Review Article

Indian J Med Res 143, June 2016, pp 696-711
DOI:10.4103/0971-5916.191927

Strategies & recent development of transmission-blocking vaccines against *Plasmodium falciparum*

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Received April 17, 2015

Transmission blocking malaria vaccines are aimed to block the development and maturity of sexual stages of parasite within mosquitoes. The vaccine candidate antigens (Pfs25, Pfs48/45, Pfs230) that have shown transmission blocking immunity in model systems are in different stages of development. These antigens are immunogenic with limited genetic diversity. Pfs25 is a leading candidate and currently in phase I clinical trial. Efforts are now focused on the cost-effective production of potent antigens using safe adjuvants and optimization of vaccine delivery system that are capable of inducing strong immune responses. This review addresses the potential usefulness, development strategies, challenges, clinical trials and current status of *Plasmodium falciparum* sexual stage malaria vaccine candidate antigens for the development of transmission-blocking vaccines.

Key words Malaria - *Plasmodium falciparum* - sexual stage antigens - transmission blocking vaccine

Malaria transmission blocking vaccine at a glance

Malaria is considered as a major global health problem, with an estimated 214 million cases and 438,000 malaria-related deaths worldwide in 2015[1]. *Plasmodium falciparum* is responsible for a majority of malaria cases in humans. The emergence of insecticide-resistant mosquitoes and increase in parasite resistance to antimalarial drugs enhanced the need for effective vaccine development[2]. Multiple stages (pre-erythrocytic, erythrocytic and sexual stage) of the life cycle of malaria parasite are being targeted for vaccine development. Transmission blocking vaccines (TBVs) are focused against sexual stages or sporogonic-specific antigens. These are designed to block the development of sporogonic stages of parasite inside the mosquito thereby reducing mosquito infectivity and prohibiting the spread of the disease[3]. The target antigens for TBVs are divided into two groups, namely, pre-fertilization and post-fertilization antigens. Pre-fertilization antigens are expressed on the surface of gametocytes and gametes of malaria parasites, such as Pfs48/45, Pfs47 and Pfs230[4]. These proteins belong to a family that contains six-cysteine domains[5]. Pfs230 and Pfs48/45 are major gamete surface antigens that induce antibody responses in naturally exposed individuals[6,7] and are associated with transmission reducing immunity[8]. Pfs25 is a post-

fertilization antigen expressed on the surface of zygote and ookinete and has shown strong immunogenicity with limited antigenic polymorphism[9,10]. The antibodies that only target conformational epitopes of these proteins depend on the proper folding of cysteine-rich proteins and exact formation of disulphide bridges[11,12]. These sexual stage antigens induce antibodies in the human host that interfere with the parasite development. Thus, transmission blocking takes place inside the mosquito vector and is antibody mediated[13]. *In vitro* studies of the transmission-reducing immune response in animal models[14,15] have shown a significant reduction in parasite development, which has led to the development of TBVs as part of malaria control and elimination strategy.

Expression of TBV target proteins in *P. falciparum*

More than hundred genes are expressed in the parasite life cycle, however, only a few of them have been cloned and studied for vaccine candidate. A unique *Plasmodium* gene superfamily encoding proteins that share six-cysteine domains are expressed during the sexual stages. The Pfs48/45 family has 12 distinct members namely, Pfs230, Pfs48/45, Pfs230p, Pfs47, P52, P36, Pf41, Pf38, Pf12, P12p, Pf92 and sequestrin[16]. Among these proteins, Pfs230, Pfs48/45 and Pfs47 play a critical role in the parasite development[17]. The six-cysteine family is conserved throughout all *Plasmodium* species and characterized by partially conserved cysteine-rich double domains having approximately 350 amino acids in length contributing to the tertiary structure of the proteins. Most of these proteins are localized on the parasite surface and some of these are known to play a role in cell-cell interaction[5,18]. The immunogenic proteins (Pfs48/45, Pfs47, Pfs230, and Pfs25) are important for fertilization process and other vital functions of parasite life cycles[13]. These antigens have the ability to boost the immune system either by vaccination or naturally during the infection[19]. These specific characteristics of the sexual stage antigens make the proteins interesting for study the development biology of the parasite in the mosquito vector and ultimately the possible vaccine targets (Tables I & II).

