DETECTION AND DIFFERENTIATION OF HAEMOSPORIDIAN PARASITES IN CHICKENS (*GALLUS GALLUS DOMESTICUS*) USING MOLECULAR DIAGNOSTIC APPROACH

Miss Thi Xuan Mai Nguyen

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การตรวจและแยกแยะเชื้อปรสิตกลุ่มฮีโมสปอริเดียนในไก่ (*แกลลัส แกลลัส โดเมสติคัส*) ด้วยวิธีการทางอณูชีววิทยา

น.ส.ธิ สวน ไหม เหงียน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	DETECTION AND DIFFERENTIATION OF HAEMOSPORIDIAN PARASITES IN CHICKENS (*GALLUS GALLUS DOMESTICUS*) USING MOLECULAR DIAGNOSTIC APPROACH
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ธิ สวน ไหม เหงียน : การตรวจและแยกแยะเชื้อปรสิตกลุ่มฮีโมสปอริเดียนในไก่ (*แกลลัส แกลลัส โดเมสติคัส*) ด้วยวิธีการทางอณูชีววิทยา. (DETECTION AND DIFFERENTIATION OF HAEMOSPORIDIAN PARASITES IN CHICKENS (*GALLUS GALLUS DOMESTICUS*) USING MOLECULAR DIAGNOSTIC APPROACH) อ.ที่ปรึกษาหลัก : มรกต แก้วธรรมสอน, อ.ที่ปรึกษาร่วม : สนธยา เตียวศิริทรัพย์

เชื้อปรสิตกลุ่มฮีโมสปอริเดียน (Haemosporidian) ในไก่ (*Gallus gallus domesticus*) ไม่เพียงแต่แพร่กระจายในวงกว้าง แต่ยังสามารถก่อให้เกิดอัตราการตายสูงและไม่มีกลุ่มอาการที่จำเพาะ ในการศึกษานี้ได้มีการพัฒนาวิธีมัลติเพล็กพีซีอาร์ (multiplex PCR) เพื่อตรวจหาและแยกแยะชนิดของเชื้อกลุ่มฮีโมสปอริเดียนในไก่จำนวน 4 ชนิด ได้แก่ *Leucocytozoon caulleryi*, *L. sabrazesi*, *Plasmodium gallinaceum* และ *P. juxtanucleare* รวมทั้งมีการเปรียบเทียบกับวิธีการวินิจฉัยด้วยกล้องจุลทรรศน์ ผลการศึกษาโดยใช้ 157 ตัวอย่างที่ให้ผลบวกพบว่า วิธีมัลติเพล็กพีซีอาร์มีอัตราการตรวจพบเชื้อกลุ่มฮีโมสปอริเดียนสูงกว่าอย่างมีนัยสำคัญทางสถิติ (p=0.000) โดยที่อัตราการตรวจพบเชื้อ *L. sabrazesi*, *P. juxtanucleare* และ *P. gallinaceum* สูงกว่าวิธีการวินิจฉัยด้วยกล้องจุลทรรศน์อย่างมีนัยสำคัญทางสถิติที่ p=0.002, 0.000 และ 0.004 ตามลำดับ วิธีมัลติเพล็กพีซีอาร์นี้ สามารถตรวจหาเชื้อได้ในจำนวนน้อยที่สุดที่ 10^9 copy ต่อไมโครลิตร และยังมีประสิทธิภาพสูงสำหรับการตรวจหาเชื้อในตัวอย่างที่มีการติดเชื้อหลายชนิด เมื่อทำการทดสอบตรวจหาเชื้อฮีโมสปอ-ริเดียนในไก่ด้วยวิธีมัลติเพล็กพีซีอาร์กับตัวอย่างภาคสนามจากจังหวัดน่าน ปราจีนบุรี และฉะเชิงเทรา พบว่าอัตราการติดเชื้อเป็น 77.2%, 93.8% และ 39.5% ตามลำดับ โดยที่มีอัตราการติดเชื้อร่วมอยู่ระหว่าง 4% ถึง 37% ในตัวอย่างจากทั้งสามจังหวัด

สาขาวิชา วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ลายมือชื่อนิสิต .....

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ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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Thi Xuan Mai Nguyen : DETECTION AND DIFFERENTIATION OF HAEMOSPORIDIAN PARASITES IN CHICKENS (*GALLUS GALLUS DOMESTICUS*) USING MOLECULAR DIAGNOSTIC APPROACH. Advisor: Assoc. Prof. MORAKOT KAEWTHAMASORN, Ph.D. Co-advisor: Assoc. Prof. SONTHAYA TIAWSIRISUP, Ph.D.

Haemosporidioses in domestic chicken (*Gallus gallus domesticus*) are not only widely distribute but causing high mortality and non-specific symptoms. The multiplex PCR assay developed in this study can concurrently detect and differentiate four haemosporidian parasites *Leucocytozoon caulleryi*, *Leucocytozoon sabrazesi*, *Plasmodium juxtanucleare*, and *Plasmodium gallinaceum*. Comparing to microscopic examination performed in parallel on 157 samples, multiplex PCR assay exhibited significantly higher detection rate in diagnosis of haemosporidians (p = 0.000). The results showed that, *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum* detection rate of multiplex PCR were all higher than that of microscopic examination with p = 0.002, 0.000, and 0.004, respectively. Detection limit of multiplex PCR on quadruple targets mixture was 10^9 copies/µl. Multiple infections were also detected more effectively by multiplex PCR. The commonness of mix infection in chicken haemosporidian parasites was further confirm as the new method have been applied to field samples from Nan province, Prachinburi province, and Chachoengsao province, in which mix infections were detected in all three sampling sites, ranging from 4% to 37%. Overall, infection rate of haemosporidian parasites in chickens from three province were 77.2%, 93.8%, and 39.5%, respectively, by using multiplex PCR. This study contributes a protocol to detect all four avian haemosporidian parasites in chicken.

Field of Study: Veterinary Science and technology — Student's Signature .....

Academic Year: 2019 — Advisor's Signature .....

Co-advisor's Signature .....

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Thi Xuan Mai Nguyen

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CHAPTER 1

INTRODUCTION

Important and rationale

According to the Food and Agricultural Organization (FAO), among the main terrestrial domestic animals in 2017, poultry has become the most consumed meat in most countries around the world (FAO, 2018). Among poultry, chicken showed rapid growing in production from 1994 to 2017 in FAO's global data. In the same manner, Thailand's chicken production sector has also been steady developed since 2005. Nevertheless, chicken industry all over the world unfailingly facing many diseases, including haemosporidioses.

Haemosporida is an order of unicellular parasites that target red blood cells. Haemosporidian divided into four families Garniidae, Haemoproteidae, Leucocytozoidae, and Plasmodiidae, which causing mild to severe disease in avian, mammals, and reptiles (Bensch et al., 2009). Similar to other animals, haemosporidioses in chicken (*Gallus gallus domesticus*) are vector-transmit diseases, involving leukocytozoonosis and avian malaria, which caused by four species from different genera, *Leucocytozoon caulleryi*, *Leucocytozoon sabrazesi*, *Plasmodium gallinaceum* and *Plasmodium juxtanucleare*. Common clinical signs are anemia, anorexia, depression, poor performance or ataxia, nervous signs and sudden death in severe cases. These parasites are widely distributed, bringing about high mortality and morbidity with non-specific symptoms.

Haemosporidian parasites have become economically important pathogens in chicken industry, especially to tropical countries. Recently, from 2014 to 2015, 56.9% prevalence of *Leucocytozoon sabrazesi* infection in free-range chicken was reported in southern China (Zhao et al., 2016). Naqvi et al. (2017) reported 69% chicken infected with haemoparasites species in Pakistan (Naqvi et al., 2017). Another study conducted by Takang and colleagues (2017) found 72.66% of backyard chicken in

Chiang Mai infected with *Leucocytozoon* (Takang et al., 2017). Monitoring and surveillance of these haemosporidian parasites in the farms require continued efforts. Knowledge and an update information on these pathogens and co-infection status are mostly unavailable for these diseases in the country to benefit the control and prevention. Additionally, parasite diversity and prevalence of avian haemosporidians are not intensively studied in the local breed and backyard populations of chickens in Thailand.

Microscopic examination and conventional PCR are normally used separately or simultaneously for detection. However, both methods are still inadequate in term of specificity and sensitivity to provide accurate, as well as timely diagnostic, which is crucial for any treatment and prevention strategy. Recently, multiplex polymerase chain reaction (PCR) technique, which is able to amplify several target sequences at the same time, have been applied to screening avian haemosporidians in wild birds. However, a reliable detection method belong to chicken industry is in need of yet still missing. In this proposal, DNA-based technique will be used to differentiate these parasites into their species. Ultimately, an in-house diagnostic tool for identification of avian haemosporidian parasites will be developed, validated and might be able to serve as detection technique for disease screening and epidemiology study.

Objective of the study

The objective of this study is to develop a molecular diagnostic technique that can give accurately, as well as speedy detect and differentiate the four common haemosporidian parasites in chickens in Thailand.

Hypothesis

Co-infections of haemosporidian parasites in chickens could be separately and/or simultaneously detected and distinguished by multiplex PCR using species-specific primers.

CHAPTER 2

LITERATURE REVIEW

2.1 Haemosporidioses

Haemosporidioses are vector borne diseases in humans and animals, caused by blood parasites that belong to the order Haemosporida. In chicken, haemosporidioses includes avian malaria and leukocytozoonosis, both cause high morbidity and mortality, exiting as burdens for the chicken industry worldwide, especially in tropical countries of Southeast and South Asia (Permin et al., 2002; Valkiunas, 2004; Williams, 2005; Naqvi et al., 2017). Compared to other vertebrates' malaria parasites, malaria parasites in avian are more widespread, prevalent, and genetically diverse (Bensch et al., 2009). *P. gallinaceum* and *P. juxtanucleare* are responsible for chicken malaria (Silveira et al., 2009; Tasai et al., 2017), while *L. caulleryi* and *L. sabrazesi* are pathogens of leukocytozoonosis (Zhao et al., 2015; Suprihati and Yuniarti, 2017). Each species has individual morphology which inevitably changes between different states of their development.

2.2 *Plasmodium* life cycle

The life cycle of the *Plasmodium* genus in mosquito is initiated when it fed on infected chicken, taking up microgametes and macrogametes, respectively. In the midgut's lumen of the mosquito, micro- and macrogametocyte fertilize and become diploid zygote. Zygotes develop into a motile form called ookinetes, which move through midgut epithelium toward the basal lamina, where ookinetes differentiate into oocysts. Lastly, oocysts produce thousands of sporozoites, which escape into the mosquito haemocoel and travel to invade the salivary gland, ready to be injected into new chickens. Infected mosquito injecting sporozoites from their salivary glands into the chicken, owing to their behavior of salivating. Sporozoites invade macrophages at the infected site and differentiate into trophozoites, then develop to

become schizonts, which later mature and release merozoites. One of the different between *P. juxtanucleare* and *P. gallinaceum* is *P. juxtanucleare* at schizonts state have lower numbers of merozoites compared to *P. gallinaceum*, which ranging from two to six merozoites (Bennett, 1970; Valkiunas, 2005). In blood circulation, merozoites infect erythrocytes, turn into trophozoites, and continue to produce several merozoites which infect other red blood cells, causing anemia symptom.

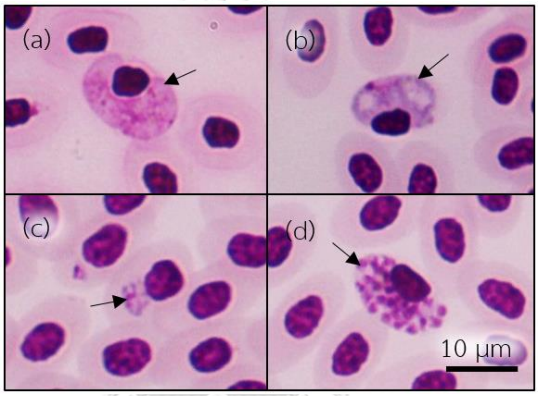

Figure 1 Different states of *P. gallinaceum*: (a) microgametocyte; (b) macrogametocyte; (c) trophozoite; (d) schizont (Kaewthamasorn, unpublished). All images were taken at the same magnification through Olympus CK31 microscope.

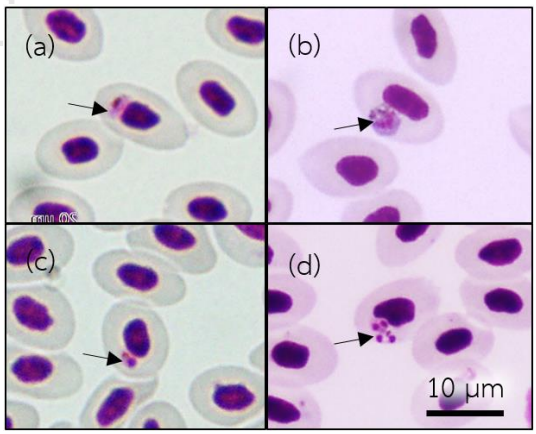

Figure 2 Different states of *P. juxtanucleare*: (a) microgametocyte; (b) macrogametocyte; (c) trophozoite; (d) schizont (Kaewthamasorn, unpublished). All images were taken at the same magnification through Olympus CK31 microscope.

The parasite was also found to infect monocytes, heterophils and thrombocytes (Macchi et al., 2013). However, monocytes can migrate in different tissues, which lead to the invading of parasites to the eyes, nerves, brain, heart, skeletal muscles, and many other organs. Furthermore, merozoites from blood circulation can invade macrophages and epithelial cells of the capillaries, which distribute to different tissues and organs, causing the secondary exo-erythrocytic state called phanerozoites. Unlike most infected mammals that have a single non-symptomatic exo-erythrocytic state, and parasitemia state as the only symptomatic phase, both parasitemia and exo-erythrocytic states in avian are malignant and lethal. Some of the invading merozoites differentiate into microgametocyte or macrogametocyte (Frevert et al., 2008; Valkiūnas and Iezhova, 2017).

