Studies on epidemiology, development of chemotherapy and genetic modification of piroplasmosis

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ピロプラズマ病の疫学,

薬剤開発および遺伝子改変に関する研究

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アリフィン　ブディマン　ヌグラハ

Contents

Abbreviations

B.	*B. bigemina*	: *Babesia bigemina*
	B. bovis	: *Babesia bovis*
	B. caballi	: *Babesia caballi*
	Bc-48	: 48-kDa rophtry protein
	B. gibsoni	: *Babesia gibsoni*
	BLAST	: Basic local alignment search tool
	B. microti	: *Babesia microti*
	B. ovata	: *Babesia ovata*
	BSD	: Blasticidin
	BW	: Body weight
C.	CCPR	: Codex committee on pesticide residues
	C. hominis	: *Cryptosporidium hominis*
	CI	: Combination index value
	CI	: Convidence interval
D.	DA	: Diminazene aceturate
	DDW	: Double distilled water
	DHFR	: Dihydrofolate reductase
	DMSO	: Dimethyl sulfoxide
	DNA	: Deoxyribonucleic acid
E.	*E. coli*	: *Escherichia coli*
	EDTA	: Ethylenediaminetetraacetic acid

	ef-1α	: *Elongation factor 1 alpha*
	ELISA	: Enzyme-linked immunosorbent assay
	EMA-2	: *Equi merozoite antigent-2*
G.	gDNA	: Genomic DNA
	GFP	: Green fluorescent protein
	Glutamyl	: *Glutamyl tRNA synthase*
H.	HCT	: Hematocrit
	HepG2	: Human liver cancer cell line
	HL60	: Human promyelocytic leukemia cell line
I.	IC_{50}	: Half maximal inhibitory concentration
	ID	: Imidocarb dipropionate
	IFAT	: Immunofluorescence antibody test
	IG	: Intergenic region
	i.p	: Intraperitoneal
	i-RBC	: Infected-red blood cells
K.	kDa	: Kilo dalton
L.	LB	: Luria bertani
M.	MDBK	: Mardin-darby bovine kidney
	MMV	: Medicine for Malaria Venture
	MRC5	: Medical research council cell strain 5
	m-RNA	: Messenger ribonucleic acid

	MQW	: Milli-Q water
	M199	: Medium 199
N.	n-PCR	: Nested-polymerase chain reaction
P.	PBS	: Phosphate buffer saline
	PBS-T	: Phosphate buffer saline-tween 20%
	PCR	: Polymerase chain reaction
	P. falciparum	: *Plasmodium falciparum*
	p.i	: Post infection
R.	RACE	: Rapid amplification cDNA ends
	RBC	: Red blood cells
	RFP	: Red fluorescent protein
	Rsds	: *Ribosomal nucleotide reductase*
	RT	: Room temperature
S.	Sc	: Subcutaneous
	S.D.	: Standard deviation
	SI	: Selectivity index
T.	*T. annulata*	: *Theileria annulata*
	T. equi	: *Theileria equi*
	T. orientalis	: *Theileria orientalis*
	T. parva	: *Theileria parva*
U.	USDA	: United States Department of Agriculture

O. OD : Optical density

Y. YFP : Yellow fluorescent protein

W. WI : Weight index

WT : Wild type

General introduction

1. Piroplasmosis

Piroplasmosis is a tick-transmitted protozoan disease caused by *Babesia* and *Theileria* in a wide range of host species. *Babesia* and *Theileria* are the most common parasites found in the bloodstream of mammals worldwide. Therefore, piroplasmosis is considered to behave significant economic, veterinary, and medical impacts (Knowles, 1996; Homer et al., 2000; Schnittger et al., 2012).

From the veterinary point of view, great attention has been paid to bovine and equine piroplasmosis. Bovine piroplasmosis associated with *Babesia bovis*, *B. bigemina*, *B. divergens*, *Theileria parva*, *T. annulata*, and *T. orientalis* and equine piroplasmosis caused by *B. caballi* and *T. equi* (formerly known as *Babesia equi*) result in huge economic losses in the cattle and horse industries, respectively. The economic damages caused by piroplasmosis are due to meat and milk production losses, treatment cost, and animal deaths (Wise et al., 2013; Yusuf, 2017). Additionally, piroplasmosis has a negative impact on international trade involving the import and export of livestock.

2. Equine piroplasmosis

Equine piroplasmosis is caused by two hemoprotozoan parasites, namely *Theileria equi* and *Babesia caballi*. These parasites infect the Equidae family, including horses, mules, zebras, and donkeys. However, most studies have only investigated the prevalence of equine piroplasmosis in horses because horses are considered more valuable for international trading and sports, and thus movement of these animals between countries is high. For those same reasons, these parasites are found worldwide, including in Southern Europe, Africa, Central and South America, and Asia (Bruning, 1996; Rothschild, 2013). According to OIE (2014), only a few countries are considered to be free from equine piroplasmosis, including Japan, the USA, and Australia.

Based on the morphology, the merozoite size of *T. equi* is smaller than that of *B. caballi*. The merozoite size of *T. equi* and *B. caballi* is approximately 2 μm and 5 μm, respectively (Soulsby, 1982).

Furthermore, the unique four-shape merozoite or “Maltese cross” is found only in the intraerythrocytic stage of *T. equi.* (Fig. 1). Therefore, during the acute phase, the morphology is easy to distinguish under microscopic examination.

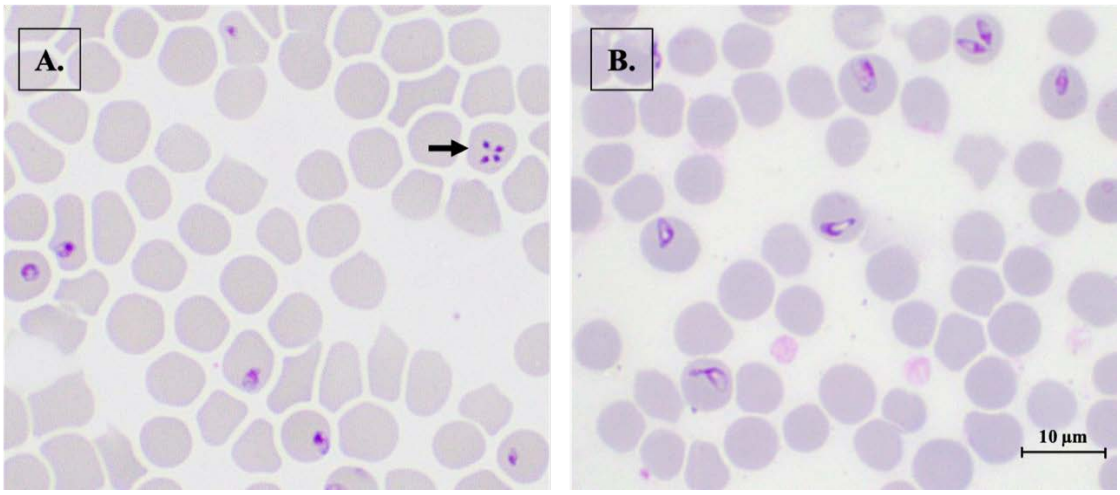

Fig. 1. Equine piroplasm parasites: (A) *T. equi*, “Maltese cross” (black arrow); (B) *B. caballi*

Several ixodid ticks have been shown to transmit equine piroplasms naturally or experimentally, including *Dermacentor* spp., *Hyalomma* spp., and *Rhipicephalus* spp. (APHIS, 2008). Furthermore, the transmission of equine piroplasmosis occurs not only by vector but also transplacentally from a pregnant mare to the fetus (Alsopp et al., 2007). Using a shared needle (mechanical infection) contaminated with infected blood could also spread the infection (Short et al., 2012).

Infection with these parasites produces some clinical symptoms in the animal, such as hemolytic anemia and another systemic illness. However, it is very difficult to distinguish between *T. equi* and *B. caballi* infection in animals by observing clinical symptoms alone. An animal that has recovered from the acute stage remains infected and serves as a reservoir for disease transmission. Horses infected with *T. equi* for their entire lives could be serologically positive, while those infected with *B. caballi* remain seropositive several years (Zweygarth et al., 1996). Additionally, the differential diagnoses of equine piroplasmosis are required with surra, dourine, equine infectious anemia, and African horse sickness (OIE, 2014).

3. Bovine babesiosis

Bovine babesiosis is a tick-transmitted disease caused by protozoan parasites, namely *B. bovis* and *B. bigemina*. These are common species that infect cattle as a major host. Bovine babesiosis is widely spread only in tropical and subtropical regions, found mainly in Africa, Asia, Australia, and Central and South America (OIE, 2018).

Based on the morphology, *B. bovis* is smaller than *B. bigemina. Babesia bovis* is approximately 1–1.5 µm long and 0.5–1.0 µm wide, while *B. bigemina* is approximately 3–3.5 µm long and 1–1.5 µm wide (Soulsby, 1982), as shown in Fig. 2.

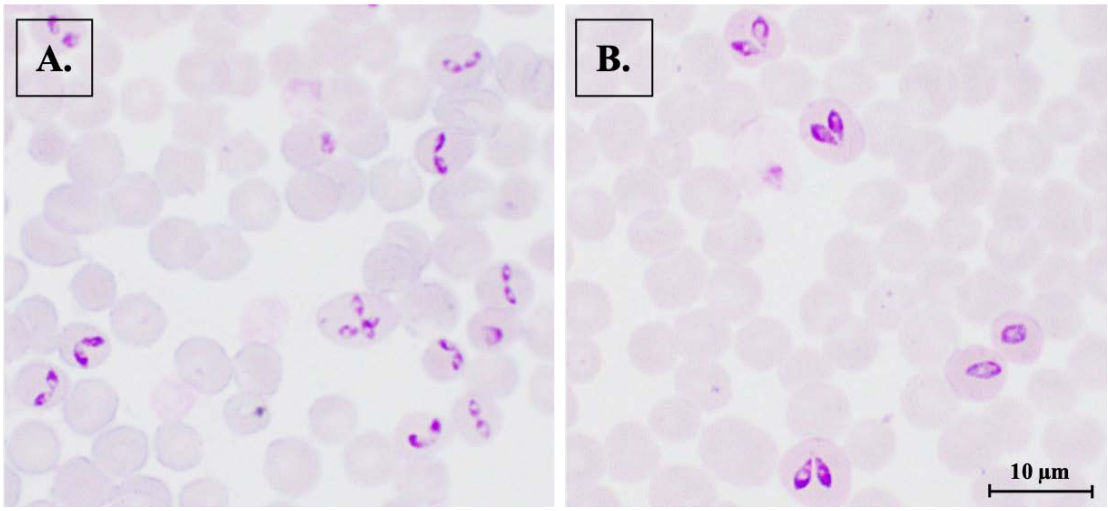

Fig. 2. Bovine babesia parasites: (A) *B. bovis*; (B) *B. bigemina*

Bovine babesiosis is transmitted by ticks, a vector belonging to the genus *Rhipicephalus*. However, bovine babesiosis is also transmitted through the use of needles contaminated with infected blood and through transplacental transmission from a pregnant cow to the fetus (Chhabra et al., 2012; Costa et al., 2016). The clinical symptoms associated with bovine babesiosis are hemolytic anemia, fever, and hematuria. Additionally, *B. bovis* has been reported to infect the brain (cerebral babesiosis); therefore, an infected animal may also display a nervous disorder, such as ataxia, and incoordination due to the accumulation of infected red blood cells in the cerebral capillaries (Beckley, 2013). In

differential diagnosis, bovine piroplasmosis could be confused with other diseases, such as anaplasmosis, trypanosomiasis, and theileriosis (OIE, 2018).

4. Life cycle and transmission of piroplasm parasites

The life cycle of piroplasm parasites involves vertebrate and invertebrate hosts (Fig. 3). There are three general successive phases of the piroplasm life cycle: merogony, gamogony, and sporogony (Schnittger et al., 2012; Jalovecka et al., 2018; Jalovecka et al., 2019). The life cycle begins when the sporozoites enter the blood stream of a vertebrate host via tick bite during the blood meal. The sporozoites invade, go directly to the erythrocytes, and divide into a trophozoite stage. The trophozoite divides asexually (merogony) into several merozoite forms. *Babesia* and *Theileria* parasites have the same life-cycle pattern in this stage; however, especially before invasion into the red blood cells, *Theileria* parasites enter the peripheral blood mononuclear cells (PBMCs) and replicate into large schizonts (schizogony). Then merozoites are released and permanently enter the red blood cells. Erythrocytes are destroyed during this stage, and merozoites are released into the blood stream and continue to invade the other new erythrocytes. After at least two cycles of the merogony stage, this form replicates and transforms into a pregametocyte form. Gamogony and sporogony will continue in the invertebrate host (tick). When the appropriate tick bite infected host and feed infected blood cells. The pregametocytes transform into gametocytes in the gut of the tick and fuse to become zygotes (sexual stage); finally, kinetes transform into sporozoites. Sporozoites move to the salivary glands via hemolymph. The transmission continues when the animal is infected with an appropriate vector.

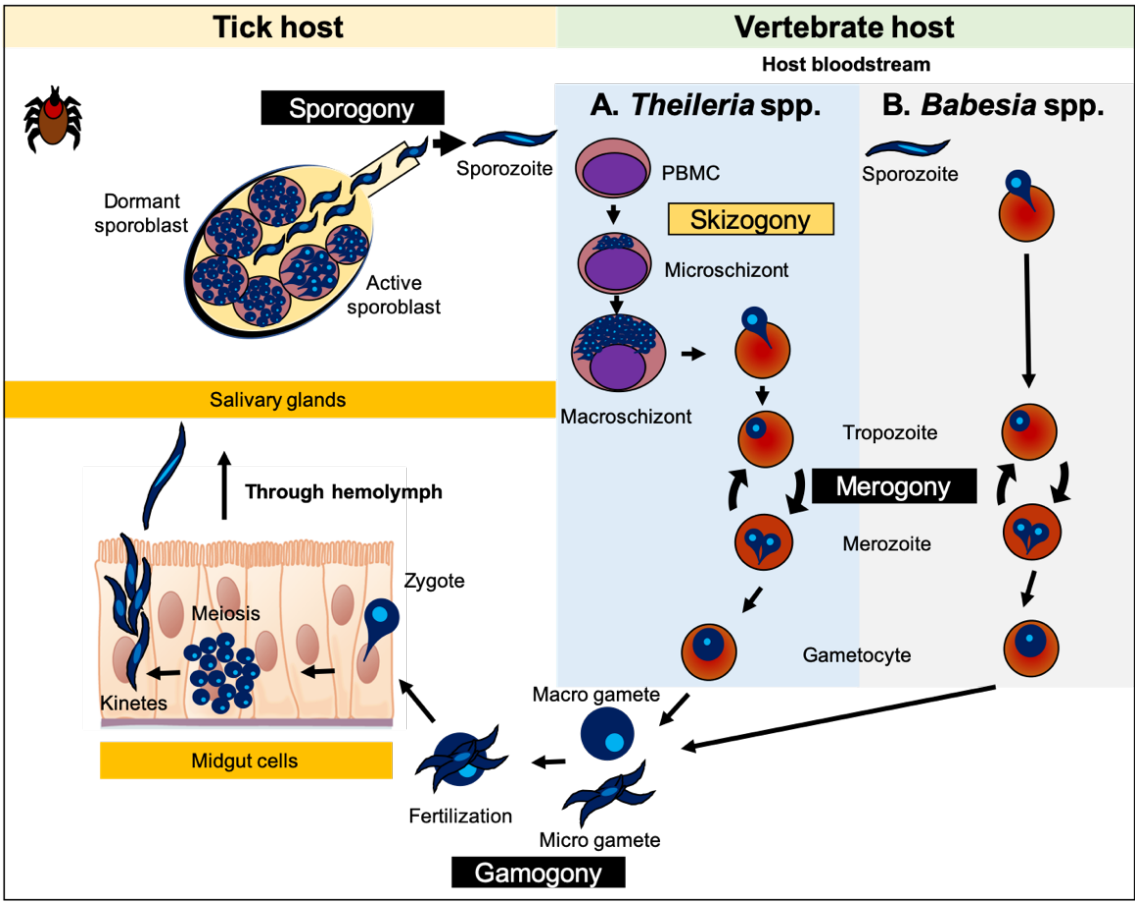

Fig. 3. Life cycle of piroplasms: (A) *Theileria* spp.; (B) *Babesia* spp.

(Figure adapted from Fig.1. in Jalovecka et al., 2019)

5. Pathogenesis and clinical signs of piroplasmosis

Piroplasms are produced in several stages of the disease, including subacute, acute, and chronic stage. The acute stage of piroplasmosis is produced due to several mechanisms, such as hemolysis and blood circulatory destruction. When sporozoites enter the blood circulation, they infect the erythrocytes and undergo rapid multiplication inside the erythrocytes. The rapid multiplication of parasites inside the erythrocytes results in destruction of the erythrocytes (Ambawat et al., 1999) and finally produces hemoglobinuria, hemoglobinemia, and fever. Therefore, in the acute stage, piroplasmosis is characterized by lethargy, anorexia, fever, anemia, hemoglobinuria, icterus, and in some cases, animal death. However, in the chronic stage of piroplasmosis, no specific clinical symptoms are observed (de Wall, 1992; Ahmed, 2002).

6. Diagnostic methods

A well-developed diagnostic test is needed to support a good prevention and control system for piroplasmosis. Several diagnostic tests are used for the diagnosis of piroplasmosis, such as stained thin blood smears, the serological test, and the molecular technique. Staining thin blood smears stained with Giemsa is a common test routinely used to detect the presence of parasites in the blood smear from the peripheral blood. This method is easy, applicable in the field, and inexpensive. Furthermore, it can be used to detect an animal in the acute stage of piroplasmosis due to the increased number of parasites. However, this method has some limitations, such as very low sensitivity in detecting a carrier or subclinically infected animal with low parasitemia. Furthermore, the discrimination between piroplasm species was difficult due to their similarity in size, shape, and morphology. In the acute stage, this technique is suitable for the detection of parasites; however, this technique also requires a well-trained person to administer it (Bose et al., 1995; Bruning, 1996).

When the number of parasites in the blood stream is very low, especially in the carrier animal, serological tests are needed to increase diagnostic sensitivity. Recently, several serological tests were developed for the detection of piroplasmosis, such as the indirect immunofluorescence antibody test (IFAT) (Tenter and Friedhoff, 1986), enzyme-linked immunosorbent assays (ELISAs) (Moloy et al., 1998; Knowles et al., 1992; Ikadai et al., 2007), and the immunochromatography test (ICT) (Huang et al., 2003; Guswanto et al., 2017). These methods are quite sensitive in detecting the chronic stage of piroplasmosis, although some limitations have been reported, such as lower sensitivity in the detection of an infected animal in the acute stage.

Polymerase chain reaction (PCR) is another technique for the detection of DNA in extracted parasitized blood. This technique is very useful when the microscopy method and serological test are not sufficient. PCR is highly sensitive and specific for the detection of piroplasm parasites (Nicolaiewsky et al., 2001). However, the limitations of this method are that it is time consuming and requires a well-established laboratory and expensive equipments. Therefore, to achieve more accurate diagnostic results and to conclude the status of the present infected animal, a combination of molecular and serological methods is necessary (Rosales et al., 2013).

7. Prevention and control of piroplasmosis

The control strategies against piroplasmosis include tick control and the treatment of infected animals. Although live attenuated vaccines are used to immunize the cattle against *B. bovis* and *B. bigemina* in some of the endemic countries, the extensive use of such vaccines is limited for various reasons, including strain-specific immunity and contamination of the vaccine with other blood pathogens. Tick control measures are severely constrained by the increasing development of acaricide resistance (George et al., 2004; Rosario-Cruz et al., 2009). Therefore, the control of piroplasmosis largely relies on treating infected animals with effective antiprotozoan agents.

Only a limited number of drugs are commercially available and used to treat piroplasmosis. Diminazene aceturate and imidocarb are the two commonly used antibabesial agents. However, toxic side effects are not uncommon in animals treated with these two drugs (Tuntasuvan et al., 2003). Additionally, the development of parasite resistance to these drugs has been reported. Similarly, the use of antitheilerial drugs is sometimes limited for various reasons, such as the presence of drug residues in the product from animals treated with buparvaquone, a commonly used antitheilerial drug (Belloli et al., 2007; Mosqueda et al., 2012).

8. Transgenic parasites as new tools to support prevention and control strategies

Piroplasm parasites are disease vectors that have important economic, medical, and veterinary impacts; however, these parasites have been poorly studied as compared with *Plasmodium*. Thus, there is an urgent need to control and treat piroplasmosis effectively. Genetic manipulation techniques offer a new approach to answer that need by developing transgenic parasites. Recently, several transgenic parasites have been successfully developed through the insertion of reporter genes, including the green fluorescence protein (GFP), the red fluorescence protein (RFP), luciferase and the yellow fluorescence protein (YFP) (Tedla et al., 2019). The transgenic parasites are new and useful tools for understanding the biology of the host–parasite interactions at the cellular level, understanding the development of the gene function, and developing fast and reliable drug screening. Several transgenic parasites have been established, including *B. bigemina*, *B. bovis*, *B. ovata*, *B. gibsoni*, and *Theileria parva* (Goeyse et al., 2015; Hakimi et al., 2016; Silva et al., 2016; Suarez and Mcelwain, 2009).

