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Characterization and antigenicity of the promising vaccine candidate *Plasmodium vivax* 34 kDa rhoptry antigen (Pv34)

Alvaro Mongui[a,b,c], Diana I. Angel[a,c], Gina Gallego[a,c], Claudia Reyes[a,c], Paola Martinez[a,c], Felipe Guhl[b], Manuel A. Patarroyo[a,c,*]

[a] *Molecular Biology Department, Fundacion Instituto de Inmunologia de Colombia (FIDIC), Carrera 50 No. 26-20, Bogota, Colombia*
[b] *Universidad de los Andes, Carrera 1#18A-10, Bogota, Colombia*
[c] *Universidad del Rosario, Calle 63D#24-31, Bogota, Colombia*

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ABSTRACT

This study describes the identification of the *Plasmodium vivax* rhoptry antigen Pv34 whose sequence was obtained based on homology comparison with the *Plasmodium falciparum* Pf34. The *pv34* gene product was characterized by molecular biology and immunological techniques. Additionally, association of Pv34 to detergent-resistant microdomains (DRMs), expression in late blood-stage parasites and recognition of recombinant Pv34 (rPv34) by sera from *P. vivax*-infected *Aotus* monkeys and patients was assessed. Lymphoproliferation and cytokine secretion was also evaluated in individuals living in malaria endemic areas. Altogether, the data support carrying out further studies to assess the immunogenicity and protection-inducing ability of rPv34 as component of a multi-antigenic, multi-stage vaccine against vivax malaria.

1. Introduction

Malaria has been the most important parasitic disease throughout history causing about 300–500 million clinical cases each year, 1.5–2.7 million of which die as a consequence of the disease [1]. Additionally, more than 2 billion people live in the 88 countries where the disease is endemic, indicating that ~48% of the world's population is currently at risk of acquiring the disease [2]. The situation is further aggravated by the gradual emergence of parasite strains resistant to antimalarial drugs, as well as of insecticide-resistant *Anopheles* mosquito populations, which make it imperative to develop an effective antimalarial vaccine.

Of the four *Plasmodium* species causing malaria in humans, *Plasmodium falciparum* is responsible for the largest annual number of clinical cases mainly in Africa [3]. This has by itself encouraged a large number of studies carried out over the last few decades on the biology and pathology of this species, most of which have focused on the development of an effective vaccine to control this scourging disease.

In parasites of the genus *Plasmodium*, the identification of possible vaccine targets has been mainly focused on parasite surface antigens and proteins contained inside apical organelles, mainly in rhoptries and micronemes. In general, these apical organelles are shared by all members of the phylum Apicomplexa since they emerged as an early evolutive trait [4] and various proteins contained inside these organelles are relatively conserved among the different parasite genera; however, each genus' tropism for invading an specific cell line (*e.g.* erythrocytes, lymphocytes and epithelial cells) has led to the functional specialization of members belonging to this unique pool of proteins to fulfill different roles during target-cell invasion [5].

Proteins of the low molecular weight complex (LMW) (RAP1, RAP2 and RAP3), the high molecular weight complex (HMW) (RhopH1, RhopH2 and RhopH3), the rhoptry-associated membrane antigen (RAMA) and reticulocyte binding ligands (RBLs) are among the best characterized *P. falciparum* rhoptry antigens. It has been suggested that these proteins might play an important role in host cell selection, erythrocyte binding or parasitophorous vacuole formation [6].

Recent studies have enabled the identification of other parasite rhoptry proteins based on their localization on detergent-resistant microdomains (DRMs), as in the case of the Pf34 protein [7]. Same as other well-studied erythrocyte-stage vaccine candidates (*e.g.* MSP1, MSP2, MSP4 and MSP5), Pf34 contains a glycosylphos-

Abbreviations: Pv34, *P. vivax* 34 kDa rhoptry antigen; rPv34, recombinant *P. vivax* 34 kDa rhoptry antigen; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SaI-1, Salvador 1 strain; VCG-1, Vivax Colombia Guaviare 1 strain; DRMs, detergent-resistant microdomains; PBMCs, peripheral blood mononuclear cells.

* Corresponding author at: Molecular Biology Department, Fundacion Instituto de Inmunologia de Colombia, Carrera 50#26-20, Bogota, Colombia. Tel.: +57 1 3244672x143; fax: +57 1 4815269.
E-mail address: mapatarr.fidic@gmail.com (M.A. Patarroyo).

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phatidylinositol (GPI)-anchor, which is a common characteristic shared by putative erythrocyte invasion proteins [8]. It has been also demonstrated that the gene encoding Pf34 is actively transcribed in late-erythrocyte stages [9], showing strong correlation with the expression of the native protein during the schizont stage. Moreover, the significant recognition of Pf34 (obtained as a recombinant protein) by hyperimmune sera from patients living in malaria endemic areas [7], highlights Pf34 as an attractive candidate for a vaccine against *P. falciparum* malaria.

On the other hand, advances in the identification and characterization of vaccine candidates in *Plasmodium vivax* (the second most prevalent *Plasmodium* species with predominant distribution in Asia and South America) has been notably delayed due to the difficulty of maintaining a long-term culture of this species *in vitro* given its preference for invading human reticulocyte subpopulations (accounting for only 1–2% of the total blood cell count). This phenomenon together with the absence of endothelial cytoadherence explains the milder symptoms seen in *P. vivax* malaria compared to malaria caused by *P. falciparum*. The unavailability of a standardized continuous culture considerably delayed the completion of the *P. vivax* genome [10] and transcriptome (of the intraerythrocytic cycle) [11] compared to *P. falciparum*, and is reflected in the current absence of proteomic data.

Aiming at developing a vaccine against *P. vivax* malaria, our research group has focused on the identification of new antigens based on homology comparison with previously characterized *P. falciparum* antigens. Using the just until recently partial *P. vivax* genomic sequence and a *P. vivax* strain adapted to *Aotus* monkeys [12], we have been able to identify various surface proteins (MSP-7, -8, -10 and Pv41) [13–16] as well as several rhoptry proteins (RAP-1, -2, RhopH3 and Pv38) [17–20], which are likely to play an important role in parasite invasion to red blood cells and are currently being tested as vaccine candidates.

