

Methodology

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Serological detection of *Plasmodium vivax* malaria using recombinant proteins corresponding to the 19-kDa C-terminal region of the merozoite surface protein-I

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Abstract

Background: Serological tests to detect antibodies specific to *Plasmodium vivax* could be a valuable tool for epidemiological studies, for screening blood donors in areas where the malaria is not endemic and for diagnosis of infected individuals. Because *P. vivax* cannot be easily obtained *in vitro*, ELISA assays using total or semi-purified antigens are rarely used. Based on this limitation, we tested whether recombinant proteins representing the 19 kDa C-terminal region of the merozoite surface protein-I of *P. vivax* (MSPI₁₉) could be useful for serological detection of malaria infection.

Methods: Three purified recombinant proteins produced in *Escherichia coli* (GST-MSPI₁₉, His₆-MSPI₁₉ and His₆-MSPI₁₉-PADRE) and one in *Pichia pastoris* (γMSPI₁₉-PADRE) were compared for their ability to bind to IgG antibodies of individuals with patent *P. vivax* infection. The method was tested with 200 serum samples collected from individuals living in the north of Brazil in areas endemic for malaria, 53 serum samples from individuals exposed to *Plasmodium falciparum* infection and 177 serum samples from individuals never exposed to malaria.

Results: Overall, the sensitivity of the ELISA assessed with sera from naturally infected individuals was 95%. The proportion of serum samples that reacted with recombinant proteins GST-MSPI₁₉, His₆-MSPI₁₉, His₆-MSPI₁₉-PADRE and γMSPI₁₉-PADRE was 90%, 93.5%, 93.5% and 93.5%, respectively. The specificity values of the ELISA determined with sera from healthy individuals and from individuals with other infectious diseases were 98.3% (GST-MSPI₁₉), 97.7% (His₆-MSPI₁₉ and His₆-MSPI₁₉-PADRE) or 100% (γMSPI₁₉-PADRE).

Conclusions: Our study demonstrated that for the Brazilian population, an ELISA using a recombinant protein of the MSPI₁₉ can be used as the basis for the development of a valuable serological assay for the detection of *P. vivax* malaria.

Background

Plasmodium vivax accounts worldwide for an estimated 70–80 million cases of malaria per year [1]. In some countries such as Brazil, *P. vivax* was responsible for approximately 79% of the 389,736 cases of the disease reported in 2001 [2].

Laboratory methods are important tools for the control of the disease progress. They can be useful for the individual diagnosis or for the patients' follow-up after specific anti-malaria treatment. Conventional light microscopy has been widely used for malaria diagnosis. However, outside areas where malaria is endemic, it is rarely performed. Also, this method is time consuming and requires trained personnel.

In attempts to overcome these problems several rapid diagnostic tests have been developed recently. These tests detect specific proteins such as HRP2, a histidine rich protein 2, or pLDH, lactate dehydrogenase, in unfractionated blood of patients with malaria. However, these assays do not differentiate *P. vivax* from *Plasmodium malariae* or *Plasmodium ovale* infections, and vary in their sensitivity and specificity (reviewed in reference [3]).

Molecular diagnosis by PCR is described as the most sensitive and specific method for *Plasmodium* detection. Genus- and species-specific primers have been used to amplify *Plasmodium* *ssrRNA* genes of the four human malaria parasites and to detect mixed infections [4-6]. However, this methodology is costly and requires trained personnel for its implementation.

Detection of antibodies by immunofluorescence or ELISA has been used for seroepidemiology of malaria. However, in the case of *P. vivax*, the difficulty of blood stage cultivation has been hindering the use of these methodologies. Production of recombinant proteins through the techniques of genetic engineering may provide sufficient *P. vivax* blood stage antigen(s) for the establishment of specific serological assays.

In the course of immuno-epidemiological studies using recombinant proteins based on the sequence of the Merozoite Surface protein-1 (MSP1) of *P. vivax*, we found that a recombinant protein representing the 19 kDa region of MSP1 (MSP1₁₉) was highly immunogenic during natural infection in humans [7,8]. This recombinant protein was recognized by antibodies of a large fraction of Brazilian individuals recently exposed to *P. vivax* [7]. This observation was subsequently confirmed in studies performed in Korea, where more than 90% of *P. vivax*-infected individuals displayed specific antibodies to *P. vivax* MSP1₁₉ [9]. Also important was the observation that the *P. vivax* MSP1₁₉ gene displays very limited allele polymorphism in

different regions of the world that does not restrict recognition by human antibodies [10,11]. Together, these results suggested that it could be possible to develop an ELISA using a single recombinant protein based on the *P. vivax* MSP1₁₉. This relatively simple and inexpensive technique could be of great applicability for epidemiological studies, the screening of blood donors and the serological diagnosis of malaria caused by *P. vivax*.

