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Cloning, expression, and characterisation of a *Plasmodium vivax* MSP7 family merozoite surface protein

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Abstract

Plasmodium vivax remains the most widespread *Plasmodium* parasite specie around the world, producing about 75 million malaria cases, mainly in South America and Asia. A vaccine against this disease is of urgent need, making the identification of new antigens involved in target cell invasion, and thus potential vaccine candidates, a priority. A protein belonging to the *P. vivax* merozoite surface protein 7 (*Pv*MSP7) family was identified in this study. This protein (named $PvMSP7_1$) has 311 amino acids displaying an N-terminal region sharing high identity with *P. falciparum* MSP7, as well as a similar proteolytical cleavage pattern. This protein's expression in *P. vivax* asexual blood stages was revealed by immuno-histochemical and molecular techniques.

Keywords: Malaria; *Plasmodium vivax*; Merozoite; MSP7; Vaccine candidate

Malaria is caused by parasites from the *Plasmodium* genus, from which four species have been found to be involved in transmission in humans: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. It has been estimated that about 300–500 million episodes of the disease occur annually around the world, causing the deaths of 1.5–2.7 million people [1]. Even though *P. falciparum* produces the highest mortality indexes mainly in Africa [2], malaria caused by *P. vivax* is one of the most widespread parasitic diseases in tropical regions, representing a huge disease burden, especially in Latin-American and Asian countries, 70–80 million new cases emerging per year [3].

Due to the gradual expansion of antimalarial-resistant parasitic strains [4], as well as insecticide-resistant transmission vectors (*Anopheles* mosquitoes) [5], developing vaccines against malaria has become an imminent priority in terms of arresting the sanitary and economic effects being suffered by countries which are endemic for this disease [6].

Sequencing the *P. falciparum* genome [7], together with the determination of the transcriptome [8] and proteome [9,10] for this specie, has led to finding and analysing drug targets and new vaccine candidate proteins. However, such type of analysis has been limited in *P. vivax* due to the difficulty of carrying out *in vitro* parasite cultures since the parasite selectively invades just the reticulocyte subpopulation (1% of red blood cells) [11]. The Institute of Genomic Research (TIGR) is currently carrying out the complete sequencing of the *P. vivax* genome. Available results have enabled us to identify genes encoding *P. vivax* antigens which are homologous to *P. falciparum* ones; the former have been shown to be important for their potential as vaccine candidates due to their association with the parasite's surface or apical organelles specialising in invasion [12–15].

Plasmodium falciparum (*Pf*MSP7) merozoite 7 surface protein, having a 42 kDa molecular weight, consists of

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351 amino acids encoded by a single exon located in chromosome 13 of the parasite. *Pf*MSP7 has been identified from a 22 kDa segment, derived from a cleavage event in mature schizonts [16], non-covalently associated with three of the fragments produced by the primary processing of *Pf*MSP1 ($MSP1_{83}$, $MSP1_{30}$, and $MSP1_{38}$), in a protein complex involved in invasion of erythrocytes [17–19]. The study identifying the MSP7 homologue in *P. yoelli* [20] has also revealed the presence of proteins which are highly related to *Py*MSP7 (merozoite surface-related proteins: MSRPs); in the same study, homologous proteins to the MSRPs were also identified in *P. falciparum*. All the sequences encoding these MSP7-related proteins are characteristically localised close to each other in the genome of these two species, meaning that they are probably localised as a gene family in *P. vivax*.

Even though the biological function of genes from the MSP7 family has not been deduced so far, producing a *P. berghei* strain lacking the *msp7* gene led to reducing the parasite's normal growth rate, meaning that it could be involved in its ability to invade red blood cells (RBC) [21]. It has also been found that polyclonal antibodies produced on immunised rabbits with the complete *Pf*MSP7 protein partially inhibited *in vitro* parasite growth, also showing cross-reactivity with other surface proteins, very possibly MSRPs. Studies of mice being immunised with *P. yoelii* MSRP2 have shown protective immune responses, meaning that MSRP2 could be involved in the erythrocyte invasion complex, together with MSP1 [22].