The present study shows the expression of the Pf34 homolog in *P. vivax*, here denoted as Pv34, its subcellular localization in late-intraerythrocytic parasite life-cycle stages, its recognition by sera from *P. vivax*-infected patients and *Aotus* monkeys when expressed as a recombinant protein (rPv34), its ability to stimulate proliferation of peripheral blood mononuclear cells (PBMCs) from individuals with a history of *P. vivax* malaria and the resulting cytokine profile.

2. Materials and methods

2.1. Bioinformatics analysis

The *P. vivax* SaI-1 strain genome (available at http://www.tigr.org/tdb/e2k1/pva1/) was scanned by tBlastn using Pf34 as query sequence (GenBank accession no. CAD49234.1; PlasmoDB accession no. PFD0955w) in order to confirm the sequence of the gene encoding Pv34. The sequence yielding the highest score was selected as the putative Pv34 sequence. Sanger Institute and J. Craig Venter Institute (JCVI) databases holding partial genome sequences from other plasmodial species were also screened searching for *pf34* or *pv34* homologous genes. Moreover, open reading frames (ORFs) adjacent to *pf34*, *pv34* and *pk34* were analyzed using GenScan and GeneComber [21,22]. Identity and similarity values between *P. vivax*–*P. falciparum* and *P. vivax*–*P. knowlesi* peptide sequences were obtained using the ALignX tool from the VectorNTI Suite 9 bioinformatics software package (Invitrogen, California, USA). The presence of a signal peptide and a GPI-anchor site was determined by using SignalP 3.0 [23] and FragAnchor [24], respectively. Tandem repeats in the Pv34 sequence were identified using the XSTREAM server [25]. The presence of lineal B epitopes in Pv34 was determined using the Bepipred (at a default 0.35 threshold and 75% of specificity) [26]. Parker's antigenicity, solvent accessibility and hydrophilicity values were evaluated using the Antheprot software [27].

2.2. Animal handling

All animals used in this study (rabbits and *Aotus* spp. monkeys) were kept at FIDIC's primate station in Leticia, Amazonas and taken care according to procedures previously established by the Office for Protection from Research Risks (OPRR, Department of Health and Human Services, USA), under the constant supervision of a primatologist. Immunization and bleeding procedures on *Aotus* monkeys were carried out in agreement with the conditions stipulated by CorpoAmazonia (resolution 00066, September 13th 2006). Ten wild-caught *Aotus* monkeys were experimentally infected with *P. vivax* (as described below) and their parasitemia levels were assessed daily by Acridine orange staining until monkeys had developed 3–5% parasitemias and 5–7 schizonts were observed per field under the microscope. Monkeys were immediately treated whenever parasitemias were $\geq$5% or before if recommended by the supervising primatologist. Treatment consisted of orally administered pediatric doses of Chloroquine (10 mg/kg on the first day and 7.5 mg/kg per day until day 5) and Primaquine (0.25 mg/kg starting on day 3 and until day 5). Once assuring total clearance of parasites from blood and excellent health conditions, monkeys were released back into their natural habitat close to the site where they had been captured with the supervision of CorpoAmazonia officials. All procedures were approved by our institute's ethical committee.

2.3. Isolation of P. vivax parasites

Parasites from the VCG-1 (Vivax Colombia Guaviare 1) strain were cultured by successive passes in *Aotus* monkeys as previously described elsewhere [12]. Briefly, infected red blood cells (primarily at the schizont stage) were extracted from 3 to 4 mL *Aotus* blood samples using a discontinuous Percoll gradient (GE Healthcare, Uppsala, Sweden), according to a previously described protocol [28]. The isolated parasite pellet was used in either of the following procedures: (1) RNA extraction, (2) genomic DNA extraction, (3) total protein extraction, (4) DRM isolation or (5) immunofluorescence assays.

2.4. RNA extraction, cDNA synthesis and genomic DNA isolation

Parasite RNA was isolated by the Trizol methodology [29] and used for reverse transcription assays. In brief, 1–5 μg RNA was synthesized into cDNA by using the one-step RT-PCR SuperScript III kit (Invitrogen), according to the manufacturer's recommendations. Genomic DNA was isolated from a parasite pellet resuspended in 300 μL 1× phosphate-buffered saline (PBS) by using the UltraClean Blood DNA Isolation kit (MO BIO, California, USA). The integrity of the purified RNA and genomic DNA was examined by electrophoresis in agarose gels.

2.5. Cloning and sequencing

Based on the SaI-1 strain *pv34* nucleotide sequence, specific forward (5′-ATGATGAATGTTTTCTTCTGC-3′) and reverse (5′-GCTGAGCAGAAAGGCGAT-3′) primers were designed to amplify the entire *pv34* gene from both cDNA and genomic DNA. These primers were included in PCR reactions with the Platinum *Pfx* DNA polymerase enzyme (Invitrogen). PCR amplification was carried out according to the following temperature profile: 1 cycle at 94 °C for 2 min, 35 cycles of 56 °C for 30 s, 68 °C for 80 s and 94 °C for 10 s, and a final extension step at 68 °C for 5 min. Each amplified fragment was purified by using the Wizard PCR preps Kit (Promega, Wisconsin, USA). Only *pv34* PCR products obtained from cDNA

were cloned into the pEXP5 CT/TOPO vector (Invitrogen) once having added adenines to their 3′ends. Finally, the integrity of the cloned fragments was verified by sequencing in an automatic ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, California, USA). Sequencing results were compared and analyzed by aligning nucleotide and amino acid sequences against the SaI-1 reference strain using ClustalW [30].

