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Identifying and characterizing a member of the RhopH1/Clag family in *Plasmodium vivax*

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ABSTRACT

Plasmodium vivax malaria caused is a public health problem that produces very high morbidity worldwide. During invasion of red blood cells the parasite requires the intervention of high molecular weight complex rhoptry proteins that are also essential for cytoadherence. PfClag9, a member of the RhopH multigene family, has been identified as being critical during *Plasmodium falciparum* infection. This study describes identifying and characterizing the *pfclag9* ortholog in *P. vivax* (hereinafter named *pvclag7*). The *pvclag7* gene is transcribed at the end of the intraerythrocytic cycle and is recognized by sera from humans who have been infected by *P. vivax*. PvClag7 subcellular localization has been also determined and, similar to what occurs with PfClag9, it co-localize with other proteins from the Rhoptry high molecular weight complex.

1. Introduction

Malaria, one of the infectious diseases having the greatest social impact, causing around 3 million deaths per year, has been considered to be a major public health problem worldwide (Snow et al., 2005). Among the five parasite species causing malaria in humans, *Plasmodium falciparum* and *Plasmodium vivax* cause the highest morbidity and mortality (Price et al., 2007; Bozdech et al., 2008), being responsible for about 90% of all malarial cases (Snow et al., 2005).

During invasion of red blood cells (RBCs) the parasite requires the intervention of specialized organelles (rhoptries, micronemes and dense granules) which release various proteins facilitating parasite entry and reorganization of the of the target cell cytoskeleton (Preiser et al., 2000). Several studies directed at elucidating transcription profiles in parasite species during their intraerythrocytic life cycle (ILC) (Bozdech et al., 2003; Bozdech et al., 2008) have been carried out as the complete genomic sequence of *P. falciparum* (Gardner et al., 2002) and *P. vivax* (Carlton et al., 2008) have become available. Conserved functional characteristics have been found for most of the protein-encoding genes depending on the biological processes they participate in (Bozdech et al., 2008; Das et al., 2009). It has thus become evident that members of the *Plasmodium* genus use similar invasion strategies relying on the use of conserved protein groups (Anantharaman et al., 2007).

The rhoptry high molecular weight protein complex (known as RhopH) (Kaneko, 2007) consists of three proteins known in *P. falciparum* as RhopH1 (155 kDa), RhopH2 (140 kDa) and RhopH3 (110 kDa). These proteins are involved in parasitophorous vacuole (PV) formation during the invasion of new RBCs (Sam-Yellowe et al., 1988), in cytoadherence and are localized close to Maurer's clefts (Ling et al., 2004; Vincensini et al., 2008). Furthermore, functional and molecular analysis has shown that antibodies directed against RhopH complex proteins partially inhibit *P. falciparum* growth *in vitro* and *in vivo* (Sam-Yellowe, 1993; Pinzon et al., 2010), highlighting their potential use as vaccine candidates.

RhopH1 protein-encoding genes, initially described in *P. falciparum* as cytoadherence linked asexual genes (*clag*), constitute the *rhoph1/clag*

Abbreviations: RBC, Red blood cells; ILC, Intraerythrocytic life cycle; *P. falciparum*, *Plasmodium falciparum*; *P. vivax*, *Plasmodium vivax*; RhopH, Rhoptry high molecular weight protein complex; PV, Parasitophorous vacuole; *clag*, Cytoadherence linked asexual genes; HMW, High molecular weight; ORFs, Open reading frames; FIDIC, Fundación Instituto de Inmunología de Colombia; VCG-1, Vivax Colombia Guaviare 1 strain; DNase, deoxyribonuclease; RT-PCR, Reverse transcription Polymerase Chain Reaction; cDNA, DNA complementary to RNA; gDNA, Genomic DNA; *w/v*, Weight/volume; RP-HPLC, Reverse Phase-High Performance Liquid Chromatography; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; SDS, Sodium dodecyl sulfate; *E. coli*, *Escherichia coli*; *M. tuberculosis*, *Mycobaterium tuberculosis*; ELISA, Enzyme-linked immunosorbent assay; PBS, Phosphate Buffered Saline; OD, Optical density; *m*, Medians; *v/v*, Volume/volume; DAPI, 4′,6-Diamidino-2-phenylindole; FITC, Fluorescein Isothiocyanate; BSA, Bovine serum albumin; kbp, kilobasepair (s); kDa, kilodalton (s); TM, Transmembrane domain.

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multigene family (Trenholme et al., 2000). These genes have been named according to their chromosomal localization and are currently reported in the plasmoDB database as: PFB0935w (*rhoph1/clag 2*), PFC0120w (*rhoph1/clag 3.1*), PFC0110w (*rhoph1/clag 3.2*), MAL7P1.229 (*rhoph1/clag 8*) and PFI1730w (*rhoph1/clag 9*).

