ความสามารถของยุงลายบ้านและยุงลายสวนในการเป็นพาหะของเชื้อพลาสโมเดียม กัลลินาเซียม

นางสาวณิชาภัทร ยุรญาติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

Vector competence of *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse) for *Plasmodium gallinaceum*

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2015

Thesis Title	Vector competence of *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse) for *Plasmodium gallinaceum*
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ณิชาภัทร ยุรญาติ : ความสามารถของยุงลายบ้านและยุงลายสวนในการเป็นพาหะของเชื้อพลาสโมเดียม กัลลินาเซียม (Vector competence of *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse) for *Plasmodium gallinaceum*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร. สนธยา เตียวศิริทรัพย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. น.สพ. ดร. มรกต แก้วธรรมสอน, 61 หน้า.

การศึกษานี้ได้เปรียบเทียบการติดเชื้อพลาสโมเดียม กัลลินาเซียมในไก่สายพันธุ์โร๊ดไอส์แลนด์เรด และอิซ่าแบ๊บค๊อก และเปรียบเทียบความสามารถของยุงลายบ้านและยุงลายสวนในการติดเชื้อและนำเชื้อ การศึกษานี้ประกอบไปด้วย 3 การทดลอง การทดลองที่ 1 ศึกษาการติดเชื้อในไก่ โดยประกอบไปด้วยไก่สายพันธุ์โร๊ดไอส์แลนด์เรดอายุ 14 วัน และไก่สายพันธุ์อิซ่าแบ๊บค๊อกอายุ 14 วัน และ 7 วัน และแบ่งออกเป็น 4 กลุ่มย่อย ไก่กลุ่มที่ 1-3 ได้รับเม็ดเลือดแดงที่ติดเชื้อจำนวน 10^6, 10^4 และ 10^2 เซลล์ ตามลำดับ กลุ่มที่ 4 เป็นกลุ่มควบคุม และทำการตรวจหาเชื้อหลังการได้รับเชื้อ การทดลองที่ 2 ศึกษาการติดเชื้อในยุง โดยแบ่งยุงออกเป็น 15 กลุ่ม แต่ละกลุ่มประกอบด้วยยุงลายสวน (>F10) ยุงลายบ้าน (>F10) และยุงลายบ้าน (F10) และยุงลายบ้าน (>F10) ยุงแต่ละกลุ่มได้ดูดเลือดจากไก่ที่มีระดับเชื้อโดยรวมคือร้อยละ 1.1-79.5 และระดับเชื้อระยะมีเพศคือร้อยละ 0.1-5.2 หลังจากนั้นทำการตรวจนับจำนวนโอโอซีสที่กระเพาะอาหารและสปอโรซอยต์ในน้ำลาย การทดลองที่ 3 ศึกษาการถ่ายทอดเชื้อโดยยุง แบ่งยุงออกเป็น 3 กลุ่ม แต่ละกลุ่มประกอบไปด้วยยุงเช่นเดียวกับการทดลองที่ 2 โดยระดับเชื้อระยะมีเพศที่ยุงได้รับคือร้อยละ 1, 3.2 และ 3.4 หลังจากนั้นทำการปล่อยให้ยุงจากทั้ง 3 กลุ่มดูดเลือดจากไก่ และทำการตรวจหาเชื้อในไก่ทุกวัน การศึกษานี้พบว่าไก่สายพันธุ์โร๊ดไอส์แลนด์เรดมีอัตราการป่วยและตายมากกว่าไก่สายพันธุ์อิซ่าแบ๊บค๊อก ระยะก่อนปรากฏที่สั้นที่สุดคือ 3 วัน โดยพบในไก่สายพันธุ์อิซ่าแบ๊บค๊อกอายุ 7 วัน เชื้อสามารถเจริญได้ในยุงทั้ง 3 ชนิด แต่จำนวนโอโอซีสมีความแตกต่างกันอย่างมีนัยสำคัญ โดยยุงทั้ง 3 ชนิดสามารถนำเชื้อได้แต่ไก่มีอัตราการติดเชื้อที่แตกต่างกัน อัตราการติดเชื้อในไก่ที่นำโดยยุงลายสวน (>F10) และ ยุงลายบ้าน (>F10) คือร้อยละ 80-100 และอัตราการติดเชื้อที่นำโดยยุงลายบ้าน (<F10) คือร้อยละ 40-60

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5675306431 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: VECTOR COMPETENCE / AEDES AEGYPTI / AEDES ALBOPICTUS / PLASMODIUM GALLINACEUM

NICHAPAT YURAYART: Vector competence of *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse) for *Plasmodium gallinaceum*. ADVISOR: ASSOC. PROF. DR. SONTHAYA TIAWSIRISUP, CO-ADVISOR: ASST. PROF. DR. MORAKOT KAEWTHAMASORN, 61 pp.

This study was conducted to compare the *Plasmodium gallinaceum* infection between Rhode Island Red and Isa Babcock chickens and compare the vector competence of *Aedes aegypti* and *Ae. albopictus*. This study consisted of three experiments. For the first experiment, a group of 14–day old Rhode Island Red, 14–day old Isa Babcock and 7–day old Isa Babcock were divided into 4 subgroups. Group 1-3 were inoculated with 10^6, 10^4, and 10^2 infected RBCs and group 4 was served as a control group. The percentage of parasite was determined post inoculation (PI). For the second experiment, it was divided into15 experiments and each of which consisted of *Ae. albopictus* (>F10), *Ae. aegypti* (>F10), and *Ae. aegypti* (<F10). The mosquitoes were allowed to feed on infected chickens at different levels of parasitemia (1.1-79.5%) and gametocytemia (0.1-5.2%). The mosquitoes were dissected, counted for oocysts, and observed for the sporozoites. The third experiment was conducted as described in the second experiment but the gametocytemia were 1, 3.2, and 3.4 %. The infected mosquitoes were allowed to feed on naïve chickens and the parasite was monitored. The finding indicated that morbidity and mortality rates in Rhode Island Red were higher than other groups. The shortest prepatent period was 3 day PI which found in 7-day old Isa Babcock. Parasite could develop in all mosquito groups but the number of oocysts were differences. All mosquito groups could transmit the parasite but infection rates in the chicken was difference. The infection rates in *Ae. albopictus* (>F10) and *Ae. aegypti* (>F10) were 80-100 % and the infection rates in *Ae. aegypti* (<F10) were 40-60 %.

Department:	Veterinary Pathology	Student's Signature .....
Field of Study:	Veterinary Pathobiology	Advisor's Signature .....
Academic Year:	2015	Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and grateful thanks to my advisors, Assoc. Prof. Dr. Sonthaya Tiawsirisup, and Assist. Prof. Dr. Morakot Kaewthamasorn for their guidance, helpful suggestions, and encouragement throughout this work.

I would like to thank my committee chair, Assoc. Prof. Dr. Theerayuth Kaewamatawong, and the committee member, Assoc. Prof. Dr. Padet Siriyasatien from the Faculty of Medicine, Chulalongkorn University for the comments. I would also thank Prof. Dr. Theeraphap Chareonviriyaphap from the Faculty of Agriculture, Kasetsart University for taking time to serve as my external examiner.

I would like to thank Prof. Dr. Jiroj Sasipreeyajan, Dr. Patchareeporn Ninvilai for providing the laboratory animals and Assist. Prof. Dr. Nareerat Viseshakul for her guidance in molecular technique.

I am thankful to the financial support of this work by grants from the H.M. the King Bhumibhol Adulyadej's 72nd Birthday Anniversary Scholarship, the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), and Special Task Force for Activating Research, Chulalongkorn University (GSTAR 57-003-31-001).

I am grateful to all the members of Parasitology Unit, and Virology Unit, Faculty of Veterinary Science, Chulalongkorn University, for their kindness, warmhearted helps, encouragement, and friendship.

Finally, I would like to thank my family for their love, understanding, and support.

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ANOVA	=	Analysis of Variance
et al.	=	et alibi, and other
IFN-rR	=	interferon-gamma receptor
iRBCs	=	infected red blood cells
MHC	=	major histocompatibility complex
PBF	=	post blood feeding
PCR	=	polymerase chain reaction technique
PfEMP 1	=	*P. falciparum* erythrocyte membrane protein 1
PI	=	post inoculation
PM	=	peritrophic matrix
TNF	=	tumor necrosis factor
var genes	=	variant gene family

CHAPTER 1

Introduction

Plasmodium gallinaceum is an intracellular protozoan parasite belonging to a phylum Apicomplexa. It is characterized by apical complex that use for invading into a host cell (Katris et al., 2014). This parasite causes avian malaria disease (Miller et al., 2002). The development of this parasite needs mosquito vector and vertebrate host to complete its life cycle. A sexual stage of parasite development occurs in mosquito consisting of fertilization and sporogony whereas an asexual stage occurs in vertebrate host comprising of tissue and blood stages. For this reason, parasites were spread through mosquito vector (Miller et al., 1994).

Avian malaria can be found in broad range of hosts for example pet birds; parrot, pheasant, and peacock and meat poultry; chicken, duck, and turkey (Njabo et al., 2009). This disease causes an economic loss in meat poultry industry. Infected birds show many clinical signs such as depression, loss of appetize, weight loss, egg drop, and death. Mortality rate is approximately 80% and internal organs cannot be served as an extra human food diet (Williams, 2005; Kumnuan et al., 2013).

The distribution of disease can be found in several countries, especially in tropical and subtropical regions (Elahi et al., 2014). The prevalence of this disease in Thailand is very high especially in the areas that have a lot of poultry farms such as Chacheongsao Province. In addition, *P. gallinaceum* parasite was often isolated from

fields (Sohsuebngarm et al., 2014). This indicates that the parasite is remained and circulated in the environment by its vector and host. Several factors support the circulation of the disease for example weather condition, season, opened housing system, treatment, and chicken breed that are suitable for mosquito development and disease transmission (Okanga et al., 2013).

The most common layer chicken breeds are Rhode Island Red and Isa group. Rhode Island Red is a pure layer chicken breed whereas Isa group is a mixed breed that has been developed for production and pathogen resistance such as viral and bacterial infections (Lamont, 1989). However, there was no information available on blood parasite infection in both breeds of chickens.

Culex gelidus and *Cx. tritaeniorhynchus* were reported as vectors for this parasite and they were found around the poultry farms in Thailand (Nithiuthai et al., 2000). However, several mosquito species might be capable of transmitting the parasite such as *Aedes* aegypti and *Ae. albopictus*. *Aedes* mosquito can be found worldwide and they can take a blood meal from several host species including human, mammal, and avian host (Valkiunas, 2005a; Richards et al., 2006). Even though avian malaria disease can be frequently found in Thailand but there was less information about vector competence of the mosquito for *P. gallinaceum* is not available in Thailand. Therefore, the study about vector competence of various mosquito species is importance. Different mosquito species might serve as potential vectors for this parasite in the environment.

