

**WANESSA CHRISTINA DE SOUZA-NEIRAS**

**ASPECTOS FILOGENÉTICOS E SOROLÓGICOS  
ENVOLVIDOS NA CARACTERIZAÇÃO DAS  
VARIANTES DA PROTEÍNA  
CIRCUNSPOROZOÍTICA DE *PLASMODIUM VIVAX***

Tese apresentada para obtenção  
do Título de Doutor em Genética

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## **WANESSA CHRISTINA DE SOUZA NEIRAS**

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Tese apresentada para obtenção do título de Doutor em Genética, junto ao Programa de Pós-Graduação em Genética do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de São José do Rio Preto.

### **BANCA EXAMINADORA**

Prof. Dr. Ricardo Luiz Dantas Machado  
Professor Assistente Doutor  
FAMERP - Faculdade de Medicina de São José do Rio Preto – São José do Rio Preto  
Orientador

Prof. Dr. Carlos Eugênio Cavasini  
Professor Assistente Doutor  
FAMERP - Faculdade de Medicina de São José do Rio Preto – São José do Rio Preto

Prof<sup>a</sup>. Dr<sup>a</sup>. Érika Cristina Pavarino Bertelli  
Professor Assistente Doutor  
FAMERP - Faculdade de Medicina de São José do Rio Preto – São José do Rio Preto

Prof<sup>a</sup>. Dr<sup>a</sup>. Dorotéia Rossi Silva Souza  
Professor Assistente Doutor  
FAMERP - Faculdade de Medicina de São José do Rio Preto – São José do Rio Preto

Prof<sup>a</sup>. Dr<sup>a</sup>. Maria Terecília V. de Azeredo Oliveira  
Professor Assistente Doutor  
UNESP – São José do Rio Preto

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Cada um que passa em nossa vida passa sozinho...  
Porque cada pessoa é única para nós,  
e nenhuma substitui a outra.  
Cada um que passa em nossa vida passa sozinho,  
mas não vai só...  
Levam um pouco de nós mesmos  
e nos deixam um pouco de si mesmos.

Há os que levam muito,  
mas não há os que não levam nada.  
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mas não há os que não deixam nada.

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Antoine de Saint-Exupéry

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## LISTA DE ABREVIATURAS E SIGLAS

AA	Refere-se a aminoácido
<i>18 SSU RNAr</i>	Gene que codifica a subunidade menor do ribossomo
<i>Alu I</i>	Enzima isolada do <i>Arthrobacter luteus</i>
AMA-1	Antígeno 1 de Membrana Apical
CEP	Comitê de Ética em Pesquisa
CONEP	Comissão Nacional de Ética em Pesquisa
CI	do Inglês <i>Confidence Intervals</i> – Intervalos de Confiança
CIM	Centro de Investigação de Microrganismos
<i>Cox 1</i>	Gene que codifica a proteína Cito-oxidase 1
<i>Cox 3</i>	Gene que codifica a proteína Cito-oxidase 3
CSP	Proteína Circunsporozoítica
<i>Cyt B</i>	Gene que codifica a proteína Citocromo B
DARC	Receptor de Antígeno Duffy para Quimiocinas
DBP	do Inglês <i>Duffy Bindin Protein</i> – Proteína de Ligação ao Antígeno Duffy
DNA	Ácido Desoxirribonucleico
<i>DPn I</i>	Enzima isolada do <i>Diplococcus pneumoniae</i>
EST	do Inglês <i>Expressed sequence tags</i> – Intervalos de Sequências Expressas
ELISA	do Inglês <i>Enzyme-Linked Immunosorbent Assay</i> – Ensaio Imunoenzimático
FAMERP	Faculdade de Medicina de São José do Rio Preto
<i>Fy [a-b-]</i>	Genótipo Duffy negativo
HLA II	Do Inglês <i>Human leukocyte antigen</i> – Antígenos Leucocitários Humanos de classe II
IBILCE	Instituto de Biociências, Letras e Ciências Exatas
IR	do Inglês <i>Index of Reactivity</i> – Índice de Reatividade, refere-se ao título de anticorpos
kDa	Kilo-Daltons

MSP	Proteína de Superfície do Merozoíto
NESTED	Refere-se à PCR semi-aninhada
PB	Pares de Bases
PCR	Reação em Cadeia da Polimerase
<i>PvCG10</i>	Gene relacionado à resistência da Cloroquina
<i>PvDHFR</i>	Gene que codifica a proteína Di-Hidrofolato Redutase
<i>PvMDR1</i>	Gene que codifica as proteínas Multi-Droga-Resistentes
QSP	Quantidade Suficiente Para
RFPL	do Inglês <i>Restriction Fragment Length Polymorfism</i> - Polimorfismo no Tamanho do Fragmento de Restrição
SIVEP	Secretaria de Vigilância em Saúde
SNP	do Inglês <i>Single nucleotide polymorphisms</i> – Polimorfismos com Troca de Nucleotídeos Únicos
SUS	Sistema Único de Saúde
<i>Taq</i>	Enzima produzida pelo <i>Thermus aquaticus</i> (DNA Polimerase)
TRSTMH	do Inglês <i>Transactions of the Royal Society of Tropical Medicine and Hygiene</i> (refere-se a um periódico científico)
UNESP	Universidade Estadual Paulista “Júlio de Mesquita Filho”
<i>vir</i>	Genes Variantes de <i>P. vivax</i>

## RESUMO

As variantes da proteína circunsporozoítica (CSP) de *Plasmodium vivax* têm sido identificadas em várias regiões do mundo. Neste trabalho foram apresentados padrões que caracterizam a diversidade genética de *Plasmodium vivax*, além da reavaliação da frequência dessas variantes e sua associação com os genótipos do grupo sanguíneo Duffy. Diferenças na frequência dos genótipos variantes da CS foram observadas entre as áreas endêmicas estudadas e, os genótipos VK247 e *P. vivax-like* foram identificados em infecções simples, pela primeira vez no Brasil. Para elucidar a implicação que as variações na região central repetida da CSP possam ter no genoma de *P. vivax*, aspectos filogenéticos e sorológicos foram avaliados. As análises filogenéticas foram realizadas a partir da amplificação e sequenciamento de domínios conservados dos marcadores *18 SSU rNA* e *Cyt B*. A resposta de anticorpos contra peptídeos do esporozíto, CSP, e do merozoíto, MSP-1 (*Pv200L*), AMA-1 e DBP ( $\alpha$ II) foi avaliada por ELISA em amostras de plasma de pacientes infectados com genótipos da CS de *P. vivax*. As análises dos dois marcadores mostraram alto grau de similaridade entre VK210 e *P. vivax-like*, evidenciando que estes genótipos são pertencentes a um mesmo clado. O perfil de resposta sorológica contra os diferentes peptídeos do parasito corrobora a idéia de que esta variação esteja restrita sua porção central, uma vez que não foram observadas associações significativas entre a presença de determinado genótipo e a frequência da resposta de anticorpos contra os três peptídeos do merozoíto analisados, e bem como, contra as frações conservadas da CSP no esporozíto, N-terminal [N] e C-terminal [C]. Apesar do genótipo VK247 não ter sido incluído nestas análises, estes resultados sugerem que a variação na porção central repetida da CSP representa apenas um marcador de diversidade intra-específica, caracterizando diferentes cepas circulantes de *P. vivax* na Amazônia brasileira.

**Palavras-chave:** Malária, *Plasmodium vivax*, Genótipos Variantes da Proteína Circunsporozoítica, Diversidade Genética, Filogenia, Resposta Imune Humoral.

## ABSTRACT

The protein circumsporozoite variants (CSP) of *Plasmodium vivax* they have been identified in several areas of the world. In this work were presented patterns that characterize the genetic diversity of *Plasmodium vivax*, besides the reevaluation of the frequency of those variants and your association with the Duffy blood group genotypes. Differences in the frequency of the CS genotypes variant were observed between the studied endemic areas and, VK247 and *P. vivax*-like genotypes were identified in single infections, for the first time in Brazil. To elucidate the implication that the variations in the CSP repeated central area can have in the genome of *P. vivax*, phylogenetics and serological aspects was appraised. The phylogenetics analyses were accomplished starting from the amplification and sequencing of conserved domains of *18 SSU RNAr* and *Cyt B*. The antibodies responses against the esporozoite, CSP, and merozoite peptides, MSP-1 (*Pv200L*), AMA-1 and DBP (rII) were detected by ELISA, in plasma samples in infected individuals with *P. vivax* CSP genotypes. The analyses of the two markers show high similarity among the *P. vivax* CS genotypes, evidencing that the genotypes, VK210 and *P. vivax*-like are members of the same clade. The evaluation of the serological response profile against the different parasite peptides corroborates the idea that this variation is restricted to central region, once significant associations were not observed between the presence certain genotype and frequency of the antibodies responses against the three analyzed merozoite peptides and against the CSP conserved fractions in the esporozoite, N-terminal and C-terminal. Although the analysis with VK247 genetics sequences has not been included, these results suggested that those variations just represent a maker of intra-specific diversity, characterizing different circulating stumps of the *P. vivax* in the Amazonian Brazilian.

**Key-words:** Malaria, *Plasmodium vivax*, Circumsporozoite Protein Genotypes Variants, Genetic Diversity, Phylogeny, Humoral Immune Response.

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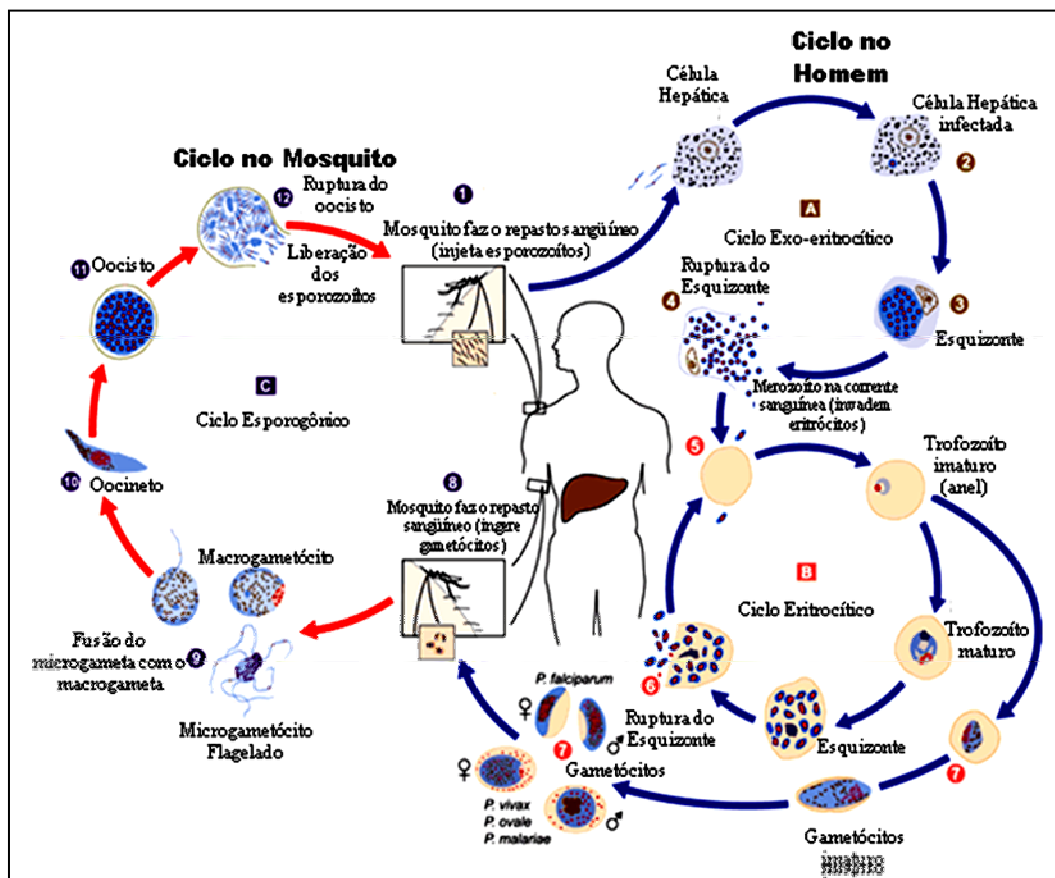
## **1 INTRODUÇÃO GERAL**

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## 1 INTRODUÇÃO GERAL

A malária é causada por protozoários pertencentes ao filo *Apicomplexa*, à família *Plasmodiidae* e ao gênero *Plasmodium*. Cinco espécies de *Plasmodium* são reconhecidas como parasitos naturais do homem: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* e *Plasmodium knowlesi*, porém somente as três primeiras ocorrem no Brasil (COX-SINGH, et al., 2008; WHITE, 2008). A infecção por essas espécies de *Plasmodium* tem suas características próprias, bem como diferenças nas suas áreas de distribuição, mas todas as cinco espécies ocasionam quadros clínicos similares, o que torna a sua diferenciação durante o início da infecção muito difícil e provavelmente impossível (BLEDSOE, 2005). Dentre essas espécies, *P. falciparum* e *P. vivax* são as mais prevalentes no mundo, sendo o *P. falciparum* considerado a espécie de maior impacto devido aos altos níveis de mortalidade, resistência a fármacos antimaláricos e dominância no continente africano. Entretanto, a infecção debilitante por *P. vivax* tem causado um profundo impacto na saúde pública, na longevidade e na prosperidade de grande parte da população humana (MENDIS et al., 2001).

O ciclo de biológico do *Plasmodium* spp. é bastante complexo e após a infecção, em poucos dias, é instalada uma parasitemia no hospedeiro (Figura 1). Os plasmódios são transmitidos por mosquitos fêmeas do gênero *Anopheles* spp. e os esporozoítos infectantes são injetados no hospedeiro vertebrado durante repasto sanguíneo. Estas formas extracelulares rapidamente migram para o fígado via sanguínea e invadem os hepatócitos. Cada esporozoíto invasor se diferencia e se divide por mitose em milhares de merozoítos, que são liberados das células do fígado e rapidamente invadem os eritrócitos, quando se inicia então, a fase assexuada do ciclo de vida do parasito. Os merozoítos nos eritrócitos diferenciam-se nos estágios de trofozoíto e esquizonte, sendo que este último se divide para liberar merozoítos filhos na corrente sanguínea e dar continuidade ao ciclo assexuado. É nesta fase que surgem os sintomas clínicos da doença (COWMAN e CRABB, 2006).



**Figura 1.** Ciclo biológico do *Plasmodium* spp. Adaptado de <http://www.med.sc.edu:85/parasitology/blood-proto.htm>

A malária é a doença parasitária mais comum em países essencialmente tropicais, e subtropicais e continua sendo o maior desafio em saúde pública em todo o mundo (DOOLAN et al., 2003; WHO, 2004; GOOD et al., 2005). Embora tenha sido descrita há mais de cento e vinte anos, o parasito da malária tem resistido a todas as formas de controle empregadas até hoje (WHO, 2004; GOOD et al., 2005).

A cada ano, pressupõe-se que trinta milhões de pessoas fiquem doentes e, que mais de um milhão dessas morram devido à infecção malárica, sendo a maioria crianças. Atualmente, também é estimado que 40% da população do mundo permaneçam sob o risco de infecção por malária (GOOD et al., 2005;

CDC, 2009). Além disso, estudos recentes sugerem que o impacto clínico causado por essa infecção tem sido subestimado e, que seu impacto econômico nunca foi adequadamente considerado (DOOLAN et al., 2003). Embora a grande maioria das mortes causadas por malária ocorra na África, a doença encontra-se amplamente distribuída na América Latina, Sudeste Asiático e Oceania.

A transmissão da malária em áreas brasileiras caracteriza-se como hipo a mesoendêmica, de transmissão instável com flutuações sazonais anuais (CAMARGO et al., 1996; COURA et al., 2006). Neste cenário, o Brasil responde por 36% do número total de casos registrados no mundo (COURA et al., 2006). Mais de 99% desses casos ocorrem na região da Amazônia brasileira, sendo que a maioria é registrada na região Norte e parte da região Centro-Oeste, nos Estados do Pará, Amazonas, Rondônia, Acre, Amapá, Maranhão, Tocantins e Mato Grosso, totalizando 807 municípios endêmicos (MINISTÉRIO DA SAÚDE, 2009). Em 2005, foram registrados cerca de 600 mil casos; entretanto, em 2008, essa casuística foi reduzida à metade (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2009).

A malária não se apresenta distribuída uniformemente em toda região amazônica, uma vez que se observam áreas com diferentes níveis de transmissão da doença. As áreas de maior transmissão são freqüentemente áreas de colonização recente, principalmente garimpos onde as condições precárias de moradia associadas aos hábitos da população proporcionam condições favoráveis para transmissão (SAWYER, 1993).

### **1.1 A importância da malária *vivax***

As infecções causadas por *P. vivax* são responsáveis por cerca de 80 milhões de casos anuais de malária, especialmente na América Latina, Ásia e Oceania (WHO, 2008). Embora a distribuição dos parasitos da malária seja ampla, observou-se que durante os últimos sete anos, as infecções causadas por *P. vivax* têm alcançado mais de 80% dos casos de malária na região amazônica brasileira

(SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2009). A habilidade deste parasito para completar seu ciclo esporogônico a uma temperatura abaixo de 16°C, quando comparada com 21°C do *P. falciparum*, contribuiu substancialmente para seu sucesso no estabelecimento de focos estáveis de transmissão em zonas temperadas (CUI et al., 2003).

Nos últimos trinta anos, a resistência dos parasitos da malária a cloroquina tem sido restrita ao *P. falciparum*, sendo que este fármaco ainda se apresenta como a melhor escolha para profilaxia e tratamento de infecções causadas por *P. vivax* (BALDASSARE et al., 1991). A primeira evidência de que o *P. vivax* estava desenvolvendo resistência a cloroquina foi reportada em Papúa Nova Guiné por Rieckmann et al. (1989). A partir daí, a redução na suscetibilidade a cloroquina tem sido observada em amostras das Ilhas Salomão (WHITBY et al., 1989), Papúa Nova Guiné (SCHURRKAMP et al., 1992; MURPHY et al., 1993), Índia (GARG et al., 1995), Ásia (PUKRITTAYAKAMEE et al., 2000) e, no Brasil (ALECRIM et al., 1999).

Diferentemente do *P. falciparum*, a infecção por *P. vivax* raramente leva a quadros letais. Entretanto, a prostração constante causada pela sua infecção não pode ser considerada benigna, sendo causa importante de morbidade e perdas sócio-econômicas (COURA et al., 2006; CUI et al., 2003). Além disso, ao contrário do *P. falciparum*, que pode ser facilmente cultivado *in vitro*, o cultivo de *P. vivax* ainda é restrito a poucos laboratórios, o que dificulta os estudos sobre os mecanismos imunológicos que operam durante este tipo de infecção (GOLENDIA et al., 1997).

Os plasmódios são geneticamente diversos e dotados de genoma nuclear, constituído por cerca de 2 a 2.5 X 10<sup>7</sup> pares de base, cuja expressão favorece a formação de uma resposta imune complexa e espécie/estágio-específica. Análises parciais do genoma do *P. vivax* revelam a presença de 14 cromossomos haplóides na maior parte do ciclo, com um conteúdo de G/C consideravelmente maior que o *P. falciparum* (DEL PORTILLO et al., 1996). O genoma mitocondrial do *Plasmodium* spp. apresenta 6 kb de comprimento e inclui apenas a presença de três genes: Cito-oxidase 1 (*Cox 1*), Cito-oxidase 3 (*Cox 3*) e

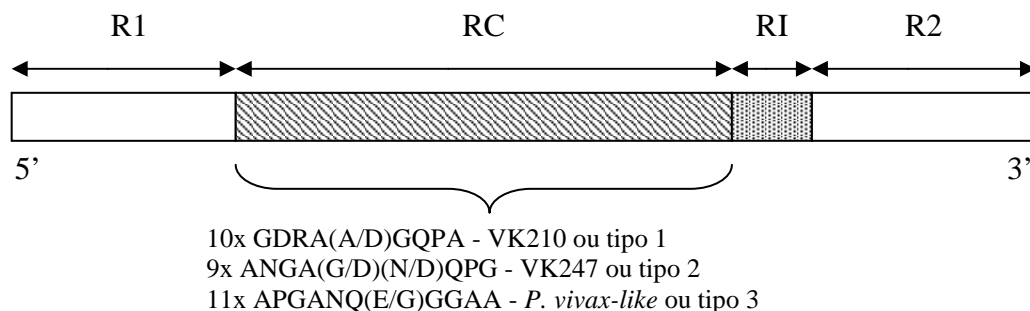
Citocromo B (*Cyt B*) (WILSON e WILLIAMSON, 1997). Estudos recentes mostram um baixo nível de polimorfismos tanto no genoma mitocondrial de *P. falciparum* (CONWAY et al., 2000) quanto de *P. vivax* (JONGWUTIWES et al., 2005). Apesar do sequenciamento completo do genoma do *P. falciparum* ter sido finalizado em 2002, o genoma do *P. vivax* ainda não foi completamente elucidado. Existem disponíveis em bancos de dados algumas seqüências originadas do sequenciamento de cromossomos artificiais de leveduras (YAC).

### **1.2 Genótipos da proteína circunsporozoítica de *P. vivax***

O principal constituinte antigênico do esporozoíto maduro, um estágio infeccioso do *Plasmodium* no hospedeiro vertebrado, é a proteína circunsporozoítica (CSP), que recobre sua superfície e, possui estrutura semelhante em todas as espécies de plasmódios conhecidas (KEMP et al., 1990).

O gene da CS (Figura 2) consiste de um terço central com blocos de nucleotídeos repetidos de maneira seqüencial, o qual constitui o epítipo imunodominante desta proteína, reconhecido primordialmente por linfócitos B (TOSTA, 1992). O papel desta região não está totalmente elucidado, porém há fortes indícios de que ela esteja relacionada a mecanismos de evasão, do sistema imune do hospedeiro (SCHOFIELD, 1991).

Nas extremidades do domínio hiper-variável da CSP existem dois segmentos que se mantêm altamente conservados, em diferentes espécies de plasmódio; região 1 (R1) que segue na direção amino-terminal e, a região 2 (R2), localizada a 54 aminoácidos da direção hidrofóbica carboxi-terminal da proteína (DE LA CRUZ et al., 1988).



**Figura 2.** Estrutura do gene da *CS* de *Plasmodium vivax*, com duas regiões terminais não repetidas altamente conservadas (R1 e R2); um domínio central repetido (RC), com um número variável de repetições sequenciais e, uma região curta de inserção (RI). Na RC indicada acima são ilustradas as variações peptídicas da proteína. De acordo com Qari et al. (1993a).

No início da década de 90, a CSP foi estudada como o principal alvo para o desenvolvimento de uma vacina antimalárica. Entretanto, a descoberta de variações na sequência repetida de sua porção central inviabilizou esses estudos. O primeiro genótipo variante identificado apresenta um nonapeptídeo repetido em sequência, GDRA(A/D)GQPA, no domínio central da proteína e, foi denominada VK210, sendo considerada a forma clássica de *P. vivax* (ARNOT, et al., 1985). Em cepas da Tailândia, Rosemberg et al. (1989) descreveram variações de dois nonapeptídeos da região repetida, ANGA(G/D)(N/D)QPG), onde essa variante ficou conhecida como VK247.

Posteriormente, em Papua Nova Guiné, Qari et al. (1993a) relataram a existência de uma nova variante denominada *P. vivax-like*. O gene do *P. vivax-like* apresenta 1182 pares de base com 16 cópias da sequência repetida de 11-mer, codificadora para APGANQ(E/G)GGAA (QARI et al., 1993b) e é diferente dos genes das *CS* conhecidas de outros parasitos de malária humana. Interessantemente, a sequência do gene desta proteína é praticamente idêntica a *CS* do *P. simiovale* que, por sua vez, é semelhante morfológicamente a outro

parasito de malária humana, o *P. ovale* (QARI et al., 1993a; ESCALANTE et al., 1995). Alguns autores acreditam que esta última variante provavelmente seja uma quinta espécie, pelo fato dela apresentar características morfológicas semelhantes à VK210 e, possuir uma porção repetida da região central da CSP diferente dos dois tipos descritos anteriormente (QARI et al., 1993b).

Diversos estudos têm sido realizados para avaliar a distribuição global das variantes de *P. vivax*. A presença da VK247 foi confirmada em áreas endêmicas da Tailândia (WIRTZ et al., 1990; KAIN et al., 1992, 1993a, 1993b), América do Sul, África (KAIN et al., 1991, GONZALEZ et al., 2001), México e Afeganistão (RODRIGUEZ et al., 2000). Amostras de sangue de outras regiões de Papua Nova Guiné, da Indonésia e Madagascar identificaram a variante *P. vivax-like* (QARI et al., 1993b).