2.6. Expression and purification of rPv34

E. coli BL21-AI bacteria (Invitrogen) transformed with the pEXP5-*pv34* plasmid were grown in 100 mL of Terrific Broth containing 0.1 mg/mL ampicillin. Once the bacteria culture had reached an optical density of 0.6 (measured in a spectrophotometer at 600 nm), 0.2% arabinose (*w*/*v*) was added as expression inductor. Cells were incubated for 5 h at 37 °C and then harvested by centrifugation at 12,000 × *g* for 30 min at 4 °C. The cell pellet was resuspended in extraction buffer containing 6–8 M urea, 15 mM imidazole, 10 mM Tris–Cl, 100 mM NaH_2PO_4 and lysozyme (10 mg/mL), supplemented with protease inhibitors (100 mM PMSF, 100 mM iodoacetamide, 0.5 M EDTA and 1 mg/mL leupeptine) and lysed by sonication. The resulting recombinant solubilized protein was recovered from the supernatant by centrifugation at 12,000 × *g* for 30 min at 4 °C. Since the pEXP5 vector adds a polyhistidine tag at the C-terminus of rPv34, this tag was used for purifying the recombinant protein by affinity chromatography using Ni^{+2}-NTA resin (Qiagen, California, USA), according to the manufacturer's recommendations. Briefly, the resin's pH was first adjusted to pH 8.0 with extraction buffer before loading the protein extract into the column. Non-retained proteins were eluted using the same extraction buffer, while an extraction buffer containing 500 mM imidazole was used to elute the recombinant protein. All individually collected fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The pure recombinant protein fractions were pooled and exhaustively dialyzed against PBS 1× pH 7.4 (protein refolding step).

2.7. Peptide synthesis

Two 20-amino-acid-long peptides were synthesized based on portions of the Pv34 deduced sequence (SaI-1). The amino acid sequences of these two peptides in single letter code were: ^{45}EVKAPKDDGGKEQDTLAGNK^{64} and ^{154}VQNEIKNNEKLNKEKKSYDE^{173}. One glycine and one cysteine were inserted at the N- and C-termini of each peptide to allow polymerization. Peptides were synthesized using the standard solid phase *t*-Boc/Bzl peptide synthesis strategy [31], lyophilized and characterized by RP-HPLC and MALDI-TOF mass spectrometry.

2.8. Peptide immunization and collection of polyclonal antibodies

Two New Zealand rabbits were immunized with a 150 μg mixture containing both polymerized synthetic peptides. The immunogen was emulsified in Freund's complete adjuvant (FCA) (Sigma, Missouri, USA) for the first dose, while two subsequent boosters administered 20 and 40 days later were emulsified in Freund's incomplete adjuvant (FIA). Sera were collected before the first immunization (pre-immune sera) and 20 days after administering the last booster dose (hyperimmune sera).

2.9. Extraction of parasite proteins

A parasite pellet was resuspended in lysis solution (5% *w*/*v* SDS, 1 mM EDTA, 10 mM PMSF and 10 mM iodoacetamide) in order to isolate its protein fraction for SDS-PAGE and Western blot analysis. To isolate parasite proteins associated to DRMs, a sample of infected red blood cells was resuspended in 0.2% saponine, centrifuged and washed thrice in PBS. Then, the parasite was resuspended in TNET buffer (1% *v*/*v* Triton X-100, 25 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) containing protease inhibitors. This product was split into two aliquots, each of which was treated as follows: (1) incubation for 30 min at 4 °C, or (2) incubation for 30 min at 37 °C. Aliquots were then centrifuged at 10,000 × *g* for 10 min at 4 °C and at room temperature, respectively. Finally, the two pellets obtained from each sample were resuspended in lysis buffer.

2.10. SDS-PAGE and Western blotting

The purified recombinant protein, total parasite lysate and extracted fractions of DRM-associated proteins were separated by 12% SDS-PAGE under denaturing conditions and then transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in PBS–0.05% Tween for 1 h at room temperature and washed thrice with PBS–0.05% Tween. The membrane containing rPv34 was cut into strips in order to evaluate: (1) recognition by sera from ten *P. vivax*-infected *Aotus* monkeys, (2) recognition by sera from ten patients with symptomatic *P. vivax* disease who voluntary agreed to participate in this study, and (3) recognition by anti-polyhistidine monoclonal antibodies (positive control). For this assay, strips were independently incubated with human or monkey sera diluted 1:100 in 5% skimmed milk–PBS–0.05% Tween for 1 h. Strips were washed thrice with PBS–0.05% Tween and then incubated for 1 h at room temperature with the secondary antibody (goat anti-*Aotus* IgG or goat anti-human IgG coupled to phosphatase) at a 1:4500 dilution. The positive control strip was incubated for 1 h with anti-polyhistidine monoclonal antibody coupled to peroxidase diluted 1:4500 in 5% skimmed milk–PBS–0.05% Tween. The membrane containing the total parasite lysate was used for assessing recognition by sera from rabbits previously immunized with the Pv34 synthetic peptides. Each rabbit's sera (pre-immune and hyperimmune) were incubated with individual strips at a 1:100 ratio in 5% skimmed milk–PBS–0.05% Tween, for 1 h at room temperature. Strips were incubated for 1 h at room temperature with goat antirabbit IgG coupled to phosphatase (1:4500) as secondary antibody. For Western blot analysis of DRM-associated proteins, a pool of rabbit hyperimmune sera was used according to the above described conditions. Blots were developed with either the VIP peroxidase substrate (Vector Laboratories, Burlingame, Canada) or the BCIP/NBT color development substrate kits (Promega) as corresponded, following manufacturers' indications.

2.11. Indirect immunofluorescence assay

A fresh parasite sample was stained using rabbit polyclonal sera as primary antibody (diluted 1:40) and goat fluorescein isothiocyanate-labeled antirabbit IgG conjugate as secondary antibody, according to a previously described protocol [13]. Fluorescence was analyzed using an Olympus BX51 microscope.

2.12. Isolation of PBMCs

Blood samples collected from another group of seven individuals who had a history of *P. vivax* malaria (age range: 19–73 years) inhabiting Tierra Alta, Córdoba, Colombia, which is an endemic region for malaria caused by this species. All individuals firmed an informed consent and were instructed about the goals of the study. None of these individuals had active malaria at the moment of collecting blood samples, as confirmed by the absence of parasites in thick blood smears. Seven individuals who had not been exposed to malaria were used as controls.

PBMCs from all individuals were isolated from 10 to 20 mL heparinized peripheral blood using a Ficoll-Hypaque density gradient (1.077 density) (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). Cells were suspended in 90% fetal bovine serum (FBS)–10% DMSO (*v*/*v*) and stored in liquid nitrogen until use.

