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FUNDAÇÃO OSWALDO CRUZ
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Doutorado em Biologia Parasitária

Rastreamento de mutações em gene marcador da quimiorresistência do *Plasmodium falciparum* à artemisinina e em genes potencialmente associados a resistência do *P. vivax* à cloroquina em isolados plasmodiais brasileiros

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Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Ciências na área de concentração de Genética e Biologia Molecular.

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RESUMO

Na ausência de uma vacina antimalárica a política de controle baseia-se no gerenciamento dos casos através do pronto diagnóstico e do tratamento dos casos com as drogas recomendadas pela OMS. Em decorrência da quimiorresistência (QR) dos parasitos da malária é fundamental o monitoramento constante da eficácia das drogas utilizadas na terapêutica dessa endemia. Em decorrência disso, realizamos um estudo descritivo sobre os polimorfismos no gene *pvmdr1* potencialmente associados à QR do *P. vivax* à cloroquina (CQ) em isolados provenientes das regiões Amazônica e Extra-Amazônica brasileiras e nos genes *pvdhfr* e *pvdhps* associados a QR a sulfadoxina/pirimetanina (SP) do *P. vivax* e *pfk13* já validado pela OMS como marcador molecular associado à QR do *P. falciparum* à artemisinina (ART) em isolados de pacientes infectados na Amazônia Legal. Independente da espécie plasmodial da infecção, todos os pacientes apresentaram boa resposta ao tratamento. Para esse fim, fizemos *nested* PCRs seguidas do sequenciamento do DNA alvo em todas as 172 amostras investigadas. Ao analisarmos a ocorrência de mutações no gene *pvmdr1* detectamos, exclusivamente, na Amazônia, o duplo mutante 976**F**/1076**L (12%)** e o mutante único 976**F (15%)** em frequências similares enquanto que a mutação no códon 958**M** foi constatada em 55% dos isolados estudados, independentemente da procedência deles. Em relação ao gene *pvdhfr,* observamos a presença de duplo F**R**T**N**I (28%), triplo F**R**T**N**L (11%) e quádruplo F**RMNL** (2%) mutantes, ao passo que o tipo selvagem não foi evidenciado. Já no gene *pvdhps* foi possível detectar frequências análogas no tipo selvagem (48%) e no mutante único 383**G** (52%). Não foi identificado a combinação quádruplo/quíntuplo mutante *pvdhfr/pvdhps*, fortemente associada ao fenótipo de QR em nenhuma das amostras analisadas. Em relação ao *P. falciparum*, todos os isolados eram do tipo selvagem nos códons do gene *pfk13* associados à QR à ART. Concluímos que: i) as mutações 976**F** e 1076**L** do gene *pvmdr1* não [arecem ser bons marcadores moleculares de QR à CQ; ii) a SP poderia ser uma droga alternativa ao tratamento da malária vivax, devido a emergência e dispersão de QR à CQ em áreas endêmicas de malária e que; iii) todos os isolados de *P. falciparum* provenientes de diferentes estados da Amazônia Legal foram do tipo selvagem, reforçando a eficácia da ART no Brasil.

ABSTRACT

In the absence of an antimalarial vaccine the control policy is based on case management through prompt diagnosis and treatment of cases using drugs recommended by WHO. Due to the malaria parasites chemoresistance (QR), the constant monitoring of drugs efficacy used in malaria treatment is fundamental. In view of the above, we conducted a descriptive study on the polymorphisms in the *pvmdr1* gene potentially associated with *P. vivax* QR to chloroquine (CQ) in isolates from the Brazilian Amazon and Extra Amazon regions and in the *pvdhfr* and *pvdhps* genes associated with sulfadoxine / pyrimetanine (SP) QR to *P. vivax* and also in *pfk13* gene already validated by the WHO as a molecular marker associated with *P. falciparum* QR to artemisinin (ART) in plasmodial isolates from Amazonia malaria patients. Regardless of the plasmodial malaria infection species, all patients had a good response to treatment. To this end, we used nested PCRs followed by DNA target sequencing of all 172 samples here investigated. When we analyzed the occurrence of mutations in the *pvmdr1* gene, we detected the double mutant 976**F** / 1076**L** (12%) and the single mutant 976**F** (15%) in similar frequencies, whereas the mutation in the 958**M** codon was found in 55% of the studied samples regardless of their origin. Concerning the *pvdhfr* gene, we observed the presence of double FRTNI (28%), triple FRTNL (11%) and quadruple FRMNL (2%) mutants, whereas wild type was not evidenced. Considering *pvdhps* gene, it was possible to detect similar frequencies between the wild type (48%) and the single mutant 383G (52%). The quadruplex / quintuple mutant *pvdhfr* / *pvdhps* combination was not identified in any of the samples analyzed. Regarding *P. falciparum*, all the isolates presented the wild type in the codons of the *pfk13* gene associated with QR to ART. We conclude that: i) the mutations 976**F** and 1076L of the *pvmdr1* gene do not seem to be good molecular markers to CQ QR; ii) SP could be an alternative drug to the treatment of vivax malaria due to the emergence and dispersion of QR to CQ in endemic areas of malaria and; iii) all isolates of *P. falciparum* from different states of the Legal Amazon were wild-type, enhancing the effectiveness of ACTs in Brazil.

ÍNDICE

1. INTRODUÇÃO

1.1. Malária - fatores epidemiológicos e incidência

A malária permanece como uma das endemias de maior prevalência no mundo ocasionando cerca de 216 milhões de novos casos, com aproximadamente 445 mil mortes por ano, sobretudo na África (WHO, 2017). A doença está presente em 91 países, sendo endêmica nas regiões tropicais e subtropicais da África, sudeste da Ásia e América Latina. Aproximadamente, 3,2 bilhões de pessoas estão expostas a doença e esses dados demonstram que quase metade da população mundial corre o risco de se infectar (WHO, 2016).

Conforme dados da Organização Mundial de Saúde (OMS), houve redução global na taxa de incidência da doença em 18%, de 76 para 63 casos por 1000 habitantes, entre 2010 e 2016. Dentre as áreas que tiveram sucesso nesse declínio podemos destacar o Sudeste Asiático (48%), seguido das Américas (22%) e África (20%), somado a isso foram registrados em 44 países no ano de 2016, 10 mil casos a menos de malária, redução esta de casos que só ocorreu em 37 países em 2010. Tais dados parecem apontar para a evolução no sentido da eliminação da doença **(Figura 1)** (WHO, 2017). Da mesma forma, com relação aos dados de 2010, o número de mortes diminuiu em todas as regiões, ressaltando-se o Sudeste Asiático (44%), a África (37%) e as Américas do Sul e Central (27%). Nas Américas, o cenário da malária mostra a sua presença em 18 países expondo, aproximadamente, 126 milhões de pessoas ao risco de transmissão. Diante desses dados a OMS almeja que até 2020, doze países, incluindo o Brasil, reduzam em 40% a incidência da doença (WHO, 2017). De fato, a redução já observada foi, em grande parte, devido ao sucesso da implementação das terapias combinadas à artemisinina (ACTs) para o tratamento do *P. falciparum* – espécie responsável pelos casos potencialmente

graves e letais desta enfermidade, corroborando que o tratamento constitui um dos maiores alicerces das medidas de controle da malária.

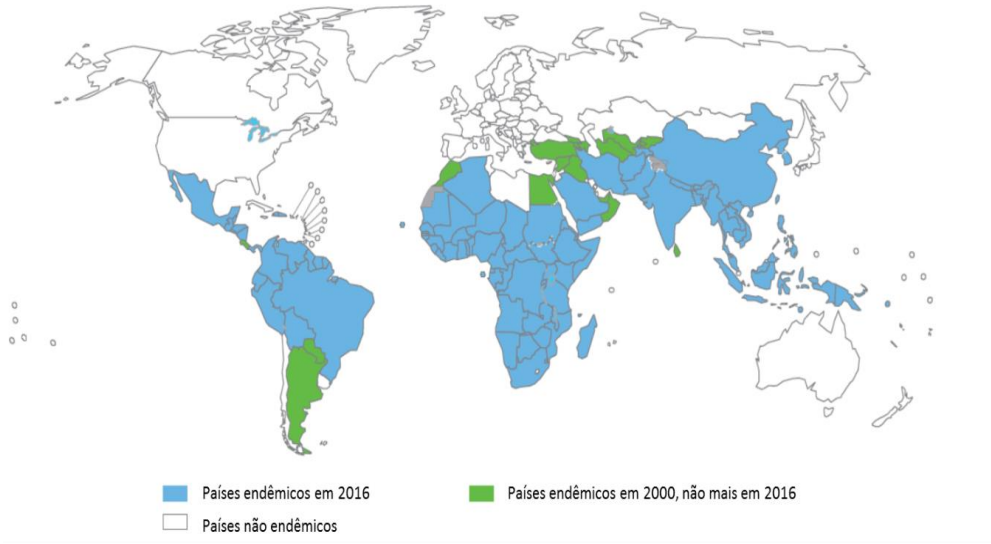

Figura 1. Distribuição mundial da malária, 2016. Adaptado de www.who.int/malaria/publications/world_malaria_report/en/

1.2. Malária no Brasil

A transmissão da malária no Brasil restringe-se quase que na sua totalidade a Amazônia Legal (Acre, Amapá, Amazonas, Maranhão, Mato Grosso, Pará, Rondônia, Roraima e Tocantins) que representa 99,7% dos casos.

No Brasil, em 2017, foram registrados 194.409 casos da doença, sendo 15 municípios dos estados do Acre e do Amazonas os detentores dos maiores números de casos. O *P. vivax* é a espécie mais prevalente com 89,2 % dos casos registrados, seguida por *P. falciparum* com 10,8 % enquanto que o *P. malariae* é raramente diagnosticado (SIVEP- Malária, 2018).

Conforme a estratificação epidemiológica de risco estabelecida pela OMS, o padrão de endemicidade em nosso país é considerado heterogêneo predominando

áreas de alto risco, como as observadas nos estados do Acre, Amazonas, Roraima e Amapá **(Figura 2)**. Além da heterogeneidade, a transmissão em áreas brasileiras é considerada instável com flutuações sazonais anuais. Nessas áreas tendem a ocorrer surtos epidêmicos que acometem adultos e crianças, podendo ser potencialmente graves quando a epidemia é originada pelo *P. falciparum*.