The objectives of this study were to investigate *P. gallinaceum* infection in purebred and hybrid bred chickens, and the vector competence of *Ae. aegypti* and *Ae. albopictus* mosquitoes for *P. gallinaceum*. The finding from this study would provide important information of the parasite, vector, and host interactions. Understanding the biology of this parasite as well as its mosquito vector is crucial for controlling and eliminating the disease.

CHAPTER 2

Objectives

There were two objectives of this study.

1. To compare the *P. gallinaceum* infection between the pure bred Rhode Island Rad and the hybrid bred Isa Babcock chickens
2. To determine the vector competence of *Aedes aegypti* and *Aedes albopictus* in response to *Plasmodium gallinaceum*

CHAPTER 3

Literature Review

Plasmodium gallinaceum (Brumpt, 1935)

Plasmodium gallinaceum is an intracellular protozoan parasite belonging to phylum Apicomplexa that includes *Babesia, Eimeria, Hepatozoon*, and *Toxoplasma* (Kappe et al., 2004). Apicomplexa is characterised by apical complex that use for invading into a host cell (Katris et al., 2014). The parasite was first described by Emile Brumpt in 1935 when he worked in Sri Lanka (Ceylon). He reported a finding of the parasites in sick hens and inoculated the blood to other chickens. At that time, parasites were distributed and maintained in malaria laboratory worldwide. Later, *P. gallinaceum* was reported in the other regions for example India, Africa, and Egypt and was also found in the other avian species (Garnham, 1966).

Life cycle and morphology of the parasite

To complete the life cycle, vector-borne pathogens need both vector and vertebrate hosts. *Plasmodium gallinaceum* in mosquito vector start its life cycle when the mosquitoes take a blood meal containing gametocytes. Microgametocytes transform into microgametes within 7-8 minutes, referred to as exflagellation. Exflagellation takes place in the mosquito midgut only (Martin et al., 1978). Macrogamete and microgamete fertilise and form a motile zygote, called ookinete. Ookinete secretes enzymes including chitinase to digest peritrophic matrix (PM) of the

mosquito midgut which is its physical barrier against invading of the parasite. Ookinete penetrates across the basement membrane of midgut and transforms into an oocyst (Langer and Vinetz, 2001; Vlachou et al., 2006). Duration of sporozoite development within the oocyst is approximately 9-10 days, which is varied among species of the parasites (Nacer et al., 2008). When the oocyst fully matured, several hundreds of readily infective sporozoites are released to haemolymph and migrated to salivary glands (Mueller et al., 2010).

Development of parasite in a vertebrate host consists of exoerythrocytic stage and erythrocytic stage. Exoerythrocytic stage is sub-categorised into pre-erythrocytic and post-erythrocytic phases. Pre-erythrocytic phase has two generations which are cryptozoite and metacryptozoite, respectively. Post-erythrocytic phase is recognised as phaneozoite (Frevert et al., 2008) (Figure 1).

During take a blood meal of infected mosquitoes, sporozoites were injected into skin of the bird. Sporozoites will invade reticuloendothelial cells surrounding the biting site and form a stage called cryptozoites (Amino et al., 2007). Fully mature cryptozoites then rupture and release a number of merozoites into blood stream. Sporozoites invade endothelial cells and macrophages of internal organs such as liver, spleen, lung, and kidney, and develop into a stage called metacryptozoites. Merozoites from both cryptozoite and metacryptozoite look physically the same but only merozoites from metacryptozoite can invade red blood cells. Merozoites in blood

circulation invade red blood cells generally classified as erythrocytic stage. This stage consists of asexual development stage called trophozoite and schizont and later develops into a sexual development stage known as gametocyte (Valkiunas, 2005b) (Figure 2). For post-erythrocytic phase, a number of merozoites released from metacryptozoite invade tissue cells such as endothelial cells of brain vessel, causing neurological sign and chronic infection (de Macchi et al., 2013).

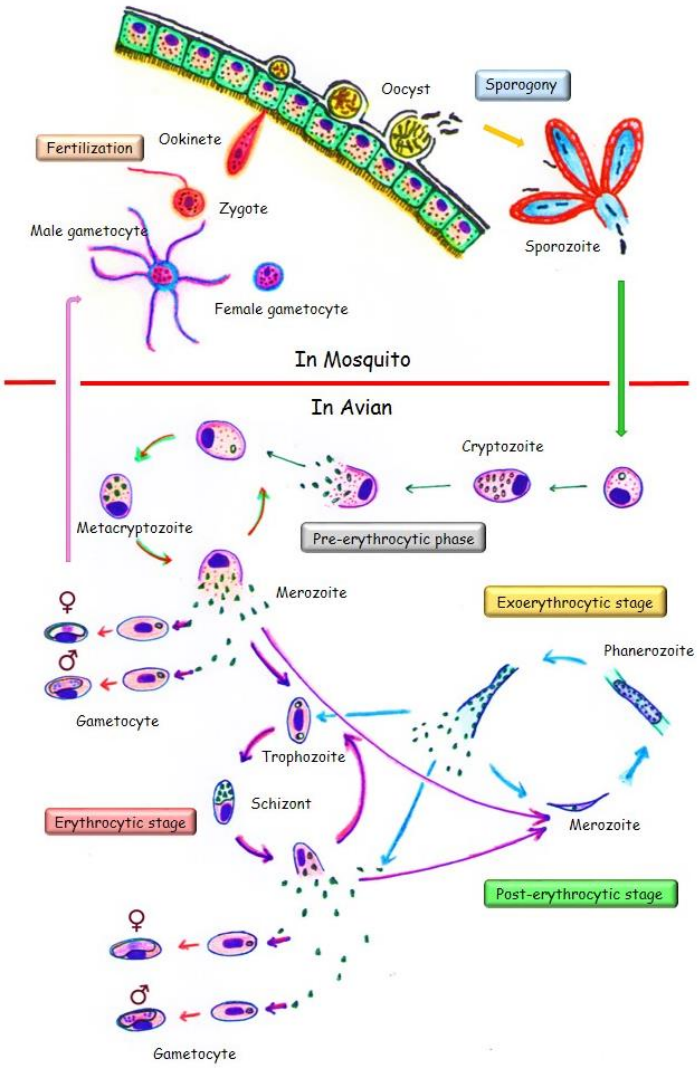


Figure 1 The *Plasmodium gallinaceum* life cycle. The male and female gametocytes fertilized in mosquito midgut and developed into zygote and ookinete, respectively. Ookinete penetrates across the midgut epithelium and forms oocyst. Sporozoites from mature oocyst migrate into salivary glands. Infected female mosquitoes inject sporozoites into dermis of host. After that, the parasites invade host cells and develop into exo-erythrocytic and erythrocytic stages (Valkiunas, 2005b).

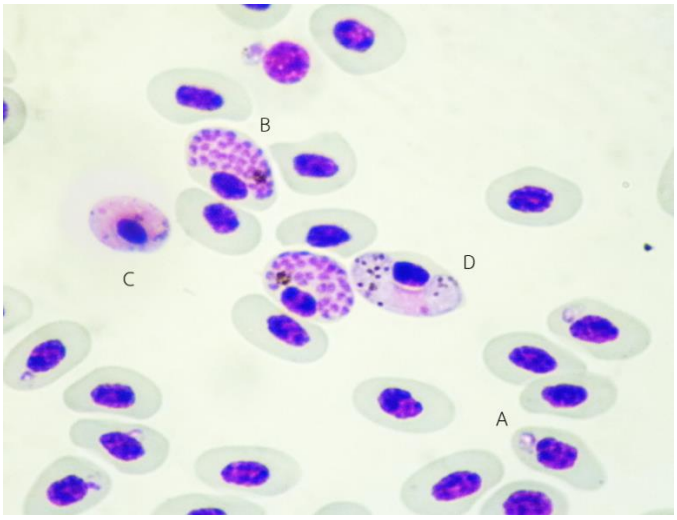


Figure 2 Morphology of erythocytic stage of Giemsa stained *P. gallinaceum*. Trophozoite has a round shape or oval or amoeboid form and does not displace a nucleus of red blood cell (A). Schizont appears in round or irregular shape (B). Mature schizont contains 8-36 merozoites and hemozoin can be found (brown color). Microgametocyte is stained pink and its nucleus is diffused. Number of basophilic granules is less seen than in macrogametocyte (C). Macrogametocyte has a pink and compact nucleus, purple cytoplasm, and more dots of basophilic granules (D). Mature schizont and gametocyte occupy more RBC space, displace nucleus and deform its shape (Valkiunas, 2005b).

Mosquito vectors

Aedes aegypti (Diptera: Culicidae)

Aedes aegypti, also known as yellow fever mosquito, is the most important mosquito vector to transmit several diseases in humans and animals, including dengue, yellow fever, chikungunya, malaria, West Nile fever, and filariasis (Lee et al., 2013; Juneja et al., 2014). The mosquito is characterized by a silver-white lyre marking on the thorax and white bands on its legs and body. *Ae. aegypti* was originated from Africa and globally distributed in subtropical and tropical areas (Eisen et al., 2014).

Aedes albopictus (Diptera: Culicidae)

Aedes albopictus, also known as Asian tiger mosquito, is originally reported from Southeast Asia (Mousson et al., 2005). The mosquito can be found in subtropical and tropical areas in Asia as well as countries around the Indian and Pacific oceans, America, and Europe (Porretta et al., 2012). The mosquito is characterized by white stripe in the middle of head and thorax, and white stripes on its body and legs, which are more visible than *Ae. aegypti* (Paupy et al., 2009) (Figure 3). *Ae. albopictus* mosquitoes were reportedly pose the ability to support the development of pathogen like *Ae. aegypti* mosquitoes (Gratz, 2004; Nelder et al., 2010).

Host preferences of *Ae. aegypti* and *Ae. albopictus*

Host preference is one of the important factor that contribute to disease transmission. If the pathogen well develops in vectors and it can cause the disease in primary focus of vector, thus probability of transmission will increase (Richards et al.,

2006). Host preference of mosquito vector depends on extrinsic and intrinsic factors. Extrinsic factors include odor, gas, body heat, humidity, body mass, gender, and climate. Intrinsic factors include host blood protein and genetic of vector (Qiu et al., 2006; Leal, 2013).

Aedes mosquitoes are anthropophilic vectors. They prefer to feed on human blood rather than other animals especially in indoor place. On the other hand, mosquitoes collected from outdoor and urban area usually fed on other mammal hosts, birds, and reptiles (Faraji et al., 2014). In addition, feeding behavior of laboratory mosquitoes maintained in many generations are lost by selective breeding, stress, and lack of contact to the nature (Takken and Verhulst, 2013).

Factors affecting the transmission of parasite in mosquito vector

An ability to transmit the parasite of the vector is contributed by several factors including host susceptibility, vector competence, and parasite itself. Blood bolus consists of several types of cells and proteins such as cytokine and complement which remain functionally active for several hours. For instance, tumor necrosis factor (TNF)–alpha has an inhibitory effect on exflagellation of microgametocyte and induced phagocytosis (Ramiro et al., 2011). A process of blood digestion resulted in releasing of oxyhaemoglobin and heme and then react with nitric oxide from macrophage leading to a production of toxic form of nitric oxide metabolite that affects to the parasite development (Peterson et al., 2007).

