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Identification of three ookinete-specific genes and evaluation of their transmission-blocking potentials in *Plasmodium berghei*

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Abstract

With a renewed hope for malaria elimination, interventions that prevent transmission of parasites from humans to mosquitoes have received elevated attention. Transmission-blocking vaccines (TBVs) targeting the sexual stages are well suited for this task. Here, through bioinformatic analysis, we selected two putative *Plasmodium berghei* ookinete-stage proteins (PBANKA_111920, and PBANKA_145770) and a previously characterized ookinete protein PBANKA_135340 (PSOP7) for evaluation of their transmission-blocking potentials. Fragments of these predicted proteins were expressed in bacteria and purified recombinant proteins were used to immunize mice. Antisera against these recombinant proteins recognized proteins of predicted sizes from ookinete lysates and localized their expression on the surface of ookinetes. Inclusion of these antisera in *in vitro* ookinete culture significantly inhibited ookinete formation. Mosquitoes fed on mice immunized with the recombinant proteins also showed significantly reduced oocyst densities (60.0 – 70.7%) and modest reductions of oocyst prevalence (10.7 – 37.4%). These data, together with the conservation of these genes in *Plasmodium*, suggest that these three ookinete proteins could be new promising targets for TBVs and are worth of future investigations in the human malaria parasites.

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Keywords

Malaria; sexual stage; transmission-blocking vaccine; *Plasmodium berghei*

1. Introduction

Malaria, a serious tropical disease caused by infections with *Plasmodium* parasites transmitted via *Anopheles* mosquitoes, continues to be an important public health problem worldwide. Though insecticide-treated bed nets, vector control, and effective chemotherapies have resulted in reduced morbidity and mortality in the past decade, an estimated 438,000 people still died of malaria in 2015 [1]. In areas of higher endemicity, the tools currently available to fight the disease are insufficient to interrupt transmission [2]. Moreover, the emergence and spread of insecticide resistance in mosquitoes and drug resistance in parasites bring further challenges to malaria control. The Malaria Eradication Research Agenda (malERA) has recognized that one key component needed for elimination of malaria is the development of novel tools capable of effective interruption of malaria transmission, among which are transmission blocking vaccines (TBVs) [3]. Unlike vaccines targeting asexual parasite stages that prevent infection and reduce disease severity, TBVs elicit specific antibodies that target the extracellular molecules of *Plasmodium* sexual stages to interrupt the disease transmission cycle [4].

The transmission of malaria parasites depends on the gametocytes that are formed in the human host. After being taken up by a mosquito during a blood meal, male and female gametes mate inside the midgut to form zygotes, which later transform into motile ookinets to invade the midgut and initiate sporogonic development. TBVs can target pre-fertilization antigens that are expressed on gametocytes and gametes as well as post-fertilization antigens that are expressed on zygotes and ookinets [5]. Immunization of the vertebrate hosts with these antigens will result in the production of antibodies targeting sexual stages, which arrest subsequent development of parasites in the mosquitoes. Transmission from humans to mosquitoes constitutes a population bottleneck in the malaria life cycle, which may be more vulnerable to control measures [6, 7]. Thus, TBVs are considered an important tool for accelerating malaria eradication [8].

Despite the potential significance of TBVs for integrated malaria control, a relatively small number of TBV candidates have been investigated to date, and only a handful of antigens including P25, P28, P48/45, P230 and HAP2 have shown clear transmission-blocking (TB) activities [9]. P25 and P28 are surface proteins found on zygotes and ookinets and are the lead TBV candidates [10]. Mouse antiserum against native [11, 12], or heterologously expressed P25 or P28 completely inhibits parasite development in mosquitoes [13-15]. To date, recombinant Pfs25 and Pvs25 have been evaluated in phase I clinical trials, which demonstrated that these protective antibodies can effectively inhibit transmission of the parasites [16, 17]. Pfs48/45 and Pfs230 belong to the six-cysteine motif gene family comprised of 10 members. These are major gametocyte and gamete surface antigens that naturally induce acquired immunity in malaria-exposed individuals [18, 19]. Thus, in view of the relatively short list of TBV candidates characterized so far, identification of additional

TBV antigens is highly desirable. The determination of proteomes of malaria parasite sexual stages provided unique opportunities for *in silico* identification of potential TBV antigens [20-23]. By datamining and reverse genetics, Ecker et al. characterized the functions of 20 *P. berghei* genes encoding putatively secreted proteins in ookinetes during parasite development in the mosquito, providing a list of ookinete genes worth of pursuing for TBV studies [24]. Recently, one of these genes, PSOP12, a member of the six-Cys family proteins, was found to be able to induce modest but significant TB activity *in vivo* by active immunization in mice [25].

Motivated by these recent TBV antigen discovery studies, we further mined the PlasmoDB database and selected two putative ookinete stage antigens and one known ookinete protein PSOP7 [24] for assessing their TBV potentials in the rodent parasite *Plasmodium berghei*. We confirmed their expression and localization on the ookinetes. Immunization of mice with the three recombinant proteins all elicited strong antibody responses, which showed substantial TB activity in *in vitro* ookinete conversion assay and *in vivo* direct feeding assay (DFA).

2. Materials and Methods

2.1. Mice, parasite, and mosquitoes

Six- to eight-week-old female BALB/c mice were used for all experiments. The *P. berghei* ANKA strain was maintained by serial passage and used for challenge infections as described previously [26]. Adult *Anopheles stephensi* mosquitoes were maintained in a 10% (w/v) glucose solution in water at 25°C and 50-80% humidity with a 12 h light-dark cycle in an insectary. Animal procedures were conducted according to the guidelines of The Animal Usage Committee of China Medical University.

2.2. Bioinformatics Analysis

We screened TBV candidate genes in the *Plasmodium* genomes (<http://www.plasmodb.org>) using a similar strategy as described in [24] with the following criteria: expression during sexual development, presence of transmembrane domain(s) and a signal peptide for potential secretion, and conservation among *Plasmodium* species. Protein domain architectures of selected genes were analyzed using SMART (<http://smart.embl-heidelberg.de/>) in order to identify target domains for the expression of recombinant proteins. B cell epitopes of selected proteins were predicted using the IEDB dataset and a previously described method [27] (<http://tools.iedb.org/bcell>). Candidate genes were used to BLAST search the GenBank to identify if homologous sequences are present in model organisms. Multiple sequences were aligned using ClustalW.