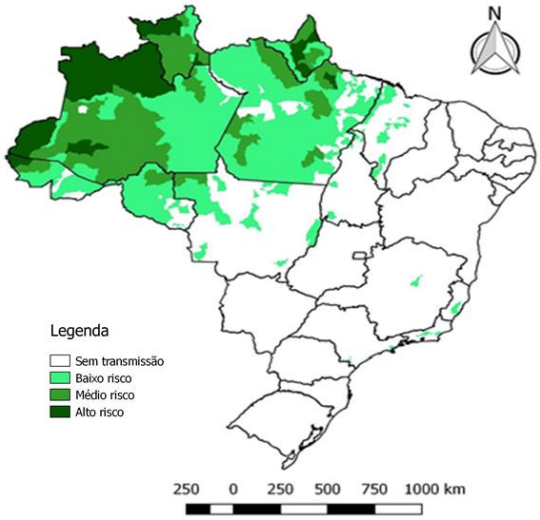

Figura 2. Mapa de risco da malária por município de infecção, Brasil, 2016.
Fonte: SIVEP.

1.3. Agente etiológico e ciclo biológico do *Plasmodium*

Existem cinco espécies de parasitos do gênero *Plasmodium* capazes de infectar o homem e causar a malária: *Plasmodium ovale*, *P. malariae*, *P. vivax*, *P. falciparum e P. knowlesi*. Dentre esses, o *P. falciparum* é a principal espécie responsável pelas formas graves e letais da doença (Miller et al, 2013; Cowman et al, 2016). O *P. vivax* responsável por considerável morbidade no mundo, tem sido negligenciado mesmo após a descrição de casos graves de malária por esta espécie

plasmodial (Mendis et al, 2001; Muller et al, 2009; Alexandre et al, 2010; Battle et al, 2012; Costa et al, 2012).

O ciclo evolutivo do parasito está dividido em duas fases bem distintas: uma no hospedeiro vertebrado - esquizogônico ou assexuado - e outra na fêmea dos anofelinos - o esporogônico ou sexuado **(Figura 3)**.

No homem o ciclo inicia-se através da picada de fêmea infectada e infectante do mosquito *Anopheles* que ao realizar seu repasto sanguíneo inocula, geralmente, na derme as formas infectantes denominadas de esporozoítos. Raramente, os esporozoítos são inoculados diretamente na circulação sanguínea; eles são injetados no tecido subcutâneo ao redor da picada, podendo ficar por alguns minutos ou horas nesse local, ou migrar diretamente para um capilar sanguíneo, seguindo depois para o fígado no qual invadem os hepatócitos. De fato, tem sido observado que os esporozoítos atravessam a barreira sinusoidal do fígado, para então invadir os hepatócitos (Mota et al, 2001; Tavares et al, 2013). Há evidências de que os esporozoítos passam por várias células hospedeiras antes de selecionar o hepatócito definitivo para prosseguir o ciclo biológico (Aly et al, 2009; Kaushansky et al, 2015). A invasão de um hepatócito depende de várias interações do tipo ligante-receptor e o processo de interação ocorre por invaginação da membrana celular dando origem a um vacúolo, denominado parasitóforo que envolve o esporozoíto.

No interior do vacúolo parasitóforo o esporozoíto se multiplica, dando origem à forma multinucleada denominada esquizonte evoluindo para os merozoítos que rompem os hepatócitos e são liberados na circulação sanguínea envolvidos por uma estrutura vesicular denominada merossomos. Tal estrutura tem como característica principal impedir o reconhecimento do parasito pelo sistema imune do hospedeiro (Sturm et al, 2006). Após romperem a membrana dos merossomos muitos merozoítos são fagocitados e destruídos pelas células de defesa, porém outros são

liberados na circulação e invadem os eritrócitos dando início ao ciclo eritrocítico assexuado, marcado pelo paroxismo típico da doença. Com efeito, no momento da ruptura de esquizontes há liberação de merozoítos e toxinas parasitárias que ativam o macrófago a produzir citocinas inflamatórias que provocaram os acessos caracterizados por febre, calafrio e sudorese intensa. Nos eritrócitos o merozoíto se transformará em trofozoíto jovem ou anel seguido por trofozoíto maduro evoluindo para esquizonte, de forma a originar novos merozoítos após um período de um a três dias dependendo da espécie parasitária (Malaguarnera & Musumeci, 2002; Garcia, 2010).

Nas espécies *P. vivax* e *P. ovale* alguns esporozoítos podem se transformar em hipnozoítos que permanecem quiescentes nas células do fígado por um longo período de tempo.

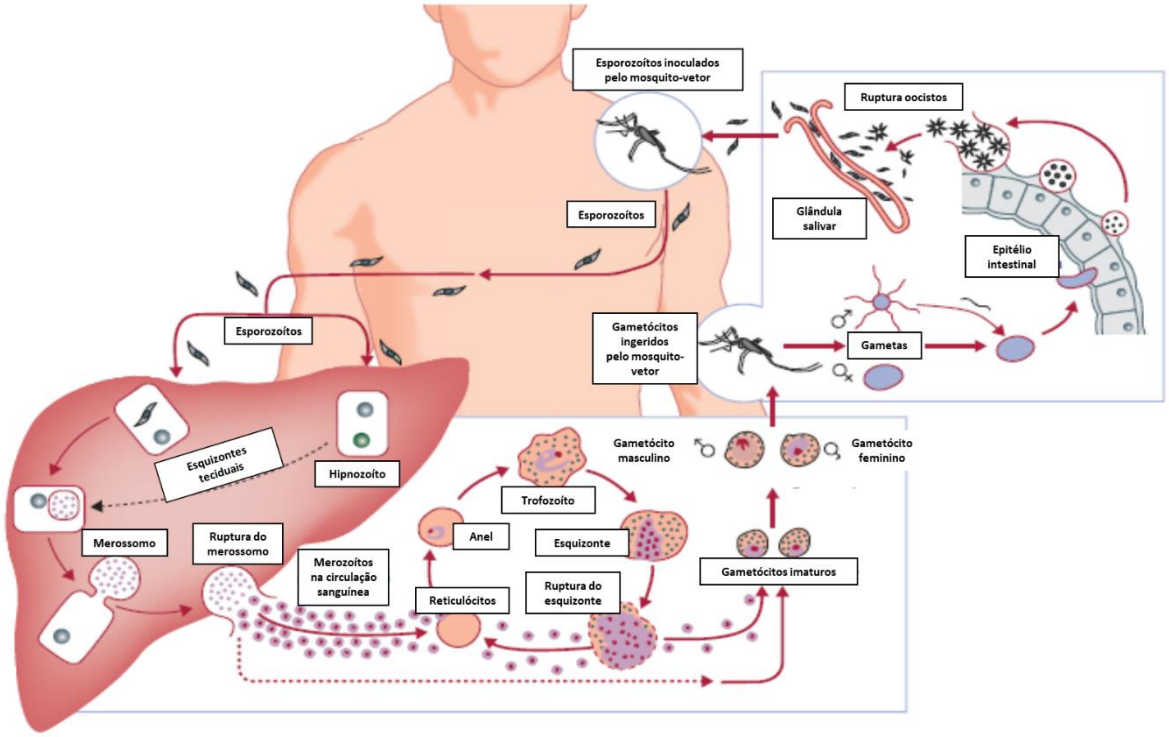

Figura 3. Ciclo evolutivo do *Plasmodium vivax* (Muller et al, 2009; adaptado)

penetração dos hepatócitos por esporozoítos, a ligação entre receptores presentes

nas membranas do parasito e do hospedeiro. No caso dos merozoítos, tal ligação permite a reorientação do complexo apical do parasito composto pelas roptrias e micronemas, estruturas necessárias para a invaginação na membrana celular do hospedeiro e a formação do vacúolo parasitóforo, local em que o parasito se replicará e se desenvolverá (Baumeister et al, 2010).

Alguns parasitos, durante o estágio sanguíneo, se diferenciam nas formas sexuadas da doença, os gametócitos (macrogametócito - fêmea e microgametócito - macho), que circulam no sangue do hospedeiro humano, sendo incapazes de se fertilizar até que sejam sugados pelo mosquito e, em seguida, encaminhados para a luz do estômago do inseto (Baker et al, 2010; Yang et al, 2017). É nesse local que ocorrerá a reprodução sexuada ou fecundação dos gametócitos, originando um zigoto, que evoluirá para a forma móvel de oocineto. Dessa maneira, o oocineto irá invadir o epitélio do estômago no qual se alojará para se transformar, posteriormente, em oocisto. No oocisto iniciar-se-ão as esporogonias, dando origem aos esporozoítos que serão liberados na hemocele do anofelino no momento da ruptura do cisto. Neste momento, irão migrar para as glândulas salivares, estando aptos a reiniciar o ciclo quando inoculados através de um novo repasto sanguíneo (Josling et al, 2015).

1.4. Estratégias de controle da malária

As primeiras medidas de controle da doença tiveram início no século XIX, que foi marcado por diversas campanhas na tentativa de controle do vetor. Na década de 50, a OMS, implementou um programa de controle para a erradicação da malária, baseado no uso do inseticida DDT (Dicloro-Difenil-Tricloroetano) e no tratamento da população. Porém, devido a problemas administrativos, financeiros e técnicos, esse programa foi abandonado em 1969, quando se reconheceu que a erradicação não era viável em todas as partes do mundo (Nájera et al, 2011). Essas circunstâncias, assim como a quimiorresistência (QR) dos mosquitos ao DDT e do *P. falciparum* às drogas antimaláricas, aliadas as migrações da população humana, levaram a um aumento substancial no número de casos em todo o mundo durante os anos 70 e 80 (Feachem et al, 2010).