All 5 *clag* genes are constituted by 9 exons, showing the highest transcription peak at the end of the intraerythrocytic cycle (Bozdech et al., 2008). Although more than one *clag* gene can be transcribed at the same time in the parasite, it is clear that only one RhopH1/Clag protein takes part in the formation of a rhoptry high molecular weight (HMW) complex. Therefore, 5 RhopH complexes would be expected to be found in *P. falciparum* merozoite rhoptries (Kaneko et al., 2005; Kaneko, 2007).

Studies aimed at identifying and characterizing proteins involved in *P. vivax* invasion of RBCs have been hampered, mainly due to the unavailability of a continuous culturing system that could allow this parasite species to be grown *in vitro*. To overcome this problem, our group has focused on identifying new *P. vivax* antigens homologous to previously characterized *P. falciparum* antigens, based on similarity searches in available databases.

To date, five *P. vivax* rhoptry proteins (RAP1 (Perez-Leal et al., 2006), RAP 2 (Patarroyo et al., 2005), RhopH3 (Mongui et al., 2007), Pv38 (Mongui et al., 2008), Pv34 (Mongui et al., 2009)) have been described by screening the complete *P. vivax* genome sequence (Carlton et al., 2008) and using a *P. vivax* strain adapted to *Aotus* monkeys as a source for parasite DNA, RNA and proteins (Pico de Coana et al., 2003). The characterized proteins might play an important role in human reticulocyte invasion and some of them have been evaluated as vaccine candidates in the *Aotus* animal model (Rojas-Caraballo et al., 2009).

One of the *P. falciparum* RhopH1/Clag homologous proteins was identified and characterized in *P. vivax* in the present study using bioinformatics tools, molecular biology and immunological assays.

2. Materials and methods

2.1. Bioinformatics analysis

The *P. vivax* complete genome sequence (available at http://gsc.jcvi.org/projects/msc/plasmodium_vivax/index.shtml) was analyzed by tBlastn using the PfClag9 amino acid sequence as query (PlasmoDB accession no: Pfl1730w) to identify the homologous gene in this parasite species. The sequence yielding the highest score (herein named *pvclag7*) was selected to be characterized. PlasmoDB and Sanger Institute databases were also screened to search for *pfclag9* orthologue genes in *P. vivax* and in other *Plasmodium* species. Upstream and downstream open reading frames (ORFs) adjacent to *pfclag9* and *pvclag7* were analyzed using GeneComber software (Shah et al., 2003). Identity and similarity values between *P. vivax*–*P. falciparum* protein sequences were obtained using the ClustalW tool (Thompson et al., 1994). A multiple alignment of the conserved genome sequence was carried out to determine possible gene inversions using MAUVE software (Darling et al., 2004). The presence of a signal peptide, transmembrane regions and functional domains were determined by using SignalP 3.0 (Bendtsen et al., 2004; Kall et al., 2004), TMHMM v 2.0 (Krogh et al., 2001) and the InterPro database (Hunter et al., 2009). Antheprot software (Deleage et al., 2001) was used for determining the presence of linear B-cell epitopes in PvClag7 by predicting the highest average values for hydrophilicity, solvent accessibility and Parker's antigenicity.

2.2. Animal handling

The experimental handling of 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 guide for the care and use of laboratory animals (National Institute of Health, USA) under the constant supervision of a veterinarian. Immunization and bleeding procedures for *Aotus* monkeys had been previously approved by our institute's ethics committee and were carried out in agreement with the conditions stipulated by CorpoAmazonia (resolution 00066, September 13th, 2006). A monkey from the *Aotus* genus was experimentally infected with the VCG-1 strain (Vivax Colombia Guaviare 1) and kept under daily supervision until reaching 2% parasitemia (assessed with Acridine Orange staining). Subsequently, pediatric doses of chloroquine (10 mg/kg on 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) were administered in order to guarantee total clearance of blood parasites. Once experiments were over, CorpoAmazonia officers supervised the primate's return to its natural habitat in excellent health.

2.3. Isolating P. vivax parasites

Parasites from the VCG-1 strain were cultured by successive passes in *Aotus* monkeys kept at our primate station in Leticia (Amazonas) according to previously described methodology (Pico de Coana et al., 2003). Infected RBCs were purified from 3 mL blood samples taken from infected animals using a discontinuous Percoll gradient, according to a previously described protocol (Andrysiak et al., 1986). The isolated parasite was used in the following procedures: (1) RNA extraction, (2) genomic DNA extraction, and (3) total protein extraction or (4) immunofluorescence assays.

