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Refractoriness of the natural malaria vector *Culex quinquefasciatus* to *Plasmodium gallinaceum*

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Abstract

Development of effective strategies to control malaria transmission is an important issue in malaria research. This study aimed to investigate refractoriness of laboratory and field strains of the malaria vector *Culex quinquefasciatus* to the avian malaria parasite *Plasmodium gallinaceum*, a model of the human malaria disease. Transmission potential was determined by feeding batches of *Cx. quinquefasciatus* on *P. gallinaceum* infected chickens with 10% and 30% parasitemia. The mosquitoes were examined for percent infectivity and numbers of oocysts. Our study showed that when the mosquitoes were fed on the infected chickens with high parasitemia, the laboratory isolate of *Cx. quinquefasciatus* was more susceptible to *P. gallinaceum* transmission and produced significantly higher oocyst numbers than the field isolate. There were, however, no differences in term of the transmission potential and infectivity of the malaria parasite when the mosquitoes were allowed to feed on the *P. gallinaceum*-infected chickens with low parasitemia. In conclusion, our study demonstrated the differences in refractoriness of the natural vector *Cx. quinquefasciatus* to the transmission of the avian malaria *P. gallinaceum*, implying the existence of the natural pathogen resistance mechanisms in the *Culex* mosquito.

Keywords: malaria, *Plasmodium gallinaceum*, transmission, *Culex quinquefasciatus*

Introduction

Climate and environmental change is a consequence of global warming. The continuous rise of the global temperature has led to the emergence and spread of insect-borne diseases to the subtropical regions [1-3]. The majority of tropical diseases are transmitted by mosquito vectors. Thus, mosquitoes are important natural carriers of various pathogens, such as viruses, parasitic protozoa and worms [4-7]. Human malaria is known to be transmitted strictly by mosquitoes in the genus *Anopheles* [8]. In addition, there are malaria parasites of experimental animals

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such as the malaria parasite of domestic chickens *Plasmodium gallinaceum* that is of great veterinary importance [9]. This avian malaria parasite can be transmitted by a wide range of mosquito vectors, such as *Anopheles*, *Aedes* and *Culex* [8-11].

Sporogonic development is an obligatory step of malaria parasite's life cycle. The developmental processes in vectors are essentially conserved in both avian and mammalian malaria parasites [8,12]. The first step is initiated by a bite of a female mosquito, taking up sexual stages, or gametocytes, of the *Plasmodium* parasites from an infected host and transferring those gametocytes to a mosquito mid-gut. Inside the midgut, gametocytes develop into gametes and the fertilization between gametes occurs, resulting in the formation of zygotes. Thereafter, zygotes transform into ookinetes that are capable of penetrating through the mid-gut's epithelial wall and developing into oocysts [13]. Inside the oocysts, the parasites undergo cell division, thereby producing numerous sporozoites that can circulate throughout mosquito body and migrate to salivary glands of mosquitoes. The infective sporozoites can transmit to another host during next blood meal. Understanding the natural processes of sporogonic development can lead to the improvement of the malaria prevention strategies [14,15].

Blocking the transmission of malaria parasite to mosquito vector is one of the important steps toward malaria eradication. Multiple environment factors and entomologic characteristics, especially genetic backgrounds (species and strains) of vectors, play critical roles in malaria transmission [16-19]. In the present work, we aimed to compare the transmission and infectivity of the malaria parasite *P. gallinaceum* to a field isolate and a laboratory strain of the mosquito *Culex quinquefasciatus* in Thailand. This study will provide basic knowledge on refractoriness of the natural vector to the malaria transmission.

Materials and methods

Malaria parasite

The avian malaria parasite used in this study was *P. gallinaceum* strain Pg01/2013MU, originated from a domestic chicken *Gallus gallus domesticus* in Chachoengsao, Thailand [20]. Blood serial passages were performed for routine maintenance. The parasites were monitored by microscopic analysis of thin blood smears stained with Giemsa's stain according to the standard laboratory procedures.

