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The GPI-anchored 6-Cys Protein *Pv*12 is Present in Detergent-resistant Microdomains of *Plasmodium vivax* Blood Stage Schizonts

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Plasmodium vivax **malaria remains one of the tropical diseases causing an enormous burden on global public health. Several proteins located on this parasite species' merozoite surface have been considered the most suitable antigens for being included in an anti-malarial vaccine, given the functional role they play during the parasite's interaction with red blood cells. The present study identifies and characterizes the *P. vivax Pv*12 surface protein which was evaluated by using molecular biology and immunochemistry assays; its antigenic potential was also examined in natural and experimental *P. vivax* malaria infections. The *P. vivax* VCG-1 strain *Pv12* gene encodes a 362 amino acid-long protein exhibiting a signal peptide, a glycosylphosphatidylinositol (GPI) anchor sequence and two 6-Cys domains. The presence of the *Pv*12 protein on the parasite's surface and its association with detergent-resistant membrane complexes, together with its antigenic potential, supports the notion that this antigen could play an important role as a red blood cell binding ligand. Further studies aimed at establishing the immunogenicity and protection-inducing ability of the *Pv*12 protein or its products in the *Aotus* experimental model are thus suggested.**

Key words: Antigen; detergent-resistant membrane; glycosylphosphatidylinositol-anchor; 6-Cys domain; malaria; *Plasmodium vivax*.

Introduction

Malaria continues being one of the public health problems having great epidemiological importance in tropical and sub-tropical regions around the world. According to the World Health Organization's (WHO) 2011 malaria report, this disease affected about 216 million individuals in 2010; an estimated 655,000 of them died as a direct consequence of this disease, of whom 86% were children aged less than 5 years old (WHO 2011). It is well-known that *Plasmodium* species are responsible for causing malaria and that only five of them are infectious for humans (*Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* (Sherman 1998) and, recently, *Plasmodium knowlesi* (Figtree et al. 2010); *P. falciparum* produces the most lethal form of the disease while *P. vivax* represents significant

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morbidity leading to socioeconomic implications in endemic communities (Mendis et al. 2001).

In spite of international control strategies and policies having been implemented during the last few years which have had limited success, malaria continues its gradual spread throughout the world. This has been attributed to malarial strains' increasing resistance to currently available anti-malarial drugs (Dondorp et al. 2009; Teka et al. 2008) and to the vector's resistance to insecticides (Hunt et al. 2011). This parasite represents an ever-present menace in Latin-American, Asian and African countries (Mendis et al. 2001; Snow et al. 2005), meaning that developing measures to control this threat has become an urgent need.

Vaccine design has been one of the best strategies to date for battling the infectious diseases caused by *P. falciparum* (Patarroyo et al. 2011; Richie and Saul 2002). It has been proposed recently that a blood-stage anti-malarial vaccine's efficacy lies mainly in blocking all merozoite molecules interacting with red blood cells (RBCs) (Patarroyo and Patarroyo 2008; Patarroyo et al. 2011); hence, several researchers have focused on the functional characterization of antigens expressed during the parasite's asexual stage. It has been shown that several *P. falciparum* merozoite surface detergent-resistant membrane (DRM) extracted proteins, such as MSP-1-4, *Pf*12, *Pf*34, *Pf*38, *Pf*41 and *Pf*92, are implicated in initial interaction with RBCs and that some of these can produce an antibody response which is able to inhibit the parasite's internalization of its target cells (Arevalo-Pinzon et al. 2010; Garcia et al. 2009; Lundquist et al. 2006; Mazumdar et al. 2010; Obando-Martinez et al. 2010; Taylor et al. 1998; Wang et al. 2001), whereby, most of these antigens have thus been considered among the best candidates for being included in an anti-malarial vaccine.

*P. falciparum Pf*12 has been described as being a member of the Ps230 family (Pradel 2007); several of these proteins have been evaluated as candidates for a transmission-blocking vaccine (van Dijk et al. 2010). *Pf*12 is highly conserved within the *Plasmodium* genus and has broad expression throughout the parasite's different life-cycle stages (schizonts, gametocytes and sporozoites) (Sanders et al. 2005). It has been predicted to contain a signal peptide, a GPI-anchor sequence and two 6-Cys domains (also known as s48/45 domains), the latter are characteristic of sexual stage surface antigens (van Dijk et al. 2010). A previous study has shown that *Pf*12 is recognized by antibodies from patients who have been naturally infected by *P. falciparum* (Sanders et al. 2005). Furthermore, it has been found that this protein has two high activity binding peptide sequences (HABPs) that partially inhibit merozoite invasion of host cells in *in vitro* assays (Garcia et al. 2009), highlighting the fact that this antigen could be important for parasite entry to RBCs.

Recently, enormous progress has been made in developing a promising vaccine against *P. falciparum* (Patarroyo et al. 2011). However, and in spite of its epidemiological importance, a vaccine against *P. vivax* has still not been developed due to limited knowledge about the antigens which this parasite uses during invasion. This has been mainly due to difficulties in maintaining a continuous *in vitro* culture system for this species (Panichakul et al. 2007; Udomsangpetch et al. 2007). Nevertheless, the publication of the *P. vivax* genome sequence, as well as studies aimed at comparing its genetic transcription profile with other species during the intra-erythrocyte life-cycle (Bozdech et al. 2008), has led to fresh possibilities of finding *P. falciparum* homologous antigens in *P. vivax* which are implicated in its development. A comparative genomic study has shown that *P. vivax* GPI-anchored proteins share great similarity with those previously identified in *P. falciparum*; 29 are homologous in both species, while one additional protein is exclusive to *P. vivax* (Carlton et al. 2008). To date, 6 of all GPI-anchored proteins have been described in *P. vivax* (*Pv*MSP1 (del Portillo et al. 1991), -8 (Perez-Leal et al. 2004), -10 (Perez-Leal et al. 2005), *Pv*38 (Mongui et al. 2008), -34 (Mongui et al. 2009), and *Pv*ASP (Moreno-Perez et al. 2011)) and only 2 of them have been experimentally assessed as vaccine candidates against the malaria blood stage (*Pv*MSP1 (Sierra et al. 2003), -10 (Giraldo et al. 2009)), highlighting that progress in this species has not achieved the same level as that for *P. falciparum*.