In mosquito factor context, the most important factor is PM which is made of chitin, proteoglycans, and proteins. It is generally formed in 12-30 hr after blood feeding (Osta et al., 2004). The PM surrounds blood bolus and works as a barricade between blood meal and midgut epithelium. It protects midgut epithelium from mechanical damage and pathogen (Okuda et al., 2005). PM plays a role in inhibiting the parasite growth involving with timing of PM formation. In *An. atroparvus*, the mosquito cannot transmit *P. falciparum* due to PM matures within 24 hr earlier than the maturation process of ookinete (ookinete needs 30 hours for its maturation process) and encapsulates the parasite. Second contributing factor is biochemical structure and thickness of the membrane that is different among mosquito species. The structures of membrane affect to ligand-receptor interaction between parasites and mosquitoes. Mosquito immunity also plays a crucial role in inhibiting oocyst development by encapsulation process. Altogether, the number of parasites will be lost in every step along the way of parasite development (Shahabuddin et al., 1995; Beier, 1998; Smith et al., 2014).

Clinical signs and pathogenesis of avian malaria

Clinical signs of avian malaria can be observed early in an erythrocytic stage due to the fact that the parasites repeatedly multiply themselves in the host's RBCs, rupture, and re-invade the other RBCs leading to a severe destruction of the cells. The ability of parasite to invade RBC relies on specific bindings between parasite ligands and receptors on RBC surface which are difference in each parasite species. For *P.*

gallinaceum, a major parasite ligand is merozoite erythrocyte-binding ligand that is commonly found in avian and mammalian malaria. This ligand binds with glycophorin A on RBC surface similar to *P. falciparum* (Martinez et al., 2013). Infected chickens show no specific signs of anemia, depression, anorexia, and slow growth rate (Permin and Juhl, 2002). Clinical outcome of the disease depends on age, breed, and immunity of the host. Young chickens are more vulnerable than adult chickens (Williams, 2005). Recovered chickens are more tolerance and show mild clinical signs because they already have protective immunity against reinfection of the parasite. (Paulman and McAllister, 2005). Anemia is caused by infected red blood cells (iRBCs) decrease their flexibility. Therefore, iRBCs cannot pass the spleen and were destroyed. Moreover, a formation of immune complex between antibody and iRBC obstructs in blood vessel and causes vasculitis, particularly in kidney (Soni and Cox, 1975). Replication process of the parasites consumes a lot of blood glucose and causes metabolic acidosis for the bird, leading to a cerebral malaria-like condition. In post-erythrocytic stage, the replication of parasites in endothelial cells causes neurological signs (de Macchi et al., 2013). In *P. falciparum,* variant gene (*var genes*) family in the parasite modifies RBC surface into knob-like structure. These genes are also responsible for antigenic variations. Antigenic determinant in knob-like structure is always changed when the parasites infect new RBCs (Voss et al., 2014). Thus, antibody raised against previous antigen cannot recognize the new antigen expressed on the new iRBC surface (Craig and Scherf, 2001; Nagao et al., 2008). The most important member from *var gene*

family is *P. falciparum* erythrocyte membrane protein 1 (PfEMP 1) (Guizetti and Scherf, 2013). PfEMP 1 interacts with receptor located in endothelial cells leading to a condition called cytoadherance. The binding makes iRBC lifespan longer and finally obstructs blood circulation especially when it takes place in brain and placenta in a pregnant woman (Carvalho et al., 2013). Therefore, the severity of the disease is worsened by these conditions (Kraemer and Smith, 2006).

Figure 3 Morphology of mosquitoes. *Ae. aegypti* (A) *and Ae. albopictus* (B) (Lounibos and O'Meara, 1999).

CHAPTER 4

Materials and Methods

Parasites

The avian malaria parasite *Plasmodium gallinaceum* was originally adapted from a field isolate from Chacheongsao Province, Thailand in August 2013. The species of parasite was identified by polymerase chain reaction technique (PCR) and DNA sequencing. The parasite was maintained and passed to chickens once every two weeks, and passed to *Aedes aegypti* once every six months to maintain the virulence of parasite.

DNA extraction and PCR

Plasmodium gallinaceum DNA was extracted from blood and tissue using commercial DNA extraction kit (NucleoSpin® Tissue, Macherey-Nagle, Germany). The genus specific primer set was used (forward primer; rPLU6 5'-TTAAAATTGTTGCAGTTAAAACG -3' and reverse primer; rPLU5 5'-CCTGTTGTTGCCTTAAACTTC -3') as described by Snounou et al. (1993). PCR reaction mixtures (total volume 25 µl) consisted of 1x High Fidelity PCR buffer (Invitrogen, USA), 0.8 mM dNTP mixture, 2 mM $MgSO_4$, 1 unit Platinum® Taq High Fidelity (Invitrogen, USA), 2 µl DNA template, and 0.4 µM of each primer. The total volume was adjusted using distilled water. The thermal cycling began with denaturation at 95 °c for 5 min, followed by 30 cycles of 94 °c for 1 min, 55 °c for 45 sec, and 72 °c for 90 sec. Final

extention step was done at 72 °c for 5 min. PCR products were analyzed on 1 % agarose gels and stained with ethidium bromide (Sigma, USA). PCR product was 1200 base pairs.

Animals

One-day old Rhode Island Red and Isa Babcock chickens were acquired from a commercial hatchery and housed in animal facility at Parasitology Unit, Department of Pathology, Chulalongkorn University. The birds were allowed to freely access to water and feed *ad libitum.* The animal use was conducted in compliance with the Chulalongkorn University Laboratory Animal Care and Use Committee (Animal Use Protocol No. 1531001).

Mosquitoes and Mosquito rearing

Aedes aegypti and *Ae. albopictus* laboratory strains were used in this study. *Aedes aegypti* mosquito generation used was two generations including less than F10 (*Ae. aegypti* <F10) and more than F10 (*Ae. aegypti* >F10). *Aedes albopictus* mosquito generation used was more than F10 (*Ae. albopictus* >F10). All mosquitoes were maintained in optimal conditions at 25°c and 80% relative humidity with 12 hour light/dark cycle. They were allowed to feed on 10% sucrose solution *ad libitum* and they were also weekly allowed to feed on the blood of mouse to collect the eggs (Clemons et al., 2010; Zheng et al., 2015).

Parasite passages

The parasite was maintained in three Isa Babcock chickens per one passage. Level of parasitemia from infected chickens was monitored daily by using 10% Giemsa stained thin blood films. Two hundred microliters of parasite seed were obtained from jugular vein and mixed with heparin as an anticoagulant. The number of iRBCs was counted and diluted with normal saline to adjust the volume of parasite inoculum (Permin and Juhl, 2002). An inoculum of 10^6 iRBCs was injected into jugular vein of chickens at seven days of age. Parasites were passaged to mosquitoes by feeding directly from an infected chicken and transmitted to a new chicken (Lacrue et al., 2005).

Experimental design

This study was divided into three experiments. The first experiment was animal infections and aim of this experiment was to estimate parasite and gametocyte levels in chickens receiving different inoculum doses. The second experiment was mosquito infections and aim of this experiment was to assess and compare the ability of *Ae. aegypti* and *Ae. albopictus* to support the development of parasites. The third experiment was transmission of *P. gallinaceum* and aim of this experiment was to investigate the ability of mosquito to transmit the parasite after taking different gametocyte levels.

Experiment 1. Animal infections

P. gallinaceum infections in Rhode Island Red and Isa Babcock chickens with different inoculum doses were investigated in this experiment. Two week–old Rhode Island Red chickens (n=20) were divided into four subgroups (n=5), three subgroups were intravenously inoculated with 10^6, 10^4, and 10^2 iRBCs, respectively and the last subgroup was served as a control group. One week–old Isa Babcock chickens (n=20) were allocated into four subgroups (n=5), each of which was intravenously inoculated with the same numbers as described in Rhode Island Red chickens. Two week–old Isa Babcock B 380 chickens (n=20) were allocated into four subgroups (n=5), each of which was intravenously inoculated with the same numbers as described in Rhode Island Red chickens. The chickens were clinically examined and monitored for the parasite levels. The percentage of asexual stage parasites as well as gametocytes were monitored from day 3 to day 21 post inoculation (PI). Blood samples were obtained from the wing vein, smeared and stained with 10% Giemsa, and observed under light microscope. The percentage of gametocyte was estimated by counting the number of gametocyte per 1,000 red blood cells.

Experiment 2. Mosquito infections

The study was divided into 15 experiments, each of which consisted of three mosquito groups including old colony of 60 *Ae. albopictus* (>F10), young colony of 60 *Ae. aegypti* (<F10), and old colony of 60 *Ae. aegypti* (>F10). All mosquitoes were 4-10 days of age. The gametocyte donors were 7-day old Isa Babcock chickens. The chickens

were intravenously inoculated with 10^6 iRBCs. The infected chicken was restrained and placed in the cage of mosquitoes and the mosquitoes of each experiment were allowed to feed on an infected chicken for 20 minutes (Alavi et al., 2003). The parasitemia ranged from 1.1 to 79.5 % and gametocytemia ranged from 0.1 to 5.2 %. Before blood feeding, the mosquitoes were starved from sugar for 12 hours. Blood-fed mosquitoes were kept and observed for mortality rate daily. On day 5 post blood feeding (PBF), 30 *Ae. albopictus* (>F10), 30 *Ae. aegypti* (<F10), and 30 *Ae. aegypti* (>F10) from each experiment were dissected, counted for oocysts on the midgut wall, and observed for the sporozoites in the salivary glands. Mosquito's midgut was stained with 0.5% mercurochrome dye, observed, and counted number of oocyst under light microscope (Coleman et al., 2007; Usui et al., 2011).

Experiment 3. Transmission of *Plasmodium gallinaceum*

This experiment was conducted as previously described in the second experiment with slight modifications for the number of each mosquito and performed on day 10 PBF only. The experiment was divided into three experiments, each of which consisted of three mosquito groups including 65 *Ae. albopictus* (>F10), 65 *Ae. aegypti* (<F10), and 65 *Ae. aegypti* (>F10). The gametocyte donors were 7-day old Isa Babcock chickens. The chickens were intravenously inoculated with 10^6 iRBCs. The parasitemia were 5.9, 60.6, and 79.5 % and the gametocytemia were 1, 3.2, and 3.4 %. On day 10 PBF, five mosquitoes were randomly selected from each mosquito group and each single mosquito was allowed to feed on a 7-day-old naïve chicken. In total, there were

15 naïve chickens bitten by *Ae. albopictus* (>F10), *Ae. aegypti* (<F10), and *Ae. aegypti* (>F10). Appearance of the parasites in blood circulation was monitored on a daily basis and recorded to calculate the percentage of infectivity.

