In vivo transmission blocking activities of artesunate on the avian malaria parasite Plasmodium gallinaceum

Rapeeporn Kumnuan\(^{a,1}\), Sittiporn Pattaradilokrat\(^{b,1}\), Kamlang Chumpolbanchorn\(^{c}\), Suntorn Pimnon\(^{a}\), Somphong Narkpinit\(^{d}\), Pongchai Hanyuttanakorn\(^{b}\), Taweek Patraradilokrat\(^{b}\), Tawee Mak CAST, Nakhon Pathom 73170, Thailand.

**Article info**

Article history:
Received 1 May 2013
Received in revised form 5 July 2013
Accepted 15 July 2013

**Keywords:**
Anti-malarial compounds
Gametocytocide
In vivo transmission blocking assays

**Abstract**

Infection and transmission of the avian malaria parasite Plasmodium gallinaceum in domestic chickens is associated with high economic burden and presents a major challenge to poultry industry in South East Asia. Development of drugs targeting both asexual blood stage parasites and sexual stages of the avian malaras will be beneficial for malaria treatment and eradication. However, current drugs recommended for treatment of the avian malaria parasites target specifically the asexual blood stage parasites, but have little or no impact to the gametocytes, the major target for development of transmission-blocking strategies. In the present work, we established a simple procedure to evaluate gametocytocidal and transmission blocking activities in a P. gallinaceum-avian model. The assays involved administration of seven consecutive daily doses of test compounds into P. gallinaceum-infected chickens with 10% parasitaemia and 1% gametocytaemia. Our studies indicated that intramuscular injection with seven daily low doses (the minimum effective dose of 10 mg/kg) of artesunate blocked the gametocyte production and transmission to the mosquito vector Aedes aegypti. This assay can be further applicable for testing new compounds against P. gallinaceum and for other parasitic protozoan infecting birds.

1. Introduction

Before the discovery of rodent malaria parasites in the 1950s, P. gallinaceum is one of the malaria parasites infecting birds that have been widely used to perform biological research of malaria parasites, such as host-parasite interactions, vaccine testing and chemotherapy (Richards, 1984; McGhee, 1988). The avian parasite also shares a number of characteristics to species of mammalian malaria parasites, including the sporogonic development in a mosquito vector (Valkiunas, 2005). Life cycle of all Plasmodium species in a mosquito vector is initiated when gametocytes from a vertebrate host are ingested during a blood meal. Inside a mosquito midgut, gametocytes develop into gametes which then undergo fertilization and form a zygote. Thereafter, the zygote transforms into a motile form called ookinete that can penetrate through a wall of midgut to develop into oocysts, within which meiotic cell division...
and asexual multiplication (sporogony) occur and result in the formation of sporozoites. Then, the sporozoites are released into hemocoel and invade salivary glands, facilitating transmission to next vertebrate hosts. Because the life cycle of all Plasmodium species in mosquito vectors is highly conserved, P. gallinaceum is useful as a model to dissect mechanisms of malaria transmission and tested for anti-malaria transmission blocking agents.

In addition to its values as a research tool, P. gallinaceum parasites in domestic chickens are often lethal and are of veterinary importance (Williams, 2005). P. gallinaceum infections are highly endemic in several tropical countries, mainly in South East Asia and South Asia (Valkiunas, 2005). Because P. gallinaceum can spread through a wide range of mosquito species, i.e. in the genera Aedes spp., Culex spp., Culiseta spp., Armigeres spp. and Mansonia spp. (Garnham, 1966), the infections can spread quickly between animals in the same or neighboring husbandry and could have strong economic burden on poultry industry. The most common and effective means to limit P. gallinaceum infections is by chemotherapy. It is widely accepted that the anti-Plasmodium drugs that target blood stages parasites can have a strong impact on the production of gametocyte stages. However, this is not always the case in P. gallinaceum. For example, chloroquine and doxycycline are the only two recommended drugs for routine treatment of P. gallinaceum infections in Thailand that reduce the asexual growth rate and mortality rate of infected chicken, but not the gametocyte production and transmission of the parasite (Sohsuebngarm, 2001). Therefore, the development of new effective drugs that can target both blood stage malaria parasites and gametocyte stages will be advantageous for the malaria treatment and eradication.