2.3. Expression and purification of recombinant proteins

Only partial domains of the selected genes (excluding the signal peptides, transmembrane domains, and low-complexity regions) were used for recombinant protein expression (Table 1). These fragments were amplified from genomic DNA using specific primers (Table S1) and cloned into the prokaryotic expression vector pET32a(+) (Novagen) at the *Bam*HI and *Not*I sites, which results in the expression of thioredoxin A fusion protein. The inserts were

verified by sequencing. Recombinant proteins were expressed in *Escherichia coli* Rosetta-gami B (DE3) cells after induction with 1 mM isopropyl β -D-1-thiogalactopyranoside (Sigma) and 1% anhydrous D-glucose at 20°C for 12 h. Bacterial cells were harvested and resuspended in a binding buffer containing 300 mM NaCl, 50 mM sodium phosphate buffer, and 10 mM imidazole, pH 8.0. After 15 cycles of sonication (20 s pulses with 30 s intervals between each cycle), the bacterial lysate was filtered through a 0.22 μ m filter and incubated with Ni-NTA His-Bind Superflow resin (Novagen) at 4°C for 2 h. The slurry was transferred into a column, washed with buffer containing 300 mM NaCl, 50 mM sodium phosphate buffer, and 20 mM imidazole (pH 8.0), and eluted in 300 mM NaCl, 50 mM sodium phosphate buffer, 250 mM imidazole (pH 8.0). Purified proteins were desalted by dialyzing extensively in 0.1 M phosphate buffered saline (PBS) at 4°C overnight. Recombinant proteins were analyzed on a 10% SDS-PAGE gel.

2.4. Immunization of mice

To obtain immune sera against recombinant *P. berghei* proteins, BALB/c mice (6 per antigen) were subcutaneously immunized with 50 μ g of recombinant proteins emulsified in complete Freund's adjuvant (Sigma). Subsequently, mice were given two booster injections of 25 μ g of proteins emulsified with incomplete Freund's adjuvant (Sigma) at 2-week intervals. Mice in the control group (n = 6) were immunized with the adjuvant formulations in PBS. Two weeks after the final immunization, blood was collected from the mice via cardiac puncture and allowed to clot at room temperature to obtain antisera. Pooled antisera were used in the subsequent trials.

Immune responses to specific recombinant proteins were followed by ELISA on day 14, 35 and 56 after the first immunization. ELISA plates were coated with purified recombinant proteins at 5 μ g/ml in 0.05 M sodium carbonate buffer (pH 9.6) at 4 °C overnight. The plates were washed twice with 300 μ l wash solution (0.1 M PBS, pH 7.4 with 0.02% Tween 20). The wells were blocked with 200 μ l PBS containing 1% bovine serum albumin (BSA, Sigma) for 1 h at 37°C. After three washes, 100 μ l mouse antisera pooled by the immunization groups were diluted 1:200 in PBS containing 1% BSA and 0.05% Tween 20 and added to appropriate wells and incubated for 2 h. The wells were washed five times and HRP-conjugated goat anti-mouse IgG antibodies (Invitrogen, 1:5000 dilution) were added and incubated for 2 h. After five washes, the plate was incubated with 100 μ l of tetramethyl benzidine (Amresco, USA). The reaction was stopped by the addition of 100 μ l of 2 N H₂SO₄ and absorbance at 490 nm was measured. For normalization, optical density (OD) value from negative control sera of non-immunized mice was subtracted from the OD values from the immunized groups.

2.5. Purification of schizonts, gametocytes and ookinetes

Purification of schizonts was achieved using a previously published protocol [28] with minor modifications. Blood-stage parasites taken from *P. berghei* infected mice on day 4 post-infection (p.i.) were placed in a blood-stage culture medium [RPMI 1640, 20% (v/v) fetal bovine serum (FBS, Thermo), 50 mg/L penicillin and streptomycin; 50 mL/0.5 mL blood] and cultured at 37 °C for 16 h with rotation at 100 rpm. Cells were harvested from each sample and a thin blood smear was used to determine the abundance of schizonts. If 70-80%

of the parasites were mature schizonts, the culture was gently loaded on top of a 55% (v/v) Nycodenz/RPMI culture medium cushion and centrifuged at $1300 \times g$ for 30 min at room temperature without braking. Schizonts concentrated at the gray layer of the interface was collected and washed with PBS.

Gametocytes were purified according to a previous study [29]. Briefly, phenylhydrazine-treated mice were infected with *P. berghei*, and beginning on day 4 p.i. mice were treated with sulfadiazine (Sigma, 20 mg/L in drinking water) for two days to eliminate asexual blood stage parasites. Blood was collected from the mice and immediately transferred to pre-warmed blood-stage culture medium at 37°C. The suspension was loaded on top of a 48% (v/v) Nycodenz/blood-stage culture medium cushion and centrifuged as described above. Cells at the interface were collected and washed twice in RPMI 1640 prior to initiation of gamete formation.

Ookinete culture and enrichment were performed according to a modified protocol [30]. Briefly, mice were pre-treated with phenylhydrazine and infected with 5×10^6 *P. berghei* parasites. Parasitemia was allowed to reach 1-3% on day 3 p.i., and blood was collected from the mice via cardiac puncture. The blood was passed through a cellulose powder CF11 column (Whatman) to deplete white blood cells and was then diluted 1:10 with complete ookinete medium (RPMI 1640, 50 mg/L penicillin, 50 mg/L streptomycin, 100 mg/L neomycin, 20% (v/v) FBS, and 1 mg/L heparin, pH 8.3) in a wide flask and maintained at 19 °C for 24 h. The ookinete cultures were spun at $500 \times g$ for 10 min at 4 °C, after which the pellet was collected and diluted in 45 ml of 0.17 M NH_4Cl for 10 min on ice to lyse erythrocytes. The resulting cell suspension was loaded on top of a 62% (v/v) Nycodenz/ookinete culture medium cushion and centrifuged as described above. Cells at the interface was removed and washed twice in PBS.

