UNIVERSIDADE FEDERAL DO AMAZONAS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA BÁSICA E APLICADA

AVALIAÇÃO DA RESPOSTA SOROLÓGICA À PROTEÍNA 1 DE SUPERFÍCIE DO MEROZOÍTO (MSP1) DE *PLASMODIUM VIVAX* EM UMA COMUNIDADE DA AMAZÔNIA BRASILEIRA

FERNANDA GUIMARÃES VERSIANI

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Dissertação apresentada ao Programa de Pós-graduação em Imunologia Básica e Aplicada da Universidade Federal do Amazonas como requisito para a obtenção do título de Mestre em Imunologia.

Orientador: Profº Dr. Paulo Afonso Nogueira

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A Deus pela vida,
ao meu querido Fran
pelo incentivo e amor incondicionais.

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RESUMO

A malária ou paludismo é uma doença parasitária de grande relevância mundial que, provocada por protozoários do gênero *Plasmodium*¸ é responsável por altos índices de morbidade e mortalidade, sobretudo nas regiões tropicais do planeta. O protozoário *P. falciparum* é o parasita relacionado aos casos mais graves da doença tendo sido mais intensamente estudado, no entanto, o *P. vivax* é geograficamente o parasita mais amplamente distribuído, responsável por 80 a 300 milhões de casos estimados, incluindo casos graves e mortes. Em regiões endêmicas, a exposição freqüente aos parasitas leva ao surgimento de indivíduos portadores de malária assintomática, cujo o desenvolvimento de uma imunidade natural os protegem dos sintomas clínicos da doença. Um dos antígenos capazes de gerar resposta imune específica é a proteína 1 de superfície do merozoíto (MSP1), conhecida por seu papel na invasão celular do hospedeiro. Neste estudo, nós analisamos a resposta imune contra as porções N e C terminais da MSP1 da espécie *P.*vivax em 312 soros de uma população proveniente de um assentamento rural localizado na região do rio Pardo, no Estado do Amazonas. 47 (15,06%) dos indivíduos foram diagnosticados com infecção malárica. Houve correlação entre o número de infecções anteriores e o tempo de residência na região (p=0,000), idade e presença de infecção (p=0,026) e número de infecções anteriores o tempo desde o último episódio malárico (p=0,000). Não foi observada correlação entre idade e número de infecções anteriores (p=0,388). 159 (51,0%) apresentaram IgG total contra a porção C-terminal (ICB-10) e 92 (29,5%) contra a porção N-terminal (ICB2-5) da proteína MSP1 (p=0,000). Não houve relação entre a presença de anticorpos IgG total contra o fragmento ICB2-5 (p=0,747) ou contra o fragmento ICB-10 (p=0,515) e proteção clínica quando efetuada análise de sobrevida com acompanhamento dos indivíduos por 360 dias. Porém, quando avaliado o período de 9 meses, a presença de IgG3 contra ICB2-5 foi relacionada à proteção clínica (p=0,039). Adicionalmente, indivíduos que não tiveram infecção malárica no período de 360 dias de seguimento, apresentaram níveis de IgG3 contra ICB2-5 significativamente maiores (p=0,003) que os indivíduos que tiveram episódio de infecção. Nossos dados sugerem que a presença de IgG_3 contra a porção N-terminal da proteína MSP1 constitui-se como marcador soro-epidemiológico de proteção clínica em indivíduos provenientes de regiões endêmicas.

Palavras chave: *Plasmodium vivax*, malária, proteína 1 de superfície do merozoíto.

ABSTRACTS

Plasmodium vivax is geographically the most widely distributed, accounting for 80 to 300 million estimated cases, including severe cases and deaths. In endemic areas, frequent exposure to parasites leads to the emergence of individuals with asymptomatic malaria, which develops a natural immunity that protects against the clinical symptoms of the disease. This immune status is important to evaluate malaria vaccine candidates. One of the antigens that can generate specific immune response is the merozoite surface protein-1 (MSP-1), known for its role in host cell invasion. We analyzed the immune response against the N-and C-Terminal Merozoite surface protein 1 of *Plasmodium vivax*, PvMSP1, in 312 sera of a population from an agricultural settlement located adjacent to the Rio Pardo, Amazonas, whom they were followed by a clinical epidemiologic study. The results showed that 51.0% had total IgG against c-terminal portion and 29.5% had total IgG against N-terminal portion of the protein. As expected antibodies against the N-terminal portion were correlated with age, number of previous episodes, time of residence in the area. There was a predominance of IgG subclass 3 against the N-terminal portion. Our data suggested that IgG3 antibodies against the N-terminus of PvMSP1 to the local repertoire of variable domains of PvMSP-1 occur in asymptomatic patients indicating as sera-epidemiological markers of clinical protection.

Keywords: *Plasmodium vivax*; malaria; merozoite surface protein -1

Lista de Abreviaturas

DNA	ácido desoxiribonucleico
ELISA	enzimed linked immunosorbent assay
GST	glutadiona S transferase
Kda	quilodaltons
mL	mililitros
MSP1	proteína 1 de superfície do merozoíto
nm	nano mol
PCR	reação em cadeia da polimerase
PvMSP1	proteína 1 de superfície do merozoíto de *Plasmodium vivax*
Real-time PCR	reação em cadeia da polimerase em tempo real
rRNA	ácido ribonucléico ribossômico
TBE	tampão tris/borato/EDTA

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Title

Plasmodium vivax merozoite surface protein 1, a vaccine candidate

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Abstract

Malaria is still a serious world public health issue which creates the necessity to look for new technologies in order to reduce morbidity and mortality rates for this disease. For a long time, the efforts to combat the disease were devoted to the *Plasmodium falciparum* species and only recently the *Plasmodium vivax* species started to have the attention of many researchers since it is geographically widely distributed throughout the world, leading the disease to millions of people. Malaria parasite antigens have been researched with a focus vaccine development. Presently, one of the most researched antigens is the merozoite surface protein 1 (MSP1) which is a protein present on the surface of the merozoite form of several species of *Plasmodium*, and particularly, in the parasitic species to man most prevalent in the world, *P. vivax* and *P. falciparum*. However, the high polymorphism between the species prevents the development of vaccines from one antigen based on one species being the immune response against the parasite species-specific. This article reviews structural and molecular bases of MSP1 of *P. vivax*, the specific immune response that is generated, and the study in search of vaccines using the protein.

Keywords

Merozoite surface protein 1, *Plasmodium vivax,* malaria, vaccine

1. Introduction

Malaria remains one of the most important infectious diseases in the world. Since the 1980's the world efforts for the development of vaccines against malaria were exclusively directed for the species *Plasmodium falciparum,* this species is responsible for the majority of the morbility and mortality of malaria. A small number of

researchers were dedicated in the last years to investigate the *P. vivax.* Today, the world recognizes that designation *"malaria* benign" several times used in scientifical research it was a mistake (Galinski and Barnwell, 2008). The *P. vivax* is geographically the most widely distributed parasite, with the risk of infection estimated in 2 to 5 billion people worldwide, responsible for 80 to 300 million estimated cases, including severe cases and deaths (Mueller et al., 2009a).

The etiological agent of malaria is the most important member belonging to the phylum *Apicomplexa*, a large group where obligatory intracellular parasites are found, able to invade cells of a wide range of hosts, depending on the species. Among those belonging to these groups there are important human and veterinary parasites such as *Toxoplasma*, *Babesia* and *Cryptosporidium.* These parasites have a complex life cicles that provide the perpetuation of the species. In the case of *Plasmodium*, the biological cycle involves an arthropod vector that transmits itself to a vertebrate host during the blood repast. There is an invasion of cells that permits the access to the parasites of a range of nutrients in a niche where they are protected from the host's own defenses (Cowman and Crabb, 2006).

The clinical symptoms of malaria are attributed to blood stages of the life cycle of parasites, including the invasion of red blood cells, intracellular multiplication and rupture of erythrocytes. This phase, in which Plasmodium merozoites are released from the liver and invade the erythrocytes , is considered a target for vaccine production, with the expected reduction of parasitemia as well as morbidity and mortality related to disease (Serrano et al., 2006). The presence of parasites in the circulation is responsible for the onset of symptoms due to release of factors such as hemozoin, glycosylphosphatidylinositol anchors (GPI) and metabolites from cell cleavage that stimulate the systemic inflammatory reaction with the production of proinflammatory

cytokines. The duration of the erythrocytic cycle determines the frequency of these symptoms, ranging among the species of *Plasmodium* (Amino et al., 2006).

The erythrocytic stage of the life cycle begins when the merozoite makes contact with the host erythrocyte, followed by reorientation and attachment. Apical organelles, including rhoptries and micronemes release their content and a junction area connects the parasite to the erythrocyte. The merozoite moves into the erythrocyte and forms the parasitic vacuole following its development to the trophozoite forms through the schizogony forming new merozoites that are released into the blood after rupture of the erythrocyte. This merozoite form has a content of proteins of membrane usually called proteins of merozoite surface, which mediate initial interactions between the parasite and the erythrocyte (Kadekoppala and Holder, 2010).

In 1982 a polypeptide of 195,000 mol associated with the blood stage of *Plasmodium falciparum* was described using an essay. In this analyses monoclonal antibodies reacted against peptides produced during schizogony obtained by developing a synchronized culture of the parasite (Holder and Freeman, 1982). The author's noted that other smaller peptides precipitated by monoclonal antibodies came from the same polypeptide described, being products of its processing. This polypeptide, termed Merozoite surface protein 1 (MSP1) (also known as Precursor of the Main Merozoite Antigen (PMMSA) or Merozoite urface antigen 1 (MSA1)), constituted by a glycoprotein of 195 kDa (gp195) has been identified in other *Plasmodium* species. These similar proteins were grouped according to some similar characteristics such as size, sub-cellular location, time of synthesis and structural similarities detected by cross-reactivity of antibodies and homologous sequence of amino acid by primary deduction. The great interest on this protein is that plays a fundamental role in the biology of the

parasite and the induction of protective immune response of the host, since it is directly involved in the process of invasion of erythrocytes (Holder et al., 1992).

