Analysis of host defense immunity and development of recombinant vaccines against *Babesia microti* infection

2017

Guanbo WANG

Doctoral Program in Animal and Food Hygiene

Graduate School of Animal Husbandry

Obihiro University of Agriculture and Veterinary Medicine

バベシアマイクロティ感染に対する宿主免疫の解析と組換えワクチンの開発

平成 29 年

(2017)

王 冠博

帯広畜産大学大学院畜産学研究科

博士後期課程　畜産衛生学専攻

Contents

Abbreviations and unit abbreviations

Abbreviations

A	Ad	- Adenovirus
B	BSA	- Bovine serum albumin
	BmAMA1	- *Babesia microti* apical membrane antigen 1
	BmRON2	- *Babesia microti* rhoptry neck protein 2
C	cDNA	- Complementary deoxyribonucleic acid
	CPE	- Cytopathic effect
D	DNA	- Deoxyribonucleic acid
	DMEM	- Dulbecco's modified Eagle's medium
	dNTP	- Deoxyribonucleotide triphosphate
E	*E. coli*	- *Escherichia coli*
	EDTA	- Ethylenediaminetetraacetic acid
	ELISA	- Enzyme linked immunosorbent assay
F	FBS	- Fetal bovine serum
H	HEK	- Human embryonic kidney
	HRP	- Horseradish peroxidase
I	IFAT	- Immunofluorescent antibody test

	IFN-γ	- Interferon gamma
	IgG	- Immunoglobulin G
	IL-4	- Interleukin-4
	IL-10	- Interleukin-10
	IL-12	- Interleukin-12
	i.p.	- Intraperitoneal
	IPTG	- Isopropyl-β-D-thiogalactopyranoside
	IU	- Infectious units
K	kDa	- Kilodalton
L	LB	- Luria broth
M	MEM	- Eagle's minimum essential medium
	MHC	- Major histocompatibility complex
N	NK	- Natural killer
O	OD	- Optical density
P	PBS	- Phosphate-buffered saline
	PBST	- Phosphate buffered saline with Tween-20
	PCR	- Polymerase chain reaction
R	r	- Recombinant
	RBC	- Red blood cell
	RNA	- Ribonucleic acid

	RT	- Reverse transcriptase
S	SDS-PAGE	- Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T	Th1	- T-helper 1
	Th2	- T-helper 2

Unit abbreviations

bp	- Base pair
µl	-Microliter
℃	- degree Celsius
µm	-Micromolar
sec	-Second
U	-Unit
min	-Minute
%	-Percentage
ml	-Milliliter

General introduction

1. Babesiosis

Babesiosis is a tick-transmitted, zoonotic disease caused by the intraerythrocytic protozoan species *Babesia*. It is one of the most important tick-borne diseases which can infect a wide range of mammals, including humans (Homer et al., 2000). Several *Babesia* species are responsible for human babesiosis according to distinct geographic distributions, with *B. microti* and *B. divergens* being the primary causative agents (Krause et al., 2003; Hildebrandt et al., 2007; Kumar et al., 2009). The transmission routes include contaminated blood transfusions and bites of infected ticks. Several factors determine the clinical manifestation, such as age, immunocompetence and coinfection with other pathogenic agents. The infection often causes mild flu-like illness but can sporadically be fatal in those patients who have undergone splenectomy (Hildebrandt et al., 2007; Krause et al., 2008; Vannier et al., 2008).

2. Life cycle of *Babesia*

The life cycle of *Babesia* parasites consists of two hosts, the tick vector and vertebrate host (Hauvin et al., 2009). Vertebrate hosts can be infected during a tick blood meal, which inoculates sporozoites with saliva. Sporozoites directly invade the red blood cells of the host and all the parasitic stages develop in red blood cells. The trophozoites produce two merozoites by binary fission (Homer et al., 2000). After escaping from the erythrocyte, each merozoite continuous their replication cycle that causes destruction of fresh erythrocytes. When *Babesia*-infected red blood cells are

ingested by a tick host, the "pre-gametocyte" stages of the parasite survive and undergo further development and mature into gametocytes (Firdaus et al., 2013). A few hours after ingestion, gametocytes fuse in the lumen of the tick's digestive tract and develop a zygote with a spike-like arrowhead organelle. Then, the zygote is converted into a motile stage, termed the ookinete. Subsequently, the ookinete escapes from the midgut epithelium and invades the tick's body tissues. Finally, sporozoites are produced in the salivary glands of ticks, which possess the capability of infecting the vertebrate host (Hauvin et al., 2009).

3. Transmission

The biological vector for *Babesia* is the ixodid tick. *Babesia* has been found in six species of ixodid ticks (Yabsley and Shock, 2013). *B. microti*, the causative agent of human babesiosis, can only infect *Ixodes* ticks, of which there are 250 species worldwide (Vannier et al., 2008). The primary mammalian hosts for *B. microti* are rodents, while other small mammals such as primates, cattle and even birds can also be infected (Hersh et al., 2012). The transmission of *B. microti* to humans occurs when an infected tick bites. *Babesia* can be transmitted by either immature or mature ticks, but mature ticks are easier to be observed and their bite prevented. Babesiosis can also be transmitted through blood transfusions (Singh and Sehgal, 2010). Therefore, *Babesia* poses a serious threat to the blood supply.

4. Clinical manifestation and pathology

In cases of mild infection, clinical signs include irregular fevers, chills, headaches, general lethargy, pain and malaise. In cases of serious infection,

symptoms are characterized by hemolytic anemia, jaundice, shortness of breath, and hemoglobinuria (Kjemtrup and Conrad, 2000). Generally, patients do not need to take medication. However, splenectomized patients are more vulnerable and may exhibit severe clinical symptoms (Vannier et al., 2008). Patients without a spleen may develop extremely high levels of parasitemia with a peak of more than 80% compared to less than 10% in individuals with healthy immune systems. Splenectomized patients undergo severe hemolytic anemia with sporadic occurrence of hepatomegaly (Vannier et al., 2008).

5. Host immune response

Humoral responses are considered to play a limited role in the protective immunity against *Babesia* infections. Transferring immune serum to immunodeficient mice infected with *B. microti* is unable to resolve the infection (Matsubara et al., 1993). However, a degree of protection can be achieved when transferring serum containing specific antibabesial antibodies (Mahoney, 1967). Immune serum can delay the progression of *B. rodhaini* infection with reduced parasitemia, whereas it can't prevent the infected mice from death (Abdalla et al., 1978). Several experiments showed that antibodies play key roles in prevention of free sporozoites or merozoites from invasion into red blood cells by inhibiting their attachment and reorientation (Abdalla et al., 1978; David et al., 1989; Hines et al., 1989; Winger et al., 1989). Therefore, antibodies have more effect on those extracellular parasites (Abdalla et al., 1978).

Cellular immunity is considered to play a critical role in resistance to babesiosis. T-cell-regulated immune responses are crucial for the resolution of the

host. Specific antigens trigger T cells to initiate differentiation. CD4+ Th1 cells are able to induce infected mice for delayed-type hypersensitivity (Ruebush et al., 1986). The infection of mice depleted of CD4+ T helper cells is more severe than the infection of normal mice (Terumasa et al., 1996; Igarashi et al., 1999). In contrast, depletion of CD8+ cytotoxic T cells in mice has no significant impact on susceptibility to *B. microti* infection (Terumasa et al., 1996; Igarashi et al., 1999). Therefore, CD4+ T helper cells are believed to play an important role in regulating protective immunity against *B. microti*.

There is evidence to show that elimination of babesial parasites in the host could be regulated through a nonspecific immune response, the innate immunity. In the past several years, several specific molecules participating in innate immunity have been determined (Frolich et al., 2012). For instance, the importance of NK cells and macrophages in regulation of protective immunity has been elucidated. Highly activated NK cells in relation to *B. microti* infection were reported (Eugui and Allison, 1980). NK cells mediate protection at the early stage of infection (Solomon et al., 1985), while another study found that NK cell activity arises during the acute and recovery phases (Aguilar-delfin et al., 2003). As for the protective role of macrophages, depletion of macrophages in mice using silica eradicates protection against *B. microti* (Terkawi et al., 2015). Moreover, inhibition (Zivkovic et al., 1985) or depletion (Saeki and Ishii, 1996) of macrophages in immunized mice leads to high mortality after challenge infection with *B. rodhaini*.

In sum, both the adaptive and innate immune systems are involved in host protective immunity to babesiosis. At the early phase, antibodies can prevent infection by neutralizing free sporozoites in the plasma prior to their successful invasion of target cells. Once babesial organisms establish intraerythrocytic infection,

parasitemia rises and severe clinical symptoms can be observed. Innate immune cells are responsible for controlling the multiplication of the parasite and therefore the duration of parasitemia. In the absence of macrophages and NK cells, a significantly elevated parasitemia takes place in a short period of time. The elimination of the parasite by innate immune cells is presumably accompanied by the production of several important cytokines: tumor necrosis factor alpha (TNF-α), nitric oxide (NO), gamma interferon (IFN-γ), and reactive oxygen species (ROSs). However, it is unclear how these molecules interact with the parasite inside the erythrocyte.

6. Current status of vaccine development against babesiosis

Currently, no vaccine is available to protect people against babesiosis (Mcallister, 2014). However, extensive research efforts have led to the development of various protozoan vaccines at the experimental level and some of them have shown an acceptable level of efficacy. Much of the research to develop protozoan vaccines has followed approaches taken for viral and bacterial diseases, such as live attenuated, subunit, DNA and vector vaccines (Meeusen et al., 2007).

Regarding the live vaccine, inoculation of susceptible animals with either virulent or attenuated parasite has been a successful approach to vaccination against several protozoan diseases (Ahmad et al., 2016; Srivastava et al., 2016), as the live organism can infect target cells and induce both cellular and humoral immunity. Generally, they do not require an adjuvant to be effective (Meeusen et al., 2007). However, they can pose a risk of residual virulence and reversion to pathogenic wild types, as well as provide a potential source of environmental contamination (Meeusen et al., 2007).

Subunit vaccines offer an attractive alternative to live vaccines for several reasons. These include the relative safety of recombinant proteins produced in bacteria, and the specificity to select those antigens which elicit protective immune responses (Jenkins, 2001). Currently, two subunit vaccines are available in the market for the prevention of canine babesiosis caused by *B. canis*. These subunit vaccines contain soluble parasite antigens and the protective efficacy reached to 88% (Comelissen and Schetters, 1996).

DNA vaccines are the third generation vaccines. The immune response can be elicited by direct injection of a naked DNA plasmid into the host cell (Article et al., 2009). Several DNA vaccines aimed at preventing protozoan infections have been tested and some of them showed promising results which can be taken to animals in the future. Immunization with plasmid DNA expressing *Toxoplasma gondii* bradyzoite antigens BAG1 and MAG1 reduced cyst burden in mice after challenge infection (Nielsen et al., 2006). Furthermore, vaccination with recombinant plasmid encoding for *T. gondii* surface antigen 1 (SAG1) has been found to confer partial protection in mice (Meng et al., 2012). It has been reported immunization with plasmid DNA encoding *P. yoelii* CSP and HEP17 conferred a degree of protection against malaria in mice (Sharma and Khuller, 2001). A DNA vaccine targeting canine babesiosis caused by *B. gibsoni* has been developed by utilizing P50 protein, which provoked protective immunity in dogs (Fukumoto et al., 2007).

Vector vaccines contain genetically modified microorganisms that generate the relevant parasite molecule. The recombinant viral vector may deliver the peptides to the MHC class I presentation pathway (Liu et al., 2000). Studies have shown that viral vector vaccines may elicit strong type 1 Th immune response (Liu et al., 2000). Viral delivery of immunogenic antigens has been tested against *T. gondii* and

Plasmodium. Immunization of mice with viruses expressing relative antigen induced both humoral and cellular immune responses (Dunachie and Hill, 2006; Jongert et al., 2009). Therefore, recombinant viral vaccines may have potential use in the prevention of babesiosis.

7. Aim of the present study

As outlined above, babesiosis is becoming an emerging zoonotic disease that poses a serious public health concern in humans and animals worldwide. The emergence of human babesiosis coupled with economic losses in livestock industry has resulted in demands for urgent preventive strategies to control the *Babesia* infection.

A better understanding of the immune response towards infection by *Babesia* parasites is important for designing a safe and efficacious vaccine. On the other hand, it is also important in order to conduct research to find effective vaccine candidates. The heterologous prime-boost immune strategy is considered a useful method for the development of a vaccine. Therefore, based on these backgrounds, I performed the current research with the aim to establish an effective preventive strategy to control *Babesia* infection.

The objectives of the present study can be summarized as follows: (1) to analyze the host defense mechanism by studying the cross-protection between *B. rodhaini* and *B. microti*; (2) to evaluate the protective efficacy of rBmAMA1 and rBmRON2 as subunit vaccines against *B. microti* infection; (3) to determine the protective effect of a heterologous prime-boost immune strategy with plasmid DNA

followed by recombinant adenovirus expressing vaccine candidate against *B. microti* infection.

Chapter 1

Analysis of the host immunity by studying the cross-protection between *Babesia rodhaini* and *Babesia microti*

1-1. Introduction

Although there has been great progress in vaccine development against viral and bacterial infections in the past decades, vaccines against protozoan diseases are still a rare item. One of the main obstacles for the development of new vaccines is the limited understanding of immune effector mechanisms (Vercruysse et al., 2004). *Babesia microti* and *Babesia rodhaini* are both rodent *Babesia* species which have been used as ideal laboratory models in many studies to understand host immune responses (Igarashi et al., 1999; Terkawi et al., 2008). Clinically, *B. microti* and *B. rodhaini* cause different diseases in mice. *B. microti* produce a self-limiting infection that is resolved within 3 weeks in mice. In contrast, *B. rodhaini* is highly virulent causing lethal infection with 100% mortality rate in mice. Interestingly, mice recovered from *B. rodhaini* infection after drug treatment exhibit a considerable protection against either *B. rodhaini* reinfection or *B. microti* challenge infection (Zivkovic et al., 1984). However, the protection mechanism remains unclear. Several studies have demonstrated the importance of antibodies and cytokines in

regulation of the immune response to *Babesia* infections (Brown, 2001; Aguilar-Delfin et al., 2003; Brown et al., 2006). The secretion of key cytokines at different stages can determine the outcome of the infection. The inflammatory cytokines gamma interferon (IFN-γ) and interleukin-12 (IL-12) secreted at the early stage are critical for controlling the initial burst of intraerythrocytic parasite multiplication. Thereafter, a switch from Th1 to Th2 response (IL-4 and IL-10) accompanied by elevated antigen-specific immunoglobulin G (IgG) appears to be crucial for parasite resolution (Chen et al., 2000; Aguilar-Delfin et al., 2003).

Therefore, in order to elucidate the protective mechanism observed in mice recovered from *B. rodhaini* infection, in this study I assessed the role of important Th1 and Th2 cytokines and the antibody response in the induction and effector phase of infection-induced resistance in mice.

1-2. Materials and methods

Experimental animals

In total, sixty six-week-old female BALB/c mice purchased from CLEA Japan (Tokyo, Japan) were used for animal experiments. All the experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine of Japan.