2.6. Western blot

Purified parasites were treated with 0.15% saponin (Sigma) in PBS for 10 min on ice, and parasites were collected by centrifugation and washed once with PBS. Protein lysates were prepared in a buffer (1% Triton X-100, 2% SDS in PBS) containing protease inhibitors (Roche) for 30 min at room temperature. Protein lysates corresponding to 5×10^7 parasites per lane were separated on a 10% SDS-PAGE gel under reducing conditions and then transferred to a 0.22 μm PVDF membrane (Bio-Rad). Purified recombinant proteins at 0.5 μg per lane were included as positive controls. Membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 2 h, and then probed with pooled antisera (1:500) or Pbs21 mAb clone 13.1 [31] for 2 h. After washing three times with TBST, the membrane was incubated with HRP-conjugated goat-anti-mouse antibodies (Invitrogen) at 1:10,000 in TBST. After washing the membrane three times, proteins were visualized using the Pierce ECL Western Blotting Kit (Thermo).

2.7. Indirect immunofluorescence assay (IFA)

Purified schizonts, gametocytes, and ookinetes were washed once in PBS and fixed with 4% paraformaldehyde (Sigma) and 0.0075% glutaraldehyde (Sigma) in PBS for 20 min at room temperature. After washing in PBS, the fixed cells were either proceeded directly for

antibody binding or permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min. The cells were then washed again in PBS and treated with 0.1 mg/ml of sodium borohydride (NaBH_4) in PBS for 10 min to reduce free aldehyde groups. Following another PBS wash, cells were blocked with 5% skim milk (Sigma) for 1 h at 37°C and subsequently washed three times in PBS. Slides were incubated first with pooled mouse antisera (1:500) or Pbs21 mAb clone 13.1 (positive control, 1:500) in 5% skim milk at 37 °C for 1 h and then with FITC-conjugated goat anti-mouse antibody (Invitrogen) at a 1:500 dilution for 1 h. Parasite nuclei were counter-stained with 1 µg/ml 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen). Stained parasites were mounted with ProLong® Gold Antifade Reagent (Invitrogen) and images were captured on Olympus BX53 and processed using Adobe Photoshop (Adobe Systems Inc., USA).

2.8. Ookinete formation inhibition assay

For the *in vitro* assay, phenylhydrazine-treated mice were injected i.p. with 5×10^6 parasites. On day 3 p.i., parasitemia was evaluated via a Giemsa-stained tail blood smear, and exflagellation of male gametocytes was assessed. Hosts were exsanguinated by cardiac puncture, and 10 µl of blood was added to 90 µl of ookinete medium containing mouse sera at a final dilution of 1:10 and incubated for 24 h at 19 °C. Cultures were harvested and mixed after 24 h, then fixed and labeled with Pbs21 mAb clone 13.1 (1:500 dilution) and FITC-conjugated goat anti-mouse antibodies (1:500 dilution). Parasite nuclei were stained with DAPI. The macrogametes/zygotes and ookinetes were counted under a fluorescence microscope at 100× magnification in 20 fields. The experiment was performed in triplicate and ookinete conversion rates were calculated as the number of macrogametes, zygotes, retorts or ookinetes)/(the total number of macrogametes, zygotes, retorts and ookinetes) × 100% as described previously [32].

To further evaluate the effect of the immune sera on ookinete conversion *in vivo*, gametocytemic mice were injected intravenously with 150 µl/mouse of pooled control or immune sera against the three recombinant proteins. One hour later, 15 female *A. stephensi* were allowed to feed on each mouse for 30 min. Ten fed mosquitoes from each group were dissected 10-12 h later, and the blood contents were used to make blood smears, fixed and probed with Pbs21 mAb as described above. Parasites at different developmental stages were enumerated under an epifluorescence microscope.

2.9. DFA

Six mice were immunized with the recombinant proteins as described above. Six mice in the control group were immunized with the adjuvant formulation in PBS. Ten days after the final immunization, all mice were treated with phenylhydrazine, and three days later injected with 5×10^6 *P. berghei* ANKA-infected red blood cells (iRBC). Three days p.i., 4-day-old female *A. stephensi* mosquitoes were starved for 12 h and then allowed to feed on immunized mice for 30 min. For each group, approximately 50 mosquitoes were used. Unfed mosquitoes were removed, and fed mosquitoes were maintained at 19–22°C and 50–80% humidity. At least 26 mosquitoes were dissected ten days later, and the midguts were stained with 0.5% mercurochrome (Sigma) to determine the prevalence (proportion of infected mosquitoes) and intensity (number of oocysts per midgut) of infection [33].

2.10. Statistical analysis

Statistical analysis was performed with the GraphPad Prism software. Ookinete conversion rates were analyzed using Student's t-tests. The intensity of infection (oocysts/midgut) was analyzed by the Mann-Whitney U test [34], and the prevalence of infection was analyzed by Fisher's exact test. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Identification of three highly conserved *Plasmodium* genes expressed in sexual stages

Using a similar bioinformatic screening strategy as in [24], we mined the PlasmoDB database for proteins potentially expressed in sexual stages with TBV potential. By including genes that 1) are only transcribed in sexual stages, 2) are conserved in *Plasmodium*, and 3) contain a putative signal peptide and transmembrane domain(s), we obtained a list of 52 genes that are conserved across different *Plasmodium* species. Most of these genes were annotated as conserved hypothetical proteins. From this short list of candidate genes, we selected three genes for further characterization (Table 1). We first selected PBANKA_135340 since it was previously characterized and annotated as a putative secreted ookinete protein PSOP7 [24]. C-myc tagging of PSOP7 localized this fusion protein in the micronemes of ookinetes and disruption of this gene led to a severe defect in midgut invasion with 100% reduction of midgut infection prevalence [24]. We then selected two most highly expressed genes in ookinete among the list (PBANKA_111920 and PBANKA_145770); the transcripts of both genes ranked in the 99th percentile of the ookinete transcriptome [35]. PSOP7 contains in the C-terminus a GBM_5_12 domain, a carbohydrate-binding domain found in different glycosyl hydrolase enzymes [36]. PBANKA_111920 also contains a predicted transmembrane region in the C-terminus (Fig. 1), while a predicted transmembrane region (5–23 amino acids) in PBANKA_145770 is located in the N-terminus and overlaps with the signal peptide. Proteomic analysis detected expression of PBANKA_111920, PBANKA_145770, and PSOP7 in *P. berghei* ookinetes [20] (Table 1). Recently, these genes have also been identified to be the targets of the ookinete stage master regulator AP2-O, an AP2 family transcription factor [37]. A BLAST search of the GenBank with candidate genes and multiple sequence alignments using ClustalW further confirmed that these selected genes are highly conserved in *Plasmodium* (Fig. S1). Following the earlier nomenclature of putative secreted ookinete proteins [24], we named the protein products of PBANKA_111920 and PBANKA_145770 as PSOP25 and PSOP26, respectively.

