Genetic diversity and molecular detection of blood parasitic infections in cattle in Northeast, Thailand

Tossapol Seerin

A Thesis Submitted in Partial Fulfillment of Requirements for
degree of Doctor of Philosophy in Veterinary Science
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ABSTRACT

Tick-borne parasites in genus *Anaplasma* spp., *Babesia* spp. and *Theileria* spp. are prevalent in cattle population in Thailand and globally. This study aimed to investigate the prevalence and to identify these tick-borne parasites using both microscopic and molecular techniques in beef and dairy cattle in the northeastern part of Thailand. A total of 187 blood samples were collected from cattle, including106 samples from beef cattle and 81 samples from dairy cattle, for the detection of *Anaplasma* spp. using PCR targeting specific genes (msp4 gene for *A. marginale* and 16S ribosomal RNA gene for *A. platys* and *A. bovis*). Additionally, 215 sample were collected for the detection of *Babesia* spp. and. *Theileria* spp., comprising 134 samples from beef cattle and 81 samples from dairy cattle, with identification based on the 18S ribosomal RNA gene. For *Anaplasma* detection, 17.6% (33/187) were positive for *Anaplasma* sp. by microscopic examination and 20.8% (39/187) were positive by DNA amplification. Of these 20.8%, 17.6% were *A. marginale* and 3.2% were *A. platys*, however, *A. bovis* infection was not discovered. Infection with *Anaplasma* sp. and *A. marginale* showed significant association with breed and gender ($p<0.05$) while age and PCV levels showed no significant statistical relationship between *Anaplasma* sp. infected and uninfected groups. For *Babesia* and *Theileria* examination, 65.58% cattle were positive for infection with *Babesia* or *Theileria*. DNA analysis revealed that infection by *Babesia bigemina, Babesia bovis, Theileria orientalis, Theileria sinensis,* and *Theileria* sp. were common piroplasms in cattle in this region. This investigation enhances our understanding on the molecular epidemiology and genetic identification of *Anaplasma* spp., *Babesia* spp. and *Theileria* spp. in beef and dairy cattle, which are crucial for effective controlling these blood parasites and updating the prevalence data of this particular area.

Keyword : Babesiosis, Theileriosis, Cattle, Blood parasite, Anaplasmosis

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CHAPTER 1
INTRODUCTION

1.1 Background and rationale

Blood parasitic diseases caused by bacteria and protozoan parasites intensely impact on livestock development, particularly among cattle in Thailand and other developing nations worldwide. These diseases affect various organs and bodily systems, leading to abnormalities in physiological functions. Common clinical manifestations include fever, weight loss, reduced growth rate, anemia, miscarriage, and infertility in affected animals (Aubry and Geale, 2011). Infected animals suffer from body functions abnormally, disability, or even death, resulting in further health complications and economic losses. Pathogenic vectors, such as blood-sucking insects including cattle ticks (*Rhipicephalus* sp.), ticks (*Ixodes* sp.), mosquitoes, flies, gnats, and house flies, play a crucial role in the transmission of blood parasites in cattle (Ahantarig et al., 2008; Ghosh and Nagar, 2014). However, ticks are considered one of the most significant vectors for transmitting disease agents, including bacteria and protozoa.

Tick-transmitted diseases pose serious health challenges to cattle in tropical and subtropical regions, including Thailand. Among bacterial pathogens, various species of *Anaplasma* such as *A. marginale*, *A. bovis*, *A. centrale*, *A. phagocytophilum*, and *A. platys* have been reported to cause bovine anaplasmosis (Dahmani et al., 2015; Battilani et al., 2017; Rjeibi et al., 2018). Regarding protozoan pathogens, the most notable species affecting cattle include *Babesia bovis*, *B. bigemina* and *B. divergens.* However, *Babesia bovis* and *B. bigemina* are primarily responsible for bovine babesiosis globally (Bock et al., 2004). In addition, three species of *Theileria* namely

T. annulata, *T. sinensis*, and *T. orientalis* (also known as *T. sergenti*) serve as primary causative agents of bovine theileriosis (Liu et al., 2010; Luo et al., 1997; Qin et al., 2106).

In Thailand, an agricultural state situated in Southeast Asia, the livestock industry has been significantly impeded by severe tick-borne haemoparasites (Jittapalapong and Lieowijak, 1988). Cattle are predominant animals in the Northeast of Thailand and serve as vital sources of meat, horns, milk products, leather, land plowing, and transportation of people and crops (Somparn et al., 2004). Two species of *Anaplasma*, namely *A. marginale* (Jirapattharasate et al., 2016), and *A. platys* (Nguyen et al., 2020) are endemic to this region, with *A. marginale* being dominant. Additionally, two species of *Babesia*, *B. bovis* and *B. bigemina*, are commonly observed. Furthermore, *T. orientalis* is the most prevalent species causing bovine theileriosis (Jirapattharasate et al., 2016). However, there remains an insufficient update information of *Anaplasma* sp., *Babesia* sp. and *Theileria* sp., infection in cattle. The aims of this study are to evaluate the prevalence of blood pathogens including *Anaplasma* sp., *Babesia* sp. and *Theileria* sp., in cattle within Northeast Thailand. Furthermore, the study aims to elucidate the genetic diversity and phylogenetic relationships of these blood parasites.

1.2 Objectives

1. To detect *Anaplasma* sp., *Babesia* sp. and *Theileria* sp. infections in cattle in the Northeast of Thailand

2. To identify *Anaplasma* sp., *Babesia* sp. and *Theileria* sp. infections in cattle in the Northeast of Thailand

1.3 Scope of the study

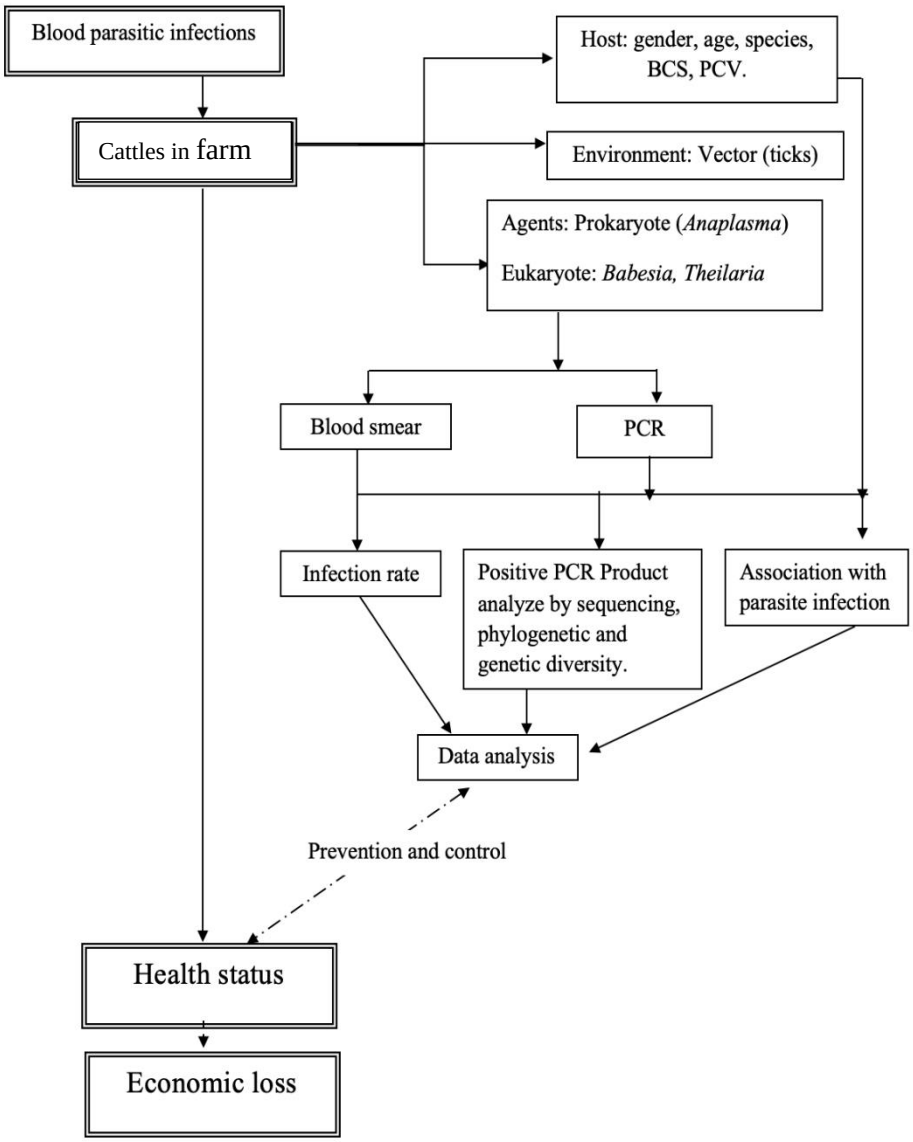
This study aimed to amplify DNA of blood parasites transmitted by ticks (*Anaplasma* sp., *Babesia* sp. and *Theileria* sp.) in cattle in the Northeast of Thailand and analyzed their genetic diversity. Blood samples were collected from cattle on smallholder farms located in Maha Sarakham, Khon Kaen, Roi Et, Ubon Ratcha Thani, Chaiyaphum and Udon Thani provinces in the northeastern part of Thailand. Data on gender, age, breed, and body condition score of the animals were also recorded. Blood samples were obtained by puncturing jugular or coccygeal veins, then levels of packed cell volume (PCV) were estimated, and blood parasites were observed under a microscope using the simple blood smear technique or PCR technique. The remaining blood were stored at –20°C until DNA extraction for long-term preservation. Blood parasites DNA were amplified using PCR or modified PCR targeting 16S rRNA, 18S rRNA gene, and other suitable genes to detect and characterize the genetic diversity of these pathogens. Positive PCR products were subjected to sequencing and phylogenetic analysis. The association between blood pathogen infection and PCV levels, as well as demographic data, was compared using statistical tests.

1.4 Anticipated Outcomes

1. The prevalence of *Anaplasma* sp., *Babesia* sp. and *Theileria* sp. in cattle in the Northeast of Thailand.

2. Species identification and phylogenetic analysis of *Anaplasma* sp., *Babesia* sp. and *Theileria* sp. infections in cattle in the Northeast of Thailand.

1.5 Conceptual framework



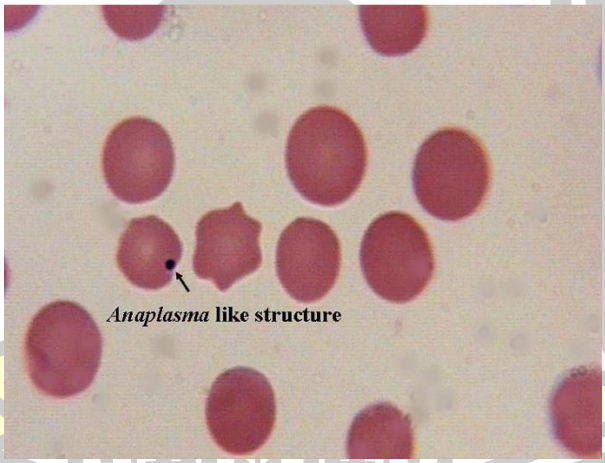
Figures 1 Conceptual framework

CHAPTER 2
LITERATURE REVIEW

2.1 *Anaplasma* sp.

2.1.1 Biology and classification

Anaplasma, an obligate intracellular bacterium belonging to the family Anaplasmataceae, is responsible for causing anaplasmosis in both domestic animals and humans (Rymaszewska et al., 2008). This pathogen is a gram-negative bacterium, resides within the blood cells of mammals (Figure 2) and is exclusively located within membrane-bound vacuoles in the cytoplasm of either vertebrate or tick hosts. The bacterial genus *Anaplasma* comprises *A. marginale*, *A. centrale*, *A. phagocytophilum*, *A. bovis* and *A. platys* (Kocan et al., 2010) (Table 1, 2).



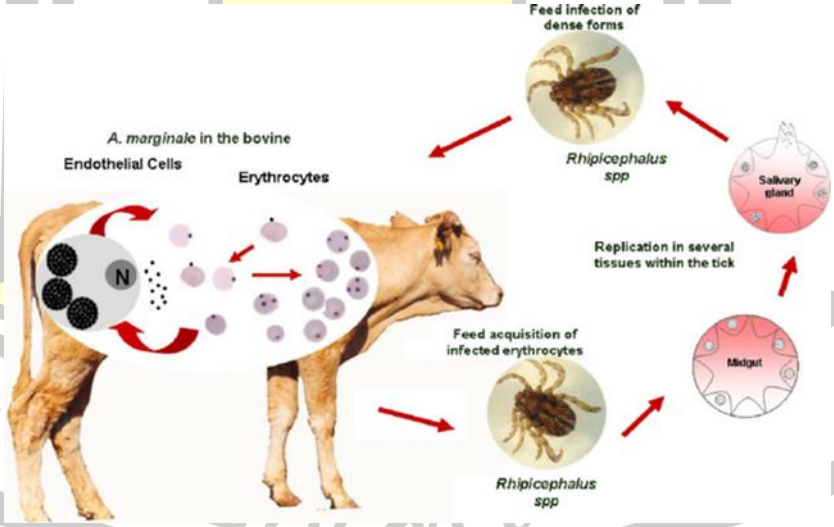
Figures 2 Blood smear by Giemsa staining of *Anaplasma* like structure. The *Anaplasma* is in the erythrocytes (arrow) (Vahid et al., 2009)

2.1.2 Life cycle of *Anaplasma* sp.

The life cycle of *Anaplasma* sp. within the ruminant host is complex and coordinated with the feeding cycle of ticks (Swift et al., 1976). Bovine red blood cells infected with *Anaplasma* sp. are ingested by ticks during their blood meals. The initial

site of infection in ticks occurs within gut cells, where the pathogen subsequently spreads to other tissues, including the salivary glands. *Anaplasma* sp. is transmitted to the vertebrate host through tick biting, after which the parasites disseminate into the bloodstream and migrate to the red blood cells of the host. Following this, *Anaplasma* multiplies within the erythrocyte and hematopoietic cells of cattle. Subsequently, the destruction of bovine reticuloendothelial cells, where the parasites inhabit, leads to hemolysis and clinical manifestations such as hemoglobinuria, fever, weight loss, abortion, and potential fatality.

In ticks, following the tick's bite on cattle, *Anaplasma* enters the tick's intestinal cells. Subsequently, the pathogen spreads to other cells within the tick's tissues and migrates to the tick's salivary glands (Figure 3), facilitating transmission of the infection through biting to other cattle (Kocan et al., 2004). The incubation period of bovine anaplasmosis ranges from 7 to 60 days, with an average of 28 days.