Potential of TBV candidates

Pfs48/45: The Pfs48/45 antigen plays a role in the male/female gamete fusion in the mosquito midgut[5]. Disruption of the *Pfs48/45* gene in *P. falciparum* and *P. berghei* has demonstrated a central role in male gamete fertility[13]. Fertilization and zygote formation are strongly reduced in *Pfs48/45* knockout parasites, but production of gametocyte and differentiation into gametes remains unaffected[17]. The Pfs48/45 antigen induces antibody responses in naturally exposed individuals which are associated with functional transmission-reducing immunity[6,8]. Transmission-blocking monoclonal antibodies that recognize B-cells epitopes on Pfs48/45 seem to block fertilization with the presence of complement proteins[11] as well as without complement[12]. The ability to stimulate the antibody response upon encounter with the natural infection as seen in the field makes the exceptionally valuable capability of vaccine boosting in the endemic areas. Data from hyperendemic Papua New Guinea (PNG) show that seroprevalence increases with age, suggesting that anti-Pfs48/45 response develops immunological memory[28]. Increasing antibody titres against Pfs48/45 have also been observed with recent exposure to malaria infection in PNG[29]. Study from Gambian and Cameroonian populations showed strong correlation between antibody response and transmission reducing activity against Pfs48/45 antigen[30,31], while anti-Pfs48/45 response in serum of Sri Lankan population did not show any correlation[32]. The antibody response against Pfs48/45 is enhanced by simultaneous exposure of gametocyte and is also related to the extent of gametocyte carriage in Tanzania[33]. Conversely, the studies carried out in Senegal and Cameroon conclude that transmission blocking immunity depends on age, antibody titres, episode of malaria infections and duration of gametocyte carriage[34]. Genetic polymorphism is a major problem in malaria vaccine development. The *Pfs48/45* gene is less polymorphic in comparison to other erythrocytic and pre-erythrocytic stage specific antigens[35-37] with a minimum number of amino acid substitutions[38]. These observations suggest that this transmission blocking antigen is conserved at the protein level, making it a good candidate for multistage, multivalent vaccine. Fusion of Pfs48/45 to glutamate rich protein (GLURP) antigen is one of the recent approaches towards the development of multi-stage malaria vaccine which can target both transmission and asexual parasite life cycle stages[39]. All these findings show that Pfs48/45 is an important candidate antigen for the development of transmission blocking vaccine.

Pfs230: Pfs230 is a 363 kDa (3135 amino acid) protein and a potent antigen of malaria transmission blocking vaccine. It is involved in the fertilization of macrogametocytes by microgametocytes[20]. Male gamete with a disrupted Pfs230 gene is incapable

Table I. Malaria sexual stage vaccine candidate antigens and their characterstics

Antigen	Gene ID	Stage	Gender	Location	Chromosome number	Nucleotides (bp)	No. of amino acids	Moleculer wt. of protein (kDa)	SP	GPI anchor	Domain	Expression time	References
Pfs48/45	PF13_0247 New PF3D7_1346700	GC,G	F, M	PV, S	13	13,40	448	48, 45	1	1	3 cysteine motif domain	Expressed on the surface of gametocytes, strating at late stage II until fertilization is completed	3, 6, 8, 11-13, 24
Pfs230	PFB0405w New PF3D7_0209000	GC, G	F,M	PV, S	2	96,36	3135	363	1	0	14 cysteine motif domain	Co-expressed in association with Pfs48/45 on the surface of gametocytes, starting at late stage II until fertilization is completed	3, 13, 20, 21, 24
Pfs47	PF13_0248 New PF3D7_1346800	GC, G	(F)	PV, S	13	13,20	439	51	1	1	3 cysteine motif domain	Expression starts at stage II gametocytes and highly expressed at stage IV and stage V gametocytes	3, 13, 22-24
Pfs25	PF10_0303 New PF3D7_1031000	G, Z, O	(F)	S	10	654	217	25	1	1	4 epidermal growth factor like domain	Expressed on the surface of gamete during the initiation of emergence and its expression persists untill the ookinete has penetrated the midgut epithelium	3, 9, 13, 24-27

Pattern of expression was homogenous in all antigens

GC, gametocyte; G, gamete; F, female; M, male; SP, signal peptide; TM, transmembrane helix; S, surface; PV, parasitophorous vacuole; 0, absent; 1, present; GPI, glycosylphophatidylinositol

Table II. Properties and current status of development of malaria transmission-blocking vaccine candidates

