Evaluation of two *Plasmodium vivax* sexual-stage antigens as transmission-blocking vaccine candidates

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Abstract

Background: *Plasmodium vivax* transmission-blocking vaccines (TBVs) have received high attention. PVX_098655 (PvPH) and PVX_101120 (PvSOP26) were predicted to be potential TBV antigens based on the studies of their orthologs in *Plasmodium berghei*.

Methods: Fragments of PvPH (amino acids 22–304) and PvSOP26 (amino acids 30–272) were expressed in the yeast expression system. The recombinant proteins were used to immunize mice to obtain the antisera. The transmission-reducing activities of these antisera were evaluated using the standard membrane feeding assay (SMFA) using *Anopheles dirus* mosquitoes and *P. vivax* clinical isolates.

Results: The recombinant proteins PvPH and PvSOP26 induced robust antibody responses in mice. With SMFA, the anti-PvSOP26 sera significantly reduced oocyst densities by 92.0% and 84.1% in two parasite isolates, while the anti-PvPH sera did not show evident transmission-reducing activity. Both PvPH and PvSOP26 showed limited gene polymorphisms in the clinical *P. vivax* isolates.

Conclusion: PvSOP26 could be a promising TBV candidate for *P. vivax*.

Background

Malaria, transmitted via *Anopheles* mosquitoes, is a widespread infectious disease in the tropics. In 2019, there were 229 million malaria cases in the world, resulting in more than 409,000 deaths [1]. *Plasmodium vivax* (*P.vivax*) is one of four human malaria parasites characterized by the recurrence of clinical symptoms every 48 hours (tertian). Although less virulent than *Plasmodium falciparum*, *P. vivax* still poses a significant burden on the economy and public health in Asia, America, and South America [2]. Some malaria-infected people are asymptomatic but infectious to mosquitoes, which may serve as an important reservoir contributing to the sustained transmission of malaria [3]. In addition, large proportions of *P. vivax* patients were due to relapse from dormant hypnozoites in the liver [4]. Vaccines hold great promise in the prevention, control, and elimination of malaria [5]

With the complex malaria parasite life cycle, multi-stage vaccines targeting antigens expressed in different parasite life stages are advocated. Transmission-blocking vaccines (TBVs) target the parasite's sexual-stage antigens or the mosquito midgut antigens, interrupting the parasite's transmission through mosquitoes. TBVs may even protect other vaccines or drugs by preventing the spread of resistant parasites, thereby greatly extending their useful lives [6]. An ideal TBV should inhibit multiple steps of sexual development, including gametocytogenesis, gametogenesis, fertilization, and ookinete's maturation and traversal of the mosquito midgut. Despite decades of research efforts, only a few TBV candidates have shown clear transmission-blocking (TB) activities. They mainly include the pre-fertilization antigens (P230, P48/45, P47, and HAP1), the post-fertilization antigens (P25 and P28), and the *Anopheles* mosquito midgut alanyl aminopeptidase N (AnAPN1) [7]. The histidine-tagged Pvs25 expressed in yeast, Pvs25H, is the first *P. vivax* TBV to enter a phase 1 clinical trial [8]. The Pvs25H protein with the adjuvant alhydrogel elicited antibodies that showed TB activity in the standard membrane feeding assay (SMFA). However, the second trial of Pvs25H with Montanide ISA51 adjuvant was halted due to unexpected reactogenicity of the vaccine candidate in volunteers [9]. The other two promising candidates, Pvs28 and AnAPN1, are still in pre-clinical trials [10, 11]. Thus, there is a pressing need to identify additional TBV candidates for *P. vivax*.

In recent years, the genomic, transcriptomic, and proteomic data available for malaria parasites provide opportunities for systematic genome-wide exploration of TBV antigens [12]. In previous studies, data mining of the PlasmoDB database led to the identification of two potential TBV candidate antigens, PbPH and PSOP26, in the rodent parasite *Plasmodium berghei* [13, 14]. In this study, the TB potentials of the orthologs of these two antigens in *P. vivax*, PvPH and PvSOP26,

were further explored. With *P. vivax* isolates from patients, PvSOP26 showed a strong effect in reducing the infection intensity using the SMFA.

