Towards Development of Effective Chemotherapy for Babesiosis: Targeting Dihydroorotate Dehydrogenase to Suppress the Growth of *Babesia* Parasites

2013

Ketsarin KAMYINGKIRD

Doctor's Course of Animal and Food Hygiene

Graduate School of Obihiro University of Agriculture and Veterinary Medicine

バベシア原虫のジヒドロオロト酸

デヒドロゲナーゼを標的としたバベシア症

に対する有効な治療法の開発

平成 **25** 年

(2013)

Ketsarin KAMYINGKIRD

帯広畜産大学大学院畜産学研究科

博士後期課程　畜産衛生学専攻

Contents

Abbreviations and prefixes

Abbreviations

A	ATV	- atovaquone
	AUD	- Australian Dollar
B	BboDHODH	- *Babesia bovis* dihydroorotate dehydrogenase
	BCA	- bicinchoninic acid
	BosDHODH	- *Bos taurus* dihydroorotate dehydrogenase
	Breq	- brequinar
	BSA	- bovine serum albumin
C	cDNA	- complementary deoxyribonucleic acid
	CO_2	- carbon dioxide
	DCIP	- 2,6-dichlorophenol-indophenol
	DHODH	- dihydroorotate dehydrogenase
	DHODHF	- forward primer for dihydroorotate dehydrogenase gene
	DHODHR	- reverse primer for dihydroorotate dehydrogenase gene
	Di	- diminazene aceturate
	DMSO	- dimethyl sulfoxide
	DW	- distilled water
E	ELISA	- enzyme linked immunosorbent assay
	EP	- equine piroplasmosis
	EGCG	- (-)-Epigallocatechin-3-gallate
F	FMN	- fumaric dibasic

H	His	- histidine
	hr	- hour
	HRP	- horseradish peroxidase
I	IC_{50}	- the half maximal inhibitory concentration
	ICR	- imprinting control region
	IFAT	- indirect fluorescent antibody test
K	KCl	- potassium chloride
	kDa	- kilodalton
	K_m	- Michaelis constant
L	L-DHO	- L-dihydroorotic acid
	LFN	- leflunomide
M	M	- molar concentration
N	nM	- nano molar concentration
O	O_2	- oxide
	OD	- optical density
	ORA	- orotic acid
	ORF	- open reading frame
P	PbDHODH	- *Plasmodium berghei* dihydroorotate dehydrogenase
	PBS	- phosphate-buffered saline
	PCR	- polymerase chain reaction
	PVDF	- polyvinylidene fluoride
Q	Q_{10}	- coenzyme Q_{10}
	Q_D	- decylubiquinone

R	rBboDHODH	- *Babesia bovis* dihydroorotate dehydrogenase recombinant protein
	RBCs	- red blood cells
	RNA	- ribonucleic acid
S	SDS-PAGE	- sodium dodecyl sulfate polyacrylamide gel electrophoresis
	Ser	- serine
T	TAZ	- 7-hydroxy-5-[1,2,4] triazolo [1,5,a] pyrimidine
	TeDHODH	- *Theileria equi* dihydroorotate dehydrogenase
	TgDHODH	- *Toxoplasma gondii* dihydroorotate dehydrogenase
	Thr	- threonine
U	UMP	- uridine monophosphate
V	V_{max}	- maximum velocity

Prefixes

	μM	- micro molar concentration
	ε	- extinction coefficient

General introduction

1. *Babesia* in general

Babesia is the haemotropic protozoal parasite of the genus *Babesia*, order Piroplasmida, phylum Apicomplexa [Levine, 1971; Allsopp *et al*., 1994; OIE, 2008]. *Babesia* infection is one of the most common infections of free-living animals worldwide and is an emerging zoonosis in human [Homer *et al*., 2000]. It was first officially known as a round intra-erythrocytic body in the infected cattle blood, described in 1888 by Vitor Babes in Romania [Babes, 1888]. *Babesia* parasites have probably been elaborated the lives of human since antiquity, which primarily infected through domestic livestock [Vial and Gorenflot, 2000].

Based on morphological and host specificity classification, more than 100 species of *Babesia* have been described. *Babesia* generally have two classes of hosts, an invertebrate and a vertebrate host, to maintain their transmission cycles. To date, only *Ixodid* ticks have been identified as invertebrate host. Variable of wild and domestic mammals as well as human, and also several avian species have been identified as vertebrate hosts of babesia, the infection been known as *Babesiosis* [Homer *et al*., 2000]. Human babesiosis is caused by *B. microti*, a rodent borne piroplasm and *B. divergens*, a bovine pathogen. *B. divergens* infection in human is rare, but more pathogenic. Animal babesiosis is caused by several species of *Babesia* parasites for example; *B. canis*, *B. gibsoni*, the dog pathogens; *B. bovis*, *B. divergens*, *B. bigemina*, *B. ovata*, the bovine pathogens; *B. caballi*, *Theileria* (*Babesia*) *equi*, the equine pathogens and etc.

Babesiosis is well-recognized as a veterinary important diseases in cattle, horses and dogs, which has gained more attention as an emerging zoonotic disease problem.

The complexity of *Babesia* parasite life cycle can be divided into three stages: 1) gamogony, a sexual stage with formation and fusion of gametes inside the gut of *Ixodid* tick vectors; 2) sporogony, asexual reproduction in salivary glands of the tick and 3) merogony, asexual dividing stage in the erythrocytes of vertebrate hosts [Kakoma and Mehlhorn, 1993] (Fig. 1). The sporozoites can directly invade vertebrate erythrocytes after a bite by infected tick. The intraerythrocytic throphozoite multiplies and forms two to four separate merozoites, and a seemingly continual cycle of asexual reproduction is established. The rapid intracellular multiplication leads to destruction of the host erythrocyte, with release of new parasites and subsequent infection and destruction of other erythrocytes. Thereafter, they are ingested when a competent *Ixodid* tick take a blood meal from an infected host and then differentiate further in the tick gut (gamogony) [Potgieter and Els, 1976; Potgieter, 1977; Mehlhorn and Shein, 1984; Friedhoff, 1988; Mackenstedt *et al*., 1995;].

The symptoms of babesiosis are often unnoticed or unexplained. Thus, in severe cases there are symptoms such as fevers up to 40.5ºC (105ºF), shaking chills and severe anemia (hemolytic anemia) in human cases. In animal cases, organ failure or obstruction of infected erythrocyte in capillaries vessels as well as neurological signs may occur. Severe cases occur mostly in immunodeficiency such as HIV/AIDS patients or spleenectomize cases (http://en.wikipedia.org/wiki/Babesiosis).

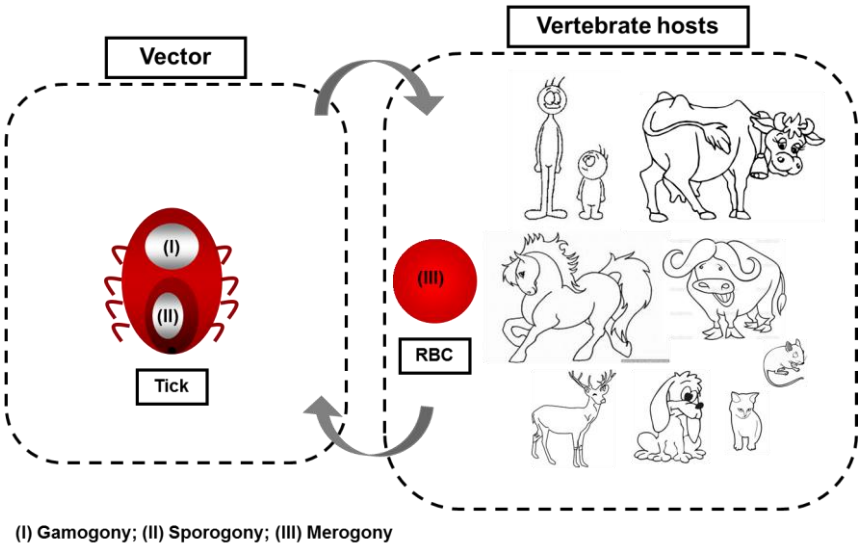

Fig. 1. *Babesia*'s life cycle

2. Bovine babesiosis

Bovine babesiosis had been known as tick fever or cattle fever. Bovine babesiosis is the major veterinary importance for livestock industry, as over half of the world's 1.2 billion cattle are at risk of infection and disease [McCosker, 1981; Bork *et al*., 2004]. At least five *Babesia* species are known to be responsible for bovine babesiosis; *B. bigemina*, *B. bovis*, *B. divergens*, *B. major* and *B. ovata* [Bock *et al*., 2004]. Bovine babesiosis is dominantly transmitted by *Ixodid* ticks. *Rhipicephalus* (*Boophilus*) *microplus*, *B. decoloratus*, *B. annulatus*, *B. geigyi* and *R. evertsi* are the vector transmitting *B. bigemina* and *B. bovis*. *Ixodes ricinus* and *I. persulcatus* are the vector transmitting *B. divergens*. *Haemaphysalis punctata* is the only vector of *B. major* while *H. longicornis* transmits *B. ovata* [Bock *et al*., 2004]. Economic losses in bovine babesiosis results from mortality, ill-thrift, abortions, reduction of milk/meat production and draft power as well as expensive control measures (such as acaricide treatments,

purchase of vaccines and therapeutics) [Bock *et al*., 2004]. It is estimated that this disease will cost the agriculture sector up to AUD 300 million in Australia [Sackett *et al*., 2006], as the cost of prevention and treatments. Moreover, approximately 23.9 million USD was calculated as losses from bovine babesiosis in many areas around the world [Bock *et al*., 2004]. Despite this estimate is likely to be an under representation of the local or global economic burden, since information of losses are often not up to date or readily available [Gohil *et al*., 2013].

This study is focusing on *B. bovis,* one of the five *Babesia* species responsible for bovine babesiosis, is characterized by extensive erythrocytic lysis leading to anemia, icterus, hemoglobinuria and death [Wright and Goodger, 1988; Wright *et al*., 1989; de Vos and Potgieter, 1994; Brown and Palmer, 1999; Ahmed, 2002]. *B. bovis* was first identified in Argentina in 1934 [Rees, 1934] and the infection usually occurs in the same areas as *B. bigemina*. However, it is generally conceded that *B. bovis* is more virulence than *B. bigemina* [Callow, 1979]. *B. bovis* infection is widely distributed and of major importance in Africa, Asia, Australia, Central and South America [OIE, 2008], where arthropod vectors are distributed. Current advances in understanding the biology of *B. bovis* are slow and difficult, with small amount of research groups working on this parasite worldwide, the biological characteristic questions remained unanswered and disintegrated [Gohil *et al*., 2013]. However, the ability to transiently and stably transform the parasite and genetically manipulate its genome have been revealed [Suarez *et al*., 2004, 2006; Suarez and McElwain, 2008, 2009, 2010]. These advances in comparative genomics, proteomic and transcriptomics, including the ability to culture the parasite *in vitro*, is now being the great potential to expand our knowledge of the biology of this parasite.

3. Equine babesiosis

Equine babesiosis or equine piroplasmosis (EP) is a tick-borne disease of horses, mules, donkeys and zebras. Characterized by acute haemolytic anemia [Maurer *et al*., 1962; Knowles *et al*., 1994; Zweygarth *et al*., 2002] and caused by two hemoprotozoan parasites; *Theileria equi* (Laveran, 1901) and *B. caballi* (Nuttall and Strickland, 1910). *B. caballi* and *T. equi* are transmitted by more than 15 species of the tick genera *Dermacentor, Hyalomma* and *Rhipicephalus* [Thompson, 1969; Friedhoff *et al*., 1990; Stiller *et al*., 2002]. *T. equi* can be also transmitted by *B. microplus* and *Amblyomma cajennese*. *B. caballi* persists in ticks throughout several generations, with transstadial and transovarial transmission. In contrast, only transstadial transmission occurs for *T. equi* within the tick vector [Knowles, 1999]. *T. equi* can also be transmitted iatrogenically by contaminated needles or surgical instruments [Brünning, 1996; Friedhoff *et al*., 1996].

The worldwide prevalence of EP is consistent with the distribution of competent tick vectors has been summarized by Rothschild [2013]. It is estimated that only 10% of horses globally and few countries in the world are free from native infection with EP. Yet, many areas currently free of EP are climatically suitable for appropriate tick vectors or already possess competent tick vectors and therefore, there is a continual threat of *T. equi* and *B. caballi* introduction into EP-free areas either by ticks or horses [Rothschild, 2013]. The typical clinical features of EP are fever, pale mucous membranes and icterus in horses, yet some cases with nonspecific to mild symptoms have reported [Zobba *et al*., 2008]. Horses infected with EP remain seropositive for several years (*B. caballi*) to life (*B. caballi* and *T. equi*) [Rothschild, 2013]. However,

acute cases, and death have been reported [Sippel *et al*., 1962; Taylor *et al*., 1969], which makes EP an important disease, with significant impact on international movement of horses [Knowles, 1996]. Economic losses associated with EP are significant and include the cost of treatment, especially in acutely infected horses, in abortion in the last trimester of gestation, loss of performance, death, and restrictions in meeting international requirements related to exportation or participation in equestrian sporting events [de Waal, 1992; Kerber *et al*., 1999; Lewis *et al*., 1999].

T. equi apparently initially develops in lymphocytes before the erythrocytic stage [Schein *et al*., 1981; Moltmann *et al*., 1901], forms the 'Maltese cross', and is resistant to babecidal drugs [Brünning, 1996]. Parasitemia level of *T. equi* infection usually ranges from 1% to 7% [Friedhoff and Soule, 1996] or maximum 95%. In *B. caballi* infection, parasitemia level typically does not exceed 1% and may be as low as 0.1% even in the clinical cases [de Waal, 1992].

B. caballi infection has been said to be self-limiting, lasting up to 4 years after infection [Holbrook, 1969]. However, many horses apparently recovered from *B. caballi* infection, later have relapse, suggesting a temporary state in which organism cannot be detected despite possible lifelong infection. Currently, reliable control methods do not exist, it is important to prevent the introduction of both infected horses and ticks into EP-free areas [Rothschild, 2013].

4. Clinical signs and diagnosis of babesiosis

4-1. Bovine babesiosis

Babesiosis caused by *B. bovis* has 10-14 days incubation time [Callow, 1984; OIE, 2008], which can be shorten by large inocula. Infection can persist for years,

perhaps even the life time of the animal. Moreover, *B. bovis* infection involves massive intravascular sequestration of infected erythrocytes [Aryeetey and Jimenez-Lucho, 2002; Allred, 2003]. Cerebral babesiosis, which is characterized by hyperaesthesia, convulsions and paralysis, often occur in acute infection when the infected erythrocytes sequestrate in cerebral capillaries, direct consequences are increased abortion rate and sterility, reduced milk and meat production and death [Bork *et al*., 2004].

B. bovis infection can be detected by clinical signs. Clinical manifestation associated with BV are typically haemolytic anemia but can vary according to agent (parasite strain) and host (immune response) [Delgliesh, 1993; Allred, 2003]. Bovine babesiosis caused by *B. bovis* is predominant in adult animal [Mahoney, 1974; Dalgliesh, 1993; Goff *et al*., 2001]. However, tolerant animals which do not show clear clinical sign or asymptomatic carrier animals such as local cattle in babesiosis endemic area, might be the limitation of clinical diagnosis. Routine laboratory diagnosis method is observation of circulating parasite in the Giemsa's stained thin blood smear under the light microscope. In addition, thick blood smear, organ smears acquired from necropsy such as cerebral cortex, kidney, liver, spleen and bone marrow can be done [Callow *et al*., 1993; Böse *et al*., 1995]. Unfortunately, *B. bovis* infection maximum parasitemia is less than 1% in acute cases [OIE, 2008]. For this reason microscopic examination has low sensitivity. Several serological diagnosis methods have been developed to detect the antibodies against parasite antigen. Enzyme-linked immunosorbent assay (ELISA) using the whole merozoite antigen was developed however, this method remains extensive evaluation. Competitive ELISAs using recombinant merozoite surface and rhobtry associated antigens have recently developed, but this method is not yet widely validated. Indirect fluorescent antibody test (IFAT) is widely used to detect antibodies

to the parasite, but cross-reaction with antibodies to *B. bovis* in the *B. bigemina* IFAT test, is a particular problem in the area where the two parasites coexist [Wright *et al*., 1987]. Complement fixation has also been used to detect *B. bovis* [Mahoney, 1964] and qualify animals for importation into some countries. In addition, dot ELISA, slide ELISA, latex agglutination and card agglutination test were developed, but none of these tests are adopted for routine diagnosis in laboratories [OIE, 2008].

4-2. Equine babesiosis

Clinical signs of EP consist of peracute, acute, subacute and chronic stage. *Babesia caballi* and *T. equi* infection in horses show similar clinical signs, although the signs associated with *B. caballi* infection tend to be milder or even unapparent [de Waal, 1992]. The majority of *Babesia*-seropositive horses are unapparent carriers with low levels of parasitemia and no obvious clinical signs. However, athletic or heavy work horses may have lower performance compared with non-infected horses and are also at risk of developing overt infection with clinical illness [Hailat *et al*., 1997]. Peracute EP primarily occurs in neonatal foals, which are infected in utero [de Waal, 1992; Lewis and Penzhorn, 1999; Phipps and Otter, 2004], in naïve adult horses suddenly introduced into areas with large numbers of infected ticks, and in adult horses infected after strenuous exercise [Hailat *et al*., 1997]. Peracute infection with *B. caballi* results in organ damage and dysfunction caused by obstruction of capillaries or other small vessels with parasitized erythrocytes. While during peracute *T. equi* infection, parasites replicate in erythrocytes, causing cell lysis and subsequently death from anemia, sudden death in adult horses is rare. Acute EP is characterized by pyrexia, moderate anorexia and malaise, frequent recumbency, dehydration, congested mucous membrane, tachypnea, tachycardia, sweating, limb edema, supraorbital edema and tearing.