3.2. Expression of recombinant proteins in *E. coli*

A fragment of each of the three selected proteins corresponding to amino acids 45–245, 315–446, and 50–254 of the predicted protein sequences of PSOP25, PSOP7, and PSOP26, respectively, were selected for expression of recombinant proteins in *E. coli* Rosetta-gami B (DE3) cells. These subdomains were selected to include at least four predicted antibody epitopes (Table S2) but to exclude the signal peptide, potential transmembrane domains and low-complexity regions. Protein induction was done at 20°C for 12 h to enhance solubility of the recombinant proteins. All His-tagged recombinant proteins were found soluble and

purified under native conditions using a Ni-NTA column. SDS-PAGE analysis showed that the purified recombinant proteins were relatively pure and their molecular weights were approximately 42, 35, and 43 kDa, consistent with those predicted for the respective proteins (Fig. 2A). All recombinant proteins were highly expressed with a yield of ~500 µg of purified proteins per 250 mL of culture.

3.3. Detection of protein expression in *P. berghei*

Purified recombinant proteins were used to immunize BALB/c mice to produce polyclonal antisera. ELISA with plates coated with respective recombinant proteins showed all recombinant proteins elicited strong antibody responses with antibody titers increased throughout the whole immunization period (Fig. 2B), and the antisera collected at two weeks after the final boost reached a titer greater than 1,024,000 (data not shown). The reactivity and specificity of the pooled antisera were determined by Western blot against denatured parasite lysates from three representative stages (schizonts, gametocytes, and ookinetes) (Fig. S1). As expected from their expression profiles, antisera against these recombinant proteins only recognized proteins bands in ookinetes, but not in gametocytes or schizonts (Fig. 3). The proteins bands identified with antibodies against recombinant PSOP25, PSOP7 and PSOP26 were approximately 40, 94, and 91 kDa, respectively, which were consistent with the predicted molecular weights of these proteins (40.0, 93.5, and 90.8 kDa, respectively) (Fig. 3). This result indicated that the antisera raised against the recombinant proteins recognized their corresponding proteins in ookinetes.

3.4. Localization of the candidate proteins

Having confirmed the expression of the three selected antigens in ookinetes, we next wanted to determine their localizations by IFA. IFA with immune sera for PSOP25, PSOP7, and PSOP26 revealed strong fluorescence on ookinetes, which was reminiscent of surface staining by monoclonal antibody against the ookinete surface antigen Pbs21 (Fig. 4). Moreover, IFA with and without membrane permeabilization showed essentially the same fluorescence patterns, indicating that these proteins are localized on the outer surfaces of the ookinetes.

3.5. Transmission-blocking activities

TB activities of the antisera were evaluated using both *in vitro* ookinete formation inhibition assay and DFA. For the *in vitro* assay, the ookinete conversion rate was determined by culturing parasites for 24 h in the ookinete culture medium containing the respective antisera at 1:10 dilution. In cultures supplemented with pooled immune sera against the recombinant PSOP25, PSOP7, and PSOP26, ookinete conversion rates were reduced by 53.0, 53.1, and 56.4% ($P < 0.01$, Fig. 5A). For all three cultures with the immune sera, significant proportions of the parasites (55.3, 55.3 and 35.4% for PSOP25, PSOP7 and PSOP26, respectively) were at the retort stage as compared to 13.6% with the control sera (Fig. 5A, Table S3). In addition, inhibition of ookinete conversion appeared to occur much earlier with the PSOP26 antisera, with 38.8% of the parasites found in earlier stages. To corroborate this finding from the *in vitro* ookinete culture assay, we also estimated ookinete conversion *in vivo* in mosquitoes fed on mice that received passive transfer of control or immune sera against the three recombinant proteins. At 10-12 h after feeding, significant inhibition of

ookinete conversion was also observed in mosquitoes fed on the mice receiving the immune sera (Fig. S3, Table S3).

To further evaluate TB activity of the antibodies against the three recombinant proteins *in vivo*, mice immunized with the respective recombinant antigens were infected with *P. berghei* and used for direct mosquito feeding three days p.i. On day 10 post-feeding, 26-30 mosquitoes were dissected in each feeding group, and the prevalence of infection and midgut oocyst density were determined. Immunization of mice with recombinant PSOP25 and PSOP26 resulted in significant reduction in both the prevalence of infection and oocyst density. Specifically, compared to the control group, the prevalence of infected mosquitoes fed on these two groups of mice was reduced by 25.0 and 33.7% respectively, while the mean number of oocysts per midgut was reduced by 64.3 and 65.4%, respectively (Fig. 5B, Table 2 and S3). Although mosquitoes fed on mice immunized with recombinant PSOP7 had only 14.3% reduction in the prevalence of infection, oocyst density was reduced by 63.1% (Fig. 5B, Table 2).

4. Discussion

TBVs are considered a high-priority research area, given its potential as a very important tool during the malaria elimination phase. The limited number of TBV candidates investigated to date demands increasing efforts in TBV antigen discovery. This study aimed to identify and characterize novel TBV candidates using the rodent parasite model. Through bioinformatic analysis we identified two putative ookinete antigens in *P. berghei* and compared their TB potentials with a known ookinete antigen PSOP7 [24]. Consistent with previous expression analysis [20, 24], western blots detected predominant expression of all three selected antigens in ookinetes, and the protein bands were consistent with the predicted molecular sizes of the respective proteins. Like the lead TBV candidates P25 and P28 [38, 39], IFA confirmed the expression of the proteins and their localizations on the surface of ookinetes, a key parameter for TBV candidate selection. Immunization of mice against these three recombinant proteins all demonstrated considerable TB activities in both *in vitro* ookinete conversion and mosquito feeding assays.

TBVs target the parasite stages inside mosquito midguts, which have a relatively long vulnerable extracellular time of ~24 h, when parasites are exposed to immune factors of the vertebrate host [40]. Gamete formation and fusion occur rapidly. After 5 to 6 h, the parasite develops into the retort stage, and the formation of a motile, invasive ookinete requires 18 to 20 h to complete in *P. berghei* [41]. Though host antibodies can persist, at potentially lethal titers, in the blood meal for at least 24 h [42], it is preferable that the antibodies target antigens that are expressed during early development and throughout the entire parasite development inside the midgut. The lead antigen P25 is expressed in multiple stages (macrogamete, zygote and ookinete), whereas P230 and P48/45 are expressed in gametocytes and are present on the surface of gametes [38, 43]. A recent comparison of four leading *P. falciparum* TBV candidates showed that anti-Pfs25 and anti-Pfs230-C provided complete blockade of mosquito infection [44]. Since the mosquito midgut is a harsh, highly proteolytic environment, antibodies against late stage ookinetes in the blood meal would have to be of relatively high titer and high avidity in order to survive for an extended period

of time to influence the final steps of midgut invasion. It is therefore important that this caveat be factored in when testing TBVs targeting late ookinete antigens.

