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Identifying and characterising the *Plasmodium falciparum* merozoite surface protein 10 *Plasmodium vivax* homologue

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Abstract

Plasmodium vivax malaria is one of the most prevalent parasitic diseases in Asia and Latin-America. The difficulty of maintaining this parasite culture in vitro has hampered identifying and characterising proteins implied in merozoite invasion of red blood cells. We have been able to identify an open reading frame in *P. vivax* encoding the *Plasmodium falciparum* merozoite surface protein 10 homologous protein using the partial sequences from this parasite's genome reported during 2004. This new protein contains 479 amino-acids, two epidermal growth factor-like domains, hydrophobic regions at the N- and C-termini, being compatible with a signal peptide and a glycosylphosphatidylinositol anchor site, respectively. The protein is expressed during the parasite's asexual stage and is recognised by polyclonal sera in parasite lysate using Western blot. *P. vivax*-infected patients' sera highly recognised recombinant protein by ELISA.

Keywords: *Plasmodium vivax*; Malaria; MSP10; Merozoite; Vaccine candidate

Plasmodium vivax malaria is one of the most prevalent parasitic diseases found in tropical regions, mainly on the South-American and Asian continents [1]. About 70–80 million new cases are reported worldwide each year, leaving about one billion inhabitants in regions at risk [2]. The difficulty of maintaining this parasite in in vitro culture has hampered identifying and characterising the blood-stage surface proteins needed for designing a vaccine against this pathogen [3].

The Institute for Genomic Research (TIGR) is currently carrying out the sequencing of the entire *P. vivax* genome with funds from the US Department of Defense and the National Institute of Allergy and Infectious Diseases [2]. Making *P. vivax* genomic sequence data available to the scientific community allows bioinformatic approaches to be used in identifying relevant proteins in the parasite–host cell interaction.

A previous comparative study between a partial 200 kb *P. vivax* sequence and a *Plasmodium falciparum* region belonging to chromosome 3 has shown a high degree of conservation in both gene order and gene structure [4]. A high degree of homology has likewise been described in chromosome gene location amongst different parasite-causing malarial species [5,6].

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Based on these results, our group has been searching for new *P. vivax* genes homologous to those previously described in *P. falciparum* which are currently considered good candidates for an anti-malarial vaccine against this parasite species.

Merozoite surface protein 10 (*Pf*MSP10) [7] represents a potential *P. falciparum* blood-stage vaccine candidate. This 524 amino-acid protein displays two important characteristics in the C-terminal region: a glycosylphosphatidylinositol (GPI) anchor and two epidermal growth factor-like motifs (also present in other *Plasmodium* merozoite surface proteins). Antigenicity studies have shown that *P. falciparum*-infected patients' sera are able to recognise *Pf*MSP10 when expressed as recombinant fragments.

Here we describe the identification and immunochemical characterisation of the *P. falciparum* merozoite surface protein 10 (*Pf*MSP10) homologue in *P. vivax*.

Materials and methods

Parasites. P. vivax VCG I (Vivax-Colombia-Guaviare) strain [3], maintained in vivo by successive passages in splenectomised *Aotus nancymaae* monkeys kept at our primate station in Leticia (Colombia), was used as a source of DNA, RNA, and parasite lysate extraction.

Plasmodium vivax genomic nucleotide sequence source. The partial *P. vivax* Sal-I strain genomic nucleotide sequences used were downloaded from the TIGR web page (http://www.tigr.org/tdb/e2k1/pva1/).

Parasite DNA. P. vivax DNA was extracted as follows. Each 1 ml blood sample, collected in a 10 ml heparinised Vacutainer tube, was centrifuged, the buffy coat removed and the cell fraction was mixed with seven volumes of 0.15% saponin (w/v) (0.131%(w/v) final concentration). This fraction was then incubated for 15 min followed by centrifuging at 3000 rpm for 20 min. The black parasite pellet was recovered and washed three times with 20 ml phosphate-buffered saline (PBS), suspended, digested with 50 μg/ml proteinase K, and then incubated at 50 °C for 18 h. Two rounds of phenol/chloroform extraction were performed, followed by precipitation with isopropanol. The nucleic acid extract was stored dry at −20 °C until use.