Vertebrate host

Six female chickens, *G. g. domesticus*, aged 2-week old, were used as vertebrate hosts in this study. The experimental animals were maintained in the designated animal facility under the standard laboratory conditions with a room temperature between 25-30˚C and a 12:12 hour dark:light cycle. Chickens were provided with commercial food and clean water *ad libitum*. All experiments were conducted in compliance with the Mahidol University-approved animal use protocols (no MUVS-2014-01) and the animal care & use regulations.

Mosquito rearing

Two batches of mosquito *Cx. quinquefasciatus* were used in this study. A laboratory-adapted strain of *Cx. quinquefasciatus* was obtained from the National Institute of Health (NIH), Ministry of Public Health, Nonthaburi, Thailand, where the colony has been established since 1978 [21]. A field isolate of *Cx. quinquefasciatus* was sampled from an endemic area in Chachoengsao Province in July 2012. Larvae were collected from the field site raised to adults and further maintained in the insectary for three generations. Both laboratory and field strains of *Cx. quinquefasciatus* were reared under the same laboratory conditions at 25-28ºC and 70-80% relative humidity. Larvae in stages 1 and 2 were provided with powered fish food (White Crane, Bangkok, Thailand). Larvae in stages 3 and 4 were supplied with grounded rodent food (Charoen Pokphand Group, Bangkok, Thailand). Larvae were raised in dechlorinated tap water at density of 100 larvae per 1 L of water.

Adult mosquitoes were transferred to a nylon cage and fed on with 10% sucrose solution *ad*

libitum. The adults, aged 5 days post emergence, were transferred to cages for blood meals. For routine maintenance of the mosquito lines, the adults were allowed to feed on uninfected chickens for 30 min. The females were allowed to lay eggs in a tray filled with dechlorinated water. Female adults were collected for species identification according to the standard illustrated keys to the mosquitoes of Thailand. II. Genera *Culex* and *Lutzia* [21].

Experimental design

An inoculum containing 10^7 *P. gallinaceum*-infected red blood cells (iRBCs) was used to inject intravenously into the jugular vein of a group of experimental chickens (n = 3). Levels of blood stage parasites and gametocytes were microscopically monitored. When the parasitemia of the infected chickens reached 10% and 30%, the chickens were anesthetized by an intramuscular injection with Tiletamine HCl/ Zolazepam HCl (Zoletil, Virbac, Thailand) at dose 10 mg/Kg at thigh muscle for mosquito feeding [22]. Before starting the blood meal, adult mosquitoes were starved for 6 hours and were allowed to feed on each anesthetized chicken for one hour. Batches of 100 female mosquitoes of laboratory strain or field isolate were allowed to feed on each infected chicken. Only fully engorged mosquitoes were maintained in the insectaries for 9-10 days.

On Days 9 and 10 post feeding, the 50 mosquitoes were randomly sampled and dissected to exam theit midguts. The mosquito midguts were stained with 0.01% mercurochrome before examination under light microscope at 100x magnifications to count the numbers of infected mosquitoes and the numbers of oocyst per midgut. The ratio of infected mosquitoes per a total number of mosquitoes dissected x 100 is referred to percent infectivity. Differences in the oocyst counts in the field and laboratory strains of *Cx. quinquefasciatus* were analyzed by Mann-Whitney U test using GraphPad Prism version for Windows (GraphPad Solfware, LaJolla, California, USA).

Results

A field strain of *Cx. quinquefasciatus* was allowed to feed on three *P. gallinaceum* infected chickens with parasitemia of 17, 14 and 10 percent (an average of 13.67±3.51%) and gametocytemia of 1, 1 and 1 percent (an average of 1±0%). At the same time, a laboratory strain of *Cx. quinquefasciatus* was permitted to feed on three *P. gallinaceum* infected chickens with parasitemia of 17, 14 and 14 percent (an average of 15±1.73%) and gametocytemia of 2, 1 and 1 (an average of 1.33±0.58%). The overall mean parasitaemia and gametocytemia were 14.33±2.58% and 1.16±0.41%, respectively.