The *Pf*12 homologous protein in *P. vivax* (here named *Pv*12) was thus studied, taking into account that knowledge of antigens is important in understanding *P. vivax* biology, aimed at continuing to identify and characterize putative proteins for this parasite species. Molecular biology and immunochemistry techniques were used for determining *Pv12* gene transcription, the protein's expression during the schizont stage and its subcellular location, together with its association with DRM and serological reactivity in patients who have suffered active episodes of *P. vivax* malaria infection and in experimentally-infected *Aotus* monkeys.

Results and Discussion

Identifying the *Pf12* Homologous Gene in *P. vivax* and Other *Plasmodium* Species

The aforementioned DRM-associated *P. falciparum* vaccine candidates were screened to identify homolgs in *P. vivax* that could be involved in the invasion of RBCs. The *Pv12* gene sequence selected for our target study (homologous to the *Pf*12 encoding gene) was found in the PlasmoDB database under the accession number PVX_113775; according to the information shown there, the *Pv12* gene from the *P. vivax* Salvador-1 strain (Sal-1) is localized in the CM000452 contig between base pairs 1,516,825 and 1,517,913 in chromosome 11. Furthermore, similar genes were found in the *Plasmodium knowlesi* (PKH_113620), *Plasmodium chabaudi* (PCHAS_011160) and *Plasmodium berghei* (PBANKA_011100) genomes, highlighting the fact that this protein is conserved in species that cause malaria in humans and rodents. Aligning these predicted hypothetical *Plasmodium* species protein sequences led to finding two strongly similar regions between amino acids 32 and 180 and 201 to 316 (Fig. 1), both containing a 6-Cys domain (see below).

It has been found that the *Pf12* (PFF0615c), *Pv12* (PVX_113775) and *Pk12* (PKH_113620) genes were localized in a syntenic region once the direction of the open reading frames (ORFs) and the exon-intron structure of the adjacent genes had been analyzed (Fig. 2); this conserved pattern was consistent with the predicted identity (Id) and similarity (S) values for the hypothetical protein products which were within a range for considering that there was homology between genes (Kuzniar et al. 2008). Previous phylogenetic analysis has shown that *P. vivax* and *P. knowlesi* are more closely related than other malaria species (Bozdech et al. 2008); the Id and S values determined in the analysis of our group (56.3% to 94.2% Id and 67.4% to 99.1% S) showed that these two organisms were similar in this particular chromosome region.

The *Pv12* Gene is Transcribed in Schizonts

Pv12 gene transcription was evaluated by PCR using cDNA from a *P. vivax* VCG-1 strain. RT-PCR and gDNA amplification (Fig. 3A, lanes 2 and 4, respectively) led to concluding that *Pv12* consisted of a single exon which was also transcribed in a schizont-enriched sample, thus coinciding with analysis of the *P. vivax* Sal-1 strain's transcriptional profile (Bozdech et al. 2008), whereas no amplification was observed in the negative controls (Fig. 3A, lanes 1 and 3). Once the *Pv12* gene sequences from the *P. vivax* Salvador 1 (Sal-1) reference strain and the *Aotus* monkey-adapted *P. vivax* VCG-1 had been aligned, the C375A nucleotide substitution was found which produced a non-synonymous change in N125K. Bearing in mind that limited variability is an important parameter when designing vaccines (Garzon-Ospina et al. 2011; Patarroyo et al. 2011), it was thus suggested that *Pv12* polymorphism should be examined to consider whether this gene, or parts of its sequence, could be used as components of a vaccine against *P. vivax*.

In silico *Pv*12 Characterization

The *P. vivax* VCG-1 strain *Pv*12 protein has 362 amino acids, having an estimated ~41 kDa molecular weight. A signal peptide having a probable cleavage site in amino acid 23 (AEG-FT) and a GPI-anchor sequence localized between amino acids 341 to 362 was predicted for the *Pv*12 protein using signalP 3.0 and FragAnchor algorithms (Fig. 3B). A tandem repeat (TR) which turned out to be exclusive for *P. vivax* was also found between amino acids 96 to 110 (Fig. 1). This TR consisted of five blocks of three amino acids having the N(A/V)(H/Q) consensus sequence.

A search of the Interpro database revealed the presence of two 6-Cys domains localized towards the *Pv*12 N and C-terminal portions (Fig. 3B), which coincided with the conserved region found in the alignment between the orthologous protein sequences (Fig. 1). This domain is characteristic of the 6-Cys protein family which has a particular 6-cysteine residue pattern leading to the formation of 3 disulfide bonds as follows: Cys 1 and 2, Cys 3 and 6, Cys 4 and 5 (Carter et al. 1995; van Dijk et al. 2001) (Fig. 3B). It has been shown that the 6-Cys domain is fundamental in carrying out the initial fertilization phase which consists of gamete surface proteins recognizing and binding to each other (van Dijk et al. 2010). The fact that several members of this family act as ligands binding to other proteins suggests that *Pv*12 probably plays an active role in interaction with cell receptors.

*Pv*12 Expression and Association with DRM

Polyclonal rabbit sera directed against two *Pv*12 synthetic peptides were produced for determining protein expression during the parasite's mature blood stage. The *Pv*12 protein was recombinantly

