Perspective

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Critical examination of approaches exploited to assess the effectiveness of transmission-blocking drugs for malaria

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In the absence of clinically proven vaccines and emerging resistance to common antimalarials and insecticides, the onus of interrupting the life cycle of *Plasmodium falciparum*, is upon the transmission-blocking drugs. Current transmission-blocking drug primaquine finds its use restricted because of associated hemolytic toxicity issues in Glucose-6-Phosphate-Dehydrogenase deficient individuals. This article provides an extensive review of the assays used by the investigators to evaluate the transmission-blocking activity of drugs. Furthermore, limitations in existing transmission-blocking assessment approaches/studies are also covered in detail. This review is expected to help in the identification of lacunae in current understanding of transmission-blocking strategies, which are hindering our efforts to develop sustainable and effective transmission-blocking interventions.

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Blocking parasite transmission through drugs

Plasmodium species are responsible for causing one of the most devastating tropical diseases on the planet, 'Malaria'. Out of the five species infecting humans, *Plasmodium falciparum* is responsible for maximum mortality and morbidity [1]. One of the main reasons for not achieving immense success in global elimination of this disease is the complex life cycle of the parasite involving two different hosts and numerous proteomically distinct parasite stages. During the blood meal an infected female *Anopheles* mosquito introduces sporozoites (haploid parasitic forms competent of invading liver cells) into the human blood stream, which subsequently reach the liver and invade hepatocytes. After completion of asexual pre-erythrocytic cycle inside the liver, merozoites are released into the circulation where they invade erythrocytes and start the asexual erythrocytic cycle (invading merozoites→ rings→ trophozoites→ schizonts→ egressing merozoites). Parasites continue to replicate asexually in the erythrocytes unless they commit to differentiate into sexually dimorphic haploid forms known as the gametocytes which are taken by the definitive host, mosquito, during another blood meal [2]. Inside the mosquito midgut, gametocytes undergo sporogonic development or sporogony which starts with the male and female gametocytes undergoing gametogenesis. During this process, each male gametocyte, after three rounds of mitosis, undergoes exflagellation and forms eight motile microgametes whereas each female gametocyte forms a single macrogamete. Male gamete invades the female gamete to form a diploid zygote which eventually matures to form a much elongated and motile form, the ookinete. Ookinete migrates through the blood bolus to reach the midgut epithelium which is subsequently invaded by the ookinete. Each ookinete invades epithelial cells before it emerges from the basal side (facing the mosquito hemocoel) and settles beneath the basal lamina to become an oocyst. The oocysts mature, get ruptured and release numerous haploid sporozoites into the hemocoel to conclude sporogony. These sporozoites migrate into the salivary glands of the mosquito, undergo maturation and become ready to perpetuate the life cycle when the mosquito takes yet another blood meal [3]. This life cycle is diagrammatically represented in Figure 1.

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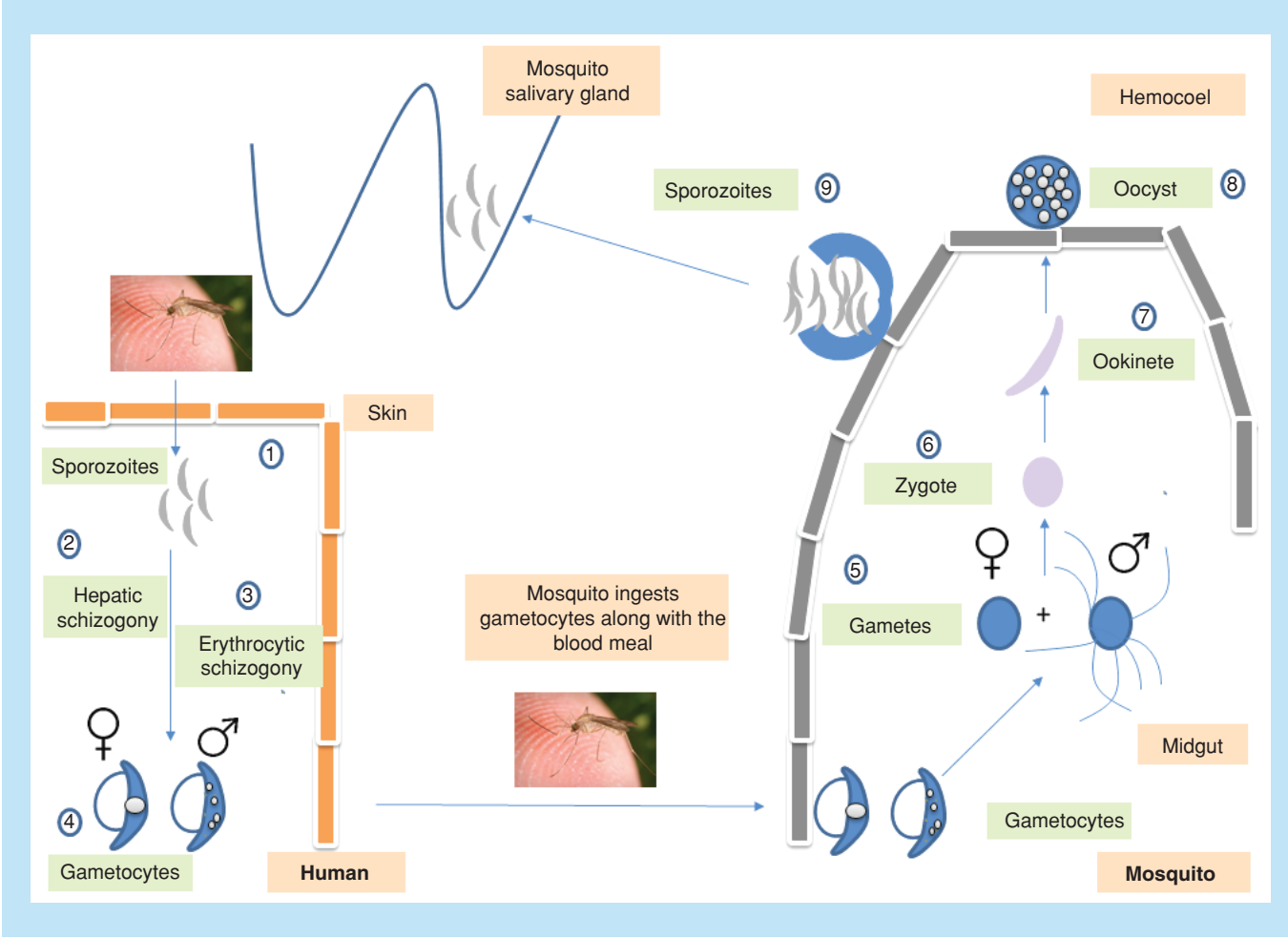

Figure 1. Life cycle of malaria parasite *Plasmodium falciparum*, focusing on gametocytes and the sporogonic stages (1–9).

In the advent of malaria elimination, it is imperative to understand the epidemiological aspects of malaria in terms of transmission, basic reproduction number and entomological inoculation rate. Malaria transmission is the process where the parasite (*Plasmodium*) is transferred from a human host to another human host through the bites of infected female *Anopheles* mosquitoes. In epidemiological terms, transmission is measured by a parameter, basic reproduction number (R_0) which is defined as the expected number of secondary cases produced by a single (typical) infection in a completely susceptible population [4]. In context of malaria, (R_0) is generally defined as the expected number of uninfected vertebrate hosts (or vectors) which would be infected by a single generation of the parasite from single infected vector (or vertebrate host) [5]. (R_0) = 1 is a threshold for malaria elimination. If in an endemic area, the basic reproduction number is reduced and kept below 1 long enough because of interventions, the infection is said to be eliminated unless there is migration of infected individuals from outside the area. In the advent of malaria elimination, transmission-blocking strategies play a crucial role in bringing down (R_0) to less than 1. (R_0) plays an essential role in providing an index of transmission intensity in malaria and other infectious diseases. The overall effectiveness of an intervention (e.g., drug intervention) is quantified by its ability to reduce the basic reproduction number, (R_0), over one round of transmission (from host to vector to host) and is termed as the effect size [6]. Another epidemiological metric which can act as a comprehensive indicator of malaria transmission level in a population is the entomological inoculation rate (EIR) [5,7]. EIR is a measure of endemicity of an area and is directly proportional to the host biting rate (the average number of mosquito bites per person per unit time) and the sporozoite prevalence rate (the proportion of biting mosquitoes which contain salivary gland sporozoites) [8]. Although, the host biting rate will be independent of the potency of the drug (exception, endectocides), but a potential transmission-blocking drug will probably, have an impact on the

sporozoite prevalence rate. Hence, apart from (R_0), experimentally investigating the drug induced reduction in EIR will also be advantageous in quantifying the transmission-blocking potential from an epidemiological perspective. However, a recent study by Churcher *et al.* predicted a dose response relationship between the sporozoite load in the mosquito's salivary glands and the probability and speed of ensuing infection in human host. Therefore, EIR which explicitly relies on the sporozoite prevalence (and not the sporozoite intensity in salivary glands) might not give correct interpretation of the transmission blocking potential of tested drugs especially used in places harboring heavily infected mosquitoes [9]. This warrants the introduction of fresh metrics for measuring the transmission blocking in the field, especially the ones which takes into account the extent of salivary gland infection in the mosquitoes, not just sporozoite prevalence.

