MINISTÉRIO DA SAÚDE

FUNDAÇÃO OSWALDO CRUZ

CENTRO DE PESQUISAS RENÉ RACHOU

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

CARACTERIZAÇÃO DO RECEPTOR DARC (Duffy antigen/receptor for chemokines) E DA RESPOSTA IMUNE ANTI DUFFY BINDING PROTEIN EM INDIVÍDUOS EXPOSTOS AO *Plasmodium vivax*.

por

Bruno Antonio Marinho Sanchez

Belo Horizonte

Fev/2011

MINISTÉRIO DA SAÚDE

FUNDAÇÃO OSWALDO CRUZ

CENTRO DE PESQUISAS RENÉ RACHOU

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

CARACTERIZAÇÃO DO RECEPTOR DARC (Duffy antigen/receptor for chemokines) E DA RESPOSTA IMUNE ANTI DUFFY BINDING PROTEIN EM INDIVÍDUOS EXPOSTOS AO *Plasmodium vivax*.

por

Bruno Antonio Marinho Sanchez

Tese apresentada com vistas à obtenção do Título de Doutor em Ciências na área de concentração de Doenças Infecciosas e Parasitárias

Orientação: Dra. Luzia Helena Carvalho
Orientação: Dra. Cristiana Ferreira Alves de Brito

Belo Horizonte

Fev/2011

Catalogação-na-fonte

Rede de Bibliotecas da FIOCRUZ

Biblioteca do CPqRR

Segemar Oliveira Magalhães CRB/6 1975

S211c Sanchez, Bruno Antonio Marinho.

2011

Caracterização do receptor DARC (Duffy Antigen/Receptor for Chemokines) e da resposta imune anti Duffy Binding Protein em indivíduos expostos ao *Plasmodium vivax* / Bruno Antonio Marinho Sanchez. – Belo Horizonte, 2011.

xix, 146 f.: il.; 210 x 297mm.

Bibliografia: f. 157 - 165

Tese (doutorado) – Tese para obtenção do título de Doutor em Ciências pelo Programa de Pós-Graduação em Ciências da Saúde do Centro de Pesquisas René Rachou. Área de concentração: Doenças Infecciosas e Parasitárias.

1. Malária Vivax/epidemiologia 2. *Plasmodium vivax*/parasitologia 3. Sistema do Grupo Sanguíneo Duffy/sangue I. Título. II. Carvalho, Luzia Helena (Orientação). III. Brito, Cristiana Ferreira Alves de (Orientação).

CDD – 22. ed. – 616.936 2

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa René Rachou
Programa de Pós-Graduação em Ciências da Saúde

Ata da quadragésima defesa de tese de doutorado do Programa de Pós-Graduação em Ciências da Saúde, do aluno Bruno Antonio Marinho Sanchez, sob a orientação das Dras. Luzia Helena Carvalho e Cristiana Ferreira Alves de Brito.

Aos vinte e três dias do mês de fevereiro do ano de dois mil e onze, às quatorze horas, realizou-se no auditório da Escola de Saúde Pública do Estado de Minas Gerais, o exame da quadragésima defesa de tese de doutorado do Programa de Pós-Graduação em Ciências da Saúde do Centro de Pesquisa René Rachou/FIOCRUZ, como parte dos requisitos para a obtenção do título de Doutor em Ciências - área de concentração Doenças Infecciosas e Parasitárias. A tese do aluno Bruno Antonio Marinho Sanchez intitula-se "Caracterização do receptor DARC (*Duffy antigen/receptor for chemokines*) e da resposta imune anti *Duffy Binding Protein* em indivíduos expostos ao *Plasmodium vivax*". A banca examinadora foi constituída pelos professores: Dra. Luzia Helera Carvalho - CPqRR/FIOCRUZ (orientadora/Presidente), Dr. Stefan Michael Geiger - CPqRR/FIOCRUZ (Titular), Dr. Mariano Gustavo Zalis - UFRJ (Titular), Dra. Simone Ladeia Andrade - IOC/FIOCRUZ (Titular) e Dra. Kézia Katiani Gorza Scopel - UFJF (titular). Após argüir o aluno e considerando que o mesmo demonstrou capacidade no trato do tema escolhido e sistematização na apresentação dos dados, a Banca Examinadora assim se pronunciou: De acordo com o regulamento do Programa de Pós-Graduação em Ciências da Saúde, o aluno foi considerado APROVADO. Uma vez encerrado o exame, eu, Luzia Helena Carvalho, presidente da Banca, assino a presente ata juntamente com os membros da Banca Examinadora. Belo Horizonte, vinte e três de fevereiro de dois mil e onze.

Dra. Luzia Helena Carvalho

Dr. Stefan Michael Geiger

Dr. Mariano Gustavo Zalis

Dra. Simone Ladeia Andrade

Dra. Kézia Katiani Gorza Scopel

Av. Augusto de Lima, 1715 Barro Preto Belo Horizonte MG Brasil Cep: 30190-002
Tel.: (31) 33497799 Fax: (31) 3295 3115 http://www.cpqrr.fiocruz.br/posgraduacao/cienciasdasaude

"Meu trabalho científico é motivado por uma irresistível ansiedade de entender os segredos da natureza e não por outros sentimentos."

Albert Einstein

Dedico este a trabalho a Raimunda Batista de Sousa (in memoriam), que fez deste momento seu próprio sonho. Aos meus pais, irmãos e sobrinhos pelo amor, companheirismo e constante incentivo. A minha esposa Karla pelo amor, ajuda e paciência.

À Dra. Luzia Helena Carvalho e a Dra. Cristiana Ferreira Alves de Brito, pela orientação, sobretudo paciência, amizade, estímulo e dedicação na minha formação científica.

Este trabalho foi realizado no Laboratório de Malária do Centro de Pesquisas René Rachou/FIOCRUZ, sob a orientação da Dra. Luzia Helena de Carvalho e Dra. Cristiana Ferreira Alves de Brito com suporte financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Organização Mundial da Saúde (TDR/WHO), a Rede de Plataformas Tecnológicas PDTIS, Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), PAPES IV/FIOCRUZ e Centro de Pesquisas René Rachou.

AGRADECIMENTOS

Eu agradeço à Deus, por ter me dado a oportunidade de uma dia iniciar minha carreira no Laboratório de Malária, onde pude terminar minha graduação e ingressar nos cursos de Mestrado e Doutorado.

Agradeço a Dra. Luzia e Dra. Cristiana pela confiança depositada em mim durante todos esses anos. Espero conseguir algum dia ser um Cientista à altura de vocês e poder retribuir tudo o que fizeram por mim.

À Karlinha por todo amor, apoio, cumplicidade, incentivos constantes e pela companhia em todos os momentos.

Aos meus pais, pelo amor incondicional, pelo apoio em todos os momentos de dificuldades e por estarem sempre presentes em todas minhas conquistas

À minha segunda família em Sete Lagoas, na pessoa dos meus amigos Carnot e Angela Guedes, pelo carinho, amizade e incentivos.

A minha eterna amiga Alice Sabatino, pelo carinho e constante ajuda dispensada.

Ao meu grande amigo Dr. Cor Fontes, pela amizade incondicional, pelos ensinamentos e por toda a ajuda dispensada.

Agradeço a minha amiga e estudante Letícia Torres, por cada momento de alegria e aprendizado nos momentos de trabalho no laboratório.

Ao meu grande amigo Dr. Fernano Varotti, pela amizade e ensinamentos.

Ao amigo Armando Menezes, pela amizade durante todos esses anos de convivência.

Eu agradeço a Dra. Flora Kano pela amizade, colaboração, críticas, sugestões, disponibilidade e parceria.

A minha amiga de Doutorado Fernanda Freire, pela convivência e amizade durante estes quatro anos.

A Dra. Tais Sousa, pelas colaborações, por toda a ajuda e pela amizade em todos esses anos de Fiocruz.

Ao Dr. Luciano Moreira, pela amizade e pelos ensinamentos.

Aos amigos do laboratório, Daniela, Flávia Alessandra, Flávia Rodrigues, Fernanda Rezende, Flávia Carolina e Ricardo Ribeiro.

Aos Mestres, por todos os ensinamentos.

À Carolina Parreira, Secretária do Laboratório de Malária, pelo apoio dado e amizade.

Ao Geraldo Felício pela amizade e ajuda técnica.

À Biblioteca do CPqRR em prover acesso gratuito local e remoto à informação técnico-científica em saúde custeada com recursos públicos federais, integrante do rol de referências desta tese, também pela catalogação e normalização da mesma.

A todos que compõe o Centro de Pesquisas René Rachou (CPqRR), na pessoa do seu diretor, o Dr. Rodrigo Corrêa Oliveira, que contribuíram não apenas para a realização deste trabalho, mas também para o meu desenvolvimento pessoal e profissional.

SUMÁRIO

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SÍMBOLOS

AMA-1 – Antígeno 1 de membrana apical (Apical membrane antigen 1)

DARC – Antígeno Duffy/receptor para quimiocinas (Duffy antigen/receptor for chemokines)

DBL-EBPs – Família de proteínas que se ligam aos eritrócitos e apresentam um domínio de ligação semelhante ao que se liga ao antígeno Duffy/DARC (Duffy binding like domain Erythrocyte binding protein)

DBL - Domínio de ligação semelhante ao que se liga ao antígeno Duffy/DARC (Duffy binding like domain)

dbp – Gene que codifica a proteína que se liga ao antígeno Duffy/DARC

DBP – Proteína que se liga ao antígeno Duffy/DARC (Duffy binding protein)

DBPII – Domínio de ligação (região II) da proteína que se liga ao antígeno Duffy/DARC (Duffy binding protein II)

dbpII – Gene que codifica o domínio de ligação (região II) da proteína que se liga ao antígeno Duffy/DARC

DNA – Ácido desoxirribonucleico

EBA-140 (BAEBL) – Antígeno de 140 kDa que se liga a eritrócito (140 kDa erythrocyte binding antigen)

EBA-175 – Antígeno de 175 kDa que se liga a eritrócito (175 kDa erythrocyte binding antigen)

EBA-181 (JESEBL) – Antígeno de 181 kDa que se liga a eritrócito (181 kDa erythrocyte binding antigen)

EBL-1 – Proteína 1 que se liga a eritrócito (erythrocyte binding ligand 1)

EBP – Proteína que se liga ao eritrócito (Erythrocyte binding protein)

ECD – Domínio amino-terminal extracelular (Extracelular amino-terminal domain)

EDTA – Ácido etilenodiaminotetracético

ELISA - Ensaio imunoenzimático (Enzyme-linked immunosorbent assay)

FIOCRUZ - Fundação Oswaldo Cruz

FY – Locus do antígeno Duffy/receptor para quimiocinas (DARC)

FY*A, FY*B – Alelos DARC que codificam os antígenos Fya e Fyb, respectivamente

$\boldsymbol{FY*A^{ES}}$**,** $\boldsymbol{FY*B^{ES}}$ – Alelos DARC silenciados na linhagem eritróide

FY*X – Alelo DARC que codifica uma expressão fraca ou qualitativamente reduzida do antígeno Fyb

GFP - Proteína de fluorescência verde (Green protein fluorescent)

IPA – Incidência parasitária anual

µg - Micrograma

µL - Microlitro

µM - Micromolar

MSP-1 – Proteína 1 de superfície do merozoíto (Merozoite surface protein 1)

pb – par de bases

PCR – Reação em cadeia da polimerase

PCR-ASP – Reação em cadeia da polimerase alelo-específica

pEGFP - Plasmídeo que codifica a proteína de fluorescência verde potencializada (Enhanced Green Fluorescent Protein)

PNG - Papua Nova Guiné, Oceania

PvDBP – Proteína de P. vivax que se liga ao antígeno Duffy/DARC (P. vivax Duffy binding protein)

RBC(s) – Célula(s) sanguínea(s) vermelha(s) (Red blood cell)

Real-time PCR – Reação em cadeia da polimerase em tempo real

RNA – Ácido ribonucleico

RT-PCR – PCR em tempo real

Sal-1 - Variante de DBPII de P. vivax de um clone de referência de laboratório isolado em El Salvador (Fang et al., 1991)

RESUMO

O processo de invasão dos eritrócitos pelos plasmódios é complexo, sendo mediado por interações moleculares específicas do tipo ligante receptor. No caso do *Plasmodium vivax*, a invasão é altamente dependente do antígeno de grupo sanguíneo Duffy (DARC), presente na superfície dos eritrócitos, que interage com uma proteína do parasito, a Duffy binding protein (PvDBP). Considerando a importância do receptor DARC como principal via de invasão do *P. vivax*, no presente estudo desenvolveu-se uma metodologia para genotipagem do receptor DARC. A técnica foi realizada através da PCR em tempo real, em sistema multiplex, o que otimizou o método de genotipagem em larga escala e reduziu o custo. Esta nova metodologia permitiu genotipar o receptor DARC dos indivíduos que participaram de um estudo epidêmico de malária e de outros estudos com indivíduos de diferentes áreas endêmicas da região Amazônica brasileira. No presente trabalho, para avaliar a influência de DARC na infecção pelo *P. vivax*, duas abordagens metodológicas foram utilizadas: um estudo de prevalência e um de incidência do tipo prospectivo. Para o estudo que buscou associação de DARC com a prevalência de malária por P. *vivax*, foram estudados indivíduos, proveniente de outros estados brasileiros, que migraram para a Amazônia. Nesta população foi observado que os indivíduos que possuíam dois alelos DARC funcionais apresentavam um maior risco de infecção ao P. vivax. Já no estudo prospectivo de base populacional, o receptor DARC foi genotipado em cerca de 800 indivíduos nativos da Amazônia, residentes em um assentamento agrícola na Amazônia. Embora nenhuma associação tenha sido encontrada entre o genótipo DARC e infecção pelo *P. vivax*, os resultados sugeriram que o receptor DARC influenciou na resposta de anticorpos. Nesta área, indivíduos com apenas um alelo funcional (*FY*B/FY*B*^{ES}) apresentaram uma resposta maior de anticorpos anti-PvDBP. Estes achados são importantes, já que a relação entre DARC e a resposta de anticorpos anti-PvDBP tem sido pouco estudada. Além disso, foram determinados os genótipos de DARC em indivíduos residentes em uma área de transmissão epidêmica de malária por *P. vivax* (surto ocorrido na região metropolitana de Belo Horizonte, MG), onde se avaliou também a resposta de anticorpos anti-PvDBP. Na área do surto, a caracterização dos genótipos de DARC dos indivíduos expostos à transmissão demonstrou que os diferentes genótipos estavam igualmente distribuídos entre aqueles que se infectaram e os não infectados. Por último, nesta área do surto epidêmico, estudou-se a cinética da resposta dos anticorpos anti-PvDBP. O resultados mostraram que anticorpos anti-PvDBP são de curta duração e específicos para a variante do parasito que causou o surto. Em conjunto, acredita-se que os resultados apresentados aqui possam contribuir para os estudos que visem o melhor entendimento da relação entre DARC, resposta imune e susceptibilidade a infecção pelo *P. vivax*.

ABSTRACT

The complex process of erythrocyte invasion by malaria parasites is mediated by specific molecular interactions. In *Plasmodium vivax*, invasion is dependent on the interaction between Duffy blood group antigen (DARC) and Duffy binding protein (PvDBP). Due to its importance in erythrocyte invasion, we used real-time PCR to investigate the genotype of the DARC receptors. The technique was performed using a multiplex system, which optimized the genotyping method on a large scale and reduced costs. This methodology was used to genotype the DARC receptor of individuals living in an area experiencing a malaria epidemic as well as other areas within the Brazilian Amazon. In this study, to evaluate the influence of DARC in *P. vivax* infection two methodological approaches were used: a prevalence study and an incidence study (prospective). In the prevalence study, it was observed that individuals who possessed two functional DARC alleles had a higher risk of infection with *P. vivax*. In a prospective study, the DARC receptor was genotyped in 800 individuals native to the Amazon. The results showed no association between DARC genotype and P. vivax infection. Nevertheless, the results suggested that the DARC receptor does influence the antibody response, with individuals possessing only one functional allele (*FY*B/FY*B*^{ES}) showing a greater response to anti-PvDBP. These findings are important because the relationship between DARC genotype and immune response to PvDBP has not previously been explored. We also determined the DARC genotypes and evaluated the response of anti-PvDBP in individuals living in a *P. vivax* epidemic area (an outbreak in the metropolitan area of Belo Horizonte, MG). In this outbreak area, different DARC genotypes were equally distributed among infected and uninfected individuals. Finally, we evaluated the kinetics of anti-PvDBP responses among individuals in this outbreak area. The results showed that anti-PvDBP antibodies were short and specific to the parasite variant responsible for the outbreak. Together, we believe that the results presented here can contribute to an improved understanding of the relationship between DARC receptor, immune response and susceptibility to *P. vivax* infection.

1 INTRODUÇÃO

A malária é causada por protozoários do filo Apicomplexa, família Plasmodiidae e gênero *Plasmodium.* Atualmente são conhecidas aproximadamente 150 espécies que infectam diferentes hospedeiros vertebrados, sendo que quatro espécies são as principais causadoras de malária no homem: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* e *Plasmodium ovale*. Recentemente foi demonstrado que uma quinta espécie, o *Plasmodium knowlesi*, que naturalmente infecta primatas não humanos, está causando infecções graves em humanos, principalmente, em algumas áreas específicas do sudeste asiático e do continente africano (White, 2008; Cox-Singh et al., 2008; Ong et al., 2009; Ménard et al., 2010)

A malária humana é uma das mais importantes causas de mortalidade e morbidade no mundo. A doença é endêmica na África subsaariana, sudeste Asiático e em áreas da América do Sul, provocando mais de 240 milhões de casos e causando cerca de 800.000 mil mortes a cada ano (World Malaria Report, 2009). A África concentra 90% dos casos de malária do mundo sendo que a mortalidade é maior do que em outras regiões, devido, principalmente, ao limitado acesso ao tratamento nas vilas. Nos últimos anos, a doença vem se propagando para outras áreas, devido ao intenso e não planejado processo de urbanização e estima-se que este processo na África subsaariana e em outros continentes, esteja alterando profundamente a epidemiologia da malaria (Siri et al., 2008; Tatem & Smith, 2010). No continente Africano, o *P. falciparum* é a espécie mais patogênica e o principal responsável pela maioria das causas de morte e morbidade (Snow et al., 2005; Hay et al., 2010).

O *P. vivax* é a espécie com mais ampla distribuição geográfica e maior prevalência fora do continente africano (Guerra et al., 2006). Estima-se que cerca de 2,85 bilhões de pessoas estão sob o risco de infecção nas diferentes áreas endêmicas do planeta (Guerra et al., 2010). O *P. vivax* causa uma doença debilitante que afeta a qualidade de vida e a produtividade econômica das pessoas afetadas (Greenwood et al., 2005). Embora freqüentemente a malária causada por *P. vivax* seja referida como

uma doença benigna e raramente fatal, estudos recentes na Papua Nova Guiné e Indonésia, e também no Brasil, tem reportado a existência de casos de malária grave por *P. vivax* (Genton et al., 2008; Tjitra et al., 2008; Andrade et al., 2010; Alexandre et al., 2010).

No Brasil, as três espécies que causam malária humana são o *P. vivax* (atualmente responsável por cerca de 80% dos casos), *P. falciparum* (responsável por aproximadamente 20% dos casos) e *P. malariae* (responsável por uma parte pequena da transmissão) (Brasil, 2001). Na região Amazônica, a transmissão está associada com a ocupação intensificada da área, que ocorreu principalmente nas décadas de 70 e 80. Indivíduos provenientes de áreas não endêmicas foram atraídos para o local devido aos projetos de colonização, expansão de fronteiras agrícolas, construção de estradas e hidroelétricas, extração de madeira e mineração (Marques 1986). As precárias condições de saneamento e moradia, a carência médica e educacional e o alto desequilíbrio ecológico, expandiram os focos de malária já existentes, levando a doença a assumir proporções alarmantes no fim das décadas 80 e 90. Como conseqüência deste processo, cerca de 600 mil casos da doença foram registrados durante este período, sendo 99,7% destes na Amazônia legal, na qual se encontram regiões de risco variado (revisto por Oliveira-Ferreira et al., 2010).

A partir do ano de 2000 o Ministério da Saúde (MS) desencadeou o processo de descentralização das ações de vigilância em saúde, que instituiu o repasse de recursos para estados e municípios certificados na modalidade fundo a fundo, por intermédio do Teto Financeiro de Vigilância em Saúde (TFVS). As ações de controle da malária englobam em cada município, principalmente, o diagnóstico e tratamento precoces dos casos, ajustados às características particulares da transmissão existentes em cada localidade. Além disso, o Ministério da Saúde iniciou uma ampla mobilização de forças multi-setorial, principalmente os gestores de saúde nos estados e municípios da região Amazônica, para coordenar movimentos populacionais e priorizar a prevenção, a vigilância e o controle da malária nestas áreas (revisto por Oliveira-Ferreira et al., 2010). Este conjunto de processos vem se efetivando anualmente com o fortalecimento da estrutura dos serviços e da capacidade de gestão

dos estados e municípios. Assim, no ano de 2009, foram registrados cerca de 300 mil casos da doença no Brasil (Brasil, 2007). Estes números mostram uma redução de 31% no número de casos comparados com o início da década de 80, justificando a eficácia das ações. Porém, de acordo com Oliveira-Ferreira e colaboradores (2010) faz-se necessário a continuidade dos programas de controle e buscar novas medidas, pois como no Brasil a doença é restrita à região Amazônica, existem vários fatores associados que favorecem a transmissão e que dificultam o uso dos procedimentos de controle padrão.

1.1 Ciclo Biológico da Malária Humana

O ciclo biológico dos parasitos da malária humana compreende uma fase de reprodução sexuada, que ocorre dentro do hospedeiro invertebrado, e outra de reprodução assexuada, que se desenvolve no hospedeiro vertebrado. Durante o repasto sanguíneo no hospedeiro vertebrado, a fêmea do mosquito infectado deposita esporozoítos, vasodilatadores e anticoagulantes sob a pele. Na picada são depositados em média 100 esporozoítos (Jin et al., 2007), que permanecem no local da picada por aproximadamente 1 a 3 horas até alcançarem os vasos sanguíneos (Amino et al., 2006; Yamauchi et al., 2007). Alguns autores descreveram uma rota diferente de migração dos esporozoítos, em que os mesmos, após atravessarem o epitélio do hospedeiro vertebrado, podem também atingir o sistema linfático. Entretanto, os parasitos não parecem atingir o fígado por esta via (Amino et al. 2006). Recentemente, um estudo demonstrou que uma fração dos esporozoítos inoculados podem diferenciar em merozoítos na epiderme, na derme e também nos folículos pilosos, entretanto, merozoítos derivados da pele, em condições normais, não contribuem significativamente para a infecção dos eritrócitos (Gueirard et al., 2010).

Os esporozoítos que chegam ao sistema circulatório atingem o fígado, onde infectam hepatócitos. Na malária de mamíferos, não está claro o mecanismo pelo qual os esporozoítos passam dos capilares sinusóides do fígado até os hepatócitos: se é através das células de Kupffer ou através das células do endotélio dos vasos

sanguíneos (Pradel & Frevert 2001, Mota et al. 2002). O processo de invasão de hepatócitos é complexo e depende de várias interações do tipo ligante-receptor. Recentemente foi demonstrado que os esporozoítos invadem vários hepatócitos, migrando através deles, antes de se desenvolverem dentro de um hepatócito (Revisto por Mota & Rodrigues 2004). Nas infecções por P. vivax e P. ovale alguns parasitos se desenvolvem rapidamente nos hepatócitos, enquanto outros, os responsáveis pelos casos de recaídas, permanecem em estado de latência no fígado, sendo assim denominadas de hipnozoítas (Krotoski et al., 1982). Uma vez dentro dos hepatócitos os esporozoítos se diferenciam em trofozoítos que, após sofrerem várias divisões por esquizogonia, formam os esquizontes. Os esquizontes maduros liberam os merozoítos teciduais através de um processo de brotamento de vesículas, os merosomas, que após atingirem a corrente sanguínea, repletos de parasitas, liberam os merozoítos (Sturm et al. 2006). Os merozoítos teciduais invadem as hemácias iniciando assim a fase eritrocítica. Para que o merozoíto invada o eritrócito é necessário que haja também o reconhecimento inicial de receptores específicos (Revisto por Barnewell & Galinski 1998). Através de mecanismos pouco esclarecidos, alguns merozoítos sanguíneos se diferenciam dando origem a formas sexuadas, os gametócitos masculinos e femininos, os quais amadurecem sem sofrer divisão celular. Ao serem ingeridos pela fêmea do mosquito do gênero *Anopheles* durante o repasto sanguíneo, inicia-se a jornada do parasita no hospedeiro invertebrado onde ocorrerá o ciclo sexuado ou esporogônico.

Dentro do estômago do mosquito, os gametócitos masculinos e femininos se diferenciam transformando-se em gametas, sob influência das condições do ambiente em que se encontra o hospedeiro invertebrado, bem como fatores internos do mosquito (revisto por Vlachou et al. 2006). No interior do intestino médio do Anopheles os gametas se fundem, formando o zigoto (Barnwell & Galinski, 1998). Aproximadamente um dia após a fecundação, o zigoto se desloca com movimentos amebóides, passando a se denominar oocineto. O oocineto por sua vez atravessa a matriz peritrófica (membrana que envolve o bolo alimentar) e, por um mecanismo trans-celular (Zieler & Dvorak 2000, Vlachou et al. 2004, Baton & Ranford-Cartwright 2004), atinge as células do intestino médio, onde se aloja entre o epitélio e a membrana basal. Então o parasita se encista, e passa a denominar-se oocisto. Inicia-se o processo de multiplicação esporogônica, e em aproximadamente duas semanas, a

parede do mesmo se rompe liberando esporozoítos que invadem a hemolinfa do inseto. Assim, muitos parasitas migrarão até atingir as glândulas salivares, sendo inoculados em outro hospedeiro vertebrado, completando o ciclo evolutivo dos plasmódios.

1.2 Aspectos biológicos e imunológicos relacionados à invasão dos eritrócitos humanos pelo *P. vivax*.

O processo de invasão dos eritrócitos pelos parasitos da malária é complexo e ocorre em diversas etapas. Muitos ligantes do parasito e diferentes receptores da membrana do eritrócito têm sido caracterizados a fim de se entender o mecanismo de invasão da célula (Gaur et al., 2004). No caso do *P. vivax* a principal via de invasão do eritrócito é através da interação entre a Duffy binding protein (PvDBP) e o seu receptor no eritrócito, o antígeno do grupo sanguíneo Duffy/receptor de quimiocinas (DARC) (Wertheimer & Barnwell, 1989).

1.2.1 A Duffy Bind Protein (PvDBP) do *P. vivax*

As proteínas do parasito que se ligam aos receptores dos eritrócitos durante o processo de invasão são caracterizadas como proteínas da superfamília EBP (erythrocyte binding protein) (Revisto por Chitnis CE 2001). A Duffy binding protein de *P. vivax* (PvDBP) é uma molécula de 140 kDa expressa durante o ciclo eritrocítico do plasmódio. Essa superfamília inclui outras proteínas que se ligam ao eritrócito: (a) as EBP α, β e γ de *P. knowlesi*, um parasito de macacos do velho mundo, que também é capaz de invadir eritrócitos humanos que expressam o receptor DARC e; (b) as moléculas de adesão de *P. falciparum* EBA-175, BAEBL (EBA-140) e JSEBL (EBA-181) (Adams et al., 1990; Adams et al., 1992; Miller et al., 1975). Também pertencem a essa superfamília os antígenos variantes de superfície de *P. falciparum* (PfEMP1), envolvidos na citoaderência dos eritrócitos infectados ao endotélio microvascular e a outros eritrócitos não infectados (Howell et al., 2006). As proteínas dessa

superfamília são caracterizadas pela presença de um domínio rico em resíduos de cisteína na região amino-terminal e funcionalmente conservado, conhecido como domínio de ligação semelhante ao que se liga ao antígeno Duffy/DARC (Duffy binding like domain, DBL) (Adams et al., 1992; Barnwell & Wertheimer, 1989). Adicionalmente, as DBL-EBPs estão localizadas nos micronemas, organela intracelular localizada na região apical do merozoíto e apresentam um segundo domínio rico em cisteína na região carboxi-terminal da proteína (Adams et al., 1992).

A PvDBP pode ser esquematicamente dividida em sete regiões, definidas a partir da similaridade da sua estrutura gênica e seqüência de aminoácidos com as outras DBL-EBPs (Figura 1): uma região que contém o peptídeo sinal (região I), duas regiões ricas em cisteínas amino e carboxiterminal (região II e VI, respectivamente), três regiões hidrofílicas (região III, IV e V), um domínio transmembrana e um curto segmento citoplasmático (região VII) (Adams et al., 1992; Fang et al., 1991). A região II da PvDBP (PvDBPII), que corresponde ao domínio DBL, compreende 330 aminoácidos contendo 12 resíduos de cisteína e onde foi mapeado o sítio de ligação da proteína ao seu receptor nos eritrócitos do hospedeiro (Adams et al., 1992; Ranjan & Chitnis, 1999). O sítio de ligação está localizado em um segmento de aproximadamente 170 aminoácidos entre as cisteínas 4 e 7 (Chitnis & Miller, 1994; Ranjan & Chitnis, 1999).

A PvDBP está localizada nos micronemas e, apenas no momento da invasão, é liberada na superfície do merozoíto, onde entra em contato com receptor de quimiocinas DARC na superfície do eritrócito humano (Wertheimer & Barnwell, 1989). Uma vez estabelecida a junção PvDBP-DARC o processo de invasão passa a ser irreversível (Barnwell & Wertheimer, 1989). A interação entre PvDBP/DARC é a principal via de invasão dessas células pelos merozoítos, pois os indivíduos que não expressam o receptor DARC na superfície de seus eritrócitos são refratários à infecção por P. vivax (Miller et al., 1976). Porém, recentemente foram reportados casos de indivíduos DARC negativos infectados por *P. vivax*, no Quênia, no Brasil e em Madagascar, o que sugere que esse parasito utilize uma via alternativa para invadir os eritrócitos, embora a minoria dos isolados do parasito parece ser capaz de

utilizar essa via alternativa ainda não identificada (Ryan et al., 2006; Cavasini et al., 2007; Ménard et al., 2010).

Diversos estudos demonstraram que a região II é a região mais polimórfica da proteína e que o padrão de variabilidade nessa região difere entre os isolados do parasito provenientes de diferentes áreas geográficas estudadas. Estudos sobre a variabilidade genética da PvDBP foram realizados na Colômbia, Coréia do Sul, Papua Nova Guiné, Tailândia e no Brasil, pelo nosso grupo (Ampudia et al., 1996; Cole-Tobian et al., 2002; Gosi et al., 2008; Kho et al., 2001; Suh et al., 2001; Tsuboi et al., 1994; Xainli et al., 2000; Sousa et al., 2006). Recentemente, um trabalho realizado pelo nosso grupo identificou os polimorfismos presentes em isolados de P. vivax de diferentes regiões da Amazônia Brasileira e demonstrou que alguns destes polimorfismos da região II da PvDBP se localizam próximos ao domínio de ligação da proteína ao eritrócito (Souza et al., 2010).

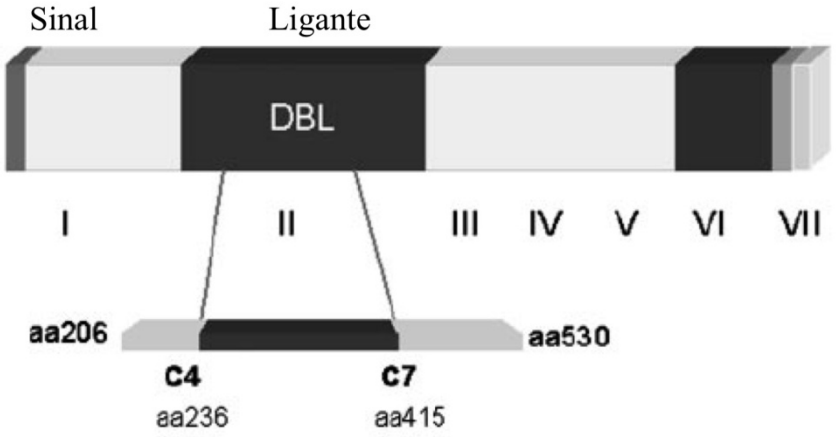


Figura 1. Representação esquemática dos domínios estruturais da proteína Duffy Bind Protein (DBP). A proteína possui as seis primeiras regiões de domínios extracelulares e a última região (VII) possui um domínio citoplasmático e transmembrana. No domínio II encontra-se a região referente ao ligante, localizada entre as cisteínas 4 e 7 (adaptado de VanBuskirk et. al, 2004).

1.2.2 Resposta imune a Duffy Binding Protein (PvDBP) do *P. vivax*

A PvDBP é expressa nos estágios eritrocíticos e se localiza em uma organela apical (micronema), sendo exposta na superfície do eritrócito somente no momento da invasão (Adams et al., 1990; VanBuskirk et al., 2004). Após ser expressa na superfície do parasito, a região do ligante da PvDBP entra rapidamente em contato com o antígeno DARC. Porém, apesar desta região do ligante da proteína entrar em contato com os eritrócitos do hospedeiro, estudos avaliando a estrutura da proteína têm sugerido que a resposta de anticorpos naturalmente adquiridos são direcionados predominantemente para as regiões opostas ao sítio de reconhecimento proposto (revisto por Chitnis & Sharma, 2008).

Na última década, os estudos de resposta imune anti-PvDBP foram realizados, principalmente, em regiões de alta endemicidade da Papua Nova Guiné e os resultados demonstraram a existência de anticorpos anti-PvDBP em indivíduos residentes nestas áreas. (Fraser et al., 1997; Michon et al., 2000; King et al., 2008). No caso do Brasil, considerado uma área de baixa a média endemicidade, foi demonstrado pelo nosso grupo que anticorpos anti-PvDBP estão presentes em indivíduos residentes na Amazônia brasileira e que parte destes anticorpos são capazes de bloquear a interação do ligante com o receptor na superfície dos eritrócitos (Cerávolo et al, 2005; Cerávolo et al, 2008). Porém, os dados encontrados na Amazônia e em outras regiões de diferentes endemicidades demonstraram que uma parte significativa da população exposta não desenvolve anticorpos anti-PvDBP (Fraser et al., 1997; Michon et al., 1998; Ceravolo et al., 2005; Souza-Silva et al., 2010). As razões para esta falta de resposta à PvDBP não são conhecidas, entretanto o fato da proteína se localizar nos micronemas pode ter contribuído para a pouca exposição da mesma ao sistema imune. Outro fator que pode contribuir para a baixa resposta é a variabilidade genética da PvDBP, pois os polimorfismos da região II parecem influenciar na resposta imune do hospedeiro (McHenry & Adams, 2006; Souza et al., 2010). Recentemente, estudos tem sido realizados a fim de identificar os epitopos de células T e B que sejam reconhecidos por anticorpos bloqueadores (Saravia et al., 2008; Chootong et al., 2010, Martinez et al., 2010).

Os estudos realizados por VanBuskirk e colaboradores (2004a) com diferentes variantes da PvDBP sugeriram que a resposta imune podia ser cepa específica. Entretanto, estes estudos foram realizados apenas in vitro com soros de coelhos imunes. É necessário entender melhor este processo de especificidade da resposta imune do hospedeiro à diversas variantes do parasito, pois isto pode contribuir nos estudos que visam a construção de uma vacina contra o *P. vivax*.

1.2.3 O antígeno Duffy/Receptor para Quimiocinas (DARC)

O receptor para a *Duffy binding protein* de *P. vivax* e *P. knowlesi* nos eritrócitos humanos é também um receptor para vários membros de quimiocinas das classes CC e CXC, conhecido também como antígeno Duffy/receptor para quimiocinas (Duffy antigen/receptor for chemokines, DARC) descrito pela primeira vez por Moore et al. (1982). Essa glicoproteína de 40-45 kDa é expressa principalmente nos eritrócitos e nas células endoteliais de vênulas pós-capilares de vários tecidos. Além do seu papel durante a invasão do *P. vivax*, o DARC tem sido muito estudado pois parece estar associado com inflamações e doenças inflamatórias, doenças infecciosas e tumorais, e com o HIV (revisto por Smolarek et al., 2010).

O receptor DARC é constituído por uma região extracelular amino-terminal (extracelular amino-terminal domain 1, ECD1) de aproximadamente 60 aminoácidos; uma região central (~250 resíduos) constituída por sete domínios transmembrana, três alças extracelulares (ECD2 a ECD4) e três alças intracelulares; e uma região citoplasmática de 28 resíduos (Figura 2) (Hadley & Peiper, 1997). A região de DARC que interage com a DBP é constituída por 35 aminoácidos (Ala8-Asp42) e está localizada na região ECD1 (Chitnis et al., 1996; Tournamille et al., 2005). O sítio de ligação para quimiocinas é formado pela associação dos quatro domínios extracelulares de DARC (ECD1-4) (Tournamille et al., 1997; 2003).

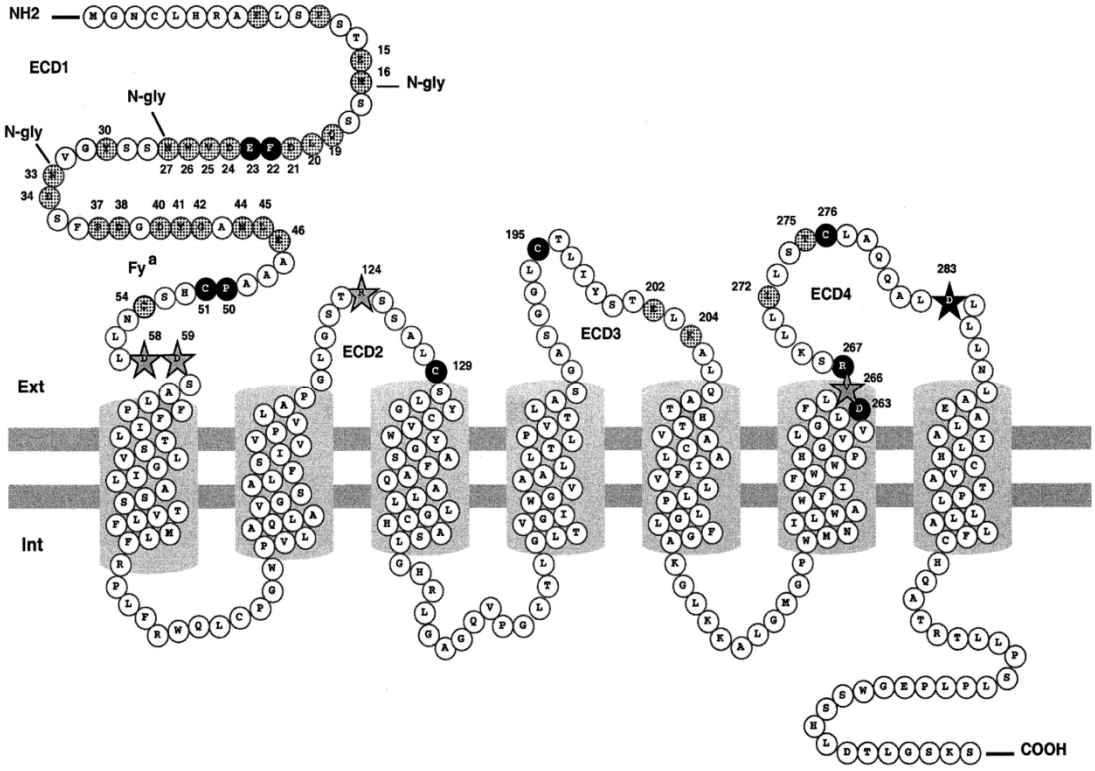


Figura 2. Representação esquemática da constituição de DARC. A figura mostra a estrutura de DARC que é constituído por uma região extracelular amino-terminal (extracelular amino-terminal domain 1, ECD1), uma região central (~250 resíduos) constituída por sete domínios transmembrana, três alças extracelulares (ECD2 a ECD4) e três alças intracelulares; e uma região citoplasmática de 28 resíduos. Figura modificada de Tournamille e colaboradores (2003).

O gene que codifica o receptor DARC é formado por dois exons e um intron de aproximadamente 450 pb (Figura 3). O primeiro exon codifica somente os sete primeiros aminoácidos da proteína. O processamento do intron produz um transcrito de RNA mensageiro (RNAm), conhecido como transcrito principal ou forma β, que codifica a forma predominante de DARC de 336 aminoácidos (Iwamoto et al., 1995). Uma forma menos abundante da proteína, com 338 aminoácidos é codificada por um RNAm, denominado de transcrito minoritário ou forma α (Chaudhuri et al., 1993; Yazdanbakhsh et al., 2000). As duas formas do transcrito de RNAm codificam proteínas funcionalmente idênticas (Yazdanbakhsh et al., 2000).

O locus Duffy/DARC (FY) está localizado no cromossomo 1, sendo caracterizado por três alelos principais: *FY*A, FY*B e FY*B*^{ES} (ES, erythroid silent; silenciado na linhagem eritróide) (Hadley & Peiper, 1997). Os dois alelos funcionais co-dominantes *FY*A* e *FY*B* codificam os antígenos Fya e Fyb, respectivamente. Esses alelos diferem em uma única mutação de ponto na posição 125 do exon 2 (G125A), que é responsável pela substituição de uma glicina em Fya por um ácido aspártico em Fyb (Gly42Asp) (Chaudhuri et al., 1995; Iwamoto et al., 1995; Mallinson et al., 1995). Esses antígenos definem os fenótipos Fy(a+b-), Fy(a-b+) e Fy(a+b+). O mecanismo molecular que forma o fenótipo Fy(a-b-) em indivíduos negros foi classicamente associado a uma mutação pontual T-33C na região do promotor do alelo *FY*B* (Tournamille et al., 1995a; 1995b). Esse fenótipo é caracterizado pela presença de dois alelos não funcionais *FY*B*^{ES}, cuja alteração impede a ligação do fator de transcrição GATA-1 à região promotora mutada, o que impede a expressão desta proteína nos eritrócitos. Porém, Tournamille e colaboradores (1995a) verificaram que apesar desta alteração fenotípicas nos eritrócitos, a expressão dessa proteína não está alterada em outros tecidos.

Um alelo raro que apresenta a mutação T-33C na região promotora do gene *FYA* (*FY*A*^{ES}), também responsável pela negatividade de DARC, foi descrito na região da Papua Nova Guiné, sempre em heterozigose com o alelo *FY*A* (Zimmerman et al., 1999). Esse alelo também foi identificado em pacientes infectados por P. vivax da Amazônia brasileira e em doadores não-infectados de regiões não endêmicas (Langhi et al., 2004a; 2004b). Outras mutações foram descritas no gene FY, como a substituição C265T (Arg89Cys) no alelo *FY*B*, que define o alelo *FY*X*, responsável por uma expressão fraca ou qualitativamente reduzida do antígeno Fyb na superfície dos eritrócitos. Tal alelo está associado ao fenótipo Fy(a+bweak) (Tournamille et al., 1998). Alguns estudos realizados também no Brasil demonstraram a presença do alelo *FY*X* na população brasileira (Castilho et al., 2004; Albuquerque et al., 2010).

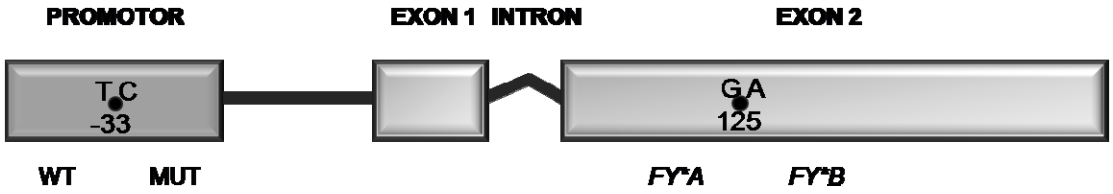


Figura 3. Representação esquemática do gene FY que codifica DARC, incluindo a região promotora, dois exons e o intron entre os exons 1 e 2. Está representado na figura: a mutação G125A, que define os alelos FY*A e FY*B, respectivamente e; a mutação T-33C, que diferencia o promotor selvagem (WT) do promotor mutado (MUT). Figura modificada de Cavasini e colaboradores (2001).

Com relação à quantidade de antígeno DARC na superfície dos eritrócitos, estudos têm demonstrado que há expressão reduzida desse antígeno nos eritrócitos de indivíduos heterozigotos para a mutação T-33C (alelos $FY*B^{ES}$ e $FY*A^{ES}$). Esses indivíduos expressavam aproximadamente duas vezes menos DARC na superfície dos eritrócitos do que os indivíduos homozigotos para o promotor não mutado (alelos *FY*A, FY*B*) (Woolley et al., 2000; Yazdanbakhsh et al., 2000; Zimmerman et al., 1999). Da mesma forma, o alelo *FY*X* é responsável por uma expressão significativamente reduzida de DARC (Yazdanbakhsh et al., 2000). Estudando esta diminuição do nível de expressão de DARC nos eritrócitos, estudos têm associado que tal fator possa estar envolvido na suscetibilidade do indivíduo à adquirir a infecção por *P. vivax*. Uma evidência indireta dessa associação foi obtida por Michon e colaboradores (2001), no qual foi observado que a expressão diminuída de DARC nos eritrócitos de indivíduos com um único alelo DARC funcional resultou na redução significativa da citoaderência em ensaios in vitro. Esses resultados sugerem que a presença de um único alelo não-funcional (FY*BES ou FY*AES) poderia reduzir a suscetibilidade à infecção por *P. vivax*. Similarmente, Zimmerman e colaboradores (1999) evidenciaram um aumento de duas vezes na prevalência de infecção por *P. vivax* em indivíduos da Papua Nova Guiné com o genótipo *FY*A/FY*A* comparado com $FY*A/FY*A^{ES}$. Entretanto, essa diferença não foi estatisticamente significativa, possivelmente devido ao número reduzido de indivíduos avaliados com o genótipo $FY*A/FY*A^{ES}$ (Zimmerman et al., 1999). A evidência mais direta foi obtida em um estudo epidemiológico conduzido na Papua Nova Guiné, onde o genótipo $FY*A/FY*A^{ES}$ estava significativamente associado à susceptibilidade reduzida à infecção por *P. vivax* (Kasehagen et al., 2007).

Além dos estudos a respeito da susceptibilidade à infecção pelo *P. vivax* em indivíduos com um ou dois alelos funcionais, alguns estudos têm sido realizados para avaliar se o tipo do genótipo do indivíduo tem influência nesta suscetibilidade à infecção. Em um trabalho realizado por Cavasini e colaboradores (2001), não se observou evidência de proteção significativa contra *P. vivax* entre indivíduos heterozigotos para a mutação T-33C (alelos $FY*B^{ES}$ e $FY*A^{ES}$). Ao contrário, um estudo recente realizado também na Amazônia demonstrou que os indivíduos heterozigotos seriam menos susceptíveis à infecção contra o *P. vivax* (Albuquerque et al., 2010).

2 JUSTIFICATIVA

A malária causada por *P. vivax* atinge milhões de pessoas no mundo, resultando em importantes perdas sociais e econômicas. No Brasil, essa é a espécie mais prevalente, tendo sido responsável por mais de 80% dos casos (Brasil, 2008). Considerando que a malária causada por *P. vivax* é um problema de saúde pública em várias regiões do mundo, e principalmente no Brasil, é fundamental o conhecimento da biologia do parasito para o desenvolvimento de medidas mais efetivas que auxiliem no controle da doença.

O processo biológico de invasão dos eritrócitos pelos merozoítos do *P. vivax* requer a presença de receptores específicos presentes na superfície do eritrócito. O *P. vivax* utiliza como via principal no processo de invasão do eritrócito a *Duffy binding protein* (PvDBP) e o seu receptor no eritrócito, o antígeno do grupo sanguíneo Duffy/receptor de quimiocinas (DARC) (Wertheimer & Barnwell, 1989). Os indivíduos que não apresentam o DARC na superfície do eritrócito são altamente resistentes à infecção pelo *P. vivax* (Miller et al., 1976). Assim, é de extremo interesse se estudar a PvDBP no processo de invasão dos eritrócitos, o que a torna um dos principais alvos de uma vacina anti-*P. vivax*.

