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Plasmodium vivax ookinete surface protein (Pvs25) is highly conserved among field isolates from five different regions of the Brazilian Amazon

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ABSTRACT

The *Plasmodium vivax* Ookinete Surface Protein (Pvs25) is one of the leading malaria Transmission-Blocking Vaccine candidates based on its high immunogenicity in animal models, transmission-blocking activity of antibodies elicited in clinical trials and high conservation among *P. vivax* isolates from endemic areas. However, the polymorphism in gene encoding Pvs25 in endemic areas from South America has been poorly studied so far. Here, we investigated the genetic polymorphism of *pvs25* in *P. vivax* isolates from five different regions of the Brazilian Amazon (*Cruzeiro do Sul*, *Mâncio Lima*, *Guajará*, *Manaus* and *Oiapoque*) and its impact on antigenicity of predicted B-cell epitopes using gene sequencing and epitope prediction tools. Firstly, only a non-synonymous substitution was found in the 657 bp amplified fragment in all sequenced samples, which represented an exchange of Gln by Lys at position 87 (Q87K) of protein amino acid sequence (domain II EGF-like). Q87K substitution was also present in all studied sites with a total frequency of 37.8%. *Cruzeiro do Sul* presented Q87K substitution in almost half of the isolates (48.4%), and an expressive frequency (40.5%) was also found in *Manaus*, while in *Mâncio Lima*, *Guajará* and *Oiapoque*, the frequencies were low (23.5%, 25% and 22.2% respectively). We also observed the Q87K mutation in a predicted B-cell epitope of *pvs25,* with no significant changes on its putative antigenicity. Our data suggest that the *pvs25* gene is conserved among isolates from different Brazilian Amazon geographic regions, an important observation considering the antigen potentiality as a vaccine candidate to cover distinct *P. vivax* endemic areas worldwide.

1. Introduction

Malaria is still an infectious parasitic disease of great epidemiological importance in several countries of tropical and subtropical regions of the world. Thus, it is extremely important to establish alternative intervention strategies capable of controlling malaria transmission, such as the development of an effective vaccine. The different cell stages developed by *Plasmodium* over its complex life cycle offer a myriad of antigenic targets that can be considered for vaccine design, including antigens expressed during sexual stage occurring in the mosquito vector (Bennink et al., 2016). In this context, transmission-blocking vaccine (TBV) has been considered an important strategy for malaria control by acting directly against the sexual and sporogonic stages of malaria parasites (Kaslow, 1997) and, consequently, preventing mosquitoes from becoming infectious (Wu et al., 2015). Thus far, these suitable targets for TBV have generated promising results.

Different proteins from sexual stage have been characterized as TBV targets because antibodies against these proteins show transmission blocking activities (Sauerwein and Bousema, 2015). Among these vaccine candidates, the 25 kDa Ookinete Surface Protein (P25) has already been described in many *Plasmodium* species (Saxena et al., 2007). The P25 protein is specifically expressed on the surface of the

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developing *Plasmodium* gametes, zygotes and ookinetes (Tsuboi et al., 1998). It is expressed before fertilization, reaching the peak of synthesis in the initial hours and later expressed in greater abundance on the surface of developing zygotes and ookinetes, being considered a post-activation target (del Carmen Rodriguez et al., 2000). This protein contains conserved structures, characterized by a secretory N-terminal signal sequence, four Epidermal Growth Factor (EGF)-like domains followed by a glycosyl-phosphatidylinositol (GPI) anchor at the C-terminal end. It is also cysteine rich and highly constrained by up to 11 disulfide bonds (Kaslow et al., 1988). Although P25 protein function has not yet been fully recognized, in *P. berghei*, it has been demonstrated that P25 protein has multiple, and partially redundant properties during ookinete and oocyst development. The protein is essential for the survival of ookinetes in the mosquito midgut and subsequent penetration of the midgut epithelium, and transformation into oocyst (Tomas et al., 2001).

Diverse platforms using sexual stage antigens as vaccine candidates demonstrated antibodies with transmission blocking activity (Carter et al., 2000). The Pvs25 has been considered one promising TB vaccine in development against *P. vivax, as* specific antibodies induced in immunized mice or *Aotus* monkeys completely block the ability of *P. vivax* to infect *Anopheles* mosquitoes (Hisaeda et al., 2000; Arevalo-Herrera et al., 2005). In humans, recombinant Pvs25 was recognized by antibodies from exposed populations in Myanmar (Kim et al., 2011). Currently, only Pfs25 and its orthologous protein in *Plasmodium vivax*, Pvs25, have been tested in Phase Ia clinical trials, its generates transmission blocking immunity in humans demonstrating the potential of this antigen as a component of a transmission blocking vaccine (Wu et al., 2008; Malkin et al., 2005).

Differently from the extensive polymorphism commonly observed in several *Plasmodium* antigens, Pvs25 is considered conserved. Sequence analyses of *pvs25* revealed a similar structural organization though only 45% amino acid identity is observed (Tsuboi et al., 1998) and to date, genetic diversity of *pvs25* gene among different isolates has been surveyed in several Asian countries. However, no study on this polymorphism has been carried out in Brazilian malaria-endemic areas. A lack of understanding of *P. vivax* population structure and transmission dynamics is one of the key problems undermining effective malaria control. Population genetic studies are, therefore, important to define the diversity, distribution and dynamics of *P. vivax* populations, being important for contributing to malaria control interventions. In this study, we aimed to analyze the genetic diversity of *pvs25* gene in *P. vivax* isolates from different regions of the Brazilian Amazon.

2. Materials and methods

2.1. Study areas and blood sample collection

The study was carried out in five different regions of the Brazilian Amazon: *Cruzeiro do Sul*, *Mâncio Lima*, *Guajará*, *Manaus* and *Oiapoque* (Fig. 1). *Cruzeiro do Sul*, Acre State, located in the North Region of Brazil, with 82,622 inhabitants, presenting latitude of 07°37′ 52" and longitude of 72° 40′ 12"; *Mâncio Lima*, Acre State, located in the North Region of Brazil, with 17,910 inhabitants, presenting latitude of 07° 36′ 51" and longitude of 72° 53′ 45"; *Guajará*, Amazonas State, with 14,074 inhabitants, presenting latitude of 2° 58′ 19″ and longitude of 57° 40′ 39″. The subset of *Manaus* and *Oiapoque* were previously described (Bitencourt Chaves et al., 2017).