Assim, durante a conferência realizada pela OMS em Amsterdã em 1992, foram estabelecidas as principais diretrizes nas quais se baseiam os atuais programas de controle da morbidade e mortalidade da malária que compreendem, entre outras, o controle do vetor e o gerenciamento dos casos.

A prevenção, através do controle vetorial, visa reduzir a população de anofelinos infectados, por meio da redução do contato com os humanos e da densidade populacional dos mosquitos. Nesse sentido, as duas intervenções mais utilizadas são as telas impregnadas com inseticidas de longa duração (*LLINS ou long-lasting insecticide-treated nets*) e a borrifação intradomiciliar de inseticidas de ação residual (*IRS ou indoor residual spraying*) (WHO, 2017).

A política de gerenciamento dos casos objetiva reduzir a morbidade e a mortalidade da doença através do diagnóstico oportuno e do tratamento adequado, de forma a impedir a progressão da doença. Essa estratégia proporciona não só benefícios de cunho individual, mas também de cunho coletivo, visto que o pronto diagnóstico promove a rapidez no tratamento impedindo o aparecimento na

circulação periférica de formas infectantes para os mosquitos, bloqueando, assim, a transmissão.

O tratamento das malárias vivax e falciparum no Brasil é o mesmo em todas as regiões da Amazônia Legal e Extra-Amazônia. O Programa Nacional de Controle da Malária (PNCM) recomenda as drogas preconizadas pela OMS que são os ACTs (*artemisin combined treatment* / terapias combinadas a derivados de artemisinina) para o *P. falciparum* e cloroquina (CQ), seguida de primaquina para o caso de *P. vivax*.

É importante ressaltar que a malária na gravidez aumenta o risco do desenvolvimento de formas mais graves da doença e é uma das principais causas de resultados adversos do nascimento. Diante disso, a OMS implementou uma intervenção profilática para este grupo considerado de risco, em áreas de elevada transmissão, como as africanas, conhecida como tratamento intermitente preventivo em grávidas (*IPTp / intermittent preventive treatment in pregnancy*). No caso de crianças, entre 3 e 59 meses de idade, a intervenção de escolha para a prevenção é a quimioprofilaxia da malária sazonal (*SMC ou seasonal malaria chemoprevention*) que visa impedir as manifestações clínicas mais graves da doença (WHO, 2017).

No contexto de gerenciamento de casos, a resistência aos medicamentos representa um dos principais obstáculos na luta contra a malária.

1.5. Antimaláricos e Quimiorresistência

A quimiorresistência (QR) na malária é definida como “a capacidade do parasito de sobreviver e/ou se multiplicar, apesar da administração e absorção de

um medicamento em doses iguais ou superiores ao recomendado, mas dentro do limite de tolerância do paciente", e é um tema recorrente na história do controle de muitas doenças infecciosas (WHO, 2010).

Constatou-se ainda na malária, a ocorrência de resistência cruzada, eis que por muitas décadas o tratamento da malária se baseou em drogas, por vezes da mesma família química **(Tabela 1)**.

Dentre as drogas utilizadas, podemos destacar a cloroquina (CQ), por ser de baixo custo, segura, pouco tóxica e com rápida ação parasiticida (White NJ, 1996), que foi utilizada por cerca de 50 anos para o tratamento da malária não complicada pelo *P. falciparum* e, nos dias de hoje, ainda é usada para o tratamento de *P. vivax*.

Drogas	*Classe química*	*Alvos das drogas*
Cloroquina, amodiaquina, piperaquina	**4-aminoquinoleínas**	**Trofozoítos e esquizontes**
Primaquina	**8- aminoquinoleínas**	**Gametócitos e esquizontes hepáticos**
Quinina, quinidina, mefloquina, lumefantrina	**Amino-álcoois**	**Trofozoítos e esquizontes **Quinina: trofozoíto e gametócitos estágios I a III**
Artemisinina, arteméter, artesunato, dihidroartemisinina	**Lactonas sesquiterpênicas**	**Todas as formas**
Tetraciclina, doxiciclina	**Antibióticos**	**Estágios sanguíneos**

Tabela 1. Principais drogas antimaláricas para o tratamento da malária e suas respectivas classes, de acordo com a classe química e o alvo parasitário.

Até a presente data, a resistência às drogas antimaláricas tem sido bem documentada em duas das cinco espécies de plasmódios que infectam o homem: *P. falciparum* e *P. vivax*.

A origem da resistência parece ocorrer em duas fases: a primeira, um evento inicial genético produz um mutante resistente (*mutação de novo*), e este novo traço promove a sobrevivência do parasito frente a droga. Na segunda fase, os parasitos

resistentes são selecionados e começam a multiplicar-se, podendo resultar em uma população de parasitos resistentes ao tratamento (Wongsrichanalai et al, 2002; Baird, 2009).

Uma das primeiras menções à resistência aos antimaláricos sintéticos foi feita no relatório da OMS de outubro de 1950, cujo documento ressaltava o fato de haver resistência, em certas regiões do continente africano, do P. falciparum ao proguanil e alguns indícios de possível resistência à mepacrina e cloroquina. Na nota técnica da OMS de 1953, já foi reconhecido que no combate à malária, a resistência a certas drogas, era um dos maiores problemas na clínica da doença, problema este associado a doses irregulares e inadequadas administradas em sua profilaxia. Até então não havia casos significativos que indicassemThe ®rst reports of con®rmed P. falciparum resistance

> (RI) to CQ came, almost simultaneously, from South America (Colombia, Brazil, Venezuela in 1960) (Moore & Lanier 1961; Wernsdorfer & Payne 1991) and South-east Asia (Thailand, Kampuchea in 1961) (Hartinuta et al. 1962). In 1973, CQ resistance had been reported in several countries in South America (Brazil, Colombia, Guyana and Venezuela) and in Asia (Burma, Cambodia, Malaysia, Philippines, Thailand and Vietnam) but not in sub-Saharan Africa (WHO 1973). In Africa, P. falciparum CQ resist-ance was ®rstly reported from the eastern region, in Kenya (Fogh et al. 1979) and Tanzania (Campbell et al. 1979), in the late 1970s and it spread from east to west.
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Os primeiros relatos confirmados de resistência a CQ por parasitos do *P. falciparum* ocorreram quase que simultaneamente, em 1961, na América do Sul (Colômbia, Brasil e Venezuela) (Wernsdorfer & Payne 1991) e no Sudoeste Asiático (Tailiandia) (Harinasuta et al, 1965), culminando na dispersão de parasitos resistentes pelo mundo nas duas décadas seguintes, que foi acompanhada por um aumento expressivo do número de casos. Posteriormente, com o amplo uso da combinação sulfadoxina pirimetamina (SP) como droga alternativa para o tratamento da malária não complicada em áreas nas quais a CQ não era mais eficaz, verificou-se também o rápido surgimento de resistência a essa combinação, no final dos anos 60 (Gama et al, 2009). Tais eventos marcaram com grande impacto o controle e modificaram a epidemiologia da malária, pois resultaram num aumento de custo e elevação considerável dos índices globais de morbidade e mortalidade a partir da década de 70 (Phillips et al, 1996; White et al 1999; Bloland, 2001).

Em consequência do aumento nos níveis de morbimortalidade e da dispersão mundial de isolados de *P. falciparum* resistentes à CQ e à SP, desde 2001 a OMS

recomenda como primeira linha de tratamento o uso de ACTs. Através dessa terapia combinada, a possibilidade de seleção de parasitos resistentes é minimizada, pois a maior parte da carga parasitária é eliminada pela rápida ação da artemisinina (ART) e/ou seus derivados, cuja concentração decai rapidamente na circulação sanguínea, nos primeiros dias de infecção. Logo, a tarefa de extinguir a parasitemia residual recai para a droga parceira que permanece mais tempo em circulação (Woodrow et al, 2017).

A introdução dos ACTs, associados a intervenções complementares como o uso de telas, mosquiteiros impregnados e a borrifação intradomiciliar de inseticidas, foi fundamental para promover a melhora no número de casos da malária. Desde o início dessa fase de monitoramento, vários países vêm registrando decréscimos no número de casos confirmados da doença, como também no número de mortes (WHO, 2017).

Entretanto, diferentes estudos vêm demonstrando a redução da susceptibilidade do *P. falciparum* aos derivados de ART em cinco países do sudeste da Ásia: Camboja, Laos, Mianmar, Tailândia e Vietnam (WHO, 2001; Maude et al, 2009; Rogers et al, 2009; Noedl et al, 2008; Dondorp et al, 2009, Phyo et al, 2012; Hien et al, 2012; Ashley et al, 2014; Amaratunga et al, 2012; Kyaw et al, 2013; Mishra et al, 2015), e na província de Yunnan e na China (Dondorp et al 2009; Ashley et al, 2014; White, 2016).

Mais precisamente, a resistência à ART surgiu no Camboja (Dondrop et al, 2009; WHO, 2010) e é clinicamente definida pela redução das taxas do clareamento parasitário e é expressa pelo aumento da meia vida do parasito (Flegg et al, 2011; White, 2011), ou pela persistência de parasitos microscopicamente detectáveis no terceiro dia de terapia após a introdução dos ACTs (WHO, 2010). O parâmetro de meia-vida se correlaciona fortemente aos resultados *in vitro* do teste de

sobrevivência das formas em anel e está associado aos resultados *ex vivo* (Baum et al, 2009). Estes testes medem a taxa de sobrevivência de parasitos no estágio inicial de anel frente a uma exposição da droga farmacologicamente relevante (700 nM por 6 h) de diidroartemisinina (DHA) - o principal metabólito de todos os ACTs.

Num estudo de eficiência terapêutica para os ACTs realizado no Suriname, foi detectada a presença de parasitos no sangue no terceiro dia de tratamento, indicando falha terapêutica pela demora no clareamento parasitário (Vredem *et al,* 2013). Essa detecção levou a preocupações sobre a suscetibilidade à ART no chamado Escudo da Guiana (Guiana Francesa, Guiana, Suriname, Brasil, Venezuela e Colômbia), pela livre circulação de pessoas entre as fronteiras desses países. Além disso a resistência a drogas, como a CQ e SP, surgiu quase que simultaneamente na América do Sul e no Sudeste Asiático (Wooton et al, 2002; McCollum et al, 2007) tornando essa QR à ART uma séria ameaça ao controle da malária também na América do Sul.