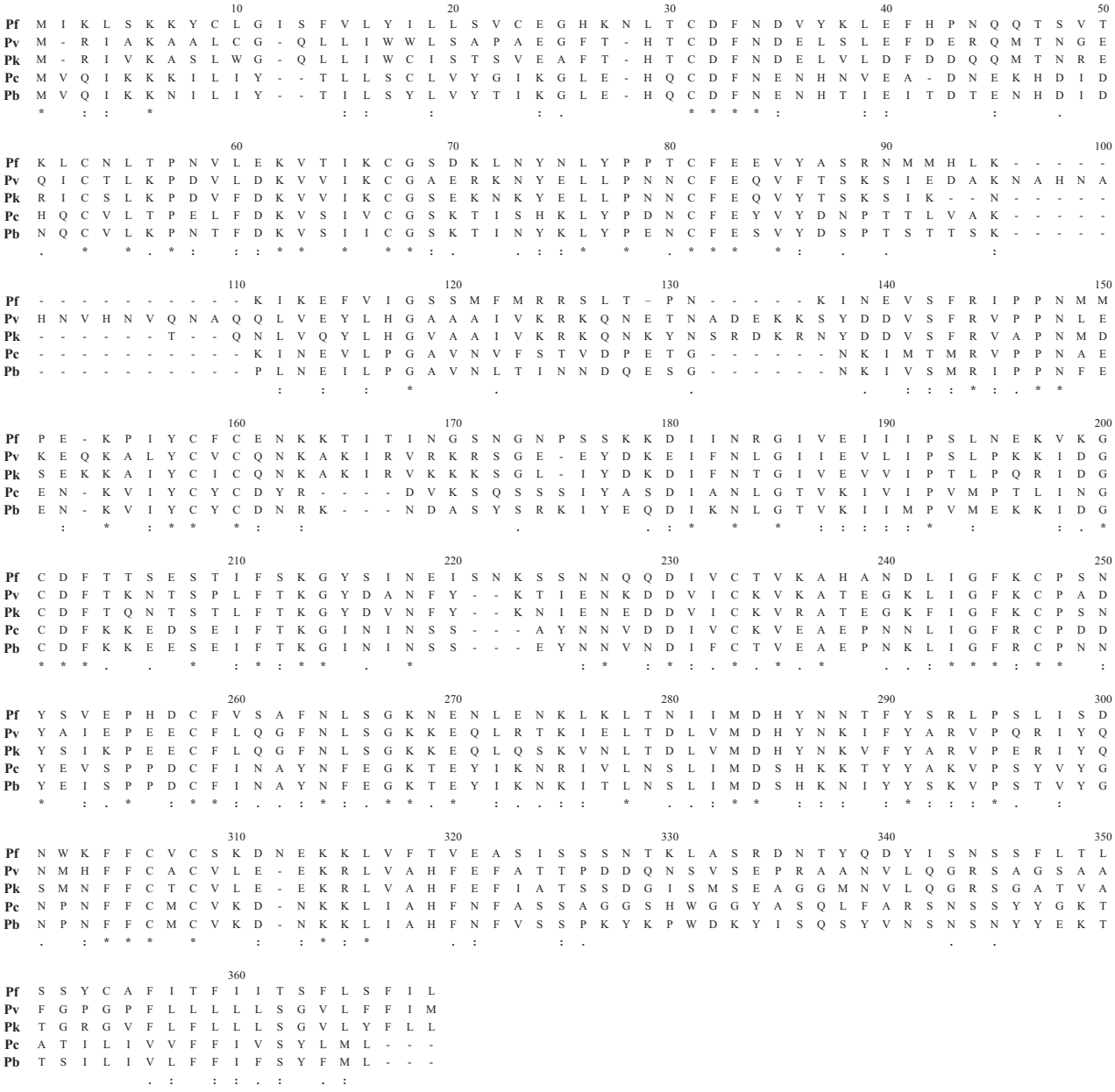

Figure 1. Alignment of *Pf*12 orthologous proteins in different malarial species using ClustalW software. This figure shows two conserved regions localized toward the N- and C-terminal ends as well as a repeat region exclusive to *P. vivax*. *Pf*: *P. falciparum, Pv*: *P. vivax, Pk*: *P. knowlesi, Pc: P. chabaudi* and *Pb*: *P. berghei.* Asterisks indicate identical amino acids; a dot refers to weakly similar amino acids and two dots indicate strongly similar amino acids.

expressed, excluding the secretion signal and the GPI anchor sequence, purified (Fig. 4A) and then used as antigen in Western blot assays. Figure 4B shows the antibodies' ability to recognize r*Pv*12 (lanes 3 and 4). Once the rabbit sera had been used as primary antibody in the blot containing *P. vivax* schizont-enriched lysate, they recognized a single ~38.6 kDa band coinciding with the expected molecular weight for the protein without the signal peptide (Fig. 4C, lane 2), thereby confirming that *Pv*12 does not present any type of proteolytic processing, the same as *Pf*12 (Bradley et al. 2005).

The parasite lysate solubilized at different temperatures was used in recognition assays with anti-*Pv*12 for confirming whether this antigen was present in the DRM; *Pv*12 was detected by the

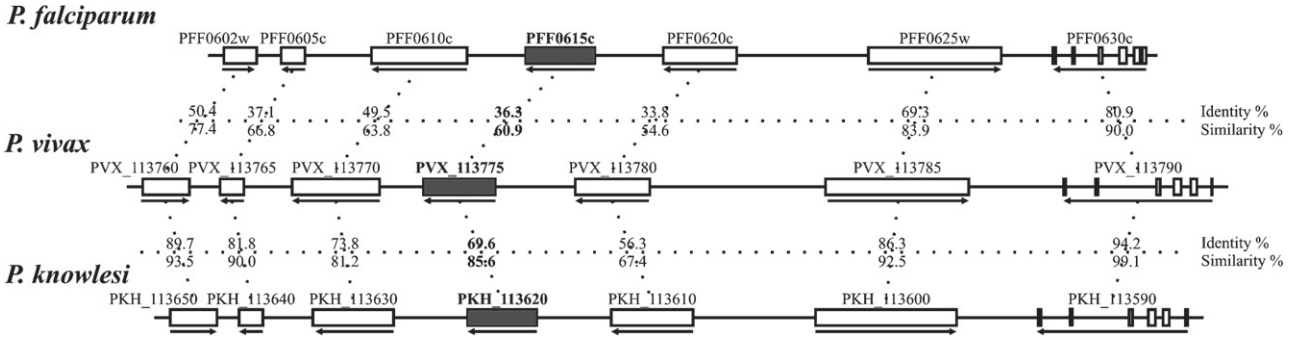

Figure 2. Schematic-scale organization showing *Pf12* (PFF0615c), *Pv12* (PVX_113775) and *Pk12* (PKH_113620) genes' localization within *P. falciparum*, *P. vivax* and *P. knowlesi* chromosome fragments. Exon-intron organization and ORF orientation are shown for each gene. Gene IDs are shown in accordance with the plasmoDB database. Identity (Id) and similarity (S) values between each hypothetical protein product contained in *P. falciparum*-*P. vivax* and *P. vivax*-*P. knowlesi* upstream and downstream contigs are shown.

antibodies as a ~38.6 kDa band (Fig. 4C, lanes 3-10). The protein was partially insoluble upon treatment at 4 °C and became soluble with Triton X-100 at 37 °C (Fig. 4C, lanes 6 and 8). The presence of a band having less intensity in the soluble fraction at 4 °C (Fig. 4C, lane 4) may have been attributable to the protein's abundant expression in the schizont-enriched sample. As it has been shown that several DRM proteins such as *Pf*12, *Pf*34 and *Pf*38 (Garcia et al. 2009) act as RBCs binding ligands, thus suggesting that *Pv*12 might play a role in interaction with such cells.