Blood samples were collected from 98 *P. vivax*-infected individuals: 31 from *Cruzeiro do Sul*, 17 from *Mâncio Lima*, 4 from *Guajará*, 37 from *Manaus* and 9 individuals from *Oiapoque*. All *P. vivax* participants were enrolled according to the following criteria sought medical assistance for clinical malaria symptoms, presented uncomplicated malaria symptoms, were > 18 years of age, and had a positive *P. vivax* malaria diagnosis. Pregnant women, patients < 18 years of age, and *P. vivax* -and *P. falciparum*- infected individuals were excluded from the study. Thin and thick blood smears were examined for the identification of the malaria parasite by a technician experienced in malaria diagnosis from the Brazilian Malaria Health Services. Thick blood smears from all the subjects were stained with Giemsa, and a total of 200 microscopic fields were examined under a 1000-fold magnification. Thin blood smears of the positive samples were examined for species identification. To increase the sensitivity of parasite detection, molecular analyses using specific primers for genus (*Plasmodium* sp) and species (*P. falciparum* and *P. vivax*) were performed in all the samples as previously described (Snounou et al., 1993). Donors positive for *P. vivax* and/or *P. falciparum* at the time of blood collection were subsequently treated by the chemotherapeutic regimen recommended by the Brazilian Ministry of Health.

2.2. Ethics considerations

The study protocol of blood samples from *Cruzeiro do Sul*, *Mâncio Lima* and *Guajará* was reviewed and approved by the *Fundação Oswaldo Cruz* Research Ethics Committee, CEP-Fiocruz CAAE 46084015.1.0000.5248. In addition, the protocol of other blood sample collection was approved by the Research Ethics Committee of each locality: *Manaus* (CEP-Fiocruz): 346–613; *Oiapoque* (Hospital Municipal do Oiapoque/AP): 68980-000.

2.3. P. vivax DNA preparation and PCR amplification of pvs25 gene

The DNA was extracted from blood samples using the QIAamp DNA Blood Midi Kit (Qiagen, Germany) according to the manufacturer's protocol and, then, stored at −20 °C until amplification. All the *pvs25* genes reported in this study were amplified by conventional polymerase chain reaction (PCR) using the following pair of primers (5′-3′): Pvs25F2 5'-CACCGACCACAAAAACTTAT-3′ (AF083502, 158–177) and Pvs25R2 5'-AACGTAAAGCCTTCCATACA-3′ (AF083502, 814–795) described previously by Han et al., 2010 (Han et al., 2010). PCR reactions of the *pvs25* gene were carried out in 25 μL volume that included 3 μL of DNA, 10 pmol/μL of each primer and the Master Mix kit (Promega) containing Taq DNA polymerase, PCR buffer and 10 nmol of each deoxynucleotide triphosphate (dNTP, Promega, Madison, WI USA). The conventional PCR reaction was carried out using a GeneAmp PCR system 9700 (Applied Biosystem) and the amplification conditions were as follows: one step at 95 °C for 2 min; 30 cycles at 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min; and a last step at 72 °C for 1 min. Ten μL of amplified products were size-fractionated by electrophoresis within 1.5% agarose gel (Sigma) in 1 × TAE buffer (0.04 M TRIS-acetate, 1 mM EDTA) in the presence of 1 × GelRed nucleic acid stain (Biotium). PCR products were visualized by ultraviolet (UV) illumination. Sizing of products was performed using a GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific). Then, PCR fragments were purified using the GE Healthcare Lifesciences kit by following the manufacturer's instructions and sequenced.

2.4. DNA sequencing and polymorphism analysis

The specificity of the assay was confirmed by sequencing the PCR products from all positive samples using a Big Dye terminator sequencing kit (Applied Biosystems) following the manufacturer's instructions. The DNA sequencing was carried out in a 3730xl DNA analyzer (Applied Biosystems) and the results were analyzed using DNASTAR's sequence alignment software to identify polymorphism against the reference strain: Sal-1 *pvs25* (AF083502) from NCBI (https://www.ncbi.nlm.nih.gov). For worldwide analysis, the following sequences were used: Mexico – GenBank: ABS70906.1-ABS70935.1; Iran – GenBank:: EU810766-EU810774, EU810766, EU810767, EU810768, EU810769, EU810770, EU810771, EU810772, EU810773, EU810774; India – GenBank: HM048519-HM048618, FJ490913-FJ490962, JF824132-JF824147; Indonesia – GenBank: AAV33639.1; North Korea: GenBank:

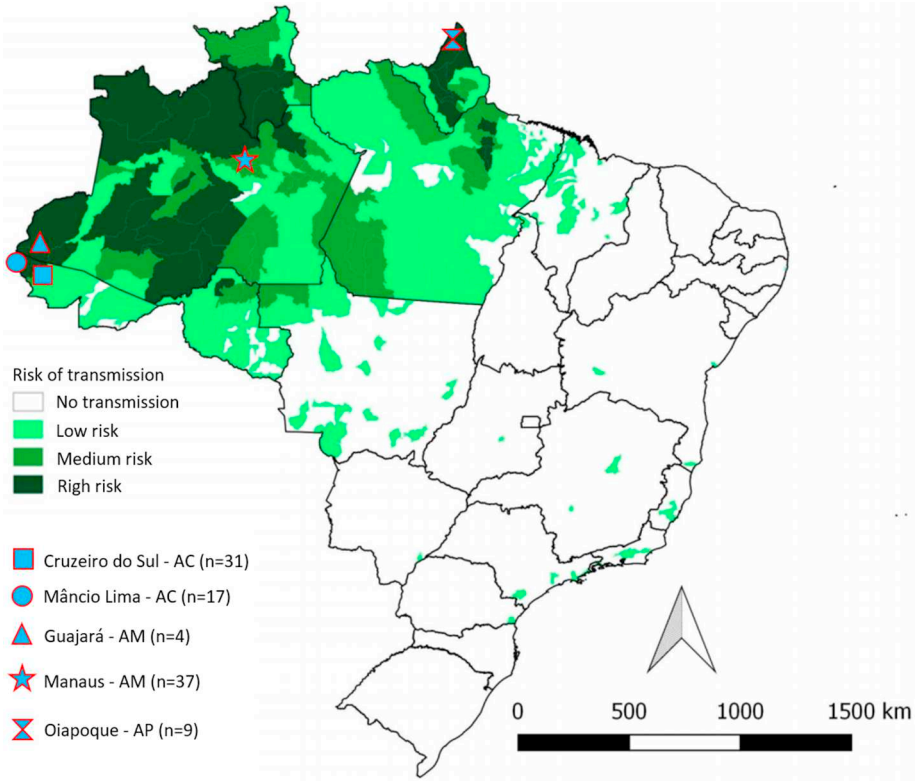

Fig. 1. Map showing the Malaria risk map by municipality of infection, Brazil, 2017 (SIVEP-Malaria).