Under the microscope, *Plasmodium* spp. form a distinguishing pigment in the erythrocytes as the result of hemoglobin metabolism, which is a crystalline called hemozoin (Goldberg et al., 1990). Still, the morphology between *P. gallinaceum* and *P. juxtanucleare* are difficult to distinguish, especially at developing trophozoite state and early state of schizont. When the next mosquitoes injects both male and female gametocytes form infected chicken, the parasite can continue their next life cycle.

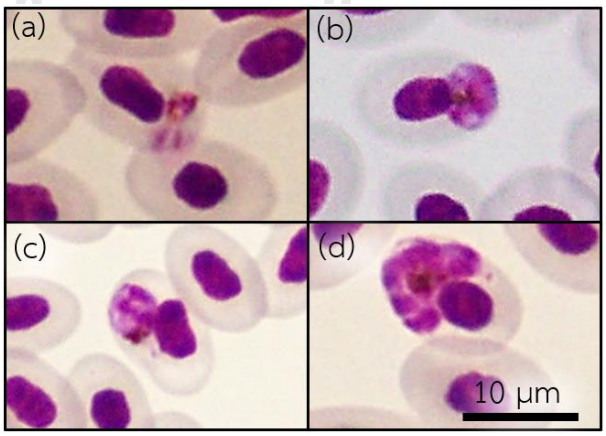

Figure 3 Early states of *P. gallinaceum* and *P. juxtanucleare* that is difficult to differentiate. *Plasmodium* spp. at trophozoite state with brown pigment (a, b) and

early state of schizont (c, d). All images were taken at the same magnification through Olympus CK31 microscope.

2.3 *Leucocytozoon* life cycle

Similar to *Plasmodium*, *Leucocytozoon* life cycle starts with infected black fly inject sporozoites into the chicken, sporozoites rapidly travel in the blood circulation to liver and differentiate into trophozoites, which develop into megaloschizonts in hepatocytes causing pressure atrophy in neighboring cells. After schizogony taking place, thousands of merozoites are released into the blood stream and invade different cell types, including red blood cells, leucocytes, macrophages, and epithelial cells, which distribute to many organs and body systems. This invasive capability is not only leading to the present of parasites in the eyes, brain, muscular system, but also in the reproductive organs like ovaries and oviducts, resulting in reducing egg productions. After invading blood cells, merozoites differentiate into either microgametocytes or macrogametocytes. Especially in *L. sabrazesi*, thrombocytes were proven to be cells housing gametocytes (Zhao et al., 2015).

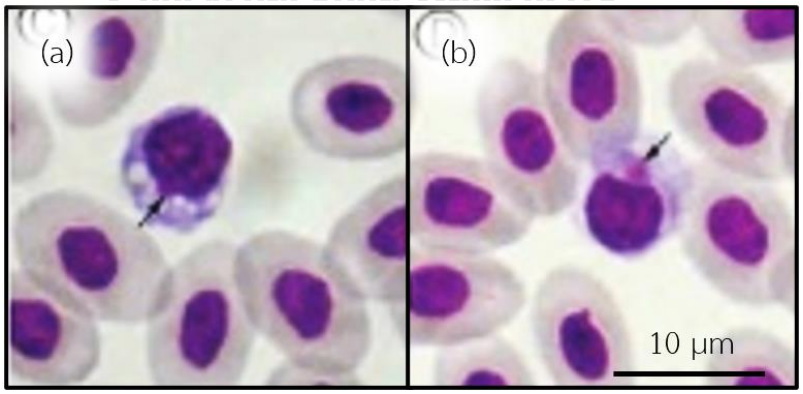

Figure 4 Putative young macrogametocyte (a) and microgametocyte (b) of *L. sabrazesi* in thrombocytes (modified from Zhao et al., 2015).

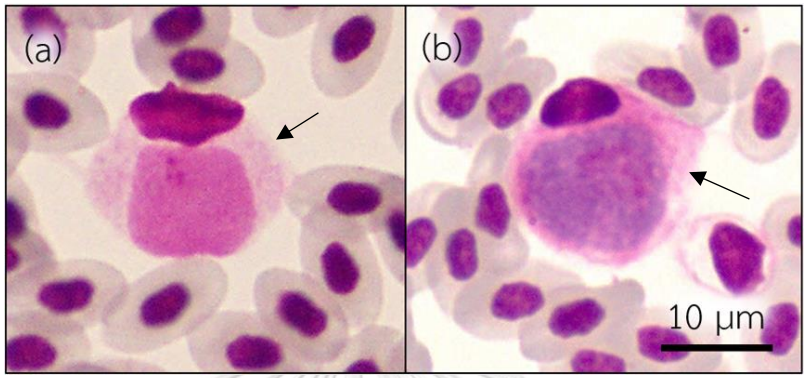

Figure 5 Microgametocyte (a) and macrogametocyte (b) of *L. sabrazesi.* All images were taken at the same magnification through Olympus CK31 microscope.

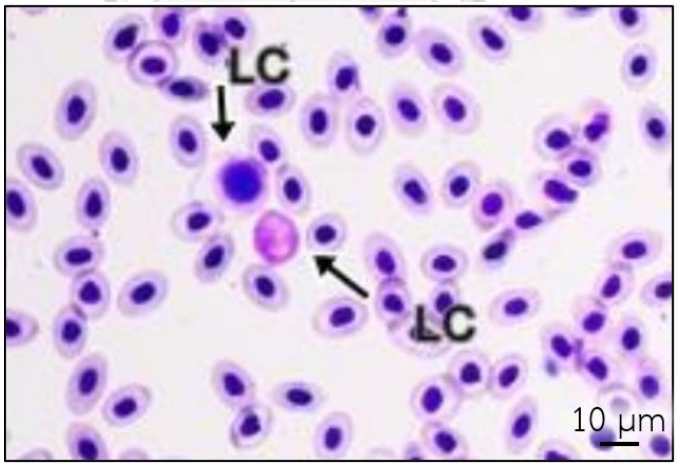

Figure 6 Microgametocyte and macrogametocyte of *L. caulleryi* (modified from Takang et al., 2017).

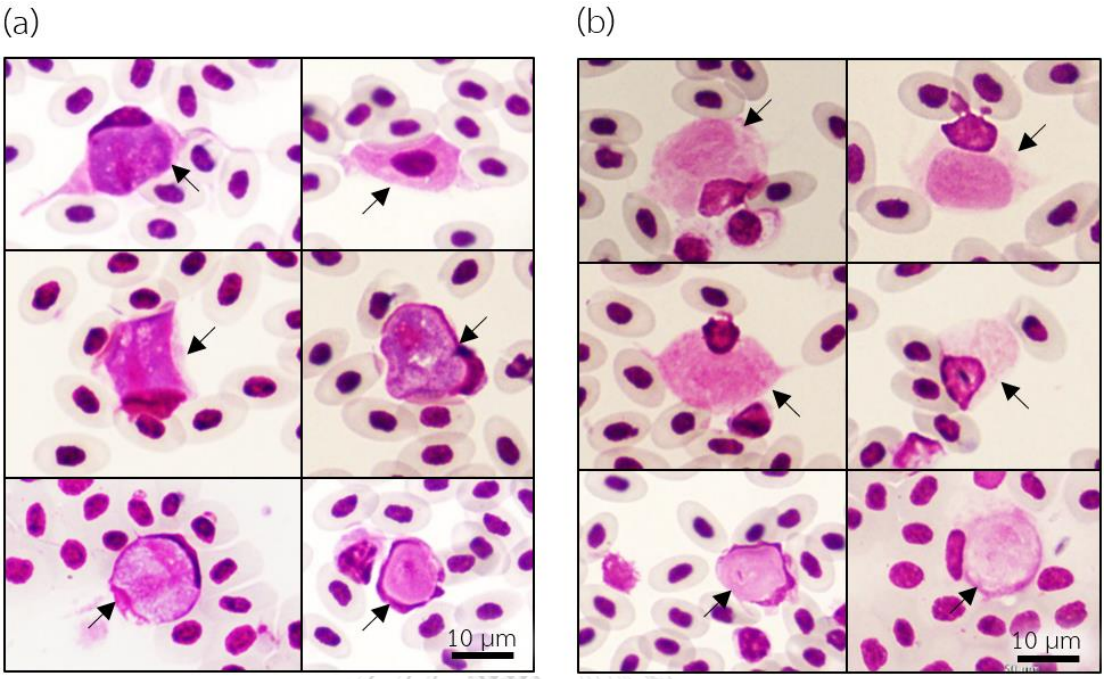

Figure 7 Different forms of *L. sabrazesi* (a) macrogametocytes; (b) microgametocytes. All images were taken at the same magnification through Olympus CK31 microscope.

When a black fly feed on the infected chicken, taking up male and female gametocytes, they fertilize and further develop into zygote and ookinete. The parasite is further undergone asexual development called sporogony similar to malaria parasites. Infected black fly then transmits leukocytozoonosis to another chicken by injecting sporozoites from their salivary gland into the new host (Valkiunas, 2004; Adler and McCreadie, 2019). Leucocytozoon do not produce pigment in their definitive host cells like *Plasmodium*, but they have rather outstanding appearance with variety morphology on the blood smear, which may lead to misdiagnosis.

2.4 Clinical symptoms

Haemosporidioses cause nonspecific clinical symptoms. Both malaria and leukocytozoonosis in chicken show depression, anorexia, reduce production

performance (Macchi Bde et al., 2010; Zhao et al., 2016) as many other diseases. Adding to these clinical signs, anemia cause by these blood parasites leading to paleness of comb and legs, making over all infected chickens appeared similar to chicken anemia virus infection (Wani et al., 2016). Above 50% parasitemia, *P. gallinaceum* also cause ataxia (Macchi Bde et al., 2010), which can be confused to tuberculosis (Graham and Tunnicliff, 1924). A side from anorectic, listlessness, and blood deficiency, *L. caulleryi* and *P. juxtanucleare* can also cause diarrhea (Silveira et al., 2009; Lee et al., 2016), which resembling coccidiosis (Soomro et al., 2001; Alnassan et al., 2014) and worm infection (Abdelqader et al., 2008) at some states. Furthermore, postmortem of *L. caulleryi* infected chickens show subcapsular hemorrhage and yellowish liver, or multifocal petechiae and lobes enlargement, which can be confused with fatty liver hemorrhagic syndrome (Lee et al., 2016).

2.5 Prevalence

Having such complex life cycle with the need of dipteral vectors, chicken haemosporidians are still widely distribute, especially in Asia countries (Yu and Wang, 2001; Imura et al., 2014). In South Korea, between 2009 and 2011, three layer farms were found to get leucocytozoonosis, and in 2013, a sudden outbreak of *L. caulleryi*, which cause the most lethal symptoms to *Gallus gallus domesticus*, happened to a commercial broiler breeder flock (Lee et al., 2013; Lee et al., 2016). In 2014, positive samples for a study in Japan were acquired from *L. caulleryi* infected chickens from Niigata, unveiling evidence of *L. caulleryi* infection in this area (Imura et al., 2014). From October 2014 to September 2015, a study in free-range chicken showed 56.9% prevalence of *L. sabrazesi* infection at seven area of southern China (Zhao et al., 2016). Recently in 2017, one study at three areas of District Layyah, Pakistan showed 69% chicken infected with haemoparasites species (Naqvi et al., 2017). In the same year, a research in backyard chickens in Chiang Mai showed high prevalence of blood

parasites (73.95%), among these, *L. sabrazesi* was 72.66% (Takang et al., 2017). However, the major update of epidemiology data about chicken haemosporidians is still lacking, especially in Thailand.

2.6 Treatment

Treatment for *Plasmodium* species infections of chicken is still limited in commercially available or approved malaria drugs. Though, some medicines such as chloroquine and doxycycline (Sohsuebngarm et al., 2014), or artesunate and tafenoquine (Tasai et al., 2017) showed effectiveness in reduce parasitemia and transmission experimentally. Exo-erythrocytic states of the parasites can lead to persistent parasitemia and relapse, which also make the successfulness of treatment varied between cases and flocks. Similarly, treatments for leukocytozoonosis are not excellently effective, pyrimethamine and sulfadimethoxine can be used for preventive medication. Several compounds such as primaquine, desipramine hydrochloride, sulfaquinoxaline and ketotifen were also tested experimentally (Zhao et al., 2016). In addition, developing vaccination for *L. caulleryi* did have some promising result in decreasing pathogenicity (Ito et al., 2013).

2.7 Diagnosis

For diagnostic of blood parasite infection, microscopic examination of blood smear is widely used due to the advantages of economical and rapid. However, the morphology variety of each species are confusing, in addiction, once species has different morphology in each state of their development. This method not only requires expert technicians, but also unpractical in fields studies with low parasitemia cases. For those reasons, several studies have applied Polymerase Chain Reaction (PCR), nested PCR, or even multiplex PCR for more accurate detection (Bensch et al., 2000; Perkins and Schall, 2002; Beadell et al., 2004; Hellgren et al., 2004;

Waldenström et al., 2004; Saiwichai et al., 2007; Zhao et al., 2016). In which, the primers were designed to detect the whole genus, or specific for one species and produce short amplicon size (Table 1). Those published primers were also applied in other studies as primers for single or nested PCR depending on researcher's objective (Bensch et al., 2009; Pattaradilokrat et al., 2015; Vanstreels et al., 2015; Lee et al., 2016; Jumpato et al., 2019). PCR is a molecular technique developed since 1983 by Kary Mullis (Bartlett and Stirling, 2003), using enzyme DNA polymerase to amplify any DNA sequence, with the addition of essential elements, in particular a pair of single strand DNA fragments that specific to the target sequence called primers, deoxynucleotide triphosphate (dNTP), buffer, and DNA template. Thermal cycling provides rotation environment of heating and cooling, which allows each PCR cycle to happen in three states. First state is DNA denaturation, in which two strands of double helix DNA separated under the effect of high temperature. Lower temperature in the second state allows primers to bind to their own complimentary sequences in the templates. During extension state, new DNA strands are formed via the assembling of free nucleotides by DNA polymerase (Porta and Enners, 2012). Nested PCR was modified from PCR with an addition of one more pair of primer, which is to reduce nonspecific amplification due to unexpected primer binding sites. In nested PCR, target DNA sequence encounters first reaction by external primers. Then PCR product from the first reaction undergoes second reaction with internal primers, which ensure the amplification of exact desired sequence (Pelt-Verkuil et al., 2008). Nonetheless, PCR techniques, which were proven both sensitivity and specificity, are not favorable and could be time consuming for screening natural infection and mixed infections, since actual infections that happen in the environment are unpredictable.