Antigen	Putative function	KO (knockout) phenotype	Antibody response in correlation with transmission blocking ability	Polymorphism	Strengths	Weakness	Current status of development	References
Pfs48/45	Plays a role in gamete fusion in the mosquito midgut. Major role in male gamete fertility.	Reduced fertilization	Camerronian and Gambian serum shows strong correlation while Sri Lankan serum shows no correlation. Antibody prevalence increased with age and recent exposure to infection and associated with the duration of carriage of gametocytes.	Limited polymorphism is found in the coding sequences. Antigenic diversity in field is low.	Antibodies to Pfs48/45 can block the transmission of *P. falciparum* to mosquitoes. Can induce transmission-blocking antibody response during an infection. Essential for fertilization in gene knockout experiments.	Problem in expressing recombinant protein in immunogenic form. Conformational epitopes requires suitable expression system.	Combinatorial peptide approach being explored Recombinant antigen expression using *Escherichia coli* (codon harmonized). Research on the expression of recombinant products in immunogenic form.	3, 6, 8, 11-13, 28-38
Pfs230	Play role in gamete-gamete interaction Central role in male gamete fertility	Reduced fertilization	Strong correlation was found in Gambian and Papua New Guinean serum. Antibody prevalence increased with age and recent exposure to infection and associated with the duration of carriage of gametocytes. Transmission blockade by anti-Pfs230 specific Antibody is isotype dependent.	Exhibits a reasonable degree of diversity. Pfs230 is under positive selection resulting in non-neutral sequence polymorphism.	Antibody to Pfs230 can block transmission of *P. falciparum* to mosquitoes. Expressed on gametocyte during infection in human host, probable boosting of transmission-blocking antibody response during an infection.	Very large molecule , difficult to determine which regions would be effective in a vaccine. Immunogenicity is poor and requires a strong adjuvant.	Fragments have been expressed in a variety of systems including plant and cell free wheat germ systems, induce some transmission-blocking activity. Research on the expression of recombinant protein in immunogenic form.	3, 13, 20, 21, 39-46

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Antigen	Putative function	KO (Knockout) phenotype	Antibody response in correlation with transmission blocking ability	Polymorphism	Strengths	Weakness	Current status of development	References
Pfs47	Central role in female gamete fertility	Parasite locking Pfs47 produce normal number of oocysts	No correlation was observed in antibody response and transmission blocking activity.	Pfs47 shows limited polymorphism in Indian field isolates. Pfs47 are under positive selection resulting in non-neutral sequence polymorphism.	Pfs47 is an essential survival factor for *P. falciparum* that allows the parasite to evade the immune system of *Anopheles gambiae*, a major mosquito vector in Africa. .	Does not induce transmission blocking immunity. Disruption of gene does not affect oocyst development.	Research on the KO phenotype study to gain the knowledge about the exact function in malaria parasite survival in mosquito.	3, 13, 22-24, 47, 48
Pfs25	Play role in ookinete survival in the midgut, penetration of the epithelium and transformation of the ookinete into the oocyst.	Reduced formation and infectivity of ookinetes.	Antibodies obtained after immunization of mice and monkeys with a yeast-produced Pfs25 showed significant transmission blocking activity. Antibodies to the second EGF-like domain of Pfs25 appear to mediate a very potent blocking activity, even at low titers.	Pfs25 are not polymorphic, as these are under no adaptive immune pressure in the human host. In Pfs25, two conserved amino acids substitutions and two silent changes were found.	Antibodies against Pfs25 can block transmission of *P. falciparum* to mosquitoes. In many zones where TBV would be utilized both *P. falciparum* and *P. vivax* are endemic. Therefore, it is very important that both Pfs25 and Pvs25 are being developed to be used together in the same vaccine. Successfully produced the small Immunogen in yeast and plants	Immunity against Pfs25 antigen is not boosted during natural infection, so the formulations that elicit long lasting immunity may need to be developed. .	Vaccination with both *P. vivax* and *P. faciparum* yeast-expressed clones induces complete transmission-blocking in model systems. Phase I human clinical trial using potent adjuvants (Pfs25-EPA/ Alhydrogel® and Pfs25 VLP-FhCMB) is ongoing.	3, 9, 10, 13, 25-27, 49-57

EGF, epidermal growth factor; TBV, transmission blocking vaccine; EPA, *Pseudomonas aeruginosa* exoprotein A

of interacting with erythrocytes and unable to form exflagellation center[20]. Evidence from a mutant analysis study has shown that in the absence of Pfs48/45, the Pfs230 protein is not retained on the surface of gametes indicating that tethering of Pfs230 is mediated by Pfs48/45[21]. Transmission reducing activity of Pfs230 is isotype dependent[40] and blocks gamete formation via complement-mediated lysis[41]. Radiolabelled antibodies against Pfs230 are able to bind to the surface of gametes and reduce the *P. falciparum* infectivity to mosquitoes[7]. Antibody responses against the Pfs230 were observed in naturally exposed individuals[42,43]. The strong correlation between transmission blocking activity and anti-Pfs230 antibody response was found in Gambian[30] and Papua New Guinean populations[44]. The antibody response in Cameroonian populations showed weak correlation[45] while no correlation was observed in Sri Lankan populations[32]. A study from Tanzania showed that the antibody level against *Pfs230* increased with age, recent exposure to infection and associated with the gametocytes carriage[33]. Pfs230 exhibits a reasonable degree of diversity[46]. These all characteristics of Pfs230 make this antigen an important promising candidate for TBVs.