No Brasil, testes sorológicos detectaram as três variantes em amostras do Estado de São Paulo (CURADO et al., 1995), em área endêmicas (OLIVEIRA-FERREIRA et al., 2004; ARRUDA et al., 2007) e em comunidades indígenas da região Amazônica (ARRUDA et al., 1996; 1998). Machado e Póvoa (2000) confirmaram, por diagnóstico molecular, a presença desses tipos variantes nos Estados de Rondônia, Amapá e Pará, descrevendo a ocorrência da VK210 em infecções puras, enquanto que as variantes VK247 e *P. vivax-like* foram evidenciadas apenas em infecções mistas.

Diante do aumento da resistência a cloroquina, Kain et al. (1993a) observaram variações na resposta a esse fármaco dependendo do genótipo de *P. vivax*, mostrando que infecções com o genótipo VK210 e infecções mistas com VK247 levam mais tempo para negativar a parasitemia do que as amostras de infecção pura. Por outro lado, Machado et al. (2003) verificaram que infecções mistas com a VK247 ou com a *P. vivax-like* levam um tempo menor para a negativação parasitária do que infecções que contém o tipo variante VK210, em amostras provenientes de Belém, no Estado do Pará. A região repetida da CSP tem sido utilizada também para identificar espécies de plasmódios em anofelinos em áreas remanescentes de Mata Atlântica do Estado de São Paulo (BRANQUINHO et al., 1997) e, no Estado do Acre (MARRELLI et al., 1998).

Em áreas endêmicas do México, Gonzales-Ceron et al. (1999) observaram a suscetibilidade do *Anopheles pseudopunctipennis* e *Anopheles albimanus* a infecções determinadas pelos genótipos VK210 e VK247, respectivamente. Interessantemente, no Pará, estudos reportaram diferenças na infectividade dos anofelinos às variantes, indicando que o *Anopheles darlingi* é mais suscetível à infecção por VK210 (DA SILVA et al., 2006). Estes achados podem ser consequência da emergência deste genótipo em regiões geográficas específicas ou podem sugerir que a variante VK210 possa estar mais adaptada no Brasil (MACHADO e PÓVOA, 2000).

### 1.3 Antígenos candidatos à vacina

A primeira habilidade do parasito da malária reconhecer e, invadir os eritrócitos, é fundamental para o processo da doença. Antígenos localizados na superfície do parasito ou especificamente em organelas apicais do merozoíto têm sido caracterizados como importantes alvos para proteção ou como possíveis candidatos a uma vacina (MILLER et al., 2002).

O sucesso de uma vacina contra a malária deve estar relacionado à intervenção contra o desenvolvimento do parasito no hospedeiro humano ou no mosquito vetor. Com o objetivo de garantir saúde e qualidade de vida a mais de 1 bilhão de pessoas em todo o mundo, vários esforços têm sido direcionados para a identificação e caracterização antigênica de diferentes antígenos de *P. vivax*, entre eles antígenos pré-eritrocíticos como a CSP (VILAI et al., 2004), proteínas de estágio sanguíneo como proteína de superfície do merozoíto 1 (MSP-1) (BARBEDO et al., 2007; ZEYREK et al., 2008), antígeno de membrana apical 1 (BARBEDO et al., 2007; LALITHA et al., 2008) e proteína de ligação ao antígeno Duffy (DBP), presente na superfície da célula hospedeira (BARBEDO et al., 2007; ARÉVALO-HERRERA et al., 2005). Atualmente, vários autores têm considerado a CSP de *P. vivax* como o maior alvo para o desenvolvimento de vacinas recombinantes, uma vez que peptídeos sintéticos construídos a partir desta

proteína induzem altos níveis de resposta imune humoral e produzem anticorpos que exibem a mesma especificidade gerada em infecções naturais (ARÉVALO-HERRERA e HERRERA, 2001; HERRERA et al., 2005; RODRIGUES et al., 2005; HERRERA et al., 2007; BEESON et al., 2007; PENNY et al., 2008; KING et al., 2008).

#### 1.4 Filogenia do *P. vivax*

Em 1949, Haldane sugeriu que a malária poderia ter agido como uma força seletiva para as populações humanas. Desde então, vários estudos têm sido propostos a fim de testar essa hipótese, a partir de tentativas de reconstrução da origem filogenética dos parasitos da malária (ESCALANTE et al., 1995).

Os primeiros relatos destas investigações estiveram focados no *P. falciparum*, causador da forma mais grave da doença, sendo que sua virulência foi atribuída ao fato deste ter se tornado um parasito humano, recentemente, por meio de uma mudança de hospedeiro (provavelmente, pássaros), há aproximadamente 5.000 a 10.000 anos atrás (BOYD, 1949; LIVINGSTONE, 1958; HOEPRICH, 1989; SNEWIN et al., 1991). Por outro lado, análises moleculares da subunidade ribossomal do gene *SSU RNAr* corroboram esses dados e revelam que o *P. reichenowi*, um parasito de chimpanzé, compartilha um ancestral comum com *P. falciparum* e, que o clado formado por esses dois parasitos está relacionado a outras espécies de *Plasmodium*, incluindo as infectantes de humanos, *P. vivax* e *P. malariae* e de pássaros (ESCALANTE e AYALA, 1995).

Estudos com microssatélites também indicam que o *P. vivax* passou a infectar humanos recentemente, cerca de 10.000 anos atrás (LECLERC et al., 2004). As análises do polimorfismo de dois genes nucleares e um gene mitocondrial colocam a origem deste parasito entre 45.000 e 81.000 anos atrás (ESCALANTE et al., 2005). A partir destas preliminares, duas principais conclusões puderam ser elaboradas: (a) os parasitos da malária tornaram-se patógenos humanos de forma independente e, (b) o *P. reichenowi*, a parasito de

chimpanzé, é a espécie que compartilha o ancestral mais comum com o *P. falciparum* (ESCALANTE et al., 1995).

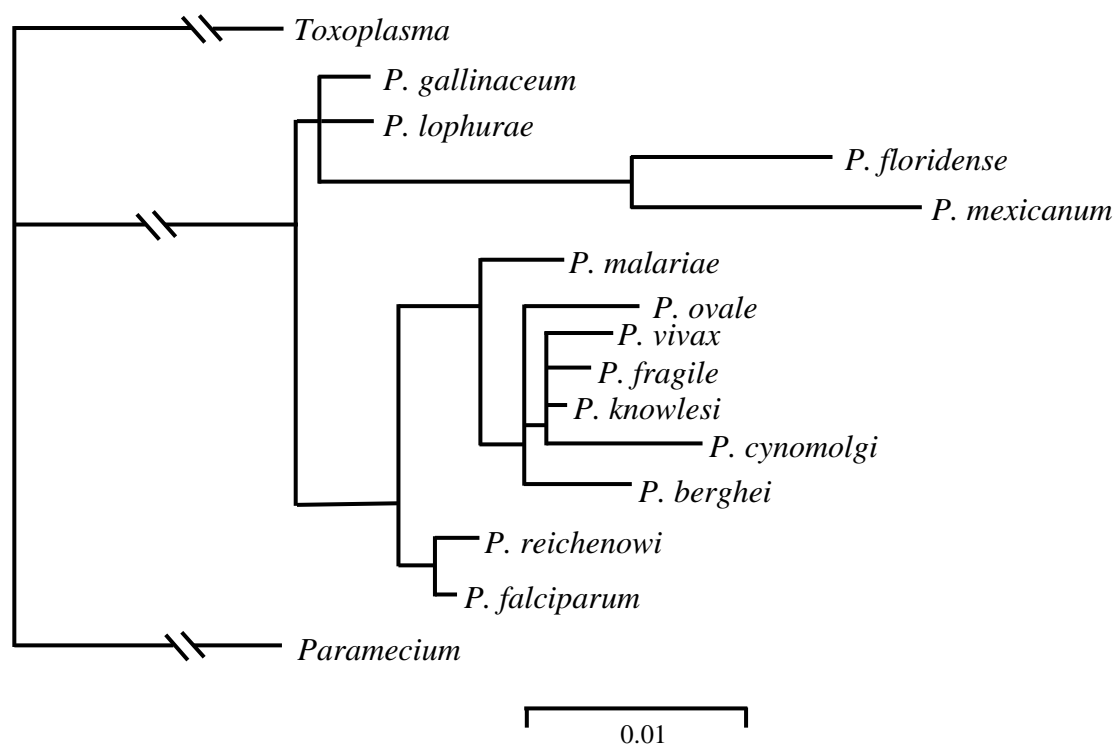
A primeira evidência da origem do *P. vivax* baseada em dados moleculares foi conduzida a partir de análises realizadas com o gene citocromo b (*Cyt B*). Os resultados mostraram fortes similaridades do *P. vivax* com plasmódios infectantes de símios oriundos da Ásia. Este estudo filogenético também sugere que o *P. vivax* se originou de um parasito de primatas não-humanos, como resultado de uma mudança de hospedeiro, provavelmente de um macaco (ESCALANTE et al., 2005; 2006). Este fato recebeu suporte adicional quando foi combinado aos resultados de análise completa do genoma mitocondrial destes parasitos (MU et al., 2005; JONGWUTIWES et al., 2005). Com isso, foi possível explicar porque o *P. vivax* é geneticamente muito semelhante ao *P. simium*, bem como, a predição de que estas espécies da América do Sul se originaram de uma mudança de hospedeiros, de macacos para humanos (ESCALANTE et al., 1998; 2005; MU et al., 2005).

Análises da porção não repetida da CSP de *P. vivax* (Figura 3) mostraram homologia com os genes das CSPs de dois plasmódios infectantes de símios, *P. cynomolgi* e *P. knowlesi* (ARNOT et al., 1985). Posteriormente, outros estudos evidenciaram que VK210 e *P. vivax-like* pertencem a um mesmo clado que inclui vários tipos de plasmódios de primatas (ESCALANTE et al., 1995; QARI et al., 1996). O gene da CS do *P. vivax-like* é diferente dos outros plasmódios que parasitam humanos, porém é praticamente idêntico ao *P. simiovale*. Interessantemente, este parasito símio é semelhante morfologicamente a outro parasito de malária humana, o *P. ovale* (QARI et al., 1993a; ESCALANTE et al., 1995).

A similaridade entre o gene da CS de *P. simiovale* e *P. vivax-like* tem provocado intensas discussões a respeito da origem evolutiva desta variante, bem como ao que se refere à sua epidemiologia clínica e aos os vetores envolvidos na transmissão (QARI et al., 1994). Escalante et al. (1995) mostraram que as semelhanças entre esses dois parasitos determinam conseqüências epidemiológicas importantes, uma vez que hospedeiros primatas podem servir de

reservatório para parasitos de malária humana. Entretanto, a utilização do gene da CS, como único marcador molecular, não é suficiente para esclarecer a possível relação evolutiva existente entre os três tipos variantes, bem como a distância genética destes com parasitos de outros primatas (ESCALANTE et al., 1995).

A caracterização filogenética e a avaliação da resposta imune humoral dos genótipos da CSP tornam-se importantes para um melhor entendimento dos aspectos evolutivos e imunológicos relacionados ao *Plasmodium vivax*, contribuindo para elaboração de novas estratégias de controle e tratamento das infecções causadas por esse parasito.



**Figura 3.** Árvore filogenética gerada a partir da porção não repetida da CS originada pelo método de máxima verossimilhança. A escala indica uma distância de 0,01 substituições nucleotídicas na seqüência. De acordo com Qari et al. (1996).

## **2 OBJETIVOS**

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## 2 OBJETIVOS

### 2.1 Objetivo geral

- Avaliar as implicações das variantes da CSP (VK210, VK247 e *P. vivax-like*), no genoma de *P. vivax* a partir de ferramentas filogenéticas e sorológicas.

### 2.2 Objetivos específicos

- Identificar a frequência dos genótipos variantes da CS de *P. vivax* em amostras de sangue de pacientes maláricos, provenientes de diferentes regiões da Amazônia brasileira;

- Inferir o perfil filogenético dos genótipos variantes da CS de *P. vivax*, a partir da análise dos marcadores moleculares: *18 SSU RNAr* e *Citocromo B*;

- Avaliar a resposta imunológica de anticorpos contra os peptídeos da CSP, MSP, AMA1 e DBP, relacionada a infecções causadas pelos genótipos da CS de *P. vivax*.

### **3 RESULTADOS**

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### 3 RESULTADOS

Os resultados obtidos encontram-se descritos, em artigos publicados ou submetidos à publicação em revistas indexadas e, organizados em forma de capítulos.

Capítulo I. The genetic diversity of *Plasmodium vivax* - A Review. Artigo de revisão publicado na revista **Memórias do Instituto Oswaldo Cruz**, v. 102, p. 245-254, 2007.

Capítulo II. *Plasmodium vivax* circumsporozoite variants and Duffy blood group genotypes in the Brazilian Amazon region. Artigo original publicado na revista **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 103, p. 672-678, 2009.

Capítulo III. *Plasmodium vivax* circumsporozoite genotypes: a limited variation or new subspecies with major biological consequences? Artigo original a ser submetido para publicação na revista **Malaria Journal**, 2009.

## **4 CAPÍTULO I**

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## 4 CAPÍTULO I

### 4.1. The genetic diversity of *Plasmodium vivax* - A Review.

Autores: Wanessa Christina de Souza-Neiras, Luciane Moreno Storti de Melo e Ricardo Luiz Dantas Machado. Artigo de revisão publicado na revista **Memórias do Instituto Oswaldo Cruz**, v. 102, p. 245-254, 2007.

### RESUMO

A diversidade genética do *Plasmodium vivax* tem sido investigada em várias áreas endêmicas, incluindo a Região Amazônica Brasileira, onde esta é atualmente a espécie causadora da malária mais prevalente em humanos. Esta revisão sumariza relatos atuais do uso de marcadores moleculares para analisar populações de *P. vivax*, destacando estudos realizados por pesquisadores brasileiros. Nós enfatizamos a importância dos estudos filogenéticos com este parasito e discutimos as perspectivas criadas para um melhor entendimento da diversidade genética e da estrutura da população deste parasito bem como a elaboração de novas estratégias de controle, incluindo vacinas e fármacos mais efetivos para o tratamento da malária *vivax*.

## The genetic diversity of *Plasmodium vivax* - A Review

Wanessa Christina de Souza-Neiras<sup>\*/+,\*</sup>, Luciane Moreno Storti de Melo<sup>†,\*</sup>,  
Ricardo Luiz Dantas Machado<sup>\*</sup>

Departamento de Biologia, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista, São José do Rio Preto, SP, Brasil \*Centro de Investigação de Microrganismos, Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto, Av. Brigadeiro Faria Lima 5416, Bloco VI, 15090-000 São José do Rio Preto, SP, Brasil

*The genetic diversity of Plasmodium vivax has been investigated in several malaria-endemic areas, including the Brazilian Amazon region, where this is currently the most prevalent species causing malaria in humans. This review summarizes current views on the use of molecular markers to examine P. vivax populations, with a focus on studies performed in Brazilian research laboratories. We emphasize the importance of phylogenetic studies on this parasite and discuss the perspectives created by our increasing understanding of genetic diversity and population structure of this parasite for the development of new control strategies, including vaccines, and more effective drugs for the treatment of P. vivax malaria.*

Key words: malaria - *Plasmodium vivax* - genetic diversity - molecular markers - phylogeny - evolution

### The importance of *vivax* Malaria

Currently, *Plasmodium vivax* is the most widely distributed human malaria species in the world causing an estimated 80-90 million cases each year. In the Americas and Asia, *P. vivax* is the most prevalent malaria species; in Brazil it represents more than 80% of clinical cases reported annually from the Amazon region (Brazilian Health Ministry 2002). The ability of this parasite to complete its sporogonic cycle at a temperature as low as 16°C, compared with 21°C for *P. falciparum*, has substantially contributed to its success in establishing stable foci of transmission in temperate zones. *Vivax* malaria is usually a non-lethal disease but its prolonged and recurrent infection can have major deleterious effects on personal well-being, growth and on the economic performance at individual, family, community, and national levels (Mendis et al. 2001). Alarming, an increasing number of clinical studies have shown the failure of treatment of the first-line *P. vivax* antimalarial agents, such as chloroquine (Baird 2004).

In response to the great importance of this type of infection, several studies have been proposed to investigate genetic diversity of *P. vivax*. The malaria parasite population structure has a significant influence on the gene flow and thus the rate at which new mutations leading to drug resistance or escape from vaccine-induced responses spread. Moreover, this information might give clues on the evolution and selection of pathogens (Rich et al. 1998). Thus, studies on malaria parasite population diversity are not only of academic interest to biolo-

gists and geneticists, but are of practical importance in the development and deployment of control strategies (Cui et al. 2003).

### Polymorphic molecular markers and genetic diversity

The majority of studies on the genetic diversity of *Plasmodium* spp. have been based on *P. falciparum*, responsible for the most severe disease form, and on genes coding for antigenic determinants such as circumsporozoite surface protein (CSP) and merozoite surface protein (MSP). These antigenic genes are non-synonymous nucleotide polymorphisms and the multiple allelic forms differ in their ability to abrogate recognition by the host's immune response (Rich & Ayala 2000). Similar approaches have been adopted to investigate *P. vivax* but this species has been less well studied at the molecular level when compared to *P. falciparum* (Cui et al. 2003).

Studies on antigenic diversity will help to predict and monitor the effectiveness of intervention strategies, such as the success of therapeutic regimens, the spread of drug resistance and the emergence of multidrug-resistant parasites. The diversity of *P. vivax* has been reported in terms of relapse patterns, morphology, and biochemistry (Figtree et al. 2000). Ortholog genes of *P. falciparum* associated to drug resistance, such as *PvDHFR* related to pyrimethamine resistance (de Pecoulas et al. 1998), *PvCG10* to chloroquine resistance (Nomura et al. 2001), and *PvMDR1* to multiple drug resistance (Sá et al. 2005) have been characterized for *P. vivax*. However, analysis does not confirm if these polymorphic genes are really related to resistant phenotypes.

### CSP

The complete or partial nucleotide sequences of CSP, the most abundant polypeptide on the sporozoite surface, have been determined. It presents a central repeat domain flanked by non-repeated amino and carboxyl sequences containing highly conserved stretches, regions I and II (Fig. 1 illustrates the CSP gene). These flanking

Corresponding author: wanejan@yahoo.com.br

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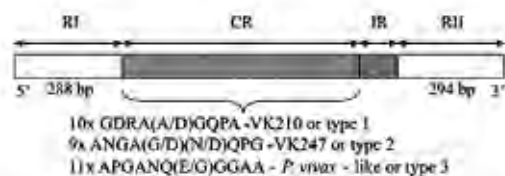


Fig. 1. Structure of the *Plasmodium vivax* CSP gene, with two highly-conserved terminal non-repeat regions (RI and RII), a central repetitive (CR) domain, with a variable number of tandem repeats, and a short IR (insertion region). In the CR are indicated below, the peptide variations from protein. According to Qari et al. (1993a).

regions also display some degree of polymorphism (Mann et al. 1994). The central repetitive domain varies in sequence and length among *Plasmodium* spp. Until some years ago, CSP was studied as the main target for antimalarial vaccine development, because it is present in the liver stages of the parasite and is the target of protective cellular and humoral immune responses. However, the existence of variations in the repetitive sequence of its central portion has made these studies impracticable.

Analysis of *P. vivax* CSP sequences revealed that parasites have repeats belonging to one of two types of nonapeptide repeat units, GDRA(A/D)GQPA or ANGA(G/D)(N/D)QPG, named VK210 or VK247 respectively (Arnot et al. 1985, Rosenberg et al. 1989). In 1993, a new human malaria parasite from a *P. vivax*-infected person was identified by Qari et al. who named it *P. vivax*-like. The CSP sequence of this parasite has an 11-mer repeat sequence, APGANQ(E/G)GGAA, and is different to the two previously described genotypes. This CSP gene sequence is similar to that of *P. simiovale*, a monkey malaria parasite originally found in *Macaca sinica* in Sri Lanka (Qari et al. 1993a). All CSP variant genotypes have a worldwide distribution (Kain et al. 1992, Gonz ales et al. 2001, Imwong et al. 2005, Zakeri et al. 2006).

In Brazil, Machado and P voa (2000) using molecular diagnosis confirmed the presence of the variant types in the states of Rond nia, Amap , and Par , describing the occurrence of VK210 in pure infections, whereas the VK247 and *P. vivax*-like variants were only evident in mixed infections. Seroreactivity tests have identified the presence of three variants in samples from the state of S o Paulo (Curado et al. 1995, 2006) and in indigenous communities of the Amazon region (Arruda et al. 1996, 1998). Oliveira-Ferreira et al. (2004a) confirmed the seroreactivity against synthetic peptides containing the CSP immunodominant epitope of *P. vivax* variants. The authors believe that antibody responses to the CSP repeats of these variants are modulated by HLA class II molecules in individuals naturally exposed to malaria.

Interestingly, studies have also reported differences in the infectivity of anophelines to the variants, indicating that *Anopheles darlingi* were more susceptible to infection by VK210 (da Silva et al. 2006). In malaria endemic areas in Mexico, Gonz ales-Ceron et al. (1999) observed the susceptibility of *An. pseudopunctipennis*

and *An. albimanus* mosquitoes to infections by the VK210 and VK247 genotypes, respectively. The VK247 genotype also was detected in anophelines originating from the states of S o Paulo (Branquinho et al. 1997) and Acre (Marrelli et al. 1998). These findings could be a consequence of differences in the emergence of this genotype in specific geographical regions or suggest that the VK210 genotype is the best adapted variant in the world (Machado & P voa 2000).

## MSP

Several *P. vivax* merozoite surface proteins (PvMSP) have been described, including PvMSP-1, the PvMSP-3abg family, PvMSP-4, PvMSP-5, and PvMSP-9 (del Portillo et al. 1991).

One of the most promising vaccine candidates against the erythrocytic forms of malaria is MSP-1. The *MSP-1* gene is very large and has 10 relatively conserved blocks alternating with regions of much higher diversity (Crewther et al. 1996). The primary structure of PvMSP-1, originally characterized from two monkey-adapted *P. vivax* strains (Bel m and Salvador-1), exhibits conserved, semi-conserved and polymorphic regions (del Portillo et al. 1991, Gibson et al. 1992).

The *MSP-1* gene, expressed as a protein of 200-190 kDa on the parasite surface (del Portillo 1988), has been cloned and sequenced (del Portillo et al. 1991). Studies performed mainly with *P. falciparum* indicate that MSP-1 is processed in two steps of proteolytic cleavage during merozoite maturation. First, it is cleaved into four major fragments of 83, 30, 38, and 42 kDa (MSP-1<sub>83</sub>, MSP-1<sub>30</sub>, MSP-1<sub>38</sub>, and MSP-1<sub>42</sub>) then, before erythrocyte invasion the MSP-1<sub>42</sub> fragment undergoes a second cleavage resulting in 33 and 19 kDa (MSP-1<sub>33</sub> and MSP-1<sub>19</sub>) fragments with the latter remaining on the merozoite surface during invasion (Pacheco et al. 2006). The MSP-1<sub>42</sub> and MSP-1<sub>19</sub> fragments have received special attention in *P. falciparum* and *P. vivax* as part of vaccine formulations as they are relatively well conserved and antibodies against these fragments inhibit parasite invasion into red blood cells (Stanisic et al. 2004). The critical role of the MSP-1<sub>19</sub> fragment in erythrocyte invasion is conserved even among distantly related species (O'Donnell et al. 2000). Studies on the naturally acquired responses against the MSP-1 protein of *P. vivax* were initiated after the primary structure of the gene encoding this antigen revealed the existence of conserved and polymorphic blocks among different *Plasmodium* species (del Portillo et al. 1991). Recombinant proteins representing conserved and polymorphic regions of the N-terminal of PvMSP-1 demonstrated that polymorphic regions, as opposed to conserved regions, are immunogenic in natural infections. The C-terminal of PvMSP-1 is the most immunogenic portion of the molecule and the presence of antibodies against it is associated with recent malaria attacks (Soares et al. 1997, 1999). Nogueira et al. (2006) demonstrated an association of clinical protection and reduced risk of infection with naturally acquired antibodies against the N-terminal but not the C-terminal portion of PvMSP-1 and that most asymptomatic individuals presented antibodies against the N-terminal. However, this association does not imply

that antibody response to the PvMSP-1 N-terminal region is in itself the mechanism of protection or simply a marker of this mechanism (Nogueira et al. 2006).