Ademais, recentemente, foi observado em isolados da África um declínio na resposta ao tratamento com os ACTs, o que poderia sugerir a emergência de parasitos com sensibilidade reduzida aos ACTs (Beshir et al, 2013; Borrmann et al, 2011).

Nesse cenário não é difícil entender como é preocupante a dispersão de parasitos resistentes aos ACTS, como o já ocorrido com a CQ e outras drogas antimaláricas, notadamente pelo *P.falciparum* o grande responsável pelas formas graves e letais da doença. Além de agravar o prognóstico de cura do paciente, a QR causa atraso ou falha na eliminação dos parasitos assexuados do sangue periférico promovendo, assim, a produção e manutenção de gametócitos responsáveis pela transmissão do genótipo resistente.

Diferentemente do *P. falciparum*, a resistência de parasitos de *P. vivax* à CQ só foi observada, aproximadamente, 28 anos depois, em 1989, na Papua Nova Guiné (Rieckmann et al, 1989), seguindo-se de relatos na Oceania, Ásia e, mais recentemente, na América do Sul (Baird, 2004).

1.6. Panorama da quimiorresistência no Brasil

No Brasil, o primeiro relato científico de QR se deu no início do século XX, quando Arthur Neiva relatou a sua dificuldade em tratar casos de malária falciparum com o quinino (Neiva, 1910). Cerca de 40 anos depois, a resistência à CQ foi notificada timidamente em um congresso não obtendo grande repercussão (Brito & Pinheiro, 1954) até que, no Rio de Janeiro, o médico José Rodrigues da Silva descreveu casos de *P. falciparum* resistentes à terapia com CQ em indivíduos que haviam retornado de construção de estradas nos estados de Rondônia, Pará, Maranhão e Bahia (Silva, 1961). A partir desses relatos foram anunciados mais casos de *P. falciparum* resistentes à CQ em Rondônia (Box et al, 1963), tal qual em outros estados brasileiros como Acre, Roraima, Amazonas (Ferraroni et al, 1981), Mato Grosso e Amapá (Reyes, 1981), demonstrando que tais parasitos resistentes estavam de fato disseminados pelo território brasileiro.

Frente a este cenário de resistência à CQ, a combinação de SP foi adotada como primeira linha no tratamento da malária *falciparum* no Brasil. Entretanto, pouco tempo após o início do uso da combinação SP foi relatado o aparecimento de parasitos resistentes à SP em pacientes provenientes de Tocantins (Almeida Neto *et al*,1972) e do Pará (Souza, 1983, Boulos et al,1986).

Da mesma forma foram observados casos de resistência ao quinino em isolados no Mato Grosso em 1997 e no Estado do Pará no ano de 1999 (Calvosa et

al, 2001). Estudos *in vitro* realizados na Bacia Amazônica evidenciaram a resistência e/ou diminuição da sensibilidade do *P. falciparum* também à mefloquina (MQ) (Souza,1983; Boulos *et al,* 1986; Di Santi et al, 1988; Cerutti et al, 1999; Calvosa et al, 2001). Diante dessa situação, a OMS recomendou os ACTs (WHO, 2001) que, atualmente, vêm sendo grandes aliados das políticas de controle e eliminação da malária no mundo.

Diferentemente do *P. falciparum*, o surgimento da QR à CQ em *P. vivax* no Brasil foi observado bem mais tarde e ainda hoje há escassos dados publicados sobre este assunto. A primeira descrição clínica da resistência à CQ proveio de um paciente atendido no Instituto de Medicina Tropical do Amazonas em 1999 (Alecrim et al, 1999), a qual foi seguida por outro relato clínico do mesmo grupo desta vez de um isolado de *P. vivax* resistente à CQ e à primaquina - uma droga hipnozoiticida usada em conjunto com a CQ (Alecrim et al, 1999). Depois disso, um estudo de acompanhamento *in vivo* foi publicado, registrando uma prevalência de 10.1% de casos de QR à CQ no Brasil, também na cidade de Manaus, Amazonas (de Santana Filho et al, 2007) e um outro relatou recorrências por *P.vivax* em 5.2% dos casos na mesma cidade (Marques et al, 2014).

De fato, devido à sua importância no controle da malária, o monitoramento da resistência no Brasil se iniciou nos anos 80 com a utilização de testes *in vitro* (Ferraroni et al, 1981; Reyes et al, 1981; Couto et al, 1995; Calvosa et al, 2001; Menezes et al, 2001), ou testes *in vitro* acompanhados de observações feitas *in vivo* (Boulos et al, 1986; Segurado et al, 1997; Cerutti et al, 1999; Neifer et al, 1991).

Mesmo que a genotipagem molecular de *P. falciparum* tenha se iniciado na década de 90, até a atualidade um número reduzido de estudos foi realizado. As primeiras análises moleculares se basearam na avaliação dos polimorfismos dos

genes *pfdhfr* e *pfdhps* (Peterson et al, 1991; Vasconcelos et al, 2000), quando foi demonstrada a presença das mutações 50**R**, 51**I**, 108**N** e 164**L** (*pfdhfr*), além das mutações 437**G**, 540**E** e 581**G** (*pfdhps*). No caso do gene *pfmdr1*, foi evidenciada a presença de SNPs como a 184**F**, 1034**C**, 1042**D** e 1246**Y** (Póvoa et al, 1998; Zalis et al, 1998) e, anos mais tarde, quando a resistência à CQ foi atribuída ao gene *pfcrt*, a genotipagem das principais SNPs também foi realizada, apontando a presença das mutações 72**S**, 74**I**, 75**E** e 76**T** em diferentes haplótipos (Vieira et al, 2004; Vieira et al, 2001; Viana et al, 2006). Posteriormente, uma nova caracterização foi conduzida, revelando a presença da mutação 76**T** no gene *pfcrt*, além das SNPs 1042**D** e 1246**Y** no gene *pfmdr1* (Ferreira et al, 2008).

Em relação ao *P. vivax*, os estudos de genotipagem molecular se iniciaram mais tardiamente e as análises conduzidas, até então, examinaram um número amostral reduzido. Nesses trabalhos iniciais, a sequência de DNA do gene *pvmdr1* foi descrita em 3 isolados (Sá et al, 2005) e as dos genes *pvmdr1* e *pvcrt-o* em 7 isolados (Orjuela-Sánchez et al, 2009).

1.7. Marcadores Moleculares de Quimiorresistência

O desenvolvimento de técnicas moleculares requer extensivas investigações sobre os mecanismos que conduzem ao fenótipo de resistência no parasito, seja através de clonagem e sequenciamento de genes homólogos e ortólogos ou de estratégias clássicas de genética reversa para analisar a progênie de cruzamentos entre cepas sensíveis e resistentes, de maneira a identificar potenciais genes candidatos a marcadores moleculares de QR. A investigação desses genes alvos permite estudos de associação entre os fenótipos *in vitro* com as alterações gênicas, tal qual mutações de um único nucleotídeo (SNP ou *single nucleotide polymorphism*),

ou mesmo alterações na expressão e no número de cópias de tais genes (Laufer et al, 2007; Plowe et al, 2007).

Desse modo, relações causais puderam ser estabelecidas entre os genes portando os marcadores identificados e a resistência *in vitro*, que foram confirmadas subsequentemente em estudos de transformação gênica nos quais substituições da sequência de DNA eram induzidas artificialmente, conferindo mudanças na susceptibilidade de cepas de *P. falciparum* cultivadas *in vitro* (Sidhu et al, 2002; Wu et al, 1996) ou ainda em células de *Saccharomyces cerevisae* recombinante (Wooden et al, 1997; Cortese et al; 1998).

Os marcadores moleculares de QR são definidos como alterações genéticas preditoras de fenótipos e representam uma nova ferramenta de saúde pública. De uma maneira geral, a genotipagem desses marcadores é realizada através das técnicas baseadas na reação da polimerase em cadeia (PCR ou *polymerase chain reaction*) seguida de sequenciamento do DNA alvo, para permitir a detecção de SNPs em genes candidatos a uma associação com o desenvolvimento de QR.

O desenvolvimento dessas técnicas foi impulsionado em resposta as limitações dos testes tradicionais *in vivo* e *in vitro* e surgiram a partir de extensivas investigações sobre os mecanismos moleculares que levavam ao fenótipo de resistência no parasito (Plowe, 2003).

Os subcapítulos que se seguem descreverão especificamente os genes canditados a resistência de *P. vivax* à SP e à CQ , bem como o gene marcador para a QR de *P. falciparum* aos derivados da ART.

1.7.1. pvcrt-o e pvmdr1

Com o surgimento da resistência à CQ em *P. vivax*, a busca por marcadores moleculares se baseou naturalmente no conhecimento obtido sobre a QR do *P. falciparum*. Sendo assim, genes ortólogos do *P. falciparum* foram descritos no *P. vivax* como candidatos a marcadores moleculares tais como: *pvcrt-o* (transportador de proteína putativa - CG10 / *chloroquine resistance transporter*) (Barnadas et al, 2008; Sá et al, 2006; Nomura et al, 2001) e *pvmdr1* (resistência a múltiplas drogas 1 / *multiple drug resistance* 1) (Barnadas et al, 2008; Marfurt et al, 2008; Kim et al, 2011; Suwanarush et al, 2007; Sá et al, 2005; Brega et al, 2005; WHO 2012).

Com relação ao *pvcrt-o*, embora tenha sido demonstrado por expressão heteróloga que a molécula codificada – K76T-CRT - participa no transporte da CQ pela membrana do vacúolo digestivo (Sá et al, 2005), diferentemente do *P.falciparum,* a presença de mutações não estava associada ao fenótipo de resistência. Esse achado sugere que o mecanismo que conduz a resistência a CQ em *P. vivax* possa ser diferente daquele observado em *P. falciparum* (Suwanarusk et al, 2008).