Os estudos de resposta imune anti-PvDBP foram realizados, principalmente, em regiões de alta endemicidade (Fraser et al., 1997; Michon et al., 2000; King et al., 2008). No caso do Brasil, considerado uma área de baixa a média endemicidade, foi demonstrado pelo nosso grupo que anticorpos anti-PvDBP estão também presentes em indivíduos residentes na Amazônia brasileira e que parte destes anticorpos são capazes de bloquear a interação do ligante com o receptor na superfície dos eritrócitos (Cerávolo et al, 2005; Cerávolo et al, 2008). Porém, a natureza polimórfica da PvDBP parece ser um fator que pode influenciar diretamente na resposta imune do hospedeiro (McHenry & Adams, 2006; Cole Tobian et al., 2009; Souza et al., 2010). Ainda permanece pouco esclarecido se a resposta de anticorpos bloqueadores anti-PvDBPII são variantes específicas. Com esses resultados, vê-se a necessidade de compreender

melhor a natureza polimórfica da PvDBP e associar essa variabilidade, à capacidade do hospedeiro de produzir anticorpos bloqueadores que sejam específicos a determinada variante.

Em adição, é crucial entender os aspectos do receptor DARC relacionados à infecção pelo *P. vivax*. Alguns trabalhos demonstraram que a expressão diminuída de DARC estava relacionada com uma menor susceptibilidade à aquisição de malária vivax e que o genótipo estava relacionado a uma maior ou menor susceptibilidade à aquisição da doença (Michon et al., 2001; Albuquerque et al., 2010). Com base nestas descobertas, era necessário desenvolver uma metodologia de genotipagem de DARC mais rápida e de baixo custo e explorar a relação entre o genótipo DARC e suscetibilidade à infecção por *P. vivax*, bem como com a resposta imune do hospedeiro vertebrado.

Tendo em vista a importância destas descobertas para o desenvolvimento de uma vacina, este trabalho estudou - do ponto de vista do hospedeiro vertebrado e do parasito - os aspectos moleculares e imuno-epidemiológicos da resposta imune anti-PvDBP, com ênfase na possibilidade de que a resposta imune anti-PvDBP, seja cepa específica, bem como os aspectos genéticos do hospedeiro que influenciam na maior ou menor susceptibilidade à infecção pelo *P. vivax*.

3 OBJETIVOS

3.1 Objetivo Geral

Avaliar a especificidade dos anticorpos anti-PvDBP no processo de reconhecimento da proteína e a influência do genótipo DARC na susceptibilidade à infecção pelo P. vivax e na resposta imune do hospedeiro.

3.2 Objetivos Específicos

Desenvolver uma metodologia rápida para genotipagem do receptor DARC.

Genotipar o antígeno DARC de uma população de uma área de assentamento agrícola no Estado do Amazonas.

Avaliar a relação entre o genótipo DARC, a susceptibilidade à infecção pelo P. vivax e a resposta imune anti-PvDBP.

Avaliar o papel dos polimorfismos de PvDBP na resposta de anticorpos bloqueadores.

4 MATERIAL E MÉTODOS

4.1 Áreas de estudo

4.1.1 Área de transmissão epidêmica

Durante o período entre Abril e Maio de 2003, foram diagnosticados 25 casos de malária autóctones por *P. vivax* na localidade de Souza, município de Rio Manso, localizado a 70 km de Belo Horizonte, capital do estado de Minas Gerais. De acordo com os dados da Secretaria de Saúde do Estado de Minas Gerais, a transmissão de malária nunca havia sido descrita naquela área. O início da infecção se deu depois que um homem da comunidade retornou da região Amazônica infectado pelo *P. vivax*, em Janeiro de 2003 (Cerbino et al., 2004; Zumpano et al., 2004). A partir de então, novos casos foram diagnosticados e estudos entomológicos na área demonstraram que o vetor responsável pela disseminação da doença foi o *Anopheles darlingi* (Cerbino et al., 2004). Os casos confirmados foram tratados conforme o esquema terapêutico recomendado para tratamento da malária vivax, determinado pelo Ministério da Saúde (Ministério da Saúde, 2001). O período da transmissão foi considerado curto (aproximadamente 50 dias), sendo o último caso de malária diagnosticado em 21 de Maio de 2003. As atividades de controle incluíram a busca ativa de malária aguda por gota espessa e a pulverização do interior e do exterior das residências com inseticida residual (Cipermetrina) (Zumpano et al., 2004).

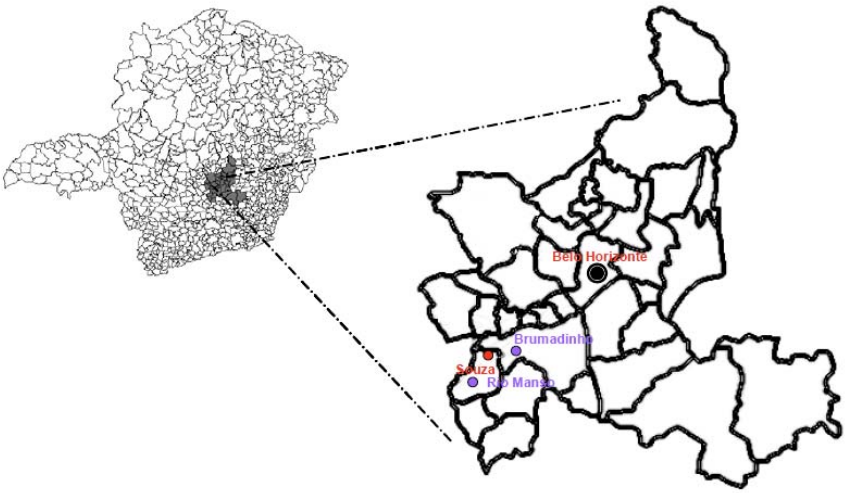


Figura 4. Mapa da região metropolitana de Belo Horizonte indicando a localização do distrito de Souza, município de Rio Manso, MG.

4.1.2 Área de transmissão endêmica

4.1.2.1 Estudo de base populacional

O estudo foi desenvolvido no assentamento agrícola de Rio Pardo (1º46'S, 1º54'S, 60º22'O, 60º10'O), localizado no município de Presidente Figueiredo, estado do Amazonas. A localidade rural de Rio Pardo fica a aproximadamente 160 km de Manaus, com acesso pela rodovia (BR-174) que liga o estado do Amazonas ao estado de Roraima (Figure 5A). O assentamento agrícola foi oficialmente criado em 1996, pelo Instituto Nacional de Colonização e Reforma Agrária (INCRA), como parte dos grandes projetos de colonização da Amazônia focados na agricultura e ocupação humana da região (deCastro et al., 2006). No assentamento de Rio Pardo, a população vive principalmente da agricultura de subsistência e pesca. A qualidade das habitações é considerada inadequada, aumentando assim a exposição ao mosquito vetor. Os serviços de saúde disponíveis são restritos, apenas um posto de diagnóstico de malária, administrado pelo município, fornece o diagnóstico e tratamento gratuito para os moradores da área. Na localidade, a temperatura média anual é de 31ºC, com clima úmido e precipitação média anual de 2.000 mm por ano. Existem duas estações bem definidas: um período chuvoso (Novembro a Maio) e um período de seca (Junho a Outubro). O assentamento é composto por 7 áreas denominadas "ramais": Principal,

Samuel, Novo Paraíso, Gusmão, Terra Preta, Taxista e Novo Progresso - que inclui as famílias assentadas de ambos os lados de vias não pavimentadas. Além disto, tem uma população ribeirinha, que vive às margens do Igarapé de Rio Pardo (Figura 5B). Nesta área, um recenseamento da população (Outubro a Setembro de 2008) identificou 701 habitantes, onde 360 (51,4%) residiam nas áreas de ramais e 341 (48,6%) no Igarapé.

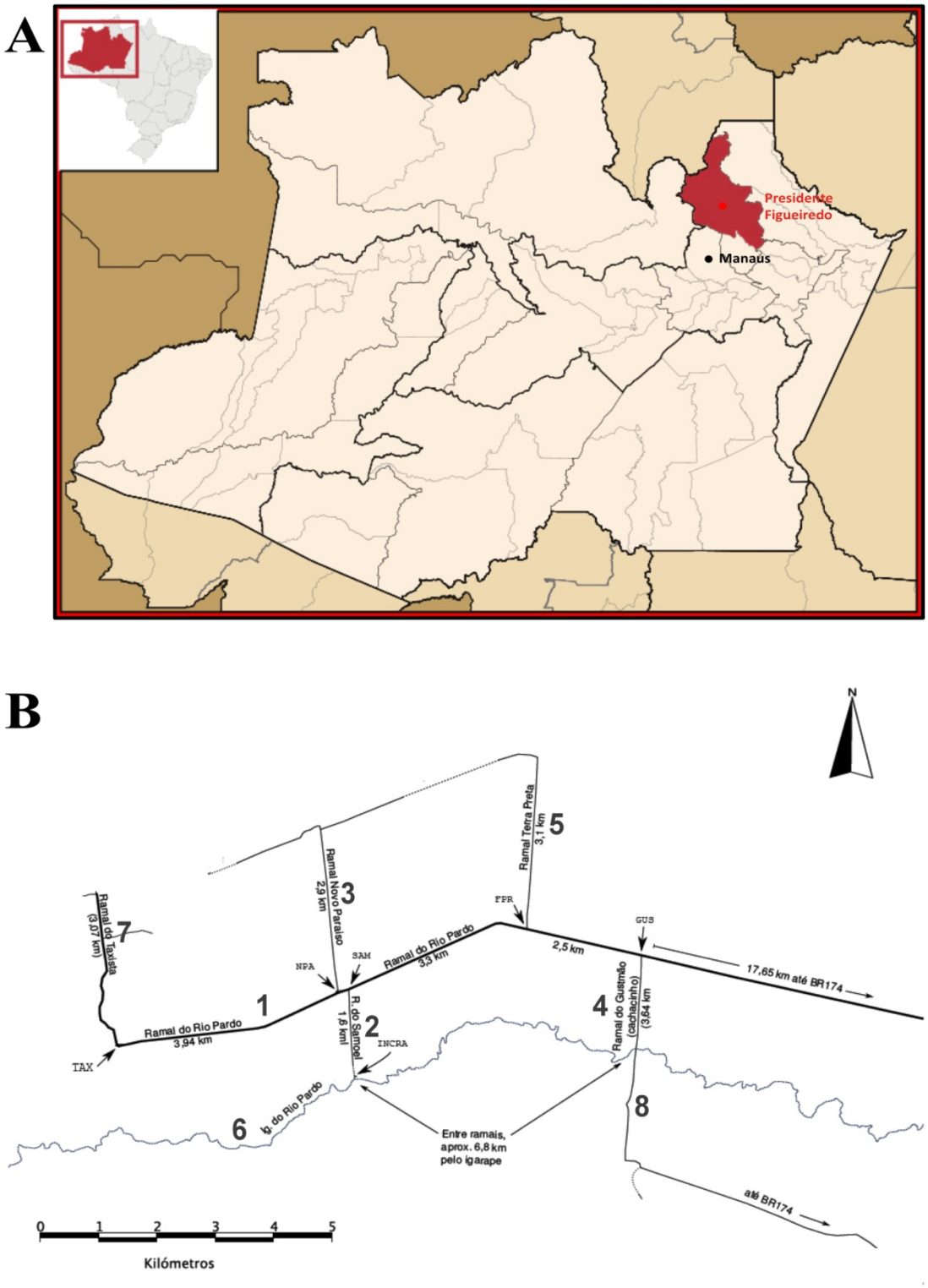


Figura 5. Mapas de localização. A - Mapa do Estado do Amazonas, indicando a localização do município de Presidente Figueiredo e a capital do Estado, Manaus. B - Mapa demonstrando o plano de assentamento agrícola de Rio Pardo. O assentamento de Rio Pardo é composto por 8 áreas denominadas Ramais: Rio Pardo ou Principal (1), Samuel (2), Novo Paraíso (3), Gusmão (4), Terra Preta (5), Taxista (7), Novo Progresso (8) e a comunidade ribeirinha do Igarapé do Rio Pardo (6).

4.1.2.2 Amostras com infecções agudas procedentes de diferentes localidades da Amazônia brasileira

As amostras de sangue periférico foram coletadas de indivíduos expostos à malária de diferentes municípios da Amazônia Legal brasileira: Acrelândia/AC, Augusto Correa/PA, Cuiabá/MT, Macapá/AP, Manaus/AM e Porto Velho/RO. As áreas de estudo são caracterizadas como regiões hipo a mesoendêmicas de malária, apresentando transmissão instável e sazonal (Brasil, 2009).

4.2 Voluntários e coleta de sangue

4.2.1 Área de transmissão epidêmica

O sangue para obtenção de plasma e de DNA foi obtido dos indivíduos de acordo com protocolos aprovados pelo comitê de ética do CPqRR (Protocolo CEPSH/CPqRR n°:002/2002 e 07/2006). Os critérios gerais de inclusão no estudo foram: (1) consentimento por escrito de acordo com as normas do Conselho Nacional de Saúde (Resolução 196/96); (2) residir no local do surto; (3) idade > 15 anos; (4) em caso do sexo feminino, um indicador de ausência de gravidez e (5) permanecer na área durante o período das subseqüentes coletas de sangue. Um total de 15 indivíduos que tiveram malária se enquadrou nos critérios de inclusão acima mencionados (média de idade 32 ± 13 anos). Foram incluídos também no estudo, 18 indivíduos, familiares e/ou vizinhos, expostos ao risco de infecção a malária (média de idade 34 ± 19 anos). De todos os indivíduos foram coletados 5 mL de sangue venoso em tubos contendo EDTA (BD Vacutainer®). No momento da coleta foram confeccionados gotas espessas e esfregaços sanguíneos. Após o primeiro corte, quatro outros cortes transversais foram realizados, com intervalo de 3, 6, 9 e 12 meses após a primeira coleta. As amostras de sangue foram utilizadas para a obtenção de plasma e extração de DNA.

4.2.2 Área de transmissão endêmica

a) Coleta de infecções agudas em diferentes localidades da Amazônia brasileira: As amostras de sangue periférico foram coletadas de indivíduos expostos à

malária de diferentes municípios da Amazônia Legal brasileira: Acrelândia/AC, Augusto Correa/PA, Cuiabá/MT, Macapá/AP, Manaus/AM e Porto Velho/RO. As áreas de estudo são caracterizadas como regiões hipo a mesoendêmicas de malária, apresentando transmissão instável e sazonal (SVS, 2008). Nessas áreas, a distribuição da malária não é homogênea, mas predominantemente focal (Atanaka-Santos et al., 2006; SVS, 2008). Nos anos de coleta das amostras, entre 2003 e 2005, o número médio de casos de malária foi de 490.000 na Amazônia Legal brasileira (SVS, 2007). As amostras de sangue sangue venoso (10ml em EDTA) foram coletadas de voluntários entre 16 e 32 anos de idade. Os aspectos éticos e metodológicos do presente estudo foi aprovado pelo Comitê de Ética em Pesquisa em Seres Humanos do Centro de Pesquisas René Rachou (CEPSH/CPqRR) / FIOCRUZ (Relatório Nº 002/2002.) de acordo com a Resolução do Conselho Nacional de Saúde - CNS 196/96.

b) Estudo de coorte na Amazônia brasileira: O sangue para obtenção de plasma e DNA foi obtido dos voluntários de acordo com protocolos aprovados pelo comitê de ética do CPqRR (Protocolo CEPSH/CPqRR No.007/2006 e No. 07/2009). Os critérios gerais de inclusão no estudo foram: (i) participação voluntária, através de consentimento por escrito; (ii) residência permanente na área de Rio Pardo. O delineamento básico do estudo envolveu uma coorte aberta, que teve seu início em Novembro de 2008 e foi encerrado em Novembro de 2009. Durante a primeira visita às áreas foram estabelecidos os seguintes procedimentos (linha de base): (i) aplicação de um questionário estruturado para todos os voluntários a fim de obter dados demográficos, epidemiológicos e clínicos; (ii) exames físicos dos indivíduos, incluindo temperatura corporal e avaliação do tamanho do fígado e do baço; (iii) busca ativa de pacientes com malária diagnosticados através de microscopia óptica (gota espessa); e (iii) coleta de sangue, sendo que dos indivíduos acima de 5 anos de idade foi coletado sangue venoso (10ml em EDTA) e das crianças com menos de 5 anos de idade, o sangue foi coletado em papel filtro no momento do diagnóstico. Dentro de um período de doze meses, após a primeira pesquisa, dois outros cortes transversais idênticos foram realizados. Durante o segundo corte (entre Maio e Junho de 2009) foram incluídos na coorte 155 indivíduos e, no terceiro corte, mais 70 indivíduos (ocorrido entre Outubro e Novembro de 2009), perfazendo um total de 766

indivíduos com 1.600 amostras. Como a maior parte da população é nativa da região Amazônica, a idade do indivíduo reflete a exposição à malária. Assim, foi estimada a exposição cumulativa do indivíduo como sendo a duração da residência em áreas endêmicas de malária (no estado do Amazonas ou em outro lugar de área endêmica) e o número de episódios prévios de malária. A infecção recente ao P. vivax foi estimada como o número de lâminas positivas para o P. vivax, registrados no posto local de diagnóstico de malária a partir dos seis meses antes da pesquisa de base (Novembro de 2008).

4.3 Extração de DNA

4.3.1 Extração de DNA a partir de sangue total

A extração do DNA genômico dos indivíduos foi realizada utilizando-se o kit QIAGEN (PUREGENE®, Gentra Systems, Minneapolis, USA) de acordo com as especificações do fabricante. Resumidamente, para cada 1mL de sangue total acrescentaram-se 3mL de solução de lise para eritrócitos em um tubo Falcon (15mL). Após lise visível (aproximadamente 10 minutos), a mistura foi centrifugada a 2000 x g, por 10 minutos, a 25ºC. O sobrenadante foi então removido e o material ressuspendido em 1mL de solução de lise celular. Nesta etapa, as amostras foram estocadas na geladeira (4ºC) e transportadas em gelo até o Laboratório de Malária/CPqRR até onde foram realizadas as etapas seguintes de extração. Nesta próxima etapa, adicionaram-se 300µL de solução de precipitação de proteína, sendo o material submetido por 30 segundos em agitador de tubos (vórtex) e centrifugado a 2000 x g por 10 minutos, a 25ºC. O sobrenadante contendo o DNA solúvel foi precipitado em um tubo contendo 1 mL de isopropanol P.A. absoluto (Merck) a 4ºC. Em seguida, o DNA foi centrifugado a 2000 x g por 3 minutos a 25ºC, sendo o sobrenadante descartado. Adicionou-se 1mL de etanol 70% (gelado) para a lavagem do DNA seguido de centrifugação a 2000 x g por 1 minuto a 25ºC. O sobrenadante foi novamente descartado e após a completa evaporação do etanol por aproximadamente 15 minutos, o DNA foi hidratado com 330µL de solução de hidratação (Tris-hidrometil aminometano, EDTA) e foi incubado por uma hora a 65ºC. O DNA extraído foi armazenado a -20ºC até o seu uso. As concentrações dos reagentes nas soluções utilizadas em cada um dos kits não são disponibilizadas pelo fabricante.

4.3.2 Extração de DNA mediante sangue total em papel de filtro

Para a extração do DNA genômico em papel de filtro utilizou-se o kit QIAGEN QIAamp® DNA mini kit (PUREGENE®, Gentra Systems, Minneapolis, USA), que apresenta grande eficiência na extração de DNA para esse tipo específico de amostra. A extração foi realizada de acordo com as especificações do fabricante. Resumidamente, um ou dois círculos de sangue em papel de filtro (aproximadamente 30 μL de sangue por círculo) foram cortados e colocados em microtubos (Eppendorf) de 1,5 mL. Foram adicionados 180 μL de tampão de lise celular ao tubo, que foi incubado a 85ºC por 10 minutos. Foram acrescentados 20 μL da solução de proteinase K e a mistura foi homogeneizada por 30 segundos em agitador de tubos (vórtex) e incubadas a 56ºC por uma hora. Foram adicionados 200 μL de tampão de lise, os tubos foram novamente homogeneizados por 30 segundos em agitador de tubos e incubados a 70ºC por 10 minutos. Em seguida, foram adicionados 200 μL de etanol P.A. (gelado) e o material foi homogeneizado. Posteriormente, todo o material (aproximadamente 500 μL de eluato) foi colocado em uma coluna QIAamp spin (agregada a um tubo de coleta de 2 mL). Os tubos foram centrifugados a 800 x g por 1 minuto a 25ºC, em seguida, os tubos contendo o filtrado foram descartados e as colunas contendo os DNAs foram colocadas em tubos novos de 2 mL. Foram adicionados 500 μL de tampão para a lavagem do DNA e o material foi centrifugado a 800 x g por 1 minuto a 25ºC. Em seguida, as colunas foram colocadas em tubos novos de 2 mL e os tubos contendo o filtrado foram descartados. Foram adicionados 500 μL de tampão para uma segunda lavagem do material e cada tubo foi centrifugado a 1500 x g por 3 minutos a 25ºC. Cada coluna foi colocada em novos microtubos do tipo eppendorf 1,5 mL e os tubos contendo o filtrado foram descartados. Foram acrescentados 150 μL de água destilada para eluir o DNA, em seguida o material foi incubado a temperatura ambiente, por 1 minuto e centrifugado a 800 x g por 1 minuto a 25ºC. Finalmente as colunas foram descartadas e o DNA foi armazenado a -20ºC até seu uso.

4.4 Diagnóstico de malária

4.4.1 Gota espessa

O diagnóstico de malária foi feito através da técnica da gota espessa de sangue corada por Giemsa em todas as amostras dos estudos (área endêmica e área epidêmica). Microscopistas bem treinados estavam encarregados de examinar o equivalente a 0,2 μL de sangue (o equivalente a 100 campos de microscopia) e estimar a densidade parasitária por microlitros de sangue. Para garantia da qualidade, uma amostra aleatória de 10% das lâminas foram analisada por um segundo microscopista treinado.

4.4.2 Diagnóstico de malária pela Reação em Cadeia da Polimerase (PCR)

Amostras de DNA foram amplificadas através da técnica de Nested-PCR descrita por Snounou e colaboradores (1993), com modificações. Este protocolo utiliza iniciadores que se anelam em uma região gênero-específicas e espécie específicas dentro da subunidade menor do RNA ribossomal, gene 18S. A Nested-PCR amplifica na primeira reação um fragmento de 1200 pb (iniciadores gênero-específica) e na segunda reação são utilizados iniciadores complementares às regiões espécie-específicas, sendo o tamanho dos fragmentos amplificados e seus iniciadores descritos na tabela 1. As reações foram realizadas em volumes de 20 μL utilizando-se: 80 ng de amostra de DNA, 250 nM de cada um dos iniciadores, 10 μL de Master Mix (PROMEGA - 0,3 U Taq DNA Polimerase, 200 μM de cada um dos dNTPs e 1,5 mM MgCl2). As amplificações foram realizadas no termociclador PTC-100™ Version7.0 - MJ Research e as condições da PCR foram, para a primeira reação: 24 ciclos de 95°C por cinco minutos, 58°C por dois minutos, 72°C por dois minutos e extensão final a 72°C por cinco minutos. A segunda reação foi realizada nas mesmas condições anteriores, porém com 29 ciclos utilizando 0,5 μL do produto da primeira reação. A visualização dos fragmentos amplificados foi feita em eletroforese em gel contendo 2% de agarose (Invitrogen) dissolvida em tampão TAE 1x (40 mM Tris-acetato, 1 mM EDTA), sendo adicionados 5 μg/mL de brometo de etídio (Invitrogen). As amostras de DNA foram misturadas em tampão de amostra (0,25% azul de bromofenol, 40% sacarose) e aplicadas em cada uma das canaletas do gel de agarose.

A corrida eletroforética foi realizada em um sistema horizontal (Bio-Rad) a 100 V, por cerca de 40 minutos. O gel foi analisado em transluminador ultravioleta (UVP - Bio-Doc it System) e arquivado em sistema digital.

Tabela 1. Sequências de iniciadores e tamanho dos fragmentos amplificados para o gene 18S ssuRNA dos parasitas da malária na Nested-PCR

Alvo	PCR	Nome dos Iniciadores	Sequência dos iniciadores (5'-3')	Tamanho do fragmento
Plasmodium sp.	1ª reação	rPLU5	CCTGTTGTTGCCTTAAACTTC	1200 pb
		rPLU6	TTAAAATTGTTGCAGTTAAAACG	
Plasmodium vivax	2ª reação	rVIV1	GTT CCT CTA AGA AGC TTT	120 pb
		rVIV2	TTAAACTGGTTTGGGAAAACCAAATATATT	
Plasmodium falciparum	2ª reação	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	205 pb
		rFAL2	ACACAATGAACTTCAATCATGACTACCCGTC	
Plasmodium malariae	2ª reação	rMAL1	ATAACATAGTTGTACGTTAAGAATACCGC	144 pb
		rMAL2	AAATTCCCATGCATAAAAAATTATACAAA	

Fonte: Snounou e colaboradores (1993)

4.4.3 PCR em tempo real para diagnóstico de malária

O protocolo de PCR em Tempo Real utilizado neste trabalho foi baseado no descrito por Mangold e colaboradores (2005), com algumas modificações realizadas pelo nosso grupo. O protocolo se baseia na detecção das quatro espécies de plasmódios humanos com a utilização de apenas um par de iniciadores consenso, desenhado para uma região gênero-específica da subunidade menor do RNA ribossomal do gene 18S. Tais iniciadores amplificam fragmentos que variam no número de nucleotídeos e esses diferentes tamanhos são analisados e discriminados por meio de curvas de dissociação (figura 6), que permitem a diferenciação das espécies de plasmódios. As reações foram realizadas em volumes de 20 µL, utilizando 100 a 200ng de amostra de DNA, 0,5 mM de cada iniciador, 2,5mM de MgSO4 e 10 µL de SYBR Green PCR master mix (Applied Biosystems, Califórnia, USA). As amplificações e a detecção da fluorescência foram conduzidas no ABI

PRISM® 7000 Sequence Detection System (Applied Biosystems). As condições da PCR foram um ciclo de 95ºC por 10 minutos, 40 ciclos de 90ºC por 20 segundos, 50ºC por 30 segundos e 60ºC por 30 segundos. As sequências dos iniciadores utilizadas nesta reação estão especificados na tabela 2. Em todos os ensaios de PCR foram utilizados controles positivos e negativos nas reações. Como controles negativos foram utilizadas amostras de DNA de indivíduos de área livre de transmissão, sabidamente negativos para malária. Como controles positivos foram utilizados: (i) DNA de *P. falciparum*, proveniente de cepa 3D7 de cultivo contínuo mantido no Laboratório de Malária (CPqRR-FIOCRUZ); (ii) DNA de *P. vivax*, proveniente de indivíduos de áreas endêmicas de malária com infecção aguda e parasitemia confirmada pela microscopia óptica; (iii) DNA de *P. malariae*, proveniente do Banco Internacional de Reagentes de Referência de Malária (www.mr4.org). Após a amplificação, os resultados foram observados através de curvas de dissociação resultantes da medição contínua de fluorescência a 530 nm, onde a temperatura foi aumentada gradativamente de 60ºC a 95°C. Os picos de fusão de cada fragmento amplificado foram visualizados plotando a derivada negativa da fluorescência em função da temperatura versus a temperatura (-dF/dTº vs Tº).

Tabela 2. Sequência dos iniciadores utilizados para a reação de PCR em tempo real.

Nome dos Iniciadores	Região	Sequência dos iniciadores	Região
P1	5'	TAA CGA ACG AGA TCT TAA	3'
P2	5'	GTT CCT CTA AGA AGC TTT	3'

Fonte: Mangold e colaboradores (2005).

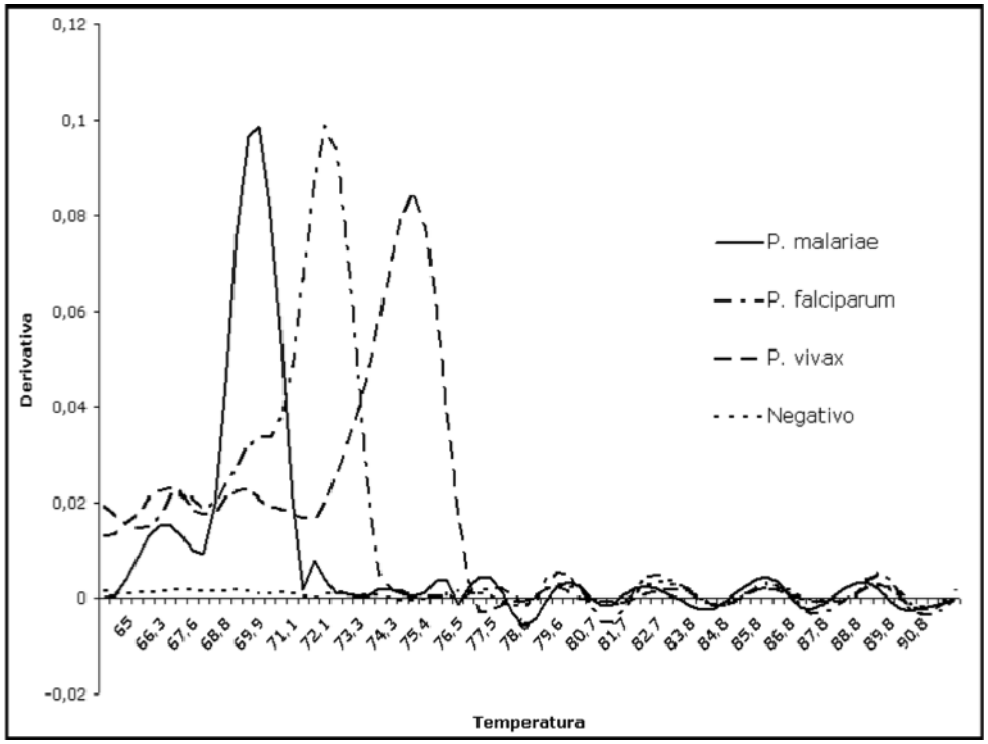


Figura 6. Curvas de dissociação obtidas por PCR em tempo real. As curvas foram obtidas a partir de DNA de cultura de P. falciparum, DNA de pacientes infectados com P. vivax e DNA de P. malariae cedido gentilmente pelo MR4 (*Malaria Research and Reference Reagent Resource Center*).

4.5 Determinação do grupo sanguíneo DARC

4.5.1 Fenotipagem do receptor DARC

O sistema ID-Card FYA/FYB (DiaMed AG, Cressier sur Morat, Switzerland) foi utilizado para a fenotipagem de DARC de acordo com as instruções do fabricante. Brevemente, uma suspensão de hemácias 0,8% foi preparada adicionando-se 1mL de solução ID-Diluent 2 com 25mL de sangue total fresco em um tubo de 1,5mL. Os anticorpos policlonais humanos anti-Fya e anti-Fyb foram adicionados aos ID-Cartões Fya e Fyb, respectivamente, seguido pela adição de 50mL da suspensão de hemácias aos microtubos de cada ID-Cartão. Após incubação por 15min a 37°C, os ID-Cartões foram centrifugados a 1030 rpm por 10min a 25°C em centrífuga ID-Centrifuge 12 S II (DiaMed AG). Os resultados positivos foram identificados através de um teste de hemoaglutinação.

4.5.2 Genotipagem do Receptor DARC pela Reação em Cadeia da Polimerase Alelo-Específica (PCR-ASP)

A amplificação dos alelos *FY*A, FY*B, FY*A*^{ES} e *FY*B*^{ES} foi realizada através da reação em cadeia da polimerase, utilizando iniciadores alelo-específicos (Tabela 3) descritos por Olsson e colaboradores (1998). Os pares de iniciadores FyAB2/FyAREV, FyAB2/FyBREV2, GATAFy2/FyAREV e GATAFy2/FyBREV2 foram utilizados para detecção dos alelos *FY*A, FY*B, FY*A*^{ES} e *FY*B*^{ES}, respectivamente. Para um volume final de reação de 25µL foi utilizado 200ng de DNA genômico, 0,2µM de cada iniciador, 200µM dNTPs, 4mM MgCl2, 1 U Taq DNA polimerase (Promega Corporation, Wisconsin, USA) e o tampão 1X fornecido com a enzima. As amplificações foram conduzidas em termociclador automático PTC100TM Programmable Thermal Controler e as condições da PCR foram: um ciclo de 5min a 95°C; quatro ciclos de 1min a 95°C, 1min a 69°C e 1min a 72°C; 31 ciclos de 1min a 95°C, 1min a 68°C e 1min a 72°C e uma extensão final de 5min a 72°C.

Tabela 3. Relação dos iniciadores utilizados na genotipagem do receptor DARC por PCR alelo-específica (PCR-ASP).

Nome dos Iniciadores	Região	Sequência dos iniciadores	Região
FyAB2	5'	CTCATTAGTCCTTGGCTCTTAT	3'
FyAREV	5'	AGCTGCTTCCAGGTTGGCAC	3'
FyBREV2	5'	AGCTGCTTCCAGGTTGGCAT	3'
GATAFy2	5'	CTCATTAGTCCTTGGCTCTTAC	3'

Fonte: Olsson e colaboradores (1998).

4.5.3 Genotipagem do antígeno de grupo sangüíneo DARC pela Reação em Cadeia da Polimerase em tempo real

As amostras foram processadas em placas óticas de 96 poços (Applied Biosystems, Foster City, CA, USA). O volume final da reação foi de 20 µL contendo 50-100 ng de DNA genômico, 5 µL SYBR® Green PCR master mix (Biosystems), e

10 pmoles de cada primer (Biosystems). A amplificação e a fluorescência foram detectadas utilizando ABI Prism® 7000 Sequence Detection System (Applied Biosystems). As condições da PCR foram: um ciclo de 95ºC por 10 minutos, seguido de 35 ciclos de 95ºC por 15 segundos e 60ºC por 1 minuto. Após a amplificação, foi realizado um ciclo de dissociação com variação crescente de temperatura (60ºC a 95ºC).

Os iniciadores foram desenhados através do programa Primer Express® v.2.0 (Applied Biosystems). Os pares de iniciadores FY/RYA e FAB/RABGATA foram utilizados na mesma reação, como uma reação de PCR multiplex, bem como os pares FY/RYB e FGATA/RABGATA. Os iniciadores RYA e RYB apresentam o polimorfismo G125A na extremidade 3' e juntamente com o iniciador senso FY amplificam os alelos *FY*A* e *FY*B*, respectivamente. Para detecção da mutação T-33C na região promotora do gene FY, foram desenhados os iniciadores FAB e FGATA, que amplificam juntamente com o iniciador RABGATA, o promotor selvagem e mutado, respectivamente. Como os iniciadores FAB/FGATA e RYA/RYB são semelhantes, foi introduzida uma alteração no nucleotídeo (sublinhado na tabela 2) imediatamente anterior ao nucleotídeo polimórfico (em negrito na tabela 2), com o objetivo de impedir a amplificação inespecífica dos alelos. A alteração impede o pareamento nucleotídico na extremidade 3' entre o iniciador não-específico e a fita molde de DNA. Uma segunda modificação no iniciador FGATA foi realizada a partir da adição de uma cauda C/G na região 5' do iniciador, com a finalidade de aumentar a diferença de tamanho e temperatura de dissociação dos produtos da PCR amplificados com o par de iniciadores FAB e FGATA.

Tabela 4. Sequência dos iniciadores utilizados para a reação de genotipagem de DARC por PCR em tempo real.

Nome dos Iniciadores	Região	Sequência dos iniciadores	Região
FGATA	5'	CCCGGGCCCGCCG CCC TCA TTA GTC GGC TCT TTC	3'
FAB	5'	CCC TCA TTA GTC GGC TCT TTT	3'
RABAGATA	5'	A GGG GCA TAG GGA TAA GGG ACT	3'
FY	5'	C TCA AGT CAG CTG GAC TTC GAA GAT	3'
RYA	5'	AC CTG CTT CCA GGT TGG CTC	3'
RYB	5'	CTG CTT CCA GGT TGG CGT	3'

4.6 Amplificação por PCR dos genes codificadores da região II da DBP

Visando avaliar a variabilidade genética da região II da PvDBP, as amostras de DNA extraídas foram utilizadas no PCR para amplificar o fragmento correspondente da DBPII (Souza et al., 2006). Após a amplificação dos fragmentos, os amplicons foram purificados conforme instruções do fabricante (kit QIAquick PCR purification - Qiagen). Seguindo a purificação, as amostras foram amplificadas com iniciadores marcados e, posteriormente, a reação precipitada para o seqüenciamento. O seqüenciamento das amostras foi realizado no seqüenciador automático de DNA MegaBace 500 (Amersham Biosciences). A análise das seqüências de nucleotídeos obtidas dos isolados do município de Souza foram analisadas pelo programa Bioedit (Biological Sequence Alignment Editor for Windows, Íbis Therapeutics, Carlsbad, CA) e alinhadas pelo programa Clustal W (EMBL-EBI, European Bioinformatics Institute, Cambridge, UK). Para identificar os polimorfismo no domínio ligante da DBPII, foram utilizados como referência as seqüências da cepa de referência Sal-1 (Fang et al., 1991) e de dois isolados P. vivax da PNG (VanBuskirk et al., 2004a).

4.7 Plasmídeos DBPII-pEGFP

As regiões II da cepa de referência Sal-1 e dos dois isolados da Papua Nova Guiné (PNG-7.18 e PNG-27.16) foram previamente clonadas no plasmídeo pEGFP-N1 (Clontech, Mountain View, CA) (Chitnis & Miller, 1994). O vetor possui um peptídeo sinal da glicoproteína de membrana D1 (HSVgD1), de herpes simplex vírus, capaz de expressar a proteína a ser clonada no vetor na superfície das células transfectadas. Além disso, o vetor possui uma região promotora que controla a expressão de uma proteína de fluorescência verde (GFP) usada como marcador de transfecção (Michon et al., 2000). Adicionalmente, foram construídos dois plasmídeos contendo as seqüências da região II da PvDBP dos parasitos isolados de pacientes do surto de Souza. As seqüências foram clonadas em novos plasmídeos pEGFP_HSVgD1, os quais foram gentilmente cedido pelo Dr. John Adams (University of South Florida, Florida, USA). O fragmento correspondente aos aminoácidos entre as posições 198 a 522 foram clonados no vetor. Para a clonagem foram usados um par de iniciadores para amplificar a região II da DBP e inserir sítios para enzimas de restrição, por PCR. Os iniciadores foram: 5' - ACT AGT GGG CCC TGT CAC AAC TTC CTG AGT - 3' e 5' - GCG GAA TTC ACG ATC TCT AGT GCT ATT - 3', continham sítios para ApaI e EcoRI, respectivamente (Chitnis & Miller, 1994). Após a reação de PCR, os amplicons e o vetor foram digeridos seqüencialmente com as endonucleases EcoRI e ApaI,. Os insertos e os vetores pEGFP–HSVgD1 digeridos foram unidos através de T4 DNA ligase (New England Biolabs, UK). Os plasmídeos recombinantes foram purificados conforme instruções do fabricante (endotoxin free plasmid DNA purification system - Qiagen,Valencia, CA, USA).

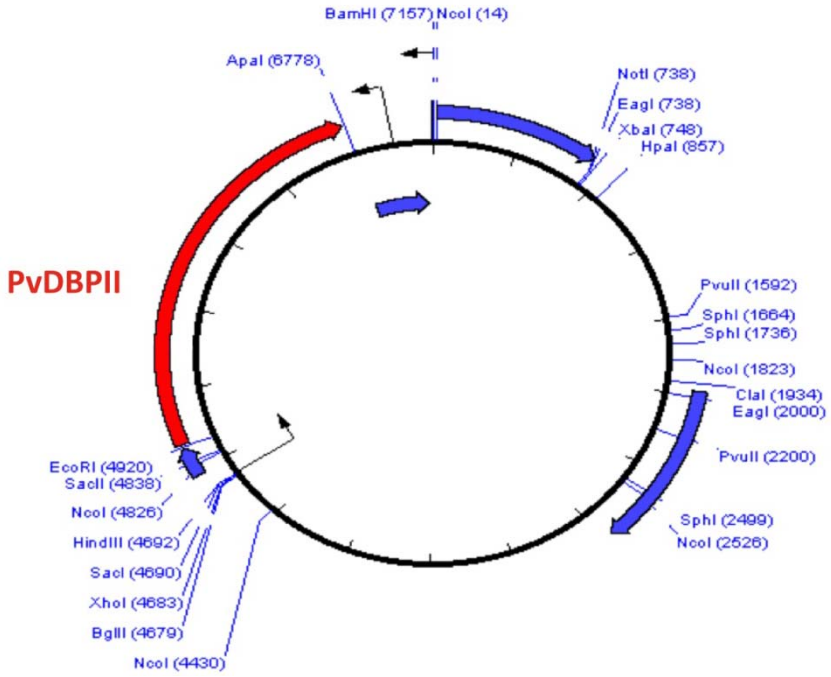


Figura 7. Representação do vetor pEGFP_HSvGD1 com o inserto PvDBPII. A seta em vermelho representa o inserto PvDBPII, com aproximadamente 2000 pb, sendo flanqueada pelos sítios de restrição das enzimas EcoRI e ApaI.

4.8 Transfecção de células COS-7 e ensaios de citoaderência

Para os ensaios de inibição da interação ligante-receptor foram utilizadas células COS-7 (American Type Culture Collection, ATCC, Manassas, VA), as quais foram transfectadas com os plasmídeos recombinantes que possuíam a região II da PvDBP de diferentes variantes. Para a transfecção, utilizou-se lipofectamina (Invitrogen Life Technologies, Carlsbad, CA) e "Plus-Reagent" (Invitrogen Life Technologies, Carlsbad, CA), como descrito por Michon et al. (2000) com modificações. Brevemente, as células COS-7 cultivadas em garrafas de cultura (75 cm2), foram transferidas para placas de seis poços (1,5 x 105 células/mL) e transfectadas com a suspensão de plasmídeos e complexos de lipossomos (5% de Plus-Reagent e 3% lipofectamina). A suspensão de cada plasmídeo com o complexo de lipossomos foi feita em meio de cultura DMEM (Gibco-BRL Life Technologies, Rockville, MD) sem soro bovino fetal (SBF). Após um período de incubação de 6 horas, das células com o complexo formado entre os lipossomos e cada um dos plasmídeos (37°C, 5% de CO_2 e 95% de umidade), o meio de transfecção foi substituído por meio DMEM contendo 10% SBF (Gibco), 2mM de L-glutamina

(Gibco), 25mM de Hepes (Sigma-Aldrich), 25mM de bicarbonato de sódio (Merck, Darmstadt, Germany), 100UI/mL de penicilina e 100µg/mL de estreptomicina (Gibco). Após 24 horas, seguido o processo de transfecção, o meio de cultura foi novamente substituído por meio fresco (DMEM 5%). No dia seguinte, os soros ou plasmas a serem testados, foram adicionados às placas, sendo essas incubadas por 1h a 37°C e 5% de CO_2. Após a incubação das células com o soro ou o plasma, uma suspensão de eritrócitos humanos O^+ e DARC positivos, foram diluídos em meio de cultura a uma concentração de 10% e adicionados às células (200µL/poço). Juntamente com os eritrócitos, as células foram incubadas por 2h a temperatura ambiente. Eritrócitos não-aderidos foram removidos por três lavagens com DMEM incompleto (sem SBF). Para avaliar quantitativamente o processo de interação ligante-receptor, as rosetas formadas pela ligação dos eritrócitos às células COS-7 transfectadas foram contadas em um microscópio de fluorescência invertido e com contraste de fase (20 campos/poço, com aumento de 200x) (Nikon, Melville, NY). Para cada ensaio de citoaderência, um pool de plasma de indivíduos não-respondedores a PvDBP no ELISA foram utilizados como controle negativo (100% de ligação). Rosetas positivas foram definidas aquelas que apresentavam mais que 50% de da superfície celular coberta por eritrócitos (figura 8). Os resultados foram expressos como porcentagem relativa de inibição (Ceravolo et al., 2008).

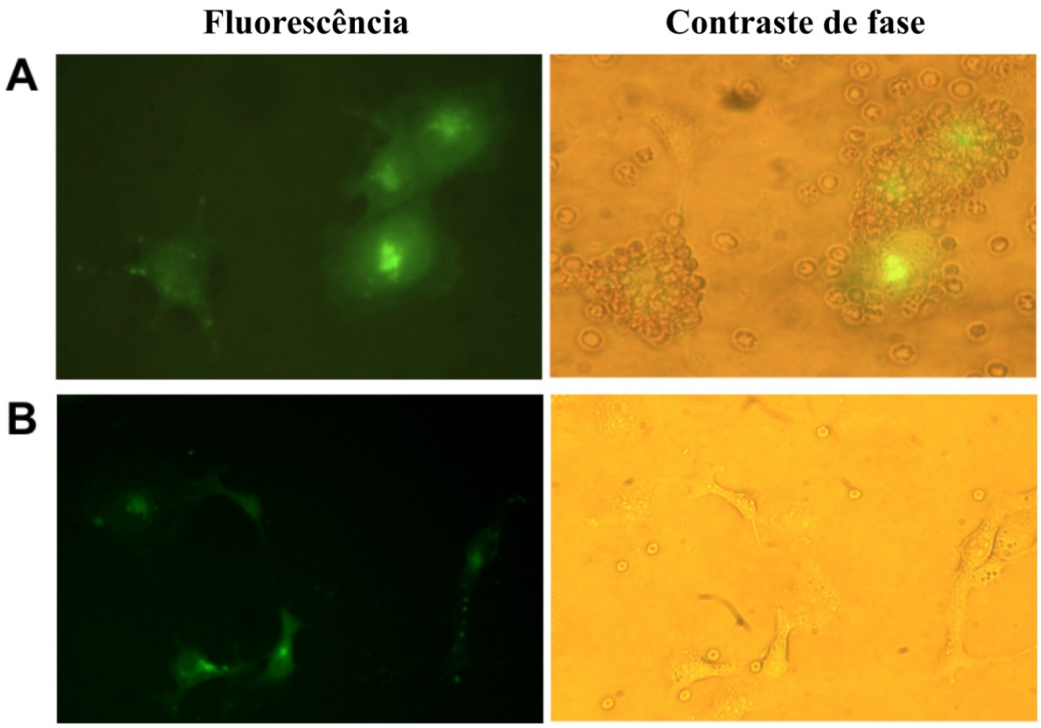


Figura 8. Ensaio de citoaderência ilustrando as células COS-7 expressando a PvDBPII. (A) A figura mostra a presença de eritrócitos aderidos às células COS-7 transfectadas (rosetas) através da ligação Duffy/DARC. (B) A figura ilustra a ausência de ligação entre eritrócitos e as células COS-7 transfectadas em função da presença de anticorpos inibitórios no soro. Visualização em aumento de 200x (Microscópio de fluorescência invertido e com contraste de fase, Nikon, Melville, NY).

4.9 Proteínas recombinantes

Foram utilizadas no estudo de resposta imune duas proteínas recombinantes de P. vivax a fim de detectar anticorpos. A porção de 19 kDa proteína recombinante merozoite surface protein-1 de formas sanguíneas do P. vivax (MSP1-19), foi gentilmente cedida Dra. Irene Soares (Instituto de Ciências Biológicas, ICB, Universidade de São Paulo, Brasil). Para a expressão da proteína recombinante PvDBP, foi utilizado o plasmídeo pGEX-2T contendo as seqüências codificadoras da região II a IV (aa-177 a 815) da PvDBP, gentilmente cedido pelo Dr. John Adams (University of South Florida, Florida, USA). O protocolo foi descrito por Ceravolo et al., 2005.