The genetic polymorphism of the *Plasmodium* spp. MSP-1 appears to be maintained by positive natural selection, both in *P. falciparum* (Hughes 1992, Escalante et al. 1998, Conway et al. 2000) and *P. vivax* (Putaporntip et al. 2006). Similar observations have been made with other malaria antigens in which the host immune system is considered to be the selective driving force that allows the accumulation and frequent switch of suitable mutations in the parasite population (Escalante et al. 2004). There is extensive allelic diversity of MSP-1 among isolates, and so this polymorphism may hamper development of effective vaccines.

The *PvMsp-1* and *PfMsp-1* genes are basically similar. The organization of these genes are represented by a mosaic organization of several interallelic variable blocks flanked by conserved blocks, dimorphic substitutions in conserved blocks and rather polymorphic substitutions in variable blocks; with allelic recombination as a mechanism for the generation of new alleles (Putaporntip et al. 2002). Analysis of specific gene regions derived from parasite isolates from Sri Lanka (Premawansa et al. 1993), Colombia (Mancilla et al. 1994), and Thailand (Putaporntip et al. 1997) suggest interallelic recombination between the sequences typified as Bel and Sal-1, supporting the notion that the MSP-1 polymorphism in *P. vivax* is dimorphic in nature, as occurs in *P. falciparum* (Tanabe et al. 1987).

The variable blocks showed variation in repeats and non-repeat unique sequences. Numerous recombination sites were distributed throughout the *PvMsp-1* gene in both conserved blocks and variable block unique sequences, without the distribution being uniform. A substantial percentage of replacements within conserved blocks result in changes of binding to HLA class II allotypes. Polymorphisms in the T cell epitope regions could enable parasites to escape the host immune response. Therefore, host immune pressure plays a crucial role in the evolution and maintenance of polymorphisms in *PvMsp-1* (Putaporntip et al. 2002). In this scenario, mutations are maintained longer in the parasite population than expected if genetic drift were the sole process acting on genetic polymorphisms.

A number of the genes encoding for *P. vivax* MSPs have been identified. *PvMsp-3a*, *PvMsp-3b* and *PvMsp-3g* are members of a multi-gene family of related MSPs (del Portillo et al. 1991). The three encoded proteins share only 35-38% amino acid identity and 48-53% similarity in pair-wise comparisons. *PvMsp-3a*, similar to *PvMsp-1*, is very polymorphic and has been used as a genetic marker in population studies of isolates from diverse geographic localities and origins (Bruce et al. 1999). Even though *PvMsp-3a* is very polymorphic, this polymorphism is restricted to the N-terminal, while the C-terminal is well conserved (Rayner et al. 2002). Investigation of *PvMsp-3b* diversity was by cloning and sequencing full-length gene fragments from fifteen *P. vivax* isolates originating from Asia, South America and the Pacific region. *PvMsp-3a* and *PvMsp-3b* are extremely poly-

morphic paralogs with multiple gene sizes and sequences present in the different isolates (Rayner et al. 2004).

Two proteins, recently described in *P. falciparum*, MSP-4 and MSP-5, are found in tandem on chromosome 2 with the synthetic region of the genome being identified in rodent malaria. In *P. vivax*, which is quite phylogenetically distant from *P. falciparum*, both MSP-4 and MSP-5 homologues can be found with their relative arrangements in respect to surrounding genes being on the whole preserved. The *PvMsp-4* and *PvMsp-5* genes have two-exon structures with the sizes of the second exons being better conserved than the first exons in both genes, when compared to structure genes of *P. falciparum* (Black et al. 2001). *PvMsp-9* is characterized along with ortholog genes from related simian malarias, are highly conserved. *P. vivax* samples from different geographical regions show that the N-terminal region of PvMSP-9 is the most conserved, while the tandem repeats have diverged only in the length and number of units (Vargas-Serrato et al. 2002). Both N-terminal and the tandem repeat regions of MSP-9 are immunogenic in mice (Oliveira-Ferreira et al. 2004b). Comparative interspecies investigations of the potential role of *Plasmodium* MSP-9 in merozoite invasion of erythrocytes and as a candidate for malaria vaccine may be useful.

#### Duffy binding protein

Duffy binding protein (DBP) erythrocyte invasion by *P. vivax* merozoites is dependent on the parasite ligand, the *P. vivax* DBP, binding to the Duffy antigen receptor for chemokines (DARC). Individuals that lack the Duffy surface antigen on their erythrocytes are naturally resistant to *P. vivax* malaria. Thus, *P. vivax* DBP provides an attractive target for vaccine-mediated immunity (Xainli et al. 2000, Souza et al. 2006).

DBP is a 140 kDa protein located within the micronemes of *Plasmodium* spp. merozoites and characterized by two functionally conserved cysteine-rich regions, Region II and Region IV. Region II (DBPII) contains the binding motifs necessary for the adherence of DBP to DARC on the erythrocyte surface. Critical binding motifs in DBPII have been mapped to a region of a 170 amino-acid (aa) stretch that includes cysteines and where some hydrophobic amino acid residues are conserved and other amino acids are highly polymorphic in the ligand domain; this diversity varies geographically. The pattern of excessive polymorphisms occurring within the ligand domain and the high rate of non-synonymous polymorphisms suggest that this allelic variation functions as a mechanism of immune evasion. Previous studies have confirmed that this strong positive selection pressure in the DBPII ligand domain acts promoting greater diversity (Xainli et al. 2000, Souza et al. 2006).

Many factors may contribute to genetic diversity in malaria populations – mutations, intragenic recombination determined by multiplicity of infections and transmission intensity, natural selection, gene flow between different regions and population size. Recombination is likely to be important to maintain diversity for *P. vivax* and may be a critical source of variations in the *dbpII* gene. This origin of the variations may be more impor-

tant for malaria than previously appreciated as the recent use of PCR (polymerase chain reaction) has demonstrated that many people in endemic areas have chronic, asymptomatic infections often consisting of multiple parasites (Bruce et al. 2000). Meiotic recombination does not appear to be an important factor contributing to the diversity of the *dbp* gene. It is also possible that the *dbp* gene may contain a mutational "hot spot" that might mask the presence of recombination in *P. vivax dbpII* (Cole-Tobian & King 2003).

The cumulative polymorphisms, which Xainli et al. (2000) identified in isolates from Papua New Guinea, show that 124 of 133 (93%) individual mutations occur in the critical binding region between cysteines 4 and 7 (aa 460-291). All but one of these mutations is non-synonymous. Compared to the Sal-I isolate, a Belém strain from the Brazilian Amazon region, most polymorphisms are located in five residues: codons 308, 384, 390, 424, and 447 (Xainli et al. 2000). In Brazil, Souza et al. (2006) analyzed the DBP variability in isolates from the Amazon region and identified 14 polymorphic residues in the DBP ligand domain compared to the Sal-I sequence, with some of them being identical to those previously described in other regions of the world. The other seven polymorphic residues seem to be unique among Brazilian Amazon isolates. By grouping these residues, the authors constructed eight partial variant families representing the haplotypes present in Brazilian isolates. This large number of isolates, consisting of a diverse population of genetically distinct clones, may reflect random recombination which occurs during frequent mixed infections observed in distinct malaria endemic areas (Souza et al. 2006).

Diverse studies provide evidence for discrete allelic families in different *P. vivax* endemic regions of the world. DBP-II diversity varies both within and between populations. Polymorphisms of this protein are under great selection pressure, and the presence of distinct allelic families in different geographic areas will complicate the development of a vaccine, emphasizing the need for better understanding on how genetic diversity is related to natural immunity. It will be critical to identify which regions of DBP are functionally constrained yet remain sufficiently immunogenic to stimulate a protective immunity so that a vaccine might not require multiple allelic forms for different geographic regions (Cole-Tobian & King 2003).

#### *vir* genes

Malaria parasites have clustered multigene families in subtelomeric regions of chromosomes, where high recombination rates facilitate their evolution and diversity. Thus, *P. vivax* contains a major subtelomeric multigene superfamily termed *vir* (*P. vivax* variant genes), which corresponds to about 10% of the coding sequences (Fig. 2). The *vir* genes are composed of different subfamilies (denominated A-F) organized by sequence similarities and expressed during intra-erythrocytic parasite development (del Portillo et al. 2001). Studies indicate that the *vir* genes are probably related to mechanisms of

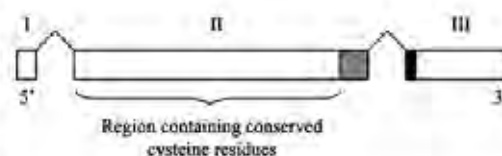


Fig. 2. organization of the *Plasmodium vivax vir* genes. This representation shows a short first exon (I), a longer second exon containing a transmembrane domain (hatched box or exon II), and the 3-exon structure (open boxes or III). Regions conserved amongst *vir* variants are indicated (filled box). Illustration modified from del Portillo et al. (2001).

antigenic variation; the host's efforts to eliminate the parasite are counteracted by the parasite's capability of constantly changing the specificity of its proteins (Brown & Brown 1965, Reeder & Brown 1996, Merino et al. 2006).

The *vir* genes and pseudogenes have similar structures. They have three-exon structures, which contrast with the 10-exon structure of the *sicavar* genes of *P. knowlesi* and with the two-exon structures of the *var*, *rvf* and *stevor* genes of *P. falciparum*. The second exon is highly variable containing a transmembrane domain and conserved cysteine residues. The region covering the point between exons 2 and 3 is extremely well conserved. Similar to the *P. knowlesi* *sicavar* protein and the *P. falciparum* PfEMP-1 protein, typical signal sequences are lacking in *vir* antigens. The major role of *vir* genes and their encoding of variant proteins in natural infections is currently unknown, although recently it has been proposed that they play a role in spleen-specific cytoadherence (macrophage-clearance escape) and in chronic infections. Thus, the function of *vir* genes is probably not directly related to the antigenic variations in the strict sense (del Portillo et al. 2004). The fact that not all their proteins are exported to the surface of infected red blood cells (Fernandez-Becerra et al. 2005) reinforces the idea that *vir* genes may have different functions related to immune evasion (Merino et al. 2006). The acquired immune responses to *P. vivax* variant antigens were also evaluated in individuals living in Brazilian malaria-endemic areas. This study showed that there is a low frequency of individuals responding to each variant antigen in these regions, which may explain host susceptibility to new episodes of the disease (Oliveira et al. 2006).

Most of the studies on the repertoire of these multigene families in natural infections, in particular those from human malaria parasites, are related to *P. falciparum* (Kirchgatter et al. 2000, Fowler et al. 2002). This knowledge is essential to understand the genetic diversity and evolution of these genes, which will contribute to elucidate chronicity in *Plasmodium*. On the other hand, some population and genetic diversity parameters can be better investigated using neutral molecular markers or those that are not undergoing strong selection processes, such as microsatellite loci (Leclerc et al. 2004).

### Microsatellites

Microsatellites are simple sequence tandem repeats that are generally hypervariable, codominant and locus specific. They are considered important neutral molecular markers as they are not directly subjected to host immunity. The neutral source of microsatellite polymorphisms is replication slippage, which is a commonly observed replication error in repetitive sequences that occurs when the new strand mispairs with the template strand (Russell et al. 2006).

Recently, several authors have been discussing the true degree of allelic diversity presented by the microsatellites (Gomez et al. 2003, Leclerc et al. 2004, Imwong et al. 2006, Russell et al. 2006). In direct contrast to the results of Gomez et al. (2003), Leclerc et al. (2004) found a low number of polymorphisms in the microsatellites they examined. In this study, 13 microsatellite sequences were isolated with 9/13 of these being completely monomorphic in eight analyzed populations, while of the remaining four loci only one showed extensive polymorphism. Using the draft of the unpublished *P. vivax* genome, Imwong et al. (2006) designed primer sets for 11 di-nucleotide microsatellites. Their data showed that *P. vivax* had a high allelic diversity. In the data of Leclerc et al. (2004), the isolated microsatellite sequences have very short repeat arrays (median = 5.5) and so would not be expected to show high levels of variation. Imwong et al. (2006), on the other hand, analyzed microsatellites with 12-18 repeats (median = 16). For this reason, the explanation given by Imwong et al. (2006) for the stark difference in their results compared to Leclerc et al. (2004) was that microsatellite variation is dependent on the length of the repeating sequence. Longer arrays are more diverse than shorter ones because slippage mutations become exponentially more common with an increase in array length. If microsatellites are stratified on the basis of length, *P. vivax* has a similar level of polymorphism to *P. falciparum* (Russell et al. 2006). These studies highlight the importance of measures standardizing genetic variations by repeat array length (Petit et al. 2005). In this context, Ferreira et al. (2007) recently standardized the use of tri- and tetranucleotide microsatellites, which usually yield more accurate allele scoring than dinucleotide markers to analyze the population structures of *P. vivax* from rural Amazonia. The authors showed that *P. vivax* isolates obtained during cross-sectional and longitudinal surveys in rural Amazonia display extensive genetic diversity and frequent multiple clone infections coexisting with strong multilocus linkage disequilibrium.

### Other polymorphic molecular markers

The gene of *PvAMA-1* encodes a very important protein for erythrocyte invasion which is highly conserved in *Plasmodium* spp. However, this gene has few predominant haplotypes, displaying very limited genetic diversity within any geographic region; a more comprehensive study of the whole gene is necessary to confirm this observation. On the other hand, the presence of single nucleotide polymorphism (SNP) has been identi-

fied in some gene regions (Cui et al. 2003). This provides many potential alleles thereby making this gene a useful marker for typing parasite populations (Fitzree et al. 2000).

The antifolate agents, sulfadoxine and pyrimethamine (SP), are commonly used to treat *P. falciparum* malaria. Nevertheless, they can also affect the *P. vivax* parasite if it co-exists with *P. falciparum* as both species have common drug targets. The dihydrofolate reductase (DHFR) enzyme of *Plasmodium* spp. is the therapeutic target of the pyrimethamine component of SP. In addition to point mutations in the target enzymes of the respective parasite, the *PvDHFR* gene also has a size polymorphism resulting from the deletion of five or six amino acids. Four allelic variants have been observed to date. However, the fact that the gene is under drug selection must be taken into account if this polymorphism is to be used as a genetic marker (Cui et al. 2003). Even less is known about associations between these specific alleles in *P. vivax* populations in Brazil.

### New tools for studies of the *P. vivax* genome

Unfortunately, due to the necessity of continuously maintaining this parasite in culture, the low parasitemias associated with natural infections and the difficulty of adapting field isolates to grow in monkeys, research on *P. vivax* remained largely neglected, up to a decade ago (Merino et al. 2003). This situation has changed dramatically in recent years. Despite the limited availability of *P. vivax* biological material, recent research has led to the construction of *P. vivax* genome in yeast artificial chromosomes (YAC). This new tool has revolutionized studies on the structure and function of the *P. vivax* genome, with the search of new polymorphic molecular markers (Camargo et al. 1997, del Portillo et al. 2001, Merino et al. 2003, 2006, Feng et al. 2003, Gomez et al. 2003, Fernández-Becerra et al. 2005). Moreover, the availability of the *P. vivax* telomeric YAC clones has helped in the identification of many antigen-encoding genes mapped to the dynamic subtelomeric domains of plasmodial chromosomes; this fact has led to a suggestion that the recombination within these domains has been recruited as a novel mechanism to generate antigenic diversity (Camargo et al. 1997).

Few polymorphic molecular markers, in particular, orthologs of previously identified *P. falciparum* antigen genes, have been used for population studies of *P. vivax*. SNPs markers have been mapped to a chromosome segment by sequencing a large insert of the YAC library (Feng et al. 2003) and as SNPs are often present at a high frequency, they are ideal genetic markers. Identification and development of large numbers of genetic markers such as SNPs from *P. vivax* will provide a framework on which studies of molecular evolution and genetic mapping can be based.

Gomez et al. (2003) identified, for the first time, a locus in a *P. vivax* telomeric YAC clone that contains simple sequence repeats. These sequences known as microsatellites were located within the second exon of the *vir13* pseudogene. In 2003, Feng et al. analyzed a contiguous chromosome segment of approximately 100

kb from five isolates, revealing 191 SNPs and 44 size polymorphisms. These analyses showed that *P. vivax* has a highly diverse genome that may represent some challenges for drug and vaccine development. This study also showed that SNPs tend to cluster in intergenic regions or even in specific genes that may be under selection (Feng et al. 2003).

Starting from genomic library constructed in YACs, using parasites obtained directly from human patients, Merino et al. (2003) described the first expressed sequence tags (ESTs), parasite genes expressed during *P. vivax* pathogenesis development. The description of EST is also a valuable resource to validate gene predictions and to create a gene index for this malaria parasite.

Recently, Imwong et al. (2006) described patterns of variations of 11 dinucleotide microsatellites in *P. vivax* populations from Colombia, India and Thailand using the YACs libraries as an important tool to better understand the parasite's genome. These data show that *P. vivax* has a highly diverse genome and provided useful information to further understand the genome diversity of the parasite.

#### Phylogenetic characteristics of the *P. vivax*

Almost 60 years ago, Haldane proposed that human malaria might act as a selective force on human populations. Since then, several studies have been proposed to test this hypothesis, starting with attempts of phylogenetic origin reconstruction of malaria parasites (Escalante et al. 1995).

The first phylogenetic reports using molecular biology analyses have focused primarily on *P. falciparum*, an agent of malignant malaria. A previous study suggested that *P. falciparum* is older than *P. vivax* (Escalante et al. 1995), and the virulence of *P. falciparum* has been attributed to the fact that it recently became a human parasite due to a host switch (probably, birds) between 5,000 to 10,000 years ago (Boyd 1949, Livingstone 1958, Snewin et al. 1991, Escalante et al. 1995). Low microsatellite and tandem repeat variability may indicate that *P. vivax* has only infected humans recently (10,000 years ago) (Leclerc et al. 2004), however a different study based on polymorphisms of two nuclear and one plastid gene places the origin at between 45,000 and 81,000 years ago (Escalante et al. 2005). Through these preliminary studies, two major conclusions were reached: (a) malaria parasites arose independently as human pathogens and, (b) *P. reichenowi*, a chimpanzee parasite, is the species that shares the most recent common ancestor with *P. falciparum* (Escalante et al. 1995). Thus, questions about the age and geographic origin of *P. vivax*, the most prevalent human malaria parasite in the world, remain largely unresolved. Recent studies have suggested possible origins for *P. vivax* but most are not based on strong phylogenetic data. Two hypotheses have been systematically discussed. One of these placed the origin of *P. vivax* in Southeast Asia, together with other *Plasmodium* parasitic species in non-human primates (Carter & Mendis 2002, Carter 2003, Jongwutiwes et al. 2005). Hence, it is possible that *P. vivax* might have fixed in this area through some hominoid lineage that had its ori-

gin there, even modern humans, since host switches seem to be common phenomena among malaria parasites. This argument was also supported by the abundance of simian malaria parasite species in Asia and the presence of several macaque parasites that shared similar morphological and biological characteristics with *P. vivax* (Carter 2003). This hypothesis has been well accepted in recent years (Escalante et al. 2005, Mu et al. 2005, Jongwutiwes et al. 2005).

It seems very probable that Duffy-negative homozygote individuals (lack of the Duffy blood group antigen), are completely protected against *P. vivax* infection (Escalante et al. 2005). Thus, the high prevalence of these genotypes among African populations, has been used to supplant *P. vivax* origin on this continent, showing that a selective pressure might have been generated in favor of the survival of the parasite organism, because the presence of the Duffy protein is essential for the merozoite invasion of red blood cells. Nevertheless, for that to have really happened, the presence of a strong selection factor would have been necessary, which seems unlikely as *P. vivax* does not exhibit high levels of virulence in terms of mortality rates. For this reason, it is probable that the Duffy-negative genotype present on the African continent, could have been fixed by another process (selection due to another pathogen or chance) and then became a barrier against the introduction of *P. vivax* (Carter 2003, Escalante et al. 2005). Also attempts to test this hypothesis starting from an investigation of two nuclear genes, *b-tubulin* and *cell division cycle 2* and a gene from the plastid genome, the elongation factor *Tu* (*TufA*) were made. In this investigation, most of the data was not compatible with the explanation that *P. vivax* was a *Homo* parasite before the expansion of the hominoid populations out of Africa. Hence, the authors suggest that *P. vivax* was probably derived from ancestral macaque parasites when hominoids colonized Southeast Asia (Escalante et al. 2005).

On the other hand, the first evidence of the origin of *P. vivax* based on molecular data using mitochondrial *cytochrome b* gene analysis showed strong similarities of *P. vivax* with a species of a simian malaria parasite from Asia, provided by an apparently recent species radiation. This phylogenetic study also suggests that *P. vivax* could have originated from a malaria parasite of non-human primates as a result of a host switch, probably from a macaque (Escalante et al. 2005, 2006). These analyses received additional support when the combined data of complete mitochondrial genomes were reported in two independent studies (Mu et al. 2005, Jongwutiwes et al. 2005). This information can maybe explain the fact that several isolates of *P. vivax* are practically identical to *P. simium* and the previous suggestion that this South American species originated from a host switch from humans to monkeys (Escalante et al. 1998, 2005, Mu et al. 2005).

As in other phylogenetic studies (Fig. 3), the *P. vivax* variant genotypes belong to a same clade that includes several types of primate *Plasmodium* species (Escalante et al. 1995, Qari et al. 1996). Analyses of the non-repetitive portion of *P. vivax* CSP (*PvCSP*) showed homology with *CSPs* genes of two simian malaria para-

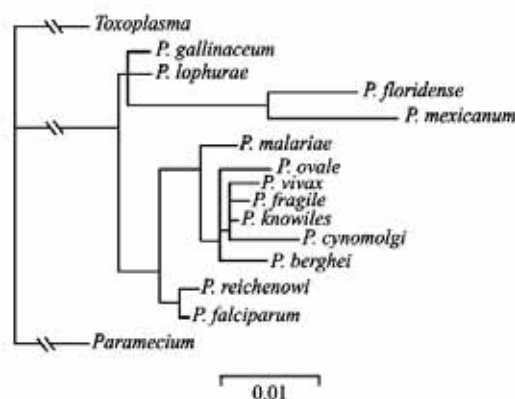


Fig. 3: phylogenetic tree of *Plasmodium* spp. derived by the maximum likelihood (fastDNAmI) method. Scale bar indicates an evolutionary distance of 0.01 nucleotide substitutions per site in the sequence. According to Qari et al. (1996)

sites, *P. cynomolgi* and *P. knowlesi* (Arnot et al. 1985). In contraposition, the *P. vivax*-like gene is different to the CSPs genes of other human malaria parasites. However, the CSP gene sequence of this variant genotype is practically identical to *P. simiovale* CSP and has similar morphologically to two other malaria parasites, *P. simium* and *P. ovale* (Qari et al. 1993b, Escalante et al. 1995). The similarity between *P. simiovale* and *P. vivax*-like has been driving intense discussions, regarding the evolutionary origin of this variant, as well as on clinical epidemiology and the vectors involved in its transmission (Qari et al. 1994). Escalante et al. (1995) also confirmed these findings through phylogenetic studies based on CSP genes, showing that the analogies between these two parasites determine important epidemiological consequences; primate hosts can serve as reservoirs for human malaria parasites. However, phylogenetic studies have been developed with the CSP gene as the only molecular marker so they cannot explain the evolutionary relationship among the three variant genotypes of CSP, or the genetic distance of these with other primate parasites that possess molecular similarities with *P. vivax* (Escalante et al. 1995).

In summary, understanding of the genetic recombination patterns and sequence variation may help to design vaccines, which represent the worldwide repertoire of polymorphic malarial surface antigens and elucidate the selection events associated to drug resistance.

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**5 CAPÍTULO II**

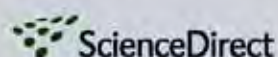
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## 5 CAPÍTULO II

**5.1. *Plasmodium vivax* circumsporozoite variants and Duffy blood group genotypes in the Brazilian Amazon region.** Autores: Luciane M. Storti-Melo, Wanessa C. de Souza-Neiras, Gustavo C. Cassiano, Ana C.P. Joazeiro, Cór J. Fontes, Cláudia R. Bonini-Domingos, Álvaro A.R. D’Almeida Couto, Marinete M. Póvoa, Luiz C. de Mattos, Carlos E. Cavasini, Andréa R.B. Rossit e Ricardo L.D. Machado. Artigo original publicado na revista **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. **103**, p. 672-678, 2009.