O gene *pvmdr1* codifica para proteínas de transporte da família ABS, homólogas a p-glicoproteína1, que estão associadas à multirresistencia a drogas no homem. Após o isolamento e a caracterização do *pvmdr1* localizado no cromossomo 10, mutações foram comparadas com os fenótipos de resistência e numa avaliação de sequências de DNA de diferentes isolados apresentando distintas susceptibilidades à CQ, não foi encontrada associação entre as diferentes mutações e o fenótipo resistente (Sá et al, 2005). Especificamente, o envolvimento da mutação Y976**F** (tirosina para fenilalanina no códon 976), inicialmente proposta como um marcador na Ásia (Sá et al, 2005; Suwanarusk et al, 2008), não foi atestado em outros locais, dado as descrições conflitantes que observaram uma discrepância

entre a frequência de ocorrência desta mutação e a prevalência de resistência clínica à CQ (Barnadas et al, 2008). Contrariamente, Lu e colaboradores (2011) observaram a presença da mutação Y976**F** associada à resistência clínica a CQ em isolados da Tailândia e de Mianmar. Da mesma forma, Mint e colaboradores (2012) demonstraram na Mauritânia, uma forte associação entre esse marcador e a resistência clínica à CQ.

Por conseguinte, até o momento os resultados ainda não são conclusivos denotando assim, a importância da realização de estudos voltados para a identificação de haplótipos do gene *pvmdr1*. Trabalhos neste sentido ainda são escassos na literatura e, no Brasil só existem três relatos com marcadores moleculares de QR do *P.vivax* a CQ sendo, um do nosso grupo (Gama et al 2009), um de um grupo em Manaus (de Santana Filho et al, 2007) e, um outro, de um grupo paulistano (Orjuela-Sanchez et al, 2009).

1.7.2. pvdhfr e pvdhps

Os antifolatos, notadamente a combinação SP, foram e têm sido muito importantes na terapia antimalárica em função do seu baixo custo e da sua segurança para grávidas. Considerava-se que os parasitos de *P. vivax* eram naturalmente refratários ao tratamento com antifolatos, dado esse que se mostrou incorreto ao serem analisadas a susceptibilidade *in vivo* (Imwong et al, 2005; Leslie et al, 2007), como também a *in vitro* (de Pecóulas, 1998).

A base molecular para a QR a SP começou a ser esclarecida em 1998, quando se deu o isolamento e caracterização do gene codificante para a enzima *dhfr* presente no cromossomo 5 de *P. vivax*. As primeiras comparações de sequências de DNA entre isolados com diferentes fenótipos revelaram a ocorrência de SNPs em

três códons: 58 (serina para arginina – S58**R**), 117 (serina para asparagina – S117**R**) e 173 (isoleucina para leucina – I173**L**) (de Pecoulas et al, 1998).

Posteriormente, quando do isolamento do gene *pvdhps* (Korsinczky et al, 2004), a análise das sequências de DNA também revelou diferentes mutações associadas à resistência para sulfadoxina em *P. vivax*. No caso do *dhps*, a ocorrência de SNPs foi observada em dois códons: 383 (alanina para glicina – A383G) e 553 (alanina para glicina/cisteína – A553G/C) (Raza et al, 2013).

Embora a terapia com a combinação SP não tivesse sido instituída na malária vivax na maioria das áreas endêmicas, vários autores verificaram a ocorrência de mutações, tanto no gene *pvdhfr* como no *pvdhps*, em isolados clínicos obtidos de várias localidades (Brega et al, 2004; Zakeri et al, 2009; Barnadas et al, 2009; Afsharpad et al, 2012; Raza et al, 2013), fato esse que possivelmente estaria ligado ao uso contínuo da SP para tratar casos de malária por *P. falciparum* resistentes à CQ. Com efeito, em áreas nas quais coinfecções por *P. falciparum* e *P. vivax* são comuns e frequentemente subdiagnosticadas, uma pressão incidental poderia ter selecionado mutações nos supracitados genes de *P. vivax* (Baird, 2009).

1.7.3. pfk13

A ART e seus derivados são as drogas preconizadas pela OMS para o tratamento do *P. falciparum* e, desde 2006, vem contribuindo para a redução significativa da morbiletalidade da malária em todo o mundo. Entretanto, diferentes estudos clínicos vem demonstrando uma demora no clareamento parasitário frente a ART. Diante disso, se intensificaram as pesquisas genéticas que detectaram uma região localizada no cromossomo 13 do *P. falciparum* fortemente associada com a demora no clareamento parasitário *in vivo* (Cheeseman et al, 2012; Takala-Harrison et al, 2013).

Por conseguinte, foi demonstrado que polimorfismos num domínio da proteína *kelch* (*k13*) *propeller* (hélice) do *P. falciparum* constituem marcadores moleculares potencialmente capazes de monitorar isolados resistentes à ART pelas seguintes razões: a) perda progressiva do tipo selvagem nos parasitos do noroeste do Camboja durante a década de emergência da resistência a ART nesta região; b) prevalência significativa de parasitos mutantes em províncias do Camboja nas quais a resistência a ART está bem estabelecida e menor frequencia onde a resistência a ART é incomum; c) múltiplas mutações, todas não-sinônimas, presentes na hélice do *k13*, refletindo a seleção positiva, em vez de um efeito carona ou de deriva genética; d) três mutações mais prevalentes do *k13* fortemente relacionadas com as taxas de sobrevivência *in vitro* e com a meia-vida de clareamento do parasito *in vivo* e; e) frequência de alelos mutantes fortemente correlacionada com a persistência de parasitos no dia 3 após o tratamento com ART no Camboja (Ariey et al, 2014).

Nesse sentido, a análise de polimorfismos do gene *Pfk13* em isolados do *P. falciparum* no Camboja resultou na descoberta de 20 códons associados ao aumento da tolerância à ART. Nesta análise genômica foram identificadas as 4 mutações na hélice *k13* de maior prevalência (C580**Y**, R539**T**, I543**T** e Y943**H**) associadas às altas taxas de sobrevivência *in vitro* e ao aumento da meia-vida do parasito (Ariey et al, 2014; Amaratunga et al, 2014). A frequência do alelo C580**Y** aumentou significativamente nos anos de 2001-2002 e 2011-2012, sinalizando sua invasão ou quase fixação na região (Ariey et al, 2014), sendo este haplótipo considerado pela OMS como o principal marcador da resistência à ART (WHO, 2017).

Desde então estudos de monitoramento de alelos mutantes no domínio *k13* têm sido realizados com o intuito de evitar ou conter a introdução de parasitos resistentes à ART onde as ACTs são eficazes. Como por exemplo, foi encontrado na Guiana o alelo mutante C580**Y** mas, interessantemente, o estudo de haplótipos dos

microssatélites flanqueadores do gene *pfk13* mostrou que este alelo possuía um perfil diferente daqueles do Camboja, sugerindo que o surgimento desses haplótipos na América do Sul ocorreu de forma independente dos da Ásia (Chenet et al. 2016).

A emergência de mutações na hélice *k13* em isolados africanos (Ashley et al 2014; Conrad et al 2014; Taylor et al 2015; Lu et al 2017) é preocupante, embora nenhuma dessas mutações tenha sido correlacionada às identificadas no Camboja e o tratamento com ACTs continue se mostrando eficaz para o tratamento da malária *falciparum* em muitas áreas africanas (Ashley, et al, 2014). Variações naturalmente ocorridas no domínio *k13* em isolados africanos nas quais não se conhece o impacto na sensibilidade à ART, merecem ser melhor investigadas (Streimer et al. 2015).

2. Justificativa e Relevância

Na ausência de uma vacina antimalárica o controle da malária se baseia, principalmente, no gerenciamento dos casos através do rápido diagnóstico e tratamento.

No Brasil, o número de infecções maláricas tem sido reduzido, sendo a redução mais significativa a das infecções pelo *P. falciparum* (Oliveira-Ferreira et al,

2010; Santelli et al, 2012), entre outros fatores, devido ao aparecimento de gametócitos nos primeiros estágios de infecção pelo *P. vivax* (Recht et al, 2017).

Desta forma, existe uma expectativa de se atingir a fase de pré-eliminação da malária, que consiste na interrupção da transmissão da malária. Para isso, a comunidade científica vem desenvolvendo novas estratégias com o intuito de impedir a reintrodução da malária em áreas devidamente controladas onde existe uma expectativa de se atingir a fase de pré-eliminação, que consiste na interrupção da transmissão da malária e na intensificação das estratégias voltadas, principalmente, para o diagnóstico, a vigilância dos casos e o tratamento (Ministério da Saúde, 2016). Contudo, durante décadas, a resistência dos parasitos da malária aos medicamentos tem sido um dos maiores obstáculos para a eliminação da doença.

Os marcadores moleculares representam ferramentas de saúde pública de grande potencial com capacidade de analisar e detectar mudanças nos padrões de susceptibilidade e / ou resistência dos isolados de *Plasmodium*, independente da observação fenotípica e, portanto, são as ferramentas ideais para o monitoramento da eficácia de drogas.

Esforços voltados para o rastreamento da QR são necessários para manter os tratamentos antimaláricos a longo prazo e evitar a emergência global da malária.

3. Objetivos

Nosso objetivo se constituiu na realização de um estudo descritivo sobre a ocorrência e prevalência de mutações em genes associados ou potencialmente candidatos à quimiorresistência em isolados de *P. vivax* (*pvmdr1*, *pvdhps* e *pvdhfr*) e de *P. falciparum* (*pfk13*) provenientes de áreas da Amazônia e/ou da Extra-Amazônia brasileiras.

Para tal, empregamos como estratégias reações de PCRs convencionais do tipo *nested*, seguidas de sequenciamento do DNA alvo, para permitir a avaliação das principais mutações, previamente descritas nesses genes, em amostras de *P. vivax* e *P. falciparum*.

4. Resultados

4.1. Artigo 1: *Plasmodium vivax mdr1* genotypes in isolates from successfully cured patients living in endemic and non-endemic brazilian areas

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Fundamentados em relatos contemporâneos do surgimento de QR à CQ no Brasil em parasitos de *P. vivax*, nosso primeiro trabalho teve como objetivo caracterizar as mutações no gene *pvmdr1*, em isolados das regiões Amazônica (Acre, Amazonas, Pará e Rondônia) e Extra-Amazônica (Rio de Janeiro).