Cloning and sequencing. P. vivax VCG-I strain genomic DNA was used as template for PCR amplification. Primer design was based on the open reading frame (ORF) found by sequence homology in the partial *P. vivax* Sal-I strain genomic sequence reported at TIGR. The primers used encompassed the entire ORF; *Bam*HI and *Pst*I cloning sites were added to the forward (5′-CGGGATCCAAACGCGCA AAGTGTAACAAATCA-3′) and reverse (5′-AACTGCAGCTAA AAAAGAAACTTGTGGAGGAGGC-3′) primers, respectively. PCR products were purified by Wizard PCR preps Kit (Promega, Madison, WI) and cloned in pGEM-T vector (Promega, Madison, WI). Plasmid DNA from recombinant bacteria was purified using the Miniprep purification kit (Promega, Madison, WI). Cloned inserts were sequenced in an automatic sequencer (ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems, Foster City, CA).

RNA extraction and cDNA synthesis. Total *P. vivax* RNA was extracted from infected *Aotus* monkey blood samples by the Trizol method [8] and treated with RQ1 RNase-free DNase (Promega, Madison, WI). One microgram of the extracted RNA was used for one-step RT-PCR using SuperScript III (Invitrogen, Carlsbad, CA) in 50 μl reactions according to manufacturer's recommendations. Briefly, cDNA was synthesised for 30 min at 55 °C and PCR was carried out for 40 cycles at the following temperatures: 94 °C for 15 s, 66 °C for 30 s, 68 °C for 90 s, and a final extension cycle of 68 °C for 5 min. Another PCR amplification using Platinum *Taq* (Invitrogen, Carlsbad, CA) and 1 μg total RNA as template was used as negative control to discard genomic DNA presence.

Recombinant protein expression and purification. The *Pv*MSP10 gene, previously cloned in pGEM-T vector, was cut with *Bam*HI and *Pst*I restriction endonucleases (New England Biolabs, Beverly, MA) according to manufacturer's instructions and further subcloned into pQE expression vector (Qiagen, Valencia, CA). This vector adds a 6-histidine tag at the N protein-terminal's portion, enabling an easier purification process and immunodetection by anti-histidine monoclonal antibodies. The recombinant plasmid was transfected into *Escherichia coli* Rosetta-Gami bacteria (Novagen, Milwaukee, WI). This strain contains a plasmid which encodes additional copies of rare *E. coli* tRNAs for obtaining accurate expression levels for foreign proteins. Protein was purified using Ni–NTA metal affinity resin (Qiagen, Valencia, CA) in accordance with manufacturer's recommendations. Protein expression was verified by either sodium dodecyl sulphate–polyacrylamide gel electrophoresis stained with Coomassie blue or Western blot. Total protein amount was quantified by bicinchoninic acid assay.

Animal and human antisera. New Zealand White Rabbits were provided by the Instituto Nacional de Salud (Bogotá, Colombia), housed, fed, and handled in accordance with the recommendations made by the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA). Three rabbits were immunised with 200 μg *Pv*MSP10 recombinant protein emulsified in Freund's complete adjuvant (FCA), inoculated subcutaneously at multiple sites. Boosting inoculations were made on days 21 and 42 with the same amount of immunogen mixed with Freund's incomplete adjuvant (FIA) [9]. Following their verbal consent, sera were collected from people who had suffered several *P. vivax* malaria episodes, as well as sera were taken from three healthy individuals from a known malaria-free region to be used as negative controls.