### RESUMO

A proteína circunsporozóica (CSP) de *Plasmodium vivax* é considerada como o maior alvo para o desenvolvimento de vacinas antimaláricas recombinantes. As moléculas do grupo sanguíneo Duffy agem como receptores das hemácias para o *P. vivax*. Nós reavaliamos a frequência das variantes da CSP de *P. vivax* e reportamos sua associação com os genótipos do grupo sanguíneo Duffy em pacientes maláricos. Amostras de sangue periférico foram coletadas de 155 indivíduos infectados com *P. vivax* de cinco áreas endêmicas brasileiras. As variantes da CSP de *P. vivax* e os genótipos do grupo sanguíneo Duffy foram diagnosticados usando PCR/RFLP. Em infecções simples, a variante VK210 foi a mais comum, seguida pela variante *P.vivax-like*. A tipagem de *P. vivax* indicou que a frequência das variantes entre as áreas estudadas foi significativamente diferente. Esta é a primeira detecção das variantes VK247 e *P.vivax-like* em infecções simples em áreas endêmicas do Brasil. A associação das variantes da CSP de *P. vivax* com os genótipos heterozigotos do grupo sanguíneo Duffy foi significativa para as infecções simples com VK210. Estas observações fornecem dados adicionais aos processos de interação *Plasmodium*-hospedeiro relacionando o grupo sanguíneo Duffy e a capacidade do *P. vivax* em causar a malária humana.

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## *Plasmodium vivax* circumsporozoite variants and Duffy blood group genotypes in the Brazilian Amazon region

Luciane M. Storti-Melo<sup>a,b,1</sup>, Wanessa C. de Souza-Neiras<sup>a,b,1</sup>,  
Gustavo C. Cassiano<sup>a,b</sup>, Ana C.P. Joazeiro<sup>b</sup>, Cor J. Fontes<sup>c</sup>,  
Cláudia R. Bonini-Domingos<sup>a</sup>, Álvaro A.R. D'Almeida Couto<sup>d</sup>,  
Marinete M. Povoá<sup>e</sup>, Luiz C. de Mattos<sup>a,b</sup>, Carlos E. Cavasini<sup>b</sup>,  
Andréa R.B. Rossit<sup>a,b,f</sup>, Ricardo L.D. Machado<sup>a,b,f,\*</sup>

<sup>a</sup> University of São Paulo State Júlio Mesquita Filho, Rua Cristóvão Colombo 2265, 15054-000 São José do Rio Preto, São Paulo State, Brazil

<sup>b</sup> Faculty of Medicine of São José do Rio Preto, Avenida Brigadeiro Faria Lima 5416, Vila São Pedro, São José do Rio Preto, São Paulo State, Brazil

<sup>c</sup> Mato Grosso Federal University, Department of Medical Clinic, Rua Luiz Phellipe Pereira Leite, 78048-902 Cuiabá, Mato Grosso State, Brazil

<sup>d</sup> SEAMA Faculty, Macapá, Amapá State, Brazil

<sup>e</sup> Evandro Chagas Institute, MS/SVS, Rodovia BR-316 km 7 s/n, Levilândia 67030-000, Ananindeua, Pará State, Brazil

<sup>f</sup> Faculty of Medicine of São José do Rio Preto Foundation, Avenida Brigadeiro Faria Lima 5416, 15090-000 São José do Rio Preto, São Paulo State, Brazil

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**Summary** The circumsporozoite protein (CSP) of the *Plasmodium vivax* infective sporozoite is considered to be a major target for the development of recombinant malaria vaccines. The Duffy blood group molecule acts as the red blood cell receptor for *P. vivax*. We review the frequency of *P. vivax* CSP variants and report their association with the Duffy blood group genotypes from Brazilian Amazon patients carrying *P. vivax* malaria. Peripheral blood samples were collected from 155 *P. vivax*-infected individuals from five Brazilian malaria-endemic areas. The *P. vivax* CSP variants and the Duffy blood group genotypes were assessed using PCR/RFLP. In single infections, the VK210 variant was the commonest followed by the *P. vivax*-like variant. The typing of *P. vivax* indicated that the frequency of variants among the study areas was significantly

\* Corresponding author. Tel.: +55 17 32015736; fax: +55 17 32015909.

E-mail address: ricardomachado@famerp.br (R.L.D. Machado).

<sup>1</sup> LMSM and WCSN contributed equally as first authors.



different from one to another. This is the first detection of the VK247 and *P. vivax*-like variants in single infections in endemic areas of Brazil. Association of the CSP *P. vivax* variants with the heterozygous Duffy blood group system genotype was significant for VK210 single infection. These observations provide additional data on the *Plasmodium*-host interactions concerning the Duffy blood group and *P. vivax* capability of causing human malaria.

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## 1. Introduction

In Brazil since 1993, the number of *Plasmodium vivax* cases has increased and they now account for more than 80% of clinical malaria cases annually reported in the Amazon region.<sup>1</sup> The circumsporozoite protein (CSP) of the infective sporozoite is considered to be a major target for the development of recombinant malaria vaccines.<sup>2-4</sup> By serological and/or molecular approaches, different authors have evaluated the occurrence of *P. vivax* variants (VK210, VK247 and *P. vivax*-like) in endemic areas of the Amazon region.<sup>5-7</sup>

The Duffy blood group acts as a receptor for *P. vivax* on the surface of the red blood cells and, therefore, its polymorphisms have an important impact where *vivax* malaria predominates.<sup>10</sup> The *FY* gene, located on human chromosome 1, encodes the Duffy proteins by the two co-dominant alleles: *FYA* and *FYB*.<sup>11</sup> The *FYB* allele is the result of a point mutation in the GATA box of the Duffy antigen/receptor for chemokines (DARC) promoter, which silences the gene encoding the Duffy system antigens in the red blood cells.<sup>12</sup>

Previous reports from Latin America<sup>8,13</sup> have suggested different abilities for vector infection between the VK210 and VK247 phenotypes. Ryan et al.<sup>14</sup> described the possibility that the VK247 CSP variant marks a *P. vivax* population capable of using receptors other than Duffy. Furthermore, two Brazilian Duffy antigen-negative individuals infected by *P. vivax* were detected, whose CSP genotypes were VK210 and/or *P. vivax*-like.<sup>15</sup> In our original study, we identified that individuals with the *FYA/FYB* genotype have higher susceptibility to *P. vivax* malaria in the Brazilian Amazon region.<sup>14</sup>

We review the frequency of *P. vivax* CSP variants and report their association with the Duffy blood group genotypes in patients infected by *P. vivax* single (only one variant) or mixed (more than one variant) infections from the Brazilian Amazon.

## 2. Materials and methods

### 2.1. Sample collection and DNA extraction

A subset of 155 patients was analysed out of 312 individuals previously evaluated by Cavasini et al.<sup>16</sup> The peripheral blood samples, which had been kept at  $-70^{\circ}\text{C}$ , were from *P. vivax* carriers who lived in five Brazilian malaria-endemic areas: Novo Repartimento, Pará State; Macapá, Amapá State; Porto Velho, Rondônia State; Plácido de Castro, Acre State and Cuiabá, Mato Grosso State (Figure 1). The patients who were enrolled in this study complied with the following criteria: they sought medical assistance for clinical malaria

symptoms, were over 18 years old and had a positive malaria diagnosis by thick blood film or molecular techniques, after written informed consent had been signed. The DNA was extracted from frozen pellets of infected erythrocytes using the Easy-DNA<sup>TM</sup> extraction kit (Invitrogen, Carlsbad, CA, USA).

### 2.2. Amplification of the *Plasmodium vivax* CSP gene fragment and RFLP analysis

The CSP *P. vivax* variants were assessed using PCR/RFLP as previously described by Alves et al.<sup>17</sup> To amplify the CSP gene a set of forward (PR1: 5'-ACT TTT ATT CGA CTT TGT TGG TC-3') and reverse (PR2: 5'-ATG GAC TCC ATG CAG TGT AAC C-3') primers (Invitrogen, Portland, OR, USA) were designed based on the conserved central portion of the CSP gene. DNA (1.5  $\mu\text{l}$ ) was amplified in a total reaction volume of 25  $\mu\text{l}$  consisting of 1  $\times$  PCR buffer (10mM Tris-HCl, pH 8.3, 50mM KCl), 1.5 mM of  $\text{MgCl}_2$ , 1.0  $\mu\text{M}$  of each primer, 200  $\mu\text{M}$  dNTPs, 2.5 U ampliTaq DNA polymerase (Invitrogen, São Paulo, Brazil), 1% betaine and water (25  $\mu\text{l}$ ). Twenty-five cycles of amplification were performed in a thermocycler (DNA MasterCycler; Eppendorf, Madison, WI, USA) after initial denaturation of DNA at  $94^{\circ}\text{C}$  for 5 min. Each cycle consisted of a denaturation step at  $93^{\circ}\text{C}$  for 60 s, an annealing step at  $41^{\circ}\text{C}$  for 90 s and an extension step at  $72^{\circ}\text{C}$  for 2 min, with a final extension at  $72^{\circ}\text{C}$  for 10 min following the last cycle. Before typing CSP variants we progressively tested lower annealing temperatures (0.5  $^{\circ}\text{C}$  lower at a time) until we reached good quality PCR products without unspecific amplification. The PCR products were analyzed by electrophoresis using 1.5% agarose gels and stained with ethidium bromide. The restriction enzymes selected were required to have at least one cleavage site in the amplification of each variant, resulting in DNA fragments that were easily visible in polyacrylamide gel. Restriction digests were set up with 10  $\mu\text{l}$  of the PCR product and 1 U of each enzyme (*AluI* and *DpnI*, Promega, Madison, WI, USA), incubated for 1 h at  $37^{\circ}\text{C}$ . Restriction fragments were separated by electrophoresis in 12.5% polyacrylamide gels. The gels were stained with ethidium bromide and analyzed with a Gel Doc 2000 illuminator (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.3. Duffy blood group genotyping

Duffy blood group genotypes were assessed using PCR/RFLP as described previously, with modifications.<sup>18</sup> Briefly, PCR was performed with 100 ng of DNA, 50 pmol of each primer



Figure 1 Study area for the *Plasmodium vivax* variants in the Amazon region of Brazil. Plácido de Castro, Acre State (AC; 10°16'33"S; 67°09'00"W); Porto Velho, Rondônia State (RO; 08°45'43"S; 63°54'14"W); Cuiabá, Mato Grosso State (MT; 15°17'05"S; 56°56'36"W); Novo Repartimento, Pará State (PA; 04°19'50"S; 49°47'47"W); Macapá, Amapá State (AP; 00°02'20"S; 51°03'59"W).

(Invitrogen, Portland, OR, USA), 2 nmol each dNTP, 1.0 U Taq DNA polymerase (Invitrogen, São Paulo, Brazil), and buffer, in a total volume of 50  $\mu$ l. The promoter region was amplified using the FYN1 and FYN2 primers that flank the GATA box motif. To determine the Duffy red blood cell polymorphism, FYAB1 sense and FYAB2 reverse sense primers were used.<sup>19</sup> The amplification conditions were performed as described by Castilho et al.<sup>18</sup> PCR products were run on 1.5% agarose gel, followed by ethidium bromide staining and photo-documentation using a Gel Doc 1000 (Bio-Rad Laboratories). The RFLP analysis was performed through 3 h of digestion with *Bln*I, *Msp*AI and *Syl*I restriction enzymes (Promega).

#### 2.4. Statistical analysis

Analyses were performed using R version 2.4.1 statistical software (The R Foundation for Statistical Computing, Vienna, Austria (<http://www.r-project.org>)). The analysis of dependency was applied to evaluate the distribution of *P. vivax* CSP variants within the five studied areas.<sup>20</sup> To obtain the independence among the proportions, Fisher's exact test was applied with a significance level of  $P < 0.05$ .

### 3. Results

The distribution of *P. vivax* CSP genotypes and the genotypic frequencies of the Duffy blood group in the 155 blood

samples obtained from malaria patients are summarized in Tables 1 and 2, respectively. The VK210 genotype was the commonest (58.7%), followed by *P. vivax*-like (9.7%) in single infections. The typing of *P. vivax* indicated that the prevalence and frequency of variants were significantly different from one area to another (Fisher's exact test;  $P = 0.046$ ). The VK210 and *P. vivax*-like genotypes were detected as single infections in all studied areas. Only in samples from Novo Repartimento were we able to detect all three CSP variants presenting as single and/or mixed infections (Table 1). However, the distribution of the *P. vivax*-like genotype seemed to be more homogeneous than VK247 in the studied areas (analysis of dependency;  $P = 0.030$ ). The VK247 variant was more frequently found in association with one of the two other variants (mixed infections) in Novo Repartimento and Macapá than in other studied areas. Additionally, this variant was found as a single infection exclusively in Pará State. Meanwhile, the VK210 variant as a single infection was more frequent in Porto Velho, Plácido de Castro and Cuiabá (Fisher's exact test;  $P = 0.033$ ).

The data show a high frequency of the FYA/FYB genotype, which was present in 55 individuals (35.5%) with all *P. vivax* CSP variants. However, the association of the *P. vivax* variants with the FYA/FYB genotype was significant only for the VK210 single infection (Fisher's exact test;  $P = 0.042$ ). As for the VK247 or *P. vivax*-like variants, either in single or mixed infections, the Duffy blood group system genotype distribution was homogeneous. There were no differences comparing the populations from the study areas for the

**Table 1** Distribution of *Plasmodium vivax* variants in five areas of the Amazon region of Brazil (2003–2005)

Study area	Single types, n (%)			Mixed types, n (%)				Total
	VK210	VK247	<i>P. vivax</i> -like	VK210+VK247	VK210+ <i>P. vivax</i> -like	VK247+ <i>P. vivax</i> -like	VK210+VK247+ <i>P. vivax</i> -like	
Cuiabá (MT)*	33 (70.2)	0	3 (6.4)	8 (17.0)	0	2 (4.3)	1 (2.1)	47
Macapá (AP)*	10 (35.7)	0	2 (7.1)	11 (39.3)	5 (17.9)	0	0	28
Novo Repartimento (PA)*	10 (29.4)	4 (11.8)	3 (8.8)	12 (35.3)	1 (2.9)	0	4 (11.8)	34
Porto Velho (RO)*	25 (96.2)	0	1 (3.8)	0	0	0	0	26
Plácido de Castro (AC)*	13 (65.0)	0	6 (30.0)	0	1 (5.0)	0	0	20
Total	91 (58.7)	4 (2.6)	15 (9.7)	31 (20)	7 (4.5)	2 (1.3)	5 (3.2)	155

MT: Mato Grosso State; AP: Amapá State; PA: Pará State; RO: Rondônia State; AC: Acre State.  
\* Significant at  $P < 0.05$ ; Fisher's exact test.

**Table 2** *Plasmodium vivax* circumsporozoite variants and their correlation with Duffy blood group system genotypes

Duffy blood group system genotypes	<i>P. vivax</i> circumsporozoite variants, n (%)							Total
	VK210	VK247	<i>P. vivax</i> -like	VK210+VK247	VK210+ <i>P. vivax</i> -like	VK247+ <i>P. vivax</i> -like	VK210+VK247+ <i>P. vivax</i> -like	
FYA/FYA	17 (11.0)	0	1 (0.6)	9 (5.9)	0	1 (0.6)	0	28 (18.1)
FYB/FYB	17 (11.0)	0	2 (1.3)	2 (1.3)	1 (0.6)	1 (0.6)	1 (0.6)	24 (15.5)
FYA/FYB	35* (22.6)	2 (1.3)	5 (3.2)	9 (5.9)	4 (2.6)	0	0	55 (35.5)
FYA/FYB-33	10 (6.5)	1 (0.6)	1 (0.6)	4 (2.6)	1 (0.6)	0	4 (2.6)	21 (13.5)
FYB/FYB-33	10 (6.5)	1 (0.6)	6 (3.9)	6 (3.9)	1 (0.6)	0	0	24 (15.5)
FYA/Fy <sup>x</sup>	2 (1.3)	0	0	0	0	0	0	2 (1.3)
FYB/Fy <sup>x</sup>	0	0	0	1 (0.6)	0	0	0	1 (0.6)
Total	91 (58.7)	4 (2.6)	15 (9.7)	31 (20)	7 (4.5)	2 (1.3)	5 (3.2)	155 (100)

\* Significant at  $P < 0.05$ ; Fisher's exact test.

Duffy blood groups (Fischer's exact test;  $P=0.221$ ; data not shown).

#### 4. Discussion

In the Brazilian Amazon region, two previous studies evaluated the molecular epidemiology of the CSP variants and detected the VK210 genotype in single infections, whereas the VK247 and *P. vivax*-like genotypes were only detected as mixed infections in three malaria-endemic areas.<sup>6,7</sup> Interestingly, after 10 years, a new scenario is observed. VK210 continues to be the most prevalent variant in all endemic areas of the Brazilian Amazon. However, regarding the VK247 and *P. vivax*-like variants, the present results suggest a change in *P. vivax* distribution dynamics since, to our knowledge, this is the first detection of both parasite forms as single infections in Brazil. These results may indicate that VK247 is not yet totally adapted in Brazilian malaria-endemic areas, unlike earlier observations in areas of Colombia,<sup>2</sup> Mexico and Asia.<sup>4,21,22</sup> The exclusive detection of VK247 in single infections only in blood samples from Novo Repartimento, Pará State, might suggest a later introduction of this variant in Rondônia, Amapá, Acre and Mato Grosso States. The results of this study demonstrate that the *P. vivax*-like variant is more widely distributed in all five studied areas than VK247. In fact, they show a more homogeneous distribution of the *P. vivax*-like variant, as a single or mixed infection, suggesting that its adaptation is happening faster than that of VK247. Another hypothesis is that the introduction of VK247 occurred more recently in Brazilian areas. These findings will allow us to continue expanding our understanding of *P. vivax* variant epidemiology in the Brazilian Amazon region.

It has been demonstrated that Duffy-negative individuals are protected against *P. vivax* infection,<sup>23</sup> although in Brazil<sup>15</sup> and in Africa<sup>14</sup> *P. vivax* malaria has been shown to affect Duffy-negative individuals. Previous results have indicated that the heterozygous genotype for Duffy antigen favours infection by *P. vivax* in the Brazilian Amazon region,<sup>16</sup> reinforcing the hypothesis that the presence of both functional Duffy alleles increases the risk of infection by this parasite.<sup>24</sup> Indeed, the FYA/FYB genotype was the most prevalent in this study, which included only malaria patients and controls (blood donors) was statistically significant (Fisher's exact test;  $P=0.035$ ), and although no qualitative or quantitative measurements of the Duffy glycoprotein expression were performed, there was a higher number of malaria episodes among FYA/FYB patients. It is possible that the FYA/FYB genotype modulates an individual's susceptibility to infection by *P. vivax* by means of quantitative and/or qualitative variations that affect the Duffy antigen expression on erythrocytes. On the other hand, the mechanism by which *P. vivax* uses the Duffy determinants to invade erythrocytes is mediated by the Duffy binding protein (DBP).<sup>12</sup> Previous reports in Papua New Guinea<sup>25</sup> and Colombia<sup>26</sup> have shown that the sequence of the DBP is highly polymorphic, suggesting that the mero-

zoite may have the capacity for rapid adaptation. Recently, Van Buskirk et al.<sup>27</sup> suggested that polymorphism in the ligand domain of DBP can alter immune recognition. In Brazil, Cerávolo et al.<sup>28</sup> showed that a recombinant DBP was immunogenic in Brazilian Amazon populations and that the immune response increased with exposure to malaria, reaching a peak in those subjects with long-term exposure. Moreover, Sousa et al.<sup>29</sup> analyzed DBP variability and identified that other polymorphic residues seem to be unique among isolates from the Brazilian Amazon region.

In the present study a higher frequency of VK210 single infections was detected among FYA/FYB subjects. Such an association between the parameters occurring with the highest frequency: FYA/FYB genotype and single infections by the VK210 variant would be expected to be found, a limitation we acknowledge. Nevertheless, other explanations are possible based on biological differences not necessarily related to the CSP protein function, as postulated before.<sup>2,14</sup> DARC is the receptor for *P. vivax* DBP and not for CSP, which is not expressed during the merozoite blood stage. Therefore, one possible explanation for the current results is that CSP and DBP variations are in linkage disequilibrium, a perspective to be explored. Likewise, *P. vivax* CSP variants could be tagging functional subset(s) of genetic diversity that modulate the efficiency of erythrocyte invasion towards a specific group of erythrocyte receptors.

The negative findings for single and mixed VK247 and/or *P. vivax*-like infections in relation to the studied Duffy genotypes point to the hypothesis that these variants characterize a *P. vivax* population capable of using receptors other than Duffy, as suggested by Ryan et al.<sup>14</sup> In the previous report of Cavasini et al.<sup>15</sup> the authors could not make this hypothesis since the presented Duffy-negative individuals were also VK247-negative.

Additional studies will be necessary to enable a better understanding of whether individuals in endemic areas of Brazil acquire *P. vivax* CSP variants that have preferential ability to bind the parasite ligand. Finally, the question remains whether the *P. vivax* CSP repeated region is a limited, mostly silent base variation,<sup>30</sup> or if these variants represent the existence of a new species or subspecies of *Plasmodium* causing human malaria, with major biological consequences.<sup>4</sup> These observations provide additional data on the *Plasmodium*-host interactions mediated by the Duffy blood group and how these affect the capability of *P. vivax* to cause human malaria. Molecular and serological investigations are currently being conducted at the Center for Microorganisms Investigation, Faculty of Medicine of São José do Rio Preto, to improve the knowledge on the role of *P. vivax* variants in malaria epidemiology.

In conclusion, the current results highlight the possibility that *P. vivax* CSP variants mark biological differences towards the preferential invasion of specific erythrocyte antigen receptors, such as the Duffy antigen receptor for chemokines.

**Authors' contributions:** RLDM and ARBR conceived and designed the study; AADC, MMP and CJF collected samples from individuals with *P. vivax* malaria; LMSM, CEC, WCSN, GCC and ACPJ carried out all the genotype assays; RLDM, ARBR, CRBD and LCM analysed and interpreted the data

and drafted the manuscript; LMSM, CEC, WCSN, GCC, ACPJ, AADC, MMP and CJF critically revised the manuscript. All authors read and approved the final manuscript. LMSM and WCSN are guarantors of the paper.

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**6 CAPÍTULO III**

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## 6 CAPÍTULO III

**6.1. *Plasmodium vivax* circumsporozoite genotypes: a limited variation or new subspecies with major biological consequences?** Autores: Wanessa C. Souza-Neiras, Luciane M. Storti-Melo, Gustavo C. Cassiano, Vanja S. C. A. Couto, Álvaro A. R. A. Couto, Irene S. Soares, Luzia H. Carvalho, Marinete M. Póvoa, Sócrates Herrera, Myrian Arévalo-Herrera, Andréa RB Rossit, Claudia M. A. Carareto e Ricardo LD Machado. Artigo original a ser submetido para publicação na revista **Malaria Journal**, 2009.

### RESUMO

Os genótipos da CS de *Plasmodium vivax* têm sido identificados em várias regiões do mundo. A real implicação desta variação no genoma de *P. vivax* permaneceu questionável por longo tempo. Embora estudos prévios do nosso grupo tenham observado associação significativa entre a VK210 e o sistema sanguíneo Duffy, nós apresentamos aqui evidências de que essa variação esteja limitada à porção central da CSP. As análises filogenéticas foram realizadas a partir da amplificação de domínios conservados de *18 SSU rRNA* e *Cyt B*. A resposta de anticorpos contra os peptídeos da CSP, MSP-1, AMA-1 e DBP foram detectados por ELISA, em amostras de plasma de indivíduos infectados com os dois genótipos da CS de *P. vivax* VK210 e *P. vivax-like*. As análises dos dois marcadores avaliados mostram alta similaridade entre os genótipos da CS de *P. vivax*, com grau de diversidade nucleotídica igual a zero, evidenciando que os genótipos VK210 e *P. vivax-like*, pertencem a um mesmo clado. Nós encontramos uma alta frequência de anticorpos contra as porções N- e C-terminal da CSP de *P. vivax* quando comparada à resposta imune das regiões repetitivas R e V ( $p=0,0005$ , pelo teste Exato de Fisher). Esta diferença foi mais pronunciada quando o genótipo *P. vivax-like* esteve presente na infecção ( $p=0,003$ , pelo teste Exato de Fisher). Uma alta frequência da resposta de anticorpos foi observada contra os peptídeos MSP-1 e AMA-1 para todos os

genótipos da *CS* em comparação à mesma frequência para o peptídeo DBP. Nossos resultados marcam que as diferenças entre as variantes da CSP estão restritas a porção central repetitiva da proteína, representando uma variação nucleotídica sem consequências sorológicas importantes.

*Plasmodium vivax* circumsporozoite genotypes: a limited variation or new subspecies with major biological consequences?