Data analysis

For the first experiment, the outcome of receiving different doses of parasite inoculum was compared by using One-way ANOVA. For the second experiment, data were analysed for a correlation between gametocyte levels and mosquito infected rate (using oocyst number as an indicator for mosquito infection) and the comparison between parasite level and oocyst number was used One-way ANOVA. For the third experiment, a correlation between mosquito infected rate and infectivity rate of the chicken host were explored to assess the ability to transmit the parasites of each mosquito strain.

CHAPTER 5

Results

Parasite identification by PCR and DNA sequencing

Parasite identity was confirmed by PCR and DNA sequencing. PCR product was 1200 base pairs (Fig 4). DNA sequence was confirmed by using BLAST in GenBank database. DNA sequence was matched with *Plasmodium gallinaceum* asexually expressed ribosomal RNA small subunit and percent identities were 99% (Fig 5).

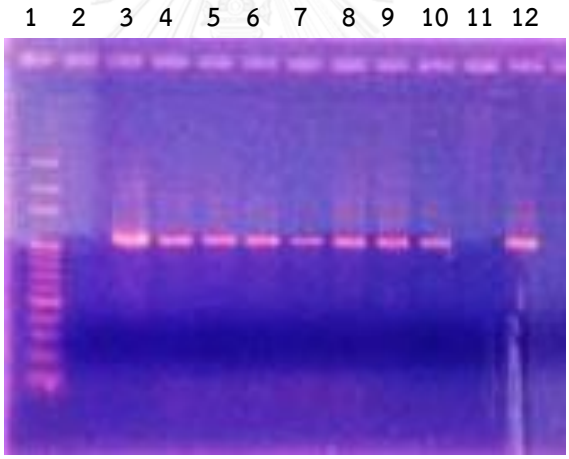
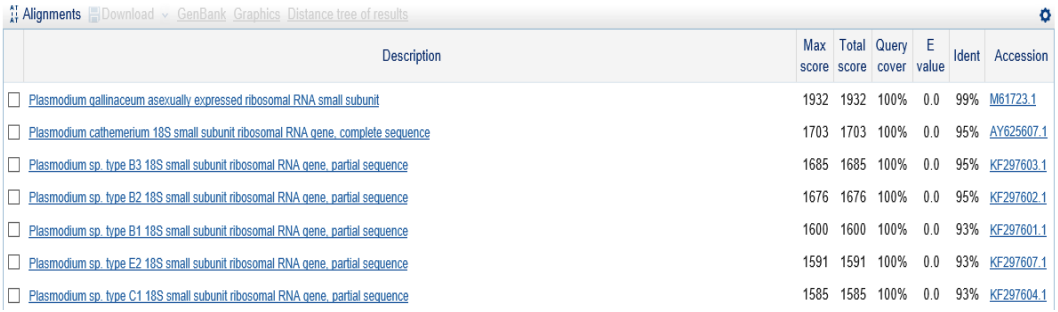


Figure 4 PCR products from infected blood and tissue samples. PCR product was 1200 base pairs. Lane: 1 = DNA marker, Lane: 2 = negative control, Lane: 3 = positive control (P. falciparum plasmid), Lane: 4 and 5 = brain, Lane: 6 = liver, Lane: 7 = spleen, Lane: 8 = kidney, Lane: 9 = lung, Lane: 10 = heart, Lane: 11 and 12= blood



Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Plasmodium gallinaceum asexually expressed ribosomal RNA small subunit	1932	1932	100%	0.0	99%	M61723.1
Plasmodium cathemerium 18S small subunit ribosomal RNA gene, complete sequence	1703	1703	100%	0.0	95%	AY625607.1
Plasmodium sp. type B3 18S small subunit ribosomal RNA gene, partial sequence	1685	1685	100%	0.0	95%	KF297603.1
Plasmodium sp. type B2 18S small subunit ribosomal RNA gene, partial sequence	1676	1676	100%	0.0	95%	KF297602.1
Plasmodium sp. type B1 18S small subunit ribosomal RNA gene, partial sequence	1600	1600	100%	0.0	93%	KF297601.1
Plasmodium sp. type E2 18S small subunit ribosomal RNA gene, partial sequence	1591	1591	100%	0.0	93%	KF297607.1
Plasmodium sp. type C1 18S small subunit ribosomal RNA gene, partial sequence	1585	1585	100%	0.0	93%	KF297604.1

Figure 5 Sequences producing significant alignments

***Plasmodium gallinaceum* infection in Rhode Island Red and Isa Babcock chickens**

Rhode Island Red and Isa Babcock chicken were inoculated with three different inoculum doses including 10^6, 10^4, and 10^2 iRBCs to examine the effect of inoculum doses and levels of parasites in the infected chickens. The chickens were examined for blood parasite for 21 days post inoculation (PI). Summary of parasite infections in chickens is shown in Table 1.

In 14-day old Rhode Island Red chicken group, the parasites were found in blood circulation of the chickens inoculated with 10^6 iRBCs only. These infected chickens also showed the signs of illness. The parasites were found on day 5 PI and the infection rate was 40% (2/5). On day 6 PI, the infection rate was 100% (5/5). The average of parasitemia were 0.4-75.4% and the parasite peaked on day 13 PI. The average of gametocytemia were 0.06-13.5% and the gametocyte peaked on day 11 PI. On day 7 PI, the infected chickens showed clinical signs, including depression, loss of appetite, green feces, and anemia. The first chicken was dead on day 12 PI (Fig 6). The

mortality rate was 100% on day 15 PI and the parasite levels before death were about 60-80%.

In 14-day old Isa Babcock chicken group, the parasites were found in blood circulation of the chickens that were inoculated with 10^6, 10^4, and 10^2 iRBCs. Infection rates of the chickens that were inoculated with 10^6, 10^4 and 10^2 iRBCs were 100, 80, and 60%, respectively. In the chickens that were inoculated with 10^6 iRBCs, the parasites were firstly found on day 4 PI and the infection rate was 40% (2/5). On day 16 PI, the infection rate was 100%. The average of parasite levels were 0.3-32.36% and the average of gametocyte levels were 0.1-2.04%. Both of the parasite stages peaked on day 8 PI and the mortality rate was 20%. The infected chickens showed moderate clinical signs compared with Rhode Island Red chickens and the chickens recovered within two weeks after inoculation. In the chickens inoculated with 10^4 iRBCs, the parasites were firstly found on day 5 PI and the infection rate was 20% (1/5). The infection rate was 80% (4/5) on day 11 PI. The average of parasite levels were 0.08-22.96% and peaked on day 13 PI. The average of gametocyte levels were 0.02-0.84% and peaked on the same day as the parasite levels. The infected chickens showed only slightly clinical signs. In the chickens inoculated with 10^2 iRBCs, the parasites were firstly found on day 9 PI and the infection rate was 20% (1/5). The infection rate was 60% (3/5) on day 12 PI. The average of parasite levels were 0.06-1.28% and peaked on day 15 PI. The average of gametocyte levels were 0.13-1.83% and peaked on the same day as the parasite levels. No clinical signs was found in the infected chickens.

In 7-day old Isa Babcock chicken group, the parasites were found in blood circulation of the chickens inoculated with 10^6, 10^4, and 10^2 iRBCs with different infection and mortality rates. In the chickens inoculated with 10^6 iRBCs, the infection rate was 100% (5/5) on day 3 PI. The average of parasite levels were 0.23-52.58% and peaked on day 7 PI. The average of gametocyte levels were 0.03-4.58% and also peaked on day 7 PI. The mortality rate was 20% (1/5) and the chicken died on day 8 PI after the peaked parasite level (74.6%). In the chickens inoculated with 10^4 iRBCs, the parasites were firstly found on day 6 PI and the infection rate was 20% (1/5). The infection rate was 100% (5/5) on day 13 PI. The average of parasite levels were 0.04-32.2% and peaked on day 12 PI. The average of gametocyte levels were 0.02-1.06% and peaked on day 12 PI. In the chickens inoculated with 10^2 iRBCs, the parasites were firstly found on day 11 PI and the infection rate was only 20% (1/5). The average of parasite levels were 0.1-23.1% and peaked on day 18 PI. The average of gametocyte levels were 0.1-2.8% and peaked on day 16 PI.

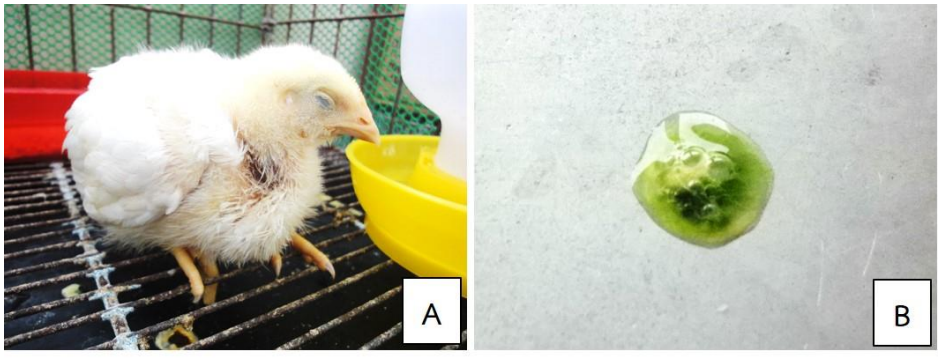


Figure 6 Clinical signs of the infected chicken. The chicken was depress, anemia, and loss of appetite (A). Green color feces of the infected chicken (B).

