Molecular epidemiologic analysis of tick-borne protozoan diseases of beef cattle from different regions of Thailand

2016

Charoonluk JIRAPATTHARASATE

Doctoral Program in Animal and Food Hygiene

Graduate School of Animal Husbandry

Obihiro University of Agriculture and Veterinary Medicine

タイの異なる地域における肉牛のマダニ媒介原虫感染症に対する分子疫学調査

平成 **28 年**

(2016)

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

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Abbreviations and unit abbreviations

Abbreviations

C	CI	- Confidence interval
D	DNA	- Deoxyribonucleic acid
	dNTP	- Deoxyribonucleotide triphosphate
E	EDTA	- Ethylenediaminetetraacetic acid
	ELISA	- Enzyme linked immunosorbent assay
H	Hb	- Hemoglobin
	HCT	- Hematocrit
I	IFAT	- Immunofluorescent antibody test
	ITS	- Internal transcribed spacer
M	MCH	- Mean corpuscular hemoglobin
	MCHC	- Mean corpuscular hemoglobin concentration
	MCV	- Mean corpuscular volume
	MPSP	- Major piroplasm surface protein
N	nPCR	- Nested PCR
O	OR	- Odd ratio
P	PCR	- Polymerase chain reaction

	Plt	- Platelet
R	RAP-1a	- Rhoptry associated protein-1a
	RBC	- Red blood cell
	RNA	- Ribonucleic acid
S	SBP2	- Spherical body protein 2
	SPSS	- Statistical package for the social sciences
T	TAE	- Tris-acetate-EDTA
	TBD	- Tick borne disease
W	WBC	- White blood cell

Unit abbreviations

bp	- Base pair	µl	-Microliter
°C	- degree Celsius	µm	-Micromolar
fl	-Femtolitre	sec	-Second
g/dl	-Gram/deciliter	U	-Unit
min	-Minute	%	-Percentage
ml	-Milliliter		
pmol	-Picomole		

General introduction

1. Bovine babesiosis

1.1. Etiology

Bovine babesiosis is a tick-transmitted intracellular heamoprotozoan disease that caused by *Babesia bovis*, *B. bigemina*, *B. divergens*, *B. major* and *B. ovata* [Bock *et al*., 2004]. *B. bovis* and *B. bigemina* are the most prevalent species that found throughout tropical and subtropical regions [Young *et al*., 1998]. Many tick vectors can transmit *Babesia* parasites. *Rhipicephalus microplus* and *R. annulatus* are the main vectors for *B. bigemnia*. In addition, *R. geigyi, R. decoloratus* and *R. evertsi* can also transmit this parasite. *R. microplus* and *R. annulatus* are recognized as vectors for *B. bovis* [Jongejan and Uilenberg, 2004].

1.2. Transmission and life cycle

The transmission of *Babesia* species occur by infected tick vectors. After ticks ingest parasites in blood of infected animals, the *Babesia* zygotes replicate and invade many tick's organs including ovaries. The parasites can pass to the offspring of tick in the egg and be transmitted from mother to progeny several generations by vertical (transovarian) transmission. When an infected tick infests to a new host, *Babesia* species are stimulated to develop their further life cycle in vertebrate host. *Babesia* sporozoites directly invade to red blood cells of the host and become to intraerythrocytic trophozoite stage. The trophozoite stages multiply and develop to merozoites by a process of

merogony (binary fission). The merozoites invade new erythrocyte and continuous their replicative cycle that cause destruction of other erythrocytes [Bock *et al*., 2004]. The larval tick can transmit *B. bovis* within 2-3 days after infection. In case of *B. bigemina* infection, the larval tick takes several days and become infective stage (approximately 9 days) and only nymphs and adults can transmit the parasite [Howell *et al*., 2007]. Beside tick vector transmission, biting insects and fomites can transmit *Babesia* spp. through direct inoculation of blood from infected animals to new hosts (mechanical transmission) [Nejash, 2016].

Babesia parasites are persisted in cattle herd by asymotomatic animals. *B. bovis* can maintain in the infected animals for many years while *B. bigemina* can survive for a few months in the hosts [Hutchings *et al*., 2007]. Calf can be infected by intrauterine infection but this phenomena is required pathological changes in placenta [Yeruham *et al*., 2003].

1.3. Clinical signs and pathology

Incubation period for *B. bovis* and *B. bigemina* are 2-3 weeks after tick infestation. Clinical manifestations of bovine babesiosis vary with age of animal, *Babesia* species and strain of the parasites. Incidence of the disease is higher in adult than in young animals. Strains of the parasites are considered in pathogenesis and pathogenicity, however, the cattle infected with *B. bovis* show more severe clinical signs than *B. bigemina* infection [Brown *et al*., 2006].

In acute phase of *B. bovis* infection, high fever (>40°C) is usually present for several days before other clinical signs can be observed. This is followed by depression, anorexia, weakness and increased respiratory rate. The fever during infection causes abortion in pregnant cattle [Carter and Rolls, 2015]. Haemoglobinuria and hemoglobinemia can be observed but these signs are not frequently found in *B. bovis* infection. Furthermore, capillaries of brain were obstructed by the sequestration of infected erythrocytes, resulting cerebral babesiosis and neurologic signs such as convulsion, posterior paralysis, coma and death are observed in *B. bovis* infection [Everitt *et al*., 1986]. The survivors may be weak and in reduced conditions such as weight loss and low milk yield, although they usually recover fully [Araújo *et al*., 1998]. In sub-acute or mild infection cases, clinical signs are less obvious and difficult to detect [Fahrimal *et al*., 1992]

Animals infected with *B. bigemina* are usually not as severely affected as those with *B. bovis* infection. The first sign is usually high fever for several days before other signs were detected. Infected animals become anorexia, depressed and separate from the herd. The manifested symptoms are caused by hemolysis and anemia. The infected animal appears pale mucous membranes and increase respiration and heart rate. Haemoglobinuria is present earlier and more consistently than *B. bovis* infection. Neurological sings are uncommon in *B. bigemina* infection and non-fatal animals are usually rapidly recovered [Carter and Rolls, 2015].

Post-mortem changes are intravascular hemolysis, anemia and jaundice. The pathological lesions include spleen and liver enlargement, gall bladder distended with thick granular bile and kidney congestion [Carter and Rolls, 2015]. The hemorrhage or

congestion was found in heart and brains lesions and surface of the brain become pink color [Demessie and Derso, 2015]. In animals infected with *Babesia* spp., clinical hematololoy is changed and the hematological parameters level related to different parasitemia rates [Mahmoud *et al*., 2008; Zulfiqar *et al*., 2012]. The decreasing hematological parameters include red blood cell (RBC), hematocrit (HCT) and hemoglobin (Hb), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) level. These results indicate microcytic-hemolytic anemia. Increase of leucocytes are commonly found in infected animals due to their immune response [Esmaeilnejad *et al*., 2012].

1.4. Diagnosis

Microscopic examination of thick and thin stained blood smear is commonly used to detect bovine babesiosis. The parasites are easily found in blood during acute infection but they may difficult to detect in chronic cases [Fahrimal *et al*., 1992]. *Babesia* parasites can be found within RBC in different stages including ring (annular), pear-shaped (pyriform) and trohozoites (single or pairs) stage [Surez and Noh, 2011].

Serological diagnosis is often used for epidemiological studies [Iseki *et al*., 2010; Terkawi *et al*., 2011]. Antibodies to *Babesia* infection are generally detected with an immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) [Mosqueda *et al*., 2012]. The presence of antibodies is a sign of previous infections, however does not relies on the present infectious status and cross-reaction

cannot differentiate of *Babesia* spp. in serological tests [Figueroa *et al*., 1992; Nayel *et al*., 2012]

Recently, polymerase chain reaction (PCR) has been widely applied to detect tick-borne diseases (TBDs) in both blood and vector samples instead of microscopy [Figueroa *et al*., 1993]. PCR assay have high sensitivity and specificity, thus considered as effective method to identify the current infections in field samples (Altay *et al*., 2008). PCR-based marker amplification of *B. bovis* and *B. bigemina* DNA has been used to directly detect the presence of the parasites in cattle blood samples. Spherical body proteins (SBP) 1, 2, 3 and 4 have been used as genetic markers to detect *B. bovis* [Silva *et al*., 2010; AbouLaila *et al*., 2010; Terkawi *et al*., 2011]. The *B. bovis* -SBP2 gene is conserved among the geographically different isolates and is not present in *B. bigemina* and it suitable to identify the infection between *B. bovis* and *B. bigemina* [AbouLaila *et al*., 2010]. Rhoptry-associated protein 1a (RAP-1a) gene is conserved and widely used to detect *B. bigemina* in cattle and water buffaloes in many countries [Terkawi *et al*., 2011; Ibrahim *et al*., 2013; Adjou Moumouni *et al*., 2015].

2. Benign bovine theileriosis

2.1. Etiology

Theileria parasite is a tick-borne protozoa of cattle in many regions. The most pathogenic species are *T. parva* and *T. annulata* that cause lymphoproliferative theileriosis with high morbidity and mortality rate [Mukhebi *et al*., 1992]. In contrast, *T. orientalis* is responsible for benign theileriosis in cattle and widely distributed in Asian countries

[Onuma *et al*., 1998]. In the past, *T. orientalis* is part of *T. sergenti/T. buffeli/T. orientalis* group of non-lymphoproliferative Theileria parasites [Watts *et al*., 2016]. Based on molecular studies, major piroplasm surface protein (MPSP) gene has been used to classify and designed *T. sergenti/T. buffeli/T. orientalis* group as one group and thereafter known simply as *T. orientalis* [Ota *et al*., 2009; Sivakumar *et al*., 2014].

The major tick vectors of *T. orientalis* are *Haemaphysalis longicornis* and *H. mageshimaensis* [Fujisaki *et al*., 1992; Yokoyama *et al*., 2012]. In addition, *T. orientalis* can be detected from other tick species such as *Hyalomma excavatum*, *Boophilus annulatus* and *R. microplus* [Altangeral *et al*., 2011a; Aktas *et al*., 2012].

2.2. Transmission and life cycle

T. orientalis is transmitted through the feeding of the infected ticks. There is a possibility that the parasite can be transmitted by sucking lice, but its life cycle cannot develop in the insects [Hammer *et al*., 2016].

Ticks are infected by feeding on an infected animal which has *Theileria* spp. in erythrocytes. Infected erythrocytes are digested and lysed in tick's gut lumen. *Theileria* parasites develop to gametocytes and fertilization occurs in the gut lumen. Zygote undergoes meiotic division and differentiates into motile kinete. The kinete travels from the tick's gut cell and pass to hemocoel. The parasites migrate and multiply in tick's salivary gland (sporogony), thereafter they develop into a multinucleate sporont. Sporozoite develops during tick feeding and slowly released into vertebrate host [Watt *et*

al., 2016]. Transtadial transmission but not transovarian transmission occurs in ticks [Stewart *et al*., 1996].

2.3. Clinical signs and pathology

After infected ticks transmit the infective sporozoite stage to animal hosts, the sporozoites invade leucocytes and develop into schizont stage within a few days. *Theileria* parasite can be detected in red blood cell at approximately 10 days post inoculation and transient pyrexia also is observed [Shimazu *et al*., 1992]. Clinical findings are weakness, icteric, pale mucous membranes, anemia, diarrhea and poor milk production [Izzo *et al*., 2010]. Severe disease is more frequently found in naïve animals when they are introduced into endemic area [McFadden *et al*., 2011]. Animals do recover from the infection but they remain infected for life [Watt *et al*., 2016].

Clinical pathology reflects a typical extravascular hemolytic anemia. There were marked decreases in hematocrit and hemoglobin. Furthermore, there are significant regenerative responses such as anisocytosis, poikilocytosis, polychromasia, basophilic stippling of RBC [Izzo *et al*., 2010]. However, the pathogenesis of anemia consequent to the infection is not clearly established [Stockham *et al*., 2000]. Leukogram of infected animals are variable, some animals have normal leucocyte counts and other has a leukocytosis [Izzo *et al*., 2010].

2.4. Diagnosis

The routine method of diagnosing theleriosis is by microscopic examination of Giemsa-stained thin blood smear [Biddle *et al*., 2013]. The morphology of *Theileria* spp. is 1.0 to 2.5 μm long, comma, signet, or rod-shaped basophilic inclusion body within the erythrocytes [Izzo *et al*., 2010]. The low number of parasite infection in erythrocytes is commonly observed in subclinical or carrier animals and difficult to detect the parasite by light microscope examination [Stockham *et al*., 2000].

Serological methods have been widely used to detect benign theileriosis in cattle [Stockham *et al*., 2000; Jenkins and Bogema, 2016] and epidemiological study [Stewart *et al*., 1992; Jeong *et al*., 2005].

Specific diagnosis of the parasite now relies on PCR assays using MPSP gene as genetic marker [Kim *et al*., 2004]. Furthermore, PCR detection based on MPSP gene also widely used for epidemiologic purposes in many countries [Ota *et al*., 2009: Jeong *et al*., 2010; Altangeral *et al*., 2011; Eamens *et al*., 2013; Sivakumar *et al*., 2013; Elsify *et al*., 2015]. This assay can detect parasite infection in cattle earlier than the presence of the parasite in blood smear and also allows differentiation of MPSP genotypes that may be associated with pathogenicity and geographic specificity [Kim *et al*., 2004; Ota *et al*., 2009]. Recently, *T. orientalis* can be distinguished into 11 genotypes (Type1-8 and type N1-N3) based on their MPSP gene sequences [Sivakumar *et al*., 2014].

3. Aim of the present study

Beef cattle represents as the largest livestock population in Thailand. Most of beef cattle farms in Thailand are located in north and northeastern parts and raised by traditional farming system [Krasachat, 2008]. Thai native cattle are classified as *Bos indicus* and this type is considered as tolerant breed when compared to exotic cattle. However, beef cattle productivity is constrained by tick-borne diseases such as babesiosis and theileriosis. The prevalence of bovine babesiosis and benign theileriosis in different parts of Thailand have been reported in various studies [Sarataphan *et al*., 2003; Altangeral *et al*., 2011a; Cao *et al*., 2012; Simking *et al*., 2013]. However, most of the epidemiological studies on *Babesia* spp. and *T. orientalis* focused on dairy cattle. This possibly explains lack of information on the burden of bovine babesiosis and benign theileriosis in beef cattle in Thailand. The cattle infected with *Babesia* spp. and *T. orientalis* may not show clinical signs but the effects of the infections on the hematological profiles of infected cattle have been reported [Ibrahim *et al*., 2009; Izzo *et al*., 2010; McFadden *et al*., 2011; Zulfiqar *et al*., 2012; Mohmmod, 2014].

The information on epidemiology of TBDs in beef cattle is required for developing the effective diagnosis, prevention and control strategies against babeisosis and benign theileriosis in beef herd in Thailand. The objectives of this study are stated as follows:

1. To determine the prevalence of *Babesia* spp. and *T. orientalis* parasites in beef cattle using PCR and to evaluate the risk factors associated with these parasites at farm level.