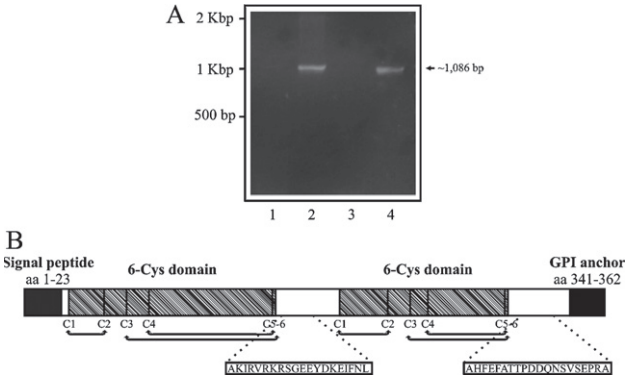

Figure 3. *Pv12* gene transcription. **A.** *Pv12* gene amplification by PCR. Lanes 1 and 2, PCR using RT (-) and RT (+) samples, respectively. Lane 3, PCR negative control using DNase-free water as template. Lane 4, gDNA amplification. **B.** *in silico* characterization of the *Pv*12 protein showing the localization of the signal peptide, the GPI anchor sequence and the 6-Cys domains with their respective Cys residues (C1-C6). The peptides selected for the immunization assay are shown in boxes.

*Pv*12 Sub-cellular Localization

A blood sample taken from a *P. vivax*-infected *Aotus* monkey was used as a source of fresh parasitized reticulocytes for evaluating *Pv*12 sub-cellular localization. Anti-*Pv*12 antibodies strongly detected the protein in schizonts while it appeared more faintly in ring and trophozoite forms (Fig. 5A-C). Double labelling was carried out to confirm its localization, using anti-*Pv*MSP10 *Aotus* antibodies and anti-*Pv*12 rabbit antibodies; both *Pv*12 and *Pv*MSP10 proteins were localized on each merozoite surface within mature schizonts as evidenced by signal overlap (Fig. 5C). The well-known *Pv*RON2 rhoptry neck marker was used to determine whether *Pv*12 was present in this organelle, as very recently described (Li et al. 2012). Figure 5D shows that the *Pv*RON2 florescence signal did not overlap with that of the VCG-1 strain *Pv*12 protein, thereby confirming that this protein is not localized in the rhoptry neck. It is worth noting that the peptides used in this study for rabbit immunization were deliberately designed outside of the 6-Cys domains to avoid antibody response cross-reactivity; tBlastn analysis confirmed that the peptides are unique to the *P. vivax* *Pv*12 protein. These results support the *Pv*12 antigen being mainly expressed at the end of the intra-erythrocyte life-cycle and having a localization pattern characteristic of surface proteins, as occurs with that previously shown for its homolog (Sanders et al. 2005).

*Pv*12 Antigenicity

Antibody response against *Pv*12 was initially evaluated by Western blot using sera from 19 *Aotus*

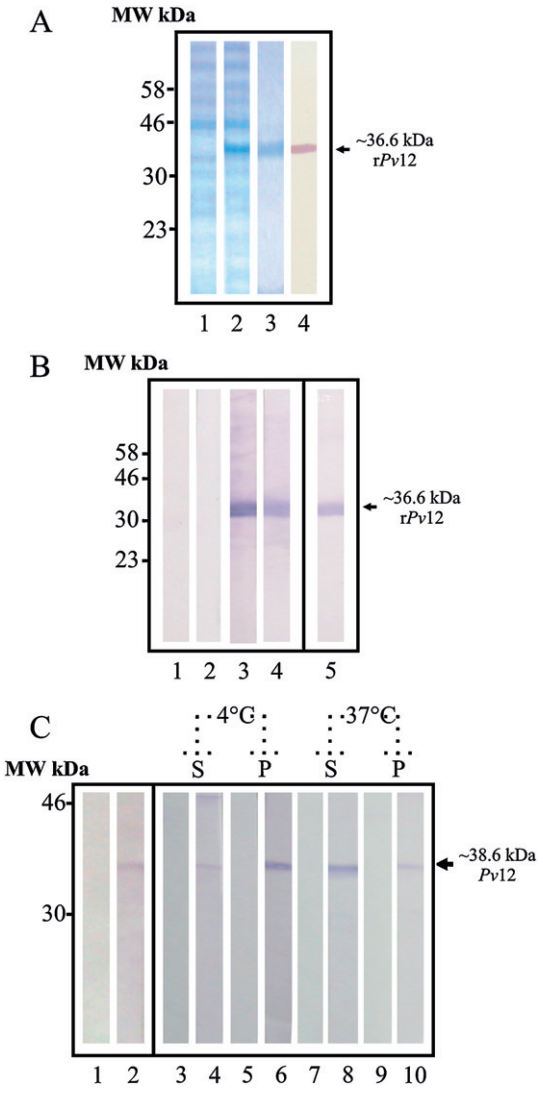

Figure 4. Detection of the recombinant and native protein by polyclonal antibodies. **A.** SDS-PAGE and Western blot analysis of r*Pv*12 expression and purification. Lanes 1-2, show the lysate supernatants from induced and un-induced cells. Lane 3, Coomassie staining of affinity purified r*Pv*12. Lane 4, Western blot analysis of purified recombinant protein. **B.** Antibody ability to recognize r*Pv*12 by Western blot. Lanes 1-2 show the absence of reactivity with rabbits 70 and 74 pre-immune sera. Lanes 3-4 indicate recognition of purified r*Pv*12 by rabbits 70 and 74 post-III sera. Lane 5 shows detection with monoclonal anti-polyhistidine antibody. **C.** Detection of the protein in total parasite lysate and in the DRM. Lanes 1 and 2, recognition of the *Pv*12 antigen in parasite lysate with pre-immune and post-III sera. Lanes 3-10, DRM fractions treated at 4 °C and 37 °C. Even numbered lanes show recognition with 70 post-III sera; odd lanes show no detection with 70 pre-immune sera. MW kDa indicates molecular weight in kDa. The letters S and P represent supernatant and pellet.

monkeys which had been experimentally infected with *P. vivax* and two *Aotus* monkeys' healthy sera as negative control (Fig. 6A). Subsequently, sera from 33 people living in malaria-endemic areas and who had presented episodes of this disease, as well as sera from 5 people who had never been infected with malaria, were used to determine *Pv*12 protein antigenicity (Fig. 6B). The Western blot analysis showed that most human and monkey immune sera detected r*Pv*12, unlike sera from the controls (Fig. 6A-B), indicating that this antigen could trigger a humoral immune response during natural or experimental malarial infections caused by *P. vivax*.