Table 1 Summary *of Plasmodium gallinaceum* infection in Rhode Island Red and Isa Babcock chickens with different inoculum doses

Type of chickens	14-day old Rhode Island Red			14-day old Isa Babcock			7-day old Isa Babcock		
Inoculum dose (iRBCs)	10^6	10^4	10^2	10^6	10^4	10^2	10^6	10^4	10^2
Group size	5	5	5	5	5	5	5	5	5
Infection rate (%)	100	0	0	100	80	60	100	100	20
Mortality rate (%)	100	0	0	20	0	0	20	0	0
Prepatent period (day PI)	5 (60%)	-	-	4 (40%)	5 (20%)	9 (20%)	3 (100%)	6 (20%)	11 (20%)
	6 (40%)			5 (20%)	11 (60%)	10 (40%)		7 (20%)	
				9 (20%)				8 (20%)	
				16 (20%)				12 (20%)	
								13 (20%)	
Maximum number of iRBCs in percent	75.4	-	-	32.36	22.96	20.7	52.58	32.2	23.1
Day of maximum number of iRBCs	13	-	-	8	13	14	7	12	18
Maximum number of gametocyte in percent	13.5	-	-	2.04	0.84	1.28	4.58	1.06	2.8
Day of maximum number of gametocyte	14	-	-	8	13	15	7	12	16

The comparison of *Plasmodium gallinaceum* infection by chicken breed, age, and inoculum dose in term of parasite level

Rhode Isa Red and Isa Babcock chicken breed were used to examine the severity of the infection. At 14 days of age, the chickens were inoculated with 10^6 iRBCs and examined for parasite levels. The parasitemia levels in Rhode Island Red were significant difference form Isa Babcock on days 10, 11, 12, 13, and 14 post inoculation (PI). Rhode Island Red also had the gametocyte levels significant difference from that of Isa Babcock on days 9, 10, 11, 12, 13, and 14 PI. Data are shown in Tables 2 and 3

For *P. gallinaceum* infection between 14-day old and 7-day old Isa Babcock chickens, parasite levels were significant difference only in the infected chickens inoculated with 10^6 iRBCs inoculum dose. The parasitemia levels in 7-day old chickens were different from 14-day old chickens on days 4, 5, 6, and 9 PI. The parasitemia levels in 14-day old chickens were different from another group on days 18 and 19 PI. The gametocytemia levels in 7-day old chickens were higher than 14-day old on days 6, 7, and 10 PI. Data are shown in Tables 4 and 5

In 14-day old Isa Babcock chickens, the parasitemia levels in infected chickens inoculated with 10^6 iRBCs inoculum dose were significant differences from infected chickens inoculated with 10^4 iRBCs inoculum dose on days 18 and 19 PI and significant differences from infected chickens inoculated with 10^2 iRBCs inoculum dose on days 14 and 16 PI. About the gametocytemia levels, there was no difference between infected chicken inoculated with 10^6 and 10^4 iRBCs inoculum dose, but there were

differences between infected chickens inoculated with 10^6 and 10^2 iRBCs inoculum dose on day 13 PI. Data are shown in Tables 6 and 7.

In 7-day old Isa Babcock chickens, the parasitemia levels in infected chickens inoculated with 10^6 iRBCs inoculum dose were significant differences from infected chickens inoculated with 10^4 iRBCs inoculum dose on days 6, 7, 8, 9, 10, and 15 PI and the gametocytemia levels in infected chickens with 10^6 iRBCs inoculum dose were differences from infected chickens inoculated with 10^4 iRBCs inoculum dose on days 7, 8, 9, and 10 PI. Data are shown in Tables 8-9.

Results in the first experiment indicated the suitable chicken breed and inoculum dose were 7-day old Isa Babcock chicken and 10^6 iRBCs inoculum dose because prepatent period of the parasites could be estimated from this chicken group.

Table 2 The comparison of the average percentage of parasitemia levels between 14-day old Rhode Island Red and Isa Babcock chickens infected with 10^6 iRBCs inoculum dose

Chicken Breed	The average percentage of parasitemia levels (mean ± SD)									
	Day post inoculation									
	3	4	5	6	7	8	9	10	11	12
Isa Babcock	NF	0.30±0.41	1.06±1.52	4.64±6.46	23.66±31.95	32.36±42.77	15.78±20.19	16.70±19.29[a]	8.43±13.17[a]	6.98±8.66[a]
Rhode Island Red	NF	NF	0.4±0.51	0.88±0.61	2.2±1.25	14.4±11.52	30.19±23.27	53.96±25.08[a]	60.54±19.68[a]	61.48±23.14[a]
	Day post inoculation									
	13	14	15	16	17	18	19	20	21	
Isa Babcock	2.33±2.45[a]	1.50±2.38[a]	1.43±1.82	1.33±1.48	2.55±2.71	2.45±0.76	3.95±1.97	11.78±11.41	15.50±19.99	
Rhode Island Red	75.4±2.83[a]	60.6[a]	NA	NA	NA	NA	NA	NA	NA	

NF, not found. NA, not alive. [a] Statistically significant differences by t-test at $p<0.05$.

Table 3 The comparison of the average percentage of gametocytemia levels between 14-day old Rhode Island Red and Isa Babcock chickens infected with 10^6 iRBCs inoculum dose

Chicken Breed	The average percentage of gametocytemia levels (mean ± SD)									
	Day post inoculation									
	3	4	5	6	7	8	9	10	11	12
Isa Babcock	NF	NF	NF	0.18±0.27	0.74±0.97	2.04±2.93	0.60±1.18[a]	0.26±0.33[a]	0.43±0.48[a]	0.08±0.10[a]
Rhode Island Red	NF	NF	0.06±0.09	0.18±0.18	0.44±0.36	1.2±0.73	4.3±2.99[a]	5.87±3.84[a]	7.81±2.73[a]	6.45±3.37[a]
	Day post inoculation									
	13	14	15	16	17	18	19	20	21	
Isa Babcock	0.20±0.16[a]	0.05 ±0.10[a]	0.08±0.10	NF	0.03±0.05	NF	NF	0.20±0.28	0.18±0.29	
Rhode Island Red	7.5±2.12[a]	13.5[a]	NA	NA	NA	NA	NA	NA	NA	

NF, not found. NA, not alive. [a] Statistically significant differences by t-test at $p<0.05$.

Table 4 The comparison of the average percentage of parasitemia levels between 14-day old and 7-day old Isa Babcock chicken infected with 10^6 iRBCs inoculum dose

Age (Day)	The average percentage of parasitemia levels (mean ± SD)									
	Day post inoculation									
	3	4	5	6	7	8	9	10	11	12
14	NF	0.30±0.41[a]	1.06±1.52[a]	4.64±6.46[a]	23.66±31.95	32.36±42.77	15.78±20.19[a]	16.70±19.29	8.43±13.17	6.98±8.66
7	1.26±0.94	5.86±4.96[a]	22.68 ±18.18[a]	45.18 ±29.34[a]	52.58±30.80	48.23±24.49	51.49±15.88[a]	28.99±15.37	21.38±15.07	8.30±5.48
	Day post inoculation									
	13	14	15	16	17	18	19	20	21	
14	2.33±2.45	1.50±2.38	1.43±1.82	1.33±1.48	2.55±2.71	2.45 ±0.76[a]	3.95 ±1.97[a]	11.78±11.41	15.50±19.99	
7	2.30±2.32	2.25±1.32	2.98±1.57	0.88±0.63	0.90±0.74	0.68±0.83[a]	0.25 ±0.26[a]	0.23±0.21	NF	

NF, not found. [a] Statistically significant differences by t-test at $p<0.05$.

Table 5 The comparison of the average percentage of gametocytemia levels between 14-day old and 7-day old Isa Babcock chicken infected with 10^6 iRBCs inoculum dose

Age (Day)	The average percentage of gametocytemia levels (mean ± SD)									
	Day post inoculation									
	3	4	5	6	7	8	9	10	11	12
14	NF	NF	NF	0.18±0.27[a]	0.74±0.97[a]	2.04±2.93	0.60±1.18	0.26±0.33[a]	0.43±0.48	0.08±0.10
7	0.04±0.09	0.30±0.25	0.68 ±0.59	1.70±1.26[a]	4.58±2.61[a]	2.83±1.78	2.33±0.97	2.26±0.69[a]	0.55±0.42	0.20±0.22
	Day post inoculation									
	13	14	15	16	17	18	19	20	21	
14	0.20±0.16	0.05±0.10	0.08±0.10	NF	0.03±0.05	NF	NF	0.20±0.28	0.18±0.29	
7	0.05±0.06	0.03±0.05	NF	NF	NF	NF	NF	NF	NF	

NF, not found. [a] Statistically significant differences by t-test at $p<0.05$.

Table 6 The average percentage of parasitemia levels in 14-day old Isa Babcock chicken

Inoculum Dose (iRBC)	The average percentage of parasitemia levels (mean ± SD)									
	Day post inoculation									
	3	4	5	6	7	8	9	10	11	12
10^6	NF	0.30±0.41	1.06±1.52	4.64±6.46	23.66±31.95	32.36±42.77	15.78±20.19	16.70±19.29	8.43±13.17	6.98±8.66
10^4	NF	NF	0.06±0.13	0.28±0.63	1.88±4.20	6.86±15.28	9.04±19.43	11.10±22.43	13.54±18.73	17.54±22.31
10^2	NF	NF	NF	NF	NF	NF	0.62±1.39	11.44±25.47	16.34±35.70	8±14.99
	Day post inoculation									
	13	14	15	16	17	18	19	20	21	
10^6	2.33±2.45	1.50±2.38[a]	1.43±1.82	1.33±1.48[a]	2.55±2.71	2.45±0.76[a]	3.95±1.97[a]	11.78±11.41	15.50±19.99	
10^4	22.96±30.13	11.68±13.51	7.68±9.01	2.08±1.51	0.88±0.72	0.50±0.42[a]	0.25±0.25[a]	0.08±0.05	0.08±0.05	
10^2	8.62±12.74	20.7±26.47[a]	15.86±20.35	6.62±7.35[a]	1.54±1.67	1.3±2.27	1.02±1.86	0.26±0.34	0.48±0.87	

NF, not found. [a] Syatistically significant differences among inoculum doses by t-test at $p<0.05$.

Table 7 The average percentage of gametocytemia in 14-day old Isa Babcock chickens

Inoculum Dose (iRBC)	The average percentage of gametocytemia level (mean ± SD)									
	Day post inoculation									
	3	4	5	6	7	8	9	10	11	12
10^6	NF	NF	NF	0.18±0.27	0.74±0.97	2.04±2.93	0.60±1.18	0.26±0.33	0.43±0.48	0.08±0.10
10^4	NF	NF	NF	0.04±0.09	0.02±0.04	0.24±0.54	0.44±0.98	0.44±0.98	0.36±0.59	0.66±1.00
10^2	NF	NF	NF	NF	NF	NF	NF	0.28±0.63	0.58±1.24	0.44±0.82
	Day post inoculation									
	13	14	15	16	17	18	19	20	21	
10^6	0.20±0.16[a]	0.05±0.10	0.08±0.10	NF	0.03±0.05	NF	NF	0.20±0.28	0.18±0.29	
10^4	0.84±1.33	0.15±0.30	0.20±0.23	0.03±0.05	NF	NF	NF	NF	NF	
10^2	0.34±0.33[a]	0.78±1.23	1.28±1.83	0.88±1.75	0.1±0.14	0.06±0.13	NF	NF	NF	

NF, not found. [a] Statistically significant differences among inoculum doses by t-test at $p<0.05$.

Table 8 The average percentage of parasitemia levels in 7-day old Isa Babcock chickens

Inoculum Dose (iRBC)	The average percentage of parasitemia (mean ± SD)									
	Day post inoculation									
	3	4	5	6	7	8	9	10	11	12
10^6	1.26±0.94	5.86±4.96	22.68±18.18	45.18±29.34[a]	52.58±30.80[a]	48.23±24.49[a]	51.49±15.88[a]	28.99±15.37[a]	21.38±15.07	8.30±5.48
10^4	NF	NF	NF	0.04±0.09[a]	0.14±0.26[a]	0.55±0.45[a]	0.92±0.89[a]	4.88±4.64[a]	20.3±20.34	32.2±29.26
10^2	NF	NF	NF	NF	NF	NF	NF	NF	0.02±0.04	0.34±0.76
	Day post inoculation									
	13	14	15	16	17	18	19	20	21	
10^6	2.30±2.32	2.25±1.32	2.98±1.57[a]	0.88±0.63	0.90±0.74	0.68±0.83	0.25±0.26	0.23±0.21	NF	
10^4	17.12±17.58	10.8±10.38	0.92± 0.52[a]	1.22±1.07	1.78±1.16	1.14±1.00	0.58±0.36	0.34±0.17	NF	
10^2	0.46±1.03	1.62±3.62	4.5±10.06	3.18±7.11	4.2±9.39	4.62±10.33	1.46±3.26	0.54±1.21	NF	

NF, not found. [a] Statistically significant differences among inoculum doses by t-test at $p<0.05$.