2. To determine genetic diversity and genetic relations of *Babesia* spp. and *T. orientalis* isolated from beef cattle.

3. To evaluate the effect of babesiosis and benign theileriosis on hematological parameters in naturally infected animals.

Chapter 1

Determination of prevalence and risk factor analysis of *Babesia* spp. and *Theileria orientalis* infection in beef cattle herd in Thailand

1-1. Introduction

The occurrence of bovine babesiosis and benign theileriosis in different parts of Thailand has been reported in various studies [Sarataphan *et al*., 2003; Altangeral *et al*., 2011; Cao *et al*., 2012; Simking *et al*., 2013]. However, most of the epidemiological studies on *Babesia* spp. and *T. orientalis* focused on dairy cattle. This possibly explains lack of information on the burden of bovine babesiosis and benign theileriosis in beef cattle in northern and northeastern Thailand. Local breeds of cattle are the dominant beef cattle in Thailand. Due to their natural tolerance to tick-borne diseases (TBDs) compared to the exotic dairy breeds, research on the epidemiology TBDs has been limited.

Previous studies have shown that native cattle possess a high degree of innate resistance to infection by tick-borne parasites as compare to exotic breeds and their crosses [Bock *et al*., 1997; Jonsson *et al*., 2006]. Consequently, native cattle that are infected with TBDs attain carrier state and become reservoir for infection in the herd [Jonsson *et al*.,

2008]. Diagnosis of field infection is usually based on identification of the parasites in thin blood smear using microscope. However, this method may not reliably detect parasites in chronic disease state due to low parasitemia [Almaria *et al*., 2001]. Recent advances in diagnostic technology have led to introduction of molecular tools as a means of overcoming low sensitivity of microscope in low parasitemia. Polymerase chain reaction (PCR) is high sensitive and specific, thus considered as an effective diagnostic tool for field samples [Altay *et al*., 2008].

The present study used PCR technique to determine the prevalence of *Babesia* spp. and *T. orientalis* parasites in beef cattle in Thailand. The risk factors associated with these parasites at farm level were evaluated with the aim of identifying effective prevention and control strategies against babesiosis and benign theileriosis in beef herds in Thailand.

1-2. Materials and methods

Sample and data collection

This cross sectional study was conducted between March 2014 and June 2015. The sample size was determined based on the method previously described [Humphry *et al*., 2004] using online epidemiological tool for sample size estimation (http://epitool.ausvet.com.au/content.php?page=PrevalenceSS). A total of 608 blood samples were collected from randomly selected herds from 9 provinces in northern, northeastern, western and central Thailand including Chiangrai, Payao, Mae hong sorn, Khon kaen, Mahasarakam, Loei, Kanchanaburi, Rachaburi and Nakhonpathom provinces (Fig. 1). The animals were restrained in crush and blood was collected from the jugular or

caudal vein and immediately transferred into 10 ml vacuum blood collection tube with EDTA-K_2. The samples were kept in cool box with ice pack and transported to the laboratory where they were stored at 4ºC until used. Data regarding the characteristics of animal and herd management were obtained through questionnaires administered by the investigators. The following data was obtained on individual animal characteristics: age, breed, gender and tick infestation. Farm based characteristics include location, herd size, contact between cattle and other domestic animals, history of tick infestation, tick control program and farming systems.

DNA extraction

Genomic DNA was extracted and purified from 200 µl of blood using the QIAamp® DNA blood Mini Kit (QIAGEN, Germany) according to the manufacturers instruction. The extracted DNA was stored at -20°C until they were used.

PCR detection of Babesia spp. and T. orientalis

The primers used for PCR amplification of specific genes are shown in Table 1. *B. bigemina* and *B. bovis* parasites were screened by nested PCR (nPCR). The final volume of 10 µl contained 1 µl of template DNA and 9 µl of reaction mixture with 0.25 mM of dNTP, 1x of PCR buffer, 0.5 pmol of each primer (Sigma-Aldrich, Japan), 0.05 U of *Taq*-polymerase (Ex-Tag DNA polymerase, Takara, Japan) and topped up with distill water to the final volume. The conditions used for amplification consisted of initial denaturing at 94ºC for 5 min, 35 cycles of a denaturing step at 94ºC for 1 min, an annealing step for *B.*

bovis at 64ºC for 1 min and *B. bigemina* at 55ºC for 1 min, and extension at 72ºC for 1 min and final extension at 72ºC for 7 min. The same concentration of buffer, dNTP and *Taq* polymerase used for amplification of 1 µl PCR product in the nPCR was the same as above. The nested PCR condition included initial denaturation at 95ºC for 5 min and 35 cycles of a denaturing step at 94ºC for 1 min, annealing temperatures of 55ºC for 1 min for *B. bigemina* and 58ºC for 1 min for *B. bovis*, and extension at 72ºC for 1 min and final extension at 72ºC for 7 min.

Identification of *T. orientalis* was performed in a total volume of 10 µl containing 1 µl of template DNA, 5 µl of Ampdirect plus® (Shimadzu corporation, Japan), 0.2 pmol of each primer (Sigma-Aldrich, Japan), 0.075U of *Taq*-polymerase (Ex-Tag DNA polymerase, Takara, Japan) and 3.725 µl of distilled water. Thermocycling consisted of initial denaturation at 94ºC for 5 min, 35 cycles of a second denaturation step at 94ºC for 30 sec, and annealing step at 58ºC for 45 sec, and extension at 72ºC for 1 min and and final extension at 72ºC for 7 min. The PCR products were separated by gel electrophoresis on 1.5% agarose in 1x TAE buffer and visualized using ethidium bromide under UV transilluminator.

Statistical analysis

Statistical analysis was done using SPSS version 21.0 software for Window (SPSS Inc, USA). Animals were grouped based on PCR results and categorized as positive or negative for *B. bigemina*, *B. bovis* and *T. orientalis*. Exposure variables included breed (native, native-brahman, native-indobrasil, native-charolaise, native-angus), gender (male

and female), age (<2, 2-10, >10years) and tick infestation (presence or absence of ticks at the time of sample collection). Farm based characteristics included location (northern, northeastern, western and central part), herd size (small to medium or large), contact between cattle and other domestic animals (yes or no), history of tick infestation (yes or no), tick control program (yes or no) and farming system (communal grazing or extensive farm).

Univariable analysis was performed using the Chi-square test or Fisher's exact test to determine the association between the presence of tick-borne protozoan infection and exposure variable. All variables with $p<0.05$ (2-sided) were selected for the backward elimination multivariable logistic regression model and model fit was assessed using the Hosmer-Lemeshow test. The interpretation value ($p<0.05$), adjusted odds ratios and 95% confidence interval (CI) were reported.

1-3. Results

Demographic characteristics of study population

Of the 608 beef cattle sampled from 61 farms, most animals were from western and central (45.9%) followed by northeastern (32.9%) and northern (21.2%) parts of Thailand. The most (85.2%) farms were small to medium and keeping between 1 to 30 heads of cattle. Majority (71.7%) of the farm are grazed their cattle on communal land while the rest (28.3%) kept cattle on their own farms under extensive system. The native (18.1%) and their crosses with brahman (47.0%) and indobrasil (23.2%) were the dominant beef breeds. Majority (72.3%) of the cattle were aged 2-10 years, followed those aged below 2 years

(24.3%) and above 10 years (2.8%). Females accounted for 81.7% of the cattle sampled while males were only 18.3%. A total of 46 farms (75.4%) had previous history of tick infestation and majority (69.6%, n=46) of them where located in northeastern Thailand. However, ticks were physically observed on only 17.3% of the cattle sampled during the study period. Majority of the farms (59.4%) had no tick control program although 40.6% used ivermectin injection for tick control. Furthermore, 84.2% of the farms had dogs and cats in close proximity to cattle.

Prevalence of single and co-infections

The prevalence of single *B. bigemina, B. bovis* and *T. orientalis* is shown in Table 2. The positive PCR products representative for the parasites species are shown in Figure 2. The PCR assay revealed that *T. orientalis* (19.2%) was the most prevalent single infection followed *B. bigemina* (10.2%) and *B. bovis* (5.4%) being the least. The overall level of co-infection with these parasites was 19.1%. However, dual co-infection between *B. bigemina* and *T. orientalis* (8.2%) was the highest compared to *B. bigemina* and *B. bovis* (7.2%), and *B. bovis* and *T. orientalis* (1.6%) co-infections. Triple co-infection with *B. bigemina, B. bovis* and *T. orientalis* was found in (2.0%) the cattle sampled (Table 3).

Risk factor analysis

Univariable analysis revealed that presence of *Babesia* spp. infection was statistically associated with previous history of tick infestation ($p<0.001$), tick infestation ($p<0.001$), herd size ($p=0.036$), lack of tick control program ($p<0.001$), communal grazing

(p<0.001) and contact with other domestic animals (p=0.006) (Table 4). However, multivariable analysis showed a strong association between *Babesia* spp. infection and communal grazing (OR=4.2, CI=2.3-7.7), contact with other domestic animals (OR=3.7, CI=1.6-8.4) and lack of tick control program (OR=4.0, CI=2.3-6.7) (Table 6). However, for *T. orientalis*, univariable analysis showed statistical significance with lack of tick control program (p< 0.001), tick infestation (p = 0.004) and communal grazing (p< 0.001) (Table 5). Multivariable analysis showed that tick infestation (OR=6.4, CI=1.5-26.6) and lack of tick control (OR=4.8, CI= 1.5-15.3) were the major risk factor for *T. orientalis* infection (Table 6). There was no significantly different (p>0.05) in the prevalence of the above parasites in the different age, gender, breeds and location and of cattle sample.

1-4. Discussion

Thailand is an endemic area for various bovine TBDs that negatively affect the health and productivity of cattle. Routine epidemiological studies therefore help in identifying the burden and risk factors for occurrence of TBDs as a basis for intervention. In this study, PCR was used to detect *Babesia* spp. and *T. orientalis* because it is reported to have high specificity and sensitivity [Altay *et al*., 2008]. Overall, 54.3% of the beef cattle sampled had at least one TBD (*B. bigemina*, *B. bovis* and *T. orientalis*). However, *T. orientalis* (19.2%) and *B. bigemina* (10.2%) were more prevalent than *B. bovis* (5.4%). These findings are consistent with previous studies in dairy cattle and water buffaloes from northern and northeast Thailand in which the prevalence of the above parasites ranged from 8.0 to 31.0% [Sarataphan *et al*., 2003; Altangeral *et al*., 2011a; Cao *et al*., 2012; Simking

et al., 2013]. The low prevalence of *B. bovis* (5.4%) compared to *B. bigemina* in this study also agrees with previous report [Amorim *et al*., 2014]. Previous studies have been reported that engorging female tick vector *Rhipicephalus (Boophilus) microlpus* is more frequently infected with *B. bigemina* than with *B. bovis* [Oliveira-sequeira *et al*., 2005; Oliveira *et al*., 2008]. Therefore, the chance of transmission of *B. bigemina* by its tick vector is higher than that of *B. bovis.*

This study also found dual and triple co-infection between *Babesia* spp. and *T. orientalis* in beef cattle. While *T. orientalis* is considered benign theileriosis [Onuma *et al*., 1998], the implication of such co-infection on its pathogenicity needs further investigation. However, previous reports suggests that co-infection with multiple TBDs often leads to fatal disease outcome especially in absence of cross immunological protection between the different parasites [Renneker *et al*., 2013; Chen *et al*., 2014].

In this study, the major risk factors for infection with *Babesia* spp. included lack of tick control program (OR=4.0, CI=2.3-6.7), communal grazing (OR=4.2, CI=2.3-7.7), contact with other domestic animals (OR=3.7, CI=1.6-8.4). Medonca Costa *et al*. [2013] also reported that cattle infested with ticks are over 18 times more likely to be infected with TBDs. High level of tick infestation indicates lack of tick control program on a farm [Regassa *et al*., 2003]. It was also observed that communally grazed cattle were at a high risk of *Babesia* spp. infection. Simuunza *et al*. [2011] attributed the above risk to sharing and increasing the spread of the tick vector leading to increased risks infection with TBDs. Contact with other domestic animal was associated with *Babesia* infections. Tick vectors can transmit the parasites to various hosts [Yeruham *et al*., 1996] and some *Babesia* spp.

can infect a wide range of animal hosts [Criado- Fornelio *et al*., 2003]. However, the role of other domestic animals in bovine babesiosis needs to be further studied.

The risk factors for *T. orientalis* infection in beef cattle were lack of tick control program (OR=4.8, CI= 1.5-15.3) and tick infestation (OR=6.4, CI=1.5-26.6). This implies that *T. orientalis* infection is associated to tick challenge. The common tick vector of *T. orientalis* is *Haemaphysalis* spp. [Fujisaki *et al*., 1992]. However, the ticks that were collected and identified were *R. microplus*. This possibly suggests that *R. microplus* may be an emerging vector for *T. orientalis* in Thailand. Previous research in Vietnam [Altangeral *et al*., 2011b] and China [Chen *et al*., 2014] found *T. orientalis* in *R. microplus*. The lack of current data on distribution of tick vectors for *T. orientalis* in Thailand warrants further research.

1-5. Summary

Beef cattle production represents the largest cattle population in Thailand. Their productivity is hampered by tick-borne diseases such as babesiosis and theileriosis. In this study, the prevalence of *B. bigemina, B. bovis* and *T. orientalis* was determined using polymerase chain reaction (PCR). Furthermore the risk factors for the occurrence of the above protozoan parasites in beef cattle from northern, northeastern, western and central parts of Thailand were assessed. A total of 608 blood samples were collected from beef cattle in 9 provinces. The study revealed that *T. orientalis* was the most prevalent (19.2%) parasite in beef cattle followed by *B. bigemina* (10.2%) and *B. bovis* (5.4%). Overall, 54.3% of the cattle screened were infected with at least one of the above parasites. Co-

infection with *Babesia* spp. and *T. orientalis* was 19.1%. *B. bigemina* and *T. orientalis* was the most prevalent (8.2%) co-infection although triple infection with the three parasites were observed in 2.0% of the samples. Lack of tick control program was the universal risk factor of the occurrence of *Babesia* spp. and *T. orientalis* infections in beef cattle in northern and northeastern Thailand. I therefore recommend training of farmers on appropriate tick control strategies and further research potential vectors for *T. orientalis* and elucidate the effect of co-infection with *Babesia* spp. on pathogenicity of *T. orientalis* infection on beef in northern and northeastern Thailand.