Human sera were screened by ELISA (Fig. 7A) for determining the anti-*Pv*12- specific antibody titer produced during natural infections; twenty eight of the thirty-three (~84.84%) sera recognized r*Pv*12, having OD values which were significantly much higher than the OD value obtained from the negative controls plus 2 SD. Evaluation of the sera having OD>0.7 revealed the presence of antibodies in up to 1:12,800 dilution for sera 15 and 19, and up to 1:102,400 dilution for sera 4, 5 and 31, whereas there were no detectable levels of anti-*Pv*12 in the control group (Fig. 7B); this result highlighted the potent immune response which *Pv*12 could produce in the host. It has been shown that antibodies directed against proteins exposed on the merozoite surface or those released during invasion are critical in inducing acquired immunity (Chuangchaiya et al. 2009; Cohen et al. 1961). Researchers working on developing a multi-valent antimalarial vaccine have established that immune responses to multiple antigenic targets may be important for effective protection (Patarroyo et al. 2011). Therefore, and taking into account the *Pv*12 protein's antigenic potential, further studies are thus needed to confirm the molecule's importance for being included in an anti-malarial vaccine against *P. vivax*.

This study has described the identification and characterization of the *P. vivax Pv*12 surface protein. The parameters identified here, such as its remarkable conservation within the genus *Plasmodium*, the presence of a signal peptide and a GPI anchor sequence, its association with DRM and the strong recognition by sera of *Aotus* monkeys and that of humans infected with *P. vivax* malaria, all support the fact that *Pv*12 could play a functional role during invasion of RBCs. It is thus recommended that its immunogenicity and protection-inducing ability be evaluated in the *Aotus* experimental model.

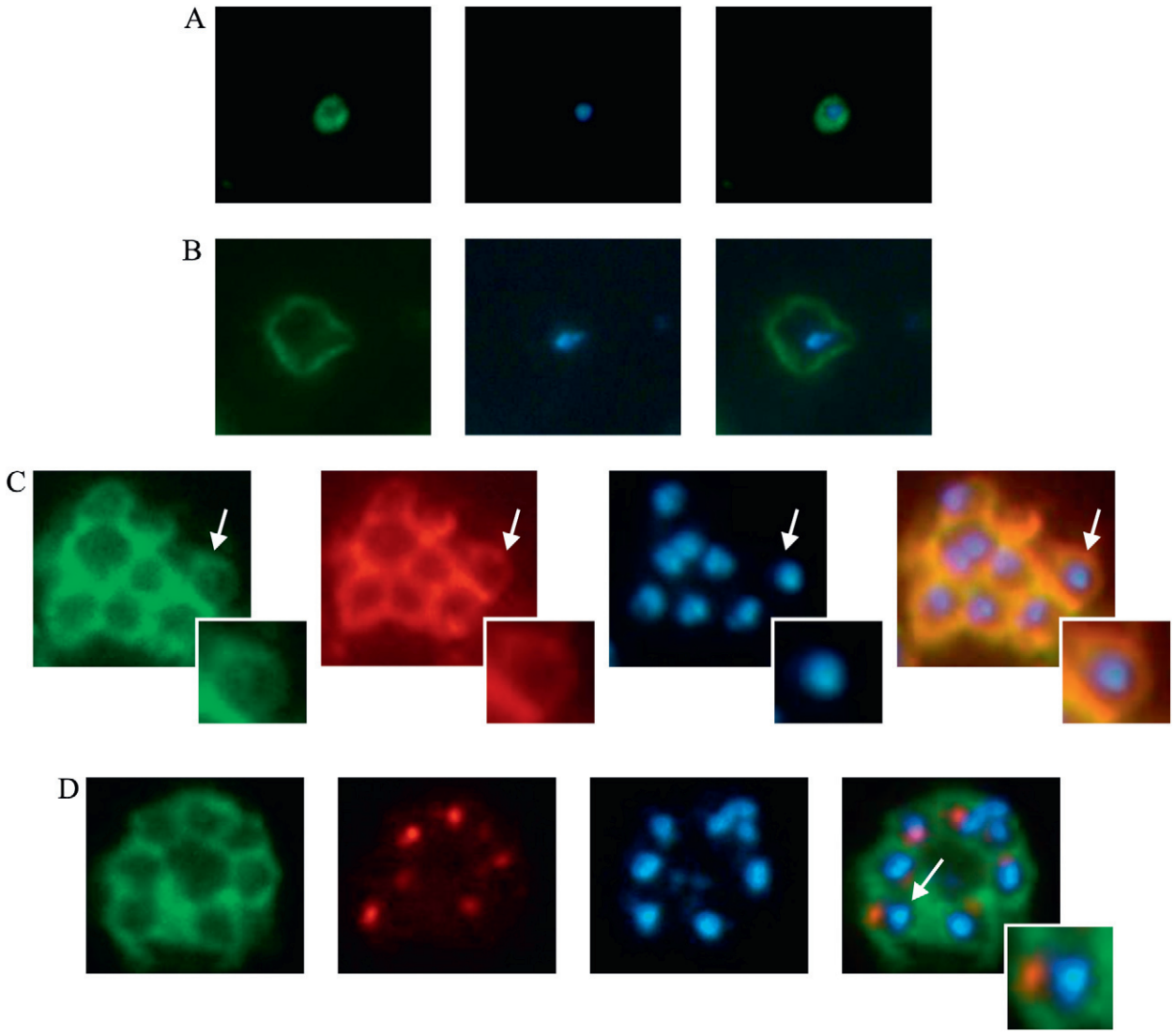

Figure 5. *Pv*12 sub-cellular localization in *P. vivax* blood stages. **A.** Protein detection in rings. **B**. Protein localization in trophozoites. **C**. Double-labeling with antibodies against *Pv*12 (green) and *Pv*MSP10 (red) proteins. **D**. *Pv*12 (green) and *Pv*RON2 (red) localization. Each small box shows a zoomed image of a single merozoite clearly indicated by an arrow (nuclei are shown in blue).

