchit et al., (2004 and 2006) demonstrated the use of truncated

form of rhoptry associated protein-1 (RAP-1) derived from *B.bovis* and *B.bigemina* as a diagnostic antigen to detect infection. Tebele *et al.* (2000) showed that the 200- kDa antigen of *B. bigemina* has the potential to be used as an antigen for ELISA. Although they found that the truncated form of this antigen could recognize only the *B. bigemina*-infected sera, field studies had not been conducted to evaluate the utility of this antigen (Tebele et al., 2000).

3.2.4. Immunochromatographic test (ICT): Recently rapid assays such as an immunochromatographic test, which can be performed at field level, have also been developed to detect the species specific antibodies in bovine sera (Kim et al., 2007).

3.3. Molecular diagnosis

3.3.1. Polymerase chain reaction (PCR): PCR assays are generally based on the amplification of species specific and highly conserved gene (Homer et al., 2000). PCR amplication of target gene is considered to be highly sensitive diagnostic method than that of light microscopy (Calder et al., 1996). The conventional PCR technique can detect up to 1 pg of DNA and the sensitivity of nested PCR is very high since it can detect as low as 1fg of the parasite DNA (Persing, 1991; Gibson and Rawlin, 1992; Belak Ballagi-Pordany, 1993). Several researchers have developed species specific primers to detect *Babesia* species by PCR technique (Figueroa et al., 1993; Criado-Fornelio et al., 2003). Although multiplex PCR assays have been developed to detect several species of *Babesia* parasites simultaneously, they lack sensitivity and therefore the accuracy of the test becomes questionable (Figueroa et al., 1992). In addition to the above mentioned diagnostic tests other test such as reverse line blot hybridization (Gubbels et al., 1999) for the diagnosis of bovine *Babesia* parasites have also been described.

3.3.2. Loop-mediated isothermal amplification (LAMP): LAMP method are recently developed novel gene amplification methods. A loop-mediated isothermal amplification (LAMP) method allows a whole reaction process at an isothermal condition and finally makes it possible to easily detect the LAMP-amplified product due to a visual confirmation of the reacted tube (Notomi et al., 2000). Furthermore, since four LAMP-specific primers are designed to recognize six distinct regions on the target gene, the reacted DNA can be amplified with high specificity (Notomi et al., 2000). Since the LAMP method does not require any complicated equipment, it may provide a cost-effective, simple, and rapid DNA amplification method. For bovine babesiosis, multi loop-mediated mediated isothermal amplification (mLAMP) method was developed (Iseki et al., 2007).

4. Treatment and vaccination

Early diagnosis and proper selection of effective drugs are essential for the successful prevention of clinical disease, while it is less likely when the treatment is delayed (Vial and Gorenflot, 2006). At present many chemotherapeutic agents are available for the treatment of bovine babesiosis such as diminazene aceturate (Berenil), Imidocarb dipropionate (Imizol) and amicarbalide isothionate (Diampron) (Vial and Gorenflot, 2006). Recently several novel antibabesial drugs, such as triclosan (Bork et al., 2003), artesunate, pyrimethamine, pamaquine (Nagai et al., 2003), heparin (Bork et al., 2004), imidazole derivatives, staurosporine (Bork et al., 2006), cysteine protease inhibitors (Okubo et al., 2007), atovaquone and azithromycin (Krause et al., 2000), have been successfully studied by using both *in vitro* and *in vivo* models. However further studies and clinical trials with field cases should be conducted to evaluate the utility of these novel anti babesial agents.

Prevention: The preventive measures to control the bovine babesiosis can be divided into three parts, vector control, chemoprophylaxis, and vaccination (De Waal and Combrink, 2006) at described below.

Tick control: Integrated control strategies must be adopted to control ticks rather than adhere to a single method. Arsenic compounds were used for the control for long time until the development of resistance (George, 2000). Although many organic pesticides were developed later, the successive evolution of resistance of ticks for the acaricides is the biggest problem in controlling the ticks by chemical means (George et al., 2004). Rotational application of pesticides in crop farming minimizes the emergence of resistance. However this strategy is still to be explored in tick control (Willadsen, 2006). Vaccine against *B. microplus* based on a recombinant antigen, Bm86, reduces the number of engorging female ticks, their weight and reproductive performance (Willadsen et al., 1995; de la Fuente et al., 1999). The study carried out in Cuba with this vaccine shows that a two-third reduction in

the number of acaricide treatment needed for tick control (de la Fuente et al., 1999). However, further studies on immunological mechanisms of ticks are essential for the potential application of vaccines as a major control measure (Willadsen, 2006). In addition to the use of chemical and vaccination methods, biocontrol methods have also been used as part of integrated management practices (Samish and Rehacek, 1999).

Vaccination: Live attenuated vaccines have been used to control bovine babesiosis in many parts of world (Shkap et al., 2007). However, vaccination failure is common because of the long use of region specific strains, which possibly results in vaccine breakthrough (De Vos and Bock, 2000). In addition, there is a risk of transmission of other blood borne pathogens during the vaccination (DeWaal, 1996). Therefore, alternative vaccination strategies, such as a choice of recombinant vaccines, must be explored.

5. Aims of the present study

Bovine babesiosis that caused by *B. bovis* and *B. bigemina* remains the leading cause of huge economical losses among cattle worldwide. The proper diagnostic methods and preventive measures are essential for the control of clinical disease to minimize the economical losses. Currently, there are no suitable drugs or effective vaccines for babesiosis and these tick born diseases (TBDs) have become major international concerns owing to the possible introduction of parasites into none endemic areas such as Japan. Therefore, it is very important to develop a more rapid, sensitive and specific method for the detection of bovine babesiosis. The objective of this study is to identify sero-diagnostic antigens for the development of ELISA for bovine babesiosis and evaluation of ELISA for the surveillance in endemic countries. A truncated 200-kDa protein (rP200/CT) of *Babesia bigemina* was identified and ELISA using the rP200/CT was evaluated for the specificity and sensitivity using cattle sera from experimentally infected with *B. bigemina* and sera collected from Ghana (Chapter 1). Novel genes (P36 and rBvP29) were isolated from the genomic DNA of *B. bovis* and their recombinant proteins were analyzed their localization and specificity of theses proteins using by the sera collected from cattle experimentally infected with *B. bovis* and *B. bigemina* (Chapters 2 and 3).

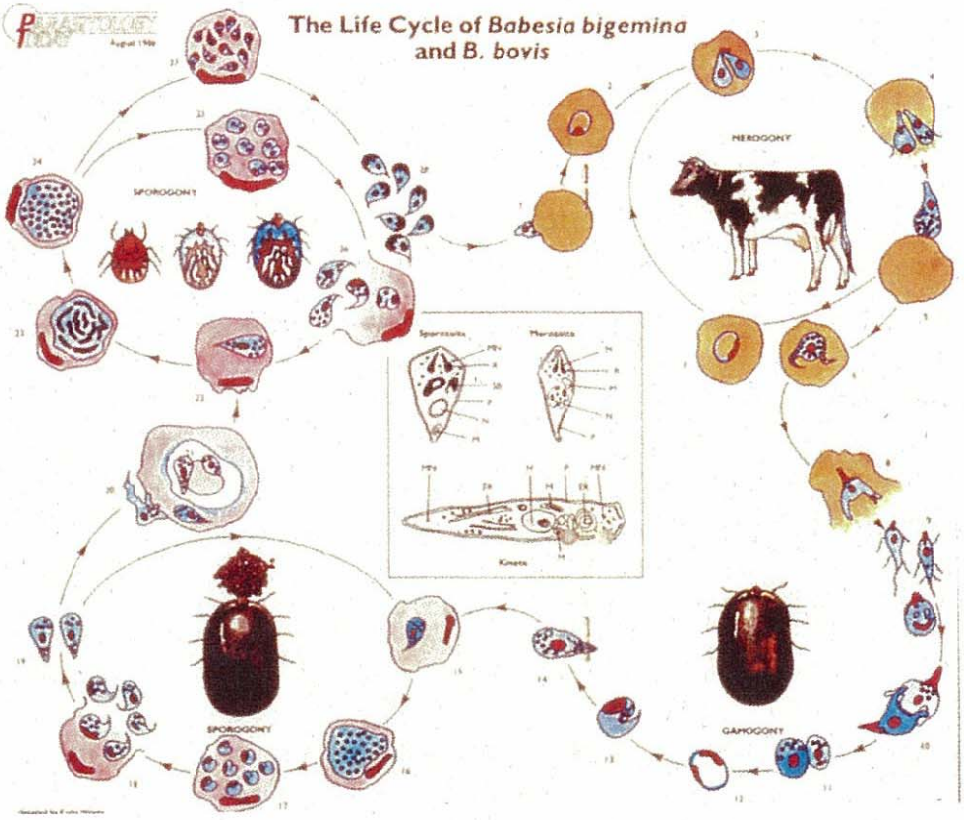

Fig. 1. Life cycle of *B. bigemina* and *B. bovis*; in tick and vertebrate hosts. 1- Sporozoite, inoculated into the cow during tick feeding via the saliva, attaches to and penetrates RBC. 2- The sporozoite becomes an intraerythrocytic trophozoite (feeding stage). 3- The trophozoite undergoes intraerythrocytic division (merogony) to form 2 daughter cells called merozoites. 4- Merozoites escape from the RBC resulting in the destruction of the latter. 5- Free merozoites penetrate new RBC and repeat the merogony (7). 6- Some forms stop dividing, these differentiate to form gametocytes. 8- Female *Boophilus* feeds on infected cow and ingests infected RBC. Only the gametocytes continue development in the gut lumen of the tick. 9- Gametes are formed from the " strahlenkorper" stage and fuse to form a zygote (10-12). 13- The zygote differentiates to form sporokinetes (17) which are liberated and are motile (18). 16- A multiple fission body is formed with many nuclei which differentiates to form sporokinetes (17) which are liberated and are motile (18). 19- The sporokinetes can initiate further sporogony in various tissues of the tick. 20- Some sporokinetes initiate the infection of the ova before the cuticle is formed and remain dormant (21) until the F_I ticks start feeding. 22-26- Sporokinetes initiate sporogony in tissues of feeding F_I ticks resulting in numerous sporokinetes. 27- Sporokinetes invade the salivary glands and intiate sporogony during tick feeding which results in many sporozoites being formed (28). These are released into the salivary ducts of the tick during feeding (Young and Morzaria, 1986).

Chapter 1

Evaluation of *Babesia bigemina* 200 kDa recombinant antigen in enzyme-linked immunosorbent assay

1-1. Introduction

Babesia bigemina is a tick-borne hemoprotozoan that causes babesiosis in cattle (Barnett 1974; McCosker 1981). The parasite is widely distributed throughout Africa, Southern Europe, Asia, Australia, Central and South America, coinciding with its main vector, the *Boophilus* tick. Economically, it is the most important cause of heavy losses as a result of infection in susceptible cattle, particularly in imported breeds (Akinboade and Akinboade 1985; Solari et al. 1992). Animals that recover from infection develop lifelong immunity and may become carriers (Böse et al. 1995).

The classical diagnosis of acutely infected animals is by light microscopic demonstration of intra-erythrocytic parasites in Giemsa-stained blood smears (Araujo et al. 1998); however, in subclinical or latent infection, parasites may not always be demonstrable because of low parasitemia (Böse et al. 1995). In epidemiological studies, exposure to *B. bigemina* is best determined by the detection of antibodies in the sera of infected cattle (Molloy et al. 1998). Several serological tests such as the indirect flourescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) have been developed for bovine babesiosis (Boonchit et al. 2006; Goff et al. 2008; Mbati et al. 2002; Ravindran et al. 2007). However, the IFAT has poor specificity because antibodies cross-react between *B. bovis* and *B. bigemina*. Furthermore, this technique is time-consuming, and the interpretation of the results is subjective and depends on individual expertise (Böse et al. 1995). On the other hand, ELISA is quite sensitive and is appropriate for testing large number of samples

especially in field surveys (Weiland and Reiter 1988). Previously, ELISAs have been evaluated for detection of antibodies to bovine *Babesia* parasites using native antigens. Although, such tests proved to be powerful tools for serological surveys, the poor quality of the antigens, and sometimes cross-reactions between the *B. bovis* and *B. bigemina* species and with closely related apicomplexan parasites resulting in false positive results have limited their application (Böse et al. 1995).

Considering these problems associated with *Babesia* infections, it is, therefore, necessary to develop a reliable diagnostic method for this disease. Although some antigens have shown potential for effective diagnosis and putative vaccine candidates in preliminary experiments, a successful antigen to diagnose and prevent this disease has not been found. Several factors have contributed to this lack of proper antigens including a complicated life cycle of the parasite, wide variety of immune responses induced by the parasite, and insufficient information concerning the relationship between the parasite and its hosts Wright et al. 1987. Cross-reactivity with antibodies to *B. bovis* is a complication in regions where the two parasites coexist (Dalrymple et al. 1992; Morzaria et al. 1992). Furthermore, *B. bigemina* immune serum has been shown to cross-react with bovine fibrinogen (El-Ghaysh et al. 1996). Hence, it is essential to develop a suitable serological test to differentiate between B. bovis and *B. bigemina*. In a review of serological tests for the diagnosis of babesiosis, Böse et al. (1995) concluded that the existing serological tests for *B. bigemina* were inadequate and that the development of an improved test was a priority. In this study, the gene encoding the 200 kDa protein of *B. bigemina* (Tebele et al. 2000) was expressed as a glutathione-*S*-transferase (GST) fused protein in *Escherichia coli* and used for serological diagnosis of *B. bigemina* infection in enzyme-linked immunosorbent assay (ELISA). In addition, corresponding DNA samples obtained from the same animals were also screened by polymerase chain reaction (PCR) for the detection *B. bigemina*.

1-2. Materials and methods

Parasites. *B. bigemina* (Argentina strain) was continuously cultured with bovine erythrocytes using a microaerophilous stationary-phase culturing system (Vega et al., 1985). When the level of parasitemia reached 5%, the infected erythrocytes were washed thrice with phosphate buffered saline (PBS), and the pellets were stored at −80°C until use.

Bovine sera and DNA. Serum samples used for the evaluation of the ELISA with rP200/CT were as follows: serum samples from cattle experimentally infected with *B. bovis* (n = 12) or *B. bigemina* (n = 12), negative serum samples (n = 12) from healthy cattle with no known past history of *Babesia* infection (Boonchit et al. 2006). To evaluate the diagnostic utility of p200/CT, field blood samples were collected from cattle in Accra Ghana. Samples were taken to the veterinary services laboratory in Accra for analysis. Blood samples were collected in EDTA tubes from the jugular vein of cattle. For each of the blood samples, Giemsa-stained blood smears were prepared for microscopic examination. Serum samples were separated after centrifugation and were tested by ELISA for detection of antibodies against *B. bigemina*. DNA was also extracted from same samples using a commercial Kit (QIAamp DNA Blood Mini-Kit Madison, WI, USA) according to the manufacturer's instructions.