Methods

Antigen selection and expression in yeast

Earlier TBV antigen discovery efforts using the rodent malaria system identified several candidate antigens, including PbPH, which contains a pleckstrin homology domain, and PbSOP26, a secreted protein from the ookinetes [13, 14]. Based on their excellent transmission-reducing (TR) activities, their orthologs in *P. vivax* PVX_098655 (PvPH) and PVX_101120 (PvSOP26) were selected for characterization. The sequences corresponding to amino acids (aa) 22–304 of PvPH and aa 30–272 of PvSOP26 of the Sal-I strain were synthesized and codon-optimized for expression in the yeast *Pichia pastoris* (GenScript Biotech Corp, China). They were cloned into the pPIC9K (Invitrogen, Carlsbad, CA, USA) vector, which was used to transform the *P. pastoris* GS115 strain [15]. The yeast strains expressing the two recombinant proteins (rPvPH and rPvSOP26) were cultured in 1 L of buffered minimal medium and induced by methanol. After lysis of the yeast cells with an ATS high-pressure homogenizer (ATS Engineering Inc, Germany), the recombinant proteins were purified using Ni-NTA columns, and the purity of the recombinant proteins was estimated by SDS–PAGE [16]. For negative control, we expressed a glutathione *S*-transferase (GST) protein as described previously [17].

Generation of anti-rPvPH and -rPvSOP26 sera

To generate antisera against the two recombinant proteins, BALB/c mice (n = 10 in each group) were injected subcutaneously with the purified rPvPH, rPvSOP26, or GST control protein (50 μg each) emulsified in the complete Freund's adjuvant (Sigma, St Louis, MI, USA). The mice were then given two booster immunizations with the same recombinant proteins (25 μg/mouse) emulsified in incomplete Freund's adjuvant (Sigma) at a 2-week interval. Two weeks after the final immunization, the antisera in each group of mice were collected via cardiac puncture and pooled.

Western blot

The recombinant proteins rPvPH and rPvSOP26 were separated in a 12% SDS–PAGE gel under reduced conditions and electro-transferred onto a 0.22 μm polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 for 2 h, the blots were probed with the pooled mouse antisera against rPvPH or rPvSOP26 as the primary antibodies and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:10,000) as the secondary antibodies. A western blot kit (Thermo, Waltham, MA, USA) was used to visualize the protein bands [18].

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to determine the antibody titers of mouse immune sera. Microtiter plates were coated with the purified rPvPH or rPvSOP26 (5 μg/mL) at 4°C for more than 8 h. The plates were first blocked with 1% bovine serum albumin (BSA) for 2 h at 37°C, then incubated with the antisera from mice immunized with rPvPH and rPvSOP26, respectively, at 37°C for 2 h. These antisera were diluted in 1% BSA in phosphate-buffered saline (PBS) from 1:200 to 1:512,000. After two washes with PBS, 100 mL HRP-conjugated goat anti-mouse IgG antibodies (Invitrogen; 1:5,000) were added to each well and incubated for 2 h. After five washes with PBS, tetramethylbenzidine was added to wells, and the plate was kept it in the dark for 10 min. The reaction was stopped by adding 2 mM H_2SO_4. An ELISA plate reader was used to measure the absorbance at 490 nm [19]. The endpoint titers were determined as the highest antiserum dilution with an optical reading greater than the average reading from a control serum (anti-GST) plus three standard deviations as the cut-off value [19].

P. vivax samples

The human-subject protocol for this study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (MUTM 2018-016).Patients with *P. vivax* malaria who were symptomatic for clinical malaria, slide-positive for *P. vivax* infection, 18 years or older, and not pregnant were considered eligible for inclusion in this study. Three *P. vivax* patients were enrolled after signing the informed consent. Before antimalarial treatment, 5–10 mL venous blood was collected into heparinized tubes and used to make blood smears and for SMFA [20].

Indirect immunofluorescent assay (IFA)

The expression and the location of PvPH were studied by IFA. The erythrocytes from the *P. vivax* patients were mixed with 47% Nycodenz/RPMI 1640 and centrifuged at 500 ×g for 25 min to obtain the parasite-infected erythrocytes at the grey interface. They were used to make thin smears, which were fixed with 4% paraformaldehyde for 30 min at 37°C. Skimmed milk (5%) in PBS was used to block the slides for 30 min. After three washes with PBS, the slides were incubated with mouse antisera against rPvPH, rPvSOP26 or the GST control for 1 h at room temperature. After three washes with cold PBS, the slides were incubated with FITC-conjugated goat anti-mouse antibodies (1:500, Invitrogen) for 1 h, and with 1 μg/mL 4'6-diamidino-2-phenylindole (DAPI; Invitrogen) for 30 min. After an additional wash with cold PBS, the slides were mounted with the ProLong®Gold Antifade Reagent kit (Invitrogen). Fluorescence images were obtained with an Olympus BX53 microscope [18, 21].