AAV33640.1; South Korea: GenBank: GU971416-GU971611; Turkey: GenBank: ABG29073; Bangladesh – GenBank: BAA94348.1-BAA94350.1; Vietnam: GenBank: ABG29072; Thailand: GenBank: AB091729-AB091731.

2.5. Prediction of linear B-cell epitopes

Full-length protein sequence was subjected to BCPreds (http://ailab.cs.iastate.edu/bcpreds/predict.html), a prediction algorithm based learning methods of the machine, with the default threshold (75%). All sequences predicted as linear B-cell epitope (20-mer) were evaluated to antigenicity of using the VaxiJen algorithm (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). This is the first server for alignment-independent prediction of protective antigens. It was developed to allow antigen classification solely based on the physicochemical properties of proteins without recourse to sequence alignment. The results page lists the selected target, the protein sequence, its prediction probability, and a statement of protective antigen or non-antigen, according to a predefined cutoff (Threshold 0.4). The impact of non-synonymous mutations in all predicted epitopes was evaluated comparing the overall score of reference sequence and mutated sequences.

2.6. Prediction of conformational B-cell epitopes

To predict the conformational B-cell epitopes we analyzed the crystal structure of Pvs25 from *Plasmodium vivax* Sal-1, obtained from Protein Databank (PDB: 1Z27). This structure was analyzed by Ellipro web server (http://tools.iedb.org/ellipro/), using its default values (Minimum score: 0.5; Maximum distance: 6 Å). This server ElliPro predicts linear and discontinuous antibody epitopes based on a protein antigen's 3D structure. This server associates each predicted epitope with a score, defined as a PI (Protrusion Index) value averaged over epitope residues. In the method, residues with larger scores are associated with greater solvent accessibility. Discontinuous epitopes are defined based on PI values and are clustered based on the distance R (in Å between residue's centers of mass). The larger R is associated with the larger discontinues epitopes being predicted. (Ponomarenko et al., 2008)

2.7. Statistical analysis

Sequences were aligned using CLUSTAL X2 and the number of segregation sites (S), number of haplotypes (h), haplotype diversity (Hd), average number of differences (K), nucleotide diversity (π) were computed using DnaSP v6 (Rozas et al., 2003). The Tajima's D test (Tajima, 1989) for determining departure from the predictions of the neutral theory of evolution was also estimated with DnaSP v6. The genetic differentiation between populations was investigated evaluating the rate of fixation (F_{ST}) by analysis of molecular variance (AMOVA) implemented in ARLEQUIN v3.5.2.2 (Excoffier and Lischer, 2010) and significances were estimated using 10,000 permutations. The significance level was adjusted by Bonferroni correction for multiple tests.

3. Results

3.1. Molecular characterization and sequence polymorphisms of the pvs25 gene

All *P. vivax* field isolates presented only one fragment corresponding to 657 base pair (bp). In addition to these samples, *P. falciparum* specimens were also tested, but proved negative for PCR amplification of the *pvs25* gene. Thus, the 98 samples from individuals infected with *P. vivax* amplified by PCR were subjected to sequencing reactions to screen the possible single nucleotide polymorphisms of the gene encoding the Pvs25.

All 98 amplified fragments were sequenced and aligned for sequence analysis. Compared to reference sequence Sal-1 (AF083502), was not observed synonymous substitution, only one non-synonymous

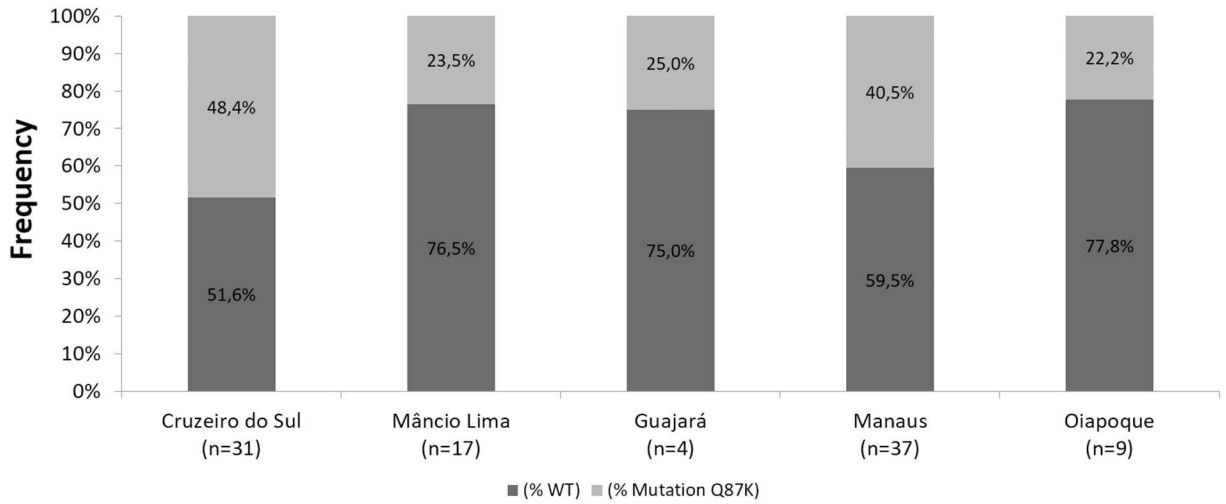

Fig. 2. Frequency of Q87K substitution in Pvs25 in *Plasmodium vivax* isolates from the Brazilian Amazon. The dark grey bar indicates the frequencies of wild type (compared with reference Sal-1 sequence) and the light grey bar indicates frequencies of the mutation Q87K.

substitution in the 657 bp amplified fragments was detected in all sequenced samples. The amino acid substitution Q87K, which represent the substitution of Gln to Lys at amino acid position 87 (EGF-like domain II), was identified with frequency of 37.8% of the isolates in the general population. In addition, regarding the areas studied, the substitution Q87K was present in *P. vivax* isolates collected in all localities. Despite being observed in all studied areas, the Q87K substitution was observed in higher frequencies in *Cruzeiro do Sul* (48.4%) and in *Manaus* (40.5%) when comparing with the frequencies observed in *Mâncio Lima* (23.5%), *Guajará* (25%) and *Oiapoque* (22.2%) (Fig. 2).