In the present study three purified recombinant proteins produced in *E. coli* (GST-MSP1₁₉, His₆-MSP1₁₉ and His₆-MSP1₁₉-PADRE) and one in *Pichia pastoris* (yMSP1₁₉-PADRE) were compared for their ability to be recognized by IgG antibodies of individuals with patent *P. vivax* infection. Serological evaluation was performed with serum samples collected from individuals living in areas endemic for malaria in the north of Brazil and compared to serum samples from individuals never exposed to *P. vivax* malaria.

Materials and methods

Study population

Sera from 430 individuals were used in this study, of which 200 were from patients with patent *P. vivax* malaria and 230 from persons not exposed to *P. vivax* malaria (negative controls). The blood samples from *P. vivax* patients were collected in the state of Pará, in the north of Brazil, in areas endemic for malaria: 103 from Belém, 21 from Marabá, 20 from Itaituba, 20 from Tailândia, 36 from Igarapé-Açu. Patent infection was documented by microscopic analysis of Giemsa-stained blood drops. These samples were obtained between January 1996 and July 1999 with informed consent of all individuals and kept at -20°C.

The 230 individuals non-exposed to *P. vivax* malaria included: (i) 49 donors to blood banks in the city of São Paulo, an area where malaria is not endemic (negative controls); (ii) 108 blood bank donors with unrelated infectious diseases, detected serologically; among them 21 with Chagas Disease, 21 with syphilis, 19 with HBV, 21 with HCV, 14 with HTLV and 12 with HIV; (iii) 10 individuals positive for antinuclear antibodies (ANA) and 10 for rheumatoid factors (RF); (iv) 53 individuals living in distinct areas in West Africa where malaria caused by *Plasmodium falciparum* is endemic: a) 26 adults without clear symptoms of malaria or other infectious disease from Senegal (n = 19) and Gambia (n = 7), b) seven children from Gambia during patent infection by *P. falciparum*, c) 20 malaria immune adults from Ghana. Aliquots of these samples were kindly supplied by Dr. Marcelo Urbano Ferreira (Universidade de São Paulo, São Paulo) and Dr. Silvia Di Santi (Superintendência de Controle de Endemias, SUCEN, São Paulo). Clinical and laboratory data were reported elsewhere [12-14].

Table 1: Recombinant proteins based on the MSP1₁₉ of *P. vivax*.

Recombinant protein	Vector	Microorganism	Molecular Weight (kDa)	Ref.
GST-MSP1 ₁₉	pGEX-3X	<i>E. coli</i>	36	15
His ₆ -MSP1 ₁₉	pET-14b	<i>E. coli</i>	18	15
His ₆ -MSP1 ₁₉ -PADRE*	pET-14b	<i>E. coli</i>	18	15
yMSP1 ₁₉ -PADRE*	pPIC-9K	<i>Pichia pastoris</i>	18	16

* PADRE epitope is composed of amino acids AKFVAAWTLKAAA.

Recombinant proteins

The recombinant proteins used in the present study represent amino acids 1616–1704 of the MSP-1 (Belem strain) of *P. vivax*. These proteins were expressed in *E. coli* (GST-MSP1₁₉, His₆-MSP1₁₉ and His₆-MSP1₁₉-PADRE, ref. 15) or *Pichia pastoris* (yMSP1₁₉-PADRE, ref. 16). The generation of the plasmids and expression/purification of the recombinant proteins produced in *E. coli* or *Pichia pastoris* were performed as described in references 15 and 16, respectively.