Thus, several researches used microscopy and molecular tecnique simultaneously for parasite detection (Bensch et al., 2009; Vanstreels et al., 2015; Zhao et al., 2015; Lee et al., 2016; Zhao et al., 2016; Suprihati and Yuniarti, 2017;

Lotta et al., 2019). In these studies, molecular technique provided high sensitivity but using non-specific primer, and microscopic examination can differentiate specific species, avoid false positive results but having low sensitivity. Multiplex PCR technique seems to be more ideal for parasite detection and epidemiology research. This technique is relatively fast, sensitive and specific as PCR yet can simultaneously detect several species (Chamberlain et al., 1988). Previous research by Ciloglu and colleagues introduced multiplex PCR into avian haemosporidians study in order to differentiate three genera *Plasmodium, Leucocytozoon* and *Haemoproteus* in birds (Ciloglu et al., 2019). However, the new method was not designed to detect parasite species in chicken, especially *Plasmodium* and *Leucocytozoon* spp. in chicken, which are no less important.

Considering the limit of low guanine and cytosine (GC) content, and highly conserved in avian malaria parasite genome (Videvall, 2018), practically the primers should be selected to be: (1) specific to each species for accurate amplification; (2) producing sequences with a sufficient different in length between different species, serving for the instant identification feature from agarose gel of the assay; (3) provide product with at least 400 bp and at most 1,500 bp to ensure informative sequences and precise amplification, respectively; (4) one external pair of primer that can amplified all four species to increase number of DNA templates, and four distinct pairs of primer specific for each species; (5) having high and similar melting temperatures (T_m) in order to bind correctly to their own template at the same annealing temperature (T_a); (6) all target sequences locate in coding region.

Table 1 Published primers used to detect avian haemosporidian parasites

Molecular method	Primers	Target		Product size (bp)	Reference
Single PCR	HaemF: 5' ATGGTGCTTTCGATATATGCATG 3' HaemR2: 5' GCATTATCTGGATGTGATAATGGT 3'	*cytb*	*Haemoproteus* and *Plasmodium*	479	Bensch et al. 2000
Nested PCR	DW2: 5' TAATGCCTAGACGTATTCCTGATTATCCAG 3' DW4: 5' TGTTTGCTTGGGAGCTGTAATCATAATGTG 3'	*cytb*	*Haemoproteus*, *Plasmodium* and *Leucocytozoon*	1,255	Perkins & Schall 2002
	DW1: 5' TCAACAATGACTTTATTTGG 3' DW6: 5' GGGAGCTGTAATCATAATGTG 3'	*cytb*		1,138	
Single PCR	3760F: 5' GAGTGGATGGTGTTTTAGAT 3' 4292Rw2: 5' TGGAACAATATGTARAGGAGT 3'	*cytb*	*Haemoproteus* and *Plasmodium*	533	Beadell et al. 2004
Nested PCR	HaemNFI: 5' CATATATTAAGAGAAITATGGAG 3' HaemNR3: 5' ATAGAAAGATAAGAAATACCATTC 3'	*cytb*	*Haemoproteus*, *Plasmodium* and *Leucocytozoon*	617	Hellgren et al. 2004
	HaemFL: 5' ATGGTGTTTTAGATACTTACATT 3' HaemR2L: 5' CATTATCTGGATGAGATAATGGIGC 3'	*cytb*	*Leucocytozoon* spp.	479	
Nested PCR	HaemNF: 5' CATATATTAAGAGAATTATGGAG 3' HaemNR2: 5' AGAGGTGTAGCATATCTATCTAC 3'	*cytb*	*Haemoproteus* and *Plasmodium*	617	Waldenström et al. 2004
	HaemF: 5' ATGGTGCTTTCGATATATGCATG 3' HaemR2: 5' GCATTATCTGGATGTGATAATGGT 3'	*cytb*		479	
Multiplex PCR	FP1: 5’ ACTTGACCGATTGTTCCTCATCGCCTTT 3’ FP2: 5’ AGTTCGTGAATATGATTTGTCTGGT 3’ RP1: 5’ TTGTTGCCTTAAACTTCCTTGTGTT 3’	small subunit ribosomal RNA	FP1-RP1: *P. gallinaceum*	1,527	Saiwichai et al., 2007
			FP2-RP1: *Plasmodium* spp.	279	
Single PCR	Ls-cytbF1: 5’ TAATCACATGGGTTTGTGGA 3’ Ls-cytbR1: 5’ GCTTTGGGCTAAGAATAATACC 3’	*cytb*	*L. sabrazesi*	248	Zhao et al., 2016
	Ls-coxIIIF2: 5’ TAACATTCTACATGATGTAGT 3’ Ls-coxIIIR2: 5’ GTAAAAGCACACTTATCTAG 3’	*coxIII*		294	

CHAPTER 3

MATERIALS AND METHODS

3.1 Reference parasites

L. sabrazesi, *P. gallinaceum*, *P. juxtanucleare,* and microfilaria positive blood samples were previously kept at Veterinary Parasitology Unit of Faculty of Veterinary Science, Chulalongkorn University as the teaching specimens or reference samples. The samples were collected in 2017, originated from free-range chickens in Nan province, Thailand. *Trypanosoma* sp. positive sample were collected in 2019 from dairy cow in Nakhonratchasrima province. Infections of these samples were confirmed by microscopic examination and DNA sequencing. DNA of *L. caulleryi* was kindly provided by Dr. Saruda Tiwananthagorn from Faculty of Veterinary Medicine, Chiang Mai University.

3.2 Sample collections

An average of 0.5 mL blood samples were collected from wing veins of free range chickens into 1.5 ml EDTA tubes. Blood samples were collected from the following sites: Nan, Prachinburi, and Chachoengsao Provinces. This study was reviewed and approved by the Chulalongkorn University Faculty of Veterinary Science Biosafety Committee (IBC 1931011), and was approved by the Institutional Animal Care and Use Committee in accordance with university regulations and policies governing the care and use of laboratory animals (No. 1931091).

3.3 Microscopic examination

Blood samples were smeared onto glass slides, air-dried, fixed with 100% methanol, and undergone Giemsa staining using standard protocol. Remaining blood was stored at -20 °C. The blood slides were screened for haematozoa. Parasitemia and gametocytemia of haemosporidian parasites were calculated as the proportion

of infected cells per 10,000 red blood cells. Parasitemia of *Trypanosoma* sp. were estimated as number of parasites per 1 µl blood.

3.4 Preparation of target DNA

3.4.1 DNA cloning of avian haemosporidian parasites

PCR products of each parasite were amplified from known infected blood samples and gDNA, using universal primers targeting mitochondrially encoded cytochrome C oxidase III (hereafter referred to as *cox 3* gene) (Table 2) with MightyAmp® PCR polymerase (Takara, Japan). Touchdown PCR condition was set as: 98 °C in initial denaturation for 2 min; 30 cycles of: 98 °C in denaturation of 20 sec, 60 °C in annealing for 20 sec, and 68 °C in extension for 1 min 30 sec; 11 cycles of: denaturation at 98 °C in 20 sec, annealing at 55 °C for 20 sec, extension at 68 °C in 1 min 30 sec; and finally 68 °C in final extension for 5 min; 12 °C was storing temperature. The PCR products then contain nucleotide A at 3'-termini for the TA cloning, the bands at the expected size from 1,137 bp to 1,168 bp were purified from agarose gel using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany). Purified PCR products were quantified by NanoDrop spectrophotometers (Thermo Scientific, USA) to adjust appropriate concentration of inserts for the ligation reaction. TArget Clone™ kit with pTA2 Vector (TOYOBO, Japan) were used for creating recombinant plasmids. Ligated products were transformed into competent cells (*E. coli* strain DH5α™) at 42 °C for 45 sec. Super Optimal broth with Catabolite repression (SOC) medium was added into transformed cells and incubated at 37 °C for 40 min in shaking incubator at 225 rpm. Pre-culture cells were spread onto LB agar containing ampicillin and X-gal for antibiotic and blue-white colony selections. The Luria-Bertani (LB) plates were incubated at 37 °C for 16 hours. White colonies were selected for colony PCR using M13F and M13R universal primers. Colonies showing expected size of insertion were propagated in 5 ml LB broth medium

containing ampicillin and incubated at 37 °C overnight in shaking incubator at 225 rpm.

3.4.2 Plasmid preparation

After *E. coli* cell propagation, plasmids were purified using NucleoSpin® Plasmid EasyPure kit (Macherey-Nagel, Germany). Bacteria cells were undergone lysis to release recombinant plasmid and other contents, including bacteria genome, using denaturing solutions. Renaturing solution was added in the next step to precipitate bacterial proteins, as well as chromosomal DNA, leaving small recombinant plasmids unbound in the solution. Following centrifugation, plasmids in the supernatant were further eluted by using ethanol to remove excess salt, then suspending with distilled water. The protocol of plasmid extraction was conducted according to the manufacturer's instruction, the only different part was the last step, where only 20 µl to 30 µl of elution buffer was added rather than 50 µl in order to increase plasmid concentration. The extracted plasmid suspensions were sent for sequencing to confirm parasite species.

3.5 Primer designs

Complete mitochondria sequences of *L. caulleryi* (GenBank™ accession number AB302215), *L. sabrazesi* (AB299369), *P. gallinaceum* (AB250690 and AB599930), and *P. juxtanucleare* (AB250415 and MG598397) were retrieved from GenBank database. Sequences were aligned using Clustal W option implemented in BioEdit or Clustal Omega software, the latter is freely access at www.ebi.ac.uk. Primers were designed with the following criteria: (1) specific to each species; (2) producing sequences with a sufficient different in length between different species; (3) provide product with at least 400 bp and at most 1,500 bp; (4) all four distinct pairs of primers specific for each species located within the same external pair of

primers that can amplified all four species; (5) having high and similar melting temperature (T_m); (6) target sequences locate in coding region. T_m and T_a were calculated with Tm Calculator program freely access at www.thermofisher.com. Primers were further tested in silico by using Primer-BLAST to confirm their specificity in GenBank database. Illustration of primer locations compared to *cox 3* gene on haemosporidian mitochondria DNA genome, specificity of forward and reverse primer sequences, as well as melting temperature, GC content, expected size of amplicon for each target are shown in Figure 8, Figure 9 and Table 2.

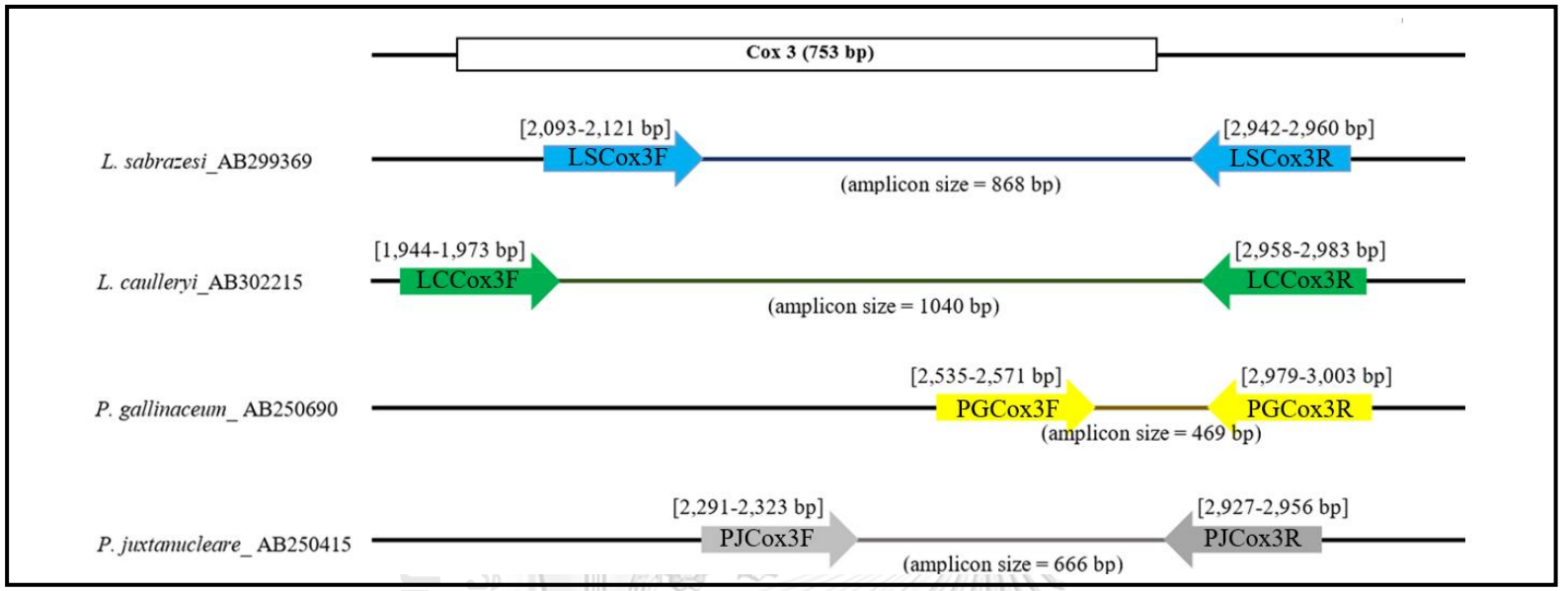

Figure 8 Diagram depicting primer locations compared to *cox 3* gene locus on haemosporidian mitochondria genome**.** Primers were designed specifically to differentiate four haemosporidian parasite species in chickens. Forward and reverse primers for each species are represented as: *L. sabrazesi* displayed in blue arrows, *L. caulleryi* in dark green arrows, *P. gallinaceum* in yellow arrows, and *P. juxtanucleare* in gray arrows. Primer locations on *L. sabrazesi* (accession number AB299369), *L. caulleryi* (AB302215), *P. gallinaceum* (AB250690) and *P. juxtanucleare* (AB250415) in the alignment are indicated accordingly in square brackets. Each pair of primers produces different amplicon size, which are written in parentheses, and their length are illustrated as: *L. sabrazesi* in dark blue line, *L. caulleryi* in dark green line, *P. gallinaceum* in dark yellow line, and *P. juxtanucleare* in dark gray line, including their primers. *Cox 3* stand for cytochrome C oxidase III encoding gene.