Although, significant progress is being made in the area of malaria vaccine technology, an effective vaccine (including transmission-blocking vaccine) is still far away from the market. Therefore, malaria control strategies available for use are antimalarial drugs (for prophylaxis, treatment and transmission blockage) and nontherapeutic prevention and control measures (insecticide treated bednets, Indoor residual spraying and other vector control measures). Despite all these strategies at our disposal, global elimination of malaria is still a formidable challenge for the entire malaria community. One of the major stumbling blocks for malaria control is continuous emergence and spread of resistance to the first line antimalarial drugs and insecticides. One way of halting the spread of drug resistance is by using safe and effective antimalarial drugs with transmission-blocking properties. Transmission-blocking antimalarial drugs can be categorized into following types and subtypes [10].

Drugs targeting the parasite in the vertebrate host

This category includes: drugs providing chemoprophylaxis by directly acting on sporozoites, hence halting establishment of infection inside the vertebrate host; drugs killing asexual stages effectively and rapidly, so that their progression to gametocytes may be stopped/reduced; drugs reducing the commitment of asexual parasites to gametocytes, the commitment blocking drugs; and drugs directly targeting stage I to stage V gametocytes in vertebrate host.

Drugs targeting the parasite in the vector

This category includes the drugs that target, mosquito stages of the parasite such as the gametocytes (ingested), male and female gametes, zygote, ookinete, oocyst and the sporozoites inside the vector.

Drugs targeting the vector

This includes a special class of drugs known as endectocides. These are those drugs which, when administered to intermediate host reach inside the vector's system while they blood feed and kill them. For example, Ivermectin.

Among different parasite stages present in the vertebrate host and the vector, gametocytes present in the vertebrate host are one of the preferred targets which can be effectively eliminated by the transmission-blocking drugs. This is because, these gametocytes are one of the bottleneck stages of parasite's life cycle, which can be directly targeted by drugs due to their presence in the bloodstream of the vertebrate host. However, gametocytes (especially stage V) are not as susceptible to common antimalarial drugs as compared to rapidly metabolizing asexual blood stages. Therefore, developing drugs against these low metabolizing stages of the parasite life cycle is a formidable challenge. Recently, it has been shown that, the stage V gametocytes are more susceptible to drugs that target redox metabolism including WHO recommended primaquine and a thiazine dye, methylene blue [11]. Although primaquine is active against stage V gametocytes and also cause reduction in transmission but its associated hemolytic toxicity concerns in patients harboring G6PD deficient genotypes preclude its use on a large scale [12]. Apart from the gametocytes, sporogonic stages such as gametes, zygotes, ookinetes and oocysts also represent attractive drug targets as they also form bottleneck population which is much smaller as compared with asexual stages or even mature gametocytes [13,14]. Drugs intended to target these mosquito stages would be administered to the human host itself, but will eventually be taken up by the mosquito in the blood meal and a prospective action will be initiated against the parasites inside the mosquito [15,16]. However, since the drug delivery to the parasite, in this case, is indirect, additional efforts are required to precisely control the dose of the drug which is intended to enter the mosquito in order to cause the desired pharmacological effect. For this purpose, pharmacokinetics of the drug in both vertebrate host and the mosquito becomes relevant along with the role of detoxifying enzymes in the mosquito, which should be extensively studied in order to achieve successful targeting of the mosquito stages of the parasite. This will not only reduce the prevalence of the infected mosquitoes but will also reduce the

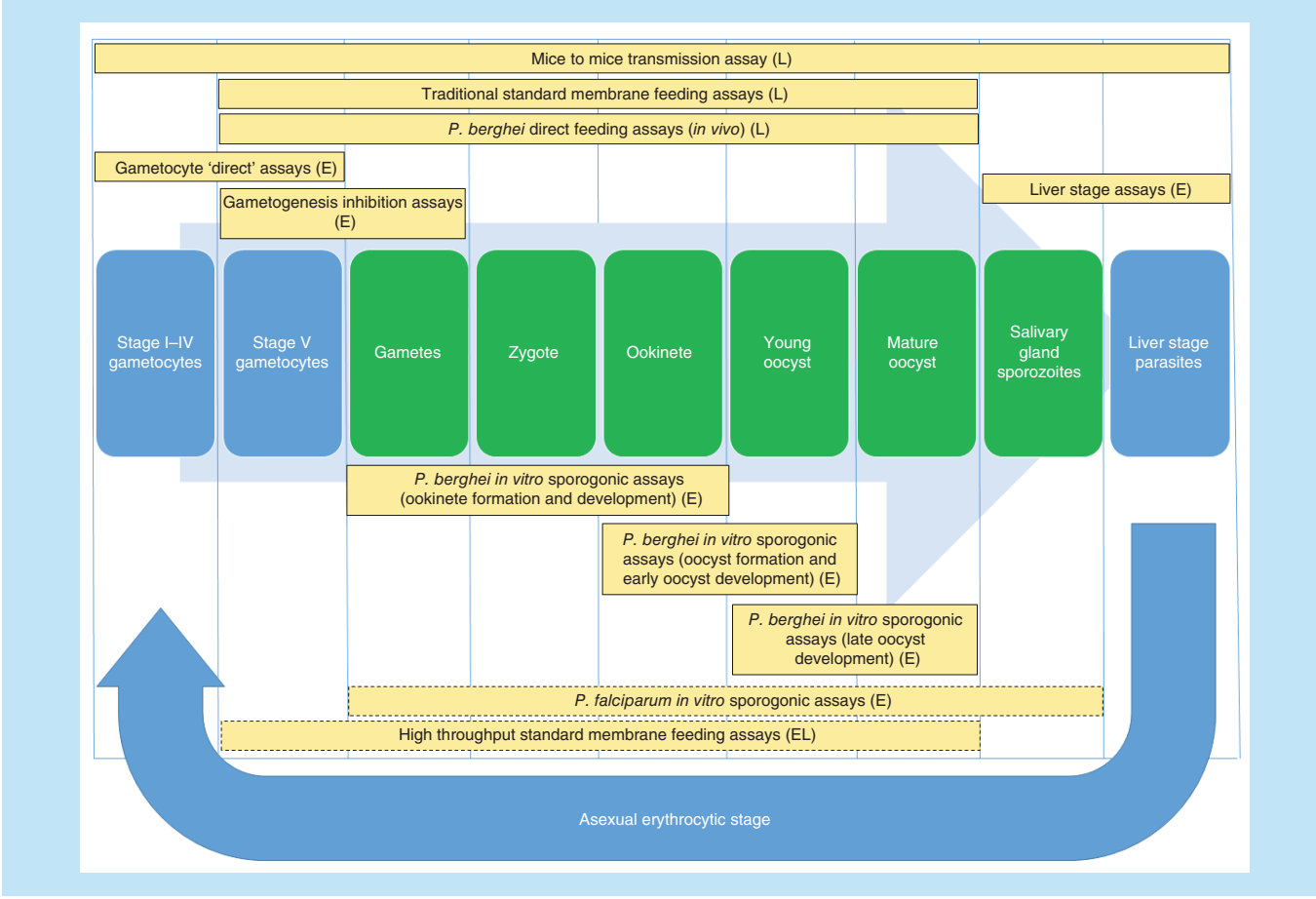

Figure 2. Diagrammatic representation of stages and pathways in the life cycle of *Plasmodium falciparum* targeted by various transmission blocking assays (Refer to the text for assay description). Available assays targeting a particular stage of the parasite such as the gametocytes or pathway such as stage V gametocyte to gamete, stage V gametocyte to oocyst are mentioned in boxes with complete borders. Assays which are unavailable at this moment are mentioned in the boxes with broken borders. Length of the box spans the biology of the life cycle covered by the assay. Mice to mice transmission assay can be used over multiple transmission cycles.
E: Indicates the applicability of the assay during early stages of drug development; L: Indicates the applicability of the assay during later stages of drug development.

parasite burden in the individual infected mosquito, thereby reducing the sporozoite prevalence rate and hence the EIR. In order to achieve the dream of malaria elimination there is an urgent need to find new drugs to target the gametocytes and sporogonic stages of the parasite which are also safe for specific and more vulnerable population such as the G6PD-deficient individuals, pregnant women and infants. In the present work, various transmission-blocking assessment approaches are critically examined for their loopholes. Approaches that are indirectly evaluating transmission-blocking *in vitro* by targeting gametocytes and gametogenesis pathways are also included in our critical analysis. But first, it is imperative to discuss various assays that are used for evaluation of the transmission-blocking activity of experimental drugs.