ELISA for detection of human antibodies

Human IgG antibodies against MSP1₁₉ were detected by ELISA as described [7]. ELISA plates were coated with 200 ng/well of each recombinant protein. The amount of recombinant protein gave the same OD₄₉₂ when we used an anti-MSP1₁₉ MAb [17]. Fifty µl of each solution were added to each of 96 well plates (High binding, Costar). After overnight incubation at room temperature (rt), the plates were washed with PBS-Tween (0.05%, v/v) and blocked with PBS-milk (5% w/v) for two hours at 37°C. Serum samples were diluted 1:100 in this same solution and 50 µl of each sample was added to each well in duplicate. After incubation for two hours at rt and washes with PBS-Tween, 50 µl a solution containing peroxidase-conjugated goat anti-human IgG (Fc specific) diluted 1:10,000 (Sigma) were added to each well. The enzymatic reaction was developed by the addition of 1 mg/ml of o-phenylenediamine (Sigma) diluted in phosphate-citrate buffer, pH 5.0, containing 0.03% (v/v) hydrogen peroxide, and was stopped by the addition of 50 µl of 4 N H₂SO₄. Plates were read at 492 nm (OD₄₉₂) with an ELISA reader (SLT SPECTRA, SLT Labinstruments, Austria). The individual values of OD₄₉₂ obtained for the recombinant proteins of MSP1₁₉ were corrected by subtraction of the individual values of OD₄₉₂ obtained against glutathione S-transferase (GST).

Statistical analysis

OD₄₉₂ from different samples were plotted using computer graphics software (GraphPad Prism, Version 3.0, San Diego, California). Cutoff values of each recombinant protein in ELISA were calculated as the mean OD₄₉₂ of

sera from 49 blood donors plus five standard deviations (SD). The values for sensitivity and specificity were estimated as described [18] with microscopy used as the gold standard. The Kruskal-Wallis test was used to test the significance of differences between the group values. Differences between proportions were analyzed by a Chi-square test.

Results

Reactivity of recombinant proteins expressed in distinct vectors with serum samples from individuals with patent *P. vivax*

Four recombinant proteins obtained as earlier described in references 15 and 16 (Table 1) were used. SDS-PAGE analysis of each recombinant protein revealed a single band of the expected molecular weight after Coomassie blue [15,16] or Silver staining (data not shown).

An IgG survey of the serum samples of individuals with patent *P. vivax* infection patients showed that the values of positivity were quite similar with the four recombinant antigens employed. Ninety to 93.5% of the 200 serum samples tested were positive for IgG with the recombinant proteins based on the MSP1₁₉.

When we compared the OD₄₉₂ values from serum samples of *P. vivax* infected individuals, we observed that values obtained for the recombinant proteins His₆-MSP1₁₉ and His₆-MSP1₁₉-PADRE were significantly higher than those obtained for GST-MSP1₁₉ (Fig. 1, $P < 0.01$, Kruskal-Wallis test). On the other hand, no statistically significant difference was observed among values obtained by the comparison of the recombinant proteins His₆-MSP1₁₉, His₆-MSP1₁₉-PADRE and yMSP1₁₉-PADRE, or GST-MSP1₁₉ and yMSP1₁₉-PADRE ($P > 0.05$, Kruskal-Wallis test).

Sera from the few individuals who tested negative towards all four recombinant proteins were also tested for the recognition of recombinant proteins representing the N-terminal region of *P. vivax* MSP1, MSP3α (C-terminal) or MSP3β(N and C-terminal and entire protein, references 19 and 20). All of them failed to recognize any of the recombinant protein tested (data not shown). However,