Quantification of TB activity

Antisera from mice immunized with rPvPH, rPvSOP26 or the GST control protein were diluted with heat-inactivated AB+ serum obtained from healthy donors in Thailand (1:1, v/v ratio). Erythrocytes of *P. vivax* patients were mixed with the diluted sera (1:1, v/v ratio) and incubated at 37^{o}C for 15 min. Each reconstituted infected blood was then introduced to a glass feeder and kept at 37 ^{o}C. One hundred starved mosquitoes were allowed to feed on the blood mixture for 30 min at 37^{o}C through the membrane feeder. After several hours, only fully engorged mosquitoes were kept on 10% sucrose solution in cotton balls at 20°C at 80% relative humidity for a week. Twenty mosquitoes from each group were randomly selected dissected on day 7 after blood feeding. Mosquito midguts were stained with 5% mercurochrome, and oocysts were counted [22, 23]. The infection prevalence, which is the proportion of oocyst-positive infected mosquitoes, was used to determine the TB activity. The intensity of infection, i.e. the number of oocysts per mosquito midgut, was used to determine the TR activity.

Analysis of genetic polymorphisms

For the parasite isolates used for SMFA, genetic polymorphisms of PvPH and PvSOP26 genes were determined. DNA was extracted from dried filter-paper blood spots using a QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). DNA fragments encoding rPvPH (22–304 aa) and rPvSOP26 (30–272 aa) were amplified by PCR [17]. The primers were designed based on the *P. vivax* Sal-1 (PVX_083235) sequence: PvPH-F (GTCCCAATTAGAATCTGTTT) and PvPH-R (GTTCCTTCTGTTGGGTGTTT); PvSOP26-F (ACCTTGTAGCCTCTACACTT) and PvPH-R (AAATTTGTTGAAAAAATTAT). All amplified DNA products were purified with a QIAquick Gel Extraction Kit (Qiagen) and sequenced using the ABI Prism® BigDye™ cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) as previously described [24]. Alignment of nucleotide sequences was done using the BioEdit software.

Statistical analyses

Statistical analyses were performed using SPSS version 22.0. The intensity of infection was calculated using a Mann–Whitney *U* test. The infection prevalence was compared using Fisher's exact test. *P* values less than 0.05 were

considered statistically significant.

Results

Identification, expression, and purification of PvPH and PvSOP26

A search of the PlasmoDB for the orthologs of PbPH and PbSOP26 identified PVX_098655 and PVX_101120 genes, designated as PvPH and PvSOP26, respectively. Multiple sequence alignment revealed that these two genes are highly conserved among the *Plasmodium* species (Fig. S1). A 282-amino acid fragment (22–304 aa) of PvPH and a 242-amino acid fragment (30–272 aa) of PvSOP26 were selected for expression in the yeast *P. pastoris* (Fig. 1A). Each of the recombinant proteins was expressed in 1 L of culture and purified using Ni-NTA chromatography. SDS–PAGE analysis showed that the purified recombinant rPvPH and rPvSOP26 were approximately 32 and 29 kDa, respectively, consistent with predicted molecular weights (Fig. 1B).

Immunization of mice with the recombinant proteins

The purified recombinant proteins were used to immunize mice to raise polyclonal antibodies. ELISA using the pooled immune sera for each antigen collected two weeks after the final booster showed that excellent immunogenicity of the recombinant proteins, and the antibody titers for both rPvPH and rPvSOP26 reached 1:32000 (Fig. 1C). Western blots showed that the antisera against rPvPH and rPvSOP26 recognized the respective recombinant proteins (Fig. 1B)

PvPH expression at the *P. vivax* gametocyte stage

IFA was performed to determine whether the PvPH was expressed at the gametocyte stage of *P. vivax*. IFA with mouse anti-rPvPH sera detected fluorescent signal in *P. vivax* gametocytes from a clinical isolate compared to the negative control with mouse anti-GST sera (Fig. 2). This result is consistent with the expression of PvPH during gametocyte development in *P. vivax*. Due to difficulties in culturing *P. vivax* ookinetes, PvSOP26 protein expression was not examined.