Transmission-blocking assays

We have categorized various nonhuman experimental assays used by the investigators for evaluating transmission-blocking potential of test compounds into the following types. Technical details of these assays are summarized in Table 1. Parasite stages and the pathways targeted by these assays are diagrammatically represented in Figure 2 along with their applicability in early or late stage drug development.

Table 1. Comparison of various lab-based methods used for transmission-blocking activity assessment.

Principle	Endpoint	Readout	Reagent/Technology	Description	Advantages†	Drawbacks†	Ref.
Luminescence	Gametocyte	Viability	ATP-bioluminescence measurement	Gametocytes treated with BacTiter-Glo™ (reagent measuring ATP levels) and bioluminescence signal detected. Signal generated is directly proportional to intracellular ATP levels, which are in turn proportional to gametocyte viability	• High/medium throughput; • Sensitive; • Genetic manipulation not a necessity	• Require stringent purification steps for obtaining highly pure gametocyte culture (free from asexual stages and uninfected erythrocytes); • May not give reliable results with endoperoxides, possibly as endoperoxides target gametocytes by interfering with ATP synthesis (such as interaction with a *P. falciparum* ATPase, *PfATP6*)	[21,28,89]
		Viability	GFP-luciferase bioluminescence measurement	Viability of gametocytes evaluated on the basis of luciferase signal obtained from parasites expressing a GFP-luciferase fusion reporter gene	• High/medium throughput; • Sensitive	• Requires use of transgenic parasite line such as NF54 ^{Pfs16} (expressing a GFP-Luciferase fusion reporter gene under the control of *Pfs16* promoter.	[18,30,31]
	Ookinete	Viability	GFP-luciferase bioluminescence measurement	GFP-Luciferase expressing *P. berghei* parasites collected and assessed for viability by measuring luciferase activity	• High/medium throughput; • Sensitive	• Requires use of transgenic parasite line stably expressing GFP-Luciferase under *P. berghei* Circumsporozoite protein (*PbCSP*) promoter	[43]
	Oocyst	Viability	GFP-luciferase bioluminescence measurement	Mosquitoes infected with GFP-Luciferase expressing *P. falciparum* parasites, homogenized and luciferase activity measured (Luciferase SMFA)	• Sensitive; • Improvement over traditional SMFA, in terms of throughput	• Requires use of transgenic parasite line stably expressing GFP-Luciferase under the control of constitutive *P. falciparum* heat shock protein (*hsp70*) or EFIalpha (*EF1a*) promoter; • Quantification of oocyst densities in individual mosquitoes might not be accurate	[17,41,62]
	Oocyst	Viability	GFP-luciferase bioluminescence measurement	Cultures of GFP-Luciferase expressing *P. berghei* parasites collected and assessed for viability by measuring luciferase activity	• High/medium throughput; • Sensitive	• Requires transgenic parasite line expressing GFP-Luciferase under *P. berghei* Circumsporozoite protein (*PbCSP*) promoter	[43]
Fluorescence /automated imaging:	Gametocyte	Viability	Flow cytometry analysis	Gametocytes stained with a nucleic acid intercalating dye such as hydroethidine (converted to ethidium by metabolically active cells which intercalates to nucleic acids) and analyzed by flow cytometry	• Sensitive; • Male and female subpopulations can be discriminated, may provide a way to separately assess drug activity for two sexes; • Genetic manipulation not a necessity	• Expensive; • Time consuming	[20,29]

†Readers are advised to take note that the comparison between the techniques described here is relative, in terms of advantages and drawbacks.
PCR: Polymerase chain reaction; pLDH: Plasmodium lactate dehydrogenase; SMFA: Standard membrane feeding assay; qRT-PCR: Quantitative real time polymerase chain reaction.

Table 1. Comparison of various lab-based methods used for transmission-blocking activity assessment (cont.).

Principle	Endpoint	Readout	Reagent/Technology	Description	Advantages†	Drawbacks†	Ref.
		Viability	Redox dyes (Alamar blue or Presto blue)	Dye reduced by the action of viable gametocytes and fluorescence signal detected by fluorescent plate reader (Excitation-525/530 ± 25 nm, Emission-598/590 ± 35 nm)	• High/medium throughput; • Sensitive; • Cost–effective; • Genetic manipulation not a necessity	• Chances of cross reactivity with test compound; • Might not give accurate results with compounds targeting redox state of the parasite or color bearing compounds such as methylene blue due to colorimetric interference	[22,33,34,91]
		Viability	Automated imaging analysis	Gametocytes stained with a fluorescent dye such as MitoTracker Red (binds to mitochondria) and assessed using high content imaging/automated microscopic analysis	• High/medium throughput; • Sensitive	• Expensive; • Requires use of transgenic parasite line such as NF54 stably expressing GFP-luciferase under the control of early gametocyte specific *Pfs16* promoter	[19,24]
		Viability	Automated imaging analysis	Gametocytes stained with a fluorescent dye such as MitoTracker Red (binds to mitochondria) after lysing the erythrocytes with saponin and assessed using high content imaging/automated microscopic analysis.	• High/Medium throughput; • Sensitive; • Genetic manipulation not a necessity	• Expensive; • More sensitive to amino alcohols such as mefloquine, lumefantrine, halofantrine which overestimates their gametocytocidal potential	[17]
	Gamete (Female)	Viability and female gamete formation inhibition	Automated imaging analysis	Digital analysis of activated gametocytes stained by a fluorescent dye, Acridine Orange	• High/medium throughput; • Sensitive; • Genetic manipulation not a necessity	• Expensive	[24]
		Viability and female gamete formation inhibition	Automated imaging analysis	Digital analysis of activated gametocytes (*Pfs25* on activated female gametocytes detected by fluorescence signal of anti-*Pfs25* antibody coupled with Cy3, using a Cy3 fluorescence filter)	• High/medium throughput; • Sensitive; • Genetic manipulation not a necessity	• Expensive	[37,38,40]
	Gamete (Male)	Male gamete formation inhibition	Automated imaging analysis	Digital analysis of exflagellation	• High/medium throughput; • Sensitive; • Genetic manipulation not a necessity	• Expensive	[38,40]
	Liver stage	Infected hepatocytes count	Fluorescence microscopy/ Automated imaging analysis	Inoculation of HepG2/ primary hepatocytes with *Plasmodium* sporozoites and quantification of infected cells achieved manually by fluorescence microscopy using Alexa Fluor 488 or by automated imaging system using Fluorescein Isothiocyanate and Alexa Fluor 680	• Sensitive; • Genetic manipulation not a necessity	• Technical limitations of working with liver stage parasites	[44,45]

†Readers are advised to take note that the comparison between the techniques described here is relative, in terms of advantages and drawbacks.
PCR: Polymerase chain reaction; pLDH: Plasmodium lactate dehydrogenase; SMFA: Standard membrane feeding assay; qRT-PCR: Quantitative real time polymerase chain reaction.

Table 1. Comparison of various lab-based methods used for transmission-blocking activity assessment (cont.).