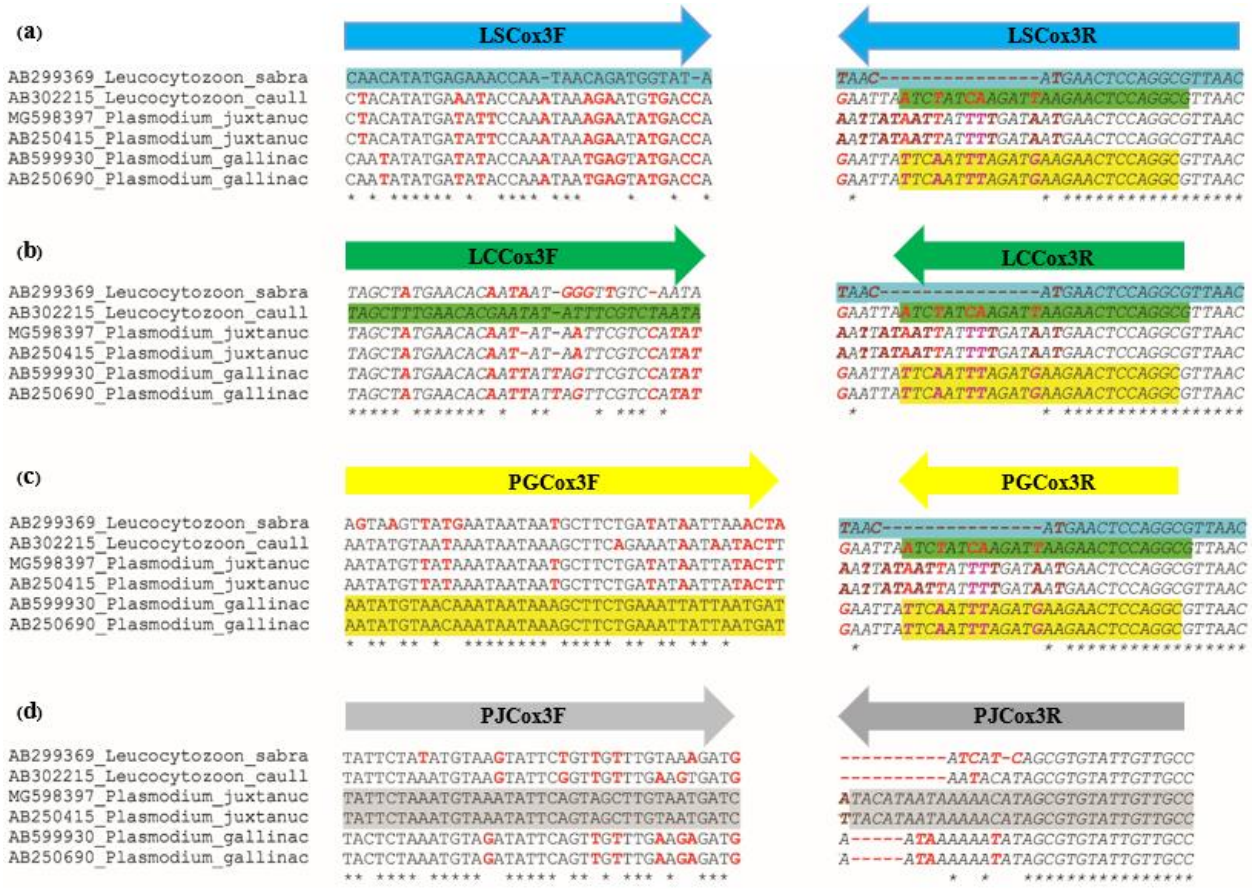

Figure 9 Forward and reverse primer sequences for specific detection and amplification of *L. sabrazesi* **(a)**, highlighted in blue, *L. caulleryi* **(b)** highlighted in dark green, *P. gallinaceum* **(c)** highlighted in yellow, and *P. juxtanucleare* **(d)** highlighted in gray. Mismatch and gap are indicated in red. Identical nucleotides are shown with asterisk.

Table 2 Primers to be used in this study.

Parasite species	Primer name and sequence (5' to 3')	No. nucleotide	%CG	T_m (°C)	T_a (°C)	Amplicon Size (bp)
Haemosporidians	LeucoPlasCox3F: ACACACTTCCCTTCTCGCC	16	62.50	61.2	64.7	At least 1,137
	LeucoPlasCox3R: CCTTTCCGGCTGTTTCC	17	58.82	62.9		
L. caulleryi	LCCox3F: GCTTTGAACACGAATATATTTCGTCTAATA	30	30.00	62.9	66.3	1,040
	LCCox3R: GCCTGGAGTTCTTAATCTTGATAGAT	26	38.46	63.4		
L. sabrazesi	LSCox3F: CATATGAGAAACCAATAACAGATGGTATA	29	31.03	62.0	65.4	868
	LSCox3R: ACGCCTGGAGTTCATGTTA	19	47.37	63.1		
P. juxtanucleare	PJCox3F: CTAAATGTAAATATTCAGTAGCTTGTAATGATC	33	27.27	61.7	65.2	666
	PJCox3R: CAACAATACACGCTATGTTTTTATTATGTA	30	26.67	61.8		
P. gallinaceum	PGCox3F: TGTAACAAATAATAAAGCTTCTGAAATTATTAATGAT	37	18.92	62.3	65.5	469
	PGCox3R: CCTGGAGTTCTTCATCTAAATTGAA	25	36.00	62.1		

T_m: Melting temperature, T_a: Annealing temperature

3.6 Primer validations

3.6.1 Ability in species-specific amplification of singleplex versus multiplex PCRs

Species-specific amplification capacity of singleplex PCR

Analytical specificity for each pair of primers was tested with conventional PCR. Either gDNA or crude blood samples of known haemosporidian infected samples were used to determine primer capacity to amplify its corresponding target. Samples with mixed infections were chosen for specificity assessment. List of samples used for specificity assessment is showed in Table 3. Genomic DNA samples infected with *L. caulleryi* were kindly provided by Dr. Saruda Tiwananthagorn from Faculty of Veterinary Medicine, Chiang Mai University. Infection of all remaining blood samples were confirmed by microscopic examination. Sterile distilled water was also used as a no template negative control.

Having similar T_m, conditions for singleplex PCRs for each pair of primers were set in the same manner. All reactions were prepared in a total volume of 12.5 µl containing 0.5 µl of template, 0.4 mM of dNTPs, 1x KOD buffer (Toyobo, Japan), 0.2 pmol/µl of each primer, as well as 0.25 unit of KOD FX Neo polymerase (Toyobo, Japan). The cycling condition was set as follow: initial denaturation at 94 ℃ for 2 min, 40 cycles of denaturation at 94 ℃ for 20 sec, annealing at 66 ℃ for 30 sec, extension at 68 ℃ for 1 min and 30 sec, and a final extension step at 68 ℃ for 3 min, then PCR products was stored at 12 ℃. PCR products from singleplex PCR with each primer pair were later sent for sequencing for verification.

Table 3 List of positive and negative samples used for specificity assessment for each primer set

Sample ID	Parasite*		
	Microscopy	Quantity or parasitemia	PCR-sequencing
THChick-LC1	*L. caulleryi*	10 ng/µl	*L. caulleryi*
THChick-LC2	*L. caulleryi*	50 ng/µl	*L. caulleryi*
THChick17-N-7	*L. sabrazesi*, *P. juxtanucleare*, *P. gallinaceum*, microfilaria	0.34%	*L. sabrazesi*, *P. juxtanucleare*, *P. gallinaceum*
THChick17-N-19	*P. juxtanucleare*, *P. gallinaceum*	0.03%	*P. juxtanucleare*, *P. gallinaceum*
THChick17-N-32	*L. sabrazesi*, *P. juxtanucleare*, *Plasmodium* spp.	0.05%	*L. sabrazesi*, *P. juxtanucleare*
THChick17-N-33	*L. sabrazesi*, *P. juxtanucleare*, *Plasmodium* spp.	0.03%	*L. sabrazesi*, *P. juxtanucleare*
THChick17-N-36	*L. sabrazesi*	0.02%	*L. sabrazesi*
THChick17-N-37	*L. sabrazesi*, *P. juxtanucleare*	0.15%	*L. sabrazesi*, *P. juxtanucleare*
THChick17-N-42	*L. sabrazesi*, *P. juxtanucleare*, *Plasmodium* spp.	0.05%	*L. sabrazesi*, *P. juxtanucleare*
THChick19-005	*P. juxtanucleare*	0.18%	*P. juxtanucleare*
THChick19-003	Negative	-	-
THDairy-008	*Trypanosoma* spp.	1,293 parasite/µl	-

Note: *based on microscopic and PCR results

Species-specific amplification capability of multiplex PCR

Plasmids containing *L. sabrazesi*, *L. caulleryi*, *P. gallinaceum*, and *P. juxtanucleare* DNA were confirmed by PCR and sequencing. Copy number of parasite DNA was calculated using an online web-based application freely access at https://cels.uri.edu/gsc/cndna.html. The formula for calculation was as follow: number of copies = (amount x 6.022 x 10^{23}) / (length x 1 x 10^{9} x 650). DNA templates of each parasite were titrated from 10^{10} to 10^{1} copies per microliter. A mixture of plasmid DNA was prepared to serve as a single and co-infection of each parasite.

Analytical specificity or potential cross amplification was evaluated using 15 sets of DNA template mixtures representing single and co-infections of each parasite mentioned above. Sterile distilled water was used as a no template negative control. All primer pairs were initially tested at concentration of 0.4 pmol/µl in multiplex PCR reactions. Each PCR reactions was prepared in a total volume of 12.5 µl containing 1 µl of DNA template, 0.4 mM of dNTPs, 1x KOD buffer (Toyobo, Japan), 0.25 unit of KOD FX Neo polymerase (Toyobo, Japan) (Figure 11). Then the primer concentrations were re-adjusted to maximize the amplification for all four targets. The concentrations of the following primer pairs; LCCox3F-R, LSCox3F-R, PGCox3F-R, and PJCox3F-R were adjusted to 0.4, 0.6, 0.7, and 0.3 pmol/µl, respectively for multiplex PCR reactions (Figure 12). The thermal cycling condition was set as follow: 94 ℃ for initial denaturation for 2 min, 40 cycles of 94 ℃ for denaturation in 20 sec, 67 ℃ for annealing in 20 sec, 68 ℃ for extension in 20 sec, and a final extension step at 68 ℃ for 3 min, then PCR product was stored at 12 ℃. Assessments of the primer specificity were carried out in triplicates.

Crude blood samples with natural infections of one or more parasites, uninfected as well as unrelated samples (*Trypanosoma* spp. positive) were also included to assess primer specificity in multiplex PCR. Thermal cycling conditions for crude samples were set as: 94 ℃ of initial denaturation for 2 min, 40 cycles of 94 ℃

for denaturation for 20 sec, 66 ℃ of annealing for 30 sec, 68 ℃ of extension for 45 sec, and a final extension step at 68 ℃ for 3 min, then PCR product was stored at 12 ℃.

3.6.2 Validation of detection limits

Analytical detection limit of singleplex PCR

The sensitivity of each pair of primer were assessed with concentrations of 0.2 pmol/µl and 0.4 pmol/µl to compare the amplification efficiency. The serial dilutions of template DNAs were made from 10^{10} to 10^{1} copies per reaction, respectively. Only the serial dilution of *P. gallinaceum* was carried out from 10^{11} to 10^{1} copies. Detection limit was assessed in triplicates. All PCR reactions was carried out in a total volume of 12.5 µl containing 1 µl of template, 0.4 mM of dNTPs, 1x KOD buffer (Toyobo, Japan), 0.2 or 0.4 pmol/µl of primers, as well as 0.25 unit of KOD FX Neo polymerase. The cycling condition was set according to a protocol used with specificity assessment mentioned earlier.

Analytical detection limit of multiplex PCR

Serial dilutions of DNA mixtures representing a quadruple infection were assessed from 10^{11} to 10^{1} copies/µl in the multiplex PCR assay. All reactions were carried out with reaction mixture and cycling condition similar to a protocol for specificity assessment.

3.6.3 Application of multiplex PCR to the field samples

After determining the suitable condition for crude blood sample, the newly established protocol was tested with field collected blood samples. A total of 352 blood samples from free range chickens were included. These samples were

originated from Prachinburi (98) and Chachoengsao (43). From Nan, 173 blood samples were collected, among these only 16 blood smears were unavailable. Multiplex PCR was applied to all 352 blood samples. Among these, only 157 samples from 3 provinces with blood smear available were used to compare with microscopic examination.

3.7 Data analysis

Data were assessed as % positivity of each pathogen or % co-infection from each collection sites by multiplex PCR and by microscopic examination. Correlation between the multiplex PCR and microscopic examination was evaluated by Kappa and McNemar Test to define if there are statistical significantly different between microscopic examination and multiplex PCR. The whole statistical analysis was conducted using IBM SPSS Statistics 22.

3.8 Places of Study

Room 1315, Veterinary Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. Henri-Dunant Rd, Patumwan District, Bangkok, 10330 THAILAND.