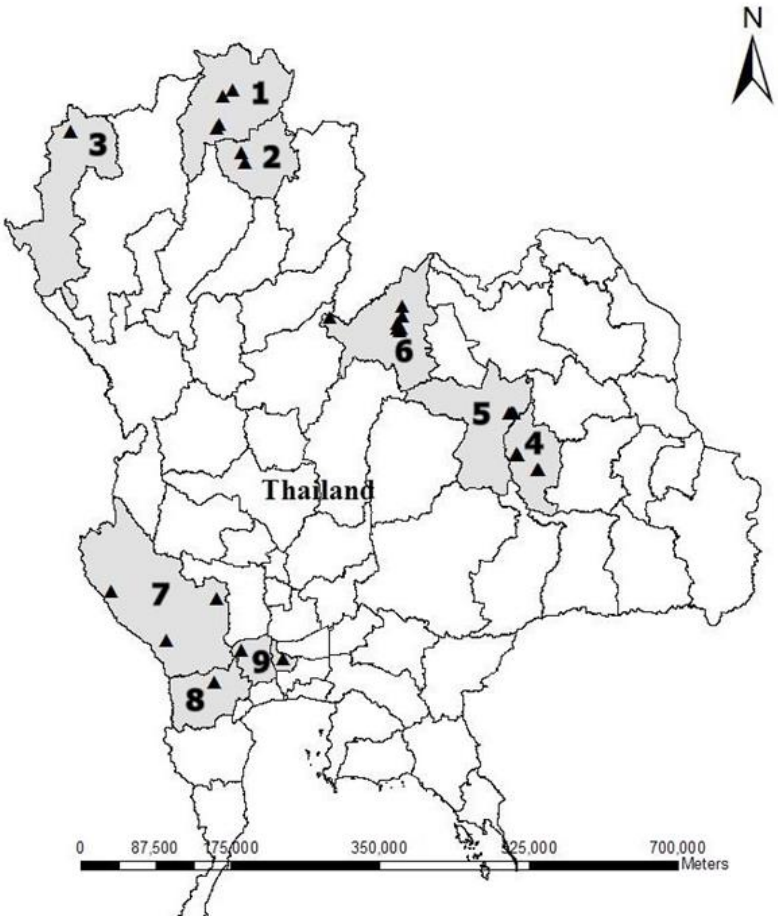

Fig. 1. Map of sampling areas in the northern, northeastern, western and central part of Thailand. The provinces are: 1, Chiangrai; 2, Payao; 3, Mae hong sorn; 4, Khon kaen; 5, Mahasarakam; 6, Loei; 7, Kanchanaburi; 8, Rachaburi; 9, Nakhonpathom.

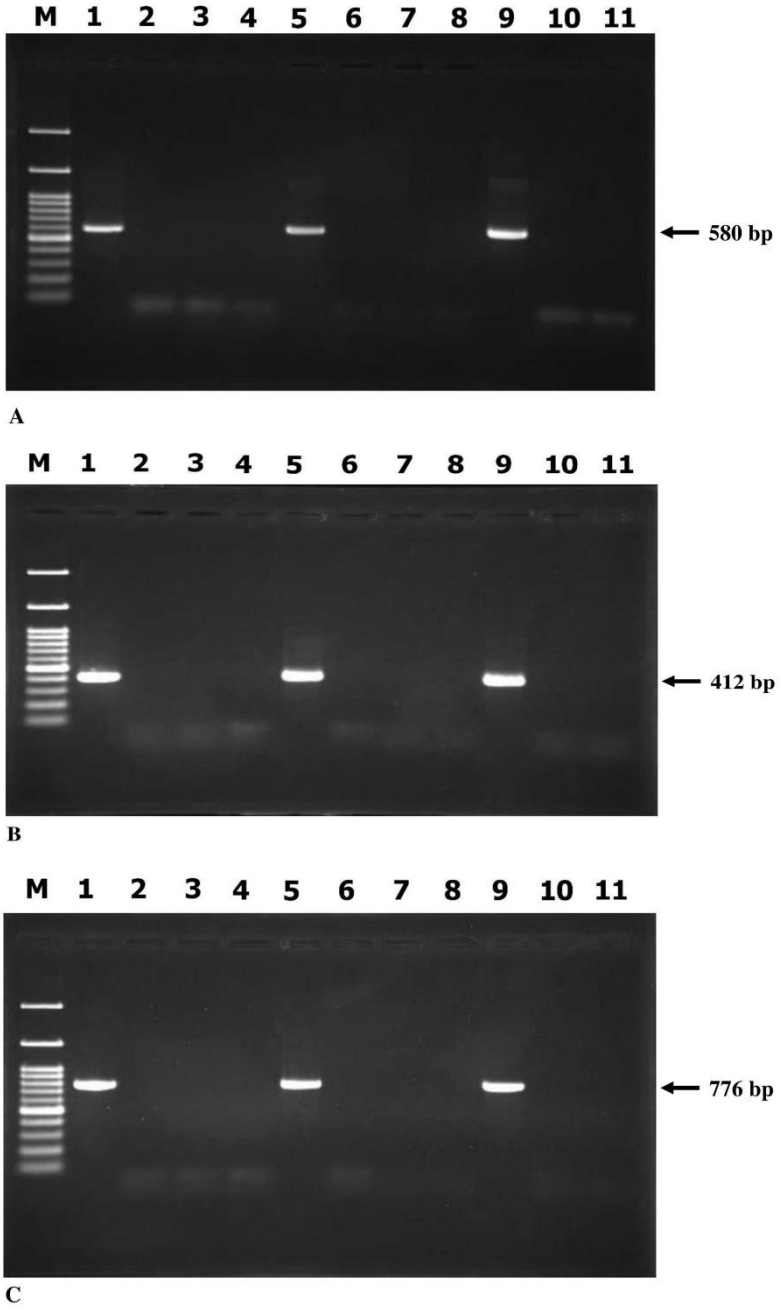

Fig. 2. PCR detection of *B. bovis* SBP2 gene (Panel A), *B. bigemina* RAP1 gene (Panel B) and *T. orientalis* MPSP gene (Panel C). M 100-bp DNA ladder. Lane 1 positive control; lanes 2-4, 6-8 and 10, negative samples, lanes 5 and 9 positive samples; lane 11 negative control.

Table 1. Primers used for the detection of *Babesia* spp. and *T. orientalis.*

Target	Assay	Sequence (5'-3')	Product size (bp)	Reference
B. bovis (SBP2 gene)	PCR	CTGGAAGTGGATCTCATGCAACC	1,236	AbouLaila *et al*., 2010
		TCACGAGCACTCTACGGCTTTGCAG		
	nPCR	GAATCTAGGCATATAAAGCAT	580	
		ATCCCCTCCTAAGGTTGGCTAC		
B. bigemina (RAP-1a gene)	PCR	GAGTCTGCCAAATCCTTAC	879	Terkawi *et al*., 2011
		TCCTCTACAGCTGCTTCG		
	nPCR	AGCTTGCTTTCACAACTCGCC	412	
		TTGGTGCTTTGACCGACGACAT		
T. orientalis (MPSP gene)	PCR	CTTTGCCTAGGATACTTCCT	776	Ota *et al*., 2009
		ACGGCAAGTGGTGAGAACT		

Table 2. Summary of *Babesia* spp. and *Theileria orientails* single species infections in cattle detected using PCR assay.

Region	Province	No. of tested cattle	*T. orientalis*		*B. bigemina*		*B. bovis*	
			Positive	%	Positive	%	Positive	%
North	Chiangrai	55	13	23.6	3	5.5	0	0
	Payao	14	3	21.4	1	7.1	2	14.3
	Mae hong son	60	13	21.7	10	16.7	10	16.7
Northeast	Khon kaen	50	11	22.0	8	16.0	4	8.0
	Mahasarakam	85	34	40.0	12	14.1	1	1.2
	Loei	65	25	38.5	9	13.8	1	1.5
Western and central	Kanchanaburi	125	9	7.2	2	1.6	6	4.8
	Rachaburi	90	8	8.9	1	1.1	3	3.3
	Nakhonpathom	64	1	1.6	16	25.0	6	9.4
	Total	608	117	19.2	62	10.2	33	5.4

Table 3. Multiple infections in cattle out of the 608 animals sampled.

Level of Co-infection	Parasite infection	Frequency	Infection rate (%)
Double	*B. bigemina* and *T. orientalis*	50	8.2
	B. bigemina and *B. bovis*	44	7.2
	B. bovis and *T. orientalis*	10	1.6
Triple	*B. bovis*, *B. bigemina* and *T. orientalis*	12	2.0
Total		116	19.1

Table 4. Univariable analysis of risk factors associated with prevalence of *Babesia* spp. infections in beef cattle.

Independent variable	Total tested	PCR-positive (%)	OR (95% CI)	*p*-value (<0.05)
1. Previous history of tick infestation				
Yes	371	134	4.75	<0.001
No	47	5	Reference	
2. Tick infestation				
Yes	51	29	3.08	<0.001
No	367	110	Reference	
3. Herd size				
Small to medium size	181	50	Reference	0.04
Large size	237	89	1.58	
4. Tick control program in farm				
Yes	288	69	Reference	<0.001
No	130	70	3.70	
5. Farming system				
Communal grazing	278	122	5.66	<0.001
Extensive farming	140	17	Reference	

Independent variable	Total tested	PCR-positive (%)	OR (95% CI)	*p*-value (<0.05)
6. Contact with other domestic animals				
Yes	352	127	2.54	0.006
No	66	12	Reference	

OR= Odd ratio, CI= Confidence interval

Table 5. Univariable analysis of risk factors associated with prevalence of *T. orientalis* infections in beef cattle.

Independent variable	Total tested	PCR-positive (%)	OR (95% CI)	*p*-value (<0.05)
1. Tick control program in farm				
Yes	267	48	Reference	<0.001
No	129	69	5.25	
2. Tick infestation				
Yes	56	34	4.79	<0.001
No	340	83	Reference	
3. Farming system				
Communal grazing	252	96	3.60	<0.001
Extensive farming	144	21	Reference	

OR= Odd ratio, CI= Confidence interval.

Table 6. Multivariable logistic regression analysis of risk factors associated with *Babesia* spp. and *T. orientalis* infections.

Independent variables	Adjusted OR	95% CI	*p*-value (<0.05)
Babesia spp. infection			
1. Farming system			
Communal grazing	4.2	2.3-7.7	<0.001
Extensive farming	Reference		
2. Tick control program in farm			
Yes	Reference		
No	4.0	2.3-6.7	<0.001
3. Contact with other domestic animals	3.7	1.6-8.4	0.002
Yes	Reference		
No			
T. orientalis infection			
1. Tick control program in farm			
Yes	Reference		
No	4.8	1.5-15.3	0.008
2. Tick infestation			
Yes	6.4	1.5-26.6	0.011
No	Reference		

OR= Odd ratio; CI= Confidence interval

Chapter 2

Molecular characterization and genetic diversity of *Babesia* spp. and *Theileria orientalis* isolated from beef cattle in Thailand

2-1. Introduction

Although various studies on genetic diversity and phylogenetic analyses of *Babesia* spp. and *T. orientalis* isolated from dairy cattle in Thailand have been reported [Altangerel *et al*. 2011a; Terkawi *et al*., 2011; Cao *et al*., 2012; Simking *et al*., 2013], genetic diversity and phylogenetic analysis of the above parasites in beef cattle have not been clarified.

Spherical body protein 2 (SBP2) gene of *B. bovis* and rhoptry-associated protein 1a (RAP-1a) gene of *B. bigemina* were widely used as target gene for the identification of filed isolates from different geographic areas because this gene is highly conserved [Terkawi *et al*., 2011; Cao *et al*., 2012; Ibrahim *et al*., 2013; Simking *et al*., 2013]. Furthermore, the rRNA internal transcribed spacer regions which including internal transcribed spacer 1 (ITS1) and an internal transcribed spacer 2 (ITS2) have greater variability in both their nucleotide sequences and length [Hill and Dixon, 1991]. Consequently, the ITS1-5.8s rRNA gene-ITS2 regions have been used as genetic markers for genetic evolutionary and taxonomic characterization studies of

Babesia spp. [Niu *et al*., 2009]. However, a few reports have revealed the nucleotide identities and similarities of these genes in Thailand.

The major piroplasm surface protein (MPSP) gene was considered as a useful epidemiology marker for genotyping *T. orientalis* in many countries [Sivakumar *et al*., 2014]. Recently, the genetic diversity of *T. orientalis* MPSP gene has been analyzed in dairy cattle and water buffalos in many regions of Thailand [Altangerel *et al*., 2011a]. The genetic diversity of *T. orientalis* MPSP gene isolated from cattle samples in Thailand was classified into 5 genotypes (types 1, 3, 5, 7, and N3). However, the genetic characterization and phylogenetic analysis of MPSP gene has not been clarified from beef cattle samples in Thailand.

The purpose of this study was to determine the identities and similarities of *B. bovis* SBP2 and *B. bigemina* RAP-1a gene for *Babesia* isolates from beef cattle in Thailand. The genetic diversity and genetic relationship of *T. orientalis* MPSP and ITS1-5.8s rRNA gene-ITS2 region of *B. bovis* and *B. bigemina* were evaluated and compared between these parasites and those from different countries.

2-2. Materials and methods

Study samples

Beef cattle samples from northern, northeastern, western and central parts of Thailand infected with *B. bovis*, *B. bigemina* and *T. orientalis* (**Chapter 1**) were randomly selected and used to characterize the above pathogens.

PCR amplification of B. bovis SBP2, B. bigemina RAP-1a and T. orientalis MPSP genes

Previously described specific gene PCR primers were used to amplify the cattle DNA samples for *B. bovis* SBP2, *B. bigemina* RAP-1a and *T. orintalis* MPSP genes [AbouLaila *et al*., 2010; Terkawi *et al*., 2011; Ota *et al*., 2009]. Nested PCRs (nPCR) were carried out for *B. bovis* and *B. bigemina*, while single PCR was used to detect *T. orientalis*. The composition of reaction mixture and cycling condition of the PCR assays were described in **Chapter 1**.

PCR amplification of ITS1-5.8s rRNA gene-ITS2 genomic region of B. bovis and B.bigemina

Genetic characterization of *Babesia* isolates was performed using ITS1-5.8s rRNA gene-ITS2 regions as marker. The specific primers used for the nPCR amplification are shown in Table 7. The PCR reaction mixtures were performed in final volume of 20 µl, containing 2 µl of DNA sample, 0.25 mM of dNTPs, 1x of PCR buffer, 1 pmol of each primer (Sigma-Aldrich, Japan), 0.1 U of *Taq*-polymerase (Ex-Taq DNA polymerase, Takara, Japan). The thermocycling conditions consisted of denaturation for 5 min at 94ºC, 35 cycles of a second denaturation step at 94ºC (1 min) and annealing step at 53ºC (1 min) for both *B. bovis* and *B. bigemina*, and extension at 72ºC (1 min), then final extension at 72ºC for 10 min. The nPCR condition was similar to first PCR condition steps, except that the annealing temperature of *B. bigemina* was 55ºC.

Cloning and sequencing

For each parasite, six PCR products were randomly selected from among the positive samples for cloning and sequencing. PCR products were extracted from agarose gel using QIAquick Gel extraction kit (Qiagen, Germany), subsequently ligated into a pGEM-T Easy Vector (Promega, USA) and transformed into *Escherichia coli* DH5α-competent cells. For each sample, 2 selected colonies were expanded in overnight cultures and DNA was extracted using Nucleospin® Plasmid QuickPure (MACHERY-NAGEL, Germany). The inserts of *B. bovis* SBP2, *B. bigemina* RAP-1a, *T. orientalis* MPSP genes, *B. bovis* and *B. bigemina* ITS regions in the purified plasmids were sequenced using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI Prism 3100 140 genetic analyzer (Applied Biosystems, USA). The sequences were analyzed using Bioedit version 7.2.5 (Tom Hall Ibis Biosciences, USA) and their identities and similarities were determined by GenBank BLASTn analysis. The percent identities between nucleotide sequences were computed by Pairwise distance using MEGA version 6.0 program [Tamura *et al*., 2013].