Principle	Endpoint	Readout	Reagent/Technology	Description	Advantages†	Drawbacks†	Ref.
Microscopy	Gametocyte	Gametocyte count	Light microscopic analysis	Counting of gametocytes on Giemsa stained thin smears and/or assessing morphological integrity of gametocytes in treated as well as control groups	• Easy to perform; • Cost–effective, hence applicable to less affluent laboratory setups; • Genetic manipulation not a necessity	• Low throughput; • Labor intensive; • Time consuming; • Operator skillset dependent; • Difficult to correlate morphology with viability	[25,92]
	Gamete (Male)	Male gamete formation inhibition	Light microscopic analysis	Manual counting of exflagellation centers under a light microscope	• Cost–effective, hence applicable to less affluent laboratory setups; • Genetic manipulation not a necessity	• Time consuming; • Low throughput; • Operator skillset dependent	[35,36]
	Ookinete	Ookinete count	Light microscopic analysis	Manual counting of *in vitro* cultured ookinetes under a light microscope	• Easy to perform; • Cost–effective; • Genetic manipulation not a necessity	• Time consuming; • Low throughput; • Operator skillset dependent	[35]
	Oocyst (Ttraditional SMFA)	Oocyst intensity and/or oocyst prevalence	Light microscopic analysis	Oocysts from infected mosquitoes manually counted under a light microscope after midgut dissection	• Cost–effective; • Genetic manipulation not a necessity	• Labor intensive; • Time consuming; • Low throughput; • Prone to intrinsic variability of SMFAs (see section Variability of traditional standard membrane feeding assays for details)	[17,26,27]
Biochemistry	Gametocyte	Viability	pLDH activity assessment	Gametocyte viability evaluated spectrophotometrically by measuring pLDH activity	• High/medium throughput; • Sensitive; • Genetic manipulation not a necessity	• LDH persists in dead cells for a long period of time, hence additional incubation over standard incubation with the test compounds is required	[22,26]
Molecular biology:	Hepatic parasitemia	Quantitative gene expression	qRT-PCR	RNA extracted from post-infected homogenized liver and converted to cDNA. Gene expression quantified by qRT-PCR with primers specific for 18S rRNA and related to the viability	• Sensitive; • Easy to implement; • Allow storage and processing of samples at a later stage; • Genetic manipulation not a necessity	• Prone to conventional variability of real-time PCR	[43]
	Gametocyte	Quantitative gene expression	qRT-PCR	RNA extracted from gametocytes and converted to cDNA. Quantitative PCR was performed with primers specific for late stage gametocyte genes (*ROM3, Pfg377, Pf77 and Pfs25*) to quantify the gene expression, which is related to the viability.	• Sensitive; • Easy to implement; • Allow storage and processing of samples at a later stage; • Genetic manipulation not a necessity	• Prone to conventional variability of real-time * PCR	[32]

† Readers are advised to take note that the comparison between the techniques described here is relative, in terms of advantages and drawbacks.
PCR: Polymerase chain reaction; pLDH: Plasmodium lactate dehydrogenase; SMFA: Standard membrane feeding assay; qRT-PCR: Quantitative real time polymerase chain reaction.

Gametocyte 'direct' assays

In these assays, test compounds/drugs are directly incubated with *in vitro* cultured *P. falciparum* gametocytes and tested against individual stages of gametocyte development (stage I to V) [17] or in combination of stages, I-III [18,19], IV-V [20–22] or isolated stage V [23]. Compounds are incubated for a specific period of time (incubation period), such as 24 [23,24], 48 [21,25] or 72 h [18,26,27] before quantifying their gametocytocidal potential. Gametocytocidal potential in some assays is quantified by the measurement of metabolic activity (acting as a viability marker) which is proportional to the intensity of bioluminescence signal [21,22,28] or signal emitted from a transgenic parasite line expressing a kind of fluorescent reporter gene such as green fluorescent protein (GFP) [29] or GFP-Luciferase [18,22,30,31]. Many investigators have also studied morphological deformations in the gametocytes [25] or plasmodium lactate dehydrogenase (pLDH) activity [26,27] or mRNA expression of genes in late stage gametocytes [32] and correlated it with viability or utilized resazurin based dyes such as alamar blue [33,34], or presto blue [22]. We refer to these assays as gametocyte 'direct' assays and these are included as a part of Table 1. Majority of these assays, although have various advantages such as the high throughput, high sensitivity and specificity but viability as a readout has certain limitations. Since these assays are only applicable to a particular stage of the parasite, such as the gametocyte, they are unable to assess the effect of the test compounds on the downstream development of the parasite. For example gametogenesis inhibition and membrane feeding assays, discussed in the next section can actually evaluate the effect of the test compounds on functional viability of gametocytes and can see whether the treated parasites are able to establish the infection within the mosquitoes.

Gametogenesis inhibition assays

These are functional viability assays which evaluate the potential of test compounds to inhibit the process of gametogenesis. This can be separately estimated for male gametogenesis/exflagellation [35,36] or female gametogenesis [37] or simultaneously evaluated in *P. falciparum* dual gamete formation assay (PfDGFA) [38,39]. Inhibition of gametogenesis may be evaluated either in indirect mode where gametocytes are incubated with the test compounds for a specific period of time (before gametogenesis induction) and then tested for their ability to form gametes or in direct mode where test compounds are added to gametocytes during gametogenesis induction. Moreover, this indirect assay can be carried out either in carryover format in which test compounds are not removed prior to gametocyte activation or in 'washout' format where test compounds are removed prior to gametocyte activation in order to discover compounds that specifically target gametocytes, thus eliminating false positives due to simultaneous action on gametogenesis/gametes [38,39]. Inhibition can be evaluated either manually, based on counting the number of exflagellation centers/activated macrogametocytes [35,36] or by automated imaging techniques [24,37,38,40]. These functional viability assays are definitely an upgrade over the gametocyte 'direct' assays, as they contain more life cycle overlap with the gold standard, standard membrane feeding assay (SMFA) as compared with gametocyte 'direct' assays. In principle, test compounds showing potential in the gametogenesis inhibition assays have more chances of being truly transmission-blocking as compared with the compounds showing promise in gametocyte 'direct' assays. Moreover, these gametogenesis inhibition assays can identify compounds having no effect on gametocyte viability per se, but with potential in inhibiting the process of gametogenesis. Also, ability of the test compounds in targeting male and female gametocytes separately, can be evaluated using one of these assays and not by majority of gametocyte 'direct' assays.

Standard membrane feeding assays

SMFAs are considered as the gold standard for evaluation of transmission-blocking activity as they cover more duration of the life cycle of the parasite (from gametocytes to oocysts) than any of the assays discussed above. These assays are further categorized into, indirect SMFA: gametocytes are incubated with the test compounds for a specific period of time (24–72 h) and then fed to mosquitoes via a membrane feeding apparatus [27,41], indirect 'washout' SMFA: gametocytes are incubated with the test compounds for a specific time period and fed to mosquitoes after removing the compounds to completely eliminate the chances of the test compounds going inside the mosquito and causing a sporontocidal effect [24]. This variation of SMFA is for evaluation of 'gametocytocidal only' effect of the test compounds and direct SMFA: this involves feeding of gametocytes and the test compound mixture to the mosquitoes without any prior incubation [27,41]. This variation is for evaluation of 'sporontocidal only' effect of the test compounds.

SMFAs can either be used independently or as part of clinical trials and end up with estimation of the oocyst burden in the mosquito midgut in test compounds-treated group and compare it with the corresponding oocyst

burden in the controls. Oocysts can either be counted microscopically like in the traditional SMFAs [27,35] or oocyst burden can be evaluated by luminescence based readouts [17,31,41]. Many investigators also escalate such assays to evaluate the salivary glands' sporozoite burden, to prevent exclusion of the compounds that may have effect on the mature oocysts and prevent the generation of sporozoites. Although SMFAs are considered to be a gold standard for evaluation of transmission-blocking drugs but these are highly variable and parasite density dependent. Another source of variation can be because of the vector related differences (intra-vector differences in immunological responses/defense mechanisms) which might influence the development of the parasites inside the vector. To increase the chances of reproducibility and make the results field-extrapolatable, apart from multiple experimental replicates, different populations of mosquitoes should be used with multiple parasite preparations corresponding to different gametocyte densities [42] (described in detail in section 'Variability of traditional standard membrane feeding assays').

Plasmodium berghei direct feeding assays (*in vivo*)

In these type of assays, instead of feeding gametocytaemic blood to the mosquitoes via an artificial membrane, mosquitoes are directly fed on the infected vertebrate host (gametocytaemic mice) and are allowed to become infected. Thereafter, the test compounds (dissolved in sugar) are administered to the infected mosquitoes. After a few days, midguts/salivary glands are dissected to enumerate the parasite burden with oocyst prevalence/density and/or sporozoites prevalence/density as readouts [42]. Alternatively, the test compounds can also be administered to the *P. berghei* infected mice; mosquitoes allowed to blood feed on the infected plus treated mice, and mosquito parasite burden quantitated [8]. Additionally, the mosquitoes from the same group can be used to feed on malaria naïve mice and hepatic parasite burden quantified by quantitative real time polymerase chain reaction (qRT-PCR) with primers specific for the *P. berghei* 18S rRNA [43]. Further, patent parasitemia may also be estimated. This assay can also be escalated over multiple host to vector to host transmission cycles [8].

Plasmodium berghei in vitro sporogonic assays

These assays take the advantage of available techniques for *in vitro* cultivation of sporogonic stages of *P. berghei* and measure the effect of test compounds using bioluminescence [43] against:

Ookinete formation & development

Test compounds are incubated with the gametes or zygotes and inhibition in ookinete formation is evaluated against untreated controls after 24 h (estimated time for ookinete formation from zygote; starting point – gamete/zygote, endpoint-ookinete) [43].

Oocyst formation & early oocyst development

Test compounds are incubated with purified ookinetes and inhibition in oocyst formation is estimated against untreated controls after 72 h, which is the estimated time for oocyst formation from ookinete (starting point – ookinete and endpoint – early oocyst) [43].