On Days 9-10 post feeding, 50 of 100 mosquitoes feeding on each chicken were dissected for examination of oocysts in the midguts, as described in *Materials and Methods*. In the field isolate of mosquitoes, the average oocyst counts were 0.58±1.59, 0.66±1.80, 0.5±1.15 oocysts per midgut for each replicate (overall of 0.58±1.53 oocysts per midgut, n total = 150) and the average percentages of infected mosquitoes (% infectivity) were 18, 22, and 26 (overall of 22±4.00%) (Table 1 and Figure 1). In the laboratory strain of mosquitoes, the average oocyst counts were 0.9±2.08, 0.82±2.15, 1.00±1.87 oocysts per midgut for each replicate (overall of 0.91±2.03 oocysts per midgut, n total = 150) and the percent infectivities were 30, 22, and 30 (overall of 27±4.61%) (Table 1 and Figure 1).This result indicated that there were no differences in the mean oocysts counts and percent infectivity of *P. gallinaceum* in the two groups of mosquitoes.

On the following day, the field strain of *Cx. quinquefasciatus* was brought to expose to a batch of three *P. gallinaceum* infected chickens with parasitemia of 20, 30 and 25 percent (an average of = 25±5%) and gametocytemia of 3, 2 and 1percent (an average of 2±1%). Similarly, a laboratory strain of *Cx. quinquefasciatus* was also allowed to feed on three *P. gallinaceum* infected chickens with parasitemia of 40, 38 and 35 percent (an average of = 37.67±2.51%) and gametocytemia of 5, 4 and 1 percent (an average of 3.33±2.08%). The overall average parasitaemia and gametocytemia were 31.33±7.79% and 2.67±1.63%, respectively.

Table 1 Oocyst count and percent infectivity in the field and laboratory strains of *Cx. quinquefasciatus* feeding on infected chickens with 10% parasitemia. Three batches of mosquitoes (Replicate (R) 1, 2 and 3) were used to feed on infected chickens.

	Cx. quinquefasciatus					
	Field strain			Laboratory strain		
	R1	R2	R3	R1	R2	R3
Maximum oocyst count	7	11	6	12	12	8
Mean oocyst count	0.58	0.66	0.5	0.9	0.82	1
Standard deviation	1.59	1.80	1.15	2.08	2.15	1.87
Number of infected mosquitoes (n= 50)	9	11	13	15	11	15
Percent infectivity	18	22	26	30	22	30
Total oocyst counts	29	33	25	45	41	50
Overall oocyst count (mean+SD)*	0.58+1.53			0.91+2.03		
Overall percent infectivity (mean+SD)	22+4.00			27+4.61		

* p=0.2697(Mann Whitney U test)

Following the same procedures, the field and laboratory strains of mosquitoes were dissected on Days 9-10 post feeding. In the field strain of mosquitoes, the average oocyst counts were 0.30±1.84, 0.36±1.19, 0.50±1.37 oocysts per midgut for each replicate (overall of 0.39±1.49 oocysts per midgut, n total = 150) and the percent infectivity were 6, 14, and 14 (an average of 11±4.61%) (Table 2 and Figure 1). In contrast, in the laboratory strain of mosquitoes, the average oocyst counts were 1.36±2.86, 0.78±1.92, 0.88±2.50 oocysts per midgut for each replicate (overall of 1.01±2.45 oocysts per midgut, n total = 150) and the percent infectivities were 32, 24, and 22 (an average of 26±5.29%) (Table 2 and Figure 1). This result indicated that the the mean oocysts counts and percent infectivities of *P. gallinaceum* in the laboratory strain of *Cx. quinquefasciatus* were significantly higher (p = 0.009) than those in the field isolate mosquitoes.

To confirm the transmission potential of *P. gallinaceum* sporozoites that develop in *Cx. quinquefasciatus*, on Day 15 post feeding, the laboratory strain (n = 100) and field strain of mosquitoes (n = 100) were allowed to bite naive chickens (n = 2), aged 2-week old. Results showed that blood stage parasites were microscopically present in all chickens on Days 7-9 post feeding (data not shown), demonstrating the complete sporogonic development of *P. gallinaceum* in *Cx. quinquefasciatus* mosquitoes.