Artesunate is the sodium salt of hemisuccinate ester of artemisinin which is recommended for treatment of severe malaria in humans. It is currently deployed in Artemisinin-based Combination Therapy (ACT) for treating uncomplicated malaria in many endemic countries (World Health Organization, 2010). It has been shown that artesunate is highly effective against the gametocytogenesis processes and the infectivity of mature gametocytes of the human malaria parasite Plasmodium falciparum using in vitro gametocyte killing assays (Pukrittayakamee et al., 2004; Chotivanich et al., 2006). In a recent field study of 17 villages in Cambodia, ACT has also shown to reduce malaria episodes after three years of a mass treatment, suggesting that a combination of schizontocidal drugs with a gametocytocidal drug may be a powerful success strategy for eliminating malaria in some endemic regions (Song et al., 2010). In addition to the clinical uses, the gametocytocidal activities of artesunate have been recently demonstrated in malaria parasites of animal models, including the rodent malaria parasite Plasmodium yoelii (Mustfa et al., 2011). Thus, it would be of interest to evaluate whether artesunate also confers the gametocytocidal activities against P. gallinaceum.

The goal of the present work is to establish a simple in vivo transmission blocking assay to screen and measure efficacy of test compounds that are active against gametocytes of P. gallinaceum. Here, we studied asexual multiplication, gametocyte production and infectivity during an early infection of a laboratory-adapted Thai isolate of P. gallinaceum. From this information, we then established an in vivo transmission blocking assay and utilized it to identify a minimum effective dose (MED) of artesunate that completely inhibits gametocyte production and transmission of P. gallinaceum. This assay can be further applied for testing new compounds against P. gallinaceum parasites and to other malaria parasites of birds.

2. Materials and methods

2.1. Plasmodium gallinaceum and laboratory hosts

The avian malaria parasite P. gallinaceum strain Pg22/2012MU was originally isolated from a layer hen from a farm in Chachoengsao province, Thailand in June 2011. The parasite species was verified by species-specific PCR amplification based on the cytochrome b gene (cytb) as previously described (Hikosaka et al., 2011) (see Supplementary Fig. 1 for detailed experiments). The parasite was cloned and maintained cyclically by weekly serial passages and occasional transmissions through Ae. aegypti mosquitoes in order to maintain high transmissibility and virulent phenotypes of the malaria parasite.

The avian hosts were female domestic chickens Gallus gallus domesticus strain CP Brown, aged 7-week-old at the time of primary infection. The chickens were raised in stainless-steel cages covered with nets to prevent entry of insects. The animals were housed under the conditions of 12:12 h dark-light cycle at room temperature of 25 ± 2 °C and 40–50% relative humidity. Food and water were provided for chickens ad libitum.

The insect vector was a laboratory colony of Ae. aegypti mosquitoes. Adult mosquitoes were reared in a nylon cage, kept in the insectary at Department of Parasitology & Entomology, Faculty of Public Health, Mahidol University. To permit the transmission of P. gallinaceum in the mosquitoes, the room conditions were set at 25 ± 2 °C, and 70–80% relative humidity and 12:12 h dark:light cycle.

Adult mosquitoes were fed on 10% sucrose solution in water on cotton pads. All experiments were conducted in compliance with the Mahidol University-approved animal study protocols and animal care and use regulations.

2.2. Blood stage infection of Plasmodium gallinaceum

Procedures to initiate a blood stage malaria infection were performed as follows (Williams, 2005). P. gallinaceum-infected blood was withdrawn from a jugular vein of a donor infected chicken and mixed with heparin to prevent blood coagulation. The bloods were used to prepare an inoculum containing 10⁶ infected red blood cells (iRBC) and injected intravenously into a jugular vein of experimental chickens.