Except for PSOP7, PSOP25 and PSOP26 have not been characterized before and the predicted proteins do not contain recognizable domains of known functions. All three genes have been shown to be under the control of the ookinete stage master transcription activator AP2-O [37, 45]. PSOP7 has previously been identified in a reverse genetic screen in *P. berghei* [24]. Knockout of *psop7* resulted in no detectable phenotype in blood stages and ookinete conversion, but completely blocked mosquito infectivity, indicating that PSOP7 is required for ookinete invasion of the mosquito midgut [24]. Using a c-myc-tagging strategy, PSOP7 was originally found to be concentrated in the tip of ookinetes suggestive of a microneme localization [24]. In comparison, our antisera raised against a recombinant fragment of PSOP7 detected PSOP7 localization that is highly similar to the ookinete surface antigen Pbs21. Whereas this discrepancy could be due to that the anti-PSOP7 antisera used here might have reacted with different surface proteins under native conditions in IFA, it could also be resulted from potential mis-tagging of the PSOP7 gene or targeting of the tagged PSOP7 to an incorrect cellular compartment, since myc tagging of PSOP7 detected a ~38 kDa protein in ookinete lysate, much smaller than the predicted molecular weight of PSOP7 (93.5 kDa) [24]. Nevertheless, PSOP7 proved to have excellent TB potentials since immunization against recombinant PSOP7 produced a strong antibody response in mice, resulting in 63.1% reduction in oocyst density and 14.3% reduction in infection prevalence in DFA. The TB activity was comparable to those of a recently studied PSOP12 (53.1% reduction in oocyst density, 10.9% reduction in prevalence) [25]. Using PSOP7 as a comparator, immunization against recombinant PSOP25 and PSOP26 produced similar levels of reduction in oocyst density, but better levels of reduction in infection prevalence (25.0 and 33.7%). These results established a solid ground for more detailed characterization of these selected antigens.

All three recombinant PSOPs generated modest levels of TB activity, leaving room for improvement. It is noteworthy that the TB activities observed in this study were the results of immunization of mice with only a small fragment of each predicted protein, which highlights the potential of these TBV candidates for inclusion in vaccine cocktails targeting multiple stages. Meanwhile, the modest TB activities could be due to the small fragments of the antigens used for recombinant proteins, where the targeting epitopes may not be ideal or sufficient. Future studies using larger protein domains may provide better TB activity. For the three proteins tested, only PSOP7 possesses a C-terminal putative carbohydrate-binding domain (GBM_5_12). Since specific carbohydrate groups have been implicated as mosquito ligands important for the recognition and binding of ookinetes to the surface of the midgut epithelium and lectins have been found to interfere with ookinete attachment to the midgut [46], it is possible that PSOP7 might act as a receptor on the ookinete. The fact that the recombinant PSOP7 tested in the present work does not include the predicted lectin domain implies that the antibodies against PSOP7 might interfere with this ligand-receptor interaction through spatial hindrance. Since the interaction of PSOP7 with midgut is completely speculative, future functional mapping and testing of PSOP7 is warranted. Furthermore, this study used a strong adjuvant (Freund's) to induce antibody responses against the recombinant proteins, which is unsuitable for clinical testing. In addition, the

intrinsic immunogenicity of the proteins is known. For subsequent studies, especially for the evaluation of functional antibody activities of the *P. falciparum* and *P. vivax* homologs, an adjuvant more suitable for clinical development (e.g., Alhydrogel or Montanide ISA-51) needs to be considered.

The AT-rich *Plasmodium* genomes often hinder abundant expression of recombinant proteins in prokaryotic expression systems [47, 48]. In addition, the presence of multiple disulfide bridges in some TBV candidates such as P25, P28, P48/45 and Pfs230 also makes prokaryotic expression difficult due to problems in achieving proper folding of the proteins [16, 49, 50]. Many strategies have been developed to overcome these difficulties [51], and *E. coli* remains a preferred host for heterologous protein expression, including the functional expression of soluble *Plasmodium* proteins [52]. In recent years, various *E. coli* strains have been engineered specifically for the cytoplasmic expression of heterologous proteins by disrupting the thioredoxin reductase (encoded by the *trxB* gene) and/or the glutathione reductase (encoded by the *gor* gene) pathways present in the bacterial cytoplasm [53, 54]. Such strains have already been successfully used for soluble expression of several heterologous proteins [54]. Moreover, expression at decreased temperatures is a common strategy to improve the solubility and stability of target proteins in *E. coli* [55]. In this study, induction at a lower temperature (20 °C) in the *trxB gor* double mutant *E. coli* strain Rosetta-gami B (DE3) was used to express the candidate *Plasmodium* proteins, resulting in the production of large amounts of soluble recombinant proteins. Further, the recombinant proteins could elicit strong antibody responses in immunized mice, and the antibodies correctly recognized the native proteins and possessed excellent TB activities. Regardless of these positive results, we do not know whether the recombinant proteins used were correctly folded even though the proteins are soluble. The small domains used also precluded understanding of potential protein folding, further highlighting the need for expressing larger domains in the future. In addition, exploration of other protein expression system may offer additional chances for optimizing expression of these antigens.