TR activity of the mouse antisera

To evaluate whether mouse anti-rPvPH and -rPvSOP26 sera had TR activity, SMFA was carried out with clinical samples obtained from three *P. vivax* patients using laboratory-reared *An. dirus* mosquitoes (Table 1). While the blood from donor #1 had an infection prevalence of 100% with the control sera (for GST) and immune sera against PvPH and PvPSOP26, the oocyst densities in all groups were low (mean 6.7-8.1 oocysts/midgut). Neither antisera showed noticeable TR activity (Table 1, Fig. 3). For the control antisera, the other two donor blood samples infected 95-100% mosquitoes and high oocyst densities (67.8-94.4 occysts/midgut). Compared with the control group, the anti-rPvSOP26 sera group showed significantly reduced oocyst density by 92.0% and 84.1%, respectively (Table 1, Fig. 3). The PvSOP26 antisera also showed TB activity and reduced the infection prevalence by 10% and 20%, respectively. In contrast, the anti-rPvPH sera showed no apparent TBR or TR activity regarding infection prevalence and oocyst density (Table 1, Fig. 3)

Genetic polymorphisms

To determine whether the variations in TR activity may be related to genetic polymorphisms of the target antigens since the antibodies were generated against the sequences of the Sal-I strain, the DNA fragments of PvPH and PvSOP26 were sequenced in the three *P. vivax* samples. Compared to the Sal-I sequence, the PvPH gene in the three samples showed no amino acid substitution, whereas all three *P. vivax* isolates had the same substitutions K263N, I355S, and L403I in the PvSOP26 gene. This result suggested that PvPH and PvSOP26 had limited genetic diversity.

Discussion

Based on the excellent TB potentials of PbPH and PbSOP26 from a TBV discovery effort using the rodent malaria system [13, 14], we evaluated the orthologs of these proteins as TBV candidates in *P. vivax*. IFA with the mouse antisera confirmed PvPH expression in the gametocyte stage of *P. vivax*. When the immune sera against the recombinant PvPH and PvSOP26 were evaluated using SMFA with *P. vivax* clinical isolates, the mouse anti-PvSOP26 antisera demonstrated considerable TR activities in reducing oocyst density.

For the development of recombinant protein-based TBVs, expression of the recombinant proteins with properly-folded conformational epitopes can be critical for inducing antibodies with TR activities [22, 25]. The yeast protein expression system has been widely used to produce many human vaccines [26, 27]. Compared to the prokaryotic expression system, the yeast system has the advantage of higher biomass expression and secretion yields. It also offers better protein-folding, disulfide-bond formation, and similar post-translational modifications as in mammals [22, 28]. For malaria vaccine development, the circumsporozoite protein, merozoite surface protein 1, and apical membrane antigen 1 expressed in yeast produced effective and protective antibodies in mice [29-33]. The yeast expression system was also used to express TBV candidates such as Pfv48/45 and Pfs25 with multiple disulfide bonds [34, 35]. In this study, the *P. pastoris* expression system was used to express PvPH and PvSOP26 with satisfactory yields, while the recombinant proteins showed high levels of immunogenicity. The resulting antibodies can recognize the native proteins expressed in *P. vivax* gametocytes, suggesting proper folding of the recombinant proteins in the expression system.

PSOP26 is predicted to be a secreted protein in ookinetes [36]. It is a highly expressed protein in *P. berghei* ookinetes, as its transcript ranked in the 99th percentile in the ookinete transcriptome. In mice infected by *P. berghei,* immunization with the recombinant PSOP26 showed significant TB and TR activities and reduced both the prevalence of infection and oocyst density in direct feeding assays (DFA) [13]. In this study, the expressed fragment of PvSOP26 covered the entire domain of the PbSOP26 used for TBV analysis [13]. The immune sera against PvPSOP26 were evaluated using SMFA with three clinical *P. vivax* isolates. When the *P. vivax* clinical isolates resulted in high oocyst density (>50 oocysts/midgut) in infected mosquitoes in the control group (as in donor #2 and #3), the anti-PvSOP26 sera significantly reduced the oocyst density by more than 84%. It is not clear why the anti-PvSOP26 antisera did not show TR activities when infection intensity in mosquitoes was low, but the differences cannot be attributed to genetic polymorphisms of *PvSOP26*, as this gene in all three samples was identical to the Sal-I reference sequence.