Pfs47: The Pfs47 antigen is a contiguous paralog of Pfs48/45 located on 1.5Kb apart from Pfs48/45 and arranged tandemly on chromosome number 13[22]. Protein expression of Pfs47 is sex-specific and expressed on the surface of female gametocyte and gamete[23,24]. Study on Pfs47 demonstrates that it is not crucial for female fertility[47]. Parasite lacking Pfs47 through targeted gene disruption produced a normal number of oocysts and anti-Pfs47 monoclonal antibodies were unable to inhibit oocysts development[47]. These characteristics reduced the potential of Pfs47 as a good TBV target. Pfs47 inhibits the Jun-N-terminal kinase (JNK) mediated apoptosis process by inhibiting the activation of caspases; this inhibition leads to inadequate nitration reaction and thus parasite becomes invisible to complement-like system in the mosquito midgut[48]. Interruption of immunomodulatory action of Pfs47 gene inside the mosquito vector may turn out to be a convincing methodology to decrease malaria transmission[47]. A study carried out at National Institute for Research in Tribal Health (NIRTH), Jabalpur on serum samples of Indian patients showed that seroprevalence against Pfs47 antigen was highest among other TBV candidates (*i.e.* Pfs48/45 and Ps230). *Pfs47* also showed limited genetic polymorphism in the Indian field isolates (Chaturvedi *et al*, unpublished observations). These findings support the candidacy of *Pfs47* as a potential target of transmission blocking vaccine. However, more studies are needed to gain the knowledge of immunomodulatory activity of Pfs47 in other mosquito vectors.

Pfs25: Pfs25 is considered as one of the most important transmission blocking vaccine candidate antigens, expressed on the surface of zygote and ookinetes. Gene knockout experiments suggest that this protein is important for the parasite to survive inside the mosquito midgut[25]. Further, double-knockout study on *P. berghei* shows that the loss of P25 antigen reduces the ookinete invasion into the midgut epithelial cells[26,27]. The Pfs25 protein is expressed only in the mosquito host and antibody raised against the recombinant Pfs25 protein stops the parasite development within the mosquito vector[49]. The significance of post-fertilization antigen is long lasting immunogenicity and less antigenic variations[9,10,50]. An immunogenic form of Pfs25 expressed in yeast[51] shows effective transmission blocking activity in membrane feeding assays[52]. To enhance the efficacy of antibody response, the chimeric form of Pfs 25-28 was expressed in yeast and tested on mice. Pfs25-28 showed the more potent response to block the oocyst formation as compared to individual proteins *i.e.* either Pfs-25 or Pfs-28 alone[53]. Intramuscular administration of Pfs25 in mice elicited the potential transmission-blocking antibodies that resulted in more than 90 per cent reduction in oocyst numbers in the mosquito midgut[54]. Phase I clinical trial of modified forms of Pfs25 called TBV25H (consisting of extra his-tag at the C- terminus of protein) in human volunteers with aluminum hydroxide adjuvant showed 50 per cent reduction in *P. falciparum* infectivity[51]. The TBV25H constructs utilized as a part of the initial tests and human trials shows antigenicity nearly to the native molecules[51]. Recombinant Pfs25 expressed in yeast linked to the outer membrane protein complex (OMPC) of *Neisseria meningitidis* serogroup B with aluminium hydroxyphosphate formulations has shown stronger anti-Pfs25 antibody response than Pfs25 alone with montanide ISA 720 at the same measurements[55]. Recombinant Pfs25H in conjugation with exoprotein A (EPA) of *Pseudomonas aeruginosa* has been produced and used to make a cGMP pilot lot to use in Phase I human clinical trials in the United States[56]. The practical assessment of Pfs25 DNA vaccine done by *in vivo* electroporation in olive baboons showed potent immunogenicity against malaria[57]. Consequences of DNA-based immunization in non-human primates give the premise to further assessment in human volunteers. Endeavours are presently centered on the

clinical advancement of Pfs25-CP VLP, containing Pfs25 fused to the alfalfa mosaic virus coat protein (CP) expressed in *Nicotiana benthamiana* plants using a tobacco mosaic virus (TMV) based "launch vector" technology. Administration of one or two doses of Pfs25-CP VLPs with adjuvant Alhydrogel® in mice stimulates the antibodies that have shown absolute transmission blocking activity[58]. A clinical trial with this immunogenic preparation is ongoing in the United States (*https://clinicaltrials.gov/ct2/show/NCT02013687*). Trials with clinical grade formulations of Pfs25 molecules involves the preparation of different constructs in immunogenic forms and the testing of safe adjuvant[59].