Due to the aforementioned evidence, characterising the members of the MSP7 gene family in *P. vivax* becomes relevant, aimed at evaluating which of them express proteins which could be used in a vaccine against this parasite specie. This study was thus aimed at characterising a protein encoded by the MSP7 gene family in *P. vivax* by searching available *P. vivax* genome data and using molecular and immuno-histochemical techniques.

Materials and methods

Parasites. Plasmodium vivax parasites, from the Vivax–Colombia–Guaviare I strain (VGC-I), were used as a source of DNA, RNA, and parasite proteins. The parasites were cultured *in vivo* by successive passes in previously splenectomised *Aotus nancymaae* monkeys which were kept at our primate station in Leticia (Amazonas). The extraction of the greatest percentage of RBC infected with late stage parasites (mainly schizonts) was done from 3 to 4 ml blood samples taken from infected animals, using a Percoll discontinuous gradient, following a previously described protocol [23].

The Plasmodium vivax genome sequence. The partial nucleotide sequence of the *P. vivax* genome used in this study (Sal-I strain) was obtained via the TIGR web page (http://www.tigr.org/tdb/e2k1/pva1/).

Parasite DNA. Once the parasite had been isolated from the sample of infected blood, *P. vivax* DNA was extracted by using a Wizard DNA purification system kit (Promega, Wisconsin, USA).

Cloning and sequencing. Plasmodium vivax (VCG-I strain) genomic DNA was used as template for PCR amplification. The primers were designed from the sequence of a putative transcript which encoded a *P. vivax* MSP7 family protein found by BLAST search in the genome reported for this specie, using the reported *Pf*MSP7 sequence as bait for finding a *P. vivax* homologue. The primers used covered the whole transcript (5′-ATGAACGGCAAAATTTTCCTT-3′ forward primer, 5′-TATCATGTTGAGCAAGTTGA-3′ reverse primer). PCR products were purified by using a Wizard PCR preps kit (Promega, Wisconsin, USA) and cloned in pEXP5-CT/TOPO vector (Invitrogen, California, USA). Recombinant plasmid DNA was purified using a Miniprep purification system kit (Promega, Wisconsin, USA). Cloned insert integrity was confirmed by automatic sequencing in an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, California, USA).

Extracting RNA and cDNA synthesis. Parasite total RNA was extracted by using the Trizol method [24] and treated with RQ1 RNase-free DNase (Promega, Wisconsin, USA). One microgram of extracted RNA was used for RT-PCR, using the SuperScript III enzyme (Invitrogen, California, USA) in 20 μl reactions, according to manufacturer's recommendations. cDNA was thus synthesised for 60 min at 50 °C, whilst PCR amplification was then done with the Platinum *Pfx* DNA polymerase enzyme (Invitrogen, California, USA) for 40 cycles at the following temperatures: 94 °C for 15 s, 54 °C for 30 s, 68 °C for 60 s, and a final extension step at 68 °C for 5 min. Additional PCR was carried out using non-reverse transcribed RNA as template (negative control) for discarding genomic DNA contamination.

Peptide synthesis. Two 20-amino-acid-long peptides were synthesised, based on portions from the *P. vivax* $MSP7_1$ deduced sequence (Sal-I). The amino acid sequences, shown in single letter code, were: ^{144}QSDNQSDQQSGAPHAGPHEG^{163} (peptide No. 32394) and ^{270}KDFMNGIYAYAKRKHYIRGT^{289} (peptide No. 32395). A glycine and cysteine were inserted at the N- and C-termini of both peptides to allow polymerisation. The peptides were synthesised using standard solid-phase t-Boc/Bzl peptide synthesis strategy [25]. The peptides were lyophilised and then characterised by RP-HPCL and MALDI-TOF MS.