SDS–PAGE and Western immunoblotting. Blood samples were taken from a *P. vivax* infected monkey (having greater than 6% parasitaemia) and passed through a CF11 cellulose column to isolate red blood cells. They were lysed with 0.25% saponin and washed three times with PBS to obtain parasites; they were then lysed with 5% SDS plus a protease inhibitor cocktail. Proteins in the extract were size-separated by electrophoresis in 7.5–15% gradient SDS–PAGE and then electroblotted onto a nitrocellulose membrane. The membrane was blocked with 0.5% Tween 20 in TBS plus 5% skimmed milk and split into rows to be independently assayed against individual sera. Primary antibodies were detected with alkaline phosphatase (AP), conjugated anti-rabbit Ig.

Indirect immunofluorescence assays. Indirect immunofluorescence assays were performed as follows: red blood cells parasitised with *P. vivax* from *Aotus* monkeys were separated from leukocytes by using a CF11 cellulose column (Whatman, Maidstone, Kent, UK); the parasite's mature forms were then concentrated using a 30–80% Percoll gradient (Sigma, St. Louis, MO). Twenty microlitres of this schizont-parasitised sample was then spread in each well of glass 8-well multi-test slides (ICN, Irvine, CA). The sample was air-dried, fixed with 4% formaldehyde in PBS for 6 min at room temperature, briefly washed with PBS, and then permeabilised with 0.2% Triton X-100 in PBS (ICN, Irvine, CA) for 6 min. The slides were washed again twice with PBS.

Each well was then blocked for 15 min with 5% bovine serum albumin (BSA) in PBS. Rabbit polyclonal sera directed against the recombinant protein were used as primary antibody at 1:100 dilution in PBS with 5% BSA. Fluorescein isothiocyanate (FITC) conjugated anti-rabbit Ig (Sigma, St. Louis, MO) was used as secondary antibody at 1:40 dilution in PBS with 5% BSA. An Olympus BX51 fluorescence microscope was used for reading immunofluorescence.

Detecting antigen-specific human IgG antibodies by ELISA. Sera from 26 individuals were tested by ELISA for reactivity with the *Pv*MSP10 recombinant protein. A sample was taken from 19 of these patients during the acute stage of the last episode prior to beginning

treatment; four had suffered from the disease but were healthy when the sample was taken whilst three had never suffered from the disease and were used as negative controls. Polysorb plates (Nunc, Rochester, NY) were coated with 1 μg/well of affinity purified recombinant protein, incubated overnight at 4 °C, and washed three times with 0.05% PBS–Tween 20. The wells were then blocked at 37 °C for 1 h with 2.5% skimmed milk in PBS. Serum samples were added to triplicate wells at 1:100 dilution and incubated for 1 h at 37 °C; unbound material was washed away with PBS-0.05% Tween 20. A 1:3500 dilution of peroxidase-conjugated goat anti-human IgG (Fc specific, ICN 634501) was added to each well as a second antibody. After 1 h incubation at 37 °C, excess-labelled antibody was removed by washing with PBS-0.05% Tween 20. The reaction was developed by using TMB Microwell Peroxidase Substrate System according to the manufacturer's instructions (KPL Laboratories, Gaithersburg, MD) and the A_{620} was read on a Labsystems Multiskan MJ ELISA reader.

Molecular modelling. A 3D model of the two *Pv*MSP10 EGF-like domains was generated by homology modelling using the *P. falciparum* $MSP1_{19}$ solution structure as template (Protein Data Bank code: 1CEJ) by using Geno3D (http://geno3D-pbil.ibcp.fr) [10], a web server that uses distance geometry, simulated annealing, and energy minimisation algorithms to build the protein model.

Model quality control involved using Insight II software (Accelrys, San Diego, CA) for calculating the root mean square deviation (RMSD) and the Verify3D program (http://shannon.mbi.ucla.edu/DOE/Services/Verify_3D/) [11].

Nucleotide and amino-acid sequence accession numbers. Nucleotide and amino-acid sequences used in the present study have been reported in GenBank under Accession No. AY587775.