Phylogenetic tree analysis

Phylogenetic trees were constructed from *B. bovis* and *B. bigemina* ITS regions and *T. orientalis* MPSP sequences obtained in this study along with corresponding sequences from other regions of the world banked in genetic databases. Multiple sequence alignment was performed for each locus with the ClustalW algorithm and then genetic relatedness was analyzed by neighbor-joining phylogenetic tree using MEGA version 6.0 program [Tamura *et al*., 2013]. Bootstrap test with 1000 replications was used to estimate the confidence of the branching pattern of the trees.

Nucleotide sequence accession numbers

The nucleotide sequences that were identified in this study were available in the GenBank database of the National Center for Biotechnology Information (NCBI). The GenBank accession number for the gene sequences are shown in Table 8.

2-3. Results

Sequence analysis of B. bovis SPB2, B. bigemina RAP-1a, T. orientalid MPSP genes, B. bovis and B. bigemina ITS1-5.8s rRNA gene-ITS2 regions

B. bovis (n= 6) and *B. bigemina* (n=6) positive samples were used to analyze the partial sequence of *B. bovis* SBP2 (580 bp) and *B. bigemina* RAP-1a genes (412 bp). The nucleotide identity of BoSBP2 sequences (KU764505-KU764510) was 90.1-99.8 % and they shared 90.2-97.9 % identity with published sequences (AB742544, AB742545, AB742547, AB772320 and AB772322). BiRAP-1a sequences in this study (KU764511-KU764516) were highly conserved (99.8-100%) and shared 99.7-100 % identity with published sequences (AB617643, AB586126, AB594817, and JX648554). Six *T. orientalis* MPSP gene sequences (776 bp) were obtained in this study and shared 73.2-98.8% nucleotide identity with each other (KU764499-KU764504).

The ITS regions were amplified from *B. bovis* and *B. bigemina* PCR positive samples to further investigate their identity and their phylogenetic relationship. Six different sequences of *B. bovis* ITS (KU841554-KU841559) were obtained and their length ranged from 520 to 544 bp (Fig. 3). The identity among these sequences was 93.1-97.1% and they shared 92.7-96.7% identity with sequences available in the GenBank (JN974304, EF547925, EF458291, EF458292 and EF458287). Furthermore, the six partial sequences of *B. bigemina* ITS (KU841548-KU841553) (Fig. 4) were

97.3-99.6% identical to each other and shared 95.7-99.4% identities with database sequences (JN974295, EF458262, EF458267, EF458249 and HM538263).

Phylogenetic analysis of B. bovis and B. bigemina ITS1-5.8s rRNA gene-ITS2 regions and T. orientalis MPSP sequences

The *B. bovis* ITS sequences were all found in the same clade (clade 3) together with sequences from China (EF457925), Thailand (JN974304) and Brazil (EF458291, EF458287 and EF458292) (Fig. 5). *B. bigemina* ITS phylogenetic tree (Fig. 6) showed that the sequences from this study were scattered in different subgroups of the same clade (clade 1). Two isolates were located in the same clade with previously reported sequences from China (HM538247, HM538227 and HM538263). Three isolates were confined to the same clade as previously published isolates from Thailand (JN 974295 and JN974296). Furthermore, one isolate showed a close relationship with a sequence from Brazil (EF458243).

Phylogenetic analysis revealed that *T. orientalis* MPSP gene sequences in this study were classified into 3 clades; type 3, type 5 and type 7 (Fig. 7). Two *T. orientalis* MPSP gene sequences were located in type 3 clade, closely related to isolates reported in cattle from Mongolia (AB571893), Thailand (AB562279) and Brazil (AB581622). The *T. orientalis* MPSP type 5 sequences were similar to China (AB571967), Thailand (KT460099) and Japan (AB491347) sequences. In addition, *T. orientalis* MPSP type 7 sequences were closely related to the type 7 sequence from Thailand (KT460098 and AB562581), Japan (AB218430) and Vietnam (AB560823).

2-4. Discussion

Information concerning the molecular characterization and genetic diversity of parasite isolates will likely influence the development of effective diagnostic tools and vaccine strategies for control of bovine tick-borne diseases. In this study, positive blood DNA samples for *B. bovis, B. bigemina,* and *T. orientalis* were used to characterize and analyze the genetic diversity of the target gene within and those from different countries. The sequence of *B. bovis* SBP2 gene was 90.1-99.8 % identity and shared 90.2-97.9% similarity with published sequences in GenBank database [AboLaila *et al*., 2010; Nagano *et al*., 2013]. In addition, *B. bigemina* RAP-1a sequences in this study also showed high identities with each other (99.8-100%) and conserved among geographic isolates (99.7-100%) [Terkawi *et al*., 2011; Ibrahim *et al*., 2013]. These results confirmed that the two genes are highly conserved and valuable gene targets to detect the parasite from different areas [AboLaila *et al*., 2010; Terkawi *et al*., 2011; Ibrahim *et al*., 2013; Nagano *et al*., 2013; Simking *et al*., 2013]. In this study I also identified and analyzed *B. bovis* and *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region sequences in beef cattle isolates. Previous studies have reported these regions are useful for identification of the new species as well as differentiation between parasite species and subspecies [Bostrom *et al*., 2008; Niu *et al*., 2009]. The sequences of *B. bovis* region were more diverse in their nucleotide length and nucleotide identity (93.1-97.1%) than *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region sequences. These results were in agreement with the previous study on *Babesia* spp. isolated from cattle in Thailand which demonstrated that the ITS1-5.8s rRNA gene-ITS2 region sequences of *B. bovis* have higher nucleotide variability than *B. bigemina* [Cao *et al*., 2012]. However, in the phylogenetic tree, ITS1-5.8s rRNA gene-ITS2 sequence of *B. bovis* formed a monophyletic clade with other known *B. bovis* ITS1-5.8s rRNA gene-ITS2

sequences (Fig. 5). Furthermore, *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region also clustered in one clade (Fig. 6). This finding suggested that *Babesia* spp. isolates might belong to the same species but different strains of *B. bovis* and *B. bigemina* [Cao *et al*., 2012; Zhou *et al*., 2016].

The MPSP gene has been recognized as an epidemiological molecular marker for identification and characterization of the genetic diversity of *T. orientalis* [Altangeral *et al*., 2011; Sivakumar *et al*., 2014). Recently, *T. orientalis* isolates from different countries have been divided into 11 genotypes (type1-8 and type N1-N3) based on their MPSP gene sequences [Sivakumar *et al*., 2014]. Previous molecular characterization of *T. orientalis* in cattle in Thailand classified MPSP gene isolates into 5 genotypes (type 1, 3, 5, 7 and N3) [Altangeral *et al*., 2011a]. However, phylogenetic analysis in this study revealed that *T. orientalis* MPSP sequences were classified into 3 clades (type 3, 5 and 7). The current study was unable to detect MPSP genotype 1 and N3 probably due to the limited number of samples, farms, or area covered. Therefore, a large-scale study with an increased number of samples from different provinces needs to be undertaken.

In conclusion, sequence analysis showed that *B. bovis* SPB2 gene and *B. bigemina* RAP-1a gene were highly conserved while *B. bovis* and *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region sequences showed genetic diversities in the different isolates. The *T. orientalis* MPSP gene from parasites isolated from beef cattle in Thailand was classified into type 3, 5 and 7 as previously reported.

2-5. Summary

The genetic diversities of *B. bovis, B. bigemina* and *T. orientalis* infecting beef cattle in Thailand were analyzed in this study. The standard and nested PCR were performed using species-specific primers based on the *B. bovis* SBP2, *B. bigemina* RAP-1a and *T. orientalis* MPSP genes. Amplicons of *B. bovis* and *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region and *T. orientalis* MPSP genes were sequenced for phylogenetic analysis. Sequence analysis revealed that *B. bovis* SPB2 gene were 90.4-99.8 % identity and shared 87.4-98.1% similarity with published sequences in GenBank database. In addition, *B. bigemina* RAP-1a sequences in this study also showed high identities with each other (99.8-100%) and conserved among geographic isolates (99.7-100%). The *B. bovis* and *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region sequences showed great genetic diversities in the different isolates. The *B. bovis* ITS1-5.8s rRNA gene-ITS2 region sequences were more diverse in their nucleotide length and nucleotide identity (93.1-97.1%) than *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region sequences. In the phylogenetic tree, ITS1-5.8s rRNA gene-ITS2 sequence of *B. bovis* formed a monophyletic clade with other known *B. bovis* ITS1-5.8s rRNA gene-ITS2 sequences. Furthermore, *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region also clustered in one clade. Six *T. orientalis* MPSP gene sequences (776 bp) were obtained in this study shared 73.2-98.8% nucleotide identity with each other. Phylogenetic analysis showed that the *T. orientalis* MPSP gene from beef cattle in Thailand was classified into type 3, 5 and 7 as previously reported.

Table 7. Specific primers of *B. bovis* and *B. bigemina* ITS1-5.8s rRNA gene-ITS2 regions [Cao *et al*., 2012].

Species	Assays	Sequences (5'-3')	Product size
B. bovis	PCR	CGTCCCTGCCCTTTGTA TATTTTCTTTTCTGCCGCTT	815
	nPCR	CACCACCAGTGGAAGCAC TTGTGCCCCATGGACACT	545
B. bigemina	PCR	CGTCCCTGCCCTTTGTA TATTTTCTTTTCTGCCGCTT	1,041
	nPCR	CGTCCCTGCCCTTTGTA TATTTTCTTTTCTGCCGCTT	495

Table 8. Accession number of DNA sequences deposited in GenBank.

Parasite	Target gene	Isolate			
		Accession number	Sequence length (bp)	Province	Region
B. bovis	*SBP-2*	KU764505	583	Mahasarakham	Northeast
		KU764506	583	Loei	Northeast
		KU764507	583	Maehongsorn	North
		KU764508	583	Maehongsorn	North
		KU764509	583	Kanchanaburi	West
		KU764510	583	Nakhonpathom	Central
B. bigemina	*RAP-1a*	KU764511	412	Loei	Northeast
		KU764512	412	Loei	Northeast
		KU764513	412	Loei	Northeast
		KU764514	412	Maehongsorn	North
		KU764515	412	Payao	North
		KU764516	412	Maehongsorn	North
B. bovis	*ITS*	KU841554	525	Khonkan	Northeast
		KU841555	542	Chiangrai	North
		KU841556	530	Rachaburi	West
		KU841557	544	Kanchanaburi	West
		KU841558	536	Payao	North
		KU841559	520	Khonkan	Northeast
B. bigemina	*ITS*	KU841548	494	Kanchanaburi	West
		KU841549	498	Khonkan	Northeast
		KU841550	498	Payao	North
		KU841551	494	Nakhonpathom	Central
		KU841552	494	Loei	Northeast
		KU841553	497	Maehongsorn	North
T. orientalis	*MPSP*	KU764499	776	Kanchanaburi	West
		KU764500	776	Nakhonpathom	Central
		KU764501	776	Chiangrai	North
		KU764502	776	Maehongsorn	North
		KU764503	776	Khonkan	Northeast
		KU764504	776	Mahasarakham	Northeast

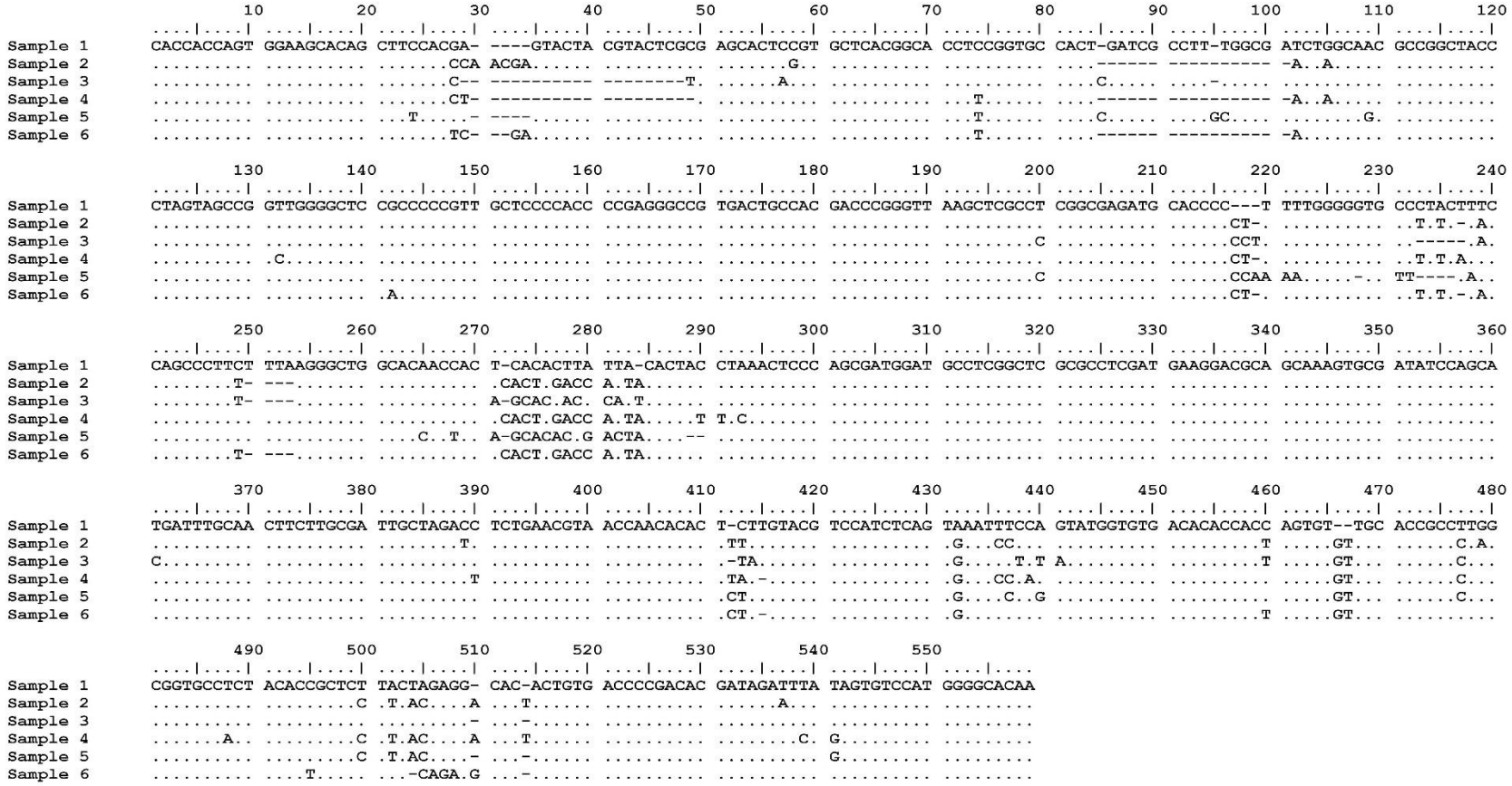

Fig . 3. Nucleotide alignment of ITS1-5.8s rRNA gene-ITS2 sequences from 6 *B. bovis* isolates from beef cattle.