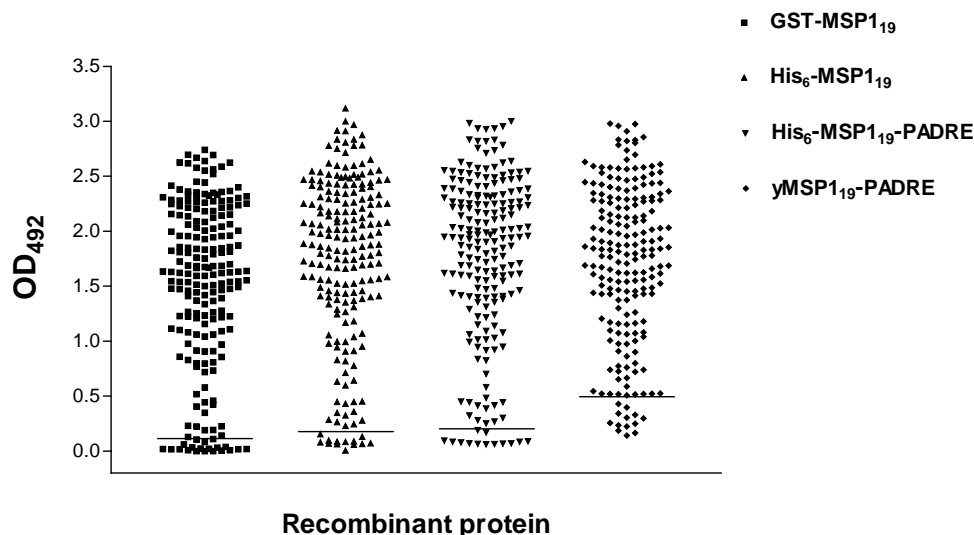


Figure 1

Distribution of OD_{492} data for 200 sera from individuals with patent malaria infection caused by *P. vivax*. The symbols represent the reactivity of each serum sample tested in duplicate at 1:100 dilution against the indicated recombinant proteins. The horizontal line inside the drops for each recombinant protein represents the cut-off values (0.127, 0.170, 0.198 and 0.500 for GST-MSP1₁₉, His₆-MSP1₁₉, His₆-MSP1₁₉-PADRE and yMSP1₁₉-PADRE, respectively).

we detected antibodies IgM anti-MSP1₁₉ in 90% (9/10) of these IgG negative individuals. If we consider the results of detection of antibodies IgG and IgM for MSP1₁₉, the sensitivity of the assay was of 99.5%. These 10 individuals were primo-infected and therefore the presence of IgM but not IgG may reflect a delay in the immunoglobulin class switch.

From the 200 individuals that we evaluated, 111 were primo-infected. One hundred and one of them had specific IgG for the recombinant proteins that we tested. Only 10 were negative for IgG and, as mentioned above, 9 of them had specific IgM.

Evaluation of the specificity of the recombinant proteins tested with serum samples from individuals exposed to *P. falciparum*, from unrelated diseases, or healthy donors

The specificity of the assay using recombinant *P. vivax* antigens was examined with 230 serum samples from individuals without previous history of *P. vivax* malaria, including individuals exposed to *P. falciparum* malaria,

individuals with unrelated diseases or healthy individuals. Due to limitations on the volume of each sample available, sera from African individuals exposed to *P. falciparum* were tested only against recombinant protein His₆-MSP1₁₉. For the calculation of the specificity of the assay using the recombinant protein His₆-MSP1₁₉, the results that was obtained did not include sera from African individuals. The specificity values determined with sera from healthy individuals and sera from individuals with other infectious diseases, were 98.3% (GST-MSP1₁₉), 97.7% (His₆-MSP1₁₉ and His₆-MSP1₁₉-PADRE) and 100% (yMSP1₁₉-PADRE). In figure 2, we compared the OD_{492} values from serum samples of *P. vivax* infected individuals, individuals exposed to *P. falciparum* and individuals with unrelated diseases.

Discussion

Due to the difficulties in cultivating blood stages of *P. vivax*, serological diagnosis of patent *P. vivax* malaria can best be accomplished with the use of recombinant proteins. In the present study, we compared for purified