Results and discussion

The *P. falciparum* genome has been shown to encode seven proteins having epidermal growth factor (EGF)-like domains at their C-terminal portion; these domains seem to be involved in both merozoite and gametocyte interaction with their target cells, making these seven proteins good vaccine candidates [7,12–16]. To date, all the *P. vivax* genes encoding the homologous proteins have been described, excepting that encoding merozoite surface protein 10 [17–20].

As TIGR has recently made the partial *P. vivax* genome sequence available, this has led to identifying relevant gene homologues becoming more economic and efficient.

We have used the Basic Local Alignment Search Tool (BLAST) in this work to identify the *Pf*MSP10 gene homologue in *P. vivax* by comparing the *Pf*MSP10 amino-acid sequence with the partially sequenced *P. vivax* 10× genome database.

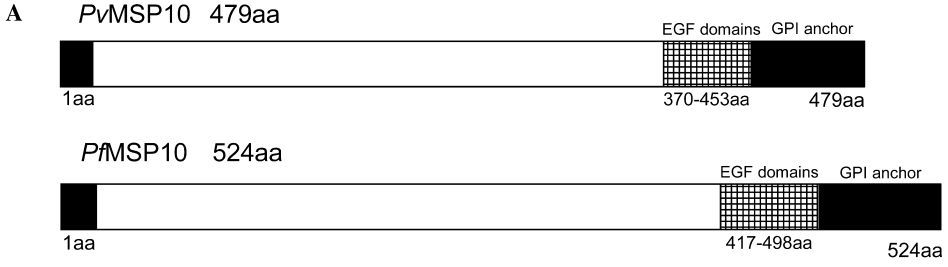
Identifying and characterising the PfMSP10 P. vivax homologue

Searching the TIGR partially sequenced *P. vivax* genome database (http://www.tigr.org/tdb/e2k1/pva1/) using the *P. falciparum* merozoite surface protein 10 (*Pf*MSP10) amino-acid sequence as the BLAST query sequence, we thus identified a 179,933 bp contig containing a 1440 bp ORF encoding a 479 amino-acid polypeptide having an estimated 52.3 kDa molecular mass.

This protein has hydrophobic regions at the N- and C-terminal extremes, these being potential sites for a signal peptide and a GPI binding site, respectively, according to sequence analysis carried out with DGPI from the Expasy Molecular Biology Server (http://au.expasy.org/tools/) (Fig. 1A).

This polypeptide also contains two EGF-like motifs in the C-terminal portion having a high degree of identity (68.6%) with those present in the *P. falciparum* protein (Fig. 1B). Aligning *Pf*MSP10 and *Pv*MSP10 complete amino-acid sequences revealed 32% identity and 49% homology; these values were similar to those found in other *P. vivax* proteins when compared with their falciparum homologues [17,20].

When confirming that the contig found was a homologous segment of *P. falciparum* chromosome 6 containing the *Pf*MSP10 sequence, it was found that *P. falciparum* *Pf*MSP10 was flanked by the

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B PvMSP10  371 CEYSKCGANARCYIVEKDKEECRCRANYMPDDSVDYFKCIPMVEK 415
  PfMSP10  417 .......P.S..........Q....P..IV.M..N.......KDM 461

  PvMSP10  416 DCSKENGNCDVNAECSIDKNKDIKCQCKFNYIGDGIFC       453
  PfMSP10  462 N...N..G.......T.VEG-AV....SHL.F...V..       498
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Fig. 1. (A) Comparing MSP10 protein structures in *P. vivax* and *P. falciparum*. The putative signal and GPI-anchor sequences are shown by solid black rectangles. The two EGF-like domains are indicated by checked rectangles. (B) Sequence alignments comparing the *Pv*MSP10 and *Pf*MSP10 EGF-like domains. Amino-acid positions are shown beside the relevant sequences. Identical amino-acids are indicated by dots.