Methods

Bioinformatics analysis: The *pv12* gene sequence, which is homologous to that of the *Pf*12-encoding gene, was obtained from the plasmoDB database; this database was also searched for orthologous genes in other malarial species. The synteny of the *P. falciparum, P. vivax* and *P. knowlesi* species' chromosome regions containing the gene of interest was evaluated by comparing open reading frame (ORF) orientation and the exon-intron organization of the genes with the hypothetic protein products. Identity (Id) and similarity (S) values were ascertained by the ClustalW software (Thompson et al. 1994). The presence of a secretion signal and a GPI anchor sequence were determined by using SignalP 3.0 (Bendtsen et al. 2004) and FragAnchor tools (Poisson et al. 2007). The Interpro database (Hunter et al. 2009) was also scanned in the search for putative domains in the whole protein sequence. Specific primer design was based on the reference gene sequence (Sal-1 strain), the same as B-cell lineal epitopes predicted with Antheprot (Deleage et al. 2001) using the amino acid sequence. The latter were exclusive to the *P. vivax Pv*12 protein once the peptide sequences had been analyzed by tBlastn.

Animal handling: The experimental handling of the animals used here was carried out in accordance with Colombian Law 84/1989 and resolution 504/1996. *Aotus* monkeys kept at FIDIC's primate station (Leticia, Amazon) and New Zealand rabbits provided by the Instituto Nacional de Salud (Bogotá, Colombia) were handled following the guidelines for the care and use of laboratory animals (National Institute of Health, USA) under the constant supervision of a veterinarian. The immunization and bleeding procedures for *Aotus* monkeys had been previously approved by our institute's ethics committee and

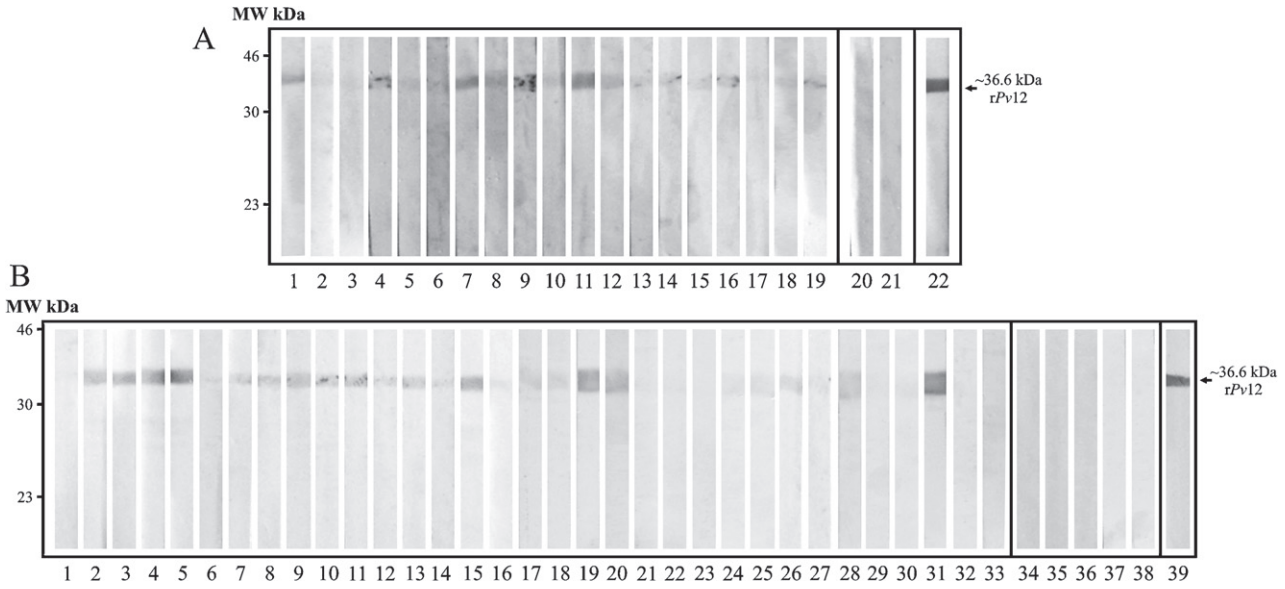

Figure 6. *Pv*12 antigenicity in natural and experimental infection. **A.** Shows the detection of the *Pv*12 recombinant protein with *Aotus* monkey sera; lanes 1-19 correspond to detection with different *Aotus* monkey sera previously infected with *P. vivax*. Lanes 20 and 21 show the absence of reactivity with sera from healthy monkeys. Lane 22, detection of the protein using anti-polyhistidine monoclonal antibody. **B.** Western blot showing recognition of r*Pv*12 by human sera. Lanes 1-33 show recognition with sera from individuals infected with *P. vivax*. Lanes 34-38, strips incubated with sera from healthy individuals. Lane 39, positive control from the assay using anti-polyhistidine monoclonal antibody.

were carried out in line with the conditions stipulated by CorpoAmazonia (resolution 00066, September 13th, 2006). A monkey from the *Aotus* genus was experimentally infected with the VCG-1 (Vivax Colombia Guaviare 1) strain and monitored daily to assess the progress of its infection during the whole study (up to day 18) using Acridine Orange staining. The monkey was treated with pediatric doses of chloroquine (10 mg/kg on the first day and 7.5 mg/kg/day until the fifth day) and primaquine (0.25 mg/kg/day from the third to the fifth day) at the end of the study to guarantee total clearance of parasites from its blood. CorpoAmazonia officers supervised the primate's return to its natural habitat in excellent health once the experiments were over.

Isolating the *P. vivax* parasite: VCG-1 strain parasites were maintained *in vivo* according to a previously described methodology (Pico de Coana et al. 2003). Mature forms of the parasite, mainly schizonts, were purified from a *P. vivax*-infected blood sample (3 mL) using a discontinuous Percoll gradient (GE Healthcare, Uppsala, Sweden) according to an already established protocol (Andrysiak et al. 1986); this sample was used as the source of parasite RNA, genomic DNA (gDNA) and total proteins.

RNA extraction and cDNA synthesis: Total RNA from the schizont-enriched sample was extracted using the TRIzol method and treated with RQ1 RNase-free DNase (Promega, Wisconsin, USA) according to the manufacturer's recommendations. 5 μL of RNA were used to synthesize complementary DNA (cDNA) with the SuperScript III enzyme (Invitrogen, California, USA) in the following conditions: 5 min at 65 °C, 1 hour at 50 °C and 15 min at 70 °C. The product was stored at -20 °C until needed for use, following a period of incubation with RNase (Promega, USA) for 15 min at 37 °C.

