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Plasmodium Sexual Stage Parasites Present Distinct Targets for Malaria Transmission-Blocking Vaccine Design

Yaser Peymanfar[1] and Andrew W Taylor-Robinson[2]*

[1]*Payesh Veterinary Laboratory, Amol, Iran*

[2]*School of Medical & Applied Sciences, Central Queensland University, Rockhampton, Australia*

***Corresponding author:** Andrew W. Taylor-Robinson, School of Medical & Applied Sciences, Central Queensland University, Bruce Highway, Rockhampton, QLD 4702, Australia, Tel: +61 7 4923 2008; **E-mail:** a.taylor-robinson@cqu.edu.au

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Abstract

Malaria is an infectious disease that in humans is caused by one of five species of the protozoan parasite *Plasmodium* and which is transmitted by mosquitoes of the genus *Anopheles*. Despite much investment over many years to eradicate or control the global incidence of malaria, infection remains a major cause of morbidity and mortality throughout the world, particularly in developing countries in tropical and subtropical regions. A large number of candidate vaccines has been developed against different antigens expressed by separate life cycle stages of *Plasmodium*. Transmission-blocking vaccines (TBVs) aim to combat the sexual stages of parasite development that occur distinctively inside the mosquito vector. This also has a potentially significant impact on the efficacy of other malaria vaccines which target stage-specific antigens expressed within humans by reducing the spread of parasites in the community, an altruistic approach that leads to a local herd immune response. In 1958 the first successful steps towards producing a TBV used an avian model to raise sexual stage-specific antibodies. In the almost 60 years that have elapsed since this proof of principle demonstration considerable effort has been expended in order to identify parasite antigens the expression of which elicits transmission-blocking activity. This review considers the significance of an anti-malaria TBV strategy, the range of potential targets, and the incremental advances which have been made to produce effective TBVs.

Keywords: Malaria; *Plasmodium*; Mosquito; *Anopheles*; Vaccine; Transmission-blocking

Introduction

Malaria is a mosquito-borne infectious disease caused by protozoan parasites of the genus *Plasmodium* which exacts a significant toll of morbidity and mortality in humans [1]. Around 3 billion people currently live in malaria-endemic areas, over the last decade leading to an estimated 250 million clinical cases and one million recorded fatalities annually [2]. In 2013, the most recent year for which data are available, 584,000 deaths and 198 million clinical illnesses were reported [3]. Susceptibility to severe manifestations of disease is increased in pregnant women and non-breastfeeding children under the age of five, especially in endemic areas [4].

The life cycle of *Plasmodium* progresses through multiple transitions in alternating hosts, sexual reproduction occurring in the midgut of an *Anopheles* mosquito and asexual replication occurring in the liver and bloodstream of a vertebrate, including humans. It is the latter cycle of intraerythrocytic vegetative growth and host cell rupture that is responsible for the pathogenesis of disease and gives rises to the characteristic influenza-like, paroxysmal symptoms of uncomplicated infection [1]. When a mosquito ingests a blood meal from a malaria-infected person, male and female gametocyte stage parasites enter the midgut where they are enclosed by a newly synthesized peritrophic matrix. Intracellular male gametocytes exit their erythrocytic environment via a process of exflagellation that is triggered cumulatively by mosquito-derived xanthurenic acid, a lowering of body temperature and a commensurate rise in pH, whereupon fertilization soon follows. Within 24 hours a zygote forms and further development and differentiation results in banana-shaped, motile ookinetes. From the midgut ookinetes penetrate the epithelium, reach the basal lamina and differentiate into oocysts. Multiplication through mitotic divisions over 7-15 days may result in tens of thousands of sporozoites. These migrate to the salivary glands and are injected into an individual's body when the now infectious mosquito next bites a human. Very rapidly after entering the peripheral blood sporozoites home to hepatocytes and after vegetative multiplication many merozoites form. These are released back into the blood, invade erythrocytes and undergo a cycle of asexual replication that repeats every 46-48 hours for *P. falciparum*, the deadliest of the human malaria species. A minority of merozoites leaves this cycle and undergoes sexual development into male and female gametocytes that are ready to transfer to a mosquito when it feeds on that person [1]. At each stage different antigen-specific immunological responses perform protective (and possibly immunopathological), sterilizing and altruistic effects (Figure 1) [2,5-7]. Harnessing each form of specific immunity plays a role in the overall strategy to control malaria, such that potentiation of a protective response underpins rational vaccine design (Figure 2) [8].