MAL6P1.222 and MAL6P1.220 genes, both of them encoding putative proteins. The amino-acid sequences of the two ORFs neighbouring the site encoding the *Pf*MSP10 homologue in *P. vivax* were thus used as query sequences for a TBLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/). High homology (53 and 75% for MAL6P1.222 and MAL6P1.220) and synteny were observed.

PvMSP10 is transcribed during asexual stage life-cycle

Evidence that this ORF is transcribed during *P. vivax* asexual stage has been found by extracting RNA and treating it with DNAse to avoid contamination with genomic DNA and then using one-step RT-PCR. We managed to amplify a fragment having the expected size in the sample which was subjected to RT-PCR; no amplification was observed in the negative control, meaning that contamination with genomic DNA could be discarded (Fig. 2). The product amplified in the RT-PCR was then confirmed by sequencing. The sequence obtained in this step was identical to the sequence of the product obtained from genomic DNA, confirming that *Pv*MSP10 is encoded by a single exon, as occurs in *Pf*MSP10.

Only three nucleotide substitutions were found along the whole of the sequence when comparing the sequence obtained from the VCG-I strain parasite against the Salvador I strain sequence obtained from the TIGR database. One substitution was synonymous and two others changed arginine amino-acid for histidine in position 316 and histidine for tyrosine in position 446. This was confirmed by sequencing two independent PCR products.

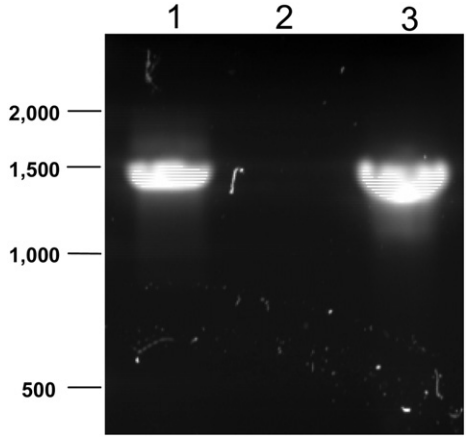

Fig. 2. One-step RT-PCR analysis of total RNA isolated from *P. vivax* asexual stage parasites. Primers corresponding to the predicted 5′ and 3′ ends of the *Pv*MSP10 ORF were used in one-step RT-PCR on DNase-treated total RNA from asexual stage *P. vivax* parasites (lane 1). Negative control for the RNA sample is shown in lane 2. The same PCR was performed with *P. vivax* genomic DNA as template as positive control (lane 3). The size standards are indicated in basepairs at the left-hand side of the panel.

PvMSP10 is expressed during the P. vivax asexual stage

*Pv*MSP10 recombinant protein was expressed in *E. coli* Rosetta-Gami strain and 500 μg per culture litre of 90% purified recombinant protein was obtained. Antibodies raised in rabbits against the recombinant protein were used in different immunochemistry experiments to show protein expression during *P. vivax* asexual stage.

Lysed *P. vivax* parasites extracted from red blood cells from an infected monkey were run on SDS–PAGE followed by immunoblotting. The antibodies raised against the recombinant protein reacted against two bands of around 75 and 58 kDa (Fig. 3A). As shown in this figure, *Pv*MSP10 seems to be proteolytically processed in a similar fashion to *Pf*MSP10, where two fragments are generated [7]. *Pv*MSP10 migrates at a higher