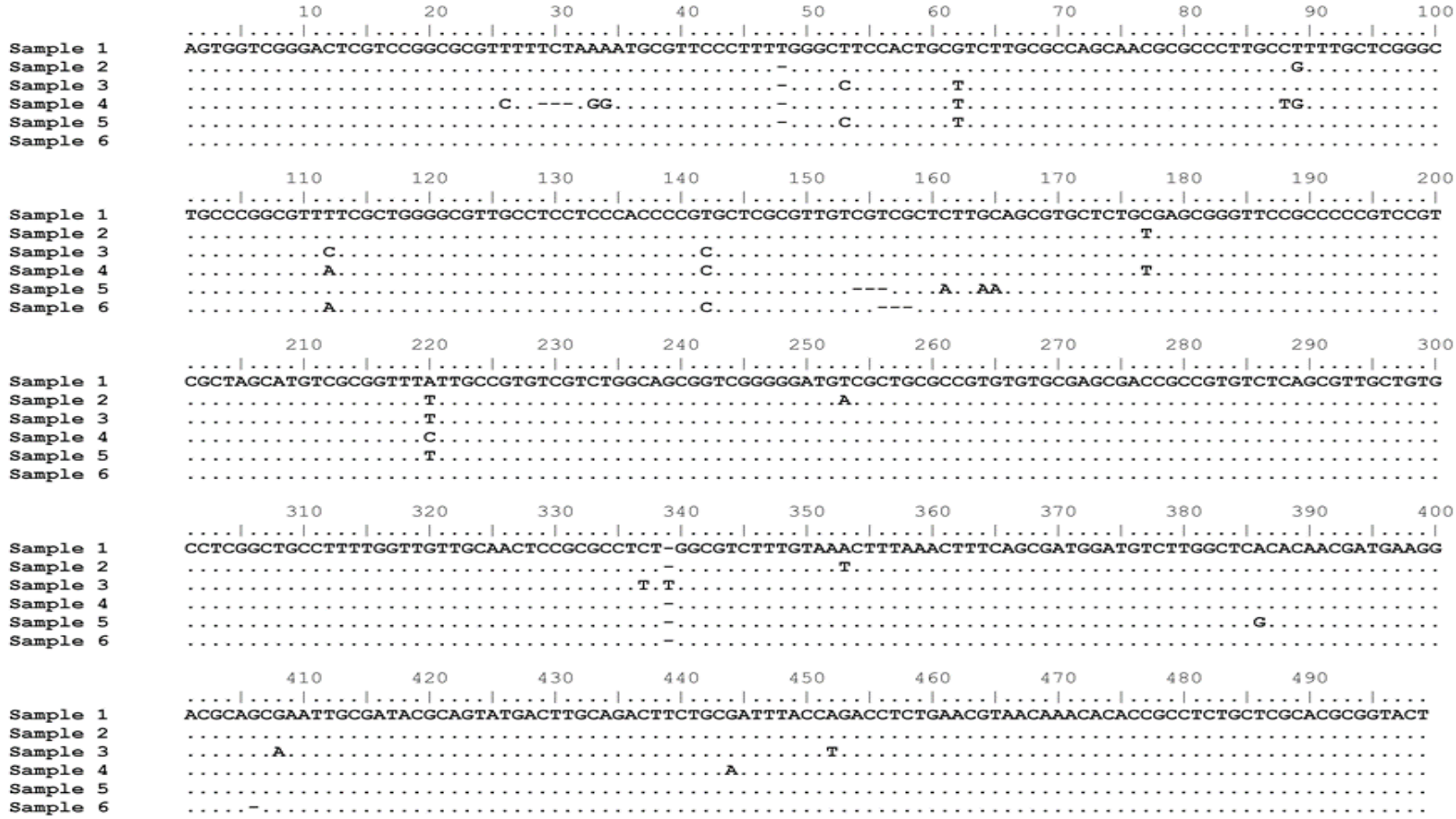

Fig. 4. Nucleotide alignments of ITS1-5.8s rRNA gene-ITS2 sequences from 6 *B. bigemina* isolates from beef cattle.

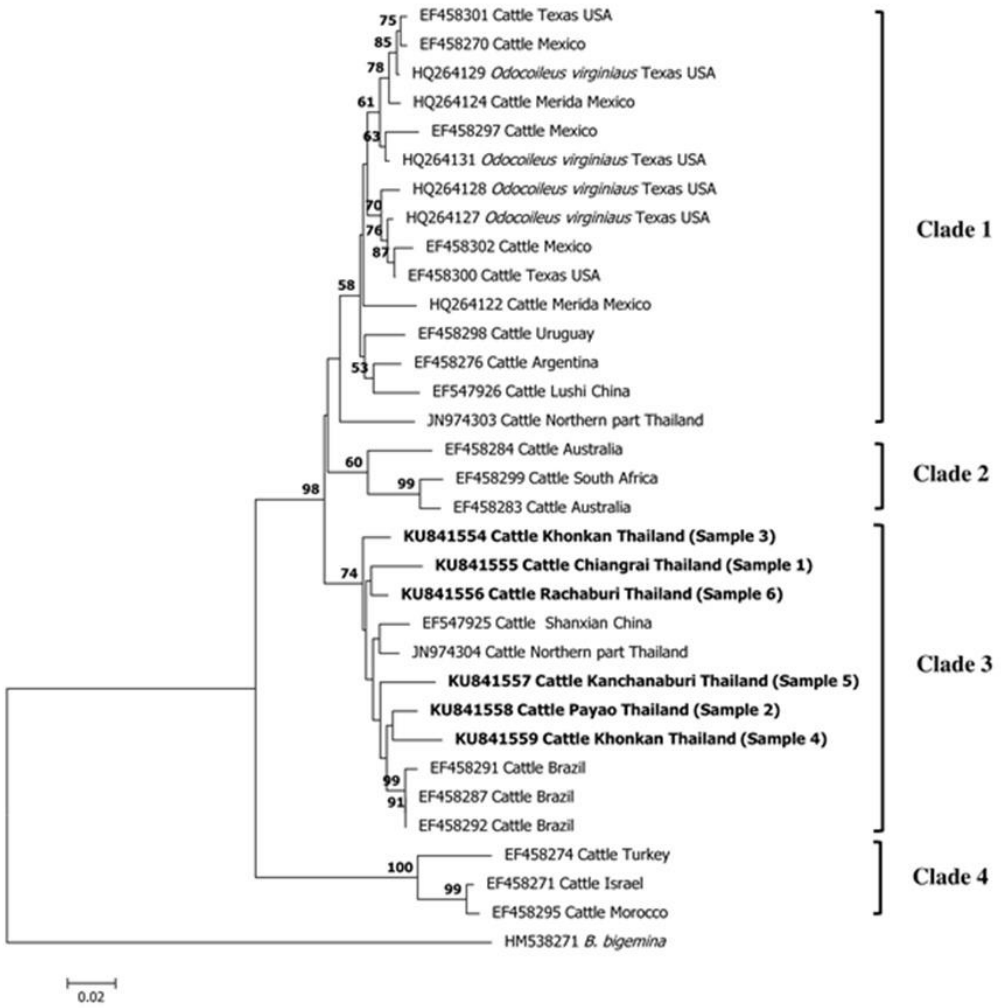

Fig. 5. Phylogenetic tree based on *B. bovis* ITS1-5.8s rRNA gene-ITS2 region sequences from this study (boldface letters) and sequences retrieved from the Genbank database. Genbank Accession Numbers for all sequences are shown. Bootstrap values are provided at the beginning of each branch. The ITS1-5.8s rRNA gene-ITS2 region sequence of *B. bigemina* (HM538271) was used as outgroup.

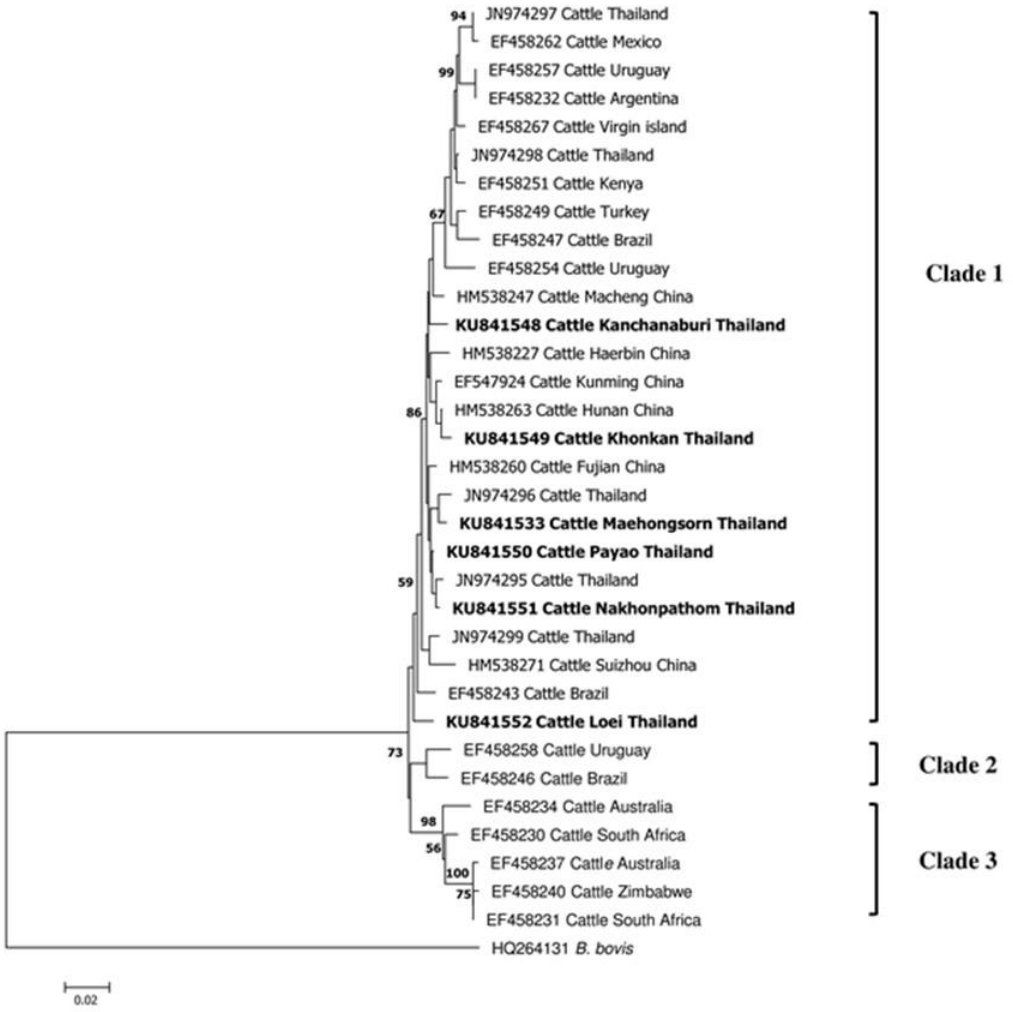

Fig. 6. Phylogenetic tree based on *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region sequences from this study (boldface letters) and sequences retrieved from the Genbank database. Genbank Accession Numbers for all sequences are shown. Bootstrap values are provided at the beginning of each branch. The ITS1-5.8s rRNA gene-ITS2 region sequence of *B. bovis* (HQ264131) was used as outgroup.

Fig 7. Phylogenetic tree based on *T. orientalis* based on MPSP gene sequences from this study (boldface letters) and sequences retrieved from the Genbank database. Genbank Accession Numbers for all sequences are shown. Bootstrap values are provided at the beginning of each branch. The Tams1 gene of *T. annulata* (JX683683) and *T. parva* (L47209) were used as outgroup.

Chapter 3

Investigation of hematological parameters in beef cattle naturally infected with *Babesia* spp. and *Theileria orientalis* in Thailand

3-1. Introduction

Babesia spp. and *Theileria* spp. are known to be tick-borne parasites that infect bovine erythrocytes and cause anemia [Bock *et al*., 2004; Izzo *et al*., 2010]. *B. bovis* and *B. bigemina* play an important role in causing anemia in affected animals. Clinical manifestations of disease associated with bovine babesiosis are high fever (>40°C), inappetence, depression and weakness [Mohmmod, 2014]. Hemolytic anemia, which is characteristically macrocytic and hypochromic, is a feature of *B. bovis* and *B. bigemina* infections. Furthermore, low hematocrit (<3.0 x 10^6 cells/ml) and hemoglobin (<50 g/l) can be observed in hematological profiles of infected animals [de Vos and Potgieter, 1994]. Severe anemia is particularly evident in chronic cases, while acute cases may die with little evidence of anemia. Circulatory stasis and hypotension cause renal and liver damage and muscle degeneration. Therefore, blood chemistry profiles of animals were changed during the infections [de Vos and Potgieter, 1994].

Benign theileriosis caused by the *Theileria sergenti/buffeli/orientalis* group is a common infection of cattle worldwide. Although the infection caused by theses parasite may be non-pathogenic in healthy cattle, but clinical disease and deaths have been reported [Izzo *et al*., 2010; Aparna *et al*., 2011; McFadden *et al*., 2011]. Clinical signs associated with benign theileria group infection included lethargy, anorexia, inappetance, pale mucous membrane and severe of anemia [McFadden *et al*., 2011].

Several studies have shown the effects of *Babesis* spp. and *Theileria* spp. infections on the hematological profiles of infected cattle [Ibrahim *et al*., 2009; Izzo *et al*., 2010; McFadden *et al*., 2011; Zulfiqar *et al*., 2012; Mohmmod, 2014]. Severe anemia was observed in naïve cattle and their cross when infected by tick-borne parasites [McFadden *et al*., 2011; Ukwueze and Orajaka, 2014]. However, the effect of tick-borne infections on hematological profiles of beef cattle has not been elucidated in Thailand. Therefore, this present study was conducted to compare the hematological profiles between the animals infected with *Babesia* spp. and *T. orientalis* and non-infected animals.

3-2. Materials and methods

Sample collection and DNA extraction

Animal samples (n=608) were randomly collected from 9 provinces in 3 parts of Thailand (**Chapter 1**). DNA was purified from 200 µl of EDTA anticoagulated blood with a QIAamp® DNA blood Mini Kit (QIAGEN, Germany) according to the manufacturers instruction. The extracted DNA was stored at -20°C until they were used.

PCR amplification

Specific gene PCR primers were used to amplify the *B. bovis*-SBP2, *B. bigemina*-RAP-1a, *T. orientalis* from cattle DNA samples (Table 1). The composition of the reaction mixture and cycling condition of the PCR assays were performed according to the methods described in **Chapter 1**.