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Plasmodium vivax mdr1 genotypes in isolates from successfully cured patients living in endemic and non-endemic Brazilian areas

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Abstract

Background: *Plasmodium vivax* is the most widely distributed species causing the highest number of malaria cases in the world. In Brazil, *P. vivax* is responsible for approximately 84 % of reported cases. In the absence of a vaccine, control strategies are based on the management of cases through rapid diagnosis and adequate treatment, in addition to vector control measures. The approaches used to investigate *P. vivax* resistance to chloroquine (CQ) were exclusively in vivo studies because of the difficulty in keeping parasites in continuous in vitro culture. In view of the limitations related to follow-up of patients and to assessing the plasma dosage of CQ and its metabolites, an alternative approach to monitor chemo-resistance (QR) is to use molecular markers. Single nucleotide polymorphisms (SNPs) in the multidrug resistance gene *pvmdr1* are putative determinants of CQ resistance (CQR), but such SNPs in *P. vivax* isolates from patients with good response to treatment should be further explored. The aim of this study is to investigate the mutations in the gene, supposedly associated to QR, in *P. vivax* isolates from successfully cured patients, living in Brazilian endemic and non-endemic areas.

Methods: Blood samples were collected from 49 vivax malaria patients from endemic (Amazon Basin: 45) and non-endemic (Atlantic Forest: four) Brazilian regions and analysed for SNPs in the CQR-related *P. vivax* gene (*pvmdr1*), using PCR-based methods.

Results: Among the 49 isolates genetically characterized for the gene *pvmdr1*, 34 (70 %) presented at least one mutation. T958**M** mutant alleles were the most frequent (73 %) followed Y976**F** (15 %) and F1076**L** (12 %). Single mutation was detected in 24 (70.5 %) isolates and double mutations in ten (29.5 %). The most common single mutant genotype was the 958**M**/Y976/F1076 (79 %), followed by 976**F**/F1076 (21 %) whereas 958**M**/Y976/1076**L** (60 %) and 976**F**/1076**L** (40 %) double mutant genotypes were detected. Single mutant profile was observed only in isolates from Amazon Basin, although double mutants were found both in the Amazon and Atlantic Forest regions. Interestingly, the genotype 958**M**/Y976/1076**L** was present in all isolates from the Atlantic Forest in the Rio de Janeiro State.

Conclusions: Considering that primaquine (PQ) efficacy is highly dependent on concurrent administration of a blood schizontocidal agent and that PQ could not circumvent CQR, together with the fact that no *pvmdr1* mutation should be expected in successfully cured patients, these findings seem to indicate that the *pvmdr1* gene is not a

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reliable marker of CQR. Further investigations are needed to define a reliable molecular marker for monitoring *P. vivax* CQR in *P. vivax* populations.

Keywords: *Plasmodium vivax*, Chloroquine resistance, *pvmdr1* gene, Brazil

Background

Almost 40 % (approximately three billion) of the world's population is presently at risk of contracting malaria. The disease causes almost 200 million clinical cases and around 600,000 deaths each year [1, 2]. *Plasmodium vivax* is the most geographically widespread of the human malaria parasites, and a serious public health concern in South and Central America, Asia and Southwest Pacific [3].

In Brazil, endemic regions are restricted to the Legal Amazon (comprising Acre, Amapá, Amazonas, part of Maranhão, Mato Grosso, Pará, Rondonia, Roraima and Tocantis States), a region that presently accounts for 99.6 % of the countrywide malaria burden [4]. *Plasmodium vivax* is the predominant species, responsible for 84 % of the reported cases [5], PNCM, SVS, MS, unpublished data 2015. Extra-Amazonian autochthonous cases account for only 0.04 % of all Brazilian total registered and correspond to the autochthonous malaria existing in the Atlantic Forest, located along the southeastern Atlantic Coast [6].

Chloroquine resistance (CQR) is the main challenge for national malaria control programmes to control vivax malaria. The first cases of *P. vivax* resistant to chloroquine (CQ) were described in Papua New Guinea [7] and thereafter observed in Indonesia [8], Oceania, Asian [9, 10] and South American countries, including Brazil [11, 12]. In Brazil, CQ treatment failures, presumably related to CQR, have been reported [13]. The latest 28 day in vivo test conducted to assess the efficacy of standard supervised CQ therapy in 109 volunteers showed a proportion of 10.1 % of treatment failure (n = 11), despite an adequate absorption of CQ in these individuals on day 2 [14].

Molecular markers can represent a valuable tool for monitoring introduction and spread of drug resistance. Contrarily to *Plasmodium falciparum*, mutations at codons in the *pfcrt* orthologue (*pvcg10*) gene do not seem to mediate CQR in *P. vivax* [15]. On the other hand, the polymorphisms at codons Y976**F** and F1076**L** in the multidrug-resistant gene 1 (*pvmdr1*) has been described as molecular marker associated to CQR [16]. Indeed, in Thailand, Indonesia [17] and Myanmar [18], as well as in Mauritania [19] and Cambodia [20], it has been shown that 976**F** mutants were associated with clinical resistance to CQ. In Nepal and India, where *P. vivax* CQR has not been recorded, prevalence of the 976**F** mutation is very low (5 %) [21] or not detected [22], while in India the presence of the F1076**L** mutation was not associated to CQR. In addition, in Madagascar, despite 5 % of clinical failures more than 90 % of Y976**F** mutant parasites were detected [33].

These polymorphisms also seem to be relatively uncommon in Latin America, where *P. vivax* CQR remains relatively infrequent [23]. In Brazil, different conclusions were drawn: either mutations in *pvmdr1* were reported in CQ-sensitive *P. vivax* parasites [24–26] or not detected in resistant *P. vivax* isolates [25, 26], as well as *P. vivax* CQR being associated with *pvmdr1* mutants only in patients with severe malaria [27].

In view of these different epidemiological data, the nucleotide polymorphisms (SNPs) of *pvmdr1* gene in successfully cured vivax malaria patients living in endemic (Amazonian) and non-endemic (Extra-Amazonian) Brazilian areas, were investigated in the present study.

Methods

Study site, blood samples and DNA extraction

Blood samples were collected between 2010 and 2014 in patients presenting vivax malaria (n = 49) at the Laboratório de Doenças Febris Agudas, INI-IPEC, Fiocruz, the Reference Laboratory for Malaria in the Extra-Amazon to the Brazilian Ministry of Health. The inclusion criterion was patients with uncomplicated vivax malaria. After obtaining informed consent, venous blood collection was performed according to protocols previously approved by the Ethical Research Committees of Fiocruz (32839013.6.00005248). Genomic DNA was extracted from 1 mL whole blood using QIAamp midi columns, as described by the manufacturer (Qiagen). *Plasmodium vivax* samples were diagnosed by microscopic examination and by polymerase chain reaction (PCR) [28]. All patients were treated with CQ plus primaquine (PQ) and followed up for 42 days and no treatment failure was detected during this period.

PCR and electrophoresis

The *pvmdr1* gene was amplified by PCR using gene-specific primers. The PCR was performed as described elsewhere [16, 29] to amplify a partial DNA sequence containing three SNPs for *pvmdr1* gene including: T958**M**, Y976**F** and F1076**L**.

DNA sequencing and SNP polymorphisms detection

After purification using the Wizard SV Gel and PCR Clean-Up System (Promega), the amplified fragments were sequenced using Big Dye® Terminator Cycle Sequencing Ready Reaction version 3.1 (Applied Biosystems) and ABI PRISM DNA Analyzer 3730 (Applied Biosystems) [30] at the Genomic Platform/PDTIS/Fiocruz. The direct DNA sequencing from PCR products was compared with the reference wild type Sal I GenBank accession n° AY571984 [24, 25]. Forward and reverse sequences were analysed using the free software Bioedit Sequence Alignment Editor version 7.2.5. Statistical significance of differences of *pvmdr1* genotypes frequencies among Brazilian localities was assessed using Fisher's tests.

Results

The *pvmdr1* gene was successfully amplified and DNA sequenced in 49 isolates from the Amazon Region (Acre, Amazonas, Pará, and Rondonia) and the Extra-Amazonian State of Rio de Janeiro.

Globally, 34 (69 %) showed non-synonymous (958**M**, 976**F** and 1076**L**) mutations. 958**M** mutant alleles were the more frequent (25/34; 73 %) while 976**F** (5/34; 15 %) and 1076**L** (10/34; 12 %) were detected at lower frequencies (Table 1). Single mutation was observed in 24 isolates (70.5 %, 24/34), while double mutations were recorded in ten (29.5 %, 10/34) *P. vivax* samples. In the isolates presenting single mutant genotype, the **M**YF profile was predominant (19/55 %) contrasting with the **FF**, which was found in only five isolates (Table 2).

The *pvmdr1* wild-type allele was prevalent in Pará (54 %) followed by Acre (40 %), Amazonas (33 %), and Rondonia (24 %) states without statistically significant difference in proportion ($p > 0.05$). Single mutants were observed only in isolates from the Brazilian Amazon: the **M**FY allele was prevalent in Rondonia (77 %), followed by Amazonas (62 %) and Acre (67 %) ($p > 0.05$), although in Pará the **FF** single mutant was more frequent (66 %) than the **M**YF ($p > 0.05$). However, when double mutants were investigated, samples presenting **FL** (12 %) and **ML** (20 %) in both Amazonian and Extra Amazonian States, were identified. Irrespective to the Brazilian State **FL** double mutant was the less frequent (Table 2) ($p > 0.05$). Interestingly, all isolates from the Extra Amazon (Rio de Janeiro State) showed double mutant genotype (**ML**) contrasting with those from the Amazon Basin (6 %) ($p = 0.01$), where most of the isolates came from.