Figures 3 Proposed life cycle of *Anaplasma* sp.
The cycle is modified from Kocan 1999. (Rodríguez et al., 2009)

Table 1 Classification of the *Anaplasma*.
(Rymaszewska et al., 2008)

Class	Order	Family	Genera	Species	Distribution	Reference
α-proteobacteria	Rickettsiales	Anaplasmataceae	*Anaplasma*	*A. marginale*	World wide	(Bock and de Vos, 2001).
				A. centrale	World wide	
				A. phagocytophilum	Europe, USA	
				A. platys	World wide	
				A. bovis	World wide	
			Ehrlichia	*E. canis*	World wide	(Dubie et al., 2014)
				E. chaffeenis	Asia, Israel, Cameroon	
				E. ruminantium	Mexico, Grenada, South Africa, Zimbabwe, Brazil, Australia	
			Neorickettsia	*N. helminthoeca*	California, Washington, Olekon, Idaho, Canada, Brazil.	(Lin et al., 2017)
				N. risticii	USA, Canada, Brazil, Uruguay	(Lin et al., 2017)
				N. senetsu	Japan, South-East Asia.	(Lin et al., 2017)

2.1.3 Prevalence and epidemiology of bovine anaplasmosis

Bovine anaplasmosis is prevalent worldwide, particularly in tropical and subtropical regions, presenting a significant constraint to cattle production in numerous countries. Common species of *Anaplasma* sp. affecting cattle include *A. marginale*, *A. centrale*, *A. ovis*, *A. phagocytophilum*, *A. bovis*, and *A. platys*. In the United States, anaplasmosis caused by *Anaplasma* spp. is endemic across the southern Atlantic states, Gulf Coast states, and various Midwestern and Western states (Jaswal et al., 2014). *A. centrale*, a less pathogenic and symptomatic in cattle compared to *A. marginale* (Rar et al., 2011), is prevalent in regions such as Europe and the USA, where it can infect both humans and animals, leading to tick-borne fever characterized by respiratory symptoms, increased white blood cell count, miscarriage, and a sudden decrease in milk production (Bakken, 2000). *A. bovis* has been reported in cattle in China and Japan (Liu et al., 2012), whereas in Korea it was found in water deer, spotted deer (Lee et al., 2009). Bovine anaplasmosis is also endemic in Asia and Africa. In South Africa, prevalence rate of *Anaplasma* infections was 15.6% (Kocan et al., 2003). In Pakistan, the prevalence of *A. marginale* has been recorded as 7.36 - 75.71% using microscopic examination of blood smears (Rajput et al., 2005). In Thailand, bovine anaplasmosis has been documented for over five decades and has resulted in substantial economic losses in livestock production (Watanasin et al., 1965; Arunyakanon et al., 1966; Jittapalapong and Lieowijak, 1988; Chethanond et al., 1995; Fungfuang et al., 2006; Worasing and Rattana, 2007; Yawongsa et al., 2013). Previous studies have indicated that *A. marginale* is the most prevalent tick-borne pathogen in the North, Northeastern, and Western Thailand, with prevalence

rates approximately ranging from 14.3% to 23.2% (Saetiew et al., 2015; Jirapattharasate et al., 2016).

During the rainy season, the populations of flies and ticks increase dramatically, thereby amplifying the potential for the propagation of *A. marginale*, as ticks serve as vectors for its transmission (Saetiewa et al., 2020). Seasonal variations, such as rainy and dry periods, significantly impact humidity and temperature, influence the habitats of vectors and the spreading of infections. The timing and duration of each season are inconsistent due to climate change (Zhang et al., 2008). Presently, the route of anaplasmosis transmission extends beyond ticks, with mechanical transmission occurring through the biting of flies or contact with blood-contaminated equipment. Furthermore, transplacental transmission from infected mothers to their offspring has also been documented (Nguyen et al., 2020).

2.1.4 Pathology and clinical sign of *Anaplasma* sp. infection

Anaplasma marginale is recognized as the most prevalent and pathogenic agent of bovine anaplasmosis (Kocan et al., 2010). Additionally, *A. centrale* and *A. bovis* are also known to induce disease in cattle, whereas *A. phagocytophilum* (formerly *Ehrlichia phagocytophilum*) serves as a causative agent of human and animal granulocytic anaplasmosis. Furthermore, *A. phagocytophilum* has been identified across a wide spectrum of animal hosts, including goats, sheep, yaks, horses, dogs, cats, rodents, wild boars, foxes, birds, and reptiles (Stuen et al., 2013). Common clinical manifestations of bovine anaplasmosis include weight loss, anemia, icterus, fever, abortion, and lethargy. However, severe cases may result in mortality. Animals infected with *A. marginale* persist as carriers throughout their lifetime.

Under conditions of stress, infected animals may deteriorate and progress to chronic infection (Kocan et al., 2010; Noaman and Bastani, 2016).

2.1.5 Diagnosis

Clinical signs of anaplasmosis in cattle vary from asymptomatic to severe symptoms. Microscopic examination is the most commonly employed diagnostic method due to its simplicity and cost-effectiveness. However, this method exhibits low accuracy, is incapable of species identification, and relies heavily on the expertise of the examiner. Serological detection, such as the Indirect Fluorescent Antibody Test (IFAT) and Enzyme-Linked Immunosorbent Assay (ELISA), is another popular method utilized in various regions for detecting antibodies against *A. marginale* and *A. centrale* infections (Molad et al., 2006). In many laboratories, molecular techniques such as Polymerase Chain Reaction (PCR) or modified PCR have been developed for diagnosing blood parasites due to their high accuracy. PCR targeting 16S rRNA and 18S rRNA is commonly employed to detect *Anaplasma* sp. infections (Terkawi et al., 2011; Adaszek and Winiarczyk, 2008; Ogata et al., 2021). Additionally, combining PCR with ELISA approaches can offer a potent tool for epidemiological investigations, providing high accuracy in the diagnosis of blood parasitic infections (Goo et al., 2008).

Several genes including 16S rRNA, *groEL*, and *msp4*, along with their corresponding amino acid sequence types have been recently utilized to illustrate the prevalence and diversity of parasites (Khumalo et al., 2016; Khumalo et al., 2018). However, for genetic diversity, the use of these conserved genes does not reliably differentiate strains of *Anaplasma* spp. For example, the highly conserved genes 16S

rRNA, *gltA*, and *groEL* are of limited utility in differentiating *A. marginale* and *A. ovis* strains, whereas the *msp4* gene, another conserved gene, is widely employed for *A. marginale* and *A. ovis* genotyping (Belkahia et al., 2017; Cabezas-Cruz et al., 2019; de la Fuente et al., 2007; Enkhtaivan et al., 2019; Selmi et al., 2020; Torina et al., 2010). It is noted that the *msp4* gene of *A. ovis* exhibits less variability compared to *A. marginale* and *A. phagocytophilum*, potentially due to a restricted host range (de la Fuente et al., 2007). Genetic diversity among *Anaplasma* spp. is predominantly characterized based on genes encoding major surface proteins (MSPs), particularly *msp1a* and *msp4*, which belong to the *msp1* and *msp2* superfamilies, respectively.

2.1.6 Treatment, prevention and control

Oxytetracycline intramuscular or intravenous injection (11 mg/kg IV for 3-5 days, or 1-2 doses of 20 mg/kg IM long acting oxytetracycline every 72 hours) is currently the treatment of choice for acute anaplasmosis (Kocan et al., 2003). If the packed cell volume (PCV) drops below 15% or lower, a blood transfusion allows for a better treatment. The treatment mentioned above is not adequate to clear the organism from a persistently infected animal in one time. Long acting oxytetracycline at 20mg/kg must be administered every 72 hours for four successive treatments to obtain complete clearance. Nevertheless, not all carrier animals will be cleared of the infection (Kocan et al., 2003). Treatment doses of blood parasitic infections may vary depends on parasitemia levels.

Prevention of ticks or flies in the endemic area and control the movement of livestock are the primary strategies for reducing the spread of infection. Moreover, adequate hygienic procedures can be used to control and eliminate anaplasmosis (De

la Fuente et al., 2006). Vaccination can prevent the development of clinical anaplasmosis. However, there is currently neither live nor killed vaccine can totally prevent infections (Shkap et al., 2002).

Table 2 Characteristics of *Anaplasma* species.
(Rar et al., 2021).

Species	Host cells	Primary vectors	Main hosts	Distribution area	Diseases
A. mar ginale	Erythrocytes	*Dermacentor* spp., *Rhipicephalus* spp., *Hyalomma spp*	Cattle, wild ruminants	Worldwide, mainly in tropical and subtropical regions	Bovine anaplasmosis
A. cen trale	Erythrocytes	*Rhipicephalus simus*	Cattle, wild ruminants	Worldwide, in tropical and subtropical regions	Mild anaplasmosis in cattle (vaccine strain)
A. ovis	Erythrocytes	*Dermacentor* spp., *Rhipicephalus* spp.	Sheep, goats, wild ruminants	Asia, Africa, Europe, North America	Ovine anaplasmosis, infection in humans*
A. bovis	Monocytes	*Amblyomma* spp., *Rhipicephalus* spp., *Hyalomma* spp., *Dermacentor* spp., *Haemaphysalis* spp.	Cattle, buffaloes, goats, sheep, wild ruminants	Asia, Africa, North and South America, South Europe	Bovine anaplasmosis, infection in humans*, infection in dogs*

Table 2 Characteristics of *Anaplasma* species. (Continue)

Species	Host cells	Primary vectors	Main hosts	Distribution area	Diseases
A. platys	Platelets	*Rhipicephalus sanguineus*	Dogs, Bactrian camels	Worldwide, mainly in tropical and subtropical regions	Cyclic thrombocytopenia in dogs, infection in humans*
A. platys-like	Platelets, granulocytes	*Rhipicephalus* spp., *Haemaphysalis* spp.	Cats, cattle, goats, sheep, camels, wild ruminants	Asia, Africa, South Europe	ND**
A. odocoilei	Platelets	*Amblyomma americanum*	Deer	The USA, Mexico	ND
A. capra	Erythrocytes	*Ixodes* spp., *Dermacentor* spp., *Rhipicephalus* spp., *Haemaphysalis* spp.	Humans, goats, sheep, cattle, wild ruminants, dogs	Western Asia	Infection in humans

ND** - not determined.
* Rare cases of infections.

2.2 *Babesia* sp.

2.2.1 Biology and classification

Bovine babesiosis is a tick-borne disease of ruminant animals. This disease caused by Apicomplexa protozoan parasites in order Piroplasmida, genus *Babesia* (Mohamad et al., 2011; Sivakumar et al., 2018; Brown et al., 2008). Vertebrate hosts are cattle, water buffalo (*Bubalus bubalis*), African buffalo (*Syncerus caffer*) and white-tailed deer (*Odocoileus virginianus*) (OIE, 2020). *Babesia* sp. is tick-borne haemoprotozoan parasites of vertebrates that have a major impact of livestock

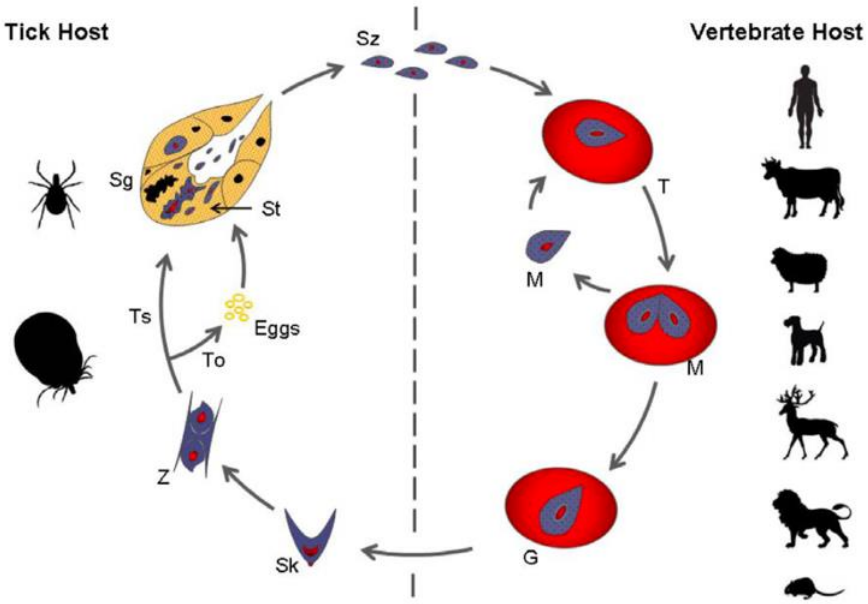
production, mainly cattle and small ruminants, in tropical and subtropical areas (Mehlhorn and Schein, 1984). The species within this genus that can infect cattle include *B. bovis, B. bigemina, B. divergens, B. major, B. ovata, B. occultans* and B. *jakimovi* (Uilenberg et al., 2006). *B. bovis* and *B. bigemina* are the mainly problem of cattle health and productivity in tropical and subtropical countries (Uilenberg et al., 1995; Iseki et al., 2010). The clinical signs of babesiosis are anemia, fever, hemoglobinuria and even death (Sharma et al., 2013). The calves up to 9-12 months of age are generally resistant to babesiosis. Clinical signs are varying influenced by several factors, such as the prevention and control program commonly used in the area, age, cattle breed and vaccination status (Vos et al., 1991). In addition, *B. bovis* and *B. bigemina* infected deer have been detected by PCR-based and serological diagnostic tests, implying that these animals might act as parasite carriers, which would have important epidemiological consequences (Holman et al., 2011). In addition, bovine babesiosis has been reported in all regions of Thailand since 1980. Moreover, approximately 100 species of *Babesia* have been reported worldwide. The predominant species affecting cattle include *B. bovis*, *B. bigemina*, *B. major*, *B. occultans*, *B. ovata*, *B. divergens*, *B. sp. Kashi*, *B. orientalis*, *B. bovis*, and *B. bigeminy* (Table 3). In Thailand, *B. bovis* and *B. bigemina* are the most prevalent causative agents of bovine babesiosis (Jirapattharasate et al., 2016).

Table 3 Species of *Babesia* infection in cattle and buffalo with the vector

Vertebrate Host	Vector	Species	Distribution	Reference
Cattle and Water Buffaloes	*R. annulatus, R.(moophylus) microplus*	*B. bovis*	World wide	Jacob, S. S et al., 2020
	R. geigyi	*B. bigemina*	World wide	Criado-Fornelio et al., 2009a; Altay et al., 2008; Liu et al., 2008
		B. major	America, Southernn Europe, Central and South Asia, Africa, Australia	Gray and De Vos et al., 1981;Zintl et al., 2003
		B. occultans	Asia, Iran, China, Nigeria	Luo et al., 2005.
		B. ovata	World wide	Jacob, S. S et al., 2020
		B. divergens	South America, Australia, North America, Africa, Europe and Asia.	Jacob, S. S et al., 2020
		B. sp. Kashi	China	Zintl et al., 2003
		B. orientalis	China	Liu et al., 1997b
		B. bovis	World wide	Liu et al., 1997a; Ferreri et al., 2012
		B. bigeminy	World wide	Liu et al., 1997a

2.2.2 *Babesia* sp. life cycle

In tick vectors, *Babesia* mates in the intestines, after that move to the salivary glands of the ticks. It develops as a sporogony and ruptures as sporozoites, and then the female tick bites the hosts. The sporozoite stage infects the hosts, after that sporozoites were migrated into the red blood cells to form the ring form before develop into the merozoite stage. Merozoite is the stage that can cause the disease to generate clinical symptoms (Tufani et al., 2009) (Figure 4).