Hematological analysis

Thin blood smears were prepared immediately after blood collection. The blood smears were air dried, fixed in methanol, stained with Giemsa and examined under light microscope for the presence of the parasites in erythrocytes. The hematological parameters of whole blood including white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), Hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet (Plt) were analyzed with fully automated Scil Vet ABC hematology analyzer (Scil animal care company, Viernheim, Germany).

Statistical analysis

All samples were divided into 2 groups based on PCR results as PCR positive and PCR negative. Statistical analysis was performed using SPSS version 21.0 for Window (Chicago, USA). The measurements obtained for each group were tested for normality with the Shapiro-Wilk and Kolmonogorov-Smirnov tests. Comparisons between the

animal with and without a positive test for single and co-infection in each hematology parameters were performed with the Mann-Whitney U test. The data were considered significant at $p<0.05$. Results are reported as min-max and medians where appropriate.

3-3. Results

PCR detection of single and co-infections

Out of total 608 blood samples, PCR based diagnosis revealed 328 animals were positive for single and co-infections with TBDs. Single infection was detected in *B. bovis* (n=33), *B. bigemina* (n=62) and *T. orientalis* (n=117). Furthermore, 116 out of 328 animal samples were co-infections with *B. bigemina* and *T. orientalis* (n=50), *B. bovis* and *B. bigemina* (n=44), *B. bovis* and *T. orientalis* (n=10) and triple infections (n=12).

Hematological changes in infected cattle

The hematological profiles of cattle infected which single infection with *B. bigemina* or *T. orientalis* were summarized in Table 9. Sixteen samples were excluded from hematological study due to red blood cell hemolysis during processing. Hematological parameters of cattle infected with *B. bigemina* such the total WBC ($p=0.001$), lymphocyte ($p=0.015$) and eosinophil count ($p=0.022$) were significantly higher than those of negative group. However, low level of hemoglobin ($p=0.005$), HCT ($p=0.018$), MCH ($p<0.001$) and MCHC ($p=0.001$) were found in *B. bigemina* positive group. In animals infected with *T. orientalis*, the mean of total WBC ($p=0.016$) and

eosinophil count (p<0.001) were significantly increase whereas mean of RBC (p=0.002) and HCT (p<0.001) were significantly decrease when compared to negative cattle group.

Hematological profiles of co-infected animals were showed in Table 10. In *B. bovis* and *B. bigemina* co-infections group, the high significance level of hematological parameters were found in mean of total WBC (p<0.001) and lymphocyte count (p=0.001) while mean of MCV (p=0.006) and MCH (p=0.004) were significantly decrease. The high significance level of total WBC (p=0.002), eosinophil count (p<0.001) and MCV (p=0.023) were found in animal's co-infections with *B. bigemina and T. orientalis*. However, HCT level (p<0.001) was significantly lower in this co-infection group. There were no differences in the mean of hematological parameters of cattle infected with *B. bovis* single infection, co-infection with *B. bovis* and *T. orientalis* and triple infections group.

3-4. Discussion

In this study, all of the animal sam*p*les were found negative in Giemsa-stained blood smear while 328 samples were found positive by PCR based diagnosis. This may be attributed to the stained thin blood smear technique that may not reliably detect parasites in subclinical animal or carriers due to the low parasitemia [Almaria *et al*., 2001]. All hematological parameter levels in both PCR-positive and PCR-negative group were within normal range when compared to reference values of bovine blood parameters. Previous studies have shown that local breed has a high degree of innate resistance to infection by

tick-borne parasites [Bock *et al*., 1997; Jonsson *et al*., 2008]. However, some parameters of infected animals showed significantly different with non-infected group.

In this study, hemogram of infected animals revealed leukocytosis, lymphocytosis and eosinophilia as indicated by significantly increase ($p<0.05$) in total WBC, lymphocyte and eosinophil counts in both single and co-infection groups. In case of *B. bigemina* infection, leukocytosis may be caused by an increase lymphocyte and eosinophil counts. Several studies have been reported that high number of lymphocyte and monocyte were noted in *Babesia* spp. infection [Ambawat *et al*., 1999; Rubino *et al*., 2006; Esmaeilnejad *et al*., 2012]. However, the number of monocyte was not significantly different between PCR-positive and PCR-negative groups in this study. Increasing lymphocyte and monocyte could be attributed to their role as active mediators in the innate immune response in infected animals [Johnson *et al*., 1996; Shoda *et al*., 2000]. This could be explained as breakdown of red blood cells by *Babesia* spp. the phagocytic cells to clean up the body from toxic remnant of rupture red blood cells [Court *et al*., 2001]. The eosinophilia in infected animal was observed in this study. This could be implied that the sensitivity to the foreign protein of parasite which may be a part of an immune response [Esmaeilnejad *et al.*, 2012].

T. orientalis is an important benign theileriosis in cattle in Southeast Asia and classified as non-lymphoproliferative theileria parasite group [Sivakumar *et al*., 2014]. However, the level of total WBC and eosinophil count was significantly increase in *T. orientali*s PCR-positive group in this study. The leukocytosis in *T. orientalis* infection in this study is consistent with Stockham *et al*. [2000] reported that animals which had infected with *T. orientalis* showed lymphoid hyperplasia and leukocytosis. The

pathogenesis of leukocytosis in benign theileriosis is not clearly established because lymphocytic schizont was not common found in non-lymphoproliferative theileria group [Choi *et al*., 2016]. Therefore, further studies to evaluate WBC indices in *T. orientalis* infection are needed.

Although, the HCT, RBC and hemoglobin value in single and co-infection were not lower than reference value but it showed significantly decrease when compared to non-infected animals. These results were consistent with previous reports that decline in HCT, Hb content and RBC observed in animals infected with *Babesia* spp. and *T. orientalis* [Ibrahim *et al*., 2009; Izzo *et al*., 2010; McFadden *et al*., 2011; Choi *et al*., 2016].

Low level of HCT and RBC in *Babesia* spp. infections indicated as anemia and may be attributed to the mechanical damage by multiplication of trophozoite in the erythrocytes [Bock *et al*., 2004] and production of toxic hemolytic factors of the parasite [Esmaeilnejad *et al*., 2012]. Previous study reported that *T. orientalis* infection causes destruction of erythrocyte and anemia [Kawamoto *et al*., 1990]. However, the pathogenesis of anemia of *T. orientalis* infection is not clearly clarified [Stockham *et al*., 2000]. In this study, increasing in MCV and decreasing in MCHC indicated regenerative anemia. This phenomena is the consequence of destruction of infected RBC [Esmaeilnejad *et al*., 2012].

In conclusion, *Babesia* spp. and *T. orientalis* infections have affected hematological parameters of the cattle. Anemia was commonly found in animals in both single and co-infections with TBDs. In addition, leukocytosis and eosinophilia were also observed in infected animal group. Although, hemograms of the animal in the present study were indicated in normal range when compared to reference values but clinical outcome and its impact on animal production should be carried out.

3-5. Summary

In the current study, a total of 608 cattle samples were examined for *Babesia* spp. and *T. orientalis* by PCR method. Furthermore, comparisons of hematological parameter between PCR-positive and PCR-negative groups were also analyzed. The PCR based diagnosis revealed 328 animals are positive for single and co-infections with TBDs. However, sixteen samples were excluded from hematological study due to red blood cell hemolysis during processing. The Mann-Whitney was used to compare the differences of hemogram between the PCR positive and negative animals. In this study, all hematological parameters levels in both PCR-positive and PCR-negative group were within normal range when compared to the reference. The hematological profiles of animals which had single infection with *B. bigemina* or *T. orientalis* had significantly lower in HCT, RBC, hemoglobin, MCH and MCHC values compared to those of animals without infection. There were significantly increase in WBC and eosinophil count in *B. bigemina* or *T. orientalis* infection. Low significance levels of HCT and MCV were found in animals that had co-infections with *B. bigemina* and *B. bovis* or *B. bigemina* and *T. orientalis*. The high level of WBC was found in animals with co-infections. Increasing of lymphocyte count was observed in animals with *B. bovis* and *B. bigemina* co-infections. Increasing of eosinophil count was found in animals with *B. bigemina and T. orientalis* co-infections.

Table 9. Hematological profile changes of single infection groups.

Variables	No	Min-Max	Median	*p*-value (<0.05)	Reference value*
B. bigemina infection					
1. WBC					
PCR negative	288	900 -22,000	5,100	0.001	4,000-12,000 cells
PCR positive	57	2,800-10,600	6,000		
2. Lymphocyte					
PCR negative	288	675-14,520	3,366	0.015	1,700-7,500 cells
PCR positive	57	1,716-6,784	3,942		
3. Eosinophil					
PCR negative	288	0-3,105	285	0.022	0-1,300 cells
PCR positive	57	0-1,920	378		
4. Hb					
PCR negative	288	3.50-17.60	10.10	0.005	8-15 g/dl
PCR positive	57	3.50-13.20	9.90		
5. HCT					
PCR negative	288	16-60	37.50	0.018	24-46%
PCR positive	57	20-52	36.00		
6. MCH					
PCR negative	288	4.70-24.50	14.60	<0.001	13.70-18.20 g/dl
PCR positive	57	9.51-22.29	12.56		
7. MCHC					
PCR negative	288	8.60-49.20	31.10	0.001	30-36 g/dl
PCR positive	57	17.40-36.90	28.00		
T. orientalis infection					
1. WBC					
PCR negative	288	900 -22,000	5,100	0.016	4,000-12,000 cells
PCR positive	113	2,000-20,700	5,700		
2. Eosinophil					
PCR negative	288	0-3,105	285	<0.001	0-1,300 cells
PCR positive	113	0-3,036	792		

Variables	No	Min-Max	Median	*p*-value (<0.05)	Reference value*
3. RBC					
PCR negative	288	2.35-15.14	7.11	0.002	5-10 x 10^6/µl
PCR positive	113	3.53-10.44	6.64		
4. HCT					
PCR negative	288	16-60	37.50	<0.001	24-46%
PCR positive	113	21-55	34.00		

*Reference value provided by the reference laboratory of livestock and wildlife animal hospital, Faculty of Veterinary Science, Mahidol University.

Table 10. Hematological profile changes of co-infections groups.

Variables	No	Min-Max	Median	*p*-value (<0.05)	Reference value
B. bigemina and B. bovis infection					
1. WBC					
PCR negative	288	900 -22,000	5,100	<0.001	4,000-12,000 cells
PCR positive	38	3,300-17,500	6,700		
2. Lymphocyte					
PCR negative	288	675-14,520	3,366	0.001	1,700-7,500 cells
PCR positive	38	1,496-9,275	4,844		
3. MCV					
PCR negative	288	31.75-69.02	46.00	0.006	40-60 fl
PCR positive	38	32.00-52.89	43.65		
4. MCH					
PCR negative	288	4.70-24.50	14.60	0.004	13.70-18.20 g/dl
PCR positive	38	8.01-19.80	12.92		
B. bigemina and *T. orientalis* infections					
1. WBC					
PCR negative	288	900 -22,000	5,100	0.002	4,000-12,000 cells
PCR positive	49	290-10,600	5,800		
2. Eosinophil					
PCR negative	288	0-3,105	285	<0.001	0-1,300 cells
PCR positive	49	0-2,014	580		
3. MCV					
PCR negative	288	31.75-69.02	46.00	0.023	40-60 fl
PCR positive	49	31.65-67.83	48.51		
4. HCT					
PCR negative	288	16-60	37.50	<0.001	24-46%
PCR positive	49	25-55	34.00		

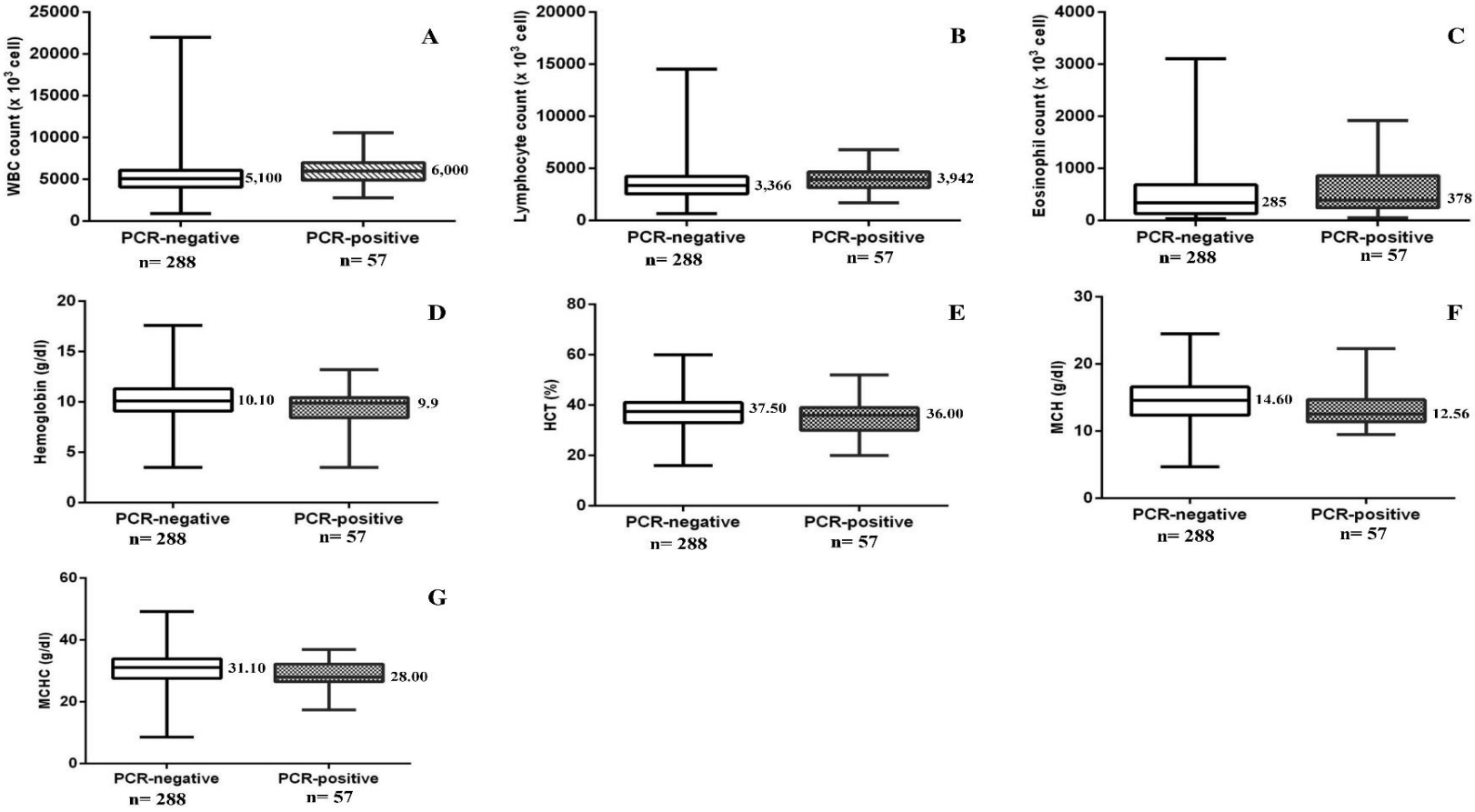

Fig 8. Comparison of hematological profiles between *B. bigemina* positive and negative group. A, Total WBC; B, lymphocyte count; C, eosinophil count; D, hemoglobin; E, HCT; F, MCH; G, MCHC.