2.13. Lymphoproliferation assays

PBMCs were thawed at 37 °C and washed twice with RPMI-1640 medium (Sigma). 2×10^5 PBMCs were seeded in 96-well culture plates in triplicate (Corning, New York, USA) with 200 μL of complete RPMI-1640 medium (supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium piruvate, 25 U/mL penicillin, 50 μg/mL streptomycin, 5 M β-mercaptoethanol and 10% autologous plasma). Cells were then pulsed with 10 μg/mL of refolded rPv34, or 20 ng/mL phorbyl-12-myristate-13-acetate (PMA) (Sigma) and 1.25 μM ionomycin (Sigma) as positive controls. After 72 h of incubation at 37 °C and 5% CO_2, culture supernatants were harvested to assess cytokine levels (as described further below) and cells were again pulsed with 1 μCi [^{3}H] thymidine for 18 h. Cells were harvested onto glass-fiber filters with a PHD Cell Harvester (Cambridge Technology Inc., Massachusetts, USA). Filters were air-dried prior to addition of 2.0 mL of Betamax ES scintillant (ICN Biochemicals Inc., California, USA). Activity (counts per minute, cpm) of [^{3}H]-thymidine was determined with a liquid scintillation counter (Beckman Instruments, California, USA) and stimulation indices (SI) calculated as the cpm ratio between stimulated cultures and nonstimulated control cultures. The mean of the control population plus 2 SD resulted in a SI of 1.57, therefore an SI ≥ 1.6 was considered positive.

2.14. Cytokine production

Levels of IL-2, IL-4, IL-5, IL-10, TNFα and IFNγ cytokines were determined by Cytometric Bead Array (CBA) (BD Biosciences, California, USA), according to the manufacturer's instructions. Briefly, 50 μL of each type of capturing beads was mixed with 50 μL of Th1/Th2 human II–PE detection reagent and 50 μL of supernatant from PBMC cultures. Ten concentrations of cytokine standards were used for plotting the calibration curve. Samples were incubated for 3 h at room temperature away from light, washed, centrifuged at 200 × *g* for 5 min and subsequently analyzed in the FACScan flow cytometer (BD Biosciences).

2.15. Accession number

Nucleotide and amino acid sequences described in the present study have been reported in the GenBank database under the accession number FJ971590.

3. Results and discussion

3.1. Identification of pv34 and possible homologs in the genus Plasmodium

A homology search was carried out using the previously reported Pf34 amino acid sequence as bait in order to confirm the sequence encoding Pv34 in *P. vivax*. This tBlastn search allowed mapping a nucleotide sequence with a high probability of encoding Pv34 that was located on a 1,370,936-bp contig (JCVI: ctg_6950; PlasmoDB: CM000446) (Fig. 1). Differences in the exon–intron structure were found between our prediction and the annotation for this gene in the SaI-1 strain genome database. This discrepancy was sorted out by sequencing *pv34* cDNA, as discussed later. A thorough search in other *Plasmodium* species' genome databases confirmed the presence of Pf34 and Pv34 homologous proteins in *P. chabaudi* (Pc34), *P. gallinaceum* (Pg34) and, as suggested previously, in *P. knowlesi* (Pk34) [7]. However, the search for protein homologues in *P. berghei*, *P. yoelii*, *P. ovale* and *P. reichenowi* did not yield positive results due to the lack of completely assembled contigs or the incomplete sequence data for this gene in these parasite species.

3.2. pv34 gene sequence analysis and localization in a syntenic chromosome region

Once the parasite's total RNA had been extracted from 3 mL-blood samples collected from infected *Aotus* monkeys (with a high percentage of schizonts), the Pv34 cDNA was synthesized and subsequently amplified (Fig. 2). Sequence integrity was verified by sequencing the product cloned into the expression vector, which indicated that there are no nucleotide substitutions in *pv34* between the *P. vivax* (VCG-1) *Aotus*-adapted strain and the SaI-1 reference sequence (available at JCVI). The sequence encoding Pv34 comprises a single exon of 1092 bp, which differs with the annotation registered in the *P. vivax* genome database pointing to *pv34* as a 2 exon gene (PlasmoDB: PVX_090075). Such difference highlights that, although usually very accurate, gene predictors sometimes fail and thus experimental confirmation is necessary.

Upstream and downstream genes were analyzed in regards to (1) reading frame orientation, (2) exon–intron composition and (3) identity and similarity values of protein products, comparing *P. vivax*–*P. falciparum* and *P. vivax*–*P. knowlesi* sequences (Fig. 1A). Protein sequence identity and similarity values among the analyzed *P. falciparum* and *P. vivax* chromosome region ranged between 28.7–71.1% and 40.2–79.3%, respectively. Identity (64.2–86.6%) and similarity (72.3–94.5%) values between *P. vivax* and *P. knowlesi* were higher, agreeing with the evolutionary closeness between these two species.

The Pv34 sequence herein reported (GenBank accession FJ971590) is slightly longer than its *P. falciparum* homologue (PlasmoDB: PFD0955w), and both sequences yielded the lowest identity and similarity values in comparison to the remaining proteins included in the synteny analysis of the three *Plasmodium* species (Fig. 1A). These identity and similarity values are similar to the ones reported previously for other protein homologs between *P. falciparum* and *P. vivax* also involved in invasion to red blood cells (MSP10, Pf38/Pv38) [15,20]. This difference might have evolutively arisen (together with other factors) as a result of the selective pressure exerted by the human host's immune system over those parasite proteins involved in invasion to red blood cells and that are eventually exposed during this process [32].