9. Aims of the present study

The importation and exportation of horses for trade and sport between countries is in increasingly high demand; however, the requirement for these animals to be free from equine piroplasmosis is a merely a priority. For several countries involved in this trade, including Indonesia, the lack of data regarding this disease is one of the basic problems. The combination of two different diagnostic techniques, including serological and molecular tests, is necessary. Other challenges in preventing and controlling this disease are the inadequate availability of chemotherapeutic agents in the market, the emergence of resistant parasites, and the emergence of toxic side effects on treated animals. The last challenge is the lack of information due to poor understanding of the fundamental biology of piroplasm parasites, especially *T. equi*. Therefore, the genetic manipulation of piroplasm parasites could improve and fill the lack of understanding basic biology of *T. equi*. The aims of this present study were to determine baseline data on equine piroplasmosis in Indonesia using serological and molecular approaches, to develop new chemotherapeutic agents for treatment against piroplasm parasites, and to develop the stable transfection of *T. equi* as a new tool to support the control of equine piroplasmosis.

Chapter 1

Serological and molecular prevalence of equine piroplasmosis in Western Java, Indonesia

1-1. Introduction

Equine piroplasmosis is an economically significant infectious disease in horses. The disease has been reported worldwide except for a few countries, including Japan, the United States, and Australia (OIE, 2014). Equine piroplasmosis is a major obstacle to the international movement of horses for trade and sport. Therefore, controlling this disease is a priority in the equine industry. The causative agents of equine piroplasmosis are *Theileria equi* and *Babesia caballi*, which are intraerythocytic hemoprotozoan parasites. Both *T. equi* and *B. caballi* are transmitted by ixodid ticks, mainly those belonging to the genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus*, which are distributed worldwide (APHIS, 2008). Once infected, horses remain carriers of *T. equi* and *B. caballi*, which can be transmitted via ticks to other susceptible host animals. Horses infected with *T. equi* remain seropositive for their entire lives, while those infected with *B. caballi* remain seropositive for up to four years (Zweygarth et al., 1996). Equine piroplasmosis caused by *B. caballi* and *T. equi* is characterized by fever, anemia, icterus, and hemoglobinuria (Zobba et al., 2008). Deaths are common if animals are not treated with the proper antiprotozoan agents in the early stage of the clinical disease. The clinical signs associated with *B. caballi* are usually milder than those induced by *T. equi* (de Waal, 1992).

The microscopic examination of a Giemsa-stained thin blood smear is a widely used diagnostic method for detecting *B. caballi* and *T. equi*. However, the use of this technique is limited by its law of specificity and sensitivity (Böse et al., 1995). Therefore, PCR assays with high sensitivity and specificity are preferred over microscopy to detect these parasite species in epidemiological surveys, which often target carrier animals (Alhassan et al., 2005; Nicolaiewsky et al., 2001). As PCR assays

detect only active infections, serological diagnostic tools, such as the enzyme-linked immunosorbent assay (ELISA) (Munkhjargal et al., 2013; Xuan et al., 2001) and the immunofluorescence antibody test (IFAT), are widely used to detect parasite-specific antibodies and, thereby, estimate seroprevalence rates, which indicate the risk of exposure to *B. caballi* and *T. equi*. Additionally, previous studies demonstrated that more informative data can be generated if a combination of molecular and serological diagnostic methods is used than if single techniques are employed (Baptista et al., 2013; Mahmoud et al., 2016; OIE, 2014; Rosales et al., 2013; Seo et al., 2011). Indonesia is a Southeast Asian country with a horse population of approximately 430,000. Various infectious diseases prevalent among horses, such as surra (Payne et al., 1991) and equine distemper, or strangles (Hidayat and Alhadi, 2012), represent significant threats to the economic success of the equine industry in Indonesia. However, *B. caballi* and *T. equi* infections have never been investigated in horses in that country. The aim of the present study was to investigate the prevalence of *B. caballi* and *T. equi* in horses in Western Java, Indonesia, using serological and molecular diagnostic tools.

1-2. Materials and Methods

Sample collection. This study was carried out in four regions of Western Java, Indonesia: Bandung, Depok, Tangerang, and Bogor (Fig. 4). Blood samples were collected randomly from 235 horses. All samples were from apparently healthy horses. The horses investigated in the present study were thoroughbreds, warmbloods, or crossbreeds. From each animal, a blood sample of approximately 3 ml was collected into a vacutainer tube with or without Ethylenediaminetetraacetic acid (EDTA) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). In addition, thin blood smears prepared from the sampled animals were stained with Giemsa and observed under a light microscope. Serum samples were prepared from blood samples collected in vacutainer tubes without EDTA. DNA samples were extracted from 200 µl of whole blood collected in EDTA-coated tubes using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions.

Enzyme-linked immunosorbent assays. ELISAs for detecting *B. caballi* and *T. equi* antibodies in equine serum samples from Indonesia were conducted using merozoite antigen 2 (EMA-2) and a

48 kDa merozoite rhoptry protein (BC48), respectively. The recombinant EMA-2 and BC48 were expressed and purified as described previously (Huang et al., 2003; Ikadai et al., 1999). ELISA was performed using these recombinant antigens as described by Xuan et al. (2001). Briefly, ELISA plates were coated with 100 µl of EMA-2 or BC48 antigens at a concentration of 2 µg/ml in a carbonate-bicarbonate buffer (50 mM, pH 9.6) and stored at 4°C overnight. The plates were washed once with 0.05% Tween 20-PBS (PBS-T) and then 100 µl of blocking solution (PBS supplemented with 3% skim milk). After washing once with PBS-T, 100 µl of each serum sample diluted to 1:100 with blocking solution was added and incubated for 1 h at 37°C. The plates were washed six times with PBS-T, and then 100 µl of the Anti-Horse IgG (whole molecule) peroxidase conjugated (Sigma-Aldrich, St. Louis, MO, USA) as a secondary antibody was diluted to 1:5000 with blocking solution and incubated for 1 h at 37°C. After washing six times, 100 µl of a substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.3 mg/ml of 2,2′-azide-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich), and 0.03% of 30% H_2O_2] was added to each well and incubated for 30 min at room temperature (RT). The optical density (OD) was measured at a wavelength of 415 nm. Positive and negative controls were previously prepared from horses experimentally infected with *T. equi* or *B. caballi*. Ten negative controls used in this study were from uninfected horses bred in Japan and confirmed to be negative for *T. equi* and *B. caballi* by ELISA and IFAT. The cutoff value was set as the sum of the mean OD value of the 10 negative sera and the threefold standard deviation. A serum sample was considered positive if the OD value was greater than the cutoff.

PCR assays. Nested PCR (nPCR) assays targeting *EMA-2* and *BC48* genes were employed to detect *T. equi* (*EMA-2* nPCR) and *B. caballi* (*BC48* nPCR), respectively. The primers for the *EMA-2* nPCR assay were designed in the present study, while previously described primer sets were used to conduct the *BC48* nPCR (Table 1, Battsetseg et al., 2001). Briefly, 20 µl of PCR reaction mixture containing 2 µl of the DNA template, 4 µl of 5× SuperFi Buffer (*Invitrogen*, Carlsbad, CA, *USA*), 2 µl of GeneAmp® dNTP Mix (Applied Biosystems, Streetsville, Ontario Canada), 0.2 µl of 0.02 U/µL Platinum SuperFi Polymerase (Invitrogen), 1 µl of 10 µM outer forward and reverse primers (Table 1), and 9.8 µl of distilled water was prepared. After an initial enzyme activation step at 98°C for 30

sec, the reaction mixture was subjected to 40 cycles, each of which included a denaturation step at 98°C for 10 sec, an annealing step at 62.8°C (*T. equi*) or 72°C (*B. caballi*) for 10 sec, and an extension step at 72°C for 30 sec. After a final extension at 72°C for 10 min, 1 µl of the first PCR product was transferred to a new PCR tube containing a reaction mixture similar to that of the first PCR except for the primers, which were replaced with inner forward and reverse primers (Table 1). The cycling condition for the second round of PCR was similar to that for the first round, except that the annealing temperature was set at 72°C (*T. equi*) or 70.3°C (*B. caballi*). The PCR products were then subjected to agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Bands detected at approximately 221 bp and 454 bp were considered positive for *T. equi* and *B. caballi*, respectively.

DNA cloning and sequencing. All of the PCR amplicons from the *EMA-2* and *BC48* nPCR assays were subjected to cloning and sequencing. Briefly, after gel electrophoresis, PCR amplicons were purified using NucleoSpin® Gel and a PCR Clean-up Kit (MACHEREY-NAGEL, Duren, Germany), ligated to a pCR 2.1 plasmid vector (TOPO, Invitrogen, Carlsbad, CA, USA), transformed into TOP 10 *E. coli* cells (Invitrogen), and plated onto Luria-Bertani (LB) agar plates (Invitrogen). Three clones were selected for each PCR amplicon and cultured in LB broth. The plasmids were extracted using NucleoSpin® Plasmid QuickPure (MACHEREY-NAGEL, Duren, Germany) and subjected to sequencing using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequence and phylogenetic analyses. The *EMA-2* and *BC48* sequences were initially analyzed using the basic local alignment search tool (BLAST) (https://blast.ncbi.nlm.nih.gov/blast.cgi). The EMBOSS needle program (http://emboss.bioinformatics.nl/cgi-bin/emboss/needle) was used to calculate the identity percentage and similarity scores among nucleotide and amino acid sequences, respectively.

The newly generated *EMA*-2 and *BC48* gene sequences, together with those retrieved from GenBank, were subjected to multiple alignment using MEGA software version 6.06. Subsequently,

maximum-likelihood phylogenetic trees were constructed based on Kimura's two-parameter substitution model using the same software program.

Statistical analysis. SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used to analyze the positive rates based on age, sex, breed, purpose for rearing, and access to pasture using a two-sided chi-squared test. The *p* values and 95% confidence intervals (CIs) were also computed. A *p* value <0.05 was considered to indicate statistical significance.

1-3. Results

Serological and molecular detection of *T. equi* and *B. caballi*. All surveyed animals were negative for *T. equi* and *B. caballi* according to microscopy. Out of 235 horses investigated in the present study, 20 (8.5%) were seropositive for *T. equi* and/or *B. caballi*. Five horses (2.1%) were seropositive for *T. equi*, and 15 (6.4%) were positive for *B. caballi* (Table 2). Of five *T. equi*–positive horses, three, one, and one were from Tangerang (14.3%), Depok (2.3%), and Bandung (0.6%), respectively. No horses were positive for *T. equi* in the Bogor district. Fourteen (8.8%) of 15 *B. caballi*–positive horses were from Bandung, while the other one was from Tangerang (4.7%). None of the surveyed horses in Depok or Bogor were positive for *B. caballi* (Table 2).

The nPCR assays detected five horses (2.1%) to be positive for *T. equi* and/or *B. caballi*. Only a single horse in Tangerang tested positive for *T. equi* (0.4%), while four horses tested positive for *B. caballi* (1.7%) in Bandung.

When ELISA and PCR results were combined, 25 horses were positive for *T. equi* and/or *B. caballi*. The single horse positive for *T. equi* according to nPCR was also positive for this parasite species according to ELISA, whereas four horses were positive for *B. caballi* according to nPCR, and two were positive according to ELISA.

Statistical analyses indicated a significant difference between seropositive rates in animals that had access to pasture and those that did not ($p<0.05$) (Table 3 and 4). Significant differences in the seropositive rates were not found for the other variable analyzed in this study.

Sequencing and phylogenetic analyses of *T. equi EMA-2* and *B. caballi BC48* genes. All of the nPCR amplicons (one *T. equi* and four *B. caballi*) were cloned, and three clones per amplicon were sequenced. However, the nucleotide sequences were identical between clones. Therefore, one *EMA-2* (accession no.: MG948458) and four *BC48* (accession nos.: MG948454, MG948455, MG948456, and MG948457) sequences were registered with GenBank. Sequence analyses showed that the Indonesian *EMA-2* gene sequence was identical to the sequences of Florida (U97169) and Washington (XM 004831448) *T. equi* strains. On the other hand, the Indonesian *BC48* sequences shared 99.8–100% and 99.3–100% identity and similarity scores among nucleotide and amino acid sequences, respectively. The Indonesian *BC48* sequences also had 96.7%, 98.2%, and 98% identity scores with those reported in Egypt (KR811096), the Philippines (KX900447), and Mongolia (AB703051), respectively.

EMA-2 gene-based phylogenesis divided the sequences into three clades, designated in the present study as clades 1, 2, and 3 (Fig. 5A). The Indonesian *EMA-2* gene sequence isolated in the present study was found in clade 1, together with sequences from the Florida and Washington strains. The *BC48* sequences occurred in four clades (designated as clades 1, 2, 3, and 4) in the phylogenesis (Fig. 5B). All four Indonesian *BC48* sequences clustered together and formed clade 2.

1-4. Discussion

Despite the clinical and economic significance of equine piroplasmosis, neither the disease nor its causative agents had ever been investigated in Indonesia. Thus, I conducted an epidemiological survey to detect *T. equi* and *B. caballi* in horses in Indonesia. The overall prevalence of *T. equi* and *B. caballi* was low, as only 8.5% and 2.1% of surveyed animals were positive for at least one parasite species according to ELISA and/or nPCR, respectively. The seropositive rates determined in Indonesia were comparable to those reported in several other endemic countries, such as Korea (Seo et al., 2011), Thailand (Kamyingkird et al., 2014), Turkey (Kizilarslan et al., 2015), Italy (Grandi et al., 2011), and Switzerland (Sigg et al., 2010). In contrast, the seroprevalence of these parasites was very high in some countries, including Brazil (Xuan et al., 2001), Costa Rica (Posada-Guzmán et al., 2015), and South Africa (Motloang et al., 2008). The difference in the prevalence of *T. equi* and *B. caballi* among

endemic countries might be due to variations in farm management practices, the diagnostic test used, and the seasonal dynamics of ticks and tick-control measures (Ferreira et al., 2016). The identification of tick species transmitting *T. equi* and *B. caballi* is key to designing successful tick-control strategies. The known potential transmission vectors of *B. caballi* and *T. equi* include *Dermacentor nitens* (Schwint et al., 2008), *Hyalomma volgense* (Enigk, 1944), *Hyalomma truncatum* (de Waal et al., 1990), and *Dermacentor reticulatus* (Neitz, 1956). In Indonesia, species of the genera *Amblyomma*, *Dermacentor*, *Rhipicephalus*, *Haemaphysalis*, and *Ixodes* are known to exist in parts of Indonesia (Durden et al., 2008). However, the tick species infecting horses in this country are not known.

The *B. caballi*–positive rate based on ELISA or nPCR was higher in Bandung than in the other districts surveyed. In particular, none of the surveyed animals were positive for *B. caballi* in Depok or Bogor. The horses in Bandung are managed by an extensive or semi-intensive system, while those in Depok, Tangerang, and Bogor are usually managed intensively. Therefore, tick infestation might be more common among horses in Bandung than in those from other districts. This could explain why the *B. caballi*–positive rate was high in horses in Bandung. Although the *T. equi*–positive rate is higher in Tangerang than in the other districts surveyed, the low number of positive animals makes a fair comparison impossible. Among the surveyed horses, thirteen samples were ELISA-positive for *B. caballi*, but the PCR assays were negative. The differences in the results might indicate the following possibility. Principally, PCR assays detect the presence of parasite DNA, while ELISA detects antibodies that can be present even in the absence of parasites in the blood. *B. caballi* causes a self-limiting infection for several years, and horses are able to eliminate the infection naturally or by drug treatment. However, horses infected with *T. equi* remain carriers for their entire lives (Friedhoff and Soule, 1996; Schwint et al., 2009). In such carrier animals, even parasites are not found in the peripheral blood but are sequestrated in the capillaries or small vessels in different organs, especially in infected animals (Kuttler, 1988; Ribeiro et al., 2013). Thus, these parasites in the organs can stimulate antibody production. Therefore, it is very possible to find tested animals that are ELISA positive but PCR negative (Jaffer et al., 2010; Mahmoud et al., 2016; Viera, et al., 2018). This phenomenon was also found in relation to the detection of bovine babesiosis (Salas et al., 2016; Carrique et al., 2000; Li et al., 2014). On the other hand, PCR-positive ELISA-negative horses were

associated only with *B. caballi* infection. The development of *B. caballi* parasitemia in horses is usually very low in peripheral blood circulation during the early stage of an acute infection, and the antibody response would be delayed. Therefore, it would be possible for PCR to detect DNA before antibodies could be detected by ELISA (Rothschild and Knowles, 2007). This could explain why two PCR-positive horses were ELISA negative for *B. caballi*.

The PCR amplicons were sequenced and subjected to bioinformatic and phylogenetic analyses to confirm the PCR findings and to investigate genetic diversity. The newly identified *EMA-2* and *BC48* sequences shared high identity scores with known *EMA-2* and *BC48* sequences in the database, confirming the PCR results. In phylogeny, the Indonesian *EMA-2* gene sequence clustered together with the Florida and Washington strains of *T. equi*. In contrast, the *BC48* sequences of Indonesian origin formed a unique clade, suggesting that the *B. caballi* population in Indonesia might represent a novel genotype. Phylogenetic analysis of large numbers of *BC-48* sequences from several endemic countries might reveal whether this novel genotype is specific to Indonesia.

In conclusion, the present study analyzed Indonesian horses and reported *T. equi* and *B. caballi* in that country for the first time. Veterinarians in Indonesia should be made aware of possible clinical piroplasmosis in horses. Further studies to investigate the prevalence of *T. equi* and *B. caballi* in various geographical territories in Indonesia using a large number of samples and to detect the tick vectors transmitting these parasite species are now a priority in Indonesia.

1-5. Summary

Equine piroplasmosis is an economically significant disease caused by *Theileria equi* and *Babesia caballi*, which are tick-borne hemoprotozoan parasites. Infections with these parasite species had never been reported in horses in Indonesia. The aim of the present study was to investigate the prevalence of *T. equi* and *B. caballi* in horses reared in parts of Western Java, Indonesia. Blood samples were collected randomly from 235 horses in four different districts (Bandung, Depok, Tangerang, and Bogor) in Western Java, Indonesia. Thin blood smears prepared from the sampled animals were stained by Giemsa and observed under a light microscope. Serum samples prepared from blood were screened by enzyme-linked immunosorbent assays (ELISAs) based on recombinant forms

of EMA-2 and BC48 antigens to determine the seroprevalence of *T. equi* and *B. caballi*, respectively. DNA samples extracted from the same blood samples were screened by *EMA-2* and *BC48* gene-based nested polymerase chain reaction (nPCR) assays for *T. equi* and *B. caballi* infections, respectively. Of 235 surveyed animals, five (2.1%) and 15 (6.4%) were seropositive for *T. equi* and *B. caballi*, respectively, whereas one and four horses were nPCR-positive for *T. equi* and *B. caballi*, respectively. All of the surveyed animals were negative for *T. equi* and *B. caballi* by microscopy. The *T. equi EMA-2* and *B. caballi BC48* gene fragments amplified by the nPCR assays were cloned, sequenced, and subjected to bioinformatic and phylogenetic analyses. The *T. equi EMA-2* gene sequence from an Indonesian horse was identical to sequences from Florida and Washington strains and clustered together with these sequences in phylogeny. On the other hand, four Indonesian *BC48* gene sequences shared 99.8–100% identity scores. This present study is the first to report *T. equi* and *B. caballi* in horses in Indonesia. My findings highlight the need for monitoring horses in Indonesia for clinical piroplasmosis caused by *T. equi* and *B. caballi*.

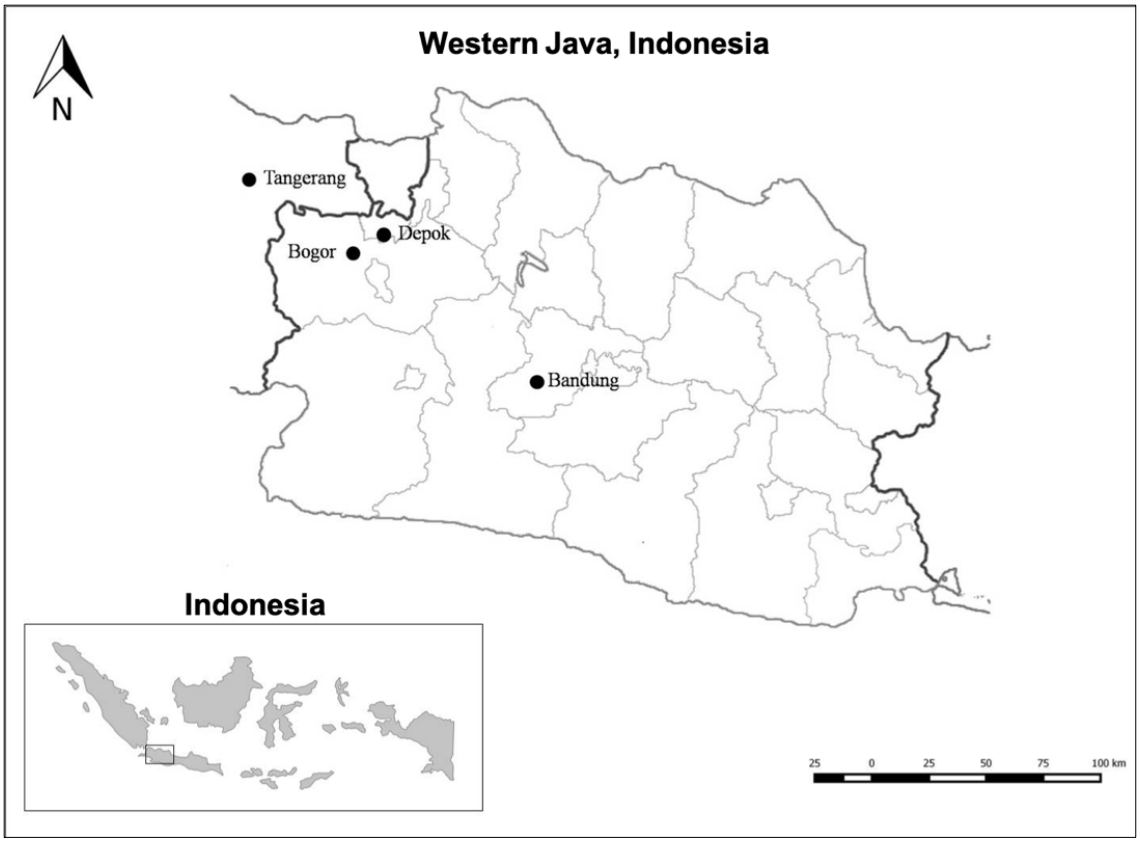

Fig. 4. Sampling locations. Blood samples were collected from horses in the Bandung (n=158), Depok (n=21), Tangerang (n=44), and Bogor (n=12) districts of Western Java, Indonesia.