Mortality can be moderate to high in untreated *T. equi* infection with intermittent pyrexia. In subacute cases EP exhibits various degree of anorexia, malaise, weight loss. Chronic EP typically presents a history of nonspecific clinical signs.

Diagnosis of EP can be done on the basis of clinical signs and the examination of blood smear. However, it is difficult to differentiate *T. equi* and *B. caballi* infections by clinical examination. Serological diagnostic such as complement fixation test (CFT), competitive enzyme-linked immunosorbent assay (cELISA), indirect IFAT, and ELISA have been developed for detection of antibodies against *T. equi* and *B. caballi* [McGuire *et al*., 1971; Knowles *et al*., 1991; Kappmeyer *et al*., 1999]. CFT is a very specific test but IFAT is more sensitive than CFT, and ELISA has improved performance compared to CFT and IFAT. However, some disadvantages have been reported for these serological methods: CFT has low sensitivity in chronic cases; IFAT is time consuming, requires large amount of antigens and is difficult to standardize. Hence, polymerase chain reaction (PCR) which is more sensitive than microscopic detection have been shown as an ideal tool for the detection of carrier infections [Bashiruddin *et al*., 1999; Nicolaiewsky *et al*., 2001; Rampersad *et al*., 2003]. *In vitro* organism cultivation method is also an alternative approach for testing the blood from suspected animals. Nevertheless, this method is laborious, expensive and inconsistent, making it inappropriate for commercial use [Rothschild, 2013].

5. Control and treatment of babesiosis

5-1. Bovine babesiosis

Current bovine babesiosis control strategies include vector control, administration of chemotherapeutic drugs and vaccination. The only available

antiprotozoan agents for bovine babesiosis are diminazene aceturate [de Vos, 1979] and imidazole dipropionate. Administration of imidocarb dipropionate (1.2-3.0 mg/kg, subcutaneous injection) can eliminate a number of bovine *Babesia* spp. And at higher dose can provide short-term (4 week's) protection against clinical babesiosis caused by *B. bovis* [Taylor and McHardy, 1979]. Nevertheless, the concern of drug residues in meat and dairy products from prolonged treatment led to the withdrawal of this drug in many European countries [Zintl *et al*., 2003; de Waal and Combrink, 2006; Mosqueda *et al*., 2012]. Moreover, repeated administration of therapeutic drugs is time consuming and costly, as well as misuse or prolonged use of these chemophylactic agents, may lead to the emergence of imidocarb-resistant parasite line [Rodriguez and Tree, 1996]. The development of vaccines against bovine babesiosis have generated [Callow, 1984; Grey *et al*., 1989; de Vos and Jorgensen, 1992; Callow *et al*., 1997], attenuated vaccines which have been produced and successfully used to control bovine babesiosis. These live attenuated vaccines are highly effective and essential in the control of bovine babesiosis, but their limitation include short shelf live, requirement of cold-chain and expensive cost of production [Wright and Riddles, 1989; Gohil *et al*., 2013]. Ideally, simultaneously use of acaricides and cattle tick vaccination programs will facilitate the control of babesiosis.

5-2. Equine babesiosis

Treatment strategy of EP aims to resolve the clinical signs during acute infection or completely clear the horse from the carrier state (sterilization). However, elimination of EP in carrier state is not recommend in endemic areas but may be desirable for animal moving into EP-free areas. The efficacy of drugs for treatment of EP is highly

variable, the thorough monitoring of horses from the beginning to the end of treatment is necessary to ensure success.

Imidocarb diproprionate (Imizol; Schering-Plough Animal Health) at 2.2 mg/kg up to 4 mg/kg intramuscular (IM) administration can be used for treatment of EP caused by *B. caballi*. Although relatively safe, toxicity with fatal outcomes in some horses can occur. Donkeys are particularly sensitive to imidocarb and treated animal have high mortality rates [Frerichs *et al*., 1973]. Diminazene aceturate (Di) (Berenil, Hoechst, Germany; Ganaseg, Squibb-Mathieson, Mexico) is effective for treatment of acute disease at 11 mg/kg (IM). But, swelling and necrosis at Di injection sites as well as primary signs of intoxication such as respiratory distress and depression have been reported. Amicarbalide (Diampron; May and Baker, England) at 9-10 mg/kg IM is often sufficient for treatment of horses with acute signs of EP. However, a delayed anaphylactic-type reaction, with respiratory and GI disturbances, periorbital, muzzle edema and subcutaneous edema over the back and flank, has been reported in some horses. Moreover, amicarbalide used at 2.2 mg/kg IM administration did not completely eliminate *B. caballi* infection [Brünning, 1996]. Euflavin (Gonacrine; May and Baker) at 4-8 ml/100 kg and tretracycline have also been reported as other treatments but do not completely eliminate the parasites.

Equine piroplasmosis caused by *T. equi* is often resistant to most of therapeutic agents. Imidocarb diproprionate (Imizol) at a dosage of 4 mg/kg IM is usually effective treatment [Grause *et al*., 2012] for clinical signs, and has also completely cleared *T. equi* in some cases. However, such a dosage causes moderate to severe signs after intoxication [de Waal, 1992], because it is near the 50% lethal dose (LD_{50}) for imidocarb. Intoxication clinical signs include salivation, restlessness, mild to moderate

colic and GI hypermotility, and local injection site reactions can also occur. Treatment in donkey can also causes adverse reactions. But, no significant adverse reactions were reported in zebras [Kumar *et al*., 2003]. Buparvaquone (Butalex; Coopers) have successfully resolved clinical signs from *T. equi* infection. Moreover, combination of buparvaquone with arteether (E-Mal, Thermis Laboratories, India), an anti-malarial drug, result in temporary clearance of infection in donkeys [Kumar *et al*., 2003] but has not been administrated in horses. Effective treatment of EP in pregnant carrier mares infected with *T. equi* or *B. caballi* has not been described. However, using imidocarb at dosages of 2.4 mg/kg in pregnant carrier mare resulted in elimination of the drug in the milk for approximately 2 hours after treatment and causes toxicity in nursing foals. In addition, safe and efficiency babesicidal drugs (e.g. imidocarb) in equine neonates do not exist. Taken together, imidocarb is recommend for treatment of mixed infection. But, the caution of toxicity and adverse side effect lead to consideration of finding new drug for EP.

6. Aims of the present study

B. bovis is a principal agent causing bovine babesiosis, the most economically important tick-borne disease affecting livestock industries worldwide [Brown and Palmer, 1999]. Infection with *B. bovis* is fatal due to its pathogenesis and neurological symptoms [Bork *et al*., 2004; Gohil *et al*. 2013; Munkhjargal *et al*., 2013]. The use of common chemotherapeutic drugs, namely imidazole diproprionate has been reported to predispose milk and meat to drug residues and diminazene aceturate causes adverse side effects and induce drug resistance [Vial and Gorenflot, 2006]. Furthermore, the lack of commercial vaccine [Homer *et al*., 2000; OIE, 2008] suggests that the intensive search

for new drug targets and new chemotherapeutic compounds as strategy to combat bovine babesiosis is required. The objective of this study is to validate a new drug target against bovine babesiosis. Molecular chemotherapeutic target namely dihydroorotate dehydrogenase have been characterized and evaluated on the most virulent species causing bovine babesiosis, *B. bovis*. Furthermore, application of this new chemotherapeutic target on equine piroplasmosis (EP) caused by *T. equi* and *B. caballi* have also been evaluated.

Chapter 1

Characterization of *Babesia bovis* dihydroorotate dehydrogenase (BvDHODH) as a drug target for babesiosis

1-1. Introduction

Babesia bovis is an intra-erythrocytic apicomplexan parasite of *Babesiidae* family, which is considered as principal agent causing bovine babesiosis, the most economically important tick-borne disease affecting livestock industries worldwide [Brown and Palmer, 1999]. Infection with *B. bovis* is fatal due to its pathogenesis and neurological symptoms [Bork *et al*., 2004; Uilenberg, 2006; Gohil *et al*., 2013]. The use of common chemotherapeutic drugs, namely imidazole diproprionate and diminazene aceturate has been reported to predispose milk and meat to drug residues, cause adverse side effects and induce drug resistance [Vial and Gorenflot, 2006]. Furthermore, the lack of commercial vaccine [Homer *et al*., 2000; OIE, 2008] suggests that the intensive search for new drug targets and new chemotherapeutic compounds as strategy to combat bovine babesiosis is required.

The pyrimidine biosynthesis pathway is essential for RNA, DNA, glycoproteins and phospholipids biosynthesis, which are important for division and growth of cells [Shambaugh, 1979; Phillips *et al*., 2008]. Six enzymes of *de novo* pyrimidine synthesis pathway have been identified from *B. bovis* homogenates, indicating self pyrimidines production ability (Fig. 2) [Gero *et al*., 1983]. Dihydroorotate dehydrogenase (DHODH) is the fourth enzyme in *de novo* pyrimidine biosynthesis pathway that

catalyzes the oxidation of dihydroorotate to orotate [Baldwin *et al*., 2002]. Inhibition of DHODH results in reduced levels of uridine 5' monophosphate (UMP), which is an essential pyrimidine precursor [Walse *et al*., 2008]. DHODHs have been identified as novel drug targets for malaria, toxoplasmosis and leishmaniasis [Heikkilä *et al*., 2006; Cheleski *et al*., 2010; Hortua Triana *et al*., 2012]. To date, no study has been carried out on *B. bovis* DHODH (BboDHODH) as chemotherapeutic target. Therefore, the study in this chapter, aimed to characterize BboDHODH.

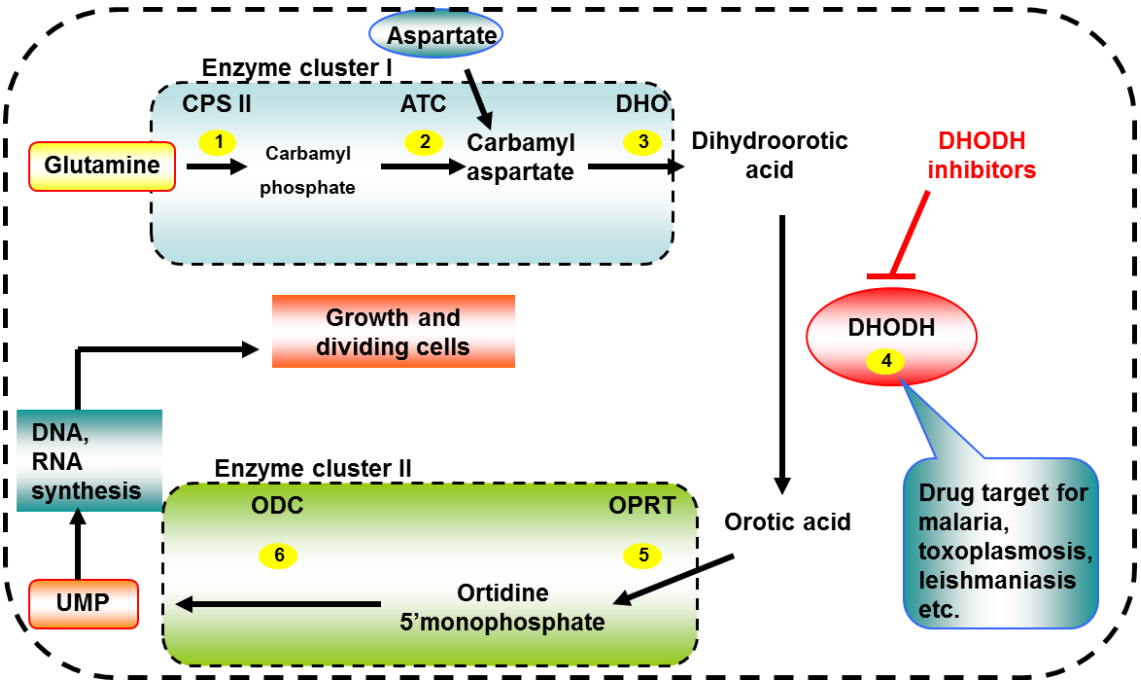

Fig. 2. Schematic of *Babesia bovis de novo* pyrimidine biosynthesis pathway. Six enzymes have been identified. 1) Carbamyl phosphate transferase [CPSII], 2) Asapartate transcarbamylase [ATC], 3) Dihydroorotase [DHO], 4) Dihydroorotate dehydrogenase [DHOHD], 5) Orotate phosphoribosyl transferase [OPRT] and 6) Orotidine 5'phosphate decarboxylase [ODC].

1-2. Materials and methods

***In vitro* cultivation of *Babesia* parasites.** *B. bovis* (Texas strain) was grown in bovine red blood cells (RBCs) using a continuous microaerophilous stationary phase culture system [Igarashi *et al*., 1998]. Cultivation of parasites was carried out using the GIT medium (Wako, Japan) supplemented with 1% penicillin and streptomycin (Sigma, USA). The overlaying medium was replaced daily. Culture plates of *B. bovis* were grown at 37°C in humidified CO_2 (5%) and O_2 (5%) incubator (BIO-LABO, Japan).

Cloning and bioinformatic analysis of BboDHODH. BboDHODH complete coding sequence gene available in GenBank database (accession no. XM001610187) was used for designing primers. The full-length BboDHODH gene was amplified from *B. bovis* cDNA template by PCR using primers with *Bam*HI and *Sac*I sites (italic), DHODH1F (5´-CGC*GGATCC*ATGTGCATTGCAGCAACCGGT-3´) and DHODH2R (5´-ACG*GAGCTC*TTACTTCTTTGTGGATTC-3´), using PCR amplification kit (Takara Ex Taq^{TM}, Japan). The thermocycling was performed as followed; initial denaturation for 5 min at 96ºC followed by 30 cycles of denaturation at 96ºC for 30 sec, annealing at 60ºC for 1 min and extension at 72ºC for 90 sec then elongation at 72ºC for 10 min. Purified PCR product was cloned into pGEM-T easy vector (Promega, USA), subsequently digested with *Bam*HI and *Sac*I and inserted into *Bam*HI and *Sac*I sites of pET-28a expression vector (Novagen, USA). The cloned BboDHODH was confirmed by sequencing using ABI PRISM 3100 sequencer (Applied Biosystems Inc., USA). Obtained nucleotide sequence was translated using Genetyx software (Genetyx Corporation, Japan), the functional domain and enzyme active sites of its polypeptide were analyzed by the BLAST search tool. Comparison of *B. bovis* DHODH with bovine and other apicomplexan parasites DHODHs gene sequence available in the GenBank

database was done using CLUSTAL X software. Phylogenetic analysis was generated using the neighbor-joining method incorporated into Mega 3.1 software.

Expression and purification of recombinant BboDHODH protein. The BboDHODH cloned in pET-28a vector was transformed into *Escherichia coli* Rosetta ™ 2 (DE3) competent cells (Novagen, Germany). Transformed *E. coli* BboDHODH-His were grown to A_{600} nm of range 0.5-0.8 in LB broth (Sigma Aldrich, Japan) with 50 μg/ml Kanamycin. Protein expression was performed according to the procedure previously described with some modifications [Baldwin *et al*., 2002]. Briefly, *E. coli* culture was induced with 1 μM IPTG, 0.25 μM FMN (riboflavin 5' phosphate sodium salt, Sigma Aldrich, Japan), and incubated at 18ºC for 20 hrs. The pellet was collected by centrifugation, suspended in 40 ml of lysis buffer (50 mM Tris-HCl pH 8.2, 0.005 mM FMN, 2 mM beta mercaptoethanol, 10% glycerol, 2% Triton X-100,), and disrupted by sonication on ice for 12 min. The supernatant was collected for protein purification. The rBboDHODH-His fusion protein was purified by a Ni-NTA affinity chromatography, according to manufacturer's instructions (Qiagen, Germany). The recombinant protein was analyzed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and its molecular weight was calculated using Gel Pro Analysis software. Protein concentration was measured using bicinchoninic acid (BCA) protein assay (Pierce Biotechnology Inc., USA) with bovine serum albumin (BSA) as a standard.

Production of mouse anti-rBboDHODH sera and characterization of native enzyme. Anti-rBboDHODH serum was produced in 6-week-old female ICR mice (Clea, Japan) following standard immunization regime. Briefly, three mice were intra-peritoneally immunized with 50 μg of purified rBboDHODH protein emulsified

with an equal volume of Freund's complete adjuvant (Sigma, USA). Thereafter, the same amount of antigen was emulsified with Freund's incomplete adjuvant (Sigma, USA) and administered to each mouse *via* the same route at days 14 and 28. Then, antiserum was collected from each mouse 14 days after the last booster [Cheleski *et al*., 2010]. To identify the native BboDHODH enzyme, *B. bovis* lysate was separated using a 12% SDS-PAGE and then probed with anti-rBboDHODH serum by Western blot analysis. In addition, an indirect fluorescence antibody test (IFAT) and confocal microscopy were performed with the same antiserum after labeling parasite's mitochondria with MitoTracker® probes (Invitrogen, UK).