2.4. RNA extraction, cDNA synthesis, cloning and sequencing

Parasite RNA was isolated using the Trizol methodology and treated with RQ1 RNase-free DNase (Promega, Wisconsin, USA). 5 μL of RNA was taken for cDNA synthesis by using the one-step RT-PCR SuperScript III kit (Invitrogen, California, USA), according to manufacturer's recommendations. In brief, cDNA was synthesized according to the following conditions: 65 °C for 5 min, 50 °C for 1 h and 70 °C for 15 min.

A PCR using the platinum *Pfx* DNA polymerase enzyme (Invitrogen, California, USA) was carried out with specific primers (A region: 5′ ATGTCCTACAAAAATGAAAACATAG 3′, 5′ GTACAGAACCTGGTCCC 3′; B region: 5′ CTACGTCAACTTTATAAAGTAC 3′, 5′ GGTCATAGATTGGTAGTCG 3′; C region: 5′ ATGCAGTACAGCTTCTTCTCC 3′, 5′ GTTCTTCACGGGGACAAAG 3′) to amplify the *pvclag7* nucleotide sequence from both cDNA and genomic DNA. Additional amplifications were made for producing two smaller sized recombinant fragments. Each amplified fragment was purified by using the Wizard PCR preps kit (Promega). Only *pvclag7* PCR products obtained from cDNA were cloned into the pEXP5 CT/TOPO vector (Invitrogen). Recombinant DNA was purified using the UltraClean mini plasmid prep purification kit (MO BIO laboratories, California, USA) and the integrity of cloned fragments obtained by independent PCR was verified by sequencing them in an automatic ABI PRISM 310 (PE Applied Biosystems, California, USA).

2.5. Extracting parasite proteins

A parasite pellet was homogenized in a lysis solution (5% *w/v* SDS, 10 mM PMSF, 10 mM iodoacetamide, and 1 mM EDTA) to isolate the protein fraction for SDS-PAGE and Western blot analysis.

2.6. Expressing and purifying recombinant fragments

Escherichia coli BL21-AI bacteria (Invitrogen) transformed with Pexp5–*pvclag7* recombinant plasmids were grown overnight and then inoculated in Terrific broth containing 0.1 mg/mL ampicillin and 0.1%

D-glucose (*w*/*v*). Cells were then incubated for 1.5 h, following which 0.2% L-arabinose (*w*/*v*) was added as an expression inductor. Cells were incubated for 5 h at 37 °C and then harvested by spinning at 13,000×*g* for 30 min at 4 °C. The cell pellet was suspended in extraction buffer (6 M urea, 10 mM Tris–Cl, 100 mM NaH_2PO_4 and 1 mg/ml lysozyme), supplemented with protease inhibitors (1 mM PMSF, 1 mM iodoacetamide, 1 mM EDTA and 1 mg/ml leupeptine) and lysed by sonication. Both recombinant proteins were purified in denaturing conditions using 6 M urea in a Ni2-NTA resin (Qiagen, California, USA), according to the manufacturer's recommendations and their expression was verified by SDS-PAGE and Western Blot. Selected protein fractions were pooled and dialyzed against PBS. The protein was ultrafiltrated and concentrated using an Amicon filtration system (Millipore, Billerica, Massachusetts, USA).

2.7. Peptide synthesis

A bioinformatics analysis was carried out to identify B-cell epitopes within the PvClag7 sequence. Based on these results, two peptides were synthesized to be later used in rabbit immunization assays (named here 36118 and 36119). The amino acid sequences in single letter code were: KQEDMPFVDKHNYTFEQTKL and WKRYYTNR-LEGKKIIPYFVY. One glycine and one cysteine were added at the N- and C-termini of each peptide to allow polymerization. Peptides were synthesized using the standard solid phase t-Boc/Bzl strategy (Houghten, 1985), then lyophilized and characterized by RP-HPLC and MALDI-TOF MS.

2.8. Peptide immunization and collecting polyclonal antibodies

New Zealand rabbits were immunized on day 0 with a 150 μg mixture containing both polymerized synthetic peptides emulsified in Freund's complete adjuvant (FCA) (Sigma, Missouri, USA). Two subsequent boosts were administered on days 20 and 40 with the same mixture, but emulsified in Freund's incomplete adjuvant (FIA) (Sigma, Missouri, USA) (Sigma, Missouri, USA). Sera were collected before the first immunization (pre-immune sera) and 20 days after administering the last boosting dose (post-III sera).