Late oocyst development

Test compounds incubated with the young oocysts and inhibition in oocyst development inhibition is estimated against untreated controls after 12 days, which is the estimated time for oocyst maturation after oocyst formation (starting point – young oocyst and endpoint – late oocyst) [43].

Liver stage assays

These type of assays involve *in vitro* culture of either liver cell lines such as HepG2 or HC-04, or primary hepatocytes. After plating the cells, they are inoculated with the sporozoites, dissected from salivary glands of *Plasmodium* infected mosquitoes. Further, the test compounds are incubated with the sporozoite infected cells for specific time period such as 24 or 48 h, and potency of the test compounds is evaluated by fluorescence microscopy [44,45]. Moreover, free sporozoites can also be incubated with the test compounds for a specific time period prior to inoculating the hepatocytes. This can be carried out either in direct format where the mixture of sporozoites and the test compounds is added to hepatocytes or in washout format in which washed sporozoites are utilized. In these inhibition invasion assays, parasite load can be evaluated by fluorescence microscopy or 18S rRNA quantification. A similar approach has been used for studying the inhibition of sporozoite invasion by anti-CSP antibodies [46].

Problems in current transmission-blocking assessment approaches/studies

Although numerous types of experimental assays as well as clinical approaches to evaluate transmission-blocking potential of test compounds are reported, which no doubt, make the best use of available technology, certain limitations hindering the development of new transmission-blocking drugs have to be addressed. This section deals with the problems in current transmission-blocking assessment approaches/studies and is subdivided into: lack of transmission-blocking assessment approaches; shortcomings in existing transmission-blocking assessment approaches and dearth of transmission-blocking assessment studies.

Lack of transmission-blocking assessment approaches

Absence of a standardized assay system covering entire life cycle of human malaria parasite

Assays that evaluate the transmission-blocking potential of test compounds are designed in such a way that the compounds in variable concentrations are incubated at a particular parasite stage such as gametocyte, and either the viability of the parasite at that particular stage is evaluated or inhibition in development of subsequent stages is recorded such as gametocyte to oocyst (SMFA) [26,27,41], gametocyte to gametes (gametogenesis assays) [27,41], gametes/zygotes to ookinetes [43], ookinetes to oocysts [43] – among others. However, it is unclear whether proposed reduction in these surrogate endpoints will translate to the similar impact in the field – that is, reduction in metrics such as basic reproduction number, entomological inoculation rate – among others. Therefore, a rodent malaria transmission model for evaluation of transmission-blocking potential of the test compounds in a mice to mosquito to mice format was developed [8,47]. Although this model is an improvement over assays that target particular stage(s) of the parasite, but, a standardized assay system simultaneously covering all/more of human host and mosquito stages of the parasite's biology (sexual erythrocytic stage → sporogonic stage → pre-erythrocytic stage → asexual erythrocytic stage) is still missing from our armamentarium [10]. These type of studies will only be possible when CHMI (controlled human malaria infection) model is routinely used for evaluation of transmission-blocking potential of drug interventions which is discussed in detail in Section 'CHMI model for evaluating transmission-blocking drug interventions: perspective and challenges'.

Absence of a standardized male gametocyte specific detection method

Molecular marker used for gametocyte detection in majority of clinical studies is only a female gametocyte specific marker- *Pfs25* whose expression can be quantified by qRT-PCR. Also, sex ratio among gametocytes is female biased and most antimalarial drugs have far greater potency against male gametocytes as compared with females [40,48]. Therefore, drug mediated reduction of male gametocytes may not always be accompanied by similar reduction in female gametocytes and hence infectivity reduction potential of a drug may be underestimated by sole measurement of its ability to reduce female gametocyte carriage by *Pfs25* based quantification. Further, *Pfs25* can also be detected in non-infectious mature gametocytes [27,49] which might further give rise to false positives. Moreover, just the presence of mature gametocytes is not the only criteria for mosquito infectivity. Other contributing factors also regulate successful establishment of mosquito infection including gametocyte maturity, appropriate sex ratio, gametocyte density inside blood meal, antimalarial drug levels, host and vector immune factors and other poorly understood intrinsic parasite factors [50]. All these factors have their own threshold for establishing a successful mosquito infection [51,52] and not meeting the threshold may result in reduced infectivity [53–56]. A simultaneous quantification of male gametocytes is expected to improve our understanding about the gametocytocidal effect of test compounds. Few clinical studies have started to incorporate qRT-PCR for male gametocyte specific marker such as *Pf3D7_1469900* or *PfMGET* [57,58] but it still requires widespread implementation. Another male gametocyte specific marker which is utilized for quantification of male gametocytes is *Pfs230p* [59], but Stone *et al.* found it to be less sensitive than *PfMGET* [58].

Absence of standardized in vitro assays for investigation of sporontocidal activity of test compounds against P. falciparum

High-throughput gametocytocidal screens of chemical libraries often miss out compounds with activity across the mosquito stages of the parasite. There might be compounds having no effect on the gametocyte viability and gametogenesis inhibition, but instead have the potential to target the later stages of parasite development, such as the zygotes, ookinetes and oocysts. Despite the availability of techniques for *in vitro* propagation of the sporogonic cycle of *P. falciparum* since 1993 [60], no *in vitro* standardized assays evaluating the activity of drugs against these stages are reported. *In vitro* assays investigating the activity of test compounds against sporogonic stages produced from the rodent malaria parasite *P. berghei* have been reported [43,61]. However, establishment of

in vitro assays for evaluating the effect of drugs against sporogonic stages of *P. falciparum* and scaling them up to a high-throughput format still remains a top priority. This becomes more important as due to cost and manpower limitations, chemical libraries containing thousands or even millions of test compounds cannot be directly screened using currently available mosquito feeding assays.

Unavailability of SMFAs or its alternatives in high-throughput format

SMFA, though is considered as the gold standard, in testing transmission-blocking drugs but absence of SMFA in high-throughput format limits its applicability to a late stage assay only. Due to this, investigators rely on high-throughput gametocytocidal screens during initial stages, which might overlook compounds in chemical libraries with sporontocidal activity. We are yet to develop a high throughput alternative to the traditional SMFAs or to scale up the traditional SMFAs to high-throughput format to ease up the drug screening process. Due to the low-throughput nature of SMFAs, they are limited to be used with a limited number of test compounds, instead of complete chemical libraries. A variation of SMFA (Luciferase-SMFA) was attempted by Stone *et al.* [62], Vos *et al.* [41] and Plouffe *et al.* [17] with an improved throughput over the traditional SMFA, but still scaling up these assays to high-throughput format or developing their high-throughput alternatives is a necessity. This will further escalate the applicability of SMFAs in early stage screening of transmission-blocking compounds and might help in identifying compounds targeting the mosquito stages of the parasite.

Lack of standardized methods for evaluation of pharmacokinetics and pharmacodynamics of drugs inside mosquitoes

Pharmacokinetics/pharmacodynamics of drugs inside the mosquitoes is an understudied area along with the role of mosquito's detoxifying enzymes which might be involved in detoxification of drugs inside the mosquitoes. More research in this area will aid in development of strategies which will help in formulation of drugs specifically to target the sporogonic stages of the parasite.

Shortcomings in existing transmission-blocking assessment approaches

No accountability of drugs that work through active metabolites during in vitro assays

In vitro assays have been extensively utilized for screening of transmission blocking drug interventions. Although these assays are very useful for evaluating stage specific activity of test compounds, but the fact that these assays do not account for drug metabolism may pose a problem as it might give rise to false-negatives. For example, 8-aminoquinolines drugs such as primaquine, whose gametocytocidal potential is mediated by the action of its active metabolites, is the WHO recommended gametocytocide. Primaquine, being a prodrug, requires prior activation by cytochrome P450 enzymes (*viz.* CYP2D6) present in the liver and is thus, inactive *in vitro* [63]. Therefore, such compounds whose activity is mediated by the means of active metabolites might be missed out during high-throughput screening (*viz.* chemical libraries) where the exact mode of action of compounds might not be completely elucidated.