Microscopic analysis. In the laboratory, blood smears were fixed in methanol for 5 min and stained for 30 min in Giemsa stain diluted with 5% buffer and observed with Bright-field light microscopy at 1,000× for the presence of *Babesia* organisms.

Cloning of the truncated P200 gene. *B. bigemina* genomic deoxyribonucleic acid (DNA) was extracted from *B. bigemina*-infected erythrocyte pellets with phenol–chloroform as previously described (Boonchit et al. 2002). The genomic DNA was used as the template in the PCR. Oligonucleotide primers were designed based on the DNA sequence of the *B. bigemina* 200 kDa gene (Gene Bank accession no AF142406) with restriction enzyme-

compatible ends for subsequent cloning. The nucleotide sequence (nt 3112–3327 nt) encoding the 200 kDa gene was obtained by PCR using primers Big-P200-F-5′-CCG GAATTCATGGTAAAACATGCATCC-3′ and Big-P200-R-5′-CCCTGCGAGCTA AGAGTCATCACC-3′ containing *Eco*RI and *Xho*I sites, respectively. The PCR conditions were 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min. Each of the PCR-amplified DNA fragments was digested with restriction enzymes, and *EcoR*I and *Xho*I were then ligated to a similarly digested pGEX-4T-1 expression plasmid (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), resulting in plasmid pGEX-P200/CT containing the truncated P200 gene fragment.

Expression and purification of *B. bigemina* rP200/CT recombinant protein. The recombinant plasmid pGEX-P200/CT was transformed in *E. coli* (BL21). Each transformed colony was cultured in LB medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl, 0.1% 5-*N* NaCl) with ampicillin sodium (50 μg/mL) at 37°C. At optical density (OD; 600 nm) 0.5, the plasmids were induced to synthesize the recombinant fused proteins by the addition of 1-M isopropyl-*b*-*d*-thiogalactopylanoside (IPTG), (Wako, Tokyo. Japan) and incubated for 4 h. The bacterial cultures were harvested by low centrifugation and sonicated with STE buffer (50-mM Tris–HCl at pH 9.5, 100 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA)) containing 50-μg/mL lysozyme and 1% Triton X-100. The supernatant containing the recombinant fused proteins (rP200/CT) was removed by centrifugation and then purified with glutathione sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech, little Chalfont, UK).

SDS–PAGE and Western blotting. The expression and purification of the recombinant proteins were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide gel according to Laemmli's method (1970). Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore,

USA), by the method of Towbin et al. (1979). Thereafter, the membranes were treated as described by Boonchit et al. (2006).

ELISA. ELISA was performed according to the method followed by Xuan et al. (2001) with some modifications. Briefly, the optimal working conditions for the rP200/CT conjugates were determined in a checkerboard assay by using serial dilutions. Pre-infection (negative control) and post-infection (positive control) cattle sera were diluted 1:100 in blocking solution before use. The conditions rendering the highest difference in OD measured for positive and negative sera were chosen for screening all the sera. Purified rP200/CT antigen was diluted to 7 μg/mL in 0.05 M carbonate–bicarbonate buffer (pH 9.6), and 50 μL of this solution was added to each well of a flat-bottomed microtiter Maxisorp plate (Nalgen Nunc, Denmark). Control wells for each of the sera were coated with 7 μg/mL purified GST. Duplicate serum samples were titered by doubling the serial dilutions in a blocking solution containing 3% skimmed milk, and the mixture was incubated at 37°C for 1 h. After blocking, the plates were washed once with PBS-T and then 50 μL of diluted sera was added to each well. The plates were incubated at 37°C for 1 h and then washed six times using PBS-T. Bovine antibodies were detected by using HRPO conjugated sheep anti-bovine immunoglobin G (IgG; 1:5,000). The plates were incubated at 37°C for 1 h and then washed six times with washing buffer. To each well, 100 μL of the substrate (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, 0.5 mg 2,2′-azino-di-3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemicals, USA) was added. The plates were further incubated at room temperature for 1 h, and the OD (415 nm) was measured by using an automatic ELISA plate reader (Corona, Japan). The net OD values were obtained by subtracting the OD for the GST control from that of the test sera. This control procedure ensured that the net OD values were due to the presence or absence of *B. bigemina* antibodies and not due to nonspecific binding. The cutoff point of OD > 0.25 was used as the mean OD for the

negative sera plus three standard deviations. *B. bigemina* RAP-1CT ELISA was performed as previously described by Boonchit et al. 2006.

PCR amplification and sequence analysis. A PCR was performed to detect *B. bigemina* DNA using previously described method by (Boonchit et al. 2006). Amplification was performed using 50-µl reactions. Each reaction contained a 1× concentration of PCR buffer (Roche), 2.5 U of Taq polymerase, 5 µl of DNA template, 10 pmol of each primer, 2 mM concentration of each deoxynucleoside triphosphate. A forward primer pair (BigF-CGAAGCAGCTGTAGAGGAAA and reverse primer BigR-TTTCTGCATCTGGAAGCTGC) were designed to amplify approximately 195 bp fragment from *B. bigemina* DNA. The PCR conditions were as follows; initial denaturation at 95°C for 5 min, followed by 35 amplification cycles (95°C for 1 min, 58°C for 1 min, and 72°C for 1 min), and a final extension step at 72°C for 7 min. The PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and visualized under UV-transillumination. Positive *B. bigemina* amplified DNA products were gel purified by a GENECLEAN kit (BIO 101, Vista, CA, USA). The nucleotide sequence of DNA fragments were directly sequenced by an ABI PRISMTM 3100 Genetic Analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) with ABI PRISMTM BigDyeTM terminator cycle-sequencing ready-reaction kit (Applied Biosystems Japan Ltd).

1-3. Results

Expression of the rP200/CT in *E. coli*. To establish an effective and improved method for the diagnosis of *B. bigemina* infection, the DNA sequence encoding a truncated 200 kDa gene (1,038–1,108 aa) was amplified by PCR and inserted into the pGEX-4 T-1 plasmid vector, resulting in the recombinant plasmid pGEX-rP200/CT. The recombinant plasmids were transformed and expressed in *E. coli* as GST fused proteins. The resultant rP200/CT was purified and observed by SDS–PAGE (Fig. 2). The protein had a molecular size of approximately 33.9 kDa. In the Western blot analysis, rP200/CT reacted strongly with the sera from cattle infected by *B. bigemina* but not with sera from *B. bovis*-infected cattle (Fig. 3).

Reactivity and specificity of rP200/CT. ELISA was used to evaluate the rP200/CT expressed in *E. coli* to ascertain whether it can be a useful antigen for the serodiagnosis of babesiosis. The OD_{415} cutoff point was set as the mean value of the negative samples plus three standard deviations. The ELISA detected antibodies (OD > 0.25) in the sera of *B. bigemina*-infected cattle but was negative (OD < 0.25) for *B. bovis*-infected cattle sera and uninfected bovine sera (Fig. 4). Similarly, when the OD values of rP200/CT were compared with those of the *B. bigemina* rhoptry-associated protein 1 (rRAP-1/CT) ELISA, the absorbance values of the rRAP-1/CT ELISA were higher than those of the rP200/CT ELISA. In addition, the absorbance values of the negative serum samples were less than the cutoff point in both ELISAs (Fig. 5).

Diagnosis of *B. bigemina* infection in cattle by the ELISA with rP200/CT and PCR. A total of 108 blood samples collected from cattle Ghana were tested for the detection of antibodies to *B. bigemina* by the ELISA with rP200/CT and for the detection of *B. bigemina* parasites in microscopy and DNA by PCR. As shown in Table 1, none of the animals were positive for *Babesia* parasites by microscopic examination of Giemsa stained blood smears.

On the other hand, 66 (61%) and 67 (62%) of the tested samples were positive by the ELISA and PCR, respectively. In addition, 42 (38.8 %) samples were positive by both the ELISA and PCR, while 25 (23.1%) PCR positive samples were ELISA negative and 22 (20.0 %) PCR negative samples were ELISA positive (Table 1). Sequence analysis of all positive field PCR samples showed a 100% identity with p200 (data not shown).

1-4. Discussion

In the search for suitable antigens to detect *B. bigemina* infection, attention has been focused on the identification of immunodominant antigens recognized by sera from animals infected with distant geographical isolates and from both acute and chronically infected animals (Boonchit et al. 2006). *B. bigemina* 200 kDa protein (P200), one of such conserved and immudominant antigens, was previously identified as a candidate diagnostic antigen for serological assaying in the detection of *B. bigemina* antibodies in infected cattle (Tebele et al. 2000).

B. bigemina p200 is present in merozoite cytoplasm, suggesting that the antigen may be a structural protein involved in forming filament structures within the cytoskeleton. In addition, P200 is immunogenic and is conserved among different strains of *B. bigemina*. In this study, a truncated fragment of the 200 kDa (rP200/CT) protein was expressed in *E. coli* for the serological diagnosis of *B. bigemina* infection. To examine whether antibodies against rP200/CT were elicited during natural infection with *B. bigemina*, recombinant rP200/CT was examined for its reactivity with known positive sera collected from infected cattle of endemic areas. As shown in Fig. 3, sera from these animals showed reactivity with the recombinant protein on Western blotting. Furthermore, in a comparative ELISA with RAP-1/CT (Boonchit et al. 2006), rp200/CT protein was recognized by *B. bigemina*-infected serum samples. However, the OD values of *B. bigemina*-infected bovine sera in the rP200/CT ELISA were slightly lower than those in the rRAP-1/CT ELISA, probably indicating that some immunodominant region capable of inducing a stronger humoral immune response could have been removed as a result of the truncation. Although the sensitivity of rP200/CT was low, as compared to RAP-1, it proved to be highly conserved and specific for the detection of antibodies against *B. bigemina* infection across different geographical regions.

In the current study, none of the cattle examined had detectable *Babesia* parasites by microscopy; however, some positive detection was obtained by ELISA and PCR methods, respectively (Table 1). The mechanisms involved in parasitemia variations in carrier infections are not well understood. However, it has been observed that parasite densities in *Babesia*-carrier animals seem to fluctuate with time and periodically fall below the detection level of microscopy and PCR (Aguilar-Delfin et al. 2001, 2003; Brown et al. 2006; de Vos and Bock 2000). The proportions of seropositive samples from *B. bigemina* naturally infected cattle compared with the PCR results (67%) are indicative of high sensitivity of rp200/CT antigen. In addition, the rp200/CT-ELISA detected antibodies against *B. bigemina* in samples that were PCR negative (Table 1). Either this could imply low level of parasite DNA below detection limit of the PCR or the persistence of the parasite antibodies for a period even after the living pathogen has been eliminated from the host. In fact, microscopy and PCR-based detection methods are less likely to detect low parasite densities because of a higher probability that no infected erythrocytes are present in the minute blood volume used. In this respect, this ELISA could be useful in screening cattle, which are exposed to *B. bigemina* infection by monitoring their antibody levels. Such a strategy is normally vital in prevention of new infections rather than in treatment of clinical infection, which require specific methods of parasite identification. On the other hand, some 20 seronegative samples tested positive for parasite DNA by PCR suggesting that the samples could have been from cattle with early parasitic infection characterized by undetectable antibody titers. Alternatively, this could have been due to the relatively higher sensitivity of PCR compared with the rp200-ELISA. The rp200/CT-ELISA detected higher proportion of positive samples than PCR in samples collected from apparently healthy cattle (Table 1). From epidemiological point of view, screening for subclinical or latent infections is important because such cases could serve as potential source of re-infection (Stegeman et al. 2003);

moreover, cases of relapses of infection are possible. Therefore, rp200/CT-ELISA could be useful in screening surveys and in identification of infected cattle that appear apparently healthy while PCR could be useful especially in cases where microscopic findings are inconclusive. Nevertheless, the ELISA and PCR each has its own advantages and disadvantages depending on the intended use. Alternatively, a repetition or a combination of different diagnostic tests might provide a more accurate picture of the true prevalence of infection.

1-5. Summary:

A truncated fragment of the gene encoding the 200-kDa protein (P200) of *Babesia bigemina* was cloned into a plasmid vector, pGEX-4 T-1 and expressed in *Escherichia coli* as a glutathione-*S*-transferase fused protein. An indirect enzyme-linked immunosorbent assay (ELISA) using the rp200/CT detected specific antibodies in cattle experimentally infected with *B. bigemina*. Furthermore, the antigen did not cross-react with antibodies to *Babesia bovis*, a closely related *Babesia* parasite indicating that rp200/CT is a specific antigen for the diagnosis of *B. bigemina* infection. Additionally, ELISA using p200/CT and polymerase chain reaction were conducted on serum and corresponding DNA samples obtained from field cattle to evaluate the diagnostic utility of the p200/CT antigen. Results from the current study suggest that p200/CT ELISA is a sensitive and specific method for improved serodiagnosis of *B. bigemina* infection.

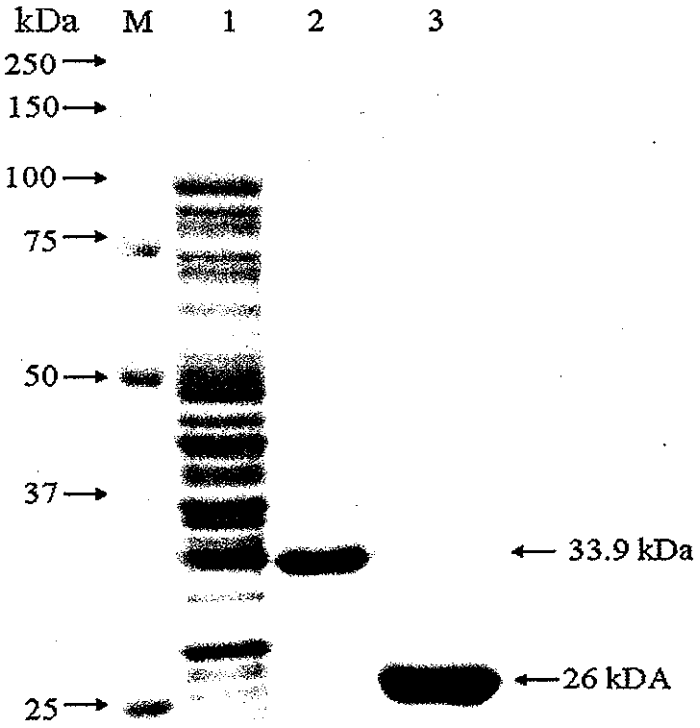

Fig. 2. Expression and purification of the GST fusion with *B. bigemina* P200/CT protein expressed in *E. coli.* Lane 1: proteins in soluble fraction; Lane 2: purified protein; Lane 3: GST. Proteins were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie brilliant blue.