Enzymatic activity of recombinant protein. The enzymatic activity of purified rBboDHODH was measured by monitoring 2,6-dichlorophenol-indophenol (DCIP) reduction [Hortua Triana *et al*., 2012]. The reaction contains 0.1 mM of L-dihydroorotic acid (L-DHO), 0.1 mM of decylubiquinone (Q_D) and 0.1 mM DCIP in DCIP buffer (50 mM Tris HCl, pH 8.0, 150 mM KCl, 0.1% Triton X-100 and 10% glycerol). After being separately incubated at 30°C for 30 min, rBboDHODH-His and reaction buffer were mixed and the DCIP reduction was measured at 600 nm (ε = 18,800 M^{-1} cm^{-1}). The reaction without recombinant protein was used as a control. The specific activity of the enzyme was measured in 96-well plate (Nunc, Denmark). Each well was filled with 200 μl of reaction mix containing 0.1 mM DCIP, 1 mM L-DHO, 0.1 mM Q_D and 0.205 μg of recombinant protein. To further evaluate the enzymatic activity of the rBboDHODH, 0.1 mM coenzyme Q_{10} and 0.1 mM sodium fumarate dibasic (FMN) were used as an alternative electron acceptor in the DCIP reduction assay. The kinetic constants of L-DHO and Q_D were determined by varying L-DHO concentration (0.01 to 8.0 mM) with Q_D fixed at 0.1 mM or by varying Q_D concentrations (0.02 to 0.8 mM) at a fixed L-

DHO concentration of 1 mM. Each reaction was repeated at least 3 times, and then, the Michealis-Menten equation was used to calculate K_m (GraphPad, USA).

1-3. Results

Bioinformatic analysis of BboDHODH. The amplified and sequenced BboDHODH gene has an open reading frame of 1,248 bp encoding 416 amino acids with a predicted 44-kDa molecular weight. The predicted amino acid residues were predominantly hydrophobic (52.64%), although 25.96% and 21.15% were hydrophilic and neutral, respectively. Computational analysis of BboDHODH revealed the presence of DHOD 2-like region, known as functional domain containing active sites, substrate binding sites, quinone interaction sites and FMN binding sites (Fig. 3A). There was no signal peptide in the BboDHODH sequence as predicted by signalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Comparison of BboDHODH with *Bos Taurus* DHODH (BosDHODH) amino acid sequence showed 42% similarity. Interestingly, the Thr103 (FMN binding site) and Thr281 (substrate binding site) observed in the parasite enzyme (BboDHODH) were replaced by Ser117 and Ser282 in the host enzyme (BosDHODH) (Fig. 3B). In addition, the comparison of *B. bovis* DHODH with other apicomplexan DHODH 2-like enzymes revealed 42%, 40%, 57% and 59% homology with *Plasmodium falcifarum* (accession no. AAC37170.1), *Toxoplasma gondii* (accession no. EPT31358.1), *Theileria orientalis* (accession no. BAM39885.1) and *T. equi* (accession no. XP004828711.1), respectively. Moreover, the phylogenetic analysis showed that *B. bovis*, *T. equi* and *T. orientalis* DHODHs belong to the same cluster, which was distinct from mammalian DHODHs (Fig. 4).

Expression and characterization of BboDHODH. The recombinant BboDHODH was successfully expressed as soluble protein with 42.4-kDa on 12% SDS-PAGE (Fig. 5A). However, the apparent molecular weight of the recombinant (42.4-kDa) was smaller than expected as calculated from amino acid including His tag (47-kDa = 44-kDa + 3-kDa). This difference of molecular weight can be explained by the interaction of SDS with hydrophobic residues, the ionic strength of denatured protein and conformation of denatured proteins which altogether may affect the migration behavior on SDS-PAGE [Rath *et al*., 2009]. Mouse anti-rBboDHODH reacted with *B. bovis* lysate yielding a specific band of approximately 44-kDa, but did not react with bovine RBC lysate. Additionally, non-immunized mice sera neither reacted with the parasite nor host's RBC lysates (Fig. 5B). Confocal laser microscopy revealed that the mice anti-serum reacted with *B. bovis* DHODH yielding the specific green fluorescence overlaid with a red fluorescence representing parasite's mitochondria (Fig. 5C). This result suggests that BboDHODH is expressed in merozoites stage and may localize in parasite mitochondria.

Enzymatic activity of recombinant protein. The BboDHODH (EC1.3.3.1)-catalyzed oxidation of dihydroorotate was measured in the presence of decylubiquinone as electron acceptor using the DCIP reduction assay. Recombinant BboDHODH exhibited enzymatic properties with specific activity of 475.7 ± 245 Unit/mg and the kinetic constant for this enzyme revealed K_m values of 276.2 μM and 94.41 μM for L-DHO and Q_D, respectively (Table 1). Histidine fusion protein did not affect the enzymatic activity as previously described (Baldwin *et al*., 2002). DCIP reduction assay with alternative electron acceptors showed that BboDHODH could oxidize dihydrootate to orotate via Q_{10} or FMN with relative activity of 64.1% and 97.7%, respectively.

1-4. Discussion

DHODH has been studied as chemotherapeutic target in certain apicomplexan parasites, but not in *B. bovis*. Therefore, this study validated *B. bovis* DHODH as a novel chemotherapeutic target. Bioinformatics analyses revealed that amino acid sequence of *B. bovis* DHODH mostly contained hydrophobic residues. The N-terminal region was highly divergent and shorter than those of *T. gondii*, *P. falciparum* and *B. taurus* DHODHs. BboDHODH contains both quinone and FMN binding sites, which support the ability of BboDHODH enzyme to use either quinone or FMN as electron acceptor. Generally, two forms of DHODHs have been documented; the cytosolic (class 1), which utilizes fumarate or NAD^+ as an electron acceptor and the membrane-bound (class 2), found in eukaryotes which use quinone as an electron acceptor [Björnberg *et al*., 1997; Sierra Pagan *et al*., 2003]. These results suggest that BboDHODH is a membrane-bound (class 2) DHODH, same as other apicomplexa DHODHs, which have been studied earlier as a chemotherapeutic target [Baldwin *et al*., 2002; Phillips *et al*., 2010]. Moreover, the differences of substrate binding sites of *B. bovis* versus the host DHODH, suggest that BboDHODH might have different biochemical properties from their host and highlight the possibility of this enzyme as novel drug target against bovine babesiosis. In addition, BboDHODH enzyme was found as a 44-kDa expressed in erythrocytic stage and was detected in parasite mitochondria. Localization result was consistent with previous finding (Rawls *et al*., 2000), which indicates that the DHODH protein is located within mitochondrial intermembrane space. This location may allow free diffusion of the DHODH substrate (dihydroorotate) and product (orotate) through the outer mitochondrial membrane from and to the proceeding and subsequent steps of

de novo pyrimidine biosynthesis [Chen and Jones, 1976]. Nevertheless, further studies using electron microscopy might be necessary to precisely confirm BboDHODH localization within parasite mitochondria.

Enzymatic assay revealed that the recombinant BboDHODH was highly active with a specific activity 95,000 fold higher than the native BboDHODH [Gero *et al*., 1983] which might be explained by the lower purity and yield of protein obtained from the parasite extracts. In addition, the specific activity of rBboDHODH was six fold higher than recombinant TgDHODH which suggests that the enzymatic activity of rBboDHODH observed in the present study was similar to the previous reports on apicomplexan DHODH [Hortua Triana *et al*., 2012].

1-5. Summary

This study aimed to characterize BboDHODH using bioinformatics analysis, recombinant production technology and enzymatic analysis. Bioinformatic analysis found that BboDHODH contains DHODH 2 like region and homologue with other apicomplexa DHODH enzyme. Differences between the parasite and its host at FMN binding site and substrate binding site were found, suggests that parasite enzyme might exhibit different enzymatic properties compared with the host enzyme. Furthermore, the recombinant BboDHODH protein was successfully produced in *E. coli* system and it was active recombinant enzyme. Native BboDHODH was also detected in the parasite lysate as well as localization showed that this enzyme exist in the parasite mitochondria.

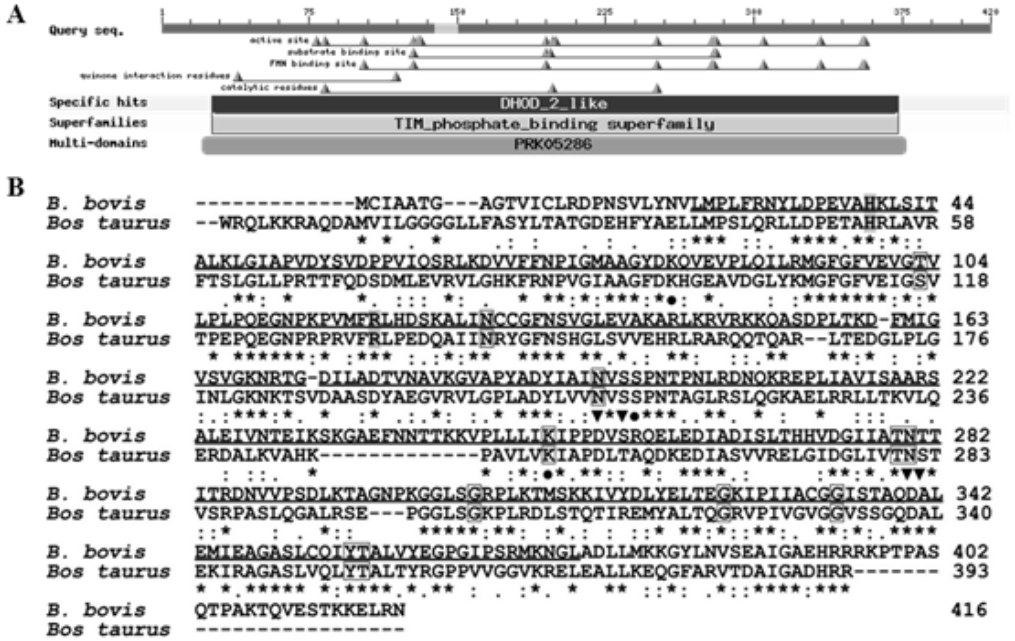

Fig. 3. Bioinformatics analysis of translated BboDHODH polypeptides. (A) The predicted functional domains of BboDHODH as shown by BLASTp. (B) Alignment of *B. bovis* DHODH with bovine DHODH enzyme. Completely conserved residues are highlighted as asterisk, DHODH 2 like region is shown as underline, quinone binding sites are shown in grey, fumarate binding sites are shown in box, active sites are shown as (●) and substrate binding sites are shown as (▼).

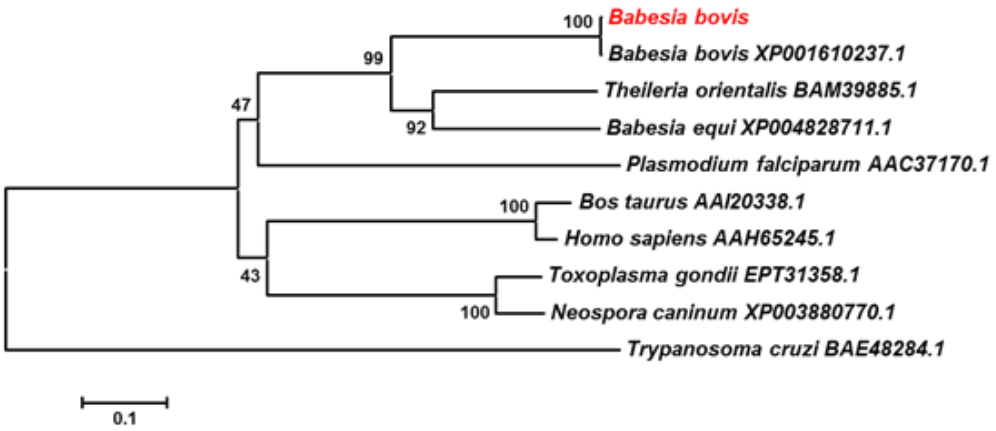

Fig. 4. A phylogenetic tree based on the DHODH amino acid sequences of apicomplexa protozoan and mammalian. The tree was generated using the neighbor-joining method incorporated into the MEGA 3 program.

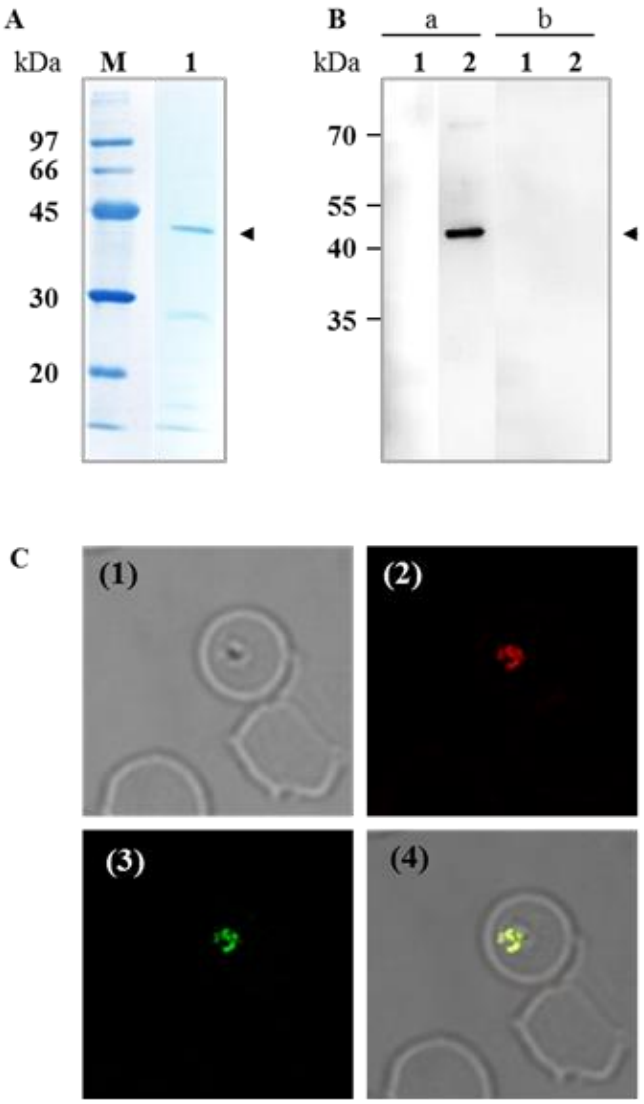

Fig. 5. Characterization of native BboDHODH. (A) SDS-PAGE showing purified BboDHODH-His stained with amide black. M: Low molecular marker; lane 1: purified rBboDHODH-His. (B) Western blot analysis showing reaction of anti rBboDHODH mice sera with parasite lysates. Lane 1, non infected bovine RBC lysates; lane 2, *B. bovis* lysates; lane a, anti-rBboDHODH mice sera; lane b, non-immunize mice sera. The arrowhead shows BboDHODH protein. (C) Localization of BboDHODH by IFAT. (1) Phase-contrast; (2) MitoTracker (red); (3) Anti-rBboDHODH (green), (4) The overlaid of fluorescence reactivity.

Table 1. Enzymatic properties of BboDHODH recombinant protein.

Substrate	K_m (μM)	V_{max} (nM min^{-1})	Specific activity (Unit/mg)
L-DHO	276.2	50.69	475.7 ± 245
Q_D	94.41	65.49	

Chapter 2

Effect of dihydroorotate dehydrogenase (DHODH) inhibitors on growth of *Babesia bovis in vitro*

2-1. Introduction

Drug residues in milk and meat product, adverse side effects on the host and resistant on drug line after long treatment have been associated with current bovine babesiosis treatment and suggest that the need of finding more efficient chemotherapeutic drug is required. The validation of DHODH a new chemotherapeutic target will be mentioned in this chapter. Dihydroorotate dehydrogenase (DHODH) shows characteristic of a potential chemotherapeutic target by playing an important role in the pyrimidine biosynthesis pathway. The discovery of new drug targeting *Plasmodium falciparum* DHODH for treatment of malaria revealed its potential as molecular target for babesiosis treatment. DHODH can be inhibited by napthoquinone analog, and its derivatives. Atovaquone (ATV), an napthoquinone analog and approved anti-malarial drug, leflunomide (LFN), an antirheumatic drug and brequinar (Breq), an immunosuppressive agent, have been identified as DHODH inhibitors [Greene *et al*., 1995; Knecht and Löffler, 1998; Löffler *et al*., 1998]. Furthermore, triazolopyrimidine derivatives have been evaluated on *P. falciparum* and showed promising inhibitory effects on parasite growth [Guijar *et al*., 2011]. Interruption of this enzyme by using chemotherapeutic compounds may affect the

growth of *Babesia* parasites. Despite the availability of compounds that successfully inhibit DHODH in other apicomplexan parasites, no study has yet been carried out on *B. bovis* DHODH (BboDHODH) as chemotherapeutic target. In this chapter, the effect of DHODH inhibitors namely ATV, Breq, LFN and TAZ have been evaluated on rBboDHODH enzymatic activity. These DHODH inhibitors were also evaluated on the growth of *B. bovis in vitro* in comparison with a control drug, diminazene aceturate (Di).

2-2. Materials and methods

***Babesia bovis in vitro* culture.** Cultivation of *B. bovis* parasite was as explained in the chapter one. The infected erythrocytes were harvested on the day fourth with high parasitemia level (5-10%) before use in the *in vitro* inhibition assay.