3.2. Population genetic analysis

The nucleotide diversity (π) for *pvs25* of 98 sequences analyzed was 0.00091. The highest nucleotide diversity was observed in the Cruzeiro do Sul group (0.00099). Among all 5 populations, Mâncio Lima sequences displayed the lowest nucleotide diversity (0.00073) followed by the Oiapoque group (0.00074) (Table 1). Similarly, parasites from Cruzeiro do Sul presented the highest estimate of haplotype diversity (Hd) (0.51613) whereas parasites from Mâncio Lima showed the lowest Hd (0.38235). Haplotype diversity was similar among the other studied areas, followed by the Oiapoque (0.38889). The Tajima's D test was performed to asses if there is selective pressure on the *pvs25* gene. The Tajima's D values ranged between −0.61237 and 1.66806, tests showed no significant departures from neutrality in all studied areas, indicating no significant selection in the *pvs25* gene. Pairwise comparisons between each parasite population were performed using the F_{ST} statistics to check whether there was indicative of genetic differentiation between parasite populations, but all F_{ST} values were non-significant, suggesting lack of genetic differentiation between the studied populations (Table 2).

Table 2
Genetic differentiation between samples from Brazil, measured by pairwise F_{ST} values.

	Cruzeiro do Sul	Mâncio Lima	Guajará	Manaus
Cruzeiro do Sul	–	–	–	–
Mâncio Lima	0.08591	–	–	–
Guajará	−0.03279	−0.2	–	–
Manaus	−0.01784	0.02444	−0.0995	–
Oiapoque	0.07847	−0.09259	−0.23077	0.01174

The F_{ST} values after Bonferroni correction.

3.3. Comparison of amino acid variations in Pvs25 among worldwide isolates

The Pvs*25* amino acid substitutions identified in worldwide isolates including those from Brazilian Amazon are resumed in Table 3. Among a total of 17 variants of Pvs25 observed in *P. vivax* isolates worldwide, the I130T, mainly detected in Asian isolates, was the most common amino acid substitution. The Q87K substitution was present in our Brazilian isolates and in the North (Mexico) (Gonzalez-Ceron et al., 2010) and South American isolates (Colombia and Venezuela) (Escalante et al., 2005), as well as in Iran (Zakeri et al., 2009), Turkey [ABG29073] and Mauritania (Escalante et al., 2005). Amino acid residue E97Q was detected in some Asian isolates such as those from Iran (Zakeri et al., 2009), India (Prajapati et al., 2011), Indonesia (Escalante et al., 2005), South Korea (Han et al., 2010), China (Feng et al., 2011), Bangladesh (Tsuboi et al., 2004) and Thailand (Sattabongkot et al., 2003), but was not found in American isolates. The Q131K substitution was present in some Asia isolates as well as in Oceanian and Papua New Guinea (PNG) (Escalante et al., 2005). The other amino acid variants of the *pvs25* gene were found but were rare in the countries.

Table 1
Comparison of genetic diversity among isolates from Brazil.

	No. of sequences	No. of segregating sites (S)	No. of haplotypes (h)	Haplotype diversity (Hd)	Average number of differences (K)	Nucleotide diversity (π)	Tajima's test (D)
Cruzeiro do Sul	31	1	2	0.51613	0.51613	0.00099	1.63773
Mâncio Lima	17	1	2	0.38235	0.38235	0.00073	0.56551
Guajará	4	1	2	0.50000	0.50000	0.00096	−0.61237
Manaus	37	1	2	0.49550	0.49550	0.00095	1.59494
Oiapoque	9	1	2	0.38889	0.38889	0.00074	0.15647
All samples	98	1	2	0.47486	0.47486	0.00091	1.66806

Statistical significance: Not significant, $P > .10$.

Table 3
Comparison of amino acid variations in the *pvs25* gene of *P. vivax* isolates between Brazilian Amazon and worldwide isolates.

	Isolates	SS	EGF-1		EGF-2		EGF-3						EGF-4					THR	Reference
		2	35	38	87	97	130	131	132	137	138	149	170	174	183	196	198	199	
	Sal-1	N	L	M	Q	E	I	Q	S	C	A	K	C	E	E	S	S	V	Tsuboi et al. (2004)
Brazilian Amazon	Cruzeiro do Sul	.	.	.	Q/K	.	.	.	.	.	.	.	.	.	.	.	.	.	
	Mâncio Lima	.	.	.	Q/K	.	.	.	.	.	.	.	.	.	.	.	.	.	
	Guajará	.	.	.	Q/K	.	.	.	.	.	.	.	.	.	.	.	.	.	Present study
	Manaus	.	.	.	Q/K	.	.	.	.	.	.	.	.	.	.	.	.	.	
	Oiapoque	.	.	.	Q/K	.	.	.	.	.	.	.	.	.	.	.	.	.	
North America	Mexico	.	.	.	Q/K	.	I/T	.	.	.	.	.	.	.	.	.	.	.	Gonzalez-Ceron et al. (2010)
Central America	Nicaragua	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	Escalante et al. (2005)
	Honduras	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Escalante et al. (2005)
	El Salvador	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Escalante et al. (2005)
South America	Colombia	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	Escalante et al. (2005)
	Venezuela	.	.	T	K	.	.	.	.	.	.	.	.	.	.	.	.	.	Escalante et al. (2005)
Asia	Iran	.	.	.	Q/K	E/Q	T	.	.	.	.	.	.	.	.	.	.	.	Zakeri et al. (2009)
	India	.	.	.	.	E/Q	T	Q/K	.	C/W	A/G	.	.	E/K	E/K	S/F	S/T	V/E	Prajapati et al. (2011)
	Indonesia	.	.	.	.	Q	T	.	.	.	.	.	.	.	.	.	.	.	Escalante et al. (2005)
	North Korea	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	Escalante et al. (2005)
	South Korea	N/D	.	.	.	E/Q	T	.	.	.	.	.	.	.	.	.	.	.	Han et al. (2010)
	China	.	L/M	.	.	E/Q	T	Q/K	.	.	.	.	.	.	.	.	.	.	Feng et al. (2011)
	Turkey	.	.	.	K	.	T	.	.	.	.	.	.	.	.	.	.	.	ABG29073
	Bangladesh	.	.	.	.	E/K	T	Q/K	.	.	.	.	.	.	.	.	.	.	Tsuboi et al. (2004)
	Vietnam	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	ABG29072
	Thailand	.	.	.	.	E/Q	T	Q/K	.	.	.	.	.	.	.	.	.	.	Sattabongkot et al. (2003)
Oceanian	PNG	.	.	.	.	.	T	K	R	.	.	N	.	.	.	.	.	.	Escalante et al. (2005)
Africa	Mauritania	.	.	.	K	.	T	.	.	.	.	.	.	.	.	.	.	.	Escalante et al. (2005)
Overall = 19 countries																			
		2	35	38	87	97	130	131	132	137	138	149	170	174	183	196	198	199	
	Sal-1	N	L	M	Q	E	I	Q	S	C	A	K	C	E	E	S	S	V	
	N countries (with substitution)	1	1	1	7	7	13	5	1	1	1	1	1	1	1	1	1	1	
	(% Frequency)	5%	5%	5%	37%	37%	68%	26%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	

The amino acid variants of the *pvs25* gene were compared to reference Sal-1 sequence (AF083502). SS, secretary signal sequence; EGF, EGF-like domain; THR, the C terminal hydrophobic region. • Indicates identical amino acid residues compared to the Salvador-1 strain.