The production of an effective vaccine would provide an ideal addition to the arsenal of tools to combat malaria. While vaccines have had a dramatic impact on many infectious diseases, to date this is not the case for malaria, a disease caused by a eukaryotic protozoan parasite that is much more complex genetically, structurally and physiologically compared to both viruses and bacteria. This complexity makes it more difficult to develop a vaccine, exemplified by stage-specific expression of antigens, phenotypic antigenic variation of cloned asexual erythrocytic parasites over time and antigenic polymorphism of geographically diverse isolates [8,9]. Over several decades from the mid twentieth century, *Plasmodium* has thwarted considerable efforts to find an effective solution to the development of an efficacious, commercially available vaccine.

All in all, four recognizably distinct approaches have been taken to prepare *P. falciparum* malaria vaccines: (a) a recombinant protein together

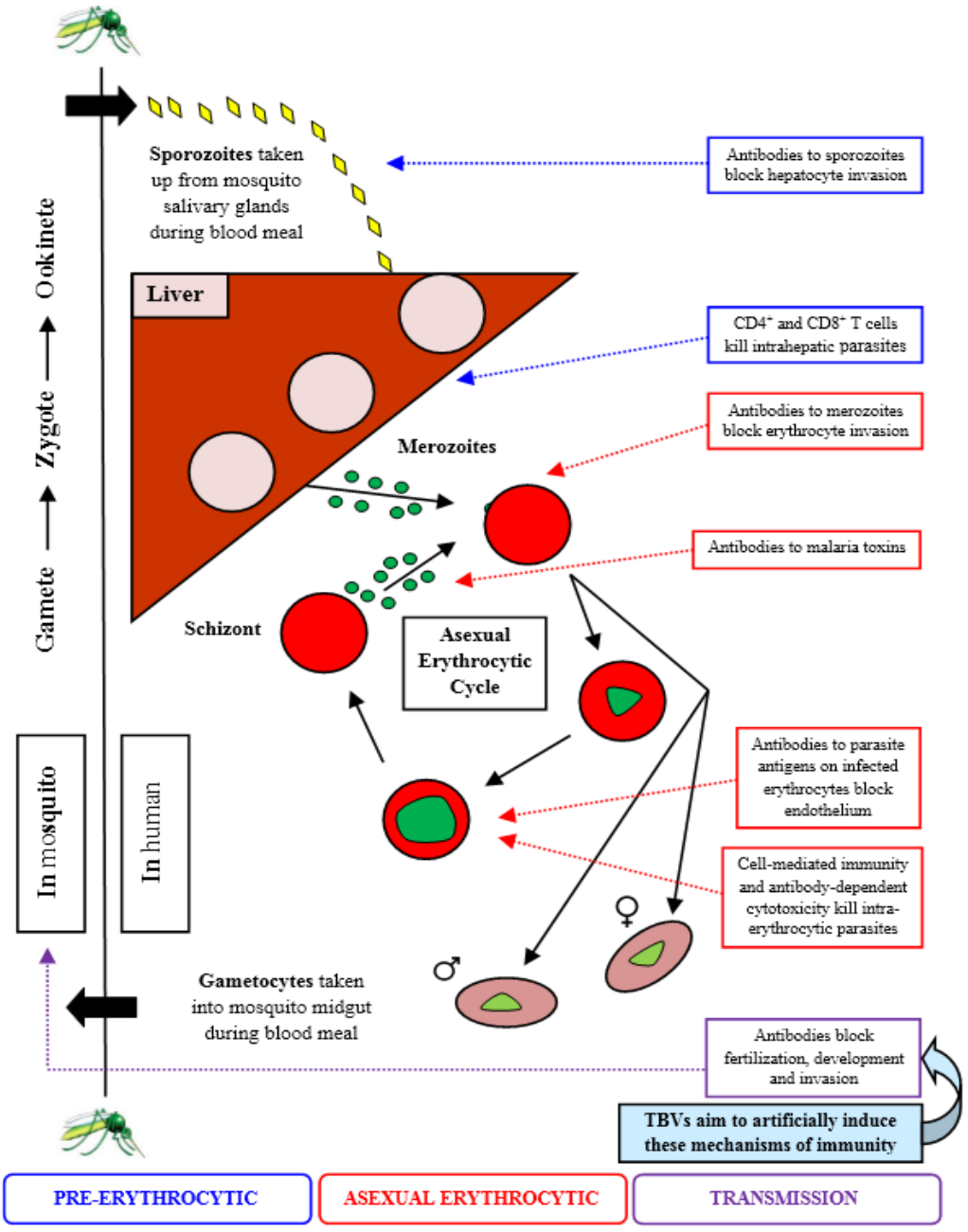

Figure 1: Schematic diagram showing the mechanisms of immunity against different life cycle stages of the major human malaria parasite *Plasmodium falciparum*, highlighting those against the sexual stages that transmission-blocking vaccines aims to induce.

with adjuvant for pre-erythrocytic stages of the parasite's life cycle (e.g. RTS,S/AS01); (b) whole sporozoite preparations for pre-erythrocytic stages (e.g. PfSPZ and PfSPZ-CVac); (c) prime-boost constructs that include recombinant DNA, viruses or bacteria; (d) recombinant protein combined with an adjuvant for sexual erythrocytic and mosquito stages [9].

Rationale for a transmission-blocking vaccine

Sexual development within the mosquito midgut may represent the most vulnerable target for vaccines to prevent transmission of malaria parasites [1]. Such a vaccine would have the potential to reduce the burden of disease, including in parts of the world's most malarious continent, Africa. In Asia and Latin America, it could help lead to the elimination of the malaria parasite. While the immunization of an individual with a transmission-blocking vaccine (TBV) cannot directly prevent that person from becoming infected with malaria via the bite of an infectious mosquito, if sufficient people in the community are vaccinated the prevalence of infected vectors will diminish. Hence, this altruistic strategy would also greatly prolong the useful life of vaccines against other stages by limiting the spread of parasites that have the potential to become resistant to these vaccines [10].

In most malaria-endemic locations, a TBV immunization program, even if partial, would reduce disease and death due to *P. falciparum* malaria. In areas of relatively low transmission, as in most endemic locations outside tropical Africa, disease would be reduced probably in direct proportion to the effective coverage with TBVs. In many situations of low endemicity, transmission could be prevented by TBVs. In more