Cloning and protein expression. BboDHODH gene was amplified from the *B. bovis* cDNA using PCR with the specific primers. The BboDHODH PCR product was digested with *BamH*I and *Sal*I restriction enzymes and cloned into pET-28a expression vector. Expression and purification of the rBboDHODH protein were as explained in the first chapter.

DHODH inhibitors and control drug. Dihydroorotate dehydrogenase inhibitors and control drug were purchased from Sigma Aldrich, included atovaquone (ATV), leflunomide (LFN), brequinar (Breq), 7-hydroxy-5-[1,2,4] triazolo [1,5,a] pyrimidine (TAZ) and Diminazene aceturate (Di), the control drug. These compounds were dissolved in either dimethyl sulfoxide (DMSO) or distilled water (DW) as the stock solution according to the manufacturer's instructions. The concentration of stock

solution of each compound was prepared as highest as possible according to the compound solubility.

Effect of DHODH inhibitor on rBboDHODH enzymatic activity. To evaluate the inhibition effect of DHODH inhibitors on rBboDHODH enzyme, the DCIP reduction assay have been performed in 96-well plate (Nunc, Denmark). Briefly, each of DHODH inhibitor was diluted in the DCIP buffer at the desired concentration. The reaction of 200 μl contains 0.1 mM of L-dihydroorotic acid (L-DHO), 0.1 mM of decylubiquinone (Q_D) and 0.1 mM DCIP in DCIP buffer (50 mM Tris HCl, pH 8.0, 150 mM KCl, 0.1% Triton X-100 and 10% glycerol). The reaction without DHODH inhibitor (relative activity was set as 100%) and the desired concentration of solvent (DMSO or DW) were used as negative controls. The relative activity of rBboDHODH was evaluated in presence of 1 μM ATV, 1 mM LFN, 0.1 mM Breq and 1 mM TAZ as the desired concentrations.

Effect of DHODH inhibitors on *B. bovis* growth *in vitro*. The effects of DHODH inhibitors on *B. bovis* growth were evaluated using 96-well plate (Nunc, Denmark), according to the procedure previously described [Salama *et al*., 2013]. Atovaquone, Breq, LFN and TAZ were dissolved in dimethyl sulfoxide (DMSO) (Wako, Japan) as stock solutions, while Di was prepared in distilled water. These compounds were individually added to the parasite cultures at the following concentrations, 0.04 to 10,903.94 nM of ATV, 19.66 to 20,133.88 nM of Breq, 0.26 to 1,000 μM of LFN, 1.95 to 2,000 μM of TAZ and 0.03 to 2,000 μM of Di. *B. bovis* cultures containing only 0.2% DMSO, 1% DMSO and 0.2% distilled water were used as controls. The inhibition assay was conducted for four days, and the overlaying medium was replaced daily with fresh medium containing the indicated concentration

of each compound. Level of parasitemia and morphological changes of parasites were monitored daily by microscopic examination of Giemsa-stained thin blood smear. The half maximal inhibitory concentration (IC_{50}) value for each compound was calculated (GraphPad, USA) based on parasitemia level recorded on the third day of *in vitro* culture.

Viability of parasites. The viability of parasites after treatment with each compound was also evaluated. Briefly, 4 μl from treated parasites and controls were sub-cultured in GIT medium with bovine RBCs without inhibitors for 10 days. Parasite recrudescence was determined under a light microscope.

Hemolytic activity assay. To evaluate toxicity of inhibitors on bovine RBCs, hemolytic activity assay was conducted as previously described [Silva *et al*., 2013]. Briefly, 10 μM ATV, 1000 μM LFN, 20 µM Breq, 1,000 TAZ, 2,000 nM Di, 0.2% DMSO and 0.2% DW were incubated with bovine RBCs at 37ºC for 30 min. The supernatant was collected by centrifugation at 5,000 rpm, then hemoglobin level at OD_{495} was measured. Distilled water and culture media were used as positive and negative controls, respectively. The hemolytic activity of each inhibitor was calculated based on the OD_{495} value.

Supplementation with ORA and UMP on ATV treated parasite. To further elucidate the action mode of DHODH inhibitor on growth of *B. bovis*, orotic acid (ORA) or uridine 5' monophosphate (UMP) and the inhibitor were simultaneously added to parasite cultures. ATV treated parasites were supplemented with 25, 50 and 100 μM of ORA or UMP. Non-treated parasites and ATV treated but non-supplemented parasite cultures were used as controls. The level of parasitemia was determined using Giemsa-stained thin blood smear at 48 hrs after supplementation.

Statistical analysis. Effects of DHODH inhibitors and ORA or UMP supplementation were analyzed by comparing treated and supplemented parasites to controls using ANOVA and Chi-square (χ^2) test (GraphPad). Data were considered statistically significant when the *P* value was less than 0.05.

2-3. Results

Effect of DHODH inhibitor on rBboDHODH enzymatic activity. rBboDHODH relative activity in the presence of 1 μM ATV, 1 mM LFN, 0.1 mM Breq and 1 mM TAZ was 19.1%, 51.5%, 106.5% and 101.0%, respectively, compared to the negative control (Table 2). The DMSO or DW solvent alone did not affect the relative activity of the recombinant BboDHODH enzyme (data not shown).

Hemolytic activity assay. Dihydroorotate dehydrogenase inhibitors at the highest concentration were incubated with bovine RBCs in order to evaluate the hemolytic activity. ATV at 10 μM, LFN at 1,000 μM, Breq at 20 μM, TAZ at 1,000 μM, Di at 2,000 μM, DMSO at 0.2%, and DW at 0.2% showed hemolytic activities lower than 10%. While, the positive control showed 100% hemolytic activity (Fig. 6). The concentration of DHODH inhibitors used in this study has no effect on bovine RBCs.

Effect of DHODH inhibitors on *B. bovis* growth *in vitro*. In order to assess the effect of DHODH inhibitors on the growth of parasites, *B. bovis* cultures were treated with ATV, LFN, Breq, TAZ and Di at different concentrations, and the parasites were monitored for 4 days. Strikingly, treatment with ATV and LFN significantly inhibited parasites growth from 48 hrs post treatment ($P<0.001$). Atovaquone at 20.76 nM decreased the growth of parasites by 92.2 ± 5.5% (Fig. 7A-1),

while at 64 μM, LFN decreased parasite growth by 49.3 ± 15.9% (Fig. 7A-2). On the other hand, the inhibitory effect by Di at 30 nM was 64.2% (P<0.001), observed from 24 hrs post treatment. However, *B. bovis* growth was not affected by Breq and TAZ treatment. The IC_{50} values of ATV, LFN and Di were 2.38 ± 0.53 nM, 52.41 ± 11.47 μM and 0.50 μM, respectively (Table 3). Morphological changes were observed 48 hrs post treatment with ATV and LFN as the treated parasites showed dot-like and shrank shapes. Similar shapes were also observed in parasite cultures treated with Di (data not show). Viability test after drug withdrawal showed that there was no re-growth of parasite in cultures previously treated with ATV, LFN and Di. At concentration of 0.48 nM ATV, 500 nM LFN and 120 nM Di (Fig. 7C), the parasites exhibited dot-like and shrank shapes (data not shown).

Effect of ORA and UMP supplementation on the growth of DHODH inhibited parasites. To confirm that the effects of DHODH inhibitors was directly related to an orotate or UMP deficiency. Atovaquone treated parasites were supplemented with ORA and UMP. Inhibitory assay results suggested that ATV was the most effective inhibitor against *B. bovis* growth. Therefore, only ATV at 100 nM was used to totally inhibit the growth of *Babesia bovis* in supplementation assay. Supplemented parasite cultures exhibited significantly higher percentage of parasitemia (>0.30%) compared to non-supplemented (control) (P<0.05) (Fig. 8). Supplementation of ORA at 25, 50 and 100 μM in ATV treated parasite cultures led to 1.20 ± 0.2%, 1.50 ± 0.3% and 1.36 ± 0.4% parasitemia, respectively. The slight decrease of parasitemia at 100 μM ORA supplementation might be related to the toxicity of ORA at high concentration. On the other hand, supplementation of UMP at 25, 50 and 100 μM in ATV treated parasite cultures led to 1.15 ± 0.3%, 1.26 ± 0.2%

and 1.93 ± 0.4% parasitemia, respectively. These results suggest that supplementation with ORA or UMP prevent the parasite starvation by probably providing pyrimidine precursors needed for *B. bovis* survival.

2-4. Discussion

The enzymatic activity of rBboDHODH could be specifically inhibited by ATV and LFN, but not by Breq and TAZ. According to previous reports, Breq is effective on human DHODH enzyme, but not on rat, *T. gondii* and *Ustilago maydis* DHODH enzymes, suggesting its species related inhibitory effect [Knecht and Löffler, 1998; Zameitat *et al*., 2007; Hortua Triana *et al*., 2012]. Similarly, the *in vitro* inhibition assay showed that ATV and LFN significantly inhibited *B. bovis* growth, while Breq and TAZ did not affect the parasites. ATV was the most effective drug in this study, as its IC_{50} value was lower than those of LFN and Di (control drug). Moreover, ATV IC_{50} was lower than those of Epoxomicin [AbouLaila *et al*., 2010a], EGCG [AbouLaila *et al*., 2010b], Fusidic acid [Salama *et al*., 2013] and Trisubstitute pyrrole [Lau *et al*., 2013] which were recently evaluated as anti-*B. bovis* compounds. However, ATV inhibitory effect was similar to Apicidin (IC_{50} = 7.1 ng/m*l*) [Munkhjargal *et al*., 2009]. Our results are consistent with previous reports on the *in vitro* inhibitory effect of ATV on *T. gondii* [Ferreira *et al*., 2012] and *B. gibsoni* [Matsuu *et al*., 2004]. Interestingly, ATV has already been successfully used to treat *P. falciparum, T. gondii, B. microti*, *B. gibsoni* and *B. divergens* infections [Pudney and Gray, 1997; Korsinczky *et al*., 2000; Birkenheuer *et al*., 2004; Matsuu *et al*., 2004; Ferreira *et al.*, 2012; Oz and Tobin, 2012]. This study has lighted up the value of ATV on treatment of bovine babesiosis. Comparison the IC_{50} value of ATV on *B. bovis* was

similar to those of *P. falciparum*, but 2,500 less sensitive compared to *T. gondii*. These indicates the species specific effect of ATV on DHODH.

De novo pyrimidine biosynthesis pathway, initiate from the glutamate amino acid precursor through six enzymes, which are responsible for biochemical reactions, for UMP production. DHODH is the fourth enzyme which oxidize dihydroorotic acid (DHO) to orotic acid (ORA). The speculation beyond using DHODH inhibitors is that inhibition of the oxidation of DHODH will result in the reduction of UMP, which may affect the survival of the parasites. In this chapter, the results showed that DHODH was inhibited using ATV and LFN. However, ATV is more effective against DHODH enzyme as it decreased the relative activity to 19%. Thereafter, to understand the mechanism of ATV inhibitor on the growth of parasites, supplementation with ORA or UMP on ATV-treated parasites have been conducted and the result showed that supplementation improved their growth. This finding agrees with a previous report [Zameitat *et al*., 2007] and suggests the supplementation compensates the growth after disruption of DHODH. This result emphasized that ATV affects the growth of parasites by interrupting orotic acid production and consequently, reduced the UMP production.

2-5. Summary

Previously, rBboDHODH was produced and its properties were confirmed, hence native BboDHODH existence in *B. bovis* parasite was clarified. Based on the previous results, the aim of this chapter was to evaluate BboDHODH enzyme as a novel chemotherapeutic target for treatment of bovine babesiosis. rBboDHODH produced from the first chapter was used for evaluation of DHODH inhibitors on the

enzymatic activity. Moreover, DHODH inhibitors effect on the growth of *B. bovis in vitro* were evaluated. The findings of this chapter suggest that BboDHODH can be inhibited by DHODH inhibitors, and that among DHODH inhibitors ATV is the most effective. The results also proved that ATV could significantly inhibited the growth of the parasites by disrupting pyrimidine biosynthesis. Taken together, these findings confirmed that BboDHODH is a novel chemotherapeutic target and ATV could be a beneficial drug for controlling *B. bovis* infection.

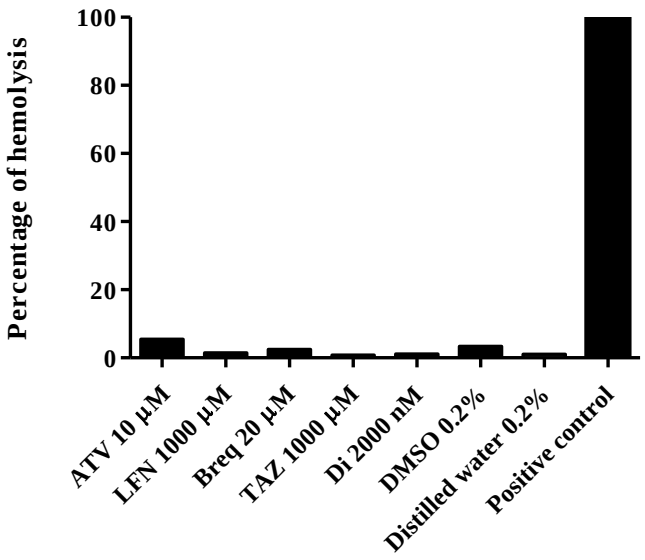

Fig. 6. Hemolytic assay showed that the DHODH inhibitor at the highest concentration used in this study did not affect bovine RBC.

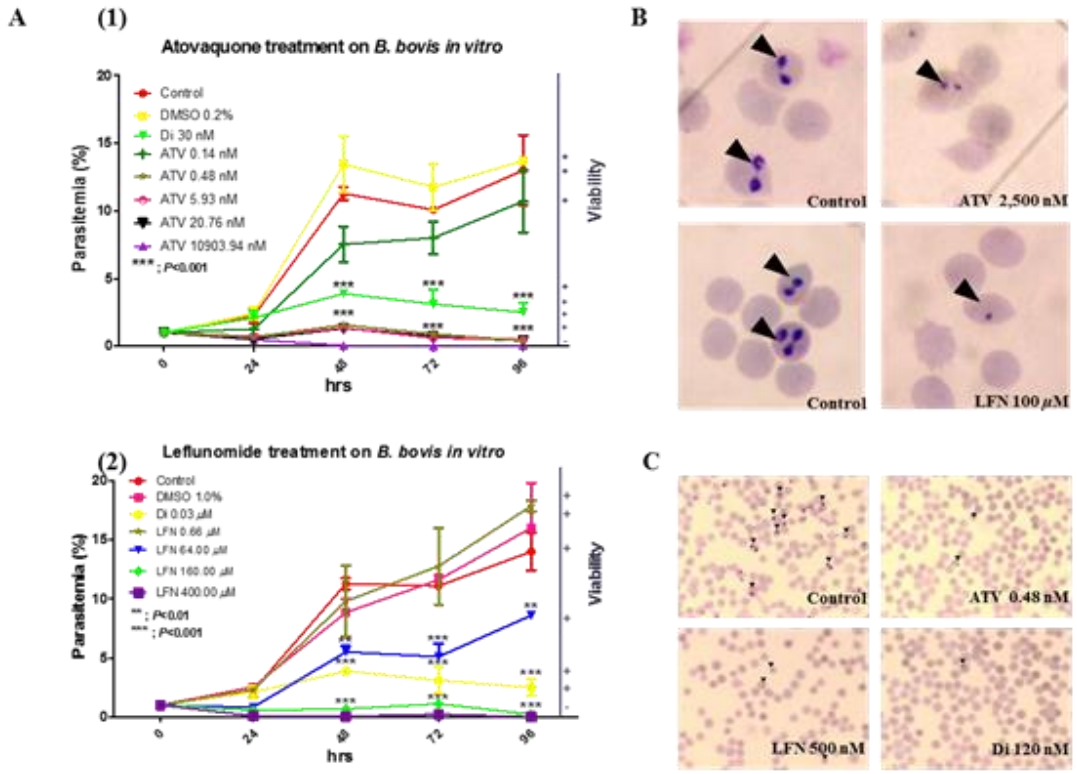

Fig. 7. *In vitro* inhibition assay. (A-1) Growth of *B. bovis* parasites was inhibited by treatment with atovaquone (ATV) and (A-2) leflunomide (LFN) from 48 hr post treatment. (B) Morphological changes of parasites after treatment with ATV and LFN, respectively. (C) Viability of parasite after treatment by ATV, LFN and Di, respectively at the significant inhibitory concentration (observed at day 10 after drug withdrawal).