2.9. SDS-PAGE and Western blotting

PvClag7 recombinant fragments and the total parasite lysate were size-separated by 8–14% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in PBS-0.05% Tween for 1 h and washed thrice with PBS-0.05% Tween. The membranes were cut into strips and incubated individually in 5% skimmed milk — PBS-0.05% Tween containing a 1:100 dilution of each rabbit sera (pre-immune and post-III sera) for 1 h at room temperature. Some sera were pre-adsorbed with PvClag7-derived synthetic peptides for 1 h at 37 °C to evaluate (by exclusion) Western blot bands corresponding to this protein. Likewise, sera were pre-adsorbed with *E. coli*, *M. tuberculosis* and RBC lysates to decrease polyclonal antibody cross-reactivity. Strips were incubated for 1 h at room temperature with a secondary goat anti-rabbit IgG antibody coupled to phosphatase at 1:4500 dilution. For Western blot analysis, a nitrocellulose strip with anti-His monoclonal antibody coupled to peroxidase, diluted 1:4500 with 5% skimmed milk — PBS-0.05% Tween, was used as positive control. Blots were revealed with either VIP peroxidase substrate (Vector Laboratories, Burlingame, Canada) or BCIP/NBT color development substrate kits (Promega) following manufacturer's recommendations.

2.10. Determining anti-rPvClag7 antibody recognition by ELISA

The antigenicity of both PvClag7 recombinant fragments was assessed by incubating each of them with human antibodies from 18 patients living in *P. vivax* endemic areas of Colombia who had suffered from *P. vivax* malaria, while five healthy individuals who had never suffered from the disease were used as negative controls (Table 1). In brief, polysorp plates (Thermo Scientific Inc) were coated with 1 μg/mL of each recombinant purified protein in native conditions and incubated for 1 h at 37 °C, followed by a 4 °C overnight period. Polysorb plates were washed thrice with PBS-0.05% Tween and blocked with 200 μL 5% skimmed milk — PBS-0.05% Tween for 1 h. A 1:100 dilution of each serum was tested in duplicate by incubating for 1 h and then washing thrice in the same previously described conditions. Incubation period lasted 1 h at 37 °C with peroxidase-coupled anti-*human* IgG as a secondary antibody in a 1:4500 dilution and the excess was removed by washing with PBS-0.05% Tween. Reactivity was detected with peroxidase substrate solution (KPL Laboratories, WA, USA). Optical density (OD) was measured at 620 nm using a MJ ELISA multiskan reader.

2.11. Statistical analysis

Differences in the OD medians for rPvClag7 recognition by *P. vivax*-infected patients' sera and in the control group were evaluated using the Wilcoxon rank-sum test. The Kruskal–Wallis test was used to evaluate differences in the medians of ODs according to the number of malarial episodes and rPvClag7 recognition. A 0.05 significance level was used for hypothesis testing.

2.12. Immunofluorescence

Test slides with *P. vivax* schizont-rich samples from infected *Aotus spp.* monkeys' blood were fixed with 4% formaldehyde (*v*/*v*). Slides were washed thrice with PBS and then blocked with 1% bovine serum albumin (BSA) (*v*/*v*) for 45 min at 37 °C. Anti-PvClag7 rabbit serum (1:30 dilution) and anti-RhopH3 mouse serum (1:40 dilution) mixed in solution with PBS-BSA1%–0.1% Triton X-100 were used as primary antibodies and incubated for 1 h at 37 °C. After three washes, slides were incubated with FITC-conjugated anti-rabbit IgG antibody (Sigma) and Red-conjugated anti-mouse IgG antibody (Millipore) using the same conditions as mentioned above. DAPI (2 μg/mL) (Invitrogen, California, USA) was added to slides and these were incubated for 20 min at room temperature. Slides were examined under an Olympus BX51 fluorescence microscope.

Table 1
Patients' clinical records. Endemic area, causal agent and number of malaria episodes suffered by each patient are described. Samples 19-23 correspond to negative controls.

Serum number	Endemic area	Causal agent	N° of episodes
1	Guaviare	*P. vivax*	>3
2	Guaviare	*P. vivax*	>3
3	Meta	*P. vivax*	1
4	Chocó (Itsmina)	*P. vivax–P. falciparum*	2
5	Chocó (Itsmina)	*P. vivax*	>3
6	Chocó (Itsmina)	*P. vivax*	>3
7	Chocó (Itsmina)	*P. vivax*	1
8	Chocó (Quibdó)	*P. vivax*	2
9	Chocó (Itsmina)	*P. vivax*	1
10	Chocó (Itsmina)	*P. vivax*	1
11	Chocó (Itsmina)	*P. vivax*	4
12	Chocó (Itsmina)	*P. vivax*	4
13	Chocó (Quibdó)	*P. vivax*	1
14	Chocó (Quibdó)	*P. vivax*	1
15	Guaviare	*P. vivax*	>3
16	Narino (Tumaco)	*P. vivax*	1
17	Narino (Tumaco)	*P. vivax*	1
18	Guaviare	*P. vivax*	1
19	Bogotá	–	–
20	Bogotá	–	–
21	Bogotá	–	–
22	Bogotá	–	–
23	Bogotá	–	–

2.13. Accession number

The nucleotide and amino acid sequences used here have been reported in the GenBank database under the accession number HQ728488.