Wanessa C Souza-Neiras<sup>1\*§</sup>, Luciane M Storti-Melo<sup>1\*</sup>, Gustavo C Cassiano<sup>1</sup>, Vanja SCA, Couto<sup>2</sup>, Álvaro ARA Couto<sup>2</sup>, Irene S Soares<sup>3</sup>, Luzia H Carvalho<sup>4</sup>, Maristela G. Cunha<sup>5</sup> Marinete M Póvoa<sup>6</sup>, Sócrates Herrera<sup>7</sup>, Myrian Arévalo-Herrera<sup>7</sup>, Andréa RB Rossit<sup>8</sup>, Claudia MA Carareto<sup>1</sup> and Ricardo LD Machado<sup>8</sup>

\*These authors contributed equally to this work

§Corresponding author

<sup>1</sup>Departamento de Biologia, Universidade Estadual Paulista “Júlio Mesquita Filho”, São José do Rio Preto, São Paulo State, Brazil,

<sup>2</sup>Faculdade SEAMA, Macapá, Amapá State, Brazil,

<sup>3</sup>Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo State, Brazil,

<sup>4</sup>Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais State, Brazil,

<sup>5</sup>Universidade Federal do Pará, Instituto de Ciências Biológicas, Belém, Pará State, Brazil.

<sup>6</sup>Instituto Evandro Chagas, MS/SVS, Ananindeua, Pará State, Brazil.

<sup>7</sup>Instituto de Imunologia, Universidade Del Valle, Cali, Colômbia,

<sup>8</sup>Centro de Investigação de Microrganismos, Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto, São Paulo State, Brazil.

Email addresses:

WCSN: wanejan@yahoo.com.br

LMSM: stortilu@yahoo.com.br

GCC: gcapatti@hotmail.com

VSCAC: vanjacouto@seama.edu.br

AARAC: alvarocouto@seama.edu.br

ISS: isoares@usp.br

LHC: lhcarvalho@cpqrr.fiocruz.br

MGC: mgcunha@ufpa.br

MMP: marinetepova@iec.pa.gov.br

SH: sherrera@inmuno.org

MAH: marami@inmuno.univalle.edu.co

ARBR: andrea@famerp.br

CMAC: carareto@ibilce.unesp.br

RLDM: ricardomachado@famerp.br

## Abstract

### Background

*P. vivax* circumsporozoite variants have been identified in several regions of the world. The real implication of the genetic variation in this region of the *P. vivax* genome has been questioned for a long time. Although previous studies of our group have observed significant association between VK210 and the Duffy blood group, we present here that evidences of this variation are limited to the CSP central portion.

### Methods

The phylogenetic analyses were accomplished starting from the amplification of conserved domains of *18 SSU RNAr* and *Cyt B*. The antibodies responses against the CSP peptides, MSP-1, AMA-1 and DBP were detected by ELISA, in plasma samples of individuals infected with two *P. vivax* CS genotypes: VK210 and *P. vivax*-like.

### Results

The analyses of the two markers demonstrate high similarity among the *P. vivax* CS genotypes and surprisingly showed diversity equal to zero between VK210 and *P. vivax*-like, positioning these CS genotypes in the same clade. We found a high frequency IgG antibody against the N- and C-terminal regions of the *P. vivax* CSP compared to the immune response to the R- and V- repetitive regions ( $p=0.0005$ , Fisher's Exact test). This difference was more pronounced when the *P. vivax*-like variant was present in the infection ( $p=0.003$ , Fisher's Exact test). A high frequency of antibody response against MSP-1 and AMA-1 peptides was observed for all *P. vivax* CS genotypes in comparison to the same frequency for DBP.

### Conclusions

Our results target that the differences among the *P. vivax* CS variants are restrict to the central repeated region of the protein, mostly nucleotide variation with important serological consequences.

## Background

The circumsporozoite surface protein (CSP) is the most abundant polypeptide present in the sporozoite covering. This protein is involved in the motility and invasion of the sporozoite during its entrance in the hepatocyte [1, 2].

Some years ago, CSP was studied as the main goal for antimalarial vaccine development; however the existence of variations in the repetitive sequence of its central portion has been hindering these studies. *P. vivax* CSP sequences analyses revealed that parasites have repeats belonging to one of two types of nonapeptide repeat units, GDRA(A/D)GQPA or ANGA(G/D)(N/D)QPG, named VK210 or VK247 respectively [3, 4]. In 1993, a new human malaria parasite from a *P. vivax*-infected person was identified by Qari et al. who named it *P. vivax*-like. The CSP sequence of *P. vivax*-like has an 11-mer repeat sequence, APGANQ(E/G)GGAA, and is different to the two previously described variants [5, 6].

All *P. vivax* CS genotypes have a worldwide distribution and have been identified for several authors [7-17]. In Brazil, the occurrence of the three genotypes in pure and mixed infections was described [11, 17]. Seroreactivity tests have identified the presence of three variant genotypes in samples from the State of São Paulo [10, 16] and in indigenous populations [8, 9] and other communities of the Amazon region [13]. Studies have also reported differences in the infectivity of anophelines to the variant genotypes, indicating that *Anopheles darlingi* and *An. pseudopunctipennis* were more susceptible to the infection by VK210 [18, 19]. These findings could be a consequence of differences in the

emergence of this genotype in specific geographical regions or suggest that the VK210 genotype is the best adapted variant in the world [11].

The successful of the vaccine against malaria can be related to the immunological intervention in the development of the parasite in the human host or mosquito vector. To improve the health and quality of more than 1 billion people around the world, several efforts have been addressed for the identification and antigenic characterization of different *P. vivax* antigens, among these the preerythrocytic antigens such as circumsporozoite protein (CSP) [20], the blood-stage proteins as merozoite surface protein 1 (MSP-1) [21, 22], apical membrane antigen 1 (AMA-1) [22, 23], and the Duffy binding protein (DBP), an merozoite antigen that interacts with the Duffy blood group in the host cells surface [22, 24]. Currently, several authors have considered the CSP of *P. vivax* as the major target for the development of recombinant malaria vaccines, since the synthetic peptides starting from this protein induce a high and specific humoral response as the induced by natural exposure of humans to malaria [25-31]. Moreover, starting from the description of the *P. vivax* CS genotypes, VK210, VK247 and *P. vivax*-like, several studies proposed the existence of differences among those that seem to go besides variations in the repetitive portion of the protein, as geographical distribution, transmission intensity, vectorial competence, immune and treatment responses and drug resistance [11, 18, 19, 32-34].

Many studies are being conducted to better understand the age and origin of the *P. vivax* as a human parasite [35, 36]. Low microsatellite and tandem repeat variability indicate that *P. vivax* infected humans recently (10,000 years

ago) [37]. Indeed, a different study based on polymorphisms of two nuclear and one mitochondrial gene places this parasite origin between 45,000 and 81,000 years ago [35]. In addition, *P. vivax* seems to be related to the clade of parasites found in Asian cercopithecines, indicating its origin in Asia via a host-switch from parasites found in non-human primates such as macaques [35, 36, 38]. Little is known about the characterization of the *P. vivax* variants, since analyses of the non-repetitive portion of *CS* gene showed that these genotypes belong to a same clade, including several types of primate *Plasmodium* species [35, 39]. Nevertheless, the important question remains whether the *P. vivax CS* repeated region is a limited, mostly silent base variation [40] or if these variants represent the existence of a new species or subspecies of *Plasmodium* causing human malaria, with major biological consequences [6].

Here, we contribute to the understanding of the implication of the central repetitive region variation of the CSP in the *P. vivax* genome by phylogenetic tools and to the evaluation of the humoral immune response against different parasite antigens.

## Methods

### Subjects

After given written informed consent, peripheral blood samples were drawn from malaria patients living in four Brazilian Amazon endemic areas (Macapá, Amapá State; Novo Repartimento, Pará State; Porto Velho, Rondônia State and Plácido de Castro, Acre State). All individuals enrolled in this study

complied with the following criteria: they sought medical assistance for clinical malaria symptoms, were over 18 years old and had a positive malaria diagnosis by thick blood film for *P. vivax*. The genomic DNA was extracted by the phenol–chloroform method [41] or using a commercially available kit (Easy-DNA™, Invitrogen, USA), and a semi-nested PCR was performed using *P. vivax*-specific small-subunit (SSU) rDNA primers to confirm the *Plasmodium* diagnosis [42]. The *P. vivax* CS genotypes were determined as described by Alves et al. [43].

## **Molecular analyses**

For the phylogenetic reconstruction, a subset of the *P. vivax* field samples was used and data for non human *Plasmodium* spp. samples were obtained from GenBank. Natural hosts type, geographic origins and GenBank accession numbers of the out groups are described in Table 1. All amplification reactions were performed in a thermocycler (DNA MasterCycler; Eppendorf, USA). The PCR-amplified products were purified by using GFX (GE Healthcare, United Kingdom) and EXOSAP (USB, USA) PCR purification kits, according to the manufacturer's protocol. DNA sequencing was performed using the Big Dye™ Terminator V3.1 Cycle Sequencing kit on ABI 3100 Genetic Analyzer (Applied Biosystems, USA).

## **Amplification of the molecular markers**

### ***18 small sub unit ribosomal RNA (SSUrRNA) gene analyses***

The amplification of a target area between variable regions 7 and 8 of the *18 SSU rRNA* gene from *P. vivax* was designed as described by Santos-

Ciminera et al. [44]. PCR was performed using the primer pairs VAR1 (5'- CTT GGA TGG TGA TGC ATG GCC - 3') and VAR2 (5'- ATC TTT CAA TCG GTA GGA GCG AC - 3'). The reaction mixture contained buffer 10mM Tris-HCl with pH 8.3, 50 mM KCl, 200  $\mu$ M of each of the four dNTPs, 10  $\mu$ M of each oligonucleotide primer, 1  $\mu$ g DNA template and 0.5 U of *ampli-Taq* DNA polymerase (Invitrogen, USA) to a final volume of 25  $\mu$ L. All amplification cycles included to an initial cycle of 95 °C for 15 min, followed by 30 cycles of 94 °C for 1 min, 68°C for 1 min, and 72 °C for 1 min, then a final extension at 72 °C for 10 min.

### **Cytochrome B gene analyses**

The *cytochrome B* (*Cyt B*) sequences were amplified by PCR using sets of primers: PC1 (5'- GCTACAGGTGCATCTCTTGTATTC - 3') and PC2 (5' – CACTTACAGTATATCCTCCACATAACCA - 3'). A reaction mixture of buffer 10mM Tris-HCl, pH 8.3, 50mM KCl, 200  $\mu$ M of each of the four dNTPs, 10  $\mu$ M of each oligonucleotide primer, 1  $\mu$ g DNA template and 0.5 U of *ampli-Taq* DNA polymerase (Invitrogen, USA). The amplification conditions were as follows: first, 1 min at 94°C, followed by 30 cycles with 0.5 min of denaturation at 94°C, annealing at 40°C for 0.5 min and elongation at 72°C for 1.5 min. After 30 cycles, a final elongation step at 72°C for 3 min was carried out. The agarose gels were stained with ethidium bromide and analyzed with a Gel Doc 2000 illuminator (Bio-Rad Laboratories, USA).

### **Sequence alignment and phylogenetic analyses**

The sequences were edited and aligned with the programs MEGA (version 4.1) and BioEdit Sequence Alignment Editor by the CLUSTAL W tool. Phylogenetic analyses were performed with neighbor-joining (NJ), using the program MEGA (version 4.1), with  $p$  distance which takes into account the possibility of high bias in the transition/transversion and in G+C content, derived of the position of the first, second and third codon [45]. The reliability of the NJ trees is assessed by the bootstrap method with 500 replications [46].

### **Assessment of the serological response against *P. vivax* CS genotypes in the current infections**

IgG antibodies against four CSP peptides (N-terminal [N] and C-terminal [C], repetitive region corresponding to the VK210 [R] and repetitive region corresponding to the VK247 [V]) [47], MSP-1 N-terminal fragment (*rPv200L*) [48], recombinant peptide of the AMA-1 [49] and of DBP region II [50] were detected by ELISA (enzyme-linked immunosorbent assay), in plasma samples in infected individuals with *P. vivax* CS genotypes.

### **Statistical analysis**

The serological data were performed using R version 2.8.1 statistical software (The R Foundation for Statistical Computing, Vienna, Austria [<http://www.r-project.org>]). Differences among the frequencies of responders

were analyzed using Pearson's chi-square or, alternatively, the Fisher's exact test. Differences were considered significant when  $p\text{-value}\leq 0.05$ .

## Results

### Phylogeny of *P. vivax* CS genotypes, VK210 and *P. vivax*-like

The analyses of the two markers show high similarity among the *P. vivax* CS genotypes, with nucleotide diversity equal to zero ( $p=0.224$ , t Student's test), positioning the genotypes VK210 and *P. vivax*-like in the same clade (Figure 1 and 2). The genetic distances between CS genotypes from the *Plasmodium* species analyzed are described in Tables 2 and 3. The blood samples infected with VK247 genotypes were not included in this study, because of the reduced numbers of VK247 samples ( $n=4$ ) and low quality of the material.

### Antibody response against the CSP, MSP-1, AMA-1 and DBP peptides

We found a higher frequency IgG antibody against the N- and C-terminal regions of the *P. vivax* CSP compared to the immune response to the R- and V- repetitive regions ( $p=0.0005$ , Fisher's exact test). Antibody responses against the peptides of the CSP repetitive central region [R] and [V] compared displayed a lower frequency against the [V] peptide, which corresponds to the central region of the VK247 variant, in individuals with VK210 genotype ( $p<0.005$ , Fisher's exact test). When *P. vivax*-like genotype was present in the infection a lower antibody response against [R] and [V] peptides was observed

( $p=0.003$ , Fisher's exact test). None other significant association was found with de *CS* genotypes in the infection (Table 4).

A high frequency of antibody response against MSP-1 and AMA-1 peptides was observed for all *P. vivax CS* genotypes in comparison to the same frequency for DBP. A high frequency of antibody response against MSP-1 (*rPv200L*) and AMA-1 peptides was observed for all the *P. vivax CS* genotypes (Table 5) in comparison to same frequency for DBP (*rII*) ( $p=0.003$ , Fisher's exact test). However, significant differences were not observed among the immune responses of individuals infected with the *P. vivax CS* genotypes for none of the analyzed peptides.

## Discussion

Starting from the description of the *P. vivax CS* genotypes, VK210, VK247 and *P. vivax*-like, several studies proposed the existence of differences among those that seem to go besides variations in the repetitive portion of the protein, as geographical distribution, transmission intensity, vectorial competence, immune and treatment responses and drug resistance [11, 18, 19, 32-34]. The real implication of the genetic variation in this region of the *P. vivax* genome has been questioned for a long time. Although previous studies of our group have observed significant association between VK210 and the Duffy blood group [17], we present here that evidences of this variation are limited to the CSP central portion.

Studies based on molecular marker analysis represent an important tool for the phylogenetic characterization of malaria parasites. Similarities

between *P. vivax*-like and *P. simiovale* have been reported in phylogenetic studies with conserved domains of the *CS* gene and, some authors suggested that this variant genotype could be a subspecies or a new species [6]. However, previous phylogenetic studies were designed with the *CS* gene as the only molecular marker in a way that prevents an explanation on the evolutionary relationship among the three *CS* genotypes as well as its relativity to other primate parasites that possess molecular similarities with *P. vivax* [35]. Our results were obtained through the phylogenetic analysis of the *18 SSU RNAr* and *Cyt B Plasmodium* spp. recognized markers and surprisingly showed diversity equal to zero between both *P. vivax CS* genotypes, VK210 and *P. vivax*-like. This analyses positioned VK210 and *P. vivax*-like as members of the same clade, in accordance with previous data [35]. Although the absence of VK247 genetic sequences, a limitation of the present study that we recognize, our results points to the fact that *P. vivax CS* genotypes merely represent markers of intra-specific genetic variations. Supporting the above mentioned hypothesis, the evaluation of the serological response profile against the different parasite peptides corroborates the idea that this variation is restricted to central portion of CSP, once significant associations were not observed between the presence of certain genotype and frequency of the antibodies responses against the three analyzed merozoite peptides, MSP1 (*Pv*200L), AMA-1, DBP (rII) and against the CSP conserved fractions in the esporozoite, N-terminal and C-terminal. Besides, when we evaluated the antibody responses against the peptides corresponding to the CSP repetitive central region, significant associations were detected against the

peptides [R] and [V], which corresponds to the protein sequences of VK210 and VK247 genotypes, respectively. In individuals infected by the VK210 genotype, a lower antibody response against [V] was observed whereas in those infected by the *P. vivax*-like genotype we observed an even lower antibody response against these two fragments ([R] and [V]). Once VK210 represents the classic *P. vivax* CS variant form and also the most prevalent in all Brazilian endemic areas [17], these results were expected. Moreover, the repeated region of the *P. vivax*-like CS is the most genetically distinct compared to the other variants [5, 6].

The report that variations in the central repetitive portion of CSP does not provide significant differences in antibody responses against *P. vivax* merozoite and esporozoite conserved regions peptides represents key information in the future design of vaccine assays. On the other hand, studies based in CSP should be consider the influences of this variation in the modulation of the epidemiology and to consider the use of chimerical constructs including the sequences of the different CS genotypes in order to obtain a vaccine indeed protecting.

## Conclusion

Our results target that the differences among the *P. vivax* CS variants are restrict to the central repeated region of the protein, mostly nucleotide variation with important serological consequences. This variation can represent intra-specific biological signatures that must be considered for *P. vivax* CSP malaria vaccine trial.

## Authors' contributions

WCS carried out the molecular genetic studies, participated in the sequence alignment, phylogenetics analyses and drafted the manuscript. LMSM carried out the immunoassays and participated in the design of the study and performed of the statistical analysis part. ISS, LHC and MGC designed serological experiments and provided reagents. GCC, VSCAC, ISS, LHC, SH, MAH, ARBR critically revised the manuscript. CMAC participated in the sequence alignment and phylogenetics analyses. RLDM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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**Table1.** Hosts type, geographic origins and GenBank accession numbers of the out groups.

<b>Parasite</b>	<b>Natural Host</b>	<b>Geographic Origin</b>	<b>Número de acesso</b>
<i>P. ovale</i>	Humans	Africa, Nigeria	L48987.1
<i>P. berghei</i>	<i>Grammomys surdaster</i>	Katanga, Congo	M14599.1
<i>P. falciparum</i> (1)	Humans	Thailand	M99416.1
<i>P. falciparum</i> (2)	Humans	Thailand	NC_002375.1
<i>P. fragile</i>	<i>Macaca radiat, M. sinisca</i>	South of India, Sri Lanka	NC_012369.1
<i>P. berghei</i>	<i>Grammomys surdaster</i>	Katanga, Congo	EU254525.1
<i>P. ovale</i>	Humans	Africa, Nigeria	FJ409567.1
<i>P. knowlesi</i>	Old World Monkeys	Asia, Africa	NC_007232
<i>P. simiovale</i>	<i>M. sínica</i>	Sri Lanka	AB434920

**Table 2.** Genetic distances between 18 SSU RNAr genes from *Plasmodium* spp.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23]	
[1]																								
[2]	0.000																							
[3]	0.000	0.003																						
[4]	0.000	0.000	0.003																					
[5]	0.000	0.003	0.000	0.003																				
[6]	0.000	0.000	0.000	0.000	0.000																			
[7]	0.000	0.000	0.003	0.000	0.003	0.000																		
[8]	0.000	0.003	0.000	0.003	0.000	0.000	0.003																	
[9]	0.000	0.003	0.000	0.003	0.000	0.000	0.003	0.000																
[10]	0.000	0.000	0.003	0.000	0.003	0.000	0.000	0.000	0.003															
[11]	0.000	0.003	0.000	0.003	0.000	0.000	0.003	0.000	0.000	0.003														
[12]	0.000	0.000	0.003	0.000	0.003	0.000	0.000	0.000	0.003	0.003	0.000													
[13]	0.000	0.000	0.003	0.000	0.003	0.000	0.000	0.000	0.003	0.003	0.000	0.003												
[14]	0.000	0.000	0.003	0.000	0.003	0.000	0.000	0.000	0.003	0.003	0.000	0.003	0.000											
[15]	0.000	0.000	0.003	0.000	0.003	0.000	0.003	0.000	0.000	0.003	0.003	0.000	0.003	0.000										
[16]	0.000	0.000	0.003	0.000	0.003	0.000	0.003	0.000	0.000	0.003	0.003	0.000	0.003	0.000	0.000									
[17]	0.000	0.000	0.003	0.000	0.003	0.000	0.003	0.000	0.000	0.003	0.003	0.000	0.003	0.000	0.000	0.000								
[18]	0.000	0.000	0.003	0.000	0.003	0.000	0.003	0.000	0.000	0.003	0.003	0.000	0.003	0.000	0.000	0.000	0.000							
[19]	0.000	0.000	0.003	0.000	0.003	0.000	0.003	0.000	0.000	0.003	0.003	0.000	0.003	0.000	0.000	0.000	0.000	0.000						
[20]	0.000	0.000	0.003	0.000	0.003	0.000	0.003	0.000	0.000	0.003	0.003	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000					
[21]	0.000	0.003	0.000	0.003	0.000	0.000	0.003	0.000	0.000	0.003	0.000	0.003	0.000	0.003	0.000	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
[22]	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183
[23]	0.129	0.131	0.134	0.131	0.134	0.132	0.131	0.134	0.134	0.131	0.134	0.131	0.134	0.131	0.134	0.131	0.131	0.131	0.131	0.131	0.131	0.131	0.134	0.212

The end 01 and 03 are corresponding of the VK210 and *P. vivax*-like genotypes, respectively. 1. 537C-01, 2. 883C-01, 3. 397C-01, 4. 542C-01, 5. 528C-01, 6. 892C-01, 7. 176C-01, 8. 531C-01, 9. 200C-01, 10. 889C-03, 11. 886C-03, 12. 888C-03, 13. 879C-03, 14. 716C-03, 15. 872C-03, 16. 337C-03, 17. 877C-03, 18. 891C-03, 19. 885C-03, 20. 875C-03, 21. 128C-03, 22. *P. berghei*, 23. *P. ovale*.



**Table 4.** Frequency of antibody response against CSP peptides in the infections with the *P. vivax* CS genotypes.

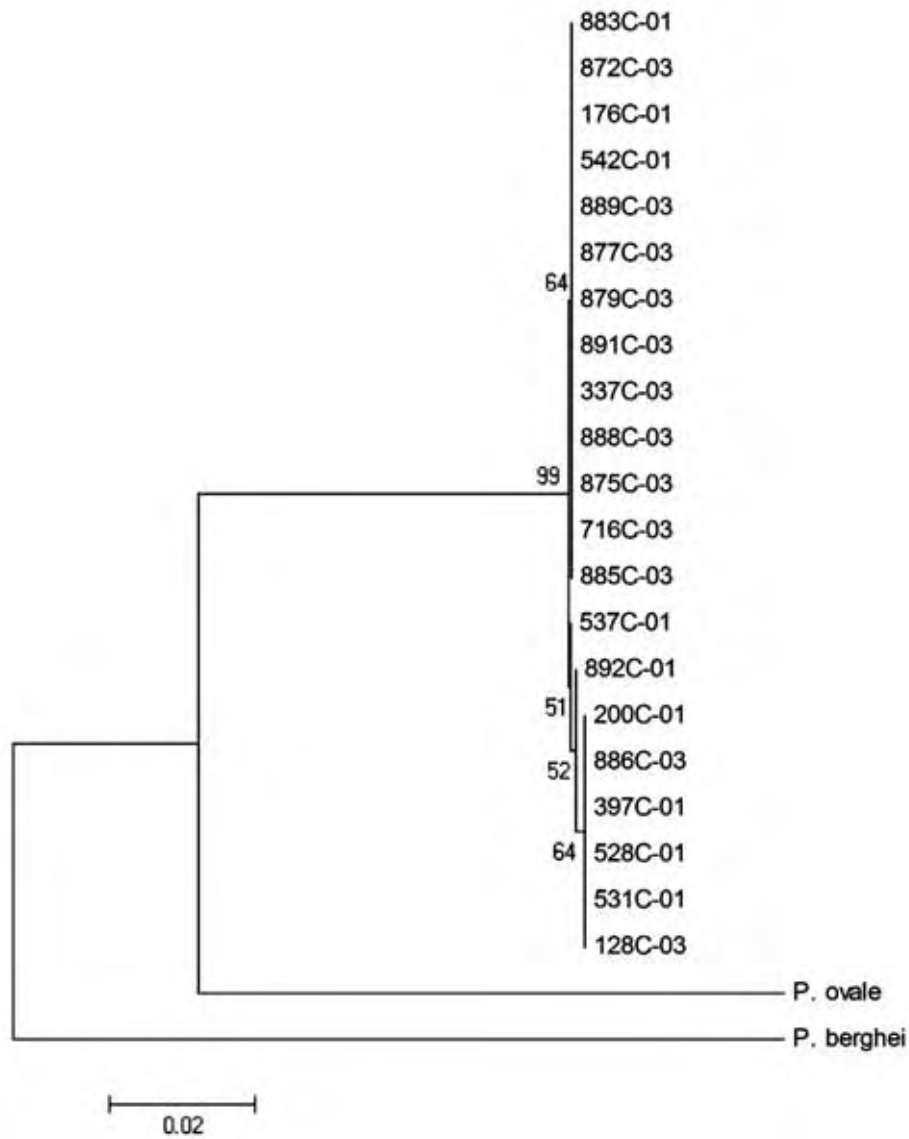
Peptides analyzed	CS genotypes present in the current infection (n)						Total
	1	2	3	1+2	1+3	1+2+3	
	(34)	(4)	(18)	(12)	(8)	(4)	(80)**
[N] - terminal	85,3	100	77,8	75	100	75	83,7
[C] - terminal	73,5	100	88,9	100	75	100	83,7
[R] - VK 210	70,6	100	38,9*	100	87,5	75	62,5
[V] - VK 247	41,1*	75	55,6*	83,3	50	50	50

\*p=0.003, Fisher's Exact test. \*\*p=0.0005, Fisher's Exact test. 1: VK210; 2: VK247; 3: *P. vivax*-like.