From 1982 to 1990, many studies were conducted using the MSP1 protein of the parasite *P. falciparum* in an attempt to elucidate the mechanisms of induction of protective immune response against the parasite. Only in 1991 del Portillo et al. first described the primary structure of MSP1 protein of *P. vivax*, whose species is geographically more distributed in the world, as well as its gene Pv200 (del Portillo et al., 1991; Mueller et al., 2009a).

Among the proteins of blood stage of *Plasmodium*, the MSP1 have been studied intensively because of the need to understand the mechanisms involved in erythrocyte invasion by parasites, in addition to its carried importance as an antigen capable of activating the host protective immunity, which becomes a potential target for the production of malaria vaccine (Babon et al., 2007; del Portillo et al., 1991). The urgency to discover potential targets for the manufacture of vaccines is the fact that the antimalarial drugs remained unchanged in the last fifty years. This is due to the occurrence of resistance to some of these in various parts of the world, reduced effectiveness of the conventional treatment and the increased number of severe cases recently (Bargieri et al., 2008).

In the last decade, the emergence of strains resistant to chloroquine and primaquine, the drugs of choice against infection by *Plasmodium vivax* species, made search urgent for alternative strategies to combat malaria, including the search for potential targets for the development of vaccines. The Asexual forms of the parasite have been investigated for vaccine production, with important studies of merozoite proteins form as reticulocyte-binding protein (PvRBP1 and 2), Duffy-binding protein (PvDBP), apical

membrane antigen 1 (AMA -1), merozoite surface protein 1 (MSP1), merozoite surface protein 3 (MSP3) and more recently, merozoite surface protein 9 (MSP9) (Lima-Junior et al., 2008).

2. Structure and function

The protozoa that cause malaria were divided by evolutionary criteria into three groups according to their nucleic acid composition. The first comprises those that cause malaria in rodents, birds and species causing human malaria *P. falciparum* (small nucleotide content 18% dG-dC). The second comprises two species that cause malaria in monkeys *P. knowlesi*, recently discovered to cause numerous cases in humans as well (Oddoux et al., 2011; Sabbatani et al., 2010; Singh et al., 2004; White, 2008) and *P. fragile*, with content of nucleotides dG-dC was high (30%). The third and last group, comprising the species *P. vivax* and *P. cynomolgi*, respectively known to cause disease in humans and monkeys with episodes of recurrence, whose genome had both dG-dC content of high and low (McCutchan et al., 1984). According to Del Portillo et al. (1991) for the MSP-1 genes and their proteins, this division only implies homology within each group to the level of nucleotides. Analyses have subdivided the MSP-1 gene in blocks based on their level of genetic diversity without using any other biological criteria, however, few studies have been conducted considering the proteolytic fragments of MSP-1 protein as functional units. Even in distant species, the fragment $MSP1_{19}$, for example, plays an important role in the erythrocyte invasion (Pacheco et al., 2007).

MSP1 protein is synthesized during schizogony forming a protein complex that is the largest component of the merozoite surface. In the merozoites released from schizont form of the species *P. falciparum*, a precursor of about 200 kDa is cleaved into an initial

processing into four fragments of ~ 83, ~ 30, ~ 38 and ~ 42 kDa that remain trapped by the C-terminal extremity, referring to the 42 kDa fragment on the surface of merozoite in a non-covalent complex, through a molecule of GPI (glycosylphosphatidylinositol) (Babon et al., 2007). In the species *P. vivax*, the protein has a similar structure. Four fragments remain non-covalently linked in a complex of the merozoite surface after the first cleavage ($MSP1_{83}$, $MSP1_{30}$, $MSP1_{38}$, $MSP1_{42}$). At the time of erythrocyte invasion, the $MSP1_{42}$ fragment is cleaved into $MSP1_{33}$ and $MSP1_{19}$ (Babon et al., 2007; Han et al., 2004; Holder et al., 1992).

Salvador and Belem alleles of MSP-1 protein of *Plasmodium vivax* species were fully characterized of strains adapted to monkeys. A study conducted in isolated Colombians (Mancilla et al., 1994) confirmed the predominance of these two alleles at a polymorphic region of the gene PvMSP-1. Gene molecular analysis in Pv200 (name of the gene for MSP-1) of Salvador -1 strain resulted in identity of 34-37% when the genetic sequence of surface antigens was compared to other three species (*P. falciparum*, *P. yoelli* and *P. chabaudi*). When compared to the Belem strain of *P. vivax* was found 81% of identity (Gibson et al., 1992).

Analysis of the primary structure of different alleles of the gene that encodes MSP-1 of *P. falciparum* led to the definition of conserved regions, semi-conserved and variables in protein molecule (Tanabe et al., 1987). This knowledge was brought to the species *P. vivax* where the high polymorphism found in the protein molecule can be used as a marker for molecular epidemiological studies. There is a wide genetic diversity within species in endemic regions (Hwang et al., 2009; Mancilla et al., 1994; Premawansa et al., 1993). Analysis of the primary structure of the gene of *Plasmodium vivax* of Belem strain showed the presence of conserved blocks between the similar proteins of species *P. vivax* (pv200), *P. yoelli* (Py230) and *P. falciparum* (pf190). These

blocks were called ICBS and contained regions of 50 or more amino acids with 50% or more identity between species. The conserved blocks may be explained by structural or functional importance, or because they are not immunogenic or because immune responses against these blocks do not block the growth of the parasite (del Portillo et al., 1991). Putaporntip et al. (2008) conducted a study which reported similarities of 56.2% to 60.8% between the amino acid sequence of MSP-1 of *P. vivax*, *P. knowlesi* and *P. cynomolgi*. Kolakovich et al. (1996) found recombination of alleles Belem and Salvador generating genetic diversity in Block 5 of the gene PvMSP-1 in isolates from Papua New Guinea. Premawansa et al. (1993) suggested that the genetic diversity of the species *P. vivax* came from three different alleles rather than two as proposed by Tanabe et al. (1987) for the species *P. falciparum*. Two would represent allele's relatives and type 3 was a recombinant form of the first two. Analysis of isolated from Colombia (Gutierrez et al., 2000) showed that there are recombination events between alleles Salvador and Belem, agreeing with the studies of Premawansa et al. (1993).

Phylogenetic analysis performed by Tanabe et al. (2007) using the alleles of MSP-1 gene of the species *P. falciparum*, *P. vivax* and *P. cynomolgi* concluded that there was a strong positive selection in the lineage *P. vivax* against *P. cynomolgi*, and that worked in different polymorphic regions when compared to *P. falciparum*, which shows a different evolutionary history for the polymorphic regions between the different species. Interestingly three of five positively selected amino acids were related to fragment $MSP1_{33}$ and no positive selection was observed in the fragment $MSP1_{19}$, whose region is considered a strong candidate for vaccine production. Sawai et al. (2010) conducted a similar study which found positive selection specific to each species (*P. vivax*, *P. cynomolgi* and *P. inui*) in the region of MSP-1 gene, which leads to an understanding of a species-specific evolutionary history of parasites of malaria.

Analyses aimed at elucidating of the molecular structure of pvMSP-1 were performed and the fragment MSP1-$_{19}$, belonging to the C-terminus of the protein, known for its antigenic properties, was proposed as a vaccine antigen against vivax malaria (Babon et al., 2007; Pacheco et al., 2007; Serrano et al., 2006). Babon et al. (2007) also carried out structural studies with MSP1-$_{19}$ fragment in different *Plasmodium* species and found similar architecture but with differences in charge distribution on the surface of the protein molecule between species. The MSP1-$_{19}$ of the species *P. vivax* has general cargo -6 while the domain 1 of the protein has a general cargo -4, due to its predominant content of waste acid. This is the best preserved fragment of the protein with only a residual variation among 40 isolated analyzed from different geographical areas. The small polymorphism found in domain MSP1 -$_{19}$ suggests that this may be a promising target for vaccine production (Pasay et al., 1995; Putaporntip et al., 2002). Pacheco et al. (2007) demonstrated through a study of the sequencing of strains from different countries that the genetic diversity for the MSP-1 gene in the species of *P. falciparum* is double that found for *P. vivax*. In addition, the MSP1$_{19}$ fragment is more conserved than the MSP1$_{33}$ in both species.

The species *P. vivax* preferentially invades reticulocytes, which are young erythrocytes, which measure up only about 2% of total of circulating erythrocytes. The invasion process is mediated by antigens such as MSP-1, the reticulocyte binding proteins (Pv-RBPs) and receiver duffy. Fourteen peptides derived from MSP-1 have high binding activity to reticulocytes in relation to the three peptides with high affinity for binding to mature erythrocytes, demonstrating that the MSP-1 plays an important role in preference for young red blood cells (Rodriguez et al., 2002). In fact, peptides with high specific binding activity (HSBA) to reticulocyte have been found in MSP1$_{33}$ fragment (Pacheco et al., 2007).

Analyses carried out by Han et al. (2004) revealed that the MSP1-$_{19}$ fragment, resulting from cleavage of MSP1-$_{42}$ producing the polypeptides MSP1-$_{33}$ and MSP1-$_{19}$ is essential for binding to the receptor of the erythrocyte and that the cytoadherence was blocked by natural antibodies. The domains 1 and 2 of the epidermal growth factor, present in MSP1-$_{19}$, when expressed alone mediated 64% and 66% of erythrocyte-binding activity, demonstrating the important function of this protein fragment. Child et al. (2010) reported that the processing steps of the MSP-1 that form the MSP1-$_{19}$ fragment are highly controlled and that this process is important for the viability of the blood stage of the parasite. The parasitic vacuole besides having the function of protecting the parasite from the host immune system and housing the processing steps of regulation enabling the maturation of merozoites before release into the bloodstream. Studies on the genetic diversity of MSP1-$_{42}$ fragment in natural infections have been conducted in Sri Lanka (Dias et al., 2011), a region of low transmission, high polymorphism was found for the fragment MSP1-$_{33}$ with 27 different haplotypes compared which the MSP1-$_{19}$ that was highly conserved among 95 samples tested. In Turkey Zeyrek et al. (2010) found very low diversity to the gene encoding the MSP-1 in 29 isolated in two cities. This could involve the acquisition of immunity more rapidly in Turkey than in other endemic regions such as Brazil and Thailand.