Immunisation in rabbits and collecting their sera. New Zealand white rabbits were provided by the Instituto Nacional de Salud (Bogotá, Colombia). They were fed, housed, and looked after according to recommendations established by the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA). Two rabbits were subcutaneously inoculated in multiple sites on day 0. Each rabbit received just one polymerised synthetic peptide. The initial dose was 150 μg of emulsified peptide in Freund's complete adjuvant (FCA), whilst the same amount of peptide mixed with Freund's incomplete adjuvant (FIA) was inoculated on days 21 and 42 as booster. Sera were collected after the first immunisation (pre-immune sera) and 21 days after the third immunisation (hyper-immune sera).

Recombinant protein expression and purification. pEXP5-CT/TOPO vector (Invitrogen, California, USA), where the *Pv*MSP7 gene was cloned, adds a six-histidine tag to the protein's C-terminal portion, thereby facilitating purification and immunodetection by anti-histidine monoclonal antibodies. The protein was purified in denaturing conditions using 6 M Urea in Ni^{2+}–NTA resin (Qiagen, California, USA), according to manufacturer's recommendations. Its expression was verified on 12% polyacrylamide gel in the presence of SDS (SDS–PAGE) which was stained with Coomassie blue or evaluated by Western blot. The total amount of protein was determined by bicinchoninic acid assay.

SDS–PAGE and Western blot. The malarial parasite, purified from *Aotus* monkeys' total blood, was lysed with a solution containing 20% SDS, 0.5 M EDTA, 100 mM PMSF, and 100 mM iodoacetamide. The proteins in lysate were size-separated on 12% polyacrylamide gel in the presence of SDS and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS-0.05% Tween. It was then washed three times with PBS-0.05% Tween for 5 min each time and cut into strips for individual assays with the sera. The sera from each rabbit were used at 1:50 dilution as primary antibody and incubated for 1.5 h at room temperature. The strips were then washed thrice with PBS-0.05% Tween. The secondary antibody (alkaline phosphatase-coupled goat anti-rabbit IgG) was diluted 1:4500 and incubated for 1.5 h at room temperature. Excess antibody was removed by washing thrice with PBS-0.05% Tween and revealed by using a VIP kit (Vector Laboratories, California, USA), according to manufacturer's recommendations.

Indirect immunofluorescence assays. RBC infected with mature blood-stage parasites (mainly schizonts) were used for indirect immunofluorescence assays, after being washed thrice with PBS to remove any Percoll remaining after extraction. Twenty microlitres of the parasitised sample was placed on an 8-well Multitest slide (ICN, California, USA). The sample was left to dry at room temperature, fixed with 4% formaldehyde in PBS, and then permeabilised with 0.2% Triton X-100 (ICN, California, USA) in PBS for 6 min. After washing the slides twice more with PBS, each well was blocked for 15 min with 5% bovine serum albumin (BSA) in PBS. Rabbit polyclonal antibodies directed against synthetic peptides at 1:100 dilution in PBS with 5% BSA were used as primary antibody. Goat anti-rabbit IgG conjugate labelled with fluorescein isothiocyanate (Sigma, Missouri, USA) diluted 1:40 in PBS with 5% BSA was used as secondary antibody. An Olympus BX51 fluorescence microscope was used for reading immunofluorescence.

Accession numbers. The accession number for the nucleotide and amino acid sequences used in the present study has been reported in GenBank as follows: EF042109.