Table 9 The average percentage of gametocytemia levels in 7-day old Isa Babcock chickens

Inoculum Dose (iRBC)	The average percentage of gametocytemia (mean ± SD)									
	Day post inoculation									
	3	4	5	6	7	8	9	10	11	12
10^6	0.04±0.09	0.30±0.25	0.68±0.59	1.70±1.26	4.58±2.61[a]	2.83±1.78[a]	2.33±0.97[a]	2.26±0.69[a]	0.55±0.42	0.20±0.22
10^4	NF	NF	NF	NF	0.04±0.09[a]	0.02±0.04[a]	0.08±0.11[a]	0.26±0.29[a]	0.64±0.60	1.06±1.06
10^2	NF	NF	NF	NF	NF	NF	NF	NF	NF	0.02±0.04
	Day post inoculation									
	13	14	15	16	17	18	19	20	21	
10^6	0.05±0.06	0.03±0.05	NF	NF	NF	NF	NF	NF	NF	
10^4	0.84±0.81	0.08±0.11	0.06±0.09	0.08±0.13	0.04±0.09	0.04±0.09	NF	0.04±0.09	NF	
10^2	0.04±0.09	0.08±0.18	0.22±0.49	0.56±1.25	0.46±1.03	0.16±0.36	0.08±0.18	0.04±0.09	NF	

NF, not found. [a] Statistically significant differences among inoculum doses by t-test at $p<0.05$.

Plasmodium gallinaceum infection in three difference mosquito vectors

All the mosquito groups, including *Aedes albopictus* (>F10), *Ae. aegypti* (>F10), and *Ae. aegypti* (<F10) were allowed to feed on the blood meal which contained different levels of *P. gallinaceum* parasites. Parasitemia levels ranged from 1.1 to 79.5% and gametocytemia levels ranged from 0.1 to 5.2%. After blood feeding, the mosquito feeding rates and mortality rates were recorded. On day five post blood feeding (PBF), the mosquitoes were dissected and counted for the oocysts on the midgut wall, and were observed for the sporozoites in salivary glands. The oocysts were round in structure and diameter was about 18 micrometers (Fig 7 A). The sporozoites had spindle shape and flew freely in the squash salivary glands (Fig 7 B). Sporozoites were found in the salivary glands of all dissected mosquito groups, therefore all mosquitoes supported the development of parasites, including oocyst formation and sporogonic stage, and the mosquito infective rates were 100%. The summary of mosquito infections of three mosquito groups are shown in Table 10 and 11. There was no correlation between oocyst formations and the parasite levels or gametocyte levels in the blood meal in all mosquito groups. R-squared of *Ae. albopictus* (>F10), *Ae. aegypti* (>F10), and *Ae. aegypti* (<F10) were 0.0004, 0.0037, and 0.0112, respectively (Fig 8).

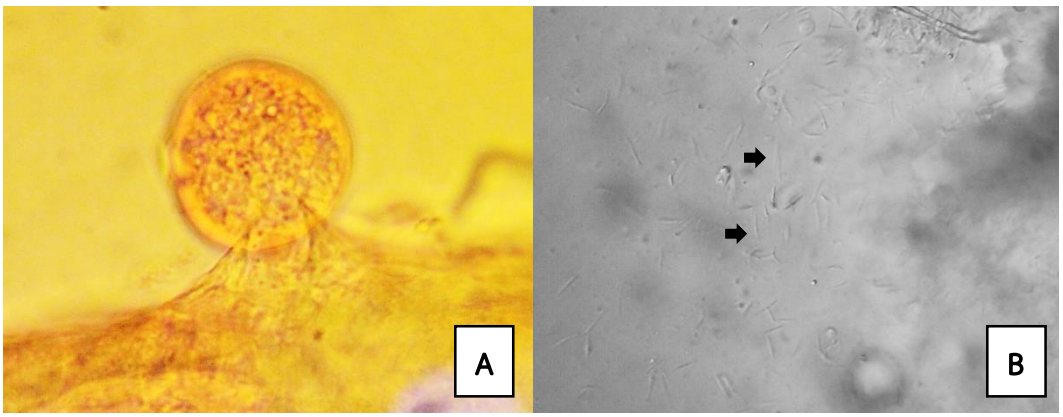


Figure 7 Oocyst and sporozoites. The oocyst on the midgut wall of Aedes albopictus (>F10), mercurochrome strained, 18 micrometers diameter, 400x objective (A). The spindle-shaped sporozoite in the salivary gland of Ae. albopictus (>F10), 400x objective (B, arrows).

Table 10 Summary of *Plasmodium gallinaceum* infection in *Aedes albpoictus* (>F10), *Aedes aegypti* (>F10), and *Aedes aegypti* (<F10) mosquitoes

Study No.	Gametocyte (%)	Parasite (%)	*Ae. albopictus* (>F10)			*Ae. aegypti* (>F10)			*Ae. aegypti* (<F10)		
			Feeding rate	Mortality rate	Oocyst Mean (SD)	Feeding rate	Mortality rate	Oocyst mean(SD)	Feeding rate	Mortality rate	Oocyst mean(SD)
1	0.1	1.1	95.65	6.82	0.60 (1.13)	77.27	11.76	0.10 (0.40)	95.45	22.5	0.30 (0.65)
2	0.2	17.8	100	3.22	18.23 (16.40)	88.89	4.17	6.13 (6.19)	92.1	18.24	3.50 (6.43)
3	0.3	17	86.67	6.92	89.20 (57.60)	92.68	8.42	43.10 (37.64)	97.06	20.97	29.13 (22.96)
4	0.5	2.1	87.5	8.57	244.23 (130.08)	89.47	5.88	155.13 (72.68)	100	19.39	119.70 (60.01)
5	0.8	7.8	96.87	10	242.80 (131.54)	95.65	9.1	135.17 (67.59)	98.04	12	80.07 (42.57)
6	1	5.9	88.24	10.34	153.10 (42.58)	97.06	11.21	80.00 (41.92)	91.18	16.67	65.90 (26.43)
7	1.3	52.6	87.65	13.64	122.07 (114.32)	87.5	0	44.53 (35.99)	91.11	17.5	10.60 (18.94)
8	1.4	26.4	82.35	0	247.37 (208.13)	96.15	9.09	91.83 (75.94)	90.57	20.08	30.77 (53.62)

Table 11 Summary of *Plasmodium gallinaceum* infection in *Aedes albpoictus* (>F10), *Aedes aegypti* (>F10), and *Aedes aegypti* (<F10) mosquitoes

Study No.	Gametocyte (%)	Parasite (%)	*Ae. albopictus* (>F10)			*Ae. aegypti* (>F10)			*Ae. aegypti* (<F10)		
			Feeding rate	Mortality rate	Oocyst mean(SD)	Feeding rate	Mortality rate	Oocyst mean(SD)	Feeding rate	Mortality rate	Oocyst mean(SD)
9	1.7	44.3	96.67	6.9	148.60 (96.62)	72.73	6.25	67.07 (36.14)	89.47	12.94	10.10 (7.85)
10	2.2	69.6	84.85	7.14	76.80 (65.56)	100	15.62	21.50 (32.68)	100	17.5	22.43 (26.90)
11	2.6	28.9	88.89	0	106.10 (74.05)	71.88	7.39	74.07 (53.18)	92.68	14.28	34.20 (24.63)
12	3.2	60.6	87.8	5.56	151.77 (70.34)	97.14	11.76	83.50 (55.95)	72.5	3.45	49.93 (32.92)
13	3.4	79.5	94.34	2.04	146.50 (49.27)	100	7.14	93.27 (47.30)	95.35	19.02	56.50 (31.33)
14	5	77.4	91.18	12.26	51.47 (54.97)	100	0	31.73 (21.95)	95.83	23.9	7.63 (7.41)
15	5.2	76.3	87.5	5.26	45.80 (52.14)	89.23	0	46.73 (35.49)	87.18	5.88	17.70 (30.22)

Figure 8 The correlation between gametocyte levels and the number of oocyst in all mosquito groups

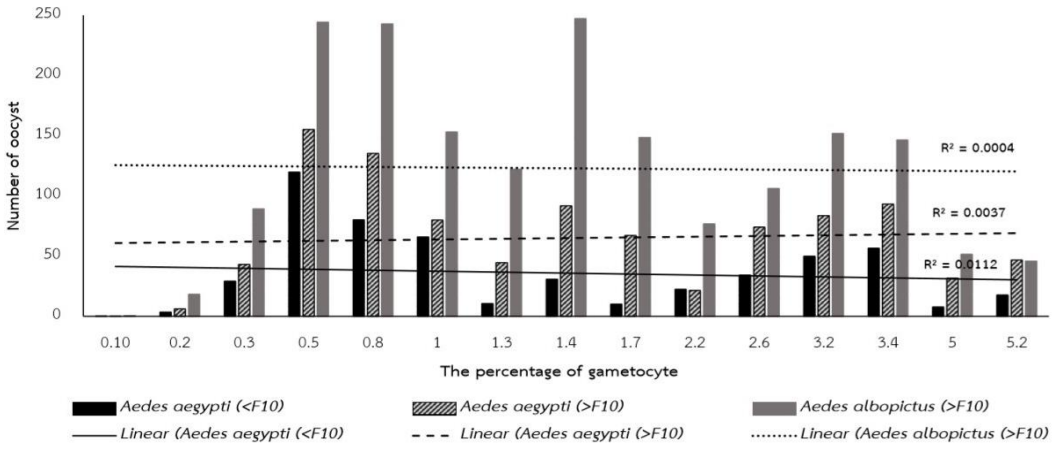


Table 12 The average number of oocyst formations from difference gametocyte levels

Mosquito Groups	Number of oocyst formations Mean (SD)														
	Gametocyte levels														
	0.1	0.2	0.3	0.5	0.8	1	1.3	1.4	1.7	2.2	2.6	3.2	3.4	5	5.2
Ae. albopictus (>F10)	0.60^{a} (1.13)	18.23^{ab} (16.40)	89.20^{ab} (57.60)	244.23^{ab} (130.08)	242.80^{ab} (131.54)	153.10^{ab} (42.58)	122.07^{ab} (114.32)	247.37^{ab} (208.13)	148.60^{ab} (96.62)	76.80^{ab} (65.56)	106.10^{a} (74.05)	151.77^{ab} (70.34)	146.50^{ab} (49.27)	51.47^{a} (54.97)	45.80^{a} (52.14)
Ae. aegypti (>F10)	0.10^{a} (0.40)	6.13^{a} (6.19)	43.10^{a} (37.64)	155.13^{a} (72.68)	135.17^{ac} (67.59)	80.00^{a} (41.92)	44.53^{ac} (35.99)	91.83^{ac} (75.94)	67.07^{ac} (36.14)	21.50^{a} (32.68)	74.07^{b} (53.18)	83.50^{ac} (55.95)	93.27^{ac} (47.30)	31.73^{b} (21.95)	46.73^{b} (35.49)
Ae. aegypti (<F10)	0.30 (0.65)	3.50^{b} (6.43)	29.13^{b} (22.96)	119.70^{b} (60.01)	80.07^{bc} (42.57)	65.90^{b} (26.43)	10.60^{bc} (18.94)	30.77^{bc} (53.62)	10.10^{bc} (7.85)	22.43^{b} (26.90)	34.20^{ab} (24.63)	49.93^{bc} (32.92)	56.50^{bc} (31.33)	7.63^{ab} (7.41)	17.70^{ab} (30.22)

$^{a-c}$ indicates statistically significant differences among mosquito groups by t-test at $p<0.05$.