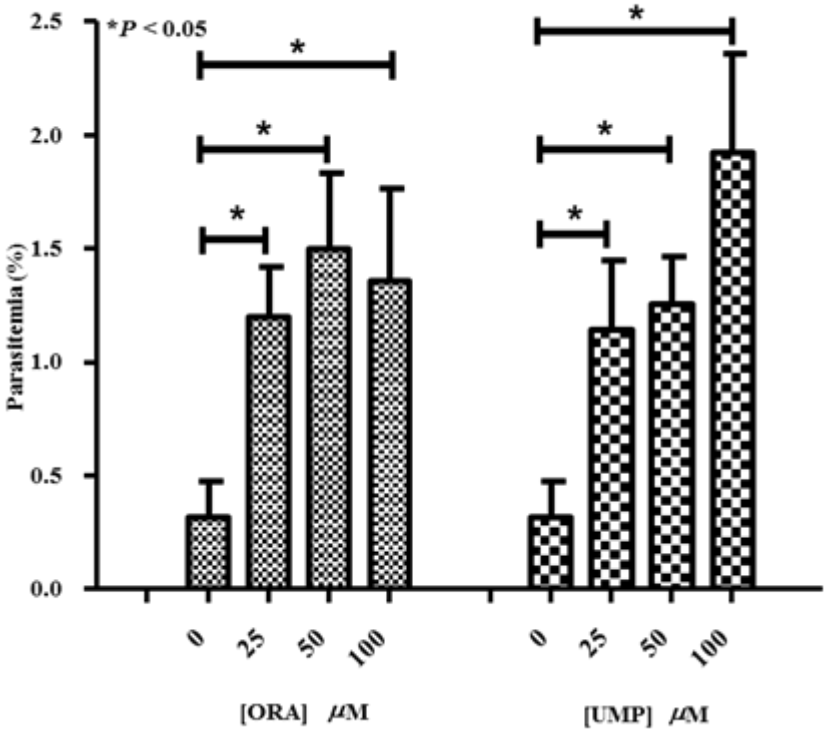

Fig. 8. Supplementation of orotic acid (ORA) and uridine 5' monophosphate (UMP) to ATV treated *B. bovis* parasites. The difference between parasitemia level of non-supplemented and supplemented groups was considered statistically significant when $P<0.05$.

Table 2. Relative activity of rBboDHODH protein in presence of different DHODH inhibitors.

Inhibitor	Relative activity (%)
ATV (1 μM)	19.10
LFN (1 mM)	51.58
Breq (0.1 mM)	106.50
TAZ (1 mM)	101.00
Without inhibitor	100.00

The reaction was measured using DCIP reduction assay, 200 μl-reactions contained 0.1 mM DCIP, 1 mM L-DHO, 0.1 mM Q_D and 0.205 μg of the recombinant protein. Relative activity of rBboDHODH in presence of inhibitor was compared to reaction of the enzyme without inhibitor which is taken as 100%.

Table 3. IC_{50} value of DHODH inhibitors on the growth of *Babesia bovis in vitro*.

Inhibitors	IC_{50}
ATV	2.38 ± 0.53 nM
LFN	52.41 ± 11.47 μM
Breq	Not effective
TAZ	Not effective
Diminazene aceturate (Di)	0.50 μM

Chapter 3

Effect of dihydroorotate dehydrogenase (DHODH) inhibitors on the growth of *Theileria equi* and *Babesia caballi in vitro*

3-1. Introduction

Equine piroplasmosis (EP) is caused by two distinct apicomplexa protozoan parasites *Babesia caballi* and *Theileria equi* and affects all members of the *Equus* genus (horses, donkeys, zebras and their cross breeds) [OIE, 2008]. It is found globally where tick vectors are present and is endemic in most tropical and subtropical areas as well as temperate climatic zones including many parts of Europe, Africa, Arabia and Asia (except Japan) [Brüning, 1996]. Horses infected with EP remain seropositive from several years (*B. caballi*) to life (*B. caballi* and *T. equi*) [Rothschild, 2013]. However, the active infection coinciding with anemia result in acute cases and death are reported which makes of EP an important disease, with a significant impact on international movement of horses [Knowles, 1996]. Economic losses associated with EP are significant and include the cost of treatment, especially in acutely infected horses, abortion in the last trimester of gestation, loss of performance, death, and restrictions in meeting international requirements related to exportation or participation in equestrian sporting events [de Waal, 1992; Kerber *et al*., 1999; Lewis *et al*., 1999].

T. equi is resistant to babecidal drugs [Brünning, 1996] and diminazene aceturate treatment in horses and mules result in mild and severe toxicity [Tantasuwan *et al*., 2004]. Currently, reliable control methods do not exist, it is important to prevent

the introduction of both infected horses and ticks into EP-free areas [Rothschild, 2013]. Therefore, this chapter aimed to find new chemotherapeutic for treatment of EP based on the inhibition of DHODH. Previous chapters have concluded that DHODH could be a novel chemotherapeutic target for treatment of bovine babesiosis, in this chapter, the benefits of targeting DHODH is applied to treatment of EP. Effect of ATV, Breq, LFN and TAZ on the growth of *T. equi* and *B. caballi in vitro* were evaluated.

3-2. Materials and methods

***In vitro* culture of parasites.** *T. equi* and *B. caballi* were used. Cultivation of *T. equi* parasites was carried out using the M199 medium (Sigma Aldrich, USA) supplemented with 1% penicillin and streptomycin (Sigma, USA), 40% horse serum, 20% horse RBC and 1% hypoxanthine. Cultivation of *B. caballi* parasites was carried out using the RPMI medium (Sigma, USA) supplemented with 1% penicillin and streptomycin (Sigma, USA), 40% horses serum and 20% horse RBC. The overlaying medium was replaced daily. Culture plates of *T. equi and B. caballi* were grown at 37°C in humidified CO_2 (5%) and O_2 (5%) incubator (BIO-LABO, Japan). The percentage of parasitized erythrocytes was estimated at day 4 by microscopic observation on Giemsa's stained slide. *T. equi* and *B. caballi* parasite lysates were prepared as mentioned in the first chapter for detection of native DHODH enzyme by western blotting.

Bioinformatic analysis. *T. equi* dihydroorotate dehydrogenase (TeDHODH) amino acid sequences (accession no. XP004828711.1), *B. bovis* DHODH (XP001610237) and *Plasmodium bergei* DHODH (PbDHODH) (accession no. XP679243.1) were obtained from GenBank database and compared using CLUSTAL

2.0. Prediction of TeDHODH molecular weight was performed using Genetyx Ver. 7.0 (Genetyx cooperation, Japan).

Detection of *T. equi* and *B. caballi* native DHODHs. Mice anti *B. bovis* DHODH serum was produced as mentioned in the first chapter. Cross-reaction between BboDHODH against *T. equi* and *B. caballi* lysates were confirmed using Western blotting. Briefly, *T. equi*, *B. caballi* and *B. bovis* parasite lysates were made to migrate in the 12% SDS-PAGE and transferred onto Immobilon PVDF membrane (Millipore, Germany). Transferred membrane was probed with mice anti rBboDHODH serum, thereafter probed with goat anti mice IgG HRP conjugate (Bethyl, Germany). Reaction of native DHODH was developed using Immun-star® HRP Chemiluminescence Kits (Biorad, USA) and visualized under the chemiluminescence visualizer (Biorad, USA).

Effect of DHODH inhibitors on equine piroplasms *in vitro*. DHODH inhibitors included ATV, Breq, LFN, TAZ and Di (control drug) were purchased from Sigma Aldrich and prepared as stock solution using either DMSO or DW as mentioned in the second chapter. These compounds were individually added to the parasite cultures at the following concentrations, 0.04 to 10,903.94 nM of ATV, 19.66 to 20,133.88 nM of Breq, 0.26 to 1,000 μM of LFN, 1.95 to 2,000 μM of TAZ and 0.03 to 2,000 μM of Di. Parasite cultures containing 0.2% DMSO, 1% DMSO and 0.2% distilled water were used as controls. The inhibition assay was conducted for four days, and the overlaying medium was replaced daily with fresh medium containing the indicated concentration of each compound. Level of parasitemia and morphological changes of parasites were monitored daily by microscopic examination of Giemsa-stained thin blood smear. The half maximal inhibitory concentration (IC_{50}) value for each compound was calculated (GraphPad) based on parasitemia level recorded on the third day of *in vitro* culture.

Viability of parasites. In order to confirm re-growth of the parasites, 4 μl from treated parasites and controls were sub-cultured in the culture medium with horse RBCs without inhibitors for 4 days. Parasite recrudescence was determined under a light microscope daily.

3-3. Results

Bioinformatic analysis. Basic Local Alignment Search Tool (Blast) of TeDHODH amino acid sequences showed that this enzyme contains DHODH 2 like domain (Fig. 6A) which belongs to class II dihydroorotate dehydrogenase. Amino acid alignment showed the same position of substrate biding sites, co-substrate binding sites, FMN binding sites and active sites for TeDHODH, BboDHODH and PbDHODH (Fig. 6B). TeDHODH was similar to BboDHODH and PbDHODH with 59% and 44% identity, respectively. Moreover, TeDHODH has high similarity with *Theileria orientalis* (BAM39885.1) and *T. annulata* (XP952548.1) DHODHs with 67% identity (data not shown). Phylogenetic analysis showed that TeDHODH belong to the same clade as *T. orientalis* and *B. bovis* DHODHs but separated from mammalian DHODHs (Fig. 7).

Native *T. equi* and *B. caballi* DHODH on Western blotting. Western blotting of *T. equi* and *B. caballi* lysates probed with mice anti BboDHODH serum showed approximately 44-kDa of native DHODH protein on the PVDF membrane (Fig. 8). The molecular weight of native *T. equi* and *B. caballi* DHODHs were similar to *B. bovis* DHODH enzyme as reported in the first chapter. These results suggest that *T. equi* and *B. caballi* DHODHs might share important domains and show cross-reaction among *Babesia* DHODHs.

***In vitro* inhibition of *T. equi* growth using DHODH inhibitors.** The growth of *T. equi* was significantly inhibited by ATV and LFN from 96 hrs post treatment at 5.93 nM and 160 μM (Fig. 9). While Di (control drug) and Breq significantly inhibited the growth of parasite from 72 hrs post treatment at 31.25 μM and 20.13 μM. However, TAZ did not affect the growth of *T. equi*. At 2,000 μM of Di, 10.9 μM of ATV and 1,000 μM of LFN, the parasites were completely killed as there was no regrowth, 4 days after drug withdrawal (Fig. 9). Unexpectedly, Breq at 157.29 nM and ATV at 0.48 nM significantly enhanced the parasitemia level as compared to the control. Treated parasites appeared dot-like and shrank (data not shown). IC_{50} of Di, ATV, Breq and LFN were 40.18±0.18 μM, 28.18±16.77 nM, 10.56±0.83 μM and 109±26.91 μM, respectively (Table 4).

***In vitro* inhibition of *B. caballi* growth using DHODH inhibitors.** The growth of *B. caballi* was significantly inhibited from 72 hrs post treatment by Di, ATV, Breq and LFN at 0.48 μM, 72.66 nM, 5.03 μM and 160 μM, respectively (Fig. 10). TAZ did not effect on the growth of *B. caballi*, but TAZ enhanced the growth of the parasites at lower concentration (data not shown). Viability of *B. caballi* after treatment with 10 μM ATV, 2,000 μM Di and 1,000 μM LFN revealed no re-growth of the parasite after drug withdrawal. Unexpectedly, *B. caballi* treated with 1.69 nM ATV, 629 nM Breq and 1.64 μM LFN showed significantly higher parasitemia level than control. This suggest that ATV, Breq and LFN at low concentration enhanced the growth of the parasites from 72 hrs post treatment. (Fig. 10). Morphological changes of treated parasites were dot-like and shrank shapes (data not show). IC_{50} of Di, ATV, Breq and LFN were 16.21±5.05 μM, 127.73±3.38 nM, 5.18±0.08 μM and 192.83±26.83 μM, respectively (Table 4).

3-4. Discussion

EP is an important tick borne disease caused by *T. equi* and *B. caballi*. Current chemotherapeutic of EP lacks choices as only diminazene aceturate and imidazole dipropionate are available in the market. Novel and effective chemotherapeutic for treatment of EP in equids is therefore needed. The first and second chapter of this study reported that BboDHODH could be a novel chemotherapeutic target for bovine babesiosis, and the use of the available DHODH inhibitors potentially inhibited the growth of *B. bovis in vitro*. The results of this chapter proved that TeDHODH was similar to other *Babesia* parasites DHODH. Detection of native DHODH in *T. equi* and *B. caballi* by mice anti BboDHODH showed their similar molecular weight on western blotting. The mice anti rBboDHODH cross-reacted with *T. equi* and *B. caballi* parasites lysates. This suggests that DHODHs among *Babesia* parasites were homologous enzyme and might exhibit similar role. Thereafter, DHODH inhibitors which had inhibited the growth of *B. bovis* in the first chapter, also effect on the growth of *T. equi* and *B. caballi in vitro*. This is an advantage of targeting DHODH enzyme for *Babesia* spp. Infection because it may be a multi-species target. IC_{50} of DHODH inhibitors, ATV, Breq and LFN sufficiently inhibited the growth of *T. equi* and *B. caballi*. Atovaquone was the most effective compound in this study though, IC_{50} of ATV varies among these parasites. IC_{50} of ATV on *T. equi* parasite was 6.5 folds higher than *B. caballi*. Effect of Breq on *B. caballi* was 2 folds higher than on *T. equi*. *Theileria equi* was 2 folds more sensitive to LFN effect than *B. caballi*. In addition, TAZ was not effective on *T. equi* and *B. caballi*. These results suggest that DHODH inhibitors have species specific inhibition effects. However, further evaluation of those inhibitors on DHODH enzymes is required. On the other hand, the results showed that ATV, Breq

and LFN enhanced the parasitemia level at particular doses. The parasitemia level was significantly higher than the control when very low concentration of compounds were used. This suggests that pyrimidine analog can be a supplementary substance for parasite growth but higher concentration of pyrimidine analog might interfere with the pyrimidine synthesis resulting in the death of the parasites. Likewise, TAZ at all concentrations used in this study enhanced parasitemia level up to 3 times compared with a control. This might suggest TAZ, a pyrimidine analog do not inhibit the parasite but supplement more pyrimidines and support the parasite growth. Moreover, this might imply that parasites not only produce pyrimidine by their own but also absorb the nutrients from surrounded environment. This study confirmed that targeting equine piroplams DHODH could be useful for the development of novel chemotherapeutic for treatment of EP. However, molecular characterization of *T. equi* and *B. caballi* DHODHs is strongly required. Moreover, horses RBC and cells toxicity of DHODH inhibitor should be investigated.

3-5 Summary

Equine piroplasmosis is caused by *Theileria equi* and *Babesia caballi* parasites of phylum Apicomplexa. *T. equi* and *B. caballi* infections have been reported as veterinary important disease, affecting the international movement of racing horses and losses of economic for the horse breeders. Toxicity of currently available drugs and *T. equi* resistance to drug recommend the finding of new chemotherapeutic for treatment of EP. Moreover, there is no effective commercial vaccine and efficient control measures for EP. This study showed that targeting dihydroorotate dehydrogenase (DHODH) using DHODH inhibitors could be a new treatment regime for controlling of

EP. The DHODH inhibitors in this study showed variable ranges of IC_{50}. However, ATV is the most effective inhibitors against *T. equi* and *B. caballi* parasites compared to diminazene aceturate, the common drug for treatment of EP. This study suggest that ATV might be an effective candidate for treatment of EP though effect of ATV *in vivo* need to be evaluated. Alternatively, combination of ATV with imidazole diproprionate might be an option to decrease the toxicity of the later drug and improve the efficacy of EP treatment.

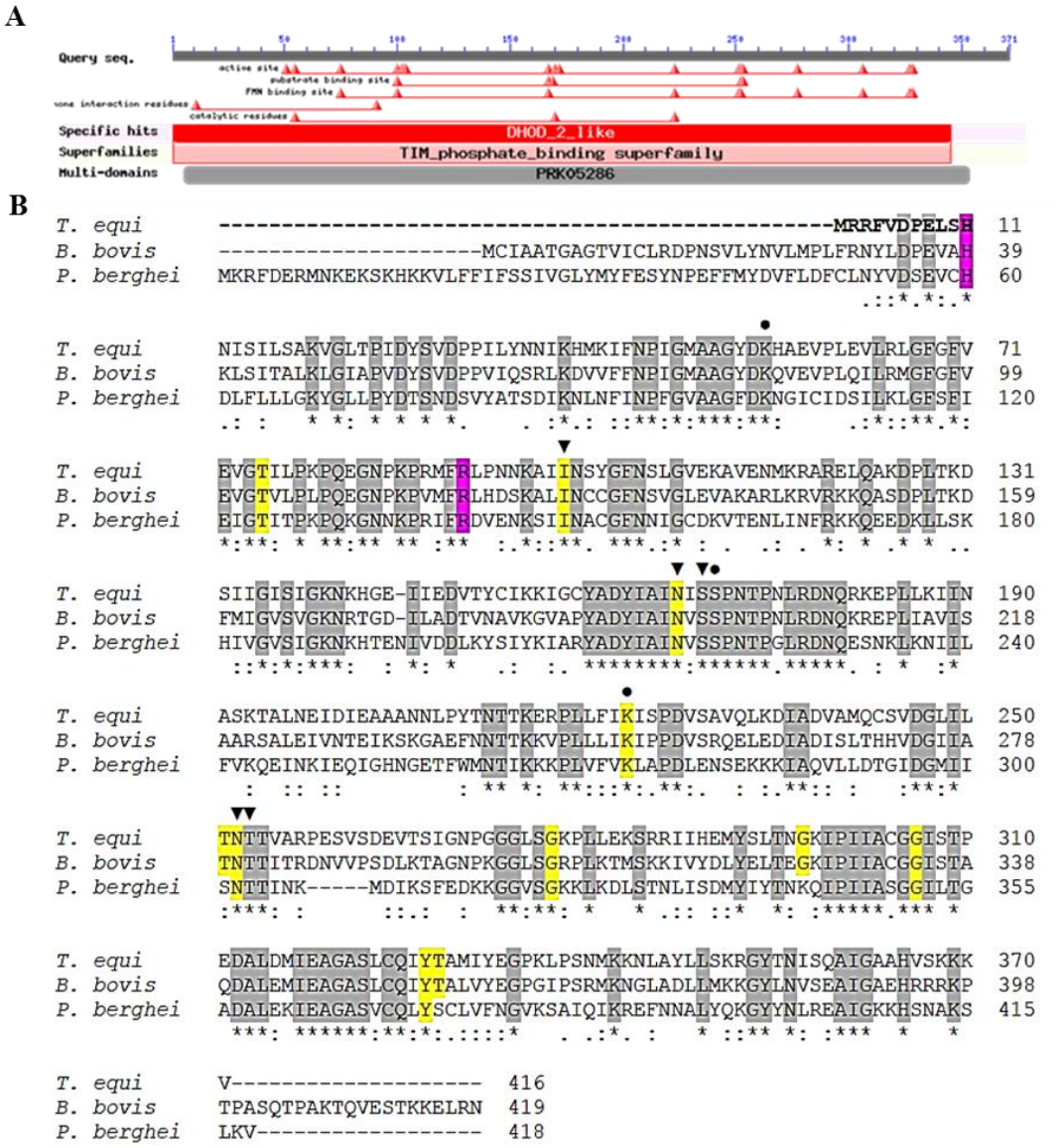

Fig. 6. Bioinformatic analysis of TeDHODH polypeptides. (A) The predicted functional domains of TeDHODH as shown by BLASTp. (B) Alignment of *T. equi* DHODH with apicomplexan DHODH enzymes. Completely conserved residues are highlighted as asterisk and grey, quinone binding sites are shown in pink, fumarate binding sites are shown in yellow, active sites are shown as (●) and substrate binding sites are shown as (▼).