3. Immune responses against MSP-1

Knowledgements of natural immunity acquired against malaria is even before the discovery of the etiologic agent of disease. By 1920, the main mechanisms of natural immunity had been described. It was assumed that natural immunity was effective in adults after prolonged exposure over a lifetime and that this immunity was lost after

exposure has ceased. Years later it was established that natural immunity was species-specific and acquired according to the degree of exposure (Doolan et al., 2009).

In endemic regions, the occurrence of clinically silent infections by *Plasmodium* reflects the ability of effector adaptive immune mechanisms in preventing the disease. Already in non-immune individuals, the infections cause clinical symptoms, where a minority of cases may progress to severe malaria and death. In general, the pattern of clinical disease depends on age and previous contact of the immune system to the antigens of the parasites (number of previous infections) (Seth et al., 2010; Soares et al., 1997). Epidemiological studies have shown that after a phase where children are susceptible to severe malaria, the development of protective immunity capable of conferring protection against new episodes of severe infection. First, the immunity is challenged by a phase where there is risk of life, after this stage is developed immunity to symptomatic infection and finally the development of partial immunity to a parasite (Schofield and Grau, 2005).

Natural immunity to malaria provides a solid protection against severe morbidity and mortality. Older children and adults rarely develop life-threatening infections, indicating that age is a significant factor. The prevention of high parasitaemia appears to be the mechanism of protection in adults. The intensity of transmission is also directly related to morbidity and mortality. Measuring the intensity of transmission in a location one can use the index vector bites per person per unit time. In localities with low transmission, there is a substantial risk of developing severe symptoms, otherwise in endemic regions, the risk is limited to travelers, young children and pregnant women (Doolan et al., 2009).

Studies on the immune response to *P. vivax* are important to identify antigens in infected patients, potential candidates for vaccine production. Seth et al. (2010)

analyzed the immune response against different synthetic antigens of *P. vivax* (MSP1, CSP, AMA1, GAM1) and the epitopes of T and B lymphocytes for each antigen allowing an association between the humoral and cellular immune responses. A total of 66% of individuals had antibodies to the four antigens and 20% for one, two or three antigens tested. They demonstrated the development of antibodies related to increased clinical protection in relation to age, the result of years of contact with immunogenic antigens of the parasite. Levitus et al. (1994) conducted serological survey in the region of Rondônia, Brazil, using 10 recombinant proteins of N-terminal region of MSP-1. The antibody response showed that small amount of antibodies recognized conserved regions of the molecule (ICBs), leading to the conclusion that conserved regions are weakly immunogenic in natural infections. When proteins were constructed containing polymorphic regions associated with the ICBs, larges quantities of sera recognized proteins ICB1-2, ICB2-3, ICB3-4 and ICB2-5. Study using polymorphism and natural antibodies in a community exposed to malaria also related the importance of polymorphic regions for the production of natural antibodies (Bastos et al., 2007). Del Portillo et al. (1992) evaluated the immune response to three fragments of the N-terminal region having found antibodies specific to the ICB-1 and ICB2-5, but interestingly did not observe correlation with the number of previous infections and age, a result similar to study carried out in Thailand (Pitabut et al., 2007) using a fraction of the C-terminal protein. Another study conducted in the same region identified clinical protection and reduction in infection risk when found in the sera of asymptomatic patients an immunoglobulin IgG subclass 3 against the N-terminus of MSP-1 (Nogueira et al., 2006). In addition, the study showed that the acquisition of immunity against the parasite was related to age of individuals. Fernandez-Becerra et al. (2010) also found the relationship between protection and the presence of IgG3 but there was no

correlation with age except for the C-terminal region. As for samples from Papua New Guinea, an increase of the IgG1 was also observed in studies conducted in Iran (Mehrizi et al., 2009) Sri Lanka (Wickramarachchi et al., 2007) and Ghana (Dodoo et al., 2008). Increased IgG1 and IgG3 was observed in populations with different levels of exposure in Brazil (Morais et al., 2005) and Turkey (Zeyrek et al., 2008).

The species *P. vivax* has a unique biology that distinguishes itself from other species. The development of a dormant form called hypnozoite in the liver can lead to subsequent infection in the body, called a relapse, which totally escapes the mechanisms currently used as insecticides, methods of diagnosis, all chemotherapy drugs used. The liver becomes an important reservoir for new infections in the blood and new transmissions (Mueller et al., 2009a). Kirchgatter and del Portillo (1998) evaluated 10 isolates from individuals in Brazil who had relapses using the gene PvMSP1 as a marker. PCR analysis showed that the recurrence of infections was from the same parasites of the first episode with the same alleles of the protein in both infections. It was also found that activation of hypnozoites is not clonal seen that two individuals contained the two alleles at first and also at the second episode. An increase of antibody titles against the C-terminal MSP-1 was observed in relapse with increased IgG1.

Cross-reactivity was found in a longitudinal study conducted by Mertens et al. (1993). Using recombinant ICB2-5 of *P. vivax*, total antigen of *P. falciparum* and *P. vivax* they found that individuals who had never had malaria episode and had the first infection with *P. falciparum* during the study acquired IgG and IgM antibodies against ICB2-5 of *P. vivax*. This possibly was due to the existing blocks conserved between species.

Humoral and cellular immune response was evaluated against subunits ICB-10 and ICB2-5 in a study carried out by Soares et al. (1997) in individuals with recent episodes

of infection by *P. vivax*. The results showed that increasing the frequency of antibodies against ICB-10 depends on the number of episodes of infection, reaching 83.3% after the fourth episode. The same pattern was not observed for ICB2-5. The frequency of antibodies against the N-terminal and C-terminal was 51.4% and 64.1% respectively. A similar study by the same group (Soares et al., 1999b) in an endemic area of transmission of *P. vivax* found antibodies frequencies of 29.8% and 42.3% respectively, with individuals with the last episode of malaria occurred less than 6 months with higher frequency for the MSP1-$_{19}$ demonstrating the high immunogenicity of this fragment, which was also described by Ladeia-Andrade et al. (2007). A more recent study conducted in four cities with different transmission rates in the Brazilian Amazon (Storti-Melo et al., 2011) evaluated individuals infected by *P. vivax* and related to the presence of natural antibodies against the N-terminal portion of Pv200L (MSP-1). The results confirmed the relationship between the number of previous infections and the presence and level of antibodies in all the cities evaluated demonstrating that this fragment is immunogenic in individuals exposed in endemic regions.

A comparative study of the humoral response in patients from the state of Pará, Brazil, infected with *P. vivax* using three recombinant proteins of erythrocytes stages MSP1$_{19}$, AMA-1 and DBP-RII revealed the marked presence of IgG antibodies to MSP1$_{19}$ (95%) compared to antibodies to other proteins (PvAMA-1 72.7% and and PvDBP-RII 44.5%) in acute infection. But after a period of 9 months of follow-up of patients, a significant decrease was observed in response to the MSP1$_{19}$ demonstrating high responsiveness in the acute period compared to the period after treatment. The reasons for this non-sustainable production of antibodies are still unknown and further studies should be performed (Barbedo et al., 2007).

The MSP-1 of *P. vivax* was also evaluated in diagnostic test method of infection in an IgM capture ELISA obtaining reliable results and high index of sensitivity (97.8%) and specificity (99.1%) making this an especially good choice for use in diagnostic in endemic regions (Park et al., 2008). The C-terminal fragment of the protein expressed in *Saccharomyces cerevisie* had already been studied by the same group (Park et al., 2001) having achieved good results in ELISA indirect methodology. This element can also be used as a diagnostic tool. In addition, Yeom et al. (2008) described methodology for IgM capture ELISA with a sensitivity of 90.6% when evaluated serological response against the PvMSP1c protein.

Searches using the species *P. falciparum* always contributed to the knowledge about other species of *Plasmodium*. A study using the MSP-1 of *P. falciparum* investigated the cellular response when used the native $MSP1_{19}$ fragment and $MSP1_{19}$ with variations in amino acid sequence. The results showed that the change of four amino acids did not affect the recognition by T cells and some of the changes also promoted an increase in response to the protein (Okafor et al., 2009). Study in individuals with a history of migration to endemic regions, reported the production of inhibitory antibodies anti-$MSP1\text{-}_{19}$ after two or more episodes of infection despite the ELISA test to detect the presence of specific antibodies after the first episode of infection. The delay in acquisition of functional antibodies is consistent with the finding that multiple infections are necessary for the acquisition of clinical immunity (Murhandarwati et al., 2008). Woehlbier et al. (2010) have done *in vitro* assays which found that specific antibodies against subunits of proteins MSP-1, MSP-6 and MSP-7 inhibited parasite growth and also affected the second protein complex processing of the merozoite surface. PfSUB2 proteolytic enzyme (membrane-bound merozoite subtilisin-like

sheddase), released by apical organelles called micronemes, catalyzes the second processing and seems to be affected by the action of antibodies.