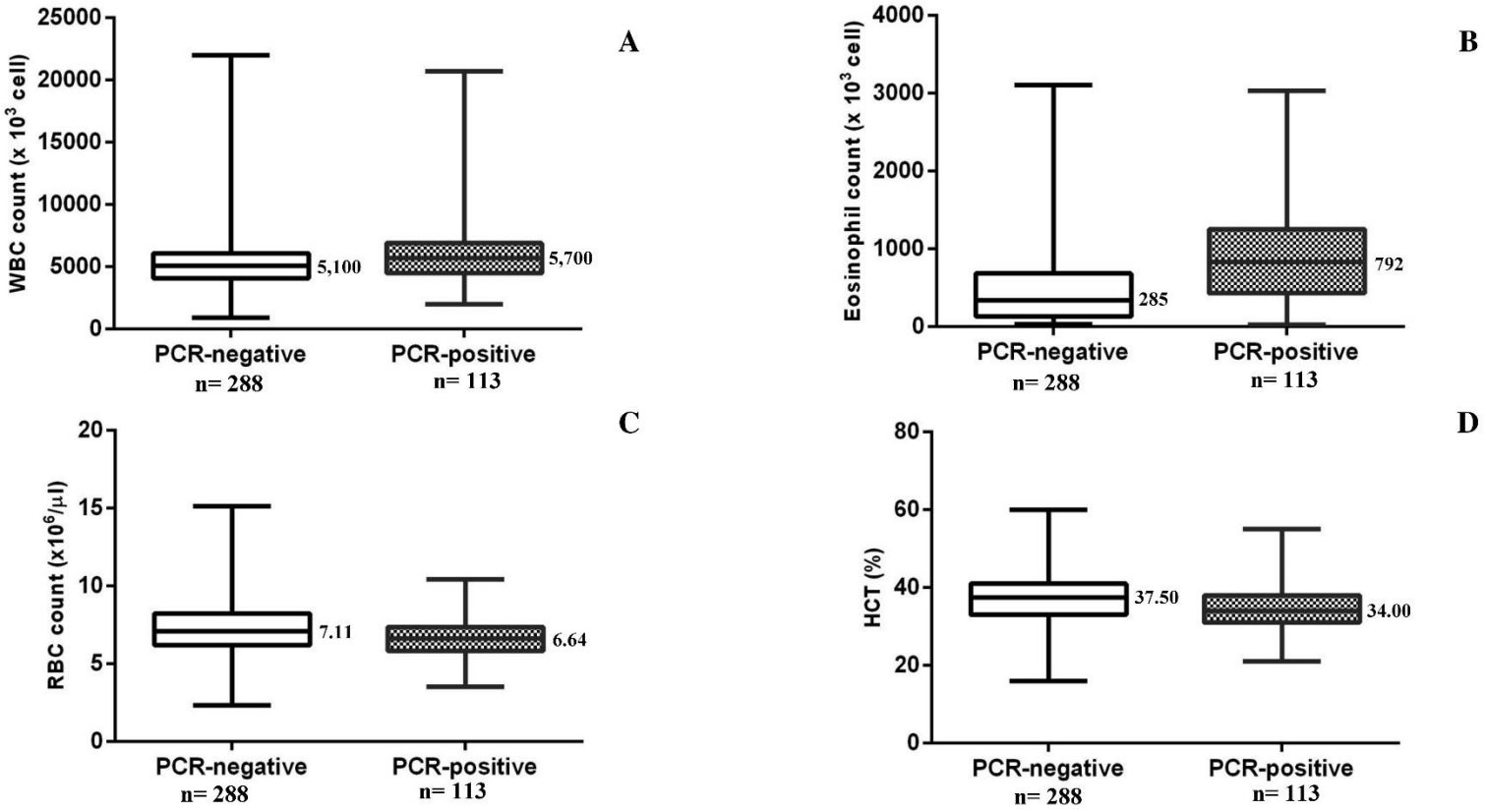

Fig 9. Comparison of hematological profiles between *T. orientalis* positive and negative group. A, Total WBC; B, eosinophil count; C, RBC; D, HCT.

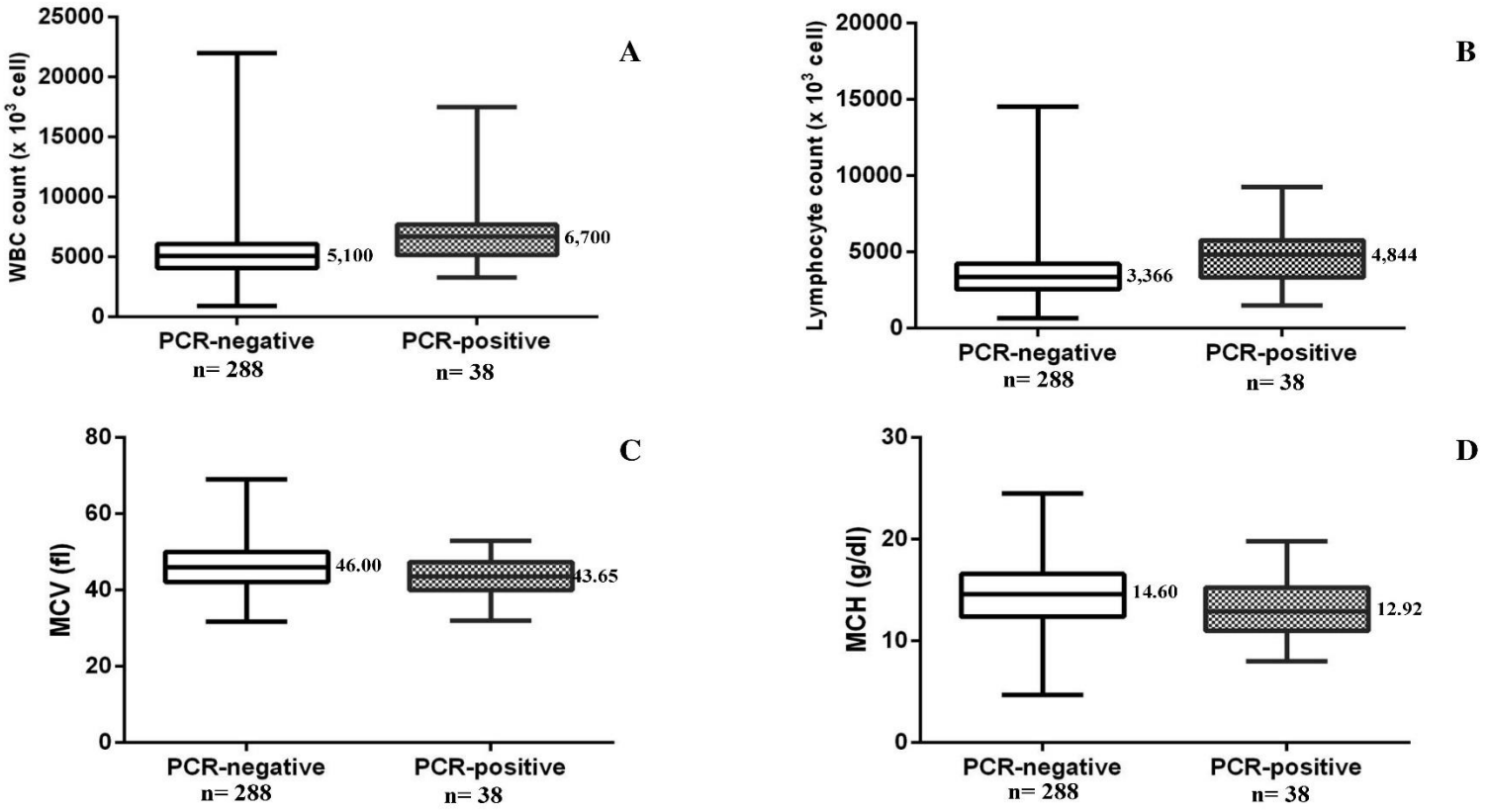

Fig 10. Comparison of hematological profiles between *B. bigemina* and *B. bovis* positive and negative group. A, Total WBC; B, lymphocyte count; C, MCV; D, MCH.

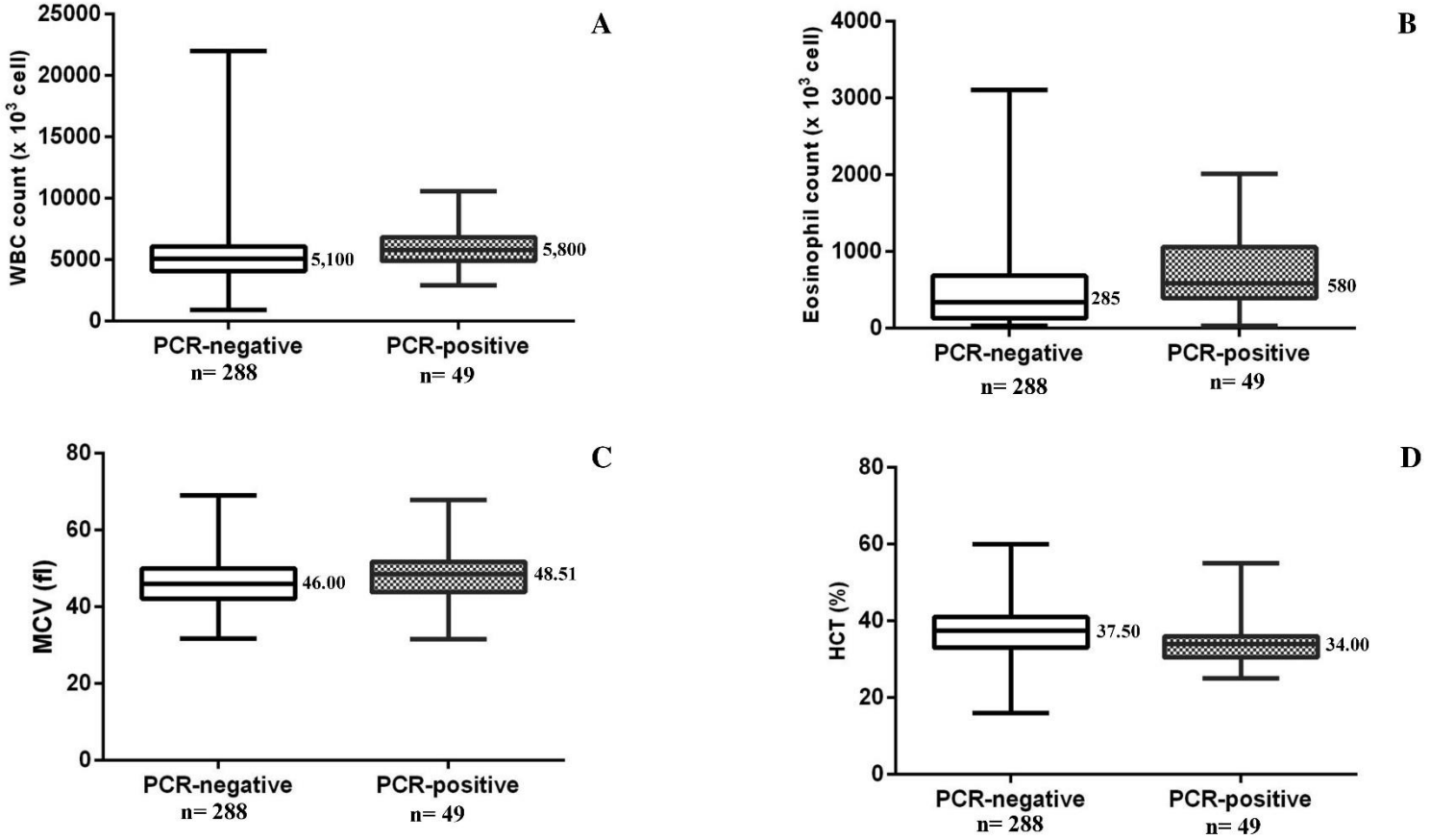

Fig 11. Comparison of hematological profiles between *B. bigemina* and *T. orientalis* positive and negative group. A, Total WBC; B, eosinophil count; C, MCV; D, HCT.

General discussion

Bovine babesiosis and benign theileriosis are economically important tick-borne diseases worldwide [De Castro, 1997]. Thailand is an endemic area for various bovine tick-borne diseases (TBDs) that negatively affect the health and productivity of cattle. Beef cattle are the largest cattle population in Thailand. Their productivity is constrained by TBDs such as bovine babesiosis and benign theileriosis. However, epidemiological studies on such diseases in the cattle have been limited. In this study, a total of 608 blood samples were collected from randomly selected beef cattle in 9 provinces located in north, northeastern, western and central parts of Thailand between March 2014 and June 2015. PCR assay was used to detect *B. bovis*, *B. bigemina* and *T. orientalis* because it is reported to have high specify and sensitivity [Altay *et al*., 2008]. The prevalence of the above parasite was determined as well as risk factor associated with the infections at farm level was evaluated (**Chapter 1**). Genetic markers used for the detection of the pathogens mentioned (*B. bovis* SBP2, *B. bigemina* RAP-1a, *T. orientalis* MPSP genes and ITS1-5.8s rRNA gene-ITS2 region of *B. bovis* and *B. bigemina*) were sequenced to determine genetic diversity and their genetic relations between isolates from different countries (**Chapter 2**). To monitor health status of infected animals, their hematological parameters were compared to those of healthy cattle (**Chapter 3**).

Overall, 54.3% of the beef cattle sampled had at least one TBD (*B. bovis*, *B. bigemina* and *T. orientalis*). The prevalence of TBDs in cattle from western and central regions were significantly lower than those form northeastern and north regions ($p< 0.001$). *T. orientalis* was the most prevalent TBD in beef cattle in north and northeastern parts

while *B. bigemina* was the most prevalent TBD in western and central parts of Thailand. The difference of infection rate among regions was probably due to tick-distribution and farm management. Co-infection with *Babesia* spp. and *T. orientalis* was found in this study. *B. bigemina* and *T. orientalis* was the most prevalent (8.2%) co-infection although triple infection with the three parasites were observed in 2.0% of the samples. Noteworthy, co-infections with *Babesia* spp. and *T. orientalis* have been found in cattle in many areas [Atlay *et al*., 2008; Elsify *et al*., 2015; Zhou *et al*., 2016].