Maintenance of the parasites and mouse infections

The *B. microti* Munich strain (Igarashi et al., 1999) and *B. rodhaini* Australian strain (Terkawi et al., 2008) were prepared from stocks in my laboratory. For the

maintenance of parasites, cryopreserved parasitized-RBCs (pRBCs) were passaged into mice by intraperitoneal (i.p.) injection. Challenge infection was performed with i.p. inoculation of 10^7 fresh *B. microti* or *B. rodhaini* infected RBCs.

Immunization of mice with alive *B. rodhaini*

To examine the protective immunity against *B. rodhaini* and *B. microti* in mice recovered from primary *B. rodhaini* infection, test mice were infected with 10^6 pRBCs and treated with 25 mg of Ganaseg® (Novartis, Japan) per kg of body weight by i.p. injection, when the parasitemia level was around 10%. Mock mice were not inoculated with pRBCs but given the drug at the same time and dose as test mice in order to evaluate the effect of the drug treatment. The parasitemia level of infected animals was monitored regularly up to one month after treatment.

Immunization of mice with dead *B. rodhaini*

To examine whether dead *B. rodhaini* could offer protection, glutaraldehyde-fixed *B. rodhaini*-infected RBCs and non-parasitized RBCs (npRBCs) were used for immunization as described before (Benach et al., 1982). Briefly, *B. rodhaini*-infected RBCs were harvested from mice when parasitemia levels reached 50%. The plasma and buffy coat were removed from the blood, and the RBCs were washed three times in sterile phosphate-buffered saline (PBS, pH 7.2). After the final wash, the RBCs were fixed with 0.25% glutaraldehyde for 15 min at room temperature, then washed three times with sterile PBS. The fixed RBCs were stored at 4℃ in sterile PBS supplemented with penicillin and streptomycin. Before inoculation, the cells were washed twice with sterile PBS. Mice were immunized three times at 2-week intervals with either 10^8 glutaraldehyde-fixed *B. rodhaini* pRBCs diluted in 0.3 ml of PBS (the test group) or an equivalent amount of glutaraldehyde-fixed npRBCs diluted in 0.3ml

of PBS (the control group). Blood samples were collected from the tail vein 2 weeks after the last inoculation to determine the specific antibody response to *B. rodhaini* antigens by an enzyme-linked immunosorbent assay (ELISA).

Challenge infection

To measure the protective effect, immunized mice were challenged with *B. rodhaini* or *B. microti* by using different time courses. For the mice immunized with alive *B. rodhaini*, challenge infection was performed at days 14 and 28 post primary infection. For the mice immunized with dead *B. rodhaini,* challenge infection was performed 2 weeks after the last immunization.

Determination of parasitemia, hematocrit value, body weight and survival rate

To evaluate the effect of drug-cured *B. rodhaini* infection in mice, parasitemia, hematocrit value, body weight and survival rate of the animals were monitored during the trials. For estimation of parasitemia, thin blood smears prepared from tail veins of mice were fixed in methanol and stained for 45 min with 10% Giemsa solution diluted in Sörensen buffer (pH 6.8). Thereafter, parasitemia was determined by examining at least 10^3 erythrocytes. For hematocrit evaluation, 10 µl of blood collected from each mice at 2-day intervals during all the course of challenge infection was transferred into plastic tubes containing 2 ml of premixed solution. A full blood cell count was made using an automatic cell counter (Nihon Kohden, Japan). In addition, the mice were observed daily for any mortality and body weight changes until day 20 post challenge infection.

Detection of specific antibodies to *B. rodhaini* P26 and *B. microti* P32

To assess the antibody levels in mice after challenge infection or immunization, recombinant *B. rodhaini* P26 (rBrP26) and *B. microti* P32 (rBmP32)

proteins were used as specific antigens for detection of the antibody response using an ELISA assay as previously described (Igarashi et al., 2000; Ooka et al., 2012). rBrP26, rBmP32 were expressed as glutathione S-transferase fusion proteins with molecular mass of 57.7 kDa and 58 kDa, respectively. The expressed fusion proteins were purified by glutathione-Sepharose 4B columns (Amersham Biosciences, USA). The levels of antibodies are measured as OD values at 415 nm.

Detection of serum cytokines

During the course of challenge infection, blood was regularly collected from the tail veins of mice and processed to obtain serum. In the case of *B. rodhaini*, blood samples were collected at 2, 4 and 6 days post challenge infection. In the case of *B. microti*, blood samples were collected at 6, 8 and 10 days post challenge infection. The cytokine concentrations were determined by ELISA assay kits using respective standard curves prepared with known concentrations of mouse recombinant IFN-γ, IL-4, IL-10, and IL-12+p40 (Pierce Biotechnology, USA), according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Prism 6; GraphPad Software, USA). The means of all variables were computed and one-way analysis of variance, followed by Tukey's multiple-comparison test was used for pairwise comparison of data from the multiple groups. Survival analyses for significant differences were done using a Kaplan-Meier nonparametric model. Results were considered to be statistically significant when the P value was <0.05.

1-3. Results

Primary infection with *B. rodhaini* offers considerable protection against *B. rodhaini* reinfection and *B. microti* challenge infection

Mice primarily infected with *B. rodhaini* exhibited a transient parasitemia from days 2 to 6 after infection. They were treated with the drug, and afterwards recovered completely, with no parasites observed in their blood till the end of trials (data not shown). Strikingly, the test mice showed a considerable protection, characterized by significantly lower parasitemia levels and no mortality compared to mock mice (Fig. 1A, B, E, F; Fig. 2A, D). In contrast, the mock mice which had received no primary infection exhibited rapid increase in the parasitemia of *B. rodhaini* and *B. microti* with a peak higher than 70% and 40%, respectively (Fig. 1A, E; Fig. 2A, D). In the case of *B. rodhaini*, the mice died within one week (Fig. 1B, F). Notably, a significant reduction in the hematocrit values and body weight which coincided with the increase of parasitemia was observed in mock mice (Fig. 1C, D, G, H; Fig. 2B, C, E, F). Test mice, however, only showed a slight decrease of hematocrit values and no significant variation in body weight.

Drug-cured *B. rodhaini*-infected mice have high levels of antibodies but low levels of cytokines post challenge infection

To determine the contribution of antibodies and cytokines to the protection conferred by drug-cured *B. rodhaini* infection, the serum antibodies and cytokines were measured in mice after the *B. rodhaini* and *B. microti* challenges. In the case of *B. rodhaini* challenge, high levels of IgG1 against *B. rodhaini* (rBrP26) were observed at days 2, 4, and 6 in test mice, whereas the IgG1 and IgG2 levels were significantly lower in mock mice at days 2 and 4 after challenge infection (Fig. 3A, B,

C). Likewise, the levels of detected IFN-γ and IL-12+p40 were significantly lower in test mice ($P<0.05$). IL-10 and IL-4 cytokines were only detected in the sera of mock mice at 6 days after challenge infection but were below the detection limit in test mice (Fig. 4A, B, C, D). In case of the challenge with *B. microti*, high level of IgG1 against *B. microti* (rBmP32) were detected in both test and mock mice at days 6, 8 and 10 after challenge infection (Fig. 3D, E, F). Similarly, INF-γ and IL-12 levels were significantly lower in test mice than those of mock mice ($P<0.05$). IL-10 was detected only in the sera of mock mice at days 6, 8, 10 after challenge infection (Fig. 4E, F, G). Moreover, the levels of IL-4 in all mice were below the detection limit (data not shown).

Immunization with dead *B. rodhaini* fails to protect the mice against *B. rodhaini* or *B. microti* challenge infections

Mice immunized with dead *B. rodhaini* developed high titers of specific antibody against rBrP26 (1:6,400 to 1:12,800), while control mice did not show antibody response (data not shown). Both immunized and control groups showed rapid increases in parasitemia after challenge infection with *B. rodhaini* and *B. microti* (Fig. 5A, E). In addition, a significant reduction in the total number of RBCs and hematological values, and loss of body weight was observed coinciding with the parasitemia increase (Fig. 5C, D, F, G). All mice succumbed to *B. rodhaini* infection and died within one week (Fig. 5B).

1-4. Discussion

Molecular evidence suggest that *B. rodhaini* and *B. microti* are genetically similar to each other (Goethert and Telford, 2003). However, the different

pathogenicities of the two parasites lead to a distinction in host immune response mechanisms. In the present study, I investigated whether mice which had recovered from *B. rodhaini* infection by drug treatment could be protected against *B. rodhaini* or *B. microti* challenge infection. The results showed that test mice acquired a robust protective immunity against *B. rodhaini* and *B. microti* infections, with significantly lower levels of parasitemia and no mortalities, as opposed to mock mice. These findings confirmed the previous study in which mice immunized with *B. rodhaini* through a drug-control method were protected from other *Babesia* species like *B. microti* (Zivkovic et al., 1984). Moreover, drug-cured *B. rodhaini* infection had a significant impact on antibody and cytokine production in response to the challenge infections with *B. rodhaini* and *B. microti* in mice. Regarding the antibody response, although high titer of antibody was produced in mice immunized with both alive and dead *B. rodhaini*, the mice immunized with dead *B. rodhaini* failed to be protected even against *B. rodhaini* reinfection, which means that antibodies induced by alive parasites may be more powerful. Generally, the function of antibodies in *Babesia* infection is to neutralize free parasites, preventing merozoite entry into host erythrocytes by lysing parasites either by complement activation or phagocytosis (Jacobson et al., 1993; Chen et al., 2000; Brown, 2001; Aguilar-Delfin et al., 2003). Therefore, dead parasites may not stimulate the host to produce antibodies targeting critical neutralization epitopes to prevent erythrocyte invasion. However, in the mice recovered from *B. rodhaini* infection, the resulting protection against *B. microti* may not be attributed to antibodies induced by alive parasites, because these were reported to lack cross-reacting antibodies (Zivkovic et al., 1984). Therefore, the components of cellular immunity may play a role in the case of this cross-protection.

For cytokine production, mock mice which received challenge infection with *B. rodhaini* or *B. microti* exhibited rapid elevation in the levels of cytokines, including INF-γ, IL-12+p40, IL-4 and IL-10, compared to test mice. These results indicate that a cytokine storm was responsible for the death or severe symptoms observed in the mock mice during the infection (Clark et al., 2004; Clark, 2007; Tisoncik et al., 2012). Normally, the host keeps an appropriate balance between the Th1 and Th2 immune responses when the immune system encounters a highly pathogenic invader, which is crucial for host resolution from parasite infection (Igarashi et al., 1999; Trinchieri, 2003; Couper et al., 2008). However, cytokine production becomes uncontrolled under the burden of parasite multiplication in mock mice. In sharp contrast, test mice kept the immune response in check with reduced expression of these cytokines. This result is similar with a related study in which mice primarily infected with *B. microti* also showed a low level of cytokine expression against the challenge infections with *B. rodhaini* (Li et al., 2012). In that case, the absence of macrophages changes the cytokine production and results in mice failing to be protected. Therefore, macrophages may play a central role in the regulation of the cross-protection between *B. microti* and *B. rodhaini* (Li et al., 2012; Terkawi et al., 2015). However, further studies are needed to understand the role of macrophages in the cross-protection conferred by drug-cured *B. rodhaini* infection.

1-5. Summary

In the present study, I investigated the protective immunity against challenge infections with *B. rodhaini* and *B. microti* in mice recovered from *B. rodhaini* infection. Six groups with 5 test mice in each group were used in this study, and were

intraperitoneally immunized with alive and dead *B. rodhaini*. The challenge infections with *B. rodhaini* or *B. microti* were performed using different time courses. Our results showed that the mice recovered from primary *B. rodhaini* infection exhibited low parasitemia and no mortalities after the challenge infections, whereas mock mice which had received no primary infection showed a rapid increase of parasitemia and died within 7 days after challenge with *B. rodhaini*. Mice immunized with dead *B. rodhaini* were not protected against either *B. rodhaini* or *B. microti* challenge infections, although high titers of antibody response were induced. These results indicate that only mice immunized with alive *B. rodhaini* could acquire protective immunity against *B. rodhaini* or *B. microti* challenge infection. Moreover, the test mice produced high levels of antibody response and low levels of cytokines (INF-γ，IL-4，IL-12，IL-10) against *B. rodhaini* or *B. microti* after challenge infection. Mock mice, however, showed rapid increases of these cytokines, which means disordered cytokine secretion occurred during the acute stage of challenge infection. The results proved that mice immunized with alive *B. rodhaini* could acquire protective immunity against *B. rodhaini* and *B. microti* infections.

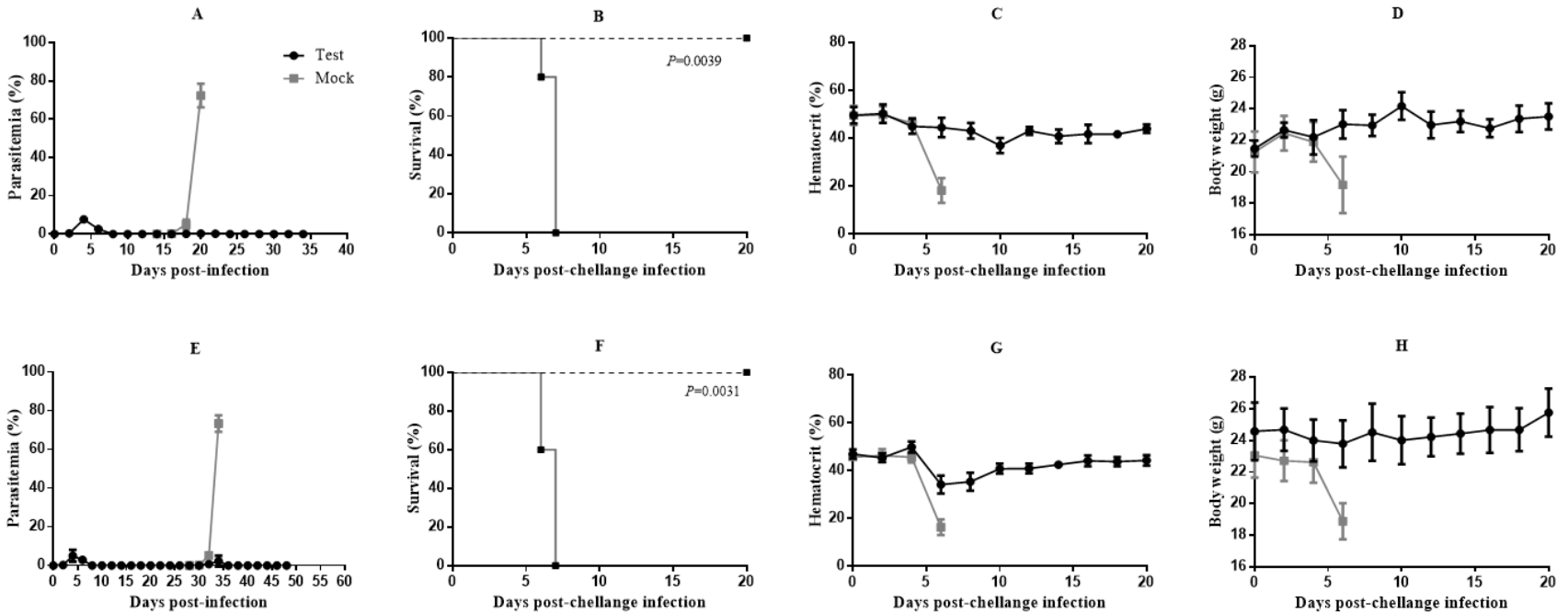

Fig. 1. Parasitemia, survival rates, hematocrit and body weight after challenge infection with *B. rodhaini* in drug-cured *B. rodhaini* infected mice. Parasitemia course (A, E), survival rates (B, F), hematocrit (C, G) and body weight (D, H) of mock and test mice are presented. Test mice were recovered from *B. rodhaini* infection followed by challenge infection with *B. rodhaini* at days 14 (A, B, C, D) or 28 (E, F, G, H) after primary infection. Mock mice received *B. rodhaini* alone. The results are expressed as a mean percent values ±the standard deviations (SD) of five mice.