Variability in the assay parameters of high-throughput gametocytocidal screens

Majority of *in vitro* gametocytocidal assays use high-throughput methods for measuring gametocyte viability [17,18,21,27,37]. These assays involve intrinsic differences in assay parameters, such as parasite strain, incubation time, gametocyte population (numbers and stage of maturity) exposed to drug, use of human serum or AlbuMAX II, measurement method – among others. Some of these assay parameters may also influence *in vitro* potencies of the compounds tested in those assays and negatively affect the robustness. For example, use of human serum or AlbuMAX II is the reason behind the potency variation in amino-alcohols [17] and ATP bioluminescence assays might be unsuitable for testing endoperoxides, as they underestimate their gametocytocidal potential [22]. Moreover, compounds targeting redox state of the parasite might give incorrect potencies in assay platforms using redox dyes such as the alamar blue assay [22] or color bearing compounds might give incorrect potencies in colorimetric assays [22]. It is easy to decipher these relationships experimentally when known drugs are tested using multiple assay platforms, as mode of action of these drugs is more or less known. However, this becomes relevant in case of chemical libraries, where the exact mode of action of most of the compounds is unclear [17,18]. This might increase the number of false positives or false negatives. So one should not rely on a single high-throughput gametocytocidal screen to identify hits and use of multiple assay platforms, including ones evaluating gametogenesis inhibition are recommended. Usually, between reported studies, potency of a drug varies and is not always directly comparable. So, while going through various studies, the focus should be on collective analysis of data rather than comparative.

Moreover, there is an urgent need to establish a standardized criteria for gametocytocidal drug screening assays where the factors including parasite strains, parasite density exposure, incubation times, hit threshold – among others. are specified. Investigators should be prompted to share the results of their test compounds when evaluated using this assay strategy. This should be the minimum requirement, otherwise investigators should be set free to experiment with their test compounds in a variety of assay formats and parameters. This will increase the chances of reproducibility and comparability without compromising much with investigators' independence.

Variability of traditional standard membrane feeding assays

The variability is not only limited to high-throughput gametocytocidal assays but also to the gold standard, standard membrane feeding assays (SMFAs) [64]. There is great inconsistency while reporting the potency of transmission blocking drugs by SMFAs due to the use of multiple readouts, such as oocyst prevalence (number of mosquitoes with at least one oocyst) and oocyst intensity (number of oocysts per mosquito) [27,35] which has made data analysis across multiple studies difficult. Epidemiologically, reduction in oocyst prevalence will have more impact on blocking transmission in an area, than the reduction in oocyst intensity as a single oocyst is still capable of producing infectious sporozoites. Oocyst intensity in control group (mean number of oocysts per mosquito) is often ignored while using oocyst prevalence as a readout. This is relevant as the number of oocysts per mosquito (oocyst intensity) observed in SMFAs in laboratory infected mosquitoes is generally higher than the oocyst intensity observed in naturally infected wild type mosquitoes (field conditions) [42] or in direct feeding assays [65]. The relationship between reduction of oocyst intensity and prevalence is predictable. Higher the mean oocyst number per mosquito, higher reduction in oocyst intensity in treated group is required to achieve the same reduction in oocyst prevalence as compared with when there is lesser mean oocyst number per mosquito. For example, it is harder for the drug to decrease the oocyst intensity in mosquitoes from 100 to 0 (hence, oocyst prevalence) than 6 to 0. In simpler terms, if a drug is unable to do a 100 to 0 in terms of oocyst intensity in laboratory based SMFA, it should not be rendered ineffective as it might have the potential to do a 6 to 0 in the field. Therefore, oocyst prevalence might not be a good readout in such cases where the mean oocysts per mosquito is high (as reduction in oocyst prevalence depends on parasite exposure) and might underestimate the potency of drugs [42].

Moreover, there is no precise means of controlling the mean number of oocysts per mosquito for every single feeding experiment. If only oocyst prevalence is to be used as a readout, the only way of overcoming the above limitation is to use the SMFA data only when the mean number of oocysts per mosquito in control group is in the specified range, as determined from field conditions or direct feeding experiments. Although, this is going to decrease the throughput level of the SMFA, but currently this is the only practical solution available [65]. It is recommended that investigators should always report oocyst intensity in control group separately for each SMFA experiment along with reduction in oocyst intensity and prevalence in the treated group(s) [42]. Oocyst intensity on the other hand is a more appropriate readout in case when, potency of a transmission blocking drug is to be evaluated against control with the mean oocyst numbers greater than that observed in field conditions. Theoretically, it appears that potency of a transmission-blocking intervention might also get influenced by density of the parasites exposed to it. However, in the analysis carried out by Churcher *et al.*, potency of transmission blocking intervention in terms of reducing oocyst intensity was not influenced by the oocyst intensity in control group [42]. This analysis was carried out for just one intervention (anti-*Pbs28* mAb 13.1), and further studies with transmission blocking drugs are needed to establish that reduction in oocyst intensity by a transmission blocking drug is independent of parasite number. Intrinsic characteristics of the blood of the host (including immunological responses) can also be a potential source of variation. Nevertheless, until the above relationships are discerned, evaluating the test compounds against a variety of parasite preparations (mostly achieved by making dilutions corresponding to various parasite densities, corresponding to variable mean oocyst numbers per mosquito), mosquito populations, and collectively analyzing the data using a statistical method such as generalized linear mixed model (GLMM) might be useful [42,66]. Moreover, investigators should always report the number of infected mosquitoes in each group along with the numbers dissected, separately for each SMFA experiment [42].

Lack of attainment of statistically secure number of oocysts for drug screening in the field conditions

The oocyst intensity observed in *Anopheles* mosquitoes during artificial/direct feeding experiments (carried out with infected blood from naturally infected gametocyte carriers [*P. falciparum*]) is generally lower compared to the oocyst intensity that is generally observed in laboratory based SMFAs using cultured parasites [42,65]. This is partly due to the presence of significantly fewer gametocytemia in the blood meal of the mosquitoes feeding

on infected human blood in contrast to when they feed on cultured gametocytes. Also, the presence of other external components in human blood plasma, or the possible differences in immune responses and other resistance mechanisms, which influence establishment and magnitude of infection (e.g., the difference in midgut microbiota) between wild type and laboratory reared mosquitoes, might also act as the potential sources of variation. In order to tackle this, feeding and dissection of a large number of mosquitoes in the field conditions is supposed to be carried out, which is labor-intensive and negatively affects the investigation in terms of throughput [67].

Peripheral gametocytemia evaluation through microscopy is a surrogate marker for evaluation of transmission-blocking potential

Majority of the clinical studies use microscopic detection of gametocytes in peripheral blood as a sole method of testing transmission-blocking potential of the test compounds. However, peripheral gametocytemia evaluation is a surrogate marker for quantifying the gametocyte load as it often underestimates the actual gametocyte prevalence in patients [68,69]. These smear positive (microscopic) gametocyte carriers represent only a subpopulation of the actual transmission reservoir which also includes a large proportion of sub-microscopic gametocyte carriers (gametocytes present at densities less than the light-microscopic detectable limit of 10–20 gametocytes/μl), which can cause substantial transmission [69–72]. Further, the studies comparing parameters such as gametocyte density, prevalence of carriers and duration of carriage using peripheral gametocytemia evaluation might not give a correct interpretation of the relationship between these parameters. Although, reduction in these parameters can be positively co-related with mosquito infectivity decline, but in many cases this relationship is not straightforward. There may still be a decrease in mosquito infectivity with no significant decrease in peripheral gametocytemia in the patient, because of the potential effect of the drug against the sporogonic stages of the parasite. On the contrary, decline in the peripheral gametocytemia may not always lead to decline in the mosquito infectivity due to presence of sub-microscopic gametocyte reservoir. To overcome the problem with sub-microscopic reservoir of gametocytes, studies have started using more specialized and sensitive gametocyte detection methods based on the quantification of *Pfs25* mRNA (expressed in stage V female gametocytes and is highly conserved among various parasite isolates) and *Pf3D7_1469900* mRNA (expressed in stage V male gametocytes) by qRT-PCR or quantitative nucleic acid sequence based amplification (QT-NASBA) but these highly sensitive techniques are still not universally adopted. These molecular methods enable the measurement of gametocyte densities when they are below the required level for microscopic detection.

Artificial membrane feeding of in vitro cultured gametocytes is not always reliable for evaluating the gametocytocidal activity of drugs as artificial membrane feeding of blood collected from the patients

In vitro produced gametocytes used for potency evaluation of the test compounds in artificial membrane feeding experiments, are often treated with compounds for a longer period of time as compared with the time for which they would be exposed to the test compounds inside the body of the human host. This might be more relevant to drugs with short half-life, such as the endoperoxides. Therefore, artificial feeding of the blood collected from the drug treated patients is more reliable for assessment of dose response relationships as compared with the drug treated cultured gametocytes [48]. However, this technique has its own limitations as several aspects of these assays are not standardized making it difficult to reproduce results between laboratories. Nevertheless, an additional clinical trial component involving mosquito feeding studies using infected blood collected from patients should be included as a confirmatory evidence regarding drug induced reduction of transmission from human host to the mosquito [55].