Figures 4 Life cycle of *Babesia* sp.
(Eman et al., 2018).

2.2.3 Clinical sign and pathology of bovine babesiosis

In general, clinical signs of bovine babesiosis divided into 2 stages which are acute and chronic stages. In the acute stage, after an incubation period or a few days after the tick bite, acute cases are characterized by high fever (up to 42 °C), red urine, ischemic, skeletal and heart muscle changes are weakness (Skotarczak et al., 2008). If

infected animals were not appropriate treatment, the disease will progress to the chronic or even dead. In chronic stage, infected animals will be found to have symptoms which are anorexia, jaundice, anemia, hepatomegaly, and splenomegaly, however, retinal detachment has been described (Vial et al., 2006).

2.2.4 Treatment, prevention and control

For medical prophylaxis, clinically affected animals should be treated with an anti-parasitic drug such as diminazene diaceturate, imidocarb, or amicarbalide, with efficacy contingent upon timely usage or early initiation in disease management. Imidocarb dipropionate stands out as the most widely available babesia lethal drug, offering dual activity for therapy and prophylaxis against babesiosis (Mosqueda et al.,2012). The development of vaccines against bovine babesiosis has indicated that infected cows are more likely to recover from the disease than non-vaccination. Following infection, animals develop long-lasting immunity; furthermore, inoculation of their blood into susceptible cattle results in a less virulent form of the disease. Thus, initial vaccine formulations consisted of blood from donor bovines that had recovered from infection (Bock et al., 2004).

Insecticides have long been utilized by farmers and disease control agencies worldwide to mitigate the detrimental impact of ticks and tick-borne pathogens on cattle health and productivity. An exemplary instance of tick control through insecticide employment is demonstrated by the tick eradication campaign undertaken in the southern USA at the beginning of the 20th century. This program, spanning four decades, culminated in the eradication of the cattle tick and its transmitted parasites, including cattle babesiosis (Sivakumar et al., 2018).

2.3 *Theileria* sp.

2.3.1 Biology and classification

Theileria is a genus of protozoan parasites belonging to the phylum Apicomplexa. These parasites are obligate intracellular pathogens that infect various vertebrate hosts, including mammals, birds, and reptiles (Bishop et al., 2004). *Theileria* species are transmitted primarily by ticks and are responsible for causing theileriosis which primarily infect lymphocytes and red blood cells of their hosts, leading to various clinical manifestations depending on the species and the host involved. In cattle, for example, *Theileria* parasites can cause bovine theileriosis, characterized by fever, anemia, jaundice, and sometimes death. In some cases, infected animals may become lifelong carriers of the parasite, serving as reservoirs for transmission to other susceptible hosts. Among various hosts, cattle are particularly susceptible to infections by *Theileria* species, leading to significant economic losses in the livestock industry worldwide. The genus *Theileria* comprises several species, for example, manifestations of *Theileria* infections in ruminants are presented in the (**Table 4**).

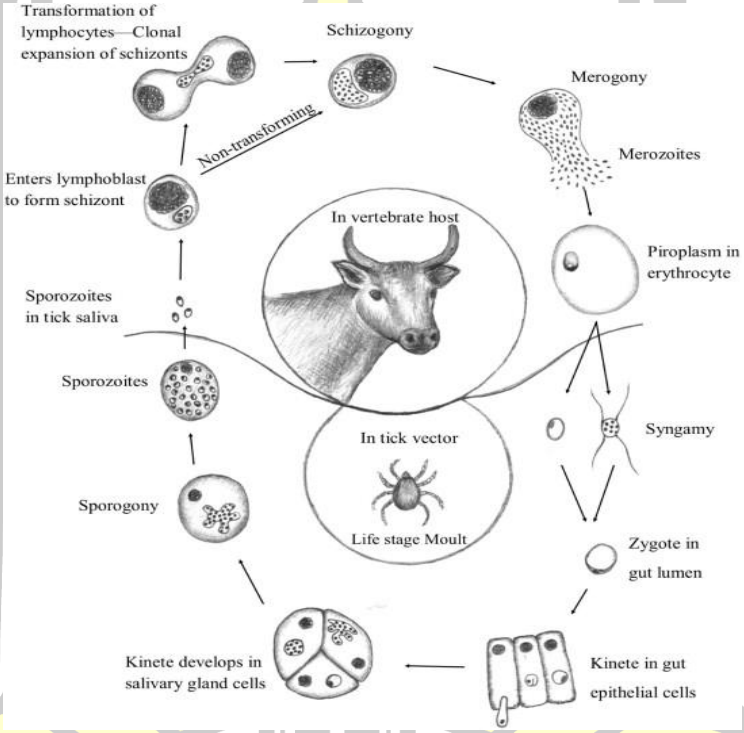
Table 4 *Theileria* species and distributions
(Mans et al., 2015)

Theileria spp.	Distribution	Reference
T. parva	South Europe, East Africa, Middle and South Asia, Kenya	Dumanli et al., 2005. Uilenberg et al., 1981.
T. mutans	Africa, Tanzania, Asia	Swai et al., 2007
T. taurotragi	South Africa	Uilenberg et al., 1981
T. velifera.	South Africa	Uilenberg et al., 1981
T. buffeli	Africa, Asia	Uilenberg et al., 1981
T. sergenti	Asia, China	Uilenberg et al., 1981
T. orientalis	Africa, Asia, Uganda,	Uilenberg et al., 1981
T. sinesis	Africa	Uilenberg et al., 1981
T. lestoquardi	Africa	Uilenberg et al., 1981
T. ovis	Asia, Africa, America, Europe	Uilenberg et al., 1981
T. separata	Africa	Uilenberg et al., 1981
T. annulata	Asia, Europe, Pakistan	Uilenberg et al., 1981

2.3.2 *Theileria* sp. life cycle

A generalized life cycle of the *Theileri*a genus involves the release of infective sporozoites during the feeding process of ticks at the feeding site. Subsequently, these sporozoites penetrate leukocytes, where they undergo multiplication through merogony. Following this phase, merozoites are released, which then invade red blood cells, thereby initiating the piroplasm stage. During following feeding cycles, larval or nymphal stages ingest the piroplasms, and the released parasites undergo syngamy within the tick gut, giving rise to a zygote, the

diploid stage in the parasite's life cycle. The zygote subsequently divides into motile kinetes, which infect the epithelial cells of the tick gut and migrate to the haemolymph, eventually infecting the salivary glands. Following the tick's feeding process, sporogony occurs, resulting in the proliferation of sporozoites within the salivary gland, which are then injected into the feeding site by nymphs or adult ticks (McKeever et al., 2009).



Figures 5 A generalized lifecycle for the *Theileria*.
The life cycle using *T. parva* as example (Mans et al., 2015)

2.3.3 Prevalence and epidemiology of Theileriosis

The spread of *Theileria* involves a complex interplay of various factors including parasite and vector distribution, socio-economic elements, climate change dynamics, host resistance and susceptibility, as well as disease control programs

(Gachohi et al., 2012). The presence of *T. orientalis* has been linked to significant animal losses in Australia and India (Aparna et al., 2011). Tropical theileriosis is prevalent in regions spanning North Africa, Southern Europe, and Asia, while East Coast fever predominantly affects regions in East, Central, and Southern Africa (Weir et al., 2010). A previous epidemiological investigation focusing on *Babesia* spp. and *T. orientalis* in beef cattle was conducted in the northern and northeastern regions of Thailand (Jirapattharasate et al., 2016).

2.3.4 Clinical signs and pathology of bovine theileriosis

Clinical manifestations encompassing weakness, icterus, pallor of mucous membranes, hemolytic anemia, diarrhea, and reduced milk production have been documented (Kamau et al., 2011). Severe presentations of the disease are notably more prevalent among animals upon their introduction into regions endemic for the pathogen (McFadden et al., 2011)

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2.3.5 Treatment, prevention and control

The primary approach for controlling and eradicating piroplasmosis is through treatment. However, the pursuit of novel chemotherapeutic agents targeting *Babesia* and *Theileria* has become increasingly imperative due to the development of parasite resistance to existing drugs. Ivermectin (IVM) stands out as the world's inaugural endectocide, exhibiting efficacy against a broad spectrum of parasites and vectors, both internally and externally (Batiha et al., 2019). Imidocarb has been established as the first-line therapeutic option for the treatment of *Theileria* infections (Bock et al., 2005).

CHAPTER 3

RESEARCH METHODOLOGY

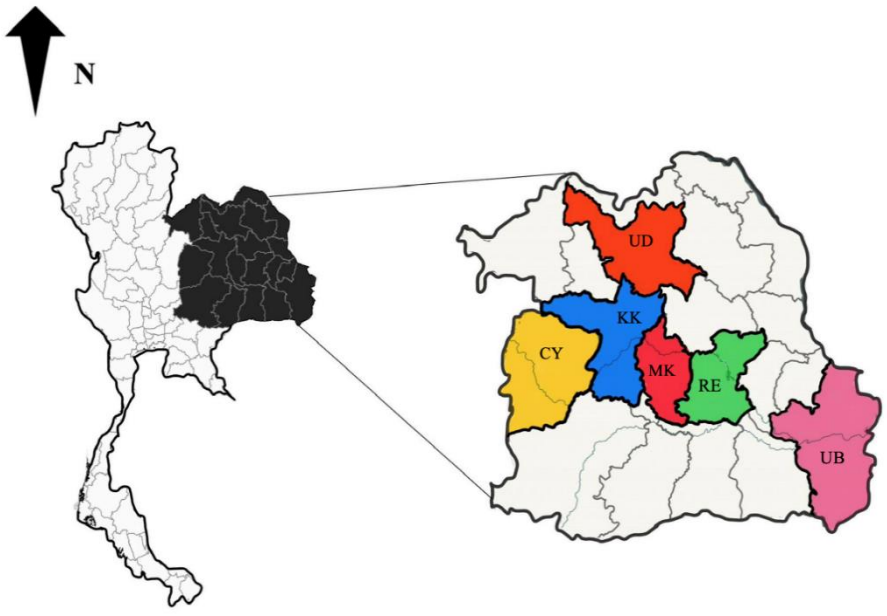
3.1 Sample collection

3.1.1 Study areas and sample collections

This cross-sectional study collected blood samples from beef and dairy cattle in smallholder farms in Maha Sarakham, Khon Kaen, Roi Et, Ubon Ratchathani, Chaiyaphum and Udon Thani provinces of Thailand during October 2022 to October 2023 (**Figure 6**). The sample size was calculated to include the appropriate number of samples from an infinite population by settling a 95% confidence level, 5% margin of error and Z equal 1.96. The power of the study was set to 80%. The sample size (N) was determine based on the following formula: $N = (Z^2 \times p \times (1-p)) / e^2$, with p represents the expected prevalence of blood parasitic infections in the study area, estimated at approximately 15% for anaplasmosis and 20% for babesiosis/theileriosis. The sample sizes in this study were 187 and 215 samples for anaplasmosis and babesiosis/theileriosis, respectively.

For the detection of anaplasmosis, a total of 187 samples were collected, comprising 106 samples from beef cattle and 81 samples from dairy cattle. In the case of babesiosis and theileriosis, a total of 215 blood samples were obtained, with 134 samples originating from beef cattle and 81 samples from dairy cattle. Blood was collected approximately 3-5 ml from the jugular vein or coccygeal vein in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes. The information (age, breed, sex, and all laboratory results) of all the field cases was also recorded. Blood samples were transported on ice to the laboratory at the Faculty of Veterinary Sciences of Mahasarakham University.

Anaplasmosis was screened for blood parasitic infections utilizing the thin blood smear technique, with concurrent measurement of packed cell volume (PCV) levels performed on the same day as blood collection. Subsequently, the remaining blood samples were stored at -20°C until DNA extraction. All procedures pertaining to animal handling and blood collection were conducted by qualified veterinarians, ensuring compliance with approval from the respective owners. Participation in the study was voluntary, and animals could be withdrawn at any time without incurring further obligation. All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Mahasarakham University (IACUC-MSU-26/2022) and (IACUC-MSU-3/2023).



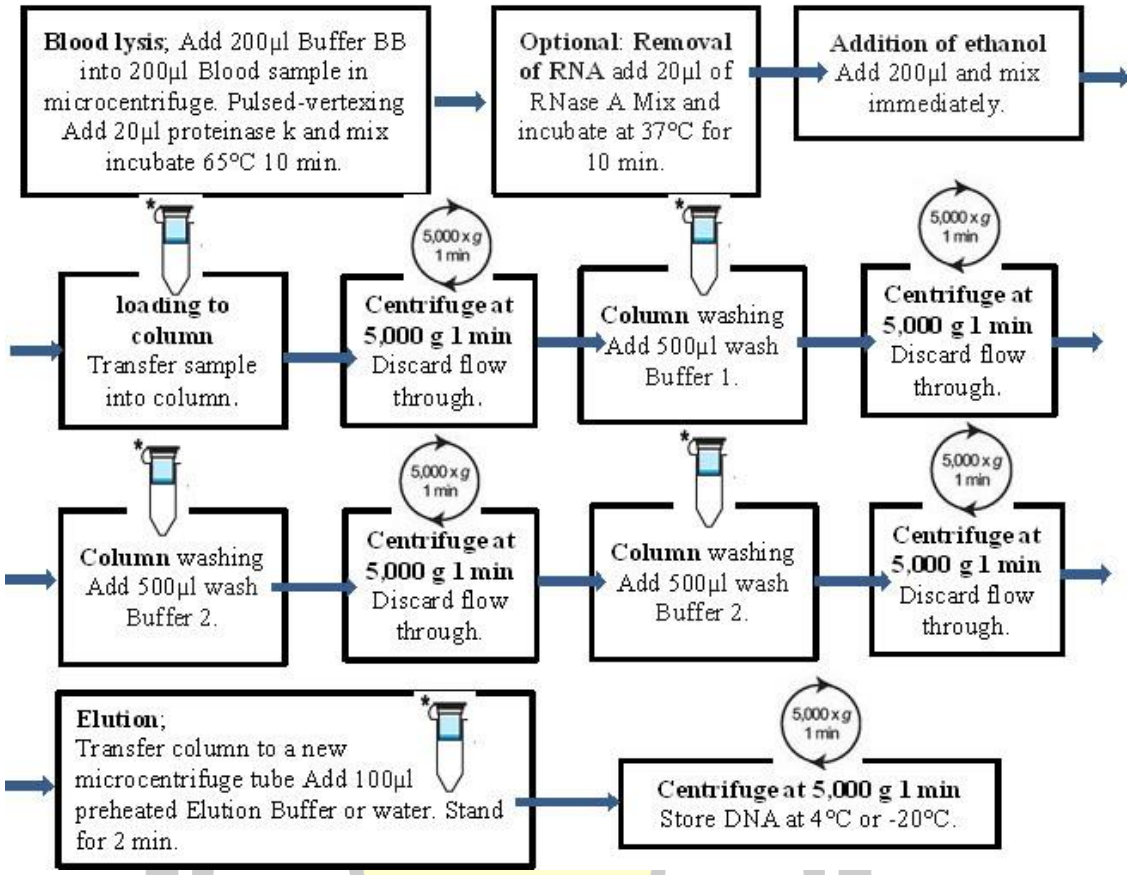
Figures 6 Map of the study area.
Cattle blood samples were collected across six provinces in the northeastern part of Thailand, consisting of Udon Thani (UD), Khon Kaen (KK), Chaiyaphum (CY), Maha Sarakham (MK), Roi Et (RE), and Ubon Rachathani (UB).