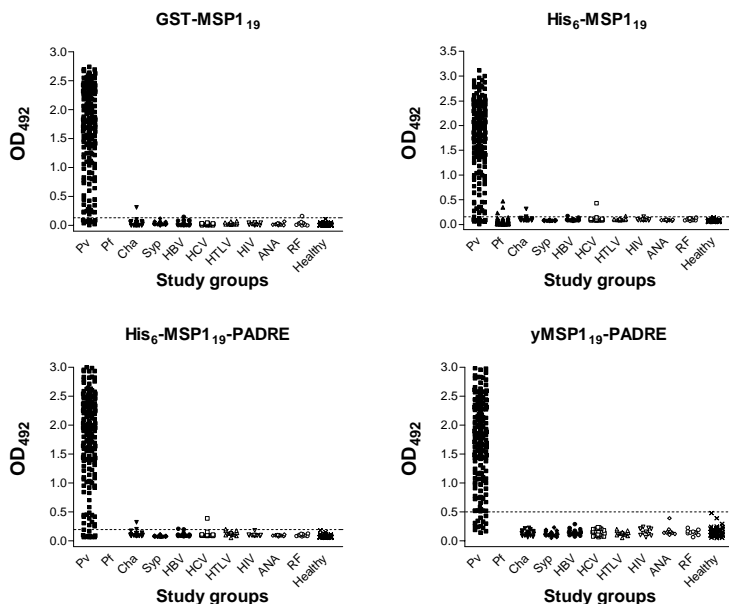


Figure 2

Distribution of the OD_{492} data for sera from individuals with patent *P. vivax* malaria, from individuals exposed to *P. falciparum*, from individuals with unrelated diseases or healthy controls. The symbols represent the reactivity of each serum sample tested in duplicate at 1:100 dilution against the indicated recombinant proteins. The abbreviations are as follow: A) Pv= individuals with *P. vivax* malaria (n = 200), B) Pf= individuals from areas where *P. falciparum* malaria is endemic (n = 53), C) Cha = individuals with Chagas Disease (n = 21), D) Syp = individuals with syphilis (n = 21), E) HBV = individuals with hepatitis B (n = 19), F) HCV= individuals with hepatitis C (n = 21), G) HTLV = individuals with HTLV (n = 14), H) HIV= individuals with HIV (n = 12), I) ANA = individuals positive for antinuclear antibodies (n = 10), J) RF = individuals positive for rheumatoid factors (n = 10), L) Healthy = Healthy individuals (n = 49). Sera from African individuals exposed to *P. falciparum* were tested only against recombinant protein His₆-MSP1₁₉. The horizontal line inside the drops for each recombinant protein represents the cut-off values (0.127, 0.170, 0.198 and 0.500 for GST-MSP1₁₉, His₆-MSP1₁₉, His₆-MSP1₁₉-PADRE and yMSP1₁₉-PADRE, respectively).

recombinant proteins produced in *E. coli* or in *Pichia pastoris* in their ability to be recognized by IgG antibodies of Brazilian individuals with patent *P. vivax* infection. Our study demonstrated that, for the Brazilian population, an ELISA using a single recombinant protein based on the *P. vivax* MSP1₁₉ kDa can serve as the basis for the development of a sensitive serological test that can be used for epidemiological studies, screening blood donors and diagnosis of *P. vivax* malaria.

So far, we do not have an estimate of the timing required after mosquito bite for the appearance of specific antibodies. To be accurate, this information should be obtained

during experimental infection in primates. Nevertheless, we were able to determine that from the 111 primo-infected individuals that we evaluated, 101 (90.9%) had specific IgG to MSP1₁₉. This information is important and suggests that IgG antibodies specific for MSP1₁₉ are suitable for testing individuals who are traveling or have traveled for the first time through malaria endemic areas. On the other hand, the persistence of the IgG antibodies specific for MSP1₁₉ is still a matter that has to be further evaluated. In previous studies we determined that the antibody titers decreased relatively rapidly after treatment [8]. However, studies are underway using these recently generated recombinant proteins.

An important observation from our study was the fact that sera from African individuals naturally exposed to *P. falciparum* failed to cross-react with the recombinant protein His₆-MSP1₁₉ (Figure 2). As a control, 39 of these sera were also tested against a recombinant protein derived of the MSP-2 of *P. falciparum*. The percentage of responders was 69.2% (data not shown). The lack of cross-reactivity may have several implications and should be further evaluated in the light of the current knowledge that both MSP1₁₉ share similarities in their predicted tertiary structures [21]. For epidemiological studies, for the screening of blood donors and the serological diagnosis of *P. vivax* malaria, the lack of cross reactivity can be a major advantage. Nevertheless, the absence of cross-recognition of *P. vivax* MSP1₁₉ by antibodies from *P. falciparum*-exposed individuals has may also have immunological consequences at the level of acquired immunity and vaccine development in areas where both malarias are prevalent. Detailed studies will be required to determine whether sera from *P. vivax* infected individuals also fail to recognize recombinant proteins representing the *P. falciparum* MSP1₁₉.

Recently, a direct sandwich ELISA to detect antibodies against the C-terminal region of MSP-1 was proposed as a potential diagnostic method for *Plasmodium vivax* exposed individuals from Korea [22]. This assay showed a high sensitivity (99.5%) indicating that recombinant proteins containing the C-terminal region of *P. vivax* MSP-1 may be used in individuals from different parts of the world.

It is also important to mention that it is very likely that serological detection of *P. vivax* malaria can be further improved by several distinct strategies. We are currently trying to improve the detection level by the combined use of other recombinant proteins based on the sequence of other blood stage antigens of *P. vivax* such as the *Plasmodium vivax* Merozoite Surface Proteins-3 α and β , Apical Membrane Antigen-1 and the Duffy Binding Protein [19,20,23,24]. Also, we are developing a chemiluminescent enzyme-linked immunosorbent assay that may greatly improve the sensitivity, eliminating the few false negative and false positive samples we had [25,26].