Artificial membrane feeding assays are not always reliable for evaluating potency of drugs as direct skin feeding assays

Reports suggest that the artificial membrane feeding technique often underestimates the infectivity of gametocyte carriers as compared with direct skin feeding [50]. Direct skin feeding demonstrated superior mosquito infection rates as compared with artificial feeding through a membrane (odds ratio = 2.39; 95% confidence interval 1.94–2.95; $p < 0.0001$) [50]. Artificial membrane feeding technique will not pose a problem in comparing the effects of two completely different treatment regimens for their transmission reducing potential (as direct feeding and artificial feeding assays are strongly correlated, $p < 0.0001$ [50]), but in case of comparing two regimens, with and without transmission-blocking drugs, it may overestimate the potency of drugs. But considering the practical implications and ethical considerations, artificial membrane feeding of blood collected from patients appear to be a more convenient approach than the direct skin feeding [50]. Such ethical limitations will have to be dealt with in CHMI trials measuring transmission-blocking potential of the test compounds.

Dearth of transmission-blocking assessment studies

There is a significant shortfall of the following types of studies:

In vivo studies directly evaluating transmission

Majority of the recent *in vivo* studies measure transmission-blocking potential indirectly in a single transmission step, in other words, from the host to the vector. Currently, there is a paucity of recent studies evaluating transmission-blocking properties of test compounds in terms of parameters, such as basic reproduction number (R_0), effect size, entomological inoculation rate – among others. An ideal transmission-blocking drug should aim to reduce the number of secondary malarial infections, therefore quantifying the transmission reducing potential in terms of reduction in parameters, such as (R_0) is essential. Blagborough *et al.* described through laboratory population model experiments that, the success or failure of a transmission-blocking drug in eliminating the infection of an area also depends on the extent of transmission (number of bites/cycle) in that area apart from the potency of the drug itself. A drug (*viz.* atovaquone) which was found to be moderately effective (57% reduction in oocyst intensity and 32% reduction in oocyst prevalence at 0.5 $\mu g\ kg^{-1}$) in the host to vector format was able to sufficiently reduce R_0 (by 20%; effect size) and eliminate the infection from both vertebrate host and vector in low transmission settings (bites <3/cycle) but has no significant impact in high transmission settings (bites >4/cycle) [8]. Studies should be designed in such a way that potential transmission-blocking effect of a test compound can be directly quantified in terms of its ability to reduce the basic reproduction number, effect size, incidence, prevalence, entomological inoculation rate – among others. [47,73]. This will also help in finding drugs that although are not that potent in a particular assay format (e.g., SMFA) during one cycle but has the potential to reduce transmission when used over multiple transmission cycles.

Studies utilizing a humanized mouse model for evaluation of transmission-blocking activity

Humanized mouse models were initially developed for studying the viruses that cause infection in humans, such as HIVs. But, these models have since been utilized to study various other infectious diseases as well, including malaria, and are a promising upcoming tool for antimalarial drug discovery, especially for the preclinical stages. In context of malaria, humanized mice is an experimental model which consists of an immunologically compromised mouse grafted with human erythrocytes or/and human hepatocytes and is capable of supporting partial/complete human component of the parasite's life cycle. Infection experiments of humanized mice with *P. falciparum*, *P. vivax* and *P. ovale* have already been successful. Various proof-of-concept studies reported successful establishment of liver [74,75], erythrocytic asexual [76] and erythrocytic sexual (gametocyte) stages [77] of the life cycle of human *Plasmodium*. Considering the biological differences between *P. berghei* and *P. falciparum* gametocytes, this model is definitely an upgrade over the classical *in vitro* and rodent models for potency evaluation of transmission-blocking compounds. Moreover, this model has also opened new avenues to design studies in order to evaluate the pharmacokinetics and pharmacodynamics of transmission-blocking drugs, which would help in predicting dosages for human consumption [78]. Further, toxicology studies using humanized mice will definitely be an advancement over studies carried using the traditional rodent model. Since, this model system is relatively new as compared with other well established models, there is a paucity of studies evaluating transmission-blocking potential of test compounds using humanized mice. Such studies can prove to be extremely valuable during the preclinical stages of transmission-blocking drug development.

Clinical studies evaluating gametocytemia as primary outcome

Majority of clinical studies testing the potency of antimalarial drug interventions evaluate asexual parasitemia rather than the patent gametocytemia as primary outcome, which is often evaluated as a secondary outcome. In such studies, patients are not recruited on the basis of gametocyte prevalence and density, and are thus not randomized as per baseline gametocytemia. Collectively, this might result in decline of study power when gametocytemia between interventions and control is compared.

CHMI model for evaluating transmission-blocking drug interventions: perspective & challenges

The controlled human malaria infection model (CHMI) or controlled human malaria infection is a model used to test antimalarial drugs and vaccines as well as to study the immunological responses to malaria parasites. CHMI experiments start with deliberately introducing *Plasmodium* infection in the form of sporozoites (sporozoite induced

malaria or SIM) or asexual blood stages (induced blood stage malaria or IBSM) in human volunteers. Introduction of sporozoites, can either be achieved, the natural way, in other words, by the bites of female *Anopheles* mosquitoes or intravenously by injecting the sporozoites by a syringe and needle (PfSPZ challenge). One advantage of IBSM over SIM is that, in IBSM, asexual stages are developed in all the volunteers at the same time, which simplifies the trial design, whereas in SIM, all volunteers might develop asexual parasitemia at different time intervals [79]. For evaluation of transmission-blocking drugs, gametocytes with appropriate sex ratio and densities sufficient to infect *Anopheles* mosquitoes are needed to be established in the participants, which is a challenge in itself. For this purpose, certain drugs (e.g., piperaquine) are administered to curtail the asexual parasitemia and to facilitate the maturation of gametocytes [80,81]. After successful establishment of stable gametocytemia in the volunteers, experimental drugs in specific doses are administered to the test group volunteers; positive control group volunteers administered with a known gametocytocidal drug, such as primaquine and negative control volunteers receive placebo. The transmission-blocking/gametocytocidal activity of experimental drugs can be evaluated using two readouts, post-feeding mosquito parasite burden (oocyst or sporozoites); and duration of gametocyte carriage in volunteers. For evaluating the mosquito parasite burden, mosquito feeding assays (SMFAs and direct skin feeding assays) can be performed in both test and control group volunteers and parasite burden in the mosquitoes quantitated. Additionally, mosquito feeding experiments may even be performed in gametocytemic volunteers pre- and post-administration of the test compounds/primaquine/placebo and mosquito parasitic burden evaluated. Duration of the gametocyte carriage can be evaluated by molecular tests such as qPCR/qRT-PCR [79,82]. CHMI based studies can be well integrated between the preclinical studies and clinical trials, in order to test the efficacy of drugs which eventually drives the dose selection for upcoming clinical studies. Few studies already evaluated transmission-blocking potential of test compounds in the CHMI model system [83–85]. Apart from being an early clinical model, it can also be utilized as a separate component in the clinical trials in order to evaluate the pharmacokinetics and pharmacodynamics of experimental drugs in infected individuals [86,87]. During evaluation of transmission-blocking drugs using CHMI, mosquitoes are always involved (unless, sporozoites or asexual blood stages are intravenously injected; and only gametocyte clearance is measured). So, there are always practical challenges of working with mosquitoes such as the requirement of an insectary near trial site, arrangement for transporting mosquitoes from the insectary to the trial site, aseptic mosquito rearing and strict compliance with CGMP regulations to overcome the theoretical risk of exposing the volunteers to microorganisms other than *Plasmodium* – among others. [88]. Moreover, naturally acquired immunity in volunteers may also impact the development of parasites or parasite multiplication rate which may increase the confounding, if study site is in high transmission zone and volunteers are not screened appropriately [87].

