A protocol for membrane feeding assays to determine the infectiousness of *P. falciparum* naturally infected individuals to *Anopheles gambiae*

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Abstract

Mosquito feeding assays play an important role in quantifying malaria transmission potential in epidemiological and clinical studies. At present, membrane feeding assays are incompletely standardised. This affects our understanding of the precision of the assay and its suitability for evaluating transmission-blocking interventions. Here, we present a detailed protocol for membrane feeding using *Anopheles gambiae* mosquitoes and naturally *P. falciparum* infected individuals.

1 Introduction

The transmission of malaria parasites from man to mosquito depends on the presence of mature *Plasmodium* gametocytes in the human peripheral blood. *P. falciparum* gametocytes undergo complex development that is characterised by five morphologically distinct stages. Only mature stage V gametocytes are observed in the peripheral blood and are accessible to feeding mosquitoes. Once ingested by a feeding female *Anopheles* mosquito, male and female gametocytes form gametes that fuse to form zygotes that develop into a motile ookinete that can penetrate the peritrophic membrane and traverse the mosquito midgut epithelium to form oocysts. The oocysts enlarge over time and rupture to release sporozoites that migrate to the mosquito salivary glands. Once the sporozoites have migrated into the salivary glands, the mosquito is infectious to humans.

The infectiousness of gametocytes is influenced by their density [1], sex-ratio [2, 3], the presence of transmission modulating factors such as antimalarial drug levels [4-6] and human and mosquito immune factors [7-10]. Other factors that can influence gametocyte infectiousness include their maturity [11, 12] and possibly poorly understood intrinsic parasite factors [13, 14]. It has been noted that whilst only mature gametocytes appear in the circulation, it may take 2-3 days before released gametocytes are infectious [15, 16] and a large proportion of gametocyte-positive individuals in a population may be non-infectious to mosquitoes [17]. As a result of this range of uncertainties and transmission-modulating factors, the infectiousness of an individual cannot be inferred based on the de-

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tection of stage V gametocytes alone. Mosquito feeding assays play an important role in quantifying malaria transmission potential in epidemiological [18, 19] and clinical studies [20]. Two field-based mosquito feeding assays are commonly used: direct skin feeding assays and membrane feeding assays. Direct skin feeding assays may have a higher efficiency and result in a higher proportion of infected mosquitoes [18]. However, there are several advantages of membrane feeding assays that may offset their lower efficiencies. Membrane feeding assays allow a large number of mosquitoes to be included in the study, thereby increasing the precision of the results, allow gametocyte quantification and, importantly, is currently the most acceptable approach for studying all age groups [21]. Membrane feeding assays are probably also less affected by inter-individual variation in innate attractiveness to mosquitoes [22, 23] and allow modifications of the blood sample (e.g. serum change [18], addition of antibodies [24], heat inactivation of gametocytes for negative control [25]).

At present, membrane feeding assays are incompletely standardised. There is variation in methods for gametocyte quantification, species and source of mosquitoes, type of membrane, starvation period of mosquitoes prior to feeding, duration of feeding, mosquito selection procedures, number of mosquitoes examined and the staining solution for oocyst detection [18]. These inter-laboratory differences in procedures affect our understanding of the precision of the assay and [18] its suitability for evaluating transmission-blocking interventions [21]. Here, we present a detailed protocol for membrane feeding.

2 Methods

A detailed protocol is provided in the online supplementary material. A 3-5 ml venous blood sample is drawn into a heparin-containing tube for mosquito feeding. Within 10 minutes after taking the blood sample, this blood is fed to local, colony-reared female *An. gambiae sensu stricto* (S-form) mosquitoes from a sufficiently narrow age range (e.g. 3-5 days). These mosquitoes are starved for a minimum of 5 hrs prior to experiments and subsequently allowed to feed for 15-20 minutes via an artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. Unfed mosquitoes are removed; fully fed mosquitoes are kept on glucose at 26-28°C and 80% humidity. One week after feeding, a minimum of 25 mosquitoes (ideally up to 50 mosquitoes) are dissected in a droplet of mercurochrome and examined by microscopy for the presence of oocysts as described in Figure 1.