3. Results

3.1. Identification the P. vivax pvclag7 encoding gene

A homology search was carried out in the *P. vivax* genome database to find genes similar to *pfclag9* by using a local alignment tool. tBlastn analysis led to identifying an homologous region having a high probability of encoding for the *P. vivax pfclag9* gene, located in the CM000448 contig of chromosome 7 (Fig. 1) coinciding with the PVX_086930 sequence reported in plasmoDB. Searching plasmoDB and Sanger Institute databases revealed the presence of *pfclag9* orthologous genes in other *Plasmodium* species (*P. knowlesi, P. yoelii, P. chabaudi* and *P. berghei*), thereby supporting the notion that this protein is part of a multigene family and that it is conserved in most fully sequenced *Plasmodium* species (Holt et al., 2001; Iriko et al., 2008).

An organizational analysis of the genomic region was carried out to determine ORFs, identity and similarity values from hypothetical protein products between *P. falciparum* and *P. vivax* homologous sequences (Fig. 1). The results obtained revealed the presence of: 1) two homologous genes adjacent to *pfclag9* and *pvclag7,* having identity and similarity values ranging from 24.78% to 43.85% and 61.74% to 80.75%, respectively, 2) four non-homologous genes, and 3) a change in the genomic orientation of the *pvclag7* gene. Adenine and thymine homopolymeric regions localized upstream and downstream of the *pfclag9* and *pvclag7* genes (Fig. 1, dotted lines) were identified through multiple alignments of conserved genomic sequences. Recent reports have shown these regions' participation in homologous recombination in *Plasmodium* species (Pologe et al., 1990; Horrocks et al., 1998) and *Eukaryotic* organisms (Meyer et al., 2010) leading us to suggest that *pvclag7* underwent a gene inversion event which occurred after the speciation of a *P. falciparum–P. vivax* common ancestor.

The previously characterized *pvclag7* gene encodes a 1328 amino acid protein, having an estimated ~159 kDa molecular weight, being twelve amino acids shorter than PfClag9 (estimated to be ~160.4 kDa). Bioinformatics analysis showed a signal peptide having a predicted cleavage site between amino acids 23 and 24 (IAC-SY) (Fig. 2A). As described for PfClag9 (Holt et al., 2001), PvClag7 seems to have a transmembrane domain (TM) located between amino acids 1154 and 1176 (Fig. 2A). A single domain called clag was predicted according to the interpro database (Hunter et al., 2009); the domain was characteristic of this multigene family and has been associated with cytoadherence, consisting of infected RBC binding to capillaries and venules from different tissues. Such phenomenon is an important factor for the pathogenesis of malaria and as an immune system evasion mechanism (Holt et al., 1999; Trenholme et al., 2000; Holt et al., 2001).

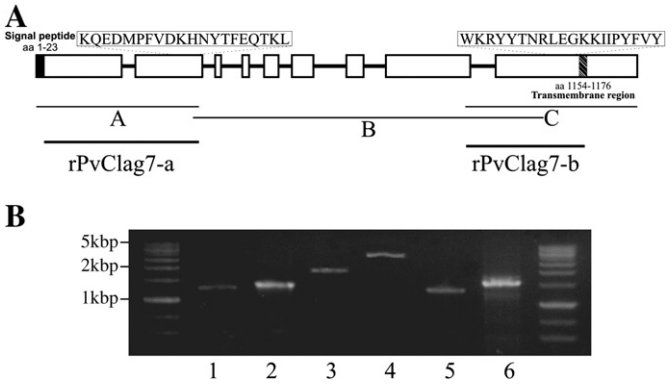

Fig. 2. A) Schematic representation of the *pvclag7* gene showing the N-terminal signal peptide, a transmembrane region and the location of amplification primers. The synthetic peptides used for immunizing rabbits are enclosed in white boxes. B) PCR amplification of *pvclag7* gene in regions A (lanes 1-2), B (lanes 3-4) and C (lanes 5-6) from cDNA and genomic DNA. Odd lanes show RT-PCR and even lanes show PCR from gDNA. The molecular weight marker is given at both sides.