The correlation between mosquito groups and oocyst formations

Mosquitoes in the second experiment were allowed to feed on the blood meal contained with the gametocytes ranging from 0.1 to 5.2%. The oocyst formations of three mosquito groups were differences and the comparison of the number of oocysts is shown in Table 12. The number of oocysts were presented as mean ± SD. *Aedes albopictus* (>F10) had more ability to support the parasite development when compared with *Ae. aegypti* (>F10) and *Ae. aegypti* (<F10) *(p*< 0.05*).* In *Ae. albopictus* (>F10) group, the number of oocysts ranged from 0.60 to 247.37 oocysts per midgut. The lowest oocyst numbers were found in the mosquitoes that fed on the blood meal with 0.1% gametocytes and the highest oocyst numbers were found in the mosquitoes that fed on the blood meal with 1.4% gametocytes.

There were only 45.80 oocysts per midgut found in the mosquito group that fed on the highest gametocyte level in the experiment.

In *Ae. aegypti* (>F10), the number of oocysts ranged from 0.10-155.13 oocyst per midgut. The lowest oocyst numbers were found in mosquito that fed on the blood meal with 0.1% gametocytes and the highest oocyst numbers were found in mosquito that fed on the blood meal with 0.5% gametocytes. There were only 46.873 oocysts per midgut found in the mosquito group that fed on the highest gametocyte level in the experiment.

In *Ae. aegypti* (<F10), the number of oocysts ranged from 0.30 to 119.70 oocysts per midgut. The lowest oocyst numbers were found in mosquito that fed on the blood

meal with 0.1% gametocytes and the highest oocyst numbers were found in mosquito that fed on the blood meal with 0.5% gametocytes. There were only 17.70 oocysts per midgut found in the mosquito group that fed on the highest gametocyte level in the experiment.

Feeding and mortality rate in experimental mosquitoes

Before blood feeding, all mosquitoes were starved from sugar for 12 hours and there were allowed to feed on the infected chicken for 20 minutes. The feeding rates of *Ae. albopictus* (>F10) ranged from 82.35 to 100% and the mean was 90.41%. In *Ae. aegypti* (>F10), the feeding rate ranged from 71.88 to 100% and the mean was 90.38%. In *Ae. aegypti* <F10, the feeding rate ranged from 72.5 to 100% and the mean was 92.57%. There was no significantly difference of the feeding rates among three mosquito groups (Fig 9).

In the experiment, all mosquitoes in each group were in the same age and generation. After blood feeding, all of them were reared at 25°c and 80% relative humidity with 12 hour light/dark cycle which was the optimum condition for mosquitoes and parasite development (Angrisano et al., 2012). Mortality rate was daily recorded to estimate the effect of parasite development for the survival of the infected mosquitoes. The mean of mortality rates in *Ae. albopictus* (>F10) was 6.58% and ranged from 0 to 13.64%. The mean of mortality rates in *Ae. aegypti* (>F10) was 7.19% and ranged from 0 to 15.62%. The mean of mortality rates in *Ae. aegypti* (<F10) was 16.29% and ranged from 3.45 to 23.9%. The mortality rate in *Ae. aegypti* (<F10)

was higher than other mosquito groups and was significant difference from *Ae. albopictus* (>F10) and *Ae. aegypti* (>F10) ($p< 0.05$). However, there was no difference of the mortality rate between *Ae. albopictus* (>F10) and *Ae. aegypti* (>F10) (Fig 10). Only gametocyte can develop to other stages in mosquitoes but the number of gametocytes in blood meal was not related to the mortality rate in infected mosquitoes. R-squared of *Ae. albopictus* (>F10), *Ae. aegypti* (>F10) and *Ae. aegypti* (<F10) were 0.0201, 0.0756, and 0.1779, respectively (Fig 11)

Figure 9 The feeding rates in different mosquito groups.

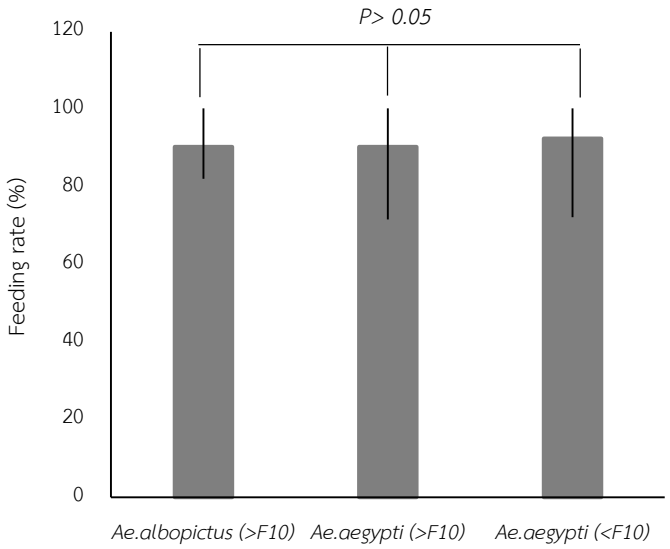


Figure 10 The mortality rates in different mosquito groups

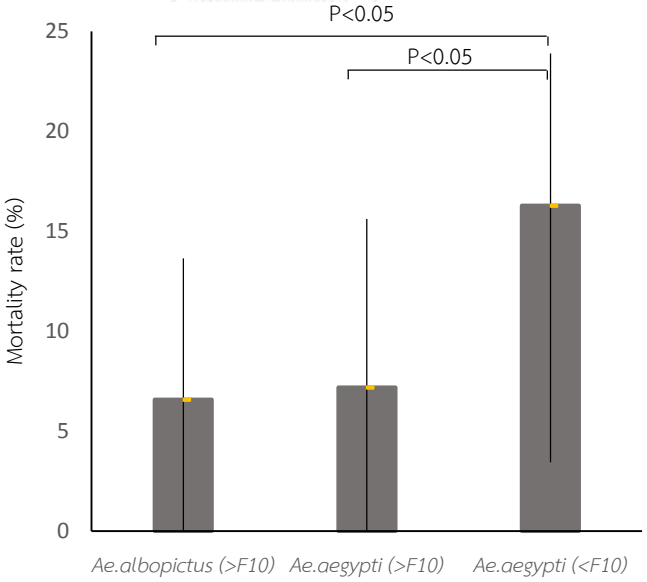
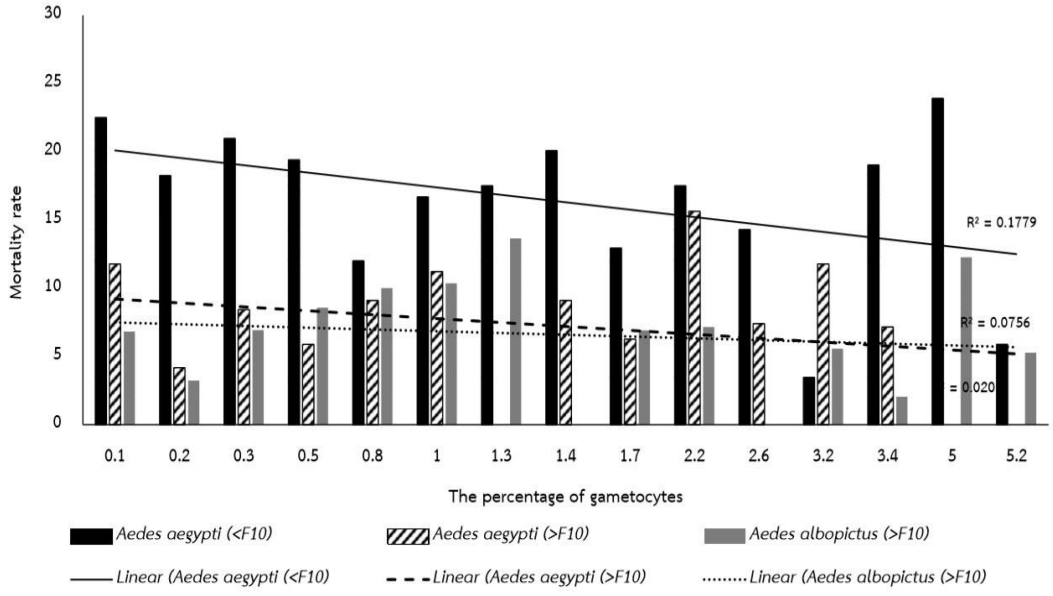


Figure 11 The correlation between gametocyte levels and mortality rates in three different mosquito groups



Plasmodium gallinaceum transmission by three different mosquito groups

The third experiment was divided into three experiments of three groups of mosquito *Ae. albopictus* (>F10), *Ae. aegypti* (>F10), and *Ae. aegypti* (<F10). Infected mosquitoes from each mosquito group were allowed to feed on 7-day old chickens. Before blood feeding, 30 infected mosquitoes in each mosquito group were sampled, dissected, counted for the number of oocysts, and observed for the sporozoites. After blood feeding, chickens were daily monitored for the parasite in the blood circulation by using thin blood smear. Mosquitoes from each experiment were infected with different gametocyte levels (1%, 3.2%, and 3.4%). The infection rate, the number of oocysts, and incubation period are shown in Table 13.

In experiment 3.1, a group of mosquitoes was allowed to feed on infected blood meal with 1% gametocytemia and 5.9% parasitemia. The number of oocysts developed in *Ae. albopictus* (>F10) was differences from other mosquito groups. Infection rates in the chickens that were bitten by the infected *Ae. albopictus* (>F10), *Ae. aegypti* (>F10), and *Ae. aegypti* (<F10) were 80%, 80%, and 40%, respectively. The shortest incubation period of the parasites was seven days, which found in the chickens that were bitten by *Ae. albopictus* (>F10) and *Ae. aegypti* (>F10). The incubation period in all chickens ranged from 7 to 10 days post mosquito biting.