Results and discussion

Identifying the gene encoding an MSP7 family protein in P. vivax

A 1,012,632 bp chromosome segment containing a 936 bp ORF encoding a 311 amino acid peptide sequence was found by using the basic local alignment tool (BLAST); the *Pf*MSP7 amino acid sequence (GenBank Accesion No: NP_705253) was used as bait for finding homologous ORFs in the reported *P. vivax* genome sequence (available in TIGR). Amongst the different ORFs showing high BLAST scores, the characterised gene was the one displaying the highest identity and similarity values to *Pf*MSP7. Nevertheless, it is worth noting that similar BLAST scores were detected for other *P. vivax* ORFs and all of them were encoded by genes adjacently located in this parasite's chromosome 13, as has already been reported for *P. yoelii* [20]. Later analysis using the encoded peptide sequences from *P. vivax* and *P. falciparum* gene family members revealed that identity and similarity values ranged from 6.2% to 29% and 9.9% to 41.8%, respectively, when comparing each sequence individually to those from the other specie (Table 1). As previously mentioned, the highest identity (21.9%) and similarity (33.1%) values to the *Pf*MSP7 protein (NP_705253) were related to the hypothetical PV082695 protein and this antigen was thus the one characterised (referred to as *Pv*MSP7$_1$ from now on).

This protein presents an estimated 34.7 kDa molecular mass in *P. vivax*, being 40 amino acids smaller than *Pf*MSP7 (estimated 41.3 kDa). According to analysis of the *Pv*MSP7$_1$ sequence, a hydrophobic region was found towards the N-terminal region, possibly encoding a signal peptide. The prediction for this region's cleavage site was localised between residues 22–23 (SHG-RT), differently to that of *Pf*MSP7 which was found between amino acids 27–28 (THS-TP), as indicated by SignalP software, v3.0 [26].

Plasmodium falciparum and *P. vivax* 75 and 65.4 kb chromosome regions, respectively, were studied to assess both the MSP7 family gene order and the gene synteny. ORFs were predicted using both GenScan and GeneComber software [27,28]. Fig. 1 shows that the closest genes flanking the MSP7 gene family upstream and downstream exhibited a relatively high level of identity (34–66.9%), similarity (44.1–78%) and conserved the same ORF orientation for homologous chromosome regions (but slightly more separated) in *P. falciparum*. It can be seen that the *P. falciparum* MAL13P1.176 and PF13_0198 genes were absent in the analysed *P. vivax* segment. These genes encode two proteins homologous to the *P. vivax* reticulocyte binding protein 2 (*Pv*RBP2) in *P. falciparum*; however, this vivax gene is localised in a different genetic locus.

Eight genes from the MSP7 gene family were found in *P. falciparum* (including *Pf*MSP7) and 11 in *P. vivax* towards the central region of the fragment being studied. Due to such numerical difference, inter-species gene correspondence was not clear amongst integrants of this gene family and homologues could not therefore be assigned based just on their chromosome position. A phylogenetic study is thus needed for establishing an evolutionary relationship between the different MSP7s and MSRPs for those *Plasmodium* species expressing them, since this type of analysis could help in determining which proteins from this family could be involved in *P. vivax* invasion of erythrocytes and which might be able to produce protection-inducing immune responses in an animal model.

PvMSP7$_1$ is transcribed in blood-stage parasites

Once RNA had been isolated from mature blood-stage parasites from *P. vivax*-infected *Aotus* monkeys it was then

Table 1
Comparing peptide sequences from integrants of the *P. falciparum* MSP7 gene family (NPs) to *P. vivax* (Pvs)