The levels of blood stage parasites and gametocytes were monitored daily as follows. A small volume of blood (1–5 μL) was collected from a wing vein and used to prepare thin blood smears. Microscopic examination on Giemsa stained thin blood smear was performed at 1000× magnification (oil immersion). The number of iRBC and the total number of RBC were counted from at least 10
Fig. 1. Asexual blood stage growth rate, gametocyte production and infectivity of Plasmodium gallinaceum. Blood stage infections of P. gallinaceum were monitored daily by microscopic examination of Giemsa stain thin blood smears for asexual stage parasitaemia (A) and gametocytaria (B). Data given is the mean of 5 chickens per group with error bars indicating the standard error of the mean (SEM). Infectivity of P. gallinaceum to Aedes aegypti mosquitoes was determined on days 3, 4 and 5 Pl when the levels of parasitaemia reached 1%, 10% and 40% and the levels of gametocytaria reached 0.1%, 1% and 3%, respectively. Three infected chickens were used to feed three batches (replicate K1, K2, K3) of mosquitoes each day (C). Data given represents the mean number of oocysts per mosquito midgut (n=30) with error bars indicating SEM. The numbers above error bars indicate percent infectivity of the mosquitoes.

2.3. Optimization of P. gallinaceum transmission

Prior to establishment of in vivo transmission blocking assays, we optimized protocols for P. gallinaceum transmission as follows. A group of chickens (n=5) were injected intravenously with an inoculum containing 10^6 iRBC and the levels of parasites were monitored daily. To encourage engorgement, mosquitoes were starved overnight before feeding. On days 3, 4 and 5 post infection (Pi), the infected chickens (weight range 0.3–0.35 kg) were injected with Tiletamine HCl/Zolazepam HCl (Zoetelit, Virbac, Thailand) at dose 10 mg/kg intramuscularly. The infected chickens were then used to feed with a batch of 40–50 female mosquitoes in a cup. The mosquitoes were allowed to bite the abdomen area of the anaesthetized chicken for 30 min and the mosquito feeding experiments was ended before the infected chicken was recovered from the anaesthesia in order to avoid unnecessary pain and distress. Three chickens were chosen randomly for mosquito blood meal to compromise variation in the levels of blood stage parasites and gametocyte production. Unfed or partially engorged mosquitoes were removed from the cages. The remaining mosquitoes were maintained for additional 8–9 days before their midguts were dissected on day 9 post blood meal. The number of infected mosquitoes and oocyst counts in the midguts were recorded.

2.4. In vivo transmission blocking assays

In an initial experiment, groups of chicken (n=4) were infected with an inoculum containing 10^6 blood stage P. gallinaceum parasites. Starting on day 4 Pl when rising parasitaemia was between 10 and 14%, the infected chickens were divided into groups and the animals were injected intramuscularly at the thighs with seven daily doses of artemunate (Guilin Pharmaceutical, Guanxi, China) in 0.9% sodium chloride at concentrations of 1, 10, 20 or 40 mg/kg, or 0.9% sodium chloride (pH 7.4). The infected chickens were monitored daily for parasitaemia and gametocytaria until day 14 Pl.

In a subsequent experiment, chickens were infected with P. gallinaceum (n=3) and monitored daily for the levels of parasitaemia and gametocytaria as above. On day 4 Pl, the infected chickens were used to feed the mosquitoes before given seven daily dose artemunate treatments at concentrations of 1 or 10 mg/kg or mock treatment (0.9% sodium chloride) as above. To evaluate the transmission blocking activities of artemunate during the course of infection, the mosquito feeding procedures were repeated in the artemunate-treated and non-treated groups on days 5, 7, 9, 11 and 13 Pl. The mosquitoes were maintained for additional 9 days prior to examinations of the midguts for percent infectivity and oocyst counts.
3. Results

3.1. Asexual parasite growth, gametocyte production and infectivity of *P. gallinaceum* in mosquitoes

A group of 5 female chickens was inoculated intravenously with $10^6$ iRBC of the Pg22/2012MU strain of *P. gallinaceum* and levels of parasitaemia and gametocytaemia were determined microscopically as described in Section 2. Blood stage infections in infected chickens were detectable at day 3 PI and continued to rise and reached peak parasitaemia of 70% and gametocytaemia of 10% on day 6 PI (Fig. 1A and B), respectively. Thereafter, both parasitaemia and gametocytaemia declined to undetectable levels by day 14 PI (an experimental end point), which was consistent with a previous observation made by Williams (2005).