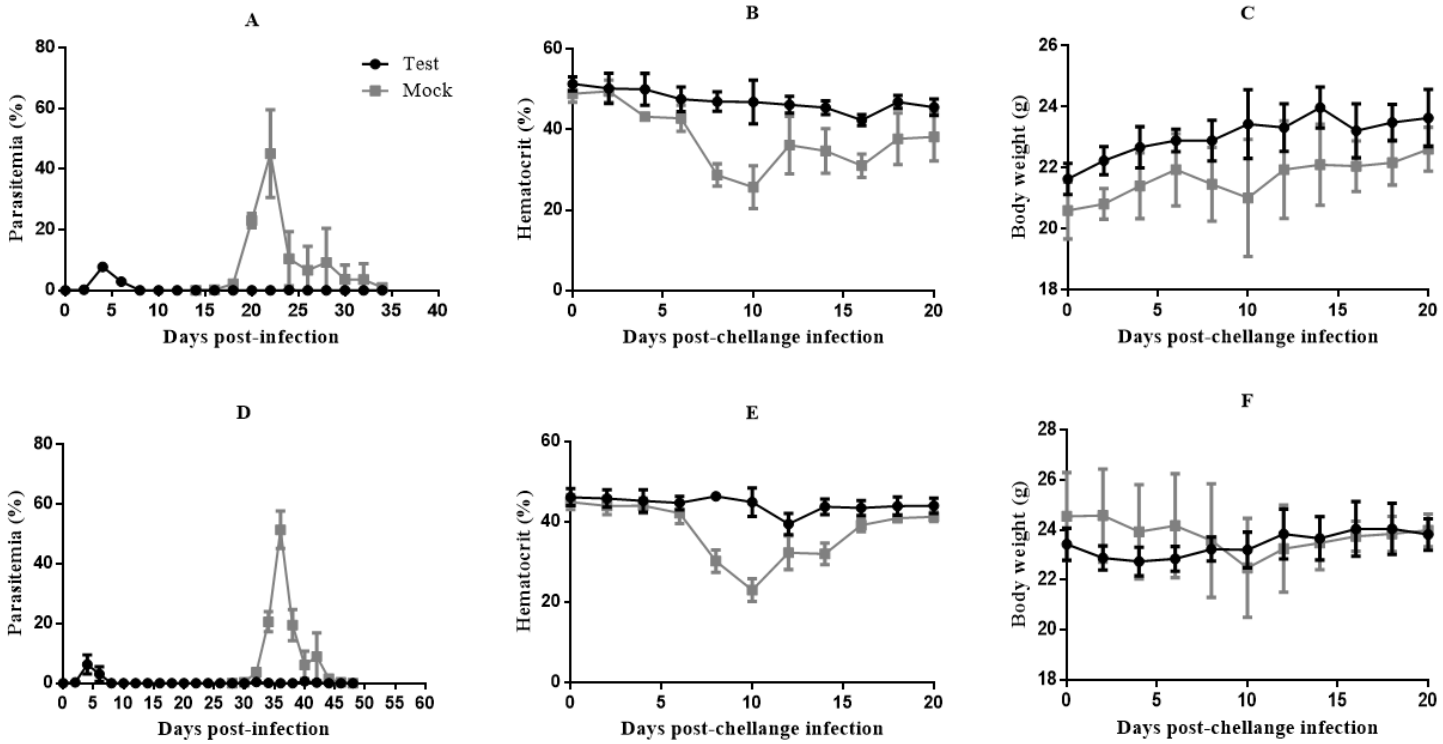

Fig. 2. Parasitemia, hematocrit and body weight after challenge infection with *B. microti* in drug-cured *B. rodhaini* infected mice. Parasitemia course (A, D), hematocrit (B, E) and body weight (C, F) of mock and test mice are presented. Test mice were recovered from *B. rodhaini* infection followed by challenge infection with B. microti at days 14 (A, B, C) and 28 (D, E, F) after primary infection. Mock mice received *B.microti* alone. The results are expressed as a mean percent values ±the standard deviations (SD) of five mice.

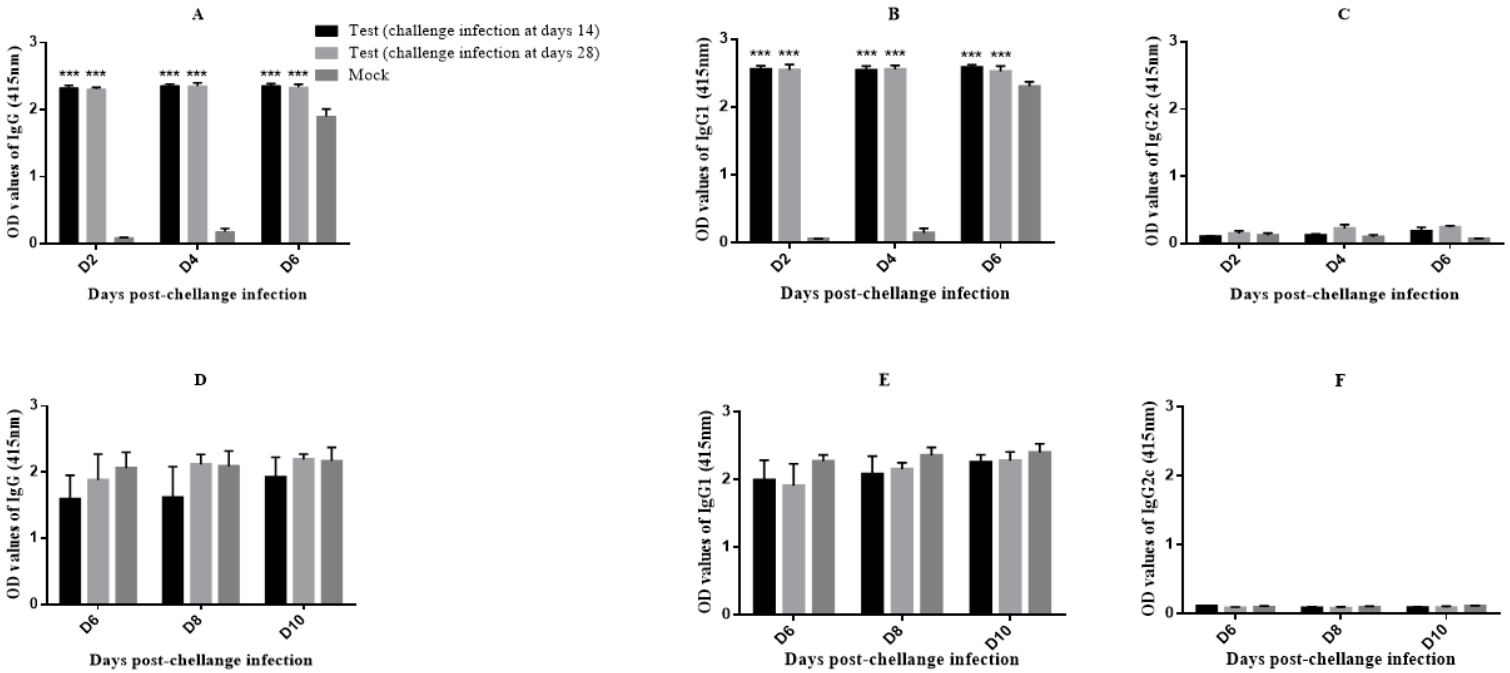

Fig. 3. Evaluation of Specific antibodies to rBrP26 and rBmP32 after *B. rodhaini* (A, B, C) or B. microti (D, E, F) challenge infection. The production of IgG (A, D), IgG1 (B, E), IgG2c (C, F) in mice after challenge infection were measured as OD values at 415nm. Test mice (drug-cured from *B. rodhaini* infection) or mock mice (which received no primary infection) were challenge infected with *B. rodhaini* or *B. microti*. Detection of IgGs was performed in the mice at days 2, 4, 6 and days 6, 8, 10 after challenge infection respectively. Asterisks indicate statistically significant differences (*, $P<0.05$; **, $P<0.005$; ***, $P<0.0001$ [compared to mock mice]). The results are expressed as mean values $\pm$ the SD of five mice.

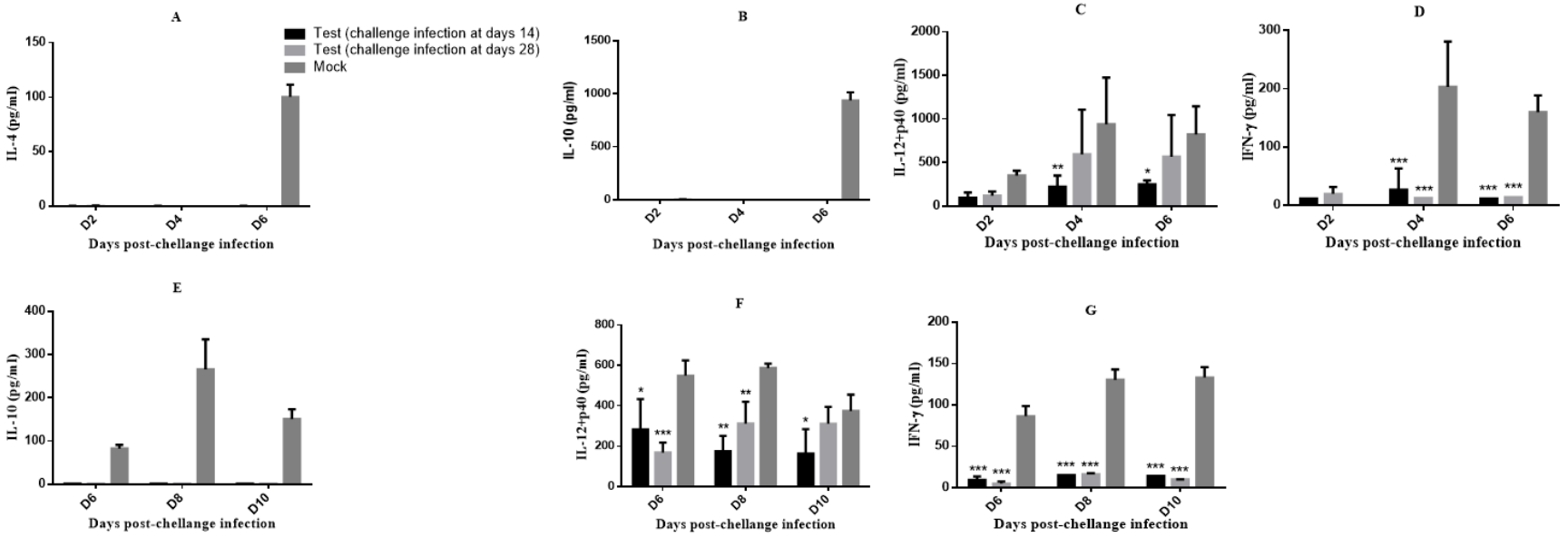

Fig. 4. Kinetics of cytokines of protected and susceptible mice after *B. rodhaini* (A, B, C, D) or *B. microti* (E, F, G) challenge infection. The production of IL-4 (A), IL-10 (B, E), IL-12+p40 (C, F), IFN-γ (D， G) in mice after challenge infection was determined. Test mice (drug-cured from *B. rodhaini* infection) or mock mice (which received no primary infection) were challenge infected with *B. rodhaini* or *B. microti*. Detection of cytokines was performed in the mice at days 2, 4, 6 and days 6, 8, 10 after challenge infection respectively. Asterisks indicate statistically significant differences (*, $P<0.05$; **, $P<0.005$; ***, $P<0.0001$ [compared to mock mice]). The results are expressed as mean values±the SD of five mice.

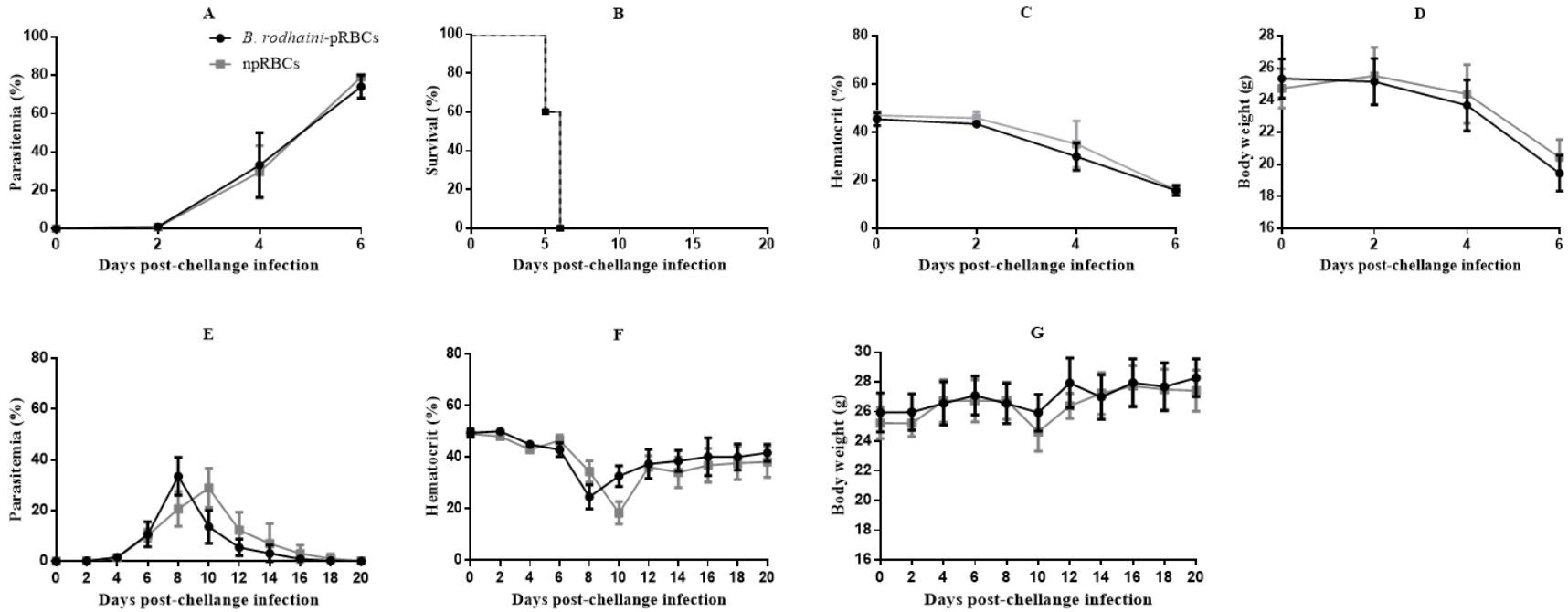

Fig. 5. Parasitemia course (A, E), survival rates (B), hematocrit (C, F) and body weight (D, G) of mice immunized with either dead *B. rodhaini* (pRBCs) or non-parasitized murine RBCs (npRBCs) and then challenge infected with *B. rodhaini* (A, B, C, D) or *B. microti* (E, F, G). The results are expressed as a mean percent values ± the standard deviations (SD) of five mice.