Other important transmission-blocking vaccine candidate antigens: Intensive research on TBVs has discovered several other potential vaccine candidates that include other surface antigens (gamete, zygote, ookinete) and a few important mosquito proteins that are required by the parasite for its maturation in a vector. Pfs28 is expressed on the ookinete surface as an antigen distinct from Pfs25 and plays a role in ookinete protection from stomach enzymes[60,61]. Knockout phenotypes of *Pfs28* significantly reduced mosquito infectivity[26]. Potent *Pfs28* was expressed as a chimeric protein in combination with *Pfs25* to enhance the transmission blocking activity[53]. Vector molecules such as carboxypeptidase B (CPB) and aminopeptidase N (APN) play an important role in the development of *P. falciparum* inside the mosquito and, therefore, are considered as TBV candidates[62-65]. These candidates are conserved and elicit transmission reducing antibodies[66, 67]. However, efforts are underway that attempt to understand the mode of action and production of these antigenic targets in immunogenic forms. Other important transmission blocking vaccine candidate genes that are currently in the development stage[68,69] are discussed in the Table III.

Production of TBVs in plant based system

The requirement for a new approach for expression of large quantities of vaccine antigen with a good safety profile is important. Plant-based expression systems may be an appropriate choice for the cost-effective production of TBVs. The plant-based expression system provides all the advantages of a eukaryotic expression system with high purity and stability. Various TBV candidates have been successfully produced in plant system[70-72] with high recovery and desired immunogenicity (Table IV). Algae are also a promising system for the production of TBV that are orally delivered to avoid expensive purification and injectible delivery[73-75]. These results sustain the feasibility of expressing TBV antigens in the plant based system.

Effective adjuvants and vaccine delivery system

An efficient malaria vaccine against important developmental phases of the parasite life cycle, required to stimulate appropriate humoral and cellular immune responses. Transmission blocking immunity is governed by both cell-mediated and antibody-mediated effector mechanisms[76,77]. However, the humoral immune response seems to be a key player in transmission blocking immunity as compared to cell-mediated response[78]. Presently authorized human immunization adjuvants like alum, may enhance the antibody generation, but are poor stimulators of cellular effector systems, while strong cell stimulants, such as Freund's adjuvant are found reactogenic for human utilization. Several methods of antigen presentation *i.e.* liposomes mediated delivery, emulsification in Freund's adjuvant and addition of bacterial protein are known to be safe and efficient strategies of vaccine delivery system that targets the different antigenic determinants to the host's immune system. Various studies have been done in analyzing the different adjuvant combination and effective delivery system to enhance the immunogenicity of vaccine candidates. Maltose binding protein fused with Pfs48/45 induces high antibody titres in mice and elicits functional antibody in standard feeding assays (SFA)[15]. This combination was found stable over a nine months period[15]. The intramuscular delivery system using novel carrier gel core liposomes encapsulated with Pfs25 showed significant and durable immune response[79]. To overcome the poor immunogenicity, recombinant Pfs25H conjugated to *P. aeruginosa* exoprotein A (EPA) has been developed and used in human clinical trials (*https://clinicaltrials.gov/ct2/show/NCT01867463*). Alhydrogel® adsorbed Pfs25-EPA nanoparticle vaccine significantly improved the Pfs25 antibody responses in mice[56]. In another study, non-classical concepts for vaccine delivery were found more suitable, in which vaccine delivery was done not only through parenteral, oral or mucosal routes but specifically via cutaneous immunization. Single inoculation and controlled release of antigen in mice, through biodegradable nano-microparticle technologies, can elicit long-lasting protective antibody titres with >85 per cent efficacy which remains effective for at least two years[80].

Table III. Other important transmission blocking vaccine candidate antigens

Antigen	Gene ID	Stage	Location	Putative function	Strengths	Weakness	Current status of development	References
Pfs28	PF10_0302 New PF3D7_1030900	Z, O	S	Play a role in ookinetes entry into the mosquito midgut and protection of ookinetes from stomach enzymes	Knockout phenotype shows reduced infectivity and formation of ookinetes	Natural boosting not possible because it is not expressed in human host. Need to develop formulations that provide prolong immune response.	Research on the production of clinical grade material. Production of chimeric protein in combination with Pfs25 to enhance the transmission blocking (TB) activity.	53, 60, 61
PfCCp proteins PfCCp2 PfCCp3	PF14_0532 New PF3D7_1455800 PF14_0067 New PF14_0067	GC, G GC, G	PV, S PV, S	Adhesion proteins play a role in development of parasite in mosquito. Support a complement mediated decrease in gametocyte emergence.	Knockout phenotype shows blocked sporozoite formation or transition from oocysts to salivary glands.	Complete functional characterization has been not done yet.	A detailed characterization of all six PfCCp proteins is currently in processing. Research on the KO phenotype study to gain the knowledge about the exact function of the protein.	68
Aminopeptidase N (APN)	PF13_813945	MMV	S	Role in ookinete invasion of the midgut.	Antibody against APN significantly reduce no. of oocysts in both *Anopheles gambaie* and *A. stephensi.* Protective epitopes is highly conserved among divergent *Anopheles* vector species.	Improper folding of protein in addition to the presence of transmission-irrelevant epitopes dilute the production of functional antibodies.	Identification of potent epitopes . Modification of existing AnAPN antigen to enhance the immune response	64-67
PYCPW-WPC-1	PBANKA_ 135250	Z, O	S	May be involved in host parasite interaction during ookinete development	Highly conserved among *Plasmodium* species Less polymorphic than blood stage vaccine targets	Targeted disruption does not reveal any essential role in mosquito stage parasite development.	Research on the KO phenotype study to gain the knowledge about the exact function of the protein family	69