4. A target for production of vaccine

Vaccination is an important part of malaria control strategy. However, the escape mechanisms of the parasite, antigenic variation and polymorphism of immunodominant antigens hinder the development of an universal vaccine that is effective and that generates long-lasting protection. However, individuals exposed to malaria in endemic regions develop immunity that protects from the clinical manifestations of infection (Arevalo-Herrera and Herrera, 2001). The mechanisms involved in the production of immunity should be explored for success in the discovery of immunogenic antigens capable of being used in vaccine production. Unfortunately for many years, studies of the species *Plasmodium vivax* were neglected and as a consequence very few antigens of this species were surveyed. Even today there are only two subunits vaccine candidates in clinical stage, the Duffy Binding Protein (DBP) and Circumsporozoite Protein (CSP) and a Phase I (sexual stage transmission blocking antigen - Pvs25) (Galinski and Barnwell, 2008; Herrera et al., 2007; Mueller et al., 2010). In recent years, researchers have made efforts to study the species and Mueller et al. (2009b) published a guide for studies of vaccines candidates against *Plasmodium vivax.*

Currently, efforts to produce vaccine against the malaria parasites have been directed to the merozoite stage, where the parasite disrupts the erythrocytes and is exposed in the bloodstream to the host immune system, to re-infect new erythrocytes. During the invasion of erythrocytes, the processing of MSP-1 leads to production of MSP1-$_{19}$, which plays an important role in binding to erythrocytes. Antibodies to this fragment or that interfere with the processing of MSP1-$_{42}$, forerunner of the MSP1-$_{19}$,

can inhibit the erythrocyte invasion or reverse the growth of the parasite directly or through dependent ways of the ion iron. So far, the main candidates to vaccine are based on C-terminal MSP1-$_{42}$ fragment, but studies *in vitro* and *in vivo* have diverged in results (Wang et al., 2009). Vaccine candidates must submit immunogenicity and generate specific antibodies that have stability and durability. Testing conducted by Lim et al. (2004) using the PvMSP1c fragment containing the MSP1$_{19}$ C-terminal region without the transmembrane domain, in regions where malaria eradication was verified the presence of natural antibodies against this protein that remained in the serum of subjects exposed for more than 30 years, indicating the durability specific immune response. However, Soares et al. (1999a) described the rapidly declining levels of antibodies to ICB2-5 and MSP1$_{19}$ and already two months after the episode of infection. These controversial results demonstrate that small differences in the construction of the protein can generate different immune responses, and production of effective vaccines depends on the progress of research in this area.

Espinosa et al. (2003) evaluated the antigenicity and variability among the Belem and Salvador strains of the MSP1$_{14}$ and MSP1$_{20}$ products (recombinant proteins produced from the MSP1$_{33}$ fragment, representing the whole molecule). In this study, variations were found only for the MSP1$_{20}$, with changes in two amino acids that were related to a peptide of low binding to reticulocyte. The serum of patients from endemic regions recognized the two fragments, and antibodies of patients responded to both native and denatured fragments, and independent of the number of previous infections, suggesting that these fragments are weakly antigenic in natural infections, and induce little incentive of the immune response. Similar results were obtained by Oliveira et al. (1999) where plasmids encoding all N-terminal region of MSP-1 were antigenic but plasmids containing conserved blocks between species of the N-terminal region, or

containing epitope for B cell of *P. vivax* or C-terminal region were not considered antigenic. DNA vaccines to become antigenic required three different recombinant plasmids.

MSP1 protein was shown to have an important role in inducing a protective immune response in the test conducted using the species *P. chabaudi* challenge infection in mice. The study showed that the MSP1 gene dominates the species-specific protective immunity (Cheesman et al., 2010). Challenge in Rhesus monkeys using *Plasmodium cynomolgi* species, which is phylogenetically related species *P. vivax* was observed that previous administration of formulations based on $PvMSP1_{42}$ induces protection decreasing parasitemia when compared to a control group that received only adjuvant (Dutta et al., 2005).

Formulations containing fragments $MSP1_{19}$ and $MSP1_{42}$ were used to test immunization in mice with induced production of specific antibodies. The formulations used CFA as an adjuvant, aluminum and Montanide ISA720 achieved better results with the highest levels of antibodies. The pattern of IgG isotype was IgG1 with higher titles, followed by IgG3 and IgG2 for both proteins (Sachdeva et al., 2004). A formulation containing a fragment of the N-terminus of MSP-1 called pv200L in Freund's adjuvant was tested in *Aotus* monkeys that developed specific IgG antibodies against the recombinant protein. Immunization conferred partial immunity to monkeys when they were challenged with infection by asexual stages of Belem strain of *P. vivax*. The protein showed high antigenicity when tested in individuals from endemic regions. Both individuals with active infection and asymptomatic often had antibodies against the recombinant protein (Valderrama-Aguirre et al., 2005). A study conducted in the Amazonian Colombian *Aotus monkeys* tested two recombinant antigens $MSP1_{14}$ and $MSP1_{20}$ administered to Freund's adjuvant. After infection, it was observed that four of

five monkeys controlled the infection and one of the monkeys had partial control of parasitemia. There was increased production of INF-γ in most monkeys and all had IgG antibodies against the tested antigens suggesting that the formulation produced antibodies that protected the vaccinated monkeys from infection by *P. vivax* (Barrero et al., 2005). The immunogenicity of recombinant PvMSP1$_{19}$ expressed in yeast containing two epitopes of tetanus toxin in a formulation containing aluminum and copolymer P1005 was found in *Saimiri* monkeys with increased IgG antibodies after three immunizations and decrease in parasitaemia after being challenged by infection of *P. vivax* (Yang et al., 1999). Test in C57BL / 6 mice using a nasal route of administration, and mucosal adjuvants generated high titles of IgG1 were prevailing high by 6 months of administration when used His6MSP1$_{19}$ and His6MSP1$_{19}$-PADRE recombinant protein (Bargieri et al., 2007). These proteins were constructed with 83 amino acid C-terminal region of MSP-1.

Another study in C57BL / 6 mouse tested vaccine formulations containing the MSP1$_{19}$ fragment transformed in the presence of a TLR5 agonist (Toll like receptors) from *Salmonella typhimurium* as an adjuvant. As a result gained a strong adaptive immune response and long duration in vaccinated mice, and demonstrate that the immunogenicity of the formulation was also augmented with adjunctive TLR9 agonists. Antibodies of patients exposed to malaria parasites clearly recognized the recombinant protein combined with adjuvant formulations containing TLR5 suggesting that these adjuvants can be a general method to develop vaccines using microbial antigens (Bargieri et al., 2008). A previous study conducted by Bargieri et al. (2007) had already demonstrated the high immunogenicity of recombinant protein when administered by intra nasal in C57BL / 6 mice in the presence of mucosal adjuvants. Cunha et al. (2001) used different expression vectors for the constructions of recombinant proteins

representing the $MSP1_{19}$. The vector pET (His6-$MSP1_{19}$) showed the highest reactivity against a panel of sera from individuals exposed to infection by *P. vivax* compared to the vectors pGEX and pMAL. Formulations using recombinant proteins were tested in experiments of immunization of mice with high antibody titers that were obtained with the TiterMax adjuvant (Sigma), MPL / TDM / CWS (monophosphoryl lipid A, trehalose dicorynomycolate and cell wall skeleton, Sigma) and aluminum compared to Freund's adjuvant-containing formulation.

Another adjuvant tested was the GNP, gold nanoparticle used as a carrier of peptides, drugs and other to target tissues. According to tests conducted by Parween et al. (2011) there was production of antibodies in mice immunized with the $PvMSP1_{19}$ and $PfMSP1_{19}$ using GNP and aluminum as an adjuvant. The adjuvant caused an increase of immunogenicity when compared to aluminum and to GNP alone.

Tests using the MSP1 of *P. falciparum* have been made in researching and manufacturing of vaccines, although protection is species-specific, may provide insights for the development of vaccines for *P. vivax*. Phase I study to evaluate two formulations of vaccines containing the portion Pf $MSP1\text{-}_{42}$ of *P. falciparum* obtained after 3 vaccinations incidence of detectable antibodies of 74% and 81%, having the formulations shown safety and tolerability. However, these elements were not immunogenic enough to obtain a significant biological effect when performed *in vitro* test (Malkin et al., 2007). Phase 1B studies using other formulations obtained immunogenicity, safety and tolerability when tested in children (Withers et al., 2006). New formulations must be tested to increase the immunogenicity of vaccines using new adjuvants.

5. Conclusion

Immunity to malaria is species-specific and for years the species *P. vivax* has been neglected, mainly due to the fact that the parasite cannot be cultivated in vitro. Thus, most studies refer to the *P. falciparum* species that causes the greatest number of lethal cases. A large knowledge about infections by *P. vivax* was brought from studies using *P. falciparum*. Today the use of molecular tools has expanded the ability to study on *P. vivax* and has made possible advances in research potential targets for the production of vaccines against infections caused by this species that is most prevalent in the Americas and Asia (Levitus and del Portillo, 1994). The mechanisms that lead to the production of immunity capable of controlling natural infections and infections after repeated years of exposure, or the incomplete protective immunity that allows the presence of asymptomatic infections may provide the key to the production of vaccines (Doolan et al., 2009; Wipasa et al., 2010). Many studies have explored the ability of specific antigens to induce immune response in natural infection. The MSP-1 protein is one of the most studied proteins today. Several studies have demonstrated the presence of anti-MSP1 antibodies in exposed individuals from endemic regions and its relation to protective immunity, as previously described. Further research about this protein and the immune response should be conducted to elucidate the mechanisms of species-specific immune response enabling knowledge that enable the production of effective vaccines and durable.

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Title

Immune responses against C- and N- terminal region of PvMSP-1 in a rural settlement in Amazonia

Short Running Title

Immunogenicity against PvMSP-1

Keywords: *P. vivax*; malaria; merozoite surface protein -1

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Summary

Studies on the immune status of individuals living in malaria-endemic areas are important to evaluate anti malaria vaccine candidates. We analyzed the immune response against the N- and C-Terminal of Merozoite surface protein 1 of *Plasmodium vivax* (PvMSP-1) in 312 sera of a population from an agricultural settlement located adjacent to the Rio Pardo, Amazonas, whom they were followed by a clinical epidemiologic study. The results showed that 51.0% had total IgG against C-terminal portion and 29.5% had total IgG against N-terminal portion of the protein. As expected antibodies against the N-terminal portion were correlated with age, number of previous episodes, time of residence in the area. There was a predominance of IgG subclass 3 against the N-terminal portion. Our data suggested that IgG_3 antibodies against the N-terminus of PvMSP-1 to the local repertoire of variable domains of PvMSP-1 occur in asymptomatic patients indicating as sera-epidemiological markers of clinical protection.