Chapter 2

Expression of truncated *Babesia microti* apical membrane protein 1 and rhoptry neck protein 2 and evaluation of their protective efficacy

2-1. Introduction

Apical membrane protein 1 (AMA1) has been considered a leading vaccine candidate for malaria and is widely conserved in the apicomplexan parasites (Mitchell et al., 2004; Wang et al., 2009). However, although high titer of AMA1-specific antibody could be induced by vaccination, it showed little efficacy in clinical trials for malaria (Spring et al., 2009). In the past few years, several studies have demonstrated a central structure formed during apicomplexan parasite invasion known as "moving junction", which is critical for a successful penetration to the host cell (Besteiro and Dubremetz, 2011; Papoin et al., 2011). This moving junction structure formed with AMA1 and rhoptry neck protein 2 (RON2) complex has been well studied in *Plasmodium* and *Toxoplasma* and is believed to be conserved among apicomplexan parasites (Chesne-seck et al., 2005). Combined vaccination with AMA1 and RON2 peptides showed enhanced protective effect compared to vaccination with AMA1 alone in *Toxoplasma* and *Plasmodium* (Zhang et al., 2015; Srinivasan et al., 2014). Recent studies identified and characterized the AMA1 and RON2 genes in *B. microti*

(Moitra et al., 2015; Orda et al., 2016). However, whether vaccination with BmAMA1 and BmRON2 is effective in protection against *B. microti* infection is unknown.

Previous studies showed the AMA1 domain II loop and the C-terminus of RON2 provides the crucial link between the AMA1 and RON complex (Tyler and Boothroyd, 2011; Delgadillo et al., 2016). The C-terminus of RON2 forms a tight interaction with the domain II loop of AMA1 by means of a loop that inserts into a hydrophobic groove (Besteiro and Dubremetz, 2011; Normand et al., 2012). Those regions were reported to be conserved among the apicomplexan parasites (Papoin et al., 2011). Therefore, in order to evaluate the potential use of BmAMA1 and BmRON2 for vaccination against *B. microti* infection, the genes encoding the BmAMA1 domains I and II and BmRON2 transmembrane regions 2 and 3 were cloned and expressed as His-tag fusion recombinant proteins in *E. coli*, and their protective efficacy against *B. microti* challenge infection was evaluated in this study.

2-2. Materials and methods

Parasites and experimental animals

In total, twenty six-week-old female Golden Syrian hamsters for the immunization trials and ten six-week-old BALB/c mice to raise antigen-specific IgG were purchased from CLEA Japan (Tokyo, Japan). Human isolated *B. microti* Gray strain (US type, American Type Culture Collection, Catalog No. 30221) was maintained in female Golden Syrian hamsters (Clea, Japan), by intraperitoneal injection with cryopreserved *B. microti*-infected erythrocytes. All the experiments were conducted in accordance with the Standards Relating to the Care and

Management of Experimental Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine of Japan.

RNA and cDNA isolation

B. microti-infected hamster RBCs were lysed with TRI reagent (Life Technologies, USA), total RNA was extracted by chloroform followed by precipitation with ethanol. cDNA was then prepared using a Superscript kit (Life Technologies, USA) following the manufacturer's instructions. The cDNA was used as a template DNA for the polymerase chain reaction (PCR).

Gene cloning of BmAMA1 and BmRON2

The gene encoding BmAMA1 (GenBank accession no. JX488467) extracellular region which contains predicted domain I and II corresponding to amino acids D84 to D455, was amplified using the primer sets: 5'-AGGATCCGATGAGGAGGATGACTATGAA-3' and 5'-ACTCGAGCTAATCCTCTAGTGGAGAACC-3' (the underlined nucleotides are *Bam*HI and *Xho*I restriction enzyme sites, respectively). The gene encoding the predicted BmRON2 (GenBank accession no. XP_012649548) transmembrane regions 2 and 3 corresponding to amino acids A1279 to A1376, was amplified using the primer sets: 5'- GGATCCTCACGTATGCTCAGTATCCAAG- 3' and 5'-GAATTCCACATCCTGCACCGCAGTTTGT -3' (the underlined nucleotides are contain *Bam*HI and *EcoR*I restriction enzyme sites). The resulting PCR products of BmAMA1 and BmRON2 were cloned into prokaryotic expression vector pET-32a and pET-28a (GE Healthcare, UK), respectively. The resulting plasmids were identified by sequencing and designated as pET-32a/BmAMA1 and pET-28a/BmRON2.

Expression, purification and refolding of rBmAMA1 and rBmRON2

rBmAMA1 and rBmRON2 were expressed as His-tagged fusion proteins in *E. coli* BL21 (DE3) strain. For recombinant expression, 1000 ml LB medium was inoculated with 10 ml *E. coli* which contain plasmids pET-32a/BmAMA1 or pET-28a/BmRON2, and the culture was grown at 37℃ to an A600nm of 0.5 and then induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 4 h cultures, cells were pelleted and stored at -80℃ until use. For purification and refolding, the cell pellet was resuspended in sonication buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaC1, 0.5 m Mdithiothreitol [DTT]) and lysed on ice with a sonicator. The inclusion bodies were resuspended in solubilization buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, 20 mM Imidazole, 8 M Urea) and incubated at room temperature (RT) for 1 h with shaking, followed by centrifugation at 15,000×g for 30 minutes. The supernatant was loaded onto a Ni2+-nitrilotriacetic acid (NTA) agarose column and incubated overnight at 4℃. The column was washed 5 times with solubilization buffer. The bound protein was eluted with refolding buffer containing 200 mM imidazole followed by dialysis overnight with phosphate-buffered saline (PBS). The approximate molecular weights of the expressed rBmAMA1 and rBmRON2 are 59 kDa and 18 kDa, respectively.

Production of anti-rBmAMA1 and anti-rBmRON2 sera

In order to characterize the native BmAMA1 and BmRON2, six-week-old female BALB/c mice (Clea, Japan) were used to prepare antiserum against rBmAMA1 and rBmRON2. Briefly, for the first immunization, mice were immunized intraperitoneally with 100 μg of purified rBmAMA1 or rBmRON2 in an equal volume of Freund's complete adjuvant (Sigma, USA). For the second and third

immunization, same volume of recombinant proteins emulsified with Freund's incomplete adjuvant (Sigma, USA) was intraperitoneally injected into mice with two weeks intervals. Mice sera were collected 2 weeks after the last immunization. The levels of antibodies were measured as OD values at 415 nm. The concentration of IgG against rBmAMA1 and rBmRON2 was 1:3,200 to 1:6,400 and 1:800 to 1:1,600, respectively.

Indirect fluorescent antibody test (IFAT) and confocal laser microscopic observation

IFAT was performed to localize native BmAMA1 and BmRON2 in the intraerythrocytic *B. microti* parasites. Briefly, thin blood smears made using blood from tail veins of *B. microti* infected hamsters were fixed in 100% methanol for 30 min at -30 ℃. The slides were incubated for 1 h at 37 ℃ with either mouse anti-rBmAMA1 or anti-rBmRON2 serum 1:20 diluted with PBS containing 3% bovine serum albumin (BSA). After four washes with PBST, Alexa-Fluor® 488 conjugated goat anti-mouse IgG (Molecular Probes, USA), 1:400 diluted in PBS-BSA, was subsequently applied as a secondary antibody and incubated for 1 h at 37 ℃. The slides were washed four times with PBST and incubated with 6.25 μg/ml propidium iodide (PI) (Molecular Probes, USA) containing 100 μg/ml RNase A (Qiagen, Germany) for 10 min at 37 ℃. After washing three times with PBS, the glass slides were mounted by adding 50 μl of a 50% glycerol-PBS (v/v) solution and then covered with a glass coverslip. The slides were examined under a confocal laser scanning microscope (TCS NT, Leica, Germany).

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

To identify the native BmAMA1 and BmRON2 in the parasite extract of *B. microti*, parasitized and non-parasitized erythrocytes of hamsters were analyzed by SDS–PAGE and Western blot analysis. Briefly, *B. microti* parasitized and non-parasitized erythrocytes were treated with 0.075% saponin in PBS (w/v) and then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins in the gel were electrically transferred to a nitrocellulose membrane. The membrane was blocked with PBS containing 5% skim milk and then incubated with anti-rBmAMA1 or anti-rBmRON2 polyclonal serum diluted in 5% skim milk at 37℃ for 60 min. Next, the membrane was washed three times with PBS and incubated with peroxidase-conjugated goat anti-mouse IgG in 5% skim milk. After washing three times with PBS, the bands on the membrane were visualized by incubation with diaminobenzine. To determine the antibody response to rBmAMA1 and rBmRON2 in the hamsters infected with *B. microti*, rBmAMA1 and rBmRON2 were also analyzed by SDS–PAGE and Western blot analysis as described above.

Immunization with recombinant proteins and challenge infection

A total of 20 six-week-old female Syrian hamsters were divided into four groups (n=5). Group 1 and group 2 were immunized with rBmAMA1 and rBmRON2 alone, group 3 was immunized with rBmAMA1+rBmRON2. Group 4 was mock immunized with PBS. For the first immunization, group 1 and group 2 were i.p. immunized with 100 μg rBmAMA1 or 200 μg rBmRON2, emulsified in an equal volume of Freund's complete adjuvant (Difco Laboratories, USA). Group 3 was immunized with rBmAMA1+rBmRON2, using the same amounts of antigens as group 1 and group 2. Group 4 was immunized with PBS alone. Thereafter, the hamsters were boosted with the same amount of antigens emulsified with Freund's incomplete adjuvant on days 28 and 49 post-primary immunization. Two weeks after

the last immunization, the hamsters were challenged i.p. with 1×10^7 *B. microti*-infected erythrocytes. The parasitemia and hematocrit levels were monitored for a month to evaluate the protective effect.

Detection of humoral response to rBmAMA1 and rBmRON2

To assess the humoral response after immunization, the sera of hamsters were collected 2 weeks after the booster immunization. The levels of BmAMA1 and BmRON2 specific total immunoglobulin G (IgG), IgG1, and IgG2 in hamster sera were measured by enzyme-linked immunosorbent essay (ELISA). Briefly, 96-well plates were coated with 50 μl of rBmAMA1 or rBmRON2 at a concentration of 4 μg/ml in a 50 mM carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4℃. The levels of total IgG, IgG1, and IgG2 in the sera which were diluted 2000-fold with 3% skim milk in PBS were measured in the plates using horseradish peroxidase-conjugated goat anti-hamster IgG, IgG1 and IgG2 (Bethyl, USA). The plates were washed five times, and then 100 μl of ABTS substrate (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, and 0.3 mg/ml 2,2'6-azide-bis [3-ethylbenzthiazoline-6-7 sulfonic acid]; Sigma, USA) was added into each well. Absorbance was measured at 415 nm using MTP-500 micro plate reader (Corona Electric, Japan).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Prism 6; GraphPad Software, USA). The means of all variables were computed and one-way analysis of variance, followed by Tukey's multiple-comparison test was used for pairwise comparison of data from the multiple groups. Results were considered to be statistically significant when the P value was <0.05.

2-3. Results

Cloning and expression of genes encoding BmAMA1 and BmRON2

The gene fragments encoding the predicted BmAMA1 and BmRON2 extracellular regions corresponding to D84 to D455 and S1279 to V1376 as shown in Fig. 1 were cloned into prokaryotic expression vectors and expressed in *E. coli* as His-tagged fusion proteins. The molecular weight of rBmAMA1 and rBmRON2 were approximately 59 kDa and 18 kDa in the SDS-PAGE analysis as shown on lane 1 and lane 2, respectively (Fig. 2). Serum from a hamster experimentally infected with *B. microti* recognized the rBmAMA1, but there was no reaction with rBmRON2 in Western blot analysis as shown on lane 3 and lane 4 (Fig. 2). In addition, no reaction was observed when incubating with non-infected hamster serum (data not shown).

Characterization of native BmAMA1 and BmRON2

Sera from mice immunized with rBmAMA1 and rBmRON2 were used to identify the native BmAMA1 and BmRON2 in *B. microti* lysate. Immunoblotting with mouse anti-rBmAMA1 serum detected distinct bands at 53 kDa and 40 kDa as shown on lane 3 (Fig. 3A). Immunoblotting with mouse anti-rBmRON2 also detected distinct bands at 170 kDa and 52 kDa as shown on lane 5 (Fig. 3A). There was no band in non-infected hamster erythrocyte lysate when probed with either anti-rBmAMA1 or anti-rBmRON2 serum as shown on lanes 4 and 6 (Fig. 3A) and no band was observed when probed with non-infected mouse serum (data not shown). In addition, specific fluorescence was observed at the apical end of the parasites when IFAT was performed using anti-rBmAMA1 or anti-BmRON2 serum (Fig. 3B) and no specific fluorescence was detected using non-infected mouse serum (data not shown).

Evaluation of the humoral responses induced by rBmAMA1 and rBmRON2

The humoral response elicited by immunization was tested by ELISA. IgG antibodies were significantly increased in groups immunized with rBmAMA1, rBmRON2 and rBmAMA1+rBmRON2, as compared with those of control mice (Fig. 4A, D). The difference in IgG levels against rBmAMA1 between the group immunized with rBmAMA1 and the group immunized with rBmAMA1+rBmRON2 was not statistically significant ($P > 0.05$) (Fig, 4A). However, the IgG level against rBmRON2 in the group immunized with rBmRON2 was significantly higher than the group immunized with rBmAMA1+rBmRON2 (Fig. 4D). Moreover, rBmAMA1, rBmRON2 and rBmAMA1+rBmRON2 immunized hamsters had a robust IgG2 response to rBmAMA1 or rBmRON2 (Fig. 4B, C, E, F).

Evaluation of the protective efficacy of rBmAMA1 and rBmRON2

The group immunized with rBmAMA1+rBmRON2 exhibited limited protection, characterized by delayed parasitemia progression with significantly lower parasitemia levels at day 6 to 10 post challenge infection and higher hematocrit values compared to the control group (Fig. 5A, B). In contrast, the group immunized with either rBmAMA1 or rBmRON2 alone did not show any significant protection after *B. microti* challenge compared to the control group, which exhibited rapid increase in the parasitemia levels and reduction in the hematocrit values (Fig. 5A, B).

2-4. Discussion

B. microti is one of the *Babesia* species most frequently found to infect humans (Krause et al., 2003). Considering the increasing threat of human babesiosis, it is necessary to develop an effective vaccine to prevent this disease.