3.2 Blood smear and microscopic examination of Anaplasmosis

Thin blood smear is the routine technique for blood parasitic detection in laboratory. To perform this technique, a drop the blood (10-20µl) on to a clean slide is spread with a clean spreader slide. The blood smear slides are completely air dried for 5-10 seconds, fixed with 100% methanol for 5 min, and stained with 10% Giemsa's solution for 15 minutes. Blood films were observed in the monolayer fields under a light microscope for the presence of the parasites. Blood smears are first scanned at low magnification (×400) for 15–20 min. Then, if parasites present, high magnification (×1,000) was used to analyses morphological traits and identify species of blood parasites.

3.3 DNA Extraction

DNA of beef and dairy cattle were extracted from whole blood (200 µL) following the GF-1 blood DNA extraction Kit procedure (Vivatis, Malaysia) (**Figure 7**). DNA of each sample was stored at 4°C until performed PCR methods or -20°C for long term preservation. Each extracted DNA sample will examine for blood parasitic infections by PCR or nested-PCR method.



Figures 7 GF-1 blood DNA extraction Kit protocol.
(Vivatis, Malaysia)

3.4 PCR methods

3.4.1 PCR primers

Each extracted DNA samples were examined for blood parasitic infections by PCR or nested-PCR method using specific primers of the parasite detection as previously described. For detection *Anaplasma* sp., primers name EHR16SD (5'-GGTACCYACAGAAGAAGTCC-3') and ESR16SR(5'TAGCACTCATTTACAGC-3') were used, which amplify the DNA belonging to genus *Anaplasma*. For the next step, positive samples for *Anaplasma* genus were amplified by species specific primers as described previously (**Table 5**).

For the detection of *A. bovis* and *A. platys*, nested PCR were preformed. First step, the EE1 (5’ TCCTGGCTCAGAACGAACGCTGGCGGC 3’) and EE2 (5’ AGTCACTGACCCAACCTTAAATGGCTG 3’) primers were utilized with an annealing temperature of 60ºC. This outer primer generated a 1,430 bp amplicon. For next step, the inner primers, Ab1f (5’-CTCGTAGCTTGCTATGAGAAC-3’) and Ab1r (5’- TCTCCCGGACTCCAGTCTG-3’) for *A. bovis*, and APf (5’ AAGTC GAACGGATTTTTGTC-3’) and APr (5’-CTTTAACTTACCGAACC-3’) for *A. platys*, had annealing temperatures of 55ºC and produced amplicons of 551 bp and 506 bp, respectively. For *A. marginale* detection, the MSP4 gene was targeted using primers MSP43 (3’-GGGAGCTCCTATGAATTACAGAGAATTGTTTAC -5’) and MSP45 (5’-CCGGATCCTTAGCTGAACAGGAATCTTGC-3’) with an annealing temperature of 56ºC. This conventional PCR method generated an 849 bp amplicon.

Each extracted DNA sample underwent examination for *Babesia* and *Theileria* infection utilizing a nested-PCR method. This method employed specific primers designed to target the 18s rRNA gene, approximately 1,500 bp in length, of the parasite, as previously described (Masatani et al., 2017). The first PCR step utilized the primer pair, namely BTH 18S 1st F (5’-GTGAAACTGCGAATGGCTCATTAC-3’) and BTH 18S 1st R (5’-AAGTGATAAGGTTCACAAAACTTCCC-3’), while the second step utilized the primer pair BTH 18S 2nd F (5’ GGCTCATTACAACAGTTA TAGTTTATTTG-3’) and BTH 18S 2nd R (5’- CGGTCCGAATAATTCACCGGAT-3’). This nested-PCR approach enabled the amplification of DNA from protozoa in genus *Babesia*, *Theileria*, and *Hepatozoon* **(Table 5).**

Table 5 Primer for *Anaplasma*, *Babesia* and *Theileria* amplification.

Parasites	**Primer**	**PCR system**	**Target gene**	**Annealing temperature (ºC)**	**PCR product (base pair)**	**References**
Anaplasma sp.	EHR16SD 5'-GGTACCYACAGAAGAAGTCC-3' ESR16SR 5'-TAGCACTCATTTACAGC-3'	PCR	16s rRNA	55	345	Ogata et al., 2021
Anaplasma (outer primer)	EE1 (5' TCCTGGCTCAGAACGAACGCTGGCGGC 3') EE2 (5' AGTCACTGACCCAACCTTAAATGGCTG 3')	nested PCR	16s rRNA	60	1430	Barlough et al.,1996
A. bovis	Ab1f 5'-CTCGTAGCTTGCTATGAGAAC-3' Ab1r 5'-TCTCCCGGACTCCAGTCTG-3'	nested PCR	16s rRNA	55	551	Kawahara et al., 2006
A. platys	APf 5'-AAGTCGAACGGATTTTTGTC-3' APr 5'-CTTTAACTTACCGAACC-3'	nested PCR	16S rRNA	55	506	Martin et al., 2005.
A. marginale	MSP43 3'-GGGAGCTCCTATGAATTACAGAGAATTGTTTAC -5' MSP45 5'-CCGGATCCTTAGCTGAACAGGAATCTTGC-3'	PCR	MSP4	56	849	Saetiew et al., 2015
Babesia sp. and *Theileria* sp.	BTH 18S 1st F (5'-GTGAAACTGCGAATGGCTCATTAC-3') BTH 18S 1st R (5'-AAGTGATAAGGTTCACAAAACTTCCC-3') BTH 18S 2nd F (5' GGCTCATTACAACAGTTATAGTTTATTTG-3') BTH 18S 2nd R (5'-CGGTCCGAATAATTCACCGGAT-3')	nested PCR	18s rRNA	55	1343-1458	Adaszek and Winiarczyk, 2008

3.4.2 PCR amplification

PCR reaction *Anaplasma* spp. detection was conducted in a final volume of 25 µL. Each reaction mixture comprised of approximately 10–50 ng of the extracted DNA or 2 µL of PCR product (nested PCR), 1 µL of each primer (10 µmol/L), 1.5 mM MgSO4, 0.2 mM deoxynucleotide triphosphate, 1× PCR buffer and 1 U of *Taq* Polymerase (Fermentas). The reaction conditions comprised 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 50-62°C and extension for 45 s at 72°C using a PCR thermocycler (Biometra, Göttingen, Germany). PCR master mixes containing only the primers with no DNA template were serve as negative controls. The PCR products were run in a 1% agarose gel stained with ViSafe Red Gel Stain (Vivantis Technologies) and visualised under ultraviolet light to check for positive amplifications.

3.5 Sequencing and phylogenetic analysis

For *Babesia* and *Theileria* we randomly selected 65 PCR amplicons from dispersed sampling sites, representing 50% of positive samples from each site, for purification and direct sequencing. The PCR products targeting the 18s rRNA genes underwent purification and sequencing at a commercial sequencing facility (1st Base, Malaysia; ATGC, Thailand). Electrograms of the sequences were meticulously examined for quality, appropriate length, and absence of double or multiple nucleotide peaks. The obtained DNA sequences were aligned and trimmed using the BioEdit sequence alignment editor program (Hall et al,.1999). Subsequently, the nucleotide sequences were analyzed for similarity to sequences in the GenBank database using the BLAST program hosted by NCBI (https://www.ncbi.nlm.nih.gov/).

Haplotype identification from the 18s rRNA sequences of *Babesia* and *Theileria* was conducted using the DnaSP6 program (Rozas et al., 2017).

The obtained sequences of the partial 18s rRNA gene of *Babesia* and *Theileria* in this study were approximately 1,405 bp in length (ranging from 1,343 to 1,458 bp). The resulting partial 18s rRNA gene sequences represented each *Babesia* and *Theileria* haplotype and were then deposited into the GenBank database with accession numbers PP380178-PP380189. Phylogenetic relationships among the 18s rRNA haplotypes from this study and 27 related sequences from various geographical locations in GenBank were inferred using the maximum likelihood method in MEGA X (Kumar et al., 2018). Bootstrap analysis with 1000 replications were employed to assess the confidence of branching patterns in the trees.

3.6 Statistical analysis

The presences of blood parasites were determined and the percentages of infection were calculated. Confidence Intervals (ICs) were also used to compare prevalence between parasitic infections. The association between blood parasite infections with other factors including gender, age, BCS, breed and PCV levels were compared with Pearson's Chi-squared test. Statistical differences were considered when *p*-value is less than 0.05.

CHAPTER 4
RESULTS

4.1 Prevalence of *Anaplasma* sp., *Babesia* sp. and *Theileria* sp. in cattles

4.1.1 Prevalence of *Anaplasma* sp.

Blood samples of beef and dairy cattle were collected from five provinces in the Northeastern region of Thailand which are Khon Kaen (n = 48), Maha Sarakham (n = 94), Roi Et (n = 12), Ubon Ratchathani (n = 13), and Udon Thani (n = 20). Blood samples of 187 cattle comprised 56.7% (106) of beef cattle and 43.3% (81) of dairy cattle. About 106 samples of beef cattle were male 13.2% and female 86.8%. The 81 samples collected from dairy cattle were male 16% and female 84%. Animal samples were in the age range from 2 months to 10 years old (21.9% were <1 year; 66.3% were 1–6 years old; and 11.8% were >6 years old). All sampling animals showed capillary refill time <2 s. Animals with BCS <3 and ≥3 were 114 and 64, respectively **(Table 6).**

4.1.2 Prevalence of *Babesia* sp. and *Theileria* sp.

Blood samples of beef and dairy cattle were collected from six provinces in the Northeastern region of Thailand which are Khon Kaen (n = 48), Maha Sarakham (n = 92), Roi Et (n = 12), Ubon Ratchathani (n = 13), Chaiyaphum (n = 30) and Udon Thani (n = 20). From a total of 215 samples, comprising 180 females and 35 males, spanning an age range from 2 months to 10 years, it was observed that 65.58% (141/215) exhibited infection with *Babesia* or *Theileria*, as determined by nested PCR analysis. Specifically, among females, 65% (117/180) were found to be infected, whereas among males, the infection rate was slightly higher at 68.57% (24/35). Chi-

square analysis revealed that the observed differences in infection rates between sexes were not statistically significant. Furthermore, when considering the distribution of infection across different production types, it was observed that 66.42% (89/134) of beef cattle and 64.20% (52/81) of dairy cattle were infected. Chi-square analysis indicates no significant difference in infection rates between beef and dairy cattle. Animals within the age range of 0-1 year showed an infection rate of 52.63% (20/38), adult animals aged between 1 and 6 years displayed an infection prevalence of 67.10% (104/155), and old animals aged over 6 years showed an infection rate of 77.27% (17/22). Despite these observed differences in infection rates across age groups, statistical analyses did not reveal any significant age-related associations with infection susceptibility within the studied population **(Table 7).**

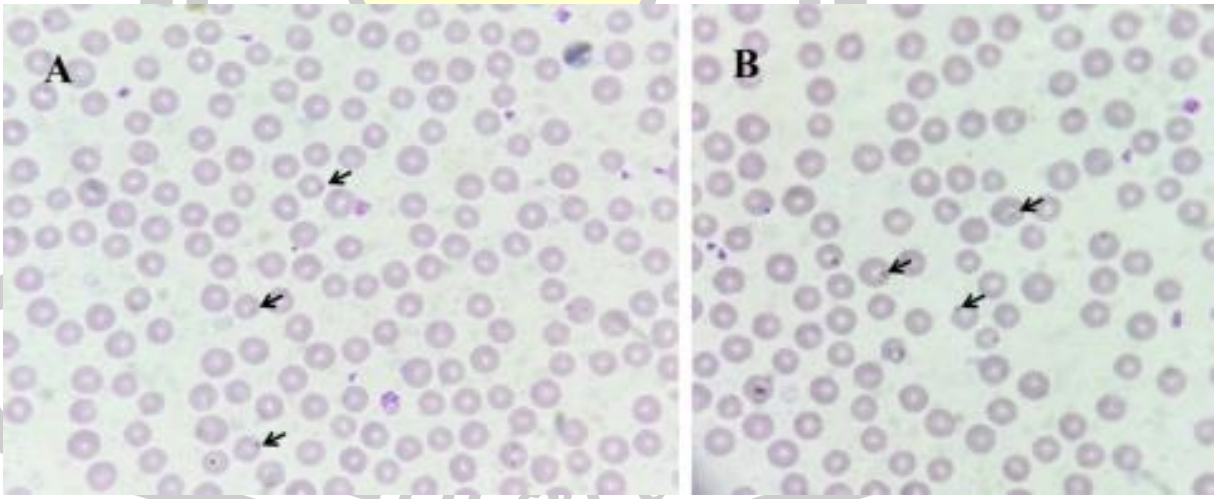
4.2 Identification of *Anaplasma* sp., *Babesia* sp. and *Theileria* sp. in cattles

4.2.1 *Anaplasma* sp. identification

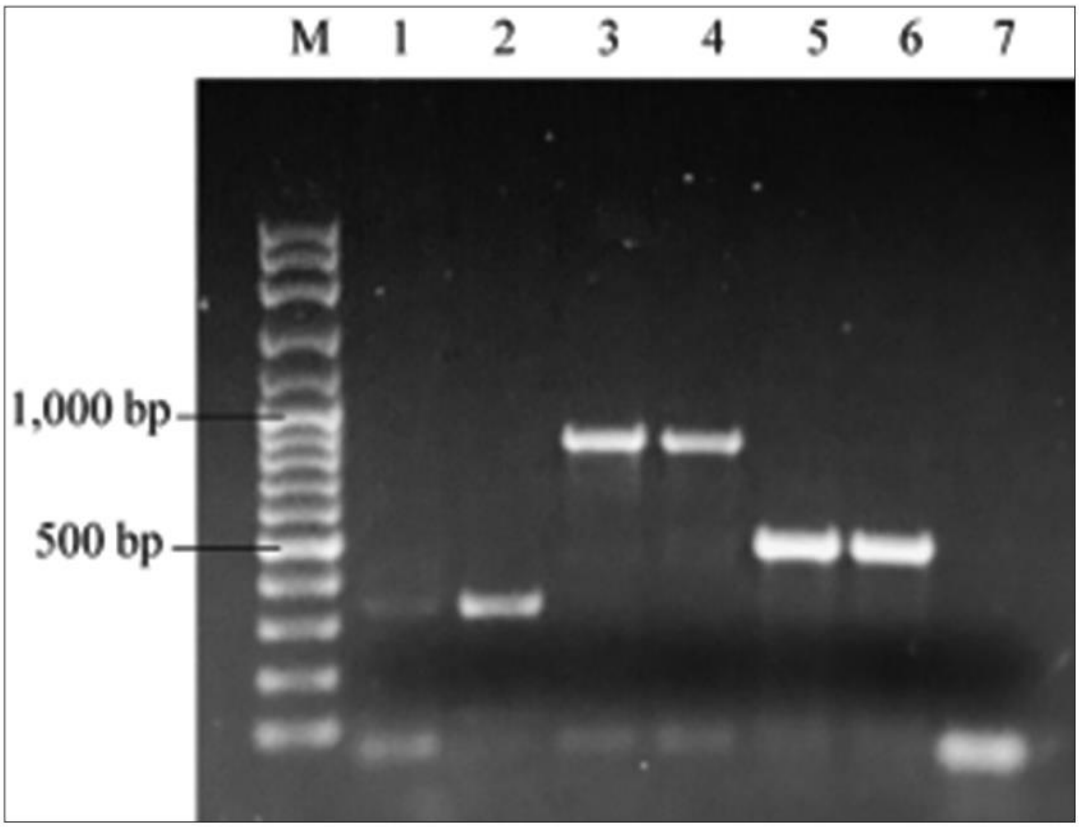
Under the light microscope, *Anaplasma* spp. infections were detected in erythrocytes **(Figure 8).** For microscope examination, the prevalence of *Anaplasma* spp. was 17.6 %. The occurrence of Anaplasmataceae was examined by PCR based on the 16S rRNA gene. Fragments of *msp4* of *A. marginale* and 16S rRNA of *A. platys* were amplified to examine infections **(Figure 9).** For PCR, the overall prevalence of anaplasmosis in cattle in Northeast Thailand was 20.8% (95% CI: 15.3–27.4) based on 16S rRNA Anaplasmataceae primers. For specific primer detection, 5.3% and 3.2% of beef cattle were infected with *A. marginale* and *A. platys.* In addition, the molecular prevalence of *A. marginale* in dairy cattle was 12.3%, while

no infection with *A. platys* was observed in this population. In addition, *A. bovis* infection was not discovered in this study.