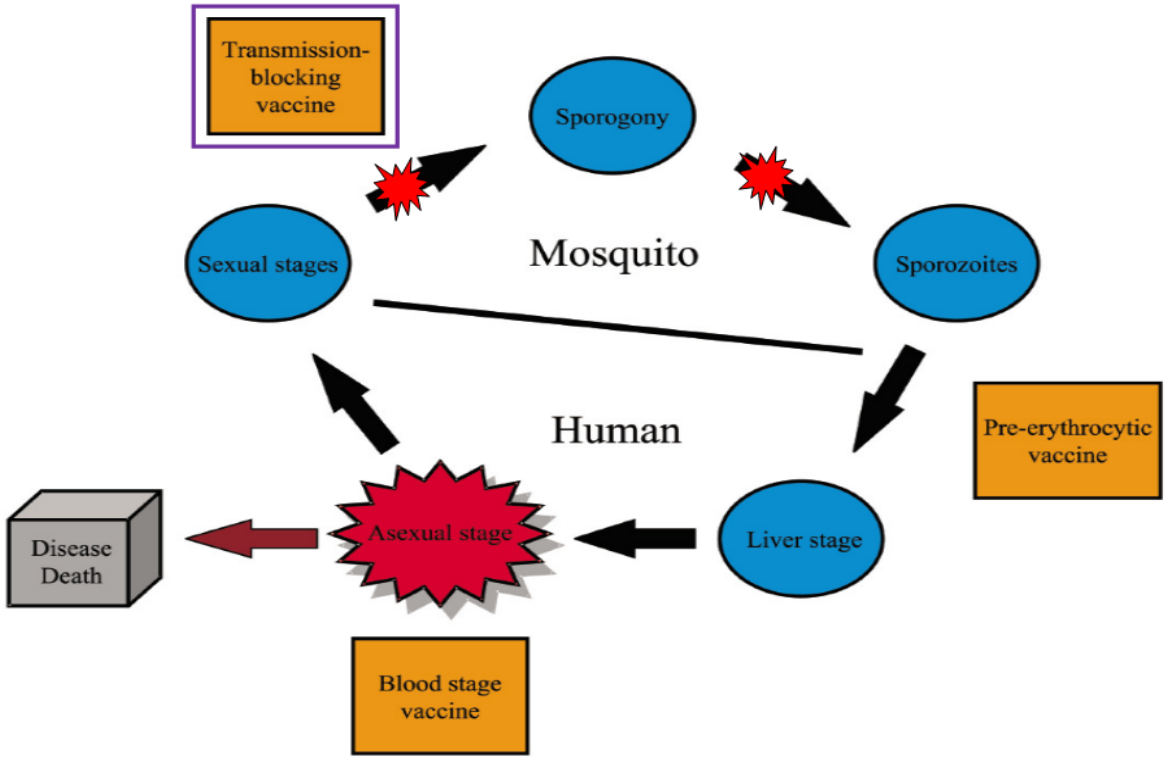

Figure 2: Schematic life cycle of *Plasmodium falciparum*, showing where vaccination may be expected to intervene. A transmission-blocking vaccine targets the parasite during its sexual development within the *Anopheles* mosquito host. In the alternate human host disease pathology is associated with the cyclical phase of asexual stage replication that occurs in the blood and which causes synchronized erythrocyte rupture and release of pyrogenic toxins.

highly endemic regions, the deployment of TBVs in conjunction with additional traditional measures such as insecticide-impregnated bed nets could bring the end of malaria transmission within reach. In some instances even incomplete TBV coverage would slow the build-up of malaria epidemics and reduce their size very substantially [11].

Induction of transmission-reducing antibodies was reported first in an avian malaria model in the late 1950s [12]. Further, in 1976 effective blocking of transmission was achieved by repeated immunization of chickens with *P. gallinaceum* gametes [13,14]. Antibodies were raised against antigens that were later characterized as P230, P48/45, P28 and P25 [15], expressed on the surface of gametes, zygotes or ookinetes [16-18].

Several principles guide TBV development [9]. Gamete antigens are an obvious source of TBV candidates; interference of gamete development and fertilization provides a source of candidates (such as Pfs25 and Pfs28 in *P. falciparum*). Epitopes recognized by transmission-blocking antibodies may show conservation between geographically distinct parasite isolates but degrees of variation exist for all candidate antigens. A principal goal of a vaccine is to induce and sustain a high titer of immunoglobulin G. The main vaccine targets elicit weak immune responses in primates, so aiming to boost immunogenicity with adjuvants but without being reactogenic is important [9].

Targets for transmission-blocking vaccine development

Most current targets for TBVs are either parasite surface antigens, ookinete-secreted proteins, mosquito components or recombinant proteins [1]. Each of these categories is discussed below.

Parasite surface antigens: Homologues of the surface antigens first found in *P. gallinaceum* were identified subsequently in *P. falciparum* and appear crucial to activation of gametes, zygotes and ookinetes. These proteins have been the primary focus of TBV development, inducing transmission-blocking monoclonal antibodies against *P. falciparum* macrogametes [19,20].