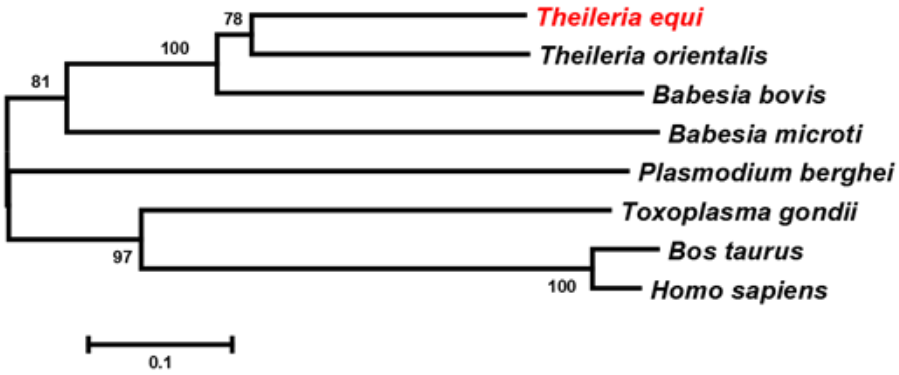

Fig. 7. Phylogenetic analysis of apicomplexan protozoan and mammalian DHODHs showing that *Theileria equi* DHODH (marked in red) is similar to DHODH of other apicomplexan but different from mammalian DHODHs.

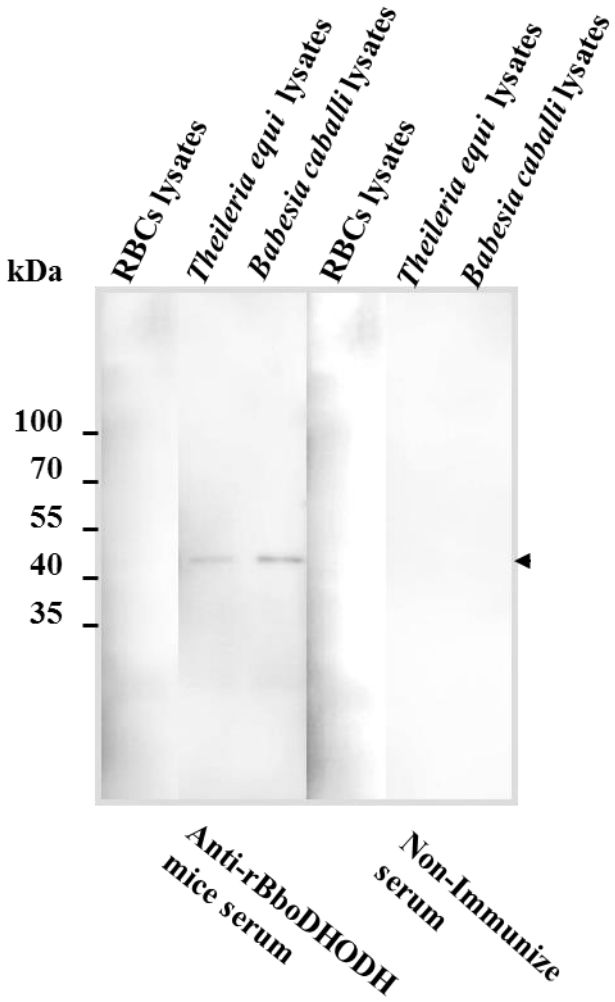

Fig. 8. Western blotting using mice anti DHODH serum probed the native DHODH in *Theileria equi* and *Babesia caballi* parasite lysates, non-immunized mice sera did not show any reaction on non-infected RBC and parasites lysates.

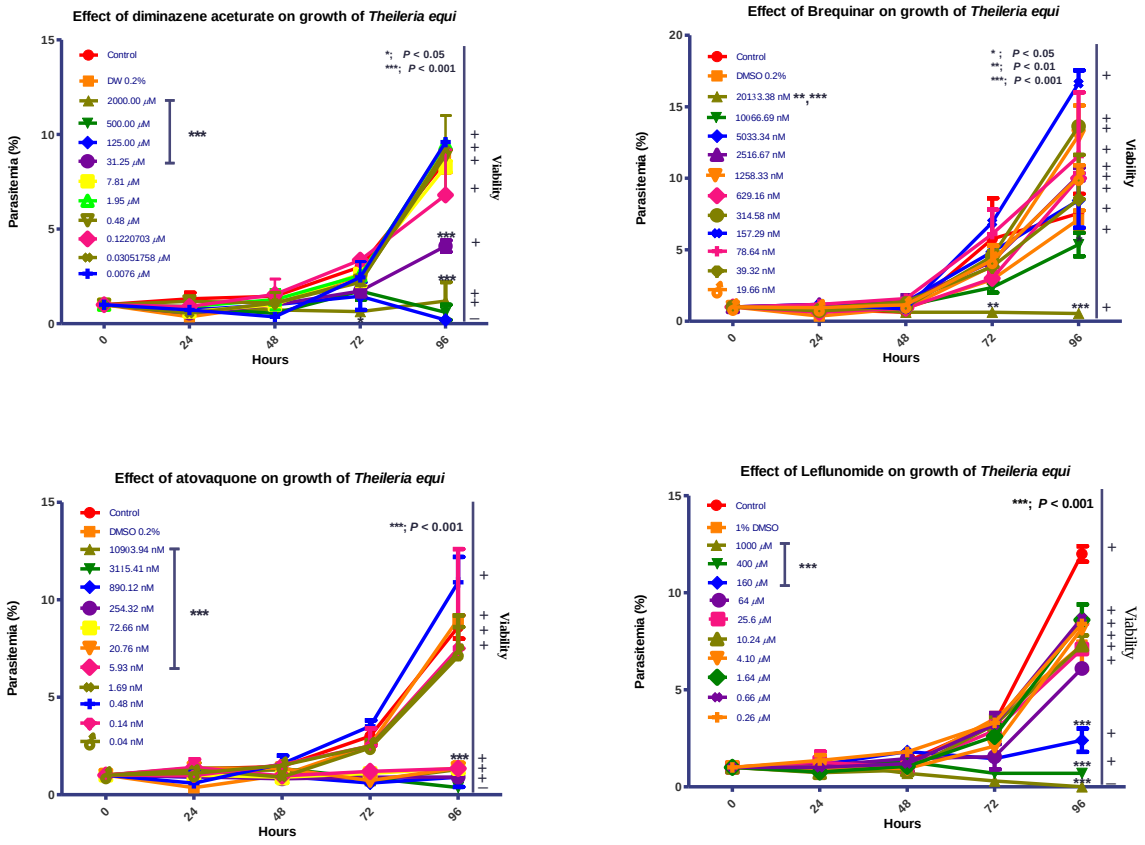

Fig. 9. *In vitro* effect of diminazene aceturate (Di), ATV and Breq on the growth of *Theileria equi* parasites.

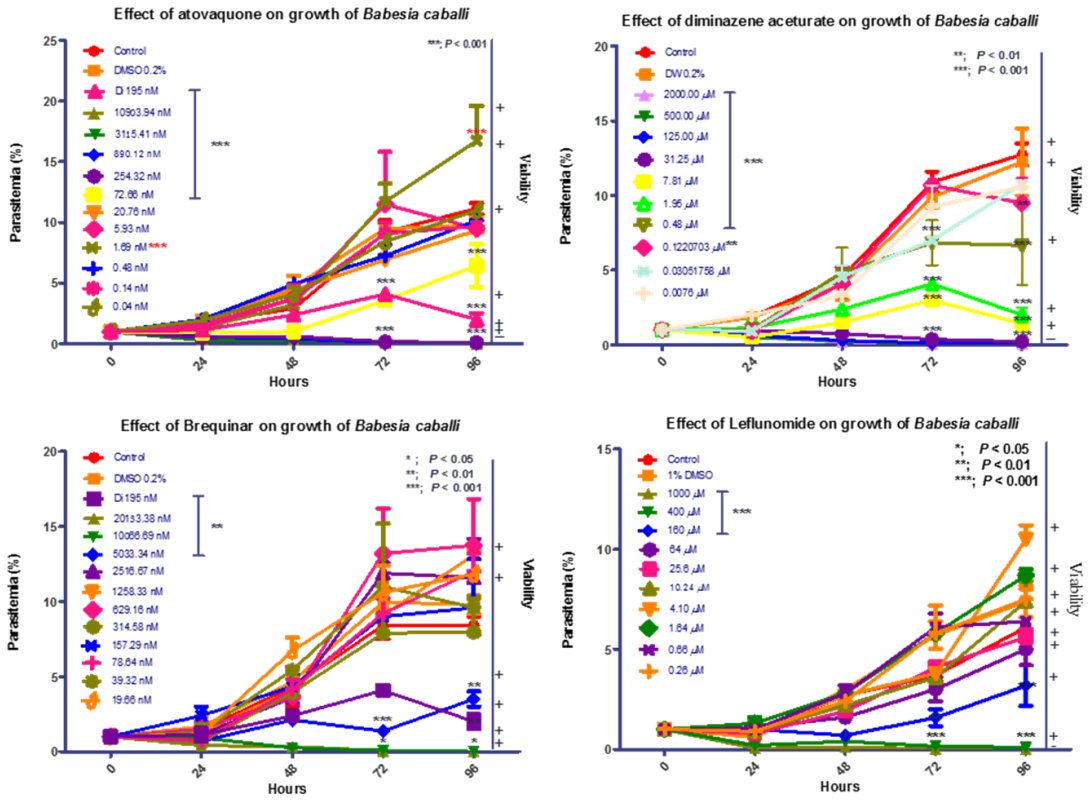

Fig. 10. *In vitro* effect of diminazene aceturate, ATV, Breq and LFN on the growth of *Babesia caballi* parasites.

Table 4. IC_{50} of DHODH inhibitors, and control drug on the growth of *Theileria equi* and *Babesia caballi* parasites.

Inhibitor	*Theileria equi*	*Babesia caballi*
Atovaquone	28.18±16.77 nM	127.73±3.38 nM
Brequinar	10.56±0.83 μM	5.18±0.08 μM
Leflunomide	109±26.91 μM	192.83±26.83 μM
Diminazene aceturate	40.18±0.18 μM	16.21±5.05 μM

General discussion

Bovine babesiosis and equine piroplasmosis (EP) are veterinary important diseases which still need explicit studies in several issues such as genomic and molecular biology, efficient procedures for transfection, conditional gene complementation and target validation to clearly understand life cycle and biology of their comparative agents. Certain understanding *Babesia*'s life cycle and biology will be very useful for better control and treatment of bovine babesiosis and EP [Vial and Gorenflot, 2006]. Meanwhile, strategies to deal with these diseases include limitation of exposure to pathogen by elimination of tick vectors, vaccination and use of chemotherapeutic for treatment. Unfortunately, to date there is no effective commercial vaccine [Homer *et al*., 2000; OIE, 2008], and common chemotherapeutic drugs have adverse side effects, toxicity, accumulation of chemical residues and drug resistance issues [Frerichs *et al*., 1973; Tantasuwan *et al*., 2004; Vial and Gorenflot, 2006]. Therefore, there is a need to find new chemotherapeutic compounds as new and better choice of treatment. Finding new chemotherapeutic compounds should be based on the identification of new drug target which generally are selected among molecules which are unique to the parasite and inexistent in the host or exist in the host but not as important as it is in the parasite. In this study, the *de novo* pyrimidine biosynthesis pathway have been chosen due to previous studies in mammalian, *Plasmodium*, *Toxoplasma* and *Trypanosomes* [Knecht and Löffler, 1998; Heillilä *et al*., 2006; Cheleski *et al*., 2010; Hortua Triana *et al*., 2012], showing that one of the six enzymes namely dihydroorotate dehydrogenase (DHODH) has an important role in the survival of protozoa. Moreover, napthoquinone analogs and some pyrimidine analogs have an

effect on this enzyme [Ferreira *et al*., 2012]. The available information on its enzymatic properties supported its choice as a novel drug target for treatment of bovine babesiosis and EP. This enzyme catalyzes dihydroorotic acid oxidation to orotic acid, a precursor for uridine monophosphate (UMP) production [Shambaugh, 1979; Rawls *et al*., 2000], which is important for RNA and DNA synthesis [Shambaugh, 1979]. The strategy of this study was to inhibit DHODH and consequently interfere with orotic acid production which as result a lack of UMP precursor, UMP deficiency and the interruption of RNA and DNA synthesis. DHODH inhibited organisms would starve and finally die due to lack of DNA synthesis, which affect the whole living systems of organisms. DHODH also exists in mammal, however, there is two sources to produce UMP precursors [Shambaugh, 1979] while *Babesia* parasite has only one source [Gero *et al*., 1983]. Therefore, the effect of DHODH inhibitors on mammalian are less than the effect on the parasite DHODHs. Moreover, many studies on human and *Plasmodium* DHODH confirmed that DHODH inhibitors have selective effect on the parasites [Phillips *et al*., 2008; Guijar *et al*., 2011]. For these reasons, DHODH is an attractive target for development of protozoan chemotherapeutic [Phillips and Rathod, 2010].

The advantage of recombinant production technology helps us to produce large amount of purified targeted protein. In this study, rBboDHODH was produced and showed enzymatic properties as reported in the chapter one and two. The recombinant BboDHODH exhibited specific activity 15,000 folds higher than the previous study using the crude enzyme from *B. bovis* culture parasite [Gero *et al*., 1983]. This confirmed that the recombinant was purer than the crude enzyme from the parasite extracts which may contain other enzymes that might inhibit its enzyme properties. On the other hand, relative activity of the recombinant significantly lowered in the reaction

contains ATV and/or LFN, suggesting that ATV and LFN are specific inhibitor, while Breq and TAZ were not specific against BboDHODH enzyme.

Among DHODH inhibitors used in *in vitro* inhibition assay in this study, ATV has previously been investigated both *in vitro* and *in vivo* [Hughes and Oz, 1995; Pudney and Gray, 1997; Matsuu *et al*., 2004; Ferreira *et al*., 2012; Hortua Triana *et al*., 2012; Oz and Tobin, 2012] and showed as one of the new effective drugs against protozoan parasites [Fry and Pudney, 1992; Baggish and Hill, 2002]. In addition, it does not affect the mammalian DHODH enzyme activity. Obviously, ATV is the most effective compound in this study with an IC_{50} value that is at nano molar level and comparable with diminazene aceturate (Di). This should be useful for the development of chemotherapeutic drug for bovine babesiosis and EP as well as other protozoa parasites infection. Atovaquone was effective against *B. bovis* with IC_{50} 2.38±0.53 nM, *T. equi* 28.18±16.77 nM and *B. caballi* 127.73±3.38 nM (Table 5). Therefore, ATV is more effective on *B. bovis* than *T. equi* and *B. caballi* among protozoa parasites. ATV is more effective against *Plasmodium* (IC_{50} 0.7 to 6.0 nM) [traveler malaria] than *Babesia* (IC_{50} 2.39 to 129 nM) but poor inhibitor of *T. gondii*. Previously, ATV has been used in clinical trials in hamster, gerbil and dogs for treatment of *B. microti* [Hughes and Oz, 1995], *B. divergens* [Pudney and Gray, 1997] and *B. gibsoni* [Matsuu *et al*., 2004]. They were shown to be effective in clearance of the parasite with no adverse side effect. Moreover, ATV is approved as a drug and used in combination with azithromycin for treatment of *B. microti* in human. Mildly side effects have been reported in some human cases but they are rare. This suggests that ATV might be safe for other mammalians such as equids and ruminant as well. Nevertheless, *in vivo* studies on the effect of ATV on infected equids and ruminants, is strongly required.