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Antigen	Gene ID	Stage	Location	Putative function	Strengths	Weakness	Current status of development	References
CPB (carboxypeptidase B1)	CPBAg1_46487999	MM	S	CPB are involved in *P. falciparum* development in its vector	Antibodies directed against CPBAg1 inhibit *P. falciparum* development in the *A. gambiae.* Induce high antibody titers and transmission-blocking activity	Selection of resistant mosquitoes due to decrease reproductive fitness of the vector	Production of the recombinant protein in immunogenic form. Research on the KO phenotype.	62, 63

PVM, parasitophorous vacuole membrane; KO, knockout; C, cytoplasmic; MMV, mosquito midgut microvilli; MM, mosquito midgut

Clinical trials

Presently, there are a small number of sexual stage vaccines under the preclinical advancement and Pfs25 is in Phase I clinical trial (*https://clinicaltrials.gov/ct2/show/NCT01867463*). Some clinical trials of Pfs25 became inactive due to systemic reactogenicity[81] and unresolved issues with FDA (*https://clinicaltrials.gov/ct2/show/NCT00977899*). As the TBVs are intended to block the parasite development within the mosquitoes the vaccine efficiency should be measured at a community level via random field trials. Due to the knowledge gap between the trial sites and epidemiological data of different endemic regions, conducting the cluster randomized trials becomes challenging. Standard membrane feeding assays (SMFA) allows investigators to assess the capability of serum to decrease the mosquito infection[14,66]. The data on the effect on transmission, immune responses in individuals and genetic diversity of sexual stage antigens are limited. Efforts are required to validate transmission inhibition and immune responses in humans living in endemic countries worldwide, as this knowledge will be important for vaccine development. Completed and ongoing clinical trials of sexual stage vaccines are discussed in Table V. Phase-I clinical trial of Pfs25 in a conjugate vaccine (Pfs25-EPA/Alhydrogel®) developed at National Institute of Allergy and Infectious Diseases (NIAID), USA was completed in healthy adults to assess the safety and immunogenicity with the collaboration of Programme for Appropriate Technology in Health-malaria Vaccine Initiative program PATH (MVI), NIAID and John Hopkins Bloomberg School of Public Health Centre for Immunization Research (CIR)[82] *(https://clinicaltrials.gov/ct2/show/NCT01434381*). This vaccine was found increasingly immunogenic with each dose and induced transmission reducing antibody responses. Some safety issues were also reported in this clinical trial such as local and systemic adverse events[82]. Phase- I clinical trial of Pfs25 using montanide ISA-51 was also stopped due to safety issues. Following each vaccination, volunteers showed adverse events. Local and systemic adverse symptoms included swelling, tenderness, erythema, fever, nausea, headache, myalgia and arthralgia[81]. Pfs25 antigen expressed in plant system known as Fraunhofer's plant-derived malaria transmission-blocking vaccine, is in Phase- I clinical trial[58] (*https://clinicaltrials.gov/ct2/show/NCT02013687*). Further human clinical trials are required to evaluate the vaccine candidate efficacy, health risk factors and cost-benefits assessments.

Table IV. Expression of transmission blocking vaccine (TBV) antigens in plant based system

Antigen	Plant based expression system	Adjuvant	Immunized animal model	Advantages	References
Pfs25MFIE (Non glycosylated mutant)	*Nicotiana benthamiana*	Alhydrogel	Mice and rabbits	Non-glycosylated form of antigen enhanced the antibody response and transmission blocking activity as compared to *Pichia* and *Saccharomyces*	70
Pfs48/45 (C-terminal antigenic region)	*Chlamydomonas reinhardtii* (chloroplast)	-	-	Chloroplast lack the machinery of N-linked glycosylation capable of folding complex protein having di-sulphide based conformation. Express antigen that has a conformation close to the native antigen and recognized by transmission blocking antibodies	73
Pfs25 fused to the subunit of cholera toxin (CtxB) Pfs25-CtxB	*Chlamydomonas reinhardtii*		Mice	CtxB domain acts as a mucosal adjuvant provides oral efficient delivery of vaccine that elicits secretory IgA antibody against pathogens that envade mucosal surfaces.	74
Pfs25 fused to modified lichenase Pfs25-Fh(MB) Plant virus based expression system	*Nicotiana benthamiana*	Lichenase (Lickm) carrier	Mice and rabbits	Induced long lasting antibody response in mice and rabbits that persisted for upto 6 months	71
Pfs25 and Co-domain of Pfs230 (Fo)	*Nicotiana benthamiana*	-	Mice	Heat treatment purification step effieciently yields recombinant protein with >90% purity and >70% recovery rate. Antibody against plant derived fo completely blocked the formation of oocysts in a malaria transmission blocking assay.	72
Pfs25 with four different human compatible adjuvants	*Chlamydomonas reinhardtii*	(Alum; TLR4 agonist (glucopyranosal lipid A, GLA) plus alum; squalene oil-in-water emulsion; GLA plus squalene oil-in-water emulsion)	-	Elicit higher antibody titre that reacts with the *P. falciparum* macrogametes and zygotes, and efficiently prevent development of parasite within a mosquito vector in a membrane feeding assays.	75