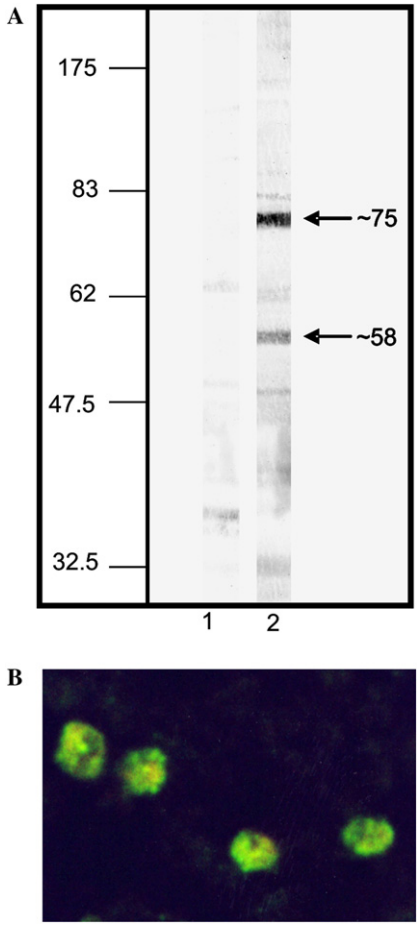

Fig. 3. (A) *Plasmodium vivax* parasite lysate was size-separated by SDS–PAGE, electroblotted, and tested with rabbit sera raised against *Pv*MSP10 recombinant protein. Lane 1, parasite lysate recognition by pre-immune rabbit sera; lane 2, parasite lysate recognition by rabbit anti-*Pv*MSP10 sera. Molecular mass standards are indicated on the left of the panel in kilodaltons. On the right-hand side, arrows indicate detected bands and their estimated molecular mass is shown in kilodaltons. (B) Indirect immunofluorescence assay using rabbit anti-*Pv*MSP10 serum showing parasite labelling of a fixed *P. vivax* schizont group.

molecular mass than expected and this phenomenon has been previously observed in other surface proteins belonging to different *Plasmodium* species [7,12,21]. According to our results, the 58 kDa band seems to correspond to one of these cleavage fragments; however, additional experiments are required to determine whether this band contains the N- or the C-termini of the protein, as well as the exact cleavage site.

IFA revealed PvMSP10 presence in the parasite

A sample of *Aotus* blood parasitised with the adapted *P. vivax* VCG-I strain was taken from a monkey kept at our primate station in Leticia, Colombia; this was used for determining *Pv*MSP10 cellular localisation. Immunofluorescence microscopy of *P. vivax* parasites in formaldehyde-fixed thin blood films with rabbit antibodies produced against recombinant *Pv*MSP10 revealed that fluorescence could mainly be seen in the schizont stage around segmented parasites within red blood cells (Fig. 3B).

Recombinant protein reactivity with human immune sera

An ELISA test was carriedout on sera from 23 patients from the Villavicencio region in eastern Colombia to evaluate the antibody response of patients who had suffered several episodes of malaria caused by *P. vivax* against recombinant *Pv*MSP10 protein. The sera from all patients showed high reactivity, having OD values three times higher than the mean plus three standard deviations from three healthy patients used as negative controls (Fig. 4). No association was observed between the number of episodes suffered and the concentration of antibodies reacting against the protein.

PvMSP10 EGF domains fold similarly to those of $PfMSP1_{19}$

A 3D structure prediction of the *Pv*MSP10 EGF domains has been carried out, based on the previously reported solution structure for the 19 kDa *P. falciparum* MSP1 C-terminal region (Protein Data Bank code: 1CEJ) [22], since the EGF-like motifs lying within this region display 32.6% amino-acid identity to those present in *Pv*MSP10. A *Pv*MSP10 EGF domain model was obtained by using the Geno3D web server and further quality controlled by the Insight II program; both structures were also superimposed for evaluating those regions displaying the most notable differences amongst them, as well as for calculating RMSD value. RMSD found was 1.85 Å (values lower than 2 Å are considered highly reliable) [23]. As observed in Fig. 5 (white arrow), the loop found within predicted model's amino-acid positions 432 and 438 was the most differing region when compared to the *P. falciparum* MSP1 19 kDa template. We have also used Verify3D for assessing struc-