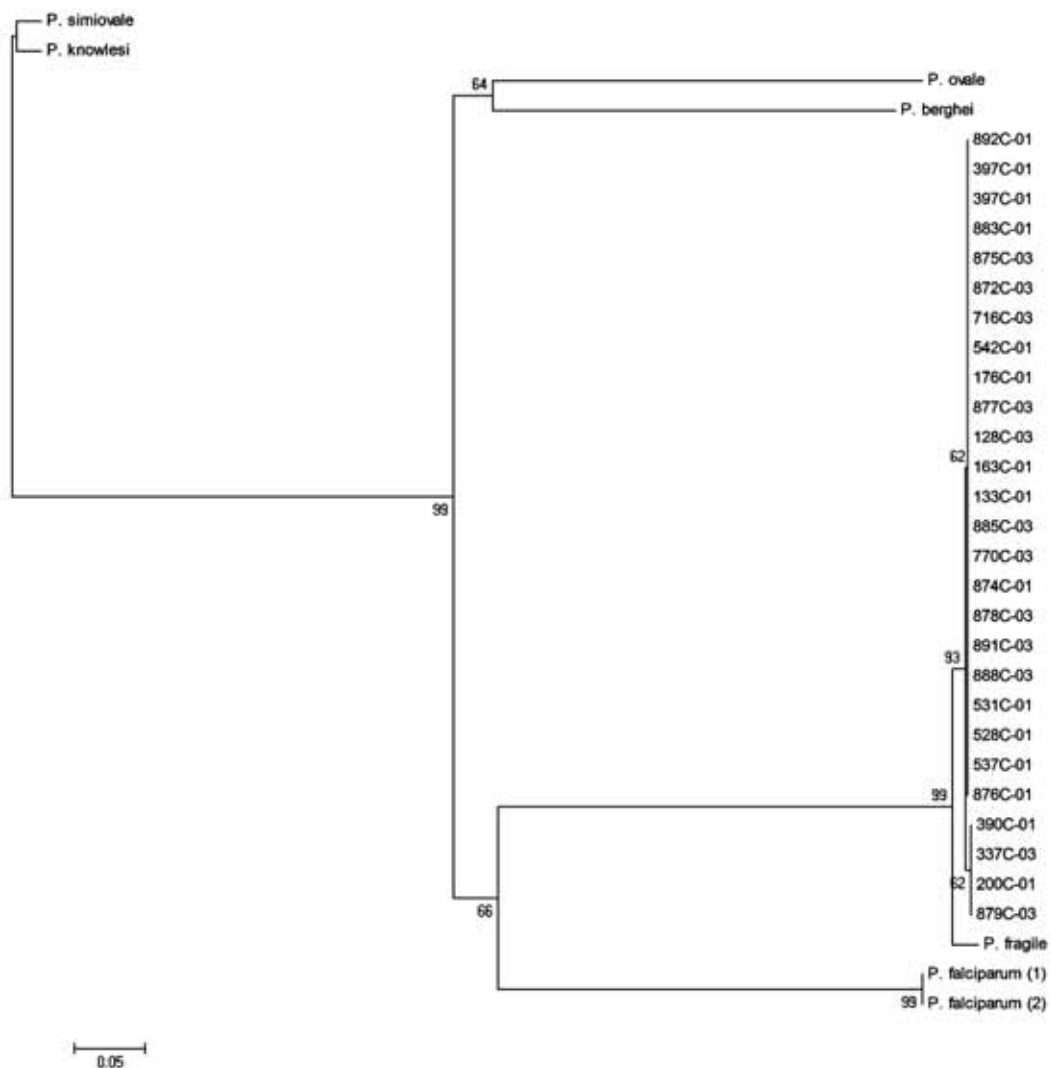
**Table 5.** Frequency of antibody response against merozoite antigens in the infections with the *P. vivax* CS genotypes.

Peptides analyzed	CS genotypes present in the current infection (n)						Total
	1	2	3	1+2	1+3	1+2+3	
	(51)	(4)	(23)	(14)	(8)	(4)	(104)
<i>Pv</i> 200L	92.2	100	78.2	100	87.5	100	90.4
	(39)	(4)	(18)	(13)	(8)	(4)	(86)
AMA-1	92.3	75	94.4	100	87.5	75	91.9
rII-DBP	41	50	33.3	38.5	50	50	40.7*

\*p=0,003 Fisher's Exact test. 1: VK210; 2: VK247; 3: *P. vivax*-like.



**Figure 1.** Neighbor-joining tree of the 18 SSU rRNA gene based in  $p$  distance, including transitions and transversions. The numbers are bootstrap percent values based on 500 replications. The end 01 and 03 are corresponding of the VK210 and *P. vivax*-like genotypes, respectively.



**Figure 2.** Neighbor-joining tree of the *Cyt B* gene based in  $p$  distance, including transitions and transversions. The numbers are bootstrap percent values based on 500 replications. The end 01 and 03 are corresponding of the VK210 and *P. vivax*-like genotypes, respectively.

## **7 DISCUSSÃO GERAL**

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Os resultados obtidos do nosso estudo evidenciaram que o genótipo variante VK210 foi detectado tanto em infecção simples quanto mista, em todas as áreas estudadas. Nossos dados também mostraram que nas amostras provenientes de Novo Repartimento, todos os três genótipos da CSP estiveram presentes em infecções mistas e simples, diferente do que foi anteriormente reportado na literatura, demonstrando a presença dos genótipos VK247 e *P. vivax-like* apenas em infecções mistas (MACHADO & PÓVOA, 2000; MACHADO et al., 2003). Interessantemente, após dez anos, observa-se uma alteração do panorama de distribuição dos genótipos variantes da CSP, no Brasil. A variante VK210 continua sendo a mais prevalente em todas as áreas endêmicas da Amazônia já analisadas. Entretanto, com relação à distribuição da VK247 e *P. vivax-like*, nossos resultados sugerem uma mudança na adaptação desses genótipos, visto que essa é a primeira detecção de ambas as formas do parasito em infecção simples no Brasil. Nossos resultados podem indicar que a VK247 ainda não esteja totalmente adaptada no nosso país, assim como observado em estudos prévios na Colômbia (GONZÁLES et al., 2001), no México e na Ásia (KAIN et al., 1992; 1993a; 1993b; QARI et al., 1992). O fato de que a VK247 foi encontrada somente em infecções simples em amostras de Novo Repartimento, no Estado do Pará, pode sugerir uma mais recente introdução desta variante nos demais Estados. Os resultados deste estudo também demonstram que o *P. vivax-like* está amplamente distribuído em todas as áreas estudadas quando comparamos com os dados observados nos indivíduos infectados com VK247. Observa-se, portanto, uma distribuição mais homogênea deste genótipo, tanto em infecção simples quanto mista, sugerindo que sua adaptação possa estar acontecendo num processo mais acelerado do que a VK247.

A partir da descrição dos genótipos variantes da CSP de *P. vivax*, VK210, VK247 e *P. vivax-like*, vários estudos têm proposto a existência de diferenças entre esses genótipos que parecem ir além de variações na porção repetida da proteína, como distribuição geográfica, intensidade de transmissão,

competência vetorial, resposta imune, resposta ao tratamento e, resistência a fármacos (BRANQUINHO et al., 1997; MARRELLI et al., 1998; GÓNZALES e CERON, 1999; MACHADO e PÓVOA, 2000; MACHADO et al., 2003; DA SILVA et al., 2006). A real implicação desta variação no genoma de *P. vivax* permaneceu questionável por longo tempo. Embora estudos prévios do nosso grupo tenham observado associação significativa entre a VK210 e o sistema sanguíneo Duffy (STORTI et al., 2009), nós apresentamos aqui evidências de que essa variação esteja limitada à porção central da CSP.

Estudos baseados na análise de marcadores moleculares representam uma importante ferramenta para a caracterização filogenética dos parasitos de malária. Similaridades entre *P. vivax-like* e *P. simiovale* têm sido relatadas em estudos filogenéticos baseados em domínios conservados do gene da CS e, alguns autores têm sugerido que este genótipo variante pudesse ser uma subespécie ou uma nova espécie (QARI et al., 1993b). Entretanto, a utilização do gene da CS, como único marcador molecular, não é suficiente para esclarecer a possível relação evolutiva existente entre os três tipos variantes, bem como a distância genética destes com parasitos de outros primatas (ESCALANTE et al., 1995). Nossos resultados foram obtidos por meio da análise filogenética dos marcadores *18 SSU RNAr* e *Cyt B* e, indicam que os genótipos variantes da CSP de *P. vivax*, VK210 e *P. vivax-like*, não apresentam nenhum grau de diversidade nucleotídica. Nossas análises mostraram que tanto VK210 quanto *P. vivax-like* pertencem a um mesmo clado e, estando de acordo com dados prévios descritos na literatura (ESCALANTE et al. 1995). Apesar de não termos incluídos análises com a seqüência da VK247, uma limitação reconhecida, estes resultados sugerem que esses genótipos representam apenas marcadores de uma variação gênica intra-específica.

Suportando essa hipótese, a avaliação do perfil de resposta sorológica contra os diferentes peptídeos do parasito corrobora a idéia de que esta variação esteja restrita à CSP, uma vez que não foram observadas associações significativas entre a presença de determinado genótipo e freqüência da resposta de anticorpos contra os três peptídeos do merozoíto analisados, MSP1 (*Pv200L*),

AMA-1 e DBP (rII) e bem como, contra as frações conservadas da CSP no esporozítio, N-terminal [N] e C-terminal [C]. Quando avaliamos a resposta de anticorpos contra os peptídeos correspondentes à região central repetida da CSP, os resultados indicaram associações significativas na resposta de anticorpos contra os peptídeos [R] e [V], os quais correspondem à seqüência peptídica das variantes VK210 e VK247 respectivamente. Nos indivíduos infectados pelo genótipo VK210 uma menor resposta de anticorpos contra o peptídeo [V] foi observada. Nas amostras infectadas pelo genótipo *P. vivax-like* observou-se menor resposta de anticorpos contra os dois fragmentos. Estes resultados seriam esperados, uma vez que a VK210 é a forma clássica e a variante mais prevalente em todas as áreas endêmicas estudadas da Amazônia brasileira (STORTI-MELO, et al, 2009). Além disso, a região repetida da CSP de *P. vivax-like* é mais geneticamente diferente das outras duas variantes.

O fato das variações na porção repetida central da CSP não apresentarem diferenças significativas na resposta de anticorpos contra os peptídeos do merozoítio bem com as regiões conservadas do esporozoítio, trazem informações promissoras nos ensaios de vacinação utilizando esses peptídeos. Por outro lado, estudos embasados na CSP devem considerar a influência dessa variação na modulação da epidemiologia e considerar a utilização de constructos quiméricos incluindo as seqüências dos diferentes genótipos da CS a fim de se obter uma vacina efetivamente protetora (YADAVA et al., 2007; BELL et al., 2009).

Diante da ausência de diversidade nucleotídica entre VK210 e *P. vivax-like* para os marcadores analisados e, da falta de associação entre resposta sorológica contra os peptídeos envolvidos tanto no estágio eritrocítico quanto extra-eritrocítico do parasito, podemos sugerir que as variações descritas previamente na CSP representem apenas uma diversidade intra-específica, restrita a esse domínio, caracterizando diferentes cepas circulantes do *Plasmodium vivax* na Amazônia brasileira.

## **8 CONCLUSÕES**

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## 8 Conclusões

O presente trabalho permitiu estabelecer as seguintes conclusões:

1. A variante VK210 continua sendo a mais prevalente em todas as áreas endêmicas da Amazônia já analisadas, entretanto as variantes VK247 e *P. vivax-like* também foram identificadas em infecções simples Brasil.
2. Os genótipos variantes da CSP de *P. vivax*, VK210 e *P.vivax-like*, não apresentam nenhum grau de diversidade nucleotídica e, são pertencentes ao um mesmo clado.
3. Os genótipos variantes da CSP de *P. vivax* não foram associados a frequência da resposta de anticorpos contra os peptídeos do merozoíto, bem como, contra as frações conservadas da CSP no esporozíto analisadas.
4. As variações na porção repetitiva central da CSP apresentam-se restritas a esse domínio, entretanto esta parece modular a epidemiologia do *P. vivax*, caracterizando diferentes cepas circulantes deste parasito na Amazônia brasileira.

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## 9 Referências Bibliográficas

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## 10 Anexos

## 10.1 Termo de aprovação do Comitê de Ética em Pesquisa (CEP)

	<b>FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO</b> Autarquia Estadual - Lei n.º 8899 de 27/09/94 (Reconhecida pelo Decreto Federal n.º 74.179 de 14/06/74)
<b>Parecer n.º 223/2006</b>	
<b>COMITÊ DE ÉTICA EM PESQUISA</b>	
<p>O Protocolo n.º 3861/2006 sob a responsabilidade de Wanessa Christina de Souza Neiras com o título "Marcadores moleculares do <i>P. vivax</i> associados a genótipos da proteína circunsporozóitica", está de acordo com a Resolução CNS 196/96 e foi aprovado por esse CEP.</p>	
<p>Lembramos ao senhor(a) pesquisador(a) que, no cumprimento da Resolução 251/97, o Comitê de Ética em Pesquisa em Seres Humanos (CEP) <b>deverá receber relatórios semestrais sobre o andamento do Estudo</b>, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, para conhecimento deste Comitê. <b>Salientamos ainda, a necessidade de relatório completo ao final do Estudo.</b></p>	
São José do Rio Preto, 10 de julho de 2006.	
 <b>Prof. Dr. Antonio Carlos Fires</b> Coordenador do CEP/FAMERP	
<small>Av. Brigadeiro Faria Lima, 5416 - 15090-000 - São José do Rio Preto - SP - Brasil Tel. (17) 3201-5700 - Fax (17) 3227-6201 - <a href="http://www.famerp.br">www.famerp.br</a></small>	

## 10.2 Técnicas de biologia molecular empregadas no estudo

### 10.2.1 Extração de DNA

O DNA genômico foi obtido de leucócitos do sangue periférico de pacientes maláricos, segundo protocolo descrito por Pena et al. (1991), com modificações. Em um tubo de polipropileno contendo 1 mL de solução de lise 1 (sacarose 0,32M; tris-HCL 10 mM; MgCl<sub>2</sub>; triton 1% 100x; água Mili-Q autoclavada q.s.p. 100 mL) foram adicionados 300 µL de sangue periférico, colhidos em EDTA. Após a homogeneização, o tubo foi centrifugado a 6.500 rpm durante 5 minutos e, desprezado o sobrenadante, deixando aproximadamente 300 µL do precipitado. Em seguida, foi adicionado ao tubo 1 mL de solução de lise 1, sendo este homogeneizado até o precipitado ser desfeito e colocado em repouso por 5 minutos. O tubo foi centrifugado novamente a 6.500 rpm por 5 minutos e o sobrenadante foi descartado. O precipitado foi dissolvido em 450 µL de solução de lise 2 (NaCl 0,075 M; EDTA 0,02 M pH 8.0; água Mili-Q autoclavada q.s.p. 500 µL), 25 µL de SDS 10% e 5 µL de proteinase K (20 mg/mL), sendo homogeneizado após este procedimento. Feito isso, a amostra ficou incubada em banho-maria por 3 horas a 42°C. Terminado o tempo de incubação, realizou-se a des-proteinização da amostra. Adicionaram-se 500 µL de fenol no tubo que foi homogeneizado por inversão e seguido por uma centrifugação por 5 minutos a 7.000 rpm. Organizou-se uma segunda série de tubos, para os quais foram transferidos os sobrenadantes da primeira série e adicionado 500 µL de clorofórmio/álcool isoamílico (24:1). O tubo foi homogeneizado e submetido a uma nova centrifugação por 5 minutos a 7.000 rpm. Este último procedimento foi repetido mais uma vez. Para a precipitação do DNA, o sobrenadante foi transferido para outro tubo contendo 50 µL de KCl (gelado), onde foram adicionados 500 µL de etanol gelado. O tubo foi invertido várias vezes até precipitar o DNA e, daí centrifugado por 30 segundos a 13000 rpm. O sobrenadante foi descartado e ao tubo foram adicionados 200 µL de etanol 70% gelado. Uma nova centrifugação foi efetuada por 30 segundos a 13.000 rpm.

Cuidadosamente o sobrenadante foi desprezado e o tubo ficou aberto para secagem por 15 minutos. O DNA foi hidratado com 100  $\mu$ L de tampão T.E. (tris-HCl 10 mM; EDTA 1 mM; água Mili-Q autoclavada q.s.p. 100 mL) sendo posteriormente armazenado a 20°C até o momento de uso.

### 10.2.2 Identificação das espécies de *Plasmodium* spp.

Para a confirmação da espécie de *Plasmodium* spp. foram realizadas reações de semi-NESTED-PCR de acordo com a técnica desenvolvida por Kimura et al. (1997). A primeira reação de PCR foi realizada para a identificação do gênero, enquanto que a segunda reação de PCR foi realizada para a identificação das espécies *P. vivax*, *P. falciparum* e *P. malariae*. Foram utilizados os seguintes oligonucleotídeos iniciadores (*primers*): P1 e P2 na primeira reação e, F2, VI e MI na segunda reação (Quadro 1).

Na primeira reação de PCR, se utilizaram 5  $\mu$ L de DNA genômico extraído e, 15  $\mu$ L da mistura de reagentes, descrita no Quadro 2. A solução foi preparada no gelo, sempre homogeneizando cada reagente inserido na solução. Após a adição dos reagentes com o DNA, os tubos foram colocados no termociclador (Eppendorf@Mastercycler, Personal) para a amplificação, como descrito no Quadro 3. Ao término do programa, as amostras foram retiradas do termociclador e os resultados da amplificação foram verificados por meio da corrida eletroforética no gel de agarose 1,5%.

**Quadro 1.** Pares de oligonucleotídeos iniciadores para reação de semi-NESTED-PCR.

Iniciador	Seqüência nucleotídica (5' → 3')	Parasito alvo	Tamanho do produto
P1*	ACGATCAGATACCGTCGTAATCTT	<i>Plasmodium</i> spp.	130 pb
P2	GAACCCAAAGACTTTGATTTCTCAT		
P1	ACGATCAGATACCGTCGTAATCTT	<i>P. falciparum</i>	100 pb
F2	CAATCTAAAAGTCACCTCGAAAGATG		
P1	ACGATCAGATACCGTCGTAATCTT	<i>P. vivax</i>	100 pb
VI	CAATCTAAGAATAAACTCCGAAGAGAAA		
P1	ACGATCAGATACCGTCGTAATCTT	<i>P. malariae</i>	110-115 pb
MI	GGAAGCTATCTAAAAGAAACACTCATAT		

Fonte: Kimura et al. (1997). \* Iniciador Universal. Pb = pares de bases.

**Quadro 2.** Reagentes para a primeira reação do semi-NESTED-PCR.

Reagentes	Concentração final
0,8 µL de P1 (47,9 nmol-Invitrogen) e P2 (48,3 nmol-Invitrogen)	0,4 µM
2 µL de Tampão 10X (Invitrogen)	1X
0,6 µL de MgCl <sub>2</sub> (Invitrogen)	1,5 M
0,25 µL de dNTPs (Pharmacia, Biotech)	125 µM
0,15 µL de <i>Taq</i> polimerase (Biotaq 5 U/1JL- Biotline)	0,75 U
10,4 µL de Água Mili-Q q.s.p.	

**Quadro 3.** Condições de amplificação para primeira reação de SEMI-NESTED-PCR.

Etapa	Nº de ciclos	Condições
Desnaturação inicial	1	92°C por 2 minutos
Amplificação	35	92°C por 30 segundos e 60°C por 90 segundos
Extensão final	1	60°C por 5 minutos

Fonte: Kimura et al. (1997).

Para a realização da segunda reação de PCR, foram preparadas soluções distintas de cada iniciador específico para as espécies. O Quadro 4 ilustra os reagentes utilizados nesta etapa.

**Quadro 4.** Reagentes para a segunda reação do semi-nested-PCR.

Solução I	
Reagentes	Concentração final
0,8 µL de P1 com 0,8 µl VI - <i>P. vivax</i> (38,8 nmol- Invitrogen), F2- <i>P. falciparum</i> (43,3 nmol- Invitrogen) e MI - <i>P.malariae</i> (40,8 nmol – Invitrogen)	0,4 µM
2 µL de Tampão 10X	1X
0,24 µL de MgCl <sub>2</sub>	1,5 mM
0,25 µL de dNTPs	312,5 µM
3,91 µL de água Mili-Q q.s.p.	
Solução II	
Reagentes	Concentração final
0,15 µL de <i>Taq</i> polimerase	0,75 U
1,25 µL Tampão 10X	1,25X
0,24 µL de MgCl <sub>2</sub>	1,5 mM
8,36 µL de água Mili-Q q.s.p.	

Fonte: Kimura et al. (1997)

### 10.2.3 Identificação dos genótipos da CS de *P. vivax*

A identificação dos três genótipos foi realizada pelo método de PCR-RFLP, baseado no protocolo descrito por Alves et al. (2007). A região central do gene da CS foi analisada a partir dos iniciadores: PR1 - 5'ACTTTTATTCGACTTTGTTGGTC 3' e PR2 - 5'ATGGACTCCATGCAGTGTAACC 3'. O DNA genômico (1,5 µL) foi amplificado em um volume total de reação de 25 µL consistindo de tampão 1x (10 mM Tris-HCl, pH 8,3, 50 mM KCl), 1,5 mM de MgCl<sub>2</sub>, 1,0 µM de cada oligonucleotídeo iniciador, 200 µM de dNTPs, 2,5 U de *ampli-Taq* DNA polimerase, betaína 1% e água (25 µL). Vinte e cinco ciclos de amplificação foram realizados em um termociclador (DNA MasterCycler, Eppendorf, USA) após desnaturação inicial do DNA a 94°C por 5 min. Cada ciclo consistiu de uma etapa de desnaturação a 93°C por 60s, uma etapa de anelamento a 41°C por 90s e uma etapa de extensão a 72°C por 2 min, com uma extensão final a 72°C por 10 min seguindo o último ciclo. Os iniciadores favoreceram a formação dos seguintes fragmentos: 694 pb para a VK210; 721 pb para a VK247 e, 736 pb para a *P. vivax-like*. Os produtos de PCR foram analisados em gel de agarose a 1,5 % corado com brometo de etídio e visualizados sob luz ultravioleta.

As enzimas de restrição foram selecionadas a fim de obter, pelo menos, um sítio de restrição na amplificação de cada variante, resultando em fragmentos de DNA que fossem facilmente visíveis em gel de poliacrilamida. A digestão enzimática foi realizada com 10 µL do produto de PCR e 1 U da respectiva enzima selecionada (*Alu* I e *Dpn* I, Promega, USA), em seguida, as reações foram incubadas por 1 h a 37°C.

A enzima *Alu* I formou fragmentos de 10, 27, 38, 54, 106 e 135 pb para a VK210; 38 e 673 pb para a VK247 e um único fragmento de 726 pb para *P. vivax-like*. A digestão com *Dpn* I resultou em fragmentos de 54, 81, 108 e 301 pb para a VK247 enquanto para *P. vivax-like* formou fragmentos de 39 e 697 pb. Esta enzima não apresentou nenhum sítio de corte para a VK210. Os fragmentos de restrição foram separados por eletroforese em gel de poliacrilamida a 12,5%. Os

géis foram corados com prata seguindo o seguinte protocolo: Inicialmente o gel foi colocado em uma solução fixadora (30 mL de etanol 100%, 2,25 mL de ácido acético e 300 mL de água destilada) por 10 minutos. Logo em seguida foi adicionado sobre a solução fixadora, 0,3 g de nitrato de prata diluído em 150 mL de água destilada. Após mais dez minutos, o gel foi lavado rapidamente com água e colocado em uma solução reveladora (4,5 g de NaOH, 450 µl de formaldeído e 150 mL de água destilada), onde permaneceu por mais 15 minutos, sendo novamente lavado com água, permitindo a análise dos fragmentos.

#### 10.2.4 Amplificação do marcador molecular *18 SSU rRNA*

A amplificação da área alvo entre as regiões variáveis 7 e 8 do marcador *18 SSU rRNA* de *P. vivax* foi desenhado como descrito por Santos-Ciminera et al. (2007) . A reação de PCR foi realizada usando os pares de oligonucleotídeos iniciadores VAR1 (5'- CTT GGA TGG TGA TGC ATG GCC - 3') e VAR2 (5'- ATC TTT CAA TCG GTA GGA GCG AC - 3'). A mistura continha tampão 10 mM Tris-HCl com pH 8,3, 50 mM KCl, 200 µM de cada um dos quatro dNTPs, 10 µM de cada oligonucleotídeos iniciadores, 1 µg de DNA molde e 0,5 U de *Taq* DNA polymerase (Invitrogen, USA) em um volume final de 25 µL. Todos os ciclos de amplificação incluíam um ciclo inicial de 95°C por 15 min, seguidos de 30 ciclos de 94°C por 1 min, 68°C por 1 min, e 72 °C por 1 min, e uma extensão final de 72°C for 10 min.

#### 10.2.5 Amplificação do marcador molecular *Cyt B*

As seqüências de *Citocromo B (Cyt B)* foram amplificadas usando o seguinte conjunto de oligonucleotídeos iniciadores: PC1 (5'- GCTACAGGTGCATCTCTTGATTC - 3') e PC2 (5' - CACTTACAGTATATCCTCCACATAACCA - 3'). A reação continha tampão 10 mM Tris-HCl, pH 8,3, 50mM KCl, 200 µM de cada um dos quatro dNTPs, 10 µM de oligonucleotídeos iniciadores, 1 µg de DNA molde e 0,5 U de *Taq* DNA

polymerase (Invitrogen, USA). As condições de amplificação foram as seguintes: primeiro, 1 min a 94°C, seguida por 30 ciclos com 0,5 min de desnaturação a 94°C, anelamento a 40°C por 0,5 min e extensão a 72°C por 1,5 min. Após esses 30 ciclos, um passo de extensão final a 72°C por 3 min foi realizada. Os géis de agarose foram fixados com brometo de etídeo e analisados no equipamento Gel Doc 2000 illuminator (Bio-Rad Laboratories, USA).

### **10.3 Testes imunoenzimáticos para avaliação da resposta de anticorpos contra os peptídeos da CSP, MSP-1, AMA-1 e DBP**

#### **10.3.1 CSP ([N], [C], [R] e [V]):**

A presença de anticorpos para os peptídeos sintéticos da CSP (N-terminal [N], C-terminal [C], região repetitiva correspondente à VK210 [R] e região repetitiva correspondente à VK247 [V] foram verificada por meio do ensaio de imunoabsorção enzimática- ELISA em amostras de plasma de indivíduos infectados pelos genótipos variantes de *P. vivax*. As análises foram realizadas em parceria com o Dr. Sócrates Herrera no Centro Internacional de Vacunas – CIV em Cali/Colômbia, segundo protocolo descrito previamente em Herrera et al., 2004 como segue: as microplacas (Nunc-Immuno Plate, Maxisorp, Roskilde, Dinamarca) foram sensibilizadas com os peptídeos da CSP à concentração de 1 mg/mL em PBS por poço overnight a 4°C. As placas foram então bloqueadas com 200 µL/poço com a solução de 5% de leite desnatado em PBS com pH 7,4 por 2h em temperatura ambiente. Após a lavagem das placas com PBS-Tween20 as amostras foram diluídas na concentração de 1:200 em solução de PBS/2,5% leite desnatado/0,05% Tween-20 em duplicata e incubadas por 1 h em temperatura ambiente. Os anticorpos IgG foram detectados através da incubação com anti-IgG humana conjugada com fosfatase alcalina (Sigma Chemical Co, St Louis, MO), a uma diluição de 1:1000. A atividade enzimática foi medida após incubação de 45 minutos em temperatura ambiente com substrato para-nitrofenil fosfato. Densidade óptica (DO) de cada poço foi medida a 450 nm

em um leitor de microplacas (Dinex Technologies, Inc., MRX Chantilly, VA). As amostras foram consideradas positivas quando a DO da amostra foi superior ao ponto de corte. O ponto de corte foi obtido através da média da DO de 20 amostras controles negativos acrescida de três desvios padrão.

### 10.3.2 MSP-1

As análises para o fragmento Pv200L foram realizadas em parceria com o Dr. Sócrates Herrera no Centro Internacional de Vacunas – CIV em Cali/Colômbia. A presença de anticorpos foi avaliada por ELISA em amostras de plasma de indivíduos infectados pelos genótipos variantes de *P. vivax* segundo protocolo previamente estabelecido (VALDERRAMA-AGUIRRE et al., 2005). Microplacas de 96-poços (Nunc-Immuno Plate, Maxisorp, Roskilde, Dinamarca) foram sensibilizadas com 100 mM of rPv200L por poço overnight a 4°C. As micoplacas foram então bloqueadas com 200 µL/poço com a solução 0,05% Tween 20 e 1% soro albumina bovina em PBS (Sigma, St. Louis, MO) por 2 h a temperatura ambiente. Após a lavagem das placas com PBS-Tween20 foram adicionadas as amostras diluídas 1:200 em solução de PBS/2,5% leite desnatado/0,05% Tween-20 em triplicata e incubadas por 1 h em temperatura ambiente. As placas foram lavadas cinco vezes com PBS-0,05% Tween 20 e adicionou-se o anticorpo secundário anti-IgG humana marcado com fosfatase alcalina (Sigma Chemical Co, St Louis, MO) em uma diluição de 1:4000 e as placas foram incubadas por 1 h a temperatura ambiente. As placas foram reveladas com para-nitrofenilfosfato (Sigma). A reação foi interrompida após 15 minutos com NaOH 1N e a densidade óptica foi obtida a 405 nm. O ponto de corte foi obtido através da média da DO de 20 amostras controles negativos acrescida de três desvios padrão.

### 10.3.3 AMA-1

A análise da resposta sorológica foi realizada por testes imunoenzimáticos, ELISA, para epítipo do Antígeno 1 de Membrana Apical de *P. vivax* (PvAMA1), segundo Rodrigues et al. (2005). A síntese dos peptídeos e as análises foram realizadas sob a colaboração da Profa. Dra. Irene Soares na Faculdade de Ciências Farmacêuticas da USP-SP. Resumidamente, a avaliação foi da seguinte forma: a princípio, o gene *pvama-1* foi clonado e seqüenciado. Em seguida, a proteína His<sub>6</sub> – AMA1 foi expressa em *Escherichia coli*. Os corpos de inclusão foram solubilizados a partir do sedimento contendo as bactérias lisadas, de acordo com Saini e colaboradores (2002). A proteína His<sub>6</sub> – AMA1 foi purificada para ser utilizada na sensibilização das placas de ELISA. As microplacas para ELISA, 96-poços, foram sensibilizadas *overnight* em temperatura ambiente com 200 ng/poço da proteína recombinante His<sub>6</sub> – AMA1. As placas foram lavadas com tampão PBS + 0,05% Tween 20 e posteriormente, foram bloqueadas com solução bloqueadora (5% de leite desnatado em PBS) por 2 horas a 37°C. Após a lavagem das placas com PBS-Tween20, as amostras foram diluídas na concentração de 1:100 em solução de PBS/1,5% leite desnatado e 50 mL de cada amostra foi adicionado em duplicata em cada poço. Após incubação de 2 horas em temperatura ambiente as placas foram lavadas com PBS-0,05% Tween 20 e foram adicionados 50 mL da solução de anti-IgG humano conjugado com peroxidase na diluição de 1:10000 (Sigma) em cada poço. A reação enzimática se deu através da adição de 1 mg/mL de dihidroclorido de fenilenodiamina (OPD) (Sigma) diluído em tampão citrato-fosfato, pH 5,0 contendo 0,03% de peróxido de hidrogênio e foi interrompida pela adição de 50 µL de 4 N H<sub>2</sub>SO<sub>4</sub>. A densidade óptica foi obtida em leitor de ELISA (SLT SPECTRA, SLT Labinstruments, Austria) a 492 nm. O ponto de corte foi obtido através da média da DO de 30 amostras de indivíduos doadores de sangue provenientes do Estado de São Paulo, Os resultados foram expressos como índice de reatividade (IR), o qual foi calculado dividindo-se a densidade óptica da

amostra teste pelo valo de ponto de corte. Valores do IR > 1.0 foram considerados como positivos.

### **10.3.3 rII-DBP**

A pesquisa de anticorpos IgG foi realizada em parceria com a Dra. Luzia Helena de Carvalho no Instituto Rene Rachou – FIOCRUZ/MG, de acordo com a metodologia previamente estabelecida (CERAVOLO et al., 2005). As microplacas para ELISA, 96-poços (Maxysorp, Nunc, Denmark) foram sensibilizadas overnight a 4°C com 5 µg/ml em PBS pH 7,4. As placas foram lavadas com tampão 0,05% Tween 20 em PBS e então incubadas por 1 h com solução bloqueadora (5% de leite desnatado em PBS). Após a lavagem das placas com PBS-Tween20 foram adicionadas as amostras diluídas 1:100 em solução de PBS/1,5% leite desnatado em duplicata e, incubadas por 1 h a 37°C. Após serem lavadas novamente, as placas foram incubadas por 1 h a 37°C com anti-IgG humana conjugado com peroxidase (Sigma-Aldrich, St. Louis, MO). A presença de anticorpos IgG foi detectada usando di-hidroclorido de fenilenodiamina (OPD) como substrato (Sigma-Aldrich). A absorbância foi lida a 492 nm usando leitor de micoplatas (Stat Fax-2100). A densidade óptica foi calculada subtraindo-se a absorbância obtida na leitura da realizada com o purificado GST (Sigma-Aldrich), usado como antígeno controle. O ponto de corte foi obtido através da média da DO de 30 amostras controles negativos acrescida de três desvios padrão.

## **11 APÊNDICES**

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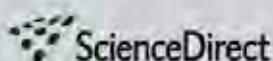
## 11 Apêndices

### Colaboração em outros trabalhos científicos

**11.1** *Plasmodium vivax* infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception? Autores: Carlos Eugênio Cavasini, Luiz Carlos de Mattos, Álvaro Augusto D'Almeida Couto, Cláudia Regina Bonini-Domingos, Sócrates Herrera Valencia, Wanessa Christina de Souza-Neiras, Renata Tomé Alves, Andréa Regina Baptista Rossit, Lilian Castilho e Ricardo Luiz Dantas Machado. Artigo de revisão publicado na revista **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 101, p. 1042-1044, 2007.

### RESUMO

Nós apresentamos evidências da infecção de *Plasmodium vivax* em indivíduos Duffy negativos em habitantes do Brasil. A identificação de *P. vivax* foi determinada por testes de genotípicos e não-genotípicos. O grupo sanguíneo Duffy foi genotipado por PCR/RFLP e fenotipado usando um kit de microtipagem. Nós detectamos dois indivíduos homocigotos *FY\*B-33* infectados por *P.vivax*, sendo estes tinham os genótipos VK210 e/ou *P. vivax-like*. Outros esforços são necessários a fim de esclarecer a evidência de que *P. vivax* está sendo transmitidos entre pacientes maláricos de grupo sanguíneo Duffy negativos provenientes da região Amazônica brasileira.

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## SHORT COMMUNICATION

## *Plasmodium vivax* infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception?

Carlos Eugênio Cavasini<sup>a,\*</sup>, Luiz Carlos de Mattos<sup>a</sup>,  
 Álvaro Augusto D'Almeida Couto<sup>b</sup>, Cláudia Regina Bonini-Domingos<sup>c</sup>,  
 Socrates Herrera Valencia<sup>d</sup>, Wanessa Christina de Souza Neiras<sup>a</sup>,  
 Renata Tomé Alves<sup>a</sup>, Andréa Regina Baptista Rossit<sup>a</sup>, Lilian Castilho<sup>e</sup>,  
 Ricardo Luiz Dantas Machado<sup>a</sup>

<sup>a</sup> Faculty of Medicine from São José do Rio Preto, Av. Brigadeiro Faria Lima, 5416, 15090-000 São José do Rio Preto, São Paulo State, Brazil

<sup>b</sup> SEAMA Faculty, Av. Nações Unidas, 1201, 68908-170 Macapá, Amapá State, Brazil

<sup>c</sup> Hemoglobin Diseases Laboratory, Universidade Estadual Paulista, Rua Cristóvão Colombo, 2265, 15054-000 São José do Rio Preto, São Paulo State, Brazil

<sup>d</sup> Malaria Vaccine and Drug Development Center, Universidad del Valle, Cra 35, 4A-53, 25573, Cali, Colômbia

<sup>e</sup> Blood Bank, Campinas University, Cidade Universitária "Zeferino Vaz", Rua Carlos Chagas, 480, Barão Geraldo 13083-878, Campinas, São Paulo State, Brazil

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 Duffy blood group;  
 Duffy antigen binding protein;  
 Protozoan proteins;  
 Brazil

**Summary** We present evidence for *Plasmodium vivax* infection among Duffy blood group-negative inhabitants of Brazil. The *P. vivax* identification was determined by both genotypic and non-genotypic screening tests. The Duffy blood group was genotyped by PCR/RFLP and phenotyped using a microtyping kit. We detected two homozygous FY\*B-33 carriers infected by *P. vivax*, whose circumsporozoite protein genotypes were VK210 and/or *P. vivax*-like. Additional efforts are necessary in order to clarify the evidence that *P. vivax* is being transmitted among Duffy blood group-negative patients from the Brazilian Amazon region.

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## 1. Introduction

Of the four human malaria parasites, only *Plasmodium falciparum*, *P. vivax* and *P. malariae* have been detected in Brazil, with 99.7% of cases occurring in the Amazon region.

\* Corresponding author. Tel.: +55 17 32105736;

fax: +55 17 32105736.

E-mail address: cecavasini@famerp.br (C.E. Cavasini).

For the past 7 years, *P. vivax* has been the most common cause of human malaria in the Brazilian Amazon region. Its variants (VK210, VK247 and *P. vivax*-like) are mainly found in mixed infections, but VK210 has also been described as a single infection (Machado and Póvoa, 2000). *Plasmodium vivax* merozoites use the Duffy blood group antigen as a receptor to invade human erythrocytes (Miller et al., 1976). However, a recent report suggests evidence for transmission of *P. vivax* among Duffy blood group-negative patients in Africa (Ryan et al., 2006). We report here two *P. vivax* human malaria cases in Duffy-negative individuals living in a Brazilian endemic area.

## 2. Materials and methods

In a study which is being conducted currently in the Center for Microorganisms Investigation, Faculty of Medicine from São José do Rio Preto, SP, Brazil to determine the Duffy blood group genotypic frequencies among *P. vivax* malaria patients from the Brazilian Amazon region, DNA has been extracted from total blood using the phenol-chloroform method, and a semi-nested PCR performed using *P. vivax*-specific small-subunit (SSU) rDNA primers (Kimura et al., 1997). The *P. vivax* circumsporozoite protein (CSP) variants were determined by spotting blood samples on glass fiber membranes and testing by PCR/ELISA (Machado and Póvoa, 2000). The *P. vivax* merozoite surface protein 1 (PvMSP1) (200L fragment) antigen was evaluated by an ELISA protocol according to Herrera et al. (1997). The Duffy antigens (Fy<sup>a</sup> and Fy<sup>b</sup>) were genotyped by PCR/RFLP and phenotyped using a microtyping kit (DiaMed-ID Micro-typing System, DiaMed AG, Cressier sur Morat, Switzerland), as described previously (Castilho et al., 2004). All patients studied were classified as a unique admixed ethnic group (African American, Caucasian and/or Amerindian descendants).

## 3. Results

Two samples from Porto Velho, Rondônia State, were phenotyped as Fy (a-b-) and genotyped as Fy<sup>a</sup>B-33/Fy<sup>b</sup>B-33 (Duffy-negative homozygous). The molecular results confirmed the *P. vivax* thick blood film diagnosis, and also showed the presence of VK210 plus *P. vivax*-like genotypes in one individual (mixed infection) and for the other the result was VK210. Antibody PvMSP1 (200L fragment) responses were present in both patients.

## 4. Discussion

Duffy-negative individuals seem to be naturally resistant to invasion by the *P. vivax* human malaria parasite (Miller et al., 1976). To our knowledge, this is the first indication of *P. vivax* infection in two Duffy-negative individuals living in the Brazilian Amazon region. The identification of *P. vivax* was made by thick blood film, amplification of *P. vivax*-specific SSU rDNA and CSP genes from sporozoites, and Fy-negative individuals also showed antibody responses against PvMSP1 (200L fragment), so it is unlikely that any parasite other than *P. vivax* was involved. As described previously (Ryan et

al., 2006), we also believe that *P. vivax* could be evolving to use receptors others than Duffy for junction formation during invasion. If this proves to be the case, we could hypothesize that the same merozoite adaptations occurred as isolated events in two geographically separated countries/continents (Kenya/Africa and Brazil/South America). As for the CSP variant analysis our results seems to contradict the possibility described by Ryan et al. (2006) that the VK247 CSP variant marks a *P. vivax* population capable of using receptors other than Duffy, since both of our study Duffy-negative *P. vivax* infected individuals were VK247-negative.

In conclusion, our findings point to the need for additional epidemiological studies of vivax malaria in the Brazilian Amazon region as well as in other endemic areas around the world. Additional efforts are necessary in order to clarify the evidence that *P. vivax* is being transmitted among Duffy blood group-negative inhabitants of the Brazilian Amazon region. Finally, the need to investigate the existence of alternative receptors is reinforced by our findings.

**Authors' contributions:** RLDM and ARBR conceived and participated in all aspects of the study and manuscript preparation; CEC carried out all the phenotype and genotype assays; AADC, CEC and WCSN collected samples from *P. vivax* malaria patients; SHV participated in the serological evaluation; WCSN and RTA participated in the SSU rDNA and CSP genes analysis; LCM, CRBD and LC participated in the design of the study and drafted the manuscript. All authors read and approved the final manuscript. CEC is guarantor of the paper.

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**Conflicts of Interest:** None declared.

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**11.2** Evaluation of the naturally acquired humoral immune response to the Pv200L: a N-terminal fragment of *Plasmodium vivax* merozoite surface protein-1 in four areas of the Amazon region of the Brazil. Autores: Luciane M. Storti-Melo, Wanessa C. Souza-Neiras, Gustavo C. Cassiano, Leonardo C. Taveira, José A. Cordeiro, Vanja S. C. A. Couto, Marinete M. Póvoa, Maristela G. Cunha, , Augusto Valderrama-Aguirre, Diana Echeverry, Andréa R. B. Rossit, Myrian Arevalo-Herrera, Sócrates Herrera and Ricardo L. D. Machado. Artigo original submetido em 05 de outubro para publicação na revista **The American Journal of Tropical Medicine and Hygiene**, 2009.

## RESUMO

Nós avaliamos a frequência dos níveis de anticorpos anti-Pv200L, um fragmento N-terminal da MSP-1, em indivíduos naturalmente expostos à malária em quatro áreas endêmicas da região Amazônica brasileiras. Amostras de plasma de 261 indivíduos infectados com malária causada por *Plasmodium vivax* de quatro áreas endêmicas de malária foram avaliados por ELISA, para a presença de anticorpos contra Pv200L. Diferenças significativas na frequência de respondedores e níveis de anticorpos entre as quatro áreas estudadas foram observadas. Os resultados encontrados neste estudo fornecem evidência de que a Pv200L é um fragmento imunogênico da porção N-terminal da MSP-1 de *P. vivax* em indivíduos naturalmente expostos da Amazônia brasileira.

Evaluation of the naturally acquired humoral immune response to the Pv200L: a  
N-terminal fragment of *Plasmodium vivax* merozoite surface protein-1 in four  
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Leonardo C. Taveira, José A. Cordeiro, Vanja S. C. A. Couto, Marinete M.  
Póvoa, Maristela G. Cunha, , Augusto Valderrama-Aguirre, Diana Echeverry,  
Andréa R. B. Rossit, Myrian Arevalo-Herrera, Sócrates Herrera and Ricardo L. D.  
Machado

*University of São Paulo State Júlio Mesquita Filho, São José do Rio Preto, São Paulo State, Brazil; Faculty of Medicine of São José do Rio Preto, São Paulo State, Brazil; SEAMA Faculty, Macapá, Amapá State, Brazil; Evandro Chagas Institute, Belém, Pará State, Brazil; Federal University of Pará, Institute of Biologic Science, Belém, Pará State, Brazil; Immunology Institute, Del Valle University, Cali, Colômbia; Faculty of Medicine Foundation of São José do Rio Preto, São Paulo State, Brazil.*

**Abstract:** We evaluated the frequency and antibody levels anti-Pv200L, a N-terminal MSP-1 fragment, in individual naturally exposed to malaria in four malaria endemic areas of the Brazilian Amazon region. Plasma samples from 261 *Plasmodium vivax* malaria infected individuals from four Brazilian malaria endemic areas were evaluated using an enzyme-linked immunosorbent assay, for the presence of the antibodies to Pv200L. Significant differences in the responders frequency and antibody levels among the four areas studied and among the number of previous malaria infections were observed. The results found in this study provide evidence that Pv200L is an immunogenic fragment of the N-terminal of the *P. vivax* MSP-1 in naturally exposed individuals from Brazilian Amazon.

**Keywords:** Malaria, *Plasmodium vivax*, Pv200L, IgG-antibody response, Brazil

## INTRODUCTION

Progress towards the development of a malaria vaccine against *Plasmodium vivax*, the main species responsible for infection in South and Central America, will require a better understanding of the immune response in regions where malaria is endemic. In Brazil, the number of *P. vivax* malaria accounts for more than 80% of clinical cases reported in Amazon region.<sup>1</sup> Naturally acquired antibodies against merozoite surface antigens of *Plasmodium* play a major role in malaria immunity. Among these antigens, the merozoite surface protein 1 (MSP-1) has received the most attention, as it is considered a malaria vaccine candidate to the asexual blood stages.<sup>2</sup> In *Plasmodium falciparum*, MSP-1 is a glycoprotein of ~190 kDa and the molecular weight varies slightly between parasite isolates due to antigen polymorphism.<sup>3</sup> MSP-1 is synthesized as a large precursor polypeptide and is then processed into smaller fragments during late schizont development.<sup>4</sup> The processed MSP-1 polypeptides contain remarkably conserved sequences<sup>5</sup>, one such, is localized in proximity of the amino-terminus of the mature MSP-1 protein and is included in the 80 kDa processing fragment, which is found associated with the surface of mature merozoites.<sup>6</sup> The most conserved part of this polypeptide is represented by a recombinant protein termed 190L (*Pf*190L)<sup>7</sup>, which contains an unusually elevated number of B- and T-cell epitopes<sup>8</sup> and showed to be highly immunogenic and partial protection inducing.<sup>9,10</sup> MSP-1 from *P. vivax* contains a region of significant sequence homology with the *Pf*190L. The fragment, termed *Pv*200L, showed immunologic relevance as a vaccine target. A preliminary seroepidemiologic study conducted

in Buenaventura, Colombia showed that 52.2% of individual previously exposed to malaria and 72.8% of *P. vivax* infected patients had antibodies to rPv200L. In addition, immunizations of BALB/c mice and *Aotus* monkeys induced IgG antibodies that cross-reacted with *P. vivax* and; immunized monkeys displayed partial protection after exposure to *P. vivax* blood stages.<sup>11</sup> Nevertheless, no immunological study of this fragment was previously conducted in other malaria endemic area. Hereby, we evaluated the frequency and levels of anti-Pv200L antibodies in naturally acquired *P. vivax* infections in four malaria endemic areas of the Brazilian Amazon region.

## MATERIAL AND METHODS

**Study subjects and areas:** The patients enrolled in this study complied with the following criteria: they sought medical assistance for clinical malaria symptoms, were over 18 years old and had a positive malaria diagnosis by thick blood film for *P. vivax*, after written informed consent had been signed. One peripheral blood samples were obtained from each of the 261 individuals living in four Brazilian malaria endemic areas: Macapá, Amapá State; Novo Repartimento, Pará State; Porto Velho, Rondônia State and Plácido de Castro, Acre State (Figure 1).

**Assessment of the antibody response:** human plasma samples were evaluated using an enzyme-linked immunosorbent assay – ELISA for the presence of naturally acquired antibodies to rPv200L, as describe elsewhere.<sup>11</sup> Briefly, Maxisorp flat-bottomed, 96-well microplates (Nunc, Rochester, NY) were coated with 100 mM of rPv200L per well and incubated overnight at 4°C. Microplates

were blocked for two hours with PBS containing 0.05% Tween 20 and 1% bovine serum albumin (Sigma, St. Louis, MO). After one washing step with T-PBS human plasma samples diluted 1:200 were added and incubated for one hour at room temperature. Plates were washed five times with PBS containing 0.05% Tween 20, and secondary anti-human IgG antibody labeled with alkaline phosphatase (Sigma) were added at a final dilution of 1:4000. Plates were incubated for one hour at room temperature, washed with T-PBS and developed with *p*-nitrophenylphosphate (Sigma). The reaction was stopped after 15 minutes with 10 $\mu$ L of 1N NaOH and read at 405 nm. Every sample was tested in triplicate. The ELISA IgG cutoff was defined as the average of the negative control plus three standard deviations. The results were expressed as an index of reactivity (IR = OD<sub>405</sub> values of tested sample divided by the value of the cutoff). Values of IR <1.0 were considered negative, values of IR  $\geq$ 1.0 and <10 were considered positive (antibody titres estimated varying from 1:200 until 1:3200) and values of IR  $\geq$ 10 were considered highly positive (antibody titres estimated varying from 1:3200 until 51200).

**Statistical analysis:** Analyses were performed using R version 2.8.1 statistical software (The R Foundation for Statistical Computing, Vienna, Austria [<http://www.r-project.org>]). Differences among the frequencies of responders were analyzed using Pearson's chi-square or, alternatively, the Fisher's exact test. Comparisons of antibody level (IR) were performed using the Kruskal-Wallis test with post hoc Bonferroni's pairwise comparisons. Relationship between the antibody responders and previous malaria episodes was analyzed by binary

logistic regression. Differences were considered significant when  $p\text{-value}\leq 0.05$ , and when Bonferroni's correction was done  $p\text{-value}\leq 0.017$  or  $p\text{-value}\leq 0.008$ , respectively to number of compared groups (3 or 4).

## RESULTS

**Frequency and level of antibodies to Pv200L in the four study areas:** For all patients the frequency of sera containing antibodies that recognized the Pv200L was 89.3%. The average of IR was 5.5, and 17.6% of samples showed high antibody titres (IR  $\geq 10$ ). Significant differences ( $p=0.000001$ , Fisher's exact test) in the responders frequency among the four studied areas were observed (Table 1).

In Macapá, the lower percentage of positive Pv200L antibodies individuals was detected (71.9%), while Novo Repartimento had the higher frequency of responders (98.7%). In *post hoc* analysis by Bonferroni's pairwise comparisons (significant  $p\text{-value}\leq 0.008$ ) Macapá frequencies were statistically lower compared to Novo Repartimento ( $p=0.000002$ ) and Porto Velho ( $p=0.0004$ ).

Taken together, the four study areas were statistically different ( $p=0.0008$ , Kruskal-Wallis test) in terms of antibody levels (estimated using IR values). The lower antibody titres were detected in Macapá patients (average of IR = 4.4) as well as the lowest frequency of highly responders sera (IR  $\geq 10$ ; 12.5%). On the other hand, the highest IR average (6.2) was found in Plácido de Castro with a frequency of 21.2% of highly positive responders (Table 1). In Figure 2A we show each individual's IR value plotted by area and bottom line concentrations

were seen only in Macapá. The IR values were utilized to construct a boxplot by area (Figura 2B) and statistic pairwise comparisons (Bonferroni's correction,  $p\text{-value}\leq 0.008$ ) confirmed that the lowest antibody responses occurred in Macapá (Figura 2C); Macapá versus Porto Velho,  $p=0.0011$ ; Macapá versus Plácido de Castro,  $p=0.0039$ ; Macapá versus Novo Repartimento,  $p=0.0000$ ).

**Relationship between frequency and level of antibodies with previous**

**malaria episodes:** We determined whether there was a correlation between the frequency and antibody levels and number of previous malaria episodes. For this, plasma from 200 individuals was separated according to the number of previous malaria and further divided into three groups: (0) primary-infected; (1 or 2) individuals with one to two previous malaria episodes; and (3 or more) individuals with at least three previous malaria episodes. We found that the frequency of Pv200L antibodies positive samples was statistically different among these three groups (Figure 3;  $p=0.006$ , Pearson Chi-Square). The percentage of individuals that do not recognize the Pv200L ( $IR<1$  or negative) was significantly higher in the (0) group (40%) compared to group 1 (1 or 2 episodes; 12.2%) and to group 3 (3 or more; 7.4%) ( $p=0.042$  and  $p=0.010$  respectively, Pearson Chi-square). None individual of the primary-infected group was positive with high titer of antibodies ( $IR>10$ ) (Figure 3). This tendency was also proven by binary logistic regression analysis. The percentage of responders in the primary infected group was compared with the other groups. The Odds Ratio (OD) for groups with 1 or 2 and 3 or more previous malaria infections were  $OD=4.8$  ( $p=0.031$ ) and  $OD=8.3$  ( $p=0.004$ ), respectively.

The correlation between Pv200L antibody levels and number of previous malaria infection showed that all primary-infected (0) had lower IR values (Figure 4A and B) with the mean IR (IR=2.0) lower than the mean IR of all patients (other three groups; IR=5.4). However, this difference was significant only between primary-infected (0) and the 3 or more malaria episodes group ( $p=0.010$ , Kruskal Wallis test, Bonferroni's correction  $p\text{-value}\leq 0.017$ , Figure 4C).

## DISCUSSION

Several studies have demonstrated the high antigenicity of different regions of the *P. vivax* MSP-1 in Brazil<sup>12-15</sup> and other countries.<sup>16-19</sup> The previously described antibodies specificity, particularly to the C-terminal region (42-kD and 19-kD subfragments), blocking parasite invasion *in vitro*<sup>20,21</sup> and inducing protective immunity in animal models<sup>9,22</sup>, reduced the interest for further investigation on other MSP-1 fragments as potential vaccine candidates. Nevertheless, Valderrama-Aguirre et al. (2005) invested on the N-terminal fragment of *P. vivax* MSP-1 (Pv200L) in terms of its antigenicity and protective efficacy in Colombian individuals.<sup>11</sup> Here, we evaluated antibody response against Pv200L in malaria naturally exposed individuals from four Brazilian endemic areas. Importantly, our results were higher (89.3%) than those observed in Colombia (72.8%)<sup>11</sup>. Both results, taken together, signalize for a high potential of this antigen as a vaccine candidate. Additionally, high antibody levels (IR>10) and IR average corroborates Pv200L high antigenicity in naturally-acquired malaria. These results are in agreement with those from others seroepidemiologic

studies using longer fragments from N-terminal of PvMSP-1.<sup>14,23,24</sup> Although the C-terminal region of MSP-1 was referred in Brazil as the most immunogenic<sup>12,13</sup>, a reduced risk of *P. vivax* infection and clinical protection against malaria were, in fact, associated with antibodies to the MSP-1 N- terminal.<sup>14</sup> In the same direction, inhibitory antibodies specific for the 19 kD C-terminal of *P. falciparum* MSP-1 were not correlated with delayed appearance of infection but, actually, declined significantly in two months after treatment which might have contributed to the risk of reinfection.<sup>15,25</sup>

In Brazil, the antibody response profile was variable in the different areas. Macapá showed the lower frequency and antibody levels among studied areas. Nevertheless, in Novo Repartimento the frequency of responders was higher (98.7%) than in the others Brazilian areas. Several reasons, not mutually exclusive, may account for the discrepancy differences observed only in Macapá. A possible explanation is the characteristic of *P. vivax* and *P. falciparum* transmission in each area. Although individuals with patent *P. falciparum* infection do not recognize the N-terminal recombinant protein of *P. vivax* MSP1<sup>24</sup> a combination of *P. vivax* and *P. falciparum* previous infections may have unpredictable outcome in the human antibody immune response.<sup>12</sup>

For that reason, we evaluated whether there was a correlation between the frequency and antibody levels and the number of past malaria infections. To answer this question, the samples were classified according to number of previous malaria in three groups, (0), (1 or 2) and (3 or more) past malaria episodes. Our analysis showed that the frequency of responders was higher in the previous

infected groups by binary logistic regression analysis, the OD values indicated that a frequency of responders tends to increase according more previous malaria infections. The antibody levels also were higher in the groups with past infection, what led us to conclude that the increase in the frequency and level of antibodies is directly proportional to the number of previous malaria episodes. Although, Brazilian previous studies did not show this tendency for the N-terminal of the *P. vivax* MSP1,<sup>12,13</sup> our data are in agreement with the studies that evaluated the C-terminal of the MSP-1<sup>16</sup> and other parasite proteins, such as DBP-II<sup>26,27</sup> and AMA-1.<sup>16,28</sup> In these studies a positive correlation was also observed between the increase in the antibody response and the number of past malaria episodes. These data taken together, suggest that multiple infections can provide a boost for the production of the specific antibodies. As observed in first evaluation of the Pv200L in Colombia, the higher antibody response in infected patients may correspond to an early immunologic boosting by parasites, after natural infection and may provide advantages for boosting of antibody response induced by vaccination.<sup>11</sup>

In summary, the results found in this study provide evidence that Pv200L is an immunogenic fragment of the N-terminal of the *P. vivax* MSP-1 in naturally exposed individuals from Brazilian Amazon. We observed difference among the study areas and in the frequency of malaria exposure that, most likely, reflecting the wide variations found in epidemiology malaria regarding the transmission pattern, parasite polymorphisms, and the genetic background of human populations. Additional studies about the factor that modulate the immune

response against other parasitic proteins, are necessary to better understanding the immunologic aspects of malaria contributed to a specific malaria vaccine development.

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**Author' addresses:** Luciane M. Storti-Melo, Wanessa C. Souza-Neiras, Gustavo C. Cassiano, Leonardo C. Taveira, José A. Cordeiro, Andréa R. B. Rossit and Ricardo L. D. Machado, Centro de Investigação de Microrganismos, Faculdade de Medicina de São José do Rio Preto, Avenida Brigadeiro Faria Lima 5416, Vila São Pedro, São José do Rio Preto, São Paulo Brasil, Telephone: 55-17-32015736, Fax: 55-17-32015909. Vanja S. C. A. Couto, Faculdade SEAMA, Av. Nações Unidas, 1201, Jesus de Nazaré, 68908-126, Macapá, Amapá, Brasil. Marinete M.

Povoa, Instituto Evandro Chagas, Rodovia BR-316 km 7, s/n, Levilândia, 67030-000, Ananindeua, Belém, Pará, Brasil. Maristela G. Cunha, Universidade Federal do Pará, Instituto de Ciências Biológicas, Av. Augusto Corrêa, No.1, 66.075 – 900, Belém, Pará, Brasil. Augusto Valderrama-Aguirre, Diana Echeverry, Myrian Arevalo-Herrera and Sócrates Herrera, Instituto de Immunología, Calle 4B# 36-00, edificio de Microbiología, 3er Piso, Facultad de Salud, Universidad del Valle, Sede San Fernando, AA 25574, Cali, Colombia.

**Reprint Request:** Ricardo L. D. Machado, Centro de Investigação de Microrganismos, Faculdade de Medicina de São José do Rio Preto, Avenida Brigadeiro Faria Lima 5416, Vila São Pedro, São José do Rio Preto, São Paulo Brasil, Telephone: 55-17-32015736, Fax: 55-17-32015909, email: ricardomachado@famerp.br.

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**Figure 1.** Study area for the *Plasmodium vivax* in the Amazon region of Brazil, Macapá, Amapá State (AP; 00°02'20''S; 51°03'59''W); Novo Repartimento, Pará State (PA; 04°19'50''S; 49°47'47''W); Porto Velho, Rondônia State (RO; 08°45'43''S; 63°54'14''W) and Plácido de Castro, Acre State (AC; 10°16'33''S; 67°09'00''W).

Table 1. Frequency and antibody levels to Pv200L in sera of infected individuals with *P. vivax* in four areas from Brazilian Amazon.

Area	Samples (n)	Antibody frequency (%)*	IR - Antibody level		
			Average	>1 (%)	>10 (%)
Macapá	64	71.9	4.4	59.4	12.5
Novo Repartimento	76	98.7**	6.0	80.3	18.4
Porto Velho	55	96.4***	5.7	78.2	18.2
Plácido de Castro	66	89.4	6.2	68.2	21.2
<b>Total</b>	<b>261</b>	<b>89.3</b>	<b>5.5</b>	<b>71.7</b>	<b>17.6</b>

\* Fisher's exact test ( $p= 0.000001$ ) analyzing the four areas. \*\* Macapá versus Novo Repartimento ( $p= 0.000002$ ); \*\*\* Macapá versus Porto Velho ( $p=0.0004$ ), Bonferroni's correction  $p\text{-value} \leq 0.008$ .

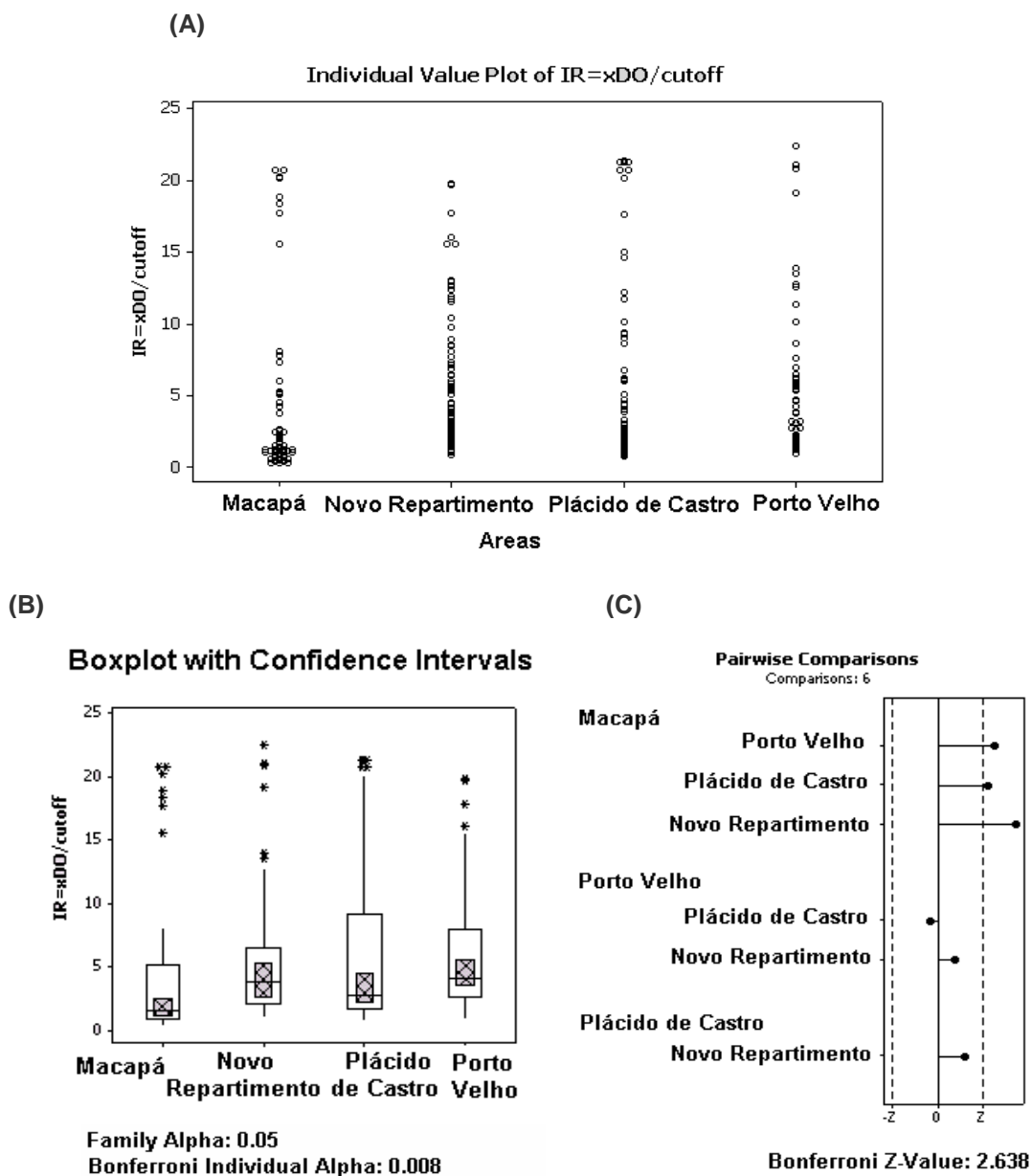


Figure 2. Antibody levels to *Pv200L* of the MSP-1 in individuals from four areas of the Brazilian Amazon region. (A) Individual value plot of IR in the four areas. Macapá had more samples with low IR values. (B) Boxplot of the median of IR in the four areas with de confidence intervals – CI (Macapá, median= 1.5 and CI= 1.1 – 2.4; Porto Velho, median= 3.8 and CI= 2.6 – 5.3; Plácido de Castro, median= 2.7 and CI= 2.1 – 4.7 and Novo Repartimento, median= 4.1 and CI= 3.4 – 5.5). The \* represent outliers. (C) *Post hoc* analysis of the Kruskal Wallis test with Bonferroni individual alpha= 0.008 and Z-value= 2.63. Macapá was statistically different of the others areas (Macapá vs Porto Velho,  $p=0.0011$  and Z-value=3.2; Macapá vs Plácido de Castro,  $p=0.0039$  and Z-value= 2.88; Macapá vs Novo Repartimento,  $p= 0.0000$  and Z-value= 4.5).

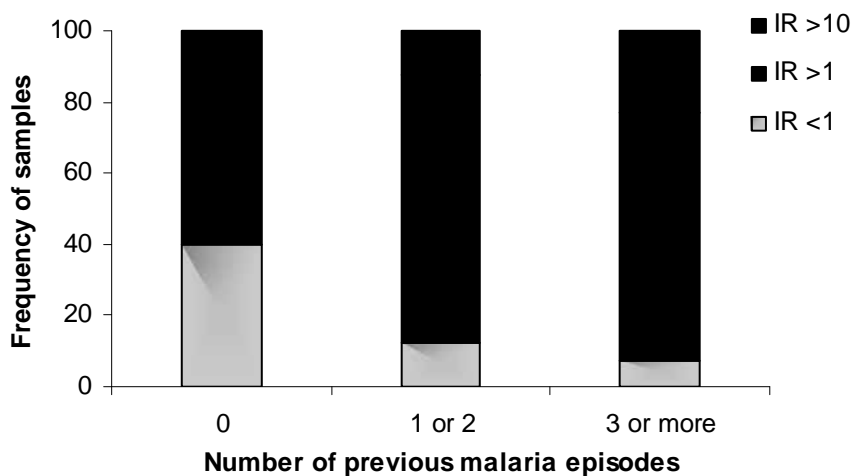


Figure 3. Correlation between the frequency of samples for IR values and the number of previous malaria episodes. Two hundred individuals were grouped according to the number of times they had a malaria infection. The number of individuals of each group was 10, 82 and 108 for zero, 1 or 2 and 3 or more previous malaria groups, respectively. The IR values are: IR <1 (negative), IR >1 (positive) and IR >10 (high positive). The frequency of sample for each IR value was statistically different between the three groups ( $p= 0.006$  Pearson Chi-Square test). The percentage of individuals that not recognized the *Pv200L* (IR<1 or negative) was significantly higher in the group without previous malaria than the groups with previous malaria (1 or 2) and (3 or more) ( $p=0.006$ , Pearson Chi-square). None individual of the primary-infected group had high titer of antibodies (IR>10).

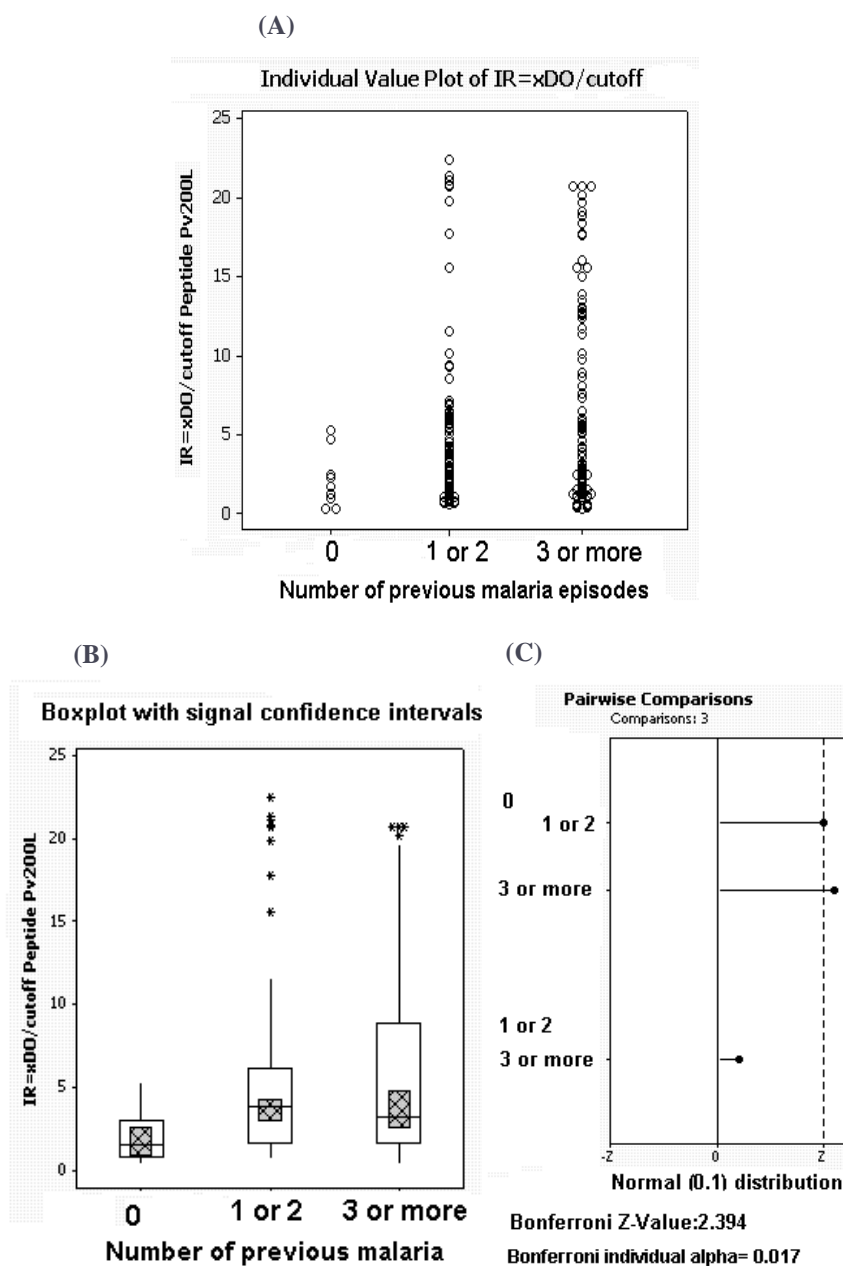


Figure 4. Correlation between antibody levels to Pv200L of the MSP-1 and number of previous malaria episodes. (A) Individual value plot of IR for primary-infected (0), with 1 or 2 and 3 or more previous malaria groups. (B) Boxplot of the median of IR for number of previous malaria groups and the confidence intervals – CI (0, median= 1.46 and CI= 0.3 – 4.7; 1 or 2, median= 3.8 and CI= 2.7 – 4.5 and 3 or more, median= 3.1 and CI= 2.5 – 5.1). The \* represent outliers. (C) *Post hoc* analysis of the Kruskal Wallis test with Bonferroni individual alpha= 0.008 and Z-value= 2.39. The difference was significant statistically between the primary-infected and the 3 or more previous malaria groups (p=0.010 and Z=2.57).

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*Vanessa Christina de Souza Neves*

Assinatura