



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
Campus de São José do Rio Preto

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

LUCIANE MORENO STORTI DE MELO

**AVALIAÇÃO DA RESPOSTA DE ANTICORPOS
CONTRA ANTÍGENOS DE *PLASMODIUM VIVAX*
RELACIONADA À FATORES GENÉTICOS DO
PARASITO E DO HOSPEDEIRO HUMANO**

**Tese apresentada para obtenção do
Título de Doutor em Genética**

**São José do Rio Preto – SP
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Tese apresentada como parte dos requisitos 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.

Orientador: Ricardo Luiz Dantas Machado

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“A coisa mais bela que o homem pode experimentar é o mistério. É esta a emoção fundamental que está na raiz de toda ciência e arte. O homem que desconhece esse encanto, incapaz de sentir admiração e estupefação, esse já está, por assim dizer, morto e tem os olhos extintos.”

Albert Einstein

RESUMO

O presente estudo avaliou a resposta de anticorpos contra diferentes antígenos de merozoíto e esporozoíto de *Plasmodium vivax*, relacionando com as variantes da porção repetitiva do domínio central do gene da Proteína Circunsporozoítica (CSP) do parasito (VK210, VK247 e *P. vivax-like*) e com os polimorfismos do *HLA-DRB1* no hospedeiro humano. A resposta de anticorpos foi avaliada para peptídeos das regiões conservadas e centrais variáveis da CSP, da porção N-terminal da Proteína de Superfície do Merozoíto 1-MSP1 (Pv200L), e recombinante do Antígeno 1 de Membrana Apical (AMA-1) e a Proteína de ligação ao Duffy (DBP) por ELISA, em amostras de plasma de pacientes naturalmente infectados com *P. vivax*. Inicialmente nós avaliamos a distribuição destas variantes da CSP em cinco diferentes áreas da Amazônia a fim de entender sua atual dinâmica de transmissão. A variante VK210 continua sendo a mais prevalente em todas as áreas estudadas. No entanto, pela primeira vez documentamos a presença das variantes VK247 e *P. vivax-like* como infecções simples na Amazônia brasileira evidenciando um novo perfil distribuição destas, o que possa sugerir um processo de adaptação das mesmas. Quando comparamos a resposta de anticorpos e a infecção pelas variantes de *P. vivax*, não foram observadas associações significativas entre a presença de determinada variante da CSP e a frequência de resposta de anticorpos contra os três peptídeos do merozoíto analisados, MSP1 (Pv200L), AMA-1 e DBP e nem contra as frações conservadas da CSP no esporozoíto, N-terminal [N] e C-terminal [C]. A falta de associações significativas entre resposta sorológica contra esses peptídeos fornece informações promissoras quanto à utilização destes antígenos para o desenvolvimento de uma vacina contra malária. Todavia, a variação na porção central da CSP deve ser considerada para utilização desta região como imunógeno, uma vez que a resposta de anticorpos parece ser variante específica. Com o intuito de avaliar a influência dos polimorfismos relacionados à resposta imunológica do hospedeiro humano nos níveis de anticorpos contra Pv200L, AMA-1 e DBP nós analisamos a frequência alélica do *HLA-DRB1* pelo método de PCR-SSP, em amostras de indivíduos infectados com *P. vivax* e de doadores de sangue não maláricos provenientes das mesmas áreas endêmicas de malária no Brasil. Observou-se significativa maior frequência do alelo HLA-DRB1*07 entre o grupo de indivíduos maláricos e este dado reforça descrição prévia da literatura de perfil de susceptibilidade à doença na presença deste alelo. Uma associação positiva foi observada entre altos níveis de anticorpos anti-Pv200L e o alelo HLA-DRB1*03, a qual não parece estar relacionada à exposição à malária, o que nos leva a sugerir que, na amostra estudada, o *HLA-DRB1* possa estar influenciando os níveis de anticorpos para MSP-1 (Pv200L), mas não para AMA-1 e DBP. Nossos dados discutem a interação parasito-hospedeiro na malária por *P. vivax* e oferecem novas informações sobre moléculas candidatas à vacina em populações brasileiras naturalmente expostas à malária.

Palavras-chave: Malária, *Plasmodium vivax*, Variantes da Proteína Circunsporozoítica, Resposta de Anticorpos, HLA classe II.

ABSTRACT

*The present study evaluated the antibody response against merozoite and sporozoite antigens of Plasmodium vivax and its relationship with the variants of the repetitive central region of the gene for Circumsporozoite protein (CSP) in parasite (VK210, VK247 and P. vivax-like) and, with the HLA-DRB1 polymorphisms in human host. The antibody response to synthetic peptides of the CSP conserved and variable regions and of the N-terminal portion of Merozoite surface protein - MSP1 (Pv200L), and, to recombinant peptides of the Apical Membrane Antigen 1 (AMA-1) and of the Duffy Binding Protein (DBP) was evaluable by ELISA in plasma samples of malaria patients naturally infected with P. vivax. Firstly, we evaluated the CSP variants distribution among five different areas from Brazilian Amazon, in order to understand their current dynamic of transmissions. VK210 variant remains the most prevalent in all study areas. However, it is the first detection of VK247 e P. vivax-like variants as simple infection in the Brazilian Amazon, showing a new distribution profile, which may suggest an adaptation process of them. When comparing the antibody response and infection by variants of P. vivax, there were no significant associations between the presence of particular CSP variant and the frequency of antibody response against all three merozoite peptides analyzed, MSP1 (Pv200L), AMA-1, DBP and against the CSP conserved fractions in the sporozoite, N-terminal and C-terminal. The lack of significant associations among immune response against these peptides provides promising information regarding the use of these antigens for malaria vaccine development. On the other hand, the central variability of CSP should be considered to employment of this region as an immunogen, since the antibody response appears to be variant-specific. In order to evaluate the polymorphisms influence of the human immune response in the antibody levels against Pv200L, AMA-1 and DBP, the HLA-DRB1 allele frequencies were evaluated by PCR-SSP method in plasma samples of P. vivax infected individuals and blood donors non-malaric from the same malaria endemic areas of Brazil. Differences were observed in HLA-DRB1*07 frequencies between malaric and non-malaric groups reinforcing the previous description in the literature of a malaria susceptibility profile in this allele presence. A positive association was observed between the highest antibody levels to Pv200L and the presence of HLA-DRB1*03, that does not seem to be related with the malaria exposure. So, we could suggest that in the sample studied, HLA-DRB1 alleles can influence antibody levels to MSP-1 (Pv200L) but not to AMA-1 and to DBP. Finally, our data argue the host-parasite interaction in malaria by P. vivax and offer new information on the vaccine candidate molecules in Brazilian populations naturally exposed to malaria.*

Key-words: Malaria, Plasmodium vivax, Circumsporozoite Protein Variants, Antibody Response, HLA class II

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LISTA DE ABREVIATURAS

AMA-1	Antígeno 1 de Membrana Apical
CEP	Comitê de Ética em Pesquisa
CIM	Centro de Investigação de Microrganismos
CS	Gene da Proteína Circunsporozoítica
CSP	Proteína Circunsporozoítica
DARC	Receptor de Antígeno Duffy para Quimiocinas
DBP	do Inglês <i>Duffy Bindin Protein</i> – Proteína de Ligação ao Antígeno Duffy
DNA	Ácido Desoxirribonucleico
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	do Inglês <i>Human Leukocyte Antigen</i> – Antígeno Leucocitário Humano.
IBILCE	Instituto de Biociências, Letras e Ciências Exatas
MAP	do Inglês <i>Multiple Antigens Peptide</i> - Peptídeos Antígenos Múltiplos
MHC	do Inglês <i>Major Histocompatibility Complex</i> – Complexo Principal de Histocompatibilidade
MSP	do Inglês <i>Merozoite Surface protein</i> - Proteína de Superfície do Merozoíto
NESTED	Refere-se à PCR semi-aninhado
PCR	Reação em Cadeia da Polimerase
RFPL	do Inglês <i>Restriction Fragment Length Polymorfism</i> - Polimorfismo no Tamanho do Fragmento de Restrição
SIVEP	Secretaria de Vigilância em Saúde
UNESP	Universidade Estadual Paulista “Júlio de Mesquita Filho”

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1 INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

1.1 Malária – Aspectos da biologia e epidemiologia

A malária ou paludismo é uma doença parasitária causada por protozoários do gênero *Plasmodium*, transmitida por mosquitos fêmeas do gênero *Anopheles* a diferentes hospedeiros vertebrados incluindo aves, répteis e mamíferos. Estes parasitos pertencem ao Reino Protista, Filo Apicomplexa, Classe Sporozoea, Ordem Hemosporidiida, Família Plasmodiidae e Gênero *Plasmodium*. Existem cinco espécies de *Plasmodium* 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). 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 drogas antimaláricas 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).

Os dados disponíveis sobre a ocorrência de malária, em todo o mundo, são bastante imprecisos. No entanto, todas as estimativas são muito elevadas (CARTER e MENDIS, 2002; SNOW et al., 2005). Segundo a Organização Mundial da Saúde, a malária é responsável por 300 a 500 milhões de novas infecções com 1,5 a 3 milhões de mortes por ano no mundo inteiro (WHO, 2008). Ainda que tenha sido descrito 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) e a malária continua exercendo formidável impacto econômico e social nas diversas áreas tropicais do planeta. Segundo a Organização Mundial de Saúde, 109 países e territórios possuem áreas de risco de malária, sendo que estas atingem exatamente as regiões mais pobres do globo, o que a torna parte integrante do círculo vicioso pobreza-doença-pobreza. Apesar

dos esforços das autoridades mundiais em saúde no sentido de controlar a doença, ela continua em franca expansão, principalmente como consequência do aumento das migrações humanas desordenadas. Ainda, em face do fenômeno do aquecimento global, a malária é hoje considerada uma doença com possibilidade de reemergir em áreas de onde já foi erradicada ou onde é controlada. 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. Nas Américas, a transmissão ocorre em 22 países, estimando-se que 137 milhões de pessoas vivem em áreas com distinta intensidade de transmissão, já que diferem em características sociais, econômicas e ecológicas. No ano de 2006 foram registrados aproximadamente um milhão de casos de malária nas Américas, e o país com maior contingente de casos notificados foi o Brasil (52,7%), seguido pela Bolívia (20,0%) e Colômbia (11,5%) (WHO, 2008).

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). A incidência da doença persiste praticamente restrita à Região Amazônica, com mais de 99% dos casos (SIVEP, 2008), sendo registrados na região Norte, nos Estados do Pará, Amazonas, Rondônia, Acre, Amapá, e Tocantins, parte da região Centro-Oeste, no Estado do Mato Grosso, e na região Nordeste no Estado do Maranhão, totalizando 807 municípios endêmicos. Na década de 80 a incidência da malária no Brasil esteve em ascensão, atingindo em 1999, aproximadamente 637.000 casos. Nos últimos anos, devido principalmente aos programas de controle governamentais, houve um declínio da incidência da doença e, no ano de 2009, foram registrados 306.908 casos de malária, sendo que 99,8% forma originários da Amazônica. (MINISTÉRIO DA SAÚDE, 2011). Apesar de endêmica da malária não ocorre uniformemente em toda região, uma vez que se observam localidades com diferentes níveis de transmissão da doença (MINISTÉRIO DA SAÚDE, 2009) (Figura 1). Na Região Extra-Amazônica, têm sido registrados surtos de malária cada vez mais freqüentes decorrentes da introdução da doença por migrantes infectados oriundos das áreas de transmissão. Esse quadro é

preocupante uma vez que toda a Região Extra-Amazônica é receptiva para transmissão de malária e os serviços de vigilância em saúde de alguns municípios são carentes de estrutura adequada para enfrentar o problema. Outro desafio que se impõe para os programas de controle é a eliminação de casos autóctones esporádicos que ocorrem em áreas focais restritas desta região. Tais casos estão localizados nas margens do lago da reserva hidrelétrica de Itaipu, em áreas cobertas pela Mata Atlântica nos Estados do Espírito Santo, Rio de Janeiro, São Paulo e Bahia (MINISTÉRIO DA SAÚDE, 2009).

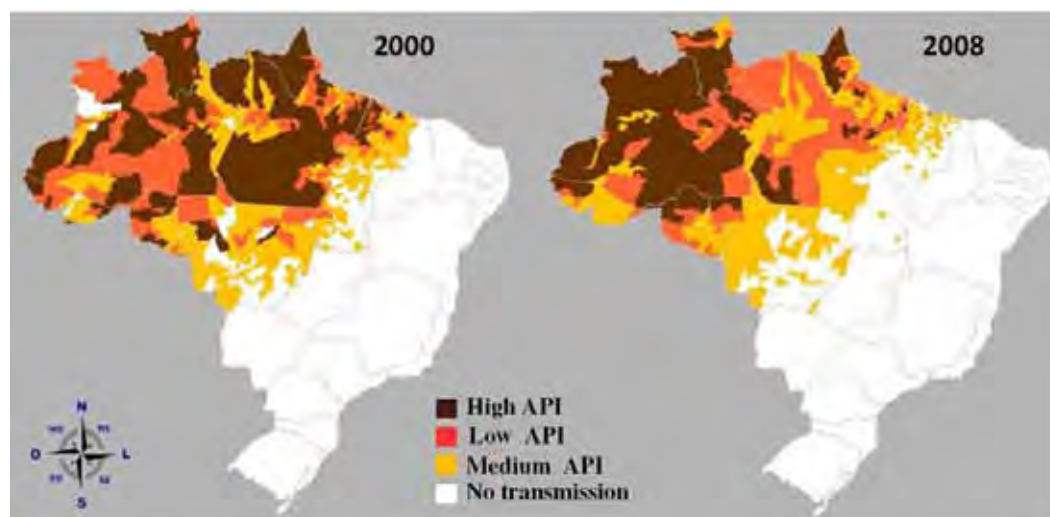


Figura 1 – Classificação das áreas de transmissão da malária no Brasil segundo o índice parasitário anual (IPA) nos anos de 2000 e 2008. O IPA corresponde ao número de casos / mil habitantes; Baixo IPA: <10 casos, Médio IPA: 10 a 49,9 casos e Alto IPA: >50 casos. (Ministério da Saúde/Secretária de Vigilância em Saúde, 2009. Adaptado de OLIVEIRA-FERREIRA et al., 2010).

1.2 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 esta droga 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 (GOLENDÁ et al., 1997).

1.3 Ciclo de biológico do *P. vivax*

O ciclo de vida do *Plasmodium spp* (Figura 2) é constituído por uma fase sexuada exógena (esporogônica), durante a qual ocorre a multiplicação dos parasitos nos mosquitos do gênero *Anopheles* (hospedeiro definitivo) e uma fase assexuada endógena (esquizogônica), onde ocorre a multiplicação no humano

(hospedeiro intermediário); esta fase ocorre em células parenquimatosas do fígado (esquizogonia hepática) ou nos eritrócitos (esquizogonia eritrocitária).

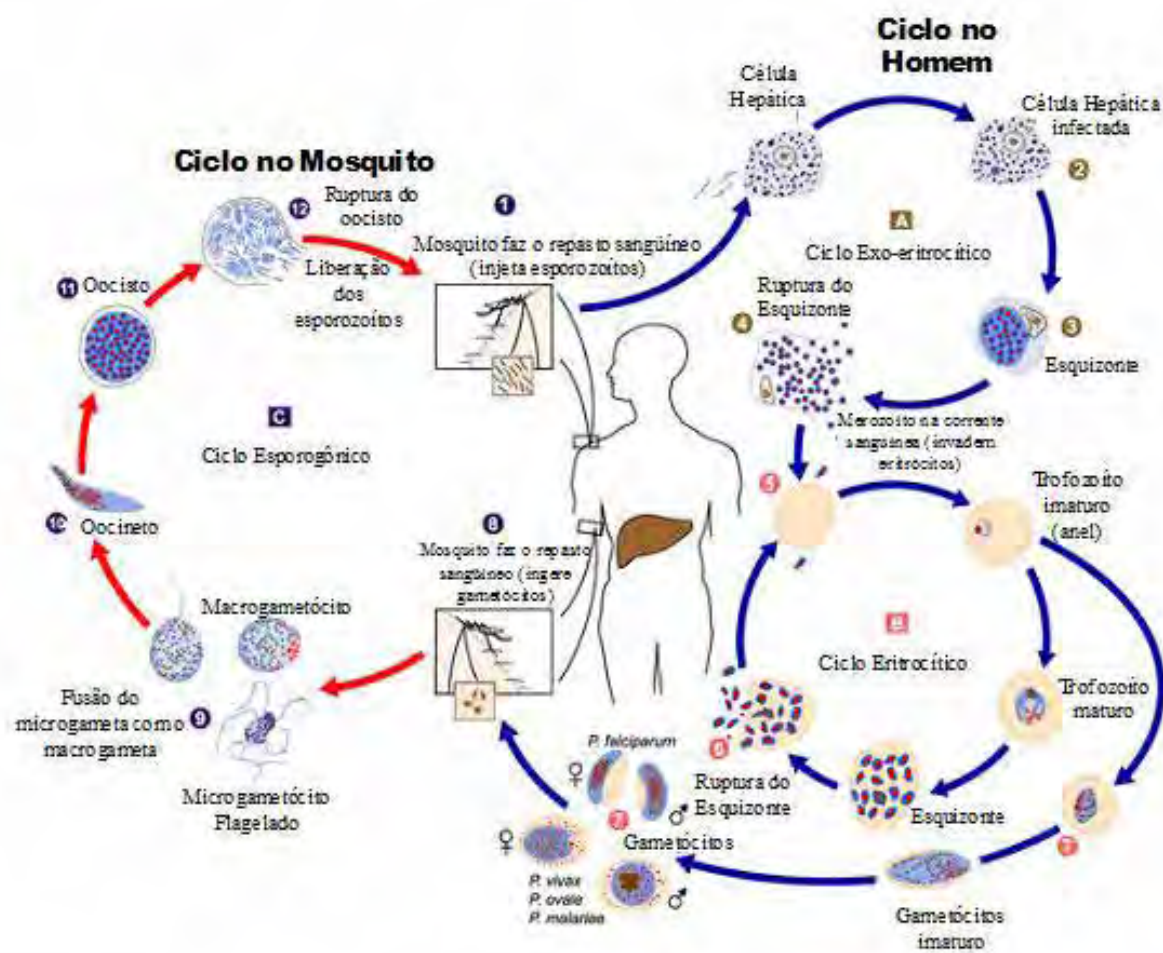


Figura 2 – Ciclo biológico de *Plasmodium spp.* Adaptado de:
[<http://www.med.sc.edu:85/parasitology/blood-prot.htm>]

A infecção inicia-se durante o repasto sanguíneo do mosquito fêmea infectado, este inocula, juntamente com a saliva, esporozoítos na corrente sanguínea (1). Os esporozoítos invadem os hepatócitos (2), onde crescem e se multiplicam, diferenciando-se em esquizontes hepáticos (3). A esta fase chama-se esquizogonia hepática (A); a sua duração depende da espécie de *Plasmodium*. No ciclo de vida do *P. vivax* e do *P. ovale* os esporozoítos podem originar

hipnozoítos, que permanecem no hepatócito em um estado dormente, durante o qual não se dividem, desenvolvendo-se meses ou anos mais tarde. No ciclo do *P. falciparum* e do *P. malariae*, inicia-se de imediato a maturação dos esquizontes hepáticos da qual resultam os merozoítos hepáticos que são liberados para a corrente sanguínea por lise dos hepatócitos (4) com conseqüente invasão dos eritrócitos. Após esta invasão (5), inicia-se a esquizogonia eritrocitária (B), na qual os merozoítos se diferenciam em trofozoítos e estes por divisões sucessivas dão origem aos esquizontes eritrocitários. Após lise do eritrócito parasitado, são liberados novos merozoítos na corrente sanguínea (6), com invasão cíclica de novos eritrócitos e sintomatologia febril associada à referida lise celular. Alguns merozoítos, após invasão dos eritrócitos, não evoluem para esquizontes, mas diferenciam-se nas formas sexuais do parasito (7), os gametócitos feminino e masculino. Estes, em fase de maturação, se e quando ingeridos pela fêmea do mosquito durante o repasto sanguíneo (8), desenvolvem-se no seu estômago, dando origem, a macrogâmetas e microgâmetas (4 a 8 microgâmetas flagelados) que após fertilização dão início ao ciclo esporogônico (C). O zigoto formado (9) evolui para oocineto (10) móvel, que invade o espaço entre as células epiteliais da parede do estômago do mosquito, fixando-se e originando o oocisto (11). Aqui, por mitoses sucessivas, resultam inúmeros parasitas individuais e alongados denominados esporozoítos (12), que após um período de maturação e por ruptura do oocisto, invadem a cavidade corporal do mosquito e migram para as suas glândulas salivares, acumulando-se nos ductos salivares e tornando-se infectantes. Quando o mosquito infectado faz nova refeição sanguínea (1), os esporozoítos são inoculados podendo começar uma nova infecção no hospedeiro vertebrado.

1.4 Proteína Circunsporozoítica – As variantes do *P. vivax*

O esporozoíto é a forma infectante do *Plasmodium*, inoculado a partir da glândula salivar do vetor em seu repasto hematofágico. Por ser a primeira

forma a entrar em contato com o sistema imune do hospedeiro, este atraiu a atenção dos cientistas envolvidos na tentativa de desenvolver vacinas antimaláricas. A estrutura da proteína majoritária que recobre a superfície do esporozoítio do *Plasmodium vivax* foi caracterizada em 1985 (ARNOT et al., 1985). Por recobrir sua superfície recebeu o nome de proteína circunsporozoítica ou, de forma abreviada, CSP.

O gene CS (Figura 2) apresenta um domínio central formado por nucleotídeos repetidos de forma seqüenciada que são flanqueados por duas regiões não repetitivas nas extremidades 3' e 5' do gene. Estes dois segmentos se mantêm altamente conservados nas diferentes espécies de plasmódios e são denominados região I (RI) que segue na direção amino-terminal e a região II (RII) a 54 aminoácidos da direção carboxi-terminal da proteína (DE LA CRUZ et al., 1988). Esta proteína é constituída por 373 aminoácidos, contendo uma região central de 19 seqüências repetidas constituídas pelo nonapeptídeo Asp-Arg-Ala-Asp/Ala-Gly-Gln-Pro-Ala-Gly (DRAD/AGQPAG). Um aspecto interessante desta proteína é sua homologia com a CSP de *P. cynomolgi* e *P. knowlesi*, dois parasitos cujos hospedeiros são símios, por outro lado, a homologia de seqüência com a CSP de outro parasito humano, o *P. falciparum*, é baixa (ARNOT et al., 1985).