To identify the risk factors that influenced the prevalence of TBDs in beef cattle farms, univariable and multivariable analyse were applied. The major risk factors for infection with *Babesia* spp. included lack of tick control program, communal grazing and contact with other domestic animals. Lack of tick control in farm may be a factor of the tick infestation on cattle in this study. Consequently, the cattle are infested with ticks are more likely to be infected with TBDs [Medonca Costa *et al*., 2013]. The high risk of *Babesia* infections was found in communal grazed cattle. The most of beef cattle farms surveyed in this study were extensive grazing system. This finding could be explained that to sharing and increasing the spread of the tick vector leading to increased risks infection with bovine babesiosis. Contact with other domestic animals is associated with *Babesia* infections. Tick vectors can transmit the parasites to various hosts [Yeruham *et al*., 1996] and some *Babesia* spp. can infect a wide range of animal hosts [Criado- Fornelio *et al*., 2003]. However, the role of other domestic animal in bovine babesiosis needs to be further studied.

The risk factors for *T. orientalis* infection in beef cattle were lack of tick control program and tick infestation. This suggests that *T. orientalis* infection is associated with

tick challenge because the natural vector, *Haemaphysalis* spp. are not abundantly found in livestock in Thailand [Changbunjong *et al*., 2009]. Therefore, the distribution of tick vector for *T. orientalis* in Thailand warrants further research.

The genetic diversity of *Babesia* spp. and *T. orientalis* were assessed as well as phylogenetic analyse of the above parasites were performed. *B. bovis* SBP2 and *B. bigemina* RAP-1a genes are considered as gene target for development of novel methods for detection and vaccine control of bovine babesiosis [Suraz *et al*., 2011]. Sequencing analyse of *B. bovis* SBP2 and *B. bigemina* RAP-1a showed high similarities and identities with previous sequences available in GenBank database. These results suggested that the two genes are highly conserved and valuable target to detect parasite from various geographical areas [Terkawi *et al*., 2011; Cao *et al*., 2012; Nagano *et al*., 2013; Yu *et al*., 2013]

Previous studies have been used ITS1-5.8s rRNA gene-ITS2 region to determine genetic variation of *Babesia* spp. [Liu *et al*., 2008; Niu *et al*., 2009; Holman *et al*., 2011]. In this study, the sequences of *B. bovis* were more divers in their nucleotide length and nucleotide identity (93.1-97.1%) than *B. bigemina*. In the phylogenetic tree, ITS1-5.8s rRNA gene-ITS2 region sequences of *B. bovis* formed a monophyletic clade with other known *B. bovis* ITS1-5.8s rRNA gene-ITS2 region sequences. Furthermore, the sequences of *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region also cluster in one clade. These results imply that *Babesia* spp. isolates might belong to the same species but different strains of *B. bovis* and *B. bigemina* [Cao *et al*., 2012; Zhou *et al*., 2016].

Phylogenetic analysis revealed that *T. orientalis* MPSP gene sequences in this study were classified into 3 clades (type 3, 5, 7). Previous molecular epidemiology of *T.*

orientalis in cattle from Thailand classified MPSP gene into types 1, 3, 5, 7, N3. This confirmed that MPSP gene is polymorphic antigen and showed a wide diversity among different field isolates [Sivakumar *et al*., 2014]. Moreover, the MPSP gene was recognized as a useful epidemiology marker for genotyping of the *T. orientalis* isolates in many countries [Yokoyama *et al*., 2011; Sivakumar *et al*., 2013].

Beef cattle breed in Thailand is native breed (*Bos indicus*) and more resistant to various diseases than dairy cattle. Furthermore, beef cattle that had infection with TBDs also resistant to clinical signs [Jonsson *et al*., 2008]. In this study, clinical examinations were not carried out to determine the health status of the animals during sampling. However, the hematological parameters of cattle blood samples were compared between PCR-positive and PCR-negative groups. The hematocrit values indicated that cattle which had infection with tick-borne pathogens (either single or co-infections) had significantly lower PCV values compared to those of cattle without infection. This finding was indicated as anemia and usually found in animals infected with *Babesia* spp. and *T. orientalis* [Ibrahim *et al*., 2009; Izzo *et al*., 2010; McFadden *et al*., 2011; Choi *et al*., 2016]. On the other hand, leukogram (WBC, eosinophil and lymphocyte counts) of infected cattle had significantly higher than those of negative groups. The pattern of leukocytosis could be attributed to their role as active mediators in the innate immune response in infected animals [Esmaeilnejad *et al*., 2012]. There were no significantly different in hematological parameters between single and co-infections groups. Although hemograms of infected cattle in this study were in normal value, TBDs still have negative impact on their health and productivity. Therefore, the investigation on clinical outcome as well as the effect on production needs to be undertaken.

General summary

Tick-borne diseases (TBDs) are among the leading causes of economic losses in cattle production, especially in tropical and subtropical areas. Babesiosis and theleriosis are the most economically important tick-borne diseases affecting cattle in many countries. Beef cattle are the largest cattle population in Thailand. Their productivity is constrained by babesiosis and theileriosis. However, epidemiological studies on such diseases in beef cattle have been limited.

This study aimed to determine the prevalence of *Babesia* spp. and *T. orientalis* in beef cattle from various parts of Thailand using PCR assay. The risk factors for the occurrence of these parasites were also assessed. The genetic markers used for their detection were sequenced to determine their genetic diversities and parasites phylogeny in relation to different countries. The effect of the parasite infections on hematological parameters in naturally infected animals was also evaluated.

In chapter 1, a total of 608 blood samples were collected from randomly selected beef cattle in 9 provinces located in north, northeastern, western and central parts of Thailand between March 2014 and June 2015. Genomic DNA was extracted and examined by standard and nested PCR. *B. bovis* spherical body protein 2 (SBP2), *B. bigemina* rhoptry associated protein-1a (RAP-1a) and *T. orientalis* major piroplasm surface protein (MSPS) were used as genetic markers. The PCR results revealed that overall, 54.3 % of the beef cattle sampled was infected with *B. bovis*, *B. bigemina* or *T. orientalis*. The highest prevalence of tick-borne diseases was found in the northeastern region followed by the

north, western and central regions. Co-infection with *Babesia* spp. and *T. orientalis* was found in this study. *B. bigemina* and *T. orientalis* was the most prevalent (8.2%) co-infection, although triple infection with the three parasites were observed in 2.0% of the samples. A lack of tick control programs was the universal risk factor for the occurrence of *Babesia* spp. and *T. orientalis* in beef cattle farms.

In chapter 2, the partial sequences of *B. bovis* SBP2 and *B. bigemina* RAP-1a were cloned and sequenced to determine identities and similarities. Furthermore, *B. bovis* and *B. bigemina* ITS1-5.8s rRNA gene-ITS2 regions and the *T. orientalis* MPSP gene were used as templates for sequencing to determine genetic diversities and their genetic relation between isolates from different countries. Sequencing analysis results revealed that *B. bovis* SBP2 and *B. bigemina* RAP-1a sequences were highly conserved and showed high homology with other geographic isolates. On the other hand, the sequences of *B. bovis* ITS1-5.8s rRNA gene-ITS2 region were more diverse in their nucleotide length and nucleotide identity than *B. bigemina* ITS-5.8s rRNA gene-ITS2 region. In the phylogenetic tree, the sequences of *B. bovis* ITS1-5.8s rRNA gene-ITS2 region formed a monophyletic clade with other known *B. bovis* ITS1-5.8s rRNA gene-ITS2 region sequences. In addition, *B. bigemina* ITS1-5.8s rRNA gene-ITS2 region also clustered in one clade. In this study, phylogenetic analysis revealed that *T. orientalis* MPSP gene sequences were classified into 3 clades (type 3, 5, 7).

In chapter 3, the hematological parameters of cattle blood samples were compared between PCR-positive and PCR-negative groups. All hematological parameter values in both PCR-positive and PCR-negative groups were within normal range when compared to the reference values. However, some parameters of infected animals showed significantly

different with the non-infected group. The hematocrit values indicated that cattle infected with tick-borne pathogens (either single or co-infections) had significantly lower PCV values compared to those of cattle without infections. In contrast, using these parameters (WBC, eosinophil and lymphocyte counts) the leukogram of infected cattle was significantly higher than those of the negative groups. These results were not significantly different in hematological parameters between single and co-infection groups.

Overall, the present study provides epidemiological information regarding *Babesia* spp. and *T. orientalis* in beef cattle and the genetic diversity of these parasites. Data about the hematological parameters in natural infection with these parasites under field conditions were also reported. These results are useful in introducing effective strategies for parasite prevention and control in cattle population in Thailand.

和文要約

マダニ媒介性疾患は特に熱帯および亜熱帯地域において経済的な損失を引き起こす。多くの国でバベシア症およびタイレリア症は経済的に最も重要なマダニ媒介性疾患である。タイにおいて肉牛は牛の飼育頭数の大半を占める。それらの生産性はバベシア症とタイレリア症によって低下するとされるが、肉牛におけるこれら感染症の疫学的調査は行われていない。

本研究はPCR法を用いてタイの異なる地域における肉牛のバベシア原虫ならびに*T. orientalis*の流行を調べることを目的とし、患畜の発症リスク要因を評価した。これらの原虫の検出に用いられた遺伝子マーカーは、異なる国の原虫との間における遺伝的多様性と原虫の系統発生を決めるためにシークエンスを行った。自然感染した動物において原虫感染の血液学的パラメーターへの影響も評価し。

第1章では、2014年3月から2015年6月の間にタイの北部、北東部、西部および中央部の９つの州の肉牛からランダムに608検体を収集した。DNAを抽出し、PCRおよびnested PCRによって解析を行った。*B. bovis* spherical body protein 2 (SBP2) *B. bigemina* rhoptry associated protein-1a (RAP-1a) および *T. orientalis* major piroplasm surface protein (MSPS) を遺伝子マーカーとして用いた。PCRの結果により肉牛の54.3%が*B. bovis*、*B. bigemina*もしくは*T. orientalis*に感染していることが明らかとなった。マダニ媒介性疾患が最も検出

されたのは北東部のエリアで、北部、西部、中央部と続いた。本研究でバベシア原虫と *T. orientalis* の共感染が明らかとなった。3 原虫に感染しているものが2.0%であったが、2原虫の感染では*B. bigemina*および*T. orientalis*の共感染が最多であった(8.2%)。マダニ制御プログラムがないことが肉牛農家におけるバベシア原虫と*T. orientalis*の発生の一般的なリスク要因であった。

第 2 章では、遺伝子相同性を決定するために *B. bovis* SBP2 および *B. bigemina* RAP-1aをクローニングし、シークエンスを行った。さらに、*B. bovis* および*B. bigemina*のITS1-5.8s rRNA遺伝子-ITS2領域と*T. orientalis*のMPSP遺伝子を他国から分離したものとの遺伝的多様性および関係性を明らかにするために鋳型として用いた。シークエンス解析では*B. bovis* SBP2および*B. bigemina* RAP-1aが高率に保存されており、他国からの分離株と相同性を示した。一方、*B. bovis* ITS1-5.8s rRNA gene-ITS2領域は*B. bigemina* ITS-5.8s rRNA gene-ITS2領域のシークエンスと比較し、塩基長と相同性という点でより多様であった。系統樹では、*B. bovis* ITS1-5.8s rRNA gene-ITS2領域は他の既知の*B. bovis* ITS1-5.8s rRNA gene-ITS2領域と単系統のクレードを形成した。さらに*B. bigemina* ITS1-5.8s rRNA gene-ITS2領域もまた、一つのクレードを形成した。本研究で系統的解析により*T. orientalis* MPSP遺伝子は3つのクレード（タイプ３、５、７）を形成することが明らかとなった。

第3章では、牛血液サンプルの血液学的パラメーターをPCRで陽性であったグループと陰性であったグループを比較した。PCR 陽性・陰性に関わらず、すべ

てのサンプルにおいて血液学的なパラメーターは基準値内であった。しかし、感染動物のいくつかのパラメーターは非感染グループと明らかに異なる結果を示した。マダニ媒介性病検体に感染している牛（単独もしくは共感染）は明らかに非感染牛と比較しPCVが低値であった。これに対し、白血球数、好酸球およびリンパ球のカウントを行った結果、感染牛の白血球数は明らかに非感染牛より高かった。これらの結果は単独あるいは共感染グループの間では明らかな差は認められなかった。

本研究はバベシア原虫および*T. orientalis*の肉牛における疫学的情報とこれら原虫の遺伝子的多様性の情報を示した。自然感染における血液学的パラメーターの情報も報告した。これらのデータはタイにおける牛群に対する原虫の予防ならびに制御に対する効果的な戦略を導くために役立つと考えられる。

Acknowledgements

This thesis marks the end of my journey in obtaining my degree in Animal and Food hygiene. There are many people who made this thesis a successful completion. First of all, I would like to express the deepest appreciation to my supervisor, Professor Xuenan Xuan, for kindly support of my Ph.D. study and research, for his motivation and great knowledge. His constant guidance helped me to improve my research and writing of the thesis. Without his support and assistance this thesis would not have been possible. I wish to express my sincere gratitude to my co-advisors, Professor Ikuo Ikarashi and Associated Professor Yoshifumi Nishikawa, for their valuable advice and support on completion of the present research. I am especially indebted to my thesis committee, Professor Makoto Igarashi of National Research Center for Protozoan Diseases and Professor Kunitoshi Imai of Diagnostic Center for Animal Health and Food Safety, for taking time out of their busy schedules to review my thesis.

My sincere thanks also goes to Dr. Theeratthawan Tobunluepob (Chiang Rai provincial livestock office), Dr. Bunjong Ardkhum (Payao provincial livestock office), Dr. Papaspong Jongchansittoe (Maehong sorn provincial livestock office), Dr. Pornsak Prasomtong (Kanchanaburi provincial livestock office), Dr. Chatchai Suebkom (Khon Kan artificial insemination and biotechnology center) and Dr. Nachai Sarataphan (Bureau of biotechnology in livestock production) for their help to arrange all facilities during field sample collection. Without their precious support, it would not have been possible to conduct this research.

I am indebted to all the staff of The Monitoring and Surveillance Center for Zoonotic diseases in Wildlife and Exotic animals (MOZWE) and Livestock and Wildlife animal hospital, Faculty of Veterinary Medicine Mahidol University for fieldwork assistants and providing laboratory facilities.

I am deeply grateful to Dr. Paul Franck Adjou Moumouni for the long discussions that helped me sort out the technical details of my Ph.D. research.

I am thankful to Dr. Patrick Vudriko for encouraging the use of correct grammar and consistent notation in my writings and for carefully reading and commenting on countless revisions of my manuscripts.

I am thankful to all of my friends at National Research Center for Protozoan Diseases (NRCPD), Obihiro University for their support and care that helped me stay sane through these difficult years. I also thank my fellow labmates in Host Defense unit for the stimulating discussions and for all the fun we have had in three years.

I am grateful to Mrs. and Mr. Yahagi, the Japanese family that helped me adjusts to a new country.

My sincerest thanks to the research funding from Grant-in-aid for Scientific Research from the Ministry of Education, Culture Sport Science, and Technology (MEXT), Japan and Faculty of Veterinary Science, Mahidol University, Thailand.

My deepest thanks, however, I owe to my family for the generosity and ingenuity of my parents, especially to my mother who gave me all the support, all the inspiration and all the best things for my life.

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