Previous studies showed that the moving junction structure formed by AMA1 and rhoptry neck proteins has a central role in host cell invasion by apicomplexan parasites (Besteiro and Dubremetz, 2011; Tonkin et al., 2014). Immunization with an AMA1-RON2 peptide complex provided complete protection against a lethal *Plasmodium yoelii* challenge in mice (Srinivasan et al., 2014). AMA1 and RON2 are conserved in apicomplexan parasites, including *Babesia* spp. (Besteiro and Dubremetz, 2011), suggesting the existence of a common host cell invasion mechanism which can be used to develop a preventative strategy for human babesiosis. In this regard, we expressed the genes encoding the predicted domains I and II of BmAMA1 and the genes encoding the predicted transmembrane regions 2 and 3 of BmRON2 and evaluated their protective efficacy against *B. microti* infection.

The molecular mass of recombinant BmAMA1 and BmRON2 was approximately 59 kDa and 18 kDa, respectively. Serum from the hamsters infected with *B. microti* could well recognize rBmAMA1 on western blot analysis. However, rBmRON2 was not detected, suggesting that the C-terminus of BmRON2 does not appear to be recognized by immune sera, which is in agreement with a related study showing that RON2 is not a highly immunodominant antigen (Orda et al, 2016). On the other hand, both mouse anti-rBmAMA1 and anti-rBmRON2 sera detected distinct bands in the *B. microti*-lysate, as shown by the presence of a band at 53 kDa, 40 kDa and 170 kDa, 52 kDa, respectively. The predicted molecular mass of BmAMA1 and BmRON2 is 69 kDa and 165.3 kDa, suggesting that the bands detected in the present study might be the possible proteolytic products of BmAMA1 and BmRON2, as previously reported (Moitra et al., 2015; Orda et al., 2016). Additionally, in accordance with earlier reports (Moitra et al., 2015; Orda et al., 2016), our

immunofluorescence studies confirmed that native BmAMA1 and BmRON2 localized to the apical organelles of the parasite.

The immune protective experiments showed that only the group immunized with rBmAMA1+rBmRON2 exhibited a limited protection against *B. microti* challenge, whereas the group immunized with rBmAMA1 or rBmRON2 alone did not show any protection compared to the control group. This result is in agreement with previous studies regarding *Toxoplasma*, where utilization of key epitopes from both proteins lead to enhanced protection against *Toxoplasma* challenge infection (Zhang et al., 2015). The absence of any significant difference in the total amount of antibodies against rBmAMA1 in the groups immunized with single and combined antigens suggests that the presence of antibodies targeting key epitopes from both antigens is more important than the total amount of antibodies produced. In addition, our result might suggest a possible role for BmAMA1 and BmRON2 during the invasion of RBCs by parasites, even though the presence of the moving junction structure has not yet been reported in *B. microti*. It is not clear whether the two proteins interact with each other in vivo or take part in the formation of a moving junction between *B. microti* merozoites and RBCs. Therefore, further investigation is needed for elucidating the presence and possible structure of a moving junction in *B. microti* infection, which may provide more clues for the development of more effective vaccines to protect against *B. microti* infection in the future.

2-5. Summary

In this study, I evaluated the protective effect of recombinant *B. microti* AMA1 (rBmAMA1) and RON2 (rBmRON2) against *B. microti* infection using a hamster

model. The genes encoding for predicted BmAMA1 Domain I and Domain II (DIDII) and the gene encoding for predicted BmRON2 transmembrane region 2 to 3 (TM2-TM3) were expressed as His fusion recombinant proteins in *Escherichia coli*. Three groups of hamsters were immunized with rBmAMA1, rBmRON2 and rBmAMA1+rBmRON2, then challenge infected with *B. microti*. The result showed that only the group immunized with rBmAMA1+rBmRON2 exhibited a degree of protection against *B. microti* challenge infection, characterized by significantly decreased parasitemia and higher hematocrit values from day 6 to 10 post infection. However, no significant protection was observed in the group immunized with rBmAMA1 or rBmRON2 alone. The protection may not be attributed to the total amount of antibodies against rBmAMA1 or rBmRON2 but both are required. These results suggest that combined immunization with rBmAMA1 and rBmRON2 is an efficient strategy to protect against *B. microti*.

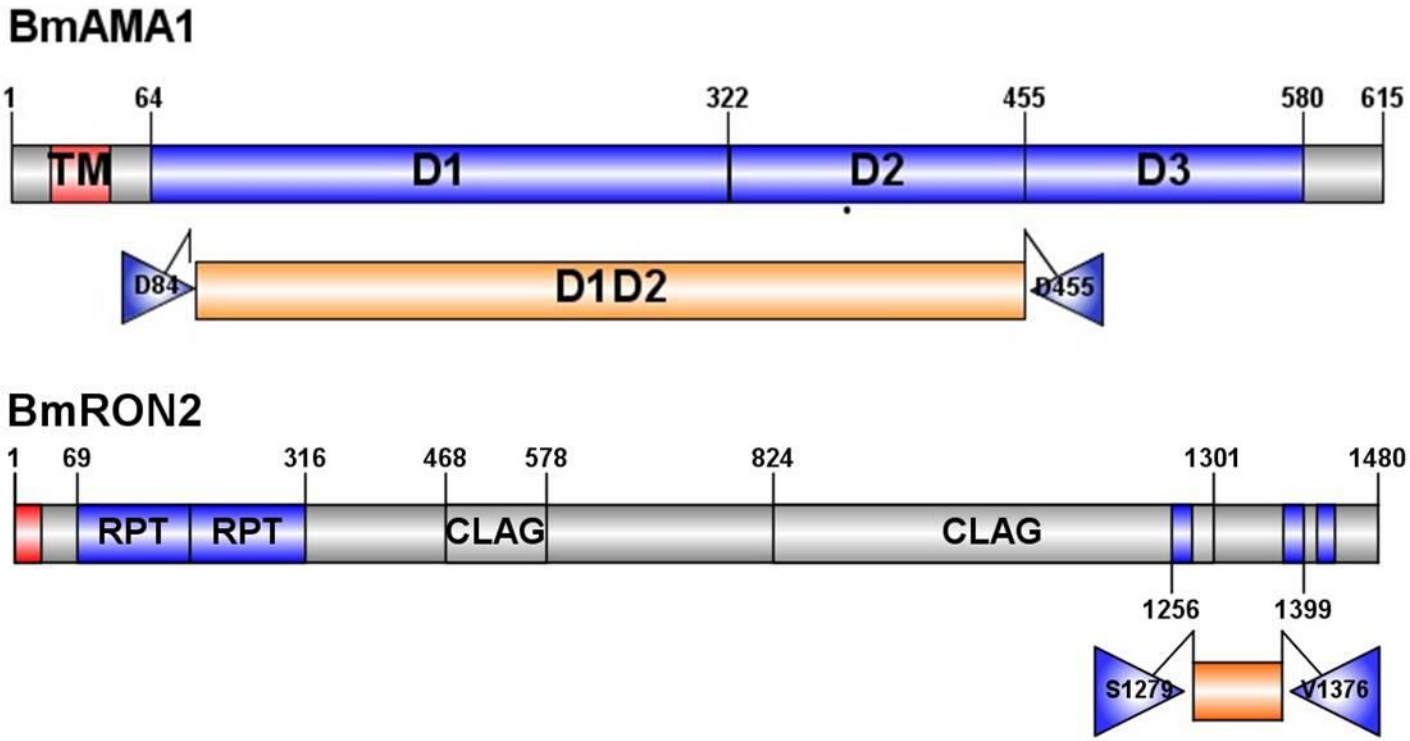

Fig. 1. Bioinformatics analysis of BmAMA1 and BmRON2 genes. The predicted domains I, II, and III of BmAMA1 and the predicted transmembrane regions of BmRON2 are demarcated.

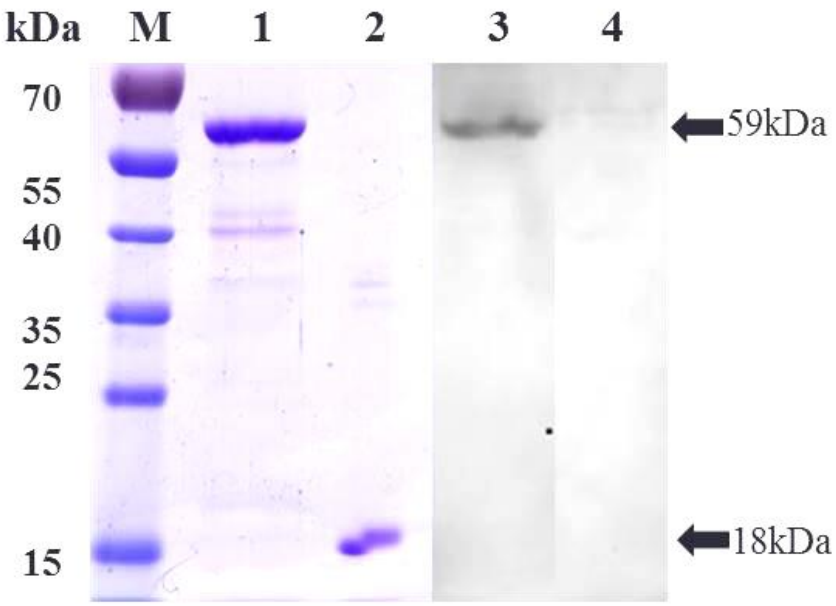

Fig. 2. SDS-PAGE, Western-blot analysis of rBmAMA1 and rBmRON2. M, molecular size markers. Lanes 1 and 2, SDS-PAGE analysis of rBmAMA1 and rBmRON2. Lanes 3 and 4, Western blot analysis of rBmAMA1 and rBmRON2 using the immune serum from a hamster infected with *B. microti*.

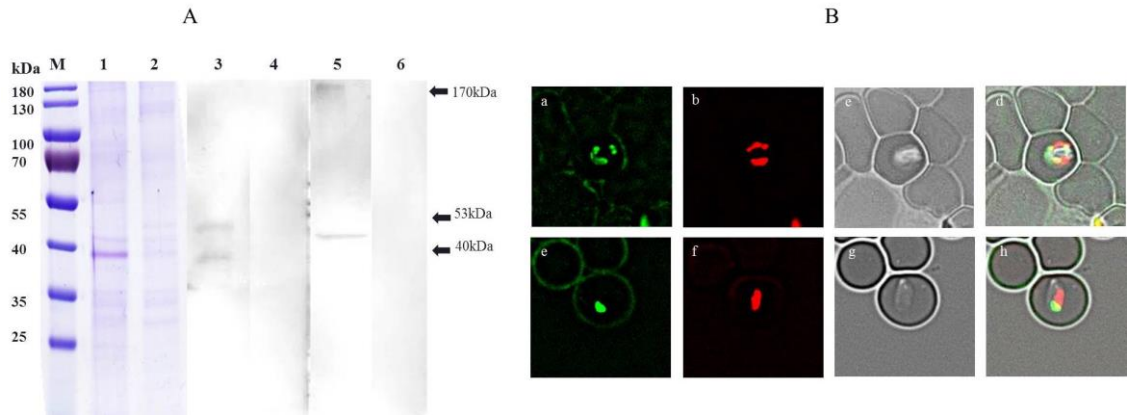

Fig. 3. SDS-PAGE, Western-blot and IFAT analysis of native BmAMA1 and BmRON2 proteins. (A) M, molecular size markers. Lanes 1 and 2, SDS-PAGE analysis of *B. microti* lysate and non-infected RBC lysate. Lanes 3 and 4, Western blot analysis of the parasite lysate and non-infected RBC lysate with anti-rBmAMA1 sera; lanes 5 and 6, Western blot analysis of the parasite lysates and non-infected RBC lysate with anti-rBmRON2 sera. (B) Observation of the native BmAMA1 and BmRON2 recognized by mice anti-rBmAMA1 and anti-rBmRON2 serum in confocal laser micrographs. (a) Immunofluorescent staining of *B. microti* merozoites with mice anti-rBmAMA1 sera. (b) Propidium iodide staining of *B. microti* merozoite nuclei. (c) Phase-contrast images of *B. microti* merozoites. (d) Panels a and b are overlaid on panel c. (e) Immunofluorescent staining of *B. microti* merozoites with mice anti-rBmRON2 sera. (f) Propidium iodide staining of *B. microti* merozoite nuclei. (g) Phase-contrast images of *B. microti* merozoites. (h) Panels e and f are overlaid on panel g. The images were derived from a single section.

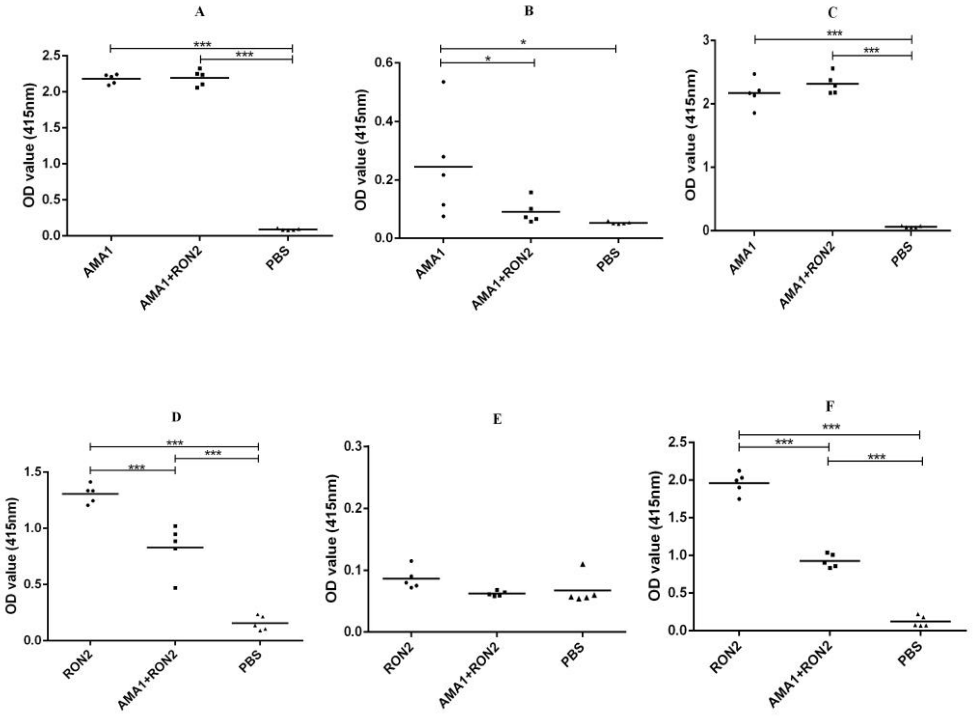

Fig. 4. The protective efficacy of vaccination with rBmAMA1, rBmRON2 and rBmAMA1+rBmRON2 on average parasitemia (A) and hematocrit (B) of hamsters after challenge infection with *B. microti*. Asterisks indicate statistically significant differences (*, $P<0.05$; **, $P<0.005$; ***, $P<0.0001$ [compared to PBS-immunized hamsters]).