Regarding breed, dairy cattle were more susceptible to *Anaplasma* infection (28.4%) than beef cattle (15.1%). Male cattle (44.4%) were more likely to be *Anaplasma* infected than females (16.9%). For PCV values, the average PCV levels in both infected and uninfected groups were in the normal range (28.5% vs. 30%). Although the infected group had a lower trend of PCV, the results showed no statistical difference between infected and uninfected groups. Moreover, there were no clinical signs in any cattle infected with *Anaplasma* spp. In addition, statistical tests of the association between *Anaplasma* infections and other factors showed infection with *Anaplasma* spp. and *A. marginale* had an association with breed and gender ($p < 0.05$) while age and PCV levels showed no significant statistical relationship between *Anaplasma* spp. infected and uninfected groups.



Figures 8 *Anaplasma marginale* infections in erythrocytes.
A. marginale infections in erythrocytes of (a) beef cattle and (b) dairy cattle.



Figures 9 Agarose gel electrophoresis of PCR products.
Lane M: 100 bp DNA ladder marker; Lanes 1–2: Positive samples for Anaplasmataceae at 345 bp; Lanes 3–4: Positive samples for *Anaplasma marginale* at 849 bp; Lanes 5–6: Positive samples for *Anaplasma platys* at 506 bp; Lane 7: Negative control.

Table 6 Characteristics of cattle and risk factors analysis of anaplasmosis.

Characteristics	No. of cattle	No. of positive with				
		Anaplasma **spp. infected (%)**	**p-value**	***A. marginale*** **infected (%)**	**p-value**	***A. platys*** **infected (%)**
Breed			0.03*		0.00*	
Beef cattle	106	16 (15.1)		10 (9.4)		6 (5.7)
Dairy cattle	81	23 (28.4)		23 (28.4)		0 (0)
Gender			0.00*		0.004*	
Male	27	12 (44.4)		10 (37)		2 (7.4)
Female	160	27 (16.9)		23 (14.4)		4 (2.5)
Age (Years)			0.51		0.42	
Calf (0-1)	39	11 (28.2)		10 (25.6)		1 (2.6)
Adult (1-6)	118	23 (19.5)		20 (16.9)		3 (2.5)
Old (>6)	21	5 (23.8)		3 (14.3)		2 (9.5)
%PCV			0.90		0.78	
Anemia (<24)	30	6 (20)		6 (20)		0 (0)
Non anemia (≥24)	157	33 (21)		27 (17.2)		6 (3.8)

Table 7 Characteristics of cattle infected with *Babesia* sp. or *Theileria* sp.

Characteristics	**NO. of cattle (n)**	**NO. of positive with** ***Babesia*** **sp. or** ***Thileria*** **sp.**	**Prevalence (%)**	**95% Confidence Interval**	**p-value**	**Chi-square value**
Breed						
Beef cattle	134	89	66.42	57.75 - 74.34	0.74	0.1103
Daily cattle	81	52	64.20	52.77 - 74.55		
Gender						
Female	180	117	65	57.55 - 71.95	0.68	0.1656
Male	35	24	68.57	50.71 – 83.15		
Age (years)						
Calf (0-1)	38	20	52.63	35.82 – 69.02	0.12	4.3131
Adult (1-6)	155	104	67.10	59.10 – 74.42		
Old (>6)	22	17	77.27	54.63 – 92.18		
Total	215	141	65.58	58.82 – 71.91		

4.2.2 *Babesia* and *Theileria* identification

Among the positive samples, a subset of 65 PCR products (representing 50% of positive samples from each province) was randomly selected for sequencing

analysis resulting in the successful sequencing of 64 specimens. Sequencing identified the presence of *Babesia bovis*, *B. bigemina*, *Thileria* sp., *T. orientalis*, and *T. sinensis* in cattle in the northeastern part of Thailand. Among obtained 64 sequences, 6 sequences corresponded to *Babesia* species (comprising 2 sequences of *B. bovis* and 4 sequences of *B. bigemina*) and 58 sequences corresponded to *Theileria* species (comprising 28 samples of *T. orientalis*, 15 samples of *Theileria* sp., and 13 samples of *T. sinensis*). All sequences underwent BLAST analysis and were aligned with other relevant sequences in the GenBank database to assess genetic similarities based on the 18S rRNA gene **(Figure 10, 11).**

Further analysis of the sequences revealed the presence of distinct haplotypes within both *Theileria* and *Babesia* species. Specifically, 58 sequences representing *Theileria* species exhibited were classified into 6 haplotypes, with 4 haplotypes associated with *T. orientalis* (accession numbers ranging from PP380178 - PP380179, PP380181 - PP380182), 1 haplotype were *Theileria* sp. (accession number PP380180) and 1 haplotype were *T. sinensis* (accession number PP380183). Whereas the remaining 6 sequences corresponding to *B. bovis* for 2 haplotypes (accession no. PP380184 - PP380185) while *B. bigemina* demonstrated differentiation into 4 distinct haplotypes (accession no. PP380186 - PP380189). Sequencing and DNA analysis revealed that infection by *B. bigemina*, *B. bovis*, *T. orientalis*, *T. sinensis*, and *Theileria* sp. were common piroplasms in cattle in this region, with sequence similarities ranging between 99-100% with homologous sequences from other countries **(Table 8)**.

For locations, samples were collected from a total of 10 farms spanning 6 provinces. The highest prevalence of infection was observed in Ubon Rachathani at 92.30% (12/13), followed closely by Roi Et at 91.66% (11/12). Subsequent infection rates were 76.66% (23/30) in Chaiyaphum, 70.83% (34/48) in Khon Kaen, 58.69% (54/92) in Maha Sarakham, and 35% (7/20) in Udon Thani.

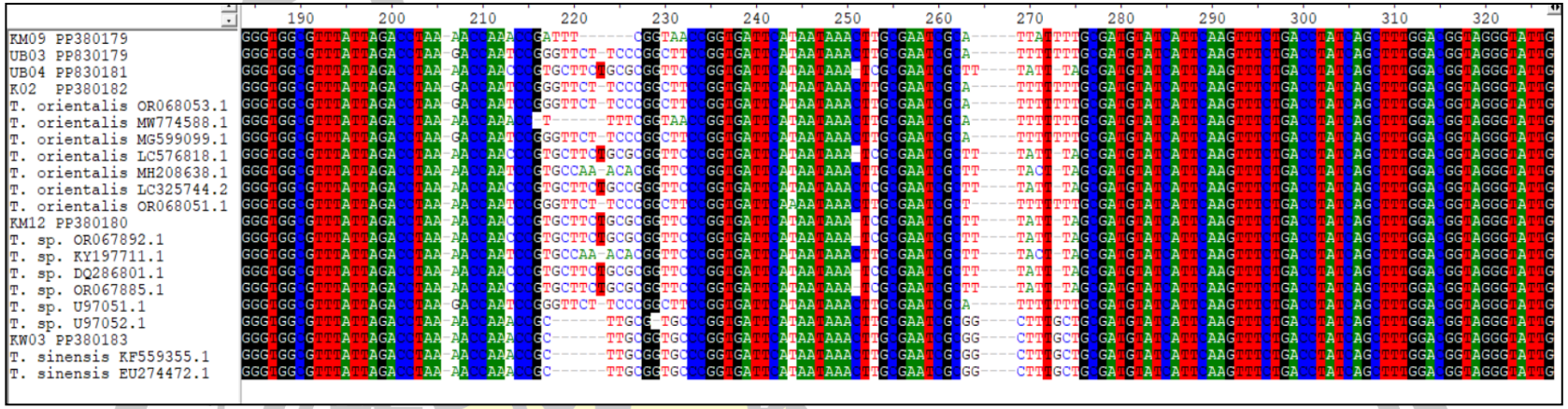
4.3 Phylogenetic tree of *Babesia* and *Theileria*

The phylogenetic analyses, utilizing the 18S rRNA gene, delineated distinct evolutionary lineages within *Theileria* and *Babesia*. *Theileria* was observed to segregate into three primary clades: *T. orientalis*, *T. sinensis*, and *Theileria* sp., while *Babesia* exhibited two main clades: *B. bovis* and *B. bigemina.*

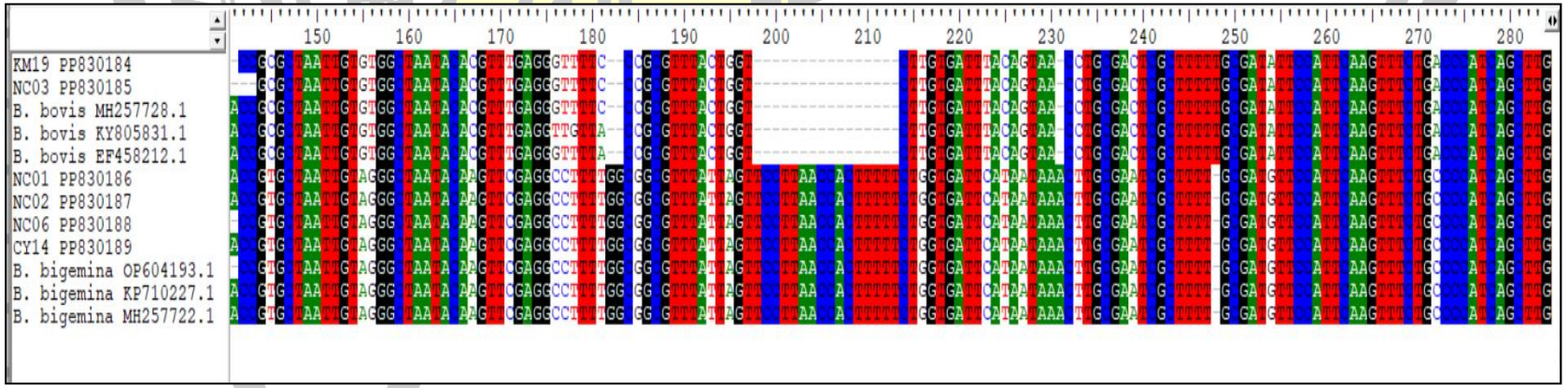
The results showed that 4 haplotypes of *T. orientalis* relevant to *T. orientalis* from Myanmar (LC602478.1), South Korea (MT889728.1), India (OR068053.1), China (KU363043.1, MMH208641.1), Pakistan (MG599099.1), and Turkey (OR211416.1). Similarly, *T. sinensis* haplotypes clustered with those from Malaysia (MT271911.1) and China (KX115427.1, KF559355.1, EU274472, HM538203.1), while *Theileria* sp. haplotypes exhibited genetic similarity with *Theileria* sp. strains from India (OR067892.1,), Myanmar (LC57817.1), and China (MN252454.1, DQ286801.1) and *T. annulata* from India (KT364499.1, MF287950.1, OR589446.1). From phylogenetic analysis, *T. orientalis* and *T. sinensis* are closely related whereas *Theileria* sp. is more distantly related and grouped with *T. annulata*.

In this study, the haplotypes of *B. bovis* from clustered closely aligned with sequences of *B. bovis* from various geographic regions, including China (KY805831.1), South Africa (MH257728.1), Brazil (EF458212.1) and USA

(L31922). Similarly, the haplotypes of *B. bigemina* exhibited clustering with sequences of *B. bigemina* originating from South Africa (MH257722.1), Taiwan (OP604193.1), and China (JX495402.1) **(Figure 12).**



Figures 10 The alignment of *Theileria* sp.



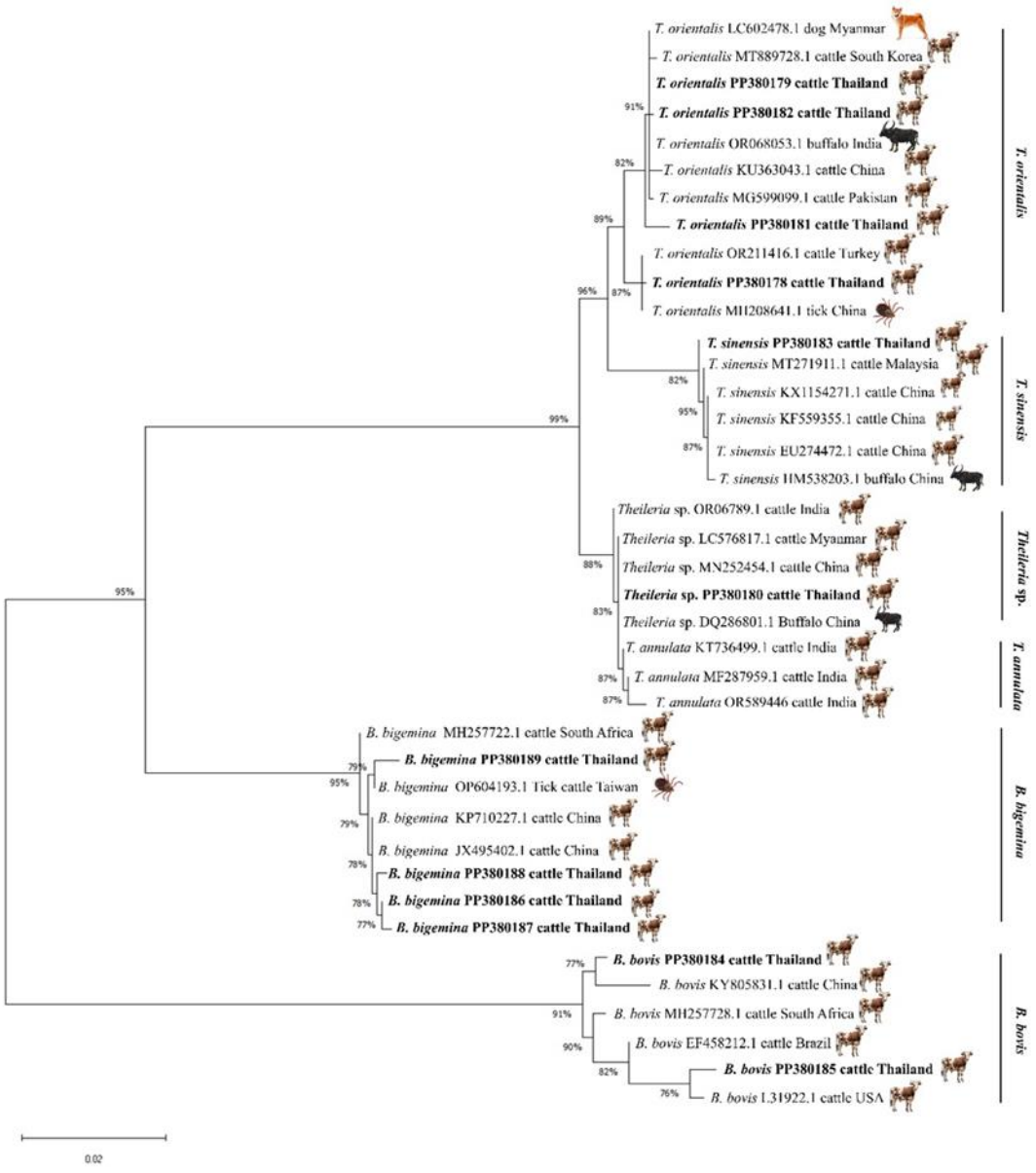
Figures 11 The alignment of *Babesia* sp.

