Opinion

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Breaking barriers: a leap ahead in Plasmodium biology

The design and interpretation of laboratory assays measuring mosquito transmission of *Plasmodium*

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Since 2010 two global reviews of malaria research have recognized that local elimination and eradication of *Plasmodium* parasites are key drivers for further experimentation. To achieve these ambitious objectives it is universally recognized we must reduce malaria transmission through the mosquito vectors. A plethora of new laboratory assays are being developed to interrogate malaria transmission from the gametocyte to the sporozoite stage: assays that augment well-established field protocols to determine the entomological inoculation rate. However, the diverse readouts of these assays are not directly comparable. Here we attempt to identify the utility of each assay and provide rational frameworks by which to compare the impacts recorded by the diverse methodologies.

Field and laboratory assays of malaria transmission are currently not comparable

It is widely accepted when describing the potential for malaria transmission in endemic populations that the entomological inoculation rate (EIR) is among the most informative measures of mosquito infection [1–3]. This parameter embraces both the probability that the mosquito is infectious to the human host and the number of bites an individual will receive in a given period of time (usually a year). The latter is the product of the number of mosquitoes per human host and the biting rate. It has been shown on repeated occasions [4,5] that the EIR correlates with the prevalence of human infections by a saturating hyperbolic relationship (Figure 1).

Crucially, in calculating the EIR it has been assumed that the presence (and not the number) of sporozoites in the salivary glands of a mosquito equates to the mosquito being infectious [6]. Doubt may be cast on this assumption in some *Plasmodium* species, notably the rodent malarias, where laboratory studies on the number of sporozoites that must be inoculated by bite or syringe to ensure a blood-stage infection have produced estimates ranging between 4 and 1000 (reviewed in [7]). Among the human parasites, laboratory studies on *Plasmodium vivax* have shown that as few as 10 sporozoites can be infectious [8,9], and the laboratory challenges undertaken in the development of *Plasmodium falciparum* sporozoite vaccines suggest bites from five mosquitoes with salivary gland burdens of >10 sporozoites (log score 2) are required to ensure the infection of a naïve volunteer [10]. As a consequence we may need to build the quantitative dimensions of sporozoite transmission into our understanding of these and future assays that seek to determine the impact of transmission-blocking interventions (TBIs).

The range of laboratory assays that have been and are being used to measure transmission, or more commonly the inhibition of transmission by drugs, vaccines and genetically modified (GM) refractory mosquitoes, is now very diverse (Table 1). The estimates of transmission

Glossary

Direct feeding assay (DFA): the numbers of oocysts are counted on the midguts of mosquitoes fed on the skin of a treated host are compared with those of a replicate group fed on the same host before treatment.
Direct membrane feeding assay (DMFA): as for the DFA except that blood is withdrawn before treatment, divided into replicate aliquots and experimental reagents, and relevant control reagents are added to separate aliquots. These are then fed to replicate groups of mosquitoes through artificial membranes.
Elongation initiation factor 1α (EIF1α): the gene encoding EIF1α is expressed at all stages of the parasite life-cycle and is highly upregulated in the female but not the male gametocytes.
Entomological inoculation rate (EIR): the number of malaria infectious bites received by an individual in a defined period (usually expressed per year).
Gametocyte: the gamete-forming cells in the malarial parasite life-cycle, formed in the peripheral bloodstream of the vertebrate host. They exist in two forms: females which form a single large gamete when the gametocyte enters the mosquito bloodmeal, and males that form eight flagellate 'sperm' – termed microgametes.
Green fluorescent protein (GFP): the gene encoding GFP is inserted behind the promoter of a gene of interest to identify the time and, if inserted into the protein-coding sequence, the place of expression of putative stage-/sex-specific proteins.
Oocyst: a vegetative stage of development formed from the ookinete after it comes to rest beneath the basal lamina of the mosquito midgut epithelium.
Ookinete: a motile stage formed from the fertilized zygote that penetrates the mosquito gut wall.
Sporozoite: the daughter cells formed by the oocyst. Each oocyst produces between 500 and 9555 sporozoites, which are carried through the hemocoel of the mosquito from where they invade the salivary glands. The female mosquito injects the sporozoites into the vertebrate host when taking a bloodmeal.
Standard membrane feeding assay (SMFA): as for the DMFA with the exception that the infectious blood is derived from cultured gametocytes.
Transmission-blocking intervention (TBI): this can be in the form of a drug, vaccine, or other measure that reduces the probability that the malaria parasite is successfully transmitted through the mosquito vectors.