3.9 Benefits of the study

This study provided a more precise method to simultaneously detect all four avian haemosporidian parasites in chickens. Additionally, the study could contribute in providing the update epidemiology data of prevalence and distribution of each chicken haemosporidian parasites.

CHAPTER 4

RESULTS

4.1 In silico validation of primers and sequence confirmation of reference DNA templates

The results from Primer-BLAST indicated that all primer pairs have low CG content, which range from 18.92% to 47.73% relatively high self-complementarity, ranging from 4 to 10, and self-3' complementarity, ranging from 2 to 8 (Table 4). No unintended targets or any other parasite sequences that might be found in chicken's blood sample are bound to the primers.

Precisely, Primer-BLAST could only find *L. caulleryi* (AB302215) and *L. sabrazesi* (AB299369) as feasible target for LCCox3F-R and LSCox3F-R, respectively. For PJCox3F-R, *P. juxtanucleare* (AB250415, MG598392, MG598393, MG598396) is the only potential target that did not present any mismatch. Other species that were listed in Primer-BLAST results like *P. atheruri* (HQ712051), *P. berghei* (LT608264, LT614642, LT608152, LK023131, AB558173, AF014115), *P. vinckei* (LR215072), *P. chabaudi* (LR215072, AB379671, AB379670, AB379669, AB379668, AB379667, AB379665, AB379664, AB379663, AF014116), *P. yoelii* (LM993670, M29000) and *Hepatocystis* spp. (LR699572) contained 8 to 10 mismatches scattered around 3' end, and they are not chicken infecting species. Result of PGCox3F-R also exhibited *P. gallinaceum* (AB250690, AB599930, LN835294) as the most possible target, other species such as *P. lutzi* (KY653816, KY653815, KC138226), *P. relictum* (AY733089), and *P. homocircumflexum* (KY653784) contained 3 to 4 mismatches scattered around 3' end, and had never been reported as natural pathogens chicken before. Furthermore, according to these results, the specific primer pairs are unlikely to cross amplify each other's target.

Table 4 Primer-BLAST Results

Primer	%CG	SC	3’ SC	Possible target templates (accession no.)	No. mismatches	Host
LCCox3F	30.00	10	6	*L. caulleryi* (AB302215)	0	Chickens, turkeys, waterfowl, and wild birds worldwide
LCCox3R	38.46	5	5			
LSCox3F	31.03	6	4	*L. sabrazesi* (AB299369)	0	Chickens
LSCox3R	47.73	6	2			
PJCox3F	27.27	6	4	*P. juxtanucleare* (AB250415, MG598392, MG598393, MG598396)	0	Birds and chickens
				P. atheruri (HQ712051),	8	Rodents
				P. berghei (LT608264, LT614642, LT608152, LK023131, AB558173, AF014115),	8	
				P. vinckei (LR215072),	9	
				P. chabaudi (LR215072, AB379671, AB379670, AB379669, AB379668, AB379667, AB379665, AB379664, AB379663, AF014116),	9	
PJCox3R	26.67	4	2	*P. yoelii* (LM993670, M29000)	9	
				Hepatocystis spp. (LR699572)	10	Primates, bats, squirrels
PGCox3F	18.92	8	8	*P. gallinaceum* (AB250690, AB599930, LN835294)	0	Chickens, birds
PGCox3R	36.00	6	6	*P. lutzi* (KY653816, KY653815, KC138226),	3	Birds
				P. relictum (AY733089),	4	
				P. homocircumflexum (KY653784)	5	

SC: Self- complementarity, 3’ SC: Self-3' complementarity

Sequencing results of the plasmid suspensions showed high identity percentage to *L. caulleryi* (AB302215), *L. sabrazesi* (AB299369), *P. gallinaceum* (LN835294), and *P. juxtanucleare* (MG598396) with 99.91%, 98.65%, 99.47%, and 99.64%, respectively (Table 5). The identities of four reference DNA templates were confirmed.

Table 5 Sequence confirmation of *cox 3* genes of reference parasites in plasmids used in this study

Sample ID	Insert length (bp)	Closely related to	Origin	% Identity	Accession No.
THChick-LC1	1,215	*L. caulleryi*	Japan	99.91	AB302215
THChick17-65	1,200	*L. sabrazesi*	Malaysia	98.65	AB299369
THChick-G2	1,122	*P. gallinaceum* (strain 8A)	Sri Lanka	99.47	LN835294
THChick19-35	1,124	*P. juxtanucleare* (strain Pj225)	Brazil	99.64	MG598396

Note: DNA fragments of all samples were amplified by LeucoPlasCox3F-R primers.

4.2 Primers' ability in species-specific amplification

4.2.1 Species-specific amplification capability of singleplex PCR

L. caulleryi, L. sabrazesi, *P. juxtanucleare*, and *P. gallinaceum* naturally infected chickens as confirmed by microscopy and DNA sequencings, were used as templates in singleplex PCRs (Table 3). All four primer pairs gave expected amplicon sizes of their corresponding targets at 1,040, 868, 666, and 469 bp for *L. caulleryi, L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum*, respectively. No non-specific band was observed either in tested samples or in a no template negative control (Figure 10).

Singleplex PCR with LCCox3F-R primer pair generated an expected amplicon size of 1,040 bp for *L. caulleryi*. No amplification product was observed in sample ID

THChick17-N-7, which was microscopically positive for *L. sabrazesi*, *P. juxtanucleare*, *P. gallinaceum*, and microfilaria (Figure 10a). Singleplex PCR using LSCox3F-R primers amplified the target fragments of 868-bp long in sample IDs THChick17-N-7, -N-33, -N-42, and -N-19 (Figure 10b). It is important to note that *L. sabrazesi* was not observed under microscopic examination for sample ID THChick17-N-19. A positive band at 868 bp suggesting that this sample was also co-infected with *L. sabrazesi*. Amplified products at 666 and 469 bp were detected in sample IDs THChick17-N-7, -N-19, -N-32, and -N-36 with primers PJCox3F-R and PGCox3F-R, respectively (Figure 10c-d) indicating a co-infection of *P. juxtanucleare* and *P. gallinaceum*. Besides *L. sabrazesi* and *P. juxtanucleare* which were observed under microscope in sample ID THChick17-N-32, early schizont state of *Plasmodium* spp. was also found. This was not be able to distinguish between *P. juxtanucleare* and *P. gallinaceum*. A clear band at 469 bp with PGCox3F-R primers suggesting that sample ID THChick17-N-32 was also co-infected with *P. gallinaceum*.

All PCR products were sent for sequencing for specificity confirmation and verification. Sequencing results confirmed that the primers were able to amplify their respective targets, even in the presence of different parasite species, host's DNA and microfilaria (Table 6). The singleplex PCR positive results for *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum* in sample IDs THChick17-N-19 and -N-36 which were undetectable by microscopy suggested that the present PCR methods were more sensitive (Figure 10c, -d).

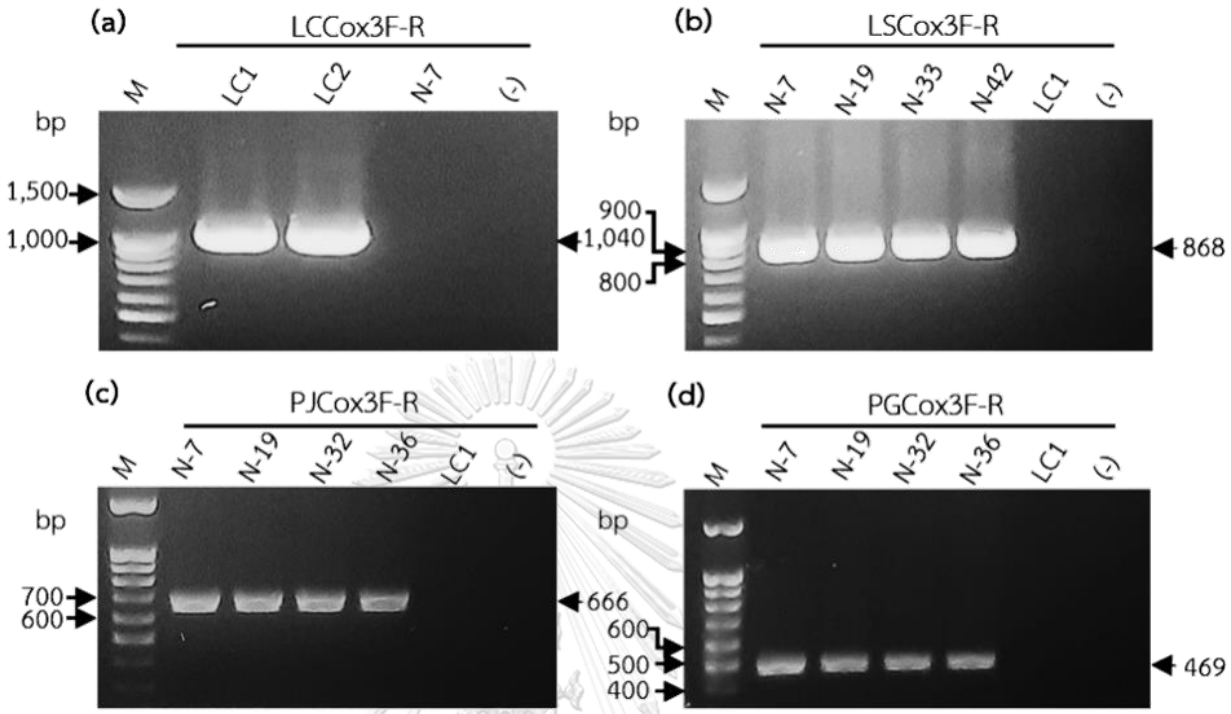

Figure 10 Singleplex PCR amplifications using samples with natural infections of *L. caulleryi*, *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum* as the templates. PCR products (1,040 bp) were amplified by primers LCCox3F-R using *L. caulleryi* positive samples (sample IDs THChick-LC1 and -LC2), and sample with co-infections of *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum* (THChick17-N-7) **(a)**. PCR products amplified by LSCox3F-R primers showing amplicon size of 868 bp which is specific to *L. sabrazesi* **(b)**. Sample IDs THChick17-N-7 and -N-19 contain *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum*, while THChick17-N-42 and THChick-LC1 have a single infection of *L. sabrazesi* and *L. caulleryi*, respectively. PCR products obtained from PJCox3F-R and PGCox3F-R primer pairs showing the expected amplicon size at 666 and 469 bp for *P. juxtanucleare* and *P. gallinaceum*, respectively **(c, d)**. Sample IDs THChick17-N-7, -N-19, -N-32, -N-36, and THChick-LC1 were included. Sterilized distilled water was used as a no-template negative control (-). M=1 Kb DNA Ladder.

Table 6 Sequence confirmation of singleplex PCR products from each species-specific primer set

Primer set	Sample ID	bp	Closely related to	Origin	% Identity	Accession No.
LCCox3F-R	THChick-LC1	354	*L. caulleryi*	Japan	99.72	AB302215
	THChick-LC2	313	*L. caulleryi*	Japan	98.40	AB302215
LSCox3F-R	THChick17-N-7	402	*L. sabrazesi*	Malaysia	98.26	AB299369
	THChick17-N-19	402	*L. sabrazesi*	Malaysia	98.26	AB299369
	THChick17-N-33	402	*L. sabrazesi*	Malaysia	98.26	AB299369
	THChick17-N-42	402	*L. sabrazesi*	Malaysia	98.26	AB299369
PGCox3F-R	THChick17-N-7	216	*P. gallinaceum* (strain 8A)	Sri Lanka	100	LN835294
	THChick17-N-19	216	*P. gallinaceum* (strain 8A)	Sri Lanka	100	LN835294
	THChick17-N-32	216	*P. gallinaceum* (strain 8A)	Sri Lanka	100	LN835294
	THChick17-N-36	216	*P. gallinaceum* (strain 8A)	Sri Lanka	100	LN835294
PJCox3F-R	THChick17-N-7	227	*P. juxtanucleare*	Japan	100	AB250415
	THChick17-N-19	227	*P. juxtanucleare*	Japan	100	AB250415
	THChick17-N-32	227	*P. juxtanucleare*	Japan	100	AB250415
	THChick17-N-36	227	*P. juxtanucleare*	Japan	100	AB250415

4.2.2 Species-specific amplification capacity PCR of multiplex PCR using plasmids containing parasite's DNA as the templates

Multiplex PCR reactions were carried out to assess a potential cross amplification of each primer pairs in a single, double, triple, and quadruple infections of each four parasites. The reactions containing four pairs of primers clearly amplified their respective targets without any non-specific band. With mixtures of two parasite's DNA as a double infection (6 different combinations), multiplex PCR were able to generate two specific amplicon sizes for all reactions. Multiplex PCR reactions using mixtures of three parasite DNAs (4 different combinations), all three expected bands were detected suggesting the amplifications were successful. Multiplex PCR reaction using a mixture of all four DNA templates gave all four expected bands. However, amplification efficiency of primer sets targeting *L. caulleryi* and *P. juxtanucleare* seems to be superior to primers targeting the rest two parasites based on amplicon intensity observed in reactions using combination mixtures of 2, 3, and 4 parasite's DNAs (Figure 11). Collectively, no cross amplification was observed in all reactions.

