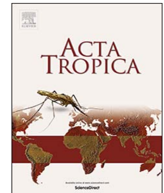
Acta Tropica 176 (2017) 263–269

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica



Modulation of transmission success of *Plasmodium falciparum* gametocytes (sexual stages) in various species of *Anopheles* by erythrocytic asexual stage parasites

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ARTICLE INFO

Keywords:
Malaria transmission
Mosquitoes
Zygote
Ookinete
Oocyst

ABSTRACT

During malaria infection, a small proportion of erythrocytic asexual stages undergo sexual differentiation. Male and female gametocytes ingested in the blood meal initiate the sexual development of malaria parasites in the mosquito midgut. During blood feeding on a host, a mosquito ingests, in addition to mature gametocytes, host immune factors present in the blood, as well as large excess of erythrocytic asexual stages. In the current study we addressed the impact of the presence of large excess of asexual stages, hitherto not known or even suspected to influence, on the infectivity of gametocytes in the mosquito. Asexual stages resulted in a dose-dependent inhibition of infectiousness of gametocytes, and some of this could be explained by the presumed effect of hemozoin and other unknown asexual-stage components on the mosquito immune system, affecting survival and maturation of parasites in the mosquito midgut. Interactions between asexual and sexual stages, maturity and ratio of male and female gametocytes, host immune factors and mosquito innate immune factors are some of the variables that determine the infectiousness of gametocytes in the mosquitoes and ultimately malaria transmission success. Understanding of determinants affecting malaria transmission will be critical to approaches directly targeting the transmission process for malaria elimination.

1. Introduction

Nearly half the world's population lives in areas endemic for malaria caused by four species of *Plasmodium*. Globally, they account for nearly 214 million infections and ~half a million deaths, and > 90% of all malaria infections result from two *Plasmodium* species, *i.e. Plasmodium falciparum* and *P. vivax* (WHO, 2015). Efforts to control and prevent malaria are continuously challenged by ever emerging resistance to anti-malarial drugs and no effective vaccines are available for mass deployment. Historically, severe malaria including malarial deaths have been associated with *P. falciparum,* there is growing realization that infection caused by *P. vivax* can also lead to similar severe complications.

A minimum requirement for successful malaria transmission includes two mosquito bites, one injects sporozoites leading to establishment of infection in the host and the other delivers circulating male and female intra-erythrocytic gametocytes in the ingested blood meal that initiate the complex events of propagating the transmission cycle through the mosquito vector. Sexual differentiation is central to malaria transmission *via* mosquito vector. A small fraction of asexually replicating erythrocytic parasites undergo commitment to sexual differentiation. These sexually committed non-dividing stages then undergo a development process resulting in the formation of mature transmission competent sexual stages (male and female gametocytes). Upon ingestion by the mosquitoes, gametocytes undergo a series of sequential development leading to completion of sporogony (formation of sporozoites) (Beier, 1998; Sinden et al., 1996). The sporogonic developmental path includes: (1) gametogenesis and exflagellation leading to emergence of free female and male gametes from erythrocytic female and male gametocytes in the midgut lumen (within 15–20 min post ingestion of gametocytes in the blood meal); (2) fertilization between female and male gametes forming zygotes (within 30 min post blood meal); (3) meiotic nuclear division of zygote and transformation into motile mature ookinetes (20–24 h post blood meal); (4) traversal of peritrophic membrane and invasion of midgut epithelial cell wall (within 24–30 h post blood meal); (5) development of ookinetes into oocysts (7–10 days post blood meal); and (6) sporozoite production in the oocyst, egress in the hemolymph and invasion of salivary glands (14–20 days post blood meal). (Baton and Ranford-Cartwright, 2005). The infectiousness of a vertebrate host to mosquito

Abbreviations: RBC, red blood cells; NHS, normal human serum; SDS, sodium dodecyl sulfate; FITC, fluorescein isothiocyanate