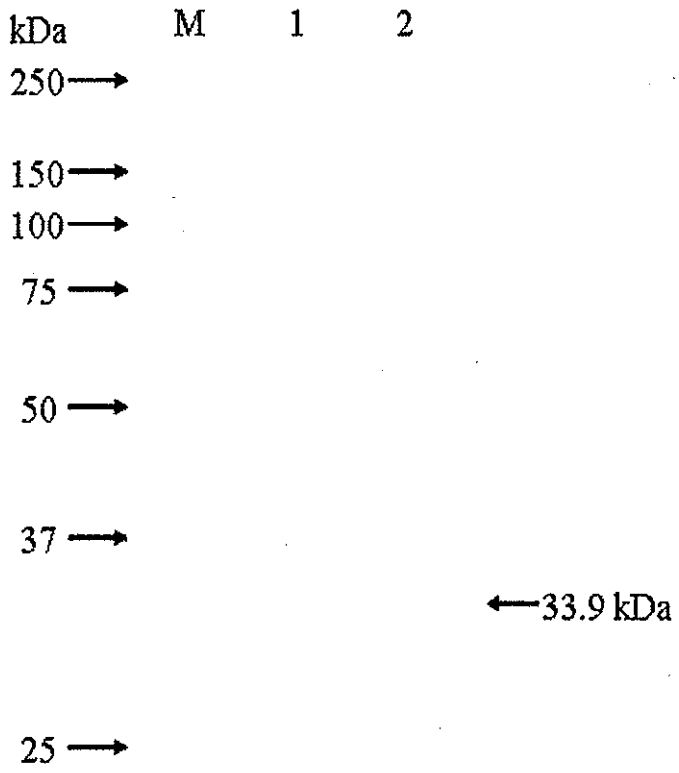

Fig. 3. Western blot analysis of *B. bigemina* P200/CT protein. The membrane was incubated with *B. bigemina*-infected cow sera (Lane 1) and *B. bovis*-infected sera (line 2).

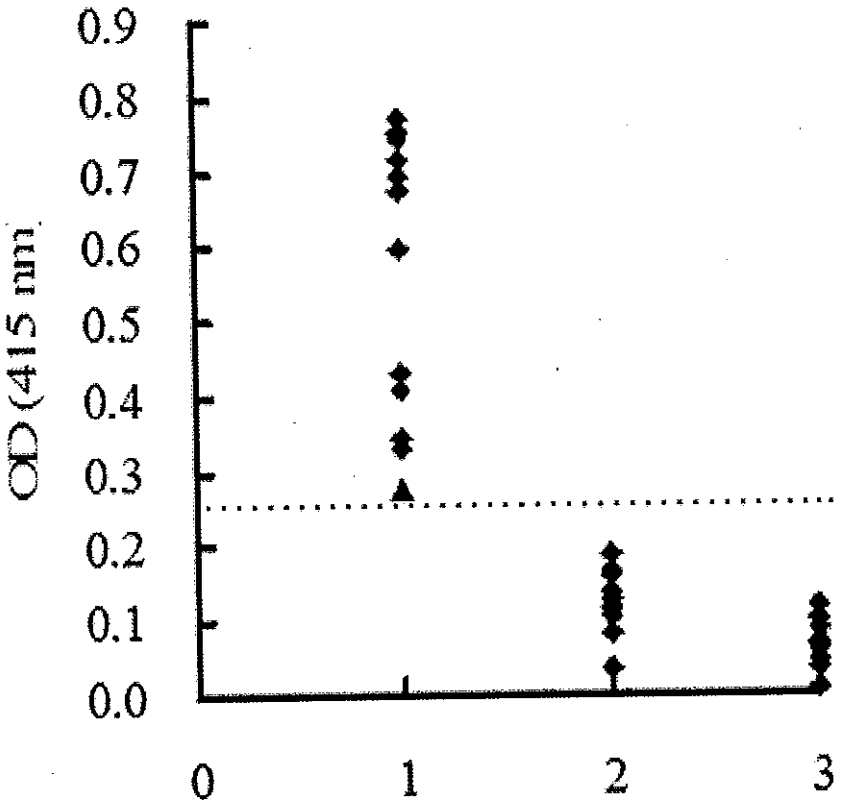

Fig. 4. Reactivity of the bovine sera with the *B. bigemina* rP200/CT in enzyme-linked immunosorbent assay (ELISA). Column 1: *B. bigemina*-infected bovine sera (n = 12), Column 2: *B. bovis*-infected bovine sera (n = 12); dotted line: cutoff point 0.25.

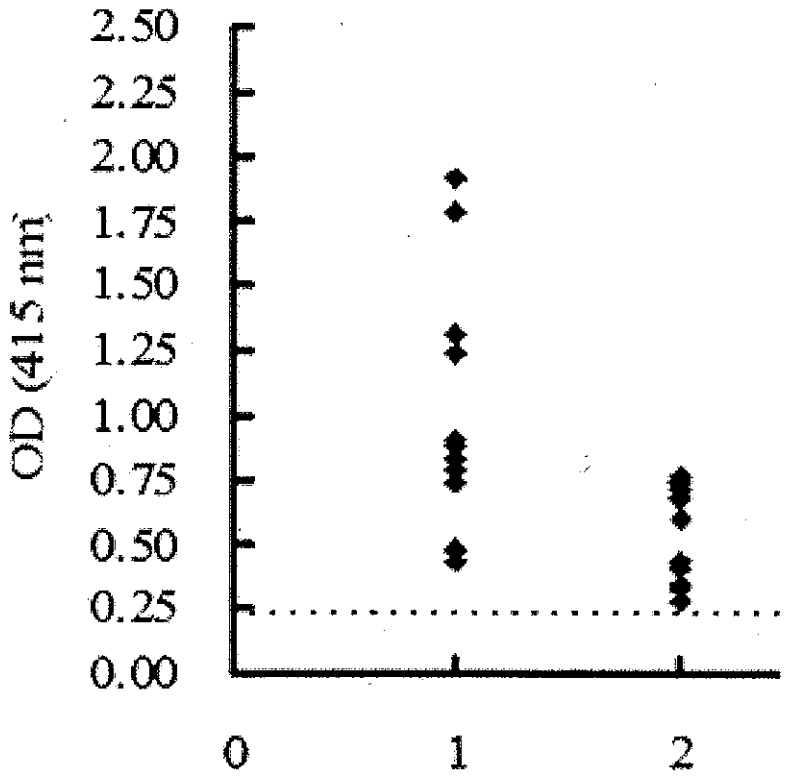

Fig. 5. Reactivity of rhoptry-associated protein 1 (rRAP-1/CT) antigen (1) and the *B. bigemina* rP200/CT (2) in the enzyme-linked immunosorbent assay (ELISA). The dotted line represents the cutoff point.

Table 1. Comparison of microscopy, PCR, and ELISA results

Number of samples	Microscopy	Elisa	PCR	ELISA-PCR[a]	ELISA-PCR[b]	PCR-ELISA[c]
108	0	66 (61%)	67 (62%)	42 (38.8%)	25 (23.1%)	22 (20.0%)

[a] Samples positive with both (GST-rP200/CT-ELISA and PCR

[b] ELISA-positive field samples but negative according to the PCR

[c] PCR-positive field samples but negative according to the ELISA

Chapter 2

A *Babesia bovis* BvP36 gene is expressed at least in the asexual stage of *Babesia* merozoite

2-1. Introduction

Babesiosis is an intraerythrocytic protozoan disease affecting a variety of wild and domestic animals. This disease is caused by the members of genus *Babesia* of apicomplexan parasites (Homer et al., 2000). *B. bovis* and *B. bigemina* have been identified as 2 economically important species causing bovine babesiosis (De Waal and Combrink, 2006). Of these 2 species, *B. bovis* is the most pathogenic causing high mortality of the affected cattle (Brown et al., 2006). Previously, it was estimated that around half-a-billion of the world's cattle population are at risk of contracting babesiosis (McCosker, P.J., 1981).

Proper treatment is essential to minimize the potentially adverse economic impact. However, delayed treatment could result in an unsuccessful treatment outcome (Vial and Gorenflot, 2006). Additionally, effective control of *Boophilus* ticks is becoming increasingly difficult because of the development of acaricide resistance and the spreading of ticks to new areas due to climatic changes (De Waal and Combrink, 2006). Despite the wide use of live attenuated vaccines in many parts of the world (Shkap *et al.*, 2007), vaccination failure was evident due to the use of region-specific strains (De Vos and Bock, 2000), short lifespan, and side effects of the vaccines (Hope *et al.*, 2005). Therefore, alternative vaccination strategies must be developed to resolve the problem of bovine babesiosis. *Plasmodium yoelii* and *P. berghei* have been used as rodent model organisms to study malarial diseases (Luke *et al.*, 2003). Recent studies indicate that the P36 antigen in the sporozoite of *P. berghei* plays an important role in hepatocyte infection (Ishino *et al.*, 2005). The P36 homologous

antigens are the members of the 6-Cys protein superfamily, which possesses a structure unique to *Plasmodium* species (Carter *et al.*, 1995). Deletion of the P36 gene from the genome of *P. yoelii* resulted in loss of the ability of the sporozoite to develop into the liver stage and complete attenuation of the sporozoite (Labaied *et al.*, 2007). More importantly, these sporozoites induced sterile immunity in mice, which completely protected the mice from subsequent challenge infections with the sporozoites (Labaied *et al.*, 2007). Structure prediction analysis showed that the 6-Cys proteins of *Plasmodium* parasites are similar to the surface antigen 1 (SAG 1) of *Toxoplasma gondii* (Gerloff *et al.*, 2005). SAG 1 is localized on the surface of tachyzoite and is involved in the host-cell attachment and immune modulation (Mineo *et al.*, 1993). In another study, mucosal protective immunity was induced in mice immunized with recombinant SAG 1 via the intranasal route (Velge-roussel *et al.*, 2000). The P36 antigen of *Neospora caninum* is closely related to the *T. gondii* SAG 1 (Sonda *et al.*, 1998). The P36 gene of *N. caninum* is expressed in the tachyzoite stage, and the antigen is involved in the invasion of host cells (Nishikawa *et al.*, 2000). Therefore, these P36 homologous antigens may be considered as commonly available new vaccine candidates against the infections caused by the apicomplexan parasites.

Recent genome project of *B. bovis* identified a homologous gene to the *Plasmodium* P36 gene (Brayton *et al.*, 2007). Because of the greater vaccine potential of P36-related antigens, Brayton *et al.* (2007) suggested that the P36 homologous antigen of *B. bovis* (BvP36) could be a potential vaccine candidate. Therefore, in the present study, we isolated the BvP36 gene and characterized BvP36 immunologically. This study was conducted as a preliminary study prior to the evaluation of the vaccine utility of the BvP36 antigen.

2-2. Materials and methods

Parasites. A continuous micro-aerophilous stationary phase culture system, which was previously established (Igarashi *et al.*, 1998; Vega *et al.*, 1985), was used for *in vitro* cultivation of the Texas strain of *B. bovis* (Hines *et al.*, 1992) and the Argentina strain of *B. bigemina* (Hotzel *et al.*, 1997). At greater than 5% parasitemia, the red blood cells (RBC) were washed with phosphate-buffered saline (PBS) and stored at -80°C until use.

DNA extraction. *B. bovis*-infected RBC were suspended in a DNA extraction buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10 mM EDTA and then digested with 1% sodium dodecyl sulfate (SDS) and 100 μg/ml of protease K. The parasite DNA was subsequently extracted by the phenol-chloroform method as described previously (Boonchit *et al.*, 2002).

Cloning and sequencing of the BboP36 gene (Accession No XM_001609815). A full-length target gene (639 bp) was amplified from the above extracted genomic DNA by PCR using a pair of forward, 5′-acgaattc ATGAGTAACGTCGAAAAGTTCC-3′ and reverse, 5′-acctcgagTCAAATTAGGTCAACCTCTG-3′ primers (lowercase letters in the forward and reverse primers represent restriction sites for *Eco*RI and *Xho*I, respectively) as described previously (Boonchit *et al.*, 2002). The PCR product was purified by a QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan), digested with 2 restriction endonucleases, namely, *Eco*RI and *Xho*I, and then ligated into a similarly pre-digested, expression plasmid vector, pGEX-4T-1 (GE Healthcare, Uppsala, Sweden). After ligation, the plasmids were transferred into *E. coli* competence cells (BL21), and subsequently plated on ampicillin-containing LB plates. After an overnight incubation, the clones were cultured overnight in LB broth at 37°C. The positive cultures were identified by the abovementioned PCR analysis. Finally, the resultant plasmids were extracted from the positive cultures (QIAprep Spin Miniprep Kit, Qiagen), and an ABI PRISM 3100 Genetic analyzer was used to confirm

the sequence of the inserted gene (Földvári *et al.*, 2005).

Production of rBvP36. The abovementioned positive culture was incubated at 37°C until they attained an optical density (OD) of 0.5 at 600 nm; subsequently, they were induced with isopropyl-ß-D-thiogalactoside (IPTG) for 4 hours to produce a glutathione S-transferase (GST)-fusion recombinant protein designated rBvP36. The rBvP36 protein was subsequently purified using a glutathione-sepharose 4B (Amersham Pharmacia Biotech, Little Chalfont, UK) (Smith and Johnson, 1988.).

Preparation of anti-rBvP36 immune sera in mice. Polyclonal antibodies against the rBvP36 antigen were produced in a group of 6-week-old female BALB/C mice (CLEA Japan, Tokyo, Japan). Primary immunization was done with 100 μg of rBvP36 together with an equal volume of Freund's complete adjuvant (Sigma Aldrich, Saint Louis, MO, USA). The first and second booster immunizations were carried out with 50 μg of rBvP36 with Freund's incomplete adjuvant (Sigma Aldrich) at 14-day intervals. The control group of mice was immunized with GST and the relevant Freund's adjuvants. Antibody production was monitored by enzyme-linked immunosorbent assay (ELISA) with rBvP36-coated plates as described below. The blood was collected on the 10th day after the last immunization, and the sera were separated and stored at –20°C until use.