Brequinar (Breq), a quinone analog, has been reported as effective compound on *Plasmodium* spp. and *T. gondii* [Walse *et al*., 2008], and confirmed as DHODH inhibitors with species-specific inhibitor effect. In this study, Breq was effective on *T. equi* and *B. caballi* but 2 folds more effective on *B. caballi* than *T. equi*. However, when compared to ATV high concentration of Breq is needed for clearing the parasites *in vitro*, as it showed IC_{50} range from 5-10 μM.

Leflunomide (LFN) had been known as an immunosuppressive agent that is effective towards several autoimmune diseases and rejection of transplants in animals [Bartlett *et al*., 1991; Thomson and Starzl, 1993]. The study of Greene *et al*. (1994) confirmed that LFN exhibited mixed-type kinetics inhibition towards L-DHO and coenzyme Q, by targeting DHODH. This compound is currently being used as anti-rheumatic drug for human. In this study, LFN significantly inhibited rBboDHODH enzymatic activity to 50%. However, the IC_{50} of LFN was higher than observed for Di. LFN was less effective compared with ATV, Breq and Di. Nevertheless, further modification on the basis of LFN structure might improve the potency, selectivity and specificity of this compound against *Babesia* parasites.

DHODH enzyme oxidized dihydroorotate to orotic acid using ubiquinone as a co-substrate as confirmed by DCIP reduction assay. Orotic acid is a precursor of UMP production in the *de novo* pyrimidine biosynthesis pathway. In this study, supplementation of ORA or UMP on ATV treated parasite showed recovery of the parasites compared to non-supplemented control. Lack of orotic acid led to reduction of UMP as predicted. Relatively, reduction of UMP led to survival of parasite. Supplementation could bring up the parasitemia level of *B. bovis* after it was inhibited with ATV at 10 nM. This result confirmed that inhibition with ATV led to lowering the

level of ORA and UMP production resulting in non-division and no growth of the parasites. Interestingly, this results also suggest that exogenous ORA and UMP passes through parasite cell membrane to fulfill the *de novo* biosynthesis pathway.

Table 5. Summary of IC_{50} value of DHODH inhibitors and diminazene aceturate on the growth of *B. bovis*, *B. caballi* and *Theileria equi in vitro*.

Inhibitor	*Babesia bovis*	*Babesia caballi*	*Theileria equi*
Diminazene aceturate	0.5±0.02 μM	16.21±5.05 μM	40.18±0.18 μM
Atovaquone	2.38±0.53 nM	127.73±3.38 nM	28.18±16.77 nM
Brequinar	Not effective	5.18±0.08 μM	10.56±0.83 μM
Leflunomide	52.41±11.47 μM	192.83±26.83 μM	109±26.91 μM
TAZ	Not effective	Not effective	Not effective

General summary

Babesia bovis is the most virulent erythrocytic protozoa causing bovine babesiosis, which affects cattle and causes huge economic losses in livestock industries worldwide. *B. caballi* and *Theileria equi,* are principle agents of equine piroplasmosis (EP), an important disease affecting horse international movement. Adverse side effects, toxicity and drug resistance have been recently reported on the current treatment of bovine babesiosis and EP. The finding of new compound for treatment of bovine babesiosis and EP is therefore required. Dihydroorotate dehydrogenase (DHODH), the fourth enzyme of *de novo* pyrimidine biosynthesis pathway, is currently a drug target for treatment of malaria, toxoplasmosis, leishmaniasis, but is yet not characterized in bovine babesiosis. This study aimed to characterize *B. bovis* DHODH (BboDHODH) and assess its potential as a chemotherapeutic target for treatment of bovine babesiosis. Furthermore, application of this target for treatment of EP have also been evaluated.

In chapter 1, BboDHODH was characterized. Bioinformatic analysis, amplification, cloning, recombinant protein production, detection of native enzyme and measurement of the enzymatic properties were conducted. Bioinformatic analysis showed that BboDHODH is a homologue enzyme among apicomplexa parasites. It has different amino acids at the FMN binding site and substrate binding site from host enzyme. Characterization of BboDHODH showed that this enzyme has DHODH 2 like region which belong to class II DHODH enzyme, it was detected in *B. bovis* lysates and locate in parasite mitochondria. The recombinant BboDHODH was an active enzyme.

In chapter 2, DHODH inhibitor including ATV, Breq, LFN and TAZ were evaluated on the recombinant rBboDHODH enzymatic properties and on the growth of *B. bovis in vitro*. ATV and LFN significantly reduced the enzymatic properties of the recombinant protein while Breq and TAZ had no effect on recombinant enzymatic activity. ATV and LFN significantly inhibited the growth of *B. bovis* 48 hrs post treatment. However, Breq and TAZ did not have effect on *B. bovis* growth. In addition, these inhibitors did not affect the bovine RBCs. This chapter revealed that BboDHODH could be drug target for treatment of *B. bovis* infection.

In chapter 3, *T. equi* DHODH (TeDHODH) amino acid was compared to BboDHODH. Detection of native *T. equi* and *B. caballi* DHODH were performed using anti rBboDHODH mice sera by Western Blotting. Effect of DHODH inhibitors were evaluated on the growth of *T. equi* and *B. caballi in vitro*. TeDHODH amino acid was similar to BboDHODH, and shared the same amino acids responsible for enzymatic activity. Native *T. equi* and *B. caballi* DHODH were detected in the parasite lysates. ATV, Breq and LFN significantly inhibited the growth of *T. equi* and *B. caballi* from 96 and 72 hrs, respectively. Therefore, DHODH might be a potential chemotherapeutic target for treatment of EP.

In summary, DHODH is a homologue enzyme among *Babesia* parasites (*B. bovis*, *B. caballi* and *T. equi*). The available DHODH inhibitors were effective against the growth of *B. bovis*, *B. caballi* and *T. equi in vitro* suggesting that DHODH could be a potential chemotherapeutic target for treatment of bovine babesiosis and EP.

和文要約

バベシア原虫のジヒドロオロト酸デヒドロゲナーゼを標的とした バベシア症に対する有効な治療法の開発

牛バベシア症の原因原虫である *Babesia bovis* は最も病原性の強い赤血球内寄生原虫の一種で、畜産業に多大な損害を与えている。一方、馬バベシア症（ピロプラズマ症）の原因原虫である *B. caballi* と *B. equi* (*Theileria equi*)も同様に赤血球内寄生原虫であり、馬の国際交易などにおいてもその影響が大きい。これらの牛・馬バベシア症に対する現行の治療薬法は、副作用等の多くの問題を抱えている。そこで、新規の治療法の開発が強く求められている。ジヒドロオロト酸デヒドロゲナーゼ (DHODH) は、原虫のピリミジンの *de novo* 生成を司る第 4 番目の酵素であり、マラリア、トキソプラズマ、リーシュマニア原虫感染症に対する新規治療法の標的として注目されている。しかしながら、バベシア原虫における DHODH の研究はまだされていないのが現状である。このような背景から、本研究では牛・馬バベシア原虫の DHODH の分子解析とこの分子を標的とした治療薬の開発を目指した。

第 1 章では、*B. bovis* の DHODH（BboDHODH）の分子解析を行った。バイオインフォマティックス解析の結果、BboDHODH はすでに公表されている他のアピコンプレックス門原虫がもっている DHODH 分子と相同性が高いことが分かった。また、牛など宿主の DHODH 分子とは異なる FMN や基質の結合部位などの機能ドメインを有することが示唆された。組換え BboDHODH に

対する特異抗体を用いた解析により、この分子は *B. bovis* 虫体のミトコンドリアに局在していることが示唆された。また、組換え BboDHODH は酵素活性を有することも確認された。

第 2 章では、DHODH の酵素抑制剤として知られる ATV、Breq、LFN、TAZ の組換え BboDHODH の酵素活性抑制試験ならびに虫体増殖抑制試験を行った。ATV と LFN は組換え BboDHODH の酵素活性を顕著に抑制し、また、*B. bovis* 原虫の増殖も著しく抑制した。Breq と TAZ にはいずれにおいても顕著な抑制作用が認められなかった。これらの酵素抑制剤には牛赤血球に対する副作用は認められなかった。これらの結果より、BboDHODH が牛バベシア症に対する治療薬の標的となりうることが示唆された。

第 3 章では、馬バベシア原虫の DHODH の分子解析を行った。TeDHODH は BboDHODH と高い相同性を有することが分かった。*T. equi* と *B. caballi* 虫体の DHODH 分子は組換え BboDHODH に対する特異抗体によって検出された。また、DHODH の酵素抑制剤である ATV、Breq、LFN は *T. equi* と *B. caballi* の増殖を著しく抑制した。これらの結果により、DHODH は馬バベシア症に対する治療薬の標的にもなりうることが示唆された。

以上の結果より、DHODH は *B. bovis*、*B. caballi*、*T. equi* などのバベシア原虫によく保存されており、DHODH の酵素抑制剤は牛・馬バベシア症の治療薬候補として有望であることが示唆された。

Acknowledgements

I am greatly grateful to Professor Xuenan Xuan for his kindness, for accepting me as one of his PhD students and offering me great opportunities during my stay and study in Japan.

I am indebted to Associated Professor Yoshifumi Nishikawa, Professor Ikuo Igarashi, Professor Noboru Inoue and Associate Professor Makoto Igarashi for their professional guidance, kind encouragement and excellent suggestions to improve my research work.

I extend my sincerest thanks to Professor Hiroshi Suzuki, and Professor Shin-ichiro Kawazu, Professor Naoaki Yokoyama, Associate Professor Shinya Fukumoto, Associate Professor Kentaro Kato and Assistant Professor Rika Shirafuji for their active support and valuable suggestion on my research study.

I am indebted to Dr. Young-Kyoun Goo, Dr. Mohamad Alaa Terkawi, Dr. Junya Yamagishi, Dr. Gabriel Aboge Oluga, Dr. Tatsunori Masatani, Dr. Shinuo Cao, Dr. Yuzi Luo, Dr. Yan Li, and Dr. Longzhang Yu for their excellent guidance, kind suggestion and laboratory supports.

My sincerest thanks to Mr. Paul Franck Adjou Moumouni for his kind support and friendship.

I would like to thank Japanese and international friends in Obihiro University for their friendship, supports and for making my life in Obihiro easier.

My gratitude to Research Unit for Host Defense, National Research Center for Protozoan Diseases (NRCPD), Obihiro University of Agriculture and Veterinary

Medicine (OUAVM) for their excellent programs, technology and supports for veterinary medical research.

My sincerest thanks to the grant from the Global COE program, the Grant-in-aid for Scientific Research, both from the Ministry of Education, Culture Sport Science, and Technology of Japan, for the funding supports, which made smoothly my research study in Japan.

I am indebted to the Yoneyama Foundation, Rotary Club, Japan for kindly providing me a scholarship and a good life during my stay in Japan. I am also grateful to Mr. Kitakawa Katsuhiro my counselor from Obihiro Nishi Rotary Club, Obihiro, Hokkaido, Japan for his kindness supports, language exchange, and being my Japanese family during my stay in Obihiro, Hokkaido, Japan.

I am deeply thanks to Miss Keiko Matsuura, Miss Hidemi Ikari, Miss Yuki Hasekawa and Miss Mizuki Chiba for coordinating, providing me with useful information for living in Japan, and giving me a hand every time I needed any kind of help.

I would like to thank Dr. Rattayaporn Ngasaman, Dr. Warangkana Fonlek, Dr. Srineenard Jiamtaweeboon, Dr. Dusit Laohasingnarong, Dr. Kwanwalai Maklon, Mr. Dulayatad Grongsang and Miss Charoonluk Jiraphatharaset, my Thai friends in Obihiro for their friendship, daily life supports, good advice, understanding and lively life even we were far from home.

I am indebted to Mr. and Mrs. Yahagi my Japanese family for their sincere help, care and cultural exchange with Thai student in Obihiro.

I am deeply indebted to Mr. Suriya Panyayos, for providing me constant inspiration, consistent love, emotional supports, valuable advice and understanding. He fully supported me as well as took care of my parent while I was away from home.

I am grateful to Buddhism for lightning the darkness in my mind, giving me the proper way to overcome obstacles and leading me to wisdom when I faced all difficulties.

Finally, I lovingly dedicate this modest output of a very challenging yet fulfilling research endeavor to Mr. Boonyeun Kamyingkird and Mrs. Juthamas Kamyingkird my ever loving parent. I thank them for giving me a wonderful life, unconditional love, utmost supports and giving me a ‘push’ every time I was down.

References

Ahmed, J.S. 2002. The role of cytokines in immunity and immunopathogenesis of piroplasmoses. *Parasitol. Res.* **88** (Suppl.): S48-S50.

Allred, D.R. 2003. Babesiosis: persistence in the face of adversity. *Trends Parasitol.* **19**: 51-55.

Allsopp, M.T., Cavalier-Smith, T., De Waal, D.T. and Allsopp, B.A. 1994. Phylogeny and evolution of the piroplasms. *Parasitology* **108**: 147-152.

Aryeetey, R. and Jimenez-Lucho, V. 2002. Babesiosis. *Curr. Treat. Options Infect. Dis.* **4**: 319-326.

Babes, V. 1888. Sur l'hemoglobinurie bacterienne du boeuf. In Bock, R., Jackson, L., de Vos, A. and Jorgensen, W. 2004. *Parasitology* **129**: 247–269.

Baggish, A.L. and Hill, D.R. 2002. Antiparasitic agent atovaquone. *Antimicrob. Agents Chemother*. **46**: 1163-1173.

Baldwin, J., Farajallah, A.M., Malmquist, N.A., Rathod, P.K. and Phillips, M.A. 2002. Malarial dihydroorotate dehydrogenase. *J. Bio. Chem.* **277**: 41827–41834.

Bashiruddin, J.B., Camma, C. and Rebelo, E. 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. *Vet. Parasitol.* **84**: 75-83.

Birkenheuer, A.J., Levy, M.G. and Breitschwerdt, E.B. 2004. Efficacy of combined atovaquone and azithromyzin for therapy of chronic *Babesia gibsoni* (Asian genotype) infections in dogs. *J. Vet. Intern. Med.* **18**: 494-498.

Björnberg, O., Rowland, P., Larsen, S. and Jensen, K.F. 1997. Active site of dihydroorotate dehydrogenase A from *Lactococcus lactis* investigated by chemical modification and mutagenesis. *Biochemistry* **36**: 16197–16205.

Bock, R., Jackson, L., de Vos, A. and Jorgensen, W. 2004. Babesiosis of cattle. *Parasitology* **129**: 247–269.

Böse, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T. and de Vos, A.J. 1995. Current state and future trends in the diagnosis of babesiosis. *Vet. Parasitol.* **57**: 61-74.

Brown, W.C. and Palmer, G.H. 1999. Designing blood-stage vaccines against *Babesia bovis* and *B. bigemina*. *Parasitol*. *Today* **15**: 275–281.

Brünning, A. 1996. Equine piroplasmosis an update on diagnosis, treatment and prevention. *Br. Vet. J.* **152**: 139-151.

Callow, L.L. 1979. Some aspects of the epidemiology and control of bovine babesiosis in Australia. *J. S. Afr. Vet. Assoc.* **50**: 353-356.

Callow, L.L. 1984. Piroplasms. In *Animal Health in Australia, Protozoal and Rickettsial Diseases*, vol. 5. *Animal Health in Australia*, 121-160. Canberra, Australian Bureau of Animal Health, AGPS.

Callow, L.L. Dalgliesh, R.J. and de Vos, A.J. 1997. Development of effective living vaccines against bovine babesiosis-the longest field trial? *Int. J. Parasitol.* **27**: 747-767.

Callow, L.L., Roger, R.J. and de Vos, A.J. 1993. Tick-borne diseases: cattle-pathology and serology. In *Australian Standard Diagnostic Techniques for Animal Diseases* (ed. Corner, L.A. and Bagust, T.J.), 1-16. East Melbourne, CSIRO Information Services.

Cheleski, J., Rocha, J.R., Pinheiro, M.P., Wiggers, H.J., da Silva, A.B.F., Nonato, M.C. and Montanari, C.A. 2010. Novel insights for dihydroorotate dehydrogenase class 1A inhibitors discovery. *Eur. J. Med. Chem*. **45**: 5899–5909.

Chen, J.J. and Jones, M.E. 1976. The cellular localization of dihydroorotate dehydrogenase: relation to *de novo* biosynthesis of pyrimidines. *Arch. Biochem. Biophys*. **176**: 82–90.

Dalgliesh, R.J. 1993. Babesiosis. In *Immunology and Molecular Biology of Parasite Infections* (ed. Warren, S.K.), 352-383. Oxford, Blackwell.

de Vos, A.J. 1979. Epidemiology and control of bovine babesiosis in South Africa. *J. S. Afr. Vet. Assoc.* **50**: 357-362.

de Vos, A.J. and Jorgensen, W.K. 1992. Protection of cattle against babesiosis in tropical and subtropical countries with a live, frozen vaccine. In *Tick Vector Biology, Medical and Veterinary Aspects* (ed. Fivaz, B.H., Petney, T.N. and Horak, I.G.) 159-174. London, Springer Verlag.

de Vos, A.J. and Potgieter, F.T. 1994. Bovine babesiosis. In *Infectious Diseases of Livestock* (ed. Coetzer, J.A.W., Thomson, G.R. and Tustin, R.C.) 278-294. Capetown, Oxford University Press.

de Waal, D.T. 1992. Equine piroplasmosis: a review. *Br. Vet. J.* **148**: 6-14.

de Waal, D.T. and Combrink, M.P. 2006. Live vaccines against bovine babesiosis. *Vet. Parasitol.* **138**: 88-96.