Cloning and sequencing: The gDNA obtained using the Wizard Genomic DNA purification kit (Promega) and the cDNA were used as templates for a 50 μL PCR reaction containing TAQXpedite PCR MasterMix (100 mM Tris-HCl (pH 8.3), 100 mM KCl, and 400 μM of each dNTP), 0.5 μM of each primer and 0.5 U of TAQXpedite (EPICENTRE, Biotechnologies). The specific primer sets used here were designed for amplifying the complete *Pv*12 gene sequence (forward 5′- ATGCGCATTGCGAAGGC-3′ and reverse 5′-CATTATAAAGAAAAGGACCC-3′) or the *Pv*12 gene without the signal peptide or the GPI anchor sequence (forward 5′- ATGACGTGCGATTTTAATGA-3′ and reverse 5′-GCCCTGCAGAACATTC-3′). The start codon was added to the forward primer's 5′ extreme in the latter case. The PCR began with a denaturing step for 5 min at 94 °C, followed by 35 cycles for 20 s at 94 °C, 10 s at 50 °C and 1 min at 68 °C. Once the quality of the amplicons had been evaluated on a 1% agarose gel, these were purified by using a Wizard PCR preps kit (Promega) and then ligated to the pEXP5 CT/TOPO vector, thereby adding a 6-histidine tag towards the C-terminal end which facilitated recombinant purification and recognition by the anti-histidine monoclonal antibody. After transformation of the *Escherichia coli* TOP10 cells (Invitrogen), each pEXP5-*Pv*12 recombinant plasmid was purified by using an Ultra Clean mini plasmid prepkit (MO BIO laboratories, California, USA); sequence integrity and its correct insertion into the vector was confirmed by automatically sequencing the two clones obtained from independent PCRs using an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, California, USA).

Parasite protein extraction: The *P. vivax* schizont pellet was treated with 0.2% saponin to remove the surrounding erythrocyte proteins and then washed 6 times in phosphate

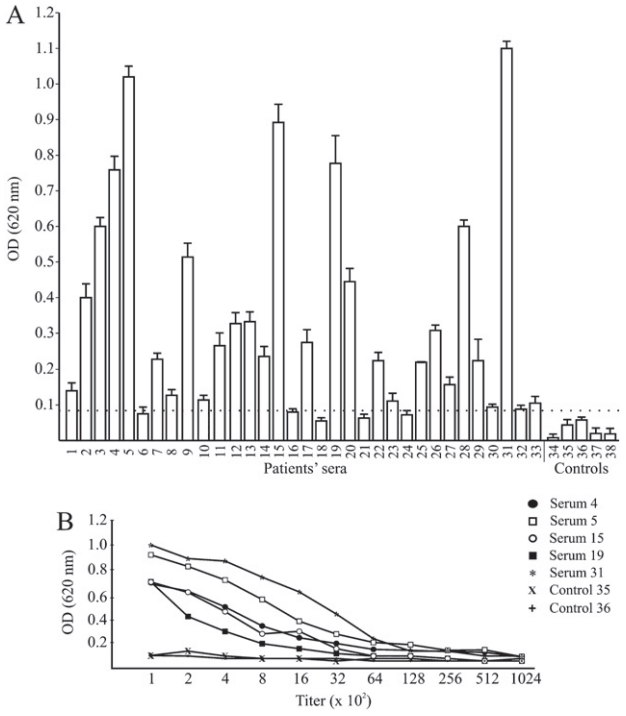

Figure 7. Anti-*Pv*12 antibody titers determined by ELISA. **A.** *P. vivax*-infected patients' sera reactivity against r*Pv*12 by ELISA. Lanes 1-33 indicate recognition by sera from patients suffering from malaria. Lanes 34-38 show detection with healthy individuals' sera. **B.** Determining anti-*Pv*12 antibody titers estimated by successive dilutions of human sera until reaching an A620 value equal to previously established cut-off absorbance.

buffered saline (PBS). The sample was homogenized in lysis buffer (5% SDS, 10 mM PMSF, 10 mM iodoacetamide, 1 mM EDTA) and then spun at 13,000 rpm. The proteins from the supernatant were quantified using a BCA protein assay kit (Thermo scientific) and then stored at -20 °C.

Peptide synthesis and polyclonal antibodies production: Two 20 amino acid-long peptides which had been designed on the Sal-1 strain *Pv*12 protein sequence (CG-AKIRVRKRSGEEYDKEIFNL-GC and CG-AHFEFATTPDDQNSVSEPRA-GC) were synthesized according to a previously established methodology (Houghten 1985), polymerized, lyophilized and then characterized by RP-HPLC and MALDI-TOF MS. A 150 μg dose of a mixture of both synthetic peptides emulsified in Freund's complete adjuvant (FCA) (Sigma, Missouri, USA) was inoculated into two New Zealand rabbits (rabbit 70 and 74) on day 0, while the same mixture emulsified in Freund's incomplete adjuvant (FIA) was inoculated on days 21 and 42. The sera were collected before the first immunization (pre-immune sera) and 20 days after the last dose (post-III sera) and then absorbed with *E. coli* proteins coupled to a Sepharose column, according to the manufacturer's recommendations (Amersham Biosciences, Buckinghamshire, UK).

Detergent-resistant microdomain (DRM) isolation: Mature *P. vivax* schizonts which had been extracted by using the Percoll method were treated with 0.2% saponin in PBS for 5 min. Following 6 washes in PBS, the pellet was homogenized in 200 μL of TNET solution (1% Triton X-100, 25 mM Tris-HCl, 150 mM NaCl and 1 mM EDTA) containing a protease inhibitor cocktail (1 mM PMSF, 1 mM iodoacetamide, 1 mM EDTA and 1 mg/mL leupeptin). The sample was divided into two aliquots and treated in the following conditions: a 100 μL aliquot was incubated for 30 min at 4 °C and then spun at 7,550 rpm for 10 min; the remaining sample was kept at 37 °C for 30 min and then spun in the same previously described conditions. The supernatant was skimmed off and the pellet was homogenized in lysis buffer (5% SDS, 10 mM PMSF, 10 mM iodoacetamide, 1 mM EDTA). Each fraction was quantified using a micro BCA protein assay kit (Thermo scientific).