Infectivity of *P. gallinaceum*-infected chickens in *Ae. aegypti* (n = 30) was determined during an early infection on days 3, 4 and 5 PI when the levels of parasitaemia were at 1%, 10% and 40% and the levels of gametocytaemia were at 0.1%, 1% and 3%, respectively. Three infected chickens were randomly chosen from the groups each day and subjected to mosquito feeding as described in Section 2. Our data showed that transmission of *P. gallinaceum* to *Ae. aegypti* was achieved between day 3 and 5 PI (Fig. 1C). Consistently high rates of mosquito infections (>60%) and oocyst counts (>10 oocysts per midgut) were obtained on days 4 and 5 PI when the rising parasitaemia and gametocytaemia were above 10% and 1%, respectively. From this data, our *in vivo* transmission block assays were established on day 4 PI when the infected chicken had at least parasitaemia of 10% and gametocytaemia of 1%.

3.2. Effects of artesunate on gametocyte production

Blood stage-induced infections were initiated by injecting i.v. groups of 4 chickens with an inoculum of $10^6$ *P. gallinaceum* parasites. *P. gallinaceum*-infected chickens with 10% (range 10–18%) parasitaemia were treated with seven daily doses of artesunate at concentration of 1, 10, 20 and 40 mg/kg and 0.9% sodium chloride solution (mock treatment). The levels of the parasitaemia and gametocytaemia were determined daily by microscopic examination of Giemsa stained thin blood smears.

As shown in Fig. 2A and B, administration of *P. gallinaceum*-infected chickens with 0.9% sodium chloride did not alter the course of infection and levels of parasitaemia and gametocytaemia were similar to those seen in the untreated group as shown in Fig. 1. In contrast, treatments of *P. gallinaceum*-infected chickens with 10, 20 and 40 mg/kg artesunate were able to suppress the blood stage infections and levels of parasitaemia and gametocytaemia were cleared after 2–3 doses of artesunate treatments and remained undetectable levels until the study end point at day 14 PI (Fig. 2C and D). However, in infected chickens treated with 1 mg/kg artesunate, the clearance of blood stage parasites and gametocytes was delayed. This was due to recrudescence of blood stage parasites seen between days 9 and 10 PI. From this data, the two doses (1 and 10 mg/kg) of artesunate were chosen to determine *in vivo* efficacy of artesunate in inhibiting transmission.

3.3. Effects of artesunate on *P. gallinaceum* infectivity

*P. gallinaceum*-infected chickens with mean parasitaemia of 10% (range 10–18%) were prepared for *in vivo* transmission blocking assays as described in Section 2. On day 4 PI the infected chickens were divided into three groups (n = 3) and all chickens in each groups were used to feed three batches of female *Ae. aegypti* mosquitoes (n = 20). The infectivity of the *P. gallinaceum*-infected chickens in all groups before the artesunate treatment was at 65–85% and the mean oocyst counts were approximately 10 oocysts per midgut (Fig. 3, day 4 PI).

One hour after the mosquito feeding, two groups of infected chickens were treated with seven consecutive daily doses of 1 and 10 mg/kg artesunate and the other group was given 0.9% sodium chloride solution. Parasitaemia and gametocytaemia of the infected chickens were monitored microscopically until day 13 PI (Supplementary Fig. 2) and the values were similar to those shown in Fig. 2. The infectivity of *P. gallinaceum*-infected chickens was evaluated after 1, 3, 5 and 7 doses of artesunate treatment or mock treatment (Fig. 3). In non-treated group, the infectivity of *P. gallinaceum* to *Aedes* mosquitoes was highest between days 5 and 7 PI, corresponding to the peak gametocytaemia (Supplementary Fig. 2). The infectivity to mosquitoes declined on day 9 PI and was maintained at low levels until an experimental end point. In contrast, a single dose of 10 mg/kg artesunate treatment was able to reduce infectivity of *P. gallinaceum* by 50%. Seven daily dose treatments with 10 mg/kg artesunate were able to block infectivity to 0%. Treatment with 1 mg/kg artesunate was able to suppress the production of gametocytes and resulted in the reduction of the mosquito infectivity and the oocyst counts, even though the treatment did not ablate the transmission as the higher artesunate concentration did. These findings indicate that treatment of *P. gallinaceum* with seven daily minimum effective dose of 10 mg/kg artesunate is effective in suppressing asexual growth, gametocyte production and blocking transmission of *P. gallinaceum* to mosquitoes.