Finally, our results support the notion that recombinant proteins based on the *P. vivax* MSP1₁₉ kDa can be useful for the development of a rapid immunochromatographic assay for field studies, small laboratories and blood banks (reviewed in references 3 and 27).

Conclusions

Our study demonstrated that for the Brazilian population, an ELISA using a single recombinant protein based on the *P. vivax* MSP1₁₉ can serve as the basis for the development of a valuable serological assay for the detection of *P. vivax* malaria.

Authors' contributions

MHCR carried out all the serological assays. MGC generated and purified the recombinant proteins His₆-MSP1₁₉ and His₆-MSP1₁₉-PADRE. RLDM collected part of the serum samples from *P. vivax* malaria individuals. OCF participated in the design of the study and the supply of the serum samples from blood bank donors and drafted the manuscript. MMR participated in the design of the study and drafted the manuscript. ISS conceived of the study and participated in all aspects of its design, execution, coordination and manuscript preparation. All authors read and approved the final manuscript.

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References

- Mendis K, Sina BJ, Marchesini P, Carter R: **The neglected burden of *Plasmodium vivax* malaria.** *Am J Trop Med Hyg* 2001, **64**:97-106.
- Fundação Nacional de Saúde-FUNASA, Ministério da Saúde. **Guia de Doenças: Malária Dados da doença – relatórios gerenciais** 2003 [<http://www.funasa.gov.br>].
- Moody A: **Rapid diagnostic tests for malaria parasites.** *Clin Microbiol Rev* 2002, **15**:66-78.
- Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosário VE, Thaitong S, Brown KN: **High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction.** *Mol Biochem Parasitol* 1993, **61**:315-320.
- Kimura M, Kaneko O, Qing L, Mian Z, Kawamoto F, Wtaya Y, Otani S, Yamaguchi Y, Tanabe K: **Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene.** *Parasitol Int* 1997, **46**:91-95.
- Rubio JM, Benito A, Roche J, Berzosa PJ, Garcia ML, Mico M, Edu M, Alvar J: **Semi-nested, multiplex polymerase chain reaction for detection of human malaria parasites and evidence of *Plasmodium vivax* infection in Equatorial Guinea.** *Am J Trop Med Hyg* 1999, **60**:183-187.
- Soares IS, Levitus G, Souza JM, del Portillo HA, Rodrigues MM: **Acquired immune responses to the N- and C-terminal regions of *Plasmodium vivax* merozoite surface protein I in individuals exposed to malaria.** *Infect Immun* 1997, **65**:1606-1614.
- Soares IS, Cunha MG, Silva MN, Souza JM, del Portillo HA, Rodrigues MM: **Longevity of the naturally acquired antibody responses to the N- and C-terminal regions of *Plasmodium vivax* MSP I.** *Am J Trop Med Hyg* 1999, **60**:357-363.
- Park JW, Moon SH, Yeom JS, Lim KJ, Sohn MJ, Jung WC, Cho YJ, Jeon KW, Ju W, Ki CS, Oh MD, Choe K: **Naturally acquired antibody responses to the C-terminal region of merozoite surface protein I of *Plasmodium vivax* in Korea.** *Clin Diagn Lab Immunol* 2001, **8**:14-20.
- Pasay MC, Cheng Q, Rzepczyk C, Saul A: **Dimorphism of the C terminus of the *Plasmodium vivax* merozoite surface protein I.** *Mol Biochem Parasitol* 1995, **70**:217-219.
- Soares IS, Barnwell JW, Ferreira MU, Cunha MG, Laurino J, Castilho BA, Rodrigues MM: **A *Plasmodium vivax* vaccine candidate displays limited allele polymorphism, which does not restrict recognition by antibodies.** *Mol Med* 1999, **5**:459-470.
- Ferreira MU, Kimura EAS, Souza JM, Katzin AM: **The isotype composition and avidity of naturally acquired anti-*Plasmodium***