3.3. In silico characterization of Pv34

The protein encoded by *pv34* is 363 amino acids in length and displays a signal sequence at its N-terminus, a GPI-anchoring sequence at its C-terminus and a previously identified conserved region [7] (Fig. 1B). Additionally, Pv34 possesses an amino acid repeat region between amino acids 296–325 (Fig. 1B) not found in the other protein homologs within the genus *Plasmodium* (including Pf34, Pk34, Pc34 and Pg34). This repeat region exclusive to Pv34 contains six characteristic 5-amino-acid-long blocks with the consensus sequence NGG[LFP]P which is predicted to contain lineal B cell epitopes (high Parker's immunogenicity values and Bepipred predictions above the 0.35 threshold). Same as in other invasion-related *Plasmodium* proteins containing sequence repeats such as the *P. falciparum* circumsporozoite protein (PfCSP), this *P. vivax*-exclusive repeat could be playing a role in evading the host's humoral immune response. In the case of PfCSP, it has been shown that its repeat region (having 4-amino acid tandem repeats) competes more efficiently for antibody binding than B epitopes located in functional regions outside the repeat [33]. Nevertheless,

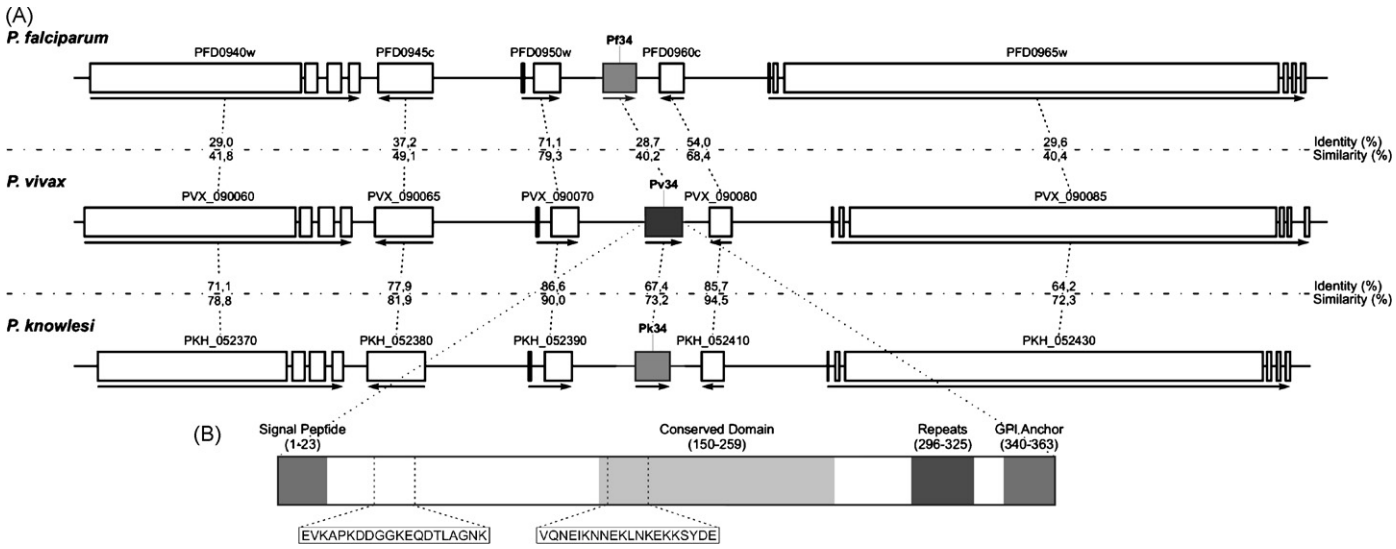

Fig. 1. (A) Scale diagram showing the localization of genes encoding Pf34, Pk34 (both in gray) and Pv34 (in bold) as well as of adjacent genes in the *P. falciparum* and *P. vivax* chromosomes fragments, respectively. PlasmoDB accession numbers, ORFs orientation and exon organization of each gene are indicated. The analyzed fragments comprise 37 kbp from *P. falciparum* chromosome 4 (864,501–901,500 bp), 37 kbp from the *P. vivax* contig ctg_6950 (1,071,001–1,108,000 bp) and 36.5 kbp from the *P. knowlesi* chromosome fragment PK4.chr05 (1,050,001–1,086,500 bp). (B) Schematic representation of the complete Pv34 protein (363 residues) showing the N-terminal signal peptide, a conserved region, a unique Pv34 repeat sequence and the C-terminal GPI-anchoring site. The synthetic peptides used for immunizing rabbits are enclosed in white boxes.

immunization assays carried out in humans have demonstrated that PfCSP repeat-specific antibodies are HLA-haplotype dependent and have short life [34], which suggests that this repeat region is involved in evading the host's immune response. Given all the above mentioned data, it would be important to assess the functional and immunological implications of this repeat region in Pv34 since a similar behavior to that of PfCSP and PvCSP might be occurring (Fig. 1B).

3.4. Expression of Pv34 as a recombinant protein and its recognition by human and Aotus sera

The complete amino acid sequence encoded by *pv34* was obtained as a recombinant protein in *E. coli* (rPv34) with a 6-histidine tag linked to its C-terminus. In order to evaluate whether a humoral immune response is raised in the course of a *P. vivax* infection against Pv34, the recognition of rPv34 was evaluated by Western blot using sera from malaria-infected patients and *Aotus* monkeys. The results of this assay showed that six out ten patients recognized rPv34 (lanes 1–5 and 8 in Fig. 3A), while seven out ten monkeys' sera reacted against rPv34 (lanes 1–3 and 6–9 in Fig. 3B), therefore highlighting this protein's antigenicity.

3.5. Evidence of Pv34 expression and localization in the parasite rhoptries

The *in silico* prediction of B epitopes in the Pv34 amino acid sequence allowed us to identify some regions, mainly located towards the protein's N-terminus and at the beginning of the conserved region, having significantly high Parker's antigenicity, solvent accessibility and hydrophilicity values, and above Bepipred's selection threshold.

Based on this analysis, two 20-mer-long peptides were selected and synthesized chemically. These peptides were inoculated in rabbits in order to obtain polyclonal antibodies that could let us recognize the Pv34 protein in malaria parasites. As can be seen in Fig. 4A, a single ~37.5 kDa band is recognized when rabbit's hyperimmune sera were tested against parasite lysate by Western blot which agrees with the molecular weight expected for Pv34 without its signal peptide.

In order to assess whether Pv34 is located on DRMs, parasite membrane solubility in non-ionic detergent at 4 and 37 °C was assessed. The partial detection of Pv34 by Western blot with rabbit hyperimmune sera on the parasite's insoluble fraction after resuspending it in TNET buffer at 4 °C, together with its complete solubilization in TNET at 37 °C, allowed us to confirm that Pv34 is targeted to DRMs (Fig. 4B).