4.10 Ensaios de ELISA

Para avaliar os anticorpos IgG totais contra as proteínas recombinantes PvDBP II–IV e MSP1-19, foram realizados ensaios de ELISA (*Enzyme-Linked Immunosorbent Assay*) conforme o protocolo bem estabelecido pelo grupo (Carvalho et al., 1997). Resumidamente, as placas de 96 poços (Maxysorp, Nunc, Denmark) foram sensibilizadas com 5 μg/mL de PvDBP II-IV ou 1 μg/mL de PvMSP1-19, diluídas em PBS 1X (formula), durante 18h a 4°C. Após a sensibilização as placas foram lavadas por três vezes com PBS contendo 0,05% de tween 20 (Sigma-Aldrich). Para o bloqueio, foram adicionados 200 μL de PBS-tween 20 com 5% de leite em pó desnatado. Após 1h de bloqueio a 37°C as placas foram lavadas três vezes com PBS-tween 20. Para cada poço, 100 μL dos soros-testes foram diluídos a 1:100 (PvDBPII-IV) ou 1:80 (PvMSP-119) em tampão PBS-tween 20 com 1,5% de leite em pó (duplicatas) e incubados a 37ºC por 1 hora. Após incubação, as placas foram lavadas por dez vezes e incubadas, novamente a 37ºC por 1 hora, com 100 μL/poço do conjugado anti-IgG humano ligada a peroxidase (específica para cadeia γ, Sigma-Aldrich) na diluição de 1:1.000. Após a lavagem, as placas foram reveladas acrescentando-se 100 μL/poço de solução contendo OPD (10 mg/tablete) (Sigma-Aldrich) diluído em 50 mL de tampão citrato de sódio (0,1M pH 5,0) na presença de 40 μL de peróxido de hidrogênio (H202) a 30% (Sigma-Aldrich). A reação foi interrompida após 15 minutos pela adição de 50 μL de uma solução de ácido sulfúrico 4N. A leitura das placas foi realizada em um leitor automático de ELISA (Stat Fax-2.100, Awareness Tecnology, Palm City, FL), onde a densidade ótica (DO) foi medida em um comprimento de onda de 492 nm. A DO obtida utilizando o antígeno controle (GST) foi subtraída da DO do antígeno-teste (PvDBPII-IV ou PvMSP-119), para que a DO específica fosse determinada.

4.11 Análise estatística

As análises estatísticas foram realizadas nos programas Epi-Info 2002 (CDC, Atlanta, GA, USA) e EpiData (Data Management and basic Statistical Analysis System, Odense Denmark). As diferenças entre as médias foram testadas utilizando o teste T de Student ou análise de variância. A significância estatística foi analisada pelo teste do qui-quadrado com correção de Yates ou pelo teste de Fisher's, sendo

considerado como significativo valores de $P < 0,05$. Foi avaliado o risco relativo (e respectivo intervalo de confiança 95%) para medir a força de associação entre os diferentes genótipos e a incidência de malária.

5 RESULTADOS

Os resultados estão descritos nos artigos publicados ou submetidos à publicação em revistas indexadas e são apresentados na seguinte ordem:

5.1 Sousa TN, Sanchez BA, Cerávolo IP, Carvalho LH, Brito CF. Real-time multiplex allele-specific polymerase chain reaction for genotyping of the Duffy antigen, the Plasmodium vivax invasion receptor. Vox Sang 2007 May; 92(4): 373-80.

5.2 Sanchez BAM, Kano FS, Torres LM, Fontes CJF, Nogueira P, Soares IS, Sousa TS, Rocha RS, Brito CFA, Carvalhio LH. Amazonian community-based study to evaluate host genotype for Duffy antigen receptor for chemokines (DARC) and immune response against the *Plasmodium vivax* Duffy binding protein (PvDBP). **Artigo submetido 2011**.

5.3 Ceravolo IP, Sanchez BA, Sousa TN, Guerra BM, Soares IS, Braga EM, McHenry AM, Adams JH, Brito CF, Carvalho LH. Naturally acquired inhibitory antibodies to Plasmodium vivax Duffy binding protein are short-lived and allele-specific following a single malaria infection. Clin Exp Immunol 2009 Jun; 156(3): 502-10.

Vox Sanguinis (2007) **92**, 373–380

DOI: 10.1111/j.1423-0410.2007.00902.x

ORIGINAL PAPER

Real-time multiplex allele-specific polymerase chain reaction for genotyping of the Duffy antigen, the *Plasmodium vivax* invasion receptor

T. N. Sousa, B. A. M. Sanchez, I. P. Cerávolo, L. H. Carvalho & C. F. A. Brito

Laboratory of Malaria, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG, Brazil

Vox Sanguinis

Background and Objectives Duffy blood group is of major interest in clinical medicine as it is not only involved in blood-transfusion risks and occasionally in neonatal haemolytic disease, but it is also the receptor for the human malaria parasite *Plasmodium vivax* in the erythrocyte invasion. The aim of this study was to develop a rapid and inexpensive approach for high-throughput Duffy genotyping.

Materials and methods This paper reported the development of a Duffy genotyping assay based on multiplex real-time polymerase chain reaction (PCR) using SYBR Green I fluorescent dye.

Results By using this approach for Duffy genotyping we obtained the same results as that for the conventional allele-specific PCR, however, in a high-throughput assay. The Duffy genotyping of field samples demonstrated that *P. vivax*-infected individuals showed a significantly higher prevalence of two functional alleles than *Plasmodium falciparum*-infected and non-infected individuals. This finding corroborates the hypothesis that the presence of two functional alleles increases the risk of *P. vivax* infection.

Conclusion This methodology may be suitable for epidemiological studies, particularly for exploring the relationship between Duffy alleles and malaria susceptibility, and also for identification of transfusional incompatibility in blood banks.

Received: 14 September 2006, revised 28 December 2006, accepted 8 January 2007, published online 15 March 2007

Key words: DARC, Duffy blood group, malaria, *Plasmodium vivax*, real-time PCR, SYBR green fluorescent dye.

Introduction

The Duffy blood group, also known as Duffy antigen receptor for chemokines (DARC), is of biological and clinical importance, as antibodies against Duffy antigens are responsible for some cases of transfusion incompatibility and haemolytic disease of the newborn [1,2]. Furthermore, besides being a receptor for various chemokines, the Duffy antigen is the obligatory receptor for invasion of *Plasmodium vivax* malaria parasite [3,4].

The Duffy antigen was first reported in a polytransfused haemophiliac patient, who had an alloantibody against an antigen denoted as Fy^a [5]. The antithetical antigen, Fy^b, was described 1 year later [6]. Fy^a and Fy^b antigens are the products of two codominant alleles *FY*A* and *FY*B* on the chromosome 1 *FY* locus [7]. The single copy *FY* gene is composed of two exons, which encodes for a major product of 336 amino acids [8]. The two common alleles in Caucasians, *FY*A* and *FY*B*, differ by a single base substitution (125G>A) resulting in the replacement of a glycine with an aspartic acid at residue 42 in the extracellular domain of Duffy antigen [9,10].

T. N. Souza and B. A. M. Sanchez contributed equally to this work.
Correspondence: Cristiana Ferreira Alves de Brito, Laboratory of Malaria, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Avenida Augusto de Lima, 1715, Belo Horizonte, Brazil, 30190-002
E-mail: cristiana@cpqrr.fiocruz.br

Most West Africans and 68% of African Americans do not express Fy^a or Fy^b on their red blood cells (RBC), which results in resistance to *P. vivax* infection, because Duffy-negative RBCs cannot be invaded by this parasite [11,12]. The total absence of Fy^a and Fy^b on erythrocytes designate the Duffy-negative phenotype, conveyed by homozygosity for the $FY*B^{ES}$ allele (ES stands for erythroid silent) [13]. The $FY*B^{ES}$ allele differs from $FY*B$ allele by one substitution, −33T>C on the gene promoter [14]. This mutation disrupted a binding site for the erythroid transcription factor, GATA1, resulting in a lack of *FY* gene expression only in the erythroid lineage [15]. Recently, the same mutation was described for a new and rare allele also responsible for Duffy antigen negativity in erythroid lineage – $FY*A^{ES}$ – in a *P. vivax*-endemic region of Papua New Guinea; all individuals with this allele were heterozygous for $FY*A$ allele [16]. Other variations that cause reduction or totally abrogate the expression of the Duffy gene have also been described but only in very low prevalences [17–19].

The molecular characterization of *FY* alleles has led to the development of Duffy genotyping by polymerase chain reaction (PCR)-based approaches. The original method was based on PCR coupled to restriction fragment length polymorphism (PCR-RFLP) [20]. Later, Olsson *et al.* [21] described a technique based on allele-specific primers (PCR-ASP) using a heat-activated DNA polymerase. More recently, a real-time PCR protocol had also been described using the LightCycler® based on adjacent hybridization of labelled probes [22]. Here, we developed a less expensive assay by using an intercalating fluorescent dye, SYBR Green by real-time multiplex allele-specific PCR to identify the three major Duffy alleles: $FY*A$, $FY*B$, and $FY*B^{ES}$. In order to validate our assay, we analyse Duffy alleles from *P. vivax*-infected, *P. falciparum*-infected, and non-infected individuals from Brazilian endemic areas.

Materials and methods

Blood samples and DNA extraction

Peripheral blood samples were collected from 252 donors: (i) 76 from our Research Institute, including five Duffy-negative individuals; and (ii) 176 field samples from Brazilian malaria endemic areas, which included 119 *P. vivax*-infected, 29 *P. falciparum*-infected, and 28 non-infected individuals. Blood was collected from these consenting volunteers as 5 ml samples in ethylenediaminetetraacetic acid. All individuals were adults of 16–72 years old, mean age of 32. DNA was extracted from whole blood samples by using the PureGene DNA olation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

The ethical and methodological aspects of this study were approved by the Ethical Committee of Research on Human Beings from the Centro de Pesquisas René Rachou/FIOCRUZ (Report no. 002/2002), according to the Resolution of the Brazilian Council on Health – CNS 196/96.

Duffy phenotyping

The antithetical Fy^a and Fy^b antigens were determined from fresh blood samples by agglutination tests with anti-Fy^a and anti-Fy^b human polyclonal antibodies using ID cards, according to the manufacturer's instructions (DiaMed AG, Cressier sur Morat, Switzerland).

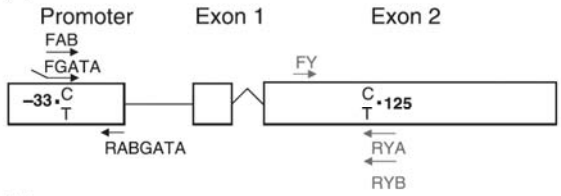
PCR-ASP

The amplification of major *FY* alleles was performed using allele-specific primers (BioSynthesis, Heiden, Switzerland) as previously described [21]. PCR-ASP protocol was modified here to replace heat-activated polymerase with conventional *Taq* DNA polymerase. For each PCR reaction we used 200 ng genomic DNA, 0·2 μM of each primer, 200 μM dNTPs, 4 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Promega Corporation, Madison, WI, USA), in a final volume of 25 μl. PTC-100™ Thermal cycler (MJ Research Inc., Watertown, MA, USA) was programmed as follows: 95 °C for 5 min, followed by four cycles of 95 °C for 1 min, 69 °C for 1 min, and 72 °C for 1 min; and 31 cycles of 95 °C for 1 min, 68 °C for 1 min, and 72 °C for 1 min, with a 5 min final extension of 72 °C. PCR products were analysed in ethidium bromide-stained agarose gels. The PCR programme takes approximately 2 h and the post-PCR analysis, such as gel preparation and visualization, takes another 1 h.

Real-time PCR

Real-time PCRs were carried out in 96-well 0·2 ml thin-wall optical PCR plates with optical sealing tapes (Applied Biosystems, Foster City, CA, USA). The 10 μl reaction mixture contained 50–100 ng genomic DNA, 5 μl SYBR® Green PCR master mix (Applied Biosystems), and 10 pmole of each primer (BioSynthesis). The primers were designed using Primer express® software version 2·0 (Applied Biosystems) with modifications that are described in the Results. Amplification and fluorescence detection were performed using ABI Prism® 7000 Sequence Detection System (Applied Biosystems). The PCR amplification profile was a cycle of 95 °C for 10 min, following by 35 cycles of 95 °C for 15 s and 60 °C for 1 min. After amplification, melting curves were observed from the dissociation curve resulting from continuous measurements of fluorescence (F) at 530 nm during which the temperature was gradually increased from 60 to 95 °C. Melting peaks of each amplified fragment were visualized by plotting the negative derivative of the fluorescence with respect to temperature against temperature ($-dF/dT°$ vs. $T°$). The real-time PCR, including sample preparation, PCR

(a)



(b)

```
FGATA     5′ CCCGGGCCCGCCG CCC TCA TTA GTC CTT GGC TCT TTC 3′
FAB                   5′ CCC TCA TTA GTC CTT GGC TCT TTT 3′
RABGATA                 5′ A GGG GCA TAG GGA TAA GGG ACT 3′
FY                    5′ C TCA AGT CAG CTG GAC TTC GAA GAT 3′
RYA                       5′ AG CTG CTT CCA GGT TGG CTC 3′
RYB                          5′ CTG CTT CCA GGT TGG CGT 3′
```

(c)

	Alleles					
Primer-Pair	*FY*A*	*FY*B*	*FY*B^{ES}*	*FY*A^{ES}*	T_m(°C)	A_L(bp)
FAB/RABGATA	+	+	–	–	**86**	**157**
FGATA/RABGATA	–	–	+	+	88	170
FY/RYA	+	–	–	+	**78**	**97**
FY/RYB	–	+	+	–	77	95

Fig. 1 Schematic diagram of primers for Duffy genotyping by real-time PCR. (a) Schematic representation of *FY* gene showing FAB/RABGATA and FGATA/RABGATA primer-pairs to detect the polymorphism −33T>C in gene promoter, amplifying the wild-type and mutated promoter, respectively. FY/RYA and FY/RYB primer-pairs to detect the polymorphism 125G>A in exon 2, which determines *FY*A* and *FY*B* alleles, respectively. (b) List of primer sequences showing the added C/G tail in italic, deliberated mismatches introduced underlined and polymorphic nucleotides in bold. (c) Table showing primer-pairs, alleles amplified (plus symbol), observed melting temperature of each amplicon (T_m) and amplicon length in base-pairs (A_L). Bold indicates that primer pairs were used together in a multiplex reaction.

program, and analysis of results takes 2 h. Forty-eight samples were analysed per plate.

Statistical analysis

Statistical analysis was undertaken using χ^2 analysis Mantel-Haenszel corrected and Fischer 2-tailed for groups with less than five samples. All analyses were performed on Epi Info statistical software, version 3·3·2 [23].

Results

Primers design and real-time PCR optimization

Four sets of primers were designed for the genotyping of the major Duffy alleles (Fig. 1). For detection of the polymorphism to distinguish the *FY*A* from *FY*B* alleles, we designed RYA and RYB primers, with the polymorphic nucleotide in their extremely 3′-end, being C and T, respectively. These primers were used with FY forward primer. In order to detect the Duffy promoter mutation, we designed primers FAB (wild-type promoter – WTp) and FGATA (mutated promoter – MUTp), with the polymorphic nucleotide in their extremely 3′-end, C and T, respectively. Each of these primers was used with RABGATA reverse primer. Because of significant homology between allele-specific primers, we introduced a deliberate mismatch between primers and template in the nucleotide exactly before the polymorphic site to prevent amplification of the non-matching allele (Fig. 1b). We added a C/G tail in the 5′-end of FGATA primer, to further enhance differences in both size and melting temperatures from the FAB primer (Fig. 1c). Because of different melting temperature of amplicons, we optimized the use of FY/RYA and FAB/RABGATA primer-pairs as a multiplex reaction in one tube, as well as FY/RYB and FGATA/RABGATA, which were used in another tube (Fig. 1c). Representative graphics of the results obtained for each genotype were shown in Fig. 2. There is an alternative genotype *FY*A^{ES}/FY*B* for the results showed on panel B (Fig. 2).

Comparison of real-time PCR and PCR using allele-specific primers

To measure the accuracy of real-time PCR, we previously identified 76 samples from ours Research Institute by Duffy phenotyping, after which these samples were codified and the Duffy genotyping performed by using PCR-ASP and real-time PCR. Our results of Duffy genotyping on real-time PCR totally agree with those results obtained by using PCR-ASP (Table 1).

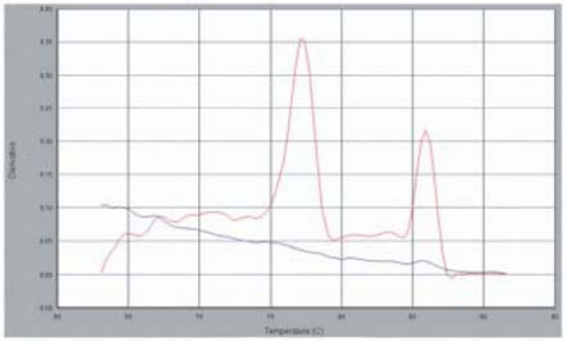
Comparative costs

Table 2 shows the comparison of costs to perform the different protocols described for the Duffy genotyping. Excluding the equipment, our methodology implies in the lowest final cost for Duffy genotyping.

Analysis of Duffy genotyping among field populations

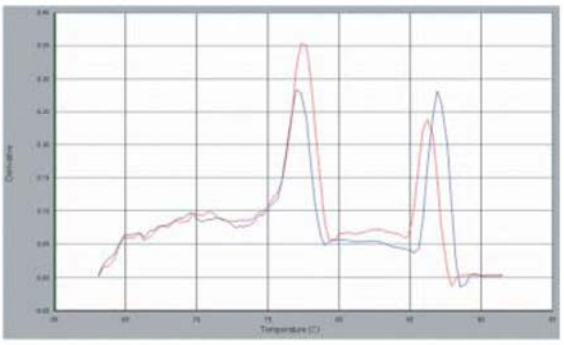
In order to validate our assay, we analysed field samples from Brazilian endemic areas, including *P. vivax*-infected, *P. falciparum*-infected, and non-infected individuals (Table 3). The last two groups were analysed together, as a control group because *P. falciparum* does not use Duffy pathway to invade erythrocytes [24]. As expected, none of the *P. vivax*-infected individuals were Duffy negative, *FY*B^{ES}/FY*B^{ES}*, however, this genotype was also not detected in our group of *P. falciparum*- and non-infected individuals from the endemic area. The primers efficacy was confirmed by using five Duffy-negative individuals from our institute. We observed a

(a)



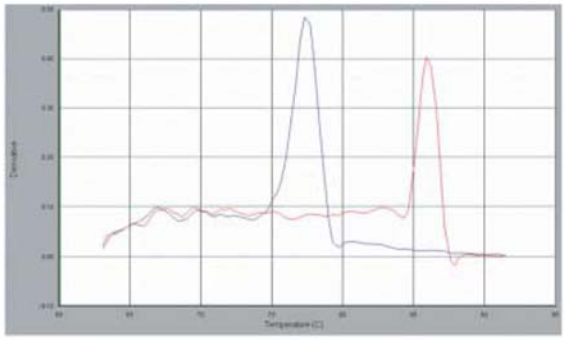
Tube 1 (red) *FY*A* + WTp +
Tube 2 (blue) *FY*B* – MUTp –
Genotype – *FY*A/FY*A*

(b)



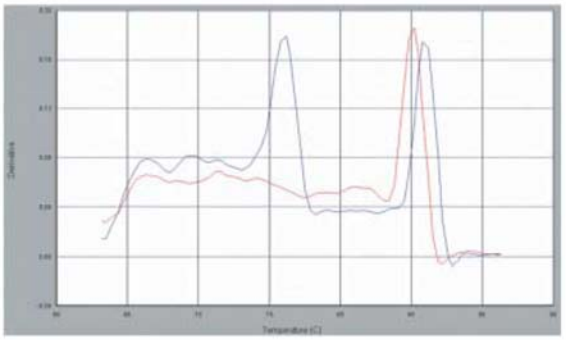
Tube 1 (red) *FY*A* + WTp +
Tube 2 (blue) *FY*B* + MUTp +
Genotype – *FY*A/FY*B*^{ES} or *FY*A*^{ES}*/FY*B*

(c)



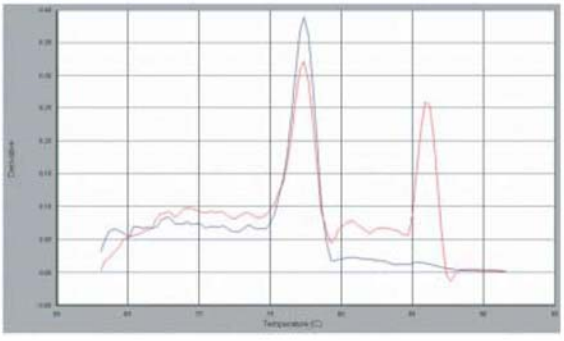
Tube 1 (red) *FY*A* – WTp +
Tube 2 (blue) *FY*B* + MUTp –
Genotype – *FY*B/FY*B*

(d)



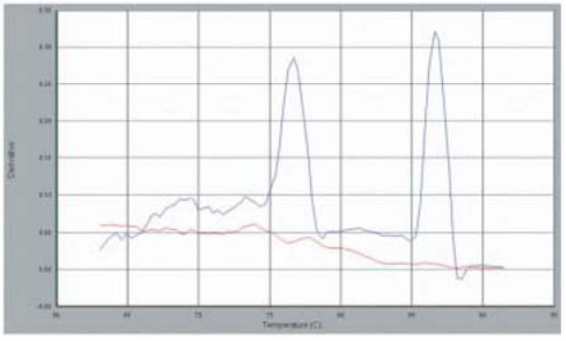
Tube 1 (red) *FY*A* – WTp +
Tube 2 (blue) *FY*B* + MUTp +
Genotype – *FY*B/FY*B*^{ES}

(e)



Tube 1 (red) *FY*A* + WTp +
Tube 2 (blue) *FY*B* + MUTp –
Genotype – *FY*A/FY*B*

(f)



Tube 1 (red) *FY*A* – WTp –
Tube 2 (blue) *FY*B* + MUTp +
Genotype – *FY*B*^{ES}*/FY*B*^{ES}

 Vox Sanguinis (2007) 92, 373–380

Table 1 Comparison of Duffy genotyping by using real-time PCR with PCR-ASP

	PCR-ASP						
Real-time PCR	***FY*A/FY*A***	***FY*A/FY*B^{ES}***	***FY*B/FY*B***	***FY*B/FY*B^{ES}***	***FY*A/FY*B***	***FY*B^{ES}/FY*B^{ES}***	**Total**
*FY*A/FY*A*	**10**[a]	0	0	0	0	0	10
*FY*A/FY*B^{ES}*	0	**7**	0	0	0	0	7
*FY*B/FY*B*	0	0	**17**	0	0	0	17
*FY*B/FY*B^{ES}*	0	0	0	**17**	0	0	17
*FY*A/FY*B*	0	0	0	0	**20**	0	20
*FY*B^{ES}/FY*B^{ES}*	0	0	0	0	0	**5**	5
Total	10	7	17	17	20	5	**76**

[a]In bold there are the coincidental results for both assays.

Table 2 Comparative costs of different approaches for Duffy genotyping (in US$)

	Conventional PCR		Real-time PCR	
	PCR-RFLP [20]	**PCR-ASP [21]**	**LightCycler [22]**	**SYBR Green I (here in)**
Equipment	20 000·00	20 000·00	60 000·00	42 000·00
PCR reagents	0·043	0·043	1·84	0·34
Polymerase	0·21	0·21/0·376[a]		
Primers	0·002	0·0023	0·8	0·0319
Enzymes	0·65[b]	NA	NA	NA
Results visualization	0·1	0·01	NA	NA
Consumables	0·098	0·098	0·18	0·075
Total/reaction[c]	1·10	0·32/0·49	2·82	0·45
Total/patient[d]	4·40	1·29/1·96	5·64	0·89

NA, not applicable.

[a]Conventional/heat-activated *Taq* DNA polymerase.

[b]By using enzymes *BanI* and *StyI*.

[c]Total cost for one reaction, except the equipment.

[d]Total cost for patient, considering four reaction for conventional PCR and two for real-time PCR.

statistically significant higher frequency of *FY*B^{ES}* allele in non-*P. vivax*-infected individuals. The percentage of individuals expressing two functional alleles for Duffy antigen was significantly higher in *P. vivax*-infected than in non-vivax individuals (74% and 55%, respectively, $P = 0{\cdot}006$ by χ^2 analysis).

Discussion

The Duffy genotyping method described here, based on SYBR Green I in real-time multiplex allele-specific PCR, can be used as a high-throughput discrimination of the three major Duffy alleles. This approach takes advantage of the fluorescent property of SYBR Green I and of the melting curve analysis for the detection and discrimination of amplicons differing in length and nucleotide content. The insertion of deliberated mutation and addition of C/G tail allowed the discrimination of variant alleles and avoid false-positive detection [25].

Of importance, besides being reliable and sensitive, our approach to Duffy genotyping is as cost-effective as other PCR-based protocols. PCR-RFLP is time consuming due to the numerous steps, and because of the use of restriction enzymes, it is also very expensive. Moreover, the interpretation of the results on an agarose gel is not always very clear because of

Fig. 2 Representative graphs of Duffy genotyping by real-time PCR. Graphs show the profiles of studied genotypes, obtained using two multiplex reactions: in tube 1 (red lines) were used FAB/RABGATA and FY/RYA primer-pairs for amplification of wild-type Duffy promoter – WTp and *FY*A* allele; and in tube 2 (blue lines) were used FGATA/RABGATA and FY/RYB primer-pairs for amplification of mutated Duffy promoter – MUTp and *FY*B* allele. In the bottom of each graph is showing the results interpretation indicating the amplified alleles (plus symbol) and the genotype obtained.

Table 3 Duffy genotyping of *Plasmodium vivax*-infected and non-vivax individuals based on real-time multiplex allele-specific PCR using the SYBR Green I fluorescent dye

Phenotype (%)			Genotype (%)			Allele (frequency)		
	P. vivax individual[a] (n = 119)	*P. falciparum*/non-infected individual[b] (n = 57)		*P. vivax* individual (n = 119)	*P. falciparum*/non-infected individual (n = 57)		*P. vivax* individual (n = 119)	*P. falciparum*/non-infected individual (n = 57)
Fy(a+b−)	27	26	*FY*A/FY*A*	15	7	*FY*A*	0·39	0·35
			*FY*A/FY*B^{ES}*	12	19	*FY*B*	0·49	0·42
Fy(a−b+)	38	37	*FY*B/FY*B*	24[c]	11	*FY*B^{ES}*	0·13[e]	0·23
			*FY*B/FY*B^{ES}*	14[d]	26	*FY*A^{ES}*	0	0
Fy(a+b+)	35	37	*FY*A/FY*B*	35	37			
Fy(a−b−)	0	0	*FY*B^{ES}/FY*B^{ES}*	0	0			

[a]*Plasmodium vivax*-infected individuals from Brazilian malaria endemic areas.
[b]*P. falciparum*-infected and non-infected individuals from the same endemic areas.
[c]χ^2 analysis P = 0·031.
[d]χ^2 analysis P = 0·036.
[e]χ^2 analysis P = 0·022.

the presence of non-specific bands, incomplete digestion, or even contamination, because every single step increases the risk of error. On the other hand, PCR-ASP has been carried out using a heat-activated DNA polymerase to avoid non-specific products. However, it is a very expensive enzyme to use in routine laboratory tests as it nearly doubles expenses (Table 2). In order to decrease this cost, we have successfully standardized the protocol using a conventional *Taq* DNA polymerase. However, although this approach was not expensive, it is still a time-consuming assay because it requires a post-PCR handling, as it is inadequate for large-scale analysis.

Duffy genotyping based on real-time PCR overcame these drawbacks because it enables specific amplification and detection of amplicons without post-PCR manipulations. Methods based on fluorescent probes are not time consuming and are easier to optimize; however, the use of labelled probes increase the expenses five times. For that reason, SYBR Green I-based methods are much less expensive.

To validate our assay we analysed field samples from Brazilian malaria endemic areas. Considering that for erythrocyte invasion the *P. vivax* parasite needs, at least, one functional Duffy antigen being expressed on RBCs, and that the merozoite invasion is significantly reduced when only one allele is expressed, we postulated that differences in Duffy allele prevalences could reflect susceptibilities to *P. vivax* infection [4,26]. Although, the Duffy phenotype prevalences of our samples were similar to the prevalences of Caucasian Portuguese, the genotype and allele frequencies were quite different, being the *FY*B^{ES}* allele more prevalent in our samples [22]. Comparing the *FY* allele frequencies of different ethnic populations from Brazil, our individuals showed a mixture of Amerindian parentage and European parentage [27,28]. Based on the lack of *FY*B^{ES}/FY*B^{ES}* genotype described here, our data differ from a previous study carried out in Brazil (Rondonia State), where the prevalence of this genotype corresponds to 12% of the *P. falciparum*- and/or *P. malarie*-infected individuals [29]. These differences might be due to the distinct genetic background of the populations that were studied, because in the Rondonia study, Amerindians were not included, and in our study, according to physical characteristics, most individuals seems to be Amerindian related. In conclusion, *P. vivax*-infected individuals have in comparison to *P. falciparum*-infected and non-infected individuals a higher prevalence of two functional alleles and lower frequency of *FY*B^{ES}* allele. These findings corroborate the hypothesis that the presence of two functional alleles increases the risk of *P. vivax* infection [26].

Since the real-time PCR is performed with the GATA-1 and the *FY*A/FY*B* analysis being performed separately, our protocol is not able to confirm whether GATA-1 mutation is in *cis* with *FY*A* or *FY*B* allele. However, we are assuming that GATA-1 mutation occurred in *cis* with *FY*B* allele because the *FY*A^{ES}* allele is rare [16]. Then, the protocol that we described here is a valuable tool to identify the most common Duffy genotypes.

Finally, this new real-time approach, which is feasible and easy to perform in a high-throughput scale, will improve and facilitate malaria epidemiological studies in different endemic areas of the world. Also, this protocol should be helpful in blood banks to prevent red blood cell alloimmunizations, including those induced by blood incompatibilities between

Fy(a+) and Fy(b+) homozygous individuals. As well, this genotyping protocol should be important to identify circumstances in which there are no immunization risk, such as FY^*A^{ES} or FY^*B^{ES} individuals receiving, respectively, Fy(a+) and Fy(b+) blood transfusions. In this case, the blood tolerance is due to the Duffy antigen expression in non-RBC tissues of recipients.

Acknowledgements

We would like to thank the financial support of Tropical Diseases Research/World Health Organization, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Programa de Apoio à Pesquisa Estratégica em Saúde/Fundação Oswaldo Cruz.

References

1 Horuk R, Chitnis CE, Darbonne WC, Colby TJ, Rybicki A, Hadley TJ, Miller LH: A receptor for the malarial parasite *Plasmodium vivax*: the erythrocyte chemokine receptor. *Science* 1993; **261**:1182–1184

2 Szabo MC, Soo KS, Zlotnik A, Schall TJ: Chemokine class differences in binding to Duffy antigen-erythrocyte chemokine receptor. *J Biol Chem* 1995; **270**:25348–25351

3 Anstee DJ, Mollison PL, Klein HG, Caplan L: *Mollison's Blood Transfusion in Clinical Medicine*, 11th edn. Oxford, Blackwell Publishing, 2006

4 Miller LH, Mason SJ, Clyde DF, McGinniss MH: The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. *N Engl J Med* 1976; **295**:302–304

5 Cutbush M, Mollison PL: The Duffy blood group system. *Heredity* 1950; **4**:383–389

6 Ikin EW, Mourant AE, Pettenkofer HJ, Blumenthal G: Discovery of the expected haemagglutinin, anti-Fyb. *Nature* 1951; **168**:1077–1078

7 Mathew S, Chaudhuri A, Murty VV, Pogo AO: Confirmation of Duffy blood group antigen locus (FY) at 1q22→q23 by fluorescence in situ hybridization. *Cytogenet Cell Genet* 1994; **67**:68

8 Pogo AO, Chaudhuri A: The Duffy blood group system and its extensions in nonhuman primates; in Blancher A, Klein J & Socha WW (eds): *Molecular Biology and Evolution of Blood Group and MHC Antigens in Primates*. Berlin, Springer-Verlag, 1997

9 Iwamoto S, Omi T, Kajii E, Ikemoto S: Genomic organization of the glycoprotein D gene: Duffy blood group Fya/Fyb alloantigen system is associated with a polymorphism at the 44-amino acid residue. *Blood* 1995; **85**:622–626

10 Mallinson G, Soo KS, Schall TJ, Pisacka M, Anstee DJ: Mutations in the erythrocyte chemokine receptor (Duffy) gene: the molecular basis of the Fya/Fyb antigens and identification of a deletion in the Duffy gene of an apparently healthy individual with the Fy(a–b–) phenotype. *Br J Haematol* 1995; **90**:823–829

11 Tournamille C, Le Van Kim C, Gane P, Cartron JP, Colin Y: Molecular basis and PCR-DNA typing of the Fy^a/Fy^b blood group polymorphism. *Hum Genet* 1995; **95**:407–410

12 Sanger R, Race RR, Jack J: The Duffy blood groups of New York negroes: the phenotype Fy(a–b–). *Br J Haematol* 1955; **1**:370–374

13 Welch SG, McGregor IA, Williams K: The Duffy blood group and malaria prevalence in Gambian West Africans. *Trans R Soc Trop Med Hyg* 1977; **71**:295–296

14 Pogo AO, Chaudhuri A: The Duffy protein: a malarial and chemokine receptor. *Semin Hematol* 2000; **37**:122–129

15 Tournamille C, Colin Y, Cartron JP, Le Van Kim C: Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. *Nat Genet* 1995; **10**:224–228

16 Zimmerman PA, Woolley I, Masinde GL, Miller SM, McNamara DT, Hazlett F, Mgone CS, Alpers MP, Genton B, Boatin BA, Kazura JW: Emergence of FY*A(null) in a *Plasmodium vivax*-endemic region of Papua New Guinea. *Proc Natl Acad Sci USA* 1999; **96**:13973–13977

17 Tournamille C, Le Van Kim C, Gane P, Le Pennec PY, Roubinet F, Babinet J, Cartron JP, Colin Y: Arg89Cys substitution results in very low membrane expression of the Duffy antigen/receptor for chemokines in Fy(x) individuals. *Blood* 1998; **92**:2147–2156

18 Rios M, Chaudhuri A, Mallinson G, Sausais L, Gomensoro-Garcia AE, Hannon J, Rosenberger S, Poole J, Burgess G, Pogo O, Reid M: New genotypes in Fy(a–b–) individuals. nonsense mutations (Trp to stop) in the coding sequence of either FY A or FY B. *Br J Haematol* 2000; **108**:448–454

19 Castilho L, Rios M, Pellegrino J Jr, Saad ST, Costa FF, Reid ME: A novel FY allele in Brazilians. *Vox Sang* 2004; **87**:190–195

20 Chaudhuri A, Polyakova J, Zbrzezna V, Pogo AO: The coding sequence of Duffy blood group gene in humans and simians: restriction fragment length polymorphism, antibody and malarial parasite specificities, and expression in nonerythoid tissues in Duffy-negative individuals. *Blood* 1995; **85**:615–621

21 Olsson ML, Hansson C, Avent ND, Akesson IE, Green CA, Daniels GL: A clinically applicable method for determining the three major alleles at the Duffy (FY) blood group locus using polymerase chain reaction with allele-specific primers. *Transfusion* 1998; **38**:168–173

22 Araujo F, Pereira C, Aleixo A, Henriques I, Monteiro F, Meireles E, Lacerda P, Cunha-Ribeiro LM: Rapid genotyping of the major alleles at the Duffy (FY) blood group locus using Real-Time fluorescence polymerase chain reaction. *Immunohematology* 2001; **17**:42–44

23 Sullivan KM, Foster DA: Useful software in epidemiology and public health. *Epidemiol Monitor* 1990; **11**:7–10

24 Gaur D, Mayer DC, Miller LH: Parasite ligand-host receptor interactions during invasion of erythrocytes by *Plasmodium* merozoites. *Int J Parasitol* 2004; **34**:1413–1429

25 Dall'Ozzo S, Andres C, Bardos P, Watier H, Thibault G: Rapid single-step FCGR3A genotyping based on SYBR Green I fluorescence in Real-Time multiplex allele-specific PCR. *J Immunol Meth* 2003; **277**:185–192

26 Michon P, Woolley I, Wood EM, Kastens W, Zimmerman PA, Adams JH: Duffy-null promoter heterozygosity reduces DARC expression and abrogates adhesion of the *P. vivax* ligand required for blood-stage infection. *FEBS Lett* 2001; **495**:111–114

27 Da Silva WA Jr, Bortolini MC, Meyer D, Salzano FM, Elion J, Krishnamoorthy R, Schneider MP, De Guerra DC, Layrisse Z,

Castellano HM, Weimer TD, Zago MA: Genetic diversity of two African and sixteen South American populations determined on the basis of six hypervariable loci. *Am J Phys Anthropol* 1999; **109**:425–437

28 Palatnik M, Silva Junior WA, Estalote AC, de Oliveira JE, Milech A, Zago MA: Ethnicity and type 2 diabetes in Rio de Janeiro, Brazil, with a review of the prevalence of the disease in Amerindians. *Hum Biol* 2002; **74**:533–544

29 Cavasini CE, Tarelho Pereira FJ, Ribeiro WL, Wunderlich G, Ferreira MU: Duffy blood group genotypes among malaria patients in Rondonia, Western Brazilian Amazon. *Rev Soc Bras Med Trop* 2001; **34**:591–595

Amazonian community-based study to evaluate host genotype for Duffy antigen receptor for chemokines (DARC) and immune response against the Plasmodium vivax Duffy binding protein (PvDBP)

Bruno A. M. Sanchez[a], Flora S. kano[a], Letícia M Torres[a], Cor JF Fontes[b], Paulo Nogueira[c], Irene S Soares[d], Tais N. Sousa[a], Roberto S. Rocha[c], Cristiana F.A. Brito[a], Luzia H Carvalho[a], *

[a] Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, MG, Brazil

[b] Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil

[c] Centro de Pesquisas Leônidas & Maria Deane, FIOCRUZ Amazônia, Manaus, AM

[d]Instituto de Ciências Biológicas,Universidade de São Paulo (USP), São Paulo, SP,Brazil

Corresponding author: Luzia Helena Carvalho, Laboratory of Malaria, Rene Rachou Research Institute, Fundação Oswaldo Cruz, Avenida Augusto de Lima, 1715, Belo Horizonte, Brazil, 30190-002. Phone: +55 31 33497793. Fax: +55 31 32953115.

E-mail: lhcarvalho@cpqrr.fiocruz.br

ABSTRACT

The *P.vivax* Duffy binding protein (PvDBP) and its erythrocytic receptor, the Duffy antigen receptor for chemokines (DARC), are involved in the major *P.vivax* erythrocyte invasion pathway. Here, in an agricultural settlement of the Brazilian Amazon area, we carried-out an open cohort study to analyzed DARC genotypes and its relationship to the PvDBP immune response. Among 687 individuals enrolled in the study, the distribution of DARC genotypes was consistent with the heterogeneous ethnic origin of the Amazon population, with a predominance of no silent FY alleles: *FY*A* > *FY*B*. In the study area, no association was found between DARC genotypes and P.vivax susceptibility. Of importance, the follow-up study demonstrated that anti-PvDBP antibodies towards to be more frequent in heterozygous carrying a DARC-silent allele (*FY*B*ES). No association was found with antibodies to $PvMSP1_{19}$, another *P.vivax* protein not associated with DARC. Together, these results suggest that DARC expression may influence the PvDBP immune response.

Kewords:

Plasmodium vivax; community-based study; DARC genotype; immune response; Duffy binding protein; Amazon area.

1. INTRODUCTION

Plasmodium vivax is the most widespread *Plasmodium* species and is a potential cause of morbidity and mortality amongst the 2.85 billion people living at risk of infection (Guerra et al. 2010). Recent evidences of multidrug-resistant *P. vivax* associated with severe and fatal disease, placing vivax infection in a higher status as a global health concern (Tjitra et al. 2008) (Genton et al. 2008*). P. vivax* infects human RBCs throw a major pathway that requires interaction between an apical parasite protein, the Duffy binding protein (PvDBP), and its receptor on erythrocytes, the Duffy antigen receptor for chemokines (DARC) (Miller et al. 1976, Fang et al. 1991, Adams et al. 1990). Although most of the individuals that lack DARC on their erythrocytes are naturally resistant to *P. vivax* (Miller et al. 1976), an DARC-independent invasion pathway has been described in some parts of the world (Cavasini et al. 2007b, Ryan et al. 2006, Ménard et al. 2010). Beyond being a receptor for *P. vivax* and various chemokines (Horuk et al. 1993), DARC proteins have clinical and biological significance, and have been reported to cause transfusion incompatibility and hemolytic disease of the newborn (Badakere and Bhatia 1970, Weinstein and Taylor 1975, Moise 2000). It is also implicated in multiple chemokine inflammation, inflammatory diseases, in cancer and might play a role in HIV infection and AIDS (Walton and Rowland-Jones 2008, Reich et al. 2009, Afenyi-Annan et al. 2008, Smolarek et al. 2010).

The two common DARC alleles in Caucasians, *FY*A* and *FY*B*, differ by a single base substitution (125 G>A) resulting in the replacement at residue 42 in the

extracellular domain of a glycine (Fya antigen) with an aspartic acid (Fyb antigen) (Iwamoto et al. 1995, Mallinson et al. 1995). The mutation in DARC that abolishes receptor expression in erythroid cells disrupt a binding site for the GATA1 erythroid transcription factor, resulting in the absence of Duffy antigens on RBCs (-33T>C; FyES, erythrocyte silent) (Tournamille et al. 1995). Although most DARC negative individuals carry the GATA mutation in the Fy*B allele (silent Fy*B allele), the presence of cis-regulatory mutation with the FY*A has been described (Kasehagen et al. 2007).

The importance of the interaction between PvDBP (region II, DBPII) and DARC to *P. vivax* infection has stimulated a significant amount of studies on anti-PvDBP antibody responses. Available data demonstrate that naturally occurring antibodies to PvDBP are prevalent in individuals living in *P. vivax* endemic areas (Fraser et al. 1997, Ceravolo et al. 2005, Souza-Silva et al. 2010), and these antibodies can block the DBPII –DARC interaction (Michon, Fraser and Adams 2000, Ceravolo et al. 2008, King et al. 2008). While inhibitory PvDBP antibodies confer some degree of protection against blood-stage infection (King et al. 2008), these antibodies are biased towards a specific allele (Ceravolo et al. 2009). Even though anti-PvDBP immune response has been well characterized, very little is known about the association between this immune response and DARC host genotype (Herrera et al. 2005, Maestre et al. 2010). Here, we presented data of a follow-up population-based study where the relationship between DARC genotypes, PvDBP antibodies and vivax malaria susceptibility were analyzed. The methodological approach included a community-based open cohort study in an agricultural settlement of the Amazon area,

Rio Pardo, Brazil, where 687 individuals were genotyping to DARC, and contributed 4560 person-month of follow-up (November 2008 – November 2009).

2. MATERIAL AND METHODS

2.1. Study area and population

The study was carried-out in the agricultural settlement of Rio Pardo (1º46'S - 1º54'S, 60º22'W, 60º10'W), Presidente Figueiredo municipality, northeast of the Amazonas state, Brazilian Amazon area. The rural locality of Rio Pardo is located at approximately 160 km from Manaus, the capital of the State, with the main access to a paved road (BR-174), that connects Amazonas to Para State. The agricultural settlement was officially created in 1996, by the National Institute of Colonization and Agrarian Reform (INCRA), as part of the large scale colonization projects focused on agriculture and wide-ranging human settlement in the Amazon area (de Castro et al. 2006). In this area, human population is living on subsistence farming and fishing along the small streams of Rio Pardo River. Housing quality is poor, thereby rendering indoor residual spraying ineffective. Curative health services are sparsely available, and a government-run malaria diagnosis outpost provides free malaria diagnosis and treatment to the inhabitants of the study site. In the Rio Pardo area, the mean annual temperature is 31^{o}C with humid climate and average annual rainfall of the 2.000 mm per year. There are two define season as rainy season (November – May) and dry season (June – October). The settlement is composed of 7 areas namely "ramais" - Principal, Samuel, Novo Paraíso, Gusmão, Terra Preta,

Taxista and Novo Progresso - which includes households on both sides of unpaved roads, and a riverine population named "Igarapé Rio Pardo". In this area, a population census (September-Octoberof 2008) identified 701 habitants, with 360 (51.4%) living in ramais area, 341 (48.6%) in Igarapé Rio Pardo and around. In this area, although Plasmodium vivax and Plasmodium falciparum are transmitted year round, P. vivax is responsible for about 90% of malaria cases (SVS/MS, 2008).

2.2. Study design and cross-sectional surveys

Cross-sectional surveys were carried out after discussions with the community about the objectives of the project and its protocols. In November 2008, from 701 residents of the settlement invited to participate in the study, 541 (77.2%) accepted by given their written informed consent, in accordance with guidelines for human research, as specified by the Brazilian National Council of Health (Resolution 196/96).

A population-based open cohort study was initiated in November of 2008, and the following procedures were performed during the first travel of the field team to the area: (i) application of a structured questionnaire to all volunteers to obtain demographical, epidemiological, and clinical data; (ii) physical examination, including body temperature and spleen/liver sizes, recorded according to standard clinical measurements; (iii) search for malaria parasites by light microscopy (Giemsa-thick blood smears); and (iii) venous blood collection in individuals aged five years or older (EDTA 10ml), or blood spotted on filter papers (finger-prick) in those aged

<5 years. At that time, geographical location of each dwelling was recorded throw a hand-held 12-channel global positioning system (GPS, Garmin 12XL, Olathe, KS, USA), with a positional accuracy within 15 meters.

After approximately six and 12 months of the first survey, two other identical cross-sectional surveys were carried out. An additional of 155 individuals were enrolled during the 2nd survey (June, 2009) and 70 individuals in the 3rd survey (October-November, 2009), respectively, giving a total of 766 subjects who were enrolled in the study. Cumulative exposure to malaria was estimated by subject´s age, the length of residence in malaria-endemic areas (either in Amazonas state or elsewhere in the endemic area) and the self-reported number of lifetime malaria episodes. Most of subjects (690, 90%) were native from the Amazon area; place of birth was not available in 14 (1.8%) individuals.

Of 766 studied subjects, 687 (89.7%) had genomic DNAs amplified to DARC genotyping and 591 (77%) had serum samples tested to *P.vivax* antibodies (Table 1); additionally, 484 (63%) individuals had paired samples. The ethical and methodological aspects of this study were approved by the Ethical committee of Research on Human Beings from the Centro de Pesquisas René Rachou (Report No.007/2006 and No. 07/2009), according to the Resolution of the Brazilian Council on Health-CNS 196/96.

2.3. Laboratory diagnosis of malaria.

Two methods were used to diagnose malarial infections: microscopic examination of Giemsa-stained thick smears and real-time PCR amplification of a species-specific segment of the 18S rRNA gene of human malaria parasites. The Giemsa-stained thick blood smear technique was used for malaria diagnosis in all samples of the present study, in which well-trained microscopists were in charge of examining the equivalent of 0.2 µL of blood (100 field microscopy), and parasite density estimated by blood microliters. For quality assurance, a random sample of 10% of the blood smears was reviewed by a second trained microscopist, who was masked to the initial smear results. Real Time PCR amplification of species-specific of 18S rRNA gene human malaria was performed, as described (Mangold et al. 2005); based on this protocol, a consensus pair of primers was used to amplify a species-specific region of the multicopy 18S rRNA gene. Briefly, each 20 µl reaction mix contained 200 ng of genomic DNA, 10 µl of 10x 5 µl Sybr® Green PCR master mix (Applied Biosystems), 2.5 mM MgSO4 and 0.5 pmole of each primer (BioSynthesis). The PCR conditions was consisted of an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 90°C for 30 seconds, and 60°C for 30 seconds; with fluorescence acquisition at the end of each extension step. After amplification, melting curves were observed from the dissociation curves and those melting curves analysis based on nucleotide variations within the amplicons provided a basis for accurate differentiation of 3 plasmodia: P. falciparum, P. vivax and P. malariae. The amplification and fluorescence detection were performed using ABI PRISM® 7000 sequence Detection System (Applied Biosystems). The melting temperature (Tm) ranges values for each Plasmodium was: P. vivax , 74 to 76°C; P. falciparum, 71 to 73°C; and P. malariae, 68 to 70°C.

2.4. The DARC genotyping

Extracted DNA was used to detect the three common alleles at the FY locus – *FY*A, FY*B, FY*B*^{ES} (ES, erythroid silent) –using real-time PCR with allele-specific primers, essentially as we have described recently (Sousa et al. 2007). Briefly, each 20 µl reaction mix contained 50-100 ng genomic DNA, 10 µL SYBR® Green PCR master mix (Biosystems), and 10 pmoles of each primer (Biosystems). The amplification and fluorescence were detected by ABI Prism® 7000 Sequence Detection System (Applied Biosystems) by using a cycle of 95 °C for 10 min, following by 35 cycles of 95 °C for 15 s and 60 °C for 1 min. After amplification, melting curves were observed from the dissociation curve resulting from continuous measurements of fluorescence (F) at 530 nm during which the temperature was gradually increased from 60 to 95 °C. Melting peaks of each amplified fragment were visualized by plotting the negative derivative of the fluorescence with respect to temperature against temperature (–dF/dT° vs. T°).