The *P. falciparum* antigen now known as Pfs230, named for its molecular weight by SDS-PAGE, and Pfs48/45, another protein named for its 48 and 45kDa structure on SDS-PAGE [21],are expressed by gametocytes and appear on the surface of gametes and newly fertilized zygotes. P230 and P48/45 are known as pre-activation targets [1]. Two other *P. falciparum* proteins, Pfs25 and Pfs28, are post-activation targets expressed on the surface of zygotes during their development to ookinetes [1]. These were identified initially as *P. gallinaceum* orthologues using monoclonal antibodies prepared from mice that had been immunized with ookinetes [16].

Several studies on genetic modification of these crucial proteins have been performed. For instance, P230 is typified by partially conserved cysteine motifs paired as cysteine-rich double domains highly constrained by disulfide bonds [22]. Deleting fragmented or entire motifs from Pfs230 did not affect the role of this protein in gamete emergence although it was no longer retained on the parasite surface [23].

An interesting feature of the human immune system is the enhancing effect on transmission-blocking anti-Pfs230 antibodies exhibited by human complement [24]. However, this complement is degraded by mosquito proteases within 3-5 hours of blood meal ingestion, so the

window of activity is short [25,26]. It may be that genetic modification of a mosquito's protease activity might prove effective in enhancing for a longer duration the efficacy of anti-P230 antibodies.

Analyzing Pfs230 orthologous genes in eight *Plasmodium* species including the human malaria parasite *P. vivax* showed structural conservation of the 14 cysteine motif/paired double domains that follow an interspecies-variable N-terminal pro-domain [22]. Sequence polymorphisms existed among the 113 isolates examined. Only a limited number of amino acid substitutions were found in a subdomain, $Pvs230_{236\text{-}943}$, containing the first four cysteine motifs. This conserved structure may be the focus of a candidate *P. vivax* TBV [22,27]. Again, a comparison of the presence and absence of human complement was made [27], which showed different mechanisms of complement fixing by these anti-Pvs230 immune sera [1].

In another study, deletion of Pfs48/45 by gene knockout did not affect the development of gametes. However, the mutated parasites produced a significantly lower number of oocysts when fed to mosquitoes [28]. Using knockouts of the Pfs48/45 orthologue in *P. berghei*, a rodent malaria model, confirmed a role for P48/45 protein in fertilization, since male gametes lacking P48/45 could not fertilize female gametes, whereas they could still be fertilized by wild type males [28].

By using a single gene deletion of *P. berghei* Pbs25 or Pbs21, effective inhibition of ookinete formation was demonstrated, through reduced parasite penetration into the midgut wall [29]. Of all sexual stage antigens, Pfs25 and its *P. vivax* orthologue Pvs25 are the only TBV antigens to be evaluated in human trials to date. In combination with another seven *P. falciparum* antigens Pfs25 was deployed with an attenuated vaccinia virus vector to generate a multi-stage vaccine candidate named NYVAC-Pf7 [30]; however, immunogenicity was poor and no transmission-blocking activity by immune sera was detected in the membrane feeding assay that is used to measure parasite transmission to mosquitoes in the laboratory setting [9,30].

Ookinete-secreted proteins: In order to continue the life cycle of *Plasmodium* within the midgut of the mosquito an ookinete in the maturation phase that is contained in the blood meal first interacts with ligands on midgut microvilli, then penetrates into the midgut epithelium and emerges on the basal side of the cell, coming to rest under the basal lamina where it forms an oocyst. Each of these processes is totally dependent upon the activities of proteins secreted from micronemes, ookinete organelles that have thus become targets for TBV development [1].

The peritrophic matrix in the midgut of an *Anopheles* mosquito's midgut comprises chitins, proteins and proteoglycans. Disruption of gene expression related to chitinases secretion (*Pfcht1* and *Pbcht1*), paralogous genes in *P. falciparum* and *P. berghei* parasites, respectively, resulted in remarkable reductions in oocyst formation in mosquitoes [31,32]. Following these revealing studies production of recombinant chitinases as the basis for a candidate vaccine was instigated.