3.4. Polymorphisms and potential B-cell epitopes

For becoming a good TBV vaccine candidate, the antigen must be hydrophilic and produce a strong B-cell mediated immunity. Full-length proteins were first analyzed for B-cell linear epitopes prediction using BCPred and all predicted B-cell epitopes were listed (Fig. 3). Four sequences of 20-mers (Pvs25: L53-A72, I80-Y99, I139-A158, L161-Q180) were predicted as linear B-cell epitopes on BCPreds. These four predicted epitopes presented high values of VaxiJen Score, which corroborate with their antigenicity (Table 4). Besides, the analyze of Pvs25 from *P. vivax* Sal-1 (PDB: 1Z27) by Ellipro server reveals 3 potential conformational epitopes (Table 5). Interestingly, all predicted B-cell linear epitopes were contained in conformational epitopes (Fig. 4), corroborating their potential as antibody targets.

About the polymorphism in predicted immunogenic epitopes of Pvs25, only the sequence LSENTCEEKNECKKETLGKA(Pvs25$_{(L53\text{-}A72)}$) was not inserted in a polymorphic region of protein. The polymorphic sequence described in Nicaragua (Escalante et al., 2005) (LKCNTDNEVRKNVEGVYKCQ), containing the SNP C170R, presented a higher combination of BCPred and VaxiJen score (Mean Score: 1.06) of all sequences identified on epitope Pvs25$_{(L161\text{-}Q180)}$. All polymorphic sequences identified on Pvs25 that were inserted in predicted epitopes presented no significant changes on predicted score (Table 4). Unfortunately, we cannot precise the real effect of mutations on structure and antigenicity of Pvs25 conformational epitopes, once that algorithms to identify these epitopes are dependents of 3D structure, which were only crystallographic to Pvs25 from *P. vivax* Sal-1. Despite this, in Fig. 4, we showed the location of predicted conformational epitopes and identified SNPs in 3D structure of Pvs25.

4. Discussion

Pvs25 is considered one important Transmission-Blocking Vaccine candidate. This protein is essential for the survival of ookinetes in the

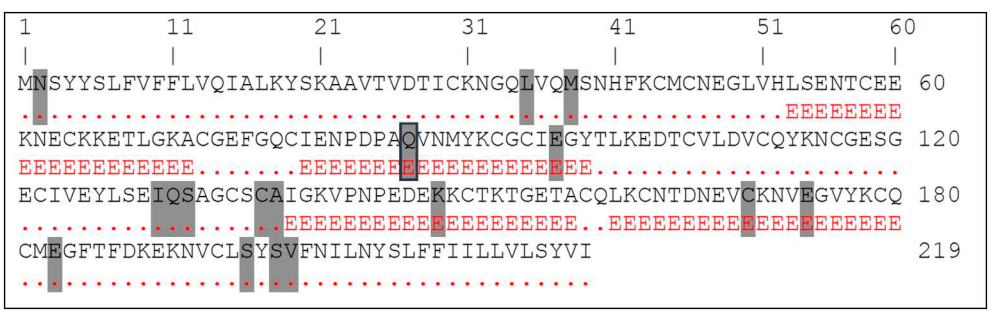

Fig. 3. B-cell epitope mapping of Pvs25 (Salvador Strain) using BCPreds and VaxiJen. Predicted epitopes were represented by letter E, non-predicted epitopes represented by red dots and grey bars indicate the polymorphic sites found in our sequences and sequences available in database from malaria endemic areas worldwide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Influence of polymorphisms in predicted B cell epitopes.

Epitope	Sequence	Epitope	BCPred score	VaxiJen score	Mean score
$Pvs25_{(L53-A72)}$	Sal-1	LSENTCEEKNECKKETLGKA	1.00	1.20	1.10
$Pvs25_{(I80-Y99)}$	Sal-1	IENPDPAQVNMYKCGCIEGY	0.95	0.65	0.80
	Q87K	IENPDPA**K**VNMYKCGCIEGY	0.95	0.70	0.82
	E97Q	IENPDPAQVNMYKCGCI**Q**GY	0.93	0.62	0.77
	Q87K; E97Q	IENPDPA**K**VNMYKCGCI**Q**GY	0.92	0.67	0.79
$Pvs25_{(I139-A158)}$	Sal-1	IGKVPNPEDEKKCTKTGETA	1.00	1.44	1.22
	K149 N	IGKVPNPEDE**N**KCTKTGETA	1.00	1.27	1.14
$Pvs25_{(L161-Q180)}$	Sal-1	LKCNTDNEVCKNVEGVYKCQ	0.95	0.76	0.85
	C170R	LKCNTDNEV**R**KNVEGVYKCQ	0.97	1.15	1.06
	E174K	LKCNTDNEVCKNV**K**GVYKCQ	0.86	0.81	0.84

The scores of BCPred and VaxiJen were listed to predicted epitopes ($Pvs25_{L53-A72}$, $Pvs25_{I80-Y99}$, $Pvs25_{I139-A158}$ and $Pvs25_{161-Q180}$). Sequences containing polymorphism inserted on predicted epitopes (Q87K, E97Q, C170R and E174K) were listed with their respective values of BCPred and VaxiJen scores. The "Mean score" represent the mean value between BCPred and VaxiJen.
Bold signifies the Sequences predicted as B-cell linear epitopes and inserted in conformational epitopes

mosquito midgut, penetration of the epithelium and transformation into oocysts (Tomas et al., 2001). However, the outstanding ability of *Plasmodium* in generating polymorphisms can culminate in immune evasion (Zakeri et al., 2009), which represents one of the major challenges for the development of a globally effective vaccine. Thus, one important strategy for malaria vaccine development is the identification and use of relatively conserved *Plasmodium* antigens. Therefore, identifying sequence variations in these candidate antigens may be helpful in designing an effective anti-malarial vaccine. Considering that the knowledge about the genetic polymorphism of *pvs25* in Brazilian Amazon areas remains unknown, we aimed to analyze this genetic diversity and to evaluate its potential impact in potential B-cell epitopes.