2.5. Recombinant proteins and serological assay

Two recombinant *P. vivax* proteins were used to detect total immunoglobulin G (IgG) antibodies. The recombinant DBP, which includes amino acids 132–771 (regions II–IV,DBPII–IV), was expressed as a soluble glutathione S transferase (GST) fusion protein of 140 kDa, as described previously (Fraser et al. 1997, Ceravolo et al. 2005). The recombinant protein representing the 19-kDa C-terminal region of the merozoite surface protein-1 of *P. vivax* (PvMSP119), which represents

amino acids 1616–1704 of the MSP1 of *P. vivax*, has been described elsewhere (Cunha, Rodrigues and Soares 2001). To assess IgG antibodies against DBPII–IV and PvMSP119, an enzyme-linked immunosorbent assay (ELISA) was carried out, as described previously (Ceravolo et al. 2005), with serum samples at 1:100. For the recombinant proteins DBPII–IV (5 mg/ml) and $PvMSP1_{19}$ (1 mg/ml), the final optical density (OD) at 492 nm was calculated by subtracting the OD obtained with GST (antigen control). The results were expressed as an index of reactivity (IR = OD492 values of test sample divided by the value of the cut-off). Cut-off points were set at three standard deviations above the mean OD492nm of sera from 30 individuals who had never been exposed to malaria. Values of IR > 1·0 were considered positive.

2.4. Statistical analysis

A database was created with Epidata software (http://www.epidata.dk). Proportions were compared in 2 × 2 tables with chi-square ($\chi 2$) tests with Yates correction for continuity or Fisher's exact tests, or trend linear $\chi 2$, as appropriate. Pairwise correlations were performed using Spearman rank correlation coefficient. Differences in the median were performed using Mann-Whitney test. The level of significance of 5% was adopted.

3. RESULTS

3.1. Prevalence of malarial infection and antibody responses

We have investigated acute malaria infection in 687 subjects aged 2 months to 90 years (median, 23 yr), with a male:female rate of 1.36:1 (Table 1). In Rio Pardo population, subject´s age basically corresponded to the time of malaria exposure in the Amazon area (r=0.96; p<0.0001, spearman´s correlation test); consequently, these subjects had between 2 months and 90 years of residence in the Amazon area (median, 20 yrs). Sixty-nine (10%) subjects had at least one symptomatic malaria infection, diagnosed by active and/or passive case detection, between November 2008 and November 2009, with 62 out of 69 (89.8%) caused by *P. vivax*. In the study population, 54.5% (322 out of 591) subjects had antibodies to PvDBP and 56.3% (333 out of 591) to $PvMSP1_{19}$. Variables associated with the presence of anti-*P.vivax* antibodies were subject´s age and the number of years of residence in the Brazilian Amazon, both reflecting cumulative exposure to malaria (Kano FS and BAM Sanchez, unpublished data).

3.2. DARC polymorphisms and incidence of P.vivax infection

Three DARC genotypes - *FY*A/FY*B*^{ES}, *FY*A*/*FY*A* and *FY*A/FY*B* - were more frequent in the study population, with the heterozygous mutant FY*A / FY*BES being the more prevalent (216 out of 687, 31.4%). As expected, DARC negative genotype (*FY*B*^{ES}/*FY*B*^{ES}) was present in the population at very low frequencies (<1%, 4 out of 687). The Demographical and epidemiological variables associated with the presence of antibodies and/or the risk of malaria infection (age and time of malaria exposure) were equally distributed among DARC genotypes (Table 2); the low number of DARC negative individuals precluded any statistical comparison, consequently, these 4 individuals were not included in these analyzes.

Further, we analyzed DARC genotypes according to the presence of acute malaria infection, including infected by P. vivax, P. falciparum or not infected individuals (Table 3). The last two groups were analyzed together, as a control group because P. falciparum does not use Duffy pathway to invade erythrocytes (Gaur, Mayer and Miller 2004). In both groups, the same three genotypes - *FY*A/FY*A, FY*A/FY*B* and *FY*A/FY*B*^{ES} - were more prevalent. With the exception of those 4 DARC negative carries- well-known as highly resistant to vivax malaria - we could not detect any statistical difference between the presence of *P. vivax* infection and DARC genotype. Despite of that, the presence of *FY*A* allele was significantly higher in both infected and non-infected groups ($p< 0.0001$; $\chi 2$ analysis). In subsequent analysis, we evaluated the relationship between DARC genotype and the annual incidence of vivax malaria, evaluated here by the incidence rate of *P. vivax* infection per 1000 persons-month (Table 4). There was no association between DARC genotypes and *P. vivax* malaria incidence. Also, no association was found by stratifying individuals according to inter-cluster transmission, i.e., proximity of dwelling to potential mosquito breeding sites (data not shown).

3.1. DARC polymorphisms and *P.vivax* antibody responses

Aiming to evaluate the influence of DARC genotype in the antibody response, we further analyzing anti-PvDBP and anti-PvMSP1$_{19}$ antibodies among individuals grouped according to DARC genotype (Table 5). The frequencies as well as the magnitudes of antibody response were similar between those different DARC genotypes, with 51 to 64% and 52 to 66% of the individuals presented antibodies to

PvDBP and PvMSP1*19*, respectively. Since the study population had been actively followed for 12 months, we subsequently analyzed the persistence of antibody response among individuals carrying different DARC genotypes. As shown in Figure 1A, there was a tendency towards increases the frequency of responders to PvDBP with DARC genotypes, with the highest proportions among individuals carrying genotypes with single functional alleles (*FY*A/FY*B*^{ES} and *FY*B/FY*B*^{ES}) ($\chi 2$ for trend=4, p=0.04). No significant difference could be obtained with antibodies to PvMSP1$_{19}$ (Figure 1B). Also, in the individuals carrying the genotype *FY*B*/*FY*B*^{ES} and categorized as persistent responder the levels of anti-PvDBP were higher than those detected in individuals carrying any other genotype (Figure 2C). Taken together these results suggested that DARC genotypes seem to influence in the stability of anti-PvDBP antibody response.

4. DISCUSSION

Populations of the Brazilian Amazonian are considered in general highly susceptible to malaria, and have different levels of acquired immunity (Carvalho, Fontes and Krettli 1999, Alves et al. 2005, Ferreira et al. 1996, Lima-Junior et al. 2011, Ceravolo et al. 2008). Since the prevalence of malaria is not homogeneous in those populations (de Castro, Sawyer and Singer 2007, da Silva-Nunes et al. 2008), it is important to investigate if host biological factors, such the DARC receptor for *P. vivax*, contribute to this heterogeneity. In our study population, DARC genotypes and its allele distributions were consistent with the heterogeneous ethnic origin of the Amazon population, with an inherent admixture of Native Americans (Amerindians),

Europeans, and Africans (Perna, Cardoso and Guerreiro 2007, Palha et al. 2010). The present results showed that three genotypes were prevalent in the study population: one showing the heterozygous 33T>C mutation on GATA box (*FY*A/FY*B*^{ES}) and two wild-type (*FY*A/FY*A* and *FY*A/FY*B*). Considering some degree of local differentiation and admixture of Caucasian/Africans, in general, these results agree with those few population-based studies carried-out in the Amazon area (Ferreira et al. 2002, Camargo et al. 2002, Cavasini et al. 2007a, Albuquerque et al. 2010). However, variations between DARC allele frequencies should be expected between our and those previous studies because most of them involved population composed predominantly of migrants, while our study community was mainly composed of natives of the Amazon area. In fact, the predominance of *FY*A* allele observed here is consistent with the results found in a small riverine community of the Western Amazon (Ferreira et al. 2002); in that community, Ameridians are present in high proportion, and the *FY*A* allele was more prevalent that *FY*B*. As expected, a different DARC profile has been described among African-Brazilian communities of the Amazon area, originally constituted from escaped slaves called Quilombolas, in which DARC negative genotype (*FY*B*^{ES}*/FY*B*^{ES}) was found with frequencies between 32% to 56% (Perna et al. 2007). At this time, scarcity of historical and genetics data of Rio Pardo population precludes any definitive conclusion on the contributions of Africans, Amerindians, and Europeans to the ethnic composition of the studied population. Therefore, it will be important for future studies to use different genetic markers that define ancestry structure and interethnic admixture in this stable and organized Amazonian population.

Based on prevalence as well as in the incidence of P.vivax infection in Rio Pardo community, no significant association was found between FY heterozygosity and protection against *P. vivax*. These results differ from those found by us and others showing that heterozygosis for GATA box mutation ($FY*B^{ES}$ or the rare $FY*A^{ES}$) seems to decrease the risk of *P. vivax* infection in the Brazilian Amazon area (Sousa et al. 2007, Cavasini et al. 2007a, Albuquerque et al. 2010) as well as in Papua New Guinea (Kasehagen et al. 2007). Although the reasons for these differences are not clear, distinct genetic background of uninfected individuals may have contributed. Specifically, in the community-based studies carried-out in Brazil, the control group was selected among volunteers from blood bank donors (Cavasini et al. 2007a, Albuquerque et al. 2010); On the other hand, in the PNG study a rare allele - FY*AES - was responsible for the *P. vivax* protection (Kasehagen et al. 2007). In conclusion, although there is little doubt that DARC negative homozygous individuals ($FY*B^{ES}$ /$FY*B^{ES}$) are highly refractory to *P. vivax* infection (Miller et al. 1976), the protection mediated by DARC negative heterozygosity remains an open question.

Despite of the importance of PvDBP as a vaccine candidate, the relationship between expression of DARC and immune response against PvDBP has been largely unexplored (Herrera et al. 2005, Maestre et al. 2010). Here, through successive epidemiological surveys, we present evidence that DARC expression may influences the antibody response to PvDBP. Of interest, individuals carrying the GATA mutated allele in heterozygosis tended to respond more frequently to PvDBP, especially those carrying the $FY*B/FY*B^{ES}$ genotype in whom the levels of anti-PvDBP antibodies were higher. Given the scarcity of data on the influence of DARC expression in the PVDBP antibody response, this profile of immune responses can be compared only

with a single study carried-out in the Caribbean coast of Colombia (Maestre et al. 2010). In accordance with our study, the Colombian study demonstrated that volunteers with one DARC negative allele were more likely to have anti-PvDBP than those with two positive alleles. An unexpected result of Colombia study was that DARC expression seems to influences in the $PvMSP1_{19}$ antibody response, a parasite blood stage protein not related to the DARC receptor. In our study, $PvMSP1_{19}$ antibodies did not show significant association with DARC expression. Nevertheless, it should be stressed that the Colombia study might not be extrapolated to other areas of Latin America because it was performed in areas where the frequencies of African-Colombians were high, consequently, also the frequencies of DARC negative trait that confer resistance to *P. vivax*. Despite of that, our results pointed to the same direction on the role of DARC expression in the acquired response to *P. vivax*. Currently, there is no clear explanation for the fact that individuals carrying one negative DARC allele have more antibodies that those with two positive alleles; especially because there is no evidence that DARC indirectly down-regulating humoral immune responses against P.vivax blood stage, as proposed by Maestre and colleagues (2010). A long-term prospective study is needed to determine the contribution of DARC expression in terms of immunity and protection against the disease.

Taken together, our results demonstrate that individuals from a stable and organized community of the Amazon area exhibit antibody response against PvDBP that varies according to the DARC expression. It is not yet clear if this finding influences in the acquisition of protection against malaria. We believe that this first

prospective study on the relationship between the profile of immune response and DARC expression will contribute to current efforts on vaccine development

FIGURE LEGENDS

Figure 1. The frequency of antibody responses to PvDBP (A) and PvMSP1$_{19}$ (B) during the follow-up of 380 individuals (Rio Pardo, Amazonas State) carrying different DARC genotypes. According to ELISA positivity in consecutive samples (November 2008-November 2009), individuals were categorized as: (i) non-responder (no antibody response at any time-point of the study); (ii) temporary responder (positive antibody response in at least one cross-sectional survey); (iii) persistent responder (positive antibody response in all cross-sectional surveys).

Figure 2. The levels of antibody responses to PvDBP and PvMSP119 during the follow-up of 380 individuals (Rio Pardo, Amazonas State) carrying different DARC genotypes. Individuals were categorized, as described in legend of Figure 1, as: (i) non-responder (no antibody response at any time-point of the study); (ii) temporary responder (positive antibody response in at least one cross-sectional survey); (iii) persistent responder (positive antibody response in all cross-sectional surveys).

REFERENCES

Adams, J. H., D. E. Hudson, M. Torii, G. E. Ward, T. E. Wellems, M. Aikawa & L. H. Miller (1990) THE DUFFY RECEPTOR FAMILY OF PLASMODIUM-KNOWLESI IS LOCATED WITHIN THE MICRONEMES OF INVASIVE MALARIA MEROZOITES. Cell, 63, 141-153.

Afenyi-Annan, A., M. Kail, M. R. Combs, E. P. Orringer, A. Ashley-Koch & M. J. Telen (2008) Lack of Duffy antigen expression is associated with organ damage in patients with sickle cell disease. Transfusion, 48, 917-24.

Albuquerque, S. R., F. e. O. Cavalcante, E. C. Sanguino, L. Tezza, F. Chacon, L. Castilho & M. C. dos Santos (2010) FY polymorphisms and vivax malaria in inhabitants of Amazonas State, Brazil. Parasitol Res, 106, 1049-53.

Alves, F. P., L. H. Gil, M. T. Marrelli, P. E. Ribolla, E. P. Camargo & L. H. Da Silva (2005) Asymptomatic carriers of Plasmodium spp. as infection source for malaria vector mosquitoes in the Brazilian Amazon. J Med Entomol, 42, 777-9.

Badakere, S. S. & H. M. Bhatia (1970) A fatal transfusion reaction due to anti-Duffy (Fya). Case report. Indian J Med Sci, 24, 562-4.

Camargo, L. M., M. M. Moura, V. Engracia, R. C. Pagotto, S. A. Basano, L. H. da Silva, E. P. Camargo, B. Beiguelman & H. Krieger (2002) A rural community in a Brazilian Western Amazonian Region: some demographic and epidemiological patterns. Mem Inst Oswaldo Cruz, 97, 193-5.

Carvalho, L. H., C. J. E. Fontes & A. U. Krettli (1999) Cellular responses to Plasmodium falciparum major surface antigens and their relationship to human activities associated with malaria transmission. American Journal of Tropical Medicine and Hygiene, 60, 674-679.

Cavasini, C. E., L. C. de Mattos, A. A. Couto, V. S. Couto, Y. Gollino, L. J. Moretti, C. R. Bonini-Domingos, A. R. Rossit, L. Castilho & R. L. Machado (2007a) Duffy blood group gene polymorphisms among malaria vivax patients in four areas of the Brazilian Amazon region. Malar J, 6, 167.

Cavasini, C. E., L. C. Mattos, A. A. Couto, C. R. Bonini-Domingos, S. H. Valencia, W. C. Neiras, R. T. Alves, A. R. Rossit, L. Castilho & R. L. Machado (2007b) Plasmodium vivax infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception? Trans R Soc Trop Med Hyg, 101, 1042-4.

Ceravolo, I., B. Sanchez, T. Sousa, B. Guerra, I. Soares, E. Braga, A. McHenry, J. Adams, C. Brito & L. Carvalho (2009) Naturally acquired inhibitory antibodies to Plasmodium vivax Duffy binding protein are short-lived and allele-specific following a single malaria infection. Clin Exp Immunol, 156, 502-10.

Ceravolo, I. P., O. Bruna-Romero, E. M. Braga, C. J. F. Fontes, C. F. A. Brito, J. M. Souza, A. U. Krettli, J. H. Adams & L. H. Carvalho (2005) Anti-Plasmodium vivax

Duffy binding protein antibodies measure exposure to malaria in the Brazilian Amazon. American Journal of Tropical Medicine and Hygiene, 72, 675-681.

Ceravolo, I. P., F. A. Souza-Silva, C. J. F. Fontes, E. M. Braga, A. P. Madureira, A. U. Krettli, J. M. Souza, C. F. A. Brito, J. H. Adams & L. H. Carvalho (2008) Inhibitory properties of the antibody response to Plasmodium vivax Duffy binding protein in an area with unstable malaria transmission. Scandinavian Journal of Immunology, 67, 270-278.

Cunha, M. G., M. M. Rodrigues & I. S. Soares (2001) Comparison of the immunogenic properties of recombinant proteins representing the Plasmodium vivax vaccine candidate MSP1(19) expressed in distinct bacterial vectors. Vaccine, 20, 385-396.

da Silva-Nunes, M., C. Codeço, R. Malafronte, N. da Silva, C. Juncansen, P. Muniz & M. Ferreira (2008) Malaria on the Amazonian frontier: transmission dynamics, risk factors, spatial distribution, and prospects for control. Am J Trop Med Hyg, 79, 624-35.

de Castro, M. C., R. L. Monte-Mór, D. O. Sawyer & B. H. Singer (2006) Malaria risk on the Amazon frontier. Proc Natl Acad Sci U S A, 103, 2452-7.

de Castro, M. C., D. O. Sawyer & B. H. Singer (2007) Spatial patterns of malaria in the Amazon: implications for surveillance and targeted interventions. Health Place, 13, 368-80.

Fang, X. D., D. C. Kaslow, J. H. Adams & L. H. Miller (1991) CLONING OF THE PLASMODIUM-VIVAX DUFFY RECEPTOR. Molecular and Biochemical Parasitology, 44, 125-132.

Ferreira, M. U., E. A. Kimura, J. M. De Souza & A. M. Katzin (1996) The isotype composition and avidity of naturally acquired anti-Plasmodium falciparum antibodies: differential patterns in clinically immune Africans and Amazonian patients. Am J Trop Med Hyg, 55, 315-23.

Ferreira, R. G., M. M. Moura, V. Engracia, R. C. Pagotto, F. P. Alves, L. M. Camargo, L. H. Silva, E. P. Camargo, B. Beiguelman & H. Krieger (2002) Ethnic admixture composition of two western Amazonian populations. Hum Biol, 74, 607-14.

Fraser, T., P. Michon, J. W. Barnwell, A. R. Noe, F. AlYaman, D. C. Kaslow & J. H. Adams (1997) Expression and serologic activity of a soluble recombinant Plasmodium vivax Duffy binding protein. Infection and Immunity, 65, 2772-2777.

Gaur, D., D. C. Mayer & L. H. Miller (2004) Parasite ligand-host receptor interactions during invasion of erythrocytes by Plasmodium merozoites. Int J Parasitol, 34, 1413-29.

Genton, B., V. D'Acremont, L. Rare, K. Baea, J. C. Reeder, M. P. Alpers & I. Muller (2008) Plasmodium vivax and mixed infections are associated with severe malaria in children: A prospective cohort study from Papua New Guinea. Plos Medicine, 5, 881-889.

Guerra, C. A., R. E. Howes, A. P. Patil, P. W. Gething, T. P. Van Boeckel, W. H. Temperley, C. W. Kabaria, A. J. Tatem, B. H. Manh, I. R. Elyazar, J. K. Baird, R. W. Snow & S. I. Hay (2010) The international limits and population at risk of Plasmodium vivax transmission in 2009. PLoS Negl Trop Dis, 4, e774.

Herrera, S., A. Gomez, O. Vera, J. Vergara, A. Valderrama-Aguirre, A. Maestre, F. Mendez, R. B. Wang, C. E. Chitnis, S. S. Yazdani & M. Arevalo-Herrera (2005) Antibody response to Plasmodium vivax antigens in Fy-negative individuals from the Colombian pacific coast. American Journal of Tropical Medicine and Hygiene, 73, 44-49.

Horuk, R., C. E. Chitnis, W. C. Darbonne, T. J. Colby, A. Rybicki, T. J. Hadley & L. H. Miller (1993) A receptor for the malarial parasite Plasmodium vivax: the erythrocyte chemokine receptor. Science, 261, 1182-4.

Iwamoto, S., T. Omi, E. Kajii & S. Ikemoto (1995) Genomic organization of the glycoprotein D gene: Duffy blood group Fya/Fyb alloantigen system is associated with a polymorphism at the 44-amino acid residue. Blood, 85, 622-6.

Kasehagen, L. J., I. Mueller, B. Kiniboro, M. J. Bockarie, J. C. Reeder, J. W. Kazura, W. Kastens, D. T. McNamara, C. H. King, C. C. Whalen & P. A. Zimmerman (2007) Reduced Plasmodium vivax erythrocyte infection in PNG Duffy-negative heterozygotes. PLoS One, 2, e336.

King, C. L., P. Michon, A. R. Shakri, A. Marcotty, D. Stanisic, P. A. Zimmerman, J. L. Cole-Tobian, I. Mueller & C. E. Chitnis (2008) Naturally acquired Duffy-binding protein-specific binding inhibitory antibodies confer protection from blood-stage Plasmodium vivax infection. Proceedings of the National Academy of Sciences of the United States of America, 105, 8363-8368.

Lima-Junior, J. C., J. Jiang, R. N. Rodrigues-da-Silva, D. M. Banic, T. M. Tran, R. Y. Ribeiro, V. S. Meyer, S. G. De-Simone, F. Santos, A. Moreno, J. W. Barnwell, M. R. Galinski & J. Oliveira-Ferreira (2011) B cell epitope mapping and characterization of naturally acquired antibodies to the Plasmodium vivax Merozoite Surface Protein-3α (PvMSP-3α) in malaria exposed individuals from Brazilian Amazon. Vaccine.

Maestre, A., C. Muskus, V. Duque, O. Agudelo, P. Liu, A. Takagi, F. B. Ntumngia, J. H. Adams, K. L. Sim, S. L. Hoffman, G. Corradin, I. D. Velez & R. Wang (2010) Acquired antibody responses against Plasmodium vivax infection vary with host genotype for duffy antigen receptor for chemokines (DARC). PLoS One, 5, e11437.

Mallinson, G., K. S. Soo, T. J. Schall, M. Pisacka & D. J. Anstee (1995) Mutations in the erythrocyte chemokine receptor (Duffy) gene: the molecular basis of the Fya/Fyb antigens and identification of a deletion in the Duffy gene of an apparently healthy individual with the Fy(a-b-) phenotype. Br J Haematol, 90, 823-9.

Mangold, K., R. Manson, E. Koay, L. Stephens, M. Regner, R. J. Thomson, L. Peterson & K. Kaul (2005) Real-time PCR for detection and identification of Plasmodium spp. J Clin Microbiol, 43, 2435-40.

Michon, P., T. Fraser & J. H. Adams (2000) Naturally acquired and vaccine-elicited antibodies block erythrocyte cytoadherence of the Plasmodium vivax Duffy binding protein. Infection and Immunity, 68, 3164-3171.

Miller, L., S. Mason, D. Clyde & M. McGinniss (1976) The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med, 295, 302-4.

Moise, K. J. (2000) Non-anti-D antibodies in red-cell alloimmunization. Eur J Obstet Gynecol Reprod Biol, 92, 75-81.

Ménard, D., C. Barnadas, C. Bouchier, C. Henry-Halldin, L. R. Gray, A. Ratsimbasoa, V. Thonier, J. F. Carod, O. Domarle, Y. Colin, O. Bertrand, J. Picot, C. L. King, B. T. Grimberg, O. Mercereau-Puijalon & P. A. Zimmerman (2010) Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people. Proc Natl Acad Sci U S A, 107, 5967-71.

Palha, T. D., E. M. Ribeiro-Rodrigues, A. Ribeiro-Dos-Santos, J. F. Guerreiro, L. S. de Moura & S. Santos (2010) Male ancestry structure and interethnic admixture in African-descent communities from the Amazon as revealed by Y-chromosome Strs. Am J Phys Anthropol.

Perna, S. J., G. L. Cardoso & J. F. Guerreiro (2007) Duffy blood group genotypes among African-Brazilian communities of the Amazon region. Genet Mol Res, 6, 166-72.

Reich, D., M. A. Nalls, W. H. Kao, E. L. Akylbekova, A. Tandon, N. Patterson, J. Mullikin, W. C. Hsueh, C. Y. Cheng, J. Coresh, E. Boerwinkle, M. Li, A. Waliszewska, J. Neubauer, R. Li, T. S. Leak, L. Ekunwe, J. C. Files, C. L. Hardy, J. M. Zmuda, H. A. Taylor, E. Ziv, T. B. Harris & J. G. Wilson (2009) Reduced neutrophil count in people of African descent is due to a regulatory variant in the Duffy antigen receptor for chemokines gene. PLoS Genet, 5, e1000360.

Ryan, J. R., J. A. Stoute, J. Amon, R. F. Dunton, R. Mtalib, J. Koros, B. Owour, S. Luckhart, R. A. Wirtz, J. W. Barnwell & R. Rosenberg (2006) Evidence for transmission of Plasmodium vivax among a duffy antigen negative population in Western Kenya. Am J Trop Med Hyg, 75, 575-81.

Smolarek, D., O. Bertrand, M. Czerwinski, Y. Colin, C. Etchebest & A. G. de Brevern (2010) Multiple interests in structural models of DARC transmembrane protein. Transfus Clin Biol, 17, 184-96.

Sousa, T., B. Sanchez, I. Cerávolo, L. Carvalho & C. Brito (2007) Real-time multiplex allele-specific polymerase chain reaction for genotyping of the Duffy antigen, the Plasmodium vivax invasion receptor. Vox Sang, 92, 373-80.

Souza-Silva, F. A., M. da Silva-Nunes, B. A. Sanchez, I. P. Ceravolo, R. S. Malafronte, C. F. Brito, M. U. Ferreira & L. H. Carvalho (2010) Naturally acquired antibodies to Plasmodium vivax Duffy binding protein (DBP) in Brazilian Amazon. Am J Trop Med Hyg, 82, 185-93.

Tjitra, E., N. M. Anstey, P. Sugiarto, N. Warikar, E. Kenangalem, M. Karyana, D. A. Lampah & R. N. Price (2008) Multidrug-resistant Plasmodium vivax associated with severe and fatal malaria: A prospective study in Papua, Indonesia. Plos Medicine, 5, 890-899.

Tournamille, C., Y. Colin, J. P. Cartron & C. Le Van Kim (1995) Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nat Genet, 10, 224-8.

Walton, R. T. & S. L. Rowland-Jones (2008) HIV and chemokine binding to red blood cells--DARC matters. Cell Host Microbe, 4, 3-5.

Weinstein, L. & E. S. Taylor (1975) Hemolytic disease of the neonate secondary to anti-Fya. Am J Obstet Gynecol, 121, 643-5.

Table 1

Demographical, epidemiological and immunological data of 687 individuals enrolled in the study (November 2008 – November 2009)

Characteristic	n = 687
Median age, years (range)	23 (0.17-90)
Gender, male:female	1.36:1
Malaria infection, n (%)[a]	
P.vivax	62 (9%)
P.falciparum	7 (1%)
Total	69 (10%)
Years of malaria exposure, median (range)	20 (0.17-90)
Previous malaria episodies, mean (range)	4 (0-73)
Antibodies, n=597 (%)[b]	
Anti-PvDBP	327 (54.7)
Anti-Pv $MSP1_{19}$	338 (56.6)

[a]Malaria infection was detected by conventional microscopy and/or real-time PCR between November 2008- November 2009.

[b]Number (%) of individuals with a positive antibody response at the time of their first blood-collection (individual baseline).

Table 2

Study population data according to DARC genotype[1]

Genotype n = 687	*FY*A / FY*B^{ES}*	*FY*A / FY*A*	*FY*A / FY*B*	*FY*B / FY*B^{ES}*	*FY*B / FY*B*
n (%)	216[a] (31.4)	138[b] (20.1)	183[b] (26.6)	64[c] (9.4)	82[c] (12.0)
Median age (range)	25.0 (0.2-68)	21.0 (0.5-90)	20.0 (0.6-87)	25.5 (0.25-69)	28.0 (0.3-74)
Gender (M:F)	1.4:1	1.3:1	1.2:1	1.8:1	1.5:1
Median years in area (range)	21 (0.17-68)	17 (0.5-90)	18.5 (0.17-85)	24 (0.25-63)	23 (0.3-63)

[1] Four (0.6%) adults males carrying the DARC negative genotype (*FY*B / FY*B^{ES}*); consequently, the low frequency of DARC negative genotype precluded any statistical comparison among this group and others genotypes.

[a-c] Different letters indicate significant difference to the level of 5% (χ^2 Yates analysis). There were no statistical differences between DARC genotypes and demographical (age/gender) and epidemiological variables (time/local of residence in the area).

Table 3

DARC genotyping of Plasmodium vivax-infected and non-vivax individuals based on real-time multiplex allele-specific

Phenotype (%)			Genotype (%)[c]			Allele (frequency)[c]	
	Pv ind[a] (n=62)	Pf/NI ind[b] (n=625)		*Pv* ind (n=62)	Pf/NI ind (n=625)	*Pv* ind (n=62)	Pf/NI ind (n=619)
Fy (a+b-)	56.4	51.0	*FY*A / FY*A*	27.4	19.4	*FY*A*	0.56	0.49
			*FY*A / FY*B^{ES}*	29.0	31.7	*FY*B*	0.26	0.30
Fy (a-b+)	16.1	21.7	*FY*B / FY*B*	8.1	12.3	*FY*B^{ES}*	0.18	0.21
			*FY*B / FY*B^{ES}*	8.1	9.4	*FY*A^{ES}*	0	0
Fy (a+b+)	27.5	26.5	*FY*A / FY*B*	27.4	26.6			
Fy (a-b-)	0	0.6	*FY*B^{ES} / FY*B^{ES}*	0	0.6			

[a]*Plasmodium vivax*-infected individuals (November 2008-November 2009).

[b]Pf/NI, includes *P. falciparum*-infected and non-infected individuals from the same study population. Regarding DARC genotypes, there was no statistical difference between groups (Pv vs. Pf/NI individuals)

[c]No statistical difference could be detected between groups (Pv vs. Pf/NI individuals); however the presence of *FY*A* allele was significantly higher in both groups of individuals (χ^2 analysis, p= 0.0001)

Table 4

Annual incidence rates of *P.vivax* infection stratified by DARC genotype in the study area, Rio Pardo, November 2008-November 2009

Genotype	*P.vivax* annual incidence		
	N[a]	Number with Pv infection[b]	Rates (cases/1000 persons-month)
Fy^a/Fy^{ES}	1416	28	19,8
Fy^a/Fy^a	954	24	25,2
Fy^a/Fy^b	1164	31	26,6
Fy^b/Fy^{ES}	480	16	33,3
Fy^b/Fy^b	546	10	18,3
Overall	4560	106	23,2

[a] 484 individuals had paired samples and they contributed for the 4560 person-month of follow-up.

[b] Number of individuals with *P.vivax* infection, detected by microscopy and/or real-time PCR.

Table 5

Antibody responses against PvDBP and $PvMSP1_{19}$ according to DARC genotype among 591 subjects of the study population

DARC Genotype	Anti-PvDBP		Anti-$PvMSP1_{19}$	
	Positive (%)[a]	IR[b] (median ± SD)	Positive (%)	(IR median ± SD)
FYA/ FYA (n=109)	56 (51.4)	0.910 ± 2.16	62 (56.9)	1.180 ± 3.59
FYA/ FYB^{ES} (n=198)	107 (54.0)	0.885 ± 1.93	107 (54.0)	0.860 ± 3.14
FYB/ FYB (n=71)	41 (57.7)	0.990 ± 1.26	37 (52.1)	0.920 ± 2.01
FYB/ FYB^{ES} (n=59)	38 (64.4)	1.100 ± 2.6	39 (66.1)	0.982 ± 3.65
FYA/ FYB (n=154)	80 (52.0)	0.895 ± 2.1	88 (57.0)	0.889 ± 2.98

[a] Number of individuals who had antibodies against PvDBP or PvMSP119, as detected by ELISA using recombinant proteins.

[b] IR= Index of Reactivity (IR = OD492 values of test sample divided by the value of the cut-off, as described in material and methods).

Figure 1

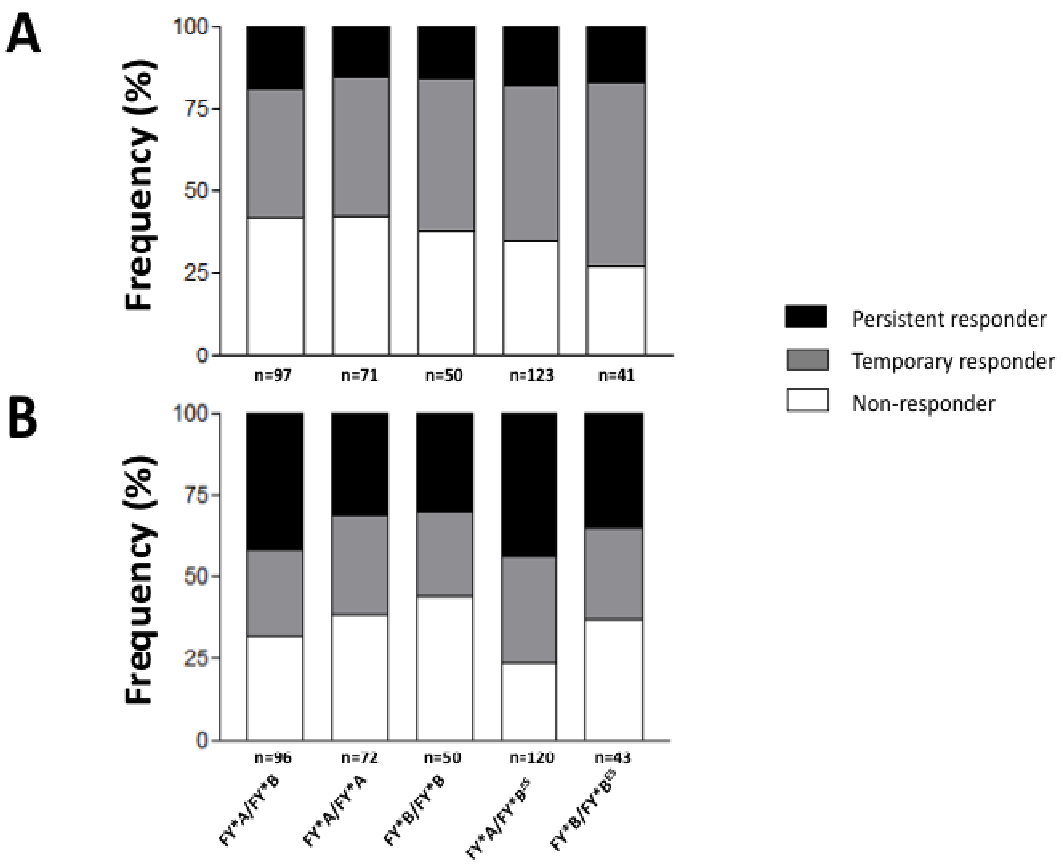
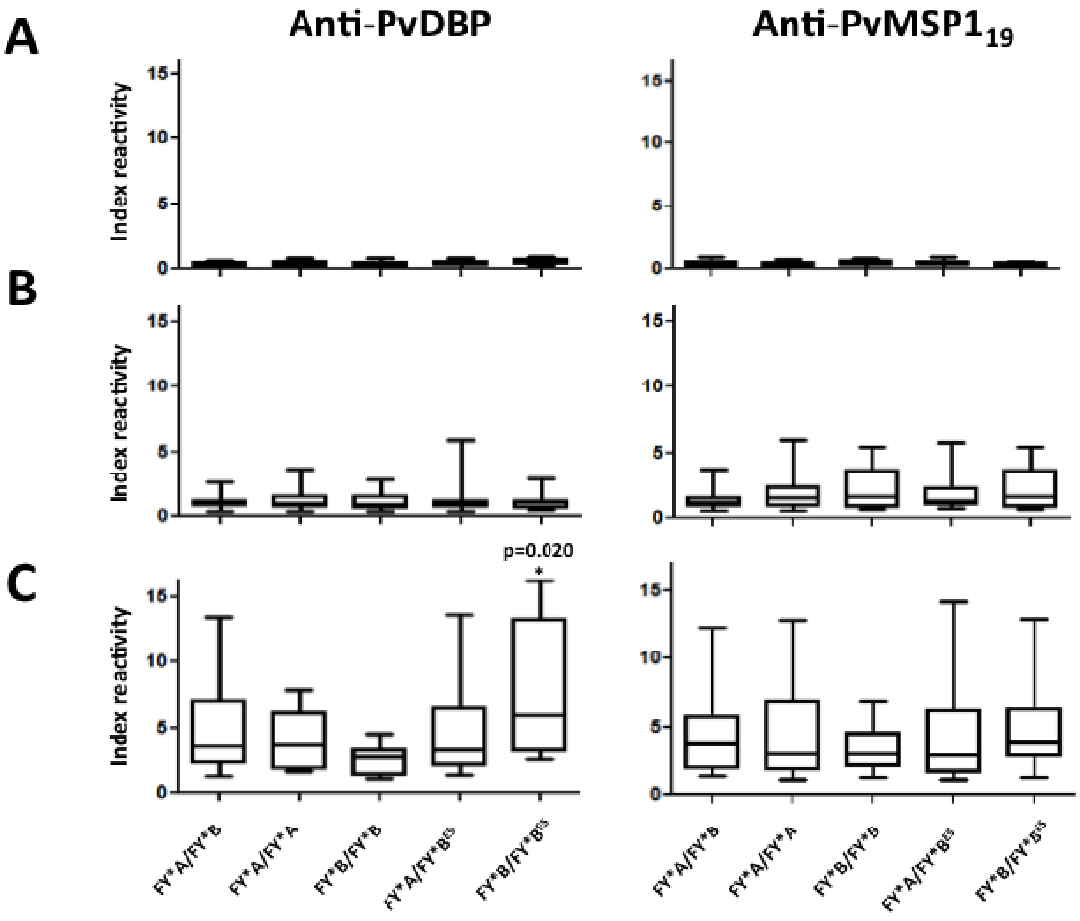

A
B
Frequency (%)
100
75
50
25
0
n=97
n=71
n=50
n=123
n=41
n=96
n=72
n=50
n=120
n=43
FY*A/FY*B
FY*A/FY*A
FY*B/FY*B
FY*A/FY*B^ES
FY*B/FY*B^ES
Persistent responder
Temporary responder
Non-responder

Figure 2


A
Anti-PvDBP
Anti-PvMSP1$_{19}$
B
C
Index reactivity
p=0.020
*
FY*A/FY*B
FY*A/FY*A
FY*B/FY*B
FY*A/FY*B^{ES}
FY*B/FY*B^{ES}

Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2009.03931.x

Naturally acquired inhibitory antibodies to *Plasmodium vivax* Duffy binding protein are short-lived and allele-specific following a single malaria infection

I. P. Ceravolo,* B. A. M. Sanchez,* T. N. Sousa,* B. M. Guerra,* I. S. Soares,† E. M. Braga,‡ A. M. McHenry,§ J. H. Adams,§ C. F. A. Brito* and L. H. Carvalho*

*Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, FIOCRUZ, Belo Horizonte, Minas Gerais, †Instituto de Ciências Biológicas, Universidade de São Paulo, São Paulo, SP, ‡Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, and §University of South Florida, Tampa, FL, USA

Accepted for publication 9 March 2009
Correspondence: L. H. Carvalho, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Malaria Laboratory, Av. Augusto de Lima 1715, 30190-002, Belo Horizonte, MG, Brazil.
E-mail: lhcarvalho@cpqrr.fiocruz.br

Summary

The Duffy binding protein of *Plasmodium vivax* (DBP) is a critical adhesion ligand that participates in merozoite invasion of human Duffy-positive erythrocytes. A small outbreak of *P. vivax* malaria, in a village located in a non-malarious area of Brazil, offered us an opportunity to investigate the DBP immune responses among individuals who had their first and brief exposure to malaria. Thirty-three individuals participated in the five cross-sectional surveys, 15 with confirmed *P. vivax* infection while residing in the outbreak area (cases) and 18 who had not experienced malaria (non-cases). In the present study, we found that only 20% (three of 15) of the individuals who experienced their first *P. vivax* infection developed an antibody response to DBP; a secondary boosting can be achieved with a recurrent *P. vivax* infection. DNA sequences from primary/recurrent *P. vivax* samples identified a single *dbp* allele among the samples from the outbreak area. To investigate inhibitory antibodies to the ligand domain of the DBP (cysteine-rich region II, DBP_{II}), we performed *in vitro* assays with mammalian cells expressing DBP_{II} sequences which were homologous or not to those from the outbreak isolate. In non-immune individuals, the results of a 12-month follow-up period provided evidence that naturally acquired inhibitory antibodies to DBP_{II} are short-lived and biased towards a specific allele.

Keywords: allele-specific, antibody response, duffy binding protein, malaria, *Plasmodium vivax*

Introduction

The Duffy binding protein of *Plasmodium vivax* (DBP) is a critical adhesion ligand that participates in merozoite invasion of human Duffy/Duffy antigen receptor for chemokines (DARC)-positive erythrocytes [1,2]. DBP belongs to a family of homologous Duffy binding-like erythrocyte binding proteins (DBL–EBP) located within the micronemes of *P. vivax* and *P. knowlesi* merozoites [3]. The functional binding domains of DBL–EBP lie in region II, and for *P. vivax* the critical binding residues have been mapped to a central 170-amino acid stretch that includes cysteines 5–8 [4–6]. The gene encoding the *P. vivax* DBP region II (DBP_{II}) is highly polymorphic, and this diversity varies geographically from region to region [7–13]. The pattern of excessive polymorphism is consistent with a high selection pressure on the DBP gene and suggests that allelic variation functions as a mechanism of immune evasion [14,15].

Invasive merozoites are believed to sequester microneme proteins until merozoites contact the target erythrocyte, presumably as a mechanism to reduce exposure of DBP to immune inhibition [16]. Currently, available data on humoral immune responses to DBP in human populations demonstrate that anti-DBP antibodies increase with exposure to *P. vivax* [17–20], and this immune response includes antibody activity that blocks adherence of DBP_{II} to its receptor on erythrocytes [18,21]. The same antibodies that block the DBP_{II}–DARC interaction also inhibit *P. vivax* erythrocyte invasion [22], which is proof-of-concept that anti-PvDBP antibodies can inhibit merozoite invasion. Of importance, children residing in hyperendemic areas for *P. vivax* develop anti-DBP inhibitory antibodies that seem to confer protection against blood-stage infection [23].

As most studies on the DBP antibody response reported to date have been carried out in areas where malaria is highly endemic, there is a scarcity of data on the responses to exposure to a single infection and about the persistence of this antibody response in the absence of reinfection. An outbreak of *P. vivax* malaria, in a village located in a non-malarious area of Brazil, offered us an opportunity to

investigate the DBP immune response among individuals who had their first and brief exposure to malaria. In the outbreak area, we hypothesized that a first exposure to *P. vivax* malaria induces an anti-DBP antibody response that blocks the interaction between DBP and its receptor on erythrocyte. To analyse this neutralizing antibody response, we used an *in vitro* cytoadherence assay that uses the putative ligand domain of the DBP (region II, DBP_{II}) expressed on the surface of cultivated mammalian cells [18]. To investigate whether neutralizing antibodies recognize DBP_{II} in a strain-specific manner, we analysed polymorphisms within the critical binding motif of *P. vivax* DBP_{II} from the outbreak isolates, and performed inhibition of cytoadherence assays with DBP_{II} sequences which are homologous or not to that from the outbreak area. In this study, carried out with non-immune individuals, we provide evidence that naturally acquired neutralizing antibodies to DBP_{II} can be strain-specific and are relatively short-lived in the absence of reinfection.

Materials and methods

The *P. vivax* malaria outbreak

Between April and May 2003, 25 cases of *P. vivax* malaria were diagnosed for the first time in a small community, Souza, located 70 km from Belo Horizonte, Minas Gerais State, a non-endemic area of Brazil [24,25]. Malaria has never been reported in this area and the Brazilian endemic region, the Amazon area, is 2000 km away. According to the Minas Gerais Department of Health, the source of the infection was a man from the community who had returned from the Amazon, infected by *P. vivax*, in January 2003. The subsequent outbreak in Souza began in April 2003, and entomological surveys incriminated the vector *Anopheles darlingi* as responsible for local malaria transmission [24]. The first human malaria case detected in the outbreak area, named S14, remained at the hospital for about 10 days, until a malaria diagnosis could be established. Because malaria infection had never been reported in the outbreak area previously, the physicians failed to consider malaria on presentation of this patient. After the first case, all patients were treated promptly with chloroquine (1·5 g for 3 days) plus primaquine (30 mg daily for 7 days), and a second round of treatment was given in case of relapses and/or recrudescence (3-day course of chloroquine and 15-day course of primaquine). Control activities also included an active search for acute malaria by thick blood smears and outdoor/indoor spraying of residual insecticide (cypermethrine) [25]. The outbreak was considered of short duration (50–60 days), with the last malaria case diagnosed on 21 May 2003; since then, local/regional Departments of Health have maintained entomological and epidemiological surveillance of the area.

Table 1. Demographic, immunological and genetic data of individuals who had been enrolled in the study carried out in the *Plasmodium vivax* malaria outbreak area.

Characteristics	Cases ($n = 15$)	Non-cases ($n = 18$)
Age, years (mean ± s.d.)*	32 ± 13	34 ± 19
Antibody response, *n* (%)†		
MSP1-19	12 (80%)	0 (0%)
DBP_{II-IV}	3 (20%)	0 (0%)
DARC functional alleles, *n* (%)‡		
One (*Fy*A* or *Fy*B*)	6 (40%)	6 (33%)
Two (*Fy*A* and/or *Fy*B*)	9 (60%)	12 (67%)
None (Fy^*B^{ES})§	0	0

*Difference not significant ($t = 0{\cdot}02$, $P > 0{\cdot}05$). †Number (%) of individuals with a positive antibody response at the time of first cross-sectional survey. ‡The frequencies of individuals bearing the functional alleles *Fy*A* and *Fy*B* (Fy^a and Fy^b antigens on erythrocytes respectively) were similar between cases and non-cases ($P > 0{\cdot}05$). §Homozygosis for the FY^*B^{ES} (*ES*, erythroid silent) allele abrogates Duffy antigen receptor for chemokines (DARC) antigen expression on the erythrocyte surface, and designates the DARC negative phenotype. DBP, Duffy binding protein; MSP, merozoite surface protein-1; s.d., standard deviation.

Volunteers and blood collection

Cross-sectional surveys were carried out after discussions with the community about the objectives of the project and its protocols. Individuals who had been infected with *P. vivax* were enrolled in the study if they met the following criteria: (i) informed written consent in accordance with guidelines for human research, as specified by the Brazilian National Council of Health (Resolution 196/96); (ii) residence in the outbreak area; (iii) a minimum age of 15 years; (iv) if women, an indicator of the absence of pregnancy; and (v) a willingness to remain in the outbreak area during the intervening year. As shown in Table 1, a total of 15 individuals met the inclusion criteria (aged 32 ± 13 years). We also included relatives and neighbours who were considered to be exposed to the risk of infection ($n = 18$; 34 ± 19 years). The latter group had had neither symptoms nor blood parasites by direct examination of Giemsa-stained thick smears. Of the 33 volunteers, 32 did not recall previous history of malaria, temporary residence in malaria-endemic areas or travel to the endemic area during their lifetime. A single volunteer with confirmed malaria in the outbreak area (S1) recalled previous malaria infection, temporary residence in the endemic (gold-mining) area, and travelling to the Amazon during the 6 months preceding the outbreak. We collected 5 ml blood samples (ethylenediamine tetraacetic acid) from all subjects. At the time of blood collection, Giemsa-stained thick blood smears were examined for parasites and nested polymerase chain reaction (PCR) assays for malaria diagnosis were conducted later in our laboratory. Blood samples were used to obtain plasma and for DNA preparation. Three, 6, 9 and 12

months after the first survey, four other identical cross-sectional surveys were carried out. The ethical and methodological aspects of this study were approved by the Ethical Committee of Research on Human Beings from the Centro de Pesquisas René Rachou/FIOCRUZ (Reports 002/2002 and 07/2006), according to the Resolution of the Brazilian Council on Health-CNS 196/96.

Microscopy and *Plasmodium* diagnosis by nested PCR

Well-trained microscopists examined 200 fields of Giemsa-stained thick blood smears. DNA was extracted from 300 µl of individual whole-blood samples by using a genomic DNA purification kit (Puregene, Gentra Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. Parasite species identification was performed by nested PCR amplification of the small subunit ribosomal RNA (18S SSU rRNA) genes, as described previously [26].