Keywords: *P. vivax*; malaria; merozoite surface protein -1

Introduction

Malaria is a parasitic disease of great global importance, being responsible for high morbidity and mortality, especially in tropical regions of the planet. According to the "World Malaria Report" 2010 (1) the number of estimated cases of malaria worldwide, in 2009, was 225 million, with 781,000 deaths. In Brazil, the mortality rate is low, but morbidity is high. According to the Brazilian Health Ministry (2) , there were 306,908 cases of malaria in the year 2009 which 4.442 cases were hospital admissions.

In the past, research related to *Plasmodium vivax* was limited, since the disease was considered benign and culture of this parasite was unavailable. However, *P. vivax* is the species most prevalent in the world and habitants of Tropical areas of the World live in high risk of infection (3, 4).

The clinical symptoms of malaria are attributed to blood stages of the life cycle of parasites, including the invasion of red blood cells, intracellular multiplication and rupture of red blood cells. This phase, in which *Plasmodium* merozoites are released from the liver and invade red blood cells, is considered a target for vaccine production, with the expected reduction of parasitemia and therefore morbidity and mortality related to disease (5). In the merozoite stage, the different *Plasmodium* species have in common a protein identified as merozoite surface protein-1 (MSP-1), a glycoprotein of 195 kDa, whose structure reveals sequences conserved between different species (6, 7).

Studies on the MSP-1 have been realized owing to the need to understand the mechanisms involved in erythrocyte invasion by parasites carried in addition to its importance as an antigen capable of activating the host protective immunity, making it a potential target for the production of malaria vaccine (6, 8). Natural immunity to malaria provides a solid protection against severe morbidity and mortality. In endemic

regions, the occurrence of clinically silent infections by *Plasmodium* reflects the ability of adaptive immune mechanisms in preventing the disease. In general, the pattern of clinical disease depends on age and previous contact of the immune system to antigens of the parasites (number of previous infections) (9-11).

Several studies related the presence of natural antibodies against fragments of PvMSP-1(12-18), and two studies (19, 20) linked the presence of specific antibodies against the N-terminal portion to reduced risk of infection. The aim of this study was characterize the humoral immune response evaluating repertoire of IgG subclass against C- and N-terminal of PvMSP-1 in individuals living in a rural settlement situated on the State of Amazonas.

Materials and Methods

Study area and population

The Rio Pardo rural settlement was established by Ordinance 274 of October 25, 1996 by the National Institute of Colonization and Agrarian Reform (INCRA), which envisaged the creation of 396 family farms (21). The region has serious problems due to lack of necessary infrastructure for storage and transportation of agricultural products. Deforestation is common, with exploration areas to the decline in production, ultimately leading producer in search of a new area because of the cost in land reclamation. About 80% of producers create small animals such as chickens, ducks and pigs. Cattle ranching is carried out extensive, and mostly cattle with low milk production. The houses are made mostly of wood, usually do not have toilets and pits are the only alternative to it. Water is obtained through bayous and eyes water, and some residents have wells (22). In Rio Pardo there is only one health post, where is the microscopist,

responsible for the diagnosis of malaria through the giemsa-stained thick blood smears. Medical care is biweekly (23). The aim was to include all residents in the study but after exclusion and subsequent exclusion criteria, 312 of 647 individuals were included in the study.

Sample collection

Participants were followed for a longitudinal study of six months, from September 2008 to February 2009 through SIVEP database (24). During the month of November of 2008 we carried out a cross-sectional study. The residents were interviewed and examined. After signing the consent form, 5 mL of peripheral blood was collected by vein puncture and blood was processed, aliquoted and stored at -20° C. At the same time, sample taken by finger prick was collected for processing the standard method of malaria diagnosis according to the Ministry of Health. Infections caused by malaria parasites were diagnosed by microscopy (Giemsa-stained thick blood smears) and the people infected received treatment recommended. DNA was extracted using the QIAGEN kit according to the manufacturer. Electrophoresis was performed in 0.6% agarose gel in 0.5 X TBE buffer to confirm the DNA extraction. Then, we performed real-time PCR for molecular diagnosis of the species *Plasmodium vivax* and *Plasmodium falciparum* by modified protocol described by Perandin et al. (25). Protocol was adapted to a multiplex reaction including primers and probes only for the species *P. falciparum* and *P. vivax* with changes in the final volume to 20 µL. The primers and probes were produced by Applied Biossystems. Sequences described by Perandin et al (25), were obtained from the 18S rRNA gene of the malaria parasites. The probes were produced having different color flag to enable multiplex reaction containing primers for both species in the same reaction. We used the "Taqman

Universal PCR Master Mix 2x" also produced by Applied Biosystems. To differentiate the species the probes was constructed using VIC dye flag for *P. vivax* probe and FAM for *P.falciparum* probe, the quencher used was TAMRA for both probes. We used the same cycle of reactions described before (25). The final concentrations were the same. The protocol was tested with 30 negative samples and 50 positive samples from the serum bank at FIOCRUZ-LMD. The study was approved by research ethics committee of the Federal University of Amazonas.

Recombinant proteins

Glutathione S-transferase (GST) fusion proteins, representing the N-terminal (ICB2-5) and C-terminal (ICB10) from de MSP-1 molecule of Belem strain was produced like described elsewhere (11). GST alone was also produced. GST and recombinant proteins were purified on Glutathione-Sepharose 4B columms (Amersham Pharmacia), and protein concentration was determined by Bradford assay (Bio-Rad).

Immunoassay (Enzime-linked Immunosorbent assay – ELISA)

The presence of naturally acquired antibodies against GST-PvMSP-1 portions has been described in detail before (11). There were evaluated IgG antibodies against N- and C- terminal of PvMSP-1. To determine if the sample was positive, we evaluated two cutoffs: First we processed 20 samples of negative malaria serum from individuals who never had malaria. The OD average of GST alone from each sample was measured as the GST-ICB2-5. The first cutoff was calculated by the OD average of ICB2-5 minus GST OD plus 2 standard deviations. The second cutoff was calculated by the OD average of GST-ICB2-5 of each sample studied minus GST OD plus 2 standard deviations (SD). If the sample studied was positive in both tests there was positive. If

one of tests results was negative, the sample was negative. The same methodology was applied for ICB10.

IgG subclasses was determined by ELISA. We used mouse monoclonal antibodies for each subclass (Sigma, St. Louis, MO) diluted according to the manufacturer's instructions using a panel of positive sera previously known to react with each subclass. All sera were tested at dilutions of 1:100 and monoclonal antibody binding was detected with peroxidase-conjugated anti-mouse immunoglobulin (Sigma). Sera positive for each isotype were determined as described (11). The calculation of cutoff was made for GST protein using the average value of duplicates for each serum tested plus 2 standard deviations and a second cutoff was calculated for the recombinant protein MSP-1 (ICB-10 and ICB2-5) using a panel of sera from negative control serum bank itself also with the mean value of duplicate plus 2 standards deviations. The average of duplicates of each serum tested were submitted to the court by two cutoff calculated values and were considered positive when greater than both cutoffs.

Statistical methods

Microsoft Excel 2007 was used for database storage. Analysis was performed with SPSS version 18.0. Associations of two categorical variables (nominal or ordinal) were tested by chi-square test with pearson or fisher correction. Continuous variables were tested for associations with Spearmans's correlation. Differences between two groups were evaluated by student T test. Some continuous variables or ordinal categorical variables analyzed in this data did not follow a normal distribution and were analyzed by Man-Whitney test.

Results

The real-time PCR results were compared with the blood smear, performed at the time of collection (Table 1). There was agreement in 265 (85.0%) negative, 11(3.5%) positive for *P. vivax* and 2 (0.6%) positive for *P. falciparum* in the analysis of two techniques. There was disagreement between 24 (7.7%) tested positive for *P vivax* in real-time PCR and negative for blood smear and 3 (1.0%) tested positive for *P. falciparum* and negative blood smear. A blood smear does not detect any case of mixed infection while PCR detected five (1.6%) cases. The blood film also obtained 2 (0.6%) *P. vivax* positives undetected by real-time PCR. We've tested the same samples using the protocol described by Rougemount et al (26) that detected the presence of *Plasmodium sp* in one of the two samples.

Table 1 - Methods used for diagnosis.

PCR	**Microscopy analysis**				
	Negative	*Plasmodium vivax*	*Plasmodium falciparum*	Mixed infection	Total
Negative	265 (85,0%)	2 (0,6%)	0 (0,0%)	0 (0,0%)	267(85,6%)
P. vivax	24 (7,7%)	11 (3,5%)	0 (0,0%)	0 (0,0%)	35(11,2%)
P. falciparum	3 (1,0%)	0 (0,0%)	2 (0,6%)	0 (0,0%)	5(1,6%)
Mixed infection	5 (1,6%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	5(1,6%)
Total	297(95,2%)	13(4,2%)	2(0,6%)	0(0,0%)	312(100%)

Association between infection and age, number of previous infections, time of residence and time of last malaria attack was evaluated by chi-square test (Table 2).

Table 2 – Summary of epidemiological results

	Rio Pardo settlement	**Associations**	***P* values**
Gender			
Male (n (%))	180 (59.2%)		
Female (n(%))	124 (40.8%)		
Total (n)	304*		
Age (median (SD)) (Minimum – Maximum)	32.78 (SD 19.259) (1 – 90)		
TR (years)		SC TR x NI	$p = 0.000$
0 - 5	99 (35.1%)		
6 – 15	98 (34.8%)	χ^2 Age x INF	$p = 0.026$
> 15	85 (30.1%)		
Total	282*	SC NI x LA	$p = 0.000$
NI (n (%))			
0	22 (7.9%)	SC Age x NI	$p = 0.388$
1 – 4	105 (37.5%)		
> 4	153 (54.6%)		
Total	180*		
LA (months)			
0 - 1	28 (9.0%)		
2 – 3	30 (9.6%)		
4 – 5	13 (4.2%)		
6 – 12	23 (7.4%)		
> 12	181 (58.0%)		
Never had malaria or didn't know	37 (11.9%)		

*information obtained trough interview, some individuals didn't know the information.