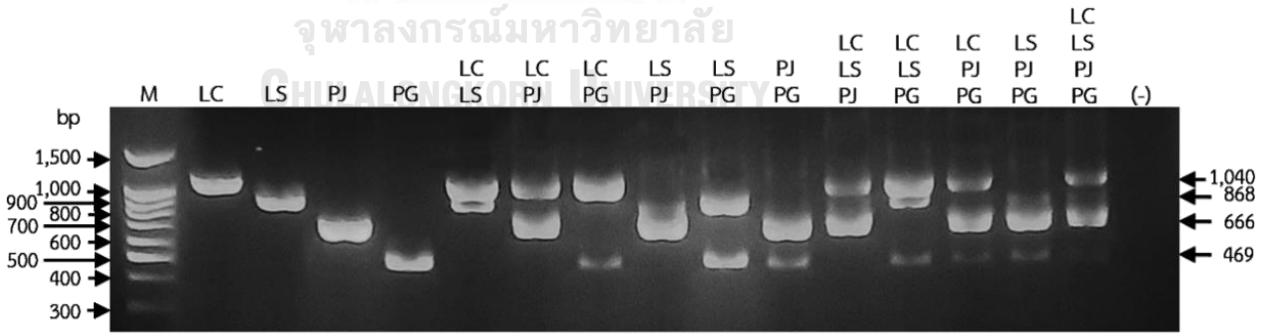

Figure 11 Multiplex PCR results with equal concentrations of each primer pair (0.4 pmol/µl). Expected amplicons corresponding to *L. caulleryi* (LC), *L. sabrazesi* (LS), *P. juxtanucleare* (PJ), and *P. gallinaceum* (PG) were detected at 1,040, 868, 666, and 469 bp, respectively. Sterilized distilled water as no-template negative control (-) was used. M: DNA Ladder.

Based on the amplification results with the preliminary reaction mixtures, concentrations of each primer pair were re-adjusted to enhance amplification efficiency or to optimize the reaction for all four targets. Concentrations of PJCox3F-R, LCCox3F-R, LSCox3F-R, and PGCox3F-R were adjusted to 0.3, 0.4, 0.6, and 0.7 pmol/µl per reaction, respectively. All four targets were successfully amplified either in reactions containing a single, double, triple, or quadruple infections as expected. After re-adjustment, it seemed that all amplicons were clearly detectable under the UV transilluminator. All amplicons appeared with the right expected sizes. Unless the right designated parasite species presented as the templates, all of four primer pairs did not produce any non-specific band. There was no PCR product appear in a no-template negative control. The single bands of each 4 single infections proved that cross amplification between primers and templates did not occur. Each band from 4 single infections were relatively clearer and brighter compared to those of quadruple infections (Figure 12). This result further confirmed the specificity of all four primer sets in the multiplex PCR reactions.

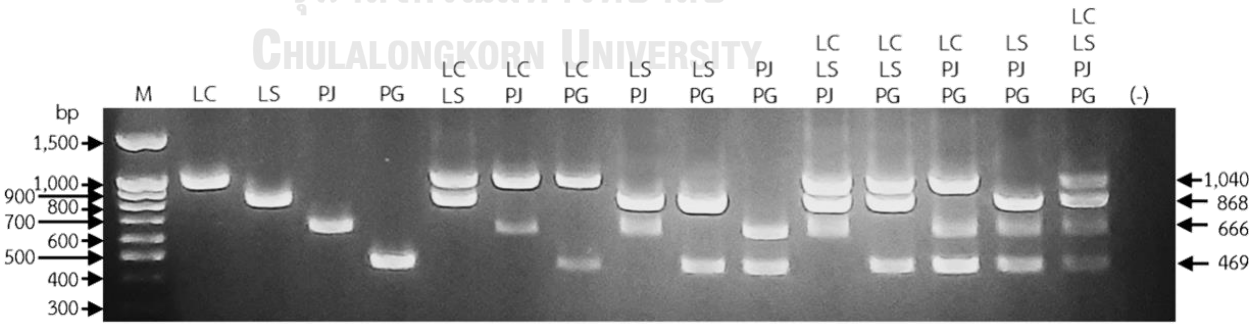

Figure 12 Multiplex PCR results with concentrations of primers PJCox3F-R, LCCox3F-R, LSCox3F-R, and PGCox3F-R adjusted to 0.3, 0.4, 0.6, and 0.7 pmol/µl, respectively. Expected amplicons corresponding to *L. caulleryi* (LC), *L. sabrazesi* (LS), *P. juxtanucleare* (PJ), and *P. gallinaceum* (PG) were detected at 1,040, 868, 666, and

469 bp, respectively. Sterilized distilled water as no-template negative control (-) was used. *M:* DNA Ladder.

4.2.3 Species-specific amplification capacity of multiplex PCR using crude and purified DNA samples

The present multiplex PCR was also assessed with several template conditions including crude, purified, single, mixed, *Trypanosoma* spp.-positive, and uninfected samples. Agarose gel electrophoresis showed that the multiplex PCR can also accurately amplified the right targets from crude and purified genomic DNA samples. There was no amplification occurred with non-infected and *Trypanosoma*-positive blood samples (Figure 13).

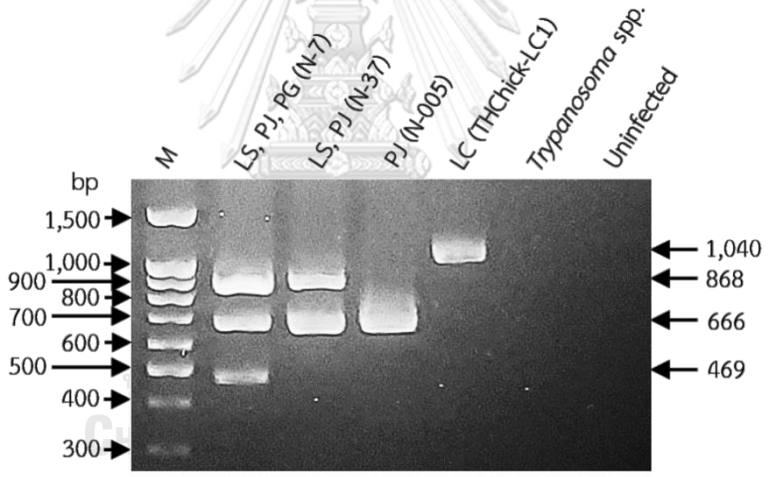

Figure 13 Specificity of multiplex PCR on crude blood and gDNA sample. Lane 1: THChick17-N-7 positive with *L. sabrazesi* (868 bp), *P. juxtanucleare* (666 bp), *P. gallinaceum* (469 bp), and microfilaria; lane 2: THChick17-N-37 positive with *L. sabrazesi* and *P. juxtanucleare*; lane 3: THChick19-005 positive with *P. juxtanucleare*; lane 4: THChick-LC1 positive with *L. caulleryi* (1,040 bp); lane 5: THDairy-008 positive with *Trypanosoma* spp.; lane 6: THChick19-003 negative with all mentioned species (Table 3); M: 1 Kb DNA Ladder.

4.3 Validation of detection limits

4.3.1 Detection limit of singleplex PCRs

Sensitivity of singleplex PCRs was determined using a serial dilution of each template ranging from 10^{10}-10^{1} copies/µl, except in *P. gallinaceum* DNA was from 10^{11}-10^{1} copies/µl. Detection limit of each primer pair was determined using two concentrations of 0.2 and 0.4 pmol/µl. At concentration of 0.2 pmol/µl, detection limits for LCCox3F-R, LSCox3F-R, PJCox3F-R, and PGCox3F-R were 10^{6}, 10^{8}, 10^{7}, and 10^{9} copies/µl, respectively (Figure 14a-d, left panel). In comparison, detection limits for LCCox3F-R, LSCox3F-R, PJCox3F-R, and PGCox3F-R primers at concentration of 0.4 pmol/µl were 10^{5}, 10^{5}, 10^{6}, and 10^{7} copies/µl, respectively (Figure 14a-d, right panel). Together, the results suggested that all primer pairs at 0.4 pmol/µl concentration had higher sensitivity than 0.2 pmol/µl. The differences were seen as high as 10^{3} times in LSCox3F-R (10^{8} copies/µl with primer concentration of 0.2 pmol/µl versus 10^{5} copies/µl with 0.4 pmol/µl of the primers). At concentration of 0.4 pmol/µl, PJCox3F-R was able to detect template at 10^{5} copies/µl, 100 times more sensitive than at concentration 0.2 pmol/µl (10^{7} copies/µl). In general, primers LCauCox3F-R, LSCox3F-R and PJuxCox3F-R showed a comparable sensitivity, but PGCox3F-R was less sensitive than the others.

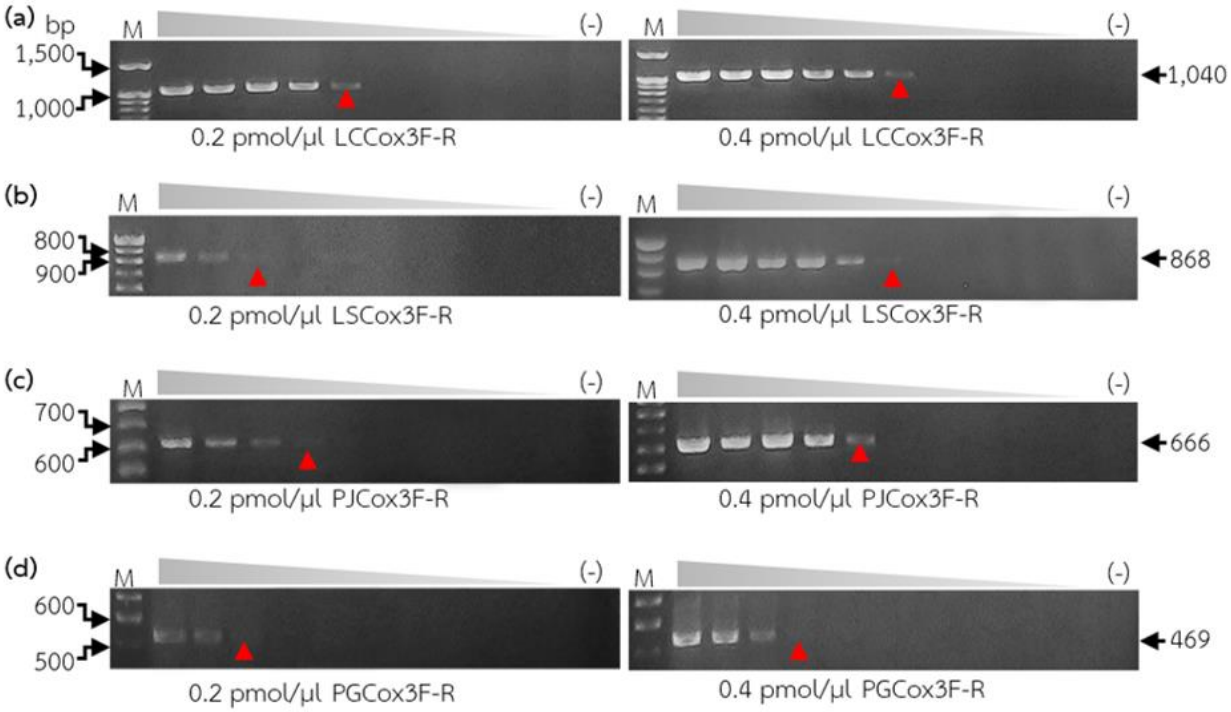

Figure 14 Detection limit for each pair of primers in singleplex PCRs (red arrowheads). Two concentrations for each primer pairs were assessed and were indicated below each agarose gel electrophoresis images **(a-d)**. Amplified products of each primer pairs were indicated by black arrows. DNA templates were diluted from 10^{10} to 10^{1} copies/µl, only *P. gallinaceum* DNA template was from 10^{11} to 10^{1} copies/µl. Sterilized distilled water as no-template negative control (-), M: DNA ladder marker.

4.3.2 Detection limit of multiplex PCRs

A mixture of four DNA templates representing a quadruple infection were used for multiplex PCRs. DNA templates were diluted from 10^{11} to 10^{1} copies/µl. The results showed that, in the presence of all four templates, detection limit of multiplex PCR was at 10^{9} copies/µl (Figure 15).

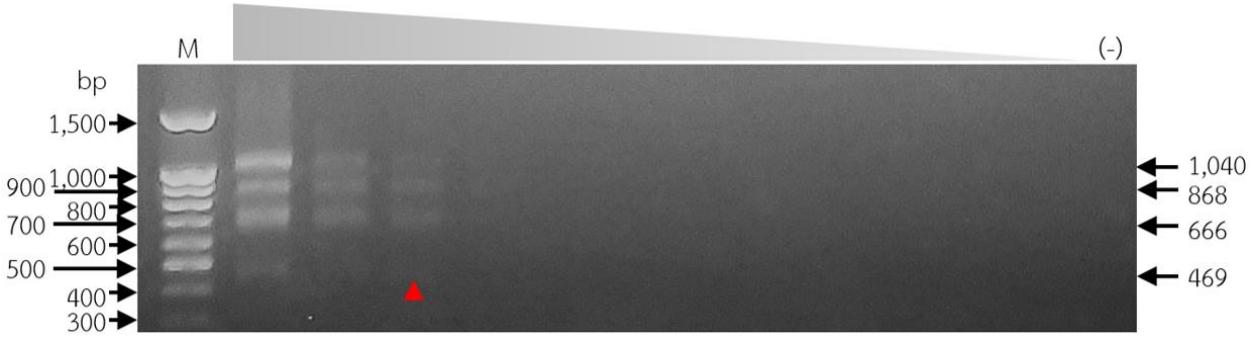

Figure 15 Multiplex PCRs using a mixture of four DNA templates representing a quadruple infection. Detection limit was at 10^9 copies/µl (red arrowhead). Templates were diluted from 10^{11} to 10^1 copies/µl. Sterilized distilled water was used as no-template negative control (-). M: DNA ladder marker.

4.4 Application of multiplex PCR to the field samples

4.4.1 Comparison between microscopic examination and multiplex PCR

Multiplex PCRs were tested with a total of 352 blood samples collected from Nan, Prachinburi, and Chachoengsao. Among these, thin blood smears from 157 samples were available for microscopic examinations. In the present study, *L. caulleryi* was not detected in any of the field isolates by either microscopic examinations or multiplex PCRs. At least one species of *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum* was observed in 69 out of 157 samples (43.9 %) by microscopy. Microscopically, only single infections of *P. juxtanucleare* and *L. sabrazesi* was observed in 54 and 2 out of 157 samples, respectively. A co-infection of *L. sabrazesi* and *P. juxtanucleare* was seen in 11 isolates, while a triple infection of *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum* was found in 1 sample by microscopy. Multiplex PCRs detected a total of 125 out of 157 samples (79.6 %). Single infections of *P. juxtanucleare* and *L. sabrazesi* was detected in 101 and 2 out of 157 samples, respectively based on multiplex PCRs. Triple infection of *L. sabrazesi*, *P.*

juxtanucleare, and *P. gallinaceum* was detected in 11 samples by multiplex PCRs (Table 7). Neither methods detected any single infection of *P. gallinaceum*, nor having double infection of *L. sabrazesi* and *P. gallinaceum* or *P. juxtanucleare* and *P. gallinaceum*.