Also, similar to clinical trials, ethical issues are needed to be addressed, especially in underdeveloped countries where vast number of people are below the level of formal education required for understanding the societal benefits of the trial as well as the associated risks and discomforts, hence unable to give *informed* consent. This, compounded with the substandard healthcare systems make it difficult to fully address the minimum requirements of conducting a CHMI study in a strictly controlled environment, close monitoring of the patients and prevention of the spread of infection to non-volunteers – among others. Moreover, specific processes of CHMI processes require extensive standardization in order to increase reproducibility and facilitate data comparison between studies. Once these considerations are addressed, CHMI studies can be routinely used for evaluating the transmission-blocking drug interventions, which the authors feel can play an important role in anti-gametocyte drug discovery. Moreover, apart from inoculating the host with sporozoites or *in vitro* asexual blood stages, a third strategy could be directly inoculating specific number of *in vitro* cultured mature gametocytes. A perquisite to conducting such studies is producing sufficient number of infectious male and female gametocytes under GMP which are safe to be delivered intravenously. First, this approach will be advantageous as all patients will be receiving gametocytes simultaneously which will simplify the trial design. Second, gametocytes will not be exhibiting any significant symptomatology, which will be a major advantage for the volunteers and clinical staff alike and will enhance compliance. After establishment of stable gametocytemia, volunteers will be given the test compounds and duration of gametocyte carriage evaluated in treated/control groups and mosquito feeding assays will be performed to evaluate the transmission-blocking activity of test compounds. However, this strategy of inoculation of gametocytes instead of sporozoites or asexual erythrocytic stages would have its own limitations. One limitation of such strategy, would be the need to absolutely make sure that the volunteers are cleared of any residual gametocytemia when they exit the CHMI facility at the end of the study to prevent any onward transmission from them. This becomes more relevant in endemic areas such as the African subcontinent where the transmission and the magnitude of G6PD

deficiency are both high and challenging due to the absence of a safe and effective gametocytocidal drug. One alternative would be measuring the G6PD activity at the time of recruitment and excluding the G6PD deficient individuals from the trial. Moreover, if the volunteers could not be given a gametocytocidal drug due to any reason, they should be kept in a strictly controlled environment in the CHMI facility till they are naturally cleared of the gametocytes. There can be other practical limitations to this technique, unknown at this point, but might adversely affect the feasibility. Nevertheless, the CHMI model can be further escalated to carry out transmission-blocking studies in a CHMI volunteer-to-vector-to-CHMI volunteer; multiple cycle type transmission model which will further facilitate the extrapolation of the results to the field.

Conclusion

In the absence of clinically proven malaria vaccines and emerging resistance to common antimalarials and insecticides, the onus of halting the transmission of the most lethal human malaria parasite, *P. falciparum* is upon the transmission-blocking drugs. In this review, we have extensively discussed various assays used by the investigators to evaluate the transmission-blocking potential of experimental drugs. Further, we have critically examined the transmission-blocking assessment approaches, to identify problems which are hindering the development of new transmission-blocking drugs. This comprehensive overview will act as a resource for the scientific community and is expected to help in designing better approaches to study the transmission blocking potential of experimental drugs.

Future perspective

With continuous emergence and global spread of drug and insecticide resistance, the dream of achieving a malaria-free world cannot be solely realized by the schizonticidal drugs and the vector control strategies. Addition of transmission-blocking drugs to existing antimalarial regimen prevents the spread of drug resistant parasites and contributes to the cause of malaria elimination. In the current scenario, mature gametocytes are the preferred targets for the transmission-blocking drugs because of the ease of drug targeting achieved, whic is due to their presence in the bloodstream of the host. Therefore, gametocytocidal drug such as primaquine is recommended along with the course of standard ACT. However, hemolytic toxicity issues associated with primaquine in G6PD deficient patients preclude its use on a large scale. Hence it is a need of the hour to develop safe and effective gametocytocidal drugs. Moreover, apart from gametocytes there are many other parasite stages (in mosquito), such as gametes, zygotes, ookinetes and oocysts which can be targeted by the antimalarial drugs, thereby preventing establishment of infection in the mosquito and halting the transmission of parasite from the vertebrate host to the vector. We feel that if a drug is intended to target only mature gametocytes (with no activity across asexual stages), it should be having a long elimination half-life. This will enable it to target mature gametocytes for a long period of time, considering the fact that *P. falciparum* gametocytes emerge at least 7/8 days after appearance of asexual parasitemia. Currently recommended gametocytocidal drug primaquine, has a short elimination half-life of 6–8 h, so it can only target mature gametocytes present in the bloodstream at the time of primaquine administration. This becomes more important when the asexual parasites reappear after a standard 3 day ACT due to ACT resistance, which may lead to the emergence of mature gametocytes a few days later.

If the drug is intended to target the mosquito stages of the parasite, it has to be present over the mosquito-stage, parasite-killing concentrations in the bloodstream of the host for a long period of time. This is so it can prevent the establishment of parasites in mosquito longer. The longer this time period will be, the longer the drug will prevent the infection in the mosquitoes. Hence, this time duration will have a great impact on malaria transmission. This time duration is either governed by host related factors or vector related factors. Host related factors include cytochrome P450 enzymes, plasma protein binding – among others. which impact the half-life of drugs. Various strategies are also available which can increase the half-life of drugs, such as use of higher than usual doses, using repeated doses, development of slow release formulations or slowing down cytochrome P450 metabolism of drugs (pharmacoenhancement, commonly used in HIV treatment with protease inhibitors). Vector related factors include the mosquito defense mechanisms (including detoxifying enzymes) and drug metabolic pathways which influences the pharmacodynamics of drugs inside mosquitoes. This part is although very crucial but understudied. Thorough understanding of these aspects might help in designing modulation strategies for mosquito defense mechanisms, which can lead to better action of drugs toward their targeted parasite stage inside the mosquito. This might open up new avenues in mosquito stage parasite drug development. Moreover, chemoprevention by antimalarial drugs is also a manifestation of transmission-blocking and prevents the transmission of parasite from vector to a

vertebrate host. However, the intractable nature of the liver stage parasites due to their experimental inaccessibility contributes to current impediments in understanding the biology of liver stages and hinders development of drugs, which can target liver stage parasites. Studies evaluating transmission-blocking potential of the test compounds use multitude of approaches involving variable assay formats, parasite population and incubation time – among others, as a result of which reproducibility and comparability is compromised. There is urgent need to develop a universally adaptable assay strategy for transmission-blocking activity evaluation. Many research groups are coming up with experimental models, such as the humanized mice and CHMI for evaluating transmission-blocking drug interventions. The authors propose the use of CHMI studies as an early clinical model in a human-to-human transmission type setting, to assess the efficacy of transmission-blocking drugs which would guide the dose selection for the upcoming clinical studies involving a large number of participants. Moreover, CHMI studies may also prove useful in evaluating pharmacokinetic and pharmacodynamics parameters of the experimental drugs in infected patients. However, balance between the risk and benefits of the CHMI trial is an ethical argument and needs to be addressed with urgency and care. Steps should be taken to ensure safety of the patients, as comprehensively as possible. This will facilitate the approvals of such trials and will eventually lead to development of promising transmission-blocking drugs.

Executive summary

Requirement of novel transmission-blocking drugs

- Since transmission-blocking approaches are a mainstay of malaria elimination, scientific community worldwide has begun to realize the importance of transmission-blocking drug interventions.
- With continuous emergence and spread of drug resistance, and absence of an effective vaccine, the role of transmission-blocking drugs becomes even more critical.
- Use of transmission-blocking drugs for malaria not only helps in interrupting the life cycle of the parasite from human-to-mosquito-to-human but also aids in containing the spread of drug resistance.
- Primaquine, which is a WHO recommended transmission-blocking drug, is not completely safe and effective for entire population, hence not extensively utilized.

Transmission-blocking assays

- Development of various high-throughput assays are a significant improvement over the traditional low-throughput assays, such as microscopic counting of gametocytes on Giemsa stained smears previously used for evaluating transmission-blocking potential of test compounds.
- Majority of such high-throughput assays only target a particular stage of the parasite, such as the mature gametocytes, or cover only some sections of the parasite biology such as early to mature gametocytes, mature gametocytes to gametes (gamete formation assays) or mature gametocytes to oocysts (membrane feeding assays) – among others.
- There is a dearth of studies reporting assays covering entire biology of the parasite, especially in *P. falciparum* and evaluating test compounds in a host-to-host or vector-to-vector transmission model.

Problems in current transmission-blocking assessment approaches/studies

- Despite the development of high-throughput drug screening techniques and novel assay methodologies, we are yet to develop a safe and effective transmission-blocking drug for falciparum malaria, although we do have some promising drug candidates in developmental pipeline.
- The limitations reviewed here provide a comprehensive overview of the problems in current transmission-blocking assessment approaches/studies, which are expected to help in the identification of lacunae in current understanding of transmission-blocking strategies and will help in streamlining the drug discovery process for finding novel transmission-blocking drugs.
- Evaluating transmission-blocking potential of test compounds in CHMI studies before clinical trials will help in early evaluation of drug efficacy and will guide the dose selection for forthcoming clinical studies.
- Presently, CHMI studies require extensive standardization of its various processes to ensure data reproducibility and also warrants careful dealing of associated ethical issues. However considering its versatility, its use as a transmission-blocking drug evaluation model is recommended to accelerate the evaluation of transmission-blocking compounds.

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Author Contributions

I Wadi, A Sinha and CR Pillai conceived the study. I Wadi collected & analyzed the data and wrote the manuscript. I Wadi, A Sinha, CR Pillai, AR Anvikar, N Valecha and M Nath carried out initial reviews. I Wadi, A Sinha and N Valecha performed the final reviews. All authors read and approved the final version of the manuscript.

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