Challenges and opportunities

A new set of malaria vaccine candidates has entered into clinical trials and several new vaccine candidates are being developed. Various hurdles encountered in the development process of transmission blocking vaccine, includes *(i)* The problem of expressing TBV antigens in an appropriate conformation which is recognized by the antibody against the same epitopes; *(ii)* There are very limited known correlates of genetic diversity and immunity for transmission blocking antigens; *(iii)* Currently, only a few immune enhancing adjuvants are available, but these are very expensive; *(iv)* Analysis of

Table V. Clinical trials of transmission blocking malaria vaccine

Sexual stage antigen	Preclinical/ clinical development	Developer's institution	Partners	Countries involved in trial	Recombinant protein/ peptide used	Expression system	Adjuvant	Outcomes	Reference
Pfs48/45	Formation process development	Radboud University, Nijmegen, LSHTM, Genova, KCMC, Moshi	Radboud University (Nijmegen) and Statens Serum Institute (Denmark)	NL, Denmark, UK, India, Tanzania	R0-PF10C (Pfs48/45 C-terminus and fused to GLURP N-terminus)	*Escherichia coli*	Maltose bindig protein	It induces uniform and high antibody titres in mice and elicits functional TB antibodies in standard membrane feeding assays in 90% of the immunized mice	15
Pfs25-AlMV VLP	Preclinical immunogenicity studies	Fraunhofer CMB	LMIV and LMVR, NIAID, NIH	USA	Pfs25 genetically fused to alfalfa mosiac virus coat protein	*N.benthamiana*	Alhydrogel	This is first report demonstrating complete transmission blocking after a single dose lasting at least 6 months	58
Pfs25-EPA	Preclinical immunogenicity studies	LMIV	LMIV and LMVR, NIAID, NIH	USA	Pfs25 conjugated to *Pseudomonas* exotoxin A	*Pichia pastoris* (Pfs25); *E. coli* (Ec EPA)	Alhydrogel	Immunogenic and induces transmission blocking antibodies	56
Pfs25 EPA/ Alhydrogel	Clinical studies (Completed)	NIAID	JSHPH	Maryland	Pfs25 conjugated to Pseudomonas ExoProtein A	*Pichia pastoris* (Pfs25); *E. coli* (Ec EPA)	Alhydrogel	This study is completed. Vaccine found increasingly immunogenic with each dose and induce transmission blocking antibody. Local and systemic adverse events were also reported in this trial	82 (*Clinical Trials*.gov ID- NCT01434381)
Pfs25 VLP	Clinical studies (Active)	Fraunhofer, USA (FhCMB)	PATH-MVI (USA) Accelovance	USA	Pfs25 genetically fused to alfalfa mosiac virus coat protein	*N. benthamiana*	Alhydrogel	This study is ongoing (results awaited)	56 (*Clinical Trials*.gov ID- NCT02013687)
Pfs25	Clinical studies (Inactive)	MVDB (NIAID)	JHSPH-CIR (USA)	USA	Recombinant protein	*Pichia pastoris*	Montanide ISA-51	Inactive due to systemic reactogenecity	81

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Sexual stage antigen	Preclinical/ clinical development	Developer's institution	Partners	Countries involved in trial	Recombinant protein/ peptide used	Expression system	Adjuvant	Outcomes	Reference
Pfs25-Pfs25 conjugate vaccine	Clinical studies (Inactive)	NICHD	(NIH-CC) (USA)	USA	Conjugate vaccine	*P. pastoris*	Adsorption of the conjugates on to aluminum hydroxide	Inactive due to unresolved issues with the FDA	(*Clinical Trials*.gov ID- NCT00977899)
Pfs230 D1M-EPA and Pfs25M-EPA	Clinical studies	NIAID	NIH-CC	USA and MALI	Recombinant protein conjugate to *Pseudomonas* exoprotein A	Pfs230 in *N. benthamiana* and Pfs25 in *P. pastoris*	Alhydrogel	This study is ongoing currently recruiting participants	56, (*Clinical Trials*.gov ID- NCT02334462)

Laboratory of Malaria Immunology and Vaccinology (LMIV), Laboratory of Cellular Imaging and Macromolecular Biophysics (LMVR), PATH - Malaria Vaccine Initiative (MVI), USA, National Institute of Allergy and Infectious Diseases (NIAID (US, NIH)), Fraunhofer, Center for Molecular Biotechnology, USA (FhCMB), Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health Clinical Center (NIHCC), Johns Hopkins School of Public Health, Baltimore, MD (USA) - Center for Immunization Research (JHSPH-CIR)

transmission blocking activity needs more rigorously qualified assays and models to access the vaccine candidate efficacy; and *(v)* Funding constrant for TBV research and development.