The DARC genotyping

Extracted DNA was used to detect the three common alleles at the *FY* locus – *FY*A*, *FY*B*, *FY*B*^{ES} (*ES*, erythroid silent) – using real-time PCR with allele-specific primers, essentially as we have described recently [27].

Recombinant proteins and serological assay

Two recombinant *P. vivax* proteins were used to detect total immunoglobulin G (IgG) antibodies. The recombinant DBP, which includes amino acids 132–771 (regions II–IV, $DBP_{II–IV}$), was expressed as a soluble glutathione S transferase (GST) fusion protein of 140 kDa, as described previously [17,20]. The recombinant protein representing the 19-kDa C-terminal region of the merozoite surface protein-1 of *P. vivax* (MSP1-19), which represents amino acids 1616–1704 of the MSP1 of *P. vivax*, has been described elsewhere [28]. To assess IgG antibodies against $DBP_{II–IV}$ and MSP1-19, an enzyme-linked immunosorbent assay (ELISA) was carried out, as described previously [20], with serum samples at 1 : 100. For the recombinant proteins $DBP_{II–IV}$ (5 µg/ml) and MSP1-19 (1 µg/ml), the final optical density (OD) at 492 nm was calculated by subtracting the OD obtained with GST (antigen control). The results were expressed as an index of reactivity (IR = OD_{492} values of test sample divided by the value of the cut-off). Cut-off points were set at three standard deviations above the mean OD_{492} of sera from 30 individuals who had never been exposed to malaria. Values of IR > 1·0 were considered positive.

The *P. vivax* DBP_{II} amplification and sequencing

Extracted DNA was used as a template in the PCR to amplify the fragment corresponding to nucleotide positions 870–1545 (amino acids 290–515) of the DBP_{II}, as described previously [13]. Platinum high fidelity *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) was used in PCR to reduce possible nucleotide mis-incorporation. Amplicons were purified using the GFX-96 PCR kit (Amersham Biosciences, Little Chalfont, UK) and sequenced directly using DYEnamic™ ET dye terminator kit (Amersham Biosciences) and MegaBace 500 automated DNA sequencer (Amersham Biosciences). The sequences were analysed using Bioedit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to identify DBP_{II} polymorphisms relative to the SAL-1 sequence [29].

The DBP–pEGFP constructs

Region II of DBP (DBP_{II}) from a *P. vivax* laboratory reference clone (Sal-1) [29] has been subcloned previously into the pEGFP–N1 plasmid (Clontech, Mountain View, CA, USA), with a flanking signal sequence from the herpes simplex virus glycoprotein D1 (HSVgD1) [18]. This targets expression to the surface of the transfected COS cells as a green fluorescent protein (GFP) fusion protein. An additional GFP construct with the DBP_{II} sequence from the outbreak *P. vivax* isolate was made by subcloning a fragment corresponding to aa 198–522 of region II into pEGFP–HSVgD1 plasmid, using primers described previously [30]. Recombinant plasmids were purified by use of an endotoxin-free plasmid DNA purification system (Qiagen, Valencia, CA, USA).

COS cell transfection and erythrocyte-binding assays

Recombinant plasmids were transfected into COS-7 cells (American Type Culture Collection, Manassas, VA, USA) using lipofectamine and PLUS-reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocols. Briefly, COS-7 cells in six-well culture plates ($1{\cdot}5 \times 10^5$ cells/well) were transfected with plasmids (0·5 µg/well)–liposome complexes (5% plus-reagent and 3% lipofectamine) in Dulbecco's modified Eagle medium (DMEM; Sigma, St Louis, MO, USA) without serum. After 6 h of cell exposure to DNA-liposome complexes (37°C, 5% CO_2), transfection medium was replaced by DMEM with 10% of fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA). At 24 h after transfection, the efficiency of transfection was assessed by fluorescence; the recombinant protein expression levels were similar between the Sal-1 and outbreak DBP_{II} variants (data not shown). Forty-eight hours after transfection, the erythrocyte-binding assays were performed as described previously [21]. For this, anti-serum was added at 1 : 20 (1 h at 37°C, 5% CO_2) followed by incubation with 10% of human O^+ erythrocytes suspension (2 h, room temperature). Unbound erythrocytes were removed by washing and binding was quantified by counting rosettes (10–20 fields, 200×). Positive rosettes were defined as adherent erythrocytes covering more than 50% of the cell surface.

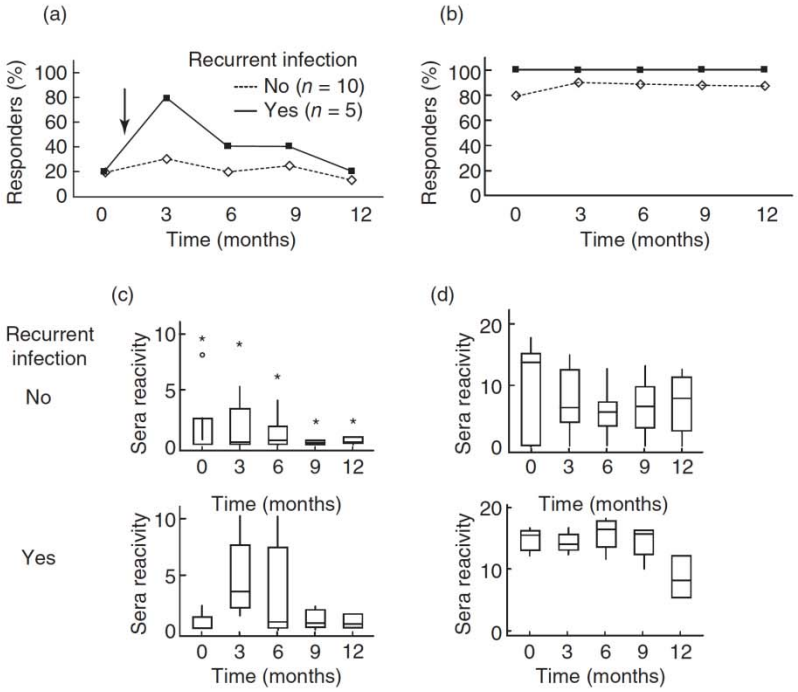


Fig. 1. Antibody responses to Duffy binding protein ($DBP_{II–IV}$) (a,c) and merozoite surface protein-1 (MSP1-19) (b,d) among individuals who had confirmed *Plasmodium vivax* infection in the outbreak area, and developed ($n = 5$) or not ($n = 10$) recurrent *P. vivax* infections (↓) during the 12-month follow-up period. In (a) and (b), the percentage of responders to $DBP_{II–IV}$ or MSP1-19, respectively, as detected by enzyme-linked immunosorbent assay (ELISA). In (c) and (d), box-plot representations of sera reactivity to $DBP_{II–IV}$ or MSP1-19 respectively; sera reactivity were expressed as index of reactivity (IR) at 492 nm, IR > 1 being considered positive. Box-plots: solid line across the box is the median, and the 25th and 75th percentiles were represented by the bottom and the top of each box respectively; the vertical lines represent the range, with outliers marked by asterisks (S14) or circle (S38).

For each assay, pooled plasma samples from Souza residents, characterized as non-responders by ELISA, were used as a negative control (100% binding). The percentage inhibition was calculated as $100 \times (Rc - Rt)/Rc$, where Rc is the average of the number of rosettes in the control wells and Rt is the average of the number of rosettes in the test wells.

Statistical analysis

Statistical analysis was performed using the Epi-Info 2002 software (CDC, Atlanta, GA, USA) or MiniTab statistical software (Minitab Inc., State College, PA, USA). Differences in means were tested by Student's t-test or one-way analysis of variance. Differences in proportions were evaluated by Yates's χ^2 or Fisher's exact tests. P-values < 0·05 were considered significant.

Results

Antibody responses to $DBP_{II–IV}$ and MSP1-19 at enrollment

Thirty-three individuals participated in the five cross-sectional surveys, 15 with previously confirmed *P. vivax* infection in the outbreak area (cases) and 18 who had not experienced malaria infection (non-cases). At the first cross-sectional survey, 20% (three of 15) of malaria cases had antibodies to $DBP_{II–IV}$ (Table 1); in contrast, the MSP1-19 was recognized initially by 80% (12 of 15) of these individuals. The remaining 18 individuals (non-case) did not develop a detectable antibody response against either anti-$DBP_{II–IV}$ or anti-MSP1-19. Although 'resistance' to vivax malaria would result from the lack of DARC glycoprotein on red blood cells, none of the individuals studied were homozygous for the allele FY^*B^{ES}. Therefore, we concluded that absence of DARC on RBCs was not responsible for refractoriness to *P. vivax* infection in this group (Table 1).

Relationship between malaria status and anti-DBP antibodies during a 12-month follow-up period

Although DBP was recognized initially by 20% of individuals who had had a *P. vivax* infection, a secondary boosting could be achieved with a new episode of malaria, making 80% into responders at this time (Fig. 1a). Nevertheless, in those individuals the frequency of responders decreased a few months after the clinical attack. By analysing the levels of anti-DBP antibodies during the 12-month follow-up, we observed a wide range of antibody responses among study participants (Fig. 1c), which made the difference between groups without statistical significance (recurrent *versus* no recurrent infection). Of interest, during the follow-up period, the levels of anti-DBP antibodies were relatively higher in a single individual (Fig. 1c, asterisk in each time-point of the follow-up); this result was not unexpected, because this patient (S14) remained at the hospital for about 10 days until a malaria diagnosis could be established. Despite individual variations, the levels of anti-DBP decreased markedly within the first 6 months of the follow-up.

Table 2. Variant amino acids in Duffy binding protein (DBPII) from the *Plasmodium vivax* outbreak isolates, compared with the *P. vivax* laboratory reference clone Sal-1.

	Codon position							
Isolate*	371	384	385	386	417†	424	437	503
Sal-1, reference clone	K	D	E	K	N	L	W	I
Outbreak								
Primary infection	E	G	K	N	K	I	R	K
Recurrent infection	E	G	K	N	K	I	R	K

*Sal-1 sequence, accession number: M61095; outbreak sequence, accession numbers: EU870443-EU870445. †Grey areas highlight polymorphisms in DBP_{II} that compromise efficiency of rabbit anti-DBP serum to inhibit DBP_{II}–Duffy antigen receptor for chemokines (DARC) interaction [7]. Also, analysis of 122 Brazilian *P. vivax* isolates demonstrates that residues 417 and 424 form part of a cluster surrounding the DARC-binding site (Sousa & Brito, unpublished results).

Altogether, eight of 15 (53%) malaria cases developed anti-DBP antibodies during the follow-up period. The serological response to MSP1-19 was distinctly different (Fig. 1b). Regardless of the occurrence of a relapse and/or recrudescence, the MSP1-19 was a relatively highly immunogenic protein for most individuals who had malaria, with 14 of 15 (93%) positives for anti-MSP1-19 IgG antibodies. However, decreasing levels of antibody reactivity to MSP1-19 was more evident in the group who did not develop a recurrent *P. vivax* infection (Fig. 1d). No one from the uninfected group (non-cases) developed antibodies against either anti-DBP or anti-MSP1-19 (data not shown).

Because the antibody response against DBP decreased few months after the clinical attack, we also investigated antibodies against another *P. vivax* apical antigen and vaccine candidate, the apical membrane antigen-1 (AMA-1) [31]. Our results demonstrated that although the profile of AMA-1 immune response was similar to that obtained with DBP, AMA-1 appears to be more immunogenic, with 53% (eight of 15) of responders at the beginning of the study and with all individuals converting into responders at the time of a new episode of malaria (see *Supporting information*, Fig. S1). However, the frequencies as well as the levels of anti-AMA-1 antibodies were lower in those individuals who did not develop a recurrent *P. vivax* infection (see *Supporting information*, Fig. S1b).

The DBP_{II} polymorphisms and inhibitory activity of naturally acquired anti-DBP antibodies

To characterize the *P. vivax* isolates responsible for the malaria outbreak, we analysed DNA sequences from primary and recurrent infections and identified a single *dbp* allele in the outbreak area (Table 2). This allele differed, at multiple codons, from the *P. vivax* laboratory reference clone Sal-1, including differences in three polymorphic codons (417, 437 and 503) suggested to play a synergistic functional effect on DBP_{II} inhibitory binding [7].

To investigate the specificities of the anti-DBP outbreak plasmas to inhibit the erythrocyte-binding function of the protein, we performed erythrocyte-binding assays using COS-7 cells expressing sequences of DBP_{II} which are identical or not to those of the outbreak isolate. Previously, this *in vitro* assay proved to be a suitable alternative tool for the live-cell invasion inhibition assay [22]. For that, plasma samples of those eight individuals who had developed conventional anti-DBP antibodies, at any time-point of the follow-up period, were tested for inhibition of DBP_{II}–DARC binding (Fig. 2). Three months after the first malaria attack, when the majority of responders were detected, seven of eight individuals had developed inhibitory antibodies against the homologous DBP_{II} sequence, while sera of two (S1 and S31) presented inhibitory activity against the heterologous sequence (Fig. 2a). Despite the occurrence of recurrent infections, most of these individuals lost their anti-DBP inhibitory antibody response within 6 months of follow-up. A single exception was an individual (S1) who had had previous malaria infection during frequent trips to the malaria-endemic area, and who developed inhibitory antibodies against homologous and heterologous DBP_{II} sequences. Beyond the frequency of response, the levels of inhibitory antibodies were also related to the DBP_{II} sequence; the greatest levels were observed with COS cells expressing the homologous DBP_{II} sequence, and no cross-reactivity could be detected at 1 : 40 sera dilution (Fig. 2b).

Discussion

Naturally occurring antibodies to DBP are prevalent in individuals living in areas where vivax malaria is endemic [17,19,20], and these antibodies can block the DBP_{II}–DARC interaction [18,21,23] and inhibit *P. vivax* erythrocyte invasion [22]. In previous studies, carried out in malaria-endemic areas, we and others have found strain-transcendent inhibitory responses to DBP_{II} [21,23]. However, those previous studies could not dismiss the possibility that DBP_{II} cross-variant inhibitory activity reflected only an accumulation of antibodies to strain-specific epitopes. Here, we have examined antibody responses of non-immune individuals after a brief initial malaria infection during a malaria outbreak outside the endemic area. Our study demonstrates that DBP has low immunogenicity

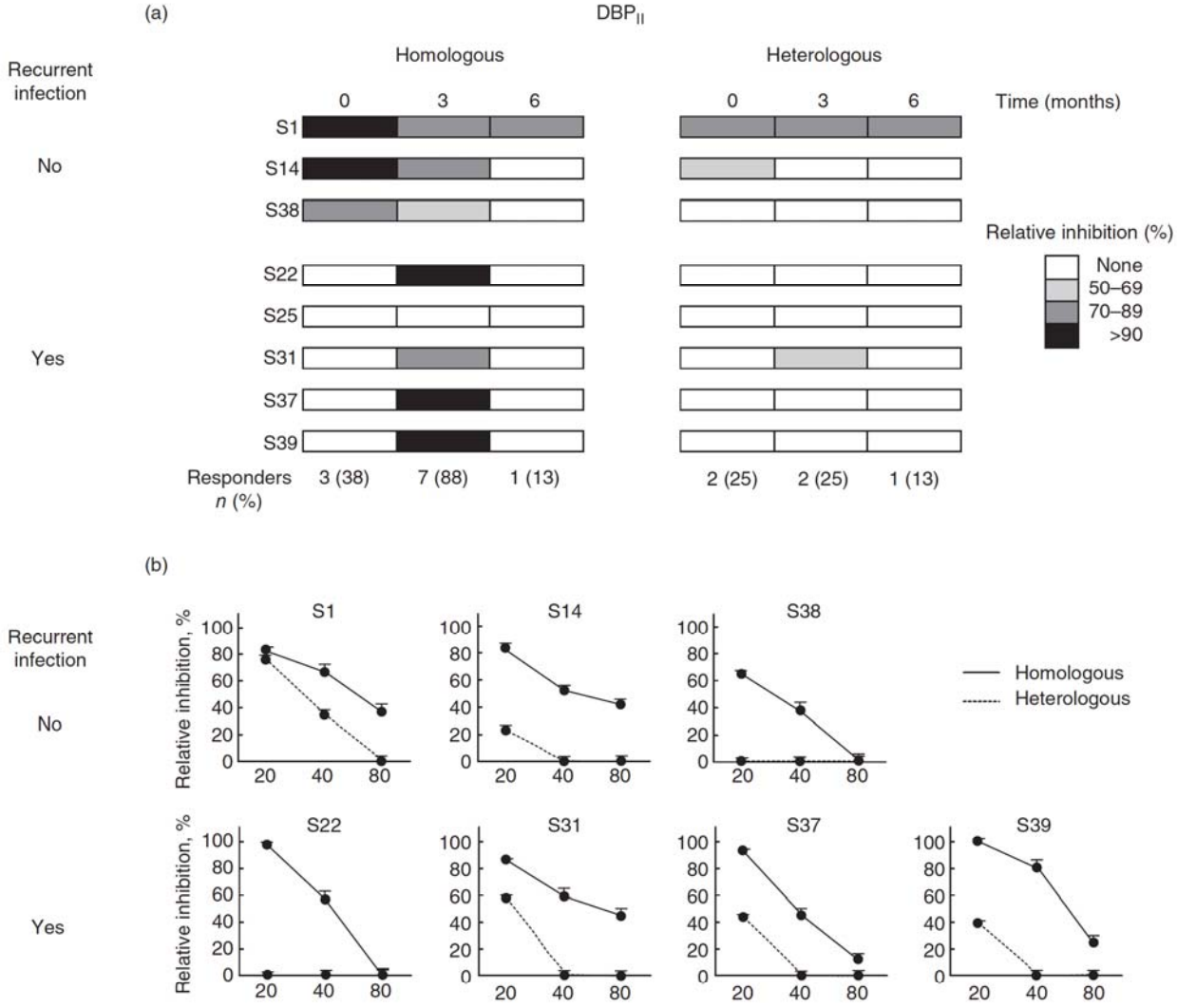


Fig. 2. Inhibition of Duffy binding protein–Duffy antigen receptor for chemokine (DBP_{II}–DARC) binding by outbreak plasma samples from eight individuals who developed conventional anti-DBP antibodies during the follow-up period. Erythrocyte binding assays were carried out with COS cells expressing the outbreak (homologous) or Sal-1 (heterologous) DBP_{II} sequences, and individual samples were grouped according to the occurrence (S22, S25, S31, S37, S39) or not (S1, S14, S38) of a recurrent *Plasmodium vivax* infection. (a) Results of each subject (at 1 : 20 plasma dilution), at enrollment (time zero), and 3 and 6 months later; values at the bottom of the figure represent the overall frequency of responders for each cross-sectional survey. (b) Titration of the inhibitory antibody responses against homologous or heterologous DBP_{II} sequences, using those seven positive samples from the second cross-sectional survey (3-month follow-up). The *x*-axis represents antibody titers.

and induces a short-term humoral immune response that is lost within 6 months of follow-up. In general, this profile of antibody response is similar to that detected to another apical *P. vivax* vaccine candidate, AMA-1, in which the ELISA positivity rates dropped during the first 9 months of follow-up (*Supporting information*). Nevertheless, during the follow-up period the frequencies of AMA-1 responders were usually higher than those obtained to DBP, especially among those individuals with recurrent *P. vivax* infections. The possibility that AMA-1 is more immunogenic than DBP could be explained by those findings demonstrating that AMA-1 is expressed in both pre-erythrocytic (sporozoite) and erythrocytic (merozoite) stages of malaria parasites [32], while DBP is a merozoite-specific protein.

Of importance, single *P. vivax* exposure appears to induce an anti-DBP inhibitory response that is biased towards a specific DBP allele. The longevity of this DBP inhibitory antibody response is different from that observed among some asymptomatic children residing in a *P. vivax* hyperendemic area [23], in which the anti-DBP inhibitory antibody response was remarkably stable over the 12-month follow-up period. Together, these findings suggest that vaccines based on DBP_{II} should consider short-term antibody responses in non-immune individuals.

The poorer, unstable antibody responses against DBP during the outbreak follow-up period is in contrast to the stronger, stable response to MSP1-19, which is a much more abundant blood-stage molecule than DBP. Regardless of the presence of recurrent *P. vivax* infections, the frequency of responders to MSP1-19 was similar at all five time-points, albeit at a lower magnitude in those without recurrent *P. vivax* infections, as described previously [33]. This longer-term stability of antibodies against *P. vivax* MSP1 has been well documented [34,35], including its persistence for 30 years after malaria exposure [36]. In none of the study individuals did the absence of DARC on erythrocytes play a role in the anti-DBP or anti-MSP1-19 responses.

In the Souza community, where the outbreak occurred, the period of malaria transmission was short (approximately 50 days), being interrupted by treatment of all patients with anti-malarial drugs (chloroquine and primaquine) and the comprehensive spraying of residual insecticide [25]. Considering that the control intervention of the outbreak was so thorough, the origin of the second attack of *P. vivax* in five individuals, about 2 months after the first malaria episode, is unclear. Typically, these infections may have had two origins: (i) a recrudescence originating from asexual blood-stage parasites that survived drug treatment; or (ii) a relapse arising from the dormant liver stages, hypnozoites [37]. The recurrences for the *P. vivax* appear to be more probably relapses, as treatment regimens used in the outbreak area were effective in clearing parasitaemias and there was a long period until the blood-stage infections reappeared. To analyse whether the isolate causing the secondary attack was genetically different from the isolate of initial infections, we compared DNA sequences from primary and recurrent *P. vivax* infections. Molecular analysis demonstrated that a single *dbp* allele was detected in the outbreak area (GenBank Accession numbers: EU870443–EU870445). The *dbp* outbreak allele belongs to allelic family VII, one of the eight DBP_{II} variant families identified in a preliminary analysis of 40 *P. vivax* Brazilian isolates [13].

Although the activation of heterologous hypnozoite populations seems to be the most common cause of relapse in patients with vivax malaria [38,39], the presence of a single *dbp* outbreak allele is consistent with either a relapse or a recrudescence. In contrast to previous studies in Asia, the *P. vivax* transmission in the outbreak area originated from a single patient who had had a *P. vivax* relapse after returning from the Amazon area [24,25]. In fact, our results are similar to a previous study of *P. vivax* relapses in Brazil which demonstrated, using the MSP1 molecule as a genetic marker, that parasites from the primary attack were identical to those in relapses [40].

An important finding of our study is the discovery of how parasite genetic diversity relates to naturally acquired neutralizing antibodies against DBP. The results demonstrated that the phylogenetically distant Sal-1 variant was significantly less sensitive to immune inhibition of its DARC binding activity than was the homologous effect against the DBP_{II} allele of the outbreak variant. Significant antibody cross-reactivity was observed in a single individual (S1), a result which was attributed to past cured infections in a gold-mine worker who had a history of previous malaria illnesses in a malaria-endemic area. Although it is not possible at this time to characterize the fine specificity of the inhibitory anti-DBP antibodies, these data demonstrate that variation in few polymorphic residues compromising the inhibitory efficacy of these antibodies. Further work will be necessary to identify the main epitopes recognized by naturally acquired antibodies to DBP in humans.

Altogether, our results indicate that polymorphisms change DBP antigenic character and can compromise immune inhibition, as suggested previously using rabbit immune sera [7]. Of importance, the outbreak and Sal-1 alleles do not share the trio of polymorphic residues (at codons 417, 437 and 503) shown to collectively alter sensitivity to inhibitory antibodies. Overall, these results point towards strain specificity in the natural immune response against DBP. Consistent with this hypothesis, the only individuals in the Amazon area who were observed previously to acquire anti-DBP antibodies that inhibit binding of different DBP_{II} variants to erythrocytes were people who had had long-term exposure [21]. Consequently, it is not surprising that only 9% of asymptomatic children residing in a *P. vivax* hyperendemic area had acquired a significant anti-DBP inhibitory antibody response that transcended strain-specificity [23].

Even though the current data demonstrate that individuals exposed briefly to *P. vivax* developed anti-DBP antibodies which exert a receptor-blocking effect, the magnitude of the inhibitory antibody response was very low compared with that from individuals with long-term exposure to malaria; in the outbreak area, inhibitory activity was achieved with immune sera diluted up to 1 : 80, whereas in the Amazon endemic area inhibitory antibodies could still be detected at a 1 : 1280 sera dilution [21]. It is possible that the low levels of immune response in the outbreak area could be due to the short and brief exposure to parasite blood stages. In fact, in this area, a secondary antibody boost was achieved with a recurrent *P. vivax* infection. Also, our previous data in the Amazon area indicate accumulative exposure to *P. vivax* as the strongest predictor of the presence of anti-DBP antibodies [21]. None the less, it is currently unclear how effective such natural antibody responses may be in preventing disease in this population. A long-term prospective study in a non-immune population is needed to determine the protective nature of the inhibitory anti-DBP_{II} antibodies in terms of anti-disease immunity.

Recently, it has been predicted that the hypervariable region of DBP_{II} is located on sites remote from the DARC binding site, implying that polymorphism cannot alter the capacity of the protein to bind DARC-positive erythrocytes [41,42]. Another line of evidence suggests that those few

polymorphic residues surrounding the DARC binding domain might elude binding of inhibitory antibody [7,16]. The second model seems to explain why antibodies to DBP can inhibit reticulocyte invasion by *P. vivax* effectively [22]. The results presented here provide strong evidence that the DARC and antibody binding sites have sufficient overlap for antibodies to inhibit binding and provide support for the role of allelic diversity in anti-DBP immune responses.

Acknowledgements

We thank Vania D. A. Cerbino (Minas Gerais Regional Department of Health) and other members of the local malaria control team from the Minas Gerais State Secretariat of Health and Sanitation for their logistic support in the fieldwork. This work is supported by the Brazilian National Research Council (CNPq) and Fundação Oswaldo Cruz, FIOCRUZ, PAPES IV (CNPq-400086/2006-9), Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG-CBB-880/06). L. H. C. is supported by a research fellowship from CNPq.

References

1 Wertheimer SP, Barnwell JW. *Plasmodium vivax* interaction with the human Duffy blood group glycoprotein: identification of a parasite receptor-like protein. Exp Parasitol 1989; **69**:340–50.

2 Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med 1976; **295**:302–4.

3 Adams JH, Hudson DE, Torii M *et al.* The Duffy receptor family of *Plasmodium knowlesi* is located within the micronemes of invasive malaria merozoites. Cell 1990; **63**:141–53.

4 Ranjan A, Chitnis CE. Mapping regions containing binding residues within functional domains of *Plasmodium vivax* and *Plasmodium knowlesi* erythrocyte-binding proteins. Proc Natl Acad Sci USA 1999; **96**:14067–72.

5 Singh SK, Singh AP, Pandey S, Yazdani SS, Chitnis CE, Sharma A. Definition of structural elements in *Plasmodium vivax* and *P. knowlesi* Duffy-binding domains necessary for erythrocyte invasion. Biochem J 2003; **374**:193–8.

6 VanBuskirk KM, Sevova E, Adams JH. Conserved residues in the *Plasmodium vivax* Duffy-binding protein ligand domain are critical for erythrocyte receptor recognition. Proc Natl Acad Sci USA 2004; **101**:15754–9.

7 VanBuskirk KM, Cole-Tobian JL, Baisor M *et al.* Antigenic drift in the ligand domain of *Plasmodium vivax* duffy binding protein confers resistance to inhibitory antibodies. J Infect Dis 2004; **190**:1556–62.

8 Tsuboi T, Kappe SH, al-Yaman F, Prickett MD, Alpers M, Adams JH. Natural variation within the principal adhesion domain of the *Plasmodium vivax* duffy binding protein. Infect Immun 1994; **62**:5581–6.

9 Xainli J, Adams JH, King CL. The erythrocyte binding motif of *Plasmodium vivax* duffy binding protein is highly polymorphic and functionally conserved in isolates from Papua New Guinea. Mol Biochem Parasitol 2000; **111**:253–60.

10 Cole-Tobian J, King CL. Diversity and natural selection in *Plasmodium vivax* Duffy binding protein gene. Mol Biochem Parasitol 2003; **127**:121–32.

11 Ampudia EM, Patarroyo A, Patarroyo ME, Murillo LA. Genetic polymorphism of the Duffy receptor binding domain of *Plasmodium vivax* in Colombian wild isolates. Mol Biochem Parasitol 1996; **78**:269–72.

12 Kho WG, Chung JY, Sim EJ, Kim DW, Chung WC. Analysis of polymorphic regions of *Plasmodium vivax* Duffy binding protein of Korean isolates. Korean J Parasitol 2001; **39**:143–50.

13 Sousa TN, Ceravolo IP, Fontes CJF, Couto A, Carvalho LH, Brito CF. The pattern of major polymorphisms in the Duffy binding protein ligand domain among *Plasmodium vivax* isolates from the Brazilian Amazon area. Mol Biochem Parasitol 2006; **146**:251–4.

14 Cole-Tobian J, King CL. Diversity and natural selection in *Plasmodium vivax* Duffy binding protein gene. Mol Biochem Parasitol 2003; **127**:121–32.

15 Martinez P, Suarez CF, Cardenas PP, Patarroyo MA. *Plasmodium vivax* Duffy binding protein: a modular evolutionary proposal. Parasitology 2004; **128**:353–66.

16 McHenry AM, Adams JH. The crystal structure of *P. knowlesi* DBPalpha DBL domain and its implications for immune evasion. Trends Biochem Sci 2006; **31**:487–91.

17 Fraser T, Michon P, Barnwell JW *et al.* Expression and serologic activity of a soluble recombinant *Plasmodium vivax* Duffy binding protein. Infect Immun 1997; **65**:2772–7.

18 Michon P, Fraser T, Adams JH. Naturally acquired and vaccine-elicited antibodies block erythrocyte cytoadherence of the *Plasmodium vivax* Duffy binding protein. Infect Immun 2000; **68**:3164–71.

19 Xainli J, Cole-Tobian JL, Baisor M *et al.* Epitope-specific humoral immunity to *Plasmodium vivax* Duffy binding protein. Infect Immun 2003; **71**:2508–15.

20 Ceravolo IP, Bruña-Romero O, Braga EM *et al.* Anti-*Plasmodium vivax* duffy binding protein antibodies measure exposure to malaria in the Brazilian Amazon. Am J Trop Med Hyg 2005; **72**:675–81.

21 Ceravolo IP, Souza-Silva FA, Fontes CJ *et al.* Inhibitory properties of the antibody response to *Plasmodium vivax* Duffy binding protein in an area with unstable malaria transmission. Scand J Immunol 2008; **67**:270–8.

22 Grimberg BTR, Udomsangpetch J, Xainli A *et al. Plasmodium vivax* invasion of human erythrocytes inhibited by antibodies directed against the Duffy binding protein. PLoS Med 2007; **4**:e337.

23 King CL, Shakri AR, Marcotty A *et al.* Naturally acquired Duffy-binding protein protein-specific binding inhibitory antibodies confer protection from blood-stage *Plasmodium vivax* infection. Proc Natl Acad Sci USA 2008; **105**:8363–8.

24 Cerbino VDA, Zumpano JF, Leopoldo FL *et al.* Follow-up of control measures during the *P. vivax* malaria outbreak in Rio Manso, Minas Gerais. Rev Bras Med Trop 2004; **37**:269.

25 Zumpano JFR, Chaves MOC, Silva RB *et al.* Study of *P. vivax* malaria relapses in Sousa District, Rio Manso municipality, Minas Gerais, Brazil. Rev Bras Med Trop 2004; **37**:267.

26 Snounou GS, Viriyakosol XP, Jarra ZW *et al.* High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 1993; **61**:315–20.

27 Sousa TN, Sanchez BA, Ceravolo IP, Carvalho LH, Brito CF. Real-time multiplex allele-specific polymerase chain reaction for

genotyping of the Duffy antigen, the *Plasmodium vivax* invasion receptor. Vox Sang 2007; **92**:373–80.
28 Cunha MG, Rodrigues MM, Soares IS. Comparison of the immunogenic properties of recombinant proteins representing the *Plasmodium vivax* vaccine candidate MSP1(19) expressed in distinct bacterial vectors. Vaccine 2001; **20**:385–96.
29 Fang XD, Kaslow DC, Adams JH, Miller LH. Cloning of the *Plasmodium vivax* Duffy receptor. Mol Biochem. Parasitol 1991; **44**:125–32.
30 Chitnis CE, Miller LH. Identification of the erythrocyte binding domains of *Plasmodium vivax* and *Plasmodium knowlesi* proteins involved in erythrocyte invasion. J Exp Med 1994; **180**:497–506.
31 Múfalo BC, Gentil F, Bargieri DY, Costa FT, Rodrigues MM, Soares IS. *Plasmodium vivax* apical membrane antigen-1: comparative recognition of different domains by antibodies induced during natural human infection. Microbes Infect 2008; **10**:1266–73.
32 Florens L, Washburn MP, Raine JD *et al.* A proteomic view of the *Plasmodium falciparum* life cycle. Nature 2002; **419**:520–6.
33 Barbedo MB, Ricci R, Jimenez MCS *et al.* Comparative recognition by human IgG antibodies of recombinant proteins representing three asexual erythrocytic stage vaccine candidates of *Plasmodium vivax*. Mem Inst Oswaldo Cruz 2007; **102**:335–9.
34 Braga EM, Fontes CJ, Krettli AU. Persistence of humoral response against sporozoite and blood-stage malaria antigens 7 years after a brief exposure to *Plasmodium vivax*. J Infect Dis 1998; **177**:1132–5.
35 Morais CG, Soares IS, Carvalho LH, Fontes CJ, Krettli AU, Braga EM. IgG isotype to C-terminal 19 kDa of *Plasmodium vivax* merozoite surface protein 1 among subjects with different levels of exposure to malaria in Brazil. Parasitol Res 2005; **95**:420–6.
36 Lim KJ, Park JW, Yeom JS *et al.* Humoral responses against the C-terminal region of merozoite surface protein 1 can be remembered for more than 30 years in persons exposed to *Plasmodium vivax*. Parasitol Res 2004; **92**:384–9.
37 Krotoski WA. The hypnozoite and malarial relapse. Prog Clin. Parasitol 1989; **1**:1–19.
38 Imwong M, Snounou G, Pukrittayakamee S *et al.* Relapses of *Plasmodium vivax* infection usually result from activation of heterologous hypnozoites. J Infect Dis 2007; **195**:927–33.
39 Chen N, Auliff A, Rieckmann K, Gatton M, Cheng Q. Relapses of *Plasmodium vivax* infection result from clonal hypnozoites activated at predetermined intervals. J Infect Dis 2007; **195**:934–41.
40 Kirchgatter K, del Portillo HA. Molecular analysis of *Plasmodium vivax* relapses using the MSP1 molecule as a genetic marker. J Infect Dis 1998; **177**:511–15.
41 Singh SK, Hora R, Belrhali H, Chitnis CE, Sharma A. Structural basis for Duffy recognition by the malaria parasite Duffy-binding-like domain. Nature 2006; **439**:741–4.
42 Chitnis CE, Sharma A. Targeting the *Plasmodium vivax* Duffy-binding protein. Trends Parasitol 2008; **24**:29–34.

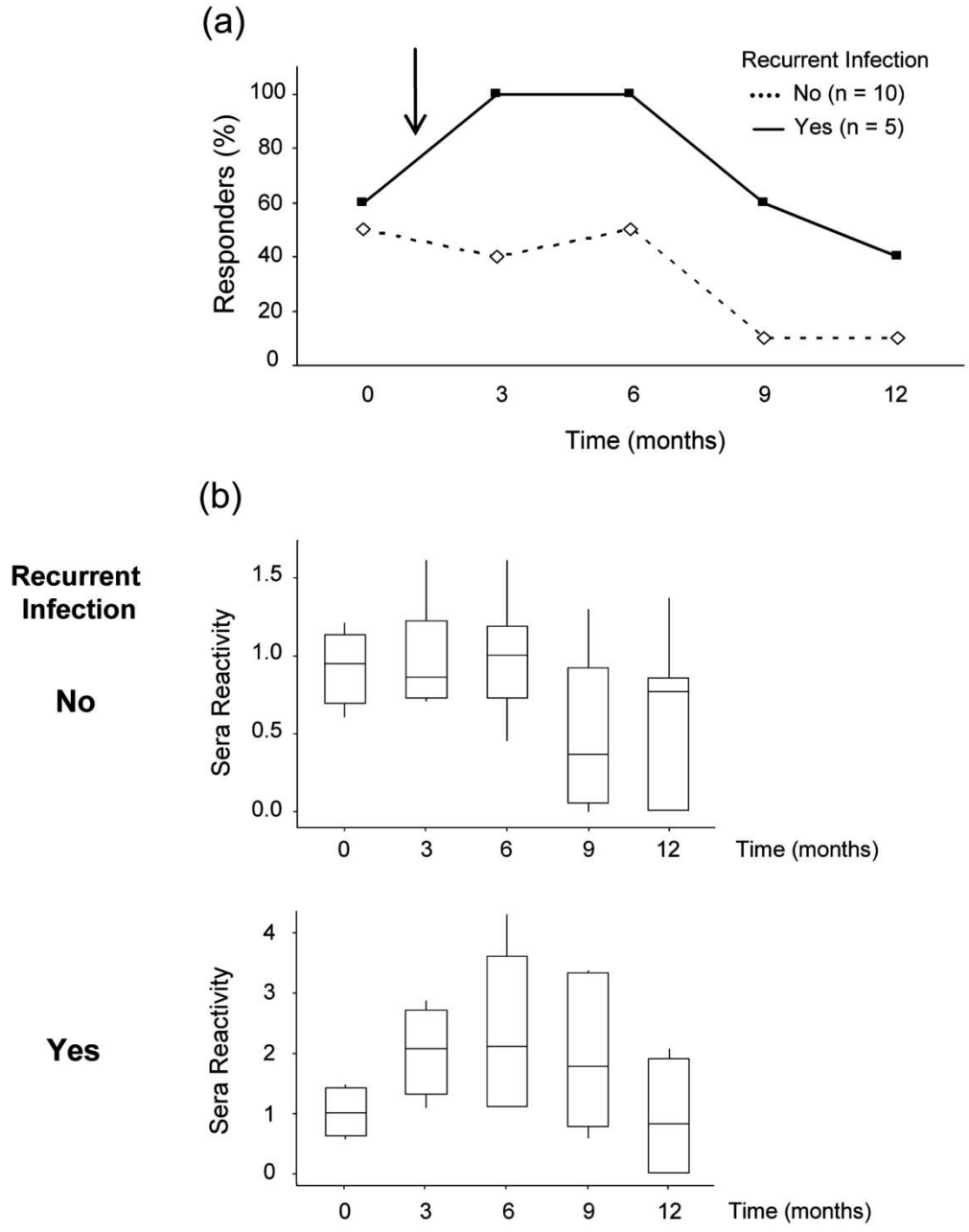
Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Antibody responses to the apical membrane antigen-1 (AMA-1) among individuals who had confirmed *Plasmodium vivax* infection in the outbreak area, and developed ($n = 5$) or not ($n = 10$) recurrent *P. vivax* infections (↓) during the 12-month follow-up period. (a) The percentage of responders to AMA-1, as detected by enzyme-linked immunosorbent assay (ELISA); (b) box-plot representations of sera reactivity; sera reactivity were expressed as index of reactivity (IR) at 492 nm, IR > 1 being considered positive. Box-plots: solid line across the box is the median, and the 25th and 75th percentiles were represented by the bottom and the top of each box respectively. The recombinant protein AMA-1 was produced as described previously [31].

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Ceravolo et al., Fig. S1



6 CONSIDERAÇÕES FINAIS

6.1 O receptor DARC e P. vivax na região Amazônica e extra Amazônica

O receptor DARC tem sido alvo de diversos estudos devido a sua importância em inúmeras doenças inflamatórias, doenças tumorais e infecciosas como na infecção pelo HIV (revisto por Smolarek et al., 2010). No caso da malária, o receptor DARC tem sido amplamente estudado devido ao fato de ser até o momento, a única via bem caracterizada de entrada do Plasmodium vivax no eritrócito humano. Sabe-se que o nível de expressão do receptor DARC na superfície dos eritrócitos está relacionado com a presença ou não de uma mutação pontual na região promotora GATA-1 (T-33C), mais frequentemente associado ao alelo *FY*B* e raramente associado ao alelo *FY*A* (Tournamille et al., 1995a; 1995b; Zimmerman et al., 1999). Esta mutação impede a expressão do antígeno DARC na superfície do eritrócito, caracterizando assim uma mutação silenciosa (não-funcional) (Woolley et al., 2000; Yazdanbakhsh et al., 2000; Zimmerman et al., 1999). Em condições de homozigose, este silenciamento é caracterizado pela presença de dois alelos não funcionais ($FY*B^{ES}$), o que impede a expressão total desta proteína nos eritrócitos. Até o momento, o alelo $FY*A^{ES}$ não foi descrito em homozigose (Kasehagen et al., 2007).

Afim de se compreender melhor se a expressão diferencial do receptor DARC influenciava na infecção pelo *P. vivax* no Brasil, foi realizado neste estudo a genotipagem de DARC em indivíduos residentes tanto na Amazônia Legal brasileira quanto em regiões não endêmicas de malária (transmissão epidêmica). Para tal, utilizou-se uma nova metodologia baseada no PCR em tempo real (Artigo 1). A técnica foi desenvolvida em um sistema multiplex com iniciadores alelo-específicos, utilizando o corante SYBR Green I em substituição às sondas marcadas, o que otimizou a metodologia e reduziu o custo em comparação aos métodos de genotipagem já descritos. Os resultados de validação da nova metodologia mostraram correlação total com os resultados de genotipagem obtidos por PCR-ASP (PCR-Allele-Specific Primer), método de referência (Olsson et al., 1998). Uma vez

padronizada e validada a PCR em tempo real, estudou-se a distribuição genotípica do receptor DARC em indivíduos expostos ao surto de malária autóctone ocorrido na região metropolitana de Belo Horizonte (área de transmissão epidêmica). Nesta área, a hipótese de trabalho foi a de que o receptor DARC era semelhante entre os indivíduos que se infectaram (casos) ou não (controles). Os resultados demonstram que nenhum dos indivíduos estudados nessa região eram DARC negativos, sendo, portanto, todos potencialmente suscetíveis à infecção pelo *P. vivax* (artigo 3). Além disto, a distribuição alélica (funcional versus não funcional) foi igual entre os grupos. Pode-se concluir com esses dados que a ausência de infecção no grupo controle foi devido à pouca exposição deste grupo, já que o surto foi controlado e a transmissão interrompida (≈ 50 dias de duração) através do tratamento de todos pacientes com drogas anti-maláricas e a aplicação de inseticidas residuais nas casas da região do surto (Zumpano et al., 2004).

Em residentes da área endêmica de malária, em um primeiro momento, caracterizou-se o genótipo DARC em indivíduos migrantes de diferentes estados brasileiros para as regiões endêmicas da Amazônia brasileira. Nesta parte do estudo, verificou-se que uma porcentagem significativamente maior de indivíduos com dois alelos funcionais pertenciam ao grupo de pessoas infectadas com *P. vivax* e não ao grupo infectado com o P. falciparum (que não utiliza esta via de invasão) ou não infectados. Esse resultado mostrou que os indivíduos com os genótipos *FY*A/FY*A*, *FY*B/FY*B* e *FY*A/FYB* (dois alelos funcionais) apresentam um risco 2,5 vezes maior de se infectarem por P. vivax em relação aos indivíduos *FY*A/FY*B*^{ES} e *FY*B/FY*B*^{ES} (um alelo funcional) (Artigo 1). Estes dados aqui encontrados foram corroborados por outros dois estudos, na Papua Nova Guiné e no Brasil, onde foi demonstrado que a presença de um alelo DARC silencioso reduzia à susceptibilidade à infecção por *P. vivax* (Kasehagen et al., 2007; Albuquerque et al., 2010).

Em uma segunda etapa, conduziu-se um estudo de base populacional, do tipo longitudinal, no assentamento agrícola de Rio Pardo, para avaliar a incidência de malária em indivíduos com expressão diferencial do receptor DARC. Neste estudo, verificou-se que os genótipos *FY*A/FY*B*^{ES}, *FY*A/FY*A* e *FY*A/FY*B* foram os

mais frequentes na população de estudo. (Artigo 2). Outros autores também encontram uma proporção significativa de diferentes genótipos de DARC, incluindo *FY*A/FY*B*, *FY*B/FY*B*, *FY*A/FY*A* e *FY*A/FY*B*^{ES}, em indivíduos da região Amazônica (Cavasini et al., 2007; Albuquerque et al., 2010). As variações entre as freqüências dos genótipos DARC nos diferentes estudos podem ser explicadas, provavelmente, pela origem diferente dos indivíduos estudados. Enquanto os estudos de Cavasini e colaboradores (2007) e Albuquerque e colaboradores (2010) foram conduzidos com populações migrantes, o estudo de Rio Pardo analisou populações nativas da área (cerca de 90% nascidos na Amazônia). Um resultado bastante interessante do presente estudo foi a alta freqüência do alelo *FY*A* encontrado na população de Rio Pardo. Por outro lado, o alelo *FY*B* parece ser predominante nas populações migrantes da Amazônia (Artigo 1; Cavasini et al., 2007; Albuquerque et al., 2010). Nossos resultados corroboram os dados encontrados em uma pequena comunidade nativa da Amazônia Ocidental, constituída de população ribeirinha (Ferreira et al., 2002), onde o alelo *FY*A* foi o mais prevalente. Assim, a variação do genótipo DARC e suas distribuições alélicas podem estar relacionadas com as diferentes origens étnicas da população em questão, particularmente na Amazônia, onde existe uma mistura inerente de ameríndios (americanos nativos), caucasianos e afro-descendentes (Perna, Cardoso & Guerreiro 2007; Palha et al. 2010). Neste momento, a falta de informações genéticas prévias da população de Rio Pardo exclui qualquer conclusão definitiva sobre a composição étnica da população. Estudos futuros fazem-se necessários nesta área para definir esta questão (estudos em andamento).

O acompanhamento prospectivo da população de Rio Pardo por 12 meses consecutivos, permitiu avaliar a relação entre a incidência de *P. vivax* e o genótipo de DARC da população. Os resultados permitiram demonstrar que não houve nenhuma associação significativa entre a expressão de DARC e a proteção contra o *P. vivax*. Estes resultados diferem daqueles encontrados pelo nosso grupo (Artigo 1) e outros grupos que demonstram que indivíduos que possuem apenas um alelo funcional parece ter menor risco de adquirir a infecção por *P. vivax* (Cavasini et., al. 2007a; Albuquerque et al., 2010; Kasehagen et al. 2007). Embora as razões para esta diferença não estejam bem estabelecidas, pode se especular que as diferenças

metodológicas entre os estudos, bem como os antecedentes genéticos dos indivíduos não infectados possam ter contribuído para tal. Em relação às abordagens experimentais, com exceção deste estudo conduzido em Rio Pardo, nenhum outro estudo brasileiro avaliou a relação entre DARC e a incidência de malária por *P. vivax*; basicamente, apenas a prevalência de malária por P. vivax foi avaliada nos estudos brasileiros. E ainda, nestes estudos que avaliaram a prevalência de malária, os grupos controles foram constituídos por poucos indivíduos (Artigo 1) ou incluíam doadores de sangue não residentes na área de exposição (Cavasini et al., 2007; Albuquerque et al., 2010). Com base nos dados encontrados neste e em outros estudos, apesar de haver pouca dúvida de que indivíduos DARC negativos ($FY*B^{ES}/FY*B^{ES}$) são altamente refratários à infecção por *P. vivax* (Miller et al. 1976), a proteção natural em indivíduos com apenas um alelo funcional permanece ainda não esclarecida. Assim, estudos futuros que avaliam a incidência da doença em comunidades da Amazônia fazem-se necessários.