TR – Time of residence in Rio Pardo settlement; NI – Number of previous infections; LA – Time in months since the last malaria attack; INF – malaria infection. We consider statistically significant *p* values lower than 0.05. The differences in proportions were evaluated by chi-square (χ^2) test. For analysis between two continuous variables we used Spearman's correlation (SC).

Here we performed a cohort of 6 months through SIVEP database, (results of thick blood smear of september to february) with a cross-sectional study (evaluated in November) when we did diagnosis by blood smear and real time PCR to select two groups, asymptomatic and symptomatic patients (Figure 1). We called symptomatic the participant that had at least one episode of infection by *P. vivax* during the six-month study (assessed by blood smear from SIVEP database or during the cross-sectional). The two cases negative in PCR but positive in blood smear were also considered symptomatic. Asymptomatic was defined as the positive case for real-time PCR performed in the month November that had no episode of infection in the previous two months and three months subsequent of the period of data collection and that did not receive anti-malarial therapy. Of a total of 312 individuals, we defined 44 (14.1%) symptomatic individuals with positive thick smears for the species *P. vivax* sometime in the six months follow-up. 24 (7.7%) individuals were positive for real-time PCR during the collection period occurred in November but negative by thick blood film and had no events during follow-up. They were considered asymptomatic. Individuals negative in thick smear and PCR and without events of infection during follow-up were hidden (Figure 1).

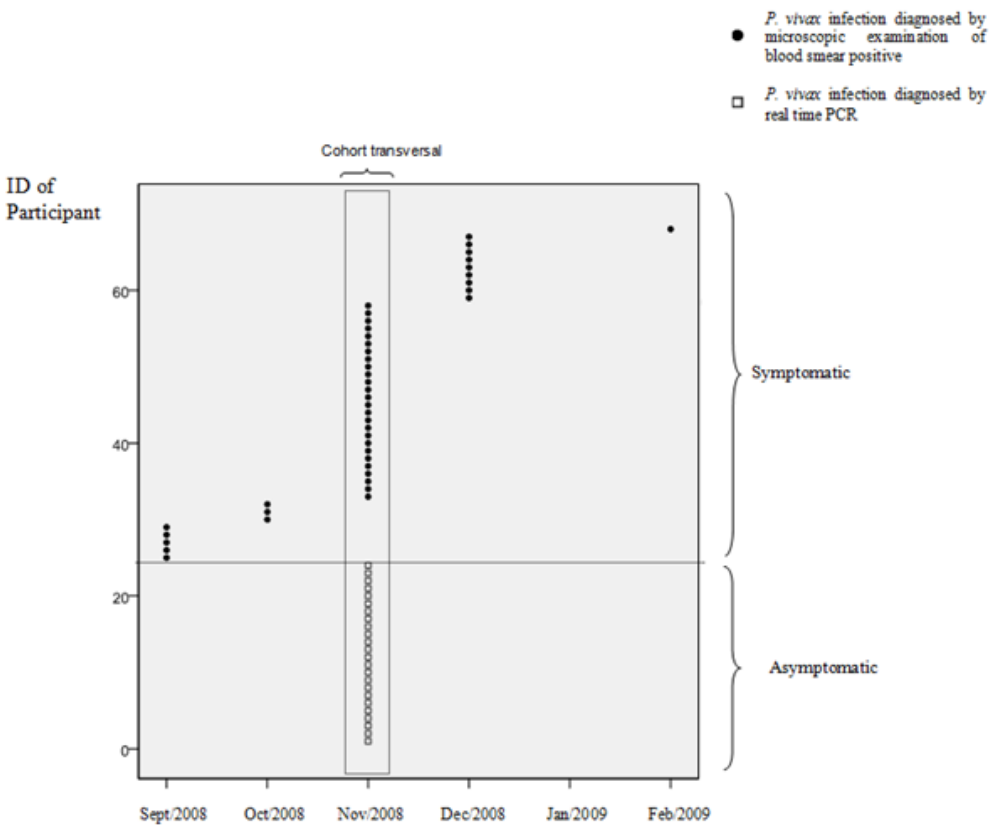

Figure 1. Outline of the monitoring study to define groups. The population of Rio Pardo was monitored for 6 months, (sept/08 to Feb/09) and a cross-sectional was performed in Nov/08. Real-time PCR and examination of thick smears were used for diagnosis of malaria. Graph shows the selection of individuals for the symptomatic group and asymptomatic group.

To determine the antibody response in individuals exposed to malaria we evaluated the acquisition immunoglobulin G anti ICB2-5 and anti ICB-10 in enzyme immunoassays with the entire population (N = 312). A total of 29.5% of participants had total IgG antibodies against ICB2-5 portion of MSP-1, while 51.0% were positive for the portion ICB-10 (p=0.00). Them, we evaluated the IgG subclasses for both fragments (data no shown) and analyzed the acquisition of antibodies in entire population and in the two groups (symptomatic and asymptomatic) separately (Figure 2) for ICB2-5. In both analysis we observed similar results with a predominantly IgG_3 response anti ICB2-5 in both the whole population and in the two study groups

consisting of individuals infected with *Plasmodium vivax*. However, we also observed a slight rise of IgG_2 anti ICB2-5 acquired in the two analysis (Figure 2). The rise of IgG_2 anti ICB2-5 in both analyses raises the question whether a relationship exists between non-cytophilic antibodies and the acquisition of clinical immunity.

To evaluate this hypothesis we compared the level of IgG_3 and IgG_2 anti ICB2-5 on the infected groups (Figure 3A). As expected we have demonstrated that elevated levels of IgG_3 anti ICB2-5 in the group of asymptomatic, average OD 0.350 ± 0.087 was statistically significant (p <0.01) compared to the group of symptomatic patients, whose sera showed very low levels of IgG_3, average of 0.049 ± 0.0257.

To evaluate the response of IgG_2 anti ICB2-5, we compare the levels in both groups. In contrast to that observed with IgG_3, asymptomatic patients had low levels of IgG_2 anti ICB2-5 and symptomatic ones, very low levels. However this difference was not significant (p = 0.06). Rio Pardo is an area of recent colonization (just over 10 years), an INCRA settlement aimed at the family farm. The community is composed of stable population and dwelling areas and stream extensions. The parasitemia annual rate recorded over the year varies between 50 and 160. Malaria was identified by residents as a major problem for occupation of the area. In the three months that followed the cohort we followed across the population and identify those who developed symptoms of malaria during this period. When we compared the levels of IgG_3 anti ICB2-5 among individuals who had symptoms with those who did not develop clinical malaria in this period we found interesting data (Figure 3B). The absence of IgG_3 was remarkable in those subjects who developed clinical malaria. As in the study of Nogueira and colleagues (19) had shown only the association between IgG anti ICB2 -5 malaria and protection, this is the first time the presence of IgG_3 anti N-terminal MSP1 is a

characteristic of acquired immunity against clinical malaria caused by P. vivax (Figure 3).

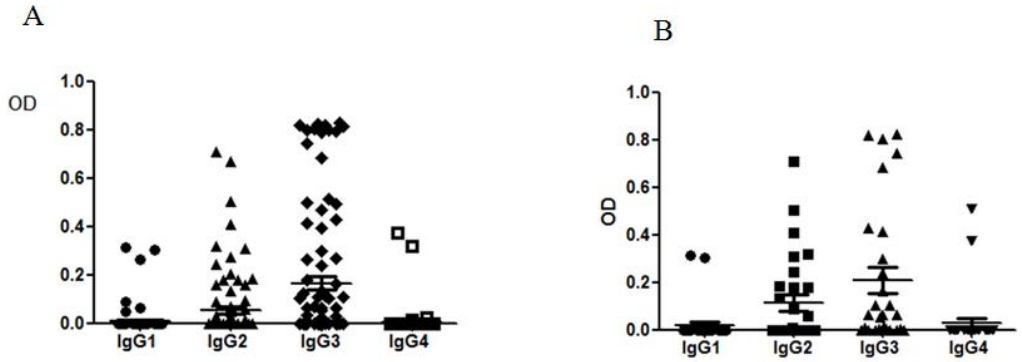

Figure 2. Humoral immune response against N-terminal Pv-MSP1. Recombinant protein corresponding to the region between ICB2 and ICB5 of allele Belem was used in this study. A) Immunoassay tests were performed with sera of the population of Rio Pardo. B) Immunoassay tests were performed with sera from both asymptomatic and symptomatic groups determined after six

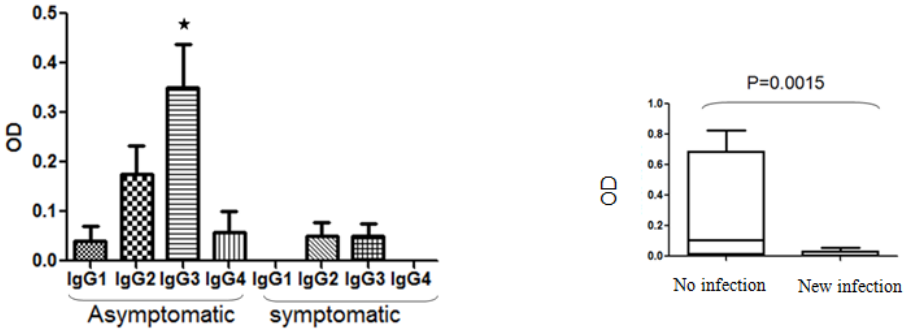

Figure 3. Comparative analysis of immunoglobulin IgG_3 and IgG_2 subclasses against N-terminal Pv-MSP1. A) Comparison of 2 and 3 subclass between asymptomatic and symptomatic groups. Star: comparison between the IgG_3 asymptomatic group versus IgG_3 symptomatic group by student t test *P* = 0.0035; asymptomatic means 0350 ± 0.0874 (N = 15) and symptomatic means 0.0496 ± 0.02576 (N = 14). B) Comparison between the level of IgG_3 anti ICB2-5 in individuals who developed clinical malaria within three months after the cross-sectional. Student t test with Welch's correction *P* = 0.015; means no-reinfection 0076 ± 0.2927 (N = 19), re-infection means 0.0138 ± 0.0105 (N = 5).