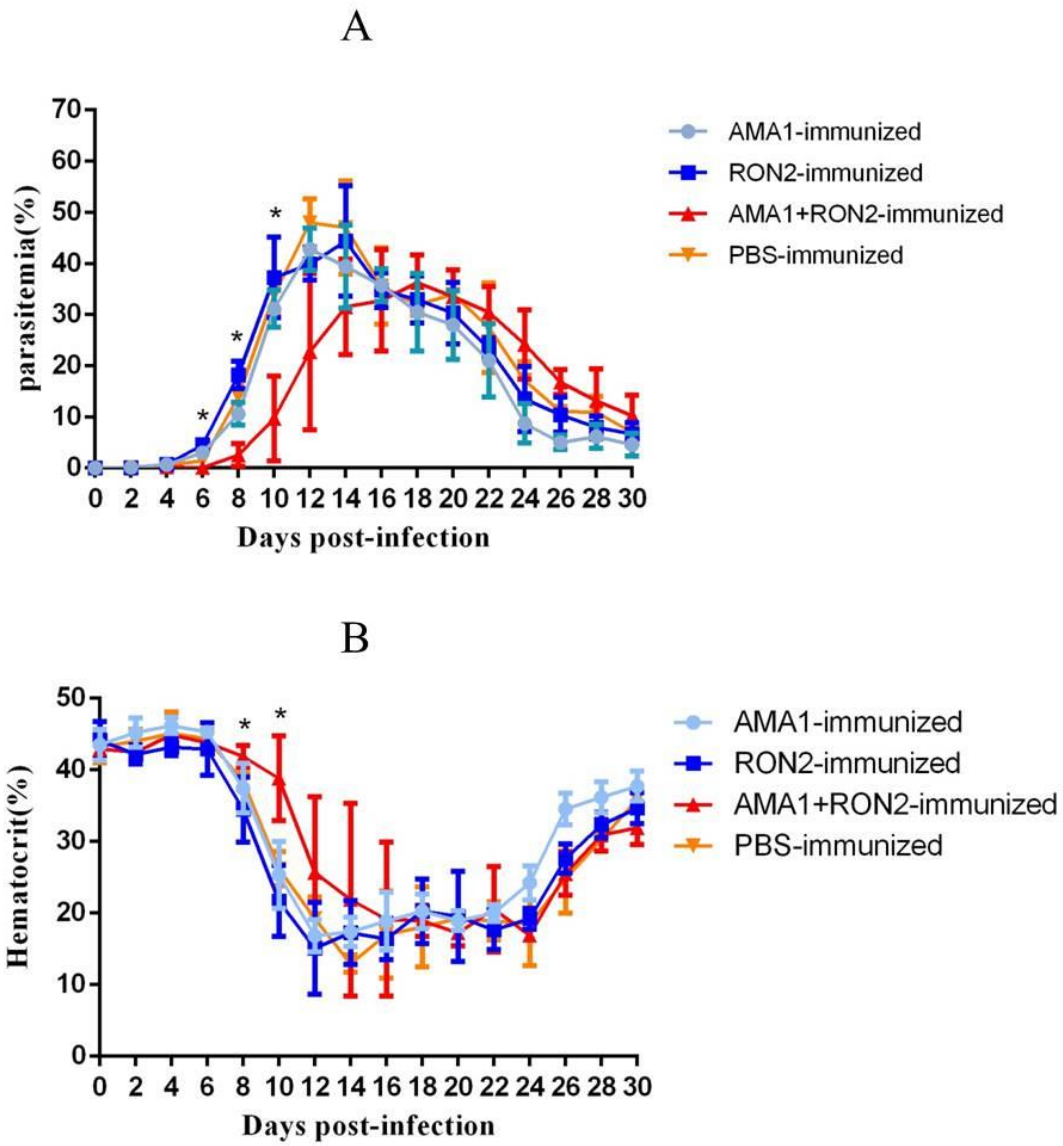

Fig. 5. The protective efficacy of vaccination with rBmAMA1, rBmRON2 and rBmAMA1+rBmRON2 on average parasitemia (A) and hematocrit (B) of hamsters after challenge infection with *B. microti*. Asterisks indicate statistically significant differences (*, P＜0.05; **, P＜0.005; ***, P＜0.0001 [compared to PBS-immunized hamsters]).

Chapter 3

Development of a heterologous prime-boost vaccination strategy against *Babesia microti* infection

3-1. Introduction

Heterologous prime-boost is an immunization strategy that utilizes unique antigen-delivery systems encoding the same epitopes or antigens (Dunachie and Hill, 2003). The advantage of the heterologous prime-boost strategy compared to the homologous boosting strategy is its ability to mediate CD8+ T-cells and Th1-type CD4+ T-cells responses (Dunachie and Hill, 2003). Heterologous prime-boost immunization may also generate memory T-cells (Anderson and Schneider, 2007; Reyes-sandoval et al., 2007). The crucial importance of the prime-boost strategy lies in the establishment of appropriate vectors that are safe, not affected by prior immunity (Woodland, 2004). Previous studies have proven several types of vectors, such as replication-defective adenoviruses, fowl pox viruses, vaccinia virus, influenza virus and naked DNA, to be effective (Ramshaw and Ramsay, 2000; Takeda et al., 2003; Woodland, 2004). Plasmid DNA vaccines and recombinant viral vectors are effective in eliciting both humoral and cellular immune responses. Immunization with a prime-boost strategy showed a promising prospect in protection against *Toxoplasma gondii* and *Plasmodium* infection (Dunachie and Hill, 2003; Caetano et al., 2006; Zhang et al., 2010).

Recent studies have identified and characterized an attractive antigen, apical membrane protein 1 (AMA1) of *B. microti*, for vaccine development (Moitra et al., 2015). AMA1 has been examined as a leading vaccine candidate for malaria, because antibodies against recombinant AMA1 are highly efficient in the blocking of parasites into RBCs (Stowers et al., 2002; Dutta et al., 2009). Although little information is ~~well~~ known regarding the function of BmAMA1, it is strongly conserved in the Apicomplexan parasites, suggesting a potential use for vaccination against *B. microti* infection (Harvey et al., 2014). A previous study has shown that immunization with recombinant BmAMA1 expressed by *E. coli* was not effective in protecting against *B. microti* challenge infection in a hamster model (Harvey et al., 2014).

Therefore, in the current study, I have evaluated the protective effect of a heterologous prime-boost strategy in hamsters administrated with plasmid DNA expressing BmAMA1 by gene gun, followed by recombinant adenovirus that expresses BmAMA1. Here, I have shown that the heterologous prime-boost strategy could enhance the protective effect of AMA1 immunization against *B. microti* infection.

3-2. Materials and methods

Experimental animals

In total, twelve six-week-old female Golden Syrian hamsters were used in the immunization trials and all experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine of Japan.

Parasite and cell lines

Human isolated *B. microti* Gray strain (US type, American Type Culture Collection, Catalog No. 30221) used for challenge infection was maintained in female Golden Syrian hamsters (Clea, Japan), by intraperitoneal injection with cryopreserved *B. microti*-infected erythrocytes. Monkey kidney epithelial cells (Vero, ATCC CCL-81) used for the transfection experiment were cultured in Eagle's minimum essential medium (MEM; Sigma, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS, Cell Cultural Bioscience, Japan) and 50 μg/ml kanamycin. Human embryonic kidney (HEK) 293 cells (Qbiogene, USA) used for recombinant adenovirus construction and amplification were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, USA) supplemented with 5% FBS and 50 μg/ml kanamycin. The cell cultures were grown at 37 ℃ in a 5% CO_2 air environment.

Plasmid construction and generation of recombinant adenovirus

To produce a construct of pBmAMA1, the gene encoding BmAMA1 was amplified from a *B. microti* gray strain cDNA library by PCR using the primer sets: 5'-AAGGATCCGCCATGGATGAGGAGGATGACTATGAA-3' and 5'-CCTCGAGCTAAAAGCCATAGAAAGTCAA-3' (the underlined nucleotides are BamHI and XhoI restriction enzyme sites), and then cloned into eukaryotic expression vector pcDNA3.1 (Invitrogen, USA). To generate the recombinant adenovirus, the BmAMA1 sequence was amplified using primer sets: 5'-CGCGTCGACATGGATGAGGAGGATGACTATGAA -3' and 5'-AAAGCGGCCGCCTAAAAGCCATAGAAAGTCAA -3' (the underlined nucleotides are *Sal* I and *Not* I restriction enzyme sites) and cloned into the expression cassette of a pCR259 transfer vector (Qbiogene, USA). The recombinant adenovirus

DNA was generated by intracellular homologous recombination between the pCR259 transfer vector carrying the BmAMA1 sequence and the plasmid Transpose-AdTM 294 (Qbiogene, USA), carrying a nonreplicative ΔE1 adenovirus type 5 genome. To obtain a recombinant adenovirus, the plasmid Transpose-AdTM 294 containing the BmAMA1 gene was transfected into the HEK 293 cells, using Lipofectamine 2000 reagents (Invitrogen, USA). The adenovirus was purified as described in a previous paper (Yu et al., 2012).

Indirect fluorescent antibody test (IFAT) and Western blot analysis

To investigate the expression of BmAMA1 *in vitro*, pBmAMA1-transfected Vero cells and Ad5BmAMA1-infected HEK293 cells were analyzed by IFAT and Western blotting. For IFAT, pBmAMA1-transfected Vero cells and Ad5BmAMA1-infected HEK293 cells were fixed in 100% methanol for 10 min at room temperature, then incubated for 1 h at 37 ℃ with mouse anti-rBmAMA1 serum 1:100 diluted with PBS containing 3% bovine serum albumin (BSA). After four washes with PBST, Alexa-Fluor® 488 conjugated goat anti-mouse IgG (Molecular Probes, USA), 1:400 diluted in PBS-BSA, was subsequently applied as a secondary antibody and incubated for 1 h at 37℃. The cells were examined under a confocal laser scanning microscope (TCS NT, Leica, Germany). For Western blotting, pBmAMA1-transfected Vero cells and Ad5BmAMA1-infected HEK293 cells were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and then electrically transferred to a nitrocellulose membrane. The membrane was blocked with PBS containing 5% skim milk and incubated with anti-rBmAMA1 polyclonal serum diluted in 5% skim milk at 37℃ for 60 min. Next, the membrane was washed three times with PBS and incubated with peroxidase-conjugated goat anti-mouse IgG in 5% skim milk. After washing three times with PBS, the bands on the membrane were

visualized by incubation with diaminobenzine.

Immunization and challenge infection

A total of twelve six-week-old female Syrian hamsters were divided into four groups as shown in Table 1 (n=3). The gene gun immunization was performed as described previously (Yu et al., 2012). The plasmid pNull (pcDNA3.1 vectors without any inserted gene) or the pBmAMA1 was affixed onto gold particles (1.0 μm diameter, Bio-Rad Laboratories, USA) using 2 μg of DNA per 1mg of gold by the addition of 1 M $CaCl_2$ in the presence of 0.05 M spermidine. Plasmid DNA-coated gold particles were loaded onto gold-coat tubing in the presence of polyvinylpyrrolidone (360,000 MW), at a concentration of 0.05 mg/ml. Plasmid DNA-coated gold particles were accelerated into the abdomen skin of hamsters using Helios Gene Gun (Bio-Rad Laboratories, USA) at a helium discharge pressure of 400 psi. Each hamster received four shots and was immunized 3 times with 2-week intervals. For the boost immunization, either Ad5AMA1 or Ad5Null recombinant adenovirus was administered 2 weeks after the last gene gun immunization by intramuscular inoculation at a dose of 5×10^8 IU per hamster. The hamsters were challenge infected i.p. with 1×10^7 *B. microti*-infected erythrocytes.

Detection of humoral response

To assess the humoral response after immunization, hamster sera were collected 2 weeks after the booster immunization. The levels of BmAMA1 specific total immunoglobulin G (IgG), IgG1, and IgG2a in hamster sera were measured by enzyme-linked immunosorbent essay (ELISA). Briefly, a 96-well plates were coated with 50 μl of rBmAMA1 at a concentration of 4 μg/ml in a 50 mM carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4℃. The levels of total IgG,

IgG1, and IgG2a in the sera which were diluted 100-fold with 3% skim milk in PBS were measured in the plates using horseradish peroxidase-conjugated goat anti-hamster IgG, IgG1 and IgG2a (Bethyl, USA). The plates were washed five times, and then 100 µl of ABTS substrate (0.1 M citric acid, 0.2 M sodium phosphate, 0.003%H_2O_2, and 0.3mg/ml 2,2'6-azide-bis [3-ethylbenzthia-zoline-6-7 sulfonic acid]; Sigma, USA) was added into each well. Absorbance was measured at 415 nm using MTP-500 micro plate reader (Corona Electric, Japan).

Determination of parasitemia, hematocrit value and body weight

To evaluate the protective effect, parasitemia and hematocrit values were monitored for a month after challenge infection. For parasitemia level estimation, thin blood smears made using blood from hamster tail veins were fixed in methanol and stained for 45 min with 10% Giemsa solution diluted in Sörensen buffer (pH 6.8). Thereafter, parasitemia was determined by examining at least 10^3 erythrocytes. For hematocrit evaluation, 10 µl of blood were collected from each hamster at 2-day intervals, and a full blood cell count was made using an automatic cell counter (Nihon Kohden, Japan). In addition, infected hamsters were observed at 2-day intervals for body weight changes until day 30 post challenge infection.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Prism 6; GraphPad Software, USA). The means of all variables were computed and one-way analysis of variance, followed by Tukey's multiple-comparison test was used for pairwise comparison of data from the multiple groups. Results were considered to be statistically significant when the P value was <0.05.

3-3. Results

Generation of the recombinant adenovirus

Cytopathic effects (CPE) were observed in HEK293 cells that were transfected with *Pac* I-linearized recombinant adenovirus plasmid DNA (Fig. 1A). This result confirmed that recombinant adenovirus particles (Ad5BmAMA1) were packaged in HEK293 cells.

In vitro expression of BmAMA1

To investigate whether pBmAMA1 and Ad5BmAMA1 express BmAMA1 in mammalian cells, pBmAMA1-transfected Vero cell and Ad5BmAMA1-infected HEK293 cells were analyzed by IFAT and Western blotting using anti-rBmAMA1 mouse sera as the primary antibody. Specific fluorescence could be observed in both pBmAMA1-transfected Vero cell and Ad5BmAMA1-infected HEK293 cells (Fig. 2A). On Western blotting, a specific band of approximately 56 kDa was detected in both pBmAMA1-transfected Vero cells and Ad5BmAMA1-infected HEK293 cells, which is slightly larger than native BmAMA1 detected in *B. microti* lysate (Fig. 2B).

Humoral response induced by prime-boost immune strategy

The humoral response elicited by vaccination was verified by ELISA. IgG antibody levels against rBmAMA1 significantly increased in test groups immunized with pBmAMA1/Ad5BmAMA1, pNull/Ad5BmAMA1 and pBmAMA1/Ad5Null compared to the control group immunized with pNull/Ad5Null (Fig. 3A). No significant difference ($P>0.05$) was found in IgG levels between these test groups. Interestingly, hamsters in the test groups exhibited a robust IgG2a antibody response compared to the control group (Fig. 3B). In contrast, both test and control hamsters did not show significantly elevated levels of specific IgG1 (Fig. 3C).