The TBV development faces formidable challenges and collaborative approach is needed to solve scientific, economic and resource obstacles. Development of clinical trial sites in different regions with different epidemiological data should be done to analyze the safety and efficacy of vaccine candidates. A new advancement in this field can defeat these challenges. Advances in manufacturing industries can increase the large-scale production of vaccines, identification of potent adjuvants that can effectively boost the immune system, techniques like structural biology can predict the defensive epitopes, genomics and proteomic for novel antigen finding. Greater collaborations, new partnerships between research groups and policymaker's organization will be critical to developing a transmission blocking vaccine that could reduce the disease burden and transmission.

Concluding remarks

Malaria is a major global health problem and currently no vaccine is available to combat the disease. A promising transmission blocking vaccines is characterized and developed with clinical grade formulations during the last two decades. The completed clinical trials with Pfs25 formulations have been found immunogenic but shown some safety outcome issues like local and systemic adverse events[81,82]. Further clinical trials are required in malaria endemic regions to assess the risk factors as well as the vaccine efficacy. Prefertilization antigens Pfs48/45 and Pfs230 have shown a strong association between sexual stage-specific antibody response and functional transmission reducing activity. Data from several studies showed that antibody response against Pfs230 and Pfs48/45 antigens was associated with the exposure and duration of gametocytes carriage[33]. Another important prefertilization antigen Pfs47 allows the parasite to evade the mosquito immune system by inhibiting the activation of apoptosis in mosquito midgut cells[48]. Beside all these characteristics, sexual stage antigens have also shown limited genetic polymorphism and strong potential for boosting the immune response[42,46,49]. Further studies suggested that transmission blocking activity could likewise be obtained by targeting the mosquito components that are needed for the successful development of parasite inside the mosquito vector. These mosquito

specific candidates include aminopeptidase N and carboxypeptidase B1, which are also able to induce an antibody response that significantly inhibits parasite development[63,65]. However, further studies are needed to strengthen the candidacy of these antigens as a potent target of transmission blocking malaria vaccines.

Currently, many efforts for the development of transmission blocking malaria vaccine are focused on the production of TBV in plant-based expression system with a good safety profile, which are capable of inducing a strong immune response to reduce the malaria transmission[71,73,74]. Completed clinical trials with Pfs25 formulations have been found immunogenic but shown some safety outcome issues like local and systemic adverse events[81,82]. Human clinical trials are further required in malaria endemic regions to assess the risk factors and to evaluate the vaccine efficacy.

Future perspectives

Current worldwide approach for malaria control and elimination needs the vaccines that directly target the malaria transmission. For eradication, it is important that the vaccine provides potential contribution to reduce the infection rates, inhibits parasite development and thereby diminishing malaria mortality and morbidity by inhibiting the transmission. Malaria transmission blocking vaccines is a tool to reduce the mosquito infection and malarian transmission by inducing the immunity that breaks the cycle of malaria parasite between humans and mosquitoes.

Future efforts for the development of malaria transmission blocking vaccines for use as crucial components in malaria riddance must include *(i)* identification and functional characterization of those potent antigens and regulatory proteins which play crucial role in the development of the parasite in a mosquito vector; *(ii)* detection of molecular markers of sexual stage development; *(iii)* development of easily transferable *in vitro* culture system to better understand the dynamics between the multiplication of parasites, gametocyte biology and malaria transmission rates; *(iv)* improvement of an effective immunization method that would maintain the high antibody titre in the blood which will significantly affect parasite development in the mosquito; *(v)* identification of safe adjuvant combinations and dose optimization of vaccines; *(vi)* enhance the interest in the large scale production of malaria transmission blocking vaccines at the industrial level; *(vii)* clinical trials should be planned at the population level to check the safety and efficacy of the TBV; and *(viii)* development of multi-stage vaccine with the fusion of sexual stage antigen will also give future insight for malaria eradication.

Acknowledgment

The first author (NC) acknowledges the support of the Indian Council of Medical Research and Department of Biotechnology, Government of India for providing junior research fellowship. Authors appreciate the support and critical review given by Dr Altaf Lal, and Drs. Nirbhay Kumar and Aparup Das for their valuable comments and suggestion.

Conflicts of Interest: None.

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