The acquisition of antibodies against ICB2-5 and ICB-10 was evaluated for associations with age, time of residence in endemic area, last malaria attack and number of previous infections (student t test). The results shown that serology against ICB2-5 was associated with age ($p < 0.01$), time of residence ($p < 0.01$), last malaria attack ($p < 0.033$) and number of previous infections ($p = 0.001$). ICB-10 did not associated with age ($p = 0.419$) and time of residence ($p = 0.14$) but was associated with last malaria attack ($p = 0.0021$) and number of previous infections ($p = 0.0021$).

The recombinant protein ICB2-5, a recombinant antigen comprising three conserved blocks (blocks 1, 3, and 5) and two variable blocks (blocks 2 and 4), was characterized and the polymorphic domains were recognized by antibodies from residents of endemic areas (11). As the protein was produced with DNA source of Belem allele, we assumed that the high prevalence of specific IgG_3 in the asymptomatic population was due to acquisition of cross-reactivity with antibodies against different domains shared between haplotypes of Block 2, known as sequence-unique or the acquisition of a repertoire of antibodies against different alleles of the polymorphic block 2 (27).

Discussion

Vivax malaria is estimated for the number of cases ranging from a minimum of 35,000,000 to 80,000,000, or perhaps more, moreover the morbidity from this disease cannot be considered inconsequential, given a propensity towards significant and severe anaemia, thrombocytopaenia, violent paroxysms and fevers of 40°C to 41.6°C that, if untreated, can last for weeks with serious morbidity (28). In Brazil, the Amazon region accounts for the largest number of cases of the disease (98.8% of cases) (24) and partial results of 2010 obtained through SIVEP database (29) shown 334,004 cases of malaria which *Plasmodium vivax* was responsible for 283,022 of that (84.74%).

Here we perform the diagnosis of malaria in the Community of Rio Pardo and found a prevalence of 11.2% of *P. vivax*, 1.6% of *P. falciparum* and 1.6% of mixed infection. The technique used was real-time PCR for malaria diagnosis of the participants along with the thick smear technique standardized by the Brazilian Ministry of Health The discrepancy between the results reflects the ability of each method, and the real-time PCR technique is more sensitive and therefore capable of detecting low parasitaemias while the thick blood films is a low cost method for parasites and quick turnaround. PCR has been used for determination of individuals with asymptomatic infections. Epidemiological studies using the molecular technique detected a high percentage of asymptomatic individuals infected with *P. vivax* in the Rio Negro, Amazonas (30) and in adjacent regions of Rondonia (31). A study in Canada to refugees found 3.1% of asymptomatic individuals, negative in thick smear, but positive for the molecular technique (32). Our study found 24(7.6%) asymptomatic demonstrating the best performance of the method although the thick smear had detected 2 samples positive for *P. vivax* and negative for real-time PCR. Another method used had detected only *Plasmodium sp* in one sample. The use of primers of other species of *Plasmodium* should be considered for elucidation of the discrepancy.

In endemic regions, most of Plasmodium infection is clinically silent, reflecting the ability of adaptive immune mechanisms in preventing the disease. In non-immune individuals, the infections cause clinical symptoms, where a minority of cases may progress to severe malaria and death. In general, the pattern of clinical disease depends on age and previous contact of the immune system to antigens of the parasites (number of previous infections) (33). Here, we observed association between age and infection,

and number of previous infections and last malaria attack and time of residence, like described before (10).

Progress towards the development of a malaria vaccine against *Plasmodium vivax*, the most widely distributed human malaria parasite, will require a better understanding of the immune responses that confer clinical protection to patients in regions where malaria is endemic. In the past few years, very few laboratories have worked to identify and pre-clinically characterize *P. vivax* vaccine candidates, and only a couple of *P. vivax* antigens are imminently poised to advance into clinical trials (4). One is an asexual blood-stage antigen, the merozoite invasion ligand protein known as the Duffy Binding Protein (DBP), whose binding domain (RII) has gone through preclinical testing in rodents and nonhuman primates (34-36). The pre-erythrocytic/sporozoite antigen, the Circumsporozoite Protein (CSP), also has under gone preclinical studies in mice and primates, testing various platforms and formulations to advance into clinical trials (37, 38). One other *P. vivax* candidate vaccine, a transmission blocking sexual stage antigen, Pvs25, has been in phase I clinical trials (39, 40).

The efforts for *vivax* blood-stage vaccine discovery and development, albeit limited in scope, have mostly followed in the footsteps of what was first being accomplished in the *P. falciparum* vaccine developmental pathway. Thus, after the Merozoite Surface Protein -1 (MSP-1) was discovered as a major merozoite surface antigen in *Plasmodium yoelii* and *P. falciparum* (41, 42), the orthologous gene for the *P. vivax* antigen was characterized (6).

The MSP1 has been intensively investigated as a malaria vaccine candidate. This protein is synthesized in a precursor form with a high molecular weight during

schizogony and during the invasion process, a proteolytic cleavage releases most of the molecule from the merozoite membrane leaving a membrane-anchored 19 kDa fragment (MSP119) on the parasite surface (43). Research led to the identification of the C-terminal end of PfMSP-1 as the target of inhibitory antigens and the equivalent PvMSP-1-p19 and p42 portions were subsequently produced and examined as *vivax* vaccine candidates (42). Pre-clinical vaccination trials carried out with rhesus monkeys showed that animals immunized with a recombinant protein based on the *P. vivax* MSP1 C-terminal region (MSP1-42 kDa) and encompassing the MSP1-19 fragment developed partial protection to infection with *P. cynolmogi*, a species closely related to *P. vivax* (44).

Currently, the search for *P. vivax* malaria parasite subunits with vaccine potential has focused toward the N-terminal region of *P. vivax* MSP-1, the fragment *Pv*200L induced partial protection of *Aotus* monkeys against experimental heterologous challenge with *P. vivax* (Salvador I) blood stages (45). Immunization with MSP1 has been performed with experimental primate challenge models (*Aotus* and *Saimiri*). Early studies demonstrated that significant protection from *P. falciparum* parasite challenge was induced by the whole _190-kDa MSP1 or a large portion of the sequence (19, 31).

Although fewer studies have focused on the rest of the MSP1 molecule the best seroepidemiologic results were found with the N-terminal block 2 region of MSP1 from both human malaria *P. falciparum* and *P. vivax*. The N-terminal block 2 region has been found to be under the strongest natural selection pressure, and antibodies specific for common allelic types of block 2 are strongly associated with a reduced risk of clinical malaria vivax or falciparum (19, 46, 47).

The occurrence of clinical protection in *P. vivax* malaria in Brazil was first reported among residents of the riverine community of Portuchuelo, in Western Amazon. Here after a cross-sectional and 6 month of follow-up clinical protection in vivax malaria was observed among residents of the settlement community of Rio Pardo in Central Amazon, an endemic region.

Naturally acquired immunoglobulin G (IgG) antibodies against merozoite surface antigens of *Plasmodium* play a major role in acquired immunity to malaria. Early study has demonstrated the presence of naturally acquired antibodies against the N terminus PvMSP1 was correlated to clinical protection against vivax malaria (19). Similarly the presence of anti- *P. falciparum* MSP-1 block 2 antibodies were shown to be associated with protection from clinical malaria episodes in several endemic regions (46), including Dielmo and Ndiop (48). The characterization of PvMSP1 showed among different recombinant proteins those had polymorphic domains were preferentially recognized by antibodies from residents of endemic areas (11).

An interesting observation of our results is that IgG_3 levels of antibodies against the N terminus of PvMSP1 were only detected in asymptomatic individuals, whereas, most of symptomatic patients had not theses antibodies. The recognition of a panel of recombinant proteins corresponding to PvMSP-1 variants commonly found in local parasites was poorly recognized by non-infected subjects in study performed in western Brazilian Amazon (49). However, after subsequent *P. vivax* infections the proportion of responders to PvMSP-1 variants increased substantially, suggesting that the poor immunogenicity of variable domains may not represent a major obstacle to variant-specific immunity. These data suggest sequence polymorphism affect antibody recognition of PvMSP-1 variants, since the panel of recombinant antigens used in this

study did not include the whole repertoire of PvMSP-1 variants found in local parasites. Or according our evidences variable antigens may be poorly immunogenic or may elicit antibody responses that are short lived in the absence of frequent boosting that is happened in asymptomatic infections.

Evidences of frequent boosting, in our previous study, long lived IgG against N-terminus Pv-MSP1 had already been shown over one year of follow-up (19). The presence of circulating mostly undetectable parasites in asymptomatic patients, which should facilitate the formation and migration of specific plasma cells to the bone marrow and sustained IgG_3 anti Pv-MSP1. Persistence of IgG_3 is usually seen only in asymptomatic infections, indicating that the presence of parasite is required for maintenance of the IgG_3 responses. These data are sustained by found with natural antibody response against *P. falciparum* GPI (glycosylphosphatidylinositol). It was shown in subjects from Papua New Guine that IgG3 responses predominated are likely to require frequent boosting to be maintained (50).

Levitus et al. (51) conducted serological survey in the region of Rondônia, Brazil, using 10 recombinant proteins of N-terminal region of MSP-1. The antibody response showed that small amount of antibodies recognized conserved regions of the molecule (ICBs), leading to the conclusion that conserved regions are weakly immunogenic in natural infections. When proteins were constructed containing polymorphic regions associated with the ICBs, large quantities of sera recognized proteins ICB1-2, ICB2-3, ICB3-4 and ICB2-5. Study using polymorphism and natural antibodies in a community exposed to malaria also related the importance of polymorphic regions for the production of natural antibodies (49).