Evaluation of the protective efficacy against challenge infection with *B. microti*

Hamsters immunized with pNull/Ad5BmAMA1, pBmAMA1/Ad5Null and pBmAMA1/Ad5BmAMA1 exhibited varying degrees of protection against *B. microti* challenge. The parasitemia decreased in pBmAMA1/Ad5BmAMA1 immunized group at 14-16 days post infection compared to control group (Fig. 4A). Although the extent of the decrease in parasitemia had no remarkable differences among immunized groups, immunization with pBmAMA1/Ad5BmAMA1 conferred better protective effect, characterized by lower parasitemia (Fig. 4A) and higher hematocrit values (Fig. 4B). In addition, no significant difference was noted on the variation of body weight between each group (data not shown).

3-4. Discussion

A heterologous prime-boost strategy with priming plasmid DNA followed by recombinant viral vectors expressing the vaccine candidate is an effective means of inducing both humoral and cellular immune responses (Harvey et al., 2014). DNA vaccines are based on bacterial plasmids that express the specific antigen using promoter elements that are active in mammalian cells (Anderson and Schneider, 2007). Viral vector vaccines could elicit a strong type 1 Th immune response by delivering the peptides to the MHC class I presentation pathway (Kochan et al., 2006). Prime-boost experiments using relative vaccine candidates delivered by gene gun and attenuated vaccinia virus have been found to be effective in protecting against *P. falciparum* and *T. gondii* (Caetano et al., 2006; Yu et al., 2012; Chuang et al., 2013). Therefore, in our study, recombinant plasmid

DNA and adenovirus expressing BmAMA1 were constructed to evaluate the protective effect against *B. microti* infection.

The recombinant BmAMA1 expressed in Ad5BmAMA1-infected and pBmAMA1-transfected mammalian cells was confirmed by IFAT and Western blotting. The molecular weight of the expressed recombinant BmAMA1 was approximately 56 kDa, which is a little larger than that of native BmAMA1 detected in *B. microti* lysate. It is probably due to the protein modification in mammalian cells.

It is known by a previous study that the segregation of IgG2a and IgG1 immunoglobulin isotypes were used as markers for Th1 and Th2 lymphocytes, respectively (Mountford et al., 1994). In the current study, pNull/Ad5BmAMA1, pBmAMA1/Ad5Null and pBmAMA1/Ad5BmAMA1 immunized hamsters produced significantly higher levels of IgG and IgG2a against rBmAMA1 compared to pNull/Ad5Null immunized hamsters. In contrast, all the hamsters produced low levels of IgG1. These results indicate that both plasmid DNA and adenovirus stimulate a strong Th1 immune response in hamsters. Consistent with previous studies, IgG antibody responses induced by a combination vaccine tend to have a bias toward IgG2a (Indresh and Margaret, 2013).

The protective immune experiments showed that hamsters immunized with pBmAMA1/Ad5BmAMA1 exhibited partial protection, characterized by significantly reduced parasitemia and slightly higher hematocrit values at the acute stage of infection compared to the control group. Immunization with pBmAMA1/Ad5BmAMA1 showed a better protective efficacy than

pBmAMA1/Ad5Null and pNull/Ad5BmAMA1 immunized groups, although this was not statistically significant. This result suggests that the heterologous prime-boost strategy is more effective in enhancing the protective immunity of the host. In chapter 2, immunization with recombinant BmAMA1 expressed by *E. coli* was not effective in protection against *B. microti* challenge. However, immunization with plasmid DNA and adenovirus expressing BmAMA1 could confer a degree of protection, which indicated an important role of cellular immunity against the intracellular parasite, because DNA and viral vector vaccines can better stimulate cellular immunity (Campos-Neto, 2005; Liu and Ulmer, 2005).

3-5. Summary

In the present study, I have investigated the protective effect of a heterologous prime-boost strategy with priming plasmid DNA followed by recombinant adenovirus expressing BmAMA1 against *B. microti* infection. Four groups of hamsters were immunized with pAMA1/Ad5AMA1, pNull/Ad5AMA1, pAMA1/Ad5Null and pNull/Ad5Null, followed by challenge infection with B. microti. Our results showed that the group immunized with pAMA1/Ad5AMA1 exhibited a better protective efficacy than those immunized with pNull/Ad5AMA1 or pAMA1/Ad5Null, characterized by significantly decreased parasitemia levels and higher hematocrit values during the acute stage of infection. Moreover, pAMA1/Ad5AMA1, pNull/Ad5AMA1 and pAMA1/Ad5Null immunized hamsters had a robust IgG and IgG2a antibody response against rBmAMA1 compared with pNull/Ad5Null immunized hamsters, suggesting that both the DNA and viral vector vaccines tend to induce a Th1-biased response. These

results demonstrate that the heterologous DNA priming and recombinant adenovirus boost strategy could enhance protective immunity against *B. microti* infection.

Table 1

The prime-boost immunization strategy.

Groups	(0 week)	(3 weeks)	(6 weeks)	(8 weeks)
	1st	2nd	3rd	Boost
pBmAMA1/Ad5AMA1	pBmAMA1	pBmAMA1	pBmAMA1	Ad5BmAMA1
pBmAMA1/Ad5Null	pBmAMA1	pBmAMA1	pBmAMA1	Ad5Null
pNull/Ad5BmAMA1	pNull	pNull	pNull	Ad5BmAMA1
pNull/Ad5Null	pNull	pNull	pNull	Ad5Null

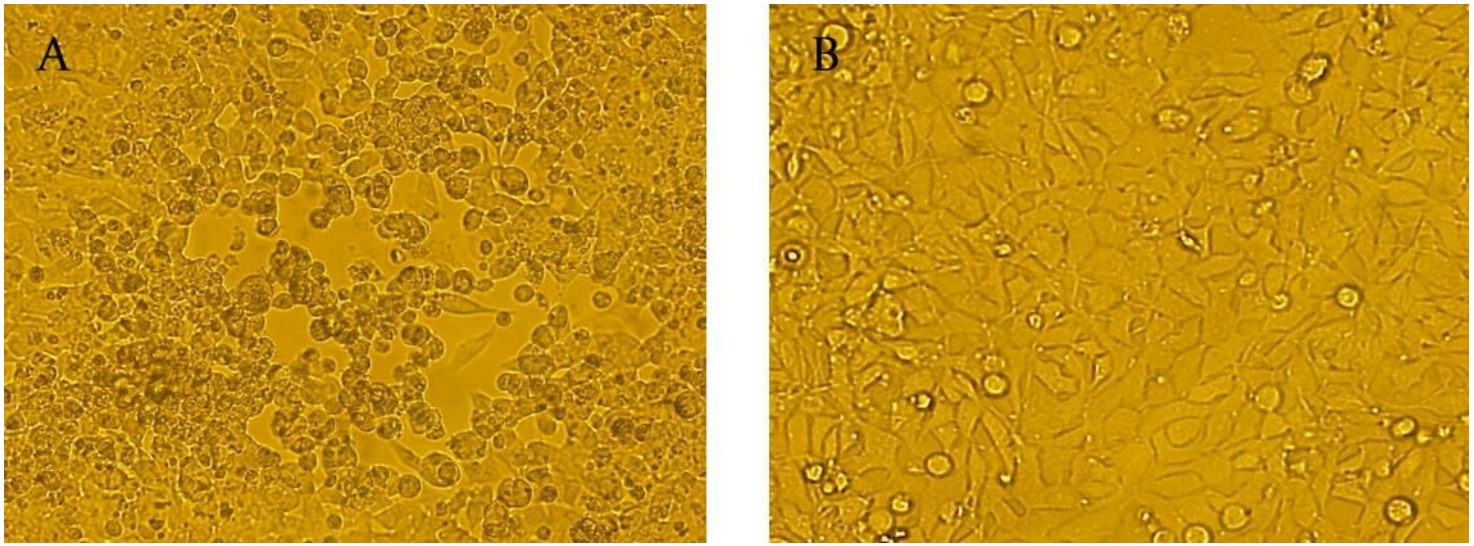

Fig. 1. Producing the recombinant adenovirus in HEK293 cells. (A) *Pac I* linearized recombinant adenovirus plasmid DNA was transfected in HEK293 cells, the cells containing viral particles break and infect neighboring cells. A plaque begins to form. (B) Normal HEK293 cells.

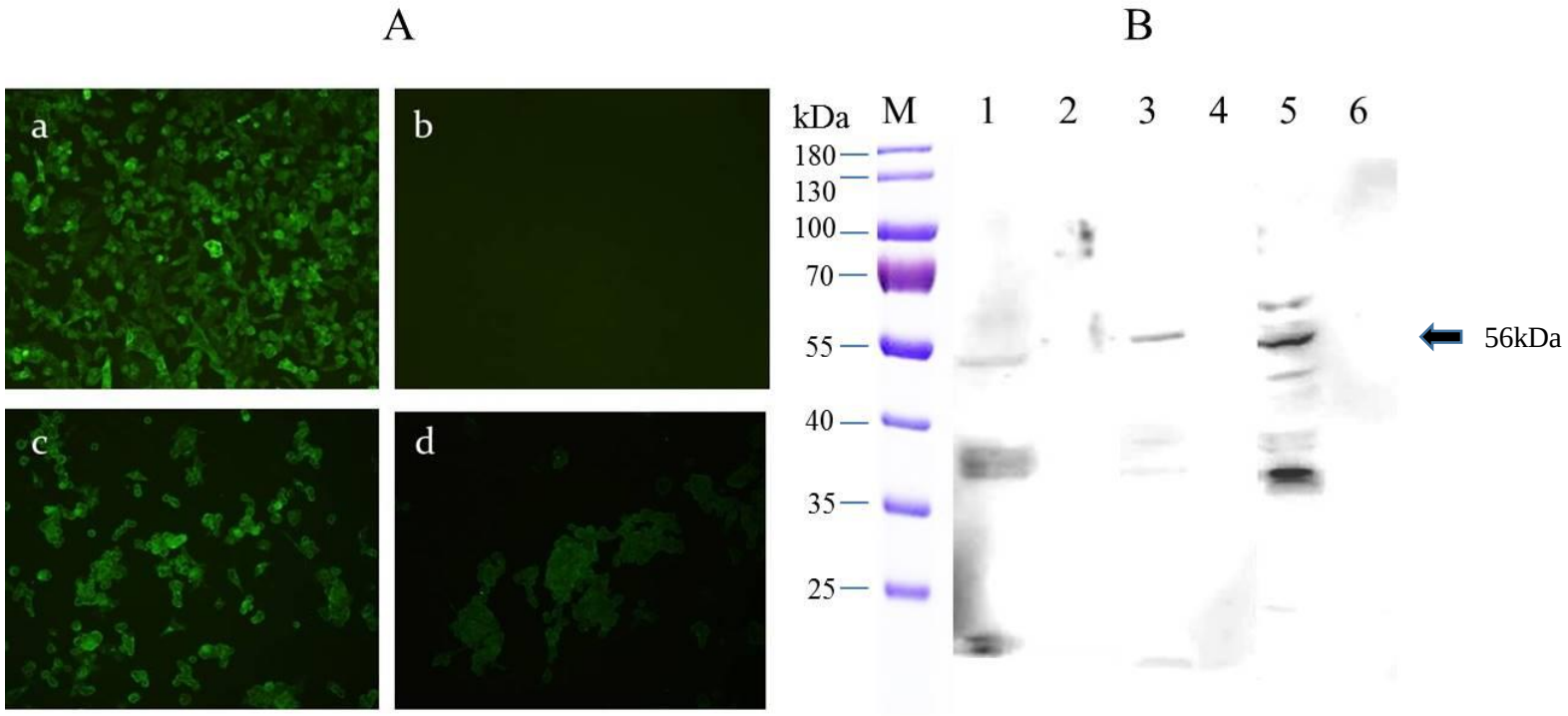

Fig. 2. IFAT and Western blot analysis of the expression of BmAMA1 *in vitro*. (A) IFAT analysis. (a) Vero cells transfected with plasmid DNA pBmAMA1. (b) Vero cells transfected with plasmid DNA pNull. (c) HEK293 cells infected with adenovirus Ad5BmAMA1. (d) HEK293 cells infected with adenovirus Ad5Null. (B) Western blot analysis. Lane M, molecular size markers. Lane 1, *B. microti* lysate. Lane 2, non-infected RBC lysate. Lane 3, pBmAMA1 transfected Vero cells. Lane 4, pNull transfected Vero cells. Lane 5, Ad5BmAMA1 infected HEK293 cells. Lane 6, Ad5Null infected HEK293 cells.

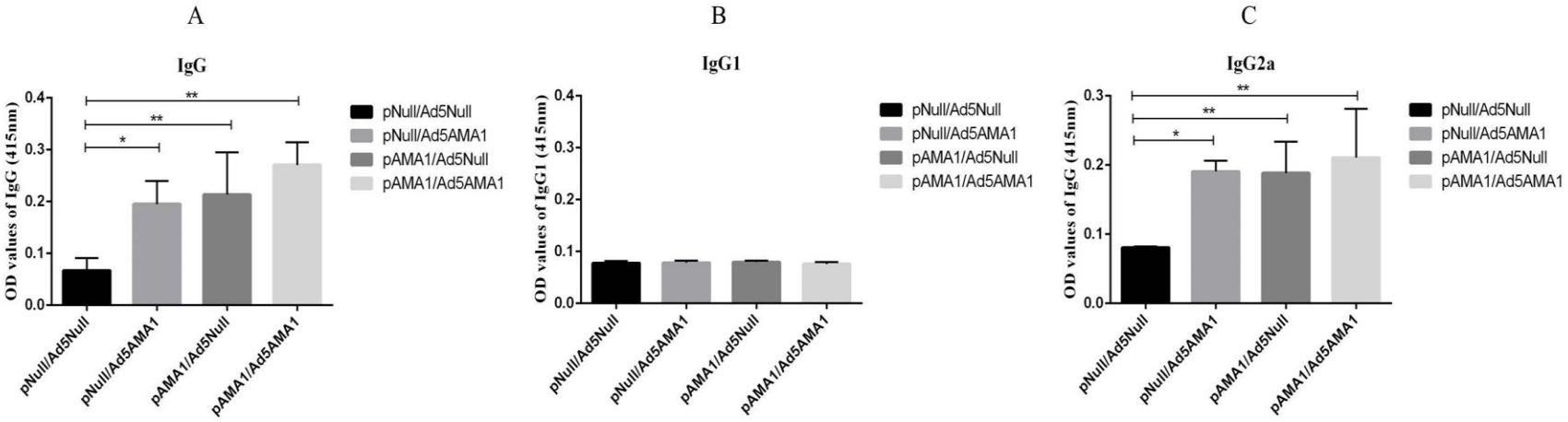

Fig. 3. Evaluation of humoral response to rBmAMA1 in the immunized hamsters. The levels of IgG (A), IgG1 (B), IgG2a (C) in hamsters immunized with pAMA1/Ad5AMA1, pNull/Ad5AMA1, pAMA1/Ad5Null and pNull/Ad5Null were measured as OD values at 415nm. Asterisks indicate statistically significant differences (*, $P<0.05$; **, $P<0.005$ [compared to pNull/Ad5Null - immunized hamsters]).