3.2. pvclag7 transcription in blood stages

The *pvclag7* gene was amplified from cDNA using specific primers. Fig. 2B shows that size differences between cDNA- and gDNA-amplified products were consistent with the presence of introns within the gene. Sequence comparison between *pvclag7* genomic DNA and cDNA showed that *pvclag7* contained 9 exons, such as *pfclag9* (Holt et al., 2001); the first exon extended 19 bp farther than previously predicted *in silico*, while the second exon started 26 bp before. Additionally, one synonymous substitution, 18 non-synonymous substitutions, and amino acid 923 deletion were found when the *P. vivax* (VCG) *Aotus*-adapted strain sequence was compared to that available for the Sal-I reference strain at the JCVI (Table 2). It is worth noting that, unfortunately, eleven out of the 18 non-synonymous substitutions came within one of the peptide sequences synthesized to induce anti-PvClag7 polyclonal sera and then assess protein expression.

3.3. PvClag7 expression during P. vivax asexual blood stage

Two peptides derived from the deduced *P. vivax* Sal-I PvClag7 sequence were chemically synthesized and inoculated simultaneously into rabbits to obtain polyclonal antibodies which allowed us to recognize the protein in the parasite lysate. Sera ability to recognize the protein was assessed by Western blot using each purified rPvClag7 fragment as antigens (Fig. 3A). The low sera recognition of rPvClag7-b might have been due to sequence differences between the VCG-1 strain and the Sal-I strain, since the latter was used as template for peptide synthesis. Between the two strains 11 non-synonymous point mutations were found just within peptide 36119 sequence (out of the 20 amino acids that this peptide has), which might have dramatically decreased the ability of polyclonal sera to recognize rPvClag7-b.

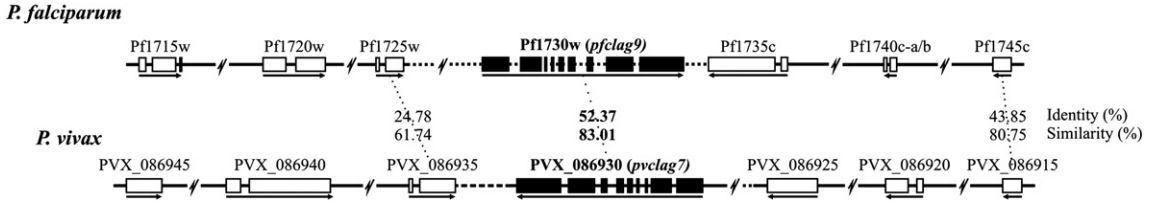

Fig. 1. Scale diagram showing the location of *pfclag9* and *pvclag7* genes (in bold) and adjacent genes in the *P. falciparum* and *P. vivax* chromosomes. Homologous genes are represented with identity and similarity values. ORF orientation and exon organization are indicated for each gene. Dashed lines show poly-A and poly-T rich regions. Gene names were assigned according to their PlasmoDB annotation.

Table 2
Mutations (red letters) found in aligning the *pvclag7* gene sequence obtained from the *P. vivax* (VCG-1) *Aotus*-adapted strain and the Sal-I reference strain.

Amino acid	Sal–I strain	VCG–1 strain
355	D (GAT)	A (GCT)
923	F (TTC)	–
1095	K (AAG)	S (AGT)
1098	N (AAT)	K (AAA)
1105	K (AAG)	T (ACG)
1107	R (AGG)	T (ACG)
1110	N (AAC)	K (AAG)
1113	E (GAG)	Q (CAG)
1115	K (AAG)	I (ATC)
1117	I (ATC)	P (CCC)
1118	I (ATA)	L (CTA)
1119	P (CCA)	Q (CAA)
1120	Y (TAT)	Y (TAC)
1123	Y (TAC)	F (TTC)
1132	A (GCA)	S (TCA)
1221	Y (TAT)	H (CAT)
1267	L (CTG)	Q (CAG)

Sera derived from rabbits recognized a ~157 kDa band in the parasite lysate, agreeing with the predicted weight for PvClag7 without its signal peptide (Fig. 3B: strip 4); further trials using sera pre-incubated with the synthetic peptide 36118 allowed us to confirm that this band corresponded to PvClag7, since reactivity was lost (Fig. 3B: strip 3). On the other hand, a non-specific band was observed at ~100 kDa which was absent in blots incubated with pre-immune sera and remained after pre-incubation with synthetic peptide 36118; the source of this band remains unknown, taking into account that sera reactivity against this band remained after pre-incubation with *E. coli*, *M. tuberculosis* and RBC lysates (data not shown).