Table 8 Haplotypes of 64 sequences of 18s rRNA of piroplasm.
BLAST results of the 18s rRNA sequences from beef and dairy cattle in Thailand and National Center for Biotechnology Information

Haplotype	Sample IDs	NCBI GenBank accession number	Closest sequences in NCBI GenBank (% similarity)	Parasites
1	KM01, KM06, KM09, RE01, RE02, RE07, KW11, KW12, UB02, K01	PP380178	MH208641.1 (99.86) OR211416.1(99.86) CP056070.2(99.86)	*Theileria orientalis*
2	KM11, UD05, K06, K11, K14, PM02, UB03, UB05, KW01, KW09, KW14, CY09, CY11, CY26	PP380179	OR068053.1(99.93) LC602478.1(99.93) AB520956.1(99.93)	*Theileria orientalis*
3	KM08, KM12, KM30, UD04, UB07, UB08, K04, K05, K08, K12, RE04, RE05, NC05, KW04, PM01, CY17	PP380180	OR067892.1(100) MN252454.1(100) DQ286801.1(100)	*Theileria* sp.
4	UB04, MM03	PP380181	OR068050.1(99.66) LC602478.1(99.59) AB520956.1(99.59)	*Theileria orientalis*
5	K02, K03	PP380182	OR068053.1(99.93) LC602478.1(99.93) MN252441.1(99.93)	*Theileria orientalis*
6	UD02, UD07, KM15, KM16, PM03, KW02, KW03, KW15, KW16, CY01, CY04, CY07, CY20, CY23	PP380183	MT271911.1(99.93) MT271902.1(99.86) KF559355.1(99.86)	*Theileria sinensis*
7	KM19	PP380184	MH257728.1(99.12) MH257734.1(99.05) KY805831.1(98.97)	*Babesia bovis*
8	NC03	PP380185	MH257726.1(99.85) MH046909.1(99.40) CP125250.1(99.33)	*Babesia bovis*
9	NC01	PP380186	AY603402.1(99.93) MH208614.1(99.93) KY805825.1(99.86)	*Babesia bigemina*

Table 8 Haplotypes of 64 sequences of 18s rRNA of piroplasm.
BLAST results of the 18s rRNA sequences from beef and dairy cattle in Thailand and National Center for Biotechnology Information (Continue)

Haplotype	Sample IDs	NCBI GenBank accession number	Closest sequences in NCBI GenBank (% similarity)	Parasites
10	NC02	PP380187	AY603402.1(99.85) KY805825.1(99.78) MH257700.1(99.78)	*Babesia bigemina*
11	NC06	PP380188	KP710227.1(99.78) OP604189.1(99.78) JX495402.1(99.78)	*Babesia bigemina*
12	CY14	PP380189	OP604193.1(99.57) KP710227.1(99.50) JX495402.1(99.50)	*Babesia bigemina*



Figures 12 Phylogenetic analyses of *Babesia* and *Theileria*.
The18s rRNA sequences obtained from Thailand cattle and related sequences in GenBank using the maximum likelihood method. The sequences determined in this study are shown in bold font and the percentage of trees in which associated taxa clustered together is shown next to the branch.

CHAPTER 5
DISCUSSION

Anaplasmosis in cattle is a worldwide veterinary health problem, especially in tropical and subtropical regions. In this study, we screened *Anaplasma* spp. infection in beef and dairy cattle using both microscopic and molecular techniques. From this study, the overall prevalence of *Anaplasma* spp. in cattle was 20.8% based on PCR and 17.6% based on microscopic results. Although microscopic examination by direct blood smear technique is common, it is suitable for the detection of anaplasmosis during the acute phase of infection and requires an expert examiner. Polymerase chain reaction is an advantageous assay over microscopic examination because it has high sensitivity and specificity and is widely used to detect all phases of anaplasmosis infection in animals. The results indicated that the PCR method exhibited much higher sensitivity for the diagnosis of this blood parasite than the microscopic method, which is the routine method in the laboratory.

In Thailand, *Anaplasma* spp. infection in large ruminants is endemic with a higher infection rate reported in water buffalo (41%) (Nguyen et al.,2020) and beef cattle in the Western region (39.1%) (Jirapattharasate et al.,2016). However, the prevalence in this study is higher than the previous studies by Junsiri *et al* (Junsiri et al.,2020). in cattle in the northern and northeastern regions of Thailand in 2020 (10.30%) and water buffaloes in Northeast Thailand (8%) (Saetiew et al.,2015). The difference in the prevalence of anaplasmosis in cattle in Thailand could be explained by the climatic condition in each region which influences the spread of tick vectors (Saetiewa et al.,2020), farm management, herd size, sampling period, sample size,

antibiotic prevention (Ola-Fadunsin et al.,2018), and diagnosis protocols (Arnuphapprasert et al.,2023). In addition, this study notices that good management practices on the farm have been observed to be the key factor in the infection rate. In other counties, the prevalence of anaplasmosis in cattle varies from 8.7% in Mongolia (Ybanez et al.,2013), 9% and 17% in Punjab (Pakistan) (Zafar et al.,2022), 11.1% in Pakistan (Asif et al.,2022), 15.7% in India (Das et al.,2022), 38.53% in Ohio (Eleftheriou et al.,2022), 49.1 % in Nigeria (Kamani et al.,2022), and 68.3% in Egypt (Al-Hosary et al.,2020). The prevalence of bovine anaplasmosis in this study is reliable in range with previous epidemiological studies.

Apart from risk factor analysis of anplasmosis, we found that the risk factors for *Anaplasma* spp. and *A. marginale* infections were significantly associated with breed and gender. Previous data also supported our finding that breed and gender had significant associations with *Anaplasma* spp. (Amorim et al.,2014). For gender, the results showed that male cattle had a higher infection rate than female cattle according to the finding in cattle in China (Zhou et al.,2019) and buffalo in Pakistan (Farooqi et al.,2018). For breed, the results revealed that dairy cattle are more susceptible to anaplasmosis than beef cattle. Although a previous study reported the age of the animals (below 1 year of age) showed a significant association with *Anaplasma* spp. infections (Amorim et al.,2014), we found adverse results that age showed no significant relationship with infections. However, similar results in this study were also reported in water buffaloes from eight provinces of Thailand (Nguyen et al.,2020). In addition, the principal clinical sign of bovine anaplasmosis was considered anemia which can be directly measured by PCV levels; however, we found PCV levels showed no significant relationship with infections according to the

report of PCV levels in infected cattle in Nigeria (Kamani et al.,2022) This phenomenon may support the evidence that most cattle, especially animals that adapt well to a tropical climate show milder symptoms on infection.

For piroplasmid infections, we demonstrated the molecular detection based on 18s rRNA in samples from beef and dairy cattle in 6 provinces in the northeastern part of Thailand. This study documented the highest prevalence of piroplasmid infection (*Babesia* or *Theileria*) reported in Thailand to date at approximately 65.58% (95% CI: 58.82-71.91%). Furthermore, there was considerable variation in infection rates observed across sampled farms, ranging from 35% to 92.3%. This variability may be attributed to multiple factors, including herd size and farm management practices, particularly those about tick control strategies (Muhanguzi et al., 2010). In comparison to previous reports, which indicated prevalence rates of *B. bovis* and *B. bigemina* in cattle of 12% and 21% respectively in 2012 (Cao et al., 2012), and 11.1% and 12.5% respectively in 2017 (Jirapattharasate et al., 2017), this study demonstrates a substantial increase. Notably, recent studies in 2022-2023 have shown a decrease in *Babesia* prevalence, ranging from 1.22% to 5.8% (Koonyosying et al., 2022; Srionrod et al., 2022; Adjou et al., 2023). Regarding *Theileria* prevalence, earlier research in 2017 reported a prevalence rate of 7.8% (Jirapattharasate et al., 2017), whereas a study conducted in 2022 recorded a higher prevalence of 36.5% (Koonyosying et al., 2022). In other countries, *Babesia* and *Theileria* are worldwide distributed with molecular prevalence of 25.26% in selected areas of China and Pakistan (Hassan et al., 2020), 36.1% in Kyrgyzstan (Aktaş et al., 2019), 52.8% in Nepal (Dhakal et al., 2023) and 87.3 % in Nigeria (Famuyide et al., 2020). The fluctuating prevalence in

each region underscores the influence of climatic and meteorological conditions which influence tick vector populations (M'ghirbi et al., 2008).

Through DNA sequencing analysis, our study identified *T. orientalis* as the dominant species of the piroplasm, followed by *Theileria* sp, *T. sinensis*, *B. bigemina* and *B. bovis* in cattle farms in Thailand. This observation is consistent with prior findings in central and northern Thailand, which also highlighted the prevalence of *T. orientalis* as the dominant species (Koonyosying et al., 2022). Furthermore, similar dominance of *Theileria* has been reported in other regions such as China (Zhou et al., 2019) and Kyrgyzstan (Aktaş et al., 2019). In addition, production type, age and sex did not exert a significant influence on the likelihood of infection with *Babesia* or *Theileria* in the studied cattle population. This finding correlated with previous studies in Malaysia and Egypt which similarly demonstrated that sex was not correlated with infection. However, factors such as production type and age exhibited significant associations ($p < 0.05$) with the prevalence of *T. orientalis* (Ola-Fadunsin et al., 2020; Selim et al., 2022).

Previous studies reported that the 18s rRNA fragments are appropriate markers to determine the genetic diversity for blood parasites (Bawm et al., 2021; Nehra et al., 2022). In this study, analysis of 18s rRNA revealed a notable degree of sequence similarity within the *Babesia* and *Theileria* of this population and GenBank database. Phylogenetic analysis showed that *Theileria* could be divided into three groups: *T. orientalis*, *T. sinensis*, and *Theileria* sp. Furthermore, our investigation identified *T. orientalis* and *T. sinensis* as genetically more similar to each other, forming a distinct cluster separate from *Theileria* sp. suggesting a closer evolutionary relationship between *T. orientalis* and *T. sinensis*, distinguishing them from *Theileria* sp. based on

18s rRNA gene. Notably, our study also confirmed the first presence of *T. sinensis* circulating within the cattle population in Thailand.

Conclusion

Epidemiology research efforts are needed to enhance our understanding of bovine blood parasitic infections in Thai cattle populations. The data presented the prevalence, associated risk factors and genetic diversity of *Anaplasma* sp., *Babesia* sp. and *Theileria* sp. in beef and dairy cattle in Thailand. The providing data holds significant potential utility for both cattle farmers and veterinarians, facilitating comprehension of blood parasite infections and improves our current understanding of the distribution of these parasites. Consequently, this enhanced understanding contributes to advancements in strategies for prevention and control of bovine anaplasmosis,babesiosism and theileriosis.

REFERENCES

REFERENCES

Adaszek, L., & Winiarczyk, S. (2008). Molecular characterization of *Babesia canis canis* isolates from naturally infected dogs in Poland. *Veterinary parasitology*, *152*(3-4), 235-241.

Adjou Moumouni, P. F., Galon, E. M., Tumwebaze, M. A., Byamukama, B., Ngasaman, R., Tiwananthagorn, S., ... & Xuan, X. (2023). Tick-borne Pathogen Detection and Its Association with Alterations in Packed Cell Volume of Dairy Cattle in Thailand. *Animals*, *13*(18), 2844.

Ahantarig, A., Trinachartvanit, W., & Milne, J. R. (2008). Tick-borne pathogens and diseases of animals and humans in Thailand. *Southeast Asian journal of tropical medicine and public health*, *39*(6), 1015.

Aktaş, M., Kısadere, İ., Özübek, S., Cihan, H., Salıkov, R., & Cirak, V. Y. (2019). First molecular survey of piroplasm species in cattle from Kyrgyzstan. *Parasitology research*, *118*, 2431-2435.

Al-Hosary, A., Răileanu, C., Tauchmann, O., Fischer, S., Nijhof, A.M. & Silaghi, C. (2020). Epidemiology and genotyping of *Anaplasma marginale* and co-infection with piroplasms and other Anaplasmataceae in cattle and buffaloes from Egypt. *Parasit Vectors.*, *13*(1): 1-11.

Altay, K., Aydin, M. F., Dumanli, N., & Aktas, M. (2008). Molecular detection of Theileria and *Babesia* infections in cattle. *Veterinary parasitology*, *158*(4), 295-301.

Amorim, L.S., Wenceslau, A.A., Carvalho, F.S., Carneiro, P.L.S. & Albuquerque, G.R. (2014) Bovine babesiosis and anaplasmosis complex: diagnosis and evaluation of the risk factors from Bahia, Brazil. *Rev. Bras. Parasitol. Vet*., *23*: 328-336

Aparna, M., Ravindran, R., Vimalkumar, M. B., Lakshmanan, B., Rameshkumar, P., Kumar, K. A., ... & Ghosh, S. (2011). Molecular characterization of *Theileria orientalis* causing fatal infection in crossbred adult bovines of South India. *Parasitology International*, *60*(4), 524-529.

Arnuphapprasert, A., Nugraheni, Y. R., Poofery, J., Aung, A., Kaewlamun, W., Chankeaw, W., ... & Kaewthamasorn, M. (2023). Genetic characterization of genes encoding the major surface proteins of *Anaplasma marginale* from cattle

isolates in Thailand reveals multiple novel variants. *Ticks and Tick-borne Diseases*, *14*(2), 102110.