The pleckstrin homology (PH) domain is predicted in most *Plasmodium* PH orthologs [14]. PH domain can bind phosphatidylinositol in biological membranes, thus recruiting or targeting the proteins to the membrane fraction [37]. Mice immunized with recombinant PbPH protein reduced both the infection prevalence and oocyst density in DFA [14]. Despite the high level of homology between the PvPH and PbPH, antibodies generated against the PvPH fragment did not show any TR activities in SMFA with *P. vivax* clinical isolates. Several reasons may account for the lack of TR activities of the anti-PvPH antibodies. While the anti-PvPH antisera could detect the antigen expression in *P. vivax* gametocytes, the antibodies may recognize the epitopes that are not critical for fertilization and sexual development. Although the rodent model offers the convenience for antigen discovery where DFA can be performed [38], the ortholog may not have identical functions in *P. vivax*. The use of transgenic *P. berghei* expressing *P. vivax* full-length target genes may offer a better prediction for the TBV potential [39, 40].

Conclusion

We evaluated two sexual-stage genes PvPH and PvSOP26 as TBV candidates using *P. vivax* clinical isolates. PvSOP26 showed prominent TR activities in reducing oocyst density for parasite isolates that caused high-intensity mosquito infections.

Abbreviations

Plasmodium vivax: *P. vivax*. TBVs: transmission-blocking vaccines. SMFA: standard membrane feeding assay. TB: transmission-blocking. AnAPN1: *Anopheles* mosquito midgut alanyl aminopeptidase N. TR: transmission-reducing. GST: glutathione *S*-transferase. ELISA: enzyme-linked immunosorbent assay. BSA: bovine serum albumin. PBS: phosphate-buffered saline. IFA: indirect immunofluorescent assay.

Declarations

Authors' contributions

EL, MW and LC conceived of the study and helped to design the study and draft the manuscript. YZ and FL carried out the function studies, statistical analysis and drafted the manuscript. YZ, WR and JS carried out the TR activity studies. FY. JB and XJ carried out the sequence alignment and statistical analysis. All authors contributed to the writing of the manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its Additional fles.

Declarations

Ethics approval and consent to participate

The human-subject protocol for this study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (MUTM 2018-016).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1. Prevalence of infection and oocyst numbers in mosquitoes fed on antisera

P. vivax samples	Group	Oocyst density Median (IQR) [a]	Mean oocyst density	% inhibition of oocyst density[b]	*P* value [c]	(Inf/Diss)	Infection rate (%) [d]	% inhibition of prevalance [e]	*P* value [f]
#1	Control	8.0 (3.3-9.0)	6.8			20/20	100		
	PvPH	8.0 (6.0-9.0)	8.1	-19.1	0.933	20/20	100	-	
	PvSOP26	6.0 (4.0-8.0)	6.7	0.7	1.000	20/20	100	-	1.000
#2	Control	88.5 (69.8-132.5)	94.4			19/20	95		
	PvPH	78.0 (56.8-127.8)	85.2	9.8	1.000	19/20	95	-	
	PvSOP26	4.5 (2.0-12.5)	7.5	92.0	0.0001***	17/20	85	10	1.000
#3	Control	66.5 (59.0-80.0)	67.8			20/20	100		
	PvPH	67.5 (49.8-86.8)	66.7	1.6	1.000	20/20	100	-	
	PvSOP26	11.5 (1.0-19.8)	10.80	84.1	0.014*	16/20	80	20	0.106

[a] IQR; inter-quartile range. [b] % inhibition of oocyst density was calculated as ($mean_{control}$ – $mean_{PvPH/PvPSOP26}$)/$mean_{control}$ × 100%. [c] Mean number of oocysts was statistically analyzed (Mann–Whitney *U* test) and *P*-values less than 0.05 were considered statistically significant. [d] Infection prevalence was calculated by number of oocyst-infected mosquitoes per 20 mosquitoes dissected in each group (Inf/Diss). [e] % inhibition of prevalence was calculated as % $prevalence_{control}$ - % $prevalence_{PvPH/PvPSOP26}$. [f] Prevalance was statistically analyzed by Fisher's exact test. *P*-values less than 0.05 were considered statistically significant.