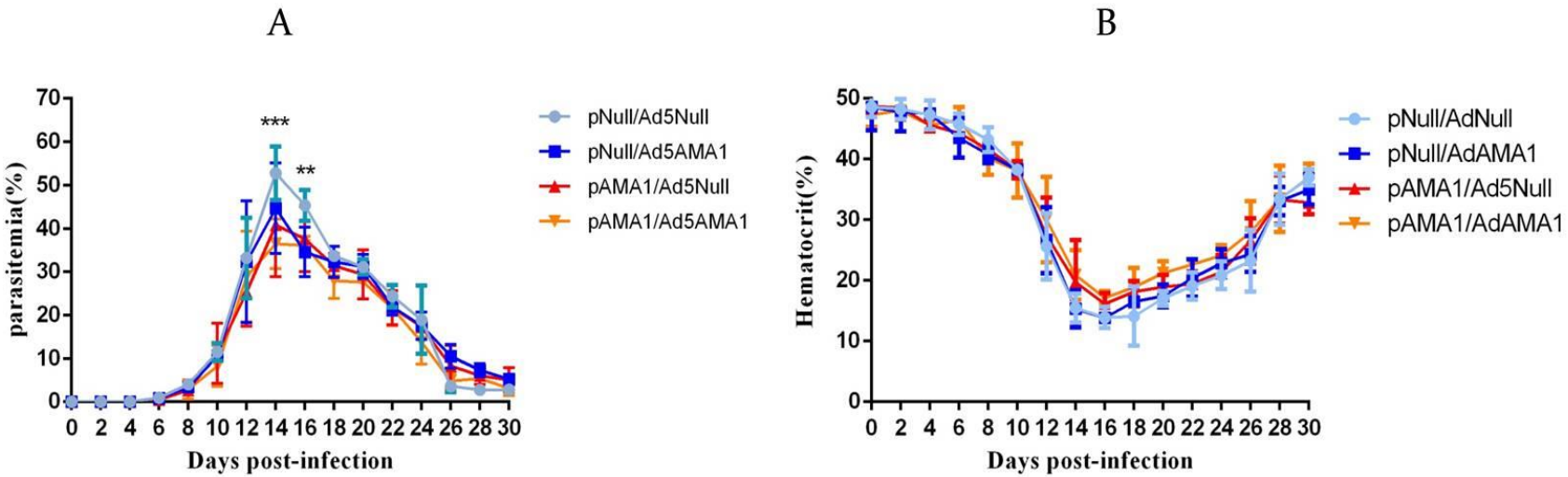

Fig. 4. The effect of immunization with pBmAMA1/Ad5BmAMA1, pNull/Ad5BmAMA1, pBmAMA1/Ad5Null and pNull/Ad5Null on the parasitemia (A) and hematocrit (B) levels of hamsters after challenge infection with *B. microti*. Asterisks indicate statistically significant differences (*, P＜0.05; **, P＜0.005; ***, P＜0.0001 [compared to pNull/Ad5Null -immunized hamsters]).

General discussion

B. microti and *B. rodhaini* are the major causative agents of babesiosis in mice. The different pathogenicities of the two parasites make them ideal for exploring the protective immunity to *Babesia* infection (Homer et al., 2000). As a step toward a better understanding of the host defense mechanism against *Babesia* infection, in chapter 1, I analyzed the host immune response by studying the cross-protection between *B. rodhaini* and *B. microti*. BALB/c mice which had recovered from *B. rodhaini* infection by drug treatment were completely protected against *B. rodhaini* reinfection and *B. microti* challenge infection, with remarkable reduced parasitemia and no mortalities compared to mock mice. These findings parallel a previous study where mice immunized with *B. rodhaini* through a drug-control method were protected from infection with other *Babesia* species (Zivkovic et al., 1984). In addition, mice immunized with dead *B. rodhaini* failed to be protected against either *B. rodhaini* reinfection or *B. microti* challenge infection, suggesting that only immunization with alive parasites could confer protective immunity. To unravel the mechanism behind this, both humoral and cellular components involved were investigated. Regarding the humoral response, high titer of antibody was produced in mice immunized with both alive and dead *B. rodhaini*. However, mice immunized with dead parasites failed to be protected, which suggests that antibodies may play a limited role in the protection. The function of antibodies is to neutralize sporozoites or merozoites at the extracellular stage (Jacobson et al., 1993; Chen et al., 2000; Brown, 2001; Aguilar-Delfin et al., 2003). Therefore, antibodies have a limited effect on those internalized parasites. On the other hand, the lack of cross-reacting antibodies leads to the conclusion that resulting protection should not be attributed to antibodies induced

by the live parasites. Regarding the cellular immune response, mice recovered from *B. rodhaini* infection produced significantly lower levels of cytokines, as opposed to mock mice. Generally, successful resolution of rodent *Babesia* is dependent on the ability of mice to mount an early proinflammatory cytokine response (IL-12 and INF-γ) and the appropriate maintenance of their kinetics during acute stage of infection (Igarashi et al., 1999; Trinchieri, 2003; Couper et al., 2008). However, the lack of any major role of antibodies and cytokines raises the question ~~that~~ whether the innate immune responses play a key role in the protection. Therefore, further studies are needed to understand the role of innate immune cells in this case of cross-protection.

For vaccine development, the first step to identifying vaccine candidates is of great importance. Therefore, in chapter 2, I have evaluated the protective effect of two attractive *B. microti* antigens, AMA1 and RON2, for the vaccination against *B. microti* infection. AMA1 and RON2 were reported to form a moving junction structure which plays an important role during the apicomplexan parasite invasion (Besteiro and Dubremetz, 2011; Tonkin et al., 2014). According to previous studies, AMA1 domain II loop and the C-terminus of RON2 is the binding region of AMA1 and RON complex and are believed to be conserved among apicomplexan parasites (Tyler and Boothroyd, 2011; Delgadillo et al., 2016). The genes encoding the predicted domains I and II of BmAMA1 and the genes encoding the predicted transmembrane regions 2 and 3 of BmRON2 were expressed and purified. Immunization of hamsters with rBmAMA1+rBmRON2 conferred partial protection against *B. microti* challenge, however, the hamsters immunized with rBmAMA1 or rBmRON2 alone did not show any protection compared to the control group. Considering the absence of significant difference in the total amount of antibodies against rBmAMA1 and rBmRON2 in the single and combined antigen immunized

groups, it appears that antibodies targeting key epitopes from both antigens are necessary for the protection. Although the presence of the moving junction structure has not been reported in *B. microti*, our result indicated the importance of BmAMA1 and BmRON2 during the invasion of parasites. Therefore, further investigation is required for understanding the function of these two proteins in *B. microti* infection.

Heterologous prime-boost vaccination, using both traditional and novel immunization approaches, provides exciting opportunities to elicit unique immune responses to allow for improved immunogenicity and/or protection (Lu, 2009). In chapter 3, therefore, the heterologous prime-boost strategy priming with the pBmAMA1 and boosting with the Ad5BmAMA1 as vaccination against *B. microti* infection was developed. IFAT and Western blotting confirmed that the recombinant BmAMA1 could be highly efficiently expressed in Ad5BmAMA1-infected and pBmAMA1-transfected mammalian cells. Immunization with pBmAMA1/Ad5BmAMA1 is capable of stimulating strong Th1-biased immune responses in hamsters, characterized by significantly elevated IgG2a isotype. This result was consistent with previous studies that plasmid DNA immunization by gene gun and viral vector vaccine leads to a Th1-biased antibody response (Kochan et al., 2006; Dautu et al., 2007). Immunization with pNull/Ad5BmAMA1, pBmAMA1/Ad5Null and pBmAMA1/Ad5BmAMA1 exhibited varying degrees of protection. Although the extent of the decrease in parasitemia had no remarkable differences among immunized groups, immunization with pBmAMA1/Ad5BmAMA1 is more effective, and was characterized by lower parasitemia and higher hematocrit values at the acute stage of infection compared to the other groups. Therefore, the heterologous prime-boost strategy is considered a useful method to control *B. microti* infection.

General summary

Babesia organisms are tick-transmitted hemoprotozoan parasites belonging to the phylum Apicomplexa, class Piroplasmea and order Piroplasmida. Pathogenesis of *Babesia* species results from the asexual erythrocytic stage, where the parasite invades, replicates and consequently causes destruction of host cells, haemoglobinuria and anemia. The increasingly emergence of human babesiosis has resulted in demands for urgent preventive strategies to control the *Babesia* infection.

In chapter 1, I investigated the host immunity involved in the cross-protection between *B. rodhaini* and *B. microti*. I found that mice which had recovered from *B. rodhaini* infection by drug treatment were completely protected against *B. rodhaini* reinfection and *B. microti* challenge infection, which was characterized by considerably reduced parasitemia, higher hematocrit values and no mortality compared to control mice. In contrast, mice immunized with dead *B. rodhaini* did not show any protection against *B. rodhaini* and *B. microti* challenge. High level of antibody response and low levels of cytokines (INF-γ，IL-4，IL-12，IL-10) were detected in the protected mice. The resulting protection should not be attributed to antibodies and cytokines induced by the live parasites, because protected mice produced low level of cytokines and there are no cross-reacting antibodies between *B. rodhaini* and *B. microti*. Therefore, these findings indicate a possible role of innate immune cells in this case of cross-protection.

In chapter 2, I evaluated the protective effect of two *B. microti* antigens, AMA1 and RON2, as subunit vaccines. The genes encoding for predicted BmAMA1 domain I and domain II (DIDII) and the gene encoding for predicted BmRON2 transmembrane region 2 to 3 (TM2-TM3) were expressed and purified. I found that

immunization with rBmAMA1+rBmRON2 conferred partial protection against *B. microti* challenge infection in hamsters, which was characterized by significantly reduced parasitemia and higher hematocrit values after challenge infection. However, immunization with rBmAMA1 and rBmRON2 alone did not show any significant protection compared to the control group. In addition, there is no significant difference in the total amount of antibodies against rBmAMA1 and rBmRON2 between the groups immunized with single and combined proteins. These results suggest that antibodies targeting key epitopes of both antigens are required for protective immunity.

In chapter 3, a heterologous prime-boost strategy using plasmid pBmAMA1 and recombinant adenovirus Ad5BmAMA1 for immunizing hamsters against *B. microti* infection was evaluated. The results showed that the heterologous prime-boost strategy stimulates a strong Th1-bias immune response. Hamsters immunized with pAMA1/Ad5AMA1 exhibited a degree of protection against *B. microti* infection, characterized by lower parasitemia and higher hematocrit values at the acute stage of infection compared to the control group. These results indicate that the heterologous DNA priming and recombinant adenovirus boost strategy could improve the protective efficacy of vaccination against *B. microti*.

Overall, the present study analyzed the host immune response against *Babesia* parasites and evaluated the protective effect of several types of vaccines against *B. microti* infection. These results will hopefully provide useful information for developing effective preventive strategies against babesiosis in the future.

和文要約

バベシア属原虫は、ダニ媒介性の血液寄生虫で、アピコンプレックス門、ピロプラズマ目に属する。バベシア属原虫は、宿主の赤血球内に侵入・増殖し、それに伴い赤血球の破壊を引き起こし、宿主の貧血、血尿症、黄疸など臨床症状の原因となっている。近年、ヒトのバベシア症例の報告は増加の傾向にあり、その予防対策が急務となっている。

第1章では、ネズミバベシアである *B. rodhaini* と *B. microti* 間の交叉防御作用に関わる宿主免疫について試験を行った。薬剤治療により *B. rodhaini* 感染から回復したマウスは、*B. rodhaini* 再感染および *B. microti* の異種感染に対して非常に強い防御能を示し、その証拠として血液寄生原虫の消失とヘマトクリット値の正常維持が挙げられる。対照的に、刹滅した *B. rodhaini* 虫体で免疫したマウスでは *B. rodhaini* および *B. microti* に対する防御能を示さなかった。さらに、防御能を示したマウスでは抗体産生のレベルは高く、サイトカイン（INF-γ、IL-4、IL-12、IL-10）産生のレベルは低かった。サイトカイン産生が低かったこと、また *B. rodhaini* と *B. microti* 間の交叉反応性抗体がなかったことから、今回示された防御能は抗体やサイトカインによるものではないと考えられた。これらの結果は、この交叉防御作用が自然免疫細胞によるものである可能性を示唆している。

第2章では、2つの *B. microti* 抗原 AMA1 と RON2 のサブユニットワクチンとしての防御効果を評価した。予測された BmAMA1 ドメインⅠおよびドメインⅡ（DIDII）をコードする遺伝子、および予測される BmRON2 膜貫通領域 2〜3

（TM2-TM3）をコードする遺伝子を発現および精製した。ハムスターにおいて、rBmAMA1 + rBmRON2 による免疫は *B. microti* 感染に対する部分的な防御効果を示した。攻撃後、寄生虫血症は有意に低下しており、またヘマトクリット値は上昇していた。しかし、rBmAMA1 および rBmRON2 単独による免疫は、対照群と比較して有意な防御効果を示さなかった。さらに、単一および組み合わせたタンパク質で免疫した群の間で rBmAMA1 および rBmRON2 に対する抗体の総量に有意差はなかった。これらの結果は、両方の抗原の重要なエピトープを標的とする抗体が防御免疫に必要であることを示唆している。

第 3 章では、プラスミド pBmAMA1 および組換えアデノウィルス Ad5BmAMA1 を用いた異種プライム - ブースト法にてハムスターを免疫し、*B. microti* に対する感染防御能を評価した。その結果、異種プライム - ブースト法が強い Th1 優勢の免疫応答を刺激することが示された。 pBmAMA1 / Ad5BmAMA1 で免疫したハムスターは対照群と比較して、感染の急性期において低寄生虫血症、高ヘマトクリット値であり、*B. microti* 感染に対する一定の防御効果を示した。これらの結果は、異種 DNA プライミングおよび組換えアデノウイルスブースト法が *B. microti* に対するワクチン接種の防御効果を改善し得ることを示している。

以上のように、本研究では *Babesia* 属原虫に対する宿主免疫応答を解析し、*B. microti* 感染に対するいくつかのタイプのワクチンの防御効果を評価した。これらの結果は、バベシア症に対する有効な予防法を開発するための有益な情報を提供するものである。

Acknowledgements

I would like to express my special gratitude to my supervisor, Prof. Xuenan Xuan (Obihiro Univ. Agri. Vet. Med.) who gave me the golden opportunity to do this wonderful project on the analysis of host defense immunity and development of recombinant vaccines against babesiosis, and also helped me in doing a lot of research and I came to know so many new things. I am really thankful to him.

Secondly, I would like to thank Associate Prof. Yoshifumi Nishikawa (Obihiro Univ. Agri. Vet. Med.) for his academic guidance, kind support, and encouragement throughout the period of my studies in Japan. My sincere thanks also goes to Prof. Ikuo Igarashi (Obihiro Univ. Agri. Vet. Med.), Prof. Makoto Igarashi (Obihiro Univ. Agri. Vet. Med.), Associate Prof. Kentaro Kato (Obihiro Univ. Agri. Vet. Med.) for their valuable advice, suggestions and comments.

I am deeply grateful to Dr. Paul Franck Adjou Moumouni and Artemis Efstratiou for their careful reading and commenting on countless revisions of my manuscripts.

I would like to acknowledge all of the former and present staff and students of the Research Unit for Host Defense as well as other colleagues in NRCPD for their kindly supporting.

Finally, my deepest thanks goes to my parents for their inspiration and support during my studies in Japan.

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