SDS-PAGE and Western blot analyse. The purified rBvP36 antigen and the relevant crude *E. coli* lysate were loaded onto the SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, U.K., 1970). For the subsequent western blot assay, polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) were used to transfer the proteins and treated with the indicated antibodies as described by Towbin *et al.* (1979) and Boonchit *et al.* (2002), respectively. Bovine sera collected from cattle experimentally infected with *B. bovis* and *B. bigemina* and murine immune serum against the rBvP36 antigen were used as primary antibodies. After blocking with 3% skimmed milk solution at 4°C overnight, the membranes were washed with 0.05% Tween 20-PBS (PBST) 3 times and treated with the

indicated primary antibody at 1:100 dilution for 1 hour at 37°C. The membranes washed with PBST were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-bovine or murine antibody (Bethyl, Montgomery, TX, USA) (1:5,000 dilution) for 1 hour at 37°C. Finally, the treated membranes were reacted with a substrate solution containing 0.5 mg/ml of diabinobenzidine and 0.005% H2O2 to detect any positive signals.

ELISA: ELISA was performed to monitor antibody production in the mice immunized with the rBvP36 antigen as described above. In brief, microtiter plates were coated with 5 μg/ml of the rBvP36 by using a coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6) and then incubated overnight at 4°C. After blocking with 3% skimmed milk solution at 37°C for 1 hour, the plates were washed 6 times with PBST and then incubated with a series of the sera collected from the immunized mice. After washing with PBST, the plates were incubated with the HRP-conjugated rabbit anti-murine antibody (1:5,000 dilution) for 1 h at 37°C. Finally, a substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H2O2, and 0.3 mg 2,2′-azide-bis (3-etylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma chemical, St. Louis, MO) per ml] was added to detect any positive reactions. The OD value of each well was measured at 415 nm by using an MTP 500 microplate reader (Corona Electric, Katsuta, Japan).

IFAT: For indirect immunofluorescence tests (IFAT), the RBC infected with *B. bovis* and *B. bigemina* were washed three times with PBS, smeared on glass slides, and then fixed in a solution containing the mixture of 50% acetone and 50% methanol at –20°C for 5 min. Subsequently, the slides were incubated with 10% skimmed milk blocking solution for 30 min at room temperature. After washing the slides thrice with PBS, the slides were incubated with anti-rBvP36 and the control GST murine immune sera at 1:100 dilution for 30 min at 37°C. The slides washed with PBS were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Southern biotechnology associates, Birmingham,

AL, USA) diluted to 1:200 at 37°C for 30 min, and then washed again with PBS. Subsequently, the slides were treated with a solution containing 25 μg/ml of propidium iodide (Wako) and 50 μg/ml of RNase A (Roche, Indianapolis, IN, USA) in PBS at 37°C for 10 min and were washed again with PBS and mounted with a fluorescent mounting medium (Dako, Carpinteria, CA, USA). The slides were finally observed under a confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

2-3. Results.

Productions of rBvP36 and anti-rBvP36 murine immune sera: A full length of the BvP36 gene with 639 bp was successfully amplified in PCR from the genomic DNA of *B. bovis* (Fig. 6). Sequence analysis confirmed that the DNA sequence of inserted BvP36 gene was completely identical to that reported previously. As detected by SDA-PAGE analysis, approximately 45-kDa of recombinant protein, designated as rBvP36, was produced as a GST-fusion form in *E. coli* (Fig. 7). The high solubility of rBvP36 was evident since it was detectable in both soluble crude *E. coli* lysate (lane 1) and the purified fraction (lane 2). Anti-rBvP36 immune sera were successfully raised in the rBvP36-immunized mice, as confirmed by ELISA (data not shown).

Immune reactivity of the rBvP36 antigen against *B. bovis*- and *B. bigemina*-infected bovine sera: During western blot analyses, the bovine serum samples collected from the cattle experimentally infected with *B. bovis* and *B. bigemina* merozoites positively recognized the rBvP36 of 45 kDa (Fig. 8, lanes B and C, respectively). Anti-rBvP36 murine immune serum also showed a positive reaction to the rBvP36 (Fig. 8, lane A).

Recognition of the anti-rBvP36 immune serum to *Babesia* merozoites: Strong reactions of the anti-rBvP36 immune serum were observed against the cultured *B. bovis* and *B. bigemina* merozoites under the confocal laser scanning microscope during IFAT. The parasites within *B. bovis*-and *B. bigemina*-infected RBC were positively recognized with the anti-rBvP36 immune serum (Fig. 9, panels A and B, respectively). Further, anti-control GST immune serum failed to give any positive reactions to both *B. bovis* (Fig. 9C) and *B. bigemina* (Data not shown) during the tests.

2-4. Discussion

In the present study, I successfully isolated a P36 homologous gene from the genomic DNA of *B. bovis*. About 45-kDa of rBvP36 was produced as a highly soluble form in *E. coli*. Importantly, the rBvP36 was recognized with bovine immune sera raised in the cattle infected with *B. bovis* and *B. bigemina* merozoites. Therefore, I assumed that the BvP36 gene is expressed at least in the asexual merozoite stage of *B. bovis*. This was further corroborated by the finding that the antisera raised in the mice against rBvP36 antigen reacted strongly to the *B. bovis* merozoites during IFAT. Interestingly, it is evident from the results of western blot and IFAT analyses that a closely related antigen to that of BvP36 is also synthesized during the merozoite stage of *B. bigemina*.

P36 of *Plasmodium* parasites, which is expressed only in sporozoites, is believed to be involved in the formation of parasitophorous vacuole in the infected hepatocytes (Labaied *et al*., 2007). When the mutant sporozoites lacking the P36 gene were injected, the sporozoites were found as free entities in the hepatocytes and became completely attenuated (Labaied *et al*., 2007). In contrast, *Babesia* sporozoites injected with the infected ticks can directly invade the host RBC of cattle and transform into merozoites in the cases of *B. bovis* and *B. bigemina* (Uilenberg, G., 2006). Therefore, the present finding that the *B. bovis* BvP36 gene can be expressed in the merozoite stage is valuable for understanding the basic biology of *Babesia* parasites. Further studies investigating the functions of BvP36 are bound to yield interesting findings.

Since several P36 homologous antigens of other apicomplexan parasites had demonstrated vaccine potential, the utility of rBvP36 should also be evaluated as a potential vaccine candidate against the bovine babesiosis. Additionally, it would be interesting to study the identification and functional analysis of the similar antigen observed in the merozoites of *B. bigemina*. Furthermore, it is of paramount importance to study whether

these antigens are well conserved among geographically different isolates of both *B. bovis* and *B. bigemina* and whether these antigens are expressed in the sporozoite stages of both the species.

2-5. Summary

Recently, *Babesia bovis* genome project identified a novel gene, which is homologous to the gene encoding sporozoite P36 antigen in *Plasmodium* parasites. In the present study, I isolated the P36 homologous gene from the genomic DNA of *B. bovis* and designated it as the BvP36 gene. The sequence of the BvP36 gene was identical to that reported previously. This gene was expressed in an *Escherichia coli* system to produce a recombinant protein (rBvP36). The rBvP36 protein was produced as a highly soluble form in *E. coli* and was detected by western blot analyses of sera collected from cattle experimentally infected with *B. bovis* and *B. bigemina* merozoites. In indirect immunofluorescence tests, sera collected from mice immunized with rBvP36 showed strong reactions to both *B. bovis* and *B. bigemina* merozoites. Our results indicate that the BvP36 antigen is expressed at least in the asexual merozoite stage of *B. bovis* and suggest that *B. bigemina* merozoite carries an antigen similar to BvP36.

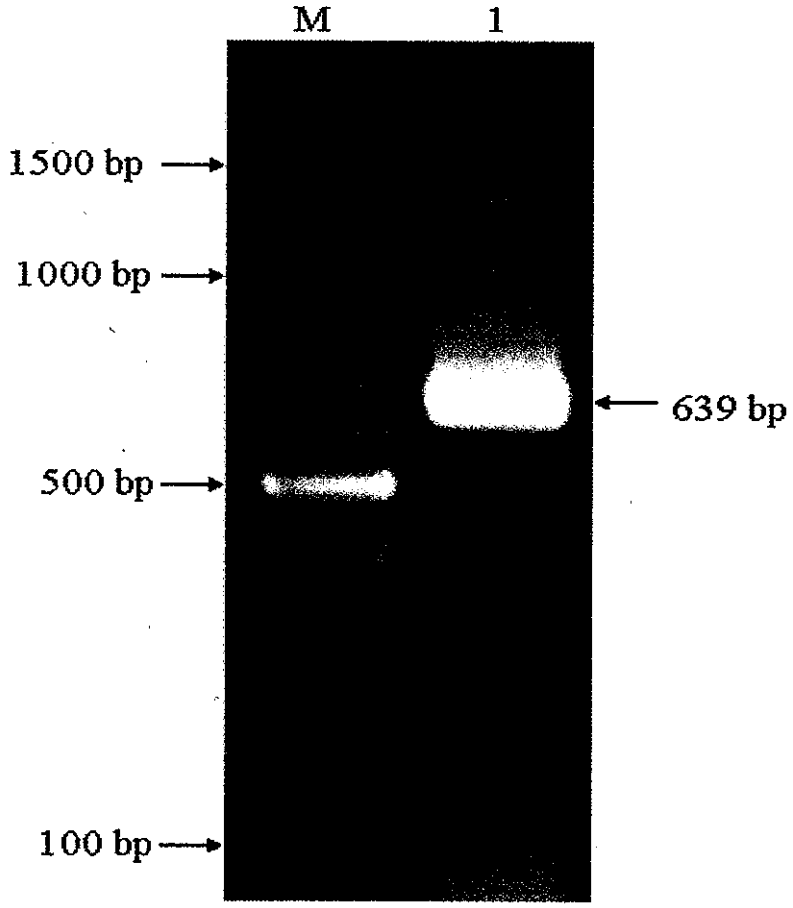

Fig. 6. PCR amplification of a full length of BvP36 gene from the genomic DNA of *B. bovis*. M, Molecular DNA ladder marker; 1, PCR product for the target BvP36 gene.

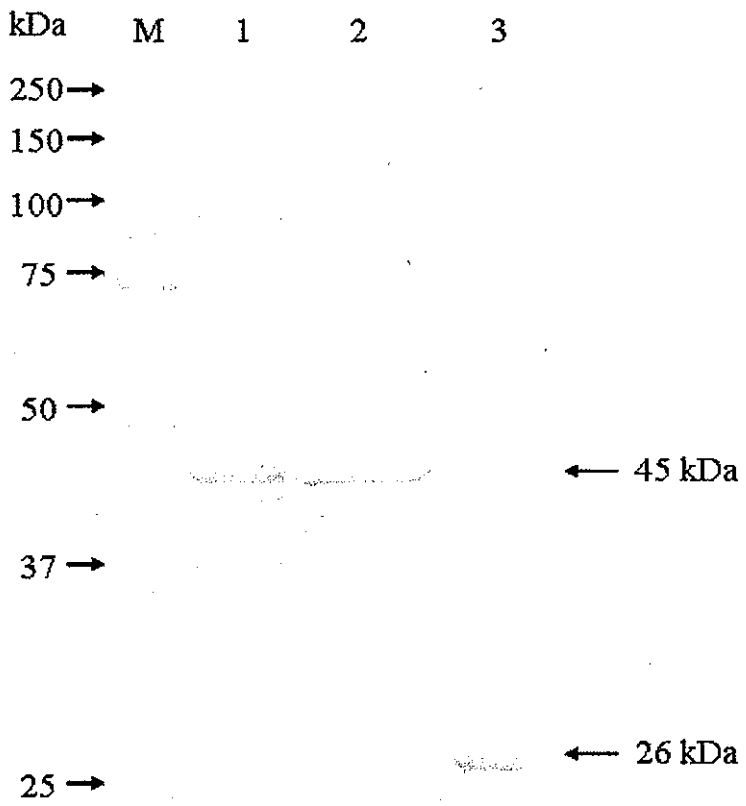

Fig. 7. Expression and purification of a recombinant GST-fusion protein (rBvP36) in *E. coli*. M, Molecular protein size marker; Lane 1, Soluble crude *E. coli* lysate; Lane 2, Purified fraction of the rBvP36 (45 kDa); Lane 3, Purified GST (26 kDa).

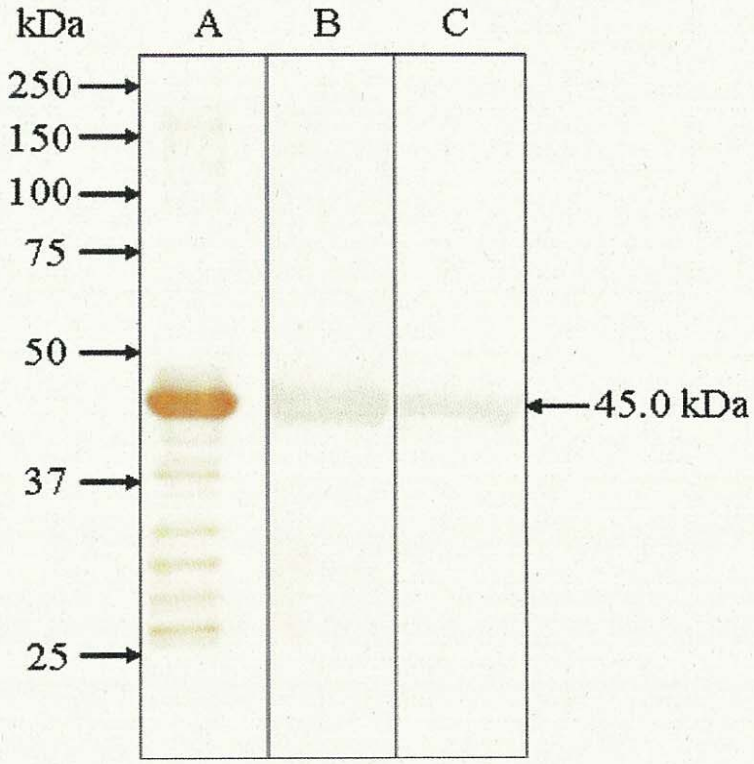

Fig. 8. Western blot analyses of rBvP36. Panel A, Treated with anti-rBvP36 serum. Panel B, Treated with bovine serum collected from the cattle experimentally infected with *B. bovis* merozoites; Panel C, Treated with bovine serum collected from the cattle experimentally infected with *B. bigemina* merozoites.