In conclusion, we identified, expressed, and evaluated two novel ookinete-specific genes as potential candidates for TBV development using the *P. berghei* model and compared with a known ookinete protein PSOP7. All proteins showed surface expression on ookinetes and recombinant proteins expressed in bacteria could induce strong TB antibodies in mice. Further, the selected candidate proteins are highly conserved in human *Plasmodium* parasites, and further evaluations of TB activities of the orthologs in *P. falciparum* and *P. vivax* are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. WHO. World Malaria Report 2015. 2015
2. Griffin JT, Ferguson NM, Ghani AC. Estimates of the changing age-burden of *Plasmodium falciparum* malaria disease in sub-Saharan Africa. *Nature Commun.* 2014; 5:3136. [PubMed: 24518518]
3. Alonso PL, Brown G, Arevalo-Herrera M, Binka F, Chitnis C, Collins F, et al. A research agenda to underpin malaria eradication. *PLoS Med.* 2011; 8:e1000406. [PubMed: 21311579]
4. Carter R. Transmission blocking malaria vaccines. *Vaccine.* 2001; 19:2309–14. [PubMed: 11257353]
5. Tsuboi T, Tachibana M, Kaneko O, Torii M. Transmission-blocking vaccine of vivax malaria. *Parasitol Int.* 2003; 52:1–11. [PubMed: 12543142]
6. Rosenberg R. Malaria: some considerations regarding parasite productivity. *Trends Parasitol.* 2008; 24:487–91. [PubMed: 18805735]
7. Sinden RE. A biologist's perspective on malaria vaccine development. *Hum Vaccin.* 2010; 6:3–11. [PubMed: 19946205]
8. Langhorne J, Ndungu FM, Sponaas AM, Marsh K. Immunity to malaria: more questions than answers. *Nature Immunol.* 2008; 9:725–32. [PubMed: 18563083]
9. Wu Y, Sinden RE, Churcher TS, Tsuboi T, Yusibov V. Development of malaria transmission-blocking vaccines: from concept to product. *Adv Parasitol.* 2015; 89:109–52. [PubMed: 26003037]
10. Tomas AM, Margos G, Dimopoulos G, van Lin LH, de Koning-Ward TF, Sinha R, et al. P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. *EMBO J.* 2001; 20:3975–83. [PubMed: 11483501]
11. Tirawanchai N, Sinden RE. Three non-repeated transmission blocking epitopes recognized in the 21 kD surface antigen of zygotes-ookinetes of *Plasmodium berghei*. *Parasite Immunol.* 1990; 12:435–46. [PubMed: 1698275]
12. Tirawanchai N, Winger LA, Nicholas J, Sinden RE. Analysis of immunity induced by the affinity-purified 21-kilodalton zygote-ookinete surface antigen of *Plasmodium berghei*. *Infect Immun.* 1991; 59:36–44. [PubMed: 1987051]
13. Kaslow DC, Bathurst IC, Lensen T, Ponnudurai T, Barr PJ, Keister DB. *Saccharomyces cerevisiae* recombinant Pfs25 adsorbed to alum elicits antibodies that block transmission of *Plasmodium falciparum*. *Infect Immun.* 1994; 62:5576–80. [PubMed: 7960139]
14. Hisaeda H, Stowers AW, Tsuboi T, Collins WE, Sattabongkot JS, Suwanabun N, et al. Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of *Plasmodium vivax* to infect mosquitoes. *Infect Immun.* 2000; 68:6618–23. [PubMed: 11083773]
15. Barr PJ, Green KM, Gibson HL, Bathurst IC, Quakyi IA, Kaslow DC. Recombinant Pfs25 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in experimental animals. *J Exp Med.* 1991; 174:1203–8. [PubMed: 1940798]
16. Malkin EM, Durbin AP, Diemert DJ, Sattabongkot J, Wu Y, Miura K, et al. Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for *Plasmodium vivax* malaria. *Vaccine.* 2005; 23:3131–8. [PubMed: 15837212]
17. Wu Y, Ellis RD, Shaffer D, Fontes E, Malkin EM, Mahanty S, et al. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. *PLoS One.* 2008; 3:e2636. [PubMed: 18612426]
18. Ouedraogo AL, Roeffen W, Luty AJ, de Vlas SJ, Nebie I, Ilboudo-Sanogo E, et al. Naturally acquired immune responses to *Plasmodium falciparum* sexual stage antigens Pfs48/45 and Pfs230 in an area of seasonal transmission. *Infect Immun.* 2011; 79:4957–64. [PubMed: 21969000]
19. Bousema T, Roeffen W, Meijerink H, Mwerinde H, Mwakalinga S, van Gemert GJ, et al. The dynamics of naturally acquired immune responses to *Plasmodium falciparum* sexual stage antigens Pfs230 & Pfs48/45 in a low endemic area in Tanzania. *PLoS One.* 2010; 5:e14114. [PubMed: 21124765]
20. Hall N, Karras M, Raine JD, Carlton JM, Kooij TW, Berriman M, et al. A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. *Science.* 2005; 307:82–6. [PubMed: 15637271]