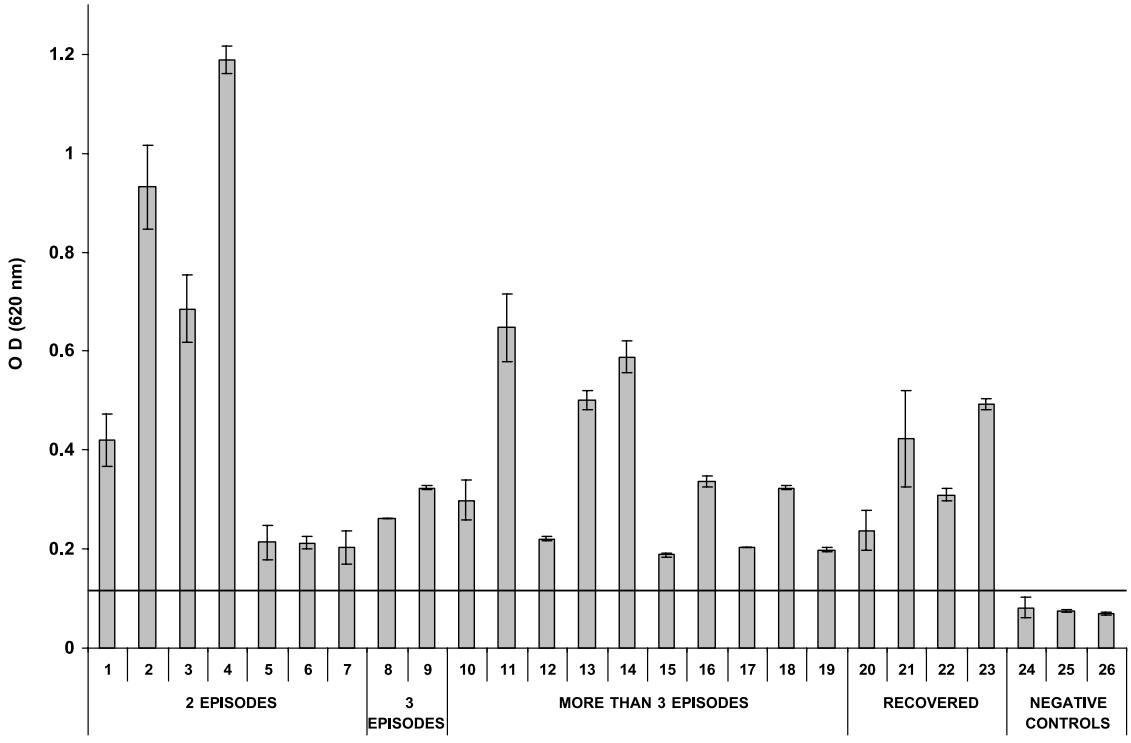

Fig. 4. ELISA displaying *P. vivax*-infected patients' sera reactivity against rPvMSP10. Sera 1–19 correspond to the last acute episode. Sera shown in column 20–23 had been infected by *P. vivax* malaria but were healthy at the time bleeding was carried out. Sera 24–26 were from three healthy individuals from a malaria-free region.