Figures

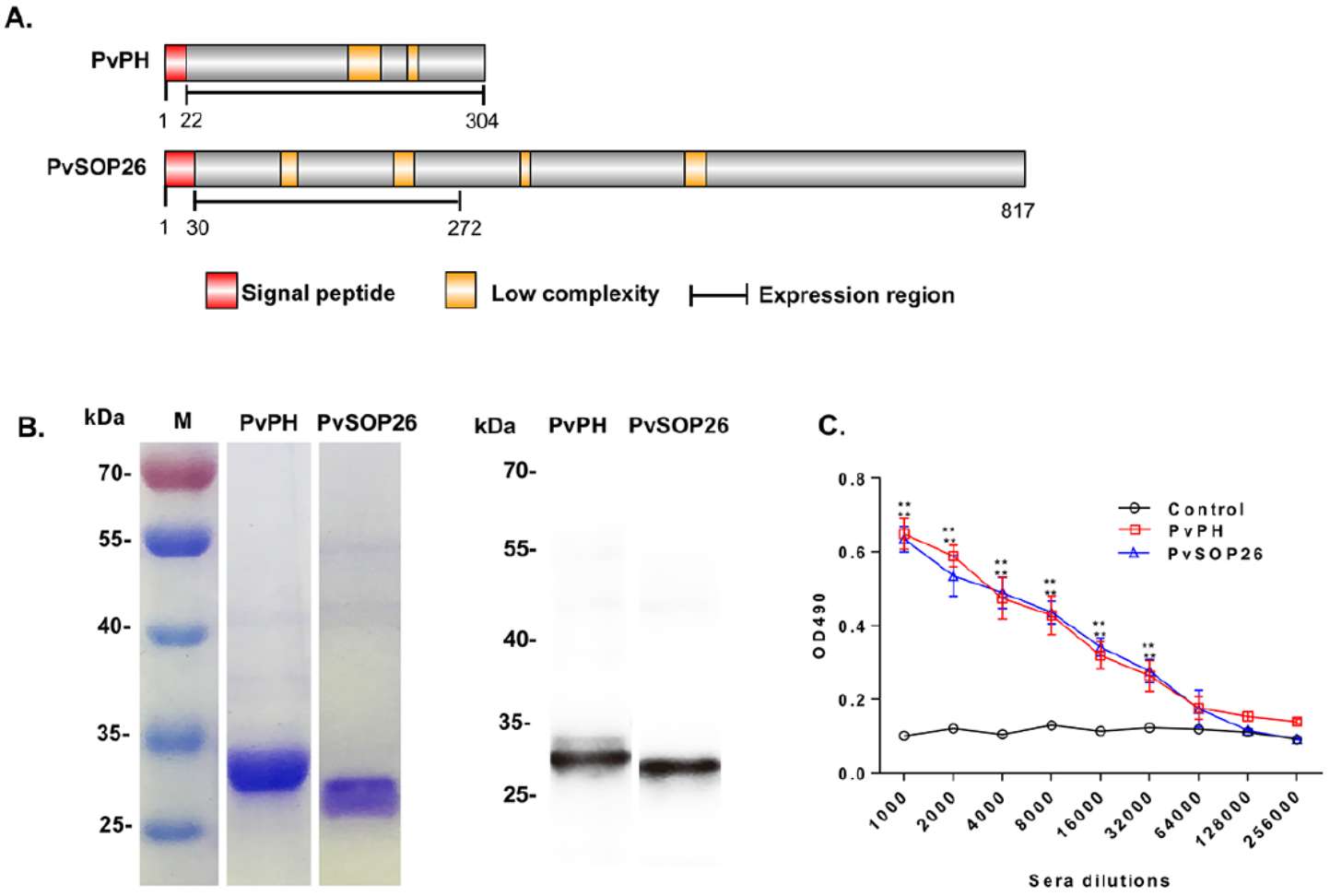

Figure 1

Domain organization, expression and immunization of PvPH and PvSOP26. (A) Schematics of PvPH and PvSOP26 showing domain organization and fragments expressed in yeast. Signal peptide, transmembrane regions, low complexity regions and the expressed segment are illustrated. (B) SDS-PAGE and western blot analysis of recombinant PvPH and PvSOP26. The Left and middle panels show Coomassie-stained SDS-PAGE gels of the purified recombinant proteins. The right panel shows the Western blot analysis using the mouse antisera against the respective recombinant proteins. M, protein marker in kDa. (C) Antibody titers against the recombinant proteins determined by ELISA. Pooled serum samples were tested at two-fold serial dilutions. Results are representative of three independent experiments. Error bars indicate mean ± SEM (n = 3). **P < 0.01 (Student's t test)