	% Identity–% Similarity																					
	Pv082645		Pv082650		Pv082655		Pv082665		Pv082670		Pv082675		Pv082680		Pv082685		Pv082695		Pv082700		Pv082710	
NP_705246.1	18.4	30.7	20.3	32.1	19.9	29.4	20.0	30.4	20.3	32.1	18.8	31.5	20.9	30.0	22.7	33.5	20.6	27.9	20.6	32.0	10.2	19.0
NP_705247.1	19.7	31.9	18.7	28.2	20.5	30.8	22.5	33.9	14.8	25.5	17.1	28.2	23.6	32.2	23.3	31.8	21.1	32.9	17.1	30.2	14.0	19.9
NP_705248.1	7.1	13.1	12.5	20.5	12.8	21.5	9.2	15.8	12.5	19.9	11.4	22.4	11.7	19.4	13.0	21.0	8.7	14.7	13.0	20.9	10.6	17.2
NP_705249.1	15.6	25.9	15.4	23.5	16.9	27.4	18.2	25.8	12.7	23.8	16.0	24.6	17.7	25.4	17.7	27.2	19.2	27.4	14.2	23.6	9.3	17.1
NP_705250.1	7.9	14.8	7.3	10.8	8.3	12.4	8.6	13.1	7.3	11.9	6.2	9.9	7.8	12.5	7.1	13.0	11.5	19.8	6.2	11.0	6.2	11.1
NP_705251.1	19.8	30.4	16.5	24.4	21.4	28.6	20.6	29.8	15.6	21.9	18.5	25.8	21.7	29.7	23.0	31.7	25.4	38.1	16.9	25.9	12.8	21.5
NP_705252.1	23.3	37.7	23.7	37.1	23.7	37.9	24.0	37.9	17.1	27.2	23.6	35.5	29.0	41.8	23.8	38.2	21.1	31.1	20.8	32.9	15.3	23.8
NP_705253.1	19.7	31.2	17.6	29.0	20.5	33.6	20.7	33.7	15.8	27.4	17.5	29.2	19.5	31.6	18.6	33.4	**21.9**	**33.1**	19.6	30.8	13.0	21.7

treated with DNAse. After RNA had been reverse transcribed, the gene encoding *Pv*MSP7$_1$ was amplified by PCR, revealing a band having the expected size (Fig. 2). No amplification product was observed in the negative control, indicating that there had been no contamination with genomic DNA. Similar band sizes for PCR and RT-PCR products suggested that *pvmsp7$_1$* consisted of a single exon and this notion was further confirmed by analysing the sequences for both amplification products. Four nucleotide substitutions were found on comparing the sequence obtained from the VCG-1 adapted strain to the Sal-1 strain sequence available in TIGR. One substitution was synonymous whilst the other three (in nucleotides 550, 654, and 877) represented substitutions in serine amino acid for alanine in position 184, glutamic acid for aspartic acid in position 218, and lysine for glutamic acid in position 293 (from Sal-1 to VCG-1, respectively, in each case). These substitutions were confirmed by using the sequence from two clones obtained from products independently amplified by PCR.

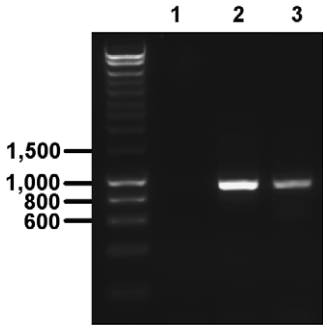

Fig. 2. *Pv*MSP7$_1$ gene amplification in *P. vivax*. Lane 1, negative control. Lane 2 shows the RT-PCR of DNase-treated total *P. vivax* RNA using the primers corresponding to the predicted 5′ and 3′ ends of *Pv*MSP7$_1$ ORF. Lane 3 shows the PCR of *Pv*MSP7$_1$ using genomic DNA as template using the same specific primers.