- falciparum** antibodies: differential patterns in clinically immune Africans and Amazonian patients. *Am J Trop Med Hyg* 1996, **55**:315-323.
13. Marsh K, Sherwood JA, Howard RJ: **Parasite-infected-cell-agglutination and indirect immunofluorescence assays for detection of human serum antibodies bound to antigens on *Plasmodium falciparum*-infected erythrocytes.** *J Immunol Methods* 1986, **91**:107-115.
 14. Marsh K, Howard RJ: **Antigens induced on erythrocytes by *P. falciparum* : expression of diverse and conserved determinants.** *Science* 1986, **231**:150-153.
 15. Cunha MG, Rodrigues MM, Soares IS: **Comparison of the immunogenic properties of recombinant proteins representing the *Plasmodium vivax* vaccine candidate MSP1₁₉ expressed in distinct bacterial vectors.** *Vaccine* 2001, **20**:385-396.
 16. Soares IS, Rodrigues MM: **Immunogenic properties of the *Plasmodium vivax* vaccine candidate MSP1₁₉ expressed as a secreted non-glycosylated polypeptide from *Pichia pastoris*.** *Parasitology* 2002, **124**:237-246.
 17. Barnwell JW, Galinski MR, DeSimone SG, Perler F, Ingravallo P: ***P. vivax*, *P. cynomolgi*, and *P. knowlesi*: identification of homologue proteins associated with the surface of merozoites.** *Exp Parasitol* 1999, **91**:238-249.
 18. Tjitra E, Suprianto S, Dyer M, Currie BJ, Anstey NM: **Field evaluation of the ICT malaria P.f/P.v immunochromatographic test for detection of *Plasmodium falciparum* and *Plasmodium vivax* in patients with a presumptive clinical diagnosis of malaria in eastern Indonesia.** *J Clin Microbiol* 1999, **37**:2412-2417.
 19. Galinski MR, Corredor-Medina C, Povoá M, Crosby J, Ingravallo P, Barnwell JW: ***Plasmodium vivax* merozoite surface protein-3 contains coiled-coil motifs in an alanine-rich central domain.** *Mol Biochem Parasitol* 1999, **101**:131-147.
 20. Galinski MR, Ingravallo P, Corredor-Medina C, Al-Khedery B, Povoá M, Barnwell JW: ***Plasmodium vivax* merozoite surface proteins-3beta and-3gamma share structural similarities with *P. vivax* merozoite surface protein-3alpha and define a new gene family.** *Mol Biochem Parasitol* 2001, **115**:41-53.
 21. Chitarra V, Holm I, Bentley GA, Petres S, Longacre S: **The crystal structure of C-terminal merozoite surface protein I at 1.8 Å resolution, a highly protective malaria vaccine candidate.** *Mol Cell* 1999, **3**:457-464.
 22. Lim KJ, Park JW, Sohn MJ, Lee S, Oh JH, Kim HC, Bahk YY, Kim YS: **A direct sandwich ELISA to detect antibodies against the C-terminal region of merozoite surface protein I could be a useful diagnostic method to identify *Plasmodium vivax* exposed persons.** *Parasitol Res* 2002, **88**:855-860.
 23. Fraser T, Michon P, Barnwell JW, Noe AR, Al-Yaman F, Kaslow DC, Adams JH: **Expression and serologic activity of a soluble recombinant *Plasmodium vivax* Duffy binding protein.** *Infect Immun* 1997, **65**:2772-2777.
 24. Kocken CH, Dubbeld MA, Van Der Wel A, Pronk JT, Waters AP, Langermans JA, Thomas AW: **High-level expression of *Plasmodium vivax* apical membrane antigen I (AMA-I) in *Pichia pastoris*: strong immunogenicity in *Macaca mulatta* immunized with *P. vivax* AMA-I and adjuvant SBAS2.** *Infect Immun* 1999, **67**:43-49.
 25. Almeida IC, Rodrigues EG, Travassos LR: **Chemiluminescent immunoassays: discrimination between the reactivities of natural and human patient antibodies with antigens from eukaryotic pathogens, *Trypanosoma cruzi* and *Paracoccidiosis brasiliensis*.** *J Clin Lab Anal* 1994, **8**:424-431.
 26. Almeida IC, Covas DT, Soussumi LM, Travassos LR: **A highly sensitive and specific chemiluminescent enzyme-linked immunosorbent assay for diagnosis of active *Trypanosoma cruzi* infection.** *Transfusion* 1997, **37**:850-857.
 27. Hanscheid T: **Current strategies to avoid misdiagnosis of malaria.** *Clin Microbiol Infect* 2003, **9**:497-504.

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