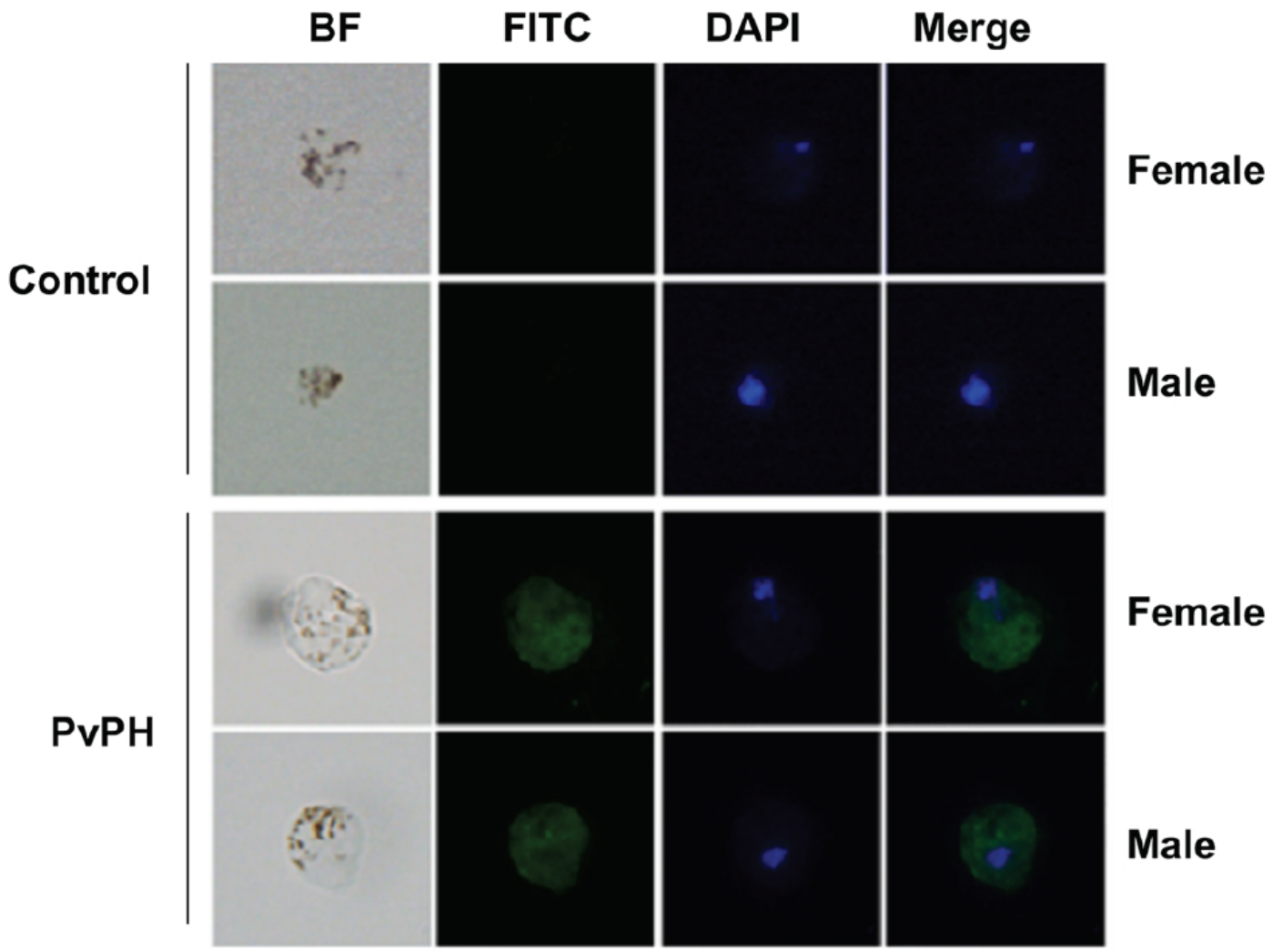

Figure 2

IFA detection of PvPH in P. vivax gametocytes. Gametocyte-infected erythrocytes were probed with the mouse anti-PvPH sera or the control anti-GST sera. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (green) was used as the secondary antibodies. Nuclei were stained with DAPI (blue). The images were magnified at ×1,000. BF, bright field;