Additionally, Pv34 localization was evaluated by indirect immunofluorescence on a peripheral blood smear of a *P. vivax*-infected monkey. This assay showed that distribution of Pv34 in mature schizonts follows a punctated pattern, typical of rhoptry proteins (Fig. 4C). Further co-localization assays with other rhop-

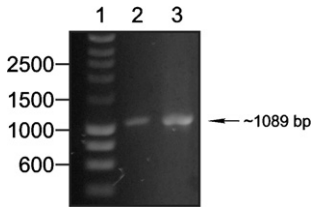

Fig. 2. PCR amplification of *P. vivax pv34* from cDNA and genomic DNA. Lane 1, molecular weight marker. Lane 2, *pv34* RT-PCR. Lane 3, *pv34* PCR from genomic DNA.

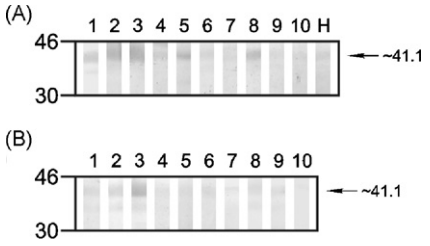

Fig. 3. (A) Western blot analysis showing antibody recognition of the purified rPv34 by sera from *P. vivax*-infected patients (lanes 1–10 correspond to sera from different patients). Lane H, recognition of purified rPv34 by monoclonal anti-polyhistidine antibodies. (B) Western blot analysis showing antibody recognition of the purified rPv34 by *Aotus* monkey's sera (lanes 1–10 correspond to sera from different monkeys). The weight estimated for the protein including the signal peptide and the histidine tag is shown.

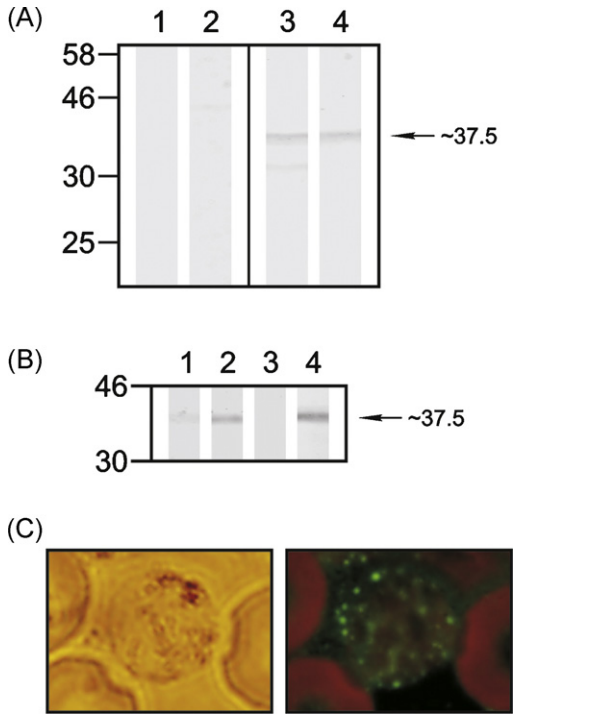

Fig. 4. (A) Western blot analysis of *P. vivax* lysate using sera raised against Pv34 synthetic peptides in rabbits. Lanes 1 and 2, protein recognition by rabbit's pre-immune sera in parasite lysate. Lanes 3 and 4, protein recognition by rabbit's hyperimmune sera in parasite lysate. The estimated weight for the parasite's protein without the signal peptide is shown. (B) Western blot detection of Pv34 in DRMs using rabbit's hyperimmune serum. Lanes 1 and 3, Pv34 recognition in the *P. vivax* insoluble fraction upon suspension in TNET buffer at 4 and 37 °C, respectively. Lanes 2 and 4, Pv34 recognition in the *P. vivax* soluble fraction upon suspension 4 and 37 °C, respectively. (C) Indirect immunofluorescence assay using rabbit's hyperimmune serum against the two Pv34 synthetic peptides showing immunolabeling of a fixed *P. vivax* schizont. The punctated fluorescence pattern observed is characteristic of rhoptry proteins. Non-infected red blood cells are seen around the schizont. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

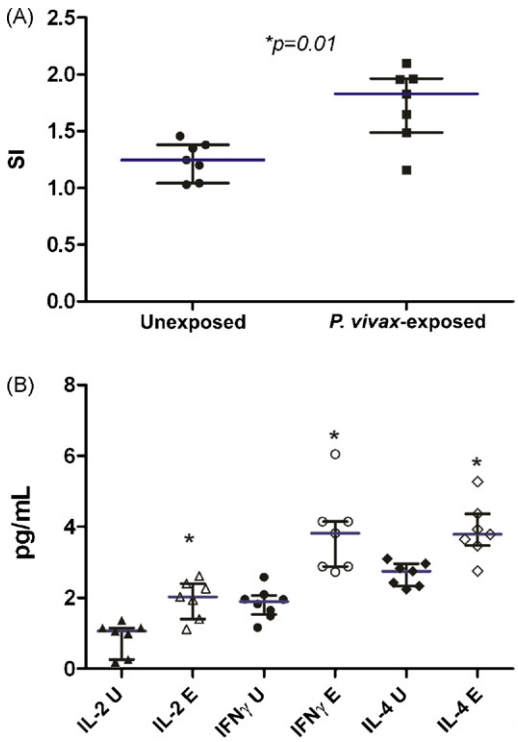

Fig. 5. T-cell response to rPv34 stimulation in *P. vivax*-exposed individuals. (A) Lymphoproliferation stimulated by rPv34 was estimated and plotted. Each point represents the stimulation index (SI) of an individual. The blue bar indicates the median and the black lines the interquartile range. Lymphoproliferation was significantly higher in the group of individuals with a history of *P. vivax* malaria in comparison to the control group (non-parametric *t*-test $p \leq 0.05$). (B) Cytokine secretion stimulated by rPv34. The concentrations of IL-2, IFNγ and IL-4 in pg/mL is shown. E, *P. vivax*-exposed; U, unexposed individuals. Asterisks indicate significant differences with regards to the unexposed group. The blue bar indicates the median and the black lines the interquartile range. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

try proteins would shed light on whether Pv34 is localized toward the rhoptry bulb or neck, as occurs with its *P. falciparum* homologue [7]. On the other hand, no evidence of Pv34 presence in newly invaded erythrocytes was observed, however it cannot be ruled out that this protein is trafficked to the parasitophorous vacuole at the beginning of a new invasion cycle since the difficulty of establishing an *in vitro* culture of this parasite species prevents obtaining a synchronous parasite population of the first 2 h post-invasion where the protein could be observed in ring stages but not after [7].