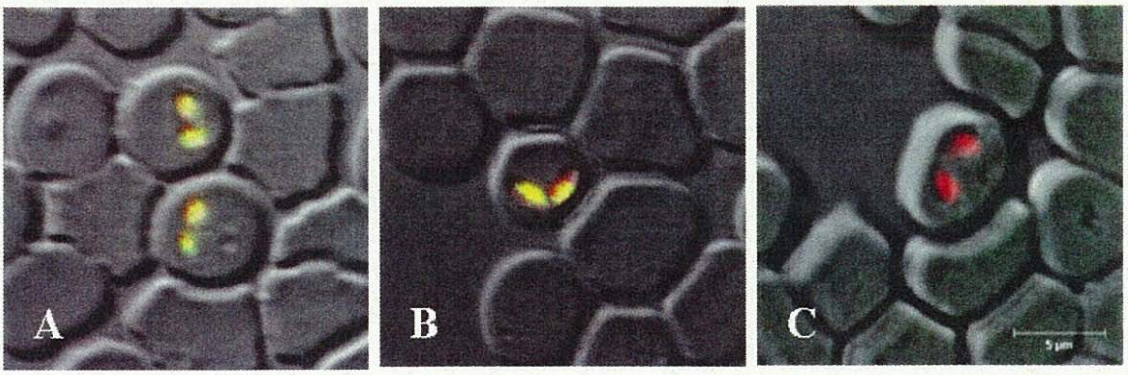

Fig. 9. Indirect Fluorescent Antibody Test analysis of rBvP36. The cultured *B. bovis* (Panel A) and *B. bigemina* (Panel B) merozoites treated with anti-rBvP36 serum; Panel C, The *B. bovis* merozoites treated with anti-GST immune serum. Note the strong fluorescence emitted in the panels A and B (5μm).

Chapter 3

Antigenic characterization of a 29-kDa cytoplasmic protein of *Babesia bovis*

3-1. Introduction

Bovine *Babesia* parasites, *B. bovis* and *B. bigemina,* are apicomplexan, tick-transmitted pathogen of cattle imposing a global risk with severe constraints to livestock industry (Barnett, 1974; McCosker, 1981). The traditional diagnostic method for babesioses has been done by the light microscopic examination of intraerythrocytic parasites in the Giemsa-stained blood smears (de Vos and Jorgensen, 1991). However, when the infections are subclinical or latent, the parasites may not always be detected by microscopy because of low levels of parasitemia (Jorgensen et al., 1994). Alternatively, the infections can be determined directly by polymerase chain reaction (PCR)-based tests (Calder et al., 1996) or indirectly by serological tests (Weiland et al., 1988). The global distribution, together with no available vaccine, babesiosis is considered as one of the most important diseases in cattle, in which over half of the world's cattle population are at the risk (McCosker, 1981).

Babesia parasites have many similarities to other apicomplexans, such as *Plasmodium* parasites. In addition, almost all of apicomplexan parasites share a set of common cytoskeletal structures that are essential for the parasite survival and pathogenesis (Morrissette et al., 2002, Dobrowolski et al., 1997). Cytoskeleton components are known to have role in maintaining the mechanical strength of the protozoan parasite (Fowler et al., 2004). The pathogenesis associated with these parasitic diseases is due to tissue damage caused by uncontrolled cycles of host-cell invasion, parasite proliferation, host-cell lysis,

and reinvasion. Furthermore, understanding the mechanisms by which these parasites are transmitted, persist, and cause disease will help optimize methods for control, including development of vaccines. Proteins located in the apical organelles are commonly involved in the selective recognition of target host cells (Sam-Yellowe et al., 1996; Barnwell and Galinski, 1998).

In *B. gobsoni* infection, the P29 was considered to play roles at the parasite invasion of the host erythrocyte by the maintenance of physical strength (Fukumoto, et al., 2003). In this study, therefore, I isolated the fragment of P29 gene from *B.bovis* genomic DNA and produced the recombinant protein (rBvP29) in order to evaluate the antigenicity.

3-2. Materials and methods

Parasites. *B. bovis* and *B. bigemina* were cultured *in vitro* using the microaerophilous stationary phase (MAPS) methods (Levi and Ristic, 1980; Vega and Buening, 1985). The parasites were used for DNA extraction as previously described (Boonchit et., al 2006).

Extraction of *B. bovis* genomic DNA and amplification of the P29 gene. Two-hundred microliters of infected red blood cells (RBC) were processed for isolation of *B. bovis* genomic DNA by using a QIAamp® DNA Blood mini kit (QIAamp DNA Blood Mini-Kit Madison, USA) according to manufacturer's instructions. Two oligonucleotide primers, Bov-P29-F- (5′-ACGAATTCATGCAGTGCTGCTCAC-3′) and Bov-P29-R- (5′-ACCTCGAGTTAGTTTGAAGATCTCTC -3′), were constructed, based on the P29 gene sequence of *B. bovis* (GenBank accession number: XM_001609876) (Brayton et al., 2007). PCR was performed in 25 µl reaction solution containing 10 pmol of each primer, 1.25 mM MgCl2, 200 µM dNTPs, 100 ng of the *B. bovis* genomic DNA and 0.5 U of ampliTaq Gold® Polymerase (Applied Biosystems, Roche, Branchburg, New jersey, USA). Reaction conditions were as follows: initial denaturation of 5 min at 94°C, 35 cycles of 60 sec at 94°C, 45 sec at 60°C, 60 sec at 72°C and final extension of 10 min at 72°C. The amplified DNA was purified by gel extraction kit (Qiagen, Tokyo, Japan) according to manufacturer's instructions.

Preparation of the recombinant BvP29 in *E. coli.* The amplified DNA was cloned into a pCR® 2.1 - TOPO® vector by using a TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) and dispensed on LB agar plate containing 50 µg/ml ampicillin in the presence of X-gal (Wako, Osoka, Japan). The positive plasmid was extracted form the culture by using a QIAprepspin miniprep kit (Qiagen), and the DNA sequence was determined using an ABI PRISM® 3100 genetic Analyzer (Applied Biosystems, Hitachi, LDT, Tokyo, Japan) and analyzed using a Genetyx® soft of version 8 (Genetyx, Corporation, Japan). A DNA

fragment containing P29 gene was isolated by digesting with two restriction endonucleases, *EcoR*I and *Xho*I, and subcloned into a pGEX-4T-1 *E. coli* expression vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The resulting plasmid was checked for the accurate insertion by sequencing and designated as pGEX-4T-1/P29. The P29 gene was expressed as a glutathione *S*-transferase (GST)-fusion protein in *E. coli* (BL21) strain according to the manufacturer's instructions (Amersham Pharmacia Biotech). The transformed *E. coli* was washed three times with phosphate-buffered saline (PBS), lysed in 1% Triton X-100–PBS, sonicated, and then centrifuged at 12,000 × g for 10 min at 4°C. The soluble GST-fusion protein, designated as rBvP29, was purified from the supernatant by glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Production of anti-rBvP29 mouse antibody. Anti-rBvP29 mouse serum was prepared as described previously (Boonchit et al., 2002). Four BALB/c female mice of 8-week old were immunized intraperitoneally with 50-μg rBvP29 in Freund's complete adjuvant (Sigma Aldrich, Saint Louis, MO, USA) followed by three booster doses with the incomplete adjuvant at 2-week interval. After the final booster, the sera were collected from the mice and stored at -20°C. The sera were subjected as anti-rBvP29 mouse antibody to immunofluorescent antibody test (IFAT) and Western blotting analysis, as described below. All mice used in the present study were handled with accordance to the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (Obihiro, Japan).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Purified rBvP29 and total *E. coli* lysate were mixed 1:1 (v/v) with 2 x SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue) (Laemmli, 1970) and boiled for 5 min. After

electrophoresis in a 12% SDS-polyacrylamide gel, the gel was subjected to a Coomassie brilliant blue (CBB) staining or Western blotting analysis. For Western blotting analysis, the proteins were transferred to nitrocellulose membranes (Immobilon transfer membrane; Millipore, Billerica, MA, USA) with a semidry blotting apparatus. The membrane was incubated in a blocking solution (3% skim milk in PBS) for 2 h at room temperature (RT) and then with experimental infected bovine sera (Boonchit et al., 2004) with *B. bovis* or *B. bigemina* or anti-rBvP29 mouse antibody (dilution 1:100) for 1 h. The membranes were washed three times with 0.05% Tween 20 in PBS (PBST) and incubated with horseradish peroxidase (HRP)-conjucated rabbit anti-bovine or anti-mouse antibody (Bethyl, Montgomery, TX, USA) (1:5,000 dilution) for 1 h at RT. The membrane was washed three times with PBST and placed into a substrate solution containing 0.5-mg/ml diabinobenzidine (Dojindo, Tokyo, Japan) and 0.005% H_2O_2 to visualize the antigen-antibody complexes (Xuan et al., 2001).

IFAT. Thin blood smear films of *B. bovis*-infected RBC were fixed with 50% acetone-50% methanol at -20°C for 5 min. Subsequently, the slides were incubated with 10% skim milk blocking solution for 30 min at RT. After washing three times with PBS, the slides were incubated with anti-rBvP29-specific or the control anti-GST mouse antibody (1:100 dilution) for 30 min at 37°C. After three-time washes with PBS, Alexa-Fluor® 488-conjugated rabbit anti-mouse immunoglobin G (IgG) (Southern biotechnology associates, Birmingham, AL, USA) was subsequently applied (1:200 dilution in 3% skim milk) and incubated for 30 min at 37°C. The slides were washed three times with PBS and incubated with 25-μg/ml propidium iodide (PI) (Wako, Japan) containing 50-μg/ml RNase A (Roche, Indianapolis, IN, USA) for 10 min at 37°C. After two washings with PBS, the slides were mounted with a fluorescent mounting medium (Dako, Carpinteria, CA, USA), and examined under a confocal laser-scanning microscope (TCS NT, Leica, Wetzlar, Germany).

3-3. Results and discussion

The aim of the present study was to clone and characterize a cytoskeleton protein (BvP29) of *B. bovis*. A full length of the BvP29 gene of 600 bp was successfully amplified in PCR from the genomic DNA of *B. bovis* (Fig. 10). The BvP29 gene was ligated in to the bacterial expression vector PGEX4T-1 and expressed as an approximately 48-kDa GST-fusion protein in *E. coli*. (Fig.11). The anti-rBvP29 mouse antibody and the experimentally infected bovine sera with *B. bovis* and *B. bigemina* were subjected to Western blot analyses using the rBvP29. As shown in Figure 12, the specific band of 48 kDa was detected by both of the infected bovine sera with *B. bovis* and *B. bigemina* (panels B and C, respectively). The rBvP29 was also clearly detected by anti-BvP29 mouse antibody (panel A).

To examine the localization of the BvP29 in the parasite, IFAT was carried out using the anti-rBvP29 mouse antibody. Figure 13 demonstrates that the immunofluorescent signals of BvP29 were strongly detected in the cytoplasm of cultured *B. bovis* and *B. bigemina* parasites. These results suggested that BvP29 and the relative homolog of *B. bigemina* might localize in the cytoplasm of parasites during their asexual phase. Consequently, I identified a gene encoding BvP29 protein, which might have homology to other protozoan cytoskeletal proteins.

In other protozoan parasites, P29 protein know to have plays a role in maintaining the mechanical strength and stability of the parasite when it exits the blood plasma for the purpose of invading new RBCs to proliferate (Nicols and Chiappino, 1987; Russel and Sinden, 1982, Fukumoto et al., 2003). The use of *B. bovis* genome sequence has provided an opportunity to identify novel drug targets. Selection of parasite proteins as drug targets is usually based on its functional importance, conservation across different species and sharing no homology with the host. Confocal microscope observation showed that P29 is localized broadly inside the parasite-infected erythrocyte. The immuno flourecence was observed in

the cytoplasm of parasite and associated with membrane-like and some other unconfirmed membrane structures (Fig.13).

Because cytoskeleton is crucial for the structural composition of the parasites, BvP29 might also play an important physiological role in asexual growth of the parasite. Thus, rBvP29 is an interesting molecule that may play a crucial role in the development of pathology and/or survival of the parasite in the host.

In summary, I cloned and characterized a *B. bovis* gene encoding a 29-kDa cytoplasmic protein (BvP29) as common antigen between *B. bovis* and *B. bigemina*. It is also important to study whether this antigen is well conserved among geographically different isolates of both *B. bovis* and *B. bigemina* and whether this antigen can be expressed in the sporozoite stages of both parasites. Hereafter, the rBvP29 proteins will be examined for development of ELISA and vaccine for bovine babesiosis.

3-4. Summary

Although a number of antigens have been described as promising vaccines for babesiosis, no fully protective vaccine has been developed yet. Therefore, a further identification of new target antigens is desired. In the present study, I characterized a 29-kDa *B. bovis* protein, named as BvP29. The BvP29 contains an open reading frame of 600 bp, corresponding to a putative 29-kDa protein of 199 amino acids. The BvP29 gene was expressed in *Escherichia coli* as a soluble glutathione *S*-transferase fusion protein (rBvP29). In Western blot analysis, approximately 48-kDa of the rBvP29 was recognized with the experimentally infected bovine sera with *B. bovis* and *B. bigemina*. Furthermore, the immunofluorescent assay with anti-rBvP29 mouse serum revealed that the positive reactions were mainly seen in the cytoplasm of *B. bovis* and *B. bigemina*. These results suggested that BvP29 might be a promising vaccine candidate for the control of bovine babesioses caused by the both parasites.

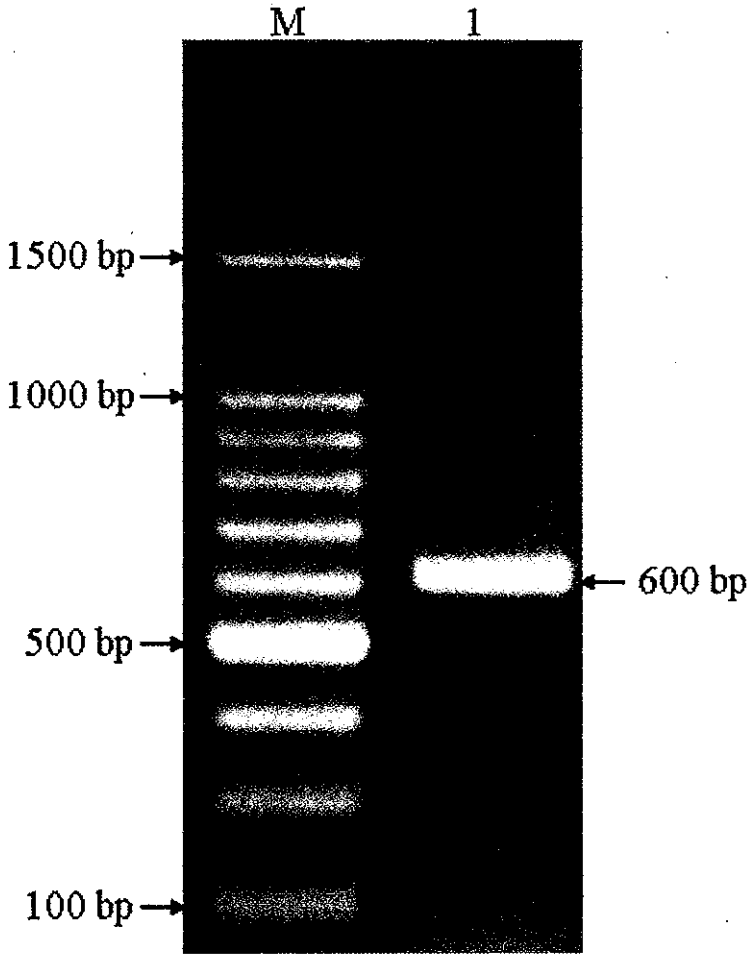

Fig.10. PCR amplification of BvP29 gene from the genomic DNA of *B. bovis*. M, Molecular DNA ladder marker; 1, PCR product for the target BvP29 gene.