Arunyakanon, P., Teerapat, K., & Dissamam, R. (1966). Study of immunity of anaplasmosis vaccine in foreign breed cattle. In *Proceedings of National Conference on Agricultural Science Fifth Session: Plant and Biological Science, Animal Science and Agricultural Economics, Kasetsart University, Bangkok, Thailand* (pp. 403-408).

Asif, M., Ben Said, M., Vinueza, R.L., Leon, R., Ahmad, N., Parveen, A., Khan, A., Ejaz, A., Ali, M., Kha, A. U., Barber, M. & Iqbal, F. (2022) Seasonal Investigation of *Anaplasma marginale* Infection in Pakistani Cattle Reveals Hematological and Biochemical Changes, Multiple Associated Risk Factors and msp5 Gene Conservation. *Pathogens*., *11*(11): 1261.

Aubry, P., & Geale, D. W. (2011). A review of bovine anaplasmosis. *Transboundary and emerging diseases*, *58*(1), 1-30.

Bakken, J. S., & Dumler, J. S. (2000). Human granulocytic ehrlichiosis. *Clinical infectious diseases*, *31*(2), 554-560.

Barlough, J. E., Madigan, J. E., DeRock, E., & Bigornia, L. (1996). Nested polymerase chain reaction for detection of *Ehrlichia equi* genomic DNA in horses and ticks (Ixodes pacificus). *Veterinary Parasitology*, *63*(3-4), 319-329.

Batiha, G. E. S., Beshbishy, A. M., Tayebwa, D. S., Shaheen, H. M., Yokoyama, N., & Igarashi, I. (2019). Inhibitory effects of Syzygium aromaticum and Camellia sinensis methanolic extracts on the growth of Babesia and Theileria parasites. *Ticks and tick-borne diseases*, *10*(5), 949-958.

Battilani, M., De Arcangeli, S., Balboni, A., & Dondi, F. (2017). Genetic diversity and molecular epidemiology of *Anaplasma*. *Infection, Genetics and Evolution*, *49*, 195-211.

Bawm, S., Myaing, T. T., Thu, M. J., Akter, S., Htun, L. L., Win, M. M., ... & Katakura, K. (2021). PCR detection and genetic characterization of piroplasms from dogs in Myanmar, and a possible role of dogs as reservoirs for *Theileria* parasites infecting cattle, water buffaloes, and goats. *Ticks and Tick-borne Diseases*, *12*(4), 101729.

Belkahia, H., Said, M. B., El Mabrouk, N., Saidani, M., Cherni, C., Hassen, M. B., ... & Messadi, L. (2017). Seasonal dynamics, spatial distribution and genetic analysis of *Anaplasma* species infecting small ruminants from Northern Tunisia. *Infection, Genetics and Evolution*, *54*, 66-73.

Bishop, R., Musoke, A., Morzaria, S., Gardner, M., & Nene, V. (2004). *Theileria*: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. *Parasitology*, *129*(S1), S271-S283.

Bock, R. E., & De Vos, A. J. (2001). Immunity following use of Australian tick fever vaccine: a review of the evidence. *Australian veterinary journal*, *79*(12), 832-839.

Bock, R., Jackson, L., De Vos, A., & Jorgensen, W. (2004). Babesiosis of cattle. *Parasitology*, *129*(S1), S247-S269.

Brown, C. Torres, A. (2008) USAHA Foreign Animal Diseases, Seventh Edition. Committee of Foreign and Emerging Diseases of the US Animal Health Association. Boca Publications Group, Inc.

Cabezas-Cruz, A., Hodžić, A., Román-Carrasco, P., Mateos-Hernández, L., Duscher, G. G., Sinha, D. K., ... & De La Fuente, J. (2019). Environmental and molecular drivers of the α-Gal syndrome. *Frontiers in Immunology*, 1210.

Cao, S., Aboge, G. O., Terkawi, M. A., Yu, L., Kamyingkird, K., Luo, Y., ... & Xuan, X. (2012). Molecular detection and identification of *Babesia bovis* and *Babesia bigemina* in cattle in northern Thailand. *Parasitology research*, *111*, 1259-1266.

Chethanond, U., Worasingh, R., & Srinuntapunt, S. (1995). Studies on parasitic infection in dairy cattle in the south. In *Proceedings of the 33rd Kasetsart University Annual Conference* (pp. 398-407).

Criado-Fornelio, A., Buling, A., Pingret, J. L., Etievant, M., Boucraut-Baralon, C., Alongi, A., ... & Torina, A. (2009). Hemoprotozoa of domestic animals in France: prevalence and molecular characterization. *Veterinary Parasitology*, *159*(1), 73-76.

Dahmani, M., Davoust, B., Benterki, M. S., Fenollar, F., Raoult, D., & Mediannikov, O. (2015). Development of a new PCR-based assay to detect Anaplasmataceae and the first report of *Anaplasma phagocytophilum* and Anaplasma platys in

cattle from Algeria. *Comparative immunology, microbiology and infectious diseases*, *39*, 39-45.

Das, D., Sarma, K., Eregowda, C.G., Roychoudhury, P., Rajesh, J.B., Behera, P., Prasad, H., Lalrinkima, H., Aktar, F., Bora, N., Thakur. N., Deka, C. & Tolenkhomba, T.C. (2022) Naturally occurring *Anaplasma marginale* infection in cattle: Molecular prevalence and associated risk factors, haemato-biochemical alterations, oxidant/antioxidant status and serum trace mineral levels. *Microb. Pathog.*, *167*: 105575.

Daszek, L., & Winiarczyk, S. (2008). Molecular characterization of Babesia canis canis isolates from naturally infected dogs in Poland. *Veterinary parasitology*, *152*(3-4), 235-241.

De la Fuente, J., & Kocan, K. (2006). Strategies for development of vaccines for control of ixodid tick species. *Parasite immunology*, *28*(7), 275-283.

De la Fuente, J., Ruybal, P., Mtshali, M. S., Naranjo, V., Shuqing, L., Mangold, A. J., ... & Kocan, K. M. (2007). Analysis of world strains of Anaplasma marginale using major surface protein 1a repeat sequences. *Veterinary Microbiology*, *119*(2-4), 382-390.Enkhtaivan, B., Narantsatsral, S., Davaasuren, B., Otgonsuren, D., Amgalanbaatar, T., Uuganbayar, E., ... & Yokoyama, N. (2019). Molecular detection of *Anaplasma ovis* in small ruminants and ixodid ticks from Mongolia. *Parasitology international*, *69*, 47-53.

De Vos, A. J. (1991). Distribution, economic importance and control measures for Babesia and Anaplasma. *Recent developments in the control of anaplasmosis, babesiosis and cowdriosis. Proceedings of*, 3-12.

Dhakal, M. (2023). Prevalence of *Babesia* spp in suspected cattle of Kathmandu Valley, Nepal (Doctoral dissertation, Department of Zoology).

Dumanli, N., Aktas, M., Cetinkaya, B., Cakmak, A., Koroglu, E., Saki, C. E., ... & Altay, K. Ü. R. Ş. A. T. (2005). Prevalence and distribution of tropical theileriosis in eastern Turkey. *Veterinary parasitology*, *127*(1), 9-15.

Eleftheriou, A., Cole, D., Kieffer, J. & Pesapane, R. (2022) Molecular prevalence of *Anaplasma marginale* and associated risk factors in beef cattle herds from Ohio: a cross-sectional study. *J. Am. Vet. Med. Assoc*, *260*(14): 1-5.

Famuyide, I. M., Takeet, M. I., Talabi, A. O., & Otesile, E. B. (2020). Molecular Detection and Identification of Piroplasms in Semi-Intensively Managed Cattle from Abeokuta, Nigeria. *Folia veterinaria*, *64*(4), 1-8.

Farooqi, S.H., Ijaz, M., Rashid, M.I., Nabi, H., Islam, S., Aqib, A.I., Hussian, K., Khan, A., Rizvi, S. N.B., Mahood, S., Mahood, K. & Zhang, H. (2018) Molecular epidemiology of bovine anaplasmosis in Khyber Pakhtunkhwa, Pakistan. *Trop. Anim. Health Prod*., *50*: 1591-1598.

Ferreri, L. M., Brayton, K. A., Sondgeroth, K. S., Lau, A. O., Suarez, C. E., & McElwain, T. F. (2012). Expression and strain variation of the novel "small open reading frame"(smorf) multigene family in *Babesia bovis*. *International journal for parasitology*, *42*(2), 131-138.

Fungfuang, W., Ruamthum, W., Lertchunhakiat, K., Khoomsab, K., & Kanjanarajit, S. (2006). *Anaplasma* infection in hog deer (Cervus porcinus) from Huay Sai wildlife captive breeding research centre in Phetchaburi province. In *Proceedings of the 44th Kasetsart University Annual Conference, Kasetsart, 30-January-2 February, 2006. Subject: Animals, Veterinary Medicine* (pp. 557-560). Kasetsart University.

Gachohi, J., Skilton, R., Hansen, F., Ngumi, P., & Kitala, P. (2012). Epidemiology of East Coast fever (Theileria parva infection) in Kenya: past, present and the future. *Parasites & Vectors*, *5*, 1-13.

Ghosh, S., & Nagar, G. (2014). Problem of ticks and tick-borne diseases in India with special emphasis on progress in tick control research: a review. *Journal of Vector Borne Diseases*, *51*(4), 259-270.

Gray, J. S., & De Vos, A. J. (1981). Studies on a bovine *Babesia* transmitted by Hyalomma marginatum rufipes Koch, 1844.

Hall, T. A. (1999, January). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic acids symposium series* (Vol. 41, No. 41, pp. 95-98).

Hassan, Z. I. (2020). Prevalence and Phylogenetic Analysis of *Babesia ovis* Isolated from Sheep and Goats in Erbil Province, Kurdistan Region-Iraq. *Polytechnic Journal*, *10*(2), 98-104.

Holman, P. J., Carroll, J. E., Pugh, R., & Davis, D. S. (2011). Molecular detection of *Babesia bovis* and *Babesia bigemina* in white-tailed deer (Odocoileus virginianus) from Tom Green County in central Texas. *Veterinary parasitology*, *177*(3-4), 298-304

Iseki, H., Zhou, L., Kim, C., Inpankaew, T., Sununta, C., Yokoyama, N., ... & Igarashi, I. (2010). Seroprevalence of Babesia infections of dairy cows in northern Thailand. *Veterinary parasitology*, *170*(3-4), 193-196.

Jacob, S. S., Sengupta, P. P., Paramanandham, K., Suresh, K. P., Chamuah, J. K., Rudramurthy, G. R., & Roy, P. (2020). Bovine babesiosis: An insight into the global perspective on the disease distribution by systematic review and meta-analysis. *Veterinary parasitology*, *283*, 109136.

Jaswal, H., Bal, M. S., Singla, L. D., Sharma, A., Kaur, P., Mukhopadhyay, C. S., & Juyal, P. D. (2014). Application of msp1β PCR and 16S rRNA semi nested PCR-RFLP for detection of persistent anaplasmosis in tick infested cattle. *Int. J. Adv. Res*, *2*(8), 188-196.

Jirapattharasate, C., Moumouni, P. F. A., Cao, S., Iguchi, A., Liu, M., Wang, G., ... & Xuan, X. (2016). Molecular epidemiology of bovine *Babesia* spp. and Theileria orientalis parasites in beef cattle from northern and northeastern Thailand. *Parasitology international*, *65*(1), 62-69.

Jittapalapong, S., & Lieowijak, C. (1988). Epidemiological survey of blood protozoa and rickettsia in dairy cow in Nongpho. In *Proceedings of 26th Kasetsart University Annual Conference* (pp. 3-5).

Junsiri, W., Watthanadirek, A., Poolsawat, N., Kaewmongkol, S., Jittapalapong, S., Chawengkirttikul, R., & Anuracpreeda, P. (2020). Molecular detection and genetic diversity of *Anaplasma marginale* based on the major surface protein genes in Thailand. *Acta tropica*, *205*, 105338.

Kamani, J., Schaer, J., Umar, A. G., Pilarshimwi, J.Y., Bukar, L., González-Miguel, J. & Harrus, S. (2022) Molecular detection and genetic characterization of *Anaplasma marginale* and *Anaplasma platys* in cattle in Nigeria. *Ticks Tick Borne Dis*., *13*(4): 101955.

Kamau, J., de Vos, A. J., Playford, M., Salim, B., Kinyanjui, P., & Sugimoto, C. (2011). Emergence of new types of *Theileria orientalis* in Australian cattle and possible cause of theileriosis outbreaks. *Parasites & Vectors*, *4*, 1-10.

Kawahara, M., Rikihisa, Y., Lin, Q., Isogai, E., Tahara, K., Itagaki, A., ... & Tajima, T. (2006). Novel genetic variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale*, and a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. *Applied and environmental microbiology*, *72*(2), 1102-1109.

Khumalo, Z. T., Brayton, K. A., Collins, N. E., Chaisi, M. E., Quan, M., & Oosthuizen, M. C. (2018). Evidence confirming the phylogenetic position of *Anaplasma centrale* (ex Theiler 1911) Ristic and Kreier 1984. *International journal of systematic and evolutionary microbiology*, *68*(8), 2682-2691.

Khumalo, Z. T., Catanese, H. N., Liesching, N., Hove, P., Collins, N. E., Chaisi, M. E., ... & Brayton, K. A. (2016). Characterization of *Anaplasma marginale* subsp. centrale strains by use of msp1aS genotyping reveals a wildlife reservoir. *Journal of Clinical Microbiology*, *54*(10), 2503-2512.

Kocan, K. M., De La Fuente, J., Blouin, E. F., & Garcia-Garcia, J. C. (2004). Anaplasma marginale (Rickettsiales: Anaplasmataceae): recent advances in defining host–pathogen adaptations of a tick-borne rickettsia. *Parasitology*, *129*(S1), S285-S300.

Kocan, K. M., de la Fuente, J., Blouin, E. F., Coetzee, J. F., & Ewing, S. A. (2010). The natural history of Anaplasma marginale. *Veterinary parasitology*, *167*(2-4), 95-107.

Kocan, K. M., De la Fuente, J., Guglielmone, A. A., & Meléndez, R. D. (2003). Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clinical microbiology reviews*, *16*(4), 698-712.

Koonyosying, P., Rittipornlertrak, A., Chomjit, P., Sangkakam, K., Muenthaisong, A., Nambooppha, B., ... & Sthitmatee, N. (2022). Incidence of hemoparasitic infections in cattle from central and northern Thailand. *PeerJ*, *10*, e13835

Lee, M., Yu, D., Yoon, J., Li, Y., Lee, J., & Park, J. (2009). Natural co-infection of Ehrlichia chaffeensis and *Anaplasma bovis* in a deer in South Korea. *Journal of Veterinary Medical Science*, *71*(1), 101-103.

Lin, M., Bachman, K., Cheng, Z., Daugherty, S. C., Nagaraj, S., Sengamalay, N., ... & Rikihisa, Y. (2017). Analysis of complete genome sequence and major surface antigens of Neorickettsia helminthoeca, causative agent of salmon poisoning disease. *Microbial Biotechnology*, *10*(4), 933-957.

Liu, E., Yan, C., Mei, X., He, W., Bing, S. H., Ding, L., ... & Fan, T. (2010). Long-term effect of chemical fertilizer, straw, and manure on soil chemical and biological properties in northwest China. *Geoderma*, *158*(3-4), 173-180

Liu, J., Yin, H., Liu, G., Guan, G., Ma, M., Liu, A., ... & Luo, J. (2008). Discrimination of *Babesia major* and *Babesia ovata* based on ITS1–5.8 S–ITS2 region sequences of rRNA gene. *Parasitology research*, *102*, 709-713.

Liu, Z., Ma, M., Wang, Z., Wang, J., Peng, Y., Li, Y., ... & Yin, H. (2012). Molecular survey and genetic identification of Anaplasma species in goats from central and southern China. *Applied and environmental microbiology*, *78*(2), 464-470.

Luo, J., & Yin, H. (1997). Theileriosis of sheep and goats in China. *Tropical animal health and production*, *29*, 8S-10S.

Luo, J., Yin, H., Liu, Z., Yang, D., Guan, G., Liu, A., ... & Chen, P. (2005). Molecular phylogenetic studies on an unnamed bovine *Babesia* sp. based on small subunit ribosomal RNA gene sequences. *Veterinary parasitology*, *133*(1), 1-6.

M'ghirbi, Y., Hurtado, A., Brandika, J., Khlif, K., Ketata, Z., & Bouattour, A. (2008). A molecular survey of *Theileria* and *Babesia* parasites in cattle, with a note on the distribution of ticks in Tunisia. *Parasitology research*, *103*, 435-442.

Mans, B. J., Pienaar, R., & Latif, A. A. (2015). A review of Theileria diagnostics and epidemiology. *International Journal for Parasitology: Parasites and Wildlife*, *4*(1), 104-118.

Martin, A. R., Brown, G. K., Dunstan, R. H., & Roberts, T. K. (2005). Anaplasma platys: an improved PCR for its detection in dogs. *Experimental parasitology*, *109*(3), 176-180.

Masatani, T., Hayashi, K., Andoh, M., Tateno, M., Endo, Y., Asada, M., ... & Matsuo, T. (2017). Detection and molecular characterization of *Babesia*, *Theileria*, and *Hepatozoon* species in hard ticks collected from Kagoshima, the southern region in Japan. *Ticks and tick-borne diseases*, *8*(4), 581-587.

McFadden, A. M. J., Rawdon, T. G., Meyer, J., Makin, J., Morley, C. M., Clough, R. R., ... & Geysen, D. (2011). An outbreak of haemolytic anaemia associated with infection of *Theileria orientalis* in naive cattle. *New Zealand Veterinary Journal*, *59*(2), 79-85.

McKeever, D. J. (2009). Bovine immunity–a driver for diversity in *Theileria* parasites?. *Trends in Parasitology*, *25*(6), 269-276.

Mehlhorn, H., & Schein, E. (1998). Redescription of *Babesia equi* Laveran, 1901 as Theileria equi Mehlhorn, Schein 1998. *Parasitology research*, *84*, 467-475.

Mohamad, A. TerkawiNguyen, X. HuyenCao, S. (2011) Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the northeast region of Thailand: Veterinary Parasitology.,178:201–207.

Molad, T., Mazuz, M. L., Fleiderovitz, L., Fish, L., Savitsky, I., Krigel, Y., ... & Shkap, V. (2006). Molecular and serological detection of A. centrale-and A. marginale-infected cattle grazing within an endemic area. *Veterinary microbiology*, *113*(1-2), 55-62..

Mosqueda, J., Olvera-Ramírez, A., Aguilar-Tipacamu, G., & J Canto, G. (2012). Current advances in detection and treatment of babesiosis. *Current medicinal chemistry*, *19*(10), 1504-1518.

Muhanguzi, D., Matovu, E., & Waiswa, C. (2010). Prevalence and characterization of *Theileria* and *Babesia* species in cattle under different husbandry systems in western Uganda. *Int. J. Anim. Vet. Adv*, *2*(2), 51-58.

Nehra, A. K., Kumari, A., Moudgil, A. D., & Vohra, S. (2022). An insight into misidentification of the small-subunit ribosomal RNA (18S rRNA) gene sequences of *Theileria* spp. as *Theileria annulata*. *BMC Veterinary Research*, *18*(1), 454.

Nguyen, A. H., Tiawsirisup, S., & Kaewthamasorn, M. (2020). Molecular detection and genetic characterization of *Anaplasma marginale* and *Anaplasma platys*-like (Rickettsiales: Anaplasmataceae) in water buffalo from eight provinces of Thailand. *BMC veterinary research*, *16*, 1-12.

Noaman, V., & Bastani, D. (2016). Molecular study on infection rates of *Anaplasma ovis* and *Anaplasma marginale* in sheep and cattle in West-Azerbaijan province,

Iran. In *Veterinary research forum* (Vol. 7, No. 2, p. 163). Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

Noaman, V., Shayan, P., & Amininia, N. (2009). Molecular diagnostic of *Anaplasma marginale* in carrier cattle.

Ogata, S., Pereira, J. A. C., Jhonny, L. V. A., Carolina, H. P. G., Matsuno, K., Orba, Y., ... & Nakao, R. (2021). Molecular Survey of *Babesia* and *Anaplasma* Infection in Cattle in Bolivia. *Veterinary sciences*, *8*(9), 188.

Ola-Fadunsin, S. D., Gimba, F. I., Abdullah, D. A., Sharma, R. S. K., Abdullah, F. J. F., & Sani, R. A. (2018). Epidemiology and risk factors associated with *Anaplasma marginale* infection of cattle in Peninsular Malaysia. *Parasitology international*, *67*(6), 659-665.

Qin, G., Li, Y., Liu, J., Liu, Z., Yang, J., Zhang, L., ... & Yin, H. (2016). Molecular detection and characterization of *Theileria* infection in cattle and yaks from Tibet Plateau Region, China. *Parasitology research*, *115*, 2647-2652.

Rajput, Z. I., Hu, S. H., Arijo, A. G., Habib, M., & Khalid, M. (2005). Comparative study of *Anaplasma* parasites in tick carrying buffaloes and cattle. *Journal of Zhejiang University. Science. B*, *6*(11), 1057.

Rar, V., & Golovljova, I. (2011). *Anaplasma*, *Ehrlichia*, and "Candidatus Neoehrlichia" bacteria: pathogenicity, biodiversity, and molecular genetic characteristics, a review. *Infection, Genetics and Evolution*, *11*(8), 1842-1861.

Rjeibi, M. R., Ayadi, O., Rekik, M., & Gharbi, M. (2018). Molecular survey and genetic characterization of *Anaplasma centrale*, *A. marginale* and *A. bovis* in cattle from Algeria. *Transboundary and emerging diseases*, *65*(2), 456-464.

Rodríguez, S. D., Ortiz, M. A. G., Ocampo, R. J., & y Murguía, C. A. V. (2009). Molecular epidemiology of bovine anaplasmosis with a particular focus in Mexico. *Infection, Genetics and Evolution*, *9*(6), 1092-1101.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular biology and evolution*, *34*(12), 3299-3302.

Rymaszewska, A., & Grenda, S. (2008). Bacteria of the genus *Anaplasma*–characteristics of Anaplasma and their vectors: a review. *Vet Med*, *53*(11), 573-584.

Saetiew, N., Simking, P., Inpankaew, T., Wongpanit, K., Kamyingkird, K., Wongnakphet, S., ... & Jittapalapong, S. (2015). Prevalence and genetic diversity of *Anaplasma marginale* infections in water buffaloes in Northeast Thailand. *J Trop Med Parasitol*, *38*, 9-16.

Saetiewa, N., Simking, P., Saengow, S., Morand, S., Desquesnes, M., Stich, R. W., & Jittapalapong, S. (2020). Spatial and seasonal variation in the prevalence of *Anaplasma marginale* among beef cattle in previously flooded regions of Thailand. *Journal of Science and Technology of Agriculture and Natural Resources*.

Selmi, R., Said, M. B., Dhibi, M., Yahia, H. B., Abdelaali, H., & Messadi, L. (2020). Genetic diversity of groEL and msp4 sequences of *Anaplasma ovis* infecting camels from Tunisia. *Parasitology international*, *74*, 101980.

Sharma, A., Singla, L. D., Tuli, A., Kaur, P., Batth, B. K., Javed, M., & Juyal, P. D. (2013). Molecular prevalence of *Babesia bigemina* and *Trypanosoma evansi* in dairy animals from Punjab, India, by duplex PCR: a step forward to the detection and management of concurrent latent infections. *BioMed research international*, *2013*.

Shkap, V., Molad, T., Fish, L., & Palmer, G. (2002). Detection of the Anaplasma centrale vaccine strain and specific differentiation from *Anaplasma marginale* in vaccinated and infected cattle. *Parasitology research*, *88*(6), 546-552.

Sivakumar, T. Tuvshintulga, B. Zhyldyz, A. Kothalawala, H. Yapa, P.R. Kanagaratnam, R. Vimalakumar, S.C. Abeysekera, T.S. Weerasingha, A.S. Yamagishi, J. Igarashi, I. Silva, S.S.P. Yokoyama, N. (2018) Genetic analysis of *Babesia* isolates from cattle with clinical babesiosis in Sri Lanka. J. Clin. Microbiol., 56, e00895–18.

Skotarczak, B., Adamska, M., Sawczuk, M., Maciejewska, A., Wodecka, B., & Rymaszewska, A. (2008). Coexistence of tick-borne pathogens in game animals and ticks in western Poland. *Veterinarni Medicina*, *53*(12), 668-675.

Somparn, P., Gibb, M. J., Markvichitr, K., Chaiyabutr, N., Thummabood, S., & Vajrabukka, C. (2004). Analysis of climatic risk for cattle and buffalo production in northeast Thailand. *International journal of biometeorology*, *49*, 59-64.

Srionrod, N., Nooroong, P., Poolsawat, N., Minsakorn, S., Watthanadirek, A., Junsiri, W., ... & Anuracpreeda, P. (2022). Molecular characterization and genetic diversity of *Babesia bovis* and *Babesia bigemina* of cattle in Thailand. *Frontiers in Cellular and Infection Microbiology*, *12*, 1065963.

Stuen, S., Granquist, E. G., & Silaghi, C. (2013). *Anaplasma phagocytophilum*—a widespread multi-host pathogen with highly adaptive strategies. *Frontiers in cellular and infection microbiology*, *3*, 31.

Swai, E. S., Karimuribo, E. D., Kambarage, D. M., Moshy, W. E., & Mbise, A. N. (2007). A comparison of seroprevalence and risk factors for *Theileria parva* and *T. mutans* in smallholder dairy cattle in the Tanga and Iringa regions of Tanzania. *The Veterinary Journal*, *174*(2), 390-396.

Swift, B. L., & Paumer, R. J. (1976). Vertical transmission of *Anaplasma marginale* in cattle. *Theriogenology*, *6*(5), 515-521.

Terkawi, M. A., Huyen, N. X., Shinuo, C., Inpankaew, T., Maklon, K., Aboulaila, M., ... & Igarashi, I. (2011). Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the northeast region of Thailand. *Veterinary Parasitology*, *178*(3-4), 201-207.

Torina, A., Galindo, R. C., Vicente, J., Di Marco, V., Russo, M., Aronica, V., ... & de la Fuente, J. (2010). Characterization of *Anaplasma phagocytophilum* and *A. ovis* infection in a naturally infected sheep flock with poor health condition. *Tropical animal health and production*, *42*(7), 1327-1331.

Tufani, N. A., Hafiz, A., Malik, H. U., Peer, F. U., & Makhdoomi, D. M. (2009). Clinico-therapeutic management of acute babesiosis in bovine. *Intas polivet*, *10*(1), 49-50.

Uilenberg, G. (1981, September). *Theilerial* species of domestic livestock. In *Advances in the Control of Theileriosis: Proceedings of an International Conference held at the International Laboratory for Research on Animal Diseases in Nairobi, 9–13th February, 1981* (pp. 4-37). Dordrecht: Springer Netherlands.

Uilenberg, G. (1995). International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Veterinary parasitology*, *57*(1-3), 19-41.0.

Uilenberg, G. (2006). Babesia—a historical overview. *Veterinary parasitology*, *138*(1-2), 3-1.

Vial, H. J., & Gorenflot, A. (2006). Chemotherapy against babesiosis. *Veterinary parasitology*, *138*(1-2), 147-160.

Watanasin, Y. (1965). Study on immunology for anaplasmosis disease. In *Proceedings of 4th national conference on Agriculture and Biology Plant and Biological Science, and Animal Science section, Kasetsart University, Bangkok, Thailand* (pp. 477-479).

Weir, W., Karagenç, T., Baird, M., Tait, A., & Shiels, B. R. (2010). Evolution and diversity of secretome genes in the apicomplexan parasite *Theileria annulata*. *BMC genomics*, *11*, 1-17.

Worasing, R., & Rattana, S. (2007). Bovine gastrointestinal and blood parasites examination in Pakpanang river basin in Nakhon Si Thammarat province. In *45. Kasetsart University Annual Conference, Bangkok (Thailand), 30 Jan-2 Feb 2007*.

Yawongsa, A., & Te-Chaniyom, T. (2013). Meta analysis: reproductive indices of Holstein Friesian cows in Thailand during 2000-2010. *Kasetsart Veterinarians*, *23*(2), 93-110.

Ybanez, A. P., Sivakumar, T., Battsetseg, B., Battur, B., Altangerel, K., Matsumoto, K., Yokayama, N. and Inokuma, H. (2013) Specific molecular detection and characterization of *Anaplasma marginale* in Mongolian cattle. *J. Vet. Med. Sci*., *75*(4): 399-406.

Zafar, S.N.U.A., Khan, A., Niaz, S., Aktas, M., Ozubek, S., Farooq, M., Adli, M.M., Zajac, Z., Iqbal, F., Alhimiadi, A.R. & Swelum, A.A. (2022) Prevalence of *Anaplasma marginale* in cattle blood samples collected from two important livestock regions in Punjab (Pakistan) with a note on epidemiology and phylogeny of parasite. *Saudi J. Biol. Sci*., *29*(3): 1515-1520.

Zhang, L., Liu, Y., Ni, D., Li, Q., Yu, Y., Yu, X. J., ... & Xu, J. (2008). Nosocomial transmission of human granulocytic anaplasmosis in China. *Jama*, *300*(19), 2263-2270.

Zhou, Z., Li, K., Sun, Y., Shi, J., Li, H., Chen, Y., Yang, H., Li, X., Wu, B., Li, X., Wang, Z., Cheng, F. & Hu, S. (2019) Molecular epidemiology and risk factors of *Anaplasma* spp., *Babesia* spp. and *Theileria* spp. infection in cattle in Chongqing, China. *PloS One*., *14*(7): e0215585.

Zintl, A., Mulcahy, G., Skerrett, H. E., Taylor, S. M., & Gray, J. S. (2003). *Babesia divergens*, a bovine blood parasite of veterinary and zoonotic importance. *Clinical microbiology reviews*, *16*(4), 622-636.

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