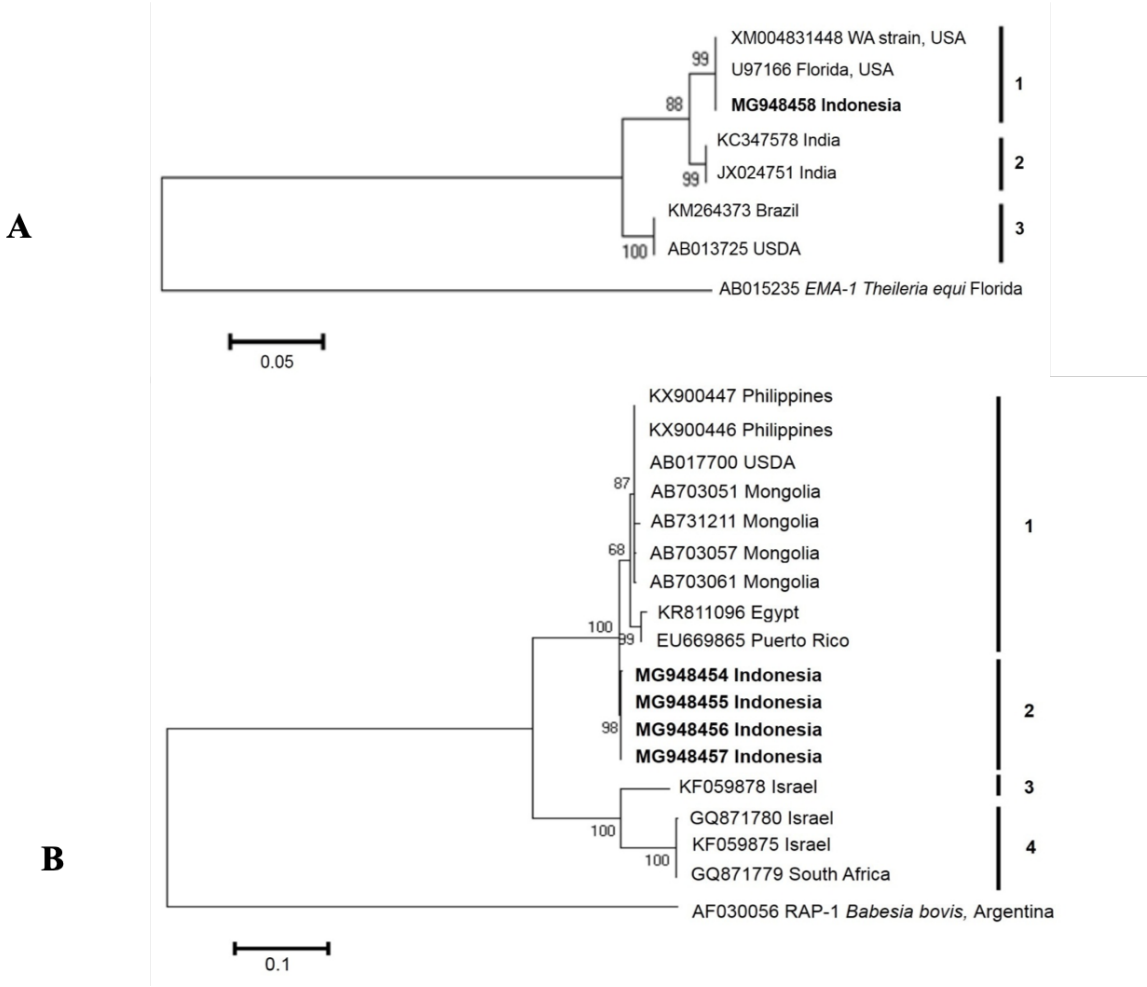

Fig. 5. Phylogenetic analyses. The *T. equi EMA-2* (Panel A) and *B. caballi BC48* (Panel B) gene sequences generated in the present study, together with those reported in other countries, were used to construct maximum-likelihood phylogenetic trees. The Indonesian gene sequences are indicated in boldface type. Note that the Indonesian *EMA-2* gene sequence isolated in the present study was found in clade 1 together with sequences from the Florida and Washington strains and that all four Indonesian *BC48* sequences clustered together and formed a new clade (clade 2).

Table 1. Primers used in this study

Primer	Target gene	Sequences (5′-3′)	Amplicon size (bp)	Annealing temperature (ºC)	Reference
EMA-2	EMA2-F	CGTTGTCACTCTCGGAGC	587	62.8	Present study
	EMA2-R	TGACCCAGGAATCACCAG			
	EMA2-NF	CGTTGTCACTCTCGGAGCCAC	221	72	
	EMA2-NR	GAGCAGGGACAACGCAGACAG			
BC48	BC48-F	ACGAATTCCCACAACAGCCGTGTT	530	72	(Battsetseg et al., 2001)
	BC48-R	ACGAATTCGTAAAGCGTGGCCATG			
	BC48-NF	GGGCGACGTGACTAAGACCTTATT	430	70.3	
	BC48-NR	GTTCTCAATGTCAGTAGCATCCGC			

Table 2. Prevalence of equine piroplasmosis as determined by ELISAs and nPCR assays

District	No. sample	No. ELISA positive (%)		No. nPCR positive (%)	
		T. equi	*B. caballi*	*T. equi*	*B. caballi*
Bandung	158	1 (0.6)	14 (8.8)	0 (0)	3 (1.8)
Depok	44	1 (2.3)	0 (0)	0 (0)	0 (0)
Tangerang	21	3 (14.3)	1 (4.7)	1 (0.6)	1 (4.7)
Bogor	12	0 (0)	0 (0)	0 (0)	0 (0)
Total	235	5 (2.1)	15 (6.4)	1 (0.4)	4 (1.7)

Table 3. Results of univariate analyses for *T. equi* and *B. caballi* seroprevalence

Variables	Category	N	*B. caballi*			*T. equi*		
			No. positive (%)	CI	*p* value	No. positive (%)	CI	*p* value
Sex								
	Male	138	8 (5.8)	2.9-11.02	0.869	2 (1.4)	0.4-5.13	0.988
	Female	97	7 (7.3)	3.5-14.15		3 (3.0)	1.06-8.7	
	Total	235	15 (6.4)	3.9-10.26		5 (2.1)	0.9-4.8	
Age (years)								
	< 5 years	27	3 (11.1)	3.8-28.06	0.064	0	0	
	6-10 years	17	3 (17.6)	6.1-41.03		0	0	
	>11 years	191	9 (4.7)	2.5-8.71		5 (2.6)	1.11-5.98	0.555
	Total	235	15 (6.4)	3.9-10.26		5 (2.1)	0.9-4.8	
Breed								
	Crossbred	195	14 (7.2)	4.3-11.69	0.422	2 (1.0)	0.2-3.66	0.067
	Thouroughbred	22	0	0		1 (4.5)	0.81-21.8	
	Warmblood	18	1 (5.9)	0.9-25.76		2 (11.8)	3.1-32.8	
	Total	235	15 (6.4)	3.9-10.26		5 (2.1)	0.9-4.8	
Purposes								
	Working	190	14 (7.4)	4.4-11.99	0.204	2 (1.1)	0.2-3.76	0.050
	Sport	45	1 (2.2)	0.3-11.57		3 (6.7)	2.2-17.8	
	Total	235	15 (6.4)	3.9-10.26		5 (2.1)	0.9-4.8	
Access to pasture								
	No.	77	1 (1.2)	0.1-3.5	< 0.05	4 (5.2)	0.9-6.33	0.073
	Yes	158	14 (8.8)	11.1-28.2		1 (0.6)	0.2-7.0	
	Total	235	15 (6.4)	3.9-10.26		5 (2.1)	0.9-4.8	

N: Number of sample tested, CI: 95% Confidence Interval.

p value < 0.05 was considered statistically significant

Table 4. Results of univariate analyses for *T. equi* and *B. caballi* PCR-positive prevalence

Variables	Category	N	*B. caballi*			*T. equi*		
			No. positive (%)	CI	*p* value	No. positive (%)	CI	*p value*
Sex								
	Male	138	1 (0.7)	0.13-3.99	0.798	1 (0.7)	0.13-3.99	0.703
	Female	97	3 (3.09)	1.06-8.7		0	0	
	Total	235	4 (1.7)	0.6-4.29		1 (0.4)	0.04-2.3	
Age (years)								
	< 5 years	27	1 (3.7)	0.6-18.28	0.619	0	0	0.891
	6-10 years	17	0	0		0	0	
	>11 years	191	3 (1.6)	0.5-4.52		1 (0.5)	0.04-2.91	
	Total	235	4 (1.7)	0.6-4.29		1 (0.4)	0.04-2.3	
Breed								
	Crossbred	195	4 (2.1)	0.8-5.15	0.841	0	0	0.075
	Thouroughbred	22	0	0		0	0	
	Warmblood	17	0	0		1 (5.9)	1.0-26.9	
	Total	235	4 (1.7)	0.6-4.29		1 (0.4)	0.04-2.3	
purposes								
	Working	190	3 (1.6)	0.54-4.54	0.764	0	0	0.068
	Sport	45	1 (2.2)	0.39-11.5		1 (2.2)	0.39-11.6	
	Total	235	4 (1.7)	0.6-4.29		1 (0.4)	0.04-2.3	
Access to pasture								
	No	77	1 (1.2)	0.1-3.5	0.739	1 (1.2)	0.1-3.5	0.151
	Yes	158	3 (1.8)	1.3-10.8		0	0	
	Total	235	4 (1.7)	0.6-4.29		1 (0.4)	0.04-2.3	

N: Number of sample tested, CI: 95% Confidence Interval; p value < 0.05 was considered statistically significant

Chapter 2

Screening the Medicines for Malaria Venture Pathogen Box against piroplasm parasites

2-1. Introduction

Piroplasmosis is a tick-transmitted disease caused by *Babesia* and *Theileria* parasites. Piroplasmosis affects humans, livestock, and wild animals worldwide. Generally, piroplasm infection is characterized by fever, icterus, hemolysis, hemoglobinuria, and death if treatment fails or is not attempted (Schnittger et al., 2012; Wise et al., 2013). Significant economic impacts of bovine babesiosis and equine piroplasmosis on the cattle and horse industries have been reported, especially in piroplasmosis-endemic countries. Bovine babesiosis caused by *Babesia bovis* and *Babesia bigemina* decreases meat and milk production and leads to the death of infected cattle (Yusuf, 2017). Equine piroplasmosis caused by *Babesia caballi* and *Theileria equi* is associated with detrimental effects in horses. Once the horse is infected by either or both *Babesia caballi* and *Theileria equi*, that animal could remain a carrier for its entire life. Consequently, such horses are restricted with regard to international movement because they can transmit disease that affects trade and equestrian sport (Knowles, 1996).

There are three widely used strategies for piroplasmosis control: vaccination, the use of antipiroplasm drugs, and vector control measures. In the past several decades, antipiroplasm drugs, including diminazene aceturate (DA) and imidocarb dipropionate (ID), have played an important role in the prevention and control of piroplasmosis (Mosqueda et al., 2012). However, recent studies on *Babesia* have reported the development of resistance to DA and documented toxic side effects in ID-treated equines (Hwang et al., 2010; Tuntasuvan et al., 2003). Furthermore, high levels of ID and DA drug residue in edible tissue have been reported in treated animals (Belloli et al., 2007; Mdachi et al.,

1995). Therefore, continuous efforts to discover and develop novel antipiroplasm drugs are urgently needed.

One alternative approach to fast-track the development of novel antiparasitic agents is large-scale screening of compounds from existing databases, such as the Medicine for Malaria Venture (MMV) Pathogen Box. The MMV foundation offers free access to compounds in the MMV Pathogen Box to researchers all over the world. The activity of compounds in the MMV Pathogen Box has been confirmed against several diseases, including tuberculosis, malaria, leishmaniasis, trypanosomiasis, helminthiasis, toxoplasmosis, and dengue. Additionally, the MMV Pathogen Box contains 26 reference compounds. Recently, several researchers have discovered effective compounds in the MMV Pathogen Box and repurposed them for treatment against other parasitic protozoa, including *Toxoplasma gondii*, *Cryptosporidium parvum*, *Giardia lamblia*, *Neospora caninum*, *Plasmodium falciparum*, and *Trypanosoma* spp. (Duffy et al., 2017; Hennessey et al., 2018; Müller et al., 2017; Spalenka et al., 2018). Moreover, all of the compounds have been tested for their cytotoxicity on mammalian cells with greater than fivefold selectivity indexes (http://www.pathogenbox.org/about-pathogen-box/supporting-information). The present study aimed to discover potent inhibitors against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* by screening 400 compounds from the MMV Pathogen Box.

2-2. Materials and Methods

Pathogen Box compounds. The Pathogen Box consists of 400 compounds provided by the MMV foundation following a request from my laboratory. The compounds were delivered in five plates, each containing 80 compounds. Each compound had a 10 µl volume diluted in 100% dimethyl sulfoxide (DMSO) to a concentration of 10 mM. In order to prepare a 1 mM stock solution, 90 µl of DMSO was added to each well and divided into two identical plates in accordance with MMV instructions. Additionally, DA (Sigma-Aldrich, Tokyo, Japan) was diluted in Milli-Q water (MQW) to make a 10 mM stock solution. All compounds were stored at -30°C until needed for the experiments. For *in vivo* studies, MMV021057 was purchased from Sigma-Aldrich, while MMV675968 was

supplied from MMV in powder form. All compounds were diluted in normal saline with 4% DMSO and 8% Tween 80 for *in vivo* studies.

Reagents. A lysis buffer containing Tris (130 mM at pH 7.5), EDTA (10 mM), saponin (0.016%; w/v), and Triton-X 100 (1.6%; v/v) was prepared and stored at 4°C. The 10,000 x SYBR Green 1 nucleic acid stain (Lonza Rockland Inc., Rockland, USA) was stored at -30°C. All reagents were purchased from Sigma-Aldrich (Tokyo, Japan).

Parasites. Four strains of parasites were used for *in vitro* studies, including *B. bovis* (Texas strain), *B. bigemina* (Argentine strain), and a USDA strain of *B. caballi* and *T. equi*. The Munich strain of *B. microti* was used for *in vivo* studies.

***In vitro* cultures.** *Babesia bovis* and *B. bigemina* were cultured in purified bovine red blood cells (RBCs) using Medium 199 (M199) supplemented with 40% bovine serum. *Babesia caballi* and *T. equi* were cultured in purified horse RBCs. The medium for *B. caballi* was GIT supplemented with 40% horse serum, while M199 supplemented with 40% horse serum and hypoxanthine (MP Biomedicals, USA) at a final concentration of 13.6 μg/ml was used for *T. equi* cultivation. An antibiotic–antimycotic solution containing 60 U/ml penicillin G, 60 μg/ml streptomycin, and 0.15 μg/ml amphotericin B (Sigma-Aldrich, Tokyo, Japan) was added to all of the media.

***In vitro* inhibition assay.** Initially, all 400 compounds were tested against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* at a single concentration of 1 μM. Each compound was tested in duplicate and repeated in three separate experiments. Compounds that showed over 60% inhibition against all of the parasites tested were selected for further analysis to determine their half maximal inhibitory concentration (IC_{50}).

To determine the IC_{50}, each compound was diluted by a twofold serial dilution ranging from 0.001 to 1 μM. DA was also included for each experiment as a comparator drug. The medium

containing 0.1% DMSO with infected RBCs (iRBCs) and uninfected RBCs were used as positive and negative controls, respectively. This experiment was repeated three times in separate experiments.

In vitro inhibition assays were carried out as previously described (Guswanto et al., 2014; Rizk et al., 2015). Briefly, the parasites were harvested after 4 days of the initial culture, when the parasitemia reached more than 3%. The iRBCs were harvested and diluted with fresh RBCs to make a 1% parasitemia. The experiment was conducted using 2.5 µl (for *B. bovis* and *B. bigemina*) or 5 µl (for *B. caballi* and *T. equi*) of iRBCs. The iRBC was added to each well in triplicate. The volume of culture medium containing the drug was added, up to a total reaction volume of 100 µl. The plates were incubated at 37ºC with 5% CO_2, 5% O_2, and 90% N_2. After 96 h, 100 µl of lysis buffer containing SYBR Green 1 was added to each well and mixed gently by pipetting. The plates were wrapped in aluminum foil to protect them from light and kept at room temperature for 6 h. The relative fluorescence values were measured using a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo Fisher Scientific, USA) excitation and emission wavelength of 485 nm and 518 nm, respectively. The relative fluorescence value was set to percentages after subtracting the mean values of the negative control.

Combination treatment with MMV675968, MMV021057, and DA. A drug combination assay was performed using a 96-well culture plate with a single drug assay at the constant ratio in accordance with Chou (2006). The two-drug combination therapy (MMV675968 + MMV021057, MMV675968 + DA, and MMV021057 + DA) was performed at concentrations of 0.25 x IC_{50}, 0.5 x IC_{50}, IC_{50}, 2 x IC_{50}, and 4 x IC_{50}. The degree of synergism was determined as the weighted average of CI values using the formula ((1 x IC_{50}) + (2 x IC_{75}) + (3 x IC_{90}) + (4 x IC_{95}))/10). The values with <0.9, 0.91 to 1.10, and >1.10 indicated synergistic, additive, and antagonistic effects, respectively. Each experiment was repeated in three separate trials.

***In vivo* inhibition assay.** The growth-inhibitory effect of the hit compounds was determined using a mouse model infected with *B. microti*, as previously described (Tuvshintulga et al., 2016). Thirty-five BALB/c mice (CLEA Japan Inc., Tokyo, Japan) were divided into seven groups, each

consisting of five mice. Groups 1 and 2 were kept as a negative control (uninfected and untreated) and positive control (infected and untreated), respectively. Groups 3 and 4 were treated by intraperitoneal (i.p.) injection of 25 mg/kg body weight (BW) MMV675968 and subcutaneous (sc) injection of 50 mg/kg BW MMV021057, respectively. Groups 5 and 6 were treated by i.p. injection of 6.25 mg/kg and 25 mg/kg BW DA, respectively. Group 7 was injected with a combination of 25 mg/kg BW MMV021057 + 6.25 mg/kg BW DA by sc and i.p. injection, respectively.

Prior to the start of the *in vivo* experiments, a frozen stock of *B. microti* was recovered from -80°C and injected intraperitoneally into a mouse. Subsequently, the parasitemia was monitored every 2 days in a Giemsa-stained blood smear. When 30% parasitemia was observed in mice, the mouse was anesthetized, and blood was collected through cardiac puncture. The blood was then diluted by 1 × PBS to acquire 2 × 10^7/ml *B. microti* iRBCs. Subsequently, all mice in the six groups except the negative control group were injected intraperitoneally with 0.5 ml of inoculum to achieve 1 x 10^7/ml iRBCs.

Parasitemia was monitored by counting iRBCs every 2 days among 10,000 RBCs in Giemsa-stained blood smears. When approximately 1% parasitemia was achieved on day 4 post-infection (p.i.), the treatment was initiated and continued for 5 consecutive days (day 4 to day 8). To analyze the effects of the treatment, 10 µl of blood from the tail was collected every 4 days and used to determine the hematological profiles using an automatic hemocytometer (Celltac α MEK-6450, Nihon Kohden, Tokyo, Japan). All parameters were monitored until day 30, and the experiment was repeated twice. The animal experiment was conducted in accordance with the Regulations for Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine, Japan (accession number 28-015-9).

Statistical analysis. The percentage of inhibition on the *in vitro* studies was calculated in Microsoft Excel. Data were calculated for the IC_{50} using a nonlinear regression sigmoidal dose-response curve fit, available in GraphPad Prism (GraphPad Software Inc., USA). For combination therapy, the combination indexes (CIs) were calculated using CompuSyn software. Statistical analysis of the parasitemia and hematological profiles was done using Student's *t*-test. The difference in

parasitemia between untreated and drug-treated groups was considered statistically significant if $P < 0.05$.

2-3. Results

***In vitro* inhibition assay.** The initial screening was performed to identify compounds showing at least 60% growth inhibition at the initial concentration of 1 μM against the four parasites tested, namely, *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*. Out of the 400 compounds, six were active against *B. bovis* and *B. bigemina* (Fig. 9) . Four compounds were active against *B. caballi* and *T. equi*. Nine compounds were active against all tested parasites (Table 5). The compound structures of active compound against four tested parasites shown in the Fig.6.

The IC_{50}s of all nine compounds were determined as shown in Table 6 and Fig. 10-19. Two compounds, MMV021057 and MMV675968, were selected as hit compounds because they showed an IC_{50} <0.3 μM and a selectivity index (SI) >100. The IC_{50}s of MMV021057 on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi* were 23 ± 8, 39 ± 11, 229 ± 62, and 146 ± 36 nM, respectively. The IC_{50}s of MMV675968 on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi* were 2.9 ± 0.3, 3 ± 0.8, 25.7 ± 6.4, and 2.9 ± 0.1 nM, respectively.