21. Khan SM, Franke-Fayard B, Mair GR, Lasonder E, Janse CJ, Mann M, et al. Proteome analysis of separated male and female gametocytes reveals novel sex-specific *Plasmodium* biology. *Cell*. 2005; 121:675–87. [PubMed: 15935755]
22. Silvestrini F, Lasonder E, Olivieri A, Camarda G, van Schaijk B, Sanchez M, et al. Protein export marks the early phase of gametocytogenesis of the human malaria parasite *Plasmodium falciparum*. *Mol Cell Proteomics*. 2010; 9:1437–48. [PubMed: 20332084]
23. Tao D, Ubaida-Mohien C, Mathias DK, King JG, Pastrana-Mena R, Tripathi A, et al. Sex-partitioning of the *Plasmodium falciparum* Stage V Gametocyte Proteome Provides Insight into *falciparum*-specific Cell Biology. *Mol Cell Proteomics*. 2014; 13:2705–24. [PubMed: 25056935]
24. Ecker A, Bushell ES, Tewari R, Sinden RE. Reverse genetics screen identifies six proteins important for malaria development in the mosquito. *Mol Microbiol*. 2008; 70:209–20. [PubMed: 18761621]
25. Sala KA, Nishiura H, Upton LM, Zakutansky SE, Delves MJ, Iyori M, et al. The *Plasmodium berghei* sexual stage antigen PSOP12 induces anti-malarial transmission blocking immunity both in vivo and in vitro. *Vaccine*. 2015; 33:437–45. [PubMed: 25454088]
26. Blagborough AM, Sinden RE. *Plasmodium berghei* HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. *Vaccine*. 2009; 27:5187–94. [PubMed: 19596419]
27. Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett*. 1990; 276:172–4. [PubMed: 1702393]
28. Janse CJ, Ramesar J, Waters AP. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat Protoc*. 2006; 1:346–56. [PubMed: 17406255]
29. Beetsma AL, van de Wiel TJ, Sauerwein RW, Eling WM. *Plasmodium berghei* ANKA: purification of large numbers of infectious gametocytes. *Exp Parasitol*. 1998; 88:69–72. [PubMed: 9501851]
30. Sinden RE, Hartley RH, Winger L. The development of *Plasmodium* ookinetes in vitro: an ultrastructural study including a description of meiotic division. *Parasitology*. 1985; 91:227–44. [PubMed: 3906519]
31. Yoshida S, Matsuoka H, Luo E, Iwai K, Arai M, Sinden RE, et al. A single-chain antibody fragment specific for the *Plasmodium berghei* ookinete protein Pbs21 confers transmission blockade in the mosquito midgut. *Molecular and biochemical parasitology*. 1999; 104:195–204. [PubMed: 10593175]
32. Reininger L, Billker O, Tewari R, Mukhopadhyay A, Fennell C, Dorin-Semblat D, et al. A NIMA-related protein kinase is essential for completion of the sexual cycle of malaria parasites. *The Journal of biological chemistry*. 2005; 280:31957–64. [PubMed: 15970588]
33. Usui M, Fukumoto S, Inoue N, Kawazu S. Improvement of the observational method for *Plasmodium berghei* oocysts in the midgut of mosquitoes. *Parasit Vectors*. 2011; 4:118. [PubMed: 21707972]
34. Blagborough AM, Yoshida S, Sattabongkot J, Tsuboi T, Sinden RE. Intranasal and intramuscular immunization with Baculovirus Dual Expression System-based Pvs25 vaccine substantially blocks *Plasmodium vivax* transmission. *Vaccine*. 2010; 28:6014–20. [PubMed: 20637303]
35. Otto TD, Bohme U, Jackson AP, Hunt M, Franke-Fayard B, Hoeijmakers WA, et al. A comprehensive evaluation of rodent malaria parasite genomes and gene expression. *BMC Biol*. 2014; 12:86. [PubMed: 25359557]
36. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, et al. CDD: NCBI's conserved domain database. *Nucleic Acids Res*. 2015; 43:D222–6. [PubMed: 25414356]
37. Kaneko I, Iwanaga S, Kato T, Kobayashi I, Yuda M. Genome-wide identification of the target genes of AP2-O, a *Plasmodium* AP2-family transcription factor. *PLoS Pathog*. 2015; 11:e1004905. [PubMed: 26018192]
38. van Dijk MR, Janse CJ, Thompson J, Waters AP, Braks JA, Dodemont HJ, et al. A central role for P48/45 in malaria parasite male gamete fertility. *Cell*. 2001; 104:153–64. [PubMed: 11163248]
39. Kaslow DC, Quakyi IA, Syin C, Raum MG, Keister DB, Coligan JE, et al. A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. *Nature*. 1988; 333:74–6. [PubMed: 3283563]

40. Sinden RE, Butcher GA, Billker O, Fleck SL. Regulation of infectivity of *Plasmodium* to the mosquito vector. *Adv Parasitol.* 1996; 38:53–117. [PubMed: 8701799]
41. Janse CJ, Mons B, Rouwenhorst RJ, Van der Klooster PF, Overdulve JP, Van der Kaay HJ. In vitro formation of ookinetes and functional maturity of *Plasmodium berghei* gametocytes. *Parasitology.* 1985; 91:19–29. [PubMed: 2863802]
42. Sinden RE. The cell biology of malaria infection of mosquito: advances and opportunities. *Cell Microbiol.* 2015; 17:451–66. [PubMed: 25557077]
43. Eksi S, Czesny B, van Gemert GJ, Sauerwein RW, Eling W, Williamson KC. Malaria transmission-blocking antigen, Pfs230, mediates human red blood cell binding to exflagellating male parasites and oocyst production. *Mol Microbiol.* 2006; 61:991–8. [PubMed: 16879650]
44. Kapulu MC, Da DF, Miura K, Li Y, Blagborough AM, Churcher TS, et al. Comparative assessment of transmission-blocking vaccine candidates against *Plasmodium falciparum*. *Sci Rep.* 2015; 5:11193. [PubMed: 26063320]
45. Yuda M, Iwanaga S, Shigenobu S, Mair GR, Janse CJ, Waters AP, et al. Identification of a transcription factor in the mosquito-invasive stage of malaria parasites. *Mol Microbiol.* 2009; 71:1402–14. [PubMed: 19220746]
46. Zieler H, Nawrocki JP, Shahabuddin M. *Plasmodium gallinaceum* ookinetes adhere specifically to the midgut epithelium of *Aedes aegypti* by interaction with a carbohydrate ligand. *J Exp Biol.* 1999; 202:485–95. [PubMed: 9929452]
47. Chung DW, Ponts N, Cervantes S, Le Roch KG. Post-translational modifications in *Plasmodium*: more than you think! *Mol Biochem Parasitol.* 2009; 168:123–34. [PubMed: 19666057]
48. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, et al. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature.* 2008; 455:757–63. [PubMed: 18843361]
49. Williamson KC, Keister DB, Muratova O, Kaslow DC. Recombinant Pfs230, a *Plasmodium falciparum* gametocyte protein, induces antisera that reduce the infectivity of *Plasmodium falciparum* to mosquitoes. *Mol Biochem Parasitol.* 1995; 75:33–42. [PubMed: 8720173]
50. Milek RL, Roeffen WF, Kocken CH, Jansen J, Kaan AM, Eling WM, et al. Immunological properties of recombinant proteins of the transmission blocking vaccine candidate, Pfs48/45, of the human malaria parasite *Plasmodium falciparum* produced in *Escherichia coli*. *Parasite Immunol.* 1998; 20:377–85. [PubMed: 9767603]
51. Sahdev S, Khattar SK, Saini KS. Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Mol Cell Biochem.* 2008; 307:249–64. [PubMed: 17874175]
52. Birkholtz LM, Blatch G, Coetzer TL, Hoppe HC, Human E, Morris EJ, et al. Heterologous expression of plasmodial proteins for structural studies and functional annotation. *Malar J.* 2008; 7:197. [PubMed: 18828893]
53. Prinz WA, Aslund F, Holmgren A, Beckwith J. The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. *J Biol Chem.* 1997; 272:15661–7. [PubMed: 9188456]
54. de Marco A. Strategies for successful recombinant expression of disulfide bond-dependent proteins in *Escherichia coli*. *Microb Cell Fact.* 2009; 8:26. [PubMed: 19442264]
55. Sorensen HP, Mortensen KK. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microb Cell Fact.* 2005; 4:1. [PubMed: 15629064]

Highlights

We mined PlasmoDB and selected three ookinete-specific genes in *Plasmodium*.