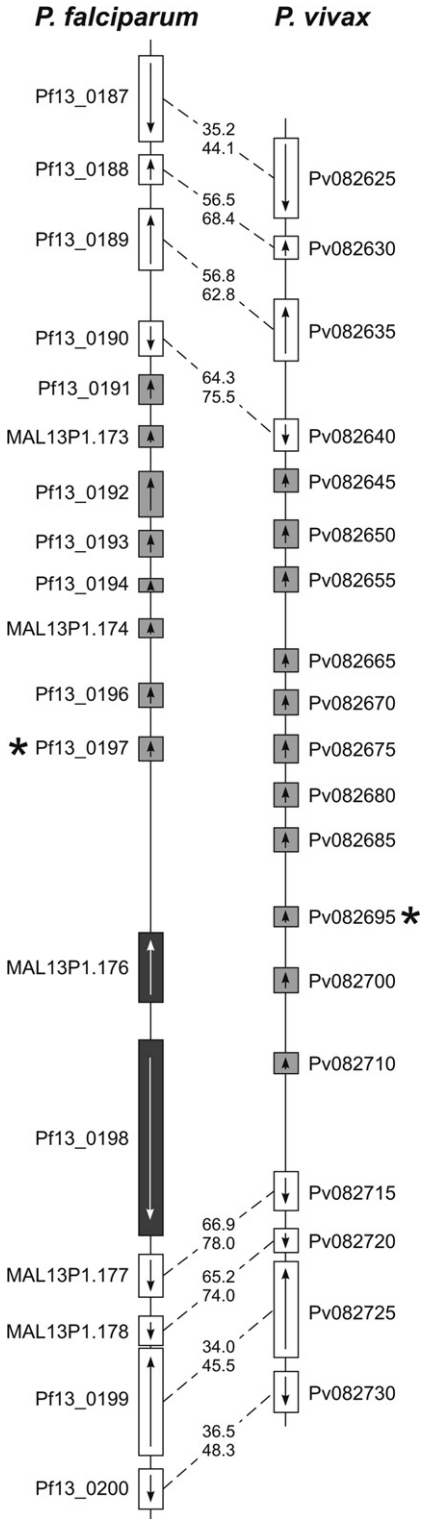

Fig. 1. Schematic diagram of the chromosome localisation of genes from the MSP7 gene family and adjacent genes in *P. falciparum* and *P. vivax*. The arrows indicate the direction of each ORF. *P. vivax* genes are named according to TIGR's genome notation. The white boxes represent homologous genes for both species, with their respective identity and similarity values. The clear grey boxes represent the genes from the MSP7 gene family. The dark grey boxes correspond to those genes only present in *P. falciparum*. Chromosome 13 fragments analysed for *P. falciparum* and *P. vivax* had 75,000 and 65,400 bp lengths, respectively. The conserved genes' identity and similarity values are shown.

PvMSP7$_1$ is expressed during P. vivax asexual stage

Polyclonal sera were produced by immunising two synthetic peptides in rabbits, each peptide being 20 residues long and derived from the amino acid sequence of the initially predicted protein. These sera were used in immunohistochemical assays for detecting *Pv*MSP7$_1$ production in asexual blood-stage parasites. Recombinant *Pv*MSP7$_1$ protein was simultaneously expressed and then purified from cultures in *Escherichia coli*.

Effective recognition by the sera was shown by their reactivity against the purified recombinant protein, after being run on SDS–PAGE and transferred to a nitrocellulose membrane (Fig. 3A). This Western blot assay revealed a recognition band having the expected molecular weight

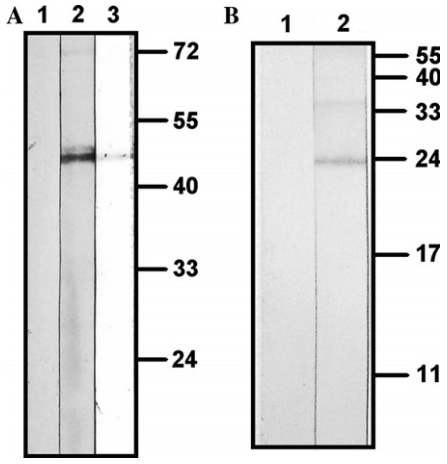

Fig. 3. *Pv*MSP7$_1$ detection with polyclonal antibodies. (A) Western blot of purified recombinant *Pv*MSP7$_1$ recognised by polyclonal antibodies against 32394 synthetic peptide. Lane 1, purified recombinant *Pv*MSP7$_1$ detected with pre-immune serum. Lane 2, purified recombinant *Pv*MSP7$_1$ detected with hyper-immune serum. Lane 3, purified recombinant *Pv*MSP7$_1$ recognition by anti-histidine monoclonal antibody (positive control). (B) *P. vivax* parasite lysate was size-separated by SDS–PAGE, electroblotted, and tested with a rabbit serum raised against 32394 synthetic peptide. Lane 1, parasite lysate recognition by pre-immune serum. Lane 2, parasite lysate recognition by hyper-immune serum.