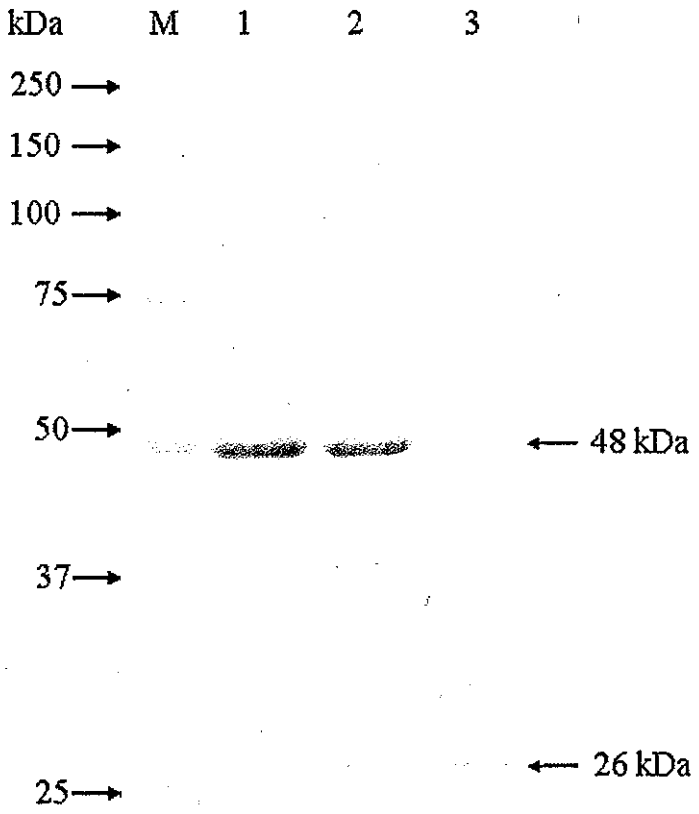

Fig.11. Expression and purification of a recombinant GST-fusion protein (rBvP29) in *E. coli*. M, Molecular protein size marker; Lane 1, Soluble crude *E. coli* lysate; Lane 2, Purified fraction of the rBvP29 (48 kDa); Lane 3, Purified GST (26 kDa).

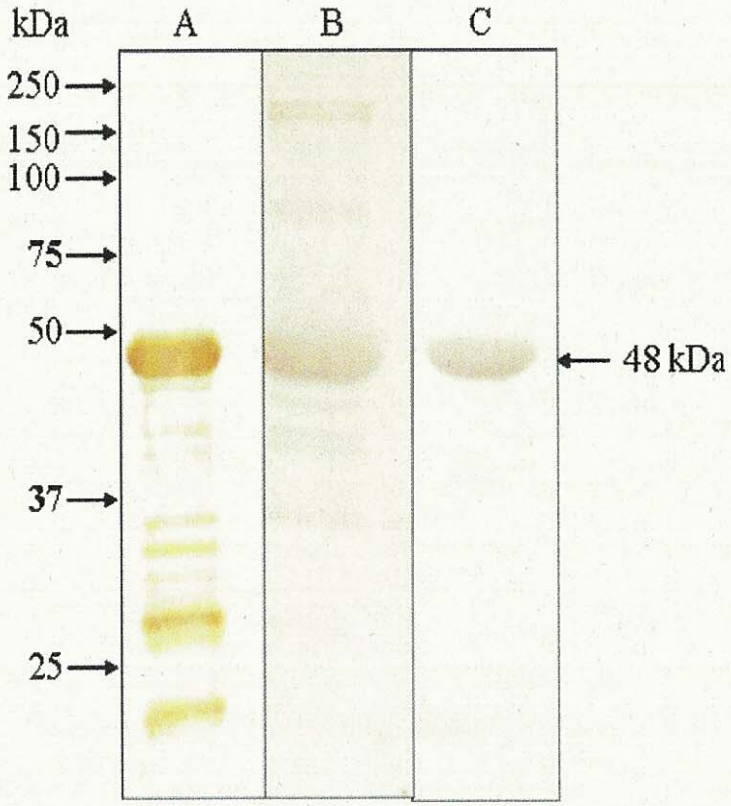

Fig.12. Western blot analyses of rBvP29. Panel A, Treated with mouse anti-rBvP29 serum. Panel B, Treated with bovine serum collected from the cattle experimentally infected with *B. bovis*; Panel C, Treated with bovine serum collected from the cattle experimentally infected with *B. bigemina.*

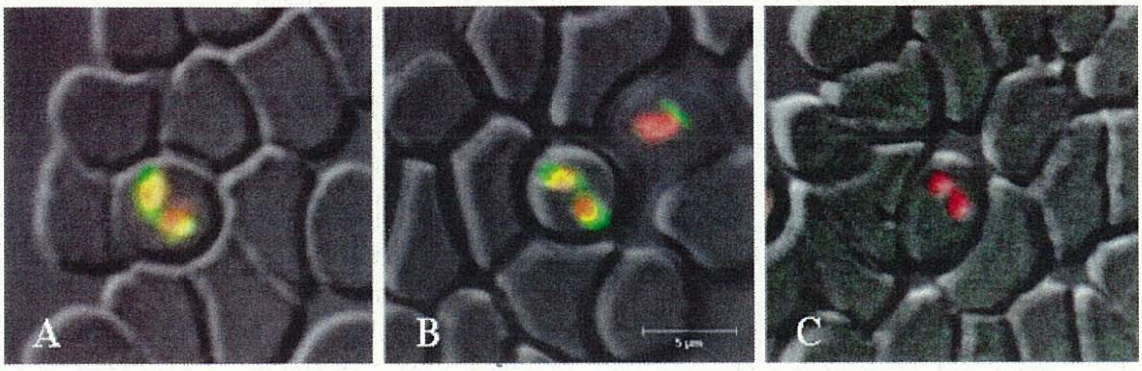

Fig.13. Indirect fluorescent antibody test analysis of rBvP29. The cultured *B. bovis* (Panel A) and *B. bigemina* (Panel B) merozoites treated with anti-rBvP29 immune serum; Panel C, The *B. bovis* merozoites treated with anti-control GST immune serum. Note the strong fluorescence emitted in the panels A and B (5μm).

General discussion

Bovine babesiosis poses greater risk to the livestock sector in tropical and subtropical regions of the world since it causes great economical losses (McCosker, 1981). The intraerythrocytic nature of the parasites induces haemolysis and results in clinical disease in susceptible animals. Early diagnosis and proper choice of anti-babesial agents are essential for the successful control of the disease.

A gold standard method for the diagnosis of acute babesiosis is an observation of parasites in Giemsa-stained thin blood smear under a light microscope (Araujo et al., 1998). However, species identification is subjective and needs experienced personnel. In addition, chronic infestations can not be diagnosed by this method (Calder et al., 1996). PCR technology has been proven as an improved diagnostic tool and more sensitive than that of light microscopy (Calder et al., 1996). Several authors have contributed for the development of specific PCR assays for the detection of *B. bovis* and *B. bigemina* (Figueroa et al., 1993, Criado-Fornelio et al., 2003). Furthermore, nested PCR, which is considered as a more sensitive technique, was also described earlier (Figueroa et al., 1993). Recently a developed real-time PCR assay allows to detect and to quantify the *Babesia* parasites in the infected animals (Kim et al., 2007). However, these PCR techniques have their own disadvantages. Multiplex PCR assay developed to detect both *B. bovis* and *B. bigemina* but showed lack of sensitivity (Figueroa et al., 1992). Contamination may become a serious problem due to extreme sensitivity of PCR techniques (Persing, 1991). In addition, presence of polymerase inhibitors in the blood may alter the results of the assay.

Detection of specific antibodies against *Babesia* parasites in bovine sera by ELISA can be used for surveillance of bovine babesiosis and monitoring of subclinical infection (Weiland and Reiter, 1988). Several authors have developed ELISA for the sero-diagnosis of

both *B. bovis* and *B. bigemina* (Boonchit et al., 2004, Tebele et al., 2000). However, some researchers pointed out that available serological test are not sufficient and stressed the need of improved assays. Tebele et al., 2000 demonstrated that P200 and its truncated form of recombinant proteins can be utilized as sero-diagnostic antigens for the diagnosis of *B. bigemina* infection.

In Chapter 1, I have produced truncated form of recombinant P200 antigen (rP200/CT) and evaluated its utility as a diagnostic antigen to detect *B. bigemina*-specific antibodies in field bovine serum samples. Western blot analysis conducted with *B. bovis* and *B. bigemina*-infected sera showed that the antigen is recognized only by *B. bigemina* infected serum. Similar results were obtained when the ELISA was performed with both antisera. These findings indicate that rP200/CT react only with *B. bigemina* infected serum and therefore can be used as an antigen for the sero-diagnosis of *B. bigemina* infected sera. However, it is essential to analyze field serum samples from different geographical areas using the rP200/CT as ELISA antigen to explore the possibility of using rP200/CT as a diagnostic antigen for surveillance and monitoring of *B. bigemina* infections. Therefore, I used serum samples and DNA samples from Ghana and performed the ELISA using rP200/CT. In addition, standard ELISAs were also conducted with *B. bovis* and *B. bigemina* specific rRAP-1/CT antigens prepared as described earlier (Boonchit et al., 2006). All of the *B. bigemina*-positive serum samples by the standard ELISA showed positive reactions in the rP200/CT coated ELISA plates. Positive serum samples for *B. bovis* and negative samples for both species identified by rRAP-1/CT antigens were negative by the ELISA conducted with rP200/CT antigen. More importantly absorbance values were higher in rP200/CT coated ELISA plates than that of rRAP-1/CT with the same field serum samples. These results indicate that the rP200/CT is very suitable antigen for the detection of the specific antibodies in *B. bigemina*-infected sera and therefore can be used for the surveillance and

monitoring of *B. bigemina* infection in susceptible cattle population in the endemic regions.

Two major preventive measures for bovine babesiosis are tick control and vaccination with live attenuated parasites (Jenkins, MC, 2001). However, tick control strategy is limited by the development of acaricide resistance and the costs associated with the use of chemicals (Palmer and McElwain, 1995). Although live attenuated vaccines are widely used to control the clinical disease (Shkap et al., 2007), certain problems such as different region specific strains, transmission of other blood borne pathogen, pathology on vaccination and short lifespan were encountered (De Vos and Bock, 2000, Hope et al., 2005). Therefore, alternative vaccination strategies have been studied; such culture-derived secreted antigens of *Babesia* parasites induced protective immunity in immunized cattle (Smith et al., 1981, Timms and Stewart, 1989) and recombinant proteins such as a 38 kDa cysteine-rich protein that elicited 90% protection level against *B. bovis* infection (Wright et al., 1992).

Recently, novel genes were identified from the genome projects expecting vaccine candidates such as a BvP36 that is a homologous antigen to P36 antigen of *Plasmodium* parasites (Brayton et al., 2007). P36 antigen in *Plasmodium* parasites is found to be involved in hepatocytes infection of sporozoites (Ishino et al., 2005). When the sporozoites lacking P36 antigen were injected to the host the formation of parasitophorous vacuoles were disturbed and the parasitic stages were found as free forms in hepatocytes (Labaied et al., 2007). In addition, the sporozoites became completely attenuated forms and produced sterile immunity (Labaied et al., 2007). Therefore, P36 gene of *Plasmodium* parasites is considered as a knock-out gene for the development of attenuated vaccine (Brayton et al., 2007). Therefore, in order to expand the spectrum for the availability of recombinant vaccine candidates, I tried to isolate a gene from *B. bovis* with similar homology to P36 gene of *Plasmodium* in Chapter 2.

I have expressed and characterized the BvP36 antigen of *B. bovis* that has homology to

the P36 antigen of *Plasmodium* parasites in the present study. I demonstrated that the BvP36 antigen is expressed in merozoite stages of *B. bovis.* Unlike the *Plasmodium* parasites, *B. bovis*-sporozoites which are injected by the infected ticks can directly invade host RBC and transform into merozoites (Uilenberg G, 2006). Therefore, use of BvP36 as a knock-out gene for the development of live attenuated vaccine against *B. bovis* infection might be possible. Further studies on the functions of this antigen is of paramount importance to reach any conclusions. Another important aspect of this antigen is the concept for the development of recombinant vaccines. The SAG-1 antigen of *T. gondii* which is similar to that of P36 antigen of *Plasmodium* parasites is well recognized as a vaccine candidate against *T. gondii* infection (Velge-roussel., 2000, Moorthy et al., 2004). Therefore, the vaccine potential of recombinant BvP36 should also be explored. Interestingly, I have demonstrated the presence of similar antigen in *B. bigemina* merozoites and therefore we can assume that if BvP36 can be used as a recombinant vaccine, the animals might be protected against *B. bigemina* infection as well as *B. bovis* infection.

Erythrocyte invasion is one of the most critical stages of the asexual growth cycle that is immunologically vulnerable. Extracellular merozoites are directly exposed to the host humoral immune components, while the antibody can't reach the parasites inside the RBC (Yokoyama et al., 2002). Therefore, the elucidation of the invasive process will lead to the successful identification of potential candidates that are applicable for the development of a *Babesia* vaccine, like *Plasmodium* parasites, where an increased number of ligands involved in the invasive stage have been identified and some of them are being explored as candidate vaccine antigens (Moorthy et al., 2004 and Mahanty et al., 2003). In apicomplexan parasites, the dense granule proteins are considered to function in host cell internalization (Blackman and Bannister, 2001; Dubremetz et al., 1998). However, the internalization processes vary within the members of the phylum Apicomlexa. In particular, parasitophorous vacuoles of

the *Babesia* species, as well as the *Theileria* species, rapidly disintegrate following the penetration step, unlike *Plasmodium* and *Toxoplasma* species, in which the parasitophorous vacuoles formation must be kept during the internalization (Sam-Yellowe, 1996). Although it has been proposed that the contents of the spherical bodies are involved in the dissolution of the PV membrane via the host cell association in *Babesia* parasites, further experiments are needed to identify their biological functions (Yokoyama et al., 2006).