3.6. Proliferation in response to rPv34 and cytokine production

The antigenicity of Pv34 was assessed by stimulating PBMCs isolated from individuals with a previous history of *P. vivax* malaria with rPv34 and determining its ability to induce proliferation of T memory lymphocytes generated in response to a natural infection. For this assay, samples with SI $\geq$ 1.6 values were considered positive for lymphoproliferation. According to this criterium, 71% of individuals showed positive lymphoproliferation, detecting a significant difference in the group of individuals compared to the control group (non-parametric *t*-test $p \leq 0.05$) (Fig. 5A).

Additionally, the presence of T helper lymphocytes subtypes was evaluated by measuring the ability of PBMCs to produce (1) three Th1-profile associated cytokines (IL-2, IFNγ and TNFα), and (2) three Th2-profile associated cytokines (IL-4, IL-5 and IL-10). A significantly higher cytokine production was observed in the group of patients compared to the controls for IL-2, ($p = 0.007$), IFNγ ($p = 0.02$) and IL-4 ($p = 0.004$). Seventy-one percent of individuals produced high levels of IL-2, 57% high levels of IFNγ and 85% high levels of IL-4 in response to stimulation with rPv34 (Fig. 5B).

Altogether, this analysis confirms the antigenicity of Pv34 as it shows its ability to induce proliferation of PBMCs from *P. vivax*-exposed individuals. In addition, the production of Th1/Th2-profile cytokines indicates the recognition of B and T epitopes in Pv34 which would be responsible of this combined immune response. The higher levels of IL-4 (85%) in *P. vivax*-exposed individuals indicates induction of an antibody-mediated immune response, which is consistent with rPv34 recognition by sera from *P. vivax*-infected *Aotus* and patients, as described in previous reports for other *P. vivax* proteins [35,36]. IFNγ secretion could be implicated in inducing isotype switch and stimulating antibody secretion by such isotype-switched B lymphocytes, however, this suggestion has to be experimentally assessed [37].

In addition to data regarding *pv34* transcription [11] and expression as a protein during late intraerythrocytic stages of the *P. vivax* life cycle, other results found in this study support postulating Pv34 as a potential candidate for a vaccine against this parasite species, such as the presence of a signal peptide, GPI-anchor, predicted lineal B cell epitopes, association with parasite DRMs, presence of *pv34* homologs in other *Plasmodium* species, strong recognition by sera from infected *Aotus* monkeys and patients and ability to stimulate proliferation of lymphocyte subpopulations in individuals with a previous history of *P. vivax* malaria. It would be therefore convenient to perform preliminary immunization assays with rPv34 in the *Aotus* animal model in order to evaluate its ability to induce

protection against experimental challenge with a *P. vivax Aotus*-adapted strain, as has been reported for some other promising vivax malaria vaccine candidates [38–40].