6.2 Resposta imune anti-PvDBP na região Amazônica e extra Amazônica

Na última década, os estudos de resposta imune anti-PvDBP foram realizados, principalmente, em regiões de alta endemicidade de malária da Papua Nova Guiné onde a imunidade clínica está presente em indivíduos com idade escolar (Fraser et al., 1997; Michon et al., 2000; King et al., 2008). Já no Brasil, considerado uma área de baixa a média endemicidade, crianças e adultos são, em geral, susceptíveis a malária clínica. Recentemente, o nosso grupo demonstrou que indivíduos residentes na área endêmica brasileira possuem anticorpos anti-PvDBP naturalmente adquiridos e que parte destes anticorpos são capazes de bloquear a interação do ligante do parasito (PvDBPII) com o seu receptor (DARC) (Cerávolo et al., 2005; Cerávolo et al., 2008; Souza-Silva et al., 2010 - Anexo 1). Visto a existência de polimorfismos da região II da PvDBP, um estudo prévio de VanBuskirk e colaboradores (2004a) sugeriu que a resposta imune anti-PvDBP poderia ser variante específica. Entretanto, como os autores trabalharam com soro de coelhos imunes, questionou-se a existência de tal especificidade de anticorpos em populações humanas (revisto por Chitnis & Sharma, 2008). Para responder esta pergunta, o presente trabalho estudou a resposta imune a

PvDBP em um surto de transmissão autóctone de *P. vivax*, ocorrido na região metropolitana de Belo Horizonte, onde a população de estudo era constituída de indivíduos sem história de exposição prévia à malária (Artigo 3).

Na região de transmissão epidêmica foi visto que apenas 20% dos indivíduos que tiveram o primeiro episódio de malária desenvolveram anticorpos anti-PvDBP. Por outro lado, os indivíduos que não apresentaram infecção aguda pelo *P. vivax* (não casos), não desenvolveram anticorpos anti-PvDBP durante o estudo. Com relação a $PvMSP1_{19}$, uma proteína extremamente abundante nos merozoítos de estágio eritrocíticos, cerca de 90% dos indivíduos que se infectaram com o *P. vivax* desenvolveram resposta humoral contra esta proteína (Artigo 3). Esta elevada resposta a $PvMSP1_{19}$ era esperada, já que anticorpos contra esta proteína são dirigidos principalmente contra epitopos conservados (Soares et al., 1999).

Como os indivíduos residentes na área do surto foram acompanhados durante 12 meses, verificou-se que novos episódios clínicos da doença ocorreram em ≈ 30% (5 de 15) dos indivíduos que tiveram malária. Considerando que a transmissão de malária por *P. vivax* foi prontamente interrompida nesta região, os novos episódios clínicos de malária foram considerados como recaídas, devido aos hipnozoítos hepáticos (Krotoski et al., 1982). Embora a resposta imune inicial anti-PvDBP foi baixa, os indivíduos que tiveram recaída apresentaram um "booster" na resposta de anticorpos, já que maioria (4 de 5, 80%) respondeu a PvDBP. Do ponto de vista da vacina contra o *P. vivax*, esse achado é relevante por que apesar da PvDBP ser pouco imunogênica na exposição primária, exposições sucessivas às formas sanguíneas induz a um aumento na frequência respondedores. Verificou-se ainda que essa resposta foi de curta duração, pois 6 meses apos a infecção inicial a frequência de respondedores caiu abruptamente. Com estes resultados permitiu-se concluir que a PvDBP é pouco imunogênica e induz uma resposta de curta duração. De fato, em estudo conduzido no Acre, em colaboração com o Dr. Marcelo F. Urbano (USP), foi Possível demonstrar uma baixa resposta a PvDBP (Souza-Silva et al., 2010). Nesta área, foi demonstrado que a chance de ter anticorpos anti-PvDBP aumenta em 2% a cada ano de exposição ao *P. vivax*.

Muitos fatores podem contribuir para a baixa imunogenicidade da PvDBP em áreas endêmicas, sendo a hipótese mais plausível a localização da proteína nos micronemas do parasito (Adams et a., 1990). Isto faz com que a proteína seja exposta ao sistema imune do hospedeiro apenas no momento da invasão do eritrócito, reduzindo assim a chance da produção excessiva de anticorpos. Porém, a AMA-1, outra proteína presente nos micronemas, parece ser mais imunogênica do que a PvDBP. A explicação para tal pode ser atribuída ao fato de que AMA-1 é expressa durante os estágios pré-eritrocítico e eritrocítico enquanto a PvDBP é expressa apenas no estágio eritrocítico, sendo liberada somente no momento exato da invasão (Florens et al., 2002). Outro fator que pode ter contribuído para a baixa resposta de anticorpos anti-PvDBP é o polimorfismo da região II (McHenry & Adams, 2006; Souza et al., 2010), portanto, na próxima etapa do estudo investigou-se a contribuição do polimorfismo da PvDBP para a resposta imune do hospedeiro.

Nesta próxima etapa, avaliou-se a especificidade da resposta imune anti-PvDBP na região do surto (primo infectados). O sequenciamento da DBPII de isolados de *P. vivax* do surto permitiu verificar qual(is) variante(s) estava(m) circulando na região. Após o sequenciamento verificou-se que apenas uma variante de PvDBPII foi detectada na região, sendo esta variante detectada tanto nas infecções primárias como nas recaídas. Considerando naquele momento que apenas uma variante circulou entre os indivíduos do surto de Souza, a pergunta sobre a especificidade da resposta imune anti-PvDBP poderia então ser respondida. Para abordar esta questão, foram realizados ensaios funcionais com células de mamíferos cultiváveis (COS-7) expressando a variante da PvDBP circulante no surto (homologa) e uma variante de PvDBP heteróloga (Sal-1). Foi observado que os anticorpos anti-PvDBP não foram capazes de inibir a ligação de eritrócitos à variante Sal-1 de PvDBP (heteróloga), porém, uma significante inibição da interação ligante-receptor foi observada com a variante homóloga (em torno de 80%). Assim, concluiu-se que os indivíduos primariamente infectados e não imunes adquirem anticorpos bloqueadores anti-PvDBP variante-específicos. Os resultados pioneiros de especificidade da resposta imune anti-PvDBP deste estudo foram posteriormente confirmados por um estudo na Papua Nova Guiné (King et al., 2008). Estes autores verificaram que em crianças que apresentaram baixos títulos de anticorpos anti-PvDBP, demonstrados por

Elisa, a resposta imune contra essa proteína também era variante específica. Juntos, esses resultados demonstram a especificidade da resposta imune anti-PvDBP, o que pode comprometer o desenvolvimento de uma vacina que utilize essa proteína como alvo.

6.3 Influência do receptor DARC na resposta imune anti-PvDBP

Apesar da importância do receptor DARC na infecção pelo *P. vivax*, estudos analisando a influência do receptor DARC na aquisição da resposta imune são escassos (Herrera et al., 2005; Maestra et al., 2010). Afim de aprofundar neste tópico, buscou-se a associação entre os genótipos do receptor DARC e a resposta imune anti-PvDBP. Para isto, a população de Rio Pardo foi escolhida, já que um estudo de base populacional prospectivo estava sendo conduzido pelo nosso grupo na área. Curiosamente, os resultados deste estudo demonstraram que os indivíduos que possuíam o genótipo $FY*B/FY*B^{ES}$ (uma alelo funcional) apresentavam significativamente mais anticorpos anti-PvDBP que os indivíduos que possuíam qualquer outro genótipo. Neste momento, este perfil de resposta imune pode ser comparado apenas a um único estudo conduzido na costa caribenha da Colômbia, que apresentou resultados semelhantes aos discutidos aqui; indivíduos do genótipo $FY*B/FY*B^{ES}$ responderam mais a PvDBP (Maestre et al. 2010). Porém, os autores observaram que a quantidade de receptores DARC influenciou também na resposta de anticorpos $PvMSP1_{19}$, uma proteína expressa no estágio sanguíneo do parasita, que não está relacionada com o receptor DARC. No estudo aqui conduzido, os anticorpos anti-$PvMSP1_{19}$ não mostraram associação significativa com o receptor DARC. Até o momento, não existe uma explicação clara para o fato dos indivíduos portadores de um alelo funcional de DARC terem mais anticorpos do que aqueles com dois alelos funcionais. Maestra e colaboradores (2010) sugeriram que a presença dos dois alelos funcionais de DARC poderiam estar exercendo um papel imuno-regulador na resposta imune anti-PvDBP. Baseado nesta hipótese, os indivíduos com dois alelos funcionais teriam uma resposta menor de anticorpos. Entretanto, até o momento, não existem estudos para comprovar esta hipótese. Em resumo, estes dados iniciais que sugerem a influencia do receptor DARC na resposta imune anti-PvDBP necessitam ser

comprovados em outras áreas. Assim, estudos prospectivos de longa duração são necessários para determinar a contribuição do receptor DARC em termos de imunidade adquirida e proteção contra a doença.

7 CONCLUSÕES

A metodologia desenvolvida para a genotipagem do receptor DARC se mostrou eficiente, com potencial para a identificação em larga escala dos principais genótipos de DARC.

A associação entre os genótipos de DARC e a susceptibilidade à infecção pelo P. vivax, identificada no estudo de prevalência, não se confirmou no estudo de incidência.

A expressão diferencial do receptor DARC parece influenciar na resposta imune anti-PvDBP, mas não na resposta imune anti-PvMSP119.

Os indivíduos primo infectados pelo P.vivax são capazes de produzir anticorpos anti-PvDBP após uma única e breve exposição ao P. vivax, mas essa resposta de anticorpos é de curta duração mesmo nos indivíduos que apresentaram recaída da doença.

A especificidade da resposta imune anti-PvDBP é variante-específica.

8 ANEXOS

8.1 Souza-Silva FA, da Silva-Nunes M, Sanchez BA, Ceravolo IP, Malafronte RS, Brito CF, Ferreira MU, Carvalho LH. Naturally acquired antibodies to Plasmodium vivax Duffy binding protein (DBP) in Brazilian Amazon. Am J Trop Med Hyg 2010 Feb; 82(2): 185-93.

8.2 Costa DC, Madureira AP, Sanchez BAM, Gomes LT, Fontes CJF, Zumpano F, Limongi JE, Brito CFA, Carvalho LH. PCR-based methods for malaria field samples: potential unreliability at low Plasmodium density. Artigo submetido 2011.

Am. J. Trop. Med. Hyg., 82(2), 2010, pp. 185–193
doi:10.4269/ajtmh.2010.08-0580

Naturally Acquired Antibodies to *Plasmodium vivax* Duffy Binding Protein (DBP) in Rural Brazilian Amazon

Flávia A. Souza-Silva, Mônica da Silva-Nunes, Bruno A. M. Sanchez, Isabela P. Ceravolo, Rosely S. Malafronte, Cristiana F. A. Brito, Marcelo U. Ferreira, and Luzia H. Carvalho*

Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, Minas Gerais, Brazil; Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo (USP), São Paulo, Brazil; Instituto de Ciências Biomédicas, USP, São Paulo, Brazil

Abstract. Duffy binding protein (DBP), a leading malaria vaccine candidate, plays a critical role in *Plasmodium vivax* erythrocyte invasion. Sixty-eight of 366 (18.6%) subjects had IgG anti-DBP antibodies by enzyme-linked immunosorbent assay (ELISA) in a community-based cross-sectional survey in the Brazilian Amazon Basin. Despite continuous exposure to low-level malaria transmission, the overall seroprevalence decreased to 9.0% when the population was reexamined 12 months later. Antibodies from 16 of 50 (36.0%) subjects who were ELISA-positive at the baseline were able to inhibit erythrocyte binding to at least one of two DBP variants tested. Most (13 of 16) of these subjects still had inhibitory antibodies when reevaluated 12 months later. Cumulative exposure to malaria was the strongest predictor of DBP seropositivity identified by multiple logistic regression models in this population. The poor antibody recognition of DBP elicited by natural exposure to *P. vivax* in Amazonian populations represents a challenge to be addressed by vaccine development strategies.

INTRODUCTION

Almost 40% of the world's population is currently exposed to *Plasmodium vivax*, with 130–435 million clinical episodes recorded each year.[1–3] The emergence of multiresistant *P. vivax* isolates associated with severe and fatal malaria[4,5] highlights the need to consider both *P. vivax* and *Plasmodium falciparum* when implementing measures designed to reduce the malaria burden in regions where both species coexist.

The Duffy binding protein (DBP) stands out as the most promising *P. vivax* vaccine candidate antigen.[6,7] The DBP plays a major role in red blood cell invasion by *P. vivax*; blocking DBP binding to the Duffy antigen/receptor for chemokines (DARC) reduces the parasite's ability to invade new erythrocytes.[8–10] Binding domains of DBP is located in the N-terminal cysteine-rich region II (DBP_{II}), which contains 330 amino acids. The critical residues map to the central, 170-amino-acid stretch of DBP_{II}, which includes cysteines 5–8.[11–13]

Naturally acquired antibodies to DBP_{II} may block DBP_{II}-DARC interaction[14–16] and inhibit erythrocyte invasion *in vitro*.[10] Antibody recognition of DBP has been described in individuals exposed to hyperendemic malaria,[14,16–18] but little is known about naturally acquired antibodies in areas where substantially lower levels of malaria transmission prevail, such as the frontier settlements across the Amazon basin.[15,19] Here, we measure levels of naturally acquired antibodies to DBP in a well-characterized population exposed to frontier malaria in Brazil,[20] and investigate whether these antibodies block DBP_{II}-DARC interaction *in vitro*. We also investigate levels of sequence diversity in DBP_{II} among local parasites and examine factors that might impair antibody recognition of DBP_{II} by populations exposed to low-level *P. vivax* transmission.

*Address correspondence to Luzia H. Carvalho, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Malaria Laboratory, Av. Augusto de Lima 1715, 30190-002, Belo Horizonte, MG, Brazil. E-mail: lhcarvalho@cpqrr.fiocruz.br

SUBJECTS, MATERIALS, AND METHODS

Study area and population. The State of Acre is located in the Western Amazon Basin of Brazil, bordering with Peru, Bolivia, and the Brazilian states of Amazonas and Rondônia (supplementary Figure 1, available at www.ajtmh.org). The study site, Granada (9°41′S–9°49′S, 67°05′W–67°07′W), was a sparsely peopled rubber tapper settlement that became part of the Pedro Peixoto Agricultural Settlement Project in 1982. The study site and local malaria transmission patterns have been described in detail elsewhere.[20] Malaria morbidity in Granada has been shown to be associated with 1) forest-related activities such as land clearing; 2) time of residence in the settlement, with the probability of having malaria decreasing with years of residence in the settlement, but it is not affected by the subject's age; and 3) place of residence in the study area, with a significant spatial clustering of malaria risk in the areas of most recent settlement.

Blood samples for laboratory diagnosis of malaria and serum separation were collected between March 2004 and May 2005. Both *P. falciparum* and *P. vivax* are transmitted year-round. Recruitment strategies have been described elsewhere, with 466 dwellers < 1 to 90 years of age (98.5% of the 473 permanent residents in the study area) enrolled at baseline and 43 individuals (mostly newcomers to the area) enrolled between September and October 2004.[20] A questionnaire was applied to all study participants to obtain demographic and clinical information and assess their cumulative exposure to malaria. Because most (60.1%) study subjects were migrants from malaria-free areas, their ages do not necessarily correlate with exposure to malaria or risk of malaria during the follow-up.[20] Cumulative exposure to malaria was therefore estimated as the length of residence in malaria-endemic areas (either in Acre or elsewhere in the Amazon area) and the self-reported number of lifetime malaria episodes. Recent exposure to *P. vivax* was estimated as the number of slide-confirmed *P. vivax* malaria episodes recorded in the three local malaria diagnosis outposts between January and December 2003.

The 425 study participants ≥ 5 years of age were invited to contribute a 5-mL venous blood sample for serum separation; 366 subjects (86.1% of the eligible; age range, 5–90 yr)

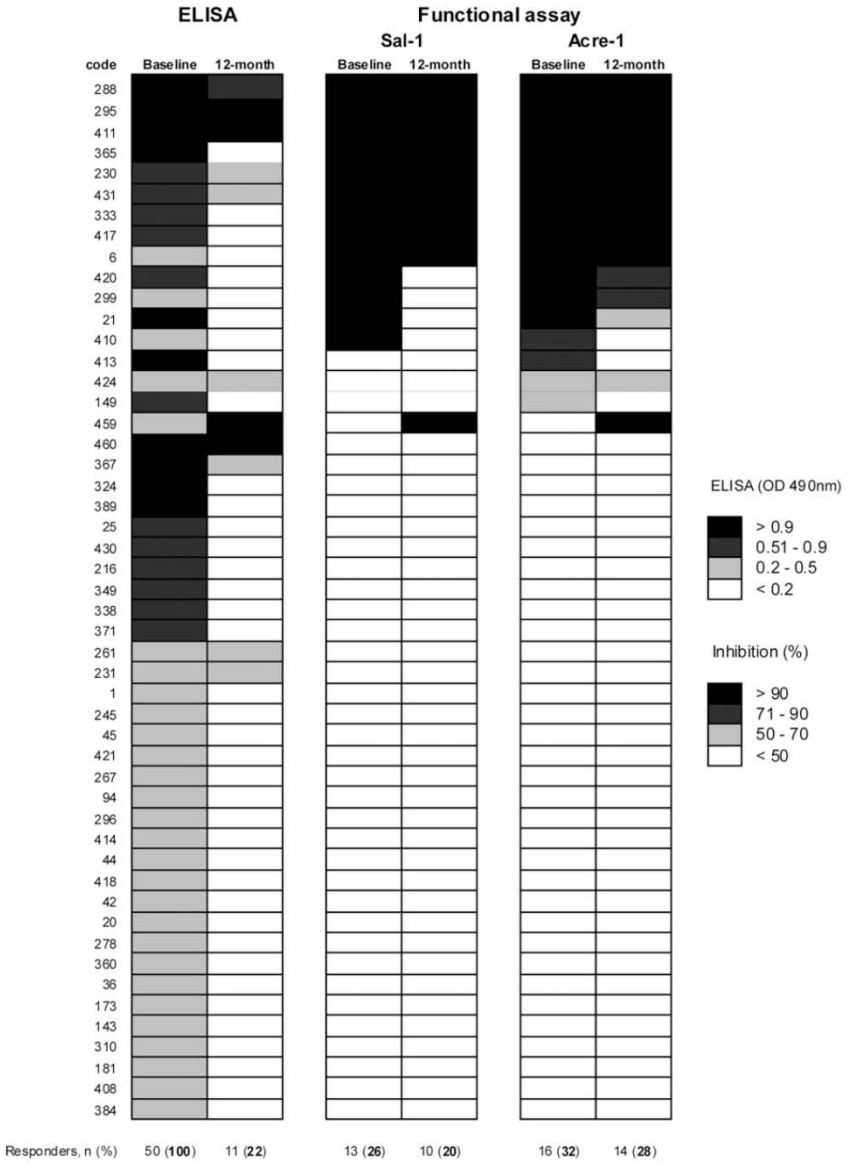


FIGURE 1. Inhibition of DBP_{II}-DARC binding in sequential samples from 50 individuals who had conventional anti-DBP antibodies by enzyme-linked immunosorbent assay (ELISA) at the time of enrollment. Each sample was assayed at baseline and ≅12-month latter. Conventional anti-DBP antibodies were detected by ELISA (at 1:100 plasma dilution), and inhibitory antibodies by erythrocyte-binding assays (at 1:40 plasma dilution) with COS cells expressing the most common DBP_{II} variant identified in the study population (Acre-1) or Sal-1 DBP_{II} (*P. vivax* laboratory reference), as described in Material and Methods. Numbers on the left refer to the individual code and values at the bottom of the figure represent the overall frequency of responders for each assay.

had their baseline serum samples tested for IgG antibodies to *Plasmodium vivax* DBP. All households were revisited in February–March 2005, when 323 venous blood samples were collected from their inhabitants 5 years of age or older and examined for IgG antibodies to DBP. Of 366 subjects enrolled at baseline, 287 (78.4%) still lived in the area and had a paired serum sample tested for anti-DBP antibodies.

The ethical and methodological aspects of this study was approved by the Ethical Committee of Research on Human Beings from the Institute of Biomedical Sciences, USP, São Paulo, SP, Brazil (Reports 318/CEP, July 19, 2002 and 538/CEP, January 7, 2004), according to the Resolution of the Brazilian Council on Health-CNS 196/96.

Malaria surveillance and acute-phase serum samples. Malaria episodes were diagnosed during 15 months of follow-up (March 2004 through May 2005) through both active and passive case detection. For active case detection, all households in the study area were visited 5 days/week by our field team and blood samples were collected from all subjects having fever or other symptoms suggestive of malaria since the last visit. Additional malaria episodes were found by passive case detection when symptomatic study participants had a malaria diagnosis confirmed at one of the three government-run malaria outposts in the study area. Asymptomatic malaria infections were detected during three cross-sectional surveys of the whole study population carried out in March–April

2004, September–October 2004, and February–March 2005. The combined active and passive case detection strategy identified 244 laboratory-confirmed *P. vivax* infections (183 symptomatic, 61 asymptomatic) among 138 subjects 5 years of age or more (mean, 1.7 episode per subject; range, 1–6). Ninety-four infections (38.5%) were missed by thick-smears microscopy, being only diagnosed by nested polymerase chain reaction (PCR); of them, 57 (60.6%) were asymptomatic. Molecular diagnosis detected both *P. falciparum* and *P. vivax* in 70 (28.7%) infections, but only three of these mixed-species infections had been diagnosed by conventional microscopy, which usually detected only the predominant species.[21,22] Acute-phase serum samples collected during 143 laboratory-confirmed *P. vivax* infections were tested for IgG antibodies to DBP.

Laboratory diagnosis of malaria. Two methods were used to diagnose malarial infections: examination of Giemsa-stained thick smears under 1,000× magnification (minimum of 100 microscopic fields examined) and nested PCR amplification of a species-specific segment of the *18S rRNA* gene of human malaria parasites.[23,24] Two sets of slides were sent for review by an expert microscopist at the National Reference Laboratory of the Ministry of Health of Brazil, in Brasília: 1) all positive slides and 2) negative slides from patients with acute febrile illness. Samples with either positive microscopy (confirmed by expert review) or positive nested PCR were considered positive for malaria parasites.

Clinical assessment. The prevalence and intensity of the symptoms associated with 174 *P. vivax* episodes diagnosed in our cohort subjects 5 years of age or more, during 15 months of follow-up, were assessed essentially as described elsewhere.[25,26] Only single-species episodes were considered. Briefly, a semiquantitative questionnaire addressing nine common symptoms (fever, chills, sweating, headache, myalgia, arthralgia, abdominal pain, nausea, and vomiting) was applied to all patients. The same medical doctor (MdSN) assessed all infections, to minimize inter-observer variation. According to the patient's perception, each clinical manifestation (except for fever) was considered to be absent, mild, moderate, or severe; fever was classified as absent, mild, or severe. Numerical scores of 0, 1, 2, or 3 were assigned to symptoms reported to be absent, mild, moderate, or severe, respectively. Asymptomatic subjects were given scores of 0 for each symptom. To minimize recall bias, patients were interviewed during the acute malaria episode or up to 1 week after treatment. Severe malaria episodes were not diagnosed in our study population during the follow-up.

Recombinant proteins and serological assay. The recombinant Duffy binding protein, which includes amino acids 132 to 771 (regions II to IV), was expressed as a soluble glutathione S-transferase (GST) fusion protein of 140 kDa.[17,19] To assess IgG antibodies against DBP an enzyme-linked immunosorbent assay (ELISA) was carried out as previously described.[19] Serum samples were assayed at 1:100 and the recombinant proteins used in the final concentrations of 5 µg/mL (DBP). Specific optical density (OD) at 492 nm was calculated by subtracting the OD obtained with GST alone (antigen control). The threshold of positivity was an OD value of 0.2 for DBP, which was based on the mean plus two standard deviations reactivity of sera from 20 non-exposed subjects.

DBP-pEGFP constructs. Region II of DBP (DBP_{II}) from a *P. vivax* laboratory reference clone (Sal-1)[27] has previously been subcloned into the pEGFP-N1 plasmid (Clontech), with a flanking signal sequence from the herpes simplex virus glycoprotein D1 (HSVgD1).[14] This targets expression to the surface of the transfected COS cells as a green fluorescent protein (GFP) fusion protein. The original Sal-1 DBP-pEGFP plasmid was kindly provided by Dr. J. H. Adams, University of South Florida, FL. An additional GFP construct with the DBP_{II} sequence from a common DBP_{II} variant circulating in the study area was made by subcloning a fragment corresponding to aa 198–522 of region II into pEGFP–HSVgD1 plasmid, using primers described previously.[28] Recombinant plasmids were purified by use of an endotoxin free plasmid DNA purification system (Qiagen, Valencia, CA).

COS cell transfection and erythrocyte-binding assays. Recombinant plasmids were transfected into COS-7 cells (American Type Culture Collection, Manassas, VA) by use of lipofectamine and PLUS-reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocols. Briefly, COS-7 cells in six-well culture plate (1.5×10^5 cells/well) were transfected with plasmids (0.5 µg/well)-liposome complexes (5% Plus-reagent and 3% lipofectamine) in Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO) without serum. After 6 hr of cell exposure to DNA-liposome complexes (37°C, 5% CO_2), transfection medium was replaced by DMEM with 10% of fetal bovine serum (Gibco-BRL, Gaithersburg, MD). At 24 hr after transfection, culture medium was replaced again and efficiency of transfection was assessed by fluorescence. Forty-eight hours after transfection, the erythrocyte-binding assays were performed as previously described.[15] Briefly, antiserum was added at 1:40, and plates were incubated for 1 hr at 37°C in 5% CO_2. The 1:40 dilution was chosen because in previous experiments this dilution provided a wide range of inhibitory activity among different plasmas. Human O^+ erythrocytes in a 10% suspension were added to each well (200 µL/well), and plates were incubated for 2 hr at room temperature. Unbound erythrocytes were then removed by washing the wells three times with phosphate buffered saline (PBS). Binding was quantified by counting rosettes observed over 10–20 fields of view (200×). Positive rosettes were defined as adherent erythrocytes covering more than 50% of the COS cell surface. For each assay, pooled plasma samples from Acre residents, characterized as non-responders by ELISA, were used as a negative control (100% binding). For this purpose, only plasma that does not inhibit erythrocyte binding (as compared with sample from unexposed Brazilian donors) has been pooled as a negative control (usually, 10 plasma samples/pool). An additional control included a pool of plasma from individuals with long-term exposure to malaria in the Amazon area (positive control). The percent inhibition was calculated as $100 \times (Rc - Rt)/Rc$, where *Rc* is the average of the number of rosettes in the control wells and *Rt* is the average of the number of rosettes in the test wells.

***Plasmodium vivax* DBP_{II} amplification and sequencing.** Extracted DNA was used as a template in the PCR to amplify the fragment corresponding to nucleotide positions 870 to 1,545 (amino acids 290–515) of the DBP_{II} encoding gene.[29] Platinum high fidelity *Taq* DNA polymerase (Invitrogen Life Technologies) was used in PCR to reduce possible nucleotide misincorporation. Amplicons were purified using the GFX-96 PCR kit (Amersham Biosciences, Little Chalfont, UK) and directly sequenced using DYEnamic ET dye terminator kit (Amersham Biosciences) and MegaBace 500 automated

DNA sequencer (Amersham Biosciences). The sequences were analyzed using Bioedit sequence alignment editor (www.mbio.ncsu.edu/BioEdit/bioedit.html) to identify DBP_{II} polymorphisms relative to the SAL-1 sequence.[27]

Statistical analyses. A database was created with SPSS 13.0 software (SPSS Inc., Chicago, IL). Proportions were compared in 2×2 tables with χ^2 tests with Yates correction for continuity or Fisher's exact tests, as appropriate. Pairwise correlations were evaluated with the Spearman's correlation coefficient ρ. Multiple logistic regression models with stepwise backward deletion were built to describe independent associations between covariates and the presence of antibodies to DBP during the cross-sectional surveys. Age, gender, time of residence in Amazonia, recent or current laboratory-confirmed episode of *P. vivax* malaria, and sector of residence within the study area were included into logistic regression models. Because of the nested structure of the data (there may be two observations per individual), we used two-level logistic models with robust standard errors, with level-1 variables corresponding to each observation (one or more per individual) and level-2 variables corresponding to each individual. Malaria transmission is heterogeneously distributed across Granada because of different patterns of land use and deforestation rates.[20] To adjust for these differences in logistic models, we divided the study area into four relatively homogeneous sectors with increasing malaria incidence: 1) sector A (92 subjects at baseline, 0.46 *P. vivax* episodes/100 person-months at risk between March 2004 and May 2005); 2) sector B (97 subjects, 0.79 *P. vivax* episodes/100 person-months at risk); 3) sector C (130 subjects, 3.44 *P. vivax* episodes/100 person-months at risk); and 4) sector D (47 subjects, 9.71 *P. vivax* episodes/100 person-months at risk). The HML software package (version 6.03, Scientific Software International, Lincolnwood, IL) was used for multilevel analysis. Only variables associated with statistical significance at the 5% level were maintained in the final models.

RESULTS

Naturally acquired IgG antibodies to DBP in cross-sectional surveys. We have studied baseline antibody responses to DBP in 366 subjects 5 to 90 years of age (median, 24.5 yr), with a male: female rate of 1.13:1 (Table 1). These subjects had between 1 month and 72 years of residence in the Brazilian Amazon area (median, 14 yr), where they are continuously exposed to *P. vivax* infections; 39 (10.7%) subjects had at least one recent symptomatic *P. vivax* malaria episode, diagnosed by passive case detection, between March 2003 and March 2004. At enrollment, 18.6% (68 of 366) study subjects had antibodies to DBP. Table 2 shows the proportions of study subjects who had IgG antibodies to DBP detected by ELISA at the cohort baseline and in February–March 2005. The overall proportion of responders differed significantly in the surveys (18.6% versus 9.0%, $P = 0.0004$, χ^2 test with Yates correction). *Plasmodium vivax*-infected subjects examined during the second cross-sectional survey had a greater prevalence of antibodies to DBP than those free of *P. vivax* infection ($P = 0.007$, χ^2 test with Yates correction), but no similar association between current *P. vivax* infection and seropositivity was found at the baseline survey. No significant association was found between recent exposure to the parasite (in the absence of current infection) and positive serology at the cohort baseline or in the second cross-sectional survey (Table 2). Similar proportions of subjects with baseline anti-DBP antibodies detected by ELISA (14 of 68, 20.6%) and those without detectable anti-DBP antibodies (50 of 298, 16.8%) had one or more *P. vivax* infections diagnosed by either passive or active case detection during the first 15 months of cohort follow-up ($P = 0.480$, Fisher's exact test).

Baseline plasma samples from 50 subjects with IgG antibodies to DBP detected by ELISA (age range, 8–75 yr) were further tested for their ability to inhibit *in vitro* the erythrocyte-binding function of DBP ligand domain. The Sal-1 DBP_{II} variant was included in these assays because it was being developed as a *P. vivax* vaccine candidate, and, of importance, Sal-1 DBP_{II} variant was present in Acre *P. vivax* isolates with a frequency greater than 10% (Table 3). We also tested the inhibitory ability of sera against the most common DBP_{II} variant identified in the study population, haplotype-1 (Table 3; renamed here Acre-1), which is quite common across the Amazon region of Brazil (Sousa TN and others, unpublished data). Overall, a plasma sample from 16 subjects (32.0% of those with anti-DBP antibodies that were tested) displayed significant (> 50%) inhibitory activity with one or both variants (13 of them had inhibitory antibodies against both DBP_{II} variants) (Figure 1). The subjects with inhibitory antibodies were slightly older (median age, 30.5 versus 26.5 years) and had a longer length of residence in the Amazon area (median length, 17 versus 14 years) than those who had ELISA-detected antibodies with no inhibitory activity ($P = 0.160$ and 0.327, respectively, Mann-Whitney test). Five baseline samples tested for inhibitory antibodies were collected from subjects with current *P. vivax* infection; two of them yielded significant erythrocyte-binding inhibition. A smaller proportion of subjects with inhibitory antibodies at the cohort baseline (3 of 16, 18.7%), when compared with those with anti-DBP antibodies that were unable to inhibit erythrocyte binding (7 of 34, 20.6%), had one or more *P. vivax* infections diagnosed during the first 15 months of follow-up, but the sample size is too small for meaningful statistical analysis. The baseline levels of IgG antibodies detected by ELISA (as estimated with corrected absorbance values) were positively correlated with the inhibitory activity of these antibodies against both Sal-1 ($\rho = 0.426$, $P = 0.003$, Spearman's correlation test) and Acre-1 ($\rho = 0.453$, $P = 0.001$, Spearman's correlation test) DBP variants.

We used multiple logistic regression models to determine whether cumulative, recent, or current exposure to *P. vivax* infection predicted the presence of anti-DBP antibodies during the cross-sectional surveys, after controlling for several covariates putatively associated with malaria risk in our population, such as age, gender, and sector of residence in the study

TABLE 1

Demographic, epidemiologic, and immunologic data of the 366 subjects who had baseline serum samples tested for IgG antibodies to Duffy binding protein (DBP)

Characteristics	
Median age, years (range)	24.5 (5–90)
Gender, male: female	1.13:1
Acute *Plasmodium vivax* infection, n (%)*	30 (8.2)
Years of malaria exposure, median (range)	14 (0–72)
Anti-DBP antibodies, n (%)†	68 (18.6)

* Fifteen out of 30 were mixed infections by *P. vivax* and *Plasmodium falciparum*.
† Positive antibody response, as detected by enzyme-linked immunosorbent assay (ELISA).

TABLE 2

Prevalence of IgG antibodies to Duffy binding protein (PvDBP) in relation to current or recent *Plasmodium vivax* infections, Acre, Brazil, 2004–2005

	Baseline (March–April 2004)				February–March 2005			
P. vivax infection	No.	Subjects with IgG antibodies		*P* (yes vs. no)	N°	Subjects with IgG antibodies		*P* (yes vs. no)
Current		No.	(%)			No.	(%)	
Yes	30*	6	(20.0%)	0.971	28†	7	(25.0%)	0.007
No	336	62	(18.5%)		295	22	(7.4%)	
Total	366	68	(18.6%)		323	29	(9.0%)	
Recent								
Yes	34‡	9	(26.5%)	0.299	40§	6	(15.0%)	0.103
No	302	53	(17.5%)		255	17	(6.7%)	
Total	336¶	62	(18.8%)		295‖	22	(7.4%)	

* Fifteen *P. vivax* infections and 15 mixed *P. vivax–Plasmodium falciparum* infections.
† Nineteen *P. vivax* infections and 9 mixed *P. vivax–P. falciparum* infections.
‡ Occurrence of one or more laboratory-confirmed *P. vivax* infections between March 2003 and March 2004.
§ Occurrence of one or more laboratory-confirmed *P. vivax* infections during the follow-up (March 2004 to March 2005).
¶ Thirty subjects with current *P. vivax* infections excluded.
‖ Twenty-eight subjects with current *P. vivax* infections excluded.

site. Because of the significant difference in seropositivity rates between surveys, the time of survey was included as one of the covariates to be controlled. The number of years of residence in the Brazilian Amazon, a surrogate measure of cumulative exposure to malaria, was a strong predictor of the presence of IgG antibodies (adjusted odds ratio [aOR], 1.02; 95% confidence interval [CI], 1.00–1.04, $P = 0.044$). In other words, each additional year of exposure to malaria increased the probability of having anti-DBP IgG antibodies by 2%. Neither age nor current or recent exposure to *P. vivax* (as defined in Table 2) were significant predictors of DBP seropositivity. As expected, however, the sector of residence was significantly associated with the presence of IgG antibodies. We conclude that the cumulative exposure to malaria, but not the recent or current exposure to *P. vivax*, was a significant independent predictor of the presence of anti-DBP IgG antibodies during the cross-sectional surveys.

Malaria surveillance and anti-DBP antibodies in sequential serum samples. We next compared the prevalence of conventional anti-DBP antibodies detected by ELISA and of inhibitory antibodies detected by erythrocyte-binding assays in paired samples (baseline versus second cross-sectional survey) obtained from subjects who experienced or did not experience one or more clinical episodes of *P. vivax* malaria between the surveys (Table 4). Subjects who had asymptomatic *P. vivax* parasitemia detected during one of the population-wide cross-sectional surveys but had no disease ($N = 11$) were not included in this analysis. No significant association was found between the presence of antibodies (either conventional or inhibitory) in the second cross-sectional survey and the occurrence of *P. vivax* malaria between the blood draws. A significant correlation between the levels of IgG antibodies detected by ELISA and the inhibitory activity of these antibodies against both Sal-1 ($\rho = 0.513$, $P < 0.0001$, Spearman's correlation test) and Acre-1 ($\rho = 0.471$, $P < 0.0001$, Spearman's correlation test) variants. Four subjects had no anti-DBP antibodies detected by ELISA during the second cross-sectional survey but maintained high-level rosette-inhibitory activity against both DBP_{II} variants (Figure 1). Although overall levels of anti-DBP antibodies tended to decrease between the first and the second surveys, serum inhibitory activity remained relatively stable in the majority of the responders (14 of 16). Of relevance, both the frequency and levels of inhibitory antibodies to Sal-1 and Acre-1 variants were quite similar (Figure 1, supplementary Figure 2, available at www.ajtmh.org).

The putative antibody boosting effect of current exposure to *P. vivax* was further assessed by analyzing acute-phase serum samples from 80 cohort participants who experienced laboratory-confirmed *P. vivax* infections diagnosed during the follow-up. Of 143 acute-phase sera tested for IgG antibodies to DBP, 34 (23.8%) were positive. The prevalence of anti-DBP antibodies increased linearly with increasing parasitemias (χ^2 for trend = 5.325, 1 degree of freedom, $P = 0.0021$), and only 10 of 67 (14.9%) acute-phase sera collected during subpatent infections had detectable antibodies. These data suggest that exposure to very low parasitemias may be poorly effective in inducing anti-DBP antibody boosting.

Finally, we compared the prevalence of anti-DBP IgG antibodies in consecutive samples collected from 53 cohort subjects who experienced one or more laboratory-confirmed *P. vivax* infections. Individual results are shown in Figure 2, with subjects categorized as 1) seroconverters (initially seronegative subjects who acquired anti-DBP antibodies at any time-point of the study; $N = 13$, Figure 2A); 2) subjects who

TABLE 3

Common *Plasmodium vivax* DBP_{II} haplotypes identified among 25 *P. vivax* isolates from the study area, State of Acre, Brazil*

AA residue	333	371	375	384	385	386	390	417	419	424	437	503	Frequency (%)
Sal-1†	L	K	N	D	E	K	R	N	I	L	W	I	12
1	–	.	.	G	.	.	H	.	.	.	.	K	20
2	.	.	.	G	K	N	H	.	.	.	R	K	12
3	.	.	.	.	.	.	.	K	.	I	R	K	12
4	F	.	D	G	K	N	H	K	.	I	R	.	16

* Eleven DBP_{II} haplotypes were identified in Acre population, and those present at a frequency greater than 10% were listed in Table 3 grey areas highlight the trio of polymorphisms in DBP_{II} that form part of a cluster surrounding the Duffy antigen/receptor for chemokines (DARC)-binding site, and which are under positive selection (Sousa TN and others, unpublished data).
† Sal-1 sequence, accession no.: M61095.[27]

TABLE 4

Prevalence and inhibitory activity of naturally acquired antibodies to Duffy binding protein (PvDBP) in consecutive cross-sectional surveys in relation to *Plasmodium vivax* malaria episodes during the follow-up; Acre, Brazil, 2004–2005*

Antibodies in consecutive surveys	*Plasmodium vivax* malaria during the follow-up			All subjects
	Yes	No.	*P*†	
Conventional ELISA antibodies				
–/–	29	194	0.358	223
+/–	8	30		38
–/+	3	8		11
+/+	2	13		15
Total	42	245		287
Inhibitory antibodies (Sal-I or Acre-1)				
–/–	6	27	1.00	33
+/–	1	2		3
–/+	1	0		1
+/+	2	11		13
Total	10	40		50

* ELISA = enzyme-linked immunosorbent assay.
† *P* for a comparison between (–/– or +/–) vs. (–/+ or +/+) with Fisher's exact test.

lost their anti-DBP antibodies ($N = 10$, Figure 2B); and 3) subjects who failed to develop antibody responses to DBP despite documented exposure to the parasite ($N = 25$, Figure 2C). Five out of 53 subjects who could not be classified following these group criteria were not included in the analysis (data not shown). DBP antibody acquisition was likely to be related with cumulative exposure to malaria, as defined by the years of residence in the Amazon area (median, 19 yr versus 12 to 13 yr) or by the number of previous malaria episodes (median, 11 episodes versus 3 to 5 episodes), but these differences were not statistically significant.

Clinical expression of *P. vivax* malaria and anti-DBP antibodies. The prevalence and severity of symptoms associated with uncomplicated *P. vivax* malaria were analyzed in 174 laboratory-confirmed single-species infections diagnosed in our cohort. Fever (67.8%), headache (71.3%), chills (58.6%), and myalgia (57.5%) were the most prevalent symptoms; 34 (19.5%) infections were symptomless and 60 (48.6%) infections, 33 of them asymptomatic, were diagnosed by PCR only.

We next examined whether the levels of anti-DBP IgG antibodies measured during *P. vivax* infections correlated to the perceived severity of symptoms. For that, each symptom was assessed separately for its correlation with levels of anti-DBP antibodies. Because of the confounding effect of parasitemias, which affect both anti-DBP responses and the clinical expression of malaria, we restricted this analysis to 57 patent infections. No significant correlation was found between ELISA absorbance values and the severity of any symptom (*P* value range, 0.161–0.943, Spearman's correlation test). Quite similar results were obtained when only the first infection experienced by each subject was analyzed ($N = 31$, *P* value range, 0.172–0.929, Spearman's correlation test).

DISCUSSION

Here, we show that a relatively small proportion of rural Amazonians exposed to low-level malaria transmission have detectable antibodies to DBP; in contrast, the $MSP1_{19}$, a much more abundant surface antigen, was previously recognized by ≅50% of these individuals.[30] Given the fact that DBP is localized in an apical secretory organelle (micronemes), and it is probably not released until erythrocyte attachment,[31] the host immune system seems to have little opportunity to mount an efficiently antibody response, particularly as the invasion process may take less than a minute to be completed.[32] It may partially explain why in Acre population a long-term exposure to malaria and, apparently, a certain level of parasitemia have to be reached until subjects acquire anti-DBP antibodies. Consistent with this hypothesis, our previous study in the Amazon area showed that high-levels of anti-DBP antibodies could be reached only among gold miners[19] whose behavioral patterns place them at a higher risk of exposure to infected mosquitoes.[33,34] In fact, malaria prevalence in Granada (3.6%)[26] is far lower than that measured in mining areas (13.8–35.0%).[34–36] However, the "just-in-time" hypothesis of DBP exposure[37] does not completely explain the large proportion of individuals who remain unresponsive to DBP after prolonged exposure to malaria, especially because a few dominant DBP_{II} haplotypes seem to account for the majority of *P. vivax* infections in areas of high malaria transmission.[38] The reasons for this are not clear, but may relate to the complexity of immune responses in terms of genetic diversity of the human and parasite populations, and stochastic events associated with the induction of a specific antibody response.[39–41] Such complexity may provide a plausible explanation for the difficulties that have been encountered in *Plasmodium* vaccine development.

The goal in developing DBP as a vaccine against blood-stages of *P. vivax* is to elicit an antibody response that inhibits the adhesion of this parasite ligand to its cognate erythrocyte receptor and thereby abrogate merozoite invasion. In the Acre population, we further analyzed whether the conventional DBP immune response, as detected by ELISA, includes antibody activity that blocks the DBP_{II}-DARC interaction. A significant inhibitory activity was detected in about one-third of those subjects, and the presence of these inhibitory antibodies was related with a long-term residence in the Amazon area (median, 19 yr). In effect, by using multiple logistic regression models, it was possible to identify cumulative exposure to *P. vivax*—estimated by the time of residence in the Brazilian Amazon—as a strong predictor of the presence of anti-DBP antibodies during the cross-sectional surveys. Nevertheless, subjects' age was not associated with the presence of anti-DBP antibodies. It was not unexpected because in this area the pattern of malaria transmission is typically from those of frontier malaria, where exposed populations consist of migrants mostly from malaria-free areas, and malaria infection affects people of all ages.[42] In conclusion, cumulative exposure, independent of host age, apparently represents a key determinant of the quantitative and qualitative nature of the IgG responses to DBP.

Recently, it has been suggested that DBP_{II} polymorphic residues surrounding the DARC binding domain might elude binding of inhibitory antibody.[6,43] In fact, individuals briefly exposed to *P. vivax* developed anti-DBP inhibitory antibodies that are biased toward a specific DBP_{II} variant.[44] Although we cannot rule out the possibility that polymorphisms at DBP_{II} could contributed to the relatively low frequency (≅30%) of inhibitory antibodies among long-term residents in Acre, it seems unlikely because 1) to reduce the potential effects of DBP polymorphism on antibody recognition, we used two

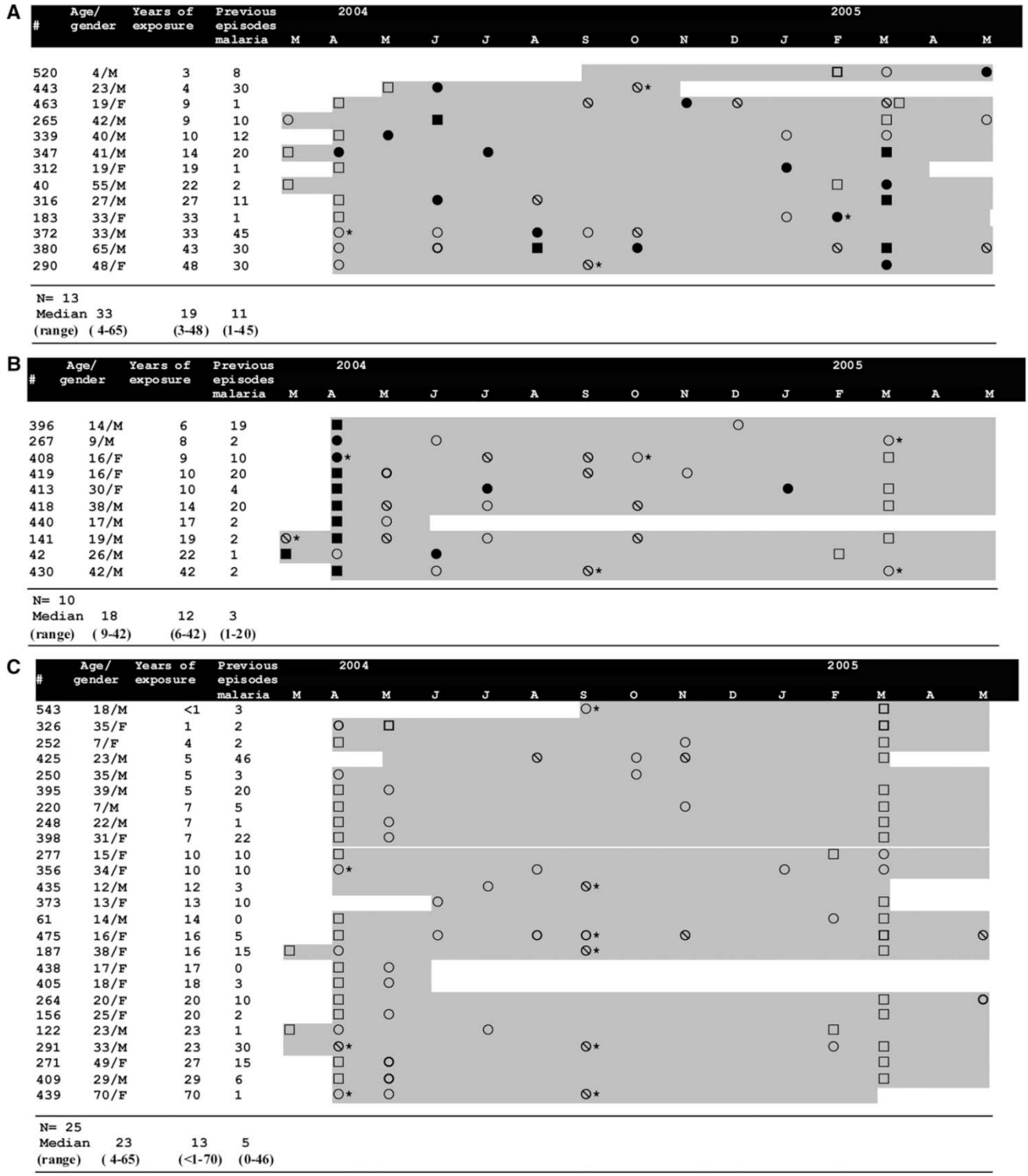


FIGURE 2. Individual results of enzyme-linked immunosorbent assay (ELISA) for IgG antibodies to Duffy binding protein (DBP) in consecutive serum samples collected from 48 malaria-exposed subjects from Acre with one or more laboratory-confirmed infections with *Plasmodium vivax* during a cohort study in Acre, Brazil. According to the DBP antibody profile throughout the study, subjects were categorized as responders who had either (**A**) acquired or (**B**) lost their antibody response, and (**C**) non-responders. The cohort was established in March 2004 (first "M") and followed until May 2005 (last "M"); the shaded area represents the duration of individual follow-up. The following symbols are used: □ = serum sample collected from a subject free of *P. vivax* infection with negative ELISA; ■ = serum sample collected from a subject free of *P. vivax* infection with positive ELISA; ○ = serum sample collected from a subject with laboratory-confirmed *P. vivax* infection with negative ELISA; ● = serum sample collected from a subject with laboratory-confirmed *P. vivax* infection with positive ELISA; ⦸ = laboratory-confirmed *P. vivax* infection without serum sample available for ELISA. Asymptomatic infections were highlighted with an asterisk (*).

different DBP_{II} variants that are commonly found in the study site (Sal-I and Acre-1) in erythrocyte binding assays; 2) Sal-1, Acre-1, and a number of Acre DBP_{II} haplotypes (5 out of 11) share the trio of polymorphisms (at codons 417, 419, and 424) that is suggested to play a role in DBP_{II} inhibitory binding (Sousa TN and others, unpublished data). Furthermore, a similar proportion of responders (39%) were found among immune children residing in a *P. vivax* hyperendemic area of Papua New Guinea (PNG), with only 18 of 208 (9%) presenting high-levels of inhibitory antibodies.[16] Consequently, the low levels of DBP inhibitory antibodies should be expected in naturally malaria-exposed populations.