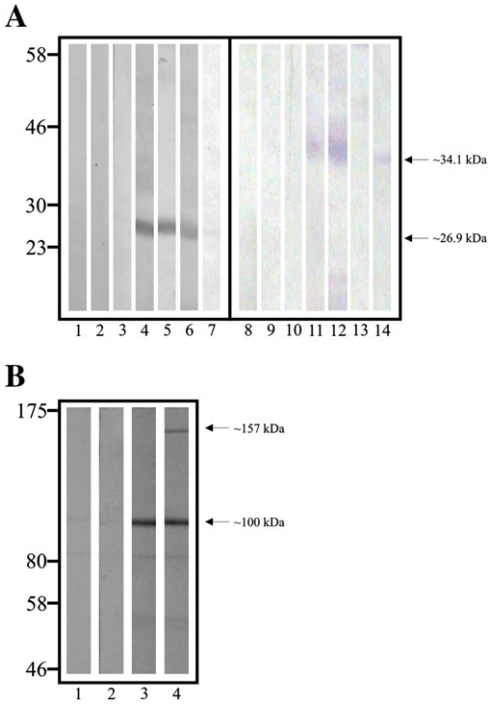

Fig. 3. A) Detection of rPvClag7 fragments by Western blot. Lanes 1-3 and 8-10 show recognition by rabbit pre-immune sera. Lanes 4-6 and 11-13, detection by post-III rabbit sera. Lanes 7 and 14, recognition of purified rPvClag7 fragments by monoclonal anti-polyhistidine antibodies. B) Western blot analysis of *P. vivax* lysate using sera directed against PvClag7 synthetic peptides in rabbits. Lanes 1 and 3 show protein recognition with rabbit pre-immune and post-III sera which were previously pre-incubated with peptide 36118. Lanes 2 and 4, protein recognition by rabbit pre-immune and post-III sera.

3.4. rPvClag7 reactivity with human sera

rPvClag7 fragment recognition by sera from patients who had suffered active episodes of the disease was assessed by ELISA (Fig. 4a). The difference between medians (m) was statistically significant (Wilcoxon rank-sum test. $Z = -2.684$, $p = 0.0073$), with $m = 0.06475$ for rPvClag7-a and $m = 0.049$ for the control group. In addition, the difference between medians for rPvClag7-b ($m = 0.0705$) and the control group ($m = 0.0515$) was statistically significant (Wilcoxon rank-sum test, $Z = -2.348$ and $p = 0.0189$).

No statistically significant difference was found regarding the relationship between the number of *P. vivax* malaria episodes and rPvClag7-a recognition (Kruskal–Wallis test $Chi2(3) = 0.184$; $p = 0.9801$). Likewise, there was no relationship between rPvClag7-b reactivity and the number of malaria episodes (Kruskal–Wallis test $Chi2(3) = 2.941$; $p = 0.4$).

3.5. Immunofluorescence assay

Double staining immunofluorescence assays were performed on slides containing *P. vivax*-infected RBC. Results clearly showed PvClag7 localization towards the merozoites' apical end and also

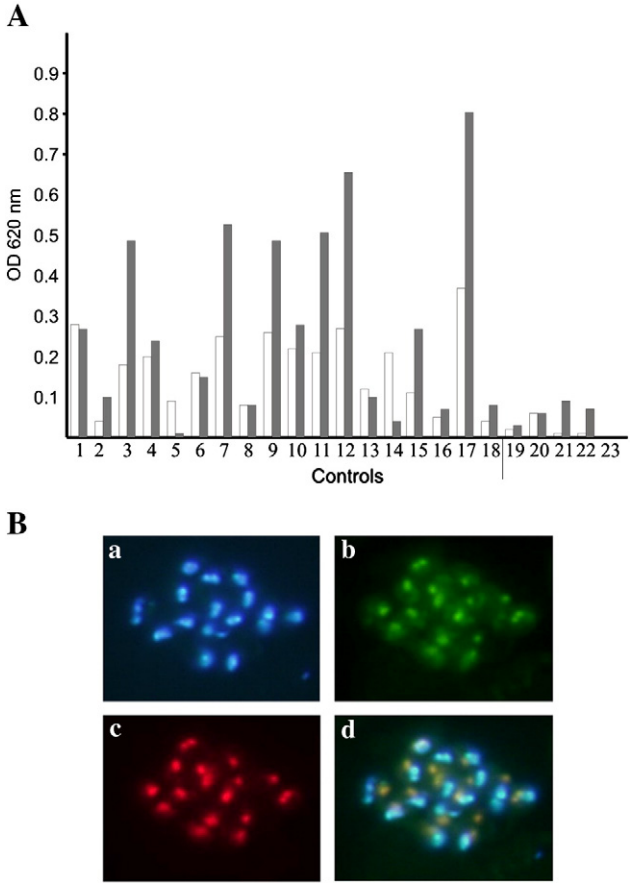

Fig. 4. ELISA recognition of purified and refolded rPvClag7-a and rPvClag7-b fragments by sera from people who had suffered previous *P. vivax* malaria episodes. White bar shows rPvClag7-a recognition and gray bar shows rPvClag7-b recognition. B) Apical expression of PvClag7 in segmented schizonts evaluated by fluorescence microscopy using 100× oil immersion objective; a) core dye with DAPI; b) PvClag7 protein recognition; c) RhopH3 protein recognition and d) the first three recognitions overlapped.

that this protein co-localized with other proteins from the high molecular weight rhoptry complex such as PvRhopH3 (Fig. 4b), similar to what occurs with PfClag9 (Gardiner et al., 2004).