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http://dx.doi.org/10.1016/j.actatropica.2017.08.027
Received 25 July 2017; Received in revised form 23 August 2017; Accepted 26 August 2017
Available online 30 August 2017
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vector has been examined by looking at oocysts in the mosquito midgut and sporozoites in the salivary gland. While absolutely critical for transmission, studies have suggested that gametocyte density alone is not a sensitive parameter that impacts overall infectiousness to mosquitoes (Graves et al., 1988; Jeffery and Eyles, 1955; Muirhead-Thomson, 1954; THOMSON, 1957). Studies employing feeding of mosquitoes on the blood from infected people or on cultured parasites have revealed that gametocyte density may have a greater impact on the proportion of infected mosquitoes rather than the actual oocyst burden (reviewed in (Churcher et al., 2012; Taylor and Read, 1997b)). During natural infection, numerous factors, including environmental, parasitological, vector and host immunity have been shown to influence overall transmission success (reviewed in (Paul et al., 2004; Talman et al., 2004).

Even though gametocytes arise from sexual differentiation of erythrocytic asexual stages (Baton and Ranford-Cartwright, 2005; Dixon et al., 2008; Lobo and Kumar, 1998; Taylor and Read, 1997a), the asexual stages, while responsible for all the malarial pathology do not directly participate in mosquito infection, and are generally present in large excess- as much as 50-fold higher in density and ~10-fold higher in prevalence as compared to gametocytes (reviewed in (McKenzie and Bossert, 1998; Taylor and Read, 1997a)). While there is no question that compared to asexual stages, *P. falciparum* gametocytes detected microscopically are not as abundant, more recent approaches based on RT-PCR detection of gametocyte transcripts have helped to identify the presence of sub-microscopic gametocytes (Bousema and Drakeley, 2011). Further complications in estimating gametocyte burden arise from the fact that the immature stages of *P. falciparum* gametocytes are believed to be sequestered in the bone marrow and any method relying on estimating gametocyte burden based on detection in the blood is most certainly going to be an underrepresentation (Aguilar et al., 2014). Whether the presence of relatively higher biomass of erythrocytic asexual stage components affects the infectivity outcome of gametocytes has never been systematically evaluated in mosquito transmission studies. Because of too many variables associated with field studies, we chose to investigate the impact of asexual erythrocytic stages on the infectiousness of gametocytes to mosquitoes using culture derived sexual stage parasites. Identification of determinants affecting transmission potential of gametocytes is expected to have significant impact on the approaches designed to monitor the success of malaria elimination efforts largely relying on the success of transmission reduction.

2. Materials and methods

2.1. Parasites and mosquito infection

P. falciparum (NF54) asexual and sexual stages were maintained in culture using O+ human red blood cells (RBC) and normal human serum (NHS) (Interstate Blood Bank, Memphis, TN, USA) (Ifediba and Vanderberg, 1981; Ponnudurai et al., 1989; Trager and Jensen, 1976; Trager and Jensen, 1997) and used for mosquito infection by membrane feeding technique. The blood meal for mosquito infection consisted of human red blood cells, normal human serum and erythrocytic sexual stages. Mature stage V gametocytes were diluted to desired final gametocytemia (0.075–0.6%) using O+ normal human serum and O+ human RBCs to a final 50% hematocrit and immediately fed to female *Anopheles* mosquitoes (4–5 day old adults, starved for 5–6 h) using parafilm covered water jacketed glass feeders maintained at 37 °C using a circulating water bath. Various species of *Anopheles* tested included *An. gambiae* (Keele strain), *An. stephensi* and *An. freeborni.* For infection with a mix of gametocytes and asexual stages, blood meals prepared as above included, in addition to gametocytes, varying concentrations (0.3%, 0.6% and 1.2%) of culture-derived erythrocytic asexual stages. Similarly, mosquitoes were fed on gametocyte blood meals that contained varying concentrations of purified hemozoin. Mosquitoes were allowed to feed for 20 min, followed by removal of any unfed mosquitoes. Blood fed mosquitoes were maintained in incubators at 26–27 °C and ~80% relative humidity for 8–10 days on 10% dextrose dipped cotton balls. Midguts from individual mosquitoes were stained with 0.1% mercurochrome and number of oocysts enumerated microscopically.