Significantly, the inhibitory ability of antibodies was relatively stable over time in Granada; 14 of 16 subjects retained their inhibitory DBP antibody response to at least one DBP_{II} variant (Acre-1) when studied 12 months after the baseline survey. To the best of our knowledge, a single study previously investigated the longevity of anti-DBP_{II} inhibitory antibodies in the endemic area.[16] King and colleagues[16] found that asymptomatic children residing in PNG developed inhibitory anti-DBP antibodies, which were remarkably stable over the 12-month follow-up period, a result corroborated by our study in Acre population. Together, these evidences imply that although the majority of people naturally exposed to *P. vivax* do not develop antibodies that inhibit the DBP_{II}-DARC interaction, once they are acquired; these inhibitory antibodies seem to be stable under continuous exposure to malaria transmission. Of note, a number of subjects with high rosette-inhibitory activity lost their conventional (ELISA-detected) anti-DBP antibodies during the follow-up. This is not completely unexpected, because standard serological assays with recombinant proteins do not account for fine epitope specificity and affinity, which are likely to be essential for inhibitory activity. However, levels of ELISA-detected antibodies correlated with levels of erythrocyte-binding inhibition, consistent with previous findings in areas with unstable or stable malaria transmission.[14,15]

Although the size of our sample was not small, the low frequency of DBP responders had precluded a number of statistical comparisons. Consequently, we were unable to test whether the presence of DBP inhibitory antibodies are associated with protection from blood-stage *P. vivax* infection, as recently suggested among children from PNG.[16]

In conclusion, our results show low immunogenicity of DBP among individuals continuously exposed to malaria in a well-consolidated settlement of the Brazilian Amazon area. Future challenges include understanding why only a few malaria exposed-individuals develop an immune response able to inhibit DBP_{II}-DARC interaction, and to establish whether DBP inhibitory immune response predicts partial protection from infection and/or disease in semi-immune populations. Although essential, those studies will be a difficult task because protection against malaria in an outbred human population may be a higher-order phenomenon related to patterns of response and not attributable to any single antigenic target.[40]

Received November 6, 2008. Accepted for publication September 15, 2009.

Note: Supplemental figures appear at www.ajtmh.org.

Acknowledgments: We thank the Municipal Government of Acrelândia, and malaria control teams in Acrelândia and Granada for their logistic support during the fieldwork. We also thank John H. Adams, South Florida University, FL, for kindly providing the Sal-1 DBP-pEGFP plasmid.

Financial support: This research was supported by the Research Foundations of São Paulo (FAPESP) and Minas Gerais (FAPEMIG) States, the Brazilian National Research Council (CNPq), and the Strategic Research Program of the Oswaldo Cruz Foundation (PAPES IV/FIOCRUZ); scholarships from FAPEMIG (F.A.S), FAPESP (M.dS.N), and CNPq (L.H.C. and M.U.F.) are also acknowledged.

Authors' addresses: Flávia A. Souza-Silva, Bruno A. M. Sanchez, Isabela P. Ceravolo, Cristiana F. A. Brito, and Luzia H. Carvalho, Laboratory of Malaria, Institute René Rachou, Fundação Oswaldo Cruz (FIOCRUZ-MG), Belo Horizonte, Minas Gerais, Brazil, E-mails: praflavia@cpqrr.fiocruz.br, bruno@cpqrr.fiocruz.br, ceravolo@cpqrr.fiocruz.br, cristiana@cpqrr.fiocruz.br, and lhcarvalho@cpqrr.fiocruz.br. Mônica da Silva-Nunes and Marcelo U. Ferreira, Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, E-mails: msnunes1@yahoo.com.br and muferrei@usp.br. Rosely S. Malafronte, Laboratory of Protozoology, Institute of Tropical Medicine of São Paulo, Cerqueira César, São Paulo, São Paulo, Brazil, E-mail: rmalafronte@usp.br.

Reprint requests: Luzia H. Carvalho, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Malaria Laboratory, Av. Augusto de Lima 1715, 30190-002, Belo Horizonte, MG, Brazil, Tel: 55-31-33497793, Fax: 55-31-32953115, E-mail: lhcarvalho@cpqrr.fiocruz.br.

REFERENCES

1. Hay SI, Guerra CA, Tatem AJ, Noor AM, Snow RW, 2004. The global distribution and population at risk of malaria: past, present, and future. *Lancet Infect Dis 4:* 327–336.
2. Guerra CA, Snow RW, Hay SI, 2006. Defining the global spatial limits of malaria transmission in 2005. *Adv Parasitol 62:* 157–179.
3. Baird JK, 2007. Neglect of *Plasmodium vivax* malaria. *Trends Parasitol 23:* 533–539.
4. Genton B, D'Acremont V, Rare L, Baea K, Reeder JC, Alpers MP, Muller I, 2008. *Plasmodium vivax* and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. *PLoS Med 5:* 881–889.
5. Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, Karyana M, Lampah DA, Price RN, 2008. Multidrug-resistant *Plasmodium vivax* associated with severe and fatal malaria: a prospective study in Papua, Indonesia. *PLoS Med 5:* 890–899.
6. McHenry AM, Adams JH, 2006. The crystal structure of *P. knowlesi* DBP alpha DBL domain and its implications for immune evasion. *Trends Biochem Sci 31:* 487–491.
7. Chitnis CE, Sharma A, 2008. Targeting the *Plasmodium vivax* Duffy-binding protein. *Trends Parasitol 24:* 29–34.
8. Miller L, Mason S, Clyde D, McGinniss M, 1976. The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. *N Engl J Med 295:* 302–304.
9. Wertheimer SP, Barnwell JW, 1989. *Plasmodium vivax* interaction with the human Duffy blood-group glycoprotein: identification of a parasite receptor-like protein. *Exp Parasitol 69:* 340–350.
10. Grimberg BT, Udomsangpetch R, Xainli J, McHenry A, Panichakul T, Sattabongkot J, Cui L, Bockarie M, Chitnis C, Adams J, Zimmerman PA, King CL, 2007. *Plasmodium vivax* invasion of human erythrocytes inhibited by antibodies directed against the Duffy binding protein. *PLoS Med 4:* 1940–1948.
11. Ranjan A, Chitnis CE, 1999. Mapping regions containing binding residues within functional domains of *Plasmodium vivax* and *Plasmodium knowlesi* erythrocyte-binding proteins. *Proc Natl Acad Sci USA 96:* 14067–14072.
12. Singh SK, Singh AP, Pandey S, Yazdani SS, Chitnis CE, Sharma A, 2003. Definition of structural elements in *Plasmodium vivax* and *P. knowlesi* Duffy-binding domains necessary for erythrocyte invasion. *Biochem J 374:* 193–198.
13. VanBuskirk KM, Sevova E, Adams JH, 2004. Conserved residues in the *Plasmodium vivax* Duffy-binding protein ligand domain are critical for erythrocyte receptor recognition. *Proc Natl Acad Sci USA 101:* 15754–15759.

14. Michon P, Fraser T, Adams JH, 2000. Naturally acquired and vaccine-elicited antibodies block erythrocyte cytoadherence of the *Plasmodium vivax* Duffy binding protein. *Infect Immun 68:* 3164–3171.
15. Ceravolo IP, Souza-Silva FA, Fontes CJF, Braga EM, Madureira AP, Krettli AU, Souza JM, Brito CFA, Adams JH, Carvalho LH, 2008. Inhibitory properties of the antibody response to *Plasmodium vivax* Duffy binding protein in an area with unstable malaria transmission. *Scand J Immunol 67:* 270–278.
16. King CL, Michon P, Shakri AR, Marcotty A, Stanisic D, Zimmerman PA, Cole-Tobian JL, Mueller I, Chitnis CE, 2008. Naturally acquired Duffy-binding protein-specific binding inhibitory antibodies confer protection from blood-stage *Plasmodium vivax* infection. *Proc Natl Acad Sci USA 105:* 8363–8368.
17. Fraser T, Michon P, Barnwell JW, Noe AR, AlYaman F, Kaslow DC, Adams JH, 1997. Expression and serologic activity of a soluble recombinant *Plasmodium vivax* Duffy binding protein. *Infect Immun 65:* 2772–2777.
18. Xainli J, Cole-Tobian JL, Baisor M, Kastens W, Bockarie M, Yazdani SS, Chitnis CE, Adams JH, King CL, 2003. Epitope-specific humoral immunity to *Plasmodium vivax* Duffy binding protein. *Infect Immun 71:* 2508–2515.
19. Ceravolo IP, Bruna-Romero O, Braga EM, Fontes CJF, Brito CFA, Souza JM, Krettli AU, Adams JH, Carvalho LH, 2005. Anti-*Plasmodium vivax* Duffy binding protein antibodies measure exposure to malaria in the Brazilian Amazon. *Am J Trop Med Hyg 72:* 675–681.
20. da Silva-Nunes M, Codeço CT, Malafronte RS, Silva NS, Juncansen C, Muniz PT, Ferreira MU, 2008. Malaria on the Amazonian frontier: transmission dynamics, risk factors, spatial distribution, and prospects for control. *Am J Trop Med Hyg 79:* 624–635.
21. Cavasini M, Ribeiro W, Kawamoto F, Ferreira M, 2000. How prevalent is *Plasmodium malariae* in Rondônia, western Brazilian Amazon? *Rev Soc Bras Med Trop 33:* 489–492.
22. Scopel K, Fontes C, Nunes A, Horta M, Braga E, 2004. Low sensitivity of nested PCR using *Plasmodium* DNA extracted from stained thick blood smears: an epidemiological retrospective study among subjects with low parasitaemia in an endemic area of the Brazilian Amazon region. *Malar J 3:* 8.
23. Kimura M, Kaneko O, Qing L, Mian Z, Kawamoto F, Wataya Y, Otani S, Yamaguchi Y, Tanabe K, 1997. Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. *Parasitol Int 46:* 91–154.
24. Win T, Lin K, Mizuno S, Zhou M, Liu Q, Ferreira M, Tantular I, Kojima S, Ishii A, Kawamoto F, 2002. Wide distribution of *Plasmodium ovale* in Myanmar. *Trop Med Int Health 7:* 231–239.
25. Karunaweera N, Carter R, Grau G, Mendis K, 1998. Demonstration of anti-disease immunity to *Plasmodium vivax* malaria in Sri Lanka using a quantitative method to assess clinical disease. *Am J Trop Med Hyg 58:* 204–210.
26. Silva-Nunes M, Malafronte RS, Luz BA, Souza E, Martins L, Rodrigues S, Chiang J, Vasconcelos P, Muniz P, Ferreira M, 2006. The Acre Project: the epidemiology of malaria and arthropod-borne virus infections in a rural Amazonian population. *Cad Saude Publica 22:* 1325–1334.
27. Fang XD, Kaslow DC, Adams JH, Miller LH, 1991. Cloning of the *Plasmodium-vivax* Duffy receptor. *Mol Biochem Parasitol 44:* 125–132.
28. Chitnis C, Miller L, 1994. Identification of the erythrocyte binding domains of *Plasmodium vivax* and *Plasmodium knowlesi* proteins involved in erythrocyte invasion. *J Exp Med 180:* 497–506.
29. Sousa T, Cerávolo I, Fernandes Fontes C, Couto A, Carvalho L, Brito C, 2006. The pattern of major polymorphisms in the Duffy binding protein ligand domain among *Plasmodium vivax* isolates from the Brazilian Amazon area. *Mol Biochem Parasitol 146:* 251–254.
30. Bastos MS, da Silva-Nunes M, Malafronte RS, Hoffmann EHE, Wunderlich G, Moraes SL, Ferreira MU, 2007. Antigenic polymorphism and naturally acquired antibodies to *Plasmodium vivax* merozoite surface protein 1 in rural Amazonians. *Clin Vaccine Immunol 14:* 1249–1259.
31. Adams JH, Hudson DE, Torii M, Ward GE, Wellems TE, Aikawa M, Miller LH, 1990. The Duffy receptor family of *Plasmodium knowlesi* is located within the micronemes of invasive malaria merozoites. *Cell 63:* 141–153.
32. Cowman A, Crabb B, 2006. Invasion of red blood cells by malaria parasites. *Cell 124:* 755–766.
33. Carvalho LH, Fontes CJE, Krettli AU, 1999. Cellular responses to *Plasmodium falciparum* major surface antigens and their relationship to human activities associated with malaria transmission. *Am J Trop Med Hyg 60:* 674–679.
34. Barbieri AF, Sawyer DO, 2007. Heterogeneity of malaria prevalence in alluvial gold mining areas in northern Mato Grosso State, Brazil. *Cad Saude Publica 23:* 2878–2886.
35. Santos E, Loureiro E, Jesus I, Brabo E, Silva R, Soares M, Câmara V, Souza M, Branches F, 1995. Diagnosis of health conditions in a pan-mining community in the Tapajós River Basin, Itaituba, Par, Brazil, 1992. *Cad Saude Publica 11:* 212–225.
36. Silbergeld E, Nash D, Trevant C, Strickland G, de Souza J, da Silva R, 2002. Mercury exposure and malaria prevalence among gold miners in Pará, Brazil. *Rev Soc Bras Med Trop 35:* 421–429.
37. Singh SK, Hora R, Belrhali H, Chitnis CE, Sharma A, 2006. Structural basis for Duffy recognition by the malaria parasite Duffy-binding-like domain. *Nature 439:* 741–744.
38. Cole-Tobian JL, Michon P, Dabod E, Mueller I, King CL, 2007. Dynamics of asymptomatic *Plasmodium vivax* infections and Duffy binding protein polymorphisms in relation to parasitemia levels in Papua New Guinean children. *Am J Trop Med Hyg 77:* 955–962.
39. Richie T, 2006. High road, low road? Choices and challenges on the pathway to a malaria vaccine. *Parasitology 133:* S113–S144.
40. Gray JC, Corran PH, Mangia E, Gaunt MW, Li Q, Tetteh KKA, Polley S, Conway DJ, Holder AA, Bacarese-Hamilton T, Riley EM, Crisanti A, 2007. Profiling the antibody immune response against blood stage malaria vaccine candidates. *Clin Chem 53:* 1244–1253.
41. Langhorne J, Ndungu FM, Sponaas AM, Marsh K, 2008. Immunity to malaria: more questions than answers. *Nat Immunol 9:* 725–732.
42. Camargo L, Ferreira M, Krieger H, De Camargo E, Da Silva L, 1994. Unstable hypoendemic malaria in Rondonia (western Amazon region, Brazil): epidemic outbreaks and work-associated incidence in an agro-industrial rural settlement. *Am J Trop Med Hyg 51:* 16–25.
43. VanBuskirk KM, Cole-Tobian JL, Baisor M, Sevova ES, Bockarie M, King CL, Adams JH, 2004. Antigenic drift in the ligand domain of *Plasmodium vivax* Duffy binding protein confers resistance to inhibitory antibodies. *J Infect Dis 190:* 1556–1562.
44. Ceravolo I, Sanchez B, Sousa T, Guerra B, Soares I, Braga E, McHenry A, Adams J, Brito C, Carvalho L, 2009. Naturally acquired inhibitory antibodies to *Plasmodium vivax* Duffy binding protein are short-lived and allele-specific following a single malaria infection. *Clin Exp Immunol 156:* 502–510.

PCR-based methods for malaria field samples: potential unreliability at low *Plasmodium* density

Daniela C. Costa[a,◊], Ana P. Madureira[b,◊], Bruno A.M. Sanchez[a], Cor J.F. Fontes[c], Francisco Zumpano[d], Jean E. Limongi[e], Cristiana F.A. Brito[a], Luzia H. Carvalho[a,*]

[a]Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, MG, Brazil, [b]Departamento de Bioengenharia, Universidade Federal de São João Del Rei (UFSJ); [c]Universidade Federal de Mato Grosso (UFMT), Cuiabá, MT, 78048-902, Brazil; [d]Faculdade de Medicina, Universidade Federal de Minas Gerais (UFMG), 30130-100, Belo Horizonte, MG, Brasil; [e]Centro de Controle de Zoonoses de Uberlândia, 38401-240, Uberlândia, MG, Brazil.

Running title: PCR-based methods for malaria diagnostic

◊These authors equally contributed to the manuscript

* *Correspondence to*: Dr. Luzia H. Carvalho, Malaria Laboratory, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ-MG). Avenida Augusto de Lima 1715, 30190-002, Belo Horizonte, MG, Brazil, Tel: 55-31-33497793, Fax: 55-31-32953115; E-mail: lhcarvalho@cpqrr.fiocruz.br

This study was presented as a poster and abstract at the XII Brazilian Meeting on Malaria Research, Ouro Preto, Minas Gerais, Brazil, October 3 to 6, 2010.

SUMMARY

The well-accepted assumption that PCR protocols detect a single parasite per microlitre of blood was largely based on titration curves of DNA templates from plasmids and/or cultured *P.falciparum*. To investigate how reproductive is PCR in field-samples with low-levels of parasitemia, we evaluated the performance of well-established PCR protocols (nested-PCR and real-time PCR) against a panel of field-samples with parasitemia varied from 0 to >1000 parasites/μL of blood, as detected by outstanding microscopists. At parasitemia of >300 parasites/μl, both PCR protocols correctly identified 100% of microscopy-positive samples; however, the performance of PCR dropped off at densities of <100/μl. The limitation of PCR amplification was restricted to field-samples because titrations of DNA templates obtained from reference isolates or species-specific plasmids were able to detect <5 parasite/μL of blood. Altogether, these data suggested that straightforward PCR protocols, as those used extensively in malaria studies, required careful interpretation, especially at low levels of parasitemia.

*Keywords***:** malaria, PCR sensitivity, diagnosis, asymptomatic infection

1. **Introduction**

Malaria still represents a diagnostic challenge to laboratories in most endemic countries. In those areas, light microscopy (LM) remains the mainstay for malaria diagnosis, and the complementary use of immunochromatographic rapid diagnostic tests (RDTs) have been restricted to special circumstances such as those where good quality light microscopy is unavailable (Wongsrichanalai et al., 2007; Bell and Perkins, 2008). Despite of that, since 1990´s important progress has been achieved in the molecular identification and characterization of *Plasmodium* parasites. PCR-based diagnostic methods targeting the small subunit rRNA (SSU rRNA) gene had great impact because its allowed specific detection of the four major human *Plasmodium* species at densities several-times lower than the limit of LM detection (Snounou et al., 1993; M. et al., 1997; Win et al., 2002) some of those protocols have evolved from straightforward species-specific amplification strategies to multiplex approaches (Rubio et al., 1999; Lim et al., 2010; Mixson-Hayden, Lucchi and Udhayakumar, 2010). The next methodological improvement was the introduction of the adapted kinetic PCR, so-called real-time PCR, that dropped the time spent on conventional PCR protocols, the risks of contamination, and also have the potential to be applied to large-scale analyses (Hermsen et al., 2001; Veron, Simon and Carme, 2009; Dormond et al., 2010). Although the applicability of PCR-based methods for diagnosis of acute malaria remains a hard task to be reached (Hänscheid and Grobusch, 2002), this technology has transformed perspectives on the field of malaria epidemiology, chemotherapy and vaccines.

While specificity of PCR results is guaranteed by the nature of the target for primers and/or probes, sensitivity can be highly variable, depending on protocols as well as the population undergoing malaria diagnosis (Berry et al., 2005; Jelinek et al., 1996). Unfortunately, in most protocols, the PCR detection limit relies on end-point titration of DNA templates obtained from 18S species-specific plasmids, *Plasmodium* reference isolates, or *P.falciparum* in culture (Perandin et al., 2004; Gama et al., 2007; Veron et al., 2009). Consequently, a significant number of publications do not address the influence of individual parasitemia on PCR positivity and reproducibility. Aiming to investigate how reproductive is PCR assays for detecting low-levels of *Plasmodium* infection in field-samples, we evaluated the performance of two well-established PCR protocols – based on nested-PCR (Snounou et al., 1993) and real-time PCR (Mangold et al., 2005) - against a panel of field-samples from individuals who had parasite densities, as detected by outstanding malaria microscopists, varied from 0 to > 1000 parasites/μL blood. Our methodological approach included to test each sample a number of times (single vs. multiple assays), and to select a PCR protocol that could be more accessible to low-income countries where malaria is endemic.

2. Material and Methods

2.1. Plasmodium field isolates from Malaria Reference Laboratories

2.1.1. Endemic area. In the study, we included 198 whole-blood samples, categorized as (Table 1): (i) 128 from symptomatic patients presenting acute malaria infection at Regional Malaria Reference Laboratories in the Brazilian Amazon area (Julio Muller

Hospital, Cuiabá, MT and CEMETRON, Porto Velho, RO); (ii) 34 samples from long-term residents of an endemic Amazon rural community, Colniza, MT, where asymptomatic malaria infections occur (CJF Fontes, unpublished results); Colniza residents were negative for malaria infection by light microscopy, but 14 (41%) were positive by a malaria-specific Nested PCR carried-out in parallel (presumptive asymptomatic malaria infection , PAM); and (iii) 36 blood-samples from volunteers living outside of the endemic area and who had never been infected by malaria parasites (negative samples).

At the time of thick blood smear preparation, five milliliters of blood was drawn into sterile tubes with EDTA, and aliquots (300 μL) of whole blood were used subsequently to purify templates for the PCR assays. To further validate an economical laboratory filter paper method to amplify plasmodia DNA, we analyzed 70 matched blood samples - liquid blood and blood spotted on filter papers (≅ 30 and 60 μL/paper) - collected from subjects under field conditions in Rondônia State, Amazon area; 54 were malaria positives and 16 negatives, as detected by light microscopy.

2.1.2. Outside the Endemic area. We tested 100 whole-blood samples collected at the Section of Malaria of Federal University of Minas Gerais Medical School and at the Regional Malaria Reference Laboratory of the Uberlândia Zoonosis Control Center, Minas Gerais State, from 70 individuals returning from areas where malaria is endemic and who presented acute febrile disease suggestive of malaria. They were prospectively collected from February to December 2009. For that, finger prick blood

samples were taken from each participating and used to prepare thick blood smears and blood spot on conventional filter papers, ≅ 60μL/paper. Dried filter paper blood spots were stored in plastic bags, with silica gel at room temperature for subsequent DNA extraction; Giemsa-stained thick blood smears were analyzed on-site by experienced microscopists, as described above. The results of light microscopy identified 22 acute infections (31%), with 12 *P.vivax*, 8 *P.falciparum* and two mixed infections due to *P. falciparum* and *P. vivax*. In thirteen out of 22 patients it was possible to collect additional blood samples to follow-up parasitological cure (Table 2). For that, blood was taken in the day of diagnosis, before chemotherapy, and up to fourth weeks after malaria specific treatment; clinical management of the patients were undertaken independently of this study.

2.2. Blood-examination. The Giemsa-stained thick blood smear technique was used for malaria diagnosis in all samples of the present study, in which well-trained microscopists were in charge of examining the equivalent of 0.2 μL of blood (≅ 200 field microscopy), in accordance with the standards of the Brazilian Ministry of Health (Secretaria de Vigilância em Saúde [SVS], 2009), and parasite density estimated by blood microliters. For quality assurance, a random sample of 10% of the blood smears was reviewed by a second trained microscopist, who was masked to the initial smear results.

2.3. Preparation of DNA template

2.3.1. Whole-blood samples. Genomic DNA (gDNA) was extracted from 1mL of individual whole-blood samples using a Qiagen genomic DNA purification kit (Puregene®, Gentra Systems, Minneapolis, MN, USA), according to the manufacturers' recommendations. The DNA was eluted to a 330 µL volume (≅100ng of DNA /µl), and stored at -20^{o}C until be used.

2.3.2. Filter Paper whole-blood Spots. In this case, two gDNA samples were isolated per individual, corresponding volumes of 30 µL and 60 µL of whole-blood spotted as circle on conventional filter paper (Consalab Com Imp Ltda, São Paulo, Brazil). The DNA samples were extracted according to the manufacturers' recommendations of the QIAamp® DNA mini kit (Puregene, Gentra Systems, Minneapolis, MN, USA). DNA samples were eluted in a final volume of 150 µl and stored at -20°C in sterile capped containers until be used.

2.4. Nested-PCR

Samples were amplified by a Nested-PCR adapted from Snounou and colleagues (Snounou et al., 1993), including the same primers. Briefly, all PCR reactions was performed in a total volume of 20 µL, with 250µM of each oligonucleotide primer, 10µL of Master Mix (Promega-0.3 unit of Taq Polymerase, 200µM of each of the four deoxyribonucleotide triphosphates and 1,5 mM of $MgCl_2$) and ≅80 ng of DNA. The PCR assays were performed using a heating block (PTC-100^{TM} version 7.0 – MJ Research Inc., USA) and the cycling parameters were: Step 1, 95°C for 5 min; step 2, annealing at 58°C for 2 min; step 3, extension at 72°C for 2

min; step 4, denaturation at 94°C for 1 min; repeat steps 2-4 for 24 times, then step 2 and 3, and finally the temperature was reduced for 4°C until be taken. The cycling parameters for the second round were the same for the first reaction, but increasing for 30 cycles. The amplified products were detected by ethidium bromide staining following agarose 2% gel electrophoresis (Invitrogen) and the specie-specifics fragments size are: 205 bp, for *P. falciparum*; 120 bp, for *P. vivax* and 144 bp for *P. malariae*. As a control of the DNA extractions, the panel of samples was previously amplified for a gene present in the ABO human system, as previously described (Olsson et al., 1998).

2.5. Real-time PCR

Malaria parasite species identification was performed by real time PCR amplification of the 18S SSU rRNA gene, as previously described (Mangold et al., 2005); based on this protocol, a consensus pair of primers was used to amplify a species-specific region of the multicopy 18S rRNA gene. Briefly, each 20 µl reaction mix contained 200 ng of genomic DNA, 10 µl of 10x 5 µl Sybr® Green PCR master mix (Applied Biosystems), 2.5 mM $MgSO_4$ and 0.5 pmole of each primer (BioSynthesis). The PCR conditions was consisted of an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 90°C for 30 seconds, and 60°C for 30 seconds; with fluorescence acquisition at the end of each extension step. After amplification, melting curves were observed from the dissociation curves and those melting curves analysis based on nucleotide variations within the amplicons provided a basis for accurate differentiation of 3 plasmodia: *P. falciparum, P. vivax and P. malariae*. The amplification and fluorescence detection were performed using ABI

PRISM® 7000 sequence Detection System (Applied Biosystems). The melting temperature (T_m) ranges values for each *Plasmodium* was: P. *vivax* , 74 to 76°C; *P. falciparum,* 71 to 73°C; and *P. malariae,* 68 to 70°C.

2.6. Statistical Analysis

The sensitivity was calculated by the ratio of the number of true positives divided by the number of true positives and false negatives combined. The specificity was calculated by the number of true negatives divided by the number of true negatives and false positives combined. The kappa coefficient of agreement was used to assess the reliability between light microscopy and molecular diagnosis with a 95% confidence interval. A chi-square analysis was performed to evaluate the positive results between Nested and Real Time PCR and between single and multiple reactions for both tests and do not shown significant difference ($p>0.05$). In the cases with less than 5 observations were realized a Fischer exact Test.

3. Results

3.1. Performances of nested PCR and real-time PCR at different malaria parasite densities

An overall comparison of PCR protocols and laboratory microscopy for malaria detection at various parasite densities is presented in Table 3. Regardless the PCR protocol, at parasite densities of >300 parasites/µl of blood, PCR correctly identified 100% (36/36) of microscopy-positive samples; however, the performance of both PCR protocols dropped off at densities of <100/µl, with only 50% (11/22) and

59% (13/22) of positive samples detected by nested and real-time PCR, respectively. Aiming to analyze if the PCR amplification efficiency could be improved by undertaking assay repetitions, we retested all 94 gDNA samples, and the results included in Table 3 represent the agreement between at least three out of five PCR reactions. Although multiple assays seem to increase the PCR efficiency at low levels of parasitemia, 50% vs. 72% and 59% vs. 68% to nested-PCR and real-time PCR, respectively, these differences were not statistically significant. Assuming light microscopy as the gold standard, both PCR protocols were specific; conversely, sensitivity decreased with parasite density, with 50-68% and 55%-73% to nested and real-time PCR, respectively, at parasite densities bellow 100/μL (Table 4).

For *Plasmodium* species-specific identification, at parasite densities ≥ 100/μL, microscopy and all PCR protocols (nested vs. real-time and single vs. multiple) were highly comparable, with PCR detecting 40 out of 41 *P.vivax* infection, and 10 out of 11 *P.falciparum* infections; in addition, PCR protocols detected a few number of mixed infections (*P.vivax* plus *P.falciparum*) that could not be detected by LM (Table 5). As expected, discordant results were obtained at low-levels of parasitemia, basically due to the high number of PCR negative samples.

Of importance, the limitation of PCR amplification, at low levels of parasitemia, was restricted to DNA from field samples because titrations of DNA templates obtained from *Plasmodium* reference isolates and or species-specific plasmid constructions were able to detect < 5 parasite/μL of blood. Figure 1 illustrates end-point DNA titrations by Real-time PCR, in which DNA templates were obtained

from *P.falciparum* continuous culture or from an artificial mixed infection obtained from high-parasitemic individuals infected with *P.falciparum* or *P.vivax* (≅10.000 parasites/μL of blood). Similar results were obtained by nested-PCR (data not shown).

3.2. Performances of PCR using liquid blood and blood spotted on filter paper

In order to assess feasibility of detecting malaria infection in an inexpensive filter paper, we analyzed DNA in 70 matched blood samples (liquid blood and dried blood on filter papers), collected from subjects under field conditions in the Amazon area, with 54 identified by light microscopy as positive thick blood smear and 16 negatives. For that, we chose the nested-PCR because it still remains as a malaria reference PCR protocol. As shown in Table 6, both protocols were able to amplify malaria parasites in 51 out of 54 (96%) microscopy-positive samples, with no difference between one (≅30 μL) or two drops (≅60 μL) of blood spotted on filter paper; those three non-amplified samples contained less than 300 parasites/μL of blood. Regarding the species-specific identification, no difference was observed between DNA extracted from filter paper or liquid blood samples; PCR assays carried-out with each protocol were able to identify 45 *P.vivax*, 8 *P.falciparum* and one mixed infection by *P.falciparum* and *P.vivax* (data not shown). Taken together, these results reinforce the feasibility of plasmodia amplification from blood spotted on non-expensive filter paper.

3.3. Evaluation of PCR among travelers returning from malaria-endemic areas

We investigated by nested-PCR samples from 70 individuals returning from the endemic area and who had history of febrile illness. These individuals, whose blood was submitted for microscopic examination, were attended at the Regional Malaria Reference Laboratories/Hospital of Minas Gerais State, non-endemic area. The results of nested-PCR were in accordance with light microscopy, since both methods identified malaria acute infections in 22 out of 70 (31%) travelers, with 12 *P.vivax*, 8 *P.falciparum* and two mixed infections due to *P. falciparum* and *P. vivax*. However, most infected travelers had parasitemia levels above of 300 parasite/μL of blood (20 out of 22).

In thirteen malaria infected travelers, additional samples were taken starting at the day of diagnostic (day 0), and collected during the next fourth weeks after malaria diagnostic (Fig. 2). During the follow-up, PCR and microscopy were concordant in 11 of 13 individuals, however, in 5 of them (MG5, MG8, MG11, MG12, MG13) it was not possible to follow-up until results become malaria negative (microscopy and/or PCR). A recurrent *P.vivax* infection was detected in one individual (MG5), around the 4^{th} week post-treatment, probably a relapse due to hypnozoites. In two *P.falciparum* infections, MG2 and MG3, LM detected parasitological cure before PCR.

3.4. Evaluation of PCR on detection of asymptomatic malaria infection

Thirty-four samples from long-term residents of an Amazon rural community - an area where asymptomatic malaria infections seems to occur (presumptive asymptomatic malaria infection, PAM) - were assayed by both PCR-based protocols. At the time of blood collection, all of them had a negative malaria thick-blood smear, but 14 out of 34 (41%) were positive by a Nested PCR assay carried-out in parallel.

In order to evaluate the reproducibility of DNA amplification by PCR-based protocols, each sample was submitted to three nested-PCR and one real-time PCR reactions; towards a molecular consensus, those discordant samples were re-amplified by additional PCR assays (Table 7). Molecular consensus was obtained with four equal PCR results, which confirmed malaria in 7 (21%) and excluded in 14 (41%) samples. In 13 (38%) samples the results of PCR assays were dubious, probably reflecting the limitation of molecular methods at low levels of parasitemia. By the other hand, no positive results were obtained with blood samples from malaria naive volunteers followed by replicate DNA extractions and PCR analysis (data not shown).

4. Discussion

Based on titration curves of malarial DNA templates obtained from either 18S species-specific plasmids or in vitro cultured *P. falciparum* parasites, PCR-based protocols has been claimed to be capable of detecting very low levels of parasitemia. Here, by analyzing field-samples, in which parasite density was quantified by certified outstanding microscopists, we demonstrated that the performance of both

nested-PCR and real-time PCR dropped off at densities <100/µl, and no significant difference could be obtained by multiple replicates.

Unfortunately, few studies have used clinical samples stratified by parasitemia to determine the PCR sensitivity, and in most of them PCR turned out to be less efficient than microscopy at low parasite densities (Nandwani, Mathur and Rawat, 2005; Aslan et al., 2007; Coleman et al., 2006; Harris et al., 2010). By follow-up malaria acute infection in a village in western Thailand, it was demonstrated (Coleman et al., 2006) a very poor performance of PCR at parasite densities <100 parasites/µl, with PCR sensitivity of 20% for *P. falciparum* and 24% for *P. vivax*. Recently, in a study conducted in Solomon Islands, among 33 microscopy positive but PCR negative samples - examined by World Health Organization (WHO) certified level 1 expert microscopists - 31 (94%) had parasite densities <100/µL, of which 18 samples were counted as 8-10/µL, which is equivalent to one parasite in 100-300 fields on a thick blood smear (Harris et al. 2010). In this study, microscopy performed better in detecting *P. vivax* than detecting *P. falciparum* as the *P. vivax* prevalence determined by microscopy was 73% of that estimated by PCR. It should be noted that the level of microscopy detection described by the authors (Harris et al., 2010) is similar to that obtained by our certified microscopists; this level of detection and accuracy would not have been achieved in many aid posts and health centers which often have less experienced microscopists and thus error in microscopy results could be greater than found here. Consequently, an apparently poor specificity of blood smears which has been described in some field studies may actually reflect poor sensitivity on the part of the PCR-based assays (Taylor et al., 2010).

Decrease in the efficiency of PCR reaction may be caused by inhibiting agents used during nucleic acid extraction or copurified from the biological sample Al-Soud (Al-Soud and Rådström, 2001). Conseguently, it might not be appropriate the assumption that standards are running with the same efficiency as field-samples (Guescini et al., 2008). In accordance with poor PCR sensitivity, blood filtration methods for removal of leukocytes has been proposed to increase PCR sensibility during malaria vaccine clinical trials (Andrews et al., 2005; Bejon et al., 2006). After using a multi-step protocol, in which 5-ml of blood were filtered for removal of leukocytes, these authors (Andrews et al., 2005) stablished a PCR cutoff point of 20 parasites/mL to follow vaccinee volunteers in Phase II trials. Taken together, these data suggested that straightforward PCR protocols, as those used extensively in malaria studies, required careful interpretation, especially at very low levels of parasitemia.

Molecular analyses of blood samples dried on filter papers is quite a routine in malaria field research (Singh et al., 1996; Mlambo et al., 2008; Lekweiry et al., 2009; Steenkeste et al., 2010), consequently, we included 70 matched blood samples (liquid blood and blood spotted on filter papers) stratified according to parasitemia. Since the results of plasmodial DNA amplification showed no difference between both DNA extraction techniques, we further choose the simple blood spot sampling onto filter paper to evaluate PCR among travelers returning from malaria-endemic areas. Although there was concordance between PCR and microscopy, the majority of those positive blood films had more than 300 parasites/µl blood. This result confirms that,

unfortunately, malaria diagnosis is often delayed in imported cases, and it is a risk factor for severe imported malaria (Lederman et al., 2006; Moulin and Gendrel, 2009; Boggild et al., 2009; Dubos et al., 2010). In effect, a *P.falciparum* patient (MG13) died soon after be admitted to the intensive care unit, reinforcing that it should be mandatory to evoke the diagnostic of malaria in any febrile traveler coming from a malaria endemic area.

Although monitoring efficacy of antimalarial treatment was out of the scope of the current study, the availability of 43 post-treatment samples from 13 patients allowed us to demonstrate a good agreement between PCR and microscopy. In only two patients, post-treatment samples remained PCR positive after negativation of microscopy (MG2 e MG3). It is possible to speculate that this positivity of PCR could be consequence of the persistence of circulating DNA from residual gametocytes or DNA from viable but drug-damaged parasites unable to initiate further infection. In fact, concerns have already been raised over the relationship between persistent gametocytes and positive PCR results (Snounou and Beck, 1998; Basco and Ringwald, 2000). Nevertheless, our study was not designed to proper investigate the relationship between PCR positivity and persistence of infection.

As part of the international target of a reversal in the incidence of malaria by 2015, it has been proposed that the treatment of asymptomatic carriers should be an essential tool for breaking the cycle of infection in some transmission settings (Ogutu et al., 2010). To reach this goal requires improving diagnostic tests that allow easier identification of asymptomatic carriers. Because a few studies have intended to

address that reproducibility and reliability of PCR-based protocols at very low levels of parasitaemia, we investigated the reproducibility of the PCR in a group of individuals with presumptive asymptomatic malaria infection (PAM), whose thick-blood smears were negative for malaria infection. Although most of PCR reactions using replicate samples were concordant (62%), in thirteen (38%) samples no consensus could be found even after running seven replicates of each DNA template (i.e., 4-times by nested-PCR and 3-times by real-time PCR). These results confirm previous observations of irreproducibility of parasite detection in samples with very low parasitemias (Singh et al., 1999). It means that any PCR template diluted past ascertain threshold copy number will experience large variations in amplification. This inherent limitation of PCR amplification, previously described (Karrer et al., 1995) as the Monte Carlo effect, has been widely underestimated (Stenman and Orpana, 2001; Soong and Ladányi, 2003). In fact, distinct factors may hinder specific parasite PCR amplification at very low parasitemias, including the biological sample itself (Guescini et al., 2008), the concentration of human genomic DNA (Andrews et al., 2005), variations in amplification conditions (Calderaro et al., 2007), and the PCR reagents, even when using premade master mixes from the same manufacturer (Bustin, 2002).

Taken together, these observations strongly advocate that the PCR results from field-samples requires careful interpretation as it has become clear that PCR –based protocols can be highly variable at low-copy-number of target templates. Further, it would be crucial the development of standardized protocols for the detection of *Plasmodium* parasites, and that these protocols should be validated by reference laboratories before their adoption as an adjunct to routine diagnosis.

Acknowledgment

The authors would like to thank the Program for Technological Development in Tools for Health - PDTIS - FIOCRUZ for use of the Real-time facilities. Maria Helena Betti and Reinando Cesar de Freitas for their help with patients at the Section of Malaria of Federal University of Minas Gerais Medical School. This work was supported by the Research Foundation of Minas Gerais State (FAPEMIG), The Brazilian National Research Council (CNPq), and Oswaldo Cruz Foundation (FIOCRUZ, PAPES V), Pronex Malaria, CNPq/DECIT/MS; scholarships from CAPES (DCO), CNPq-FIOCRUZ (APM), and CNPq (LHC, CJFF, and CFAB) are also acknowledged.

References

Al-Soud WA, Rådström P (2001) Purification and characterization of PCR-inhibitory components in blood cells. J Clin Microbiol 39:485-493.

Andrews L, Andersen R, Webster D, Dunachie S, Walther R, Bejon P, Hunt-Cooke A, Bergson G, Sanderson F, Hill A (2005) Quantitative real-time polymerase chain reaction for malaria diagnosis and its use in malaria vaccine clinical trials. Am J Trop Med Hyg 73:191-198.

Aslan G, Seyrek A, Kocagoz T, Ulukanligil M, Erguven S, Gunalp A (2007) The diagnosis of malaria and identification of plasmodium species by polymerase chain reaction in Turkey. Parasitol Int 56:217-220.

Basco L, Ringwald P (2000) Molecular epidemiology of malaria in Yaounde, Cameroon. VII. Analysis of recrudescence and reinfection in patients with uncomplicated falciparum malaria. Am J Trop Med Hyg 63:215-221.

Bejon P, Mwacharo J, Kai O, Mwangi T, Milligan P, Todryk S, Keating S, Lang T, Lowe B, Gikonyo C, Molyneux C, Fegan G, Gilbert S, Peshu N, Marsh K, Hill A (2006) A phase 2b randomised trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. PLoS Clin Trials 1:e29.

Bell D, Perkins M (2008) Making malaria testing relevant: beyond test purchase. Trans R Soc Trop Med Hyg 102:1064-1066.

Berry A, Fabre R, Benoit-Vical F, Cassaing S, Magnaval J (2005) Contribution of PCR-based methods to diagnosis and management of imported malaria. Med Trop (Mars) 65:176-183.

Boggild A, Page A, Keystone J, Morris A, Liles W (2009) Delay in diagnosis: malaria in a returning traveller. CMAJ 180:1129-1131.

Bustin S (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23-39.

Calderaro A, Piccolo G, Perandin F, Gorrini C, Peruzzi S, Zuelli C, Ricci L, Manca N, Dettori G, Chezzi C, Snounou G (2007) Genetic polymorphisms influence Plasmodium ovale PCR detection accuracy. J Clin Microbiol 45:1624-1627.

Coleman R, Sattabongkot J, Promstaporm S, Maneechai N, Tippayachai B, Kengluecha A, Rachapaew N, Zollner G, Miller R, Vaughan J, Thimasarn K, Khuntirat B (2006) Comparison of PCR and microscopy for the detection of asymptomatic malaria in a Plasmodium falciparum/vivax endemic area in Thailand. Malar J 5:121.

Dormond L, Jaton-Ogay K, de Vallière S, Genton B, Bille J, Greub G (2010) Multiplex real-time PCR for the diagnosis of malaria: correlation with microscopy. Clin Microbiol Infect.

Dubos F, Dauriac A, El Mansouf L, Courouble C, Aurel M, Martinot A (2010) Imported malaria in children: incidence and risk factors for severity. Diagn Microbiol Infect Dis 66:169-174.

Gama B, Silva-Pires FE, Lopes M, Cardoso M, Britto C, Torres K, de Mendonça Lima L, de Souza J, Daniel-Ribeiro C, Ferreira-da-Cruz MF (2007) Real-time PCR versus conventional PCR for malaria parasite detection in low-grade parasitemia. Exp Parasitol 116:427-432.

Guescini M, Sisti D, Rocchi M, Stocchi L, Stocchi V (2008) A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. BMC Bioinformatics 9:326.

Harris I, Sharrock W, Bain L, Gray K, Bobogare A, Boaz L, Lilley K, Krause D, Vallely A, Johnson M, Gattom, M, Shanks G, Cheng Q (2010) A large proportion of asymptomatic Plasmodium infections with low and sub-microscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: challenges for malaria diagnostics in an elimination setting. Malar J 9:254.

Hermsen C, Telgt D, Linders E, van de Locht L, Eling W, Mensink E, Sauerwein R (2001) Detection of Plasmodium falciparum malaria parasites in vivo by real-time quantitative PCR. Mol Biochem Parasitol 118:247-251.

Hänscheid T, Grobusch M (2002) How useful is PCR in the diagnosis of malaria? Trends Parasitol 18:395-398.

Jelinek T, Proll S, Hess F, Kabagambe G, von Sonnenburg F, Loscher T, Kilian A (1996) Geographic differences in the sensitivity of a polymerase chain reaction for the detection of Plasmodium falciparum infection. Am J Trop Med Hyg 55:647-651.

Karrer E, Lincoln J, Hogenhout S, Bennett A, Bostock R, Martineau B, Lucas W, Gilchrist D, Alexander D (1995) In situ isolation of mRNA from individual plant cells: creation of cell-specific cDNA libraries. Proc Natl Acad Sci U S A 92:3814-3818.

Kimura M, Osamu K, Qing L, Mian Z, Fumihiko K, Yusuke W, Shuzo O, Yoshiko Y, Kazuyuki T (1997) Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. Parasitology International 46, 91-154.

Lederman E, Sutanto I, Wibudi A, Ratulangie L, Rudiansyah I, Fatmi A, Kurniawan L, Nelwan R, Maguire J (2006) Imported malaria in Jakarta, Indonesia: passive surveillance of returned travelers and military members postdeployment. J Travel Med 13:153-160.

Lekweiry K, Abdallahi M, Ba H, Arnathau C, Durand P, Trape J, Salem A (2009) Preliminary study of malaria incidence in Nouakchott, Mauritania. Malar J 8:92.

Lim Y, Mahmud R, Chew C, T T, Chua K (2010) Plasmodium ovale infection in Malaysia: first imported case. Malar J 9:272.

Mangold K, Manson R, Koay E, Stephens L, Regner M, Thomson RJ, Peterson L, Kaul K (2005) Real-time PCR for detection and identification of Plasmodium spp. J Clin Microbiol 43:2435-2440.

Mixson-Hayden T, Lucchi N, Udhayakumar V (2010) Evaluation of three PCR-based diagnostic assays for detecting mixed Plasmodium infection. BMC Res Notes 3:88.

Mlambo Mlambo G, Vasquez Y, LeBlanc R, Sullivan D, Kumar N (2008) A filter paper method for the detection of Plasmodium falciparum gametocytes by reverse transcription polymerase chain reaction. Am J Trop Med Hyg 78:114-116.

Moulin F, Gendrel D (2009) [Imported malaria: diagnostic traps and rapid tests]. Arch Pediatr 16 Suppl 2 S89-92.

Nandwani S, Mathur M, Rawat S (2005) Evaluation of the polymerase chain reaction analysis for diagnosis of falciparum malaria in Delhi, India. Indian J Med Microbiol 23:176-178.

Ogutu Ogutu B, Tiono A, Makanga M, Premji Z, Gbadoé A, Ubben D, Marrast A, Gaye O (2010) Treatment of asymptomatic carriers with artemether-lumefantrine: an opportunity to reduce the burden of malaria? Malar J 9:30.

Olsson M, Hansson C, Avent N, Akesson I, Green C, Daniels G (1998) A clinically applicable method for determining the three major alleles at the Duffy (FY) blood group locus using polymerase chain reaction with allele-specific primers. Transfusion 38:168-173.

Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, Medici M, Arcangeletti M, Snounou G, Dettori G, Chezzi C (2004) Development of a real-time PCR assay for detection of Plasmodium falciparum, Plasmodium vivax, and Plasmodium ovale for routine clinical diagnosis. J Clin Microbiol 42:1214-1219.