Table 1 Frequency of 958M, 976F and 1076L mutants in *pvmdr1* gene among 49 Brazilian *P. vivax* isolates

SNPs	Number of Isolates (%)
No mutation	15 (31)
958**M**	25 (51)
976**F**	5 (10)
1076**L**	4 (8)

SNPs single nucleotide polymorphisms

Discussion

CQ and PQ remain the drugs of choice to treat vivax malaria, but recent studies have reported *P. vivax* cases of resistance to CQ in different regions of the world [9, 31], including Brazil [11–13]. Therefore, monitoring the efficacy of CQ in the treatment of vivax malaria is essential for early warning systems to promote drug policies.

To circumvent the limitations of in vivo and in vitro studies and to assess chemo-resistance, identification of mutations in target genes has been proposed, such as those in the *pvmdr1* gene at codons 976 and 1076, as well as the increased expression of *pvcrto* transcripts [12, 32]. Similar to previous studies performed with samples from western Brazilian Amazon [24, 25], in this work the T958**M** mutant was found to be the more frequent in the Brazilian Amazon and even in isolates from the Extra Amazonian regions. Additionally, in Madagascar [33], Nepal [21] and Thailand [34], most of the samples

Table 2 Proportion of the 4 alleles observed among 49 Brazilian *P. vivax* isolates, according to the sampling location

Genotypes	*Pv* Isolates N (%)	Localities				
		Rondônia (n = 17)	Pará (n = 11)	Amazonas (n = 8)	Acre (n = 5)	Rio de Janeiro (n = 4)
Wt Sal1 type	15 (31)	4	5	4	2	0
Single **FF**	5 (15)	1	4	0	0	0
Double **FL**	4 (12)	2	0	2	0	0
Single **M**YF	19 (55)	10	2	5	2	0
Double **ML**	6 (18)	0	0	1	1	4
		13 (76 %)	6 (54 %)	8 (67 %)	3 (60 %)	4 (100 %)

Pv, *Plasmodium vivax*

presented mutations at 958 position, although all isolates have been obtained from individuals with successful response to CQ therapy. No later than November 2015, Schousbe and colleagues [35] reported a high prevalence of 958**M** (97.6 %) among *P. vivax* samples from six different geographical sites, suggesting that this allelic variant is most likely not associated with CQR and could be an allele characteristic of Asia and Africa isolates. The present data reinforce the lack of association of 958**M** with CQR, but are not in agreement with Asian and African geographical characteristic of this allele, since this allele was present in 51 % of the Brazilian (South American) samples.

Previous studies seemed to indicate that both SNP and amplification of *pvmdr1* are associated with variation in in vitro CQ susceptibility of *P. vivax* [17, 32]. It has been shown that the geometric mean of the CQ inhibitory concentration 50 % (IC50) was significantly higher in isolates carrying the Y976**F** mutation when compared to wild-type isolates in samples from Indonesia and Thailand [17]. However, the clear association between the clinical outcome following a three-day CQ treatment and non-synonymous mutations in this gene has never been demonstrated elsewhere. In fact, the single 976**F** mutant was not very common worldwide (7.4 %) [22], and the **FF** double mutant genotype was detected only in endemic regions of three countries: Brazil [36, 37], Honduras [38] and Papua New Guinea [39]. In the samples herein analysed, no significant difference was observed between the presence of double 976**F**/1076**L** mutant (12 %) and single 976**F** mutant (15 %) and no single 1076**L** mutant was noted. These findings suggest that polymorphisms at codons 976 and 1076 may not be strong indicators of CQ resistance since all *P. vivax* isolates were obtained from patients with good response to CQ therapy. In addition, 976**F** and 1076**L** mutants were also detected in *P. vivax* isolates in several countries in Africa and in South America from patients with no history of CQ recrudescence [23]. Probably, these mutations might have been introduced in these countries from Asia where these mutations are prevalent [23]. Interestingly, the 976**F** mutation in *P. vivax* isolates from Extra-Amazonian were not detected in areas where autochthonous malaria cases from Brazilian Atlantic Forest can occur. Thus, it seems that 976**F** mutations are more associated to geographical characteristics than to CQR.

Concerning codon 958, only samples from the Amazon Basin showed the **MYF** single mutant genotype, and double **MYL** mutants were observed in Amazonas and Acre State isolates. On the other hand, all isolates from the Extra Amazon State of Rio de Janeiro had the double mutant 958+1076 (**MYL**) genotype and these samples were wild type for codon 976. Once again, the heterogeneity in these *P. vivax* populations could reflect the genetic diversity rather than an association with CQR in endemic areas with different endemic profiles [6].

Considering that *pvmdr1* mutations should not be expected in CQ-sensitive parasites and that PQ efficacy is highly dependent on concurrent administration of a blood schizontocidal agent [40] and thus PQ could not circumvent CQR, the present findings seem to indicate that the *pvmdr1* gene is not a reliable marker of CQR.

Conclusion

This study provides new data concerning the molecular characterization of *P. vivax* isolates from Brazilian Atlantic Forest. The SNP diversity observed in samples from New World is similar to those from Asia and Africa, and probably reflects a capacity for great functional variation, as already suggested [41]. Very little is known about the molecular mechanisms underlying drug resistance in *P. vivax* and most *loci* that have been suggested to be responsible for *P. vivax* CQR derived from orthologue *P. falciparum* drug CQR genes. Further investigations are needed to define a reliable molecular marker for monitoring CQR in the Brazilian *P. vivax* population.

Authors' contributions

MFFC carried out the study and the manuscript. LRG performed and analysis DNA sequencing and statistical analysis and drafted the manuscript. DM and CTDR participated in the discussions and reviewed the final manuscript. NKAO, SRFL and AL performed DNA extraction and PCRs. APC and PB recruited the patients. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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4.2. Manuscrito 2: Lack of quadruple and quintuple mutant alleles associated with SP resistance in *Plasmodium vivax* isolates from Brazilian endemic areas.

Submetido à Antimicrobial, Agents and Chemotherapy

Postulava-se que o *P. vivax* parecia ser naturalmente resistente à combinação terapêutica sulfadoxina / pirimetanina (SP), combinação esta que nunca foi utilizada para o tratamento dessa espécie plasmodial no Brasil. Na realidade, estudos recentes sinalizaram para a efetividade da SP no tratamento da malária *vivax* assim como foi estabelecida uma associação das mutações nos genes *pvdhfr* (diidrofolato redutase) e *pvdhps* (diidropteroato sintase) com a resposta clínica à SP. Dessa maneira, investigamos esses genes em nosso segundo trabalho em amostras provenientes da região Amazônica.

Lack of quadruple and quintuple mutant alleles associated with SP resistance in *Plasmodium vivax* isolates from Brazilian endemic areas.

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Abstract

Brazil is responsible for the larger number of *Plasmodium vivax* cases in the Americas. Given the emergence of *P. vivax* parasites resistant to Chloroquine and the effectiveness of antifolates in malaria vivax treatment together with a strong correlation between mutations in *P. vivax dhfr* and *dhps* genes and SP treatment failure, the pattern of point mutations in *pvdhfr* and *pvdhps* genes in Brazilian isolates was investigated here. **Subjects and Methods:** Blood samples were collected from 54 patients presenting vivax malaria from 2010 to 2016. Genomic DNA was extracted from the isolates using a DNA extraction kit (QIAGEN). Nested PCR amplification of

pvdhfr and *pvdhps* were carried out and ten point mutations were investigated: F57**L**/**I**, S58**R**, T61**M**, S117**T**/**N** and I173**F**/**L** for the *pvdhfr* gene, and S382**A**, C383**G**, K512**M**/**T**/**E**, A553**G** e V585**G**/**C** for the *pvdhps* one. **Results:** All the 54 isolates from Amazonian Region sequenced for *pvdhfr* gene showed non-synonymous mutations: 117**N** (54 out 54; 100 %) and 58**R** (25 out 54; 46%) mutant alleles were the more frequent. Double 58+117 haplotype (F**R**T**N**I / 28 %) was the more prevalent while triple mutant alleles were observed at lower rates - FST**NL** (117+173 / 4 %), F**RMN**I (58+61+173 / 5 %) and F**R**T**NL** (58+117+173 / 11 %) - and the quadruple F**RMNL** (58+61+117+173 / 2 %) mutant was detected in only one isolate. Concerning *pvdhps* gene, 26 out 54 (48%) presented only a mutation at codon C383**G** and no alleles were detected at codons 382**A**, 512**M**, 553**G** and 585**C**. The wild-type SCKAA (52%) and the single haplotype S**G**KAV (48%) were observed at similar frequencies. **Conclusion:** No molecular evidence of *P. vivax* SP resistance was observed in Brazilian samples. The prevalence of point mutations on these genetic markers of SP resistance should be assessed in different endemic Brazilian localities for providing information for future treatment policy with alternative antifolate drugs.

Key words : *P.vivax;* malaria; *pvdhfr; pvdhps*; chemoresistance

BACKGROUND

Plasmodium vivax is geographically widespread, and it is found through Asia, South and Central America, Middle East and some parts of Africa. Venezuela and Brazil are responsible for the greater number of cases in the Americas. Malaria transmission occurs almost entirely (> 99% of the registered cases) within the northern Brazilian Amazon region and both *P. falciparum* and *P. vivax* infections co-exist, but *P. vivax* is the predominant species, responding to 89.2% of the 194.409 malaria cases reported in 2017 (1).

Emerging resistance to Chloroquine (CQ) greatly contributed to explosive *P. falciparum* malaria outbreaks across the Amazon in the 1980s (2). The emergence of *P. vivax* strains resistant to CQ occurred later than those to *P.falciparum* and was first documented in Papua New Guinea in 1989 (3) where CQ monotherapy remains ineffective (4). From then on cases of CQ failure have been reported in Southeast Asia (5,6) and South America (7,8,9,10), complicating the current international efforts for malaria control and elimination and signaling to the need to alternative drug to vivax malaria treatment.

Antifolates, most notably drug combination sulfadoxine-pyrimethamine (SP), have been used as an anti-malarial *P. falciparum* treatment throughout the world because they are inexpensive, relatively safe and treatment requires only a single dose. It was claimed that antifolates are ineffective against *P. vivax*, however, this assumption seems to be incorrect (11). Although resistance to antifolates for *P. falciparum* treatment has been well documented in many parts of the world, *P. vivax* chemoresistance to SP is scarcely studied (12).