In our study, all the 98 field isolates from five different localities presented only one PCR fragment corresponding to 657 bp as seen in previous studies in other localities (Han et al., 2010). Sequencing analysis revealed that only Q87K substitution was found in the Brazilian isolates when comparing with reference Sal-1 strain. However, no work had yet been done in such diverse Brazilian endemic areas, and this is the first time that this mutation is described in *P. vivax* isolates from regions of east, middle and west of the Brazilian Amazon. The polymorphism was observed in all studied locations and the frequencies presented no statistical difference between the studied localities. In addition, this non-synonymous mutation has been previously reported in studies conducted in North (Mexico) (Gonzalez-Ceron et al., 2010) and South (Colombia and Venezuela) American isolates (Escalante et al., 2005) as also Iran (Zakeri et al., 2009), Turkey [ABG29073] and Mauritania (Escalante et al., 2005). Interestingly, in South America, the wild type genotype was found only in our studied areas. However, in sequences available worldwide, the Q and K genotypes at position 87 were also observed in isolates from Iran (Zakeri et al., 2009). This finding can corroborate the evidences that *P. vivax* in the Americas does not result from a single introduction into the continent (Taylor et al., 2013), and that some of the American haplotypes could derive from Asian populations (Cornejo and Escalante, 2006; Culleton et al., 2011).

In order to compare our findings with the Pvs25 sequences around the world, we also considered the frequencies of mutations in all continents. Despite the Q87K mutation is present in 5 out of 7 regions studied, the overall distribution of Pvs25 non-synonymous polymorphism was not restricted to this mutation. The comparison of our data and sequences available showed at list 17 variants in Pvs25 sequences observed in worldwide isolates. The frequencies of Pvs25 polymorphism in different countries ranged from 5% to 68%, being I130T (68%), Q87K (37%) and E97Q (37%) the most frequent polymorphism found globally. These data corroborate the studies of Chaurio et al., which suggested that some parasite haplotypes may have

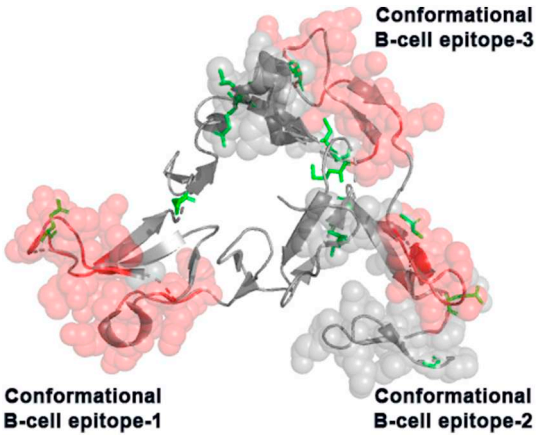

Fig. 4. Conformational epitopes and identified mutations in Pvs25 – 3D structure. Pvs25 representation as cartoon (grey), in which predicted conformational epitopes where represented by spheres. Predicted linear B-cell epitopes were located in conformational epitopes and were signalized by red spheres. Identified SNPs were represented by green sticks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Conformational B-cell epitopes on Pvs25.

Epitope	Residues	Number of residues	Ellipro score
1	**K39, N40, E41, C42, K43, K44, E45, T46, L47, G48, K49,** C57, **I58, E59, N60, P61, D62, P63, A64, Q65, V66, N67, M68, Y69, K70**	25	0.785
2	T3, V4, D5, T6, **L139, K140, C141, N142, T143, D144, N145, E146, V147, K149,** Q158, C159, M160, E161, G162, F163, T164, F165, D166, K167, E168, K169, N170, V171, C172, L173, G174, P175	32	0.679
3	K92, N93, C94, G95, E96, S97, E103, Y104, L105, S106, E107, I108, Q109, S110, A111, A116, **I117, G118, K119, V120, P121, N122, P123, E124, D125, E126, K127, K128, C129, T130, K131, T132, G133, E134, T135**	35	0.667

Mers that compound each Conformational epitope were listed as residues, where the letter indicates the one letter code of amino acid, and the number indicates the position of residue in the sequence. Sequences predicted as B-cell linear epitopes and inserted in conformational epitopes were highlighted by bold letters.

different geographic clusters (Chaurio et al., 2016). Moreover, even with good conservation rates, *pvs25* showed higher genetic variability compared to other sexual stage antigens reported in *P. vivax* as *pvs48/45* and the Willebrand factor A domain-related protein (WARP) (Chaurio et al., 2016), but lower polymorphisms when compared to well stablished vaccine candidates against sporozoites and merozoites, such as CSP and AMA-1 (Takala et al., 2009).

As observed in previous studies (Feng et al., 2011; Han et al., 2010), the EGF2 and EGF3 sites presented higher variability, and most conserved areas were located at EGF1 and EGF4 domains. Interestingly, Pvs25 the EGF2 and EGF3 like domains have been already described as sites targeted for blocking antibodies (Saxena et al., 2006). In addition, the EGF-like domains in the orthologous protein Pfs25 have also shown immune blocking activity, indicating that the EGF2 domain might be a good target for TBV. Considering that an epitope corresponding to the B-cell receptor plays an important role in vaccine design aiming at antibody production, full-length Pvs25 were analyzed to determine whether SNPs in Pvs25 antigen are involved in antigenic regions and the potential impact of all observed polymorphisms in predicted B-cell epitopes. The four linear B-cell epitopes predicted in Pvs25 presented conservation degrees ranging from 90 to 95% between the epitope sequences of isolates and reference strain. However, only $Pvs25_{(L53\text{-}A72)}$ was fully conserved among our isolates, while the epitopes $Pvs25_{(I80\text{-}Y99)}$, $Pvs25_{(I139\text{-}A158)}$ and $Pvs25_{(L161\text{-}Q180)}$ presented similar prediction scores for reference and mutant epitopes. However, we cannot estimate the real effect of identified mutations in the recognition or affinity of specific antibodies against Pvs25, mainly due the low number of known monoclonal antibodies against Pvs25 of *P. vivax*. Besides, the observation that all mutate epitopes were still predicted as immunogenic suggest that, beyond the low selective pressure in this immunogenic regions and the limited polymorphism of TBV candidates compared to target antigens expressed in asexual stage parasites (Barry et al., 2009; Tsuboi et al., 1998; Kaslow et al., 1989; Shi et al., 1992). In fact, during this sexual stage, the expressed proteins may adapt to diverse microenvironments into the human and mosquito hosts where parasites have to go through in order to complete their life cycle (Moreno-Garcia et al., 2014). It is also important to mention, that for *P. vivax* sexual antigens, the exposure to the immune system is expected to be longer than in *P. falciparum* due to the early appearance of *P. vivax* gametocytes in circulation (Vallejo et al., 2016). However, this increased exposure does not seem to result in relevant Pvs25 polymorphism (as a potential evasion mechanism) in response to a selective pressure exerted by the immune response to Pvs25, since the protein remains relatively conserved and immunogenic under natural conditions (Kim et al., 2011).