We confirmed their expression and localization on *P. berghei* ookinetes.

Antisera against these recombinant proteins had transmission blocking activity.

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Fig. 1.

Identification and domain organization of three ookinete genes in *P. berghei*. The signal peptide, transmembrane region, low complexity, predicted protein domain, and internal repeat regions are highlighted in red, blue, pink, green, and light blue, respectively. Scale bar, 100 aa.

Fig. 2.

(A) Recombinant protein expression and purification from *E. coli*. Purified proteins were separated on a 10% SDS-PAGE gel. Molecular weight markers in kDa are shown on the left. (B) Induction of antibodies in immunized mice. BALB/c mice (6 per antigen) were immunized with recombinant proteins and serum samples were collected post-immunization on days 14, 35, and 56. Antibody titers to the respective recombinant proteins were assessed by ELISA using pooled sera from control and immunized mice. The data are representative of two separate experiments. Data are presented as mean $\pm$ SD. SD only indicates the assay variance, not variance between individual mice. * and ** indicate significant difference at $p < 0.05$ and $p < 0.01$, respectively (Student's *t* test).

Fig. 3.

Western blot analysis of purified *P. berghei* lysates from schizonts (Sch), gametocytes (Gam), and ookinetes (Ook) probed with respective pooled mouse antisera. Purified recombinant protein lanes (R-P) were included as positive controls. The blots probed with Pbs21 mAb clone 13.1 and PbHSP70 polyclonal antiserum serve as a positive control and a loading control, respectively.

Fig. 4.

Localization of the candidate proteins in ookinetes. Cultured mature ookinetes were probed with the three respective antisera and then with the FITC-conjugated goat anti-mouse IgG (green). Nuclei were stained with DAPI (blue). BF, bright field. IFA with Pbs21 mAb clone 13.1 serves as a positive control. Scale bar, 5 μ m.

Fig. 5.

TB effects of antisera against respective recombinant proteins. **(A)** Effects of the respective antisera on *P. berghei* macrogamete/zygote/retort/ookinete conversion rates *in vitro*. Both control and antisera were used at a dilution of 1:10. Means were calculated from three independent experiments. Error bars indicate mean $\pm$ SD, and ** indicates significant difference at $p < 0.01$. Typical forms of the respective parasite stages probed with the Pbs21 mAb clone 13.1 are illustrated. Scale bars = 5 μ m. **(B)** Direct mosquito feeding assay. Individual data points represent the number of oocysts found in individual mosquitoes 10 days post-feeding. Horizontal bars indicate the mean number of oocysts per midgut and error bars indicate SEM within individual treatments. Three independent experiments were performed. *, $p < 0.05$; **, $p < 0.01$.

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Table 1

Characteristics of three predicted ookinete-specific genes in *P. berghei*.

<i>P. berghei</i> gene ID	Protein size (aa)	Recombinant protein (aa)	Functional domain	Protein expression *	<i>P. falciparum</i> ortholog	<i>P. vivax</i> ortholog
PBANKA_111920 (PSOP25)	350	45-245	-	Ookinete (gametocyte)	PF3D7_0620000 (PFF0975c)	PVX_114125
PBANKA_135340 (PSOP7 [#])	812	315-446	CBM_5_12	Ookinete	PF3D7_1340000 (MAL13P1.203)	PVX_082920
PBANKA_145770 (PSOP26)	774	50-254	-	Ookinete	PF3D7_1242000 (PFL2135c)	PVX_101120

* *P. berghei* expression information is based on the *P. berghei* proteomic data [30].

[#] PBANKA_135340 was previously characterized and annotated as a putative secreted ookinete protein PSOP7 [28]. This nomenclature was followed when naming the other two proteins as PSOP25 and PSOP26, respectively.

Table 2

in vivo evaluation of transmission-blocking effects of anti-PSOPs immunization in direct mosquito feeding experiments.

Exp.	Treatment ^a	Median oocyst number (IQR) ^b	Mean oocyst number (SEM) ^c	% reduction ^d	P ^e	Infected/Dissected (%) ^f	% reduction ^g	P ^h
# 1	Control	62.5 (8.3-139.5)	81.3 (14.42)			27/28 (96.4)		
	PSOP25	4.5 (0-39)	30.6 (10.69)	62.3	0.002	17/26 (65.4)	31	0.004
	PSOP7	17.0 (1.8-46)	31.8 (6.858)	60.8	0.009	22/28 (78.6)	17.8	0.051
	PSOP26	6.0 (0-60.5)	32.5 (9.153)	60.0	0.002	18/29 (62.1)	34.3	0.001
# 2	Control	62.5 (4.5-146.5)	94.6 (19.08)			25/28 (89.3)		
	PSOP25	7.5 (0-43.3)	32.1 (10.64)	66.0	0.009	18/26 (69.2)	20.1	0.067
	PSOP7	11.0 (1-49.8)	30.1 (6.714)	68.1	0.013	22/28 (78.6)	10.7	0.234
	PSOP26	2.0 (0-39.8)	27.7 (8.134)	70.7	0.002	18/30 (60)	29.3	0.011
# 3	Control	49.0 (3.5-124.3)	72.6 (15.14)			25/28 (89.3)		
	PSOP25	6.0 (0-42.3)	25.5 (8.347)	64.8	0.008	17/26 (65.4)	23.9	0.036
	PSOP7	7.0 (0.25-46.5)	28.8 (7.571)	60.3	0.031	21/28 (75)	14.3	0.148
	PSOP26	2.0 (0-44)	24.9 (8.009)	65.7	0.003	14/27 (51.9)	37.4	0.002

^a *In vivo* TB activity assay was carried out using mice immunized with respective recombinant proteins in three experiments.

^b IQR, interquartile range.

^c SEM., standard error of arithmetic mean.

^d % reduction in oocyst intensity was calculated as (mean_{control} - mean_{PBANKA})/mean_{control} × 100%.

^e P-values from Mann-Whitney U test comparing the control and immunized groups.

^f Prevalence of infection was calculated as the number of mosquitoes with oocysts/total mosquitoes dissected.

^g % reduction in prevalence was calculated as % prevalence control - % prevalence immunization.

^h P-values from Fisher's exact test for comparing the control and immunized groups.