Combination treatment. The combination of two drugs including MMV021057, MMV675968, and DA was performed as described by Chou (2006). A combination of MMV021057 and DA showed synergistic effects against *B. caballi* and *B. bovis*, while an additive effect was observed against *B. bigemina* and *T. equi*. On the other hand, drug combinations of MMV021057 and MMV675968 and of MMV675968 and DA showed antagonistic effects in all tested parasites (Table 7 and Fig.20).

***In vivo* inhibition assay.** *In vivo* experiments were conducted to evaluate the chemotherapeutic efficacy of single treatments with 50 mg/kg BW MMV021057, 25 mg/kg BW MMV675968, 25 mg/kg DA, and 6.25 mg/kg BW DA and combination treatments with MMV021057+DA (25 + 6.25 mg/kg BW) against *B. microti*. The groups treated with 50 mg/kg BW MMV021057 and 25 mg/kg BW

MMV675968 showed a higher inhibition level and more rapid reduction of parasitemia than the untreated group (Fig. 7A). The peak parasitemia in MMV021057- and MMV675968-treated mice was reached on day 10 p.i., while that in the untreated and DA-treated group was reached on day 8 p.i. The percentage of growth inhibition of 50 mg/kg BW MMV021057, 25 mg/kg BW MMV675968 and 25 mg/kg BW DA, compared with that of the untreated group, was 54, 64, and 83%, respectively. Furthermore, the inhibitory effect was improved to 91.6% when a combination of 25 mg/kg BW MMV021057 and 6.25 mg/kg BW DA was used (Fig. 7B). The hematocrit indexes were stable in the treated groups as compared to the positive control group (infected and untreated) (Fig. 8).

2-4. Discussion

Although diminazene aceturate and imidocarb dipropionate are still readily available for use against piroplasmosis, it has been reported that they have toxic side effects and leave residue in animal products and that parasites are developing resistance (Belloli et al., 2007; Tuntasuvan et al., 2003). Thus there is a need to find new drugs with potent antipiroplasm activity and low toxic side effects to the host.

In the present study, 400 compounds from the MMV Pathogen Box were screened against *Babesia* and *Theileria* parasites. Nine hit compounds were identified as effective against *Babesia* and *Theileria* parasites with IC_{50}s of less than 1 μM. The most interesting hit compounds were MMV021057 and MMV675968, with IC_{50}s of less than 0.3 μM and an SI of more than 100. The two hit compounds identified from the Pathogen Box were fewer than the four hits identified from the Malaria Box in a previous study under the same conditions (Van Voorhis et al., 2016). Interestingly, one of the reference compounds, known as buparvaquone, showed a lower IC_{50} on *T. equi* than on *Babesia* parasites. This finding was comparable to that of a previous report showing that buparvaquone could be useful for the treatment of equine theileriosis in the field. However, this drug was unable to clear carrier infections alone (Zaugg and Lane, 1992).

MMV675968 was the most effective antipiroplasm compound, with IC_{50}s of less than 10 nM in *B. bovis*, *B. bigemina*, and *B. caballi*, but not in *T. equi*. This result revealed that MMV675968 is more effective against *Babesia* than *Theileria*. Moreover, the IC_{50} of this compound was 5.6 times

lower than that of DA. MMV675968 is known as an inhibitor of the dihydrofolate reductase (DHFR) enzyme in *Cryptosporidium*. The mechanism of action (MOA) of MMV675968 has been reported the substituted two-atom linker interacts with Cys 113 of *C. hominis*-DHFR enzyme and extends the phenyl group into two different hydrophobic pockets, therefore, inhibited an enzyme activity (Popov et al., 2006). DHFR is an essential enzyme in nucleic acid and amino acid and also is highly conserved in all protozoan parasites, including *Babesia* and *Theileria* (Anderson, 2005; Begley et al., 2011). MMV675968 was also identified as a potent inhibitor against *Plasmodium falciparum* and *Toxoplasma gondii*, with IC_{50}s of 0.03 μM and 0.02 μM , respectively (Duffy et al., 2017; Spalenka et al., 2018). In concordance with previous studies, I assumed that the findings in the current study indicate that MMV675968 also inhibited the DHFR enzyme in *Babesia* and *Theileria* parasites.

The compound MMV021057, also known as azoxystrobin, was the second best after MMV675968. MMV021057, also known as a broad-spectrum fungicide, has been reported to function as an inhibitor of the mitochondrial respiration process by binding at the Q_o site of cytochrome *b*. Eventually, there is blockage of the electron transfer between cytochrome *b* and *c*, which blocks the production of ATP (Bartlett et al., 2004). In addition, the MOA of MMV021057 (azoxystrobin) has been reported binding at the Q_o site in the haem b_l-proximal region or in the distal site of cytochrome *b* in *Plasmodium falciparum*. Furthermore, MMV021057 that targeted *bc 1* complex was a potent inhibitor of *P. falciparum*, with ranged IC_{50} 15 to 30 nM (Duffy et al., 2017; Witschel et al., 2012). Although further studies are needed to elucidate the exact mode of action, it is possible that MMV021057 exerted this effect against the growth of *Babesia* and *Theileria*.

The present study investigated the growth-inhibitory effects of MMV675968 and MMV021057 against *B. microti* in mice. The findings showed that MMV675968 and MMV021057 inhibited the growth of *B. microti* by 64% and 54%, respectively, compared to the untreated group. Furthermore, the mice treated with MMV675968 and MMV021057 showed peak parasitemia on day 10, whereas the untreated mice and DA-treated group had peak parasitemia on day 8. Even though the MMV675968 and MMV021057 single treatments were less effective than DA against *B. microti* in mice, they delayed the peak of parasitemia by 2 days (Fig. 2A). This implied that MMV675968 and MMV021057 slowed the growth of *B. microti* and could be potentiated to increase their efficacy.

To test the effect of potentiation with an effective drug (DA) and the possibility of reducing the therapeutic dose of DA in the field, I conducted a combination treatment of 25 mg/kg BW MMV021057 with a lowered dose of DA 6.25 mg/kg BW. Importantly, the combination treatment showed a stronger inhibitory effect than single treatments with 50 mg/kg BW MMV021057 and 25 mg/kg BW DA. Comparatively, the MMV021057 and DA combination was more effective than combination treatments of DA with clofazimine, 17-DMAG, allicin, and thymoquinone (El-Sayed et al., 2019; Guswanto et al., 2018; Salama et al., 2014; Tuvshintulga et al., 2017). This emphasized that MMV021057 in combination with DA could reduce the toxic effect of DA, since only 6.25 mg/kg BW was used in the combination therapy. The synergistic and additive effect of other known cytochrome *b* inhibitor (atovaquone) also observed when combined with DA against piroplasm parasites (Guswanto et al., 2018). This finding strongly suggest that drug targeted to cyctochrome *b* could be develop for combination treatment with DA as approved drug against piroplasm parasites. Moreover, MMV021057 was reported to be nongenotoxic, nonteratogenic, and nonneurotoxic by the Codex Committee on Pesticide Residues (CCPR). In addition, MMV021057 is metabolized quickly and excreted in both feces and urine (FAO, 2005).

The current study identified two hit compounds from the MMV Pathogen Box that possess potent growth-inhibitory effects against *Babesia* and *Theileria* parasites *in vitro*. Further analysis *in vivo* confirmed the effectiveness observed *in vitro*. Furthermore, one of the compounds, MMV021057, produced outstanding growth-inhibitory effects when combined with DA, which was superior to the previously reported combination of DA and other test compounds, namely, clofazimine, 17-DMAG, allicin, and thymoquinone. Taken together, this study has identified compounds that could be repurposed for treatment against piroplasmosis. Most importantly, the combination of MMV021057 and DA could be an alternative treatment with higher chemotherapeutic efficacy and could reduce the side effects of single treatment with DA. Future studies are needed to evaluate the mode of action of MMV675968 and MMV021057 and to evaluate the efficacy of derivatives related to these compounds against *Babesia* and *Theileria* parasites

2-5. Summary

Diminazene aceturate (DA) and imidocarb dipropionate are commonly used in livestock as antipiroplasm agents. However, toxic side effects are common in animals treated with these two drugs. Therefore, evaluations of novel therapeutic agents with high efficacy against piroplasm parasites and low toxicity to host animals are of paramount importance. In this study, the 400 compounds in the Pathogen Box provided by the Medicines for Malaria Venture foundation were screened against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*. A fluorescence-based method using SYBR Green 1 stain was used for initial *in vitro* screening and determination of the half maximal inhibitory concentration (IC_{50}). The initial *in vitro* screening performed using a 1 μM concentration as baseline revealed nine effective compounds against four tested parasites. Two "hit" compounds, namely MMV021057 and MMV675968, that showed IC_{50} <0.3 μM and a selectivity index >100 were selected. The IC_{50}s of MMV021057 and MMV675968 against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were 23, 39, 229, and 146 nM, and 2.9, 3, 25.7, and 2.9 nM, respectively. In addition, a combination of MMV021057 and DA showed additive or synergistic effects against four tested parasites, while combinations of MMV021057 with MMV675968 and of MMV675968 with DA showed antagonistic effects. In mice, treated with 50 mg/kg MMV021057 and 25 mg/kg MMV675968 inhibited the growth of *Babesia microti* by 54 and 64%, respectively, as compared to the untreated group on day 8. Interestingly, a combination treatment with 6.25 mg/kg DA and 25 mg/kg MMV021057 inhibited *B. microti* by 91.6%, which was a stronger inhibition than that by single treatments with 50 mg/kg MMV021057 and 25 mg/kg DA, which showed 54 and 83% inhibition, respectively. My findings indicated that MMV021057, MMV675968, and the combination treatment with MMV021057 and DA are prospects for further development of antipiroplasm drugs.

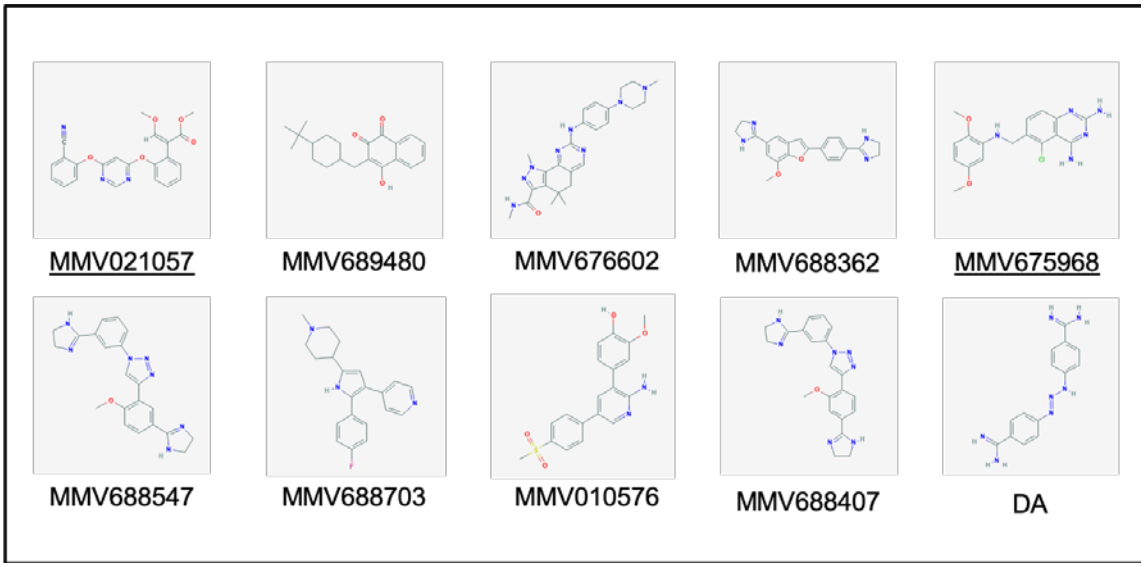

Fig. 6. Structures of the nine hit compounds identified by the initial *in vitro* screening using 1 µM as the highest concentration. The most effective compounds are indicated by underlined letters. These compound structures were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov).

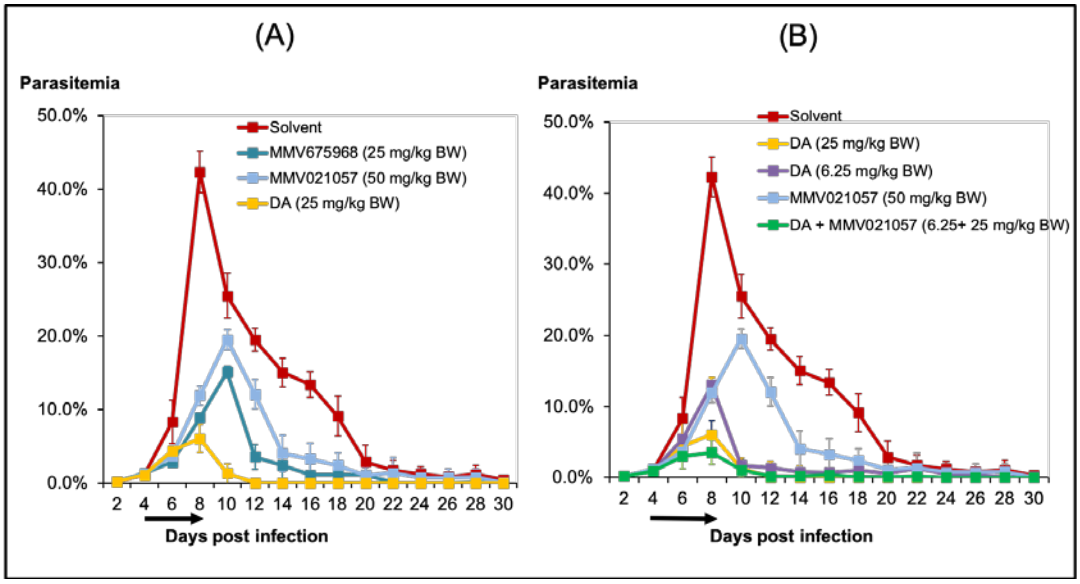

Fig. 7. The growth-inhibitory effects of *B. microti* in mice treated with the test compounds MMV675968, MMV021057, and DA and monitored for 30 days. (A) The parasitemia in mice administered with single treatments of MMV675968, MMV021057, and diminazene aceturate (DA) at doses of 25, 50, and 25 mg/kg BW, respectively. (B) The parasitemia in mice administered with combination treatments of 25 mg/kg BW MMV021057 and 6.25 mg/kg BW DA via subcutaneous and intraperitoneal injections, respectively. The arrow indicates the 5 consecutive days of treatment beginning on day 4 and continuing through day 8 p.i. Each value represents the mean and standard deviation (S.D.) from two separate experiments of five mice per experimental group. The significant differences (P <0.05) between untreated and MMV021057-treated mice are indicated with asterisks

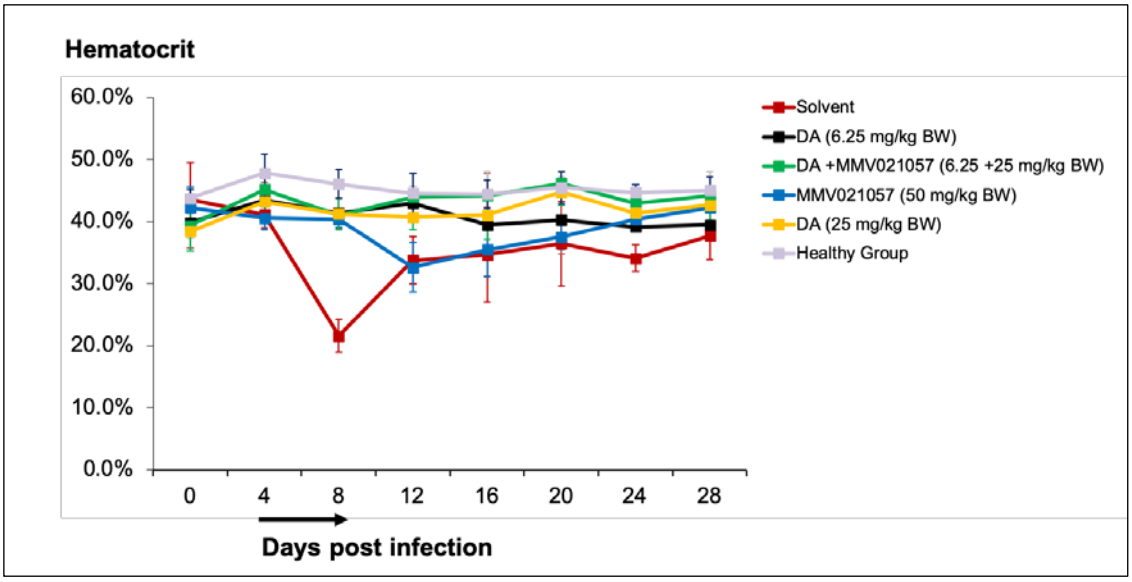

Fig. 8. The hematocrit levels in uninfected and untreated mice and in *B. microti*-infected and treated mice administered with 6.25 mg/kg BW DA, 25 mg/kg BW DA, 50 mg/kg BW MMV021057, and the combination of DA and MMV021057 (6.25 + 25 mg/kg BW). Drug treatment was performed on days 4–8 p.i. (arrow). The hematocrit levels were the mean and S.D. from two separate experiments of five mice in each experimental group. Asterisks indicate statistically significant differences (P <0.05) between infected and treated mice and uninfected and untreated mice.

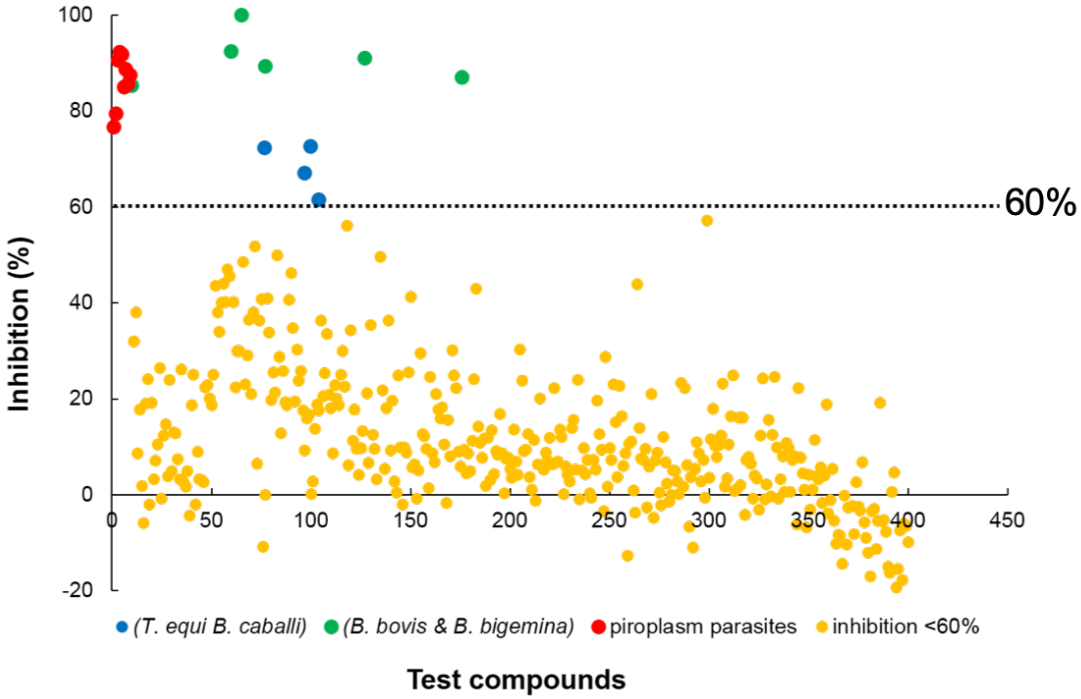

Fig. 9. Scatter plot of tested compounds after initial screening at single concentration of 1 µM. The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed at single concentration of 1 µM each compounds for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). Compounds with a growth reduction of more than 60% (dot line) were considered compounds able to inhibit piroplasm parasites growth. The red, blue and green dot color indicated compounds could inhibited the growth of all tested parasites, *T. equi* & *B. caballi*, and *B. bovis* & *B. bigemina*, respectivey.