4. Discussion and conclusion

The spread of the avian malaria parasite *Plasmodium gallinaceum* in South East Asian countries contributes to substantial economic loss to poultry industry. Efforts to control transmission and elimination of *P. gallinaceum* parasites have been hampered, partly due to the lack of gametocytocidal compounds. Common drugs for treatment of *P. gallinaceum*-infected chickens are chloroquine and doxycycline that can alleviate mortality and morbidity, but not the spread of disease (Soohsuebgarn 2001). The main objective of the present work was to develop a simple procedure to test gametocytocidal activities and transmission blocking activities of test compounds against *P. gallinaceum* in mosquito vector *Ae. aegypti*.

Prior to the establishment of the transmission blocking assays, we evaluated the influence of the number of
Fig. 2. Effect of artesunate on the asexual growth and gametocyte production of *Plasmodium gallinaceum*. Starting on day 4 PI when *P. gallinaceum*-infected chickens showed parasitaemia of 10%, the infected chickens were administrated with seven daily doses of 0.9% sodium chloride (A, B) solution or artesunate at concentrations of 1, 10 (C, D), 20 and 40 (E, F) mg/kg. Area labeled in gray represents a period from days 4 to 10 PI that the animals received the mock or artesunate treatments. The infected chickens were monitored daily for the levels of parasitaemia (A, C, E) and gametocytaemia (B, D, F). Data given is the mean of 4 chickens per group with error bars indicating the standard error of the mean (SEM).

Blood stage malaria parasites (parasitaemia) and the number of gametocytes (gametocytaemia) on transmission to mosquito vectors. During an early infection, the number of infected mosquitoes and the oocyst counts increased in proportion with the rise of asexual blood stage parasites and gametocytes. In our *in vivo* transmission blocking assay, the infected animals were selected for inclusion in the experiments when the levels of parasitaemia rose to approximately 10% and the levels of gametocytaemia were approximately 1%. This condition was frequently obtained during the early infection (on day 4 PI) when the animals were injected with $10^6$ iRBC as described in Section 2. Although the high infectivity rates and the high number of oocysts were detected in *Ae. aegypti* mosquitoes fed upon the *P. gallinaceum*-infected chickens between days 4 and 7 PI (the minimum rate of 65% on day 4 to the maximum rate of 100% on day 7 PI, see Figs. 1C and 3), the infectivity and oocyst counts were declined and prevailed at very low levels, probably due to influence of host immune responses. Thus, in order to minimize the inference of host immunity in suppressing the parasitaemia, gametocytaemia and transmission, the infected animals were chosen to
establish in vivo transmission blocking assays when the rising parasitaemia and gametocyte reached 10% and 1% in the infected chickens on day 4 PI, respectively.

In our transmission blocking assays, *P. gallinaceum*-infected chickens with 10% parasitaemia and 1% parasitaemia were subjected to seven daily doses artesunate and mock treatments. Our studies indicated that treatment with lower doses (1 mg/kg) artesunate could not prevent malaria transmission, although it was sufficient to inhibit blood stage malaria parasites. In contrast, the seven daily dose treatments with artesunate at 10 mg/kg were sufficient to block the gametocyte production and the transmission to mosquito vectors, thereby indicating the activities of artesunate against sexual stages of *P. gallinaceum*. Thus, it is critical to determine the activities of anti-malaria drugs at different doses (concentrations) and treat *P. gallinaceum*-infected chicken with sufficient amounts of gametocytocidal drugs so that asexual blood stage growth, gametocyte production and transmission could be blocked completely.

In addition to its veterinary importance, advances in the studies of avian malaria *P. gallinaceum* has led to the development of transmission blocking vaccines and identification of chemicals or drugs with gametocytocidal activities (Gwadz, 1976; Carter et al., 1979a,b). Several proteins on the surface of sexual stages identified in the avian malaria had functional homologs in the human malaria parasites that are used today as components of transmission blocking vaccines (Grotendorst et al., 1984; Duffy et al., 1993; Patra et al., 2008). In addition to the surface antigens, *P. gallinaceum* ookinetes were also found to secrete enzymes, such as chitinase and plasmepsin 4, that are essential for survival in mosquito midguts (Vinnet et al., 2000; Li et al., 2010). Introduction of peptides or antibodies that inhibit the mosquito’s enzymes can block invasion and subsequent sporogonic development of ookinetes in the mosquito midgut (Langer et al., 2002; Bhatnagar et al., 2003).