2.2. Purification of hemozoin

Parasite derived hemozoin (malaria pigment) was purified by SDS-urea method (Chen et al., 2001). Briefly, cultured asexual stage parasites were treated with 0.15% saponin to lyse red blood cells and freed parasites stored at −80 °C. Frozen parasite pellets were resuspended in 2% SDS and sonicated to completely lyse parasites. The pellet obtained after centrifugation (20,000 rpm, 20 min, 25 °C) was extracted with 2% SDS, 3–5 times or until the supernatant was clear and colorless. The pellet was then incubated (overnight at 37 °C) with 2 mg/ml proteinase K in 10 mM Tris buffer, pH 8.0 containing 0.5% SDS and 1 mM $CaCl_2$, followed by three washes using 2% SDS as above. Finally, the pellet was treated (3–4 h at room temperature) with 6 M urea using a tube rotator. After an additional 3 washes with 2% SDS, the purified hemozoin pellet was washed 8–10 times using deionized water and stored at −20 °C until further use. The concentration of hemozoin was determined using hemin as standard. Hemin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO to prepare a 6.5 mg/ml solution (10 mM concentration). The stock solution of hemin was serially diluted in 2% SDS and 10 mM NaOH to yield (0.1–1000 μM standards). Purified hemozoin after sonication was likewise diluted in 2% SDS and 10 mM NaOH and the absorbance was recorded at 400 nm. The concentration of hemozoin was determined based on hemin extinction coefficient of 10^5.

2.3. Immunofluorescence detection of P. falciparum stages in the mosquito midgut

Two different approaches were used to detect parasites undergoing development in the mosquito midgut. Antibody against Pfs25 protein expressed in zygotes and ookinetes (Lobo and Kumar, 1998) was used for immunofluorescent detection of mosquito stage parasites. Initially, blood-fed midguts were dissected in PBS and gently homogenized in 20 μl of the 3% acetic acid to lyse the red blood cells. The midgut contents were washed once with PBS and resuspended in 10 μl PBS for spotting (2 μl) in 8-well slides. Air dried spots were fixed with pre-chilled methanol (−20 °C) for 30 min, blocked with 5% milk in PBS (1 h, room temperature) and followed by incubation with mouse anti-Pfs25 antibody in a moist chamber at room temperature for 30 min. Following 3–5 washes in PBS, FITC-conjugated goat anti-mouse Ig (Southern Biotech, Birmingham, AL, USA) was added and the slides incubated for 30 min in the dark. Following PBS washing, slides were dried, mounted and examined using fluorescent microscope (Chege and Beier, 1994; Zollner et al., 2005). Multiple fields from 2 to 3 replicate spots were examined for enumeration of fluorescent parasites designated as zygote (round), retort (immature form of ookinete) and ookinete (elongated) and the numbers averaged and extrapolated to the total number of parasites per midgut. The counts were used to estimate proportion of each stage at different time periods. In order to evaluate parasites at time points, later than 24 h, we adapted the procedure established for murine malaria parasite *P. berghei* (Han et al., 2000). Briefly, mosquito midguts were dissected in 1% paraformaldehyde in PBS and cut longitudinally one minute later using a sharp scalpel blade. After one more minute continued incubation in 1% paraformaldehyde, the midgut epithelium was peeled away from blood bolus and incubated for 1 h in 4% paraformaldehyde (50 μl in a 96 well plate). The midguts were carefully washed 2 times with PBS, incubated with PBST (PBS + 0.1% Tween 20) for 2 min followed by 3 washes with PBS. Midguts were incubated in the blocking solution (10% goat serum, 0.2% BSA in PBST) for 1 h. Washed midguts were incubated with anti-

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