In summary, the present study explored the genetic polymorphism of *pvs25*, uncovered the amino acid substitution at the protein among Brazilian Amazon isolates and the frequent amino acid substitutions observed shared by American isolates. Despite the observed amino acid changes in natural populations worldwide, the antibody targets may not be significantly affected, since the genetic diversity of the Pvs25 vaccine candidate observed globally is limited and the predicted antigenicity was preserved.

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Author's contribution

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Investigation: LBC, DdSPdS, RNRdS.
Methodology: LBC, RNRdS, DdSPdS, BdOB, ABLS.
Resources: DMB, RLDM, PRRT, RMdS, CTDR, EKPR, JdCLJ.
Writing – original draft: LBC, JdCLJ, RNRdS, CTDR.
Writing – review & editing: DdSPdS, LRPR, RLDM, DMB, PRRT, EKPR, CTDR and RMdS.

References

Arevalo-Herrera, M., Solarte, Y., Yasnot, M.F., Castellanos, A., Rincon, A., Saul, A., Mu, J., Long, C., Miller, L., Herrera, S., 2005. Induction of transmission-blocking immunity in Aotus monkeys by vaccination with a *Plasmodium vivax* clinical grade PVS25 recombinant protein. Am. J. Trop. Med. Hyg. 73, 32–37.

Barry, A.E., Schultz, L., Buckee, C.O., Reeder, J.C., 2009. Contrasting population structures of the genes encoding ten leading vaccine-candidate antigens of the human malaria parasite, *Plasmodium falciparum*. PLoS One 4, e8497.

Bennink, S., Kiesow, M.J., Pradel, G., 2016. The development of malaria parasites in the mosquito midgut. Cell. Microbiol. 18, 905–918.

Bitencourt Chaves, L., Perce-da-Silva, D.S., Rodrigues-da-Silva, R.N., Martins da Silva, J.H., Cassiano, G.C., Machado, R.L., Pratt-Riccio, L.R., Banic, D.M., Lima-Junior, J.D., 2017. Plasmodium vivax cell traversal protein for Ookinetes and Sporozoites (PvCelTOS) gene sequence and potential epitopes are highly conserved among isolates from different regions of Brazilian Amazon. PLoS Negl. Trop. Dis. 11, e0005344.

Carter, R., Mendis, K.N., Miller, L.H., Molineaux, L., Saul, A., 2000. Malaria transmission-blocking vaccines–how can their development be supported? Nat. Med. 6, 241–244.

Chaurio, R.A., Pacheco, M.A., Cornejo, O.E., Durrego, E., Stanley Jr., C.E., Castillo, A.I., Herrera, S., Escalante, A.A., 2016. Evolution of the transmission-blocking vaccine candidates Pvs28 and Pvs25 in *Plasmodium vivax*: geographic differentiation and evidence of positive selection. PLoS Negl. Trop. Dis. 10, e0004786.

Cornejo, O.E., Escalante, A.A., 2006. The origin and age of *Plasmodium vivax*. Trends Parasitol. 22, 558–563.

Culleton, R., Coban, C., Zeyrek, F.Y., Cravo, P., Kaneko, A., Randrianarivelojosia, M., Andrianaranjaka, V., Kano, S., Farnert, A., Arez, A.P., Sharp, P.M., Carter, R., Tanabe, K., 2011. The origins of African *Plasmodium vivax*; insights from mitochondrial genome sequencing. PLoS One 6, e29137.

del Carmen Rodriguez, M., Gerold, P., Dessens, J., Kurtenbach, K., Schwartz, R.T., Sinden, R.E., Margos, G., 2000. Characterisation and expression of pbs25, a sexual and sporogonic stage specific protein of *Plasmodium berghei*. Mol. Biochem. Parasitol. 110, 147–159.

Escalante, A.A., Cornejo, O.E., Freeland, D.E., Poe, A.C., Durrego, E., Collins, W.E., Lal, A.A., 2005. A monkey's tale: the origin of *Plasmodium vivax* as a human malaria parasite. Proc. Natl. Acad. Sci. U. S. A. 102, 1980–1985.

Excoffier, L., Lischer, H.E., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10, 564–567.

Feng, H., Zheng, L., Zhu, X., Wang, G., Pan, Y., Li, Y., Yang, Y., Lin, Y., Cui, L., Cao, Y., 2011. Genetic diversity of transmission-blocking vaccine candidates Pvs25 and Pvs28 in *Plasmodium vivax* isolates from Yunnan Province, China. Parasit. Vectors 4, 224.

Gonzalez-Ceron, L., Alvarado-Delgado, A., Martinez-Barnetche, J., Rodriguez, M.H., Ovilla-Munoz, M., Perez, F., Hernandez-Avila, J.E., Sandoval, M.A., Rodriguez Mdel, C., Villarreal-Trevino, C., 2010. Sequence variation of ookinete surface proteins Pvs25 and Pvs28 of *Plasmodium vivax* isolates from southern Mexico and their association to local anophelines infectivity. Infect. Genet. Evol. 10, 645–654.

Han, E.T., Lee, W.J., Sattabongkot, J., Jang, J.W., Nam, M.H., An, S.S., Suh, I., Lim, C.S., 2010. Sequence polymorphisms of *Plasmodium vivax* ookinete surface proteins (Pvs25 and Pvs28) from clinical isolates in Korea. Tropical Med. Int. Health 15, 1072–1076.

Hisaeda, H., Stowers, A.W., Tsuboi, T., Collins, W.E., Sattabongkot, J.S., Suwanabun, N., Torii, M., Kaslow, D.C., 2000. Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of *Plasmodium vivax* to infect mosquitoes. Infect. Immun. 68, 6618–6623.

Kaslow, D.C., 1997. Transmission-blocking vaccines: uses and current status of development. Int. J. Parasitol. 27, 183–189.