Recombinant protein expression and purification: The pEXP5-*Pv*12 recombinant plasmid containing the gene without the signal peptide or the GPI anchor sequence was transfected in *E. coli* BL21-AI bacteria (Invitrogen) which were grown overnight at 37 °C in Luria Bertani (LB) medium containing 100 μg/mL ampicillin and 0.1% (w/v) D-glucose with constant shaking (~300 rpm). Once a 0.6 OD_{600} had been reached, 0.2% L-arabinose (w/v) was added to induce expression for 4 hours. The culture was centrifuged at 13,000 rpm for 30 min and lysed by sonication after having been homogenized in extraction buffer (EB) (6 M Urea, 12 mM imidazol, 10 mM Tris-Cl, 100 mM NaH2PO4 and 10 mg/mL lysozyme) supplemented with protease inhibitors (1 mM PMSF, 1 mM iodoacetamide, 1 mM EDTA and 1 mg/mL leupeptin). Protein expression was verified by Western blot and r*Pv*12 was then purified by solid phase affinity chromatography using Ni+2-NTA resin (Qiagen, California, USA), according to the manufacturer's recommendations. Briefly, total lysate was incubated with the resin overnight at 4 °C. The r*Pv*12 protein coupled to the resin was poured into a column and then washed several times with EB to eliminate weakly-coupled proteins. The r*Pv*12 was eluted with EB containing 500 mM imidazole, in 3 mL fractions, which were analyzed by Comassie blue staining to verify the presence of a single band and then dialyzed in PBS at pH 7.0 to achieve protein refolding. After the protein had been concentrated by using an Amicom filtration system (LabX scientific marketplace, Midland, Canada) it was quantified by using a micro BCA protein assay kit (Thermo scientific) using bovine serum albumin (BSA) as standard.

SDS-PAGE and Western blot: The purified r*Pv*12 (1 μg), total lysate (50 μg) and DRM fractions (50 μg) were run on 12% SDS-PAGE gels and then transferred to nitrocellulose membranes. After blocking with a 5% skimmed milk PBS-0.05% Tween solution for 1 hour, each membrane was cut into strips and individually analyzed as follows. The strips with the recombinant protein were incubated with a 1:100 dilution of rabbit sera, *Aotus* monkey or human sera (stored in FIDIC's serumbank) containing 5% skimmed milk PBS-0.05% Tween for 1 hour at room temperature (RT). Likewise, the total parasite lysate strips and those containing the DRM fractions were incubated for 1 hour at RT in 5% skimmed milk PBS-0.05% Tween, including a 1:100 dilution of rabbit sera. Following 3 washes, the strips were incubated for 1 hour with phosphatase-coupled goat anti-rabbit IgG (1:4,500) secondary antibody, goat anti-monkey IgG (1:1,000) or goat anti-human IgG (1:10,000) at RT. A strip which had been incubated with peroxidase-coupled anti-histidine monoclonal antibody (1:4,500) was used as positive control for r*Pv*12 Western blotting. The blots were revealed with a VIP peroxidase substrate kit (Vector Laboratories, Burlingame, Canada) or BCIP/NBT color development substrate kits (Promega), following the manufacturer's indications.

Indirect immunofluorescence assay (IFA): *P. vivax*-parasitized RBCs were washed 3 times with PBS and then diluted in this solution until 5-7 schizonts per field were obtained, as evaluated by acridine orange staining. 20 μL of the sample were placed in each well on a glass 8-well multitest slide (Biomedicals, Inc) and the supernatant was then skimmed off 10 min later. Once they had dried, the samples were fixed in 4% formaldehyde for 5 min and then blocked for 10 min at RT with 1% skimmed milk-PBS solution (v/v). After three more washes, each sample was incubated with a mixture of anti-*Pv*12 rabbit 70 with anti-*Pv*MSP10 *Aotus* S034 sera or with mouse sera directed against a rhoptry neck marker (anti-*Pv*RON2) (previously obtained in our institute (Arevalo-Pinzon et al. 2011; Giraldo et al. 2009)), each one at 1:100 dilution for 1 hour (such dilution being the highest one giving positive peptide recognition by ELISA). The samples were then incubated with FITC-conjugated anti-rabbit IgG antibody (Sigma) (1:20) and Red-conjugated anti-*Aotus* IgG antibody (1:40) or Red-conjugated anti-mouse IgG antibody (1:200) for 30 min in the previously described conditions. Slides were stained with DAPI (0.5 μg/mL) for 10 min at RT and two washes with 0.05% PBS-Tween and others with PBS were used to remove the excess reagent. An Olympus BX51 fluorescence microscope was used to observe the slides using 100× oil immersion objective and Volocity software (5.3.2 version) was then used to overlay the images.

Enzyme-linked immuno sorbent assay (ELISA): 96-well polysorb plates were covered with 1 μg r*Pv*12 per well overnight at 4 °C and then incubated for 1 hour at 37 °C. The wells were blocked with 200 μL 5% skimmed milk-PBS-0.05% Tween for 1 hour at 37 °C. The antibodies' reactivity against r*Pv*12 was evaluated by incubating the plates with a 1:100 dilution of human sera in 5% skimmed milk-PBS-0.05% Tween for 1 hour at 37 °C. A peroxidase substrate solution was added (KPL Laboratories, WA, USA) to reveal the reaction, according to the manufacturer's recommendations, after incubating the wells with peroxidase-coupled anti-human IgG secondary antibody (1:10,000) diluted in 5% skimmed milk-PBS–0.05% Tween for 1 hour at 37 °C. The 620 nm specific optical density (OD) detected by the MJ ELISA multiskan reader was calculated by subtracting the OD value from the control well value (no antigen). The cut-off value for evaluating the positivity threshold was determined as being the mean of the OD plus twice the standard deviation of healthy patients' sera reactivity. Sera having OD>0.7 were further tested to determine antibody titers by successive half dilutions of the primary antibody until reaching an A620 value equal to the cut-off value previously established for the controls.

Sample source: Sera were obtained from 33 patients who were living in malaria-endemic areas of Colombia and who had suffered episodes of *P. vivax* infection as well as from healthy individuals who had never been affected by the disease. All individuals signed an informed consent form after receiving detailed information regarding the study goals. Sera were also obtained from monkeys experimentally infected with the parasite and from healthy monkeys kept in our institute.

Accession number: The nucleotide and amino acid sequences used here have been reported in the GenBank database, under accession number JN572105.

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