The following endpoints can be determined using membrane feeding assays:

a) the proportion of infectious individuals: the proportion of individuals infecting ≥1 mosquito;
b) the proportion of infected mosquitoes: the number of infected mosquitoes divided by the total number of examined mosquitoes. This can be calculated per experiment or for groups of participants. If calculated for groups of participants, analyses have to adjust for clustering of observations derived from the same feeder and from the same individual.

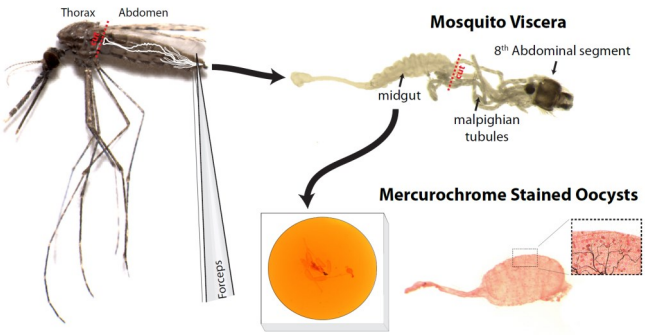

Figure 1. Outline of the procedure for detecting and counting *Plasmodium* oocysts in the mosquito midgut. (Steps are described clockwise). First, an anesthetised mosquito is placed in a drop of PBS and placed under a dissecting microscope (top left). The posterior tip of the abdomen is then slowly pulled from the remainder of the body with a pair of forceps, while a second pair of forceps is used to grasp and stabilise the thorax. In cases when the midgut does not pull out with the abdominal tip, the midgut and foregut may be separated by cutting through the mosquito with a surgical knife or razor at the thoraco-abdominal junction (top left, dotted red line) and the viscera can then be pulled out of the resulting hole in the anterior abdomen. During this process the visceral organs are normally pulled from the body along with the last abdominal segments (top right). The Malpighian tubules as well as any debris are then cut and removed from the midgut using either a knife or forceps (top right). The midguts are then soaked in a convex glass well containing mercurochrome or individually in a drop of mercurochrome on the slide for the desired time (bottom middle; between 5- 15 minutes, depending on the concentration of mercurochrome made up in distilled water). After staining the midguts are mounted in PBS on a glass slide, a cover slip is added, and then samples are visualised under a compound microscope (bottom right). Either phase contrast or bright field can be used depending on the model of microscope. (Inset) Close-up of the oocysts on the mosquito midgut.

c) the oocyst density in infected mosquitoes. This should be recorded for individual mosquitoes (and not summarised per experiment) to allow the most powerful statistical analysis [26]

3 Discussion

The association between gametocyte density and mosquito infection rates is, at best, described as “loose”. While there is a positive association between gametocyte density and the likelihood that a person is infectious to mosquitoes and the proportion of infected mosquitoes [19, 27-29], it was already noted in the 1950s that high gametocyte densities sometimes do not result in mosquito infections while very low densities efficiently infect mosquitoes [17]. The considerable variability between endemic settings in both the proportion of gametocyte carriers that are infectious to mosquitoes and the proportion of mosquitoes they infect [18], and the variation in outcomes in bloodmeals that are offered to mosquitoes in different conditions [18], suggest that field-based membrane feeding assays are characterised by limited precision. A stochastic element in biologi-

cal experiments is not unexpected and has been described before for feeding assays using cultured *P. falciparum* gametocytes [30, 31]. Variability in infection rates between mosquitoes feeding on aliquots of the same blood sample may also be related to heterogeneity in gametocyte density [32] and variation in temperature or feeding efficiency between feeders [26, 28].

Before the variability in mosquito feeding assays can be addressed, consensus has to be reached on membrane feeding procedures. In this manuscript, we provide a detailed protocol with contributions of researchers from 10 research institutes that share an interest in malaria transmission research.

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