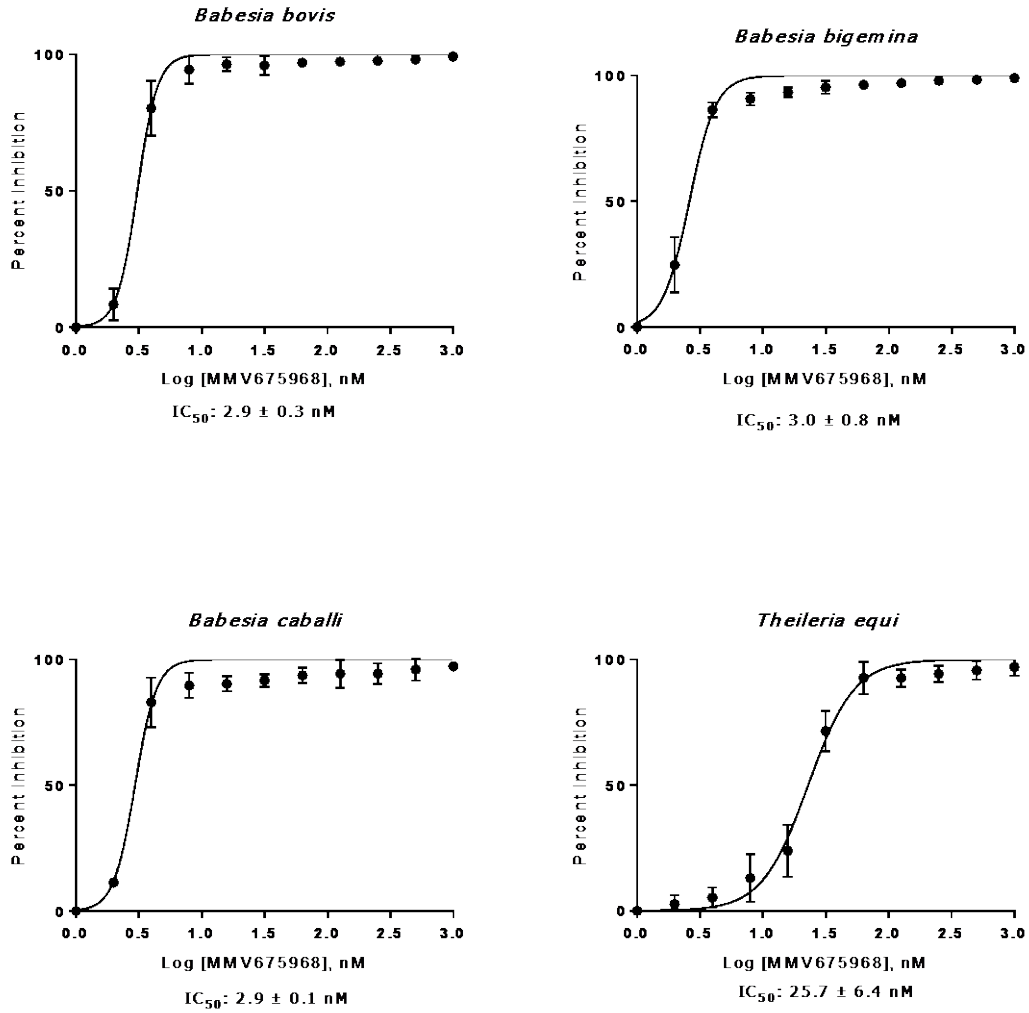

Fig. 10. Dose response curve of MMV675968 on the *in vitro* culture.

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of MMV675968 for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

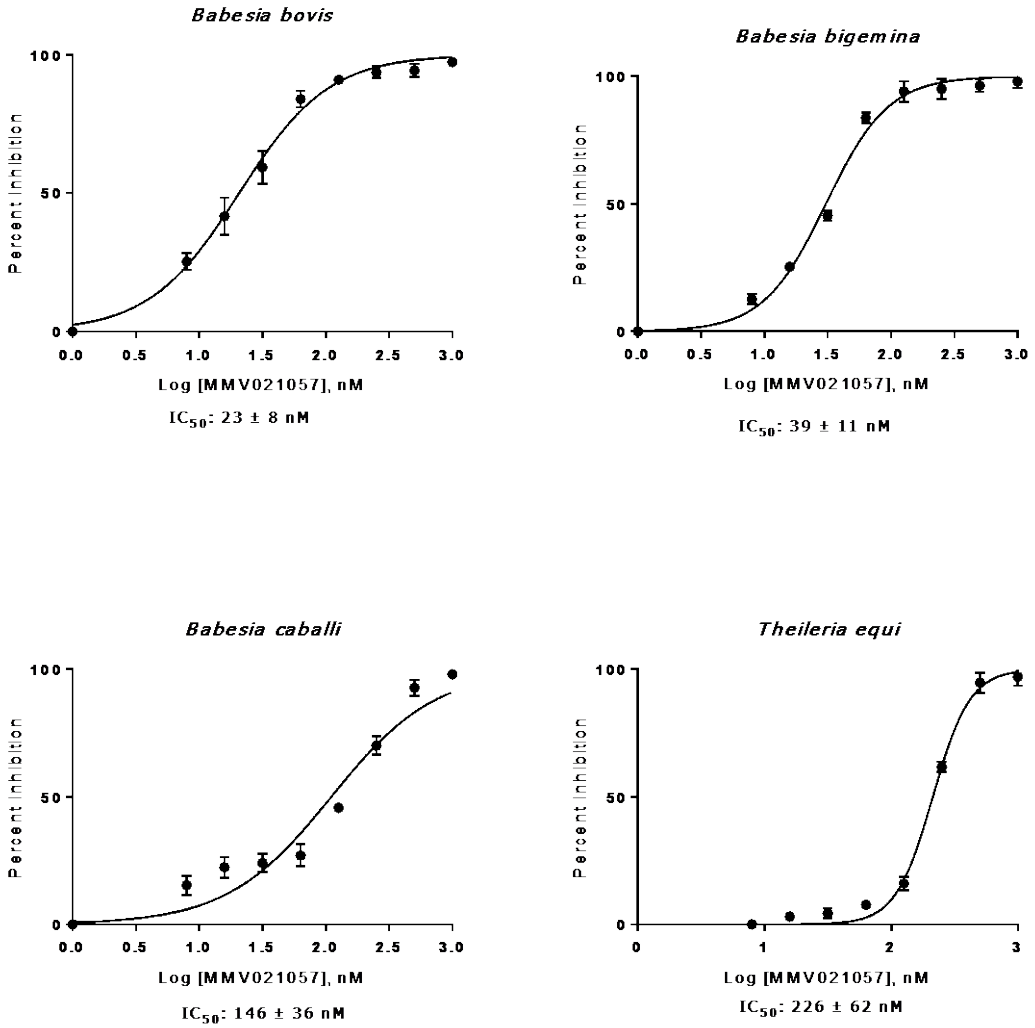

Fig. 11. Dose response curve of MMV021057 on the *in vitro* culture

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of MMV021057 for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

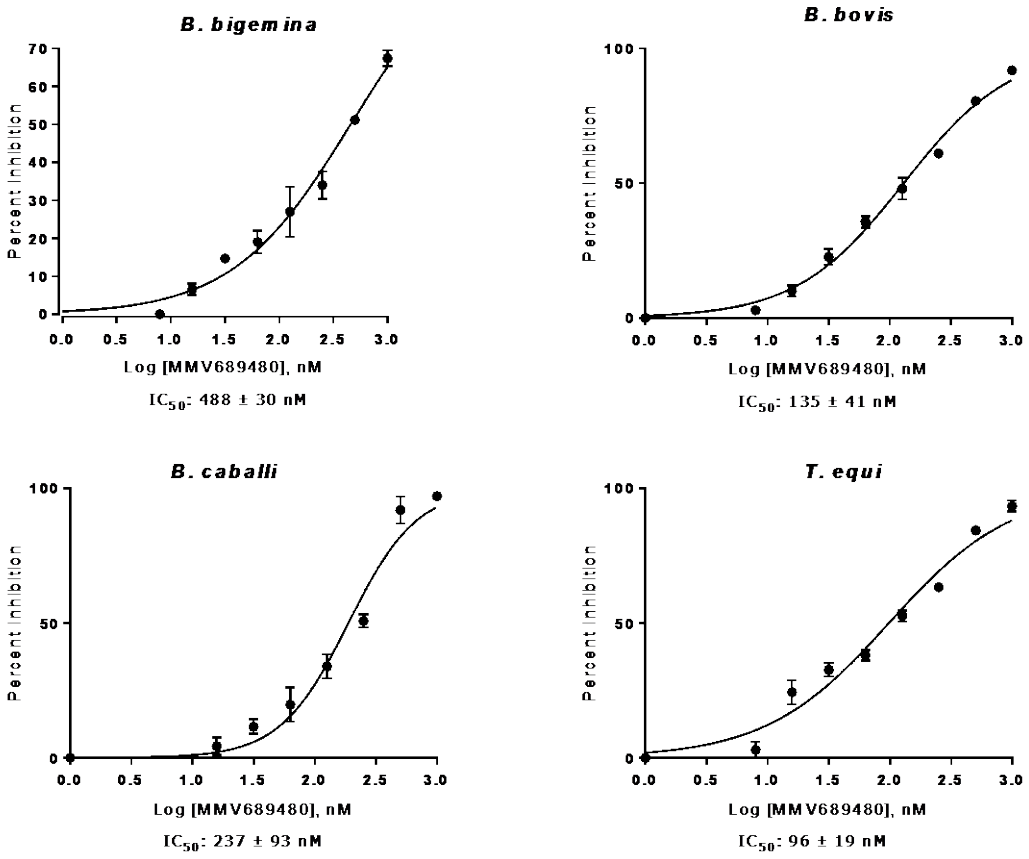

Fig. 12. Dose response curve of MMV689480 on the *in vitro* culture

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of MMV689480 for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

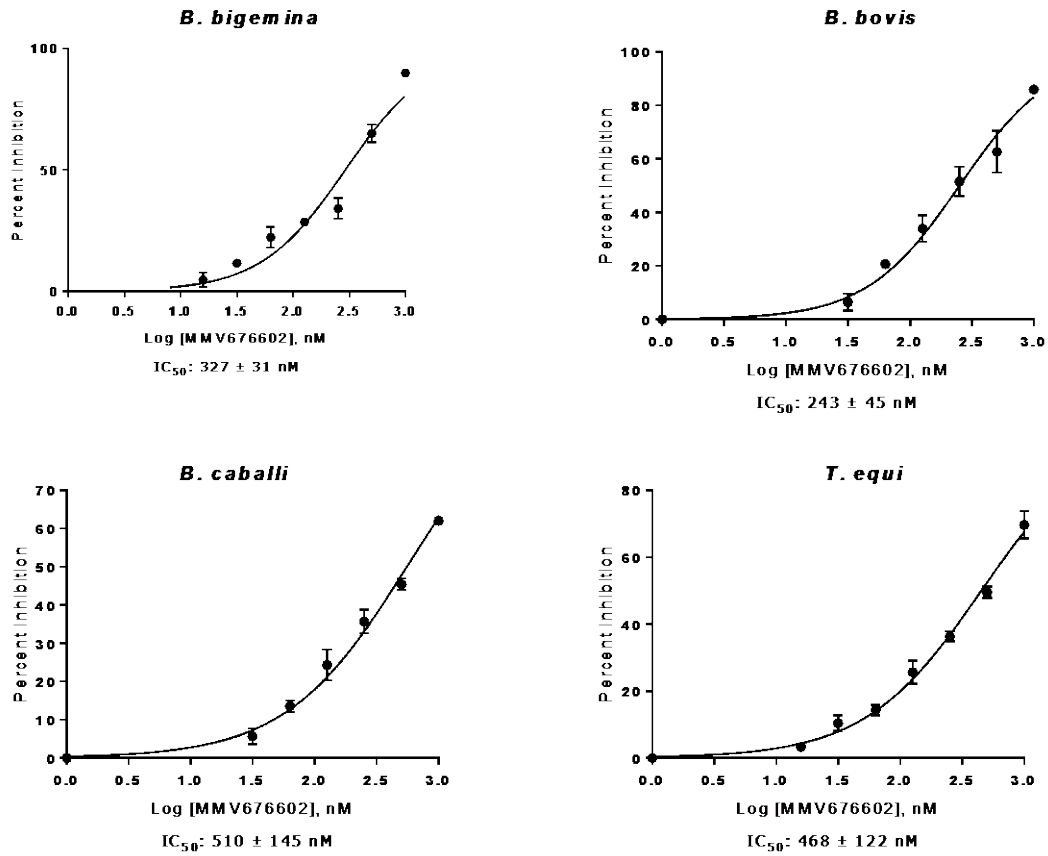

Fig. 13. Dose response curve of MMV676602 on the *in vitro* culture

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of MMV676602 for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

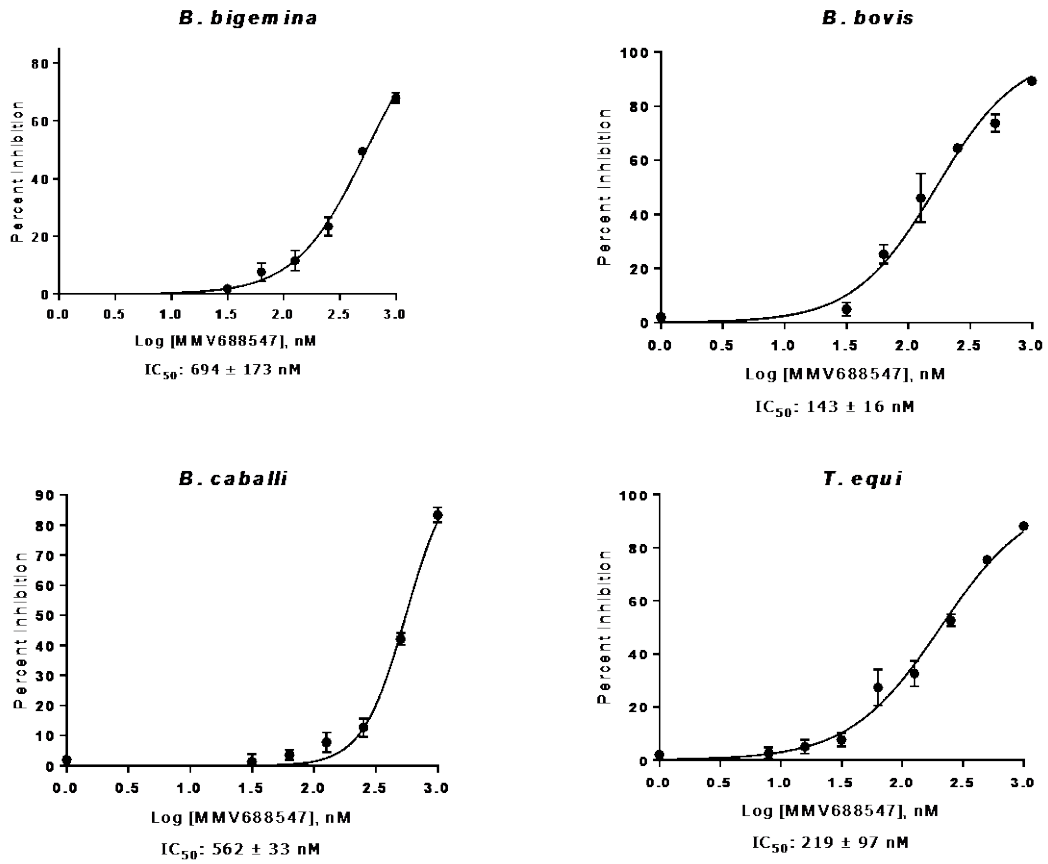

Fig. 14. Dose response curve of MMV688547 on the *in vitro* culture

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of MMV688547 for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

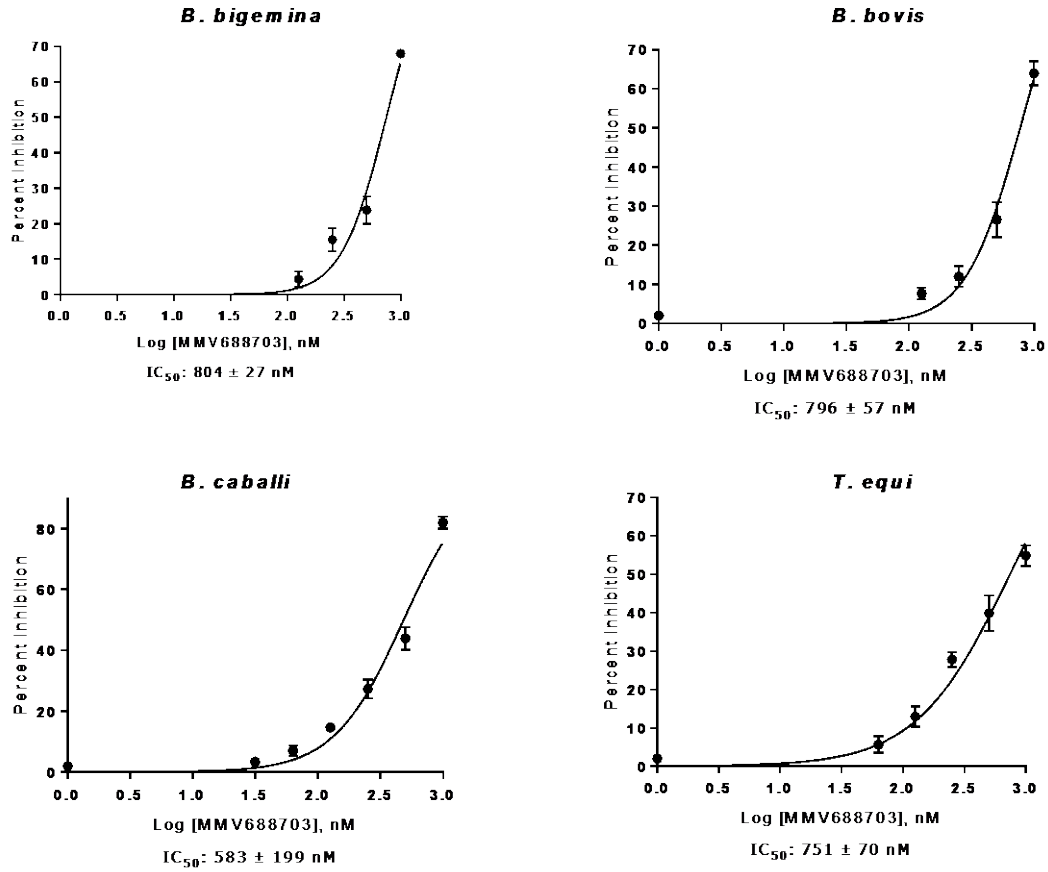

Fig. 15. Dose response curve of MMV688703 on the *in vitro* culture

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of MMV688703 for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

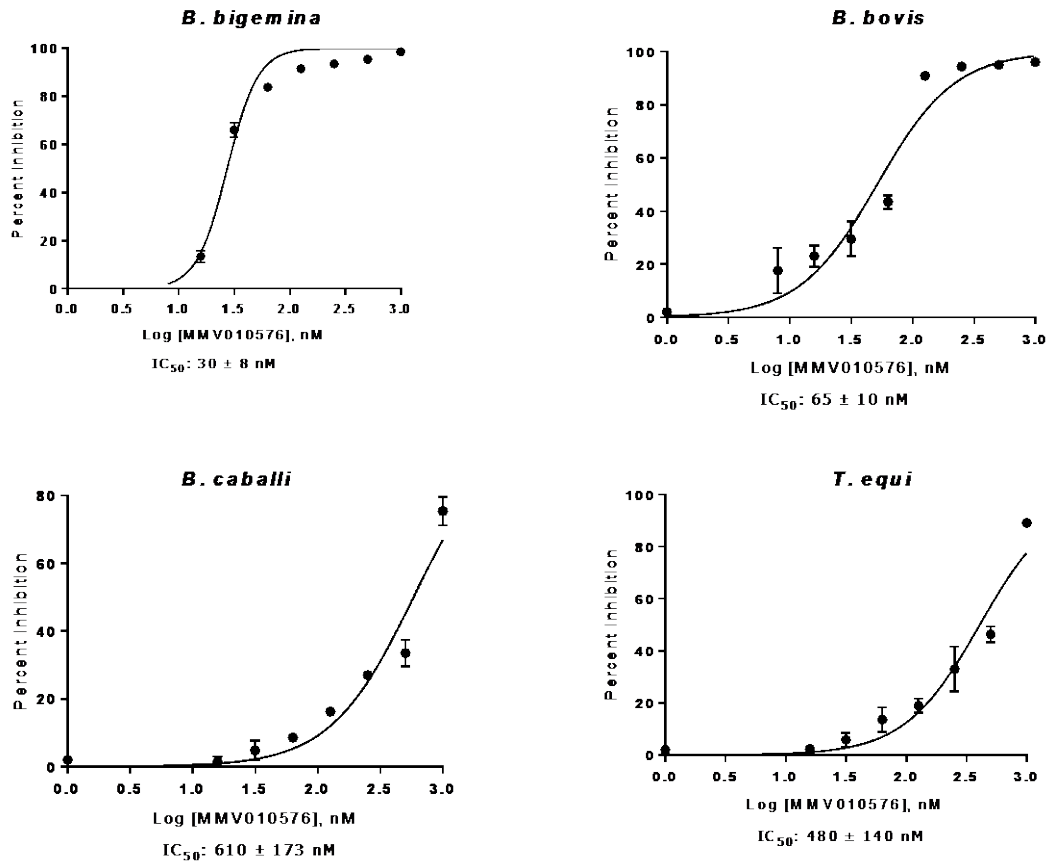

Fig. 16. Dose response curve of MMV010576 on the *in vitro* culture

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of MMV010576 for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

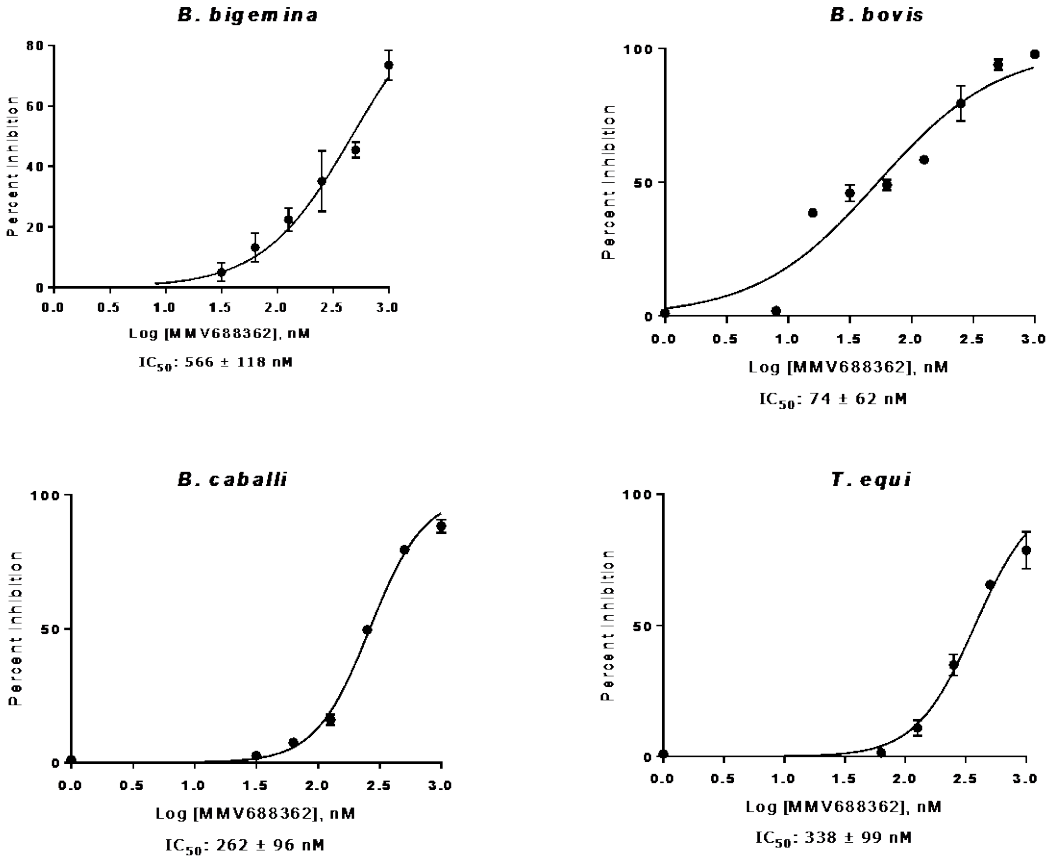

Fig. 17. Dose response curve of MMV688362 on the *in vitro* culture

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of MMV688362 for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