One of the *Plasmodium* perforin-like proteins (PPLPs), so-called membrane-attack ookinete protein (MAOP), which is located in the micronemes of ookinetes, has been analyzed [33,34]. Disruption of MAOP gene expression in *P. berghei* parasites inhibited ookinete penetration through the midgut epithelium. Further studies on the activity of antibodies to MAOP should be performed to extend this evaluation [1]. Moreover, secreted ookinete adhesive protein (SOAP) and cell-traversal protein for ookinetes and sporozoites (CelTOS) are two additional ookinete microneme proteins that genetic modification of the expression of which has a crucial effect on the penetration of ookinetes into midgut epithelium [35,36]. Subsequent research revealed that immunization with recombinant PfCelTOS may protect mice against challenge with lethal *P. berghei* sporozoites, leading to consideration of GMP CelTOS as a candidate vaccine antigen [37,38]. Recently, improving the immunogenicity of PfCelTOS by administration with an adjuvant has been examined [39].

Mosquito components: Vaccines that target components of the *Anopheles* midgut are attractive candidates because the resultant reduction in vector competence has the potential to simultaneously block transmission of multiple *Plasmodium* species [1].

Lectin proteins of mosquitoes, such as jacalin, known to adhere to glycoproteins of cell walls, reduce ookinete attachment to the mosquito midgut by binding glycan ligands [40]. A mosquito salivary gland protein, saglin, was identified as a putative target the blocking of which caused a 70% decrease in sporozoite levels [41]. By injection of anti-saglin antibodies into the mosquito haemocoel or disrupting its expression by RNAi, significant reduction of sporozoites in salivary glands was obtained. Hence, saglin is considered by some to be a potential target for TBV [1].

Recent analysis suggests that mammalian antibodies delivered in the blood meal may up regulate the mosquito immune response. Antibodies to *A. gambiae* serine protease inhibitor (serpin)-2 reduced *P. berghei* oocyst numbers by 54% [42], a finding which warrants further investigation.

Recombinant proteins: On the basis of evidence that P230, P48/45, P25 and P28 antigens induce antibodies, efforts have been made to attain the native conformation of these proteins by recombinant technology in a vector system. In particular, the yeast *Saccharomyces cerevisiae* has been used extensively as an expression platform. Initial research showed that Pfs25 proteins expressed in *S. cerevisiae* have a partially native conformation [1]. Trials of a Pfs25 candidate expressed in this system and formulated with the adjuvant alum were terminated early owing to reactogenicity, considered likely to be due to an unbound antigen in the formulation. However, Pvs25 expressed in *S. cerevisiae* and formulated with alum was found to be well tolerated in humans [43].

Recently, Pfs25 was expressed successfully in a plant-based vector system as a fusion to the lichenase carrier molecule (Pfs25eLiKM) [44], and as a fusion to the Alfalfa mosaic virus coat protein (Pfs25eCP) [45]. In each case, recombinant products were purified to a high level of homogeneity.

A dual expression system has been developed based on the baculovirus *Autographa californica nucleopolyhedrosis*, which possesses strong adjuvant properties that can activate dendritic cell-mediated innate immunity [1]. This vector offers much promise for future TBV development.

Conclusion

In the continuing drive to produce an effective malaria vaccine, abroad range of studies has been conducted on antigens expressed during different stages of the *Plasmodium* life cycle. Blocking of sexual development inside the *Anopheles* mosquito provides a means to combat the parasite outside of the human body. In this article various potential targets for the development of TBVs have been described and progress with different TBV strategies has been reviewed.

Further research is merited to evaluate the added value of TBVs in reducing or even eliminating locally the spread of *Plasmodium*, which would thereby augment the protective effect of anti-malaria vaccines that target directly parasite life cycle stages in the human host.

Conflict of interest

The authors declare that they have no competing issues of interest.

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