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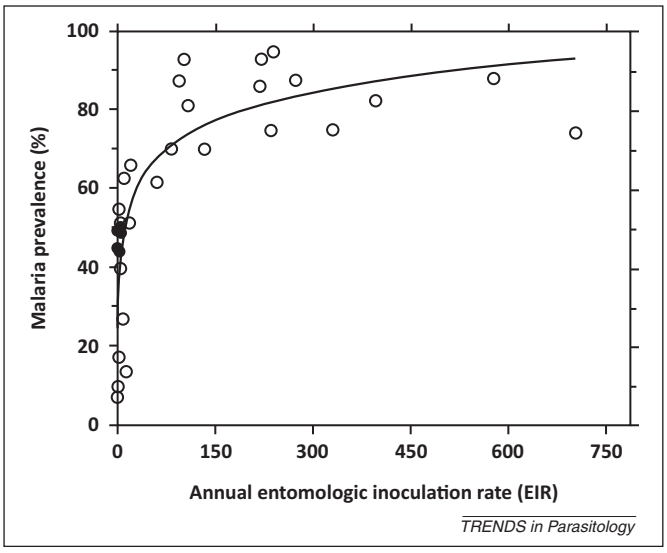

Figure 1. Relationship between entomological inoculation rate (EIR) and the parasite prevalence of *Plasmodium falciparum* in the human host. Based on data obtained from 31 sites throughout Africa (reproduced from [4]).

blockade reported by these assays could be considered equivalent by the casual reader, however, a deeper appreciation of the significant disparities between these readouts requires an understanding of the molecular biology, cell biology, and population dynamics of the parasites as they pass through the vectors.

The biology of transmission

Transmission of *Plasmodium* to the mosquito vector is mediated exclusively by sexually mature gametocytes circulating in the peripheral bloodstream of the host. In the laboratory the male:female ratio is variable both within and between parasite species and clones (~0.05 to ~0.62; estimated from Figure 2 in [11]), in other words, often exhibiting a significant female excess. Immature gametocytes are metabolically highly active – substantially replicating the metabolism of the trophozoite – and schizonticides kill both asexual parasites and immature gametocytes [12,13]. Immature gametocytes can be distinguished from trophozoites by their expression of the proteins P27 or P16 [14]. By contrast, mature gametocytes, which can be identified by the upregulation of late genes *Pfs48/45* or *mal8p1.16* [15], enter metabolic/cell-cycle arrest [12,16]. The viability of these arrested cells can nonetheless be determined by their production of ATP [16]. Gametocytes of most *Plasmodium* spp. (all of the subgenus *Plasmodium*) develop virtually simultaneously with the asexual blood stages. Conversely, gametocytes of species in the subgenus *Laverania* (e.g., *P. falciparum*) take an additional ~9 days to mature, and then appear in the peripheral circulation only as mature stage five (V) male and female cells. In the field, patients will attend clinic when they suffer fever induced by high asexual parasitaemia. These persons will in all probability harbor mature (schizonticide-insensitive) gametocytes. Consequently there will always remain the need to develop interventions that inhibit the onward transmission of mature stage V gametocytes from infected hosts to the mosquito vector. Whereas each human host may carry as many as 10^9 gametocytes in the peripheral circulation, only ~10^3 are ingested by a single female mosquito, and of these commonly less than 10 will develop into oocysts.

The mature female gametocyte is comparable to the vertebrate egg cell, full of protein and nucleic acid precursors [17], including translationally blocked mRNAs from ~370 genes (including that encoding the surface antigen P25) required for gamete/zygote development [18]. Mature females can be distinguished by the expression of these repressed mRNA species, and by stored proteins such as P48 and P230. The mature male, by contrast, is loaded with the tools to undergo three rapid endomitoses and the assembly of eight flagellate microgametes. These cells express high levels of α-tubulin II [19,20]. Male gamete production may be readily quantified by recording the motility of microgametes, and female gamete production by the release from the host erythrocyte of round gametes expressing P25 or P28 on their surface. Formation of both male and female gametes is regulated by temperature and xanthurenic acid via calcium-dependent pathways [21] (Box 1).

Macrogamete formation from mature intracellular female gametocytes can be very efficient. If fertilized, each gives rise to a single ookinete. This transition is density dependent, saturating at high parasite burdens (Figure 2) [7]. Microgamete formation is of variable efficiency, and can be suppressed by a wide range of host-derived factors including cytokines, blood pH and lactic acid (most of which can be overridden by appropriate laboratory methods) [22–24].

Fertilization usually occurs within 1 h, after which time the male gametes are exhausted/immotile, thus placing practical constraints on measuring their activity. Fertilization is readily achieved *in vitro* for all species of malaria examined to date. Fertilization requires that the gametes make physical contact either by impact of the swimming male gamete [25] or possibly via nanotube extensions of the macrogamete [26]; the gametes undergo molecular (but not necessarily species-specific) recognition involving proteins of the six-cysteine family [27,28], and then HAP2-mediated membrane fusion [29]. To date there are no reported markers that can identify the recently fertilized zygote other than the DNA content being twice the haploid value. New markers will emerge once the appropriate transcriptomic and proteomic data are made public. Such markers would usefully facilitate a single assay to monitor simultaneously and specifically the viability of both male and female gametes.

Differentiation of the zygote into the ookinete occurs in the mosquito bloodmeal and takes between 12 and 36 h; it requires first that the cell undergoes meiosis (producing a tetraploid, uni-nucleate cell), the 'activation' of the male genome, and the assembly of a single motile ookinete. Structural, metabolic, and drug studies indicate this is a multifaceted and highly vulnerable transformation [30]. The mature ookinete possesses many organelles of unique molecular composition (e.g., the micronemes and apical complex) offering a wide range of molecular indicators of 'maturity'. The mature ookinete migrates from the bloodmeal, and invades the mosquito midgut tissues by lysing cells in the midgut epithelial monolayer. *In vivo*, the