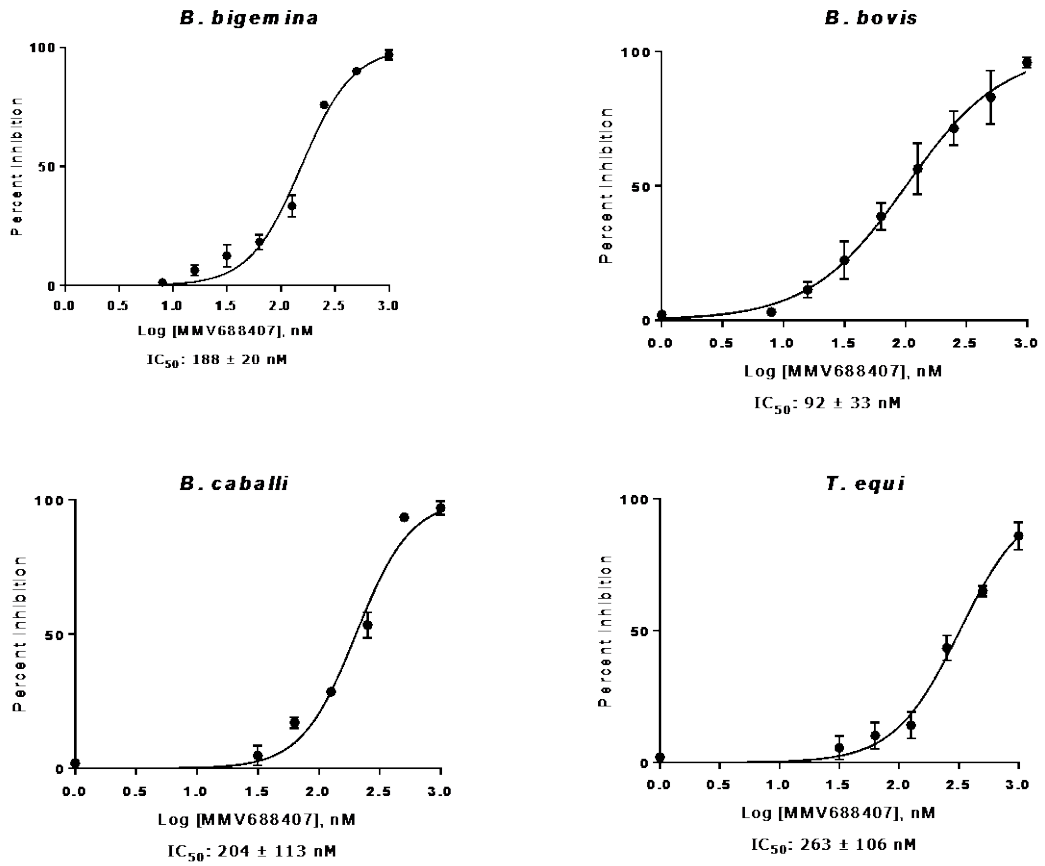

Fig. 18. Dose response curve of MMV688407 on the *in vitro* culture

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of MMV688407 for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

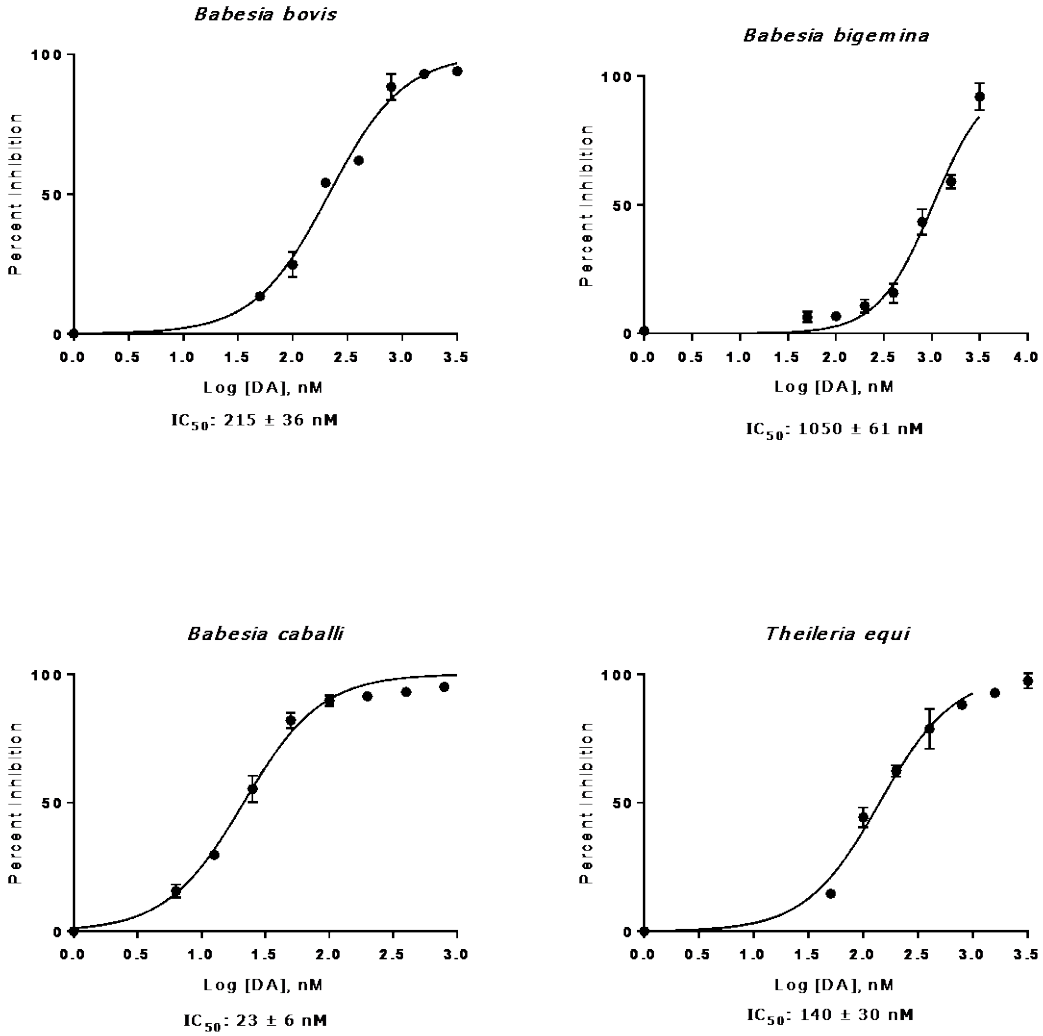

Fig. 19. Dose response curve of diminazene aceturate (DA) on the *in vitro* culture

The growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* after exposed to various concentrations of DA for 96 h as determined by fluorescence-based method using SBYR Green 1 stain. The percent inhibition is presented as percentage of inhibited parasites compared with positive control (untreated wells) after subtracted with negative control (un-infected RBCs). The IC_{50s} were determined from dose response curve using non-linear regression (curve fit analysis). The values from triplicate experiments are shown.

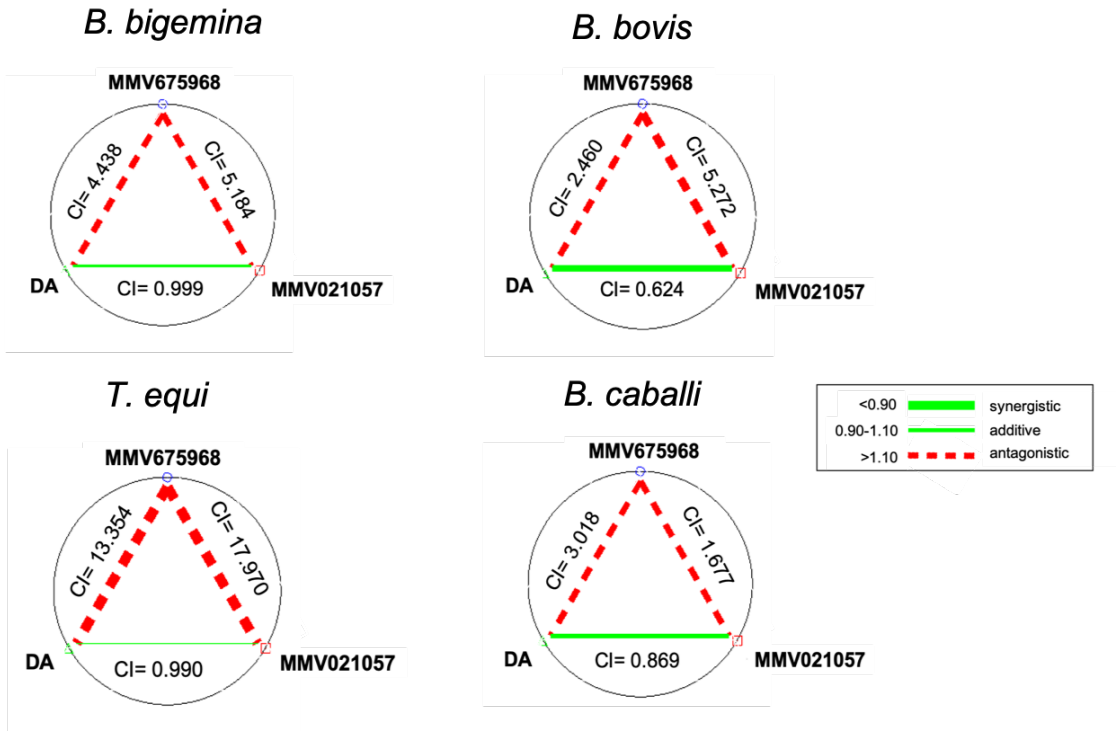

Fig. 20. Polygonogram of the degree of synergism. The polygonogram of synergism between MMV675968, MMV021057, and diminazene aceturate (DA) based on the weighted average of combination index values (CIs) at IC_{50}, IC_{75}, IC_{90}, and IC_{95} using the formula ((1 x IC_{50}) + (2 x IC_{75}) + (3 x IC_{90}) + (4 x IC_{95}))/10 (Chou, 2006). The degree of synergism was determined based on the following CI value: < 0.90 (synergistic), 0.90–1.10 (additive), and > 1.10 (antagonistic).

Table 5. The nine lead compounds in all tested parasites after primary screening at 1 µM

Compound ID	Disease set within the Pathogen Box	Compound class	Mechanism of action in other organisms
MMV021057	Malaria	β-Methoxyacrylate analogue (azoxystrobin)	Mitochondrial cytochrome bc_1 complex
MMV689480	Reference compound (buparvaquone)	Hydroxynaphthoquinone	Q_o quinone-binding site of mitochondrial cytochrome *b*
MMV676602	Kinetoplastids	Milciclib	Cyclin-dependent kinase 2 inhibitor
MMV688547	Kinetoplastids	Bisarylamidine	DNA minor groove binding at AT-rich DNA sequences
MMV688703	Toxoplasmosis	Trisubstituted pyrrole	cGMP-dependent protein kinase
MMV010576	Malaria	2-Amino-3,5-diaryl pyridine	Kinase
MMV688362	Kinetoplastids	Bisarylamidine	DNA minor groove binding at AT-rich DNA sequences
MMV688407	Kinetoplastids	Triazole	Not available
MMV675968	Cryptosporidiosis	Quinazoline-2,4-diamine	Dihydrofolate reductase

Table 6. Half maximum inhibition concentrations (IC_{50}s)

Compound ID	IC_{50} (nM)				CC_{50} (µM)	SI*
	B. bovis	*B. bigemina*	*T. equi*	*B. caballi*		
MMV021057***	23 ± 8	39 ± 11	229 ± 62	146 ± 36	>28[a]	>1217; >717; >122; >192
MMV689480	135 ± 41	488 ± 30	96 ± 19	237 ± 93	8.66[d]	64; 18; 90; 37
MMV676602	243 ± 45	327 ± 31	468 ± 122	510 ± 145	1.1[b]	5; 3; 2; 2
MMV688547	143 ± 16	694 ± 173	219 ± 97	562 ± 33	>32[b]	>224; >46; >146; >57
MMV688703	796 ± 57	804 ± 27	751 ± 70	583 ± 199	>50[c]	>63; >62; >67; >86
MMV010576	65 ± 10	30 ± 8	480 ± 140	610 ± 173	>10[a]	>153; >333; >21; >16
MMV688362	74 ± 62	566 ± 118	338 ± 99	262 ± 96	>32[b]	>432; >57; >95; >122
MMV688407	92 ± 33	188 ± 20	263 ± 106	204 ± 113	>32[b]	>348; >170; >122; >157
MMV675968***	2.9 ± 0.3	3.0 ± 0.8	25.7 ± 6.4	2.9 ± 0.1	5.5[d]	1896; 1833; 214; 1896
DA**	215 ± 3.6	1050 ± 61	140 ± 30	23 ± 6	>40[e]	>186; >38; >286; >1739

[a] The CC_{50} values on HepG2 cells

[b] The CC_{50} values on MRC5 cells

[c] The CC_{50} values on HL60 cells

[d] The CC_{50} values on Vero cells (Spalenka et al., 2018)

[e] The CC_{50} values on MDBK cells (Guswanto et al., 2018)

*Selectivity index (SI) = CC_{50}/IC_{50} on *B. bovis*, *B. bigemina*, *B. caballi* and *T. equi* ratio

**Diminazene aceturate (DA) as a control drug

***Two selected compounds which showed IC_{50}< 0.3µM and SI> 100 in four tested parasites.

The cytotoxicity values on HepG2, MRC5, and HL60 were obtained from (http://www.pathogenbox.org/about-pathogen-box/supporting-information).

Table 7. Combination indexes among MMV675968, MMV021057, and DA

Parasites	Drug combinations	CI value at				WI[a]	Degree of synergism[b]
		IC_{50}	IC_{75}	IC_{90}	IC_{95}		
B. bovis	MMV675968 + MMV021057	12.872	7.214	4.293	3.135	5.272	Antagonistic
	MMV675968 + DA	5.795	3.255	2.009	1.568	2.460	Antagonistic
	MMV021057 + DA	0.691	0.646	0.615	0.602	0.624	Synergistic
B. bigemina	MMV675968 + MMV021057	20.143	7.681	3.102	1.756	5.184	Antagonistic
	MMV675968 + DA	15.61	6.727	2.908	1.648	4.438	Antagonistic
	MMV021057 + DA	2.05	1.303	0.872	0.68	0.999	Additive
B. caballi	MMV675968 + MMV021057	1.66	1.652	1.674	1.697	1.677	Antagonistic
	MMV675968 + DA	0.493	1.109	2.649	4.88	3.018	Antagonistic
	MMV021057 + DA	1.162	0.989	0.842	0.755	0.869	Synergistic
T. equi	MMV675968 + MMV021057	27.663	21.66	16.996	14.433	17.970	Antagonistic
	MMV675968 + DA	6.877	9.54	13.357	16.878	13.354	Antagonistic
	MMV021057 + DA	0.913	0.953	0.994	1.025	0.990	Additive

[a] Weight average (WI) of combination index (CI) values

[b] WI values: synergistic (<0.90), additive (0.90–1.10), antagonistic (>1.10)

Chapter 3

Expression of a green fluorescent protein in the erythrocytic stage of *Theileria equi*

3-1. Introduction

Equine theileriosis is a disease caused by a hemoprotozoan parasite, namely *Theileria equi*. It has a significant economic impact on the horse industry, particularly for international trade and sport. The distribution of *T. equi* is found in subtropical and tropical regions due to the variety of ticks as vectors and the global movement of horses between countries (Knowles, 1996). Infected horses in the acute or subacute stage may develop severe clinical signs such as fever, anemia, icterus, and hemoglobinuria (Wise et al., 2013). Although most of the animals can recover from that acute stage, they could become carrier hosts for several years. *Theileria equi* can persist in several organs, such as bone marrow and the liver, spleen, lung, heart, and brain. Therefore, the carrier animal could act as a source for transmitting the disease (Alhassan et al., 2007; Ribeiro et al., 2013).

The challenges raised for controlling equine theileriosis include the emergence of acaricide-resistance, inadequate equine antitheilerial drugs, and toxic side effects in treated animals. Therefore, a deeper understanding of the basic biology of *T. equi* is necessary to develop a new approach to controlling the disease. An alternative approach is through gene manipulation. In the past 10 years, the genetic manipulation of apicomplexan parasites through transfection has been developed, including that of *Babesia bovis*, *B. bigemina*, *B. gibsoni*, *B. ovata*, *Theileria parva*, *T. annulata*, *Plasmodium falciparum*, *Toxoplasma gondii*, and *Cryptosporidium parvum*, but not *Theileria equi* (Adamson et al., 2001; Goeyse et al., 2015; Hakimi et al., 2016; Li et al., 2009; Liu et al., 2018; Nishikawa et al., 2008; Silva et al., 2016; Suarez et al., 2006; Talman et al., 2010).

In order to drive and control a foreign gene in the parasite genome, I selected one of strongest promoters used in several transfection systems in mammalian cells (Wang et al., 2017) and piroplasm

parasites, including *B. bovis*, *B. bigemina*, *B. ovata*, and *T. parva*, namely *elongation factor-1 alpha* (*ef-1α*) (Goeyse et al., 2015; Hakimi et al., 2016; Silva et al., 2016; Suarez and McElwain, 2009). This gene plays an important role in protein translation and is highly expressed in eukaryotic cells (Mateyak and Kinzy, 2011). The highest activity of this gene was in the intergenic region.

Therefore, I selected *ef-1α* as a promoter gene to establish GFP expression in *T. equi*. In this study, I developed a stable transfection system of *T. equi* using the *ef-1α* gene as a promoter to drive the GFP–BSD gene.

3-2. Materials and Methods

Parasite culture. *Theileria equi* (USDA strain) was maintained in continuous microaerophilic stationary phase cultures. *T. equi* was cultured in purified horse RBCs and M199 supplemented with 40% horse serum, and hypoxanthine (MP Biomedicals, USA) at a final concentration of 13.6 μg/ml was used for *T. equi* cultivation. An antibiotic–antimycotic solution containing 60 U/ml penicillin G, 60 μg/ml streptomycin, and 0.15 μg/ml amphotericin B (Sigma-Aldrich, Tokyo, Japan) was added to the medium.

Evaluation of *T. equi* sensitivity to blasticidin. The experiment was conducted specifically by cultured *T. equi* with 200 μl containing 10% horse RBC in a 96-well plate. To determine the sensitivity of *T. equi* to blasticidin, the latter was diluted by a twofold serial dilution ranging from 0.1 to 50 μM. Media containing 0.025% purified water with infected RBCs (iRBCs) or uninfected RBCs were used as positive and negative controls, respectively. The initial parasitemia was 1%, and on day 4 the parasitemia was calculated by examining 5000 RBCs. This experiment was conducted in triplicate for each drug concentration and repeated three times in separate experiments.

RACE analysis: Analysis of *T. equi ef-1 alpha* cDNA. The *T. equi* (USDA strain) *ef-1α* cDNA was generated using SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). I designed universal primers based on the conserved region of the *ef-1α* gene of *T. equi* (WA strain), *B. bovis*, *B. bigemina*, and *Theileria parva* obtained from the GenBank, namely Teq-Race

(For-5'- GATTACGCCAAGCTTCGTGAGCATGCCTTGTTGGCCTTC -3') and Teq-Race (Rev-5'- GATTACGCCAAGCTT GGTCCTCCTTGTAGTCGCACTTG -3'). 3Prime-CDS-Primer-A (5'- GTACTCTGCGTTGATACCACTGCTT-3') was used for RACE analysis to amplify cDNA of 5' and 3' prime. Subsequently, the amplified 5' end and 3' end products were cloned into Stellar competent cells using an In-Fusion HD Cloning Kit and sequenced.

Long fragment amplification and sequencing. To identify the long fragment amplicons of the *ef-1α* locus, which includes 5' and 3' orf- *ef-1α*, intergenic region (IG) of *ef-1α* and terminator regions in *ef-1α* locus. One set primer was designed to amplify the *ef-1α* locus. The forward and reverse primers were designed based on ribonucleotide reductase (Teq-Prom-EF1a-F) and glutamyl tRNA synthase (Teq-Prom-EF1a-R), respectively. The expected size of this fragment was ~5.5 kb. Subsequently, the amplified amplicon was cloned into a pCR4-TOPO Cloning Vector to produce plasmid-EF1-EQ.