Furthermore, chemotherapy is an important alternative strategy for control. A few approaches have been developed to evaluate the efficacy of anti-malarial compounds in inhibiting maturation and infectivity of gametocyte stages and development of sexual stages in mosquitoes. The original approach to screen transmission-blocking drugs in *P. gallinaceum* and *Ae. aegypti* model had been developed by Gerberg et al. (1966). By this method, the adult female mosquitoes were allowed to feed on a suspension of test compounds in 10% glucose for 2 days and then starved for 2 days before being fed on *P. gallinaceum* infected chickens. Following the blood meal, the mosquitoes were maintained for 9–10 days prior to examinations of the presence and quantity of oocysts. Since the technique bypassed the in vivo treatment of test compounds in chickens, it provided a platform for high-throughput screening of over 100,000
compounds (Gerberg, 1971). However, it was found later that the technique was too sensitive as many compounds appeared to be active, but this action could not be confirmed in vivo model. Subsequently, Gwadz et al. (1983) developed an alternative approach to determine in vivo gametocytoidal and sporontocidal activities of three reference anti-malaria drugs chloroquine, primaquine and pyrimethamine and other test compounds by administrating single maximum tolerant doses of test compounds to P. gallinaceum-infected chickens and then allowing the mosquitoes to feed on the treated chickens. The study had led to identification of pyrimethamine as a potential transmission blocking drug. However, in our study, activities of test compounds were tested at various concentrations and the mosquito feeding assays were performed at multiple time points, thereby providing a platform for observing gametocytoidal activities of anti-malarial compounds during the course of infection and also for the evaluation of minimum effective dose (MED). Indeed, our approach is complementary to the Gwadz’s approach (Gwadz et al., 1983), which will facilitate rapid discovery of compounds for inhibiting asexual blood stage growth and gametocyte production and blocking malaria transmission.

The findings in the present study have demonstrated the effectiveness of artesunate treatment in P. gallinaceum-infected chickens in blocking blood stage growth, gametocytes and transmission potential of the malaria parasite. Our results are also in general agreement with the two previous studies, which indicated that artesunate is a potent inhibitor of gametocytogenesis of P. falciparum (Puikrittayakamee et al., 2004; Chotivanich et al., 2006). Despite enormous research efforts on artesunate and its derivatives, there is still much debate concerning its mode of action on malaria parasites (e.g. Cui and Su, 2009; O’Neill et al., 2010; Dondorp et al., 2010). Nevertheless, because human malaria parasites and the avian parasites are both sensitive to artesunate, it is possible that the target molecules of artesunate in both malaria species are conserved. Hence, our study may present a new opportunity to employ P. gallinaceum as a supportive research tool to dissect mechanisms of artesunate in inhibiting parasite multiplication and transmission. It is also an area of our current research interest.

In summary, the present work established a simple and inexpensive platform for screening transmission blocking compounds against P. gallinaceum in Aedes aegypti model. This assay can be applied directly for testing new compounds against P. gallinaceum and also for other parasitic protozoa infecting birds. Our study also demonstrated that artesunate is effective against P. gallinaceum malaria parasites in domestic chickens. Further clinical evaluation of artesunate in blocking malaria transmission of P. gallinaceum in the field may provide new weapons for the current effort of malaria eradication.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

**Acknowledgements**

The study was partially supported by (1) the China Medical Board (CMB), Faculty of Public Health, Mahidol University, (2) Faculty of Graduate Studies, Mahidol University, Bangkok, Thailand and (3) the Thailand Research Fund (grant number MRG5680134 for Dr Sittiporn Pattaradilokrat). We thank Dr Wang Nguitrakool (Faculty of Tropical Medicine, Mahidol University) for critical reading of the manuscript. We also gratefully acknowledge the help of Mr Phumin Simpalipan for molecular characterization of P. gallinaceum malaria parasites.

**Appendix A. Supplementary data**

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2013.07.024.

**References**