Rubio J, Benito A, Roche J, Berzosa P, García M, Micó M, Edú M, Alvar J (1999) Semi-nested, multiplex polymerase chain reaction for detection of human malaria parasites and evidence of Plasmodium vivax infection in Equatorial Guinea. Am J Trop Med Hyg 60:183-187.

Secretaria de Vigilância em Saúde (2009) Manual de Diagnóstico Laboratorial da Malária. 116p. Brasília, DF Mnistério da Saúde.

Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah M, Rahman H (1999) A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg 60:687-692.

Singh B, Cox-Singh J, Miller A, Abdullah M, Snounou G, Rahman H (1996) Detection of malaria in Malaysia by nested polymerase chain reaction amplification of dried blood spots on filter papers. Trans R Soc Trop Med Hyg 90:519-521.

Snounou G, Beck H (1998) The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. Parasitol Today 14:462-467.

Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown K (1993) Identification of the four human malaria parasite species in field samples by the polymerase chain

reaction and detection of a high prevalence of mixed infections. Mol Biochem Parasitol 58:283-292.

Soong R, Ladányi A (2003) Improved indicators for assessing the reliability of detection and quantification by kinetic PCR. Clin Chem 49:973-976.

Steenkeste N, Rogers W, Okell L, Jeanne I, Incardona S, Duval L, Chy S, Hewitt S, Chou M, Socheat D, babin F, Ariey F, Rogier C (2010) Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: implication for malaria elimination. Malar J 9:108.

Stenman J, Orpana A (2001) Accuracy in amplification. Nat Biotechnol 19:1011-1012.

Taylor S, Juliano J, Trottman P, Griffin J, Landis S, Kitsa P, Tshefu A, Meshnick S (2010) High-throughput pooling and real-time PCR-based strategy for malaria detection. J Clin Microbiol 48:512-519.

Veron V, Simon S, Carme B (2009) Multiplex real-time PCR detection of P. falciparum, P. vivax and P. malariae in human blood samples. Exp Parasitol 121:346-351.

Win T, Lin K, Mizuno S, Zhou M, Liu Q, Ferreira M, Tantular I, Kojima S, Ishii A, Kawamoto F (2002) Wide distribution of Plasmodium ovale in Myanmar. Trop Med Int Health 7:231-239.

Wongsrichanalai C, Barcus M, Muth S, Sutamihardja A, Wernsdorfer W (2007) A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). Am J Trop Med Hyg 77:119-127.

Figure 1

Real-time PCR melting curve analysis obtained from DNA template dilutions from *P.falciparum* continuous erythrocyte culture (A) and artificial *P.falciparum* and *P.vivax* infection (B). The graph was generated by using Sequence detection system (SDS) Applied Biosystems Software ABI Prism ® 7000. The dilution was done in 3,000; 300; 30; 3; 0.3 and 0 parasite/μL of blood.

Figure 2R. Malaria follow-up by light microcopy (LM) and Nested-PCR for 13 patients infected with (A) *P.falciparum,* Pf, (B) *P.vivax,* Pv, and (C) both plasmodium species, Pf + Pv, and whose antimalarial treatment was in accordance with the standards of the Brazilian Ministry of Health (Secretaria de vigilância em Saúde [SVS], 2009). Follow-up samples were taken from time zero (1^{st} sample, on day of diagnosis and before therapy) and during any time of the following weeks (2^{nd}, 3^{rd}, 4^{th} and 5^{th} samples). LM results are given by stratified parasitemia (parasites/μl of blood) and nested-PCR by positive (+) or negative (-) species-specific amplification. Numbers on the left refer to the individual code (MG1 to MG13).

Table 1

Distribution of study samples according to Light Microscopy (LM) results carried-out by outstanding microscopists from Malaria Reference Laboratories in the Amazon area

Samples	**LM[a]**	**N (%)**
Positive	>1000	49 (25)
	301-1000	22 (11)
	101-300	30 (15)
	1-100	27 (14)
PAM[b]	0	34 (17)
Negative[c]	0	36 (18)
Total (%)		198 (100)

[a] Giemsa-stained blood thick smears were stratified by different levels of parasitemia, and results were expressed as parasites/μl blood.

[b] PAM= Presumptive asymptomatic malaria infection.

[c] Negative samples from non endemic area.

Table 2

Light Microscopy (LM) results for the first malaria diagnosis and parasitological follow-up of thirteen cases of imported malaria in Minas Gerais State, non-endemic area, 2009

Case	First LM diagnosis	Follow-up by LM		
		Thick blood smears	Results	
	(parasite species)	(n)	Positive	Parasite species
MG 1	Pv[a]	4	0	-
MG 2	Pf[b]	3	0	-
MG 3	Pf	4	1	Pf
MG 4	Pv	3	1	Pv
MG 5	Pv	3	0	Pv
MG 6	Pf	3	2	Pf, Pf
MG 7	Pf plus Pv[c]	2	0	-
MG 8	Pf	2	2	Pf, Pf
MG 9	Pv	2	0	-
MG 10	Pv	1	0	-
MG 11	Pf plus Pv	1	1	Pf plus Pv
MG 12	Pf	1	1	Pf
MG 13	Pf	1	1	Pf[d]
Total		30	9	-

[a] Pv = *Plasmodium vivax.*

[b] Pf = *Plasmodium falciparum.*

[c] Pf plus Pv = mixed infection by *P.vivax* and *P.falciparum.*

[d] Patient evolved to death on the 3rd day of malaria specific treatment (Coartem, as recommended by Brazilian Ministry of Health).

Table 3

Performance of PCR protocols at different parasite densities, as detected by Light Microscopy, with single or multiple assays realized per PCR protocol

		Nested-PCR positive (%)		Real-time PCR positive (%)	
Samples	**Parasites/μl (n)**[a]	**Single**[b]	**Multiple**	**Single**	**Multiple**
Positive	>1000 (20)	20 (100%)	20 (100%)	20 (100%)	20 (100%)
	301-1000 (16)	16 (100%)	16 (100%)	16 (100%)	16 (100%)
	101-300 (16)	15 (94%)	16 (100%)	15 (100%)	15 (100%)
	1-100 (22)	11 (50%)	16 (72%)	13 (59%)	15 (68%)
Total	>1 (74)	62 (84%)	68 (92%)	64 (86%)	66 (89 %)
Negative	0(20)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

[a] Parasitaemia was stratified based on Light Microscopy results; Single, the result of the first PCR reaction, and Multiple, the results of three concordant PCR reactions.

[b] There were not statistical difference between positive results from single and multiple Nested-PCR, from single and multiple Real Time PCR, or from Nested and Real Time PCR for single or multiple reactions ($p>0.05$).

Table 4

Sensitivity and Specificity of Nested-PCRand Real-time PCR using light microscopy as the gold standard for different parasitemia

		PCR[a]	Parasite/µL of blood (n)			
			≤ 100 (22)	101-300 (16)	301-1000 (16)	>1000 (20)
Nested-PCR	Sensitivity	Single	0.50	0.94	1.0	1.0
		Multiple	0.68	1.0	1.0	1.0
	Specificity	Single	1.0	1.0	1.0	1.0
		Multiple	1.0	1.0	1.0	1.0
Real-time PCR	Sensitivity	Single	0.55	0.94	1.0	1.0
		Multiple	0.73	0.94	1.0	1.0
	Specificity	Single	1.0	1.0	1.0	1.0
		Multiple	1.0	1.0	1.0	1.0

[a] Single, the result of the first PCR reaction; Multiple, the results of three concordant PCR reactions.

Table 5

PCR performance for the detection of P.falciparum and/or P.vivax, at different parasite densities detected by light microscopy (LM), with single or multiple PCR reactions performed per protocol

Molecular Method	PCR [c]	*Plasmodium* Specie	Number of positive samples (%)			
			≥ 100/µL [a], n=52		< 100/µL, n=22	
			LM	PCR	LM	PCR
Nested	Single	Pv	41 (79) [b]	40 (77)	9 (41)	4 (18)
		Pf	11 (21)	9 (17)	12 (55)	5 (23)
		Mixed	0	2 (4)	1(4)	2 (9)
		Negative	0	1 (2)	0	11 (50)
	Multiple	Pv		40 (77)		8 (36)
		Pf		10 (19)		6 (27)
		Mixed		2 (4)		2 (9)
		Negative		0		6 (27)
Real Time	Single	Pv		40 (77)		10 (45)
		Pf		10 (19)		3 (14)
		Mixed		1 (2)		0
		Negative		1 (2)		9 (41)
	Multiple	Pv		40 (77)		11 (50)
		Pf		10 (19)		4 (18)
		Mixed		1 (2)		0
		Negative		1 (2)		7 (32)

[a] Parasites/µl blood, as detected by light microscopy (LM).

[b] Frequencies (%) were calculated assuming LM results as true positives, which included fifty-two samples positive at parasite density of 100 µL/blood or greater, and twenty-two at low levels of parasitemia.

[c] Single, the first PCR reaction, and multiple, the results of three concordant PCR reactions.

Table 6

Performance of PCR protocols at different parasite densities, using liquid blood and blood spotted on filter paper

Samples	LM	Different protocols of DNA extration (%)		
		Liquid blood	Blood spotted on filter papers	
	Parasites/µl (n)[a]		30 µL	60 µL
Positives	>3000 (16)	16 (100)	16 (100)	16 (100)
	3000-1001 (13)	13 (100)	13 (100)	13 (100)
	1000-301 (6)	6 (100)	6 (100)	6 (100)
	300-101 (14)	12 (86)	12 (86)	12 (86)
	1-100 (5)	4 (80)	4 (80)	4 (80)
Total	1->3000 (54)	51 (96)	51 (96)	51 (96)
Negatives	0 (16)	0 (0)	0 (0)	0 (0)

[a] Parasites/µl blood, as detected by light microscopy (LM).

Table 7

Reproducibility of PCR-based protocols among 34 long-term residents of an Amazon rural community (Colniza, MT) whose thick blood smears were negative for malaria infection

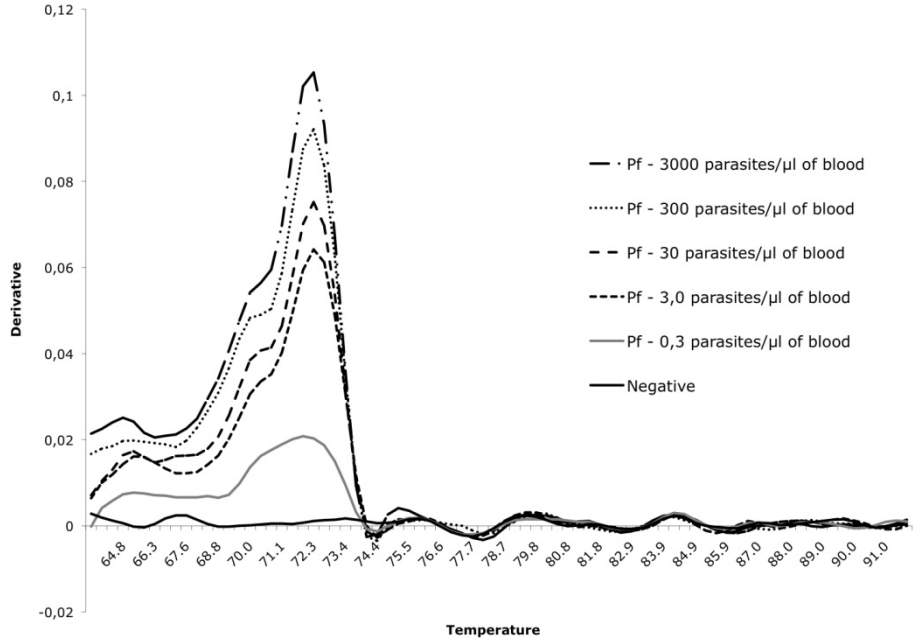
Code	Nested-PCR [a]				Real-time PCR [b]			Consensus result
	1st	2nd	3rd	4th	1st	2nd	3rd	
903	-	-	-	ND	-	ND	ND	
906	-	-	-	ND	-	ND	ND	
907	-	-	-	ND	-	ND	ND	
909	-	-	-	ND	-	ND	ND	
911	-	-	-	ND	-	ND	ND	
912	-	-	-	ND	-	ND	ND	
913	-	-	-	ND	-	ND	ND	Negative, 14 (41%)
918	-	-	-	ND	-	ND	ND	
920	-	-	-	ND	-	ND	ND	
922	-	-	-	ND	-	ND	ND	
923	-	-	-	ND	-	ND	ND	
934	-	-	-	ND	-	ND	ND	
935	-	-	-	ND	-	ND	ND	
936	-	-	-	ND	-	ND	ND	
902	+	+	+	ND	+	ND	ND	
904	+	+	+	ND	+	ND	ND	
916	+	+	+	ND	+	ND	ND	
921	+	+	+	ND	+	ND	ND	Positive, 7 (21%)
924	+	+	+	ND	+	ND	ND	
925	+	+	+	ND	+	ND	ND	
929	+	+	+	ND	+	ND	ND	
901	+	-	-	-	-	-	-	
908	+	-	-	-	-	-	-	
915	-	-	+	-	-	-	-	
928	-	-	+	-	-	-	-	

919	-	-	+	+		-	-	-	
905	+	-	-	+		+	+	+	Doubtful, 13 (38%)
910	-	+	-	+		+	+	+	
917	-	+	+	+		+	+	+	
930	+	-	+	-		+	+	+	
926	+	-	-	+		+	-	-	
927	-	+	+	+		+	-	-	
932	+	+	+	ND		-	-	+	
933	+	-	-	-		-	-	+	

[a] Each sample was submitted to three Nested-PCR reactions (1st, 2nd and 3rd), and those whose results were discordant, a fourth Nested-PCR reaction (4th) was realized.

Figure 1

A


0,12
0,1
0,08
0,06
0,04
0,02
0
-0,02
Derivative
64.8
66.3
67.6
68.8
70.0
71.1
72.3
73.4
74.4
75.5
76.6
77.7
78.7
79.8
80.8
81.8
82.9
83.9
84.9
85.9
87.0
88.0
89.0
90.0
91.0
Temperature
Pf - 3000 parasites/µl of blood
Pf - 300 parasites/µl of blood
Pf - 30 parasites/µl of blood
Pf - 3,0 parasites/µl of blood
Pf - 0,3 parasites/µl of blood
Negative

B

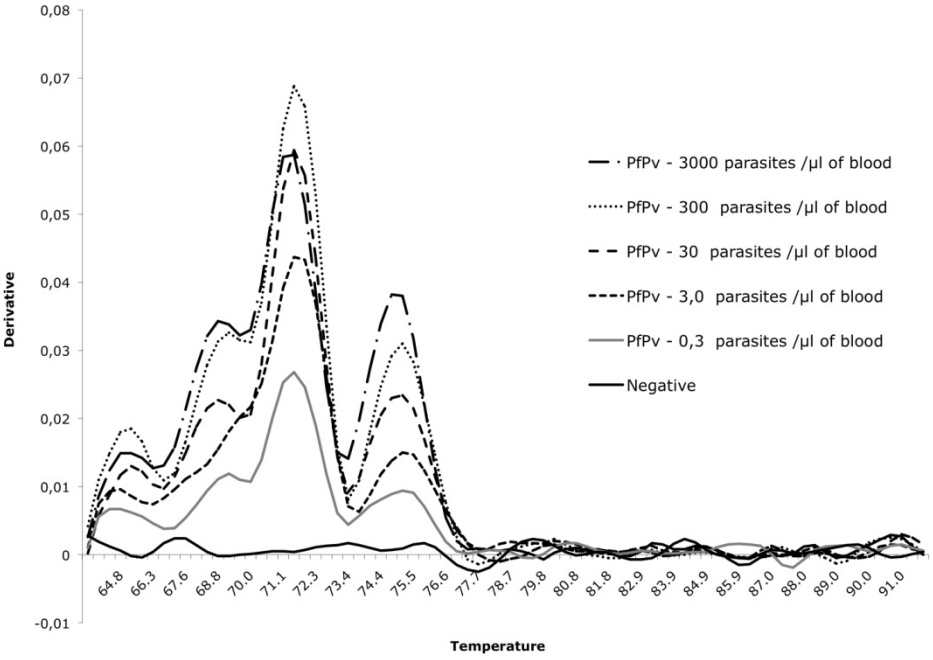
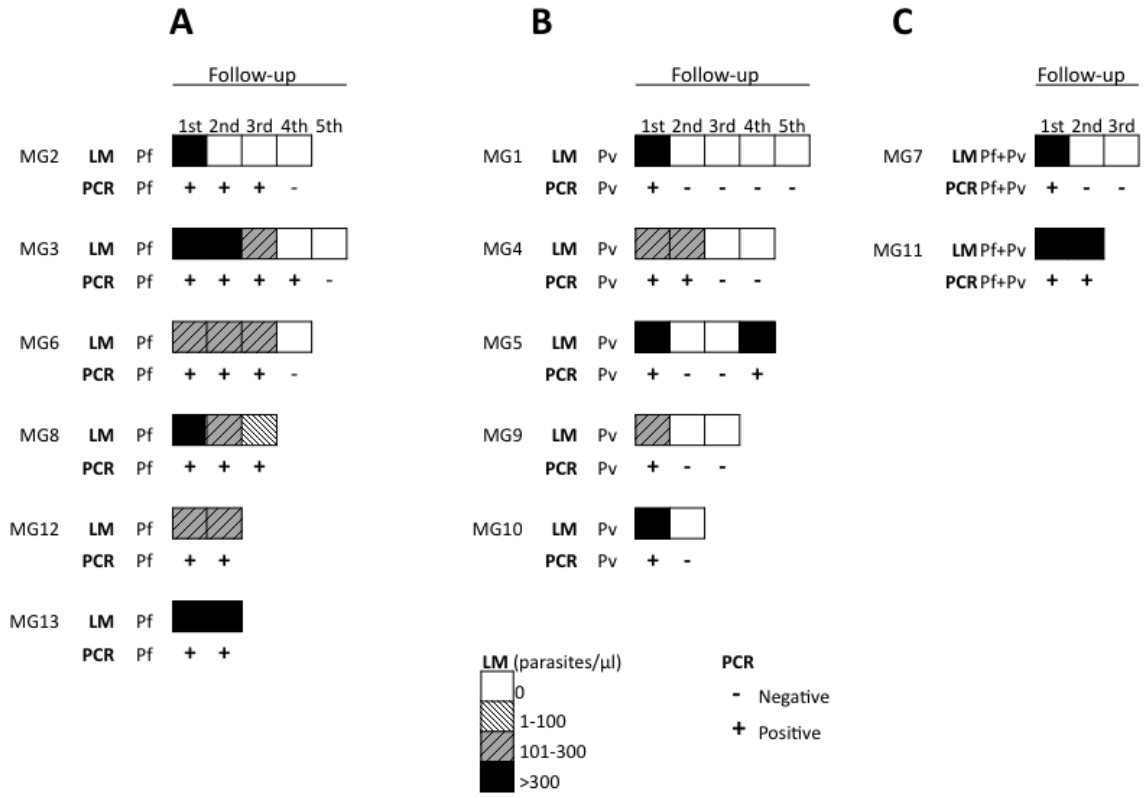

0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
-0,01
Derivative
64.8
66.3
67.6
68.8
70.0
71.1
72.3
73.4
74.4
75.5
76.6
77.7
78.7
79.8
80.8
81.8
82.9
83.9
84.9
85.9
87.0
88.0
89.0
90.0
91.0
Temperature
PfPv - 3000 parasites /µl of blood
PfPv - 300 parasites /µl of blood
PfPv - 30 parasites /µl of blood
PfPv - 3,0 parasites /µl of blood
PfPv - 0,3 parasites /µl of blood
Negative

Figure 2


A
Follow-up
1st 2nd 3rd 4th 5th
MG2 LM Pf
PCR Pf + + + -
MG3 LM Pf
PCR Pf + + + + -
MG6 LM Pf
PCR Pf + + + -
MG8 LM Pf
PCR Pf + + +
MG12 LM Pf
PCR Pf + +
MG13 LM Pf
PCR Pf + +
B
Follow-up
1st 2nd 3rd 4th 5th
MG1 LM Pv
PCR Pv + - - - -
MG4 LM Pv
PCR Pv + + - -
MG5 LM Pv
PCR Pv + - - +
MG9 LM Pv
PCR Pv + - -
MG10 LM Pv
PCR Pv + -
C
Follow-up
1st 2nd 3rd
MG7 LM Pf+Pv
PCR Pf+Pv + - -
MG11 LM Pf+Pv
PCR Pf+Pv + +
LM (parasites/µl)
0
1-100
101-300
>300
PCR
- Negative
+ Positive

9 REFERÊNCIAS BIBLIOGRÁFICAS

Adams JH, Hudson DE, Torii M, Ward GE, Wellems TE, Aikawa M, Miller LH The Duffy receptor family of Plasmodium knowlesi is located within the micronemes of invasive malaria merozoites. Cell 1990 Oct 5; 63(1): 141-53.

Adams JH, Sim BK, Dolan SA, Fang X, Kaslow DC, Miller LH. A family of erythrocyte binding proteins of malaria parasites. Proc. Natl. Acad. Sci USA 1992 Aug 1; 89(15): 7085-9.

Albuquerque SR, Cavalcante FO, Sanguino EC, Tezza L, Chacon F, Castilho L, dos Santos MC. FY polymorphisms and vivax malaria in inhabitants of Amazonas State, Brazil. Parasitol Res 2010 Apr; 106(5): 1049-53.

Alexandre MA, Ferreira CO, Siqueira AM, Magalhães BL, Mourão MP, Lacerda MV, Alecrim MG. Severe Plasmodium vivax malaria, Brazilian Amazon. Emerg Infect Dis 2010 Oct; 16(10): 1611-4.

Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, Menard R. Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nat Med 2006 Feb; 12(2): 220-4.

Ampudia E, Patarroyo MA, Patarroyo ME, Murillo LA. Genetic polymorphism of the Duffy receptor binding domain of Plasmodium vivax in Colombian wild isolates. Mol Biochem Parasitol 1996 Jun; 78(1-2): 269-272.

Andrade BB, Reis-Filho A, Souza-Neto SM, Clarêncio J, Camargo LM, Barral A, Barral-Netto M. Severe Plasmodium vivax malaria exhibits marked inflammatory imbalance. Malar J 2010 Jan; 13: 9-13.

Barnwell J and Wertheimer S. Plasmodium vivax: merozoite antigens, the Duffy blood group, and erythrocyte invasion. Prog Clin Biol Res 1989 313: 1-11.

Barnwell JW, Galinski MR. Invasion of vertebrate cells: Erythrocytes. In: IW Sherman, Malaria: Parasite biology, pathogenesis, and protection. Washington DC: ASM Press: 1998. Cap.7. P. 93-120.

Baton LA, Ranford-Cartwright LC. *Plasmodium falciparum* ookinete invasion of the midgut epithelium of Anopheles stephensi is consistent with the time bomb model. Parasitology 2004 Dec; 129(pt-6): 663-76.

Brasil. Ministério da Saúde. Fundação Nacional de Saúde. Manual de Terapêutica da Malária. 6. ed. Brasília: FUNASA, 2001. 102 p. (Vigilância Epidemiológica). Exercendo uma de suas principais funções dentro do SUS, que é, a normatização e a assessoria técnica a Estados e Municípios, a FUNASA disponibiliza o presente documento como contribuição ao tratamento dos doentes maláricos, beneficiando o controle da endemia. Disponível em: < http://netra.cpqrr.fiocruz.br/phl82/index.html >. Biblioteca de Ciências da Saúde Prof. Zigman Brener. Acesso em: 16 mar. 2011.

Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Diretoria Técnica de Gestão. Vigilância em Saúde: Situação Epidemiológica da Malária no Brasil. Brasília: MS, 2007. 12 p. Disponível em: < http://netra.cpqrr.fiocruz.br/phl82/index.html >. Biblioteca de Ciências da Saúde Prof. Zigman Brener. Acesso em: 16 mar. 2011.

Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Diretoria Técnica de Gestão. Vigilância em Saúde: Situação Epidemiológica da Malária no Brasil. Brasília: MS,2008.2p.Disponívelem:<http://portal.saude.gov.br/portal/arquivos/pdf/folder_malaria_2008_final.pdf>.Acesso em:16 mar.2011.

Brasil. Ministério da Saúde. Coordenação Geral do Programa Nacional de Controle da Malária. Casos confirmados de Malária, segundo mês de notificação. Brasil, Grandes Regiões e Unidades Federadas. 2009. In: _____. Malária. Brasília: MS. [s.d.]. Disponívelem:<http://portal.saude.gov.br/portal/saude/profissional/area.cfm?id_area=1526>. Acesso em: 16 mar. 2011.

Castilho L, Rios M, Pellegrino JJ, Saad S, Costa F, Reid M. A novel FY allele in Brazilians. Vox Sang 2004 87: 190-5.

Cavasini CE, Tarelho Pereira FJ, Ribeiro WL, Wunderlich G, Ferreira MU. Duffy blood group genotypes among malaria patients in Rondônia, Western Brazilian Amazon. Rev Soc Bras Med Trop 2001 Nov-Dec; 34(6): 591-5.

Cavasini CE, de Mattos LC, Couto AA, Couto VS, Gollino Y, Moretti LJ, Bonini-Domingos CR, Rossit AR, Castilho L, Machado RL. Duffy blood group gene polymorphisms among malaria vivax patients in four areas of the Brazilian Amazon region. Malar J 2007 Dec; 19(6): 167.

Ceravolo IP, Bruña-Romero O, Braga EM, Fontes CJ, Brito CF, Souza JM, Krettli AU, Adams JH, Carvalho LH. Anti-Plasmodium vivax duffy binding protein antibodies measure exposure to malaria in the Brazilian Amazon. Am J Trop Med Hyg 2005 Jun; 72(6): 675-81.

Ceravolo IP, Souza-Silva FA, Fontes CJ, Braga EM, Madureira AP, Krettli AU, Souza JM, Brito CF, Adams JH, Carvalho LH. Inhibitory properties of the antibody response to Plasmodium vivax Duffy binding protein in an area with unstable malaria transmission. Scand J Immunol 2008 Mar; 67(3): 270-8.

Cerbino VDA, Zumpano JF, Leopoldo FL. Follow-up of control measures during the P. vivax malaria outbreak in RioManso, Minas Gerais. Rev Soc Bras Med Trop 2004 37: 269.

Chaudhuri A, Polyakova J, Zbrzezna V, Williams K, Gulati S, Pogo A. Cloning of glycoprotein D cDNA, which encodes the major subunit of the Duffy blood group system and the receptor for the *Plasmodium vivax* malaria parasite. Proc Natl Acad Sci U S A 1993 Nov; 90(22): 10793-7.

Chaudhuri A, Polyakova J, Zbrzezna V, Pogo A. The coding sequence of Duffy blood group gene in humans and simians: restriction fragment length polymorphism, antibody and malarial parasite specificities, and expression in nonerythroid tissues in Duffy-negative individuals. Blood 1995 feb; 85(3): 615-21.

Chitnis CE, Miller LH. Identification of the erythrocyte binding domains of Plasmodium vivax and Plasmodium knowlesi proteins involved in erythrocyte invasion. J Exp Med 1994 Aug; 180(2): 497-506.

Chitnis CE, Chaudhuri A, Horuk R, Pogo A, Miller L. The domain on the Duffy blood group antigen for binding Plasmodium vivax and P. knowlesi malarial parasites to erythrocytes. J Exp Med 1996 Oct 1; 184(4): 1531-6.

Chitnis CE. Molecular insights into receptors used by malaria parasites for erythrocyte invasion. Curr Opin Hematol 2001 Mar; 8(2): 85-91.

Chitnis CE, Sharma A. Targeting the Plasmodium vivax Duffy-binding protein. Trends Parasitol 2008 Jan; 24(1): 29-34.

Chootong P, Ntumngia FB, VanBuskirk KM, Xainli J, Cole-Tobian JL, Campbell CO, Fraser TS, King CL, Adams JH. Mapping epitopes of the Plasmodium vivax Duffy binding protein with naturally acquired inhibitory antibodies. Infect Immun 2010 Mar; 78(3): 1089-95.

Cole-Tobian JL, Cortés A, Baisor M, Kastens W, Xainli J, Bockarie M, Adams JH, King CL. Age-acquired immunity to a Plasmodium vivax invasion ligand, the duffy binding protein. J Infect Dis 2002 Aug 15; 186(4): 531-9.

Cole-Tobian JL, Michon P, Biasor M, Richards JS, Beeson JG, Mueller I, King CL. Strain-specific duffy binding protein antibodies correlate with protection against infection with homologous compared to heterologous plasmodium vivax strains in Papua New Guinean children. Infect Immun 2009 Sep; 77(9): 4009-17.

Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. Cell 2006 Feb 24; 124(4): 755-66.

Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, Rahman HA, Conway DJ, Singh B. Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. Clin Infect Dis 2008 Jan 15; 46(2): 165-71.

de Castro MC, Monte-Mór RL, Sawyer DO, Singer BH. Malaria risk on the Amazon frontier. Proc Natl Acad Sci U S A (2006) Feb 14; 103(7): 2452-7.

Fang XD, Kaslow DC, Adams JH, Miller LH Cloning of the Plasmodium vivax Duffy receptor. Mol. Biochem. Parasitol 1991 Jan; 44(1): 125-32.

Ferreira RG, Moura MM, Engracia V, Pagotto RC, Alves FP, Camargo LM, Silva, Camargo EP, Beiguelman B, Krieger H. Ethnic admixture composition of two western Amazonian populations. Hum Biol 2002 Aug; 74(4): 607-14.

Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, Moch JK, Muster N, Sacci JB, Tabb DL, Witney AA, Wolters D, Wu Y, Gardner MJ, Holder AA, Sinden RE, Yates JR, Carucci DJ. A proteomic view of the Plasmodium falciparum life cycle. Nature 2002 Oct 3; 419(6906): 520-6.

Fraser T, Michon P, Barnwell JW, Noe AR, Al-Yaman F, Kaslow DC, Adams JH. Expression and serologic activity of a soluble recombinant Plasmodium vivax Duffy binding protein. Infect Immun 1997 Jul; 65(7): 2772-7.

Gaur D, Mayer DC, Miller LH. Parasite ligand-host receptor interactions during invasion of erythrocytes by Plasmodium merozoites. Int J Parasitol 2004 Dec; 34(13-14): 1413-29.

Genton B, D'Acremont V, Rare L, Baea K, Reeder JC, Alpers MP, Müller I. Plasmodium vivax and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. PLoS Med 2008 Jun 17; 5(6):e127.

Gosi P, Khusmith S, Khalambaheti T, Lanar DE, Schaecher KE, Fukuda MM, Miller SR. Polymorphism patterns in Duffy-binding protein among Thai Plasmodium vivax isolates. Malar J. 2008 Jun 26; 7:112.

Gann VR, Ziv E, Joseph CK, Neugut AI, Wei Y, Jacobson JS, Horwitz MS, Bowman N, Beckmann K, Hershman DL. Duffy (Fy), DARC, and neutropenia among women from the United States, Europe and the Caribbean. Br J Haematol 2008 Oct; 143(2): 288-93.

Greenwood BM, Bojang K, Whitty CJ, Targett GA.Malaria. Lancet 2005 Apr 23-29; 365(9469): 1487-98.

Gueirard P, Tavares J, Thiberge S, Bernex F, Ishino T, Milon G, Franke-Fayard B, Janse CJ, Ménard R, Amino R. Development of the malaria parasite in the skin of the mammalian host. Proc Natl Acad Sci U S A (2010) Oct 26; 107(43): 18640-5.

Guerra CA, Snow RW, Hay SI. Defining the global spatial limits of malaria transmission in 2005. Adv Parasitol 2006 62: 157–179.

Guerra CA, Snow RW, Hay SI. Mapping the global extent of malaria in 2005. Trends Parasitol 2006 Aug;22(8):353-8..

Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, Temperley WH, Kabaria CW, Tatem AJ, Manh BH, Elyazar IR, Baird JK, Snow RW, Hay SI. The international limits and population at risk of Plasmodium vivax transmission in 2009. PLoS Negl Trop Dis 2010 Aug 3; 4(8): e774.

Hadley TJ, Peiper SC. From malaria to chemokine receptor: the emerging physiologic role of the Duffy blood group antigen. Blood 1997 May 1; 89(9): 3077-91.

Hay SI, Okiro EA, Gething PW, Patil AP, Tatem AJ, Guerra CA, Snow RW Estimating the global clinical burden of Plasmodium falciparum malaria in 2007. PLoS Med. 2010 Jun 15;7(6):e1000290.

Howell DP, Samudrala R, Smith JD. Disguising itself--insights into Plasmodium falciparum binding and immune evasion from the DBL crystal structure. Mol Biochem Parasitol 2006 Jul; 148(1): 1-9.

Iwamoto S, Omi T, Kajii E, Ikemoto S. Genomic organization of the glycoprotein D gene: Duffy blood group Fya/Fyb alloantigen system is associated with a polymorphism at the 44-amino acid residue. Blood 1995 Feb 1; 85(3): 622-6.

Jin Y, Kebaier C, Vanderberg J. Direct microscopic quantification of dynamics of Plasmodium berghei sporozoite transmission from mosquitoes to mice. Infect Immun 2007 Nov; 75(11): 5532-9.

Kasehagen LJ, Mueller I, Kiniboro B, Bockarie MJ, Reeder JC, Kazura JW, Kastens W, McNamara DT, King CH, Whalen CC, Zimmerman PA. Reduced Plasmodium vivax erythrocyte infection in PNG Duffy-negative heterozygotes. PLoS One 2007 Mar 28; 2(3): e336.

Kho WG, Chung JY, Sim EJ, Kim DW, Chung WC. Analysis of polymorphic regions of Plasmodium vivax Duffy binding protein of Korean isolates. Korean J Parasitol 2001 Jun; 39(2): 143-50.

King CL, Michon P, Shakri AR, Marcotty A, Stanisic D, Zimmerman PA, Cole-Tobian JL, Mueller I, Chitnis CE. Naturally acquired Duffy-binding protein-specific binding inhibitory antibodies confer protection from blood-stage Plasmodium vivax infection. Proc Natl Acad Sci USA 2008 Jun 17; 105(24): 8363-8.

Krotoski WA, Collins WE, Bray RS, Garnham PC, Cogswell FB, Gwadz RW, Killick-Kendrick R, Wolf R, Sinden R, Koontz LC, Stanfill PS. Demonstration of hypnozoites in sporozoite-transmitted Plasmodium vivax infection. Am J Trop Med Hyg 1982 Nov; 31(6): 1291-3.

Langhi DM, Aalbuquerque S, Covas DT, Perez CA, Bordin JO. The presence of FYA null allele in inhabitants of an endemic region and in blood donors from Brazil. Rev Bras Hemat Hemot 2004a 26: 268-269.

Langhi DM, Albuquerque S, Covas DT, Perez CA Bordin JO. The Presence of FYAnull Alele of Duffy Blood Group System in Blood Donors and Individuals from a Malarial Endemic Region of Brazil. In: ASTMH Annual Meeting; 2004b;Poster Session 927-II (ed. 927-II, P. S.).

Maestre A, Muskus C, Duque V, Agudelo O, Liu P, Takagi A, Ntumngia FB, Adams JH, Sim KL, Hoffman SL, Corradin G, Velez ID, Wang R. Acquired antibody responses against Plasmodium vivax infection vary with host genotype for duffy antigen receptor for chemokines (DARC). PLoS One 2010 Jul 15; 5(7): e11437.

Mallinson G, Soo K, Schall T, Pisacka M, Anstee D. Mutations in the erythrocyte chemokine receptor (Duffy) gene: the molecular basis of the Fya/Fyb antigens and identification of a deletion in the Duffy gene of an apparently healthy individual with the Fy(a-b-) phenotype. Br J Haematol 1995 Aug;90(4):823-9.

Mangold KA, Manson RU, Koay ES, Stephens L, Regner M, Thomson RB Jr, Peterson LR, Kaul KL. Real-time PCR for detection and identification of Plasmodium spp. J Clin Microbiol 2005 May; 43(5): 2435-40.

Marques AC. Migration and dissemination of malaria in Brazil. Mem Inst Oswaldo Cruz 1986 81(2): 17-30.

Martinez P, Lopez C, Saravia C, Vanegas M, Patarroyo MA. Evaluation of the antigenicity of universal epitopes from PvDBPII in individuals exposed to *Plasmodium vivax* malaria. Microbes Infect 2010 Dec; 12(14-15): 1188-97.

McHenry AM, Adams JH. The crystal structure of P. knowlesi DBPalpha DBL domain and its implications for immune evasion. Trends Biochem Sci 2006 Sep; 31(9): 487-91.

Ménard D, Barnadas C, Bouchier C, Henry-Halldin C, Gray LR, Ratsimbasoa A, Thonier V, Carod JF, Domarle O, Colin Y, Bertrand O, Picot J, King CL, Grimberg BT, Mercereau-Puijalon O, Zimmerman PA. Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people. Proc Natl Acad Sci USA 2010 Mar 30; 107(13): 5967-71.

Michon PA, Arevalo-Herrera M, Fraser T, Herrera S, Adams JH. Serologic responses to recombinant Plasmodium vivax Duffy binding protein in a Colombian village. Am J Trop Med Hyg 1998 Oct; 59(4): 597-9.

Michon P, Fraser T, Adams JH. Naturally acquired and vaccine-elicited antibodies block erythrocyte cytoadherence of the Plasmodium vivax Duffy binding protein. Infect Immun 2000 Jun; 68(6): 3164-71.

Michon P, Woolley I, Wood EM, Kastens W, Zimmerman PA, Adams JH. Duffy-null promoter heterozygosity reduces DARC expression and abrogates adhesion of the P. vivax ligand required for blood-stage infection. FEBS Lett 2001 Apr 20; 495(1-2): 111-4.

Miller LH, Mason SJ, Dvorak JA, McGinniss MH, Rothman IK. Erythrocyte receptors for (Plasmodium knowlesi) malaria: Duffy blood group determinants. Science 1975 Aug 15; 189(4202): 561-3.

Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med 1976 Aug 5; 295(6): 302-4.

Mota MM, Hafalla JC, Rodriguez A. Migration through host cells activates Plasmodium sporozoites for infection. Nat Med. 2002 Nov; 8(11): 1318-22.

Mota MM, Rodriguez A. Migration through host cells: the first steps of Plasmodium sporozoites in the mammalian host. Cell Microbiol 2004 Dec; 6(12): 1113-8.

Moore S, Woodrow CF, McClelland DB. Isolation of membrane components associated with human red cell antigens Rh(D), (c), (E) and Fy. Nature 1982 Feb 11; 295(5849): 529-31.

Oliveira-Ferreira J, Lacerda MV, Brasil P, Ladislau JL, Tauil PL, Daniel-Ribeiro CT. Malaria in Brazil: an overview. Malar J 2010 Apr 30; 9: 115.

Olsson ML, Hansson C, Avent ND, Akesson IE, Green CA, Daniels GL. A clinically applicable method for determining the three major alleles at the Duffy (FY) blood group locus using polymerase chain reaction with allele-specific primers. Transfusion. 1998 Feb; 38(2): 168-73.

Ong CW, Lee SY, Koh WH, Ooi EE, Tambyah PA. Monkey malaria in humans: a diagnostic dilemma with conflicting laboratory data. Am J Trop Med Hyg 2009 Jun; 80(6): 927-8.

Palha TD, Ribeiro-Rodrigues EM, Ribeiro-Dos-Santos A, Guerreiro JF, de Moura LS, Santos S. Male ancestry structure and interethnic admixture in African-descent communities from the Amazon as revealed by Y-chromosome Strs. Am J Phys Anthropol 2010 Dec 29.

Perna SJ, Cardoso GL, Guerreiro JF. Duffy blood group genotypes among African-Brazilian communities of the Amazon region. Genet Mol Res 2007 Mar 29; 6(1): 166-72.

Pradel G, Frevert U. Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion. Hepatology 2001 May; 33(5): 1154-65.

Ranjan A, Chitnis CE. Mapping regions containing binding residues within functional domains of *Plasmodium vivax* and *Plasmodium knowlesi* erythrocyte-binding proteins. Proc Natl Acad Sci U S A 1999 Nov 23; 96(24): 14067-72.

Ryan JR, Stoute JA, Amon J, Dunton RF, Mtalib R, Koros J, Owour B, Luckhart S, Wirtz RA, Barnwell JW, Rosenberg R.Evidence for transmission of *Plasmodium vivax* among a Duffy antigen negative population in western Kenya. Am J Trop Med Hyg 2006 Oct; 75(4): 575-81.

Saravia C, Martinez P, Granados DS, Lopez C, Reyes C, Patarroyo MA. Identification and evaluation of universal epitopes in Plasmodium vivax Duffy binding protein. Biochem Biophys Res Commun 2008 Dec 26; 377(4): 1279-83.

Siri JG, Lindblade KA, Rosen DH, Onyango B, Vulule J, Slutsker L, Wilson ML. Quantitative urban classification for malaria epidemiology in sub-Saharan Africa. Malar J 2008 Feb 25; 7: 34.

SMOLAREK D, BERTRAND O, CZERWINSKI M, COLIN Y, ETCHEBEST C, de Brevern AG. Multiple interests in structural models of DARC transmembrane protein. Transfus Clin Biol 2010 Sep; 17(3): 184-96.

Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol Biochem Parasitol 1993 Apr; 58(2): 283-92.

Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature 2005 Mar 10; 434(7030): 214-7.

Soares IS, Barnwell JW, Ferreira MU, Gomes Da Cunha M, Laurino JP, Castilho BA, Rodrigues MM. A Plasmodium vivax vaccine candidate displays limited allele polymorphism, which does not restrict recognition by antibodies. Mol Med 1999 Jul; 5(7): 459-70.

Sousa TN, Cerávolo IP, Fernandes Fontes CJ, Couto A, Carvalho LH, Brito CF. The pattern of major polymorphisms in the Duffy binding protein ligand domain among Plasmodium vivax isolates from the Brazilian Amazon area. Mol Biochem Parasitol 2006 Apr; 146(2): 251-4.

Sousa TN, Tarazona-Santos EM, Wilson DJ, Madureira AP, Falcão PR, Fontes CJ, Gil LH, Ferreira MU, Carvalho LH, Brito CF. Genetic variability and natural selection at the ligand domain of the Duffy binding protein in Brazilian Plasmodium vivax populations. Malar J 2010 Nov 22; 9: 334.

Souza-Silva FA, da Silva-Nunes M, Sanchez BA, Ceravolo IP, Malafronte RS, Brito CF, Ferreira MU, Carvalho LH. Naturally acquired antibodies to Plasmodium vivax

Duffy binding protein (DBP) in Brazilian Amazon. Am J Trop Med Hyg 2010 Feb; 82(2): 185-93.

Sturm A, Amino R, van de Sand C, Regen T, Retzlaff S, Rennenberg A, Krueger A, Pollok JM, Menard R, Heussler VT. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. Science 2006 Sep 1; 313(5791): 1287-90.

Suh IB, Hoffman KJ, Kim SH, Song KJ, Song JW, Lee JS, Lim CS. The analysis of Plasmodium vivax Duffy receptor binding domain gene sequence from resurgent Korea isolates. Parasitol Res. 2001 Dec; 87(12): 1007-10.

Tatem AJ, Smith DL. International population movements and regional Plasmodium falciparum malaria elimination strategies. Proc Natl Acad Sci U S A 2010 Jul 6; 107(27): 12222-7.

Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, Karyana M, Lampah DA, Price RN. Multidrug-resistant Plasmodium vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia. PLoS Med 2008 Jun 17; 5(6): e128.

Tournamille C, Colin Y, Cartron JP, Le Van Kim C. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nat Genet 1995a Jun; 10(2): 224-8.

Tournamille C, Le Van Kim C, Gane P, Cartron JP, Colin Y. Molecular basis and PCR-DNA typing of the Fya/fyb blood group polymorphism. Hum Genet 1995b Apr; 95(4): 407-10.

Tournamille C, Le Van Kim C, Gane P, Blanchard D, Proudfoot AE, Cartron JP, Colin Y. Close association of the first and fourth extracellular domains of the Duffy antigen/receptor for chemokines by a disulfide bond is required for ligand binding. J Biol Chem 1997 Jun 27; 272(26): 16274-80

Tournamille C, Le Van Kim C, Gane P, Le Pennec PY, Roubinet F, Babinet J, Cartron JP, Colin Y. Arg89Cys substitution results in very low membrane expression of the Duffy antigen/receptor for chemokines in Fy(x) individuals. Blood 1998 Sep 15; 92(6): 2147-56. Erratum in: Blood 2000 May 1; 95(9): 2753.

Tournamille C, Filipe A, Wasniowska K, Gane P, Lisowska E, Cartron JP, Colin Y, Le Van Kim C. Structure-function analysis of the extracellular domains of the Duffy antigen/receptor for chemokines: characterization of antibody and chemokine binding sites. Br J Haematol 2003 Sep; 122(6): 1014-23.

Tournamille C, Filipe A, Badaut C, Riottot MM, Longacre S, Cartron JP, Le Van Kim C, Colin Y. Fine mapping of the Duffy antigen binding site for the Plasmodium vivax Duffy-binding protein. Mol Biochem Parasitol 2005 Nov; 144(1): 100-3.

Tsuboi T, Kappe SH, al-Yaman F, Prickett MD, Alpers M, Adams JH. Natural variation within the principal adhesion domain of the Plasmodium vivax duffy binding protein. Infect Immun 1994 Dec; 62(12): 5581-6.

VanBuskirk KM, Cole-Tobian JL, Baisor M, Sevova ES, Bockarie M, King CL, Adams JH. Antigenic drift in the ligand domain of Plasmodium vivax duffy binding protein confers resistance to inhibitory antibodies. J Infect Dis 2004a Nov 1; 190(9): 1556-62.

VanBuskirk KM, Sevova E, Adams JH. Conserved residues in the Plasmodium vivax Duffy-binding protein ligand domain are critical for erythrocyte receptor recognition. Proc Natl Acad Sci USA 1994b Nov 2; 101(44): 15754-9.

Vlachou D, Zimmermann T, Cantera R, Janse CJ, Waters AP, Kafatos FC. Real-time, in vivo analysis of malaria ookinete locomotion and mosquito midgut invasion. Cell Microbiol 2004 Jul; 6(7): 671-85.

Vlachou D, Schlegelmilch T, Runn E, Mendes A, Kafatos FC. The developmental migration of Plasmodium in mosquitoes. Curr Opin Genet Dev 2006 Aug; 16(4): 384-91.

Wertheimer SP, Barnwell JW.Plasmodium vivax interaction with the human Duffy blood group glycoprotein: identification of a parasite receptor-like protein. Exp Parasitol 1989 Nov; 69(4):340-50.

White NJ. Plasmodium knowlesi: the fifth human malaria parasite. Clin Infect Dis 2008 Jan 15; 46(2): 172-3.

WHO. World Malaria Report 2009, Geneva, World Health Organization.

Woolley IJ, Hotmire KA, Sramkoski RM, Zimmerman PA, Kazura JW. Differential expression of the duffy antigen receptor for chemokines according to RBC age and FY genotype. Transfusion 2000 Aug; 40(8): 949-53.

Xainli J, Adams JH, King CL. The erythrocyte binding motif of plasmodium vivax duffy binding protein is highly polymorphic and functionally conserved in isolates from Papua New Guinea. Mol Biochem Parasitol 2000 Dec; 111(2): 253-60.

Yamauchi LM, Coppi A, Snounou G, Sinnis P. Plasmodium sporozoites trickle out of the injection site. Cell Microbiol 2007 May; 9(5): 1215-22.

Yazdanbakhsh K, Rios M, Storry JR, Kosower N, Parasol N, Chaudhuri A, Reid ME. Molecular mechanisms that lead to reduced expression of duffy antigens. Transfusion 2000 Mar ;40(3): 310-20.

Zieler H, Dvorak JA. Invasion in vitro of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells. Proc Natl Acad Sci U S A 2000 Oct 10; 97(21): 11516-21.

Zimmerman PA, Woolley I, Masinde GL, Miller SM, McNamara DT, Hazlett F, Mgone CS, Alpers MP, Genton B, Boatin BA, Kazura JW. Emergence of FY*A(null) in a Plasmodium vivax-endemic region of Papua New Guinea. Proc Natl Acad Sci U S A 1999 Nov 23; 96(24): 13973-7.

ZUMPANO JFR, CHAVES MOC, SILVA RB. Study of P. vivax malaria relapses in Sousa District, Rio Manso municipality, Minas Gerais, Brazil. Rev Soc Bras Med Trop 2004 37: 267.