Plasmid constructs. The schematic diagram for plasmid constructs is shown in Fig. 21. A plasmid was constructed for stable transfection as well as for genome integration in the *ef-1α* locus. The 5' region, 3' region, small part-terminator region *ef-1α*, and promoter region of *ef-1α* IG were amplified by PCR using four set primers, *ef-1α* -IG, 5'- *ef-1α*, 3'- *ef-1α*, and Ter- *ef-1α* from plasmid-EF1-EQ, respectively. The GFP–BSD fused protein was used as a reporter gene and selectable marker for drug selection and was amplified by PCR from plasmid CMV-BSD-lacZ using set primer of GFP-BSD-EQ. The primers used for plasmid constructs are listed in Table 8. All prepared fragments were inserted into SacI and XmaI restriction enzyme sites of pBS-SKII using an In-Fusion HD Cloning Kit. The constructed plasmid was named pBS-EF1-GFP-EQ. The plasmid was purified using a Qiagen Plasmid Maxi Kit (Qiagen, MD, USA) according to the manufacturer's instructions. The inserted fragments were sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Transfection of parasites. The *T. equi*–infected RBCs (iRBCs) at 10% parasitemia were harvested for transfection. Prior to transfection, iRBCs were washed twice with PBS. In order to promote genome integration and produce *T. equi* stably expressing GFP, the pBS-EF1-GFP-EQ plasmid was linearized by incubation overnight with Apal1. Twenty micrograms of linearized pBS-EF1-GFP-EQ in 100 µl of Lonza buffer SF was mixed with 100 µl of washed iRBCs. The *T. equi* iRBCs were transfected with linearized plasmids using an Amaxa Nucleofector™ device (Lonza, Cologne, Germany) with a program v-024 and immediately transferred into 1 ml of culture containing 10% fresh RBCs. Twenty-four hours post transfection, 70 nM blasticidin was added to the culture medium to select GFP-expressing transgenic parasites. In order to select a single transgenic parasite, the limited dilution was conducted after 2 weeks of drug pressure. One microliter of iRBCs from cultured *T. equi*, transfected or wild-type (non-transfected parasites), was observed under fluorescence microscopy (1000x magnification). The iRBC was stained using Hoechts 33342 to confirm the parasite nucleus.

Confirmation of stable integration. To confirm integration of the transfected plasmid into the *ef-1α* gene locus, three sets of primers were designed (Table 9). First, I designed a set of primers to analyze the genome integration site, whether in the *ef-1α* gene locus A with *ribosomal nucleotide reductase* (*rsds*) or the B site with *glutamyl tRNA synthase* (*glutamyl*). Two set primers were used for this analysis, namely GFP-*rsds* and GFP-*glutamyl*, and the expected amplicon size was ~2.7 kb. Second, to examine whether one of the *ef-1α genes* was replaced with the plasmid construct based on the 5'orf and 3'orf of *ef-1α gene*. Two set primers were used for this analysis, namely 5'- *ef-1α* -F and 3'- *ef-1α*-R, and the double amplicons were expected (~485 bp and ~2.7 bp). Subsequently, another set of primers was used to confirm the order of all five inserted fragments integrated within the genome of *T. equi*. Each forward primer of 5'- *ef-1α*-F, *ef-1α* -IG-F, GFP-BSD-EQ-F, 3'- *ef-1α*-F, and Ter- *ef-1α*-F was paired with *glutamyl*-R.

Southern blot analysis. Genomic DNA was extracted from wild-type and integrated transfected parasites. Two micrograms of gDNA was digested overnight with 10 units/µl of SacI and XmaI restriction enzymes. The digested gDNA was run on 0.8% electrophoresis gel, stained with ethidium bromide, and transferred onto Hybond N^+ (GE Healthcare, Buckinghamshire, UK). A probe was prepared using a set of primer GFP-probe (For-5'-CCAAAGGAGAAGAACTTTTCACTGG-3') and GFP-probe (Rev-5'-TTTGTGTCCGAGAATGTTTCC-3'). The probe was labeled and hybridized with the AlkPhos Direct Kit (GE Healthcare) in accordance with the manufacturer's protocols. The signal was detected using CDP Star detection reagent (GE Healthcare).

Evaluation of *in vitro* growth. *T. equi* (transfected) parasites and wild-type (non-transfected) parasites were cultured in 1 ml of culture medium with or without 70 nM blasticidin. The initial parasitemia was 1%, containing 10% purified horse RBCs, and parasites were cultured in duplicate in 24-well plates. The medium was replaced daily, and the parasitemia was monitored up to 72 hr using Giemsa-stained RBC smears. The parasitemia was calculated by examining 5000 RBCs. This experiment was conducted three separate times.

3-3. Results

Sensitivity of *T. equi* to blasticidin. In order to know the suitable concentration of blasticidin for the selection of transgenic parasite, *T. equi* was cultured in the presence and absence of different concentrations of blasticidin ranging from 0.5 to 100 µM, and the parasitemia was calculated daily until 96 hr. The calculated IC_{50} was 7 µM and at 10 times higher concentration IC_{50} of BSD was completely inhibited the growth of *T. equi* (Fig. 22a). Therefore, I chose a concentration of 70 µM for selection of the transgenic parasite.

RACE analysis and ~5.5 kb fragment from *the genomic region of* ef-1*α*. Due to the unavailability of data on the whole genome sequences of *T. equi* (USDA strain), I conducted a RACE analysis to determine the open reading frame (ORF) of the *ef-1α* gene in *T. equi* (USDA strain) (Fig. 23). From the RACE analysis, I identified 633 bp and 962 bp for 5'orf and 3'orf, respectively.

Furthermore, the mRNA sequence of the *ef-1α* gene in *T. equi* shared 98.3% identity with the sequence of *T. equi* (WA strain) retrieved from the GenBank and revealed presence of two *ef-1α* orfs using a BLAST. The ~5.5 kb long fragment was amplified from *rsds*- and *gltmyl*-based primers. Furthermore, from sequencing analysis I can identify the promoter and terminator region in the *ef-1α* gene. In addition, based on these results, I used all identified fragments for plasmid constructs.

Stable transfected line. *Theileria equi* was transfected with plasmid pBS-EF1-GFP-EQ and immediately cultured. Twenty-four hours post transfection, the medium was added to blasticidin to select transgenic parasites. The transgenic parasites that were resistant to blasticidin and showed a green fluorescence signal under a fluorescence microscope emerged 12 days after transfection (Fig. 22b and Fig. 24). The transgenic parasites were maintained for at least 2 months without drug pressure before confirmation of the genome integration by PCR, and examination of the growth curve rate of parasites. Based on the limited dilution, I selected a clone D3-2 parasite line. In addition, I evaluated the *in vitro* growth rate of the transfected parasite as compared with wild-type parasites. The result showed that the growth rate of transfected parasites was comparable with that of wild-type parasites. Moreover, the D3-2 line parasite reached peak parasitemia of 11% on day 4 (Fig. 26).

PCR and Southern blot confirmation of stable transfection parasites. The transfected parasites were maintained for at least 2 months without drug pressure. The genome integration was checked using PCR amplification. The PCR amplification was performed using three set primers, and the first primer was examined at the integrated genome site. The result revealed genome integrated in *ef-1α* locus B, which proved that ~2.7 kb can amplify only from a set of primer based (*glutamyl tRNA synthase*) site. As I expected, no amplicons were detected from the wild-type parasite or from the primer-based *rsds* site (*ef-1α* locus A) (Fig. 25). I also performed PCR amplification to confirm whether *ef-1α*-B had been replaced or integrated with the plasmid. The results showed that double amplicons (~2.7 kb and ~485 bp) were detected only from integrated line parasites, while only a single amplicon (~485 bp) was detected from the wild-type parasite. Additionally, I confirmed the five fragments that were inserted into the *ef-1α* gene locus. In Southern blot analysis, both gfp and promotor

probes detected a single 5.26 kb band for D3 parasite, while the promotor probe detected a single 2.99 kb band, and the gfp probe did not detect any band for the WT parasite (Fig. 26).

3-4. Discussion

Several studies have reported the limitations and challenges of preventing and controlling *T. equi* infection, including the lack of an available vaccine and the inadequacy of chemotherapeutic antitheilerial drugs (Wise et al., 2013). To solve these challenges, a study of the basic biology of *T. equi* is needed. However, there is no reported study on the basic biology of *T. equi*, such as the replication and invasion activities of *T. equi* in the erythrocytic stage. Thus, in this study to fill that gap, I established a stable transfection of *T. equi* expressing GFP.

The *ef-1α* gene has been reported as one of the strongest promoter genes that can drive a foreign gene for the purpose of developing a transgenic parasite in several *Babesia* and *Theileria* species. Therefore, I selected this promoter gene to develop a transgenic parasite in *T. equi*. However, I used *T. equi* (USDA strain), and there is no available data for whole-genome sequencing of *T. equi* (USDA strain) in the GenBank. Therefore, I performed a RACE analysis and successfully determined the open reading frame of the *ef-1α* gene. The mRNA sequence of the *ef-1α* gene in *T. equi* shared 98.3% identity with the sequence of *T. equi* (WA strain) retrieved from the GenBank, and a BLAST revealed the presence of two *ef-1α* orfs. These findings are similar to those in reported studies of *B. bovis, B. bigemina, B. ovata,* and *Plasmodium* in which the *ef-1α* gene also was a bidirectional gene (Hakimi et al., 2016; Silva et al., 2016; Suarez et al., 2006; Vinkenoog et al., 1998). Furthermore, I succeeded in identifying ~5.5 kb of the genomic region containing the *ef-1α* locus; therefore, I were able to amplify the promoter and terminator region of *ef-1α* in *T. equi* (USDA strain), which I used for plasmid constructions.

In this study, I applied blasticidin to the drug selection of a transgenic *T. equi* parasite. The growth inhibitory (IC_{50}) of blasticidin after 4 days of drug pressure was 7 µM. This IC_{50} was five times higher than that reported in *B. bovis* and *B. bigemina* (Suarez and McElwain, 2009). The transfected parasites emerged 12 days after transfection under a drug pressure of 70 µM, with no growth of untransfected parasites. Thus, BSD was suitable for drug selection of the stable transfection of *T. equi*.

Fluorescent microscopy revealed the expression of GFP in different morphology shape of *T. equi* in the erythrocytic stage, including the trophozoite and merozoite (Maltese cross) stages. This finding proved that the transfected parasite was able to grow normally. Moreover, the growth curve

of transgenic *T. equi* was comparable to that of the wild-type parasite; therefore, as I expected, the replaced part of the *ef-1α* gene did not affect the growth of *T. equi*. My findings are also in accordance with previous reported studies in *B. bovis*, *B. bigemina*, and *B. ovata* (Hakimi et al., 2016; Silva et al., 2016; Suarez et al., 2006).

This present study is the first successful development of a stable transfection system for expressing the green fluorescent protein in *T. equi*. My finding will facilitate better understanding of the basic biology of the *T. equi* parasite.

3-5. Summary

The aim of this study is to establish stable GFP expression in *T. equi*. I introduced a foreign gene expressing the green fluorescent protein (GFP) into *T. equi*. To drive that gene, I selected an effective promoter of the *elongation factor-1 alpha* (*t*) gene. In this regard, I sequenced and identified the open reading frame of the *ef-1α* gene in *T. equi* (USDA strain) using RACE analysis. I designed a plasmid containing the IG of the *ef-1α* gene, a GFP–blasticidin (BSD) fusion gene, and a terminator fragment of the *ef-1α* gene. The designed plasmid was linearized and then transfected into the *ef-1α* gene locus of the *T. equi* genome using an Amaxa Nucleofector 2d device. Twenty-four hours after transfection, the cultures were treated with 10 × IC_{50} blasticidin for transgenic parasite selection and consistently expressed the GFP for at least 2 months without drug pressure. PCR analysis revealed that the transfected plasmid integrated with the *ef-1α* genome, particularly in the B-direction gene of *ef-1α*. Furthermore, the transfected *T. equi* grew normally and comparably with the wild-type parasite. This result is the first establishment of a stable GFP expressing *T. equi* as a useful tool for understanding the biology of parasite and parasite–host interactions.

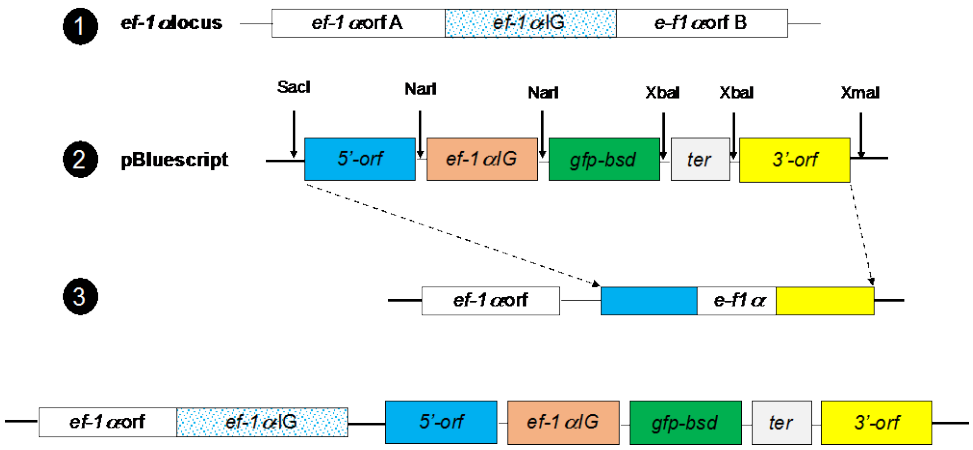

Fig. 21 Schematic of the plasmid constructs used for the transfection of *T. equi*. Original scheme of *ef-1α* **(1)**. The restriction enzymes in plasmid construction are indicated by black arrows **(2)**. The plasmid constructs for the stable integration into the *ef-1α* locus **(3)**

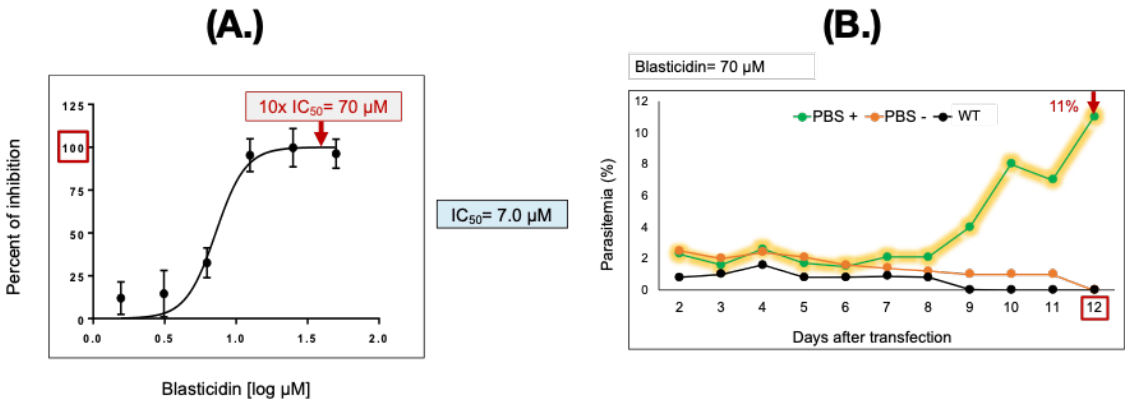

Fig. 22 Growth inhibition rate of *T. equi* in the different concentrations of blasticidin. The error bars indicate the mean ± SD from triplicate experiments **(A)**. The growth of transfected parasites expressing the green fluorescent protein emerged 12 days after transfection **(B)**.

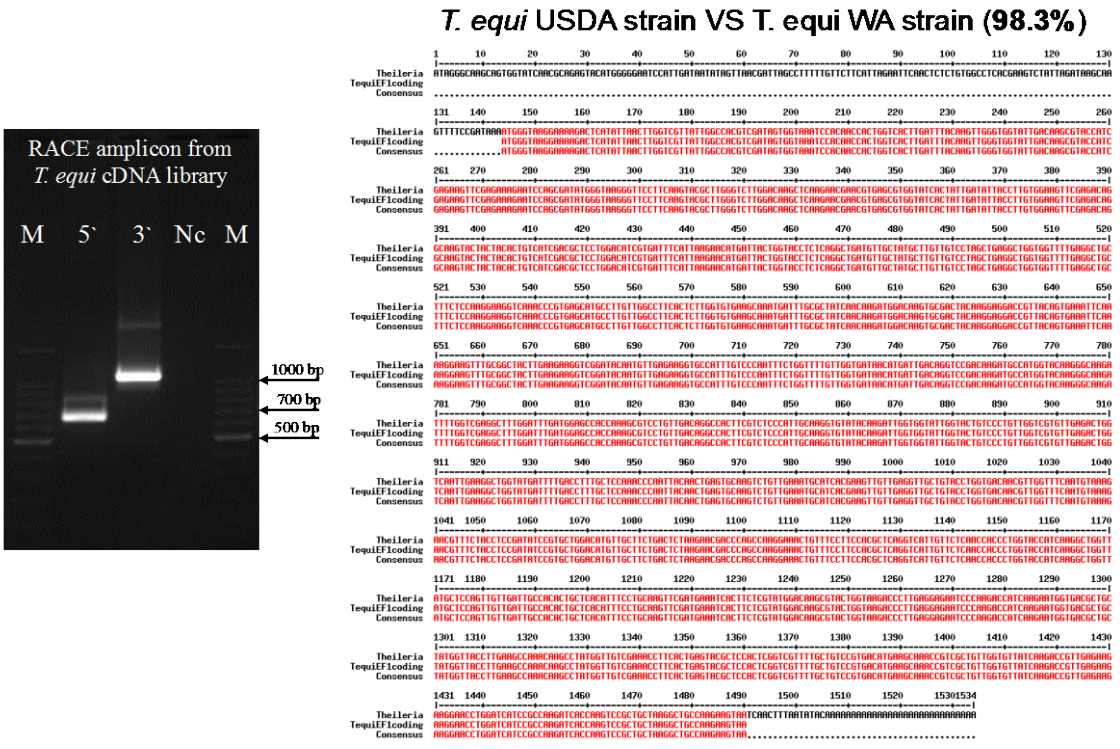

Fig. 23 RACE analysis confirmed the open reading frame (orf) of the *ef-1α* gene locus in *T. equi* USDA strain. The identity score compared with the *T. equi* WA strain (available in the GenBank) was 98.3%. The *ef-1α* locus showed a bidirectional gene in the *T. equi* USDA strain.

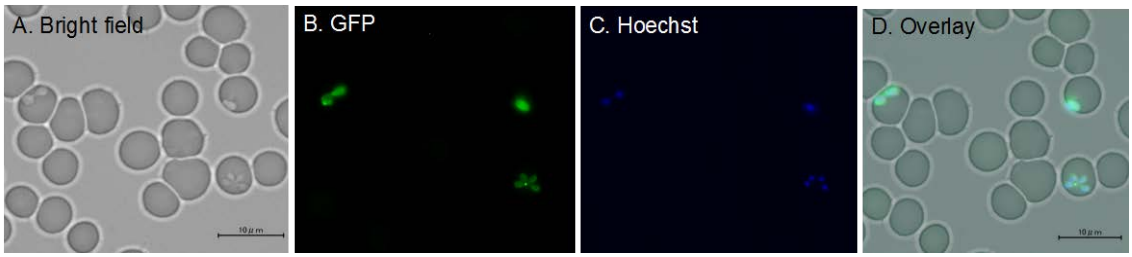

Fig. 24 Fluorescence microscopy images of GFP-expressing parasites. Panel **D** shows overlay images from the bright field **(A)**, green laser **(B)**, and blue laser **(C)**. The nucleus was stained with Hoechst 33342.

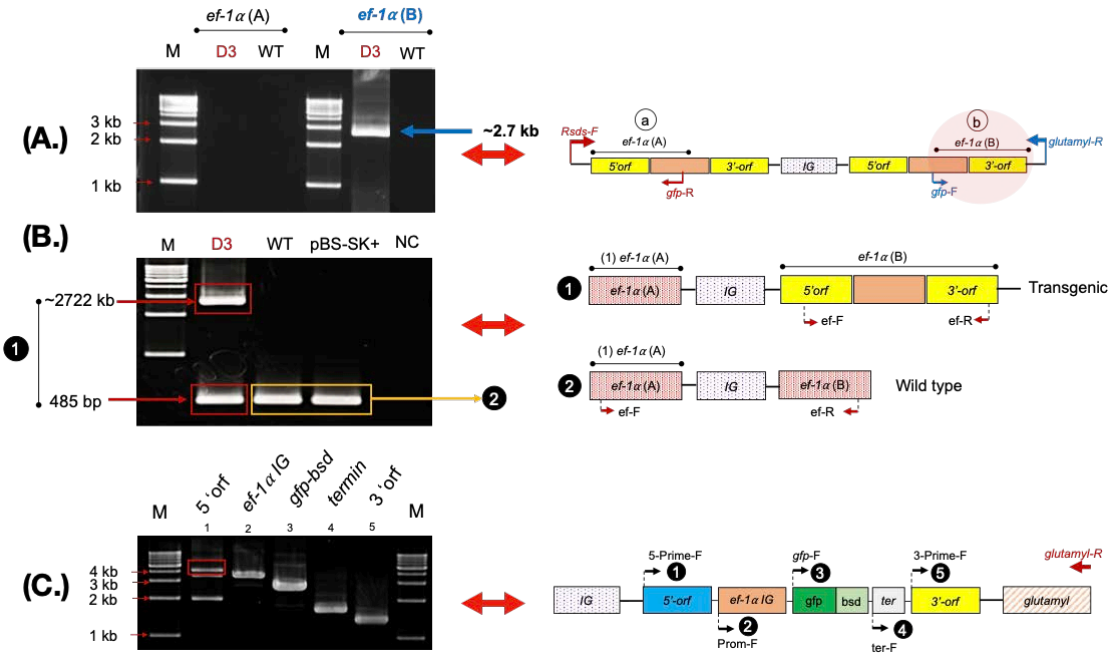

Fig. 25 Schematic diagram and PCR results to confirm genome integration into the *ef-1α* locus, particularly into *ef-1α* part B.