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References

[1] Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 2005;434(March (7030)):214–7.
[2] Hay SI, Guerra CA, Tatem AJ, Noor AM, Snow RW. The global distribution and population at risk of malaria: past, present, and future. Lancet Infect Dis 2004;4(June (6)):327–36.
[3] Carter R, Mendis KN. Evolutionary and historical aspects of the burden of malaria. Clin Microbiol Rev 2002;15(October (4)):564–94.
[4] Anantharaman V, Iyer LM, Balaji S, Aravind L. Adhesion molecules and other secreted host-interaction determinants in Apicomplexa: insights from comparative genomics. Int Rev Cytol 2007;262:1–74.
[5] Zhou XW, Kafsack BF, Cole RN, Beckett P, Shen RF, Carruthers VB. The opportunistic pathogen Toxoplasma gondii deploys a diverse legion of invasion and survival proteins. J Biol Chem 2005;280(October (40)):34233–44.
[6] Kaneko O. Erythrocyte invasion: vocabulary and grammar of the Plasmodium rhoptry. Parasitol Int 2007;56(December (4)):255–62.
[7] Proellocks NI, Kovacevic S, Ferguson DJ, Kats LM, Morahan BJ, Black CG, et al. *Plasmodium falciparum* Pf34, a novel GPI-anchored rhoptry protein found in detergent-resistant microdomains. Int J Parasitol 2007;37(September (11)):1233–41.
[8] Gilson PR, Nebl T, Vukcevic D, Moritz RL, Sargeant T, Speed TP, et al. Identification and stoichiometry of glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite *Plasmodium falciparum*. Mol Cell Proteomics 2006;5(July (7)):1286–99.
[9] Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. PLoS Biol 2003;1(October (1)):E5.
[10] Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, et al. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. Nature 2008;455(October (7214)):757–63.
[11] Bozdech Z, Mok S, Hu G, Imwong M, Jaidee A, Russell B, et al. The transcriptome of *Plasmodium vivax* reveals divergence and diversity of transcriptional regulation in malaria parasites. Proc Natl Acad Sci USA 2008;105(October (42)):16290–5.
[12] Pico de Coana Y, Rodriguez J, Guerrero E, Barrero C, Rodriguez R, Mendoza M, et al. A highly infective *Plasmodium vivax* strain adapted to Aotus monkeys: quantitative haematological and molecular determinations useful for *P. vivax* malaria vaccine development. Vaccine 2003;21(September (25–26)):3930–7.
[13] Mongui A, Perez-Leal O, Soto SC, Cortes J, Patarroyo MA. Cloning, expression, and characterisation of a *Plasmodium vivax* MSP7 family merozoite surface protein. Biochem Biophys Res Commun 2006;351(December (3)):639–44.
[14] Perez-Leal O, Sierra AY, Barrero CA, Moncada C, Martinez P, Cortes J, et al. *Plasmodium vivax* merozoite surface protein 8 cloning, expression, and characterisation. Biochem Biophys Res Commun 2004;324(November (4)):1393–9.
[15] Perez-Leal O, Sierra AY, Barrero CA, Moncada C, Martinez P, Cortes J, et al. Identifying and characterising the *Plasmodium falciparum* merozoite surface protein 10 *Plasmodium vivax* homologue. Biochem Biophys Res Commun 2005;331(June (4)):1178–84.
[16] Angel DI, Mongui A, Ardila J, Vanegas M, Patarroyo MA. The *Plasmodium vivax* Pv41 surface protein: identification and characterization. Biochem Biophys Res Commun 2008;377(December (4)):1113–7.
[17] Perez-Leal O, Mongui A, Cortes J, Yepes G, Leiton J, Patarroyo MA. The *Plasmodium vivax* rhoptry-associated protein 1. Biochem Biophys Res Commun 2006;341(March (4)):1053–8.
[18] Patarroyo MA, Perez-Leal O, Lopez Y, Cortes J, Rojas-Caraballo J, Gomez A, et al. Identification and characterisation of the *Plasmodium vivax* rhoptry-associated protein 2. Biochem Biophys Res Commun 2005;337(November (3)):853–9.
[19] Mongui A, Perez-Leal O, Rojas-Caraballo J, Angel DI, Cortes J, Patarroyo MA. Identifying and characterising the *Plasmodium falciparum* RhopH3 *Plasmodium vivax* homologue. Biochem Biophys Res Commun 2007;358(July (3)):861–6.
[20] Mongui A, Angel DI, Guzman C, Vanegas M, Patarroyo MA. Characterisation of the *Plasmodium vivax* Pv38 antigen. Biochem Biophys Res Commun 2008;376(November (2)):326–30.
[21] Burge CB, Karlin S. Finding the genes in genomic DNA. Curr Opin Struct Biol 1998;8(June (3)):346–54.
[22] Shah SP, McVicker GP, Mackworth AK, Rogic S, Ouellette BF. GeneComber: combining outputs of gene prediction programs for improved results. Bioinformatics 2003;19(July (10)):1296–7.
[23] Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 2004;340(July (4)):783–95.
[24] Poisson G, Chauve C, Chen X, Bergeron A. FragAnchor: a large-scale predictor of glycosylphosphatidylinositol anchors in eukaryote protein sequences by qualitative scoring. Genomics Proteomics Bioinformatics 2007;5(May (2)):121–30.
[25] Newman AM, Cooper JB. XSTREAM: a practical algorithm for identification and architecture modeling of tandem repeats in protein sequences. BMC Bioinformatics 2007;8:382.
[26] Larsen JE, Lund O, Nielsen M. Improved method for predicting linear B-cell epitopes. Immunome Res 2006;2:2.
[27] Geourjon C, Deleage G, Roux B. ANTHEPROT: an interactive graphics software for analyzing protein structures from sequences. J Mol Graph 1991;9(September (3)):188–90, 67.
[28] Andrysiak PM, Collins WE, Campbell GH. Concentration of *Plasmodium ovale*- and *Plasmodium vivax*-infected erythrocytes from nonhuman primate blood using Percoll gradients. Am J Trop Med Hyg 1986;35(March (2)):251–4.
[29] Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 1993;15(September (3)):532–4, 6–7.
[30] Thompson JD, Gibson TJ, Higgins DG. Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinformatics; 2002 (Chapter 2, Aug:Unit 2 3).
[31] Houghten RA. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. Proc Natl Acad Sci USA 1985;82(August (15)):5131–5.
[32] Frank SA, Barbour AG. Within-host dynamics of antigenic variation. Infect Genet Evol 2006;6(March (2)):141–6.
[33] Burkot TR, Da ZW, Geysen HM, Wirtz RA, Saul A. Fine specificities of monoclonal antibodies against the *Plasmodium falciparum* circumsporozoite protein: recognition of both repetitive and non-repetitive regions. Parasite Immunol 1991;13(March (2)):161–70.
[34] Nardin EH, Oliveira GA, Calvo-Calle JM, Castro ZR, Nussenzweig RS, Schmeckpeper B, et al. Synthetic malaria peptide vaccine elicits high levels of antibodies in vaccinees of defined HLA genotypes. J Infect Dis 2000;182(November (5)):1486–96.
[35] Tran TM, Oliveira-Ferreira J, Moreno A, Santos F, Yazdani SS, Chitnis CE, et al. Comparison of IgG reactivities to *Plasmodium vivax* merozoite invasion antigens in a Brazilian Amazon population. Am J Trop Med Hyg 2005;73(August (2)):244–55.
[36] Wickramarachchi T, Illeperuma RJ, Perera L, Bandara S, Holm I, Longacre S, et al. Comparison of naturally acquired antibody responses against the C-terminal processing products of *Plasmodium vivax* Merozoite Surface Protein-1 under low transmission and unstable malaria conditions in Sri Lanka. Int J Parasitol 2007;37(February (2)):199–208.
[37] Metzger DW, McNutt RM, Collins JT, Buchanan JM, Van Cleave VH, Dunnick WA. Interleukin-12 acts as an adjuvant for humoral immunity through interferon-gamma-dependent and -independent mechanisms. Eur J Immunol 1997;27(August (8)):1958–65.
[38] Sierra AY, Barrero CA, Rodriguez R, Silva Y, Moncada C, Vanegas M, et al. Splenectomised and spleen intact Aotus monkeys' immune response to *Plasmodium vivax* MSP-1 protein fragments and their high activity binding peptides. Vaccine 2003;21(October (27–30)):4133–44.
[39] Barrero CA, Delgado G, Sierra AY, Silva Y, Parra-Lopez C, Patarroyo MA. Gamma interferon levels and antibody production induced by two PvMSP-1 recombinant polypeptides are associated with protective immunity against *P. vivax* in Aotus monkeys. Vaccine 2005;23(July (31)):4048–53.
[40] Rojas Caraballo J, Delgado G, Rodriguez R, Patarroyo MA. The antigenicity of a *Plasmodium vivax* reticulocyte binding protein-1 (PvRBP1) recombinant fragment in humans and its immunogenicity and protection studies in Aotus monkeys. Vaccine 2007;25(May (18)):3713–21.