Discussion

This study focused on investigating different transmission potential of the avian malaria parasite *P. gallinaceum* to the laboratory strain and field isolate of the mosquito vector *Cx. quinquefasciatus*. Comparison of malaria transmission in the field and laboratory strains of the *Culex* mosquito was the focus of this study since *Cx. quinquefasciatus* is abundant and acts as a major vector in endemic areas of *P. gallinaceum* in Thailand [23]. Our study showed that there were no differences in the oocyst counts and percent infectivity between the two groups of mosquitoes when the mosquitoes fed on blood with 10% parasitemia. However, the oocyst counts and the percent infectivity of the parasite were significantly higher in mosquitoes of

Table 2 Oocyst count and percent infectivity in the field and laboratory strains of *Cx. quinquefasciatus* feeding on 30% parasitemia. Three batches of mosquitoes (Replicate (R) 1, 2 and 3) were used to feed on infected chickens.

	Cx. quinquefasciatus					
	Field strain			Laboratory strain		
	R1	R2	R3	R1	R2	R3
Maximum oocyst count	13	7	6	14	10	15
Mean oocyst count	0.30	0.36	0.50	1.36	0.78	0.88
Standard deviation	1.84	1.19	1.37	2.86	1.92	2.50
Number of infected mosquitoes (n= 50)	3	7	7	16	12	11
Percent infectivity	6	14	14	32	24	22
Total oocyst counts	15	18	25	68	39	44
Overall oocyst count (Mean+SD)*	0.39+1.49			1.01+2.45		
Overall percent infectivity (mean+SD)	11+4.61			26+5.29		

* p=0.009 (Mann Whitney U test)

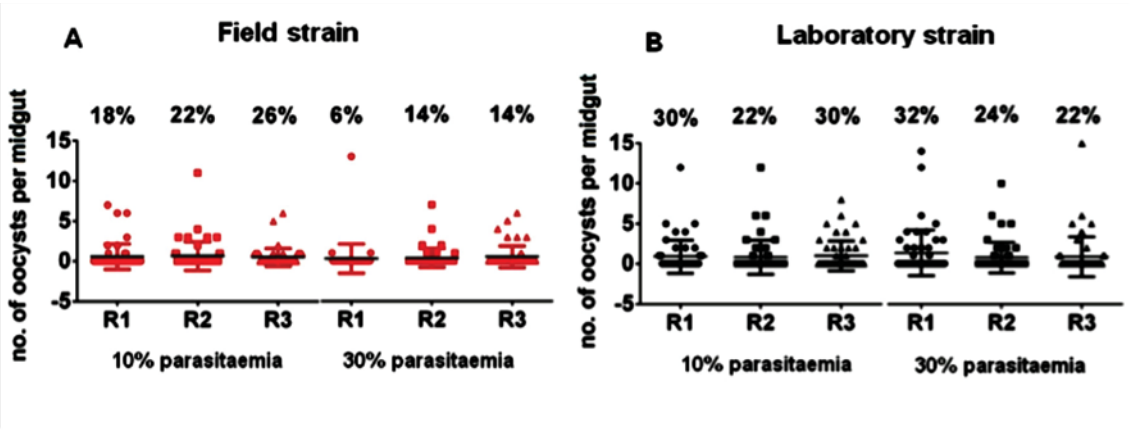

Fig 1 Mean oocyst count and percent infectivity of *Plasmodium gallinaceum* in field and laboratory strains of *Culex quinquefasciatus* feeding on blood with mean parasitemia of 10 and 30 percent. Three batches (replicate (R)1, R2, R3) of each mosquito strain were used to feed three infected chickens. Data given shows the mean oocyst counts per mosquito (n = 50) with error bars indicating standard deviation. The numbers above error bars represent percent infectivity of the mosquitoes.

laboratory strain when feeding on blood with 30% parasitemia, indicating the marked differences in susceptibility of laboratory and field strains of mosquitoes to *P. gallinaceum*.