Interestingly, even though microscopic examination and multiplex PCR had the same results of 2 *L. sabrazesi* infected samples and 11 double infected samples of *L. sabrazesi* and *P. juxtanucleare*, not all these samples were the same. The 2 *L. sabrazesi* infected samples detected by microscopic examination were detected to have triple infection by multiplex PCR, and the 2 samples infected with only *L. sabrazesi* detected by multiplex PCR were negative in microscopic examination. Likewise, several samples found double infection by microscopic examination were detected to have triple infection by multiplex PCR, including a sample positive with two *Plasmodium* species, and a number of double infected samples detected by multiplex PCR were negative or have single infection found by microscopic examination (Table 7).

Both methods did not detect any samples infected with only *P. gallinaceum*, nor having double infection of *L. sabrazesi* and *P. gallinaceum*, nor *P. juxtanucleare* and *P. gallinaceum* in these 157 samples. There were 88 negative samples according to microscopic results, and only 32 samples marked as negative by multiplex PCR. There were no samples positive in microscopic examination but negative by multiplex PCR (Table 7, 8).

Table 7 Prevalence of avian haemosporidian infections based on microscopic examinations and multiplex PCRs

	Prevalence (%)	
	Microscopy (n=157)	Multiplex PCR (n=157)
Single infection		
L. sabrazesi	2 (0.1)	2 (0.1)
P. juxtanucleare	54 (34.4)	101 (64.3)
P. gallinaceum	0	0
Double infection		
L. sabrazesi and *P. juxtanucleare*	11 (7.0)	11 (7.0)
L. sabrazesi and *P. gallinaceum*	0	0
P. juxtanucleare and *P. gallinaceum*	1 (0.6)	0
Triple infection		
L. sabrazesi, *P. juxtanucleare*, and *P. gallinaceum*	1 (0.6)	11 (7.0)
Overall	**69 (43.9)**	**125 (79.6)**

Statistical analysis was performed using SPSS Statistic version 22 to compare detection rate for each parasite species based on microscopy and multiplex PCR. Kappa statistics were tested to confirm the agreement between the two methods. In which, K = 0 means the agreement was observed by chance, K = 1 means the results from two methods were perfectly agree with each other (Cohen, 1960), and K value ranging from 0 to 1 represent different degrees of agreement (McHugh, 2012). McNemar tests were used to determine if the differences in the results obtained from microscopy and multiplex PCR were significant ($p < 0.05$), under the null hypothesis of agreement in the results of two methods. A total of 10 samples (6.4%) were positive for *L. sabrazesi* by multiplex PCR but found negative by microscopic examination. *L. sabrazesi* detection rate by both methods was considered moderate agreement (K = 0.703). Detection rate by microscopy (14 of 157 or 8.9%) significantly lower than multiplex PCR (24 of 157 or 15.3%) based on McNemar test ($p = 0.002$). Multiplex PCR detected 123 *P. juxtanucleare* infected samples out of 157 (78.3%), while only 67 (42.7%) were observed by microscopy. Multiplex PCRs detected 11

infected samples (7%), while microscopy was able to detect only 2 samples (1.3%). Detection rate of multiplex PCR was significantly higher than microscopy in *P. juxtanucleare* (p = 0.00) and *P. gallinaceum* (p = 0.004). Detections by the 2 methods for *P. juxtanucleare* and *P. gallinaceum* were with minimal agreement (K value = 0.341 and 0.292, respectively) (Table 8). Overall, 56 samples were detected by multiplex PCR but negative by microscopy (44.8%), resulting in the fair agreement between two methods. McNemar test suggested that microscopic examination has significantly lower detection rate compared to multiplex PCR (p = 0.000).

Table 8 Statistical comparisons between detection results obtained from microscopic examination and multiplex PCR

Parasite			Multiplex PCR			Kappa* (agreement)	McNemar
			Negative	Positive	Total		
L. sabrazesi	Microscopy	Negative	133	10	143	0.703 (moderate)	0.002
		Positive	0	14	14		
		Total	133	24	157		
P. juxtanucleare		Negative	34	56	90	0.341 (minimal)	0.000
		Positive	0	67	67		
		Total	34	123	157		
P. gallinaceum		Negative	146	9	155	0.292 (minimal)	0.004
		Positive	0	2	2		
		Total	146	11	157		
Overall		Negative	32	56	88	0.334 (minimal)	0.000
		Positive	0	69	69		
		Total	32	125	157		

*Kappa values were evaluated based on McHugh (2012)

4.4.2 Haemosporidian parasites in all field isolates

A total of 352 samples were assessed by multiplex PCR only (among these 157 blood smears were unavailable). Collectively, 211 samples were from Nan province, 98 from Prachinburi province, and 43 from Chachoengsao province. Samples from Nan province dominate in number of double and triple infections. There were 163 out of 211 samples (77.25%) from Nan province found positive by at least one species of haemosporidian parasites. A total of 85 (40.3%), and 46 (22%) samples were found to be single and double infections, respectively. In this finding, 31 (67.4%) of double infections samples occurred between *L. sabrazesi* and *P. juxtanucleare*. Majority of the triple infections (31 samples or 97%) were *L. sabrazesi*, *P. juxtanucleare* and *P. gallinaceum* (Table 9). In Prachinburi province, a high prevalence of haemosporidian parasites were detected (92/98 or 93.8%). Most of the chickens (88/98 or 89.8%) were infected by *P. juxtanucleare.* Double infection of *L. sabrazesi* and *P. juxtanucleare* was detected in 4 out of 98 samples or 4.1%. Single infection was the main finding in Chachoengsao province with 14 out of 43 tested samples (32.5%). Among these, 12 and 2 samples were infected with *P. juxtanucleare* and *L. sabrazesi,* respectively (Table 9). Overall, infection rate in this province was 39.5% (17/43). A few samples (3/43 or 7%) had double infection of *L. sabrazesi* and *P. juxtanucleare*. Generally, Nan and Prachinburi province had high prevalence of chicken haemosporidian parasite, 77.25% and 94%, respectively. Chachoengsao province had a lower prevalence of 39.5%. There was no quadruple infection nor mix infection of *L. caulleryi* and *L. sabrazesi* found in three provinces. The summary of prevalence data by province based on multiplex PCR is presented in Table 9.

Table 9 Summary of haemosporidian parasite in the field samples based on three sampling sites using multiplex PCR

Parasite	Prevalence (%)		
	Nan province (n = 211)	Prachinburi province (n = 98)	Chachoengsao province (n = 43)
Single infection	**85 (40.2)**	**88 (89.8)**	**14 (32.5)**
L. caulleryi	18 (8.5)	0	0
L. sabrazesi	10 (4.7)	0	2 (4.6)
P. juxtanucleare	55 (26)	88 (89.8)	12 (27.9)
P. gallinaceum	2 (0.9)	0	0
Double infection	**46 (21.8)**	**4 (4.1)**	**3 (7)**
L. caulleryi and *L. sabrazesi*	0	0	0
L. caulleryi and *P. juxtanucleare*	1 (0.5)	0	0
L. caulleryi and *P. gallinaceum*	1 (0.5)	0	0
L. sabrazesi and *P. juxtanucleare*	31 (14.7)	4 (4.1)	3 (7)
L. sabrazesi and *P. gallinaceum*	5 (2.4)	0	0
P. juxtanucleare and *P. gallinaceum*	8 (3.8)	0	0
Triple infection	**32 (15.2)**	**0**	**0**
L. caulleryi, *L. sabrazesi* and *P. juxtanucleare*	0	0	0
L. caulleryi, *L. sabrazesi* and *P. gallinaceum*	0	0	0
L. caulleryi, *P. juxtanucleare* and *P. gallinaceum*	1 (0.5)	0	0
L. sabrazesi, *P. juxtanucleare* and *P. gallinaceum*	31 (14.7)	0	0
Overall (%)	**163 (77.2)**	**92 (93.8)**	**17 (39.5)**

CHAPTER 5

DISCUSSION

Avian haemosporidian parasites are distributed worldwide causing severe damages or ultimately death to infected individuals. If survive, haemosporidian parasites remain persistently in the chickens' body and serve as the gametocyte donors for the next transmission to the other birds. Haemosporidian parasites in chicken are widely distribute and causing high mortality with non-specific symptoms. Normal clinical signs such as red blood cell depletion, depression, cyanosis, anorexia, diarrhea, weight loss, reducing of egg production, etc. (Macchi Bde et al., 2010; Zhao et al., 2016). Microscopic examination and conventional PCR are normally either used separately or in combination. However, both methods are still inadequate in term of specificity and sensitivity to provide accuracy as well as timely diagnosis. While every conventional PCR reaction can only detect one target, microscopic examination must be performed by a skilled investigator and can provide more data such as parasitemia, gametocytemia, mix infections or specific species. Microscopy is economical and a reliable method for blood parasite diagnosis (Valkiū̄nas et al., 2008). However, it is generally accepted that microscopy has lower sensitivity than molecular techniques (Richard et al., 2002; Rantala et al., 2010; Doctor et al., 2016; Zhao et al., 2016). Thus, the present study was conducted to establish a multiplex PCR to be an alternative or to overcome the disadvantages of microscopy and conventional PCR. Microscopic examination was also carried out in parallel with multiplex PCR for comparison.

Among candidate genes from nucleus, apicoplast, and mitochondria those were used as the diagnostic markers, mitochondria genes are frequently chosen over the others because of its highly conserve nature and high copy number. While the copy number of several genes from nucleus and apicomlast can dicrectly represent the number of parasites, high copy number of mitochondria genes can effectively increase sensitivity of molecular detection methods, which is actually bring more

remarkable data for disease control, prevention, and epidemiology studies. Primers targeting mitochondria *cox3* gene of *Leucocytozoon* spp. were considered to be more species-specific compared to *cytb* (Zhao et al., 2016). It is known that avian malaria parasite has high adenine and thymine (AT) content in its genome (Videvall, 2018). In the sequence alignment of mitochondria genomes, at least two in four haemosporidian species always has identical sequence. As a result, species-specific primers chosen in this study contain low GC content, high number in both self-complementarity and self-3' complementarity. However, the selected primers in the present study have preeminent high annealing temperatures, ranging from 64.7 to 66.3 °C, and were able to generate expected amplicons for all four parasite species. Previously, several primers were used for detecting avian blood parasites, but they either target a group of bird-infecting species (Hellgren et al., 2004; Bensch et al., 2009; Bell et al., 2015; Ciloglu et al., 2019), or specific but produce unfavorable amplicon size (Saiwichai et al., 2007; Zhao et al., 2016).

The current multiplex PCR demonstrated that it capable of simultaneously detect DNA mixture of *L. caulleryi*, *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum* in recombinant plasmids and natural infected samples. The optimal cycling condition for plasmids and host originate samples were different. 20 seconds at 67 °C for annelling, and 20 seconds at 68 °C for extension prevented non-specific binding when using recombinant plasmids as template. 30 seconds at 66 °C for annealing, and 45 seconds at 68 °C for extension allowed better amplification of all four species in the presences of host DNA, proteins, anticoagulants, and other substrates that might interfere PCR reaction. The protocol from supplier also recommended the KOD polymerase need different extension time for crude and purified samples. Since the multiplex PCR assay in this study was developed to work with crude samples, different cycling conditions for plasmids and host originate samples were unavoidable. Detection of quadruple infection by multiplex PCR was achievable at detection limit of 10^9 parasite copies per reaction. Detection limit of the current

multiplex PCR seems to be high compared to other studies with only 10^3 or 10^4 copies (Lung et al., 2017; Yao et al., 2019). This might be partly explained by the low CG content of the target causing the longer sequence of the primers, since typical primers would contain 45% to 55% CG (Shen et al., 2010). Increasing concentration of primers or adding Mg^{2+} may help improve amplification efficacy. However, natural quadruple infection was not detected by both multiplex PCR and microscopy in this study, and detection rates on three, two, and one target are generally higher than on four targets. Multiplex PCR was found to has lower sensitivity than microscopy in another study targeting gastrointestinal parasites (Sri-Hidajati et al., 2018). Nevertheless, the results in this study showed that multiplex PCR was more efficient than microscopy for the detection of *L. sabrazesi*, *P. juxtanucleare*, *P. gallinaceum*, as well as in co-infections. Several samples with *L. caulleryi* positive by PCR were not have blood smears. Therefore, a comparison between microscopy and PCR was not allowed. However, detection rate of multiplex PCR in detecting *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum* were all significantly higher than microscopic examination.

The current multiplex PCR detected a high prevalence of haemosporidian parasites in Nan and Prachinburi (77.25% and 94%, respectively). Infections of *L. sabrazesi* and *P. juxtanucleare* were more prevalent than the other co-infections. Among 7 combinations of co-infections, samples from Nan also showed the highest co-infection rate accounting for 37%. Co-infections between *L. caulleryi* and *L. sabrazesi* was not detected in this study. This observation of a co-infection between *L. caulleryi* and *L. sabrazesi* was contradictory to a previous study by Takang and colleagues (Takang et al., 2017). Taken together, co-infections seem to be common in avian haemosporidians. Either microscopic diagnosis or conventional PCR alone might, therefore, overlook details of this co-infections. The present multiplex PCR was successfully established and worked well with crude samples. Therefore, this

protocol would be a time and cost-saving that might be able to apply in the resource-poor setting.