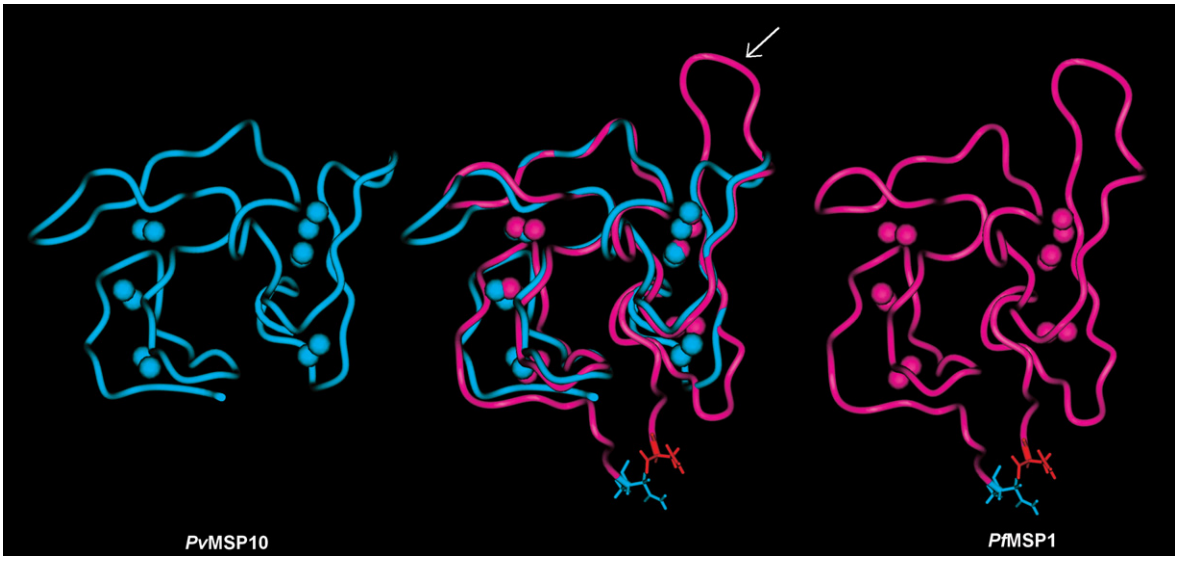

Fig. 5. *Pv*MSP10 EGF-like motif structure prediction. *Pv*MSP10 EGF-like motifs (blue) were obtained by homology modelling. The *Pf*MSP1 19 kDa 3D structure obtained by NMR is shown at the right-hand side; it contains two EGF-like motifs. A superimposition of both structures is displayed in the centre. Spheres indicate disulphur bridges. The white arrow indicates the region differing most between both structures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

tures at the residue level, allowing us to reveal correctly folded protein regions; this analysis showed that 68.3% of all amino-acids forming the model has been correctly localised [24].

Previous work has shown that the 19 kDa *Pf*MSP1 fragment actively participates in merozoite invasion of red blood cells by specifically binding to band 3 [25]. Based on high amino-acid conservation between *Pv*MSP10 and *Pf*MSP10 EGF domains, it could be suggested that this protein region might be mediating the same host cell adhesion function. This suggestion is also supported by the high three-dimensional structure similarity predicted.

In a previous study [7], rabbit sera raised against several *Pf*MSP10 fragments have shown in vitro *P. falciparum* growth inhibition, as well as low polymorphism within this protein. As reported here, the high degree of homology found amongst *Pv*MSP10 and *Pf*MSP10, as well as its antigenicity, leads us to consider this protein as a good anti-*P. vivax* malaria vaccine candidate.

A study of genes transcribed during *P. falciparum* erythrocyte stage has been published recently [26]. It is worth noting that this work statistically analyses the pattern of expression of all the genes transcribed, with respect to seven genes encoding merozoite invasion proteins being considered as good vaccine candidates (MSP1, AMA1, MSP3, MSP5, EBA175, RAP1, and RESA1) in an attempt to identify new proteins which are expressed during the time prior to red blood cell invasion. This would make them good candidates for inclusion in a multi-component anti-malaria vaccine. This analysis showed that there are 262 genes having a similar expression pattern to the seven good vaccine candidates, the *Pf*MSP10 gene being found amongst them.

Carrying out similar work in *P. vivax* once its genome has been completely sequenced will be hampered due to the difficulty of culturing the parasite. It can be suggested that those genes found to be good vaccine candidates according to the transcription pattern in *P. falciparum* should also be found in *P. vivax*. This would aid the search for important antigens in the process of parasite interaction with the host cell and their use as part of a multi-component vaccine against malaria caused by *P. vivax*.

Acknowledgments

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