Our study also indicated that the rate of transmission of the malaria parasite *P. gallinaceum* could be influenced by the origin (source) of the mosquito vectors. It was shown in a previous study that natural isolates of malaria vectors in the genus *Anopheles sp*. were highly resistant to malaria

infections [24]. The mosquito midgut, like the human intestinal tract, contained a diverse range of microbial flora, which compromised the ability of *Plasmodium* to establish the infection. The authors demonstrated that bacteria, *Acinetobacter* sp., *Pseudomonas putida*, and *Enterobacter* sp. that were present in natural isolates of *An. arabiensis* in southern Zambia interfered with *P. falciparum* development by generating reactive oxygen species [24]. In addition, another study showed that *Wolbachia* infections in *Cx. pipiens* mosquito in natural setting could also affect the density of malaria parasites *Plasmodium relictum,* the related species of *P. gallinaceum* [25]. *Wolbachia* was commonly detected in *Cx. quinquefasciatus* and *Cx. pipiens* collected from many localities in Brazil and Argentina [26]; similarly in Thailand, at least 11 species of naturally caught *Culex* mosquitoes, including *Cx. quinquefasciatus*, were found to be positive for *Wolbachia* infections [27,28]. However, whether the bacteria *Wolbachia* and gut microbes (*Acinetobacter*, *P. putida* and *Enterobacter*) were prevalent in our field isolates or laboratory strain of *Culex* mosquitoes is not yet determined and this will require further investigations.

In addition, the genetic backgrounds of the mosquito could also affect the susceptibility and refractoriness to the malaria transmission. Although very little has been demonstrated in the *P. gallinaceum*/*Culex* mosquito model, several factors are known from studies of *Plasmodium* in *Anopheles* mosquitoes. For example, the rodent malaria parasite *P. yoelii* and *P. berghei* was transmissible in the *An. stepheni* mosquitoes, but the sporogonic development was arrested in the *Culex* or *Aedes* mosquitoes after the parasite reached the midgut [29]. The difference in susceptibility and refractoriness was attributed to differences in xanthurenic acid (XA), a metabolite that requires for exflagellation of the malaria parasite. XA was highly abundant in the gut of *Anopheles* mosquitoes, but not in the *Culex* or *Aedes* mosquitoes [30]. Moreover, several studies also showed that natural isolates of *An. gambiae* in West Africa exhibited extensive genetic variation and such variation could lead to the different susceptibility to *P. falciparum* development in the mosquito vector [31]. To date, a small number of genetic loci has been identified and shown to be linked with the natural pathogen resistance mechanisms [31-33]. Yet, it is not known whether the diversity of such genes does exist in the natural populations of *Culex* mosquitoes in Thailand. Further investigation into the genetic diversity between the laboratory and field strains of the *Culex* mosquito that accounts for variation in the malaria transmission rate between these mosquitoes is of our great interest.

Consistent with our previous observation [22], the present study also showed that different levels of parasitemia or gametocytemia could affect the rate of malaria transmission. At the time, the exposure to the parasites during the blood meal can also trigger mosquito's innate immune responses. It is known that the mosquito innate immune system can eliminate *Plasmodium* parasite inside the epithelial midgut. These mechanisms involve the production of reactive oxygen species (ROS) and protein nitration mediated by a peroxidase and a NADPH oxidase system [34-36]. It is likely that these mechanisms may be enhanced in the field isolate mosquitoes, but not in the laboratory mosquito, and it is may be parasite-density dependent. Quantitative measure of genes involved in ROS responses and protein nitration in the midguts of field and laboratory strains of *Culex* mosquitoes could also be performed to test the above hypothesis.

In conclusion, our study indicated that *Cx. quinquefasciatus* could act as the natural vector of *P. gallinaceum* in Thailand. This study also pointed that the natural isolates of the *Culex* mosquito were more refractory to *P. gallinaceum* development as compared to the laboratory strain that fed on the *P. gallinaceum*-rich blood. This finding suggests the existence of potential natural pathogen resistance mechanisms in the wild-caught *Culex* mosquitoes. Understanding the natural pathogen resistance mechanisms could have important implications for development of strategies aimed at interrupting malaria transmission during parasite development in the vector.

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