for this antigen on the membrane when using hyper-immune sera but not pre-immune sera.

It has been described that MSP7 undergoes a proteolytic cleavage in *P. falciparum* producing a 22 kDa fragment; since this fragment is solely observed on the surface of *P. falciparum* merozoites, it has been proposed that MSP7 conversion to MSP7$_{22}$ occurs before schizont rupture [19]. The 22 kDa fragment is then further cleaved to produce a 19 kDa polypeptide. Western blot analysis for identifying the MSP7$_1$ protein in *P. vivax* parasite lysate revealed that the hyper-immune serum from rabbit 412 (immunised with the synthetic peptide No. 32394) recognised ~34 and ~20 kDa bands (Fig. 3B). This suggested that *Pv*MSP7$_1$ had a proteolysis pattern similar to that of *Pf*MSP7, since the serum recognised the complete protein and a peptide fragment having similar size to that observed following *Pf*MSP7 primary processing [16]. However, secondary proteolysis could not be determined due to the similar weight of the cleaved and non-cleaved bands. Another synthetic peptide localised at the *Pv*MSP7$_1$ C-terminal region was also designed (peptide No. 32395) and used for immunising a rabbit to produce polyclonal antibodies. Western blot results showed that this serum cross-reacted with several proteins at different molecular weights (data not shown). Aligning the 11 MSP7 gene family-derived *P. vivax* putative proteins showed that all peptide sequences had a conserved region towards their C-terminal extreme; this could explain the cross-reactivity observed on using polyclonal antibodies directed towards the second peptide. This phenomenon has been previously described with sera obtained from immunisation with the *Pf*MSP7 recombinant protein [19], having an equally conserved region in relation to the other proteins from the *P. falciparum* MSP7 family [20].

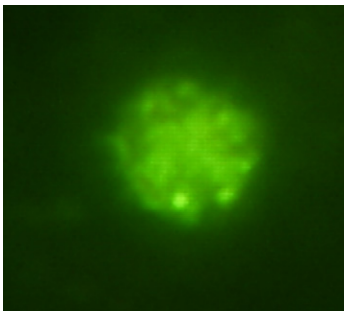

Fig. 4. Indirect immunofluorescence assay using rabbit 412 hyper-immune serum showing parasite labelling of a fixed *P. vivax* schizont.

The immunofluorescence assay revealed that the parasite expressed PvMSP7$_1$

Independently taken blood samples, obtained from *Aotus* monkeys infected with the *P. vivax* VCG-1 adapted strain, were used for obtaining fresh parasite. Once extracted, live parasites were used for determining the cellular localisation of *Pv*MSP7$_1$ by immunofluorescence microscopy. Hyper-immune serum from rabbit 412 was thus used as primary antibody. This assay led to revealing fluorescence within infected RBC (iRBC), seeming to be mainly on the surface of the merozoites, during malarial parasite schizont stage (Fig. 4).

This study led to characterising a protein from the MSP7 family in *P. vivax*, displaying a similar proteolysis pattern to that observed for *Pf*MSP7, suggesting that it could be involved in the erythrocyte invasion complex, together with MSP1. As it could not be exclusively correlated to *Pf*MSP7, *Pv*MSP7$_1$ should be studied further for evaluating its potential as a candidate for a vaccine against *P. vivax*, the same as other proteins from this gene family whose expression on the parasite's surface has also yet to be shown.

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