Kaslow, D.C., Quakyi, I.A., Syin, C., Raum, M.G., Keister, D.B., Coligan, J.E., McCutchan, T.F., Miller, L.H., 1988. A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. Nature 333, 74–76.

Kaslow, D.C., Quakyi, I.A., Keister, D.B., 1989. Minimal variation in a vaccine candidate from the sexual stage of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 32, 101–103.

Kim, T.S., Kim, H.H., Kim, J.Y., Kong, Y., Na, B.K., Lin, K., Moon, S.U., Kim, Y.J., Kwon, M.H., Sohn, Y., Kim, H., Lee, H.W., 2011. Comparison of the antibody responses to *Plasmodium vivax* and *Plasmodium falciparum* antigens in residents of Mandalay, Myanmar. Malar. J. 10, 228.

Malkin, E.M., Durbin, A.P., Diemert, D.J., Sattabongkot, J., Wu, Y., Miura, K., Long, C.A., Lambert, L., Miles, A.P., Wang, J., Stowers, A., Miller, L.H., Saul, A., 2005. Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for *Plasmodium vivax* malaria. Vaccine 23, 3131–3138.

Moreno-Garcia, M., Recio-Totoro, B., Claudio-Piedras, F., Lanz-Mendoza, H., 2014. Injury and immune response: applying the danger theory to mosquitoes. Front. Plant Sci. 5,

451.
Ponomarenko, J., Bui, H.H., Li, W., Fusseder, N., Bourne, P.E., Sette, A., Peters, B., 2008. ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinforma. 9, 514.
Prajapati, S.K., Joshi, H., Dua, V.K., 2011. Antigenic repertoire of *Plasmodium vivax* transmission-blocking vaccine candidates from the Indian subcontinent. Malar. J. 10, 111.
Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.
Sattabongkot, J., Tsuboi, T., Hisaeda, H., Tachibana, M., Suwanabun, N., Rungruang, T., Cao, Y.M., Stowers, A.W., Sirichaisinthop, J., Coleman, R.E., Torii, M., 2003. Blocking of transmission to mosquitoes by antibody to *Plasmodium vivax* malaria vaccine candidates Pvs25 and Pvs28 despite antigenic polymorphism in field isolates. Am. J. Trop. Med. Hyg. 69, 536–541.
Sauerwein, R.W., Bousema, T., 2015. Transmission blocking malaria vaccines: assays and candidates in clinical development. Vaccine 33, 7476–7482.
Saxena, A.K., Singh, K., Su, H.P., Klein, M.M., Stowers, A.W., Saul, A.J., Long, C.A., Garboczi, D.N., 2006. The essential mosquito-stage P25 and P28 proteins from Plasmodium form tile-like triangular prisms. Nat. Struct. Mol. Biol. 13, 90–91.
Saxena, A.K., Wu, Y., Garboczi, D.N., 2007. Plasmodium p25 and p28 surface proteins: potential transmission-blocking vaccines. Eukaryot. Cell 6, 1260–1265.
Shi, Y.P., Alpers, M.P., Povoa, M.M., Lal, A.A., 1992. Single amino acid variation in the ookinete vaccine antigen from field isolates of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 50, 179–180.
Snounou, G., Viriyakosol, S., Zhu, X.P., Jarra, W., Pinheiro, L., do Rosario, V.E., Thaithong, S., Brown, K.N., 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol. Biochem. Parasitol. 61, 315–320.
Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.
Takala, S.L., Coulibaly, D., Thera, M.A., Batchelor, A.H., Cummings, M.P., Escalante, A.A., Ouattara, A., Traore, K., Niangaly, A., Djimde, A.A., Doumbo, O.K., Plowe, C.V., 2009. Extreme polymorphism in a vaccine antigen and risk of clinical malaria: implications for vaccine development. Sci. Transl. Med. 1, 2ra5.
Taylor, J.E., Pacheco, M.A., Bacon, D.J., Beg, M.A., Machado, R.L., Fairhurst, R.M., Herrera, S., Kim, J.Y., Menard, D., Povoa, M.M., Villegas, L., Mulyanto, A., Snounou, G., Cui, L., Zeyrek, F.Y., Escalante, A.A., 2013. The evolutionary history of *Plasmodium vivax* as inferred from mitochondrial genomes: parasite genetic diversity in the Americas. Mol. Biol. Evol. 30, 2050–2064.
Tomas, A.M., Margos, G., Dimopoulos, G., van Lin, L.H., de Koning-Ward, T.F., Sinha, R., Lupetti, P., Beetsma, A.L., Rodriguez, M.C., Karras, M., Hager, A., Mendoza, J., Butcher, G.A., Kafatos, F., Janse, C.J., Waters, A.P., Sinden, R.E., 2001. P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. EMBO J. 20, 3975–3983.
Tsuboi, T., Kaslow, D.C., Gozar, M.M., Tachibana, M., Cao, Y.M., Torii, M., 1998. Sequence polymorphism in two novel *Plasmodium vivax* ookinete surface proteins, Pvs25 and Pvs28, that are malaria transmission-blocking vaccine candidates. Mol. Med. 4, 772–782.
Tsuboi, T., Kaneko, O., Cao, Y.M., Tachibana, M., Yoshihiro, Y., Nagao, T., Kanbara, H., Torii, M., 2004. A rapid genotyping method for the vivax malaria transmission-blocking vaccine candidates, Pvs25 and Pvs28. Parasitol. Int. 53, 211–216.
Vallejo, A.F., Garcia, J., Amado-Garavito, A.B., Arevalo-Herrera, M., Herrera, S., 2016. *Plasmodium vivax* gametocyte infectivity in sub-microscopic infections. Malar. J. 15, 48.
Wu, Y., Ellis, R.D., Shaffer, D., Fontes, E., Malkin, E.M., Mahanty, S., Fay, M.P., Narum, D., Rausch, K., Miles, A.P., Aebig, J., Orcutt, A., Muratova, O., Song, G., Lambert, L., Zhu, D., Miura, K., Long, C., Saul, A., Miller, L.H., Durbin, A.P., 2008. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. PLoS One 3, e2636.
Wu, Y., Sinden, R.E., Churcher, T.S., Tsuboi, T., Yusibov, V., 2015. Development of malaria transmission-blocking vaccines: from concept to product. Adv. Parasitol. 89, 109–152.
Zakeri, S., Razavi, S., Djadid, N.D., 2009. Genetic diversity of transmission blocking vaccine candidate (Pvs25 and Pvs28) antigen in *Plasmodium vivax* clinical isolates from Iran. Acta Trop. 109, 176–180.