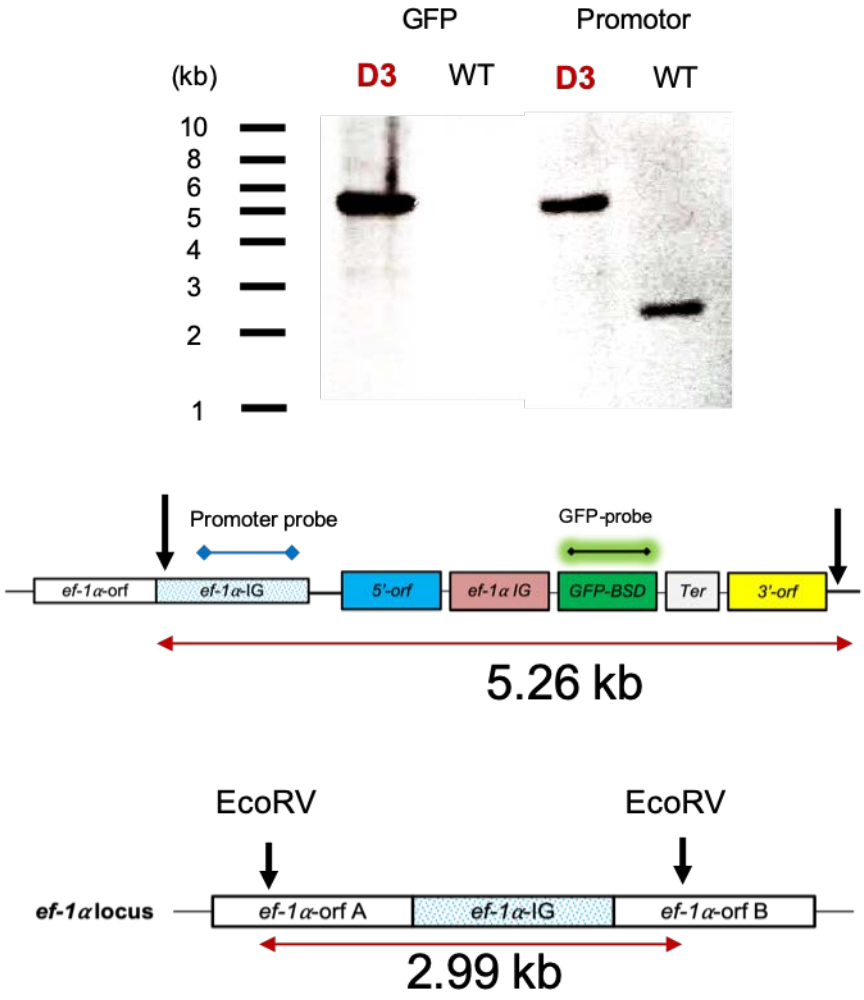

Fig. 26 Schematic diagram and Southern blot analysis to confirm the integration of pBS-EF1-GFP-EQ into ef-1α locus. One point five μg of samples genomic DNA were digested with EcoRV and hybridized with gfp probe and promoter probe.

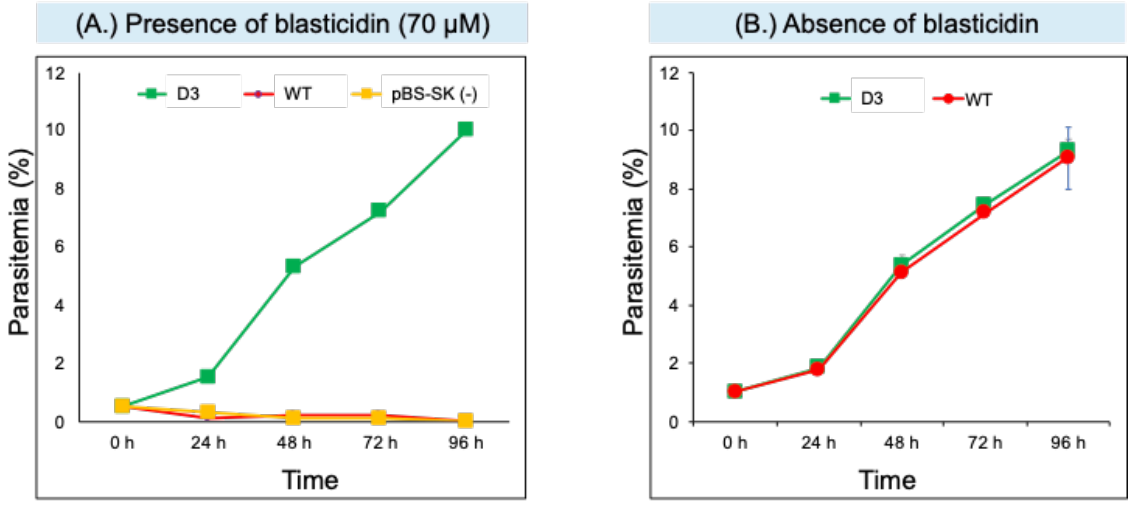

Fig. 27 Growth curve of the transfected line parasite (D3) compared with the wild-type (WT) parasite in **(A)** the presence and **(B)** absence of blasticidin (70 µM).

Table 8. List of primers for plasmid constructs

Primer	Sequence
5'-orf-SacI-F	caagctcgaaattgGAGCTCatgggtaaggaaaagactc
5'-orf-NarI-R	GGCGCCtgtaccatggcatcttgt
EF1a-IG-NarI -F	acaagatgccatggtacaGGCGCCtgcatcataatcctgacg
EF1a-IG-NarI-R	GGCGCCtttatcggaaaacttgct
GFP-BSD- NarI -F	tttccgataaaGGCGCCatggcctccaaaggagaag
GFP-BSD-XbaI-R	atataaaagttgattTCTAGAttagccctcccacacata
Ter-EF1a- XbaI-F	gttatgtgtgggagggctaaTCTAGAaatcaacttttatattcaacg
Ter-EF1a- XbaI-R	ccaaaatcttgccctTCTAGAtatcaatataataaatag
3'orf- XbaI-F	ttattatattgataTCTAGAagggcaagattttggtcgag
3'orf-XmaI-R	TCGAATTCCTGCAGCCCGGGttacttcttggcagcctta

Table 9. List of primers for confirmation of genome integration

Primer	Sequence
WATequi180116-R	CCCAAAGCAACCCAAGCCATCTATG
5'-orf-F	CAAGCTCGAAATTGGAGCTCATGGGTAAGGAAAAGACTC
EF1a-IG-F	ACAAGATGCCATGGTACAGGCGCCTGCATCATAATCCTGACG
GFP-BSD-F	TTTCCGATAAAGGCGCCATGGCCTCCAAAGGAGAAG
Ter-EF1a-F	GTTATGTGTGGGAGGGCTAATCTAGAAATCAACTTTTATATTCAACG
3'orf-F	TTATTATATTGATATCTAGAAGGGCAAGATTTTGGTCGAG

General discussion

The lack of epidemiological data on equine piroplasmosis in Indonesia, the inadequacy of chemotherapy agents, and the limited information on genetic tools to understand the biology of *Theileria equi* led us to try to fill those information gaps in this study. In chapter 1, the surveillance of equine piroplasmosis in Indonesian horses was conducted in West Java, Indonesia. The DNA and serum samples were collected and screened using PCR assays and ELISAs, respectively. The overall prevalence of *T. equi* and *B. caballi* was low, as only 8.5% and 2.1% of surveyed animals were positive for at least one parasite species according to ELISA and/or nPCR, respectively. The *T. equi* and *B. caballi*–positive rate based on ELISA or nPCR was higher in Tangerang and Bandung, respectively. The PCR amplicons were sequenced and subjected to bioinformatic and phylogenetic analyses to confirm the PCR findings and to investigate genetic diversity and show shared high identity scores with known sequences in the database.

The present study analyzed Indonesian horses and reported *T. equi* and *B. caballi* in that country for the first time. Although the prevalence of *T. equi* and *B. caballi* in this study was low when compared with that reported in several endemic countries, I confirmed the presence of these parasites in my country. Furthermore, I detected early- and chronic-stage infection with equine piroplasmosis based on the combined results of PCR and ELISA. Therefore, veterinarians in Indonesia should be made aware of the possibility of clinical piroplasmosis in horses. Moreover, if suitable tick vectors are also present in the surveyed area, the transmission of equine piroplasmosis might be possible from infected horses to other horses. Thus, further studies to investigate the prevalence of *T. equi* and *B. caballi* in various geographical territories in Indonesia using a large number of samples and to detect the tick vectors transmitting these parasite species are now a priority in Indonesia.

In chapter 2, 400 compounds from the MMV Pathogen Box were screened against *B. bovis*, *B. bigemina*, *B. caballi*, and *Theileria equi*. A fluorescence-based method using SYBR Green 1 staining was used for initial *in vitro* screening and determination of the half maximal inhibitory concentration (IC_{50}). I identified two hit compounds against *Babesia* and *Theileria* parasites with low

IC_{50}s and high SI, namely MMV021057 and MMV675968. MMV675968 was the most effective antipiroplasm compound but was more effective against *Babesia* than *Theileria*. MMV675968 is known as an inhibitor of the dihydrofolate reductase (DHFR) enzyme in *Cryptosporidium* and also has been identified as a potent inhibitor of *Plasmodium falciparum* and *Toxoplasma gondii*. MMV021057, known as a broad-spectrum fungicide, has been reported to function as an inhibitor of the mitochondrial respiration process by binding at the Q_o site of cytochrome *b*. Moreover, MMV021057 produced outstanding growth-inhibitory effects when combined with DA, which was superior to the previously reported combination of DA and other test compounds. Overall, this study has identified compounds that could be repurposed for treatment against piroplasmosis. Most importantly, the combination of MMV021057 and DA could be an alternative treatment for piroplasmosis and could reduce the side effects of single treatment with DA.

In chapter 3, I established a stable transfection of *T. equi* that expresses GFP. I performed a RACE analysis and determined the open reading frame of the *ef-1α* gene. The mRNA sequence of the *ef-1α* gene in *T. equi* shared 98.3% identity with the sequence of *T. equi* (WA strain) retrieved from the GenBank, and a BLAST revealed the presence of two *ef-1α* orfs. These findings are similar to those reported in studies of *B. bovis*, *B. bigemina*, *B. ovata*, and *Plasmodium*, in which the *ef-1α* gene also had bidirectional genes (Vinkenoog et al., 1998; Suarez et al., 2006; Hakimi et al., 2016; Silva et al., 2016). Furthermore, I successfully identified ~5.5 kb of the genomic region containing the *ef-1α* locus; therefore, from these results, I were able to amplify the promoter and terminator region of *ef-1α* in *T. equi* (USDA strain), which is used for plasmid construction. The growth inhibitory concentration (IC_{50}) of blasticidin after 4 days of drug pressure was 7 µM. The transfected parasites emerged 12 days after transfection under a drug pressure of 10 x IC_{50} (70 µM), with no growth of the untransfected parasite. This finding proved that the transfected parasite was able to grow normally. Moreover, the growth curve of transgenic *T. equi* was comparable to that of the wild-type parasite; therefore, as I expected, the replaced part of the *ef-1α* gene did not affect the growth of *T. equi*. In future studies using these transgenic parasites, I will investigate the gene function and pursue a better understanding of parasite biology.

General summary

Piroplasmosis is a disease caused by infection with apicomplexan intraerythrocytic parasites of the genera *Babesia* and *Theileria*. These are the most common parasites found in the bloodstream of mammals. Therefore, piroplasmosis is considered to be of significant economic, veterinary, and medical importance. This study investigated the prevalence of equine piroplasmosis in West Java, Indonesia, using serological and molecular diagnostic methods. To support the challenges of inadequate chemotherapeutic agents against piroplasm parasites, I evaluated novel therapeutic agents with high efficacy against piroplasm parasites by screening 400 compounds obtained from the MMV Pathogen Box against *Babesia* and *Theileria* parasites *in vitro* and *in vivo*. Furthermore, establishment of a gene modified *T. equi* was studied to study biology of parasites.

In chapter 1, I conducted surveillance of *T. equi* and *B. caballi* in horses reared in parts of West Java, Indonesia. Blood samples were collected randomly from 235 horses in four different districts (Bandung, Depok, Tangerang, and Bogor) in West Java. Of 235 surveyed animals, 5 (2.1%) and 15 (6.4%) were seropositive for *T. equi* and *B. caballi*, respectively, whereas 1 and 4 horses were nPCR-positive for *T. equi* and *B. caballi*, respectively. The *T. equi EMA-2* and *B. caballi BC48* gene fragments amplified by the nPCR assays were confirmed via bioinformatic and phylogenetic analyses. The *T. equi EMA-2* gene sequence from an Indonesian horse was identical to sequences from Florida and Washington strains and clustered together with these sequences in phylogeny. On the other hand, four Indonesian *BC48* gene sequences shared 99.8–100% identity scores. The present study is the first to report *T. equi* and *B. caballi* in horses in Indonesia. My findings highlight the need to monitor horses in Indonesia for clinical piroplasmosis caused by *T. equi* and *B. caballi*.

In chapter 2, I evaluated novel therapeutic agents with high efficacy against piroplasm parasites. In this study, the 400 compounds in the Pathogen Box provided by the Medicines for Malaria Venture foundation were screened against *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi*. The initial *in vitro* screening performed using a 1 μM concentration as baseline revealed nine effective compounds against four tested parasites. Two hit compounds, namely

MMV021057 and MMV675968, that showed an IC_{50} <0.3 μM and a selectivity index >100 were selected. The IC_{50}s of MMV021057 and MMV675968 against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were 23, 39, 229, and 146 nM and 2.9, 3, 25.7, and 2.9 nM, respectively. In addition, a combination of MMV021057 and DA showed additive or synergistic effects against four tested parasites, while combinations of MMV021057 with MMV675968 and of MMV675968 with DA showed antagonistic effects. In mice, treatment with 50 mg/kg MMV021057 and 25 mg/kg MMV675968 inhibited the growth of *Babesia microti* by 54 and 64%, respectively, as compared to the untreated group on day 8. Interestingly, a combination treatment with 6.25 mg/kg DA and 25 mg/kg MMV021057 inhibited *B. microti* by 91.6%, which was a stronger inhibition than that by single treatments with 50 mg/kg MMV021057 and 25 mg/kg DA, which showed 54 and 83% inhibition, respectively. My findings indicated that MMV021057, MMV675968, and combination treatment with MMV021057 and DA are prospects for the further development of antipiroplasm drugs.

In chapter 3, I established a stable expression of GFP in *T. equi*. I selected an effective promoter of the *elongation factor-1 alpha* (*ef-1α*) gene. In this regard, I sequenced and identified the open reading frame of the *ef-1α* gene in *T. equi* (USDA strain) using RACE analysis. The mRNA sequence of the *ef-1α* gene in *T. equi* shared 98.3% identity with the sequence of *T. equi* (WA strain) retrieved from the GenBank and revealed the presence of two *ef-1α* orfs using a BLAST. Based on sequencing analysis, an intergenic region (IG) between the *ef-1α* orfs and the terminator from gDNA were amplified and sequenced. I designed a plasmid containing the IG of the *ef-1α* gene, a GFP–blasticidin (BSD) fusion gene, and a terminator fragment of the *ef-1α* gene. The designed plasmid was linearized and then transfected into the *ef-1α* gene locus of the *T. equi* genome using an Amaxa Nucleofector 2b device. Twenty-four hours after transfection, the cultures were treated with 10 × IC_{50} blasticidin for transgenic parasite selection. Expression of the green fluorescence parasite was observed within 24 h post transfection under a fluorescence microscope, and the GFP was consistently expressed for at least 2 months without drug pressure. PCR analysis revealed that the transfected plasmid integrated with the *ef-1α* genome, particularly in the B-direction gene of *ef-1α.* Moreover, the transfected *T. equi* grew normally and comparably with the wild-type parasite. This result is the first

time a stable GFP-expressing *T. equi* as a useful tool for understanding the biology of parasite and parasite–host interactions

和文要旨

ピロプラズマ病は、アピコンプレックス門、バベシア属とタイレリア属の赤血球内寄生原虫の感染による疾病である。これらの寄生虫はほ乳類の血流中に最もよく認められる寄生虫であり、ピロプラズマ病は、経済的、獣医学および医学的に重要と考えられている。この研究は、インドネシアの西ジャワにおける馬ピロプラズマ病の感染状況について血清学的ならびに遺伝子検査法によって研究する子を目的とした。さらに、ピロプラズマ原虫に対する不適切な治療の課題を解決するために、MMV Pathogen Box より得られた 400 種類の化合物をバベシアとタイレリア原虫を試験管内ならびに動物を用いて新規の治療薬を効率よく評価した。さらに、タイレイア原虫の生物学的な機能解析を進めるため、遺伝子改変原虫の作製についても検討を行った。

第 1 章では、インドネシアの西ジャワにおいて飼育されている馬の *T. equi* と *B. caballi* の感染状況について検討を行なった。西ジャワの異なる 4 地域（Bandung、Depok、Tangerang ならびに Bogor）で無作為に 235 頭の馬から血液試料を採集した。調査した 235 頭の馬で、*T. equi* と *B. caballi* がそれぞれ血清試験で 5 頭と 15 頭、nPCR で 1 頭と 5 頭陽性だった。PCR で増幅した *EMA-2* と *BC48* 遺伝子断片は生命情報学と系統樹解析で確認された。インドネシアの馬からの *EMA-2* 遺伝子配列は、フロリダとワシントン系統の配列と全く同一で、1 つの系統樹的に 1 つの集団を形成していた。一方で、4 種類の *BC48* 遺伝子配列は 99.8-100%の相同値であった。本研究は、インドネシアの馬における *T. equi* と *B. caballi* の最初の報告である。この知見は、インドネシアの梅における *T. equi* と *B. caballi* による臨床的ピロプラズマ病を監視する必要性を示している。

第 2 章では、ピロプラズマ原虫に対する新規の治療薬を効率的に評価した。本研究では、*Babesia bovis*、 *Babesia bigemina*、 *Babesia caballi* および *Theileria equi* に対して Medicine for Malaria Vaccine 財団から提供を受けた Pathogen Box の 400 種類の化合物が評価された。1 μm の基準濃度で行なわれた最初の評価で 4 種類の原虫に対して効果のある 9 種類の化合物が認められた。その中で、50%阻害濃度（IC_{50}）が 0.3 μM 以下、選択指数が 100 以上の 2 種類のヒット化合物、MMV021057, MMV695968 が選択された。MMV021057 と MMV695968 の *B. bovis*、 *B. bigemina*、 *B. caballi* と *T. equi* に対する IC_{50} は、それぞれ、23, 39, 229, 146 nM と 2.9, 3.0, 25.7, 2.9 nM であった。さらに、MMV021057 とジミナゼン・アセチュレート（DA）との併用では、4 種類の原虫に対して相加あるいは相乗効果を示した。しかし、MMV021057 と

MMV695968、MMV695968 と DA の併用では、敵対的効果を示した。マウスを用いた実験では、50mg/kg の MMV021057、25mg/kg の MMV695968 投与により、未投与対照群に比較して、感染後 8 日目に *Babesia microti* の増殖がそれぞれ 54%および 64%抑制された。興味深いことに、6.25mg/kg の DA、25mg/kg の MMV021057 併用投与により、*B. microti* の増殖が 91.6%抑制された。この併用は、50mg/kg の MMV021057 およ 25mg/kg の DA 単独投与による 54%および 83%抑制より強い増殖抑制効果である。これらの知見により、MMV021057、MMV695968、さら MMV021057 との DA 併用投与は更なる抗ピロプラズマ病薬開発が期待される。

第３章では、*T. equi* における安定的な GFP の発現を構築した。そのため、*elongation factor-1 alpha*（*ef-1α*）遺伝子と名付けられた効果的なプロモーターが選択された。RACE 分析により、*T. equi*（USDA 株）の *ef-1α* 遺伝子の配列を決定し、オープンリーディングフレームを同定した。*ef-1α* 遺伝子の mRNA 配列はジェンバンクから取得した *T. equi*（WA 株）遺伝子配列と 98.3%の相同性を有し、BLAST 解析により 2 個の *ef-1α* オープンリーディングフレームが存在することが判明した。遺伝子配列分析により、*ef-1α* の オープンリーディングフレームと gDNA 間の遺伝子間領域が増幅され、遺伝子配列が決定された。次に *ef-1α* 遺伝子の遺伝子間領域、GFP-ブラスチジン（BSD）融合遺伝子、*ef-1α* 遺伝子、ターミネーター断片を含むプラスミッドを設計した。設計されたプラスミッドは直線化され、AMAXA Nucleofector 2b 装置を使って *T. equi* ゲノムに遺伝子導入を行った。２４時間後、培養は遺伝子導入をされた原虫を選択するため、培養を IC_{50} の 10 倍の濃度のブラスチジンで処理した。遺伝子導入後２４時間以内に、蛍光顕微鏡下で原虫の緑色蛍光の発現が認められ、薬剤の負荷なしに少なくとも２ヶ月間緑色蛍光タンパク質（GFP）が安定的に発現されている。PCR 解析により、導入プラスミッドは *ef-1α* 遺伝子特に B 方向遺伝子に挿入されていた。さらに遺伝子導入原虫は、野生型原虫と同様の正常発育をした。この結果は、安定的に GFP を発現する *T. equi* の作出に世界で最初に確立した例であり、将来的に原虫の生物学や原虫―宿主関係の理解に役立つ手法である。

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