In Chapter 3, BvP29 gene was isolated from *B. bovis* and the recombinant BvP29 (rBvP29) was expressed as a GST fusion protein. Approximately 48-kDa of rBvP29 protein was recognized by the both serum samples from cattle experimentally infected with *B. bovis* or *B. bigemina*. BvP29 was mainly seen during the ring stage of *B. bovis* and *B. bigemina* merozoites. These results suggest that rBvP29 protein is a common antigen between *B. bovis* and *B. bigemina*. The BvP29 antigen of *B. bovis* has homology to the P29 antigens of *B. gibsoni* and *T. gondii*. The P29 antigen of *B. gibsoni* is found to be involved in invasion of merozoites into canine erythrocytes (Fukumoto et al., 2003; Mann et al., 2001). Therefore, it will be very interesting to study the functional of the BvP29 antigen for involvement of parasite invasion. These studies might lead to the vaccine development based on the invasion factors of *B. bovis*.

In summary, I have produced a recombinant antigen rP200/CT and demonstrated its potential as a sero-diagnostic antigen by ELISA. Chapters 2 and 3 dealed with expressions and characterizations of BvP36 antigen which is homologous to the P36 antigen of *Plasmodium* parasites and similar to SAG-1 of *T. gondii* and BvP29 antigen which is homologous to the P29 antigen of *B. gibsoni* and *T. gondii* parasites, respectively. I also demonstrated that BvP36 and BvP29 are expressed in *B. bovis* merozoites. Further study will be necessary to examine the potential usefulness of these antigens for diagnostics and control of bovine babesiosis in the future.

Acknowledgments

This study was carried out at the National Reasearch Center for Protozoan Diseases (NRCPD), Obihiro University of Agriculture and Veterinary Medicine and supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), a Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), the industrial Technology Reasearch Grant program from the New Energy industrial Technology Development Organization (NEDO) of Japan, and 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) and the Japan International Cooperation Agency (JICA).

I am deeply indebted to my supervisor, Professor Ikuo Igarashi for intellectual instruction, direction, constructive criticisms, invaluable suggestions, patience revising this dissertation, and tireless support during this doctoral course.

My deepest thanks are expressed to Associate Professor Dr. Naoaki Yokoyama (Obihiro Univ. Agri. Vet.Med. NRCPD), Associate Professor Dr. Noboru Inoue (Obihiro Univ. Agri. Vet.Med. NRCPD), Dr. Andy allhassan (NRCPD), Dr. Damdinsuren Boldbaatar (Kagoshima University) for their valuable advice, helpful suggestion, comments and critical reading of the manuscripts as well as teaching the basic techniques.

Futhermore, my especial thanks to Professor Dr. Shin-ichiro Kawazu (Obihiro Univ. Agri. Vet.Med. NRCPD), Professor Dr. Hiroshi Suzuki (Obihiro Univ. Agri. Vet.Med. NRCPD), Professor Dr. Xuenan Xuan (Obihiro Univ. Agri. Vet.Med. NRCPD), Professor Dr. Hirotaka Kanuka (Obihiro Univ. Agri. Vet.Med. NRCPD), Associate Professor Dr. Makoto Igarashi, Assistant Professor Shinya Fukumoto (Obihiro Univ. Agri. Vet.Med. NRCPD) for their valuable advice and encouragement.

I am also grateful to all staff of the Mongolian Government and Mongolian Ministry

of Food and Agriculture and Department of Veterinary Services.

I extend my gratitude to Dr. Noriyuki Takabatake (NRCPD), Dr. Hiroshi Iseki (NRCPD), Dr. Mohamed Alaa Terkawi (NRCPD), Mr. Mahmoud AbouLaila (NRCPD), Mr. Thillaiampalam Sivakumar (NRCPD) and other former and present staff and students in the National Research Center for Protozoan Diseases for their assistance in many ways.

My sincere gratitude to Dr. Zayat Batsukh (Mongolian Veterinary Institute), Dr E.B.M. Koney (Vet.Ser.Lab, Ghana) and all members of their laboratories for collaboration and helping in both academic and non-academic ways.

Finally, I would like to thank my family, and my friends for their encouragement and support.

Reference

1. Aguilar-Delfin, MJ., Homer, PJ., Wettstein and Persing., DH. (2001). Innate resistance to *Babesia* infection is influenced by genetic background and gender. Infect. Immun. 69, 7955–7958.
2. Aguilar-Delfin, PJ., Wettstein and Persing., DH. (2003). Resistance to acute babesiosis is associated with interleukin-12- and gamma interferon-mediated responses and requires macrophages and natural killer cells. Infect. Immun. 71, 2002–2008.
3. Akinboade, OA., Akinboade, CY., (1985). The effect of *Babesia bigemina* infections caused by cattle ticks on Nigerian economy. Rev. Elev. Med. Vet. Pays. Trop. 38, 250–252.
4. Araujo, FR., Madruga, CR., Leal, CR., Schenk, MA., Kessler, RH., Marques, AP., Lemaire, DC., (1998). Comparison between enzyme-linked immunosorbent assay, indirect fluorescent antibody and rapid conglutination tests in detecting antibodies against *Babesia bovis*. Vet. Parasitol. 74, 101-108.
5. Avarzed, A., Igarashi, I., Kanemaru, T., Hirumi, K., Omata, Y., Saito, A., Oyamada, T., Nagasawa, H., Toyoda, Y., and Suzuki, N. (1997). Improved in vitro cultivation of Babesia cabali. J.Vet Med. Sci. 59, 479-481.
6. Babes, V., (1888). Sur l'hemoglobinurie bactérienne du boeuf. C. R. Acad. Sci. Ser. III Sci. Vie, 107, 692-694.
7. Barnett, SF., (1974). Economical aspects of protozoal tick-borne diseases in livestock in parts of the world other than Britain. Bull Off int. Epiz. 8, 183–196.

8. Barnwell, JW., and Galinski, MR., (1998). Invasion of vertebrate cells: erythrocytes. In Sherman,I.W. (ed.), Malaria: Parasite Biology, Pathogenesis and Protection. ASM. Press, Washington, DC, 193–120.

9. Belák, S., Ballagi-Pordány, A., (1993). Experiences on the application of the polymerase chain reaction in a diagnostic laboratory. Mol. Cell. Probes 7, 241-8.

10. Blackman, M.J., and Bannister, LH., (2001). Apical organelles of Apicomplexa: biology and isolation by subcellular fractionation, Mol. Biochem. Parasitol. 117, 11–25.

11. Boonchit, S., Alhassan, A., Chan, B., Xuan, X., Yokoyama, N., Ooshiro, M., Goff, WL., Waghela, SD., 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.

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

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

14. Bork, S., Das, S., Okubo, K., Yokoyama, N., Igarashi, I., (2006). Effects of protein kinase inhibitors on the in vitro growth of *Babesia bovis*. Parasitol. 132, 775-779.

15. Bork, S., Yokoyama, N., Ikehara, Y., Kumar, S., Sugimoto, Ch., and Igarashi, I., (2004). Growth-Inhibitory Effect of Heparin on *Babesia* Parasites Antimicrob. Agents Chemother. 48, 236–241.

16. Bork, S., Yokoyama, N., Matsuo, T., Claveria, FG., Fujisaki, K., Igarashi, I., (2003). Growth inhibitory effect of triclosan on equine and bovine *babesia* parasites. AJ. Trop. Med. Hyg. 68, 334-340.

17. Bose, R., Jorgensen, WK., Dalgliesh, RJ., Friendhoff, KT., de Vos, AJ., (1995). Current state and future trends in the diagnosis of babesiosis. Vet. Parasitol. 57, 61–74.

18. Brayton, KA., Lau, AO., Herndon, DR., Hannick, L., Kappmeyer, LS., Berens, SJ., Bidwell, SL., Brown, WC., Crabtree, J., Fadrosh, D., Feldblum, T., Forberger, HA., Haas, BJ., Howell, JM., Khouri, H., Koo, H., Mann, DJ., Norimine, J., Paulsen, IT., Radune, D., Ren, Q., Smith, RK., Suarez, CE., White, O., Wortman, JR., Knowles, DP., McElwain, JrTF., and Nene, VM., (2007). Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS. Pathog. 3, 1401-13.

19. Brown, WC., Norimine, J., Knowles, DP., Goff, WL., (2006). Immune control of *Babesia bovis* infection. Vet. Parasitol. 138, 75–87.

20. Calder, JA., Reddy, GR., Chieves, L., Courtney, CH., Littell, R., Livengood, JR., Norval, RA., Smith, C., Dame, JB., (1996). Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. J. Clin. Microbiol. 34, 2748–2755.

21. Carson, CA., and Philips, RS., (1981). Immunologic response of the vertebrate hosts to *babesia* In: RISTIC, M. and KREIER, J.P. (eds). Babesiosis. Academic Press, 411-443.

22. Carter, R., Coulson, A., Bhatti, S., Taylor, BJ., and Elliott, JF., (1995). Predicted disulfide-bonded structures for three uniquely related proteins of *Plasmodium falciparum*, Pfs230, Pfs48/45 and Pf12. Mol. Biochem. Parasitol. 71, 203-210.

23. Criado-Fornelio, A., Martinez-Marcos, A., Buling-Saraña, A., Barba-Carretero, JC., (2003). Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part I. Epizootiological aspects. 113, 189-201.

24. Dalrymple, BP., Jorgensen, WK., de Vos, AJ., Wright, IG., (1992). Analysis of the composition of samples of *Babesia bovis* and the influence of different environmental conditions on genetically distinct subpopulations. Int. J. Parasitol 22, 731–737.

25. De Vos, AJ., Bock, RE., (2000). Vaccination against bovine babesiosis. Ann. NY. Acad. Sci. 916, 540–545.

26. De Vos, AJ., Jorgensen, WK., (1991). Manual of recommended diagnostic techniques and requirements for biological products. Paris, France: Office Intern. des Epizootics. Bovine babesiosis. 1/14–14/14.

27. De Waal, DT., (1996). Vaccination against babesiosis. Acta. Parasitol. Turcica 20, 487 −499.

28. De Waal, DT., and Combrink, MP., (2006). Live vaccines against bovine babesiosis. Vet. Parasitol. 138, 88-96.

29. De la Fuente, J., Rodriguez, M., Montero, C., Redondo, M., Garcia-Garcia, JC., Mendez, L., Serrano, E., Valdes, M., Enriquez, A., Canales, M., Ramos, E., Boue, O., Machado, M., and Lleonart, R., (1999). Vaccination against ticks (*Boophilus* spp.) the experience with the Bm86-based vaccine Gavac (TM), Genet. Anal.: Biomol. Eng. 15, 143–148.

30. Dobrowolski, J., Sibley, LD., (1997). The role of the cytoskeleton in host cell invasion by *Toxoplasma gondii*. Behring. Inst. Mitt. 99, 90-6.

31. Dubremetz, JF., Garcia-Reguet, N., Conseil, V., and Fourmaux, MN., (1998). Apical organelles and host-cell invasion by Apicomplexa, Int. J. Parasitol. 28, 1007–1013.

32. El-Ghaysh, A., Sundquist, B., Christensson, DA., Hilali, M., Nassar, AM., (1996). Observations on the use of ELISA for detection of *Babesia bigemina* specific antibodies. Vet. Parasitol. 62, 51–61.

33. Etkind, P., Piesman, J., Ruebush, II T., Spielman, A., and Juranek, DD. (1980). Methods for detecting *Babesia microti* infection in wild rodents. J. Parasitol. 66, 107-110.

34. Figueroa, JV., Chieves, LP., Johnson, GS., and Buening, GM., (1993). Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood, Vet. Parasitol. 50, 69–81.

35. Figueroa, JV., Chieves, LP., Johnson, GS., Buening, GM., (1992). Detection of *Babesia bigemina*-infected carriers by polymerase chain reaction amplification. J. Clin. Microbiol. 30, 2576–2582.

36. Fowler, RE., Margos, G., (2004). The cytoskeleton and motility in apicomplexan invasion Mitchell GH. Adv. Parasitol. 56, 213-63.

37. Friedhoff, KT., and Smith, RD., (1981). Transmission of *babesia* by ticks. In: RISTIC, M., and KREIER, JP., (eds). Babesiosis. Acad. Press, 268-321.

38. Fukumoto, S., Xuan, X., Inoue, N., Igarashi, I., Sugimoto, Ch., Fujisaki, K., Nagasawa, H., Mikami, T., Suzuki, H., (2003). Molecular characterization of a gene encoding a 29-kDa cytoplasmic protein of *Babesia gibsoni* and evaluation of its diagnostic potentiality, Mol. Biochem. Parasitol. 131, 129–136.

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

40. George, JE., (2000). Present and future technologies for tick control. Ann. NY. Acad. Sci. 916, 583-8.

41. George, JE., Pound, JM., Davey, RB., (2004). Chemical control of ticks on cattle and the resistance of these parasites to acaricides. Parasitol. *129*, S353–S366.

42. Gerloff, DL., Creasey, A., Maslau, S., and Carter, R., (2005). Structural models for the protein family characterized by gamete surface protein Pfs230 of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. 102, 13598-13603.

43. Gibson, CA., Rawlin, GT., (1992). Biotechnology in the diagnosis of animal diseases. Australas Biotechnol. 2, 292-6.

44. Goff, WL., Johnson, WC., Molloy, JB., Jorgensen, WK., Waldron, SJ., Figueroa, JV., Matthee, O., Adams, DS., McGuire, TC., Pino, I., Mosqueda, J., Palmer, GH., Suarez, CE., Knowles, DP., McElwain, TF., (2008). Validation of a competitive ELISA for detection of *Babesia bigemina* antibodies in cattle. Clin. Vac. Immunol, 15, 1316-1321.

45. Grande, N., E. Precigout, M. L. Ancelin, K. Moubri, B. Carcy, J. L. Lemesre, H. Vial, and A. Gorenflot. (1997). Continuous *in vitro* culture of *Babesia divergens* in a serum-free medium. Parasitology 115: 81–89.

46. Gubbels, JM., de Vos, AP., van der Weide, M., Viseras, J., Schouls, LM., de Vries, E., Jongejan, F., (1999). Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 37, 1782-9.

47. Hines, SA., Palmer, GH., Jasmer, DP., McGuire, TC., and McElwain, TF., (1992). Neutralization-sensitive merozoite surface antigens of *Babesia bovis* encoded by members of a polymorphic gene family. Mol. Biochem. Parasitol. 55, 85-94.

48. Holman, PJ., Frerichs, WM., Chieves, L., and Wagner, GG., (1993). Culture confirmation of the carrier status of *Babesia caballi* infected horses. J. Clin. Microbiol. 31, 698–701.