The molecular targets of SP action are, respectively, dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*), two major proteins of the folate biosynthesis pathway parasites (13,14). Polymorphisms surrounded by the genes that encode these active enzymes are the major factor in SP resistance.

Data on *pvdhfr* and *pvdhps* genotypes are available for many Southeast Asian countries. Such reports remain limited in some *P. vivax* endemic areas, notably South America. In Brazil, only one study characterizing *pvdhfr* gene was communicated (15) and there is no report on the frequency of SNPs-haplotypes in the *dhps* gene in *P. vivax* clinical isolates from Brazilian endemic areas.

Given the emergence of *P.vivax* CQ resistant parasites and the effectiveness of antifolates in malaria vivax treatment together to a strong correlation between mutations in *P. vivax dhfr* and *dhps* genes and SP treatment failure (12,16) the present paper reports an investigation on the pattern of point mutations in *pvdhfr* and *pvdhps* genes in Brazilian isolates.

METHODOLOGY

Parasites isolates and DNA extraction

Blood samples were collected from 54 patients presenting vivax malaria at the *Laboratório de Doenças Febris Agudas*, INI-IPEC, Fiocruz, the Reference Clinical Laboratory for Malaria in the Extra-Amazon to the Brazilian Ministry of Health, from 2010 to 2016. All of the clinical isolates were diagnosed as single *P. vivax* infections by light microscopic examination of Giemsa-stained blood smears and PCR (17). Genomic DNA was extracted from all the isolates using a commercially available DNA extraction kit (QIAGEN), following the manufacturer's instructions. This study was performed according to protocols previously approved by the Ethical Research Committees of Fiocruz (32839013.6.00005248). All the patients were treated with CQ plus primaquine, according to the Brazilian Ministry of Health recommendation for uncomplicated malaria vivax treatment (18), followed up for 42 days and no treatment failure was detected during this period.

Nested Polymerase chain reaction (PCR) and electrophoresis

Nested PCR amplification of *pvdhfr* and *pvdhps* were carried out using a method described previously (19). Ten point mutations were investigated: F57**L**/**I**, S58**R**, T61**M**, S117**T**/**N** and I173**F**/**L** for the *pvdhfr*, and S382**A**, C383**G**, K512**M**/**T**/**E**, A553**G** e V585**G**/**C** for the *pvdhps*. PCR products were analyzed by ethidium bromide-stained agarose-gel (2%) electrophoresis.

DNA sequencing and SNP polymorphisms detection

After purification using the Wizard SV Gel and PCR Clean-Up System (Promega), the amplified fragments were sequenced using Big Dye® Terminator Cycle Sequencing Ready Reaction version 3.1 (Applied Biosystems) and ABI PRISM DNA Analyzer 3730 (Applied Biosystems) (20) at the Genomic Platform/PDTIS/Fiocruz. The direct DNA sequencing from PCR products were compared with the reference wild type to: *pvdhfr* (GenBank X98123) and *pvdhps* (GenBank AY186730). Forward and reverse sequences were analyzed using the free software Bioedit Sequence Alignment Editor version 7.2.5.

RESULTS

All the 54 isolates from Amazon Region (Acre, Amapá, Amazonas, Rondonia and Pará) sequenced for *pvdhfr* gene showed non-synonymous mutations: 117**N** (54 out 54; 100 %) and 58**R** (25 out 54; 46%) mutant alleles were the more frequent, while 173**L** (9 out 54; 17%) and 61**M** (4 out 54; 7%) were detected at lower frequencies. Mutation at position 57**L** was not found in the analyzed samples **(Table 1)**.

Table 1 *Plasmodium vivax dhfr* and *dhps* mutated codons in 54 *P. vivax* isolates from Brazilian endemic areas

Gene	SNPs	Prevalence N (%)
dhfr	58**R**	25 (46)
	61**M**	4 (7)
	117**N**	54 (100)
	173**L**	9 (17)
dhps	383**G**	26 (48)

The most common single mutant allele was the 117**N** that was recorded in 27 isolates (50%;). Such single mutant was identified in similar prevalence in Acre (10 out 15; 66%), Amazonas (11 out 23; 52 %) and Pará states (4 out 8; 50 %), followed by Rondonia state (1 out 7; 14 %) **(Table 2)**.

Table 2 Number of alleles in *dhfr* and *dhps* genes observed among 54 Brazilian *P. vivax* isolates, according to sampling location.

Gene	SNPs	Amazonas (n=23)	Acre (n=15)	Amapá (n=1)	Pará (n=8)	Porto Velho (n=7)
dhfr	117**N**	12	10	-	4	1
	58**R**/117**N**	8	2	-	-	5
	117**N**/ 173**L**	-	-	-	1	1
	58**R**/117**N**/173**L**	1	3	1	1	-
	58**R**/61**M**/117**N**	1	-	-	2	-
	58**R**/61**M**/117**N**/173**L**	1	-	-	-	-
dhps	383**G**	13	8	-	5	-

Double 58+117 haplotype (F**R**T**N**I / 28 %) was the more common, contrasting with the frequencies of other *dhfr* double, triple or quadruple mutant alleles, which were observed at lower rates: FST**NL** (117+173/ 4 %), F**RMN**I (58+61+173/ 5 %) and

F**R**T**NL** (58+117+173/ 11 %) and F**RMNL** (58+61+117+173/ 2 %). Independently of the locality the *pvdhfr* wild-type FSTSI was not found amongst *P. vivax* isolates **(Table 3)**.

Table 3 Deduced *dhfr* and *dhps* haplotype profiles in 54 *P. vivax* isolates from Brazilian endemic areas

Gene	Haplotypes	N	%
dhfr	FST**N**I	27	50
	F**RTN**I	15	28
	FST**NL**	2	4
	F**RTNL**	6	11
	F**RMN**I	3	5
	F**RMNL**	1	2
dhps	S**G**KAV	26	48
	SCKAA **(wild type)**	28	52

58**R+**117**N** mutant alleles were detected in isolates from Acre (2 out 15; 13,3%), Rondonia (5 out 7; 71%) and Amazonas (8 out 23; 35%) states. All localities, except Rondonia, presented 58**R+**117**N+**173**L** triple mutant profile, and quadruple mutant was only noted in one *P. vivax* isolate from the Amazonas state (1 out of 23; 4%) **(Table 4)**. Pará and Rondonia states showed the unusual 117**N**+173**L** *pvdhfr* haplotype **(Table 4)**.

Table 4 Prevalence of *P. vivax dhfr* and *dhps* mutants in 54 *P. vivax* isolates from Brazilian endemic areas

Gene	Mutants	SNPs	Prevalence N (%)
dhfr	**Single**	S117**N**	27 (50)
	Double	S58**R**/S117**N**	15 (28)
		S117**N**/ I173**L**	2 (4)
	Triple	S58**R**/S117**N**/I173**L**	6 (11)
		S58**R**/T61**M**/S117**N**	3 (5)
	Quadruple	S58**R**/T61**M**/S117**N**/ I173**L**	1(2)
dhps	**Single**	C383**G**	26 (48)

Concerning *pvdhps* gene, 26 out 54 (48%) presented only a mutation at codon C383**G** and no alleles were verified at codons 382**A**, 512**M**, 553**G** and 585**C**. The wild-type SCKAA (52%) and the single haplotype S**G**KAV (48%) were observed at similar frequencies. The single mutant 383**G** was observed in isolates from Amazonas (13 out 23 / 56%), Acre (8 out of 15 /53%) and Pará (5 out of 8 – 62%) but not in Rondonia state **(Table 2)**.

Combining *pvdhfr* and *pvdhps* alleles, only one haplotype double for p*vdhfr* and single for *pvdhps* (F**R**T**N**I + S**G**KAV) was seen. The *pvdhps* and *pvdhfr* wild type combination was not observed because all isolates were mutated in at least one of the five *pvdhps* codons investigated. F**R**T**N**I + S**G**KAV combined haplotype were detected in three of the four study sites, but it was most frequent in Amazonas state where the only *pvdhfr* quadruple mutant was noted **(Table 5)**.

Table 5 Combined molecular *dhfr* and *dhps* gene profiles in *P. vivax* samples from Brazilian endemic areas

Gene	Haplotypes	Mutated	
dhfr* + *dhps		**NP/N (%)**	**Type**
58**R**/117**N** + 383**G**	F**R**TN**N**I + S**G**KAV	26/54 (48)	Double and single

NP: number of positive

No *pvdhfr* or *pvdhps* quadruple or quintuple mutant allele, which might result in poor clinical response against antifolate drugs, was detected in any of the Brazilian localities investigated.

DISCUSSION

Mutations in the *pvdhfr* and *pvdhps* genes have been found to be associated with antifolate drug resistance in both *in vivo* (21,22) and *in vitro* assays (23,12,24), suggesting that molecular markers may provide information about the trends of SP resistance in *P. vivax*. Here, a lookout of SP resistance was explored to determine specific point mutations in five codons of p*vdhfr* and five codons of *pvdhps.*

It has been postulated that *pvdhfr* 117**N** mutation might occur first, followed by the S58**R** mutation (25). In this study *pvdhfr* S117**N** was detected in all isolates followed by 58**R** (74%), 173**L** (17%) and 61**M** (7%) polymorphisms, supporting that S117**N** mutation is the first step in the drug selection process. These data are similar to other observations done in areas where *P. falciparum* and *P. vivax* parasites co-exist (25,26,27,28).

The predominance of S117**N** followed by the double mutant 58**R** + 117**N** (28%) was also analogous to those reported in Iran (29), India (26), Papua New Guinea (21), Pakistan (30), Afghanistan (31), China (32), Nepal (33), Thailand (34), Indonesia (12), Colombia (35,36), French Guyana (34) and Brazil (15). The triple 58**R** + 117**N** + 173**L** *pvdhfr* mutant, not seen in *P. vivax* samples from Southeast Asian, - where non-synonymous mutation in codon 173 comprises the change of