CHAPTER 6

CONCLUSION AND SUGGESTION

6.1 Conclusions

Multiplex PCR, developed in this study, is a reliable and effective method for detections of haemosporidian parasites which are endemic in Thailand. With one PCR reaction mixture, multiplex PCR was capable of simultaneously detect four haemosporidian species comprising of *L. caulleryi*, *L. sabrazesi*, *P. juxtanucleare*, and *P. gallinaceum.* The current method worked either with crude or purified samples and was able to differentiate all forms of infections i.e. single, double, and triple infections from the field samples. Diagnosis using the current protocol can be carried out in shorter time compared with conventional PCR. This multiplex PCR assay also displayed higher detection rates compared to microscopic examination.

6.2 Suggestions

It would be useful if the other conventional or nested PCRs using established primers are tested in comparison with the current method. For the future studies, this multiplex method might be further modified to achieve lower detection limit.

REFERENCES

Abdelqader A, Gauly M, Wollny CBA and Abo-Shehada MN 2008. Prevalence and burden of gastrointestinal helminthes among local chickens, in northern Jordan. Preventive Veterinary Medicine. 85(1): 17-22.

Adler PH and McCreadie JW 2019. Chapter 14 - Black Flies (Simuliidae). In: Medical and Veterinary Entomology (Third Edition). Gary R. Mullen and Lance A. Durden (eds). Academic Press. 237-259.

Alnassan AA, Kotsch M, Shehata AA, Kruger M, Daugschies A and Bangoura B 2014. Necrotic enteritis in chickens: development of a straightforward disease model system. Vet Rec. 174(22): 555.

Bartlett JM and Stirling D 2003. A short history of the polymerase chain reaction. Methods Mol Biol. 226: 3-6.

Beadell JS, Gering E, Austin J, Dumbacher JP, Peirce MA, Pratt TK, Atkinson CT and Fleischer RC 2004. Prevalence and differential host - specificity of two avian blood parasite genera in the Australo - Papuan region. Molecular Ecology. 13(12): 3829-3844.

Bell JA, Weckstein JD, Fecchio A and Tkach VV 2015. A new real-time PCR protocol for detection of avian haemosporidians. Parasites & Vectors. 8(1): 383.

Bennett G 1970. Gallinaceous species of Plasmodium. Journal of Parasitology. 56: 27.

Bensch S, Hellgren O and Perez-Tris J 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol Ecol Resour. 9(5): 1353-1358.

Bensch S, Stjernman M, Hasselquist D, Hannson B, Westerdahl H and Pinheiro RT 2000. Host specificity in avian blood parasites: a study of Plasmodium and Haemoproteus mitochondrial DNA amplified from birds. Proceedings of the Royal Society of London. Series B: Biological Sciences. 267(1452): 1583-1589.

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN and Caskey CT 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic acids research. 16(23): 11141-11156.

Ciloglu A, Ellis VA, Bernotiene R, Valkiunas G and Bensch S 2019. A new one-step multiplex PCR assay for simultaneous detection and identification of avian haemosporidian parasites. Parasitol Res. 118(1): 191-201.

Cohen J 1960. A Coefficient of Agreement for Nominal Scales Educ Psychol Meas. SAGE Publications Inc. 20: 37-46.

Doctor SM, Liu Y, Whitesell A, Thwai KL, Taylor SM, Janko M, Emch M, Kashamuka M, Muwonga J, Tshefu A and Meshnick SR 2016. Malaria surveillance in the Democratic Republic of the Congo: comparison of microscopy, PCR, and rapid diagnostic test. Diagnostic Microbiology and Infectious Disease. 85(1): 16-18.

FAO 2018. Meat consumption. [Online]. Available: https://data.oecd.org/agroutput/meat-consumption.htm, Accessed July 29, 2020.

Frevert U, Späth GF and Yee H 2008. Exoerythrocytic development of Plasmodium gallinaceum in the White Leghorn chicken. International journal for parasitology. 38(6): 655-672.

Goldberg DE, Slater A, Cerami A and Henderson GB 1990. Hemoglobin degradation in the malaria parasite Plasmodium falciparum: an ordered process in a unique organelle. Proceedings of the National Academy of Sciences. 87(8): 2931-2935.

Graham R and Tunnicliff E 1924. Tuberculosis of fowls. University of Illinois Agricultural Experiment Station. 285: 4-5.

Hellgren O, Waldenström J and Bensch S 2004. A new PCR assay for simultaneous studies of Leucocytozoon, Plasmodium, and Haemoproteus from avian blood. Journal of Parasitology. 90(4): 797-802.

Imura T, Sato S, Sato Y, Sakamoto D, Isobe T, Murata K, Holder AA and Yukawa M 2014. The apicoplast genome of Leucocytozoon caulleryi, a pathogenic apicomplexan parasite of the chicken. Parasitology research. 113(3): 823-828.

Ito A, Gotanda T, Himeno N, Itchoda N, Tabayashi N, Ike K, Sugimoto C and Matsumura T 2013. Booster responses by oral vaccination with transgenic plants against chicken leucocytozoonosis. Avian Pathol. 42(3): 215-220.

Jumpato W, Tangkawanit U, Wongpakam K and Pramual P 2019. Molecular detection of Leucocytozoon (Apicomplexa: Haemosporida) in black flies (Diptera: Simuliidae)

from Thailand. Acta Trop. 190: 228-234.

Lee D-H, Jang J-H, Kim B-Y, Kwon Y-K, Gomis S, Lee J-B, Park S-Y, Choi I-S and Song C-S 2013. Diagnosis of Leucocytozoon caulleryi Infection in Commercial Broiler Breeders in South Korea. Vol 58. In: BIOONE. 183-186, 184.

Lee HR, Koo B-S, Jeon E-O, Han M-S, Min K-C, Lee SB, Bae Y and Mo I-P 2016. Pathology and molecular characterization of recent Leucocytozoon caulleryi cases in layer flocks. Journal of biomedical research. 30(6): 517-524.

Lotta IA, Valkiūnas G, Pacheco MA, Escalante AA, Hernández SR and Matta NE 2019. Disentangling Leucocytozoon parasite diversity in the neotropics: Descriptions of two new species and shortcomings of molecular diagnostics for leucocytozoids. International journal for parasitology. Parasites and wildlife. 9: 159-173.

Lung O, Ohene-Adjei S, Buchanan C, Joseph T, King R, Erickson A, Detmer S and Ambagala A 2017. Multiplex PCR and Microarray for Detection of Swine Respiratory Pathogens. Transboundary and emerging diseases. 64(3): 834-848.

Macchi Bde M, Quaresma JA, Herculano AM, Crespo-Lopez ME, DaMatta RA and do Nascimento JL 2010. Pathogenic action of Plasmodium gallinaceum in chickens: brain histology and nitric oxide production by blood monocyte-derived macrophages. Vet Parasitol. 172(1-2): 16-22.

Macchi BM, Miranda FJB, Souza FS, Carvalho ECQ, Albernaz AP, Nascimento JLM and DaMatta RA 2013. Chickens treated with a nitric oxide inhibitor became more resistant to Plasmodium gallinaceum infection due to reduced anemia, thrombocytopenia and inflammation. Veterinary research. 44(1): 8.

McHugh ML 2012. Interrater reliability: the kappa statistic. Biochemia medica: Biochemia medica. 22(3): 276-282.

Naqvi MA-u-H, Khan MK, Iqbal Z, Rizwan HM, Khan MN, Naqvi SZ, Zafar A, Sindhu ZuD, Abbas RZ and Abbas A 2017. Prevalence and associated risk factors of haemoparasites, and their effects on hematological profile in domesticated chickens in District Layyah, Punjab, Pakistan. Preventive Veterinary Medicine. 143: 49-53.

Pattaradilokrat S, Tiyamanee W, Simpalipan P, Kaewthamasorn M, Saiwichai T, Li J and

Harnyuttanakorn P 2015. Molecular detection of the avian malaria parasite Plasmodium gallinaceum in Thailand. Vet Parasitol. 210(1-2): 1-9.

Pelt-Verkuil Ev, Belkum Av and Hays JP 2008. Principles and technical aspects of PCR amplification. In: Springer Science & Business Media. 330.

Perkins SL and Schall J 2002. A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. Journal of Parasitology. 88(5): 972-978.

Permin A, Esmann JB, Hoj CH, Hove T and Mukaratirwa S 2002. Ecto-, endo- and haemoparasites in free-range chickens in the Goromonzi District in Zimbabwe. Prev Vet Med. 54(3): 213-224.

Porta AR and Enners E 2012. Determining Annealing Temperatures for Polymerase Chain Reaction. The American Biology Teacher. 74(4): 256.

Rantala A-M, Taylor SM, Trottman PA, Luntamo M, Mbewe B, Maleta K, Kulmala T, Ashorn P and Meshnick SR 2010. Comparison of real-time PCR and microscopy for malaria parasite detection in Malawian pregnant women. Malaria Journal. 9(1): 269.

Richard FA, Sehgal RN, Jones HI and Smith TB 2002. A comparative analysis of PCR-based detection methods for avaian malaria. J Parasitol. 88(4): 819-822.

Saiwichai T, Harnyuttanakorn P, Sukhumavasi W, Buddhirakkul P, Bhumiratana A, Rojanapremsuk J and Nithiuthai S 2007. Diagnosis of Plasmodium gallinaceum in infected mosquitoes by multiplex PCR. Journal of Tropical Medicine and Parasitology. 30: 76-80.

Shen Z, Qu W, Wang W, Lu Y, Wu Y, Li Z, Hang X, Wang X, Zhao D and Zhang C 2010. MPprimer: a program for reliable multiplex PCR primer design. BMC Bioinformatics. 11(1): 143.

Silveira P, DaMatta RA and Dagosto M 2009. Hematological changes of chickens experimentally infected with Plasmodium (Bennettinia) juxtanucleare. Veterinary Parasitology. 162(3): 257-262.

Sohsuebngarm D, Sasipreeyajan J, Nithiuthai S and Chansiripornchai N 2014. The efficacy of artesunate, chloroquine, doxycycline, primaquine and a combination of artesunate and primaquine against avian malaria in broilers. The Journal of veterinary medical science. 76(6): 813-817.

Soomro N, Rind R, Arijo A and Soomro SJIJAB 2001. Clinical, gross and histopathological studies of coccidial infection in chicken. 3(4): 426-427.

Sri-Hidajati B, Basuki S, Pusarawati S, Kusmartisnawati K, Rossyanti L, Sulistyowati SW, Kartikasari DP, Arwati H, Tantula I and Darma A 2018. Comparison of multiplex single round PCR and microscopy in diagnosis of amoebiasis. African journal of infectious diseases. 12(1S): 120-126.

Suprihati E and Yuniarti WM 2017. The phylogenetics of Leucocytozoon caulleryi infecting broiler chickens in endemic areas in Indonesia. Veterinary world. 10(11): 1324-1328.

Takang P, Pikulkaew S, Awaiwanont N and Numee SJVIS 2017. Prevalence and risk factors of blood parasites infection in backyard chickens in Chiang Mai. 15(3): 157-167.

Tasai S, Saiwichai T, Kaewthamasorn M, Tiawsirisup S, Buddhirakkul P, Chaichalotornkul S and Pattaradilokrat S 2017. Artesunate-tafenoquine combination therapy promotes clearance and abrogates transmission of the avian malaria parasite Plasmodium gallinaceum. Vet Parasitol. 233: 97-106.

Valkiunas G 2004. Avian malaria parasites and other haemosporidia. In: CRC press.

Valkiunas G 2005. Avian malaria parasites and other haemosporidia. In: CRC press.

Valkiūnas G and Iezhova TA 2017. Exo-erythrocytic development of avian malaria and related haemosporidian parasites. Malaria journal. 16(1): 101-101.

Valkiūnas G, Iezhova TA, Križanauskienė A, Palinauskas V, Sehgal RN and Bensch S 2008. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. Journal of Parasitology. 94(6): 1395-1401.

Vanstreels RET, da Silva-Filho RP, Kolesnikovas CKM, Bhering RCC, Ruoppolo V, Epiphanio S, Amaku M, Ferreira Junior FC, Braga ÉM and Catão-Dias JL 2015. Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. Veterinary research. 46: 30-30.

Videvall E 2018. Plasmodium parasites of birds have the most AT-rich genes of eukaryotes. Microbial genomics. 4(2): e000150.

Waldenström J, Bensch S, Hasselquist D and Östman Ö 2004. A new nested polymerase

chain reaction method very efficient in detecting Plasmodium and Haemoproteus infections from avian blood. Journal of Parasitology. 90(1): 191-194.

Wani MY, Dhama K and Malik YS 2016. Impact of virus load on immunocytological and histopathological parameters during clinical chicken anemia virus (CAV) infection in poultry. Microbial Pathogenesis. 96: 42-51.

Williams RB 2005. Avian malaria: clinical and chemical pathology of Plasmodium gallinaceum in the domesticated fowl Gallus gallus. Avian Pathol. 34(1): 29-47.

Yao M, Zhang X, Gao Y, Song S, Xu D and Yan L 2019. Development and application of multiplex PCR method for simultaneous detection of seven viruses in ducks. BMC veterinary research. 15(1): 103-103.

Yu CY and Wang JS 2001. Role of chicken serum in inhibiting Leucocytozoon caulleryi development in Culicoides arakawae infected by membrane-feeding of infective blood meals. Parasitol Res. 87(9): 698-701.

Zhao W, Liu J, Xu R, Zhang C, Pang Q, Chen X, Liu S, Hong L, Yuan J, Li X, Chen Y, Li J and Su X-Z 2015. The Gametocytes of Leucocytozoon sabrazesi Infect Chicken Thrombocytes, Not Other Blood Cells. PloS one. 10(7): e0133478-e0133478.

Zhao W, Pang Q, Xu R, Liu J, Liu S, Li J and Su X-Z 2016. Monitoring the Prevalence of Leucocytozoon sabrazesi in Southern China and Testing Tricyclic Compounds against Gametocytes. PloS one. 11(8): e0161869-e0161869.

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