Frerichs, W.M., Allen, P.C. and Holbrook, A.A. 1973. Equine piroplasmosis (*Babesia equi*): therapeutic trials of imidocarb dihydrochloride in horses and donkeys. *Vet. Rec.* **93**: 73-75.

Friedhoff, K.T. 1988. Transmission of *Babesia*. In *Babesia of Domestic Animals and Man* (ed. Ristic, M.) 23-52. Boca Raton, Florida, CRC Press. Inc.

Friedhoff, K.T. and Soule, C. 1996. An account on equine babesioses. *Rev. Sci. Tech.* **15**: 1191-1201.

Friedhoff, K.T., Tentor, A.M. and Muller, I. 1990. Haemoparasites of equines: impact of international trade of horses. *Rev. Sci. Tech.* **9**: 1187-1194.

Fry, M. and Pudney, M. 1992. Site of action of the antimalarial hydroxynapthoquinone, 2-[*trans*-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-napthoquinone (566C80). *Biochem. Pharmacol.* **43**: 1545-1553.

Gero, A.M., O'Sullivan, W.J., Wright, I.G. and Mahoney, D.F. 1983. The enzymes of pyrimidine biosynthesis in *Babesia bovis* and *Babesia bigemina*. *Aust. J. Exp. Biol. Med. Sci.* **61**: 239–43.

Goff, W.L., Johnson, W.C., Parish, S.M., Barrington, G.M., Tuo, W. and Valdez, R.A. 2001. The age-related immunity in cattle to *Babesia bovis* infection involves the rapid induction of interleukin-12, interferon-gamma and inducible nitric oxide synthase mRNA expression in the spleen. *Parasite Immunol.* **23**: 463-471.

Gohil, S., Herrmann, S., Günther, S. and Cooke, B.M. 2013. Bovine babesiosis in the 21st century: advances in biology and functional genomics. *Int. J. Parasitol.* **43**: 125–132.

Gray, J.S., Langley, R.J., Brophy, P.O. and Gannon, P. 1989. Vaccination against bovine babesiosis with drug-controlled live parasites. *Vet. Rec.* **125**: 369-372.

Hailat, N.Q., Lafi, S.Q., al-Darraji, A.M. and al-Ani, F.K. 1997. Equine babesiosis associated with strenuous exercise: clinical and pathological studies in Jordan. *Vet. Parasitol.* **69**: 1-8.

Heikkilä, T., Thirumalairajan, S., Davies, M., Parsons, M.R., McConkey, A.G., Fishwick, C.W.G. and Johnson, P. 2006. The first *de novo* designed inhibitors of *Plasmodium falciparum* dihydroorotate dehydrogenase. *Bioorg. Med. Chem. Lett.* **16**: 88–92.

Holbrook, A.A. 1969. Biology of equine piroplasmosis. *J. Am. Vet. Med. Assoc.* **155**: 453-454.

Homer, M.J., Aguilar-Delfin, I., Telford III, S.R., Krause, P.J. and Persing, D.H. 2000. Babesiosis. *Clin*. *Microbiol*. **13**: 451–469.

Hortua Triana, M.A., Huynh, M.H., Garavito, M.F., Fox, B.A., Bzik, D.J., Carruthers, V.B., Löffler, M. and Zimmermann, B.H. 2012. Biochemical and molecular characterization of the pyrimidine biosynthetic enzyme dihydroorotate dehydrogenase from *Toxoplasma gondii*. *Mol*. *Biochem*. *Parasitol*. **184**: 71–81.

Hughes, W.T. and Oz, H.S. 1995. Successful prevention and treatment of Babesiosis with atovaquone. *J. Infect. Dis.* **172**: 1042-1046.

Igarashi, I., Njonge, F.K., Kaneko, Y. and Nakamura, Y. 1998. *Babesia bigemina*: *in vitro* and *in vivo* effects of curdlan sulfate on growth of parasites. *Exp. Parasitol*. **90**: 290–293.

Kakoma, I. and Mehlhorn, H. 1993. *Babesia* of domestic animals. In Vial, H.J. and Gorenflot, A. 2006. *Vet. Parasitol.* **138**: 147-160.

Kappmeyer, L.S., Perryman, L.E., Hines, S.A., Baszler, T.V., Katz, J.B., Hennager, S.G. and Knowles, D.P. 1999. Detection of equine antibodies to *Babesia caballi* by recombinant *B. caballi* rhobtry associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **37**: 2285-2290.

Kerber, C.E., Ferreira, F. and Pereira, M.C. 1999. Control of equine piroplasmosis in Brazil. *Onderstepoort J. Vet. Res.* **66**: 123-127.

Knecht, W. and Löffler, M. 1998. Species-related inhibition of human and rat dihydroorotate dehydrogenase by immunosuppressive Isoxazol and Cinchoninic acid derivatives. *Biochem. Pharmacol.* **56**: 1259–1264.

Knowles, D. Jr. 1996. Equine babesiosis (piroplasmosis): a problem in the international movement of horses. *Br. Vet. J.* **152**: 123-126.

Knowles, D.P. Jr., Kappmeyer, L.S. and Perryman, L.E. 1994. Specific immune responses are required to control parasitemia in *Babesia equi* infection. *Infect. Immun.* **62**: 1909-1913.

Knowles, D.P. Jr., Perryman, L.E., Kappmeyer, L.S. and Hennager, S.G. 1991. Detection of equine antibody to *Babesia equi* merzoite proteins by a monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **29**: 2056-2058.

Kumar, S., Gupta, A.K., Pal, T. and Dwedevi, S.K. 2003. *In-vivo* therapeutic efficacy trial with artemisinin derivative, buparvaquone and imidocarb diproprionate against *Babesia equi* infection in donkeys. *J. Vet. Med. Sci.* **65**: 1171-1177.

Laveran, A. 1901. Contribution to the study of equi piroplasma. *C.R. Seances. Soc. Biol. Paris* **53**: 385-386. In Rothschild, C.M. 2013. *J. Equin. Vet. Sci.* **33**: 497-508.

Levine, N.D. 1971. Taxonomy of the piroplasms. *Transact. Amer. Microscop. Soc.* **90**: 2-33.

Lewis, B.D., Penzhorn, B.L. and Volkmann, D.H. 1999. Could treatment of pregnant mares prevent abortions due to equine piroplasmosis? *J. S. Afr. Vet. Assoc.* **70**: 90-91.

Mackenstedt, U., Gauer, M., Fuchs, P., Zapf, F., Shein, E. and Mehlhorn, H. 1995. DNA measurements reveal differences in the life cycles of *Babesia bigemina* and *B. canis*, two typical members of the genus *Babesia*. *Parasitol. Res.* **81**: 595-604.

Mahoney, D.F. 1964. Bovine babesiosis: an assessment of the significance of complement fixing antibody based upon experimental infection. *Aus. Vet. J.* **40**: 369-375.

Mahoney, D.F. 1974. The application of epizootiological principals in the control of Babesiosis in cattle. *Bulletin of Office International Des Epizooties* **81**: 123-138.

Maurer, F.D. 1962. Equine piroplasmosis-another emerging disease. *J. Am. Vet. Med. Assoc.* **141**: 699-702.

McCosker, P.J. 1981. The global importance of babesiosis. In *Babesiosis* (ed. Ristic, M. and Kreier, J.P.), 1-24. New York, Academic Press.

McGuire, T.C.,Van Hoosier, G.L. Jr. and Henson, J.B. 1971. The complement-fixation reaction in equine infectious anemia: demonstration of inhibition by IgG (T). *J. Immunol.* **107**: 1738-1744.

Mehlhorn, H. and Shein, E. 1984. The piroplasms: life cycle and sexual stages. *Adv. Parasitol.* **23**: 37-103.

Moltmann, U.G., Mehlhorn, H., Schein, E., Rehbein, G., Voigt, W.P. and Zweygarth, E. 1983. Fine structure of *Babesia equi* Laveral. 1901 within lymphocytes and erythrocytes of horses: an *in vivo* and *in vitro* study. *J. Parasitol.* **69**: 111-120.

Mosqueda, J., Olvera-Ramirez, A., Aguilar-Tipacamu, G. and Canto, G.J. 2012. Current advances in detection and treatment of babesiosis. *Curr. Med. Chem.* **19**: 1504-1518.

Nicolaiewsky, T.B., Richter, M.F., Lunge, V.R., Cunha, C.W., Delagostin, O., Ikuta, N., Fonseca, A.S., da Silva, S.S. and Ozaki, L.S. 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. *Vet. Parasitol.* **101**: 9-21.

Nuttall, G.H.F. and Strickland. 1910. The parasites of the horse piroplasmosis reponse of biliary fever. *Zentralb Bakteriol* **1**: 524-525. In Rothschild, C.M. 2013. *J. Equin. Vet. Sci.* **33**: 497-508.

Phillips, M.A. and Rathod, P.K. 2010. *Plasmodium* dihydroorotate dehydrogenase: a promising target for novel anti-malarial chemotherapy. *Infect. Disord. Drug Targets* **10**: 226–239.

Phillips, M.A., Gujjar, R., Malmquist, N.A., White, J., Mazouni, F.E., Baldwin, J. and Rathod, P.K. 2008. Triazolopyrimidine-based dihydroorotate dehydrogenase inhibitors with potent and selective activity against the malaria parasite *Plasmodium falciparum*. *J. Med. Chem*. **51**: 3649–3653.

Phipps, L.P. and Otter, A. 2004. Transplacental transmission of *Theileria equi* in two foals born and reared in the United Kingdom. *Vet. Rec.* **154**: 406-408.

Potgieter, F.T. 1977. The life cycle of *Babesia bovis* and *Babesia bigemina* in ticks and in cattle in South Africa. PhD thesis, Rand Afrikaans University.

Potgieter, F.T. and Els, H.J. 1976. Light and electron microscopic observations on the development of small merozoites of *Babesia bovis* in *Boophilus microplus* larvae. *Onderstepoort J. Vet. Res.* **43**: 123-128.

Rampersad, J., Cesar, E., Campbell, M.D., Samlal, M. and Ammons, D. 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. *Vet. Parasitol.* **114**: 81-87.

Rath, A., Glibowicka, M., Nadeau, V. G., Chen, G. and Deber, C. M. 2009. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Biochemistry* **106**: 1760-1765.

Rawls, J., Knecht, W., Diekert, K., Lill, R. and Löffler, M. 2000. Requirements for the mitochondrial import and localization of dihydroorotate dehydrogenase. *Eur. J. Biochem.* **267**: 2079–2087.

Rees, C. 1934. Characteristics of the piroplasms *Babesia argentina* and *B. bigemina* in the United States. *Univ. Agri. Res.* **45**: 427-438.

Rodriguez, R.I. and Tree, A.J. 1996. *In vitro* responsiveness of *Babesia bovis* to imidocarb diproprionate and the selection of a drug-adopted line. *Vet. Parasitol.* **62**: 35-41.

Rothschild, C.M. 2013. Equine pirplasmosis. *J. Equin. Vet. Sci.* **33**: 497-508.

Sackett, D., Holmes, P., Abbott, K., Jephcott, S. and Barber, M. 2006. Assessing the economic cost of endemic disease on the profitability of Australian beef cattle and sheep producers. Meat & Livestock Australia, Sydney. Limited Final Report AHW.087. Available from <http://www.mla.com.au/Research-and-development/Final-report-details?projectid=3578>.

Schein, E., Rehbein, G., Voigt, W.P. and Zweygarth, E. 1981. *Babesia equi* (Laveran 1901) Development in horses and in lymphocyte culture. *Tropenmed Parasitol.* **32**: 223-227.

Shambaugh, G.E. 1979. Pyrimidine biosynthesis. *Am. J. Clin. Nutr.* **32**: 1290–1297.

Sierra Pagan, M.L. and Zimmermann, B.H. 2003. Cloning and expression of the dihydroorotate dehydrogenase from *Toxoplasma gondii*. *Biochem. et Biophys. Acta.* **1637**: 178–181.

Silva, M.G., Domigos, A., Alexandra Esteves, M., Crus, M.E.M. and Suarez, C.E. 2013. Evaluation of the growth-inhibition effect of trifluralin analogues on *in vitro* cultured *Babesia bovis* parasites. *Int. J. Parasitol.: Drugs and Drug Resistance* **3**: 59-68

Sippel, W.L., Cooperrider, D.E., Gainer, J.H., Allen, R.W., Mouw, J.E. and Teigland, M.B. 1962. Equine piroplasmosis in the United States. *J. Am. Vet. Med. Assoc.* **141**: 694-698.

Smith, T. and Kilborne, F.L. 1893. Investigation into the nature, causation and prevention of Southern cattle fever. In *Ninth Annual Report of the Bureau of Animal Industry for the Year 1892*, 177-304. Washington Government Printing Office.

Stiller, D., Goff, W.L., Johnson, L.W. and Knowles, D.P. 2002. *Dermacentor variabilis* and *Boophilus microplus* (Acari: *Ixodidae*): Experimental vectors of *Babesia equi* to equids. *J. Med. Entomol.* **39**: 667-670.

Suarez, C.E. and McElwain, T.F. 2008. Transient transfection of purified *Babesia bovis* merozoites. *Exp. Parasitol.* **118**: 498-504.

Suarez, C.E. and McElwain, T.F. 2009. Stable expression of a GFP-BSD fusion protein in *Babesia bovis* merozoites. *Int. J. Parasitol.* **39**: 289-297.

Suarez, C.E. and McElwain, T.F. 2010. Transfection systems for *Babesia bovis*: a review of methods for the transient and stable expression of exogenous genes. *Vet. Parasitol.* **167**: 205-215.

Suarez, C.E., Norimine, J., Lacy, P. and McElwain, T.F. 2006. Characterization and gene expression of *Babesia bovis* elongation factor-1alpha. *Int. J. Parasitol.* **36**: 965-973.

Suarez, C.E., Palmer, G.H., LeRoith, T., Florin-Christensen, M., Crabb, B. and McElwain, T.F. 2004. Intergenic regions in the rhobtry associated protein-1 (rap-1) locus promote exogenous gene expression in *Babesia bovis*. *Int. J. Parasitol.* **34**: 1177-1184.

Taylor, R.J. and McHardy, N. 1979. Preliminary observations on the combined use of imidocarb and *Babesia* blood vaccine in cattle. *J. S. Afr. Vet. Assoc.* **50**: 326-329.

Taylor, W.M., Bryant, J.E., Anderson, J.B. and Willers, K.H. 1969. Equine piroplasmosis in the United States-a review. *J. Am. Vet. Med. Assoc.* **55**: 915-919.

Thompson, P.H. 1969. Ticks as vectors of equine piroplasmosis. *J. Am. Vet. Med. Assoc.* **155**: 454-457.

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

Vial, H.J. and Gorenflot, A. 2006. Chemotherapy against babesiosis. *Vet. Parasitol.* **138**: 147–160.

Walse, B., Dufe, V.T., Svensson, B., Fritzson, I., Dahlberg, L., Khairoullina, A., Wellmar, U. and Al-Karadaghi, S. 2008. The structures of human dihydroorotate dehydrogenase with and without inhibitor reveal conformational flexibility in the inhibitor and substrate binding sites. *Biochemistry* **47**: 8929–8936.

World Organization for Animal Health (OIE). Manual of diagnostic tests and vaccines (online). Paris 2008. (Bovine babesiosis: http://www.oie.int/fr/nomes/mmanual/A_00059.htm).

Wright, I.G. and Goodger, B.V. 1988. Pathogenesis of babesiosis. In *Babesiosis of Domestic Animals and Man* (ed. Ristic, M.) 99-118. Boca Raton, Florida, CRC Press. Inc.

Wright, I.G. and Riddles, P.W. 1989. Biotechnology in tick-borne diseases: present status, future perspectives. In *FAO Expert Consultation of Biotechnology for Livestock Production and Health*, 325-340. Rome, FAO.

Wright, I.G., Gooder, B.V., Leatch, G., Aylward, J.H., Rode-Bramanis, K. and Waltisbuhl, D.J. 1987. Protection of *Babesia bigemina*-immune animals against subsequent challenge with virulent *Babesia bovis*. *Infect. Immun.* **55**: 364-368.

Wright, I.G., Goodger, B.V., Buffington, G.D., Clark, I.A., Parrodi, F. and Waltisbuhl, D.J. 1989. Immunopatholophysiology of babesial infections. *Trans. R. Soc. Trop. Med. Hyg.* **83** (Suppl.): S11-S13.

Zameitat, E., Fraymark, G., Dietz, C.D., Löffler, M. and Bölker, M. 2007. Functional expression of human dihydroorotate dehydrogenase (DHODH) in *pry4* mutants of *Ustilago maydis* allows target validation of DHODH inhibitors *in vivo*. *Appl. Environ. Microbiol.* **73**: 3371–3379.

Zintl, A., Mulcahy, G., Skerrett, H.E., Taylor, S.M. and Gray, J.S. 2003. *Babesia divergens*, a bovine blood parasite of veterinary and zoonotic importance. *Clin. Microbiol. Rev.* **16**: 622-636.

Zweygarth, E., Lopez-Rebollar, L.M. and Meyer, P. 2002. *In vitro* isolation of equine piroplasms derived from Cape Mountain zebra (*Equus zebra zebra*) in South Africa. *Onderstepoort J. Vet. Res.* **69**: 197-200.