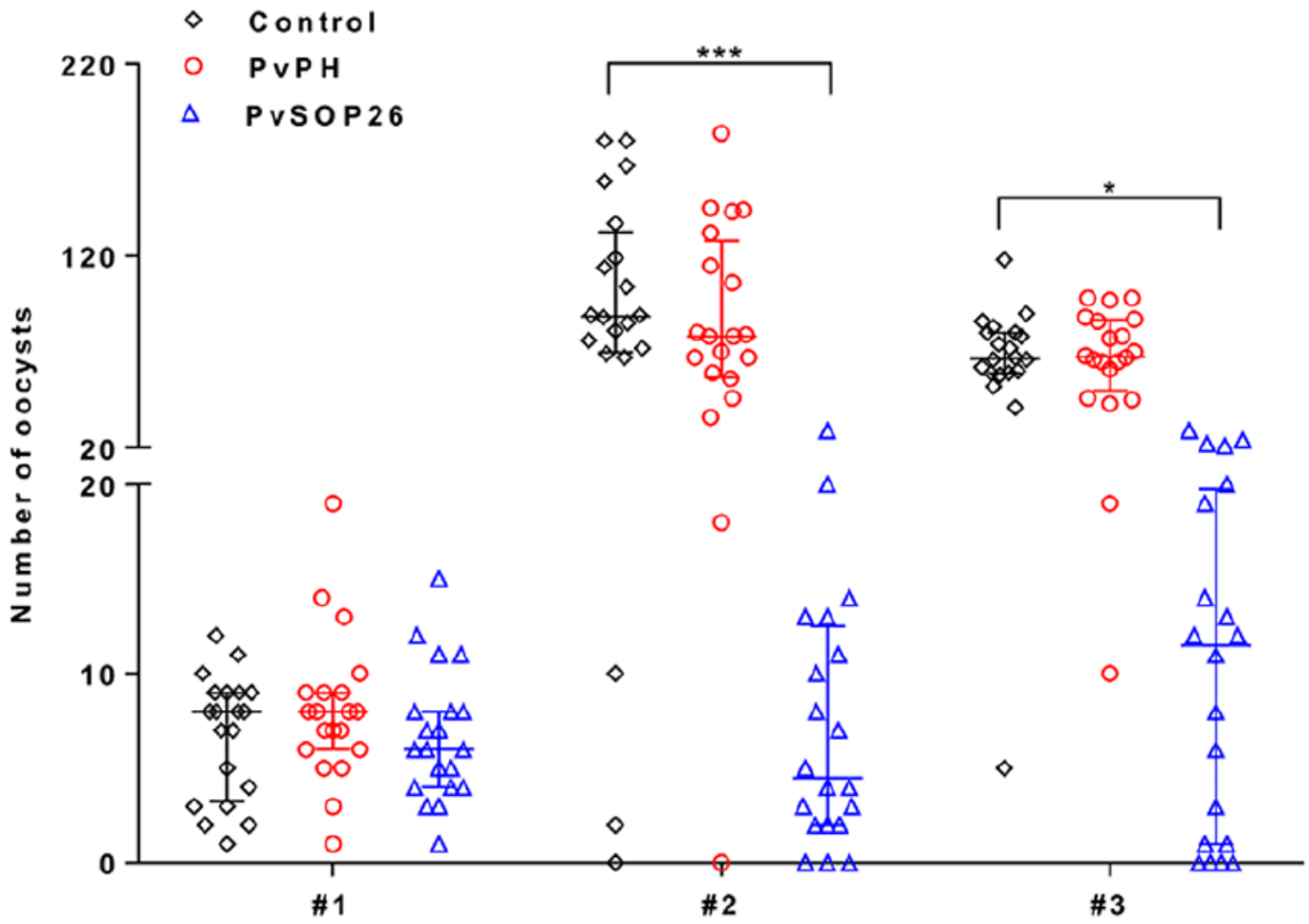

Figure 3

Evaluation of transmission-reducing activities of mouse antisera against PvPH, PvSOP26, and GST with P. vivax clinical isolates. SMFA was performed for three P. vivax isolates (shown on the x-axis) with reconstituted blood consisting of mouse antisera mixed with heat-inactivated AB+ healthy human serum at 1:1. The numbers of oocysts on individual mosquito midguts are shown in the scatter dot plot. The long horizontal bar designates the median number of oocysts, while the two short horizontal bars indicate interquartile ranges in each group. * and *** indicate $P < 0.05$ and $P < 0.001$, respectively (Mann–Whitney U test).

Supplementary Files

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