49. Holman, P. J., L. Chieves, W. M. Frerichs, D. Olson, and G. G. Wagner. (1994). *Babesia equi* erythrocytic stage continuously cultured in an enriched medium. J. Parasitol. 80: 232–236.

50. Homer, MJ., Aguilar-Delfin, I., Telford, SR., Krause, PJ., and Persing, DH., (2000). Babesiosis. Clin. Microbiol. 13, 451-69.

51. Hope, M., Riding, G., Menzies, M., Colditz, I., Reverter, A., and Willadsen, P., (2005). Potential for recombinant *Babesia bovis* antigens to protect against a highly virulent isolate. Parasit. Immunol. 27, 439-445.

52. Hotzel, I., Suarez, CE., McElwain, TF., and Palmer, GH., (1997). Genetic variation in the dimorphic regions of RAP-1 genes and rap-1 loci of *Babesia bigemina*. Mol. Biochem. Parasitol. 90, 479-89.

53. Howell, J., Massaro, M., Ueti, W., Palmer, H., Scoles, A., and Donald., Knowles, P., (2007). Transovarial Transmission Efficiency of *Babesia bovis* Tick Stages Acquiredby *Rhipicephalus* (*Boophilus*) *microplus* during Acute Infect. j. clin. Microbiolog. 45, 426–431.

54. Igarashi, I., Njonge, FK., Kaneko, Y., and Nakamura, Y., (1998). *Babesia bigemina*: *in vitro* and *in vivo* effects of curdlan sulfate on growth of parasites. Exp. Parasitol. 90, 290-3.

55. Iseki, H., Alhassan, A., Ohta, N., Thekisoe, O., Yokoyama, N., Inoue, N., Nambota, A., Yasuda, J., and Igarashi, I., (2007). Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites. J. Microbiol.Methods. 71, 281-287.

56. Ishino, T., Chinzei, Y., and Yuda, M., (2005). Two proteins with 6-Cys motifs are required for malarial parasites to commit to infection of the hepatocytes. Mol. Microbiol. 58, 1264-1275.

57. Jenkins, MC., (2001). Advances and prospects for subunit vaccines against protozoa of veterinary importance. Vet. Parasitol. 101, 291-310.

58. Jorgensen, W., de Vos, AJ., Dalgleish, JR., (1994). Methods for diagnosis of babesiosis in developing countries. Present and future trends. In: Uilenberg, G., Permin, A., Hansen, JW., editors. Use of applicable biotechnological methods for diagnosing haemoparasites. FAO, 65–84.

59. Kim, Ch., Iseki, H., Herbas, MS., Yokoyama, N., Suzuki, H., Xuan, X., Fujisaki, K., and Igarashi, I., (2007). Development of Taqman-Based Real-Time PCR Assays for Diagnostic Detection of *Babesia bovis* and *Babesia bigemina* Am. J. Trop. Med. Hyg, 77, 837-841.

60. Krause, PJ., Lepore, T., Sikand, VK., Gadbaw, JJr., Burke, G., Telford, SR., Brassard, P., Pearl, D., Azlanzadeh, J., Christianson, D., McGrath, D., Spielman, A., (2000). Atovaquone and azithromycin for the treatment of babesiosis N Engl. J. Med. 16, 1454-8.

61. Labaied, M., Hurupa, A., Dumpit, RF., Coppens, I., Mikolajczak, SA., and Kappe, SHI. (2007). *Plasmodium yoelii* sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection. Infect. Immun. 75, 3758-3768.

62. Laemmli, UK., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685.

63. Levi, MG., and Ristic, M., (1980). *Babesia bovis*: Continuous cultivation in a microaerophilus stationary phase culture. Science, 207, 1218-1220.

64. Levine, ND., (1985). Veterinary protozoology. Lowa State University Press, Ames, 288-313.

65. Luke, TC., Hoffman, SL., (2003). Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated *Plasmodium falciparum* sporozoite vaccine. J. Exp. Biol. 206, 3803–3808.

66. Mahanty, S., Saul, A., and Miller, LH., (2003). Progress in the development of recombinant and synthetic blood-stage malaria vaccines, J. Exp. Biol. 206, 3781–3788.

67. Mann, T., Beckers, C., Inoue, N., Igarashi, I., Nagasava, H., (2001). Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite *Toxoplasma gondii*. Mol. Biochem. Parasitol. 115, 257–268.

68. Mbati, PA., Hlatshwayo, M., Mtshali, MS., Mogaswane, KR., de Waal, TD., Dipeolu, OO., (2002). Ticks and tick-borne diseases of livestock belonging to resource-poor farmers in the eastern Free State of South Africa. Exp. Appl. Acarol. 28, 217–224.

69. McCosker, PJ., (1981). The global importance of babesiosis. In: RISTIC, M., and KREIER, JP., (eds). Babesiosis. Acad. Press. 2-25.

70. Mehlhorn, H., and Schein, E., (1984). The piroplasms: life cycle and sexual stages, Adv. Parasitol. 23, 37–103.

71. Mineo, JR., MacLeod, R., Mack, D., Smith, J., Khan, IA., Ely, KH., and Kasper, LH., (1993). Antibodies to *Toxoplasma gondii* major surface protein (SAG-1, P30) inhibit infection of host cells and are produced in murine intestine after peroral infection. J. Immunol. 150, 3951-3964.

72. Moorthy, S., Good, MF., and Hill, AV., (2004). Malaria vaccine developments, Lancet. 363, 150–156.

73. Morrissette, NS., Sibley, LD., (2002). Cytoskeleton of apicomplexan parasites. Microbiol. Mol. Biol. 66, 21-38.

74. Morzaria, S., Katende, J., Kairo, A., Nene, V., Musoke, A., (1992). New methods for the diagnosis of *Babesia bigemina* infection. Mem. Inst. Oswaldo. Cruz. 87, 201–205.

75. Nagai, A., Yokoyama, N., Matsuo, N., Bork, S., Hirata, H., Xuan, X., Zhu, Y., Claveria, FG., Fujisaki, K., and Igarashi, I., (2003). Growth-Inhibitory Effects of

Artesunate, Pyrimethamine, and Pamaquine against *Babesia equi* and *Babesia caballi* in *In Vitro* Cultures. Antimicrob. Agents Chemother. 47, 800–803.

76. Nichols, BA., Chiappino, ML., (1987). Cytoskeleton of *Toxoplasma gondii*. J. Protozool. 34, 217–26.

77. Nishikawa, Y., Xuan, X., Nagasawa, H., Igarashi, I., Fugisaki, K., Otsuka, H., and Mikami, T (2000). Monoclonal antibody inhibition of *Neospora caninum* tachyzoite invasion into host cells. Inter. J. parasit. 30, 51-58.

78. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T.,Watanabe, K., Amino., (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

79. Okubo, K., Yokoyama, N., Govind, Y., Alhassan, A., Igarashi, I., (2007). *Babesia bovis*: effects of cysteine protease inhibitors on in vitro growth. Exp. Parasitol. 117, 214-7.

80. Palmer, GH., McElwain, TF., (1995). Molecular basis for vaccine development against anaplasmosis and babesiosis. Vet. Parasitol. 57, 233-53.

81. Persing, DH., (1991). Polymerase chain reaction: trenches to benches. J. Clin. Microbiol. 29, 1281-5.

82. Ravindran, R., Mishra, AK., Rao, JR., (2007). Slide enzyme-linked immunosorbent assay for the diagnosis of *Babesia bigemina* infection in bovines. Vet. Res. Commun. 31, 999–1004.

83. Ristic, M (1988). Babesiosis of Domestic Animals and Man, CRC Press, Boca Raton, FL. 275.

84. Ristic, M., and levy, MG., (1981). A new era of research toward solution of bovine babesiosis. In: In: RISTIC, M., and KREIER, JP., (eds). Babesiosis. Acad. Press. 509-549.

85. Rodriguez, S., Buening, G., Green, T., and Carson, C., (1983). Cloning of *Babesia bovis* by *in vitro* cultivation, Infect. Immun. 42, 15–18.

86. Rudzinska, MA., Spielman, A., Riek, RF., Lewengrub, SJ., and Piesman, J., (1979). Intraerythrocytic "gametocytes" of *Babesia microti* and their maturation in ticks. Can. J. Zool. 57, 424-434.

87. Rudzinska, MA., Lewengrub, S., Spielman, A., and Piesman, J., (1983). Invasion of *Babesia microti* into epithelial cells of the tick gut. J. Protozool. 30, 338-346.

88. Russell, DG., Sinden, RE., (1982). Three-dimensional study of the intact cytoskeleton of coccidian sporozoites. Int. J. Parasitol. 12, 221–6.

89. Sam-Yellowe, TY., (1996). Rhoptry organelles of the Apicomplexa: their role in host cell invasion and intracellular survival. Parasitol. Today 12, 308–316.

90. Samish, M., and Rehacek, J., (1999). Pathogens and predators of ticks and their potential in biological control, Annu. Rev. Entomol. 44, 159–182.

91. Shkap, V., Leibovitz, B., Krigel, Y., Hammerschlag, J., Marcovics, A., Fish, L., Molad, T., Savitsky, I., Mazuz, M., (2005). Vaccination of older *Bos taurus* bulls against bovine babesiosis. Vet. Parasitol. 129, 235-42.

92. Shkap, V., de Vos., AJ., Zweygarth, E., and Jongejan, F. (2007). Attenuated vaccines for tropical theileriosis, babesiosis and heartwater: the continuing necessity. Trends. Parasitol. 23, 420-426.

93. Smith, T., and Kilborne, FL., (1893). Investigations into the nature, causation, and prevention of Texas or southern cattle fever, 8th and 9th Repts., Bur. Anim. Industr. U.S. Dept. Agric. 177–304.

94. Smith, DB., and Johnson, KS., (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene 67, 31-40.

95. Smith, RD., James, MA., Ristic, M., Aikawa, M., Vega, Y., Murguia, CA., (1981). Bovine babesiosis: protection of cattle with culture-derived soluble *Babesia bovis* antigen. Science, 212, 335–338.

96. Solari, MA., Nari, A., Cardozo, H., (1992). Impact of *Babesia bovis* and *Babesia bigemina* on the production of beef cattle in Uruguay. Mem. Inst. Oswaldo. Cruz. 87, 143–149.

97. Sonda, S., Fuchs, N., Connolly, B., Fernandez, P., Gottstein, B., Hentrich, B., and Hemphill, A., (1998). The major 36 kDa *Neospora caninum* tachyzoite surface protein is closely related to the major *Toxoplasma gondii* surface antigen. Mol. Biochem. Parasitol. 97, 97-108.

98. Stegeman, JR., Birkenheuer, AJ., Kruger, JM., Breitschwerdt, EB., (2003). Transfusion associated *Babesia gibsoni* infection in a dog. J. Am. Vet. Med. Assoc. 222, 959-963.

99. Tatchell R., (1984). Ticks and Tick born Disease Control: A practical Field Manual. Vol II Tick-born Disease Control. Food Agriculture Organisation of the United Nations (FAO), Rome, Italy.

100. Tebele, N., Skilton, RA., Katende, J., Wells, CW., Nene, V., McElwain, T., Morzaria, SP., Musoke, AJ., (2000). Cloning, characterization, and expression of a 200-kilodalton diagnostic antigen of *Babesia bigemina*. J. Clin. Microbiol. 38, 2240–2247.

101. Tenter, AM., and Friedhoff, KT., (1986). Serodiagnosis of experimental and natural *Babesia equi* and *B. caballi* infections, Vet. Parasitol. 20, 49–61.

102. Timms, P., Stewart, NP., (1989). Growth of *Babesia bovis* parasites in stationary and suspension cultures and their use in experimental vaccination of cattle. Res. Vet. Sci. 47, 309-14.

103. Towbin, H., Staehelin, T., Gordon, J., (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. 76, 4350–4354.Uilenberg, G., (2006). *Babesia* – A historical overview. Vet. Parasitol. 138, 3-10.

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

105. Vega, CA., Buening, GM., Green, TJ., and Carson, CA., (1985). *In vitro* cultivation of *Babesia bigemina*, Am. J. Vet. Res. 46, 416–420.

106. Velge-roussel, F., Marcelo, P., Lepage, AC., Buzoni-gatel, D., and Bout, DT., (2000). Intranasal immunization with *Toxoplasma gondii* SAG1 induces protective cells into both NALT and GALT compartments. Infect. Immun. 68, 969-972.

107. Vial, HJ., and Gorenflot, A., (2006). Chemotherapy against babesiosis. Vet. Parasitol. 138, 147-60.

108. Waltisbuhl, DJ., Goodger, BV., Wright, IG., Mirre, GB., Commins, MA., (1987). *Babesia bovis*: vaccination studies with three groups of high molecular weight antigens from lysate of infected erythrocytes. Parasitol. Res. 73, 319-23.

109. Weiland, G., Reiter, I., (1988). Methods for the measurement of the serological response to *Babesia*. In: Ristic, M., editor. Babesiosis of domestic animals and man. Boca Raton, Fla: CRC Press, Inc. 143–162.

110. Wenyon, CM., (1926). Protozoology, A Manual for Medical Men, Veterinarians and Zoologists, vol. II, Hafner Publish. Co. 986.

111. Willadsen, P., Bird, P., Cobon, GS. and Hungerford, J., (1995). Commercialization of a recombinant vaccine against *Boophilus microplus*, Parasitol. 110, S43–S50.

112. Willadsen, P., (2006). Tick control: thoughts on a research agenda. Vet. Parasitol. 138, 161-8.

113. Wright, IG., Goodger, BV., Leatch, G., Aylward, JH., Rode-Bramanis, K., Waltisbuhl, DJ., (1987). Protection of *Babesia bigemina*-immune animals against subsequent challenge with virulent *Babesia bovis*. Infect. Immun. 55, 364–368.

114. Wright, R., Casu, MA., Commins, BP., Dalrymple, KR., Gale, BV., Goodger, PW., Riddles, DJ., Waltisbuhl, I., Abetz, DA., Berrie, Y., Bowles, C., Dimmock, T., Hayes, H., Kalnins, G., Leatch, R., McCrae, PE., Montague, IT., Nisbet, F., Parrodi, JM., Peters, PC., Scheiwe, W., Smith, K., Rode-Bramanis, MA., White, (1992). The development of a recombinant *Babesia* vaccine, Vet. Parasitol. 3–13.

115. Xuan, X., Larsen, A., Ikadai, H., Tanaka, T., Igarashi, I., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., (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.

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

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

118. Young, AS., Morzaria, SP., (1986). Biology of *Babesia* parasit. Today. 8, 211-219.

119. Zweygarth, E., C. J. van Niekerk, and D. T. de Waal. (1999). Continuous *in vitro* cultivation of *Babesia caballi* in serum-free medium. Parasitol. Res. 85:413–416.