4. Discussion

A large number of proteins expressed during *Plasmodium* genus parasites' intraerythrocytic life cycle have been identified as being potential vaccine candidate antigens due to their role during RBC invasion and their ability to induce protective immunity in a host (Rodriguez et al., 2008). Among the main antigens presented by the parasite, high molecular weight proteins have shown interesting features, especially RhopH1 protein (Topolska et al., 2004). Recent studies have demonstrated that this protein is encoded by different genes; amongst them, *clag9* has been studied not only for its involvement in adhesion to endothelial cells, but also for its participation in host-parasite interactions (Trenholme et al., 2000; Pinzon et al., 2010). According to the above, a *pfclag9* homologous gene was identified in *P. vivax* using bioinformatics tools, which was called *pvclag7* due to its chromosomal location. Furthermore, other two genes similar to *pfclag9* were found in the *P. vivax* genome (data not shown) which led to suggesting that, as occurs in *P. falciparum* (Kaneko et al., 2001), the PvRhopH1 protein is encoded by more than one gene from the clag multigene family. However, it is clear that experimental support is needed to confirm such suppositions.

Bioinformatics algorithms, validated along with a set of vaccine candidate proteins confirmed experimentally in *P. falciparum,* have led to several relevant characteristics being found regarding these antigens (Vivona et al., 2008). Some of these parameters, such as the presence of a signal peptide, transmembranal regions presumably located in the RBC membrane and a conserved putative domain, were found once the hypothetical PvClag7 sequence had been exhaustively analyzed (Fig. 2A); these parameters make PvClag7 protein identification and characterization relevant.

Based on this, recombinant plasmids having *pvclag7* gene fragments were generated to facilitate their sequencing. Once *pvclag7* genomic and complementary DNA sequence integrity was analyzed, it was found that the exons–introns organization was conserved, as has been demonstrated for orthologous genes found in other *Plasmodium* species reported in the plasmoDB database. In turn, 19 mutations were observed throughout the encoding sequence. Interestingly, the location of most substitutions found in *pvclag7* gene alignment analysis of SAL-I and VCG-1 strains was congruent with polymorphic regions found for the *P. falciparum* clag multigene family (Iriko et al., 2008), suggesting that the *pvclag7* gene probably contains a variable region. However, studies about *rhoph1/clag* genetic variability in different *P. vivax* strains are needed to determine its polymorphism level.

PvClag7 protein was recognized in parasite schizont stage as being a ~157 kDa polypeptide by immunochemical assays carried out with polyclonal sera obtained from previous peptide immunization in rabbits (Fig. 3B; strips 3 and 4). Subcellular localization was also determined. According to Gardiner et al. (2004), PfClag9 presents an apical localization pattern characteristic of rhoptry proteins. The PvClag7 protein also showed that it is expressed in a native form in merozoite rhoptries, according to the immunochemical assays carried out here (Fig. 4b).

During a blood-stage malaria infection, antigenic proteins trigger production of antibodies in the host (Beeson et al., 2008) which play a major role in acquired immunity against this pathogen (Persson et al., 2006; Rodriguez et al., 2008). Several studies have shown that such antibodies inhibit target cell invasion by the parasite and their protein targets have thus been considered as potential candidates for creating an effective anti-malarial vaccine. Taking the above into account, PvClag7 protein antigenicity was thus assessed here. Antibody responses observed against both rPvClag7 proteins indicated that the native parasite antigen could trigger an immune response in *P. vivax*-infected patients (Fig. 4a), as occurs with other antigenic proteins (Chen et al., 2010). It can therefore be presumed that PvClag7 protein is a good candidate for being assessed in immunological and functional studies aimed at establishing its role during RBC invasion.

5. Conclusions

A member of the *P.vivax* RhopH1/clag family has been identified and characterized in this study. Additionally, the Pvclag7 protein transcription and expression profile and its sub cellular location were determined. As shown here, this protein is antigenic since antibody responses against it were generated during natural infection with *P. vivax*. Therefore, further studies aimed at assessing this protein's immunogenicity and protection-inducing ability in the *Aotus* monkey experimental model are recommended.

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