Our data suggest that antibodies to the local repertoire of variable domains of PvMSP-1 are elicited after several repeated infections and require frequent boosting, as occur in asymptomatic patients. Our studies point to the potential value of the IgG_3 against the N terminus of PvMSP1, as sero-epidemiological markers that can indicate the existence of clinically protected individuals in a population. Our studies have validated the N termini of PvMSP1 as a solid subunit vaccine candidate against *P. vivax*.

Deciphering the factors that constrain the MSP-1 block 2 response might help in the design of efficient malaria vaccines based on this antigen. One of the main obstacles to the acquisition of antimalarial immunity is the polymorphism in potential target antigens, which enables parasites to evade immune responses elicited by past exposure to variant forms of the same antigen.

The results have implications for vaccine design because they demonstrate the importance of the block 2 polymorphic repeat sequences in naturally acquired immunity and provide an assessment of the cross-reactivity of human antibodies to semi conserved domains.

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Anexos

Ms. Ref. No.: MEEGID-D-11-00294 Title: Plasmodium vivax merozoite surface protein-1, a vaccine candidateInfection, Genetics and Evolution

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DINÂMICA DAS INFECÇÕES MALÁRICAS EM COMUNIDADE DO AMAZONAS SOB O ASPECTO DE MARCADORES MOLECULARES DE PARASITAS E DO POLIMORFISMO DE PROTEINAS CANDIDATAS A VACINAS

Andamento do projeto - CAAE - 3640.0.000.115-07

Situação	Data Inicial no CEP	Data Final no CEP	Data Inicial na CONEP	Data Final na CONEP
Aprovado no CEP	13/11/2007 11:46:48	28/11/2007 10:42:38		

Descrição	Data	Documento	Nº do Doc	Origem
1 - Envio da Folha de Rosto pela Internet	01/11/2007 12:50:20	Folha de Rosto	FR164515	Pesquisador
2 - Recebimento de Protocolo pelo CEP (Check-List)	13/11/2007 11:46:48	Folha de Rosto	3640.0.000.115-07	CEPV
3 - Protocolo Aprovado no CEP	28/11/2007 10:42:37	Folha de Rosto	305/07	CEP

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Pedimos sua autorização para realizar um exame médico e preencher um formulário contendo perguntas sobre malária, leishmaniose e viroses e sobre as atividades e hábitos que poderiam ajudar a entender como essas doenças acontecem na sua região.

Pedimos também autorização para coletar sangue da sua veia, que será utilizado para o diagnóstico e atividades de pesquisa sobre estas doenças transmitidas por insetos, que são comuns em Rio Pardo. Se você autorizar esta coleta, seu sangue será examinado para averiguar se você tem ou não o parasito que causa a malária e se você teve alguma vez a virose causada por um vírus chamado Mayaro (que causa dor de cabeça, dor de corpo, dor nas juntas e, às vezes, erupção na pele). O sangue que não for usado será guardado na coleção de soros do laboratório da FIOCRUZ, em Manaus. Esta coleta de sangue poderá causar uma leve dor e/ou uma pequena mancha roxa que desaparecerá em 3 a 4 dias.

Mesmo após sua autorização, você terá o direito e a liberdade de retirar seu consentimento em qualquer fase da pesquisa, independentemente do motivo e sem prejuízo do atendimento fornecido pela equipe.

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Você será informado pessoalmente dos resultados dos exames e receberá orientação e, se for necessário, será tratado pela equipe ou encaminhado para o sistema de saúde local.

Ao auxiliar na realização deste estudo, você ajudará no fornecimento de informações para a prevenção das doenças estudadas.

Os resultados serão analisados e divulgados em relatórios e artigos científicos; a equipe do projeto também realizará atividades de divulgação dos resultados na própria comunidade. Contudo, sua identidade será mantida em sigilo para sempre, e não aparecerá em nenhum relatório, artigo ou qualquer outro meio de divulgação.

Se você quiser saber mais detalhes e os resultados da pesquisa, pode fazer contato com os pesquisadores Paulo Afonso Nogueira ou Sergio Luiz Bessa Luz pelo telefone (92) 3621-2304 ou 3621 2337 ou diretamente na FIOCRUZ à Rua Teresina 476, Adrianópolis, Manaus.

Eu, _____, por me considerar devidamente informado e esclarecido sobre o conteúdo deste documento e da pesquisa a ser desenvolvida, livremente dou meu consentimento para a minha inclusão como participante da pesquisa DINÂMICA DAS INFECÇÕES MALÁRICAS EM COMUNIDADE RIBEIRINHA DO AMAZONAS EXPOSTA À MALARIA SOB O ASPECTO DE MARCADORES MOLECULARES DE PARASITAS E DO POLIMORFISMO DE PROTEINAS CANDIDATAS A VACINAS e atesto que me foi entregue uma cópia deste documento.

Rio Pardo, _____ de novembro de 2008.

Assinatura do paciente ou responsável:

_____

DIGITAL

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz
Instituto Leônidas e Maria Deane

EXAME CLÍNICO
Rio Pardo / P. Figueiredo

Identificação

Nome Completo _____ Indivíduo Nº _____

Nº Questionário_____ Idade _____ Data nascimento ____/____/____ Sexo ☐M ☐F

Anamnese

Sintomas nas ultimas 48 horas	Ausente	Leve	Moderado	Intenso
Febre				
Calafrios				
Sudorese				
Dor nas costas				
Dor no corpo				
Fraqueza				
Dor na barriga				
Diarréia				
Enjôos				
Vômitos				
Falta de apetite				
Dor de cabeça				
Tontura				
Tosse				
Falta de ar				
Alteração cor da urina				

Exame físico

Temperatura _____ ° C	Pulso _____ bpm	PA _____ mmHg
Mucosas ( ) Normocoradas ( ) Hipocoradas	Icterícia ( ) Sim ( ) Não	Lesões / Cicatrizes (LTA) ( ) Não ( ) Sim [descrever nº e local]
Fígado_____ cm do RCD	Baço _____ cm do RCE	
_____ de novembro de 2008.	Médico _____	

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INQUÉRITO EPIDEMIOLÓGICO DA MALÁRICA EM RIO PARDO – P. FIGUEIREDO

Identificação

Nome Completo _____ Indivíduo Nº _____

Nº Questionário_____ Idade _____ Data nascimento ____/____/____ Sexo ☐M ☐F

		Código não preencher
Estado Civil *[Marque com um X a quadrícula ao lado que corresponde ao estado civil respondido pelo entrevistado.]*	☐ Solteiro ☐ Casado ☐ Viúvo ☐ Separado ☐ União consensual	
História migracional *[Escreva no espaço ao lado a seqüência das siglas das Unidades da Federação em que residiu o entrevistado]*	_____	
Há quanto tempo você mora neste neste município? *[Escreva no espaço ao lado o tempo que o entrevistado exerce a atividade no município do estudo. Se não tem ocupação respondida anteriormente, marque Não se Aplica]*	____anos ____meses ☐ Não sabe ☐ Não se aplica	
Nos últimos 30 dias você usou algum meio de diminuir as picadas de mosquitos? *[Marque a quadrícula correspondente à resposta do entrevistado. Liste para ele os métodos: repelentes, tela em janela mosquiteiro, alho, vitamina B, etc.]*	☐ SIM , todos os dias ☐ SIM, esporadicamente, quando tem mais mosquito ☐ Não usou Qual(is):	
Que idade você tinha quando teve a sua primeira malária? *[Marque no espaço ao lado a resposta do entrevistado.]*	____anos ☐ Não sabe Não se aplica	
Quantas malárias você já teve na vida? *[Escreva no espaço ao lado o número de malária referida pelo entrevistado. Caso ele não precise o número, descreva a faixa que seja mais adequada. Mesmo que ele suspeite de ser a mesma malária que recrudesceu/recaiu, registre sempre a quantidade que ele referir. Se nunca teve malária, preencha com 0]*	_____ ☐ 5 a 10 ☐ 11 a 20 ☐ 20 a 50 ☐ 50 ou mais ☐ Não sabe	
Qual foi a última vez (dias, meses ou anos) que você fez lâmina para malária e deu positiva? *[Anote, no espaço ao lado, o tempo referido pelo paciente. Atenção, não esquecer da unidade: dias, meses ou anos. Caso não seja exato, registre o tempo aproximado]*	___ dias ___meses __anos ☐ Não sabe ☐ Não se aplica	
Qual foi o tipo de sua última malária (falciparum, vivax ou as duas juntas? *[Marque a quadrícula correspondente à espécie referida pelo entrevistao]*	☐ *P.falciparum* ☐ *P.vivax* ☐ Mista ☐ Não sabe ☐ Não se aplica	

Nos últimos 7 dias você fez uso de algum remédio ou chá caseiro para tratar de malária ou alguma outra doença? *[Marque a quadrícula correspondente à resposta do entrevistado]*	☐ SIM ☐ NÃO ☐ Não sabe
Qual o nome do remédio que você tomou? *[Se a resposta anterior foi SIM, tente identificar o produto e marque, no espaço ao lado, o produto usado como antimalárico]*	_____ ☐ Não sabe ☐ Não se aplica
Você tem algum problema de saúde ou está tratando de alguma doença hoje? *[Marque na quadrícula ao lado e se a resposta for SIM, especifique o problema relatado pelo paciente]*	☐ SIM ☐ NÃO _____
Você já pegou leishmaniose alguma vez? *[Marque a quadrícula correspondente à resposta do entrevistado]*	☐ SIM ☐ NÃO ☐ Não sabe
Você fez o tratamento médico para leishmaniose? *[Marque a quadrícula correspondente à resposta do entrevistado]*	☐ SIM ☐ NÃO ☐ Não sabe ☐ Não se aplica
Como era o medicamento usado? *[Marque a quadrícula correspondente à resposta do entrevistado]*	☐ Comprimidos ☐ Injeções ☐ Pomada ☐ Não sabe ☐ Não se aplica