In experiment 3.2, a group of mosquitoes was allowed to feed on infected blood meal with 3.2% gametocytemia and 60.6% parasitemia. The number of oocysts in all mosquito groups was different. Infection rate in the chickens that was bitten by

Ae. albopictus (>F10), *Ae. aegypti* (>F10) and *Ae. aegypti* (<F10) were 100%, 100%, and 60%, respectively. The shortest incubation period was seven days, which found in all groups of the chickens. The incubation period in all chickens ranged from 7 to 12 days post mosquito biting.

In experiment 3.3, a group of mosquitoes was allowed to feed on infected blood meal with 3.4% gametocytemia and 79.5% parasitemia. The number of oocysts in all mosquito groups was different. Infection rate in the chickens that was bitten by *Ae. albopictus* (>F10), *Ae. aegypti* (>F10) and *Ae. aegypti* (<F10) were 100%, 80%, and 60%, respectively. The shortest incubation period was seven days, which found in the chickens that were bitten by *Ae. albopictus* (>F10), and *Ae. aegypti* (>F10). The incubation period in all chickens ranged from 7 to 12 days post mosquito biting. Infection rate in chickens in all experiments were not different ($p>0.05$).

Table 13 Summary of *Plasmodium gallinaceum* transmission by three different mosquito groups

		The percentage of *P. gallinaceum* transmission by three different mosquito groups								
		Experiment 3.1			Experiment 3.2			Experiment 3.3		
		Oocyst Mean (SD)	Infection rate (%)	Incubation period	Oocyst Mean (SD)	Infection rate (%)	Incubation period	Oocyst Mean (SD)	Infection rate (%)	Incubation period
Mosquito groups	*Ae. albopictus* (>F10)	153.10^{ab} (42.58)	4/5 (80%)	7-9	151.77^{ab} (70.34)	5/5 (100%)	7-12	146.50^{ab} (49.27)	5/5 (100%)	7-12
	Ae. aegypti (>F10)	80.00^{a} (41.92)	4/5 (80%)	7-10	83.50^{ac} (55.95)	5/5 (100%)	7-12	93.27^{ac} (47.30)	4/5 (80%)	7-8
	Ae. aegypti (<F10)	65.90^{b} 26.43	2/5 (40%)	8-9	49.93^{bc} (32.92)	3/5 (60%)	7-9	56.50^{bc} (31.33)	3/5 (60%)	8-10

$^{a-c}$ Indicates statistically significant differences among mosquito groups by t-test at $p< 0.05$. Frequency indicates a proportion of positive infection/total number of chicken groups.

CHAPTER 6

Discussion

Over the past few years, there were several studies on the relationship among genetics and breed line of animal host and the host susceptibility and resistance to pathogens. Genetic is an important factor that can regulate the body physiology, including immune response to pathogen infection in birds (Zekarias et al., 2002). For example, Rhode Island Red chickens are more susceptible to Marek's disease, colibacillosis, and coccidiosis than other chicken breeds due to the major histocompatibility complex (MHC) encoded genes (Powell et al., 1980; Lamont, 1989).

Rhode Island Red and Isa Babcock were the chicken breeds that were examined in this study. Rhode Island Red is a layer purebred chicken and Isa Babcock is a hybrid bred layer chicken. Isa Babcock is a crossed breed among numbers of different chicken breeds. The parasite infections between both breeds of chickens were different. Parasite lode, morbidity rate, and mortality rate in Rhode Island Red chickens were more severe than those in Isa Babcock chickens. These results were similar to previous studies of purebred chickens that were more susceptible to pathogens than hybrid bred. In addition, *Plasmodium* infections in other hosts were also different. *Plasmodium yoelii* infection in BALB/C mice was more severe than in BALB.D2 mice because BALB.D2 had different T-cell receptor from BALB/C mice (Burt, 1999).

The severity of disease including clinical signs, morbidity rates, and mortality rates was related to inoculum doses (Permin and Juhl, 2002). The mortality rate in infected chickens that were inoculated with 10^6 iRBCs inoculum dose was higher than other groups. Clinical signs were observed in inoculated chickens and each group of chickens showed different severity of clinical signs.

About the course of infection, this study showed infected chickens that were inoculated with the higher inoculum dose had a shorter prepatent period than other groups. On the other hand, patent period or erythocytic stage in all group of chickens and inoculum doses were about two weeks after the first appearance of parasites. Interestingly, *P. gallinaceum* infection in another chicken breed and age had the patent period similar to the results showed in this study. For the parasite infection in 9-day old white leg horn chicken, prepatent period was three days and patent period was 10-14 days (Macchi Bde et al., 2010). For the parasite infection in 35-day old Hubbard chicken, prepatent period was six days and patent period was 13-14 days. Moreover, prepatent period or exoerythocytic stage depends on the chicken breed and inoculum dose (de Macchi et al., 2013). In addition, the course of infection of *Plasmodium* spp. in other animals is similar to avian malaria. *P. chabaudi chabaudi* infection in wild type mice and interferon-gamma receptor (IFN-rR) deficient mice had patent period around two weeks but parasitemia peaked in IFN-rR deficient mice was two times higher than that in wild type mice. IFN-r is an inflammatory cytokine that works with tumor necrotic

factor to reduce the erythocytic stage parasites (Favre et al., 1997; Wedekind et al., 2005).

Sporogony is a development of malaria parasite in mosquito vector and it is an important stage that causes the disease transmission. Sporogonic development is divided into three stages including early stage, mid stage, and late stage. Early stage consists of gametogenesis, zygote formation, ookinete, and oocyst. Mid stage is an oocyst development and sporozoite differentiation. Late stage is a migration of sporozoites. During this stage, there are many factors or mechanisms that interfere with the parasite development. The population dynamics of parasite will be decreased in every stage like a bottleneck (Zollner et al., 2006).

The development of *P. falciparum* in *An. gambiae* in the early sporogonic stage was a threshold pattern. Density of ookinetes in midgut must be at least 30 ookinetes to transit into oocyst stage but the development in *An. freeborni* was a linear pattern (Vaughan et al., 1994).

Another pattern was density dependent that regulated the parasite population to balance the parasite input and output for parasite and vector survival (Dietz, 1988; Sinden et al., 2007). Density dependent was found in the development of *P. berghei*, *P. chabaudi*, and *P. yoelli* in *An. stephensi.* The oocyst formation depended on density of the ookinete but the ookinete was not related with density of the gametocyte (Poudel et al., 2008).

For the development of parasite in the late stage, the number of salivary gland sporozoites and oocyst formation were linear relationship, however sporozoites were destroyed by hemocyte and immune peptides during the migration (Sinden et al., 2007).

In this study, all mosquito groups could support the parasite development but the number of oocysts in each group was difference. The density of gametocytes in an infected blood meal was not related to the number of oocysts in the mosquito. The results were similar to other studies but more data about ookinete formation need to be investigated. In addition, there was a study showed that offspring generation of the mosquito did not affect the mosquito vector competence (Moncayo et al., 2004).

An ability of mosquito vector to transmit the parasite is more important than an ability of mosquito vector to support the parasite development. In this study, the mosquitoes infected with different gametocyte levels were allowed to feed on the chickens. All mosquito groups could transmit the parasites but the infection rates and incubation periods were differences in each mosquito groups and each gametocyte levels. The study about the transmission of *P. yoelii* by *An. stephensi* in mice by Medica and Sinnis (2005) indicated that the sporozoites that were injected into a host were vary in sizes or sometime the sporozoites were not injected into a host when the infected mosquitoes took a blood meal. Moreover, the number of injected sporozoites did not correlate to the number of sporozoites in the salivary glands. Less than 1% of the sporozoites in the salivary glands were injected into a new host. The number of

sporozoites at the biting site was related to the parasite development in the blood stage and the course of disease in vertebrate host depended on the number of injected sporozoites (Kebaier et al., 2009).

Blood meal is an essential protein source for reproductive fitness and egg development in female mosquitoes. However, host preferences in mosquitoes are differences because nutrient requirements are vary in different species of the mosquitoes (Takken and Verhulst, 2013). Host preference of *Aedes* mosquitoes is humans rather than other mammals and birds. On the other hand, *Culex* mosquitoes prefer to feed on avian rather than other hosts (Faraji et al., 2014).

In this study, feeding rates in all mosquito groups were not differences which more than 90% of the mosquitoes fed on the chickens. All experimental mosquitoes were reared in the laboratory for many generations and they were allowed to feed on a variety of hosts such as mice and birds. Therefore, the mosquitoes probably change their feeding behavior. The study of Takken and Verhulst (2013) also showed that laboratory mosquitoes reared for many generations might change their feeding behavior (Takken and Verhulst, 2013).

The survival of mosquitoes is an important factor that affect the period of mosquitoes to transmit the pathogens. Previously, there were many theories that were used to explain the interaction between pathogen and vector mortality including parasite density dependent and vector age dependent (Vaughan, 2007). High parasite density or heavy infection can damage the vector tissue. In case of *Plasmodium*

infections, there are many mechanisms that cause the damage to the mosquito tissue for example when ookinetes penetrate to the midgut wall, the physical barrier is damaged and will be increased the susceptible to bacterial infection. Moreover, parasite infection affects to normal physiology and behavior of the mosquitoes (Ferguson and Read, 2002). Another theory is age dependent, mosquito mortality depends on its age but does not depend on the parasites. Mortality rate in young mosquito is less than an older mosquito (Styer et al., 2007).

In this study, the mortality rates in all mosquito groups were not related to the gametocyte density. The finding in this study is similar to the previous study which indicated that *P. gallinaceum* development in mosquito did not influence the mosquito mortality (Ferguson and Read, 2002). In other *Plasmodium* species, both theories can be used to explain the mosquito mortality for example *P. berghei* infection in *An. stephensi* and *P. falciparum* infection in *An. gambiae*, survival of the mosquito depended on its age but did not depend on the parasite lode except when the parasite lode was very high (Anderson et al., 2000; Dawes et al., 2009). Mortality rate in *Ae. aegypti* (<F10) was difference from other groups but an ability to support the development of parasite was not difference. Generation of mosquito did not influence the vector competence but influenced the mosquito morphology such as wing length (O'Donnell and Armbruster, 2010).

CHAPTER 7

Conclusion

Chicken breed, chicken age, and inoculum dose affected the parasite infections including prepatent period, parasite level, clinical sing, and mortality rate of the chickens. All mosquito groups could support the parasite development. Generation of the mosquitoes did not affect the vector competence. There was no difference of the vector competences between *Aedes albopictus* and *Ae. aegypti* for *Plasmodium gallinaceum*. Mosquito transmission rate did not depend on the number of oocysts in infected mosquitoes. Incubation period of *P. gallinaceum* infection in the chickens ranged from 7 to 12 days post mosquito biting.

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