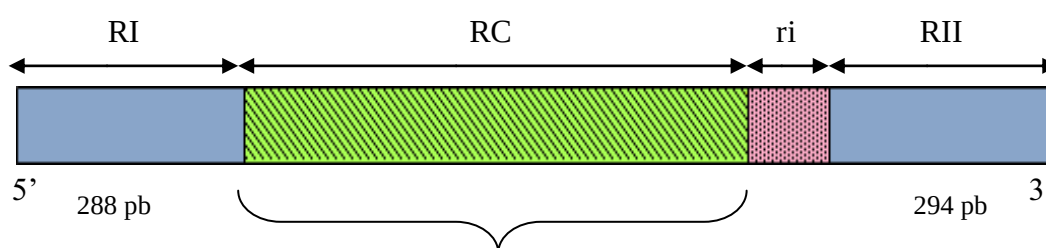


Figura 2 – Estrutura do gene CS de *P. vivax*, com duas regiões terminais não repetidas altamente conservadas (RI e RII); um domínio central repetido (RC), com um número variável de repetições seqüenciais e, uma região curta de inserção (ri). Na RC indicada acima são ilustradas as variações peptídicas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

Posteriormente, novos estudos revelaram que a sequência desta proteína não estava conservada em todos isolados de *P. vivax*. Na Tailândia, esporozoítos obtidos da glândula salivar de mosquitos cujo repasto havia sido realizado no sangue periférico de indivíduos infectados com *P. vivax* não eram reconhecidos por anticorpos específicos pela técnica de Ensaio de imunoabsorção-enzimática ou “Enzyme Immunosorbent Assay” (ELISA). Esta ausência de reatividade ocorreu em 14% dos casos humanos avaliados. Esta proteína variante tem 17 sequências repetidas de um nonapeptídeo diferente: Ala-Asn-Gly-Ala-Gly/Asp-Asn/Asp-Gln-Pro-Gly (ANGA(G/D)(N/D)QPG) ((ROSENBERG et al., 1989) e foi denominada de VK247. Mais tarde, novos estudos revelaram que o número de repetições na porção repetida da CSP, tanto de *P. vivax* clássico (VK210) quanto de VK247, não é constante, variando entre 15 e 21 repetições (MANN et al., 1995; LIM et al., 2001; KIM et al., 2002).

Qari et al. (1993a) avaliando a identidade de oito isolados de *P. vivax* de Papua Nova Guiné relataram a existência de uma nova variante, denominada *P. vivax-like*. O sequenciamento da CSP revelou 16 cópias da sequência repetida APGANQEGGAA (QARI et al., 1993b). Um fato a se destacar é que a sequência do gene desta proteína é praticamente idêntica a CSP do *P. simiovale*, um parasito originalmente identificado em macacos do Sri Lanka (*Macaca sínica*) que, por sua vez, é semelhante morfológicamente a outro parasito de malária humana, o *P. ovale* (QARI et al., 1993a; ESCALANTE et al., 1995). A homologia com *P. vivax* clássico e com a VK247, entretanto, era de apenas 83% (QARI et al., 1993a). Devido a estas evidências, alguns autores acreditam que esta variante possa ser uma espécie diferente de *Plasmodium*.

1.5 Distribuição das variantes do *Plasmodium vivax*

Diversos estudos foram realizados com o intuito de avaliar a distribuição global das variantes do *Plasmodium vivax*. O genótipo VK247 foi encontrado em áreas endêmicas da Tailândia (WIRTZ et al., 1990; KAIN et al.,

1992, 1993a, 1993b), na América do Sul, África (KAIN et al., 1991; GONZALEZ et al., 2001), México e Afeganistão (KAIN et al., 1992; RODRIGUEZ et al., 2000). Qari et al. (1993b) evidenciaram a ampla distribuição da variante *P. vivax-like* analisando amostras de sangue oriundas de regiões geográficas distantes como Papua Nova Guiné, Brasil, Indonésia e Madagascar.

A presença de anticorpos contra a CSP atesta a ocorrência de inoculação das formas infectantes pelos mosquitos. Estudos sorológicos evidenciaram a presença de anticorpos contra peptídeos sintéticos confeccionados a partir da sequência repetida do *P. vivax-like* em 33% de 80 soros de Papua Nova Guiné e em 24% de 156 soros do Brasil (QARI et al., 1993b). Em áreas brasileiras, foram identificadas as três variantes em amostras de sangue de habitantes do Estado de São Paulo (CURADO et al., 1995) e de comunidades indígenas da Amazônia brasileira (ARRUDA et al., 1996, 1998). Oliveira-Ferreira et al. (2004) também confirmaram a presença destes anticorpos em indivíduos provenientes de Candeia do Jamari no Estado de Rondônia.

Através do diagnóstico molecular do gene *CS* de *P. vivax*, estudos prévios confirmaram a presença das três variantes em nosso país a partir de amostras dos Estados de Rondônia, Amapá e do Pará, mostrando que a variante VK210 pode ser encontrada em infecções simples e em concomitância com as outras duas variantes, enquanto a VK247 e a *P. vivax-like* foram detectadas apenas em infecções mistas (MACHADO e PÓVOA, 2000; MACHADO et al., 2003).

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).

Diante do aumento da resistência a cloroquina, Kain et al. (1993a) observaram variações na resposta a essa droga 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.

1.6 Antígenos candidatos à vacina

Apesar de décadas de pesquisas e promessas, ainda não há uma vacina efetiva disponível para malária. As dificuldades apresentadas para o seu desenvolvimento estão relacionadas aos múltiplos estágios do ciclo de vida e número elevado de genes do parasito que apresentam uma diversidade de antígenos em potencial (BEENSON & CRABB, 2007). O sucesso de uma vacina contra a malária deve estar relacionado à intervenção imunológica no 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*. A habilidade do parasito da malária de reconhecer e, então 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 alvo para proteção ou como possível vacina contra malária (MILLER et al., 2002). Dentre estes, destacam-se antígenos pré-eritrocíticos como a CSP (VILAI et al., 2004), proteínas de estágio sanguíneo como proteína de superfície do merozoíto 1

(MSP-1) (ZEYREK et al., 2008; BARBEDO et al., 2007), a Proteína de Ligação ao antígeno Duffy (DBP), presente na superfície da célula hospedeira (BARBEDO et al., 2007; ARÉVALO-HERRERA et al., 2005) e o Antígeno 1 de Membrana Apical (AMA1) (BARBEDO et al., 2007; LALITHA et al., 2008) e

O papel crucial que a resposta imune humoral desempenha na proteção contra a infecção pelos esporozoítos já foi amplamente descrito. Anticorpos monoclonais direcionados contra a região repetitiva do maior antígeno de superfície do esporozoíto, a CSP, podem bloquear a invasão da célula hospedeira e passivamente conferir imunidade (VANDERBERG et al., 1969; POTOENJAK et al., 1980). 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; BEESON et al., 2007; PENNY et al., 2008; KING et al., 2008).

Diferentes segmentos recombinantes derivados da proteína de superfície do merozoíto (MSP) têm sido avaliados quanto ao potencial de estimular resposta sorológica. Trabalhos com dois polipeptídeos recombinantes, rPvMSP₁₄ e rPvMSP₂₀ mostraram que esses fragmentos foram capazes de estimular a produção de anticorpos contra a proteína MSP, entretanto o rPvMSP₂₀ apresentou maiores níveis de resposta (BARRETO et al., 2005). Valderrama-Aguirre et al. (2005) observaram que 52,2% dos indivíduos previamente expostos ao *P. vivax* e 72,8% dos infectados com *P. vivax* desenvolveram anticorpos contra um fragmento N-terminal da MSP1 denominado Pv200L. Nos ensaios de imunização em macacos observou-se proteção parcial contra os estágios sanguíneos, sugerindo que o fragmento Pv200L represente um novo candidato a desenvolvimento de uma vacina.

A proteína de ligação ao antígeno Duffy (DBP) é uma proteína conservada expressa na superfície do merozoíto que interage com antígenos do grupo sanguíneo Duffy, necessários para invasão dos eritrócitos. Arévalo-Herrera

et al. (2005) demonstraram que a imunização com um peptídeo recombinante da DBP de *P.vivax*, a rPvRII, induz altos níveis de anticorpos específicos, mas não fornece imunidade protetora. Em indivíduos vivendo em área de transmissão instável de malária no Brasil, observou-se que a exposição ao *P. vivax* por mais de dez anos induz a produção de anticorpos anti-DBP, capazes de inibir a interação eritrócito-DBP (CERAVOLO et al., 2009).

Estudos têm demonstrado que o Antígeno 1 de Membrana Apical (AMA-1) desempenhe uma função no início do processo de invasão do eritrócito, fazendo parte da reorientação do merozoíto (MITCHELL et al., 2004). Recentemente, demonstrou-se que esta proteína não é somente uma parte essencial no movimento da junção, mas também que a fosforilação do domínio citoplasmático pode ser um pré-requisito para invasão de sua célula alvo (TREECK et al., 2009). A imunização ativa de macacos ou ratos com formas de AMA-1 nativa (DEANS et al., 1988) ou recombinante (ANDERS et al., 1998; COLLINS et al., 1994) tem protegido estes animais contra parasitos de símios e roedores, respectivamente. Anticorpos anti-AMA-1 humanos naturalmente adquiridos reconhecem cepas específicas e epítomos conservados, e, ainda que, anticorpos direcionados são capazes de inibir a invasão de eritrócitos pelo merozoíto de três linhagens de *P.falciparum* expressando diferentes formas de AMA-1, fornecendo fortes evidências de que esta proteína é um alvo natural da resposta de anticorpos protetora e (HODDER et al., 2001).

1.4 A resposta de anticorpos e o Complexo Principal de Histocompatibilidade

Os diversos estudos analisando resposta sorológica a diferentes peptídeos do parasito têm obtido resultados variáveis de acordo com o antígeno utilizado e a população estudada. No entanto, deve-se destacar que, fatores genéticos da resposta imune, correspondentes aos genes do Complexo Principal de Histocompatibilidade (MHC), parecem controlar a eficiência da resposta humoral

nas vacinações. (NARDIN et al., 2000). O MHC na espécie humana é denominado Antígeno Leucocitário Humano (HLA), sendo determinado por genes que se localizam no braço curto do cromossomo 6. Os antígenos de histocompatibilidade podem ser divididos em três classes: HLA de classe I, HLA de classe II e HLA de classe III com diferenças baseadas na distribuição dos mesmos nos tecidos e suas funções. As principais proteínas do HLA II são chamadas DR, DP e DQ e são expressas principalmente pelas células imunocompetentes, incluindo macrófagos, células dendríticas, linfócitos B e são responsáveis pela ativação dos linfócitos T CD4. A principal função das moléculas do HLA II é ligar peptídeos antígenos derivados de proteínas exógenas e apresentá-los na superfície celular para o reconhecimento por linfócitos T CD4. Esses por sua vez são necessários para a iniciação da resposta imune antígeno específica e a produção de anticorpos pelos linfócitos B. O complexo principal de histocompatibilidade é conhecido como o sistema genético humano mais polimórfico, sendo atualmente conhecidos 1374 alelos diferentes que podem codificar a molécula de HLA (CHOO, 2007). A região de classe II consiste de uma série de sub-regiões, cada uma contendo genes *A* e *B*, que codificam as cadeias α e β respectivamente. O gene da família DR consiste de um único gene *DRA* e nove genes *DRB* (*DRB1* a *DRB9*). O gene *DRA* codifica uma cadeia invariável e liga a várias outras cadeias β codificadas pelos genes *DRB*. Nas famílias DP e DQ, a expressão é feita por um gene das cadeias α e β e um pseudogene adicional não expresso. Produtos do gene *DQA1* e *DQB1* se associam para formar moléculas DQ, e produtos do gene *DPA1* e *DPB1*, para formar moléculas DP (Figura 3). (CHOO, 2007). Os polimorfismos nos genes das moléculas de HLA II podem alterar a habilidade para ligar peptídeos e apresentá-los às células T, dessa forma, tendo potencial para modular a resposta de anticorpos (PATARROYO et al., 1991).

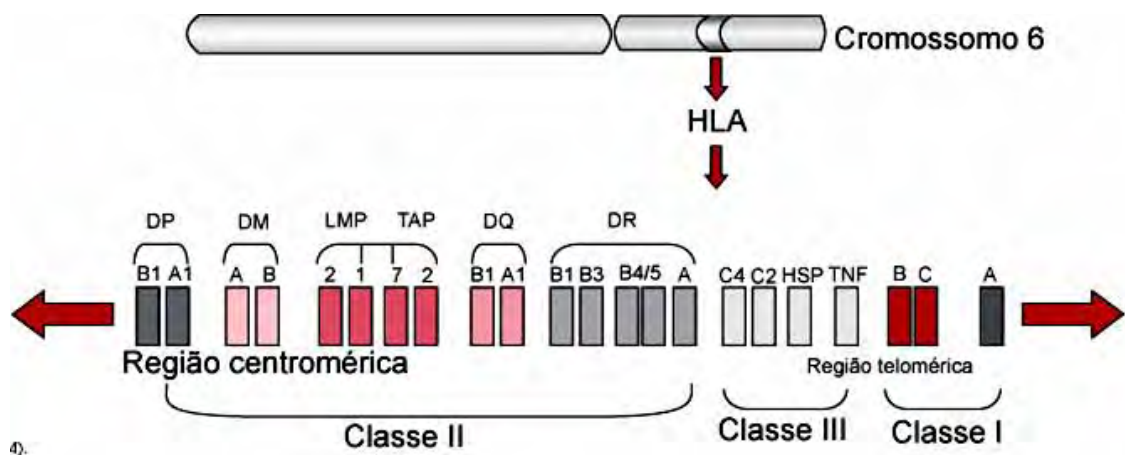


Figura 3. Estrutura gênica do MHC humano. Identificando os genes HLA de classe I (HLA-A, B e C), de classe II (HLA-DR, DQ e DP) e os de classe III. Os genes TAP, LMP e HLA-DM codificam proteínas não expressas nas superfícies celulares.

Estudos prévios têm descrito associações entre diferenças alélicas nos genes do HLA II e a resposta sorológica nos ensaios de vacinas para a malária (PATARROYO et al., 1991; NARDIN et al., 2001). Stephens et al., (1995) descreveram acentuada resposta de anticorpos em indivíduos tailandeses vacinados com antígenos de esporozoítos e a presença do alelo HLA DPB1*0501. Ensaios de vacinas utilizando peptídeos antígenos múltiplos (MAP) têm obtido altos níveis de imunização para anticorpos anti-esporozoítos. Entretanto, essa resposta mostrou-se geneticamente restrita a três genótipos HLA de classe II, sendo eles DRB1*0401, *1101 e DQB1*0603, os quais são encontrados em aproximadamente 25 a 35% da população dependendo da formação étnica. Por outro lado, o alelo DRB1*0701 tem sido associado a baixos níveis de resposta de anticorpos (NARDIN et al., 2000).

A pesquisa por imunógenos ideais tem sido direcionada na identificação de antígenos expostos na superfície celular, regiões conservadas envolvidas na invasão da célula hospedeira e a caracterização de epítomos de célula B. Os dados de ensaios clínicos de vacinas para malária têm mostrado imunogenicidade limitada e falha ao induzir resposta de longa duração, sugerindo a falta de epítomos de célula T efetivos nos construtos usados como imunógenos.

Um dos maiores impedimentos para identificação e utilização de epítomos de célula T é o alto nível de polimorfismos das moléculas do HLA de classe II (CARO-AGUILAR et al., 2002). Diferentes epítomos da MSP-1 foram avaliados quanto à interação com as moléculas do HLA, sendo testados 86 peptídeos da PvMSP-1, dos quais oito ligaram-se competitivamente a quatro alelos do *HLA-DRB1* em ensaios experimentais (CARO-AGUILAR et al., 2002).

A prevalência de anticorpos contra antígenos da CSP de *P. vivax* tem sido avaliada em diferentes populações, utilizando peptídeos recombinantes contendo diferentes seqüências da região não repetitiva assim como para as variantes da região repetitiva central (FRANKE et al., 1992; KREMSNER et al., 1992). A utilização de peptídeos sintéticos lineares permitiu a identificação de vários epítomos de células B e T ao longo da proteína. Herrera et al. (1997) identificaram três epítomos de célula T, sendo um da região central e dois da região carboxil, amplamente reconhecidos por linfócitos humanos.

A resposta sorológica contra as variantes da CSP de *P. vivax* foi analisada em indivíduos de uma comunidade endêmica do Estado de Rondônia que declararam apresentar média de dois episódios de malária nos últimos dois anos. Os polimorfismos nas moléculas de HLA II dessa população foram avaliados, sugerindo a relação entre o alelo HLA-DR16 e resposta de anticorpos para a variante *P. vivax* VK247, enquanto o alelo HLA-DR7 esteve relacionado à ausência de resposta de anticorpos contra a variante VK210. Nenhuma associação foi evidenciada para o locus HLA-DQ (OLIVIERA-FERREIRA et al., 2004). Os polimorfismos do HLA têm sido associados à variações sorológicas nos ensaios de vacinação, no entanto, são poucas as avaliações desses polimorfismos frente a resposta de anticorpos para antígenos importantes do parasito em populações naturalmente expostas.

1.5 Justificativa

O Brasil apresenta alta miscigenação, com populações etnicamente diversas e grande heterogeneidade entre suas regiões devido ao padrão peculiar de colonização. A região Norte exibe maior contribuição ameríndia, enquanto no sul do país observa-se a maior proporção de caucasóides. Além disso, o padrão regional de miscigenação foi consideravelmente alterado pelas correntes de migração interna (GUERREIRO et al., 1988; NOVARETTI et al., 2000). A imunoprevenção efetiva contra a malária requer uma vacina que seja capaz de estimular a imunidade humoral e celular parasito-específica em indivíduos de diferentes tipos de HLA. O epítipo ideal deve ser universal, capaz de interagir com um grande número, se não todas as moléculas de classe II. Além disso, a avaliação entre a infecção pelos genótipos descritos a partir da CSP do *P. vivax* e a resposta imune contra diferentes peptídeos do parasito, incluindo os da região repetitiva da CSP, pode fornecer avanços para compreensão do comportamento da resposta imunológica frente a essas variantes. Enquanto diversos estudos estão focados na busca por vacinas eficazes, considerações da influência das frequências alélicas do HLA de classe II em populações etnicamente diversas podem ser importantes, antes de ensaios de vacinas em pessoas naturalmente expostas aos parasitos da malária (OLIVEIRA-FERREIRA et al., 2004).

1.6 Hipótese

Os genótipos variantes da proteína circumsporozoita de *P. vivax* e/ou os polimorfismos nos genes das moléculas do HLA de classe II podem influenciar a resposta imune humoral contra os diferentes antígenos de *P. vivax* em indivíduos naturalmente expostos à malária em áreas endêmicas da Amazônia brasileira

2 OBJETIVOS

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a resposta de anticorpos contra antígenos de merozoíto e esporozoíto de *P. vivax* e sua relação com a frequência das variantes genéticas da CSP no parasito, bem como com os polimorfismos do HLA de classe II no hospedeiro humano.

2.2 Objetivos específicos

1º - Avaliar a distribuição das variantes do gene *CS* de *P. vivax* por diagnóstico molecular em amostras de indivíduos infectados provenientes de cinco diferentes regiões da Amazônia brasileira;

2º - Avaliar a resposta imunológica de anticorpos contra os peptídeos da CSP, MSP1, AMA1 e DBP e sua relação com as infecções causadas pelas variantes genéticas da CSP de *P. vivax*.

3º - Avaliar as frequências alélicas dos polimorfismos do *HLA-DRB1* em amostras da região norte do Brasil e sua influencia na resposta de anticorpos contra os peptídeos da MSP1 (*Pv200L*), AMA-1 e DBPII em indivíduos naturalmente infectados com *P. vivax*.

3 RESULTADOS

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 publicado na revista **Malaria Journal**, v. 9, n. 178, p. 1-8, 2010.

Capítulo IV. Evaluation of the naturally-Acquired Antibody Immune Response to the Pv200L N-terminal Fragment of *Plasmodium vivax* Merozoite Surface Protein-1 in Four Areas of the Amazon Region of the Brazil. Artigo original aceito para publicação na revista **American Journal of Tropical Medicine and Hygiene**.

Capítulo V. Evaluation of HLA-DRB-1 alleles in the immune response against MSP-1, AMA-1 and DBP in Brazilian individuals naturally infected with *Plasmodium vivax*. Artigo original submetido à publicação e em fase de revisão na revista **Acta Tropica**.

3.1 CAPÍTULO I

A fim de melhor entender a variabilidade existente no genoma do *Plasmodium vivax* e assim compreender a diversidade genética dos seus principais antígenos, foi elaborado o trabalho de revisão descrito a seguir:

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 drogas mais efetivas para o tratamento da malária *vivax*.

The genetic diversity of *Plasmodium vivax* - A Review

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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

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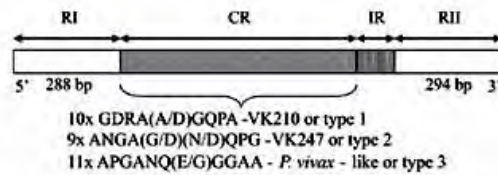


Fig. 1: structure of the *Plasmodium vivax* CSP gene, with two highly-conserved terminal non-repeat regions (RI and RII); a central repetitive (CR) domain, with a variable number of tandem repeats, and a short IR (insertion region). In the CR are indicated below, the peptide variations from protein. 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  les et al. 2001, Inwong 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  les-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-1₈₃, MSP-1₃₀, MSP-1₃₈, and MSP-1₄₂) then, before erythrocyte invasion the MSP-1₄₂ fragment undergoes a second cleavage resulting in 33 and 19 kDa (MSP-1₃₃ and MSP-1₁₉) fragments with the latter remaining on the merozoite surface during invasion (Pacheco et al. 2006). The MSP-1₄₂ and MSP-1₁₉ 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-1₁₉ 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

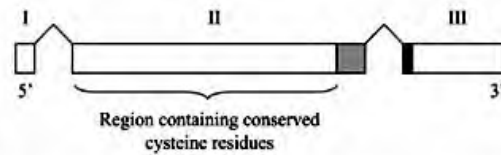


Fig. 2: organization of the *Plasmodium vivax vir* genes. This representation shows a short first exon (I), a longer second exon containing a transmembrane domain (hatched box or exon II), and the 3-exon structure (open boxes or III). Regions conserved amongst *vir* variants are indicated (filled box).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

in some gene regions (Cui et al. 2003). This provides many potential alleles thereby making this gene a useful marker for typing parasite populations (Figtree 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, Fernandez-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-

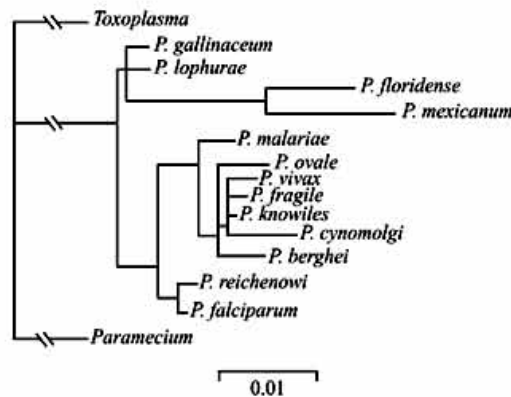


Fig. 3: phylogenetic tree of *Plasmodium* spp. derived by the maximum likelihood (fastDNAML) method. Scale bar indicates an evolutionary distance of 0.01 nucleotide substitutions per position in the sequence. According to Qari et al. (1996)

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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3.2 CAPÍTULO II

Uma vez que, os objetivos específicos deste trabalho incluíram avaliar a associação entre a frequência das variantes da CSP de *P. vivax* na resposta de anticorpos, a distribuição destas variantes da CSP em cinco diferentes áreas da Amazônia foi inicialmente avaliada, buscando entender sua atual dinâmica de transmissão e assim atendendo ao primeiro objetivo específico da tese. Os resultados estão apresentados na Tabela 1 do artigo a seguir.

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 circunsporozoítica (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 CS 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 a 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

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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

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different from one to another. This is the first detection of the VK247 and *P. vivax*-like variant 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.¹ The circumsporozoite protein (CSP) of the infective sporozoite is considered to be a major target for the development of recombinant malaria vaccines.²⁻⁴ 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.⁵⁻⁹

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.¹⁰ The FY gene, located on human chromosome 1, encodes the Duffy proteins by the two co-dominant alleles: FYA and FYB.¹¹ 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.¹²

Previous reports from Latin America^{9,13} have suggested different abilities for vector infection between the VK210 and VK247 phenotypes. Ryan et al.¹⁴ 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.¹⁵ 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.¹⁶

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.¹⁶ The peripheral blood samples, which had been kept at -70°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™ 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.¹⁷ 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 µl) was amplified in a total reaction volume of 25 µl consisting of 1 × PCR buffer (10mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM of MgCl₂, 1.0 µM of each primer, 200 µM dNTPs, 2.5 U ampli-Taq DNA polymerase (Invitrogen, São Paulo, Brazil), 1% betaine and water (25 µl). Twenty-five cycles of amplification were performed in a thermocycler (DNA MasterCycler; Eppendorf, Madison, WI, USA) after initial denaturation of DNA at 94°C for 5 min. Each cycle consisted of a denaturation step at 93°C for 60s, an annealing step at 41°C for 90s and an extension step at 72°C for 2 min, with a final extension at 72°C for 10 min following the last cycle. Before typing CSP variants we progressively tested lower annealing temperatures (0.5°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 µl of the PCR product and 1 U of each enzyme (*AluI* and *DpnI*, Promega, Madison, WI, USA), incubated for 1 h at 37°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.¹⁸ 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 µL. 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.¹⁹ The amplification conditions were performed as described by Castilho et al.¹⁸ 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 *Ban*I, *Msp*AI and *Sty*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.²⁰ 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 ^d	2 (1.3)	0	0	0	0	0	0	2 (1.3)
FYB/FY ^d	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.^{6,7} 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,² Mexico and Asia.^{4,21,22} 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,²³ although in Brazil¹⁵ and in Africa¹⁴ *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,¹⁶ reinforcing the hypothesis that the presence of both functional Duffy alleles increases the risk of infection by this parasite.²⁴ Indeed, the FYA/FYB genotype was the most prevalent in this study, which included only malaria patients. Possibly, variations in FYA and FYB allelic frequencies in populations exposed to *P. vivax* may determine differential susceptibility. Cavasini et al.¹⁶ showed that the difference in the FYB allelic proportion between 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).¹² Previous reports in Papua New Guinea²⁵ and Colombia²⁶ 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.²⁷ suggested that polymorphism in the ligand domain of DPB can alter immune recognition. In Brazil, Cerávolo et al.²⁸ 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.²⁹ 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.^{2,14} 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.¹⁴ In the previous report of Cavasini et al.¹⁵ 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,³⁰ or if these variants represent the existence of a new species or subspecies of *Plasmodium* causing human malaria, with major biological consequences.⁴ 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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Conflicts of interest: None declared.

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3.3 CAPÍTULO III

Com o intuito de avaliar as implicações da variação na porção central da CSP de *P. vivax*, nós buscamos comparar os níveis de resposta de anticorpos contra diferentes antígenos de merozoíto e esporozoíto e a frequência das variantes da CSP, de forma a atender ao objetivo específico número 2. O manuscrito apresentado neste capítulo faz uma análise em nível sorológico e molecular das consequências biológicas desta variação.

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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 RNAr 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 as

variantes da CS, 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 CS estão restritas a porção central repetitiva da proteína, representando uma variação nucleotídica sem consequências sorológicas importantes.



RESEARCH

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

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Abstract

Background: *Plasmodium vivax* circumsporozoite variants have been identified in several geographical areas. 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 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 rRNA* 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: These 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. A high frequency IgG antibody against the N- and C-terminal regions of the *P. vivax* CSP was found as 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: This 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 anti-malarial vaccine development; however the existence of variations in the repetitive sequence of its central portion has been hindering these studies. *Plasmodium 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* [5], 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

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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 *Anopheles 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 one 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 it 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 simple 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, this work contributes to the understanding of the implication of the central repetitive region variation of the CS 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 additional file 1: Hosts type, geographic origins and GenBank accession numbers of the out groups. 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 (SSU rRNA) 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 10 mM Tris-HCl with

pH 8.3, 50 mM KCl, 200 μ M of each of the four dNTPs, 10 μ M of each oligonucleotide primer, 1 μ g DNA template and 0.5 U of *ampli-Taq* DNA polymerase (Invitrogen, USA) to a final volume of 25 μ 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'- GCTACAGGTG-CATCTCTTGTATTC - 3') and PC2 (5' - CACTTACAG-TATATCCTCCACATAACCA - 3'). A reaction mixture of buffer 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μ M of each of the four dNTPs, 10 μ M of each oligonucleotide primer, 1 μ 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 analysed 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 the DBP [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 [51]. Differences among the frequencies of responders were analysed using Pearson's chi-square or, alternatively, the Fisher's exact test. Differences were considered significant when *p*-value ≤ 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 Figure 2). The genetic distances between CS genotypes from the *Plasmodium* species analysed are described in Additional file 2 and Additional file 3: Genetic distances between 18 *SSU rRNA* and *Cyt B* genes from *Plasmodium* spp. 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.

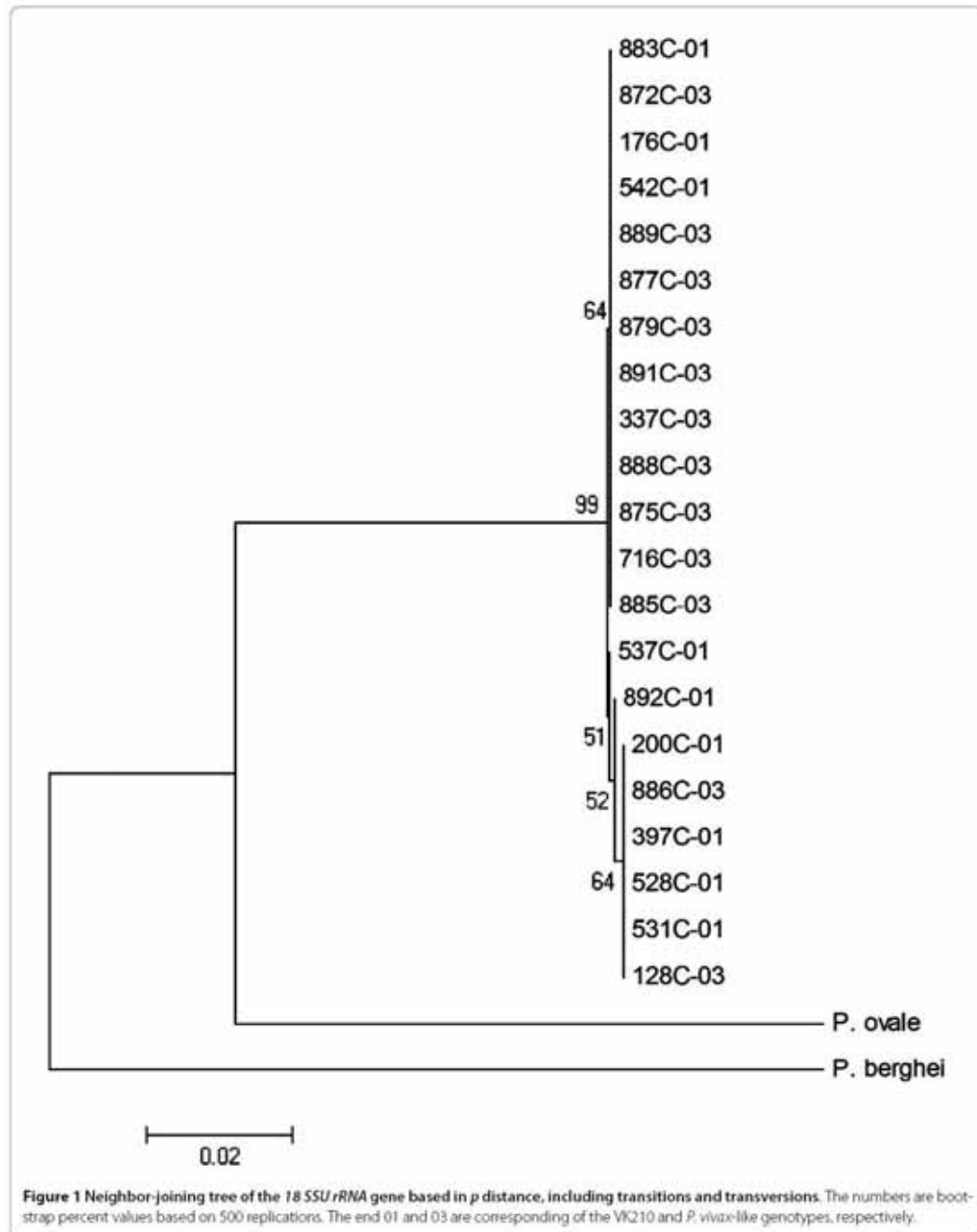
Antibody response against the CSP, MSP-1, AMA-1 and DBP peptides

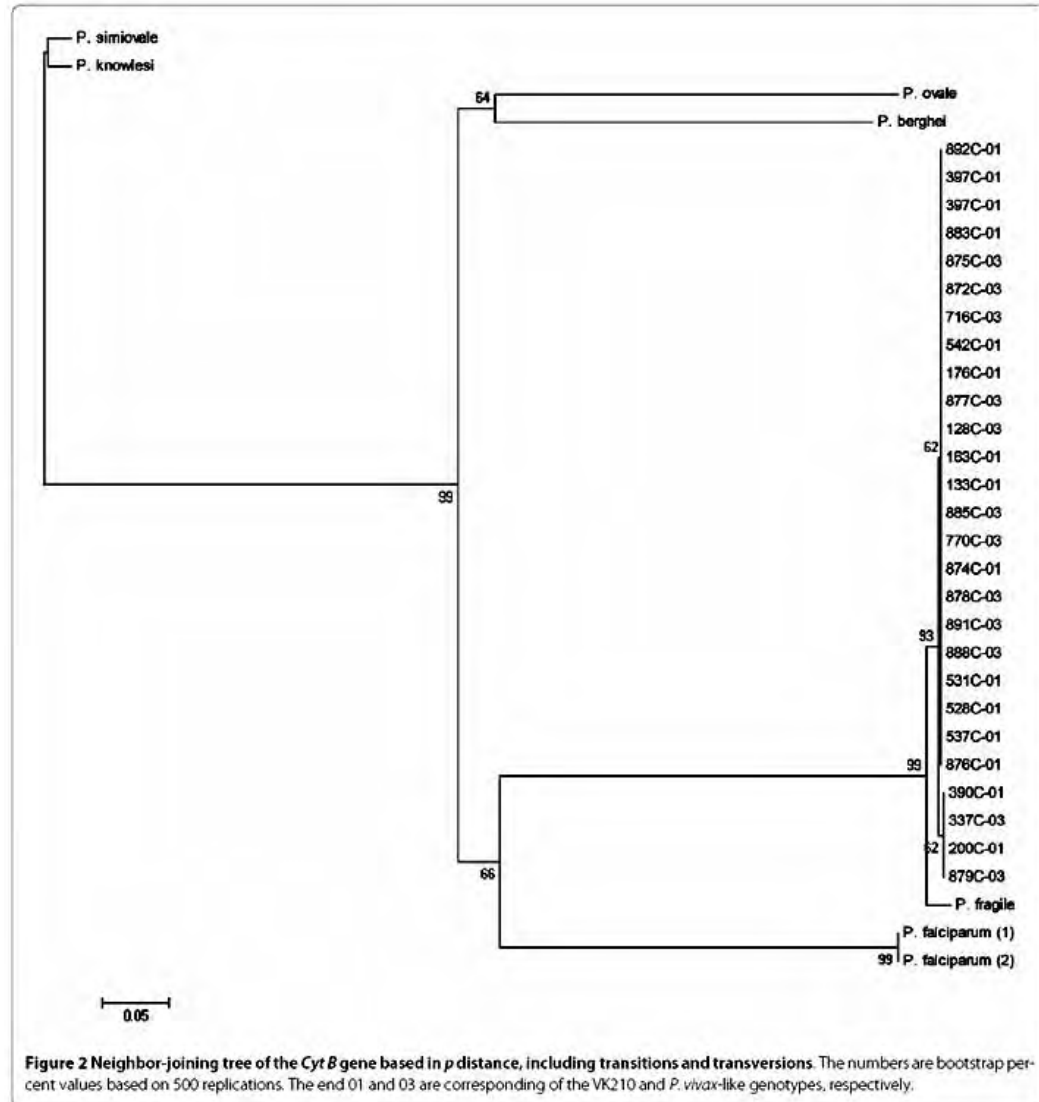
A higher frequency IgG antibody against the N- and C-terminal regions of the *P. vivax* CSP was found as compared to the immune response to the R- and V- repetitive regions ($p = 0.0005$, Fisher's exact test). Antibody responses against the peptides of the CSP repetitive central region [R] and [V] compared 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 the CS genotypes in the infection (Table 1).

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 2) in comparison to same frequency for DBP ($p = 0.003$, 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 analysed 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], this work presents here evidences of this variation is 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 *Plasmodium 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]. In this study, the results were obtained through the phylogenetic analysis of the 18 *SSU rRNA* 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

Table 1: 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)						
	1 (34)	2 (4)	3 (18)	1 + 2 (12)	1 + 3 (8)	1 + 2 + 3 (4)	Total (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.

same clade, in accordance with previous data [35]. Although the absence of VK247 genetic sequences, a limitation of the present study, the results point 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 analysed merozoite peptides, MSP1 (*Pv*200L), AMA-1, DBP and against the CSP conserved fractions in the sporozoite, N-terminal and C-terminal. Besides, when the antibody responses against the peptides corresponding to the CSP repetitive central region were evaluated, 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 observed an even lower antibody response against these two fragments ([R] and [V]) were observed. 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 do not provide significant differences in antibody responses against *P. vivax* merozoite and sporozoite conserved regions peptides represents key information in the future design of vaccine assays. On the other hand, studies based in CSP should 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

Conclusion
These results target that the differences among the *P. vivax* CS variants are restrict to the central repeated

Table 2: 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)						
	1 (51)	2 (4)	3 (23)	1 + 2 (14)	1 + 3 (8)	1 + 2 + 3 (4)	Total (104)
Pv200L	92.2	100	78.2	100	87.5	100	90.4
	1 (39)	2 (4)	3 (18)	1 + 2 (13)	1 + 3 (8)	1 + 2 + 3 (4)	Total (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.

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.

Additional material

Additional file 1 Hosts type, geographic origins and GenBank accession numbers of the out groups. GenBank accession numbers.

Additional file 2 Genetic distances between 18 SSU rRNA genes from *Plasmodium* spp. Genetic distances.

Additional file 3 Genetic distances between Cyt B genes from *Plasmodium* spp. Genetic distances.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WCSN 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 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. RLD 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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3.4 CAPÍTULO IV

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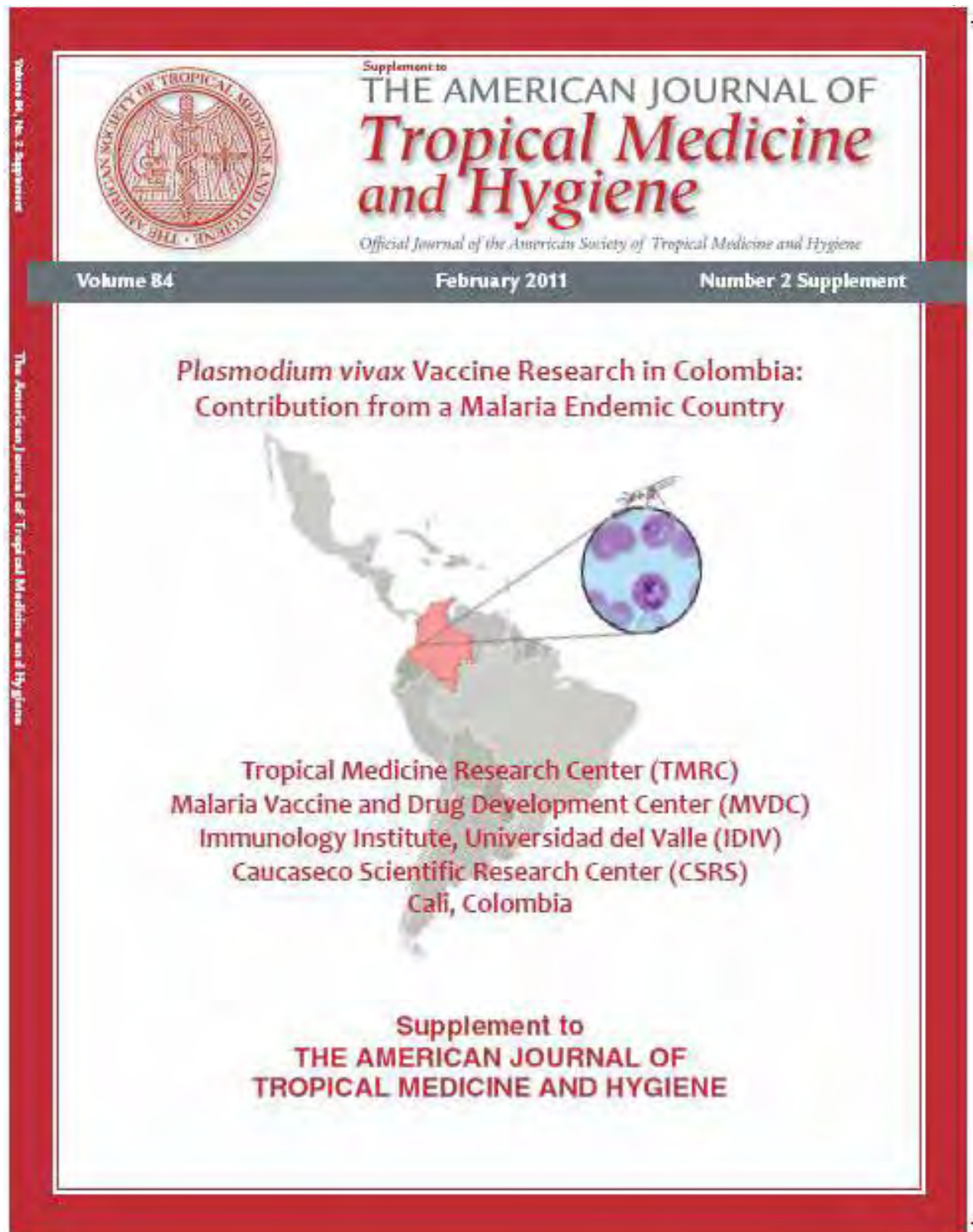
Dentre as proteínas estudadas nesta pesquisa incluí-se a MSP1, mais especificamente o peptídeo correspondente a sua região N-terminal, descrito em 2005 por VALDERRAMA-AGUIRRE na Colômbia. Este fragmento denominado Pv200L havia sido avaliado quanto sua antigenicidade e imunogenicidade apenas pelo grupo Colombiano. Todavia para obtermos um perfil de reconhecimento e resposta de anticorpos contra este fragmento nas populações brasileiras naturalmente expostas a malária elaboramos manuscrito abaixo.

Evaluation of the naturally-Acquired Antibody Immune Response to the Pv200L N-terminal Fragment of *Plasmodium vivax* Merozoite Surface Protein-1 in Four Areas of the Amazon 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, Diana M. Echeverry, Andréa R. B. Rossit, Myriam Arévalo-Herrera, Sócrates Herrera, and Ricardo L. D. Machado Artigo original publicado no suplemento do periódico1 **American Journal of Tropical Medicine and Hygiene**.

RESUMO

Neste trabalho avaliou-se a frequência e os níveis de anticorpos IgG para um fragmento N-terminal da proteína de superfície do Merozoíto (MSP-1) de *Plasmodium vivax* (Pv200L) por ELISA, em indivíduos naturalmente expostos à malária provenientes de quatro áreas endêmicas do Brasil. Foram analisadas amostras de plasma de 261 indivíduos infectados com *P. vivax* das comunidades de Macapá, Novo Repartimento, Porto Velho, e Plácido de Castro na região Amazônica, as quais apresentam diferentes intensidades de transmissão. Um grande número dos indivíduos estudados (89,3%) apresentou níveis de anticorpos

contra o Pv200L, os quais foram relacionados com o número de infecções prévias de malária. Observou-se diferenças significantes na frequência de respondedores (71,9–98,7) e nos níveis de anticorpos (1:200–1:51.200) entre as quatro áreas estudadas. Os resultados deste estudo fornecem evidências de que o Pv200L é um fragmento naturalmente imunogênico da MSP-1 de *P. vivax*, sendo que os níveis de resposta parecem estar associados com o grau de exposição ao parasito. Atualmente a especificidade dos anticorpos anti-Pv200L esta sendo avaliada.



Evaluation of the Naturally Acquired Antibody Immune Response to the Pv200L N-terminal Fragment of *Plasmodium vivax* Merozoite Surface Protein-1 in Four Areas of the Amazon Region of Brazil

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Abstract. Frequency and levels of IgG antibodies to an N-terminal fragment of the *Plasmodium vivax* MSP-1 (Pv200L) protein, in individuals naturally exposed to malaria in four endemic areas of Brazil, were evaluated by enzyme-linked immunosorbent assay. Plasma samples of 261 *P. vivax*-infected individuals from communities of Macapá, Novo Repartimento, Porto Velho, and Plácido de Castro in the Amazonian region with different malaria transmission intensities. A high mean number of studied individuals (89.3%) presented with antibodies to the Pv200L that correlated with the number of previous malaria infections; there were significant differences in the frequency of the responders (71.9–98.7) and in the antibody levels (1:200–1:51,200) among the four study areas. Results of this study provide evidence that Pv200L is a naturally immunogenic fragment of the PvMSP-1 and is associated with the degree of exposure to parasites. The fine specificity of antibodies to Pv200L is currently being assessed.

INTRODUCTION

Plasmodium vivax is the main species responsible for malaria infection in the American continent and in vast areas of the Middle East, East and Southeast Asia, Oceania, and the Eastern Mediterranean region.^{1,2} In America, Brazil and Colombia are the most malaria-endemic countries with *P. vivax* accounting for more than 70% of the malaria clinical cases in the region with a high percentage of cases originating in the Amazon region.^{3,4} Vaccines against malaria are considered a potentially cost-effective measure for malaria control and elimination, therefore, significant effort is currently being invested in vaccine development.⁵ In addition to the recent progress toward the development of a vaccine against *P. falciparum*, further development of a functional vaccine for most endemic areas of the world where both *P. falciparum* and *P. vivax* co-exist requires a better understanding of the protective immune responses induced by both malaria parasites. Individuals permanently exposed to malaria infection in endemic areas eventually develop clinical immunity; analyses of such immune responses may contribute to the identification of target antigens with vaccine potential.^{6–9}

The merozoite surface protein 1 (MSP-1), a glycoprotein of ~195 kDa expressed in all *Plasmodium* species studied, is considered to have great vaccine potential.¹⁰ It has been shown that in *P. falciparum*, MSP-1 is synthesized as a large precursor polypeptide that is then proteolytically processed into smaller fragments during late schizont development.¹¹ Its molecular weight varies slightly among parasite isolates caused by antigen size polymorphism,^{12,13} however the processed MSP-1 polypeptides contain remarkably conserved sequences¹⁴; one such

sequence is localized in proximity of the amino terminus of the protein within a 80 kDa processing fragment that includes a region termed 190L (Pf190L).^{15,16} The latter peptide region has an elevated number of B- and T-cell epitopes¹⁷ and has shown to be highly immunogenic and partially protective in monkey and human trials.^{18,19} *Plasmodium vivax* MSP-1 has a region with significant sequence homology to the Pf190L fragment that has been termed Pv200L.²⁰ Although most studies in the PvMSP-1 have concentrated on the carboxyl region, a few have been designed to analyze the antigenicity of the amino terminus of the protein that appears to be associated with clinical protection.⁷ A longitudinal study conducted in individuals from Rondonia State, Brazil indicated that more than 90% of the sera from patients having experienced more than three previous malaria infections contained antibodies to the ICB2-5 fragment, located on the amino terminus of the PvMSP-1 protein, which comprises the Pv200L fragment.²¹ Additionally, the Pv200L fragment was first tested in a seroepidemiological study conducted in Colombia and indicated that the majority of individuals previously exposed to *P. vivax* malaria infection had antibodies to rPv200L.²⁰ Moreover, immunizations of BALB/c mice and *Aotus* monkeys with the Pv200L fragment indicated great immunogenicity and partial protection against experimental challenge with *P. vivax* blood stages.²⁰

Herein, we have 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 with different *P. vivax* transmission intensities. These findings have allowed us to better define the immunological relevance of this protein fragment and its potential as a vaccine target.

MATERIAL AND METHODS

Study areas. In the Brazilian Amazon region malaria predominates in Mesoendemic conditions with wide variations in transmission, as can be observed by the non-immune or semi-immune status of the adult population and by presence

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of the asymptomatic carriers.^{22,23} Because of this, the samples were collected in four different sites of the Amazon region of Brazil: Macapá, (Amapá State); Novo Repartimento (Pará State); Porto Velho (Rondônia State); and Plácido de Castro (Acre State). Macapá is the capital of the state of Amapá and is located on the Amazon River with a tropical forest. Its estimated population is 366,486 inhabitants and its annual parasitic index (API) was 6.0 in 2009. Porto Velho is the capital of the State of Rondônia in the upper Amazon River basin, with 383,425 inhabitants and an API of 53.7 last year. Plácido de Castro is located at the border of Rondônia and Amazonas states, it has a population of 18,235 inhabitants and its API was 20.6 in 2009. Novo Repartimento is a gold mining area in southeastern Pará State. Its population is estimated as 55,759 habitants and had an API of 15.4 last year. The climate in these areas is characterized as tropical with no dry season; the mean monthly precipitation level is at least 60 mm.

Study subjects and blood samples. A total of 261 adult individuals were evaluated in this study following a protocol approved by the Institutional Review Board (IRB) of the Faculty of Medicine, São José do Rio Preto. All participants were enrolled according to the following criteria: sought medical assistance for clinical malaria symptoms, were > 18 years of age, and had a positive *P. vivax* malaria diagnosis by thick blood film. We excluded from the study pregnant women, patients < 18 years of age, and no other concomitant illness. Participants were asked to sign a written consent form before blood samples were drawn. Epidemiological data such as age, gender, past history of malaria, and current infection information were obtained from a specific interview and also from medical records. Five mL of peripheral blood was collected by venipuncture from each of the individuals using tubes containing EDTA. Approximately 3 mL of plasma was then stored at -80°C until use, and the remaining cell volume was used for malaria diagnose confirmation by using a polymerase chain reaction (PCR) technique.

Parasite detection. Patients were examined to confirm the *P. vivax* malaria diagnosis by using Giemsa stained thick blood smear. Slides were kept at room temperature and parasite density was quantified after examination of 200 microscopic fields at 1,000× magnification under oil-immersion. All slides were examined by two independent well-trained microscopists who were unaware of each result according to the Ministry of Health in Brazil recommended procedures.²⁴ Additionally, diagnose was confirmed in all samples by using an PCR technique.²⁵ For the PCR technique DNA samples were extracted from frozen pellets of infected erythrocytes using the Easy-DNATM extraction kit (Invitrogen, Carlsbad, CA), and a semi-nested PCR using specific small-subunit (SSU) ribosomal RNA (rRNA) primers.²⁵ Briefly, two rounds of amplification were performed with oligonucleotide primers that target small sub-unit rRNA genes of *Plasmodium*. The first round (35 cycles) used a pair of universal (i.e., genus-specific) primers, P1 and P2, whereas the second round (18 cycles) used the species-specific reverse primers F2, V1 and M1 combined with the universal forward primer P1. Oligonucleotide primer sequences and detailed PCR protocols are given elsewhere.

Pv200L protein. The Pv200L protein was expressed as a recombinant protein in *Escherichia coli* as described previously.²⁰ The protein encodes the fragment containing amino acid residues 121–416 of the *P. vivax* MSP-1. The fragment was amplified, cloned, and expressed

in *E. coli* according to standard methods,²⁰ and purified by immobilized metal ion affinity chromatography (IMAC) with Ni-nitrilotriacetic acid matrix (Qiagen, Brazil), dialyzed against phosphate-buffered saline (PBS) at 4°C, and reprocessed several times to reduce *E. coli* contaminants. Final batches of rPv200L were analyzed for homogeneity and purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analytical reverse-phase high-performance liquid chromatography (HPLC).

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 described elsewhere.²⁰ Briefly, flat-bottomed, 96-well microplates (Nunc Maxisorp, Rochester, NY) were coated with 100 mM of rPv200L per well and incubated overnight at 4°C. Microplates were blocked for 2 hours with PBS containing 0.05% Tween 20 and 1% bovine serum albumin (Sigma, St. Louis, MO). After one wash with T-PBS, samples diluted 1:200 were added and incubated for 1 hour at room temperature. Plates were washed five times with PBS containing 0.05% Tween 20, and secondary antihuman immunoglobulin G (IgG) antibody labeled with alkaline phosphatase (Sigma) was added at a final dilution of 1:4000. Plates were incubated for 1 hour at room temperature, washed with T-PBS, and developed with *p*-nitrophenylphosphate (Sigma). The reaction was stopped after 15 minutes with 10 uL of 1N NaOH and read at 405 nm. Every sample was tested in triplicate. The ELISA IgG cutoff was defined as the average of negative control samples plus three standard deviations. The results were expressed as an index of reactivity (IR = OD₄₀₅ values of tested sample divided by the value of the cutoff). Values of IR < 1.0 were considered negative, values of IR ≥ 1.0 and < 10 were considered positive (antibody titers estimated to vary from 1:200 until 1:3,200), whereas values of IR ≥ 10 were considered highly positive (antibody titers estimated to vary between 1:3,200–51,200).

Statistical analysis. Analyses were performed using R version 2.8.1 statistical software (The R Foundation for Statistical Computing, Vienna, Austria [http://www.rproject.org]). Differences among the frequencies of responders were analyzed using Pearson's χ^2 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* value ≤ 0.05, and when Bonferroni's correction was performed *P* value ≤ 0.017 or *P* value ≤ 0.008, respectively, to the number of compared groups (3 or 4).

RESULTS

Frequency and level of antibodies to Pv200L in the four Amazonian communities. An average of 65 individuals per study area was evaluated for a total of 261 subjects. The overall frequency of antibodies that recognized the Pv200L in ELISA was 89.3%. The average IR was 5.5 and 17.6% of samples showed high antibody titers (IR ≥ 10). Significant differences (*P* = 0.000001, Fisher's exact test) in responder frequency among the four study sites were observed (Table 1).

In Macapá, the lowest percentage of positive Pv200L antibodies in individuals was detected (71.9%), whereas Novo

TABLE 1

Frequency and antibody levels of Pv200L in sera of infected individuals with *Plasmodium vivax* in four areas from Brazilian Amazon region

Area	Samples (n)	Antibody frequency (%) ^a	Index of reactivity (IR) – antibody level		
			Average	> 1 (%)	> 10 (%)
Macapá	64	71.9	4.4	59.4	12.5
Novo Repartimento	76	98.7 [†]	6	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
Total	261	89.3	5.5	71.7	17.6

^a Fisher's exact test ($P = 0.00001$) analyzing the four areas

[†] Macapá vs. Novo Repartimento ($P = 0.00002$).

[‡] Macapá vs. Porto Velho ($P = 0.0004$), Bonferroni's correction P value ≤ 0.008 .

Repartimento had the highest frequency of responders (98.7%). In the *post hoc* analysis by Bonferroni's pairwise comparisons (significant P value ≤ 0.008), Macapá frequencies were statistically lower compared with 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 lowest antibody titers were detected in Macapá subjects (average of IR = 4.4) and the lowest frequency of high responders (IR ≥ 10 ; 12.5%). On the other hand, the highest average IR (6.2) was found in Plácido de Castro with a frequency of 21.2% of high responders (Table 1). Figure 1 indicates each individual IR value plotted by endemic area. Individuals from Macapá were the only ones presenting bottom line antibody concentrations. The IR values were used to construct a boxplot by area that can also be seen in Figure 1, Macapá presented lower median and confidence interval values than others. Statistical pairwise comparisons (Bonferroni's correction, P value ≤ 0.008) confirmed that the lowest antibody responses occurred in Macapá; 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. Findings showed a correlation between the frequency and antibody levels and number of previous malaria episodes. For this, plasma samples from 200 individuals were separated and categorized according to the number of previous malaria episodes and then further divided into three groups: (0) primary infected, (1 or 2) individuals

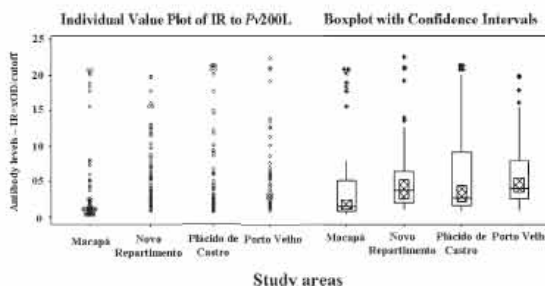


FIGURE 1. Antibody levels to Pv200L in individuals from four areas of the Brazilian Amazon region. Individual value plot of index of reactivity (IR) and boxplot of the median values of IR in each area with confidence intervals: CI (Macapá, median = 1.5 and CI = 1.1–2.4; Porto Velho, median = 3.8 and 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 * represents outliers.

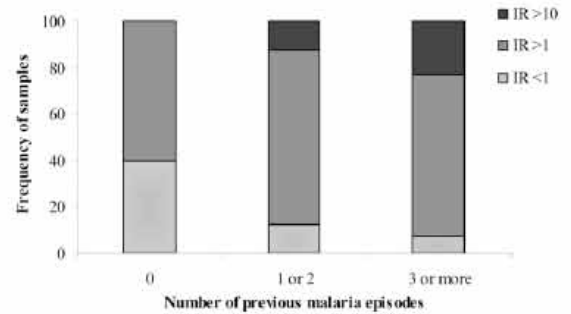


FIGURE 2. Correlation between the frequency of samples for IR values and the number of previous malaria episodes. Individuals ($N = 200$) were grouped according to the number of previous malaria infections. The number of individuals of each group was 10, 82, and 108 for zero, 1 or 2, and 3 or more previous malaria groups, respectively. The index of reactivity (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 χ^2 test).

with one or two previous malaria episodes, and (3 or more) individuals with at least three previous malaria episodes. We found that the frequency of Pv200L antibody-positive samples was statistically different among these three groups (Figure 2; $P = 0.006$, Pearson χ^2). The percentage of individuals that did not recognize Pv200L (IR < 1 or negative) was significantly higher in the (0) group (40%) as compared with 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 χ^2). None of the individuals of the primary-infection group presented high antibody titer (IR > 10) (Figure 2). This tendency was also confirmed by binary logistic regression analysis. The percentage of responders in the primary-infected group was compared with the other groups. The odds ratio (OR) for groups with 1 or 2 and 3 or more previous malaria infections was OR = 4.8 ($P = 0.031$) and OR = 8.3 ($P = 0.004$), respectively.

Correlation between Pv200L antibody levels and the number of previous malaria infections showed that all primary infected (0) had lower IR values (Figure 3) with average of IR (IR = 2.0) lower than average of IR of all patients (other three

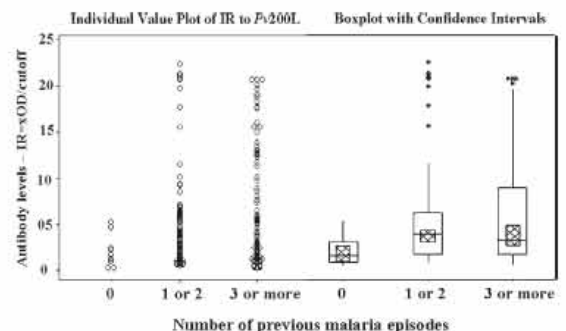


FIGURE 3. Correlation between antibody levels to Pv200L and number of previous malaria episodes. Individual value plot of index of reactivity (IR) for primary infected (0), with 1 or 2, and 3 or more previous malaria groups and boxplot of the median of IR for number of previous malaria groups and the 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 * represents outliers.

groups; IR = 5.4). However, this difference was significant only between primary infected (0) and the three or more malaria episode groups ($P = 0.010$, Kruskal-Wallis test, Bonferroni's correction P value ≤ 0.017).

DISCUSSION

In this study, we confirmed the high prevalence of individuals in malaria-endemic areas of Brazil who develop antibodies specific to the Pv200L fragment of the *P. vivax* MSP-1 upon exposure to natural infection. The mean number of individuals presenting antibodies to the Pv200L in this study was higher (89.3%) than that previously reported in Colombia (72.8%) where transmission intensity is lower than the Amazon region. There were significant differences in the frequency of responders and in antibody levels among the four study sites. Although we could not reliably record the number of previous episodes in individuals with multiple previous infections, the surprisingly high difference in antibody titers, which ranged from negative (IR < 1 = 10.7%) to highly positive (IR > 10 = 17.7%), indicates that some of the individuals may have been exposed to numerous *P. vivax* infections, certainly more than in Colombian individuals who presented significantly lower titers (10^3 – 5×10^5)^{20,26}. In the latter study, individuals were not only exposed to lower transmission intensity but a proportion was Fy- volunteers with only a transient exposure to limited amounts of circulating merozoites.²⁶

Several studies in Brazil^{7,9,27,28} and other countries^{29–32} have demonstrated the high antigenicity of different regions of the *P. vivax* MSP-1. The previously described antibody specificity, particularly to the C-terminal region (42-kD and 19-kD sub-fragments), correlates with inhibition of blocking parasite invasion *in vitro*^{33,34} and induction of protective immunity in animal models.^{19,35} We have focused considerable efforts on the genetic and immunological characterization of the N-terminal (Pv200L) fragment of *P. vivax* MSP-1²⁰ and have found that it has not only significant antigenicity both in human and animals, but it displays high immunogenicity in mice and monkeys and induces partial protection against experimental parasite challenge in *Aotus* monkeys vaccinated with the same protein tested here.

These results are in agreement with those of other seroepidemiologic studies that used longer fragments from N-terminal of PvMSP-1.^{7,21,36} Although the C-terminal region of MSP-1 was referred to in Brazil as the most immunogenic,^{9,28} high antibody levels (IR > 10) and IR average corroborates Pv200L high antigenicity in naturally acquired malaria. Moreover, reduced risk of *P. vivax* infection and clinical protection against malaria were, in fact, associated with antibodies to a specific N-terminal fragment from MSP-1.⁷ We must point out that maybe the same protection may not occur with other antigens from the N-terminal region, however, it is an extrapolation that should be considered. 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 2 months after treatment, which might have contributed to the risk of reinfection.^{27,37} Herein, all individuals were infected at the time of sampling.

In Brazil, the antibody response profile was variable in the different areas, whereas Macapá showed the lowest frequency and antibody levels among studied areas; Novo Repartimento displayed the highest frequency of responders (98.7%). Several reasons, not mutually exclusive, may account for the

discrepant differences observed. Macapá had the lowest API (6.0 in 2009) that can justify the low frequency and the low level of antibody that was found. On the other hand, Porto Velho presented the highest API (53.7) followed by Plácido de Castro (20.6) and Novo Repartimento (15.8). Interestingly, Novo Repartimento but not Porto Velho showed the highest frequency of responders. Another possible explanation is the characteristics of *P. vivax* and *P. falciparum* transmission in each site. Although individuals with patent *P. falciparum* infection do not recognize the N-terminal recombinant protein of *P. vivax* MSP-1,³⁶ a combination of previous *P. vivax* and *P. falciparum* infections may have an unpredictable outcome in terms of the human antibody response.⁹

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 the number of previous malaria in three groups: (0), (1 or 2), and (3 or more) past malaria episodes. As expected, the analysis showed that the frequency of responders and the antibody levels to Pv200L fragment was higher in those individuals with a greater number of previous malaria infections. Although two previous Brazilian studies did not show this tendency for the N-terminal of the *P. vivax* MSP-1,^{9,28} our data are in agreement with another study in which the proportion of responders to PvMSP-1 variants increased during subsequent infections.³⁸ Moreover, in studies that evaluated the C-terminal of the MSP-1³¹ and other parasite proteins, such as DBP-II^{39,40} and AMA-1^{31,41} 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 the initial evaluation of Pv200L in Colombia, the higher antibody responses in infected patients as compared with healthy individuals with previous exposure to malaria may be explained by an immune boosting by parasites after natural infection and may provide advantages for boosting of antibody response induced by vaccination.²⁰

In summary, results of this study provide evidence that Pv200L is an immunogenic fragment of the N-terminal region of the *P. vivax* MSP-1 in naturally exposed individuals from the Brazilian Amazon. Observed differences among the study areas may be explained by the frequency of malaria exposure in each region and that it might also be associated with the wide variations found in the malaria transmission pattern, parasite polymorphisms, and the genetic background of human populations. Additional studies regarding the fine specificity of antibodies to the Pv200L fragment and factors that modulate immune responses against heterologous parasitic proteins, especially in other geographical areas, are warranted to improve the understanding of the immunology of malaria with an eye toward identification of viable malaria vaccine candidates.

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3.5 CAPÍTULO V

Atendendo ao terceiro objetivo específico, o qual previa avaliar as frequências alélicas dos polimorfismos do *HLA-DRB1* em amostras da região norte do Brasil e sua influencia na resposta de anticorpos contra os peptídeos da MSP1 (Pv200L), AMA-1 e DBP em indivíduos naturalmente infectados com *P. vivax*, descrevemos o trabalho a seguir:

Evaluation of HLA-DRB-1 alleles in the immune response against MSP-1, AMA-1 and DBP in Brazilian individuals naturally infected with *Plasmodium vivax*. Autores: Luciane Moreno Storti-Melo, Daniela Reis da Costa, Wanessa Christina Souza-Neirasa, Gustavo Capatti Cassiano, Vanja Suely Calvosa D’Almeida Couto, Marinete Marins Póvoa, Irene da Silva Soares, Luzia Helena de Carvalho, Myrian Arevalo-Herrera, Sócrates Herrera, Andrea Regina Baptista Rossit, José Antonio Cordeiro, Luiz Carlos de Mattos e Ricardo Luiz Dantas Machado. Artigo original submetido à publicação e em fase de revisão na revista **Acta Tropica**.

RESUMO

Nós avaliamos a frequência alélica do HLA-DRB-1 na aquisição e níveis de anticorpos contra Pv200L, AMA-1 e DBP usando amostras de plasma de indivíduos infectados com *P. vivax* provenientes de áreas endêmicas de malária do Brasil. Os alelos mais frequentes observados na amostra da população analisada foram HLA-DRB1*04, *08, *07 e *13. Observou-se diferenças nas frequências do alelo HLA-DRB1*07 entre os grupos de indivíduos maláricos e não-maláricos, o que pode sugerir um perfil de susceptibilidade à doença na presença deste alelo. Uma associação positiva foi observada entre altos níveis de anticorpos anti-Pv200L e o alelo HLA-DRB1*03, a qual não parece estar relacionada à exposição à malária, o que nos leva a sugerir que, na amostra estudada, o HLA-DRB1 possa estar influenciando os níveis de anticorpos para MSP-1 (Pv200L), mas não para AMA-1 e DBP. Este estudo oferece novas informações sobre a regulação genética

da resposta imune contra importantes antígenos de *P. vivax* candidatos à vacina em populações brasileiras naturalmente expostas à malária.

View Letter

Date: Aug 03, 2010
To: "Luciane M Storti-Melo" stortilu@yahoo.com.br
From: Klavs Berzins klavs@imun.su.se
Subject: Your Submission

Ms. Ref. No.: ACTROP-D-10-00189

Title: Evaluation of HLA-DRB-1 alleles in the immune response against MSP-1, AMA-1 and DBP in Brazilian individuals naturally infected with Plasmodium vivax
Acta Tropica

Dear Mrs. Storti-Melo,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

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Evaluation of HLA-DRB-1 alleles in the immune response against MSP-1, AMA-1 and DBP in Brazilian individuals naturally infected with *Plasmodium vivax*

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ABSTRACT

We evaluated the influence of the HLA-DRB-1 allelic frequency on the acquisition and level of the antibody to Pv200L, N-terminal of MSP-1 protein, recombinant AMA-1 and DBP proteins using plasma samples of individuals infected with *Plasmodium vivax* from malaria endemic areas of Brazil. The most frequent alleles in North population samples were HLA-DRB1*04, *08, *07 and *13. Differences were observed in HLA-DRB1*07 frequencies between malaric and non-malaric groups suggesting a malaria susceptibility profile in this allele presence. HLA-DRB1 alleles can influence antibody levels to MSP-1 (Pv200L) but not to AMA-1 and DBP since a positive association was observed between the highest antibody levels to Pv200L and the presence of HLA-DRB1*03 that does not seem to be related with the malaria exposure. Finally, this study offers new information about the genetic regulation of the immune response against important vaccine candidate antigens of *P. vivax* in Brazilian population naturally exposed to malaria.

Key words: *Plasmodium vivax*, HLA class II, Antibody response, MSP1, AMA-1 and DBP

1. Introduction

Human leukocyte antigen (HLA) class II genes were originally called genes of the immune response, since their alleles are known to influence the antibody production (Germain, 1999). Population studies have reported the association of the HLA antigens with the susceptibility or resistance in the pathogenesis of infectious diseases, such as leprosy (Todd et al., 1990), mucosal leishmaniasis (Petzl-Erler et al., 1991) and hepatosplenomegaly in schistosomiasis (Assaad-Khalil et al., 1993). Various studies have searched for the influence of the HLA alleles in the malaria immunology. In 1989, the relationship between the HLA-DR types and the immune responses to circumsporozoite protein (CSP) of *Plasmodium falciparum* (Brown et al., 1989) and to sporozoite and gametocytes epitopes in Papua New Guinea (Graves et al., 1989) couldn't be established. Two years later, the protection against *P. falciparum* severe malaria was associated with the presence of the HLA-Bw53, DRB1*1302 e DQB1*0501 alleles in African children (Hill et al., 1994). In the beginning of 2000's, some HLA-DR alleles have been associated with a better antibody response to Nt47 (p126 amino-terminal portion) (Banic et al., 2002); apical membrane antigen-1 (AMA-1) (Johnson et al., 2004) of *P. falciparum* and the VK247 CSP repetition of *P. vivax* (Oliveira-Ferreira et al., 2004).

Additionally, several studies have reported the association of specific HLA alleles with the immune response to malaria antigens in human vaccine trials (Patarroyo et al., 1991; Nardin et al., 2001). In different populations, HLA-DR4 (Patarroyo et al., 1991) and HLADR15/DQB1*0601 (Beck et al., 1995) were associated with lower response to the synthetic peptide SPf166 vaccine. High levels of responsiveness were found in the presence of HLA DPB1*0501 in Thailand individuals vaccinated with circumsporozoite antigens (Stephens et al., 1995). Similarly, in a phase I clinical Multiple Antigens Peptides (MAP) vaccine trial higher anti-sporozoite antibodies titers were restricted to three HLA class II alleles, DRB1*0401, DRB1*1101 and DQB1*0603, on the other hand, HLA-DRB1*07 allele has failed to develop high antibodies levels (Nardin et al., 2000).

Furthermore, a poor responsiveness to hepatitis B surface (Desombere et al., 1998), measles (Gelder et al., 2002) and influenza (Polland, 1999) vaccines has been documented in the HLA-DR7 presence, and it has also been incriminated like a weak responder to the VK210 CSP repetition of *P. vivax* in naturally infected individuals (Oliveira-Ferreira et al., 2004). So, the knowledge of the influence of HLA class II in the immune recognition of vaccine candidate antigens among people naturally exposed to malaria is an imperative for the development of an effective vaccine (Johnson et al., 2004).

Several asexual-stage molecules are being considered as vaccine candidates, because the proteins expressed in the merozoite parasite form play critical roles during the invasion of red blood cells (RBC) and are responsible for the perpetuation of the parasite life cycle (Remarque et al., 2008). Among these the merozoite surface protein 1 (MSP-1), the AMA-1 and the Duffy binding protein (DBP) are being intensively studied regarding their immune potential (Good et al., 2005). An 80 kDa amino-terminal fragment of the PvMSP-1 (Pv200L) showed immunologic relevance since 72.8% of *P. vivax* infected Colombian patients developed antibodies, and BALB/c mice and *Aotus* monkeys that were immunized with this fragment displayed partial protection against *P. vivax* blood stages (Valderrama-Aguirre et al., 2005). The AMA is recognized by 72.7% of the sera samples of infected individuals from North of Brazil (Barbedo et al., 2007). Active immunization of experimental animals with recombinant forms of this protein showed a protective effect against infection (Collins et al., 1994), and a significant pro-inflammatory response was elicited by PvAMA-1, which plays a direct role on innate immune response (Bueno et al., 2008). The DBP of *Plasmodium vivax* (PvDBP) is a 140 kDa protein with functionally conserved region rich in cysteine, which is referred as region II. Despite of the lowest antibody frequency observed in naturally infected people (Barbedo et al., 2007) the currently data have demonstrated that the anti-DBP antibodies response increases with exposure to *P. vivax* and some of these antibodies have a blocked adherence activity of DBP on the receptor of the reticulocytes (Grimberg et al., 2007; Ceravolo et al., 2008; Ceravolo et al., 2009).

The majority of the studies about the influence of the HLA alleles and malaria immune response were conducted with *P. falciparum* antigens because of its higher mortality, but *P. vivax* is the main specie responsible for infection in South and Central America. In Brazil this specie accounts for more than 80% of the clinical cases reported in the Amazon region (Brazilian Health Ministry, 2007). Thus, it is important to understand the factors that control the immune response to vaccine candidate antigens, as the asexual-blood stage proteins of *P. vivax*, among people naturally exposed to parasite. Hereby, we evaluated the influence of the HLA-DRB-1 allelic frequency on the acquisition and on the level of the antibody against Pv200L, N-terminal of MSP-1, recombinant AMA-1 and DBP peptides using plasma samples of infected individuals with *P. vivax* from malaria endemic areas of Brazil.

2. Materials and Methods

2.1 Study subjects and areas

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 for *P. vivax*. All patients signed a written informed consent. Peripheral blood samples were obtained from individuals distributed in four Brazilian malaria endemic areas: Macapá in Amapá state; Novo Repartimento in Pará state; Porto Velho in Rondônia state and Plácido de Castro in Acre state. A control group consisted of blood donors and according to the Brazilian blood bank policy they complied with the following criteria: they were over 18 years old, their place of birth was in the study area, they reported never suffering from malaria attacks and had no signs of malaria during the initial interview and had negative results for thick blood film and molecular diagnosis were used for HLA-DRB frequencies comparisons. All the studied groups showed no statistically significant difference in mean ages or ethnicity, indicating a well-matched population. The same results were obtained

when we compared both groups in each area. DNA samples were extracted from frozen pellets of erythrocytes using the Easy-DNA™ extraction kit (Invitrogen, Carlsbad, CA, USA), and a semi-nested PCR using specific small-subunit (SSU) rDNA primers (Kimura et al., 1997) was performed to confirm the malaria diagnosis. The protocol for this study was reviewed and approved (Process number 235/2006) by the Research Board of the Faculty of Medicine from São José do Rio Preto, São Paulo state, Brazil.

2.2 HLA class II alleles

A sub-set of DNA samples (n=73) of the malaria patients and of the control group (n=29) were selected to HLA-DRB-1 alleles genotyping. The DNA concentrations were obtained in a spectrophotometer at 260 and 280 nm and content measured of 100 ng/mL was used for low resolution typing of the HLA-DRB1 by polymerase chain reaction with sequence-specific primers (PCR-SSP) as previously described (Olerup and Zetterquist, 1992).

2.3 Assessment of the serological response

The presence and levels of IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA) according to what was previously described: to N-terminal fragment (Pv200L) of MSP-1 (n=261) (Valderrama-Aguirre et al., 2005), to recombinant peptide of the AMA-1 (n=119) (Rodrigues et al., 2005) and to regions II-IV of DBP (n=119) (Cerávolo et al., 2005) in plasma samples of the infected individuals with *P. vivax*. The ELISA IgG cutoff was defined as the OD average from Amazon region outside individuals (n=30) plus three standard deviations. The results were expressed as an index of reactivity ($IR = OD_{405}$ 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 and values of $IR \geq 10$ were considered highly positive.

2.4 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>)). Student's *t*-test was used to analyze differences in mean values. Antigen and gene frequencies were calculated respectively by the formula $af = n/N$, where *n* is the number of positive samples for the antigen, *N* is the total number of samples, and $gf = 1 - \sqrt{1(1 - af)}$ (Baur and Danilovs, 1980). The heterogeneity of HLA antigen frequencies between control and malaric groups and between responder and nonresponder groups was evaluated by Chi-square with Yate's correction test or, alternatively, the Fisher's exact test. Differences were considered significant when $p\text{-value} < 0.05$.

3. Results

3.1 HLA-DRB1 alleles frequency in Brazilian Amazon populations

HLADRB1 genotyping was performed in a total of 102 individuals, including malaric and non-malaric DNA samples from Brazilian North region. As summarized in Table 1, thirteen different alleles were found in our casuistic and the frequencies observed for all samples and for each group are also described there. The most frequent alleles in all samples group were HLA-DRB1*04, *08, *07 and *13. In the malaric group these same alleles were frequent, but the HLA-DRB1*07 was the most frequent in this group following, *04, *08 and *13. In the control group the HLA-DRB1*04, *08, and *13 were the most frequent as well, but the HLA-DRB1*07 was one of the least frequent alleles in this group. When we compared both the malaric and the control group, in the first one HLA-DRB1*07 frequency was higher than in the control group and this difference was statistically significant ($p=0.006$, Chi-square test).

3.2 Antibody response to MSP-1, AMA-1 and DBP in individuals naturally exposed to malaria.

The immune response was analyzed in plasma samples of 261 individuals for presence of anti-MSP-1 antibodies and of 119 individuals for anti-AMA-1 and anti-DBP antibodies presence. The frequency of responders to MSP-1 and AMA-1 was higher than 89%, and high antibody levels ($IR \geq 10$) were observed in 17.6% and 15.1% of the samples respectively. For DBP, 37% of the samples responded positively for antibody presence and only 1.7% was highly positive. The frequencies of responders and IR values are shown in Table 2.

We verified whether or not the frequency and the level of antibody increase with the exposure to infection. For this, the individuals were separated according to the number of previous malaria into three groups: (0) primary-infected; (1 or 2) individuals with one or two previous malaria episodes; and (3 or more) individuals with at least three previous malaria episodes. The frequencies of not responder (Neg), responders (Pos) and highly responders (High Pos) in each previous malaria group are in Table 3. A positive correlation with the number of previous malaria episodes and the antibody response was observed for MSP-1 ($p=0.006$) and AMA-1 ($p=0.005$), in which the frequencies of responders and highly responders significantly increase with more exposition. For DBP, there was not a significant correlation ($p>0.05$).

3.3 HLA-DRB1 allele frequency and antibody response against MSP-1, AMA-1 and DBP

The HLA-DRB1 frequencies for each antibody response group: not responders (Neg), responders (Pos) and highly responders (High Pos) against MSP-1, AMA-1 and DBP were evaluated (Table 4). A significant association between high response to MSP-1 and the presence of HLA-DRB1*03 ($p=0.048$) was observed. In fact, among individuals with DRB1*03, the frequency of highly responders to MSP-1 (35.7%) was significantly ($p=0.040$) higher than in the

people with other alleles (14.4%). Because the prevalence and the levels of anti-MSP-1 antibodies seem to be related to previous infection, we examined the possibility of DRB1*03 people being more exposed to malaria, but no difference ($p>0.05$) was observed in the number of previous infection between these individuals and other people. The HLA-DRB1 frequencies were similar between responders and not responders to AMA-1 and DBP, and no significant association between any HLA-DRB1 allele and antibody response against these proteins was found.

4. Discussion

Brazilian population is genetically diverse, formed by contribution of three important groups: Caucasians, Africans and Amerindians, resulting in a mixed population (Moraes et al., 1993). Because of the large territorial size of Brazil and the diversities in its colonization, different regions of the country present more prevalence of one or other groups described (Alves-Silva et al., 2000, Carvalho-Silva et al., 2001). We found a high frequency of HLA-DRB1*04 in our sample according to previous studies (Polland, 1999; Parolín and Carnese, 2001), since this allele is characteristic of the American indigenous population its prevalence reflects the Amerindians contribution in Brazilian population, especially in the North of the country (Zago et al., 1996). Additionally, the high frequency of HLA-DRB1*04 that was observed in both, malaric and non-malaric groups indicates that this allele was not associated to susceptibility or protection to *P. vivax* malaria. On the other hand, the significant higher frequency of the HLA-DRB1*07 in the malaric group than in the non-malaric group could suggest a malaria infection susceptibility associated to this allele. Oliveira-Ferreira et al., (2004) proposed a bad responder profile for HLA-DRB1*07 against the VK210 repetitive region of the *P. vivax* CSP. In fact, previous studies with viral vaccine (Desombere et al., 1998; Polland, 1999) and vaccine trials with CSP of *P. falciparum* (Nardin et al., 2000) have been incriminate this allele in fail in the

developed immune response. These findings may explain the susceptibility suggested to malaria that was observed in this allele presence.

The levels of antibody response that were observed for blood stage antigens are in accordance to previous reports (Olerup and Zetterquist, 1992; Cerávolo et al., 2005; Valderrama-Aguirre et al., 2005; Barbedo et al., 2007). We had firstly documented the antibody response profile for *Pv200L* in naturally exposed Brazilian individuals and found a high recognition by sera samples with an increase in the frequency and in the level of antibodies directly proportional to the number of previous malaria episodes (Storti-Melo et al., in press). The high antibody response against *Pv200L* that was observed confirms the potential of this MSP1 N-terminal fragment like an immunogenic candidate vaccine. Although the increase of the antibody response according to more infection had been previous reported to AMA-1 (Rodrigues et al., 2005; Barbedo et al., 2007) and to DBP (Cerávolo et al., 2005; Grimberg et al., 2007), in our data, this tendency was observed for antibody levels to AMA-1, but not to DBP. The lowest frequency of responders against DBP was in agreement with previous studies in endemic areas of Brazil (Cerávolo et al., 2005; Barbedo et al., 2007), and once the inhibitory properties have been attributed to anti-DBP antibodies this protein remains like an important immunogenic molecule (Grimberg et al., 2007; Ceravolo et al., 2008; 2009).

All these three *P. vivax* asexual-stage antigens are reported like targets of parasitic invasion-inhibitory or growth-inhibitory antibodies and because, all of them are candidates for vaccine development, we investigated the possible influence of the HLA-DRB1 polymorphisms in the frequency and in the levels of antibodies against them. We found that HLA-DRB1 can influence antibody levels to MSP-1 (*Pv200L*) but not to AMA-1 and to DBP. Since the frequency of responders to *Pv200L* was high in the population (89.3%), restrictions to antibody production against this antigen do not seem to be a problem. However, a positive association was observed between the highest antibody levels to *Pv200L* and the presence of HLA-DRB1*03. This antibody response did not seem to be related to exposure, since any difference was found between previous malaria infections and

this allele presence. In contrast, a similar study, evaluating the HLA influence in the antibody response to *P. falciparum* asexual-stage antigens found no association with any HLA-DRB1 or DQB1 alleles for antibody levels to MSP1-190l and to MSA2, but positive individuals for HLA-DRB1*1201 had the highest antibody levels to PfAMA-1 (Johnson et al., 2004). However this work was conducted in an African population, where the genetic composition, the condition of malaria transmission and the *Plasmodium spp.* epidemiology are different from Brazil.

The lack of association observed in this work, may mean that the HLA-DRB1, in fact, does not modulate the antibody response against AMA-1 and DBP of *P. vivax*, or can be a limitation of the size of the sample that needs to be better investigated. Another possibility is the presence of other intervening factors, such as genes linked to the histocompatibility complex or elsewhere. The genetic regulation of antigen-specific antibody responses does not seem to be only by HLA class II genes (Duah et al., 2009). Recently works had shown that genetic factors regulate the different antibody isotype and subclass responses to malaria antigens, mainly due to undefined non-HLA-linked genes (Duah et al., 2009). An earlier analysis of IgG levels to parts of MSP1 and of MSP2 of *P. falciparum* had surprisingly shown that the contribution of non-HLA encoded genes exceed that of the HLA genes (Jepson et al., 1997). The heritability of responses to vaccines (BCG, polio, hepatitis B, diphtheria, pertussis and tetanus) in infants has been reported (Newport et al., 2004), indicating that antibody responses are influenced mainly by non-HLA genes whilst cellular responses to BCG are regulated more by genes within the HLA class II region. It has been shown that although genetic factors control early antibody responses to vaccines, the persistence and avidity maturation of these responses are regulated by environmental factors such as frequent exposure to the microorganisms that influences the subsequent activation of memory B cells (Marchant et al., 2006). The positive influence of the HLA-DRB1*03 in the MSP1 antibody response that was found in our data needs to be confirmed in other populations and as well in functional studies about the interaction of these antigenic molecules. A genome-wide approach could

contribute to the discovery of other genetic determinants of the specific antibody responses, which remain very limited (Malaria Genomic Epidemiology Network, 2008). Finally, this study offers new information about the genetic regulation of the immune response against important vaccine candidate antigens of *P. vivax* in Brazilian population naturally exposed to malaria.

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Table 1

Frequencies of HLA-DRB1 alleles in samples from Brazilian Amazon region, including control and malaric groups.

HLA-DRB-1 Alleles	Allele frequencies		
	All samples (n=102)	Malaric group (n=73)	Control group (n=29)
*01	0.147	0.151	0.138
*03	0.196	0.192	0.207
*04	0.304	0.315	0.276
*07	0.255	0.342 [†]	0.034
*08	0.265	0.260	0.276
*09	0.029	0.014	0.069
*10	0.078	0.068	0.103
*11	0.098	0.110	0.069
*12	0.029	0.028	0.034
*13	0.235	0.220	0.276
*14	0.147	0.136	0.172
*15	0.127	0.110	0.172
*16	0.088	0.054	0.172

[†] p=0.006, Chi-square test with Yates' correction compared control and malaric groups.

Table 2

Frequency and antibody levels to MSP-1, AMA-1 and DBP in sera of infected individuals with *P. vivax* from Brazilian Amazon.

Protein	n	Antibody response (%)	IR - antibody level		
			average	>1 (%)	>10 (%)
MSP-1	261	89.3	5.5	71.7	17.6
AMA-1	119	89.1	6.6	73.94	15.1
DBP	119	37	1.24	35.29	1.7

Table 3

Relationship between the frequency of antibody response for MSP-1, AMA-1 and DBP and the number of previous malaria episodes.

Past malaria episodes	MSP-1[†]			AMA-1[‡]			DBP	
	Neg	Pos	High Pos	Neg	Pos	High Pos	Neg	Pos
0	40	60	0	42.8	42.8	14.4	71.4	28.6
1 or 2	12.2	75.6	12.2	11.1	83.3	5.6	63.9	36.1
3 or more	7.4	69.5	23.1	3.6	72.7	23.7	60	40

Neg: not responders (IR <1); Pos: responders (1 ≤ IR <10); High Pos: Highly responders (IR ≥10)

[†] Fisher's exact test. p= 0.006

[‡] Fisher's exact test. p= 0.005

Table 4

Frequencies of HLA-DRB1 alleles in responders and not responders to MSP-1, AMA-1 and DBP.

HLA-DRB1 Alleles	MSP-1			AMA-1			DBP	
	Neg (n=2)	Pos (n=59)	High Pos (n=12)	Neg (n=6)	Pos (n=43)	High Pos (n=11)	Neg (n=35)	Pos (n=25)
*01	0	0.169	0.083	0.334	0.162	0.09	0.229	0.080
*03	0	0.153	0.416 [†]	0.334	0.14	0.364	0.143	0.280
*04	0	0.288	0.500	0.666	0.372	0.272	0.371	0.400
*07	0.5	0.356	0.250	0	0.302	0.364	0.257	0.320
*08	0.5	0.237	0.333	0.334	0.256	0.182	0.257	0.240
*09	0	0.017	0	0	0	0	0	0
*10	0	0.068	0.083	0	0.094	0.09	0.086	0.080
*11	0	0.102	0.167	0	0.14	0.09	0.086	0.160
*12	0	0.034	0	0	0.024	0	0.029	0
*13	0.5	0.237	0.083	0.166	0.162	0.364	0.171	0.240
*14	0.5	0.136	0.083	0	0.162	0.182	0.143	0.160
*15	0	0.136	0	0.166	0.094	0	0.14286	0
*16	0	0.068	0	0	0.094	0	0.086	0.040

Neg: not responders (IR <1); Pos: responders (1 ≤ IR <10); High Pos: Highly responders (IR ≥10)

[†] p= 0.048, Chi-square test.

4 DISCUSSÃO GERAL

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Apesar de diversas tentativas de controle, a malária ainda continua sendo uma doença com alta prevalência e elevado índice de mortalidade (COWMAN & CRABB, 2006), resultando em um milhão de mortes no mundo a cada ano (CDC, 2009). No Brasil já foram registrados cerca de 600 mil casos, mas hoje esse quadro vem mudando, os casos notificados estão diminuindo, isso devido a intervenções do governo no combate e prevenção a esta doença (MINISTÉRIO DA SAÚDE, 2009). A malária causada pelo *P. vivax* é responsável por cerca de 80% dos casos brasileiros, embora a infecção por esta espécie dificilmente acarrete a morte do paciente, ela causa morbidade e perdas sócio-econômicas relevantes (MINISTÉRIO DA SAÚDE, 2009). As três variantes da CSP (VK210, VK247 e *P. vivax-like*) desta espécie de plasmódio têm sido detectadas no Brasil por métodos moleculares (MACHADO et al., 2003; STORTI-MELO, et al., 2009) e, além disso, anticorpos naturalmente adquiridos que reconhecem a porção repetida desta proteína já foram demonstrados em várias áreas endêmicas do Brasil (MARRELLI et al., 1998, OLIVIERA-FERREIRA et al., 2004; ARRUDA et al., 2007; CERUTTI et al., 2007) e do mundo (COCHRANE et al., 1990; WIRTZ et al., 1990; FRANKE et al., 1992; GONZÁLEZ et al., 2001). Neste trabalho, a distribuição destas variantes tanto em infecções simples como mistas não se apresentou de forma homogênea nas diferentes regiões da Amazônia brasileira. No entanto a variante VK210 permanece como a mais prevalente em todas as áreas analisadas. As variantes VK247 e *P. vivax-like* atualmente parecem estar mais adaptadas à infecção nas populações brasileiras do que anteriormente, uma vez que em nossos dados ambas puderam ser identificadas em infecções simples, diferentemente do que foi reportado em 2000 e 2003, quando estas variantes foram observadas apenas em infecções mistas (MACHADO e PÓVOA, 2000; MACHADO et al., 2003). Interessantemente, após dez anos, observa-se uma alteração do panorama de distribuição das variantes da CSP, no Brasil. Embora a VK210 continue sendo a mais prevalente em todas as áreas analisadas, a relação de distribuição com a VK247 e *P. vivax-like*, sugere uma mudança na adaptação

dessas variantes, 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 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). Uma vez que esta variante foi encontrada em infecções simples somente em amostras de Novo Repartimento, no Estado do Pará, sugerindo uma introdução mais recente nos demais Estados. Os resultados deste estudo também demonstram que o *P. vivax-like* está amplamente distribuído em todas as áreas estudadas sugerindo que esta variante esteja mais bem adaptada do que VK247 nas áreas brasileiras.

A partir da descrição dos genótipos variantes da CSP de *P. vivax*, vários estudos têm proposto a existência de diferenças entre essas variantes 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 drogas (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, mas os dados atuais trazem evidências filogenéticas de que essa variação esteja limitada à porção central da CSP (SOUZA-NEIRAS, et al., 2010). A avaliação do perfil de resposta sorológica contra os diferentes peptídeos do parasito relacionado às variantes da CSP corrobora a idéia de que esta variação esteja restrita a porção central da proteína, uma vez que, em nossos resultados não foram observadas associações significativas entre estas variantes e frequência de resposta de anticorpos contra os três peptídeos do merozoíto analisados, MSP1 (Pv200L), AMA-1 e DBP, bem como, contra as frações conservadas da CSP no esporozíto, N-terminal [N] e C-terminal [C]. Por outro lado, quando avaliamos a resposta imune humoral contra os peptídeos correspondentes à região central repetida da CSP, os resultados indicaram associações significativas na frequência de respondedores para os peptídeos [R] e [V], os quais correspondem à sequência peptídica da CSP das variantes VK210 e VK247 respectivamente. Nos indivíduos

infectados pela variante 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. De forma geral, a frequência de respondedores contra o peptídeo [R] correspondente a VK210 foi significativamente maior que para o [V] (VK247). Levando-se em consideração a existência de uma correlação positiva entre o número de exposições prévias e a maior resposta sorológica, um fato a ser considerado é que a variante VK210 é a forma clássica e a mais prevalente em todas as áreas endêmicas estudadas (STORTI-MELO, et al., 2009) o que justificaria a maior frequência de respondedores observada contra o peptídeo correspondente à esta variante. Ademais, deve-se lembrar que a região repetitiva da CSP de *P. vivax-like* é geneticamente distinta das demais variantes (QARI, et al., 1993a), dado que pode explicar a significativa diminuição na resposta contra os peptídeos correspondentes à VK210 e VK247 em indivíduos infectados por essa variante.

A CSP representa um importante alvo no desenvolvimento de uma vacina para malária, uma vez que ela recobre toda a superfície do esporozoito, estágio infectante do parasito (KEMP et al., 1990). A alta frequência de resposta de anticorpos contra os peptídeos correspondentes às regiões conservadas N-terminal e C-terminal indica a alta imunogenicidade destes peptídeos na população brasileira naturalmente exposta à malária, confirmando ainda, seu maior potencial imunogênico em relação às regiões centrais repetitivas (HERRERA et al., 2004). O fato da infecção pelas diferentes variantes da CSP não apresentar diferenças significativas na resposta de anticorpos contra os peptídeos do merozoíto nem com as regiões conservadas do esporozoíto, fornece informações promissoras nos ensaios de vacinação utilizando esses peptídeos como imunógenos. Por outro lado, estudos embasados na CSP devem analisar sua variabilidade genética para a utilização da porção central desta proteína como antígeno para desenvolvimento de uma vacina anti-*P. vivax*. Considerando-se a influência dessa variação na modulação da epidemiologia do parasito e, a utilização de constructos quiméricos incluindo as diferentes seqüências da CSP, a

fim de se obter uma vacina efetivamente protetora contra esta espécie de parasito (YADAVA et al., 2007; BELL et al., 2009).

O desenvolvimento de resistência à droga pelos parasitos e resistência a inseticidas pelos vetores têm comprometido os métodos tradicionais de tratamento e controle, reforçando a necessidade crítica do desenvolvimento de uma vacina para malária. Nas ultimas décadas, diversos estudos têm sido realizados para identificar e testar antígenos candidatos para uma vacina efetiva (HERRERA, et al., 2005; CÉRAVOLO et al., 2005), obtendo resultados variáveis de acordo com o antígeno e a população analisada. No entanto, fatores genéticos do hospedeiro humano têm sido considerados para explicar tais variações. Estudos prévios têm descrito associações entre as frequências alélicas dos genes do HLA de classe II e a resposta sorológica nos ensaios de vacinas para a malária (PATARROYO et al., 1991; NARDIN et al., 2000, 2001). Em população naturalmente exposta da Amazônia brasileira, evidenciou-se que a resposta de anticorpos contra os peptídeos variantes da CSP sejam modulados pelos polimorfismos do *HLA-DRB1* (OLIVEIRA-FERREIRA et al., 2004).

A população brasileira é geneticamente diversa, formada pela contribuição de três importantes grupos: Caucasianos, Africanos e Ameríndios, resultando em uma população altamente miscigenada (MORAES et al., 1993). Ademais, devido à grande extensão territorial do Brasil e as diversidades em sua colonização, diferentes regiões do país apresentam prevalência de um ou outro dos grupos descritos (ALVES-SILVA et al., 2000, CARVALHO-SILVA et al., 2001). A alta frequência do alelo *HLA-DRB1*04* observada em nossa casuística esta de acordo com trabalhos anteriores (POLLAND, 1999; PAROLÍN AND CARNESE, 2001) e, uma vez que, este alelo é característico de populações indígenas Americanas sua prevalência reflete a contribuição desta população na região norte do Brasil (ZAGO et al., 1996). A maior frequência do alelo *HLA-DRB1*07* no grupo de indivíduos maláricos comparado aos doadores, não maláricos pode estar associado a um perfil de baixa resposta imune sugerindo uma susceptibilidade à doença, relacionada à este alelo. O perfil de mal respondedor deste alelo já foi anteriormente proposto por Oliveira-Ferreira et al., (2004) que

evidenciaram menor resposta contra o peptídeo da variante VK210 da CSP de *P. vivax*. Além disso, outros trabalhos com vacinas virais (DESOMBERE et al., 1998; POLLAND, 1999) e ensaios de vacinação com a CSP de *P. falciparum* (NARDIN et al., 2000) têm incriminado este alelo nas falhas de desenvolvimento da resposta imune.

Os antígenos do estágio asexual têm sido reportados como alvos de anticorpos inibidores da invasão e do crescimento parasitário de forma que investigar uma possível modulação pelos polimorfismos do HLA de classe II na resposta imune humoral para estes antígenos fez-se relevante. Em nossa amostragem encontramos evidências de que os alelos do *HLA-DRB1* podem influenciar os níveis de anticorpos para MSP-1 (Pv200L), mas não para AMA-1 e DBP, uma vez que, uma associação positiva foi observada entre o alelo *HLA-DRB1*03* e altos níveis de anticorpos contra o Pv200L. Em contraste, um estudo similar avaliando a influência do HLA na resposta de anticorpos contra antígenos do estágio asexual de *P. falciparum* não encontrou associações significativas entre nenhum alelo *HLA-DRB1* ou *HLA-DQB1* e os níveis de anticorpos para MSP1-190l e para MSA2, todavia, uma associação positiva foi observada entre altos níveis de anticorpos anti- PfAMA-1 e a presença do alelo *HLA-DRB1*1201* (JOHNSON et al., 2004). Entretanto este trabalho foi conduzido em uma população Africana, a qual possui composição genética e condições epidemiológicas e de transmissão das espécies de *Plasmodium* diferentes do Brasil. A falta de associação observada para os demais antígenos em nossa casuística pode significar que de fato os alelos *HLA-DRB1* não estejam modulando a resposta de anticorpos contra AMA-1 e DBP de *P. vivax*, ou podem ser uma limitação do tamanho da amostra, que necessita ser reavaliada. No entanto outra possibilidade a ser investigada é que outros fatores genéticos possam estar interferindo nesta resposta, tais como outros genes ligados ou não ao complexo principal de histocompatibilidade. De fato, a regulação genética na resposta de anticorpos antígeno-específica parece não ser exclusiva dos genes HLA de classe II (DUAH et al., 2009). Um estudo pioneiro em gêmeos na Libéria indicou que os níveis de anticorpos contra o antígeno RESA de *P. falciparum* foram

influenciados por genes não-HLA (SJOBERG et al., 1992). Recentemente, Duah et al. (2009) também demonstraram que os níveis de anticorpos contra diversos antígenos do *P. falciparum* são influenciados principalmente por genes não-HLA. Trabalhos recentes têm mostrado que fatores genéticos regulam a expressão dos diferentes isotipos e subclasses de anticorpos na resposta contra antígenos da malária, principalmente relacionados a genes não ligados ao HLA. A análise dos genes candidatos que possam afetar a resposta imune na malária ainda requer melhor elucidação. Um SNP (*Single Nucleotide Polymorphism*) na região promotora do gene *IL-4* (-50C/T) parece afetar os níveis de transcrição do gene (LUONI et al., 2001) sendo que a variante alélica T está associada a maiores níveis de IgE em pacientes com malária grave (VERRA et al., 2004). Além disso, foram reportadas variações nos níveis de IgG2 contra antígenos do *Plasmodium* devido a polimorfismos no receptor FcγRIIa em indivíduos do Sudão (NASR et al., 2009). Estes polimorfismos podem explicar apenas uma parte das variações genéticas que modulam a resposta imune na malária, e muitos outros também são prováveis de ter diferentes implicações. Uma abordagem ampla do genoma poderá contribuir para a descoberta de outros determinantes genéticos da especificidade da resposta de anticorpos, a que ainda permanece bastante limitada (MALARIA GENOMIC EPIDEMIOLOGY NETWORK, 2008).

5 CONCLUSÕES

5 Conclusões

O presente trabalho permitiu estabelecer as seguintes conclusões:

1. As frequências de distribuição das três variantes da CSP de *P. vivax*, tanto em infecções simples quanto mistas são variáveis ao longo das diferentes áreas endêmicas da Amazônia brasileira. No entanto a variante VK210 permanece como a mais prevalente em todas as áreas analisadas.
2. A variante, *P. vivax-like* parece estar mais bem adaptada nas áreas brasileiras comparada à VK247.
3. As variantes da CSP de *P. vivax* não estão relacionadas à resposta de anticorpos contra os peptídeos do merozoítio, bem como, contra as frações conservadas da CSP no esporozítio, no entanto essa variabilidade genética deve ser considerada para a utilização da porção central desta proteína como antígeno para desenvolvimento de uma vacina anti-*P. vivax*.
4. As frequências alélicas do HLA-DRB1 parecem estar relacionadas à susceptibilidade malária na população amazônica estudada.
5. Na amostra analisada os alelos HLA-DRB1 parecem estar influenciando os níveis de anticorpos para MSP-1 (Pv200L), mas não para AMA-1 e DBP.

6 REFERÊNCIAS

6 Referências

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7 Anexo

7.1 Termo de aprovação do Comitê de Ética em Pesquisa (CEP)



FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO

Autarquia Estadual - Lei n.º 8899 de 27/09/94
(Reconhecida pelo Decreto Federal n.º 74.179 de 14/06/74)

Parecer n.º 235/2006

COMITÊ DE ÉTICA EM PESQUISA

O Protocolo n.º 3784/2006 sob a responsabilidade de Luciane Moreno Storti de Melo com o título "Genótipos da Proteína Circunsporozoítica de *Plasmodium vivax* associados ao Complexo Principal de Histocompatibilidade", está de acordo com a Resolução CNS 196/96 e foi aprovado por esse CEP.

Lembramos ao senhor(a) pesquisador(a) que, no cumprimento da Resolução 251/97, o Comitê de Ética em Pesquisa em Seres Humanos (CEP) deverá receber relatórios semestrais sobre o andamento do Estudo, 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. Salientamos ainda, a necessidade de relatório completo ao final do Estudo.

São José do Rio Preto, 10 de julho de 2006.

Prof. Dr. Antonio Carlos Pires
Coordenador do CEP/FAMERP

8 Apêndices

8.1 Frequency of ABO blood group system polymorphisms in *Plasmodium falciparum* malaria patients and blood donors from the Brazilian Amazon region. Autores: D.B. Carvalho, L.C. de Mattos, W.C. Souza-Neiras, C.R. Bonini-Domingos, A.B. Cósimo, L.M. Storti-Melo, G.C. Cassiano, A.A.A. Couto, A.J. Cordeiro, A.R.B. Rossit e R.L.D. Machado. Artigo original publicado na revista **Genetics and Molecular Research**, v. 9, n. 3, p. 1443-1449, 2010.

RESUMO

Nós investigamos os genótipos ABO e a heterogeneidade dos alelos *O* em indivíduos infectados não-infectados pelo *Plasmodium falciparum* da região Amazônica Brasileira. A coleta das amostras de sangue ocorreu entre Maio de 2003 a Agosto de 2005 de pacientes com malária por *P. falciparum* provenientes de quatro regiões endêmicas da Amazônia brasileira. O grupo controle foi composto por doadores de quatro bancos de sangue nas mesmas áreas. O DNA foi extraído utilizando o kit de extração Easy-DNATM. A genotipagem do ABO foi realizada por PCR / RFLP. Houve uma alta frequência do ABO*O01O01. ABO*AO01, foi o segundo genótipo mais frequente, e o terceiro mais frequente foi o genótipo ABO * BO01. Observaram-se baixas frequências dos genótipos ABO*O01O02, ABO*AA, ABO*AB, ABO*BB e ABO*O02O02. Foram analisados os alelos do fenótipo O; o alelo *O1variant* foi o mais frequente, tanto nos casos de malária quanto no grupo de não-malária, conseqüentemente, o genótipo homozigoto *O^{1v}O^{1v}* foi o mais frequentemente observado. Não observou-se o alelo O2 em homozigose. Diferenças significativas não foram detectadas na frequência dos alelos na comparação entre os pacientes com malária e a população em geral (doadores de sangue).



Frequency of ABO blood group system polymorphisms in *Plasmodium falciparum* malaria patients and blood donors from the Brazilian Amazon region

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ABSTRACT. We investigated the ABO genotypes and heterogeneity of the *O* alleles in *Plasmodium falciparum*-infected and non-infected individuals from the Brazilian Amazon region. Sample collection took place from May 2003 to August 2005, from *P. falciparum* malaria patients from four endemic regions of the Brazilian Amazon. The control group consisted of donors from four blood banks in the same areas. DNA was extracted using the Easy-DNA™ extraction kit. ABO genotyping was performed using PCR/RFLP. There was a high frequency of *ABO***O01O01*. *ABO***AO01* was the second

most frequent genotype, and the third most frequent genotype was *ABO*BO01*. There were low frequencies of the *ABO*O01O02*, *ABO*AA*, *ABO*AB*, *ABO*BB*, and *ABO*O02O02* genotypes. We analyzed the alleles of the O phenotype; the *O^{ivariant}* allele was the most frequent, both in malaria and non-malaria groups; consequently, the homozygous genotype *O^{iv}O^{iv}* was the most frequently observed. There was no evidence of the homozygous *O²* allele. Significant differences were not detected in the frequency of individuals with the various alleles in the comparison of the malaria patients and the general population (blood donors).

Key words: Malaria; *Plasmodium falciparum*; ABO blood system; Brazilian Amazon region; Genetic polymorphism

INTRODUCTION

The ABO system consists of the A, B and H carbohydrate antigens synthesized by a series of enzymatic reactions catalyzed by glycosyltransferase and antibodies against these antigens. The *A*, *B* and *O* genes are at the same genetic locus on chromosome 9 at q34, and the *A* and *B* alleles are co-dominant against the recessive *O* allele (Yamamoto et al., 1995). Several point mutations on the *A* gene have been described. They cause a number of amino acid changes and alter the glycosyltransferase from A to B. Single guanine deletions at position 261 on the *ABO* gene result in a truncated, enzymatically inactive O protein (referred to as *O¹*) (Roubinet et al., 2004). Another *O* allele (*O²*), which lacks this deletion, has been identified. A commonly occurring variant of the *O¹* gene, *O^{ivariant}*, has also been reported (Olsson and Chester, 1996). The combination of these alleles offers several genotypes, which result in four phenotypes, but the alleles have now been shown to be highly polymorphic (Chester and Olsson, 2001; Yamamoto, 2004). The importance of ABO histo-blood groups is supported by the observation that their geographical distribution varies significantly, suggesting that positive selective factors may have influenced gene spread (Loscerales et al., 2007).

Plasmodium falciparum infections can be linked to the severest form of human malaria, and virulence is associated with parasite reproduction and erythrocyte invasion (Chotivanich et al., 2000). In Brazil, its distribution is localized: it is more common than *P. vivax* in some areas, but very rare or absent in others (Camargo et al., 1999). The ABO blood group system is the most important system for blood group transfusion medicine. Its association with malaria disease has been proposed (Carlson et al., 1990; Lell et al., 1999), and the hypothesis that *P. falciparum* malaria shaped the distribution of ABO blood group in humans is supported by the literature (Athreya and Coriell, 1967). Additionally, there is evidence that the ABO histo-blood group is not related to the incidence of *P. falciparum* malaria (Fischer and Boone, 1998), but it has been linked as a co-receptor in parasite and vascular cytoadherence, with higher rosette rates among non-group O compared to group O erythrocytes (Barragan et al., 2000; Cserti and Dzik, 2007).

We, therefore, investigated the ABO genotypes and the heterogeneity of the *O* alleles in *P. falciparum*-infected and non-infected individuals from the Brazilian Amazon region.

MATERIAL AND METHODS

Study population and sample collection

Sample collection took place from May 2003 to August 2005 in four endemic regions of the Brazilian Amazon: Macapá, Amapá State (00°02'20"S; 51°03'59"W); Novo Repartimento, Pará State (04°19'50"S; 49°47'47"W), Porto Velho, Rondônia State (-08°45'43"S; 63°54'14"W), and Plácido de Castro, Acre State (10°16'33"S; 67°09'00"W). The malaria group was composed of male and female patients with positive thick blood film and molecular diagnosis results for *P. falciparum* single infection (N = 98). These individuals were randomly recruited to participate in this study at the health clinics in each area studied. They were all over the age of 18 with age ranging up to 65 years old. All had an absence of cerebral malaria, severe malarial anemia, and multiple organ dysfunction syndromes. We excluded pregnant women, patients under the age of 18 years, and patients with concomitant illnesses from the study. The control group consisted of both male and female donors from four blood banks in the areas studied (N = 142), and, following the policies for Brazilian blood banks, the donors met the following criteria: over 18 years old (ranging from 19 up to 57 years old), no previous malaria attack, and no signs of malaria reported during the initial interview. The consent form was co-signed by a staff member of the health clinic and a staff member of the blood bank. The control subjects were matched to the patients with respect to age (± 5 years), gender and ethnicity. All control subjects were genetically independent. The protocol for this study was reviewed and approved by the Research Board of the Faculty of Medicine of São José do Rio Preto.

DNA preparation and ABO histo-blood group system genotyping

DNA was extracted from frozen pellets of infected and non-infected erythrocytes using the Easy-DNA™ extraction kit (Invitrogen, Carlsbad, CA, USA). The ABO genotyping was assessed in 240 samples by amplifying fragments covering exons 6 and 7 using polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP). Moreover, a subset of peripheral blood samples from 30 *P. falciparum*-infected individuals and 27 blood donors were evaluated for the heterogeneity of the *O* alleles as previously described (Mattos et al., 2001). Briefly, four PCR were used. The *A*, *B*, *O*^L, and *O*^V genes were identified in three amplifications using the fy46/fy47, fy43/fy31 and fy47/fy29 primer pairs, respectively. Additionally, the *O*^L and *O*^V genes were distinguished using the fy47/fy31 primer pair in the last reaction. PCR was performed in a 25- μ L total reaction volume containing 200 ng genomic DNA, 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M of each dNTP, 0.25 μ M of each primer and 0.5 U Taq DNA polymerase; 35 amplification cycles were carried out (94°C for 120 s, 56°C for 60 s and 72°C for 60 s). After PCR analysis, the amplified DNA was digested with the restriction enzymes *Kpn*I, *Nar*I, *Alu*I, and *Dde*I, respectively, for the four reactions, and the product was analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide and examined under UV light.

Statistical analyses

Analyses were performed using the R version 2.4.1 statistical software (The R Foun-

dation for Statistical Computing ISBN 3-900051-070 - <http://www.r-project.org>, Vienna, Austria). The Hardy-Weinberg equilibrium was tested by applying a hidden Markov chain with 10,000 steps (Guo and Thompson, 1992). To achieve independence among the proportions, the Fisher exact test was applied with a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

The genotype and allele frequencies of the ABO histo-blood group of the 142 blood donors and 98 patients infected with *P. falciparum* as determined by PCR/RFLP are summarized in Table 1. The data show a high frequency of the *ABO*O01O01* in 134 of the 240 individuals (55.83%) of the population studied. *ABO*AO01* was the second most frequent genotype in 46 of the individuals studied (19.16%). The third most frequent genotype was *ABO*BO01*, which was found in 26 individuals, or 10.84% of the population studied. Low frequencies were detected for the *ABO*O01O02*, *ABO*AA*, *ABO*AB*, *ABO*BB*, and *ABO*O02O02* genotypes.

Table 1. Comparison of genotyping results and allele frequency of the ABO histo-blood system among blood donors and patients infected with *Plasmodium falciparum* from the Brazilian Amazon region.

Genotype	Predicted phenotype of donors/patients	Total sample (N = 240)	Donors (N = 142)	<i>P. falciparum</i> patients (N = 98)	P
<i>ABO*AA</i>	A	5	4	1	0.6184
<i>ABO*AO01</i>	A	46	25	21	0.5668
<i>ABO*AB</i>	AB	8	5	3	0.8645
<i>ABO*BB</i>	B	5	4	1	0.6184
<i>ABO*BO01</i>	B	26	17	9	0.6370
<i>ABO*O01O01</i>	O	134	81	53	0.7476
<i>ABO*O01O02</i>	O	13	4	9	0.0641
<i>ABO*O02O02</i>	O	3	2	1	0.7451
Allele frequencies					
<i>ABO*A</i>		0.13	0.13	0.13	0.9709
<i>ABO*B</i>		0.09	0.11	0.07	0.2646
<i>ABO*O01</i>		0.74	0.73	0.74	0.9399
<i>ABO*O02</i>		0.04	0.03	0.06	0.1916

Among blood donors and patients, the frequency of individuals with the *ABO*A* allele was 13%, and the frequency of individuals with the *ABO*O01* allele was around 75%. Significant differences were not detected in terms of the frequency of individuals with all alleles studied between the two groups.

The Brazilian population has a highly heterogeneous ethnic composition, which results from the hybridization of the numerous native indigenous populations and immigrants from Europe, Africa, and Asia. Waves of immigration occurred in unequal proportions in the different regions of the country (Zago et al., 1983). The homozygous genotype *ABO*O01O01* showed an expected higher frequency in the population studied, since phenotype studies of the ABO blood groups in Amerindian-descent populations have revealed that most individuals are exclusively of the O group (Franco et al., 1994; Olsson et al., 1998). The indigenous contributions in the ethnic formation of the North Brazil region are unquestioned, and the molecular basis of the O phenotype in Indians from the Brazilian Amazon was reported by Franco et al. (1994) as being the same G261 - single base deletion (O^1), and they found the absence of the O^2 allele in this population. We also observed a lower frequency of the O^2 allele in our sample,

which is in agreement with the studies in Amerindians (Franco et al., 1994; Olson et al., 1998).

According to data in Table 2, of 27 blood donor samples, 5 were homozygous for the O^I allele, and 7 were $O^I O^{I\text{variant}}$. One sample was $O^I O^2$, and 3 were $O^{I\text{variant}} O^2$ heterozygotes. The 30 samples from the *P. falciparum*-infected patients included 12 $O^{I\text{variant}}$ homozygous individuals, 6 $O^I O^{I\text{variant}}$ individuals, 2 $O^I O^2$, and 4 $O^{I\text{variant}} O^2$ heterozygote. In the samples studied, there was no evidence of the homozygous O^2 allele. We have shown here that these individuals rarely have the O^2 allele, but do have a higher frequency of the $O^{I\text{variant}}$ allele. Thus, the gene frequency for the $O^{I\text{variant}}$ allele in these samples is 57% in the *P. falciparum*-infected patients and 60% in the control group. Comparing the genotype and O allele frequencies of the groups studied, no statistically significant difference between the blood donors and the individuals suffering from malaria was found. The loci were in Hardy-Weinberg equilibrium in the present sample.

Table 2. Comparison of genotyping results and allele frequency of the O histo-blood system among blood donors and patients infected with *Plasmodium falciparum* from the Brazilian Amazon region.

	<i>P. falciparum</i> patients (N = 30)	Donors (N = 27)	P (Fisher exact test)
$O^I O^I$	6 (20%)	5 (18.5%)	0.5781
$O^I O^2$	2 (6.66%)	1 (3.7%)	0.5402
$O^n O^I$	6 (20%)	7 (25.9%)	0.4136
$O^n O^2$	4 (13.33%)	3 (11.1%)	0.5607
$O^n O^n$	12 (40%)	11 (40.74%)	0.5840
Allele frequency			
O^I	0.33	0.33	1.000
O^2	0.10	0.07	0.9066
O^{Iv}	0.57	0.60	0.9918

When we analyzed the alleles of the O phenotype, the $O^{I\text{variant}}$ allele was the most frequent both in malaria and non-malaria groups with a consequent highest frequency of the homozygous genotype $O^{Iv} O^{Iv}$. Indeed, previous studies have shown that several isolated Indian populations in South America comprise only blood group O individuals and that they rarely have the originally described O allele (O^I) or O^2 allele, but instead have almost exclusively the O^{Iv} allele (Franco et al., 1995). In contrast, the frequencies found for this allele in Brazilian whites and blacks are around 40 and 30%, respectively (Olsson et al., 1998). Comparing the Brazilian population data, our results show that $O^{I\text{variant}}$ allele frequencies are lower than the frequencies reported for some Amerindians and higher than those for populations of Caucasian and African ancestry (Olsson et al., 1998), probably due to the influence of Portuguese colonization in the northern region of Brazil as well as the presence of Amerindians (Batista dos Santos et al., 1999). On the other hand, previous studies in Southeast Brazil (Mattos et al., 2001) have shown lower $O^{I\text{variant}}$ allele frequencies than those found in our sample, probably because the indigenous contribution in this region of country is the smallest. Along the same line, the frequency of the A phenotype reaches values around 35%, and the A allele frequency is more than 0.25 in these populations (Mattos et al., 2001), while in our sample of the North region the frequencies were 21.2 and 0.13%, respectively.

To our knowledge, this is the first evaluation of ABO genotyping of malaria patients in Brazil. In regions that are highly endemic for *Plasmodium falciparum* malaria, it is well recognized that a range of red blood cell polymorphisms associated with resistance to severe diseases have undergone positive selection (Min-Oo and Gros, 2005). Moreover, as mentioned

before, the blood group O proved to be a protective factor against severe malaria (Barragan et al., 2000; Chung et al., 2005). However, since in the Brazilian Amazon region malaria predominates in Mesoendemic conditions with wide variation in transmission, malaria endemicity could be viewed as a selective pressure for maintenance of the observed frequencies of genotypes of the ABO system, which could be very interesting as the focus of a new investigation, including analysis of the genotypes in severe and non-severe malaria patients, as well as in individuals living in non-endemic areas.

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Host erythrocyte polymorphisms and exposure to *Plasmodium*

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8.2 Development of PCR-RFLP assay for the discrimination of *Plasmodium* species and variants of *P. vivax* (VK210, VK247 and *P. vivax*-like) in *Anopheles* mosquitoes. Autores: Gustavo Capatti Cassianoa, Luciane Moreno Storti-Melo, Marinete Marins Póvoa, Allan Kardec Ribeiro Galardo, Andréa Regina Baptista Rossit e Ricardo Luiz Dantas Machado. Artigo original submetido à publicação e em fase de revisão na revista **Acta Tropica**, 2010.

RESUMO

A identificação das espécies de *Plasmodium* em mosquitos *Anopheles* é um componente essencial do controle da malária. Nós desenvolvemos uma nova abordagem para identificação de *P. falciparum*, *P. malariae* e das variantes de *P. vivax*. Foram desenhados *primers* específicos para hibridizar regiões específicas do gene *CS*. Uma PCR-RFLP foi utilizada para distinguir as variantes de *P. vivax*, VK210, VK247 e *P. vivax-like*. Obteve-se um bom grau de concordância entre a nova técnica de PCR-RFLP comparada com uma nested PCR usando mosquitos *Anopheles* infectados artificialmente. Este sensível método de PCR pode ser útil para a detecção das espécies de *Plasmodium* e das variantes de *P. vivax* contribuindo para o melhor entendimento da dinâmica de transmissão da malária pelas espécies de *Anopheles*.

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Abstract: The identification of Plasmodium species in Anopheles mosquitoes is an integral component of malaria control. We developed a new assay for the identification of P. falciparum, P. malariae and P. vivax variants. Specific primers were designed to hybridize to CS gene specific regions. A PCR-RFLP was used to distinguish the P. vivax variants VK210, VK247 and P. vivax-like. The new PCR-RFLP assay, compared with a nested PCR using artificially infected Anopheles mosquitoes, revealed good agreement between the two. This sensitive PCR method can be useful when Plasmodium species and P. vivax variants detection are required and may be employed to improve the understanding of malaria transmission dynamics by Anopheles species.

10th January 2011

To Editor

Acta Tropica

Dear Editor,

We are sending the manuscript “**Detection of human malaria parasites in *Anopheles* mosquitoes by PCR-RFLP using specific sequences on circumsporozoite gene**” after second modification requested by Journal Manager to publish in the ACTA TROPICA as article. The authors hereby certify that none of the material in this manuscript has been or will be published and none is currently under consideration for publication elsewhere.

Thank you very much

Yours faithfully

Gustavo Capatti Cassiano MSc

**Development of PCR-RFLP assay for the discrimination of
Plasmodium species and variants of *P. vivax* (VK210, VK247 and *P. vivax*-
like) in *Anopheles* mosquitoes**

Gustavo Capatti Cassiano^{a,*}, Luciane Moreno Storti-Melo^a, Marinete
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ABSTRACT

The identification of *Plasmodium* species in *Anopheles* mosquitoes is an integral component of malaria control. We developed a new assay for the identification of *P. falciparum*, *P. malariae* and *P. vivax* variants. Specific primers were designed to hybridize to CS gene specific regions. A PCR-RFLP was used to distinguish the *P. vivax* variants VK210, VK247 and *P. vivax*-like. The new PCR-RFLP assay, compared with a nested PCR using artificially infected *Anopheles* mosquitoes, revealed good agreement between the two. This sensitive PCR method can be useful when *Plasmodium* species and *P. vivax* variants detection are required and may be employed to improve the understanding of malaria transmission dynamics by *Anopheles* species.

Keywords: Malaria diagnosis, circumsporozoite gene, *P. vivax* variants, *Anopheles*.

1. Introduction

The correct identification of the human-specific *Plasmodium* species in the mosquito host is an essential component for planning and monitoring malaria control. According to the Brazilian Ministry of Health, *P. vivax* is the predominant species in this country (83.5% of all cases) followed by *P. falciparum* (15.47%), mixed species infection (1.0%) and *P. malariae* (0.03%) (Brazilian Ministry of Health, 2009). Additionally, the *P. vivax* circumsporozoite protein (CS) genotypes, VK210, VK247 and *P. vivax*-like, has been found in different Brazilian Amazon regions, in both pure and mixed infections (Machado and Póvoa, 2000; Storti-Melo et al., 2009). These different human malaria species may differ in infectivity of anophelines (Gonzales-Ceron et al., 1999), transmission potential and responses to anti-malarial drugs (Machado et al., 2003). Information about the geographical distribution of the parasite and vector species is important for the accurate interpretation of epidemiological data.

For many years, detection of malaria parasites in mosquitoes was performed by dissection and visualization of the midgut and salivary glands under a microscope. Although microscopic examination is reliable, it requires fresh material, experienced microscopists and is time consuming (Lulu et al., 1997). Another limitation of this methodology is that it cannot distinguish among *Plasmodium* species. A major breakthrough was the discovery of specific CS protein antigen. The sequencing of this protein and its corresponding gene revealed the existence of specific repetitive sequences for some species of *Plasmodium* (Ozaki et al., 1983; Dame et al., 1984; Arnot et al., 1985; Lal et al., 1988), allowing their discrimination by enzyme-linked immunosorbent assay (CS-ELISA) using monoclonal antibodies (Wirtz et al., 1987). Although CS-ELISA has been widely used due to its high sensitivity and specificity (Sattabongkot et al., 2004; Hasan et al., 2009), there are some limitations: overestimation of true salivary gland infection rates (Robert et al., 1988; Fontenille et al., 2001), false positive results (Hasan et al., 2009) and failure to detect low-level infections (Arez et al., 2000). Ryan et al. (2001) developed a rapid dipstick assay (VecTest™ Malaria), which determines the presence or absence of specific CS peptide epitopes of *P. falciparum* and VK210 and VK247 *P. vivax* genotypes, but it is less sensitive compared with the PCR assay (Moreno et al., 2004).

PCR-based assays have been considered the most efficient methods for the identification of human malaria parasites (Snounou et al., 1993a,b). In fact, currently, the most widely used PCR assay is a nested-PCR designed by Snounou et al. (1993b) using the small subunit ribosomal RNA, generally accepted as “the gold standard” for human malaria species identification. Recently, a real-time TaqMan PCR assay (Bass et al., 2008) and a novel single step PCR based on the amplification of the mitochondrial cytochrome b (*Cyt b*) gene (Hasan et al., 2009) were developed. These methods are sensitive and specific for the detection of infectivity in mosquitoes. Nevertheless, they are unable to distinguish *Plasmodium* species other than *falciparum*.

Herein, we describe a novel PCR test using primers for specific regions in the sequences of the *CS* gene to identify human *Plasmodium* species, including *P. vivax* genotypes, in the mosquito vector (CS-PCR/RFLP).

2. Materials and Methods

2.1 Preparation of mosquito samples

Laboratory-infected mosquitoes were kindly provided by Dr Willian Collins at the Malaria Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention. Thirty *Anopheles dirus* mosquitoes were artificially infected with *P. vivax* and 30 with *P. falciparum*. Thirty *An. gambiae* mosquitoes were artificially infected with *P. malariae*. Mosquitoes were storage on silica gel before being frozen at -20°C. The assay developed with these mosquitoes was approved by the Animal Research Board of the Faculty of Medicine of the São José do Rio Preto.

2.2 Extraction of malaria parasite DNA from mosquitoes and plasmid clones

DNA was extracted from individual mosquitoes using DNAzol (Invitrogen, U.S.A.), with slight modifications. Briefly, the head and thorax of single mosquitoes were placed in 1.5 mL Eppendorf tubes and macerated using a new sterile pipette tip in 100 µL of DNAzol. The product was suspended in 100 µl 8 mM NaOH and stored at - 20°C until use. Three plasmid clones carrying a PCR insert of the *CS* gene amplified from the *P. vivax* variants VK210, VK247 and *P. vivax*-like (BlueScript, Stratagene, U.S.A.), used for PCR-RFLP standardization, were kindly provided by Dr Ira Goldman from the Center for Disease Control and Prevention.

2.3 Primer design

We designed one PCR reaction to amplify the conserved region of the CS gene from *P. falciparum* and *P. malariae* and a second one to amplify the internal variable region of the *P. vivax* CS gene. The sequence of *P. falciparum* was amplified using primer pairs PFCSP1 (5' CCAGTGCTATGGAAGTTCGTC 3') and PFCSP2 (5' CCAATTTTCCTGTTTCCCATAA 3'). We used PMCSP1 (5' ATATAGACTTGCTCCAACATGAAGAA 3') and PMCSP2 (5' AATGATCTTGATTTCGTGCTATATCTG 3') for *P. malariae* and PVCSP1 (5' AGGCAGAGGACTTGGTGAGA 3') and PVCSP2 (5' CCACAGGTTACACTGCATGG 3') for *P. vivax*. The primers were selected using the web-based software Primer3 v.0.4.0 (<http://frodo.wi.mit.edu/primer3/>). A conformational analysis investigated the possibility of primer secondary structure formation, annealing temperature and GC content using the software programs Primer3 and IDT OligoAnalyzer 3.1 (<http://www.idtdna.com>).

Nucleotide alignment of the CS gene sequences from *Plasmodium* species and variants of diverse geographic origin, available in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>), was performed to ensure that no variation was reported in the primer annealing regions.

2.4 PCR amplification

All PCR amplifications were carried out in a 25 µL reaction mixture containing 3 µl genomic DNA for *P. falciparum* and *P. vivax* and 5 µL for *P. malariae*, 1 x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 µM of each primer and 2.5 U of *Taq* polymerase (Invitrogen, U.S.A.). A separate reaction was carried out with every sample for the detection of each *Plasmodium* species. Only the primers corresponding to each one of the species were used in each reaction mixture. The amplification was performed in a thermal cycler (DNA MasterCycler, Eppendorf, Germany) as follows: an initial cycle of 94°C for 15 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, then a final extension at 72°C for 10 min.

min. DNA of *P. falciparum*, *P. malariae* and *P. vivax* were included as positive controls while sterilized water and DNA extracted from colonized, malaria-free *Anopheles darlingi* were used as a negative controls.

2.5 PCR product analysis

Five µL PCR product was electrophoresed at 100 V for 50 min with 50 or 100 bp DNA molecular weight markers (Invitrogen, U.S.A.) in 1.5% agarose gel stained by ethidium bromide and the target DNA was visualized on an ultraviolet transilluminator.

2.6 Sensitivity and specificity of the assay

The infected blood samples from patients with parasitemia ranging from 300 to 12,500 parasites per microliter were used. These samples were serially diluted in blood from an uninfected donor to a final level of parasitemia corresponding to 10^{-6} and further processed for PCR amplification. DNA samples of *P. falciparum*, *P. malariae* and *P. vivax* were diluted to 10 ng/µL in sterile water (determined using a NanoDrop® ND-1000 UV-Vis spectrophotometer) and then serial dilutions were made down to 1 in 1×10^6 to determine the sensitivity of the PCR assay.

To define PCR specificity, genomic DNA obtained from patients' blood infected with *P. vivax*, *P. falciparum* and *P. malariae* was used. In addition, DNA from *Anopheles stephensi* infected with *P. ovale*, *Anopheles gambiae* infected with *P. malariae*, *Anopheles dirus* infected with *P. falciparum* and *P. vivax*, as well as DNA from uninfected *Anopheles darlingi*, were used.

2.7 Restriction digests of PCR products

P. vivax variants were typed by RFLP analysis based on PCR products from all variants displaying at least one cleavage site for the restriction enzymes

selected by the software RestrictionMapper (<http://www.restrictionmapper.org/>). The restriction reaction was performed in a final volume of 20 µL, using 10 U of *AluI* (Invitrogen, U.S.A.), 2 µL of recommended restriction buffer, 10 µL of the PCR product and 7 µL of sterilized water. Reactions took place at 37°C for 2 h. Digested products were electrophoretically separated on 12.5% polyacrylamide gels, in the presence of 50 bp DNA molecular weight markers (Invitrogen, U.S.A.) and the gels were subsequently silver stained.

2.8 Statistical analysis

Statistical comparison between CS-PCR and the nested PCR described previously by Snounou et al. (1993b) was made using Cohen's Kappa (*k*) measure of test association with a 95% confidence interval. Analyses were performed using the BioEstat program version 5.0 (Ayres et al., 2003). The nested PCR was considered the reference method of choice of test accuracy for determination of CS-PCR sensitivity and specificity. Sensitivity was calculated as the proportion of mosquitoes that were nested PCR positive with a positive CS-PCR and the specificity was calculated as the proportion of mosquitoes that were tested PCR negative with a negative CS-PCR). Positive predictive value was calculated as the number of true positive test results among all positive test results observed.

3. Results

3.1 Amplification of the *P. malariae*, *P. falciparum* and *P. vivax* variants CS gene fragments

The size of fragments amplified by plasmids correspond to 789 bp for *P. vivax* variant VK210 and 834 bp for *P. vivax* variants VK247 and *P. vivax*-like. PCR products had lengths of 199 bp for *P. malariae* and 118 bp for *P. falciparum* in 1.5% agarose gel (Figure 1A).

3.2 PCR-RFLP analysis

The product amplified with the primers PVCSP1 and PVCSP2 for the identification of *P. vivax* was subjected to RFLP. The patterns observed with the *AluI* enzyme are shown in Figure 1B. PCR-RFLP for *P. vivax* variant VK210 showed fragments of 135, 106, 100, 54, 43 and 27 bp. Three fragments (691, 100 and 43 bp) were specific for *P. vivax* variant VK247, while for *P. vivax*-like fragments of 731, 62 and 41 bp were detected. Fragments below 50 bp are not easily visible on the polyacrylamide gel; however the differences among the variants are easily determined based on larger fragments (Fig. 1B).

3.3 Sensitivity and Specificity of CS-PCR

The CS-PCR showed a sensitivity for *P. vivax* at a 1:10,000 dilution, for *P. falciparum* at 1:5,000 dilution and for *P. malariae* at 1:1,000 dilution. Control genomic DNAs from *P. malariae*, *P. falciparum* and *P. vivax* as well as from *Anopheles stephensi* infected with *P. ovale* and unfed mosquitoes were used to confirm the specificity of each primer pair. No amplification was obtained when each species of *Plasmodium* DNA was submitted to PCR with a primer pair designed to amplify a different species. *Anopheles* PCR products were never obtained from CS-PCR reactions (Figure 2).

3.4 Evaluation of the CS-PCR

CS-PCR and the nested PCR were tested in artificially infected mosquitoes. A total of 120 mosquitoes were screened, consisting of 30 infected with *P. vivax*, 30 infected with *P. falciparum*, 30 infected with *P. malariae* and 30 unfed mosquitoes. The results are shown in Table 1. All infected mosquitoes, as determined by CS-PCR, were also determined as *Plasmodium* positive by the nested PCR, except for one mosquito positive for *P. vivax* by the first methodology. No infection was found in any of the 30 unfed mosquitoes using

both methods. The comparison revealed a close agreement between the “gold standard” nested PCR and CS-PCR ($\kappa = 0.723, 0.867$ and 0.657 , respectively for *P. vivax*, *P. falciparum* and *P. malariae*).

The CS-PCR assay presented a good sensitivity for *P. vivax* and *P. falciparum* sporozoites (84.2% and 87.5%, respectively) and less sensitivity for *P. malariae* sporozoites (76.2%). The specificities were high for *P. vivax*, *P. falciparum* and *P. malariae* (90.9%, 100% and 100%, respectively). The predictive positive value was 94.5% for *P. vivax* and 100% for *P. falciparum* and *P. malariae*.

4. Discussion

Correct identification of *Plasmodium* species that occur in a malaria endemic area together with the infection rate of *Anopheles* species, help to understand local disease epidemiology. Precise survey data reduces inappropriate use of resources and insecticides. Thus, the identification of *Plasmodium* species in *Anopheles* mosquitoes is an integral component for malaria control. The value of this parameter in the understanding of malaria transmission dynamics resides on its accuracy.

Traditionally, the detection of a parasite occurs under microscope, but is laborious, demands fresh materials and cannot distinguish between *Plasmodium* species. A rapid diagnostic test that detects CS antigen with monoclonal antibodies allows the identification of *P. falciparum* and variants VK210 and VK247 (Ryan et al., 2001). Although simple, fast and specific (Bangs et al., 2002), may overestimate infection rates, since CS antigen can be found freely in the haemolymph of insect. The CS-ELISA, although widely adopted, has similar limitations (Robert et al., 1988; Fontenille et al., 2001; Hasan et al., 2009).

Usually PCR-based assays can discriminate between different *Plasmodium* species using two rounds of amplification (Snounou et al., 1993a; Singh et al., 1999; Rubio et al., 2002) and are more sensitive than other methods (Wilson et al., 1998; Póvoa et al., 2000; Moreno et al., 2004). We have developed a method in

which sequences of the *CS* gene are exploited for use in a PCR, allowing the detection and identification of the three variants of *P. vivax*, VK210, VK247 and *P. vivax*-like. Moreover, we used specific conserved sequences for identification of *P. falciparum* and *P. malariae*.

The CS-PCR showed high specificity and positive predictive values for the three *Plasmodium* species tested. The concordance between our CS-PCR and the nested PCR (Snounou et al., 1993b) for detecting human malaria parasites (*P. vivax*, *P. falciparum* and *P. malariae*) and sporozoites in mosquitoes infected in the laboratory was good for *P. vivax* and *P. malariae* ($\kappa = 0.723$ and 0.657 respectively) and high for *P. falciparum* ($\kappa = 0.867$). This may be due to the fact that nested PCR uses two rounds of PCR. Moreover, the nested PCR targets the small subunit ribosomal RNA gene, which is present in four copies per haploid genome, and thus improves the efficiency of the PCR (Hasan et al., 2009). However, the advantage of using the *CS* gene as a target is the ability to identify the *P. vivax* variants.

The *CS* gene is present in one copy per haploid genome and encodes a circumsporozoite protein (CSP) that varies in length between different *Plasmodium* species, but, in general, has about 400 amino acids (Hughes, 1991). The CSP contains a central repeat region bracketed between nonrepetitive sequences. In *P. falciparum*, most single nucleotide polymorphisms (SNPs) occur in the 3' nonrepeat region and in the repeat region (Putaporntip et al., 2009). In *P. malariae*, the 5' nonrepeat region is highly conserved and the polymorphisms in CSP are limited to the central repeat region (Tahar et al., 1998). *P. vivax* displays three major types of central repeat region, thereby classifying field isolates into VK210, VK247 or *P. vivax*-like (Qari et al., 1993).

The *CS* gene has been extensively studied because its protein is the main target for vaccine development (Sócrates et al., 2007). Consequently, variations in its nucleotide sequence are documented and deposited in databases. Since the presence of mutations in the primer binding sites can preclude primer-binding during PCR, we investigated multiple *CS* gene sequences isolated from different regions in the world, available in the GenBank database to ensure that there was

no change in the binding sequence of newly designed primers (data not shown). In the case of *P. vivax*, nonrepeat regions were selected for the choice of primers. After alignment sequences we found that there was no variation in the binding sequence of newly designed primers of any sequence. For *Plasmodium malariae*, there is not variation in the 5' region of the gene of 16 sequences analyzed and this region was used for the choice of primers. Regarding *P. falciparum* sequences, most of the variation is found in the central region and 3' region. When we realize the choice of primers, we selected 5' region. In fact, we found only one single base substitution in this region for *P. falciparum* (accession nos. U20969). This is favorable since it suggests that this method will be useful in different endemic areas around the world.

P. vivax malaria has been endemic in many countries and its CSP genotypes are found worldwide, so its effective diagnosis is very important. Indeed, *P. vivax* malaria variants may have different characteristics with respect to the intensity of symptoms and the response to drugs, which could result in the failure of control measures (Kain et al., 1993; Machado and Póvoa, 2000). Besides this, some species of *Anopheles* have differential susceptibility to *P. vivax* variants (Gonzales-Ceron et al., 1999, 2001; Silva et al., 2006). Thus, it is important to identify *P. vivax* variants in *Anopheles* mosquitoes to better target appropriate mosquitoes for vector control.

The choice of restriction enzymes was also influenced by our objective of creating an efficient test with optimal resolution of restriction profiles. Based on the sequence analysis of *P. vivax* variants available in the GenBank database, the *AluI* endonuclease was found to be the most suitable enzyme and it showed optimal discriminatory power to distinguish all variants.

Obviously, all PCR based methods have some limitations. In the method described herein, the requirement for separate PCRs for each species increases the time required and the assay cost, and therefore it may not be suitable for large-scale epidemiological surveys. However, this PCR-RFLP is very useful when *P. vivax* variant detection is required, since there is no need for sequencing.

However, the CS-PCR does not identify *P. ovale* and it may not be so useful if employed in countries where this species exists.

In conclusion, this comparative study showed a close agreement between the novel CS-PCR and the 'gold standard' nested PCR. Moreover, the CS-PCR-RFLP described here was highly specific to each *Plasmodium* species and the variants. Because of its low detection threshold, especially for *P. vivax*, this assay can be used for detection even at low parasite levels. The CS-PCR-RFLP is the first molecular diagnostic, to our knowledge, that can identify *P. vivax* variants in *Anopheles* mosquitoes.

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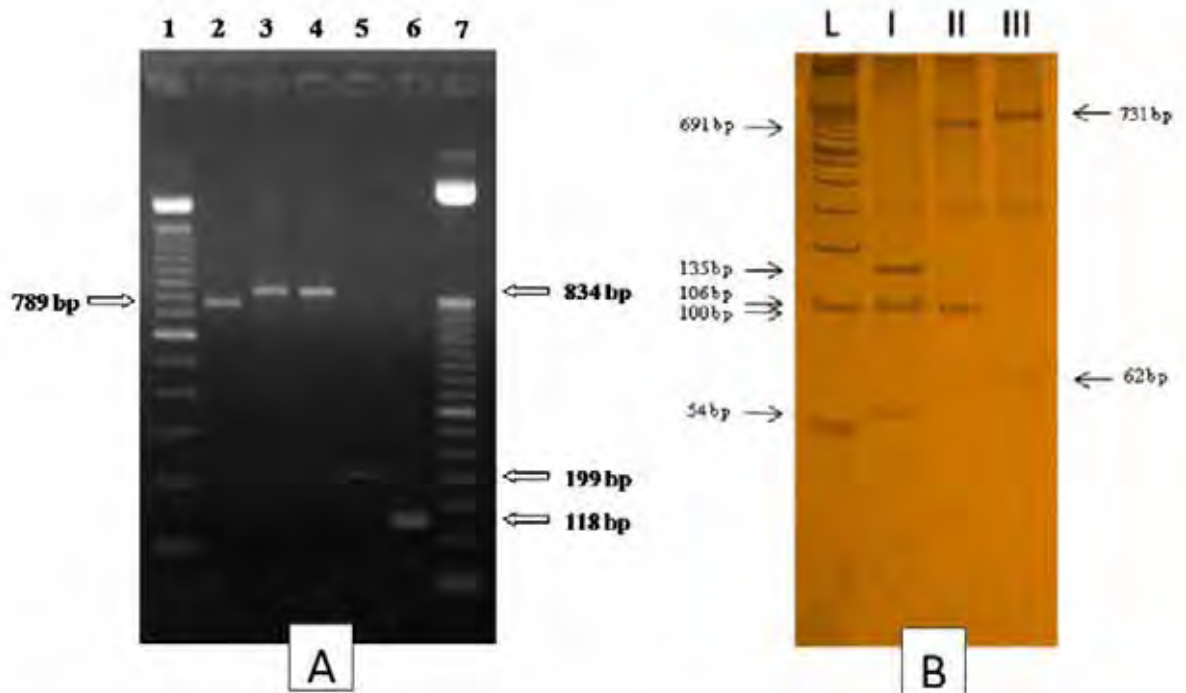


Fig. 1. Banding pattern of the CS-PCR-RFLP. (A) Lane 1, 100 bp DNA ladder (Invitrogen, U.S.A.); Lane 2, VK210 plasmid; Lane 3, VK247 plasmid; Lane 4, *P. vivax*-like plasmid; Lane 5, *P. malariae*; Lane 6, *P. falciparum*; Lane 7, 50 bp DNA ladder (Invitrogen, U.S.A.). Band sizes from 2 to 6, 789, 834, 834, 199, and 118 bp, respectively. (B) Digestion of products amplified of *P. vivax* variants VK210, VK247 and *P. vivax*-like. Image showing the fragments of digestion with *AluI*. L: 50 bp ladder; I: VK210; II: VK247; III: *P. vivax*-like. The products were run on 12.5% polyacrylamide gel.

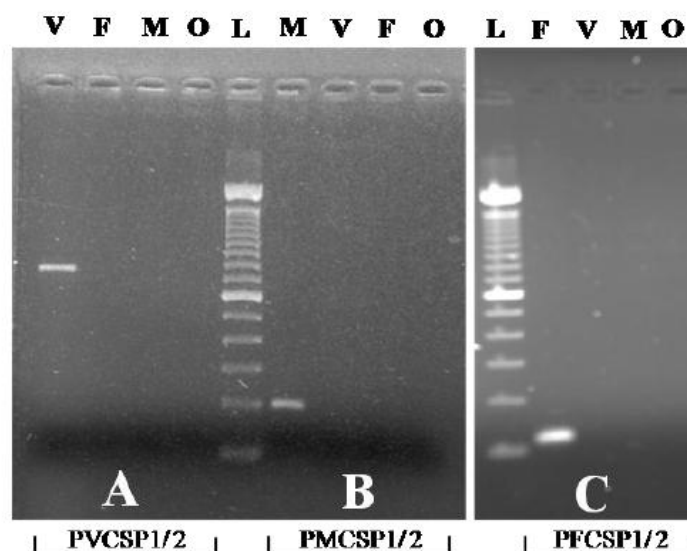


Fig. 2. Specificity of the amplification assays. Amplification of PCR products using the indicated primer pairs (PVCSP1/2, PMCSP1/2, PFCSP1/2). (A) A fragment specific for *P. vivax*. (B) A fragment specific for *P. malariae*. (C) A fragment specific for *P. falciparum*. None of *Anopheles* gene was amplified in any reaction. V: *P. vivax*; F: *P. falciparum*; M: *P. malariae*; O: *P. ovale*; L: 100 bp ladder as a molecular size marker. Electrophoresis was in a 1.5% agarose gel.

Table 1

Results of the CS-PCR and nested PCR using *Plasmodium* artificially infected and uninfected mosquitoes.

	CS-PCR		Nested PCR		Cohen's Kappa value
	Positive	Negative	Positive	Negative	
Mosquitoes infected with <i>P. vivax</i>	17	13	19	11	0.723
Mosquitoes infected with <i>P. falciparum</i>	14	16	16	14	0.867
Mosquitoes infected with <i>P. malariae</i>	16	14	21	09	0.657
Uninfected mosquitoes	0	30	0	30	1

The sensitivity for *P. vivax*, *P. falciparum* and *P. malariae* sporozoites were 84.2%, 87.5% and 76.2% respectively. The specificities were 90.9% for *P. vivax* and 100% for *P. falciparum* and *P. malariae*. The predictive positive value was 94.5% for *P. vivax* and 100% for *P. falciparum* and *P. malariae*.

8.3 Giardiasis as Zoonosis: between proof of principle and paradigm in the Northwestern region of the São Paulo State, Brazil. Autores: Aline Cardoso Caseca Volotão, Nathália Motta Delvaux Ramos, Maria Fantinatti, Marcus Vinícius Proença de Moraes, Halim Atique Netto, Luciane Moreno Storti-Melo, Juarez Elias Santos Junior, Andréa Regina Baptista Rossit, Octávio Fernandes, Ricardo Luiz Dantas Machado. Artigo original submetido à publicação e em revisão na revista **Asian Pacific Journal of Tropical Medicine**.

RESUMO

A fim de avaliar o potencial zoonótico de *Giardia duodenalis*, isoladas de humanos e cães na região noroeste do Estado de São Paulo, Brasil foram caracterizados com base no gene β giardina. Cem amostras de fezes confirmaram o diagnóstico de giardíase por métodos parasitológicos e imunológicos (83 humanos e 17 cães) e, sessenta e duas amostras (55 humanos e 7 cães) foram analisadas por seqüenciamento dos produtos de Nested-PCR. Os subgenótipos A1 e A2 foram detectados em humanos e cães. Cistos das linhagens B, C e D não foram encontrados em nenhum isolado. Dessa forma, destaca-se um risco transmissão de *G. duodenalis* via zoonótica entre os habitantes do noroeste do estado de São Paulo, encorajando-nos para uma reavaliação crítica de um conjunto maior de dados, incluindo a relação parasito-hospedeiro com os aspectos do quadro clínico / fecal e também visando à origem da infecção na comunidade.

De: Ricardo Luiz Dantas Machado

Enviada em: sexta-feira, 8 de outubro de 2010 17:31

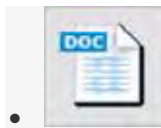
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Thanks for all,

Ricardo Machado

Giardiasis as Zoonosis: between proof of principle and paradigm in the Northwestern region of the São Paulo State, Brazil

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Abstract

Objective: In order to evaluate the potential zoonotic transmission of *Giardia duodenalis*, isolates from humans and dogs in the Northwestern region of the São Paulo State, Brazil were characterized based on the β -

giardin gene. **Methods:** One hundred stool samples confirmed the giardiasis diagnosis by parasitological and immunological methodology (83 human and 17 dogs) and, sixty-two samples (55 human and 7 dogs) were analyzed by sequencing of the Nested-PCR products. **Results:** The A1 and A2 subgenotypes were detected in human and dogs. Cysts of assemblage B, C and D have not been found in any isolates studied. **Conclusions:** Therefore, the risk of *G. duodenalis* zoonotic route among the inhabitants of Northwestern of São Paulo state was observed, encourage us for a critical re-evaluation of a larger data set, including the correlation between host–parasite relationship with clinical/fecal aspects and will also shed light on the origin of infection into the community. This is consistent with the view that giardiasis in the largest endemic region of the Brazil should not be seen as a single entity.

keywords: *Giardia duodenalis*, *beta giardin* gene, genotypes, zoonosis, Brazil

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1. Introduction

Giardia duodenalis is the protozoan frequently found in the intestinal infection causing gastroenteritis worldwide. The *G. duodenalis* is complex specie with at least seven different assemblages. The A and B assemblages have been associated with infections in human as well as in other mammals, while the other assemblages have demonstrated to prefer different animal species ^[1]. In spite of this, the zoonotic potential of the *G. duodenalis* remains unclear, mainly among domestic animals ^[2]. Moreover, new assemblages of the *G. duodenalis* are been described yet ^[3,4].

Around of 200 millions of people worldwide are infected with this protozoan and each one can release 900 millions of cysts for year^[5]. In Brazil, a large variation in this parasite frequency has been observed and in some regions of the São Paulo state, up to 40% of the population carries this protozoan^[6]. Few molecular studies were conducted in Brazil and discordant results were reported. In Rio de Janeiro city, Southeastern region of Brazil, only A1 and A2 subgenotype were found in human fecal samples and A1 subgenotype was the unique detected in the domestic animals isolate^[5]. In the study carried out in the Southeastern of the São Paulo state, the A2 subgenotype and assemblage B were detected in human, A1 and F genotypes were found in cat and C and D were observed in dog isolates^[7]. In São José do Rio Preto city, located in the Northwestern of São Paulo state, two studies in HIV-1 soropositive

population; one in adults^[8] and once more in a child population^[6] have shown that the *G. duodenalis* frequency ranged from 4.1% to 9%. Hereby, we evaluate the potential zoonotic transmission of giardiasis, in the Northwestern region of the São Paulo State, Brazil.

2. Material and methods

2.1. Sampling

From July 2007 until March 2008 were selected 150 individuals with positive fecal samples for this protozoan in parasitological test among the 4,300 samples analyzed by the staff of the Central Laboratory of the Hospital de Base of São José do Rio Preto (HB). The HB is a tertiary care hospital catering to a large population from the Northwestern region of São Paulo state, Brazil. All individuals received explanations about the objectives of this work and after they had signed the written informed consent, fecal samples were collected from each one of the 145 individuals that agreed to participate in the study. Five *G. duodenalis* positive fecal samples from HIV-1 infected individuals, which were storage at Centro de Investigação de Microrganismos (CIM) from Faculdade de Medicina de São José do Rio Preto (FAMERP) were included. We also evaluated 52 pet dog stool samples collected at Hospital Veterinário do Centro Universitário de Rio Preto (UNIRP). All stool samples were transported for microscopic and immunological analysis at CIM. The

genotyping analysis was conducted at Laboratório Interdisciplinar de Pesquisas Médicas do Instituto Oswaldo Cruz – Fundação Oswaldo Cruz, Rio de Janeiro. The parasitological survey developed with this population was approved by Human (process 3456/2007) and animal (process 109509/2007) research board from FAMERP.

2.2. Parasitological examination

Fecal samples were examined for *Giardia* cysts using a standard methodology by a centrifugal flotation in 33% zinc sulphate solution, in which parasite form were detected by light microscopy. To confirm the parasitological results, a commercially available immunoenzymatic assay (Alexon BIOBRÁS, Brazil) processed according to the manufacturer's recommendations was used^[6]. Samples were stored for 1-6 months at 4°C or at -20°C before DNA extraction.

2.3. DNA isolation and PCR amplification

Cyst disruption and genomic DNA extraction/purification from frozen feces were performed using QIAamp DNA mini kit (Qiagen GmbH, Alemanha), according manufacture procedures, with modifications. The lise temperature was changed to 95°C, and the final AE buffer volume was reduced to 100 µL. In the primary polymerase chain reaction (PCR) a 753 bp fragment of the *β-giardin* gene was amplified using the forward primer G7 and the reverse primer G759 as previously described ^[9]. In the

sequential nested PCR, a 511 bp fragment was amplified using BGIA1F/BGIA1R primer pair according to Lalle et al.(2005)^[1].

PCR were performed in 50 µL total reaction volume containing 100 ng of genomic DNA, 10mM Tris-HCl pH8.5, 50mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP, 10 µM of each primer and 2.5 U AmpliTaqGold polimerase (Applied Biosystems, EUA); 94°C for 5 minutes followed by 35 cycles of amplification (94°C for 30s, 65°C for 30s and 72°C for 60s) were carried out, with a final elongation step of 7 minutes at 72 °C. The product was analyzed by electrophoresis in 1.2% agarose gel, stained with 0.5µg/mL ethidium bromide (Invitrogen, USA) and examined under UV light (GelDoc, Bio-Rad, Hercules, CA, USA). As positive controls we used WB strain (ATCC 50803) and GM-1 isolate for A1 and B genotypes, respectively. As negative control, DNA of the *Entamoeba histolytica*, *Trichomonas vaginalis* axenic culture and *Cryptosporidium parvum* clinical sample were used.

2.4. Molecular characterization

Amplified products were purified using the Wizard® SV Gel and Pcr Clean-Up System (Promega, Madison, WI, USA). DNA fragments were sequenced in both directions, in triplicate using ABI Prism™ v 3.1 Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 3730 automatic DNA sequencer (Applied Biosystems, Foster City, CA).

Sequences were analyzed using Chromas (<http://www.technelysium.com.au/chromas.html>) (2005/09/25) and compared with know β -giardin of *G. duodenalis* sequences obtained from GenBank using ClustalW algorithm ^[10] in the MEGA version 3.1 (<http://www.megasoftware.net>) (2007/05/22). Phylogenetic analyzes of 350 nucleotides length β -giardin sequences were performed through MEGA 3.1, on alignments obtained from ClustalW. The distance estimations were carried out using Jin and Nei (1990)^[11] equation (Kimura 2-parameter model). The phylogenetic trees were constructed using a neighbor-joining algorithm^[5]. For each calculation branch reliability was assessed using bootstrap analysis (1000 replicates). The nucleotide sequence of β -giardin from *Giardia muris* (Genebank accession number AY258618) was used as an out group.

3. Results

Only one hundred stool samples confirmed the giardiasis diagnosis by parasitological and immunological methodology (83 human and 17 dog) and, sixty-two samples (55 human and 7 dogs) were amplified by Nested-PCR β -giardin. As summarized in table 1, the analysis of the 59 sequences of the 511 bp fragment of the β -giardin gene shown that all *G. duodenalis* isolates were assemblage A; 41 subgenotype A1 (38 human and 3 de dogs) and 18 subgenotype A2 (16 human and 2 dogs). Cysts of assemblage B, C and D have not been found in any of the Northwestern

region of São Paulo state isolates studied to date. The β -giardin sequences of the different genotypes found in this study were compared to already known sequences obtained from GenBank. Phylogenetic analysis of the β -giardin gene of *G. duodenalis* isolates from human provided strong bootstrap support (100%) for the placement of genotypes A and B in different clusters (Figure1).

4. Discussion

The Southeastern Brazilian region concentrates 46.5% of inhabitants of the Brazil and São Paulo state shows the majority of that. Although the *G. duodenalis* subgenotypes prevalence reported here can be representative of the human and household dog community-disseminated giardiasis in São José do Rio Preto, the biggest city of the Northwestern region of São Paulo state, the obtained data can update the information about these genotypes distribution for further comparisons within the diverse Brazilian regions and also within other developing countries. Additionally, this data serves as a springboard for a future large-scale analysis to the understanding, prevention, and control of such relevant disease around the world.

In this work, we studied 55 human and 7 domestic dog fecal samples by sequencing analysis of the Nested-PCR products of the β -giardin gene. Interestingly, the human and pet dogs of this city displayed only assemblage A (subgenotypes A1 and A2) and neither assemblage B

(human, cat, horse and dog genotypes) nor C and D assemblages (canine-specific genotypes) were detected. Although previous study did not show this tendency for the assemblage A as single infection^[7,12], our data are in agreement with other studies which had showed that this assemblage was the unique detected^[1,5]. Moreover, since all individuals had diarrhea, our data support previous evidence that assemblage A are more virulence than assemblage B. Despite of the fact that dogs are very important in human life, they can be a source of parasite for environment and consequently for human infection^[13]. Although A and B assemblages have been described in dogs previously, it has been documented that these animals are more infected by C and D assemblages than other canine population^[4]. Curiously, in our work two individuals and their pet dogs were infected with cysts of subgenotype A1. The non-detection of the dog-specific assemblages in our work supports the fact that in non-urban settings, they are more prevalent in the canine population, possibly because of a better host adaptation^[1]. In addition, in human populations living with domestic animals, where the giardiasis prevalence is moderate, the detection of zoonotic assemblages is more usual, than canine-specific assemblages, which are observed mainly in areas where the relationship is more restricted^[14]. Sequencing of the one human and two canine isolates were not able to be confirmed by the analysis (table 1), showing sequences with both low quality and overlapping nucleotide peaks, which

can be attributed to mixed infections or to allelic sequence heterozygosity of single cysts^[5].

Therefore, we have three hypotheses to explain these data: (1) the genotype homogeneity in Northwestern of São Paulo state is probably because of their origin, since all fecal samples came from the same clinical laboratory and the individuals live in close geographic areas^[12]. (2) the huge variation of social, climatic, and geographic features in Brazil has been reported in several studies regarding diarrhea etiologic agents as important factors that adjust the frequency of different pathogens^[6], which can explain the difference observed between our results and the ones found by Souza et al., (2007)^[7] (450 km apart from the study area). (3) since the Brazilian population has a highly heterogeneous ethnic composition, as result of the hybridization of the numerous native indigenous populations with immigrants from Europe, Africa and Asia and the immigration flow was not uniform in the country^[15], the distribution of these genotypes could be related to the host-parasite relationship.

Even though the detection of the assemblage A in humans could not be incriminated as zoonotic fecal-oral route^[1,7], the presence of the A1 and A2 subgenotypes in all isolates appears to prevail the potential zoonotic in this region, supporting the fact that, giardiasis in the largest endemic areas of the Brazil should not be seen as a single entity.

Authors' contributions: RLDM and ACCV conceived and participated in all aspects of the study and manuscript preparation; MVPM, JSJ and LMSM carried out all the parasitological and immunological assays; HAN selected and participated in the dog parasitological evaluation; and ACCV, MMDR and MF participated in the *β-giardin* gene analysis; ARBR and OF participated in the design of the study and drafted the manuscript. All authors read and approved the final manuscript. ACCV is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: Research board of the FAMERP (Human process - 3456/2007 and animal process - 109509/2007).

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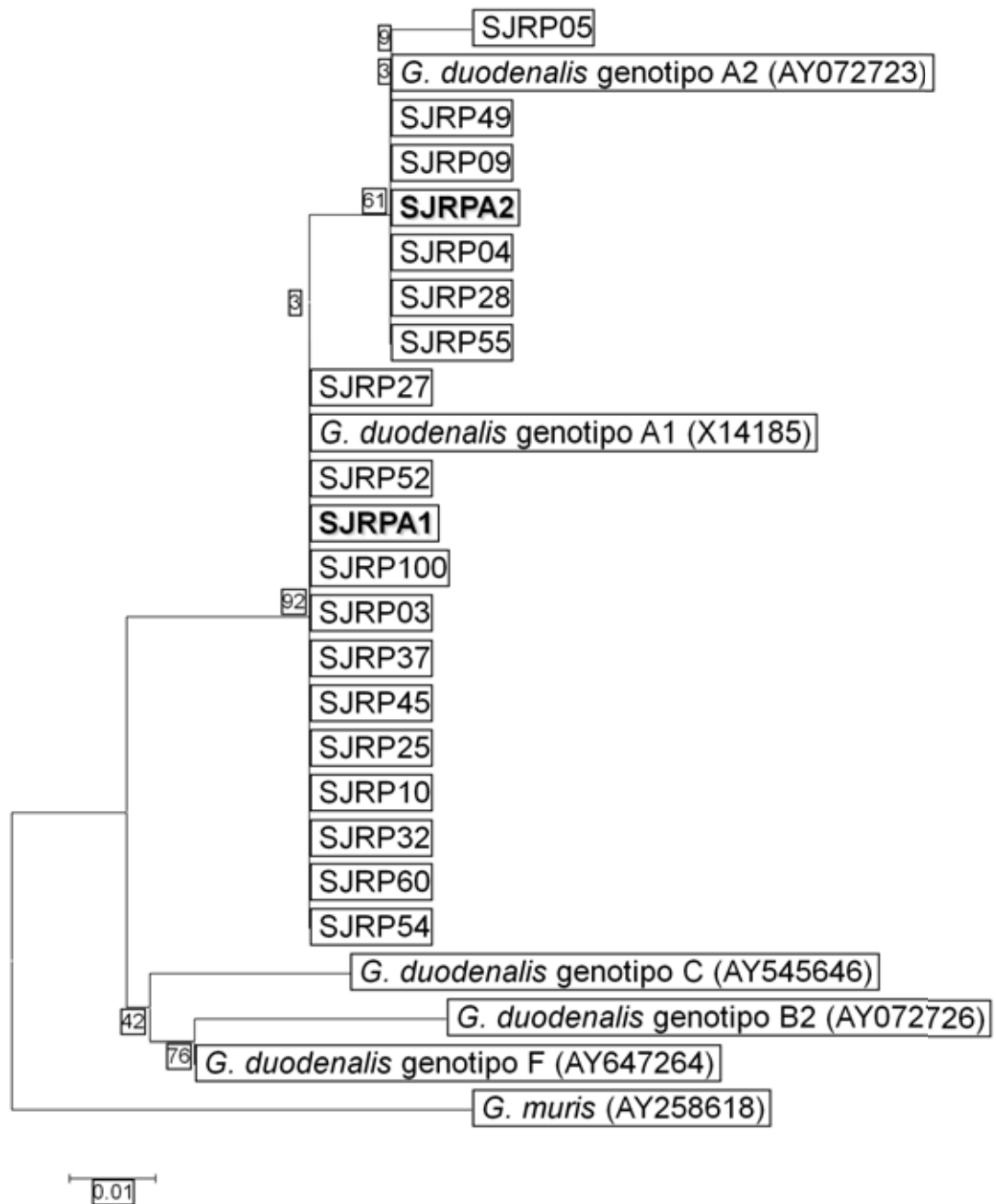


Figure 1. Phylogenetic tree of the *Giardia* isolates based on sequences of the β -giardin gene from human and domestic animals inferred by neighbor-joining algorithm using Kimura two-parameter. Sequences

obtained in GenBank are indicated by their accession numbers in parentheses.

Table 1 - Frequency of *Giardia duodenalis* genotypes in feces from human and pet dog population of the Northwestern of the São Paulo state, Brazil.

Sample	Nested-PCR positive	Genotyping and sequencing samples		
		A1	A2	No identified
HIV-1 human Positive (n=5)	5 (100%)	2 (40%)	2 (40%)	1 (20%)
HIV-1 human Negative (n=78)	50 (64.1%)	36 (31.0%)	14 (15.4%)	-
Pet Dogs (n=17)	7 (41.2%)	3 (17.6%)	2 (11.8%)	2 (11.8%)
Total (n=100)	62 (46.7%)	41 (36%)	18 (16%)	3 (7%)

Autorizo a reprodução deste trabalho.

São José do Rio Preto, 11 / 02 / 2011

Luciane Moreno Storti de Melo