PCR-BASED DETECTION AND DIFFERENTIATION OF PIROPLASMS AND MALARIA PARASITES FROM GOATS IN THAILAND

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Science and technology
Common Course
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2019

การตรวจและแยกแยะเชื้อไพโรพลาซึมและเชื้อมาลาเรียจากแพะในประเทศไทยด้วยวิธีพีซีอาร์

น.ส.ตู กั้ม ลู เฮิน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

Thesis Title	PCR-BASED DETECTION AND DIFFERENTIATION OF PIROPLASMS AND MALARIA PARASITES FROM GOATS IN THAILAND
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การตรวจและแยกแยะเชื้อไพโรพลาซึมและเชื้อมาลาเรียจากแพะในประเทศไทยด้วยวิธีพีซีอาร์. (PCR-BASED DETECTION AND DIFFERENTIATION OF PIROPLASMS AND MALARIA PARASITES FROM GOATS IN THAILAND) อ.ที่ปรึกษาหลัก : มรกต แก้วธรรมสอน, อ.ที่ปรึกษาร่วม : สนธยา เตียวศิริทรัพย์

การศึกษาเรื่องการพัฒนาวิธีการตรวจด้วยวิธี มัลติเพล็กซ์ พีซีอาร์ (mPCR) เพื่อใช้ในการระบุและจำแนกเชื้อมาลาเรีย (*Plasmodium caprae*) และเชื้อจากกลุ่มไพโรพลาส 2 ชนิด ได้แก่ *Babesia bigemina* และ *Theileria luwenshuni* ในแพะในประเทศไทย ไพร์เมอร์ที่จำเพาะต่อเชื้อปรสิตทั้ง 3 ชนิดถูกออกแบบให้มีความจำเพาะต่อยีนในไมโตรคอนเดรียที่เรียกว่า ไซโตโครม ซี ออกซิเดส ซับยูนิต ที่หนึ่ง (COI) พลาสมิดที่มีดีเอ็นเอ ของปรสิตแต่ละชนิดถูกทำการเจือจางลงครั้งละ 10 เท่า เพื่อประเมินความไวของวิธีการปฏิกิริยาลูกโซ่แบบธรรมดา (single PCR) และ mPCR ความจำเพาะของชุดไพรเมอร์ได้ถูกทดสอบ โดยการใช้ตัวอย่างจากดีเอ็นเอจากเชื้ออื่นๆ ที่เป็นปรสิตในเลือด ตัวอย่างเลือดแพะ 100 ตัวอย่าง จาก 5 จังหวัดในประเทศไทย ถูกนำมาใช้เพื่อทดสอบความน่าเชื่อถือของการพัฒนาเทคนิคทางอณูชีวโมเลกุลนี้ ผลการศึกษาพบว่าขีดจำกัดการตรวจหาเชื้อโดยใช้วิธี mPCR ในการศึกษานี้คือ 10^8 สำเนาของพลาสมิด และ ความจำเพาะของการตรวจหา *P. caprae* และ *T. luwenshuni* พบว่ามีความจำเพาะถึง 94.9%-100% พบระดับความไว 100% ในการการตรวจของเชื้อ *T. luwenshuni* อย่างไรก็ตาม การทดสอบความไวของ mPCR ถูกพบว่าต่ำ (50%) ในการตรวจวินิจฉัยหาเชื้อ *P. caprae* จากตัวอย่างเลือดแพะ ผลการศึกษาแสดงให้เห็นถึงความสำเร็จในการออกแบบเครื่องมือการตรวจโดยใช้วิธี mPCR ที่สามารถแสดงความไว เท่ากับ การใช้การตรวจด้วยวิธี single PCR อีกทั้งยังสามารถตรวจหาเชื้อทั้งสามชนิดได้ในปฏิกิริยาเดียวกัน นอกจากนี้ในการศึกษาครั้งนี้เป็นครั้งแรกที่ตรวจพบและ จำแนกเชื้อ *T. luwenshuni* และ *B. bigemina* ได้จากตัวอย่างแพะของประเทศไทย

สาขาวิชา วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6175402831 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY
KEYWORD: detection vector goat malaria piroplasm

Tu Cam Luu Huynh : PCR-BASED DETECTION AND DIFFERENTIATION OF PIROPLASMS AND MALARIA PARASITES FROM GOATS IN THAILAND. Advisor: Assoc. Prof. MORAKOT KAEWTHAMASORN, Ph.D. Co-advisor: Assoc. Prof. SONTHAYA TIAWSIRISUP, Ph.D.

A novel multiplex PCR (mPCR) was developed to identify and differentiate malaria parasite (*Plasmodium caprae),* and two piroplasms (*Babesia bigemina* and *Theileria luwenshuni)* in Thai goats. Three pairs of primer were designed to be species-specific, targeting *cytochrome c oxidase subunit I (COI)* gene in mitochondria. Plasmids containing each parasite's DNA was serially diluted to evaluate the sensitivity of singleplex and mPCR assays. The specificity of the primer sets was confirmed by testing for amplification from DNA of each pathogen and other related hemoparasites. A total of 100 goat blood samples from five provinces of Thailand was used to validate the reliability and application of the assay. The detection limit of the mPCR in this study was 10^8 parasite copies and the diagnostic specificity in detection of *P. caprae* and *T. luwenshuni* was found to be high (94.9%-100%). The high level of diagnostic sensitivity (100%) in the detection of *T. luwenshuni* was also recorded. However, the diagnostic sensitivity of mPCR was found to be low (50%) in the detection of *P. caprae* from field-collected blood samples. These results demonstrated the successful establishment of the mPCR presented by its same level of sensitivity to each single PCR counterparts and its ability to simultaneous detection all three parasites in one reaction. This study also describes the first report of *Theileria luwenshuni* and *Babesia bigemina* in goats of Thailand.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my research supervisor Assoc. Prof. Dr. Morakot Kaethamosorn for his continuous support of my study, his motivation, as well as patient and dedicated involvement in every step throughout the process. I am greatly thankful to you for your understanding and enthusiasm over the past two years. I also wish to express my deep gratitude to my co-advisor Assoc. Prof. Dr. Sonthaya Tiawsirisup for all of his kind support and valuable comments.

Additionally, I would like to thank undergraduate and graduate veterinary students and staffs in Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University Bangkok, Thailand for their kindly supporting in sample collection. My heartfelt thanks also go to the International Graduate Course of Veterinary Science and Technology (VST), the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University for providing me with an opportunity to access the research fund as well as laboratory and research facilities.

Finally, I also express my profound gratitude to my parents and my friends for offering me unfailing support and continuous encouragement through the process of researching and writing this thesis. This accomplishment would not have been possible without their incentive.

Tu Cam Luu Huynh

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CHAPTER 1 INTRODUCTON

As goats are the primary meat production source in South-East Asia and Africa, most of the world's goat population is raised in these areas. Goat meat is leaner compare with meat from other domestic animals as well as relatively equivalent in terms of its nutritional constituents. In addition, goat meat is one of the meats that acceptable by many cultures and religions. (Dhanda et al., 2003). The world goat population in 2013 is accounted for 1 billion, with Asia continually getting the highest contribution about 59.4%. China has the highest goat meat production that makes up 35.89% of the total world goat meat production while India, Bangladesh, Pakistan and Sudan are the world's largest producers of goat milk (Skapetas and Bampidis, 2016). In Thailand, goats play significant roles in the source of food and income for several decades (Khamseekhiew and Pompi, 2016). Goat raising in Thailand is generally carried out by smallholders; however, commercial production gradually becomes an attractive trend for agriculture enterprises (Wattanachant, 2008). In recent decades, it has seen significant steady growth in goat production in Thailand due to a rise in demand for goat meat and milk (Pralomkarn et al., 2011). The distribution of the goat population in Thailand is associated with the society of Thai Muslims: the southernmost region and outskirts of Bangkok.

Vector-borne pathogens, present in tropical and sub-tropical countries, infect a wide range of ruminants. *Theileria* and *Babesia* parasite are tick-borne pathogens, responsible for a common complex disease, known as piroplasmosis (Naqid, 2011). This disease is recognized as one of the most detrimental economic impacts, threatening the livestock industry throughout the world (Gou et al., 2012). *Theileria luwenshuni* has been reported as highly pathogenic species in goat, which has been described in parts of Asia, Europe and the Middle East while *Babesia ovis* and *Babesia motasi* are the two most important species in small ruminants in the Middle East, Southern Europe and some Africa and Asian countries (Schnittger et al., 2003; Ranjbar-Bahadori et al., 2012; Haghi et al., 2017; Zhou et al., 2017). *Plasmodium* was

first identified in a goat in 1923 (de Mello F, 1923) and later reported in Myanmar, Thailand, Iran, Sudan and Kenya (Kaewthamasorn et al., 2018). This parasite is probably transmitted by mosquito, but its natural vector remains unknown.

The accurate identification of vector-borne pathogens provides a significant benefit for an effective counter control measure. Conventional diagnosis based on microscopic examination is known for its low sensitivity, requiring well-trained technicians and unable to differentiate physically identical parasites. Compared to microscopic examination, molecular techniques give more reliable and specific results, improving the sensitivity for the diagnosis. Several PCR assays (conventional, nested, real-time PCR) are enabled to distinguish vector-borne pathogens. However, these PCR methods are specifically designed to detect parasites which are endemic in each studied site or region. Goat infections with the vector-borne pathogens in many regions in Thailand remain mostly uninvestigated. Vector-borne pathogens and the co-infection among them in each geographic location might be varied according to the vector abundance and environmental factors. In order to address the current infection and co-infection status among vector-borne pathogens in the Thai goats, the present study is conducted to investigate the tick-borne piroplasms (*Theileria* spp. and, *Babesia* spp.) and malaria parasite (*Plasmodium caprae*) in goat.

Objective of the study:

This study aims to develop and validate the technique for simultaneous detection of the vector-borne parasites as well as investigate their infection status in Thai goats.

Keywords (Thai): การตรวจ แพะ มาลาเรีย ไพโรพลาสซึม พาหะ
Keywords (English): Detection, goat, malaria, piroplasm, vector

Hypothesis:

Co-infection of tick and mosquito-borne parasites in goats could be separately and/or simultaneously detected and distinguished by PCR using species-specific primers.

CHAPTER 2 LITERATURE REVIEW

2.1. Goat

Goat is the most prolific ruminant of all domesticated ruminants in tropical and sub-tropical regions (Dhanda et al., 2003). Goats are pliable animals for livestock producers due to their being capable of maintaining an equilibrium between metabolism heat and environment heat to avoid hyper- and hypothermia. They are unique in terms of their characteristics such as water conservation capability, higher sweating rate, lower basal heat metabolism, higher respiration rate, higher skin temperature, and constant heart rate and constant cardiac output (Patton, 1962; Robertshcaw, 1968; Quartermain and Broadbent, 1974; Shkolnik et al., 1975; Borut et al., 1979; Dmi'el et al., 1979; Feistkorn et al., 1983). Goats thus have successfully adapted to desert, mountainous and tropical regions where other livestock species would not flourish (Amills et al., 2017). Most of the goat production in Thailand is used for meat (more than 90%) and only less than 10% of the goat industry for milk production. Native goats in Thailand are crossed with exotic breeds to produce rangeland goats to get more benefits in production. There are two local and nine exotic goat breeds in Thailand. The Northern Native Thai goats also known as “Bangala” have characteristics of large but thin body, long pendulous ears and a straight face profile while the other in the south of Thailand referred to as “Katjang” which is small with short upright ears. Five dairy goat breeds are Saanen, Alpine, Toggenburg, Shami and Laoshan. Saanen, raised mainly in central and southern regions, is the most favorite breed of dairy goats due to its high milk production (Nakavisut and Anothaisinthawee, 2014). Though local goats are smaller than exotic breeds, they are well-adapted to the harsh environments, making them excellent resources for smallholders. Native goats are also non-seasonal breeding and prolificacy. Litter size of native southern Thai goat is 1.40 ± 0.05 kids per litter (Nakavisut et al., 2007). These characteristics are one of their most critical economic traits. The native goats have a high tolerance with hot and humid climate and excellent resistance to tropical diseases and parasites. However, they have low meat

and milk yield. Exotic breeds can cover those drawbacks traits of local breeds with high meat and milk production, but they are not tolerating the tropical environment, parasites, and diseases. Therefore, crossbreeding of native and exotic goats brings many benefits to production and income (Anothaisinthawee et al., 2010).

2.2. Vector-borne parasites

2.2.1. *Theileria*

2.2.1.1. Background of *Theileria* spp.

Theileria species are important tick-borne parasites, belonging to the Apicomplexa and transmitted by ixodid ticks. *Theileria* was first described in Egypt in 1914 in Sudanese sheep; some species circulated in animals with few or no clinical signs while the others'infection can be very severe with high morbidity and mortality and substantial economic impact on the livestock industry. *T. annulata*, *T. parva*, *T. lestoquardi* and *T. luwenshuni* are considered high pathogenic (Uilenberg, 1981; Schnittger et al., 2000; Bishop et al., 2004; Yin et al., 2007). Nevertheless, the other species such as *T. ovis*, *T. separata* and *T. buffeli/orientalis/sergenti* are considered as benign or non-pathogenic to small ruminants (Uilenberg, 1981; Alani and Herbert, 1988; Sarataphan et al., 1999). One unique feature of several members of genus *Theileria* is their ability to transform the cell they infect reversibly, resulting in the uncontrollable cancer-like proliferation of the parasite and the host cell. The pathway of how parasites mediating the transformation is still unclear, but the understanding of this mechanism may give insights into cancer treatment (Ahmed et al., 2008; Florin-Christensen and Schnittger, 2018). *T. luwenshuni* has been first reported in China as *Theileria* sp. China 1 and considered as one of the most virulent species in goat, transmitted by *Haemaphysalis qinghaiensis* (Yin et al., 2002; Li et al., 2007; Yin et al., 2007). Although *T. luwenshini* has been detected in parts of Asia, Europe and the Middle East, there was no report about this species from goat in Thailand.

2.2.1.2. Morphology and Life cycle

Merozoites of *Theileria* species are pear-shaped while piroplasms are coma-, bacillary-, ring- or oval-shaped. In general, *Theileria* spp have homogenous morphology in most of the mammalian stages. *Theileria* spp have cellular organelles as other eukaryotes including nucleus, mitochondria, ribosomes and vacuoles. A unique structure of either *Theileria* spp or most other apicomplexans is apicoplast, a vestigial plastid-like organelle (Florin-Christensen and Schnittger, 2018). This structure thus provides an excellent target for the development of non-toxic anti-*Theilerial* drugs for the host (Moore et al., 2008; Lizundia et al., 2009).

Theileria spp. have a complex life cycle due to their need to infect two hosts, ticks and ruminants. The life cycle in mammalian host starts when sporozoites are released from the salivary gland of the tick during blood feeding. Sporozoites then enter leukocytes via zippering mechanism (Florin-Christensen and Schnittger, 2018). The host cell parasitophorous vacuolar membrane (PVM) surrounding the invading sporozoite is dissolved at 24-hour post-infection, leaving the parasites residing freely in the cytoplasm where it can interact with the host's spindle apparatus (Tilney and Tilney, 1996). Upon internalization, sporozoites develop into macroschizont, which induces transformation and reversible cancer-like proliferation (Williams and Dobbelaere, 1993). During the merogony, macroschizont continues differentiating into merozoites which are released upon leukocytes rupture and then infect erythrocytes. Inside red blood cells, merozoites develop into piroplasm and continue replicating to invade other erythrocytes (Ahmed et al., 2008). Piroplasm stage infects tick through blood feeding. The life cycle in a tick begins when piroplasms are released from erythrocytes in the tick gut. *Theileria* piroplasms differentiate into ray bodies, some of them develop to form microgametes, and some develop into spherical structures and form macrogametes. Diploid spherical zygotes are formed by the fusion of gametes in syngamy and then mature into motile kinetes. After that, kinetes following hemolymph invade acinar cells in tick salivary gland and subsequently become sporozoites (Florin-Christensen and Schnittger, 2018).

2.2.1.3. Diagnosis

Theileria species are usually first diagnosed by clinical signs such as fever and enlargement of lymph nodes and by the presence of tick infestation. Following tests are indirect fluorescence antibody test (IFAT) and, microscopic examination using Giemsa-stained blood smear. Molecular techniques are needed when the species-level diagnosis is required (Kiara et al., 2018).

2.2.1.3.1. Microscopy

Due to the similarity in the morphology of schizonts and piroplasms, differential diagnosis cannot be made by Giemsa staining in blood smear or lymph node. There are two exceptions for this which are *T. velifera* (rectangular veil piroplasms) and *T. taurotragi* (bar structure in infected erythrocytes). Lymph node biopsy smears showing multinucleate intracytoplasmic and free schizonts in leukocytes indicate acute infection with *T. annualata, T. parva* and *T. lestoquadi*. The present of schizonts in cattle blood smears, lymph node impression smear or histological sections shows diagnostic of East Coast fever (OIE, 2008).

2.2.1.3.2. Molecular technique

There are several molecular tests that can be used for detection of *Theileria* species including PCR-restriction fragment length polymorphism (RFLP), conventional PCR, nested PCR, real-time PCR followed by hybridization with radio - isotope-labelled probes. Nested PCR- RFLP was developed for detection and differentiation of *Theileria lestoquardi*, *T. ovis* and *T. annulata* in goat blood (Rahmani-Varmale et al., 2019). Conventional PCR and nested PCR were also developed for *T. luwenshuni* based on 18S rRNA (Bawm et al., 2018; Begam et al., 2019; Phipps et al., 2016). There are also PCR assays for *T. ovis, T. parva* and *T. annulata* (Altay et al., 2005; Moni et al., 2019; Silatsa et al., 2020). Many multiplex PCRs have also described for simultaneous detection of multiple parasites (Bilgiç et al., 2013). Real-time PCRs are more sensitive than conventional PCR which has been used to detect *T. parva* on the 18S rRNA and the cytochrome coxidase gene (Sibeko et al. 2008; Papli et al. 2011; Chaisi et al. 2013). Another new method for detection

parasites is loop-mediated isothermal amplification (LAMP) in which DNA is amplified under isothermal conditions. A LAMP assay has been developed for *T. parva* targeting PIM and p150, two for *T. annulata* based on GeneDB TA04795, 18S ribosomal RNA gene and internal transcribed spacer (ITS) sequences, and another for *T. lestoquardi* based on the clone 5 sequence (Salih et al., 2008; Thekisoe et al., 2010; Liu et al., 2012; Salih et al. 2012).

2.2.1.4. Prevention and Treatment

Acaricides are the most common tool to prevent tick infestation and theileriosis. However, resistance to the available acaricides is growing, demanding more research for this prevention. Live vaccines have been developed for *T. parva*, *T. annulata*, and lately for *T. lestoquardi* and *T. annulata* (Pipano et al., 2000). However, there are some obstacles to vaccine delivery in rural areas and the feasible for commercial production of vaccine. Parvaquone and buparvaquone are two essential drugs for the treatment of *T. parva* and *T. annulata*. Buparvaquone is more efficient for treating *T. parva* and *T. annulata* than parvaquone. However, Buparvaquone can be detected in muscles for at least 35 days and in the liver and injection site of some cows until at least 328 days after injection (McDougall et al., 2016).

2.2.2. *Babesia*

2.2.2.1. Background of *Babesia* spp.

Babesia species are intraerythrocytic apicomplexan parasites distributing in tropical and subtropical regions. They are responsible for an emerging tick-transmitted, malaria-like disease, babesiosis. Being a zoonotic disease, some *Babesia* spp. infect humans (*B. microti, B. divergens, B. duncani, B. venatorum*) (Cornillot et al., 2012). *Babesia* spp. and *Theileria* spp. are usually co-infected in cattle due to their pyriform (pear-shaped) structure visible during intracellular stages in the host erythrocytes. *Babesia* spp. infect many mammals, including cattle, buffalo and small ruminants. The clinical signs in ruminant of this parasite are associated with fever, anaemia, hemoglobinuria, abortion, neurological symptoms and even death. Caprine

babesiosis is caused mainly by *B. ovis, B. motasi* and *B. crassa* which is important in the Middle East, Southern Europe and some African and Asian countries. Recently, several *Babesia* strains in sheep and goat, *Babesia*. sp. BQ1 (Niu et al., 2016a), *Babesia*. sp. Xinjiang (Liu et al., 2007; Guan et al., 2009; Niu et al., 2017), *B. motasi*-like species (Niu et al., 2009; Niu et al., 2016b) have been isolated in China. However, there have not been any published reports of caprine babesiosis in Thailand.

2.2.2.2. Life cycle and morphology

The life cycle of *Babesia* spp. is similar to that of *Theileria* spp. but they do not have the leukocyte-infective stage. Sporozoites invade erythrocytes of a host during blood meal of an infected tick and then develop into trophozoites. Trophozoites undergo merogony multiplying into two or four merozoites which invade new erythrocytes during cell lysis. Some merozoites transform into gamonts and are taken up by a tick. Gamonts differentiate into gametes in tick's gut that then fusing of male and female gametes and forming diploid zygotes. Zygotes undergo meiosis developing into motile haploid kinetes. Kinetes transform into sporozoites in sporogony stage, infecting several tick organs including salivary glands. Kinetes also invade the tick ovaries and eggs, and infective sporozoites are formed in the salivary glands of the next-generation larvae (transovarial transmission) (Florin-Christensen and Schnittger, 2018).

2.2.2.3. Diagnosis

Microscopic examination by Giemsa staining of blood or tissue fluids is usually used to confirm the presence of babesiosis. However, this method is time and labor-consuming, low sensitivity, and it cannot differentiate between morphologically similar species (Maharana et al. 2016). Molecular detection can improve the obstacles of microscopic technique due to its high sensitivity and specificity for the parasite of interest. Several of the direct, nested, and quantitative PCR assays have been described for the detection of ruminant *Babesia* spp (He et al. 2011; Ramos et al. 2011; Horta et al. 2014; Romero-Salas et al. 2016; Erster et al. 2016). The 18S rRNA gene is the most common target because of its adequate

species-related degree of polymorphism and repetitive arrangement within the genome (Lempereur et al. 2017). Mitochondrion cytochrome b gene is also a good target for detection because of its high copy numbers. LAMP, loop-mediated isothermal amplification, is cost-effective, simple, and fast, which makes it suitable for field diagnostics. Amplification is carried out at a constant temperature in a water bath, and results can be observed by the naked eye (OIE 2014). LAMP has been developed for the molecular diagnosis of babesiosis in cattle, buffalo, and small ruminants (Guan et al., 2008; He et al. 2009; Yang et al., 2016). Reverse line blot hybridization (RLB) test is also used to simultaneously detect DNA from different piroplasm in blood or tick samples. Although this technique is technically elaborate, it has been applied to a high number of epidemiological studies in cattle, small ruminants, and ticks around the world (Hurtado 2015; Schötta et al., 2017).

2.2.2.4. Prevention and treatment

Vaccines consisting of live-attenuated strains of *B. bovis* and *B. bigemina* are produced in several countries which is used when animals are transported from a tick-free to a tick-infested area (Ganzinelli et al., 2018). Most *B. bovis* and *B. bigemina* live vaccines are produced in government-supported production facilities as a service to the livestock industries, in particular in Australia, Argentina, South Africa, and Israel (OIE 2014). However, the risk of contamination with other microorganisms makes precise quality control essential, and the high cost of these tests can obstruct their routine application (de Waal and Combrink 2006; Florin-Christensen et al., 2014). Acaricides have long been applied by farmers around the world to reduce the deleterious impact of ticks and tick-borne diseases. However, it brought some drawbacks to the environment, and the increase of tick resistance to multiple acaricides required the development of new alternative drugs, which is a lengthy and costly process (Florin-Christensen et al. 2014). Early diagnosis with prompt administration of chemotherapeutic drugs is critical for successful treatment of babesiosis. Diminazene aceturate and imidocarb dipropionate are two available anti-*Babesia* drugs (de Waal and Combrink 2006; Gohil et al. 2013). Imidocarb became the

product of choice in some countries because of its therapeutic utility and effective prophylactic at twice the therapeutic dose (Zintl et al., 2003; Mosqueda et al., 2012).

2.2.3. *Plasmodium*

2.2.3.1. Background of *Plasmodium* spp.

Plasmodium spp. are intracellular mosquito-borne parasites, belonging to Apicomplexa. Malaria in human caused by this parasite is one of the most severe diseases in the world. *Plasmodium* affects a wide range of hosts, including non-human primate, rodent, ungulate, chiroptera, avian, and reptile (Ott, 1967). In 2016, three published papers reported the first molecular analysis of malaria parasite in ungulates. The first paper described *Plasmodium odocoilei* clade 1 and 2 in white-tailed deer in North America (Martinsen et al., 2016). The second paper reported *Plasmodium* sequences from duiker antelope (*Cephalophus* spp.) in Africa (Boundenga et al., 2016). Finally, the third paper provided data on *Plasmodium* parasites from water buffalo in Vietnam and Thailand and one sequence isolated from domestic goat in Zambia (Templeton et al., 2016). Most recently, *Plasmodium caprae* has been detected in Sudan, Kenya, Iran, Myanmar and Thailand (Kaewthamasorn et al., 2018). However, except for some DNA sequences and morphology, little is understood for this parasite.

2.2.3.2. Life cycle of *Plasmodium* spp.

The malaria parasite life cycle involves two hosts (mosquito and vertebrate host). The parasite from female anopheline mosquito enters the vertebrate during the blood meal. Sporozoites then infect the liver or other tissue. *P. vivax* and *P. ovale* can form dormant stage and persist in liver for years (Vaughan and Kappe, 2017). However, for most *Plasmodium,* the parasites develop into merozoites and invade the erythrocytes. Erythrocyte infection may cause disease, called malaria, which sometimes can be severe, rapidly followed with the death of the host (*P. falciparum* in humans). In other hosts, this invasion can be asymptomatic (Vernick et al., 2005). In red blood cell, merozoites become larger with a ring-shaped form called trophozoites. Trophozoites, then mature and differentiate to schizonts which

develop into new merozoites. The new merozoites release from ruptured red blood cell, enter to the blood stream and infect new erythrocytes. Some merozoites differentiate into male and female sexual forms called gametocytes. Gametocytes are then taken up from the blood by the mosquito during blood feeding. In the mosquito, gametocytes move to the mosquito's midgut. Here, they develop into zygote through the fertilizing of male and female gametes. The zygotes, in turn, become motile and elongated form called ookinetes which invade the midgut wall of the mosquito where they develop into oocysts. Oocysts divide and produce sporozoites which then migrate to salivary gland of mosquito and be inoculated into a new vertebrate host (Kreier, 2013).

2.2.3.3. Diagnosis

Malaria can be diagnosed by various techniques, including conventional microscopic diagnosis by staining thin and thick peripheral blood smears and molecular diagnostic methods, such as polymerase chain reaction (PCR). However, the staining and interpretation processes of microscopic diagnosis are labor intensive, time-consuming, and require expertise and trained technician, particularly for identifying species accurately at low parasitemia or in mixed malarial infections. The most critical shortcoming of microscopic examination is its relatively low sensitivity, particularly at low parasite levels (Erdman and Kain, 2008). PCR-based techniques have proven to be one of the most specific and sensitive methods to the diagnosis of malaria, particularly for lowe parasitemia or mixed infection cases. PCR has shown higher sensitivity and specificity than conventional microscopic examination of stained peripheral blood smears, and now seems the best method for malaria diagnosis. However, due to the high cost and sophisticated methodologies, this technique may not be suitable for malaria diagnosis in remote rural areas or in routine clinical diagnosis (Hänscheid et al., 2002). LAMP technique is considered to be easy, sensitive, quick and lower in cost than PCR. However, reagents require cold storage, and it should be validated about the feasibility and clinical utility by further clinical trials (Erdman et al., 2008).

2.2.3.4. Treatment and control

Antimalarial drugs can be used to prevent and treat malaria, but parasites frequently develop resistance to these agents. Vector management such as insecticides, environmental modification, and bed nets have significantly contributed to successful malaria control.

2.2.4. Diagnosis of vector-borne parasites

For many years, microscopic examination based on morphology is a method to diagnose blood parasites in ruminants. However, it requires qualified laboratory technicians and may be challenging to use for species identification. Molecular-based approaches provide higher sensitivity and specificity. Currently, identification of vector-borne parasites relies mainly on single PCR and nested PCR (Altay et al., 2004; Yin et al., 2008; Bawm et al., 2018; Kaewthamasorn et al., 2018). PCR is a well-known biological technique that is widely used by researchers for genomic studies as a rapid and very sensitive method. It is based on the ability of DNA polymerase to amplify a small number of DNA molecules into millions of copies. Amplification is achieved by a series of three steps: denaturation, annealing and extension. With each repetition of these three steps, the number of copied DNA molecules increased (Garibyan and Avashia, 2013). Nested PCR is a variation of standard PCR that improves the specificity and sensitivity, which involves the use of two sets of primer and two PCR reactions. The first reaction is performed with primers that cover the target sequence, which is the template for the second reaction. Unfortunately, those methods are time-consuming and expensive when applied in large numbers of field samples where co-infection of different parasites usually exist. Multiplex PCR allows amplification of multiple targets using more than one set of primers in a single PCR tube, which provides significant advantages in the diagnosis of several parasitic infections simultaneously. Recently, several multiplex PCRs for rapid and simultaneous detection of parasites have been widely used to examine co-infections at the species level (Zhang et al., 2013; Miguel-Oteo et al., 2016; Cui et al., 2017). This method requires primer sequences to be unique to their target both in individual pairs and in combinations of many primers. In addition, methods must be available for the

analysis of each amplification product which has a distinct size to be resolved by gel electrophoresis (Markoulatos et al., 2002). Multiplex PCR has provided considerable advantages for saving time, resource or reagent, sample and effort without compromising test utility (Elnifro et al., 2000).

CHAPTER 3 MATERIALS AND METHODS

3.1. Blood sample collection

Goat blood samples have been collected from goat farms in five provinces of Thailand (Phetchaburi, Chonburi, Rayong, Kanchanaburi, and Nan) (Table 1). The blood samples were taken from individual goats through the jugular vein using Eclipse™ 21G needles and BD Vacutainer® blood collection tubes containing Acid Citrate Dextrose as the anticoagulant (BD Franklin Lakes, NJ, USA) as previously explained by Kaewthamasorn and colleagues (2018). This study was approved by Chulalongkorn University Animal Care and Use Committee (Approval No. 1731069).

Table 1. Sampling location and period

Site number	Sampling site	Sampling period	No. of sample
1.	Phetchaburi	September 2017	10
		September 2019	10
2	Kanchanaburi	June 2019	20
3	Chonburi	July 2017	20
4	Rayong	July 2017	20
5	Nan	January 2016	20

3.2. Primer designs

The *cytochrome c oxidase* subunit I (hereafter referred to as *COI*) gene in mitochondria of *Theileria luwenshuni*, *Babesia* spp. and *Plasmodium caprae* was retrieved from GenBank™ database and aligned by Clustal Omega. The software is freely accessible at www.ebi.ac.uk. The GenBank accession numbers included for primer design in the present study were as follows: JQ518296 and JQ518295 (*T. luwenshuni)*; LC326032 and LC090215 (*P. caprae*) and JQ518305, JQ518304, JQ518303, JQ518302, JQ518301, KX698108, KT224420, EU075182 (*Babesia* spp). Conserved DNA regions universally specific for *T. luwenshuni, Babesia* spp. and *P. caprae* were chosen as the target for the outer primers and named as follow:

PiroPlasmoCoxIFout and PiroPlasmoCoxIRout. For the species-specific PCR for *T. luwenshuni* detection, the primer set was designed to amplify a 320-bp fragment of the mitochondrion COI gene (TluweCoxIFinn and TluweCoxIRinn). The *P. caprae* specific primer set (PcapraeCoxIFinn and PcapraeCoxIRinn) was designed to amplify a 664-bp sequence of the mitochondrion COI gene. The primer set to specifically detect *Babesia* spp. (BabeCoxIFinn and BabeCoxIRinn) was designed to amplify a 533-bp sequence of the mitochondrion COI gene. All primer sets were designed with similar annealing temperatures. In silico validation of the primers were carried out by using Primer-BLAST in GenBank database. The details of the primer set and expected amplicon size are provided in Table 2.

Table 2. List of primer sequences used in this study

Primers	Target	Sequences (5'-3')	No. Nucleotide	Ta (°C)	Expected size (bp)
PiroPlasmoCoxIFout	*T. luwenshuni,*	GWRRWGGWACWGGATGGAC	19		
PiroPlasmoCoxIRout	*Babesia* spp. and *P. caprae*	CWAYDARHACWCCAGTWGTACCWCC	25	52	717
PcapraeCoxIFinn	*P. caprae*	CCTTTAAGTACATCTTTAATGTCTTTATCTCCAG	34		
PcapraeCoxIRinn		CAAAAGTAAATGTACATATAAATAATAATGCTAATAAA GATG	42	61	664
BabeCoxIFinn	*Babesia* spp.	GAGTGGTGCTAATTTTGTTGTTACTTTTGGAG	32	61	553
BabeCoxIRinn		TTTACAACAAATAGAATAGCCATC	24		
TluweCoxIFinn	*T. luwenshuni*	CTATAGGTTACGTTATAGATAGAATTCTTCCAACT	35	61	320
TluweCoxIRinn		CAAACATCCCAATAAAGCGATAGAGGC	27		

Ta = Annealing temperature

3.3. Primer validations

3.3.1. Preparation of reference parasite DNA

Genomic DNA of *T. luwenshuni* and *P. caprae* were obtained from our previous study with confirmation of sequencing results of COI gene. *B. bigemina* was kindly provided by Dr. Montakan Jiratanh from National Institute of Animal Health of Thailand. DNA fragments from PCR positive products of *T. luwenshuni*, *B. bigemina* and *P. caprae* were inserted into pCR®2.1 plasmid vectors (Invitrogen, USA) and used for primer validation. Concentrations of plasmids containing the parasite's DNA were measured by NanoDrop™ spectrophotometer (Thermo Scientific, USA). The PCR reactions were conducted using outer primers. These reactions were performed in the total volume of 12.5 µl containing 1 µl of DNA template and master mix including 0.25 µl KOD FX Neo Polymerase (TOYOBO, Japan), 6.25 µl of 2X PCR Buffer, 2.5 µl of 2 mM dNTPs, 0.375 µl of each primer and 1.75 µl of sterile distilled water (SDW). The PCR condition comprises one step of initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 15 sec, annealing at 52 °C for 30 sec and extension at 68 °C for 25 sec. The final extension was performed with one cycle at 68 °C for 7 min. PCR products were electrophoresed on a 1.5 % agarose gel and then gel purification was conducted using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). The following steps of the process composed of DNA quantification using NanoDrop and adding A-tail into the PCR products. The A-tail PCR products were then ligated to plasmid vector pTA2 (TOYOBO, Japan) and transformed to competent high DH5α cell (TOYOBO, Japan) then incubated at 37°C in 18 hours. Single white colonies were suspended into 3 ml LB broth with ampicillin. Plasmid DNA (pDNA) of each parasite was extracted from LB broth medium using NucleoSpin® Plasmid EasyPure (Macherey-Nagel, Germany). To confirm that the plasmids contained right insert, pDNA was verified by PCR and DNA sequencing. These clones served as positive controls and templates in sensitivity and specificity test.

3.3.2. Specificity and sensitivity assessments

Analytical specificity of each pair of primers was tested in conventional PCR, using the adjusted copies of parasite DNA from the reference collection. Genomic DNA samples containing single infection of *T. luwenshuni*, *P. caprae* and *B. bigemina* were assessed in this step. Genomic DNA from an uninfected goat, *Anaplasma* sp. and *Trypanosoma* sp. positive blood samples were also included to assess primer specificity. Potentially cross amplification with the other unintended targets was evaluated in silico with Primer BLAST available online at https://www.ncbi.nlm.nih.gov/tools/primer-blast/. Artificial mixtures of DNAs from *T. luwenshuni*, *P. caprae* and *B. bigemina* were used as the template to assess the cross amplification for each primer set. Sterile distilled water was used as the negative control. The primer specificity tests were carried out in triplicates. Number of the parasite copies was calculated using an online web-based application freely accessed at https://cels.uri.edu/gsc/cndna.html. Analytical sensitivity of the currently developed protocols was tested using serial dilutions of parasite DNA. The dilutions were as follow: 10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4}, 10^{3}, 10^{2}, 10^{1}, and 10^{0} copies per reaction, respectively. Detection limit was assessed for each primer set.

3.4. Multiplex PCR (mPCR) optimization

According to our previous study, goat malaria infections showed very low parasitemia; for this reason, microscopic examination (the standard method) is not the reliable method in this case (Kaewthamasorn et al. 2018). Therefore, nested PCR (nPCR) amplification is necessary. The outer primers were used in the first amplification with the reaction mixture containing 1 µl of DNA, 0.25 µl of KOD FX Neo Polymerase (Toyobo, Japan), 6.25 µl of 2X KOD FX NEO PCR Buffer, 2.5 µl of 2 mM of dNTPs mixture, 0.375 µl of each primer and 1.75 µl of SDW in the final volume of 12.5 µl. The thermal cycling profile was set at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 15 sec, annealing at 52 °C for 30 sec and extension at 68 °C for 25 sec. The final extension was set for one cycle at 68 °C for 7 min.

mPCR amplification can be influenced by multiple parameters including annealing temperature, primer concentration and the number of cycles. Therefore, in order to facilitate amplification for all targets in a multiplex reaction, variations of these parameters are tested. Annealing temperatures were tested from 55 °C to 62 °C. Primer concentrations were tested from 0.3 to 2 µM. Amplification cycles were tested from 30 to 40 rounds.

3.5. Examination of field collected samples

A total of 100 goat blood samples collected from goat farms in five provinces of Thailand were used as templates to investigate for the presence of *P. caprae*, *Babesia* sp. and *T. luwenshuni*. The collected blood samples were kept at -20 °C for further DNA extraction. The DNA samples were extracted using NucleoSpin Blood kit (Macherey-Nagel, Germany). The DNA extraction procedure was carried out according to the instruction described by the manufacturer. DNA yield was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Three published PCR detection methods for *P. caprae*, *Babesia* spp. and *T. luwenshuni* (Templeton et al., 2016; Masatani et al., 2017; Henker et al., 2020) were used for comparison of the efficiency of the mPCR detection (Table 3).

Table 3. Oligonucleotide primers of published PCR methods used in this study

Parasite	Target gene	Primer name	Oligonucleotide sequences (5'-3')	References
P. caprae	Cytochrome b	DW2	TAATGCCTAGACGTATTCCTGATTATCCAG	(Templeton et al.,
		DW4[a]	TGTTTGCTTGGGAGCTGTAATCATAATGTG	2016)
		NCYBINF	TAAGAGAATTATGGAGTGGATGGTG	
		NCYBINR[b]	CTTGTGGTAATTGACATCCAATCC	
T. luwenshuni	18S rRNA	BTH 18S 1st F	GTGAAACTGCGAATGGCTCATTAC	(Masatani et al., 2017)
		BTH 18S 1st R[c]	AAGTGATAAGGTTCACAAAACTTCCC	
		BTH 18S 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	
		BTH 18S 2nd R[d]	CGGTCCGAATAATTCACCGGAT	
B. bigemina	18S rRNA	GAU5	TGGCGGCGTTTATTAGTTCG	(Henker et al., 2020)
		GAU8	GCCAGCGAAAAGACCCAAC	

[a, c] Primer sets were used in the first round of amplification for *P. caprae* and *T. luwenshuni*, respectively.

[b, d] Primer sets were used in the second round of amplification for *P. caprae* and *T. luwenshuni*, respectively

3.6. DNA sequencing and statistical analysis

All the positive PCR products of *P. caprae, Babesia* spp. and *T. luwenshuni* from the field samples were sequenced to re-confirm the specificities of each pair of primers. Nucleotide sequences from naturally infected goats were also analyzed by sequencing and submitted for a BLAST research. The clinical sensitivity and specificity of three primer sets were calculated using 100 field samples with the published singleplex PCR as the reference method. Sensitivity was calculated as (number of true positives)/(number of true positives + number of false negatives), and specificity was calculated as (number of true negatives)/(number of true negatives + number of false positives). Results from the comparison between mPCR and sPCR assay are shown in a 2 x 2 table by the SPSS program Agreement of results between the two methods was assessed using Cohen's Kappa-test for concordance and McNemar's test for discordance. The influence of amplicon sizes on diagnostic sensitivity and specificity were calculated by Pearson correlation coefficient (R) and coefficient of determination (R^2).

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CHAPTER 4
RESULTS

4.1. Positive control

In the present study, DNA fragments from COI genes of *P. caprae, B. bigemina* and *T. luwenshuni* were successfully cloned into the plasmids. COI genes of *P. caprae*, *B. bigemina* and *T. luwenshuni* shared 97.75, 99.57, and 88.01% sequence identity with and most closely related to Thailand (THGoat16-18), Japanese Kochinda, Chinese Ninxian isolates (GenBank accession no. LC326032, AB499085, and JQ518295, respectively) (Table 3). Plasmids at concentrations of 358.2, 244.7, and 209.9 ng/µl were recovered from bacteria culture containing DNAs of *P. caprae*, *T. luwenshuni* and *B. bigemina*, respectively and were used as positive controls hereafter.

Table 4. Sequencing results of the reference of three plasmid DNA parasites used in this study.

Sample ID	Insert (bp)	Closely related to	Origin	% Identity	Accession No.
THGoat18-68	717	*P. caprae*	Thailand	97.75	LC326032
BbiNIAH	703	*B. bigemina*	Japan	99.57	AB499085
THGoat17-536	710	*T. luwenshuni*	China	88.01	JQ518295

Note: PiroPlasmoCoxIF-Rout was used to amplify DNA fragments of positive samples. BboNIAH was *B. bigemina* DNA kindly provided by National Institute of Animal Health of Thailand.

4.2. Optimization of mPCR

An annealing temperature for mPCR reaction was set at 57 °C with amplification cycle of 40 rounds. Primer concentrations of 1.8, 0.6, 0.8 µM for *P. caprae*, *T. luwenshuni* and *B. bigemina*, respectively were chosen for the mPCR

conditions. The reaction mixture was carried out in a final volume of 12.5 µl containing 0.0625 µl of TaKaRa Ex Taq Polymerase (Takara, Japan), 1.25 µl of 10X Ex Taq Buffer, 1 µl of dNTPs mixture, and 0.5 µl of first PCR product mixture after undergone a 10-fold dilution in distilled water. The PCR condition consisted of an initial denaturation step at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 15 sec, annealing for 30 sec at 57 °C and extension at 72 °C for 28 sec with final extension at 72 °C for 5 min. The PCR products were then subjected to electrophoresis on a 1.5% agarose gel and visualized under UV light.

4.3. Primers designed *in silico* validation

After the design, three primer sets in this study were re-checked by Primer-BLAST (Table 5). The primer pair of *P. caprae* showed perfect matches to COI gene of *Plasmodium* sp. from goat (LC326032 and LC090215) as well as other *Plasmodium* spp. from buffalo, deer and white-tailed deer (MK518339, LC326034, KU133759, respectively). Some other potential amplicons were also detected including: *Haemoproteus* spp. (KY653805, KY653797, KY653752) and *Polychromophilus* spp. (KT750541, KT750457). However, these parasites are pathogens of birds and bats, respectively. On the other hand, primer pair of *Babesia* spp. showed several matches to *B. bigemina* (AB499085, JQ518300), *B. ovata* (JQ518306, JQ518307), *B. motasi* (JQ518303, KT224420) and *Babesia* spp. (JQ518308, MF078482). None of them is perfectly matched, but there are less than 5 mis matches in each primer, which is probably sufficient for the effective amplification. There were not any amplicons detected by primer pair of *T. luwenshuni*. This might be explained by the lack of *T. luwenshuni*'s COI gene sequence deposited in the database. Indeed, the preference plasmid DNA of *T. luwenshuni* just shared 88.01% sequence identity with Chinese Ninxian isolates from sheep. The results revealed no evidence of amplification interference between three pairs of primers as well as non-specific amplification of each primer set.

Table 5. Primer-BLAST results of three primer sets

Primers	GC %	SC	3' SC	Potential target (accession no.)
PcapraeCoxIFinn	33.33	5	2	*Plasmodium* sp. (LC326034), *P. caprae* (LC326032), *P. odocoilei* (KU133759),
PcapraeCoxIRinn	18.75	6	4	*P. bubalis* (MK518339), *Haemoproteus* spp. (KY653805, KY653797, KY653752),
				Polychromophilus spp. (KT750541, KT750457)
BabeCoxIFinn	37.50	4	0	*B. bigemina* (AB499085, JQ518300), *B. ovata* (JQ518306, JQ518307), *B. motasi*
BabeCoxIRinn	29.17	4	2	(JQ518303, KT224420), *Babesia* spp. (JQ518308, MF078482)
TluweCoxIFinn	31.43	8	5	No unintended target
TluweCoxIRinn	44.44	4	2	

SC: Self-complementarity, **3' SC:** 3' self-complementarity

4.4. Specificity and sensitivity of singleplex PCR

A successful amplification with amplicon sizes of 664, 553, and 320 bp were detected in DNA samples of *P. caprae*, *B. bigemina* and *T. luwenshuni,* respectively (Fig. 1). Cross amplification with the other unintended target DNAs was not observed indicating that the three primer pairs in this study were specific to their respective targets. The sensitivity of primer pairs in this study was determined using parasite DNA templates in the plasmids. The detection limit for *P. caprae, B. bigemina* and *T. luwenshuni* was 10^5, 10^5 and 10^4 copies, respectively (Fig. 2).

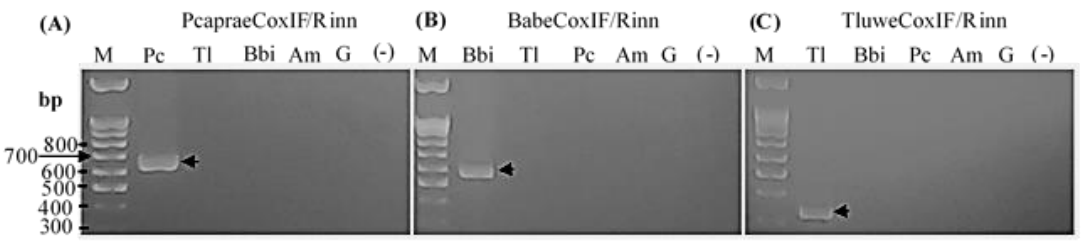

Figure 1. Analytical specificity of each primer set targeting *P. caprae* **(A)**, *B. bigemina* **(B)** and *T. luwenshuni* **(C),** respectively. DNA ladder marker (M). The specificity assay for *P. caprae* (A), samples included in the assessment were as follow: *P. caprae* (Pc); *T. luwenshuni*; (Tl); *B. bigemina* (Bbi); A. marginale (Am); uninfected goat (G); sterile distilled water (-). Expected size of 664-bp fragment was detected only with *P. caprae* (arrowhead).The specificity assay for *B. bigemina* (B), samples included in the assessment were as follow: *B. bigemina* (Bbi); *T. luwenshuni* (Tl); *P. caprae* (Pc); A. marginale (Am); uninfected goat (G); sterile distilled water (-). Expected size of 553-bp fragment was detected only with *B. bigemina* (arrowhead).The specificity assay for *T. luwenshuni* (C), samples included in the assessment were as follow: *T. luwenshuni* (Tl); *B. bigemina* (Bbi); *P. caprae* (Pc), A. marginale (Am); uninfected goat (G); sterile distilled water (-). Expected size of 320-bp fragment was detected only with *T. luwenshuni* (arrowhead).

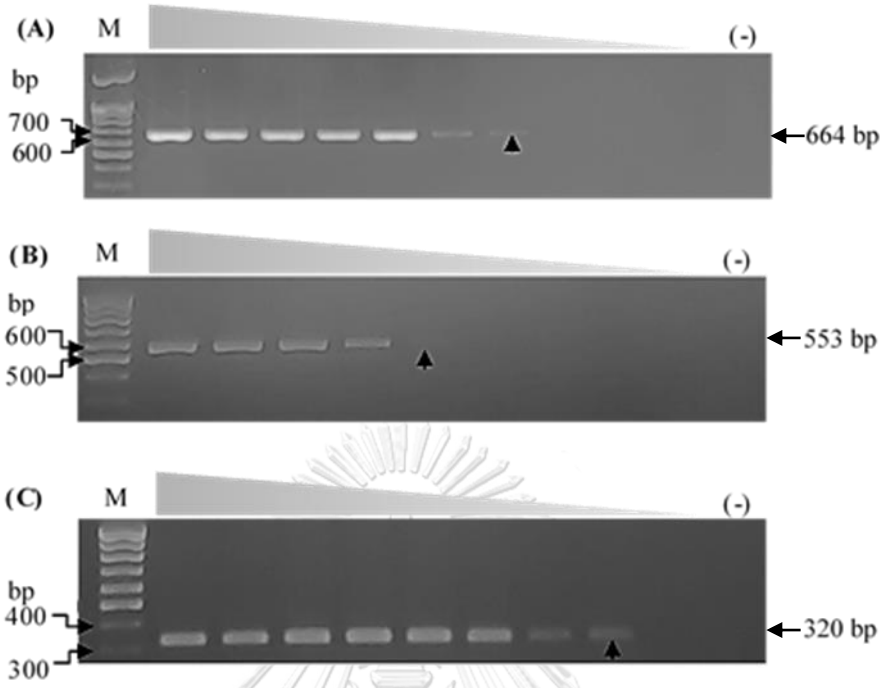

Figure 2. Analytical sensitivity of primer sets targeting each of DNA template of *P. caprae. B. bigemina* and *T. luwenshuni.* Analytical sensitivity of primer sets targeting each of DNA template of *P. caprae, B. bigemina* and *T. luwenshuni.* M: DNA ladder marker; Red safe-stained agarose gel electrophoresis images showing amplicons of using 10-fold serial dilutions of DNA template of *P. caprae* **(A)**, *B. bigemina* **(B)** *and T. luwenshuni* **(C)**, respectively (from 10^{10} to 10^{2} copies). Detection limits for *P. caprae* (10^{4} copies), *B. bigemina* (10^{6} copies), and *T. luwenshuni* (10^{3} copies) are indicated by arrowheads. Sterile distilled water was used as a no template negative control (-).

4.5. Simultaneous detection of *P. caprae, T. luwenshuni* and *B. bigemina* by nested mPCR assay

A mixture of two and three DNA of *P. caprae, T. luwenshuni* and *B. bigemina* was used as the templates. Two and three bands of expected sizes were observed in both artificially made double and triple infections (Fig. 3). No amplification product in either unintended target or in negative control. The result showed that no cross

reactivity or competition among the multiplex PCR primer sets. This indicates that the developed nested mPCR in this study was highly specific to its target and the amplicons of 664, 553, and 320 bp were distinguishable by gel electrophoresis.

Analytical sensitivity of mPCR for simultaneous detection of the three targets with concentrations adjusted to the same level are shown in Fig. 4. The reaction mixture containing DNA templates of each parasite ranging from 10^{10} to 10^{8} copies was clearly seen positive amplicons for all three targets. This concentration of plasmid templates was therefore the optimal set up for the mPCR assay for all three parasites, in the present study.

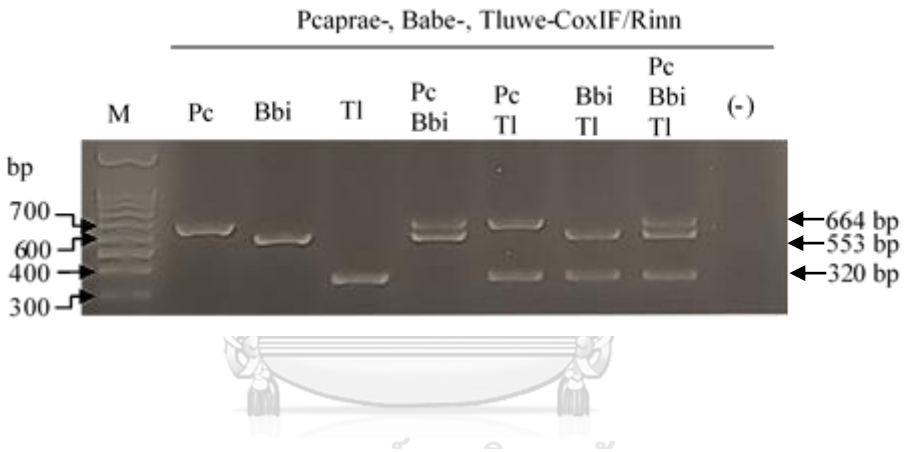

Figure 3. Simultaneous detection of *P. caprae, B. bigemina* and *T. luwenshuni* by mPCR**.** A mixture of three specific primer sets (PcapraeCoxIF/Rinn, BabeCoxIF/Rinn, and TluweCoxIF/Rinn) was able to detect all single, double and triple infections of *P. caprae* (Pc), *B. bigemina* (Bbi) and *T. luwenshuni* (Tl), respectively with expected amplicon sizes of 664, 553 and 320 bp, respectively. M, 1.5 kb DNA ladder marker. Sterile distilled water was used as negative control (-).

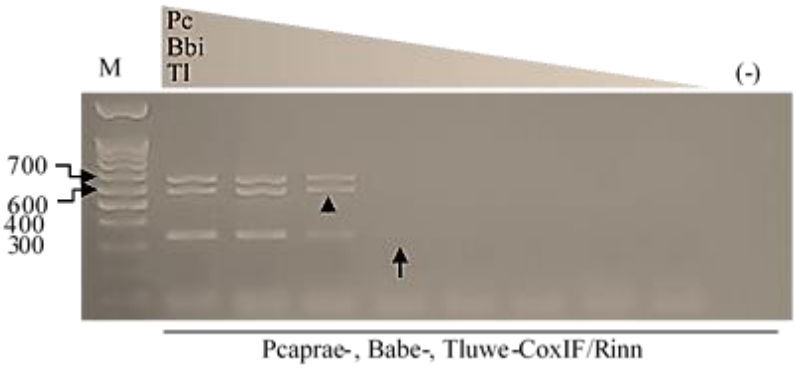

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Figure 4. Analytical sensitivity of nested mPCR assay for *P. caprae, B. bigemina* and *T. luwenshuni* detection. M, 1.5 kb DNA ladder marker. Red safe-stained agarose gel electrophoresis images showing 10-fold serial dilution of artificially made co-infections of *P. caprae, B. bigemina and T. luwenshuni* using DNA template ranging from 10^{10} to 10^{3} copies. Sterile distilled water (-) was used as a no-template negative control. The detection limit of the mPCR with mixed infections was observed at 10^{8} copies for both *P. caprae* and *B. bigemina* (arrowhead), while *T. luwenshuni* (arrow) appeared faintly at 10^{7} copies of the parasite's DNA template.

4.6. Detection *of P. caprae, Babesia* spp. and *T. luwenshuni* infection in goat blood samples

Singleplex PCRs targeting *P. caprae* and *T. luwenshuni* detected 2 and 5 positive samples, respectively. No positive sample has been found with sPCR for *B. bigemina*. To confirm the application and reliability of the nested mPCR, 100 goat blood samples were tested for the presence of *P. caprae, T. luwenshuni* and *Babesia* spp., and the amplicons were confirmed by DNA sequencing (Table 6). With nested mPCR method, 6, 5, and 2 samples were positive for *P. caprae*, *T. luwenshuni* and *B. bigemina,* respectively. Among the positive samples, co-infection was not detected. The details of the results are shown in Table 7.

Table 6. Sequencing results of field samples from each species-specific primer set

Primer set	Sample ID	Size (bp)	Closely related to	Origin (Isolate)	% Identity	Accession No.
NCYBINF-R	THGoat19_Kancha_133	752	*P. caprae*	Thailand (THGoat16-18)	99.08	LC326032
	THGoat17_Chon_434	768	*P. caprae*	Thailand (THGoat16-18)	98.69	LC326032
PcapraeCoxIF-Rinn	THGoat19_Phetcha_151	600	*P. caprae*	Thailand (THGoat16-18)	99.17	LC326032
	THGoat19_Kancha_124	573	*P. caprae*	Thailand (THGoat16-18)	99.3	LC326032
	THGoat17_Chon_349	616	*P. caprae*	Thailand (THGoat16-18)	98.21	LC326032
	THGoat17_Chon_438	611	*P. caprae*	Thailand (THGoat16-18)	99.84	LC326032
	THGoat16_Nan_125	604	*P. caprae*	Thailand (THGoat16-18)	99.34	LC326032
BabeCoxIF-Rinn	THGoat19_Kancha_149	492	*B. bigemina*	Japan (Kochinda)	98.17	AB499085
	THGoat17_Rayong_538	558	*B. bigemina*	Japan (Kochinda)	98.99	AB499085
PiroPlasmoCoxIF-Rout	THGoat17_Rayong_529	632	*T. luwenshuni*	China (Ninxian)	88.13	JQ518295
	THGoat17_Rayong_530	595	*T. luwenshuni*	China (Ninxian)	88.07	JQ518295
	THGoat17_Rayong_534	602	*T. luwenshuni*	China (Ninxian)	88.37	JQ518295
	THGoat17_Rayong_536	603	*T. luwenshuni*	China (Ninxian)	88.37	JQ518295

Table 7. Summary positive goat blood samples of nested mPCR and the cPCR methods.

Province	No. of	*P. caprae*			*T. luwenshuni*			*B. bigemina*		
	sample	cPCR	mPCR	95% CI	cPCR	mPCR	95% CI	cPCR	mPCR	95% CI
Phetchaburi	20	0	1	1.3%-11%	0	0	0.7%-9%	0	0	-0.8%-4.8%
Kanchanaburi	20	1	1	(E= 0.047)	0	0	(E=0.043)	0	1	(E=0.028)
Chonburi	20	1	3		1	1		0	0	
Rayong	20	0	0		4	4		0	1	
Nan	20	0	1		0	0		0	0	
Overall	**100**	**2**	**6**		**5**	**5**		**0**	**4**	

E- Margin of Error (95% CI Upper limit - 95% CI Lower limit/2)

4.7. DNA sequencing and statistical analysis

The diagnostic sensitivity and specificity of the mPCR were determined by screening 100 goat blood samples collected from the field. The results obtained from mPCR were compared with sPCR protocols. Due to *B. bigemina* was not detected by sPCR, therefore either sensitivity or specificity of mPCR was not be able to calculate. However, *B. bigemina* was detected by mPCR in two samples. The PCR products were confirmed by sequencing. This suggested that the current mPCR has higher sensitivity than sPCR for *B. bigemina* detection. The diagnostic sensitivity of mPCR for *P. caprae*, *T. luwenshuni* was at 50%, 100%, respectively. The diagnostic specificity of mPCR for *P. caprae*, *T. luwenshuni* was at 94.9%, and 100%, respectively. Comparing sPCR and the current mPCR for *P. caprae* detection, McNemar's test confirmed that there was no significant difference between the two methods (p= 0.219), despite the fact that Kappa value (0.227 ± 0.202) indicated fair agreement. The efficiency of sPCR and mPCR in detection of *T. luwenshuni* was the same. Also, the Kappa value revealved very good agreement (Kappa value: 1 ± 0) (Table 8). The coefficient of determination (R^2) between PCR product size and sensitivity and specificity of mPCR was calculated as 1, which indicates that there is a full influence of PCR product size on determining sensitivity and specificity of mPCR. The Pearson correlation coefficient (R) between amplicon size and sensitivity and specificity of mPCR was calculated as -1, which suggests the great negative correlation of amplicon size with sensitivity and specificity of this mPCR assay.

Table 8. Diagnostic sensitivity and specificity of the mPCR in comparison with cPCR using the goat blood samples collected from the field.

Parasites			Singleplex PCR			McNemar's Test	Kappa ± SE	Sensitivity	Specificity
			Positive	Negative	Total	*p* value			
P. caprae	Multiplex PCR	Positive	1	5	**6**	0.219	0.227 ± 0.202 (Fair agreement)	50%	94.9%
		Negative	1	93	**94**				
		Total	**2**	**98**	**100**				
T. luwenshuni		Positive	5	0	5	1	1 ± 0 (Very good agreement)	100%	100%
		Negative	0	95	95				
		Total	**5**	**95**	**100**				

CHAPTER 5
DISCUSSION

Several vector-borne pathogens cause substantial negative economic impact, threatening the livestock industry worldwide. Co-infections of *Theileria* and *Babesia,* the causative agents of theileriosis and babesiosis, respectively are commonly found in ruminant, responsible for a complex disease, collectively known as piroplasmosis. Caprine piroplasmosis is considered highly pathogenic in sheep and goat, having significant economic importance throughout the world, particularly in tropical and subtropical regions (Bock et al.,2004). *T. luwenshuni* is one of *Theileria* species that cause malignant theileriosis of sheep and goat, which is an acute and highly fatal disease (Abutarbush, 2010). *B. ovis, B. motasi* and *B. crassa* are causative agents of caprine babesiosis in the Middle East, Southern Europe, and in some African and Asian counties. *B. bovis* and *B. bigemina* are important causative agents of bovine babesiosis in Asia, including Thailand. *P. caprae* has been reported in Sudan, Kenya, Iran, Myanmar and Thailand (Kaewthamasorn et al.,2018) but little is known about its pathogenicity, natural vector and co-infection status with other vector-borne parasites. Each blood parasite infection requires specific management. Therefore, accurate diagnosis and species-level detection are of importance for disease control.

Vector-borne parasites are traditionally identified by microscopic examination of Giemsa-stained blood smears, but this method does not allow for discrimination of individual species. Moreover, its difficulty in the detection of a low level of parasitemia is the potential risk for an outbreak and does not permit its use in the epidemiological investigation. Single PCR can overcome those limitations due to it poses high sensitivity and specificity, which could differentiate species to reveal the presence of parasites in the animals. This diagnostic method, however, solely targets a single pathogen which relatively time and reagent consuming. Real-time PCR has become a popular technique, but it remains costly for routine diagnosis. Meanwhile,

mPCR has provided simultaneous detection of multiple targets in one reaction. The development of this technique aims to overcome the inherent drawbacks of cost, time and diagnosis capability of singular PCR assay. In addition, mPCR has been confirmed as a valuable tool for diagnosis pathogen, where co-infection cases exist (Bilgiç.,2013; Zhang et al.,2014). In the present study, a specific, sensitivity, mPCR method was developed using three pairs of primer for simultaneous detection of *P. caprae*, *T. luwenshuni* and *Babesia* spp.

Several studies have revealed the detection of *P. caprae, T. luwenshuni* and *Babesia* spp. by single PCR methods (Zhou et al., 2017; Templeton et al., 2016; Kaewthamasorn et al., 2018; (Ranjbar-Bahadori et al., 2011; Bawm et al., 2018; Ozubek & Aktas, 2017; Mamatha et al., 2017). However, except one paper was made by Kaewthamasorn et al., 2018, there have not been any report about the presence of *T. luwenshuni* and *Babesia* spp. in goat in Thailand. Furthermore, the literature on mPCR based detection of blood parasites in goat are limited, and none of the mPCR assays was evaluated of their detection on different intraerythrocytic apicomplexan parasites. Cui et al. (2017) attempted a duplex PCR for detection of *Theileria* spp. and *Anaplasma* spp. based on 18S rRNA and 16S rRNA, respectively. Another mPCR was designed for the simultaneous detection of three *Theileria* species (5.8S rRNA gene for *T. luwenshuni* and *T. ovis*; 18S rRNA for *T. uilenbergi*) (Zhang et al., 2014). However, the sensitivity of both those mPCR assay tended to be less sensitive than single PCR method. Three blood parasites of goat (two piroplasms and one malaria parasite) were selected as targets of the mPCR in the present study.

The COI gene is one of the most popular markers for population genetic and phylogeographic studies across the animal kingdom (Derycke et al., 2010). Species-level identification can routinely be achieved by COI analysis (Hebert et al., 2003). COI gene is often used asa target in several studies for a large number of copíe and DNA barcoding methodology (Armstrong et al., 2005; Kerr et al., 2007; Rach et al., 2008). COI gene is suitable for this role because of its fast mutation rate for distinguishing the closely related species and its conserved sequence among

conspecifics. In this study, mPCR assay using three pairs of primers, which are based on the COI gene, was developed for identification of three blood parasites in goats. After optimizing the reaction and PCR condition, the three primer sets have successfully amplified their specific target genes in one reaction. No cross dimers were found, and the reaction products were easily distinguishable by gel electrophoresis. The detection limit of the novel mPCR is 10^8 copies of the plasmid of all three parasites.

From the results of primer designed in silico validation, primer pair for *P. caprae* detection could match to *P. caprae* and other ungulate *Plasmodium* sequences in GenBank which is probably due to their high genetic similarity. The close relationship among ungulate *Plasmodium* is in agreement with a previous study (Templeton et al., 2016). Although there was amplicon detection of other species, these pathogens do not cause disease in goat. The result indicates that the primer for *P. caprae* was particular for its target. Primer set for *Babesia* spp. could match with vary *Babesia* species (*B. bigemina, B. motasi*, *B. ovata*). However, only *Babesia* genus amplicons have been detected, which showed the ability of this primer pair in the detection of universal *Babesia* spp. with high specificity. This pair of primer could be useful for the study of *Babesia* spp. prevalence in Thai goat. In silico validation, primer set of *T. luwenshuni* showed no match with any amplicons in GenBank database because there may not have been any published COI gene from *T. luwenshuni*. No non-specific amplicon detected indicates the high specificity of this primer set.

In order to evaluate the reliability of the mPCR, 100 field blood samples were used to detect the presence of *P. caprae, T. luwenshuni* and *B. bigemina*. Three published singleplex PCR methods for the detection of *P. caprae, T. luwenshuni* and *B. bigemina* were used as references (Templeton et al., 2016; Masatani et al., 2017; Henker et al., 2020). Based on the results, 5 out of 100 samples (5%) from Phetchaburi, Chonburi, Kanchanaburi, Rayong and Nan were positive for *T. luwenshuni* by using both mPCR and sPCR methods. *B. bigemina* was detected from

Kanchanaburi and Rayong by mPCR (2/100) while none was detected by sPCR. Six out of a hundred samples (6%) with *P. caprae* infection was detected in five investigated provinces with mPCR. Meanwhile, only 2% (2/100) was positive by sPCR. No co-infection has been found by either sPCR or mPCR methods in all provinces. According to the present results obtaining with the field samples, the mPCR seems to have equivalent to sPCR sensitivity for *T. luwenshuni*. Similarly, no significant difference in the sensitivity between mPCR and sPCR for detection of *P. caprae* was observed. However, the current mPCR seems to be superior to sPCR for the detection of *B. bigemina*. Generally, mitochondrial genes are multi-copy compared to nuclear genes. The improvement in the detection limit of diagnostic assays were partly due to primers targeting multi-copy genes (Gruenberg et al., 2018; Lloyd et al., 2018; Polley et al., 2010). The sensitivity of mPCR tends to be lower than in single PCR assays, which were reported in several previous studies (Bilgiç et al., 2013; Cui et al., 2017; Zhang et al., 2014). The competition between primers as well as the amount of DNA template used in the reaction mixture for the finite amount of reagents leads to reduce the sensitivity in the reaction (Edwards et al., 1994; Henegariu et al., 1997). Therefore, single PCR with a high amount of DNA template and no competition between primers would generate a large amount of amplicon resulting in a greater sensitivity than in the mPCR assay (Bilgiç et al., 2013). Nevertheless, the mPCR in this study was able to amplify simultaneously the mixture of three DNA templates in one reaction with a similar sensitivity to the sPCR. Therefore, the present novel mPCR would serve as an alternative tool for epidemiological studies.

It is important to note that, detection of *P. caprae* by mPCR was not as sensitive as in *T. luwenshuni*, this probably due to its larger amplicon size (664 bp versus 320 bp, respectively). This observation was also in agreement with a previous report (Quemelo et al., 2009). In addition, the regression analysis in this study indicates the remarkable role of the size of PCR amplicon in determining the sensitivity and specificity of mPCR. The Pearson correlation coefficient (R) between PCR product size and sensitivity and specificity of mPCR was calculated as -1, which

patently suggests that the PCR amplicon size is in negative correlation with the sensitivity and specificity. In order to improve the sensitivity and specificity, the smaller the PCR amplicon size is recommended.

According to the results of infection in the field samples, *P. caprae* positive goats were found in 4 out of 5 investigated provinces (Phetchaburi, Kanchanaburi, Chonburi and Nan), suggesting a wider distribution of this parasite in Thailand than in the previous report by Kaewthamasorn (2018). The low prevalence of malaria parasite (6%) confirmed again that its presence is sporadic in Thai goats. By contrast, the infection of *T. luwenshuni* was found only in two investigated provinces (Chonburi and Rayong). *B. bigemina* was detected only in 2% of samples were from Kanchanaburi and Rayong. The present study is confirming the infections of *T. luwenshuni* and *B. bigemina* in Thai goats.

In conclusion, the novel nested mPCR in the present study offers simultaneous detection of *P. caprae, T. luwenshuni* and *B. bigemina* from goats. This study also confirmed the findings of *T. luwenshuni* and *B. bigemina* in Thai goats and revealed the new geographical distribution of *P. caprae.* The mPCR method offers an alternative diagnostics for addressing the current infection and co-infection of three parasites in goats.

CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study was conducted to develop a novel nested multiplex PCR assay for the detection of *P. caprae, B. bigemina* and *T. luwenshuni* in goats of Thailand. The results of primer validation showed that the three primer sets and the nested mPCR assay of the present study were highly specific to their targets, and the detection limit for *P. caprae, B. bigemina* and *T. luwenshuni* and mPCR was quite high, which is 10^5, 10^5, 10^4 and 10^8 copies, respectively. These findings indicate that the nested multiplex PCR method for simultaneous detection of those three blood parasites was successfully established with high sensitivity and specificity. From the field sample examination results, the mPCR for *P. caprae* and *T. luwenshuni* was able to amplify their target genes with the same sensitivity as the published sPCR. Meanwhile, the mPCR of *B. bigemina* revealed a higher sensitivity because it could detect two positive in the field-collected samples in comparison to none by sPCR method. Therefore, the nested mPCR, in general, is as capable of amplifying parasite infections as the sPCR but with the advantage of simultaneous identification of all three pathogen species in a single assay. This mPCR method could give a promising application in epidemiological studies which provide benefits in controlling the outbreak in future. In the present study, we report the first identification of *T. luwenshuni* and *B. bigemina* from goats in Thailand by nested mPCR and sequencing of the COI gene.

6.2 Recommendations

All three vector-borne parasites, *P. caprae*, *B. bigemina* and *T. luwenshuni*, were detected by nested mPCR in this study. While *B. bigemina* and *T. luwenshuni* were first reported in Kanchanaburi, Chonburi, and Rayong of Thailand, the results of *P. caprae* detection revealed a wider distribution of this parasite when compared with the previous article. Thus, a broader investigation about the prevalence of those

parasites should be conducted to understand better the distribution and evolution of vector-borne parasites in Thai livestock. In addition, a further study about the vector investigation of those three parasites should also be done to contribute more to the knowledge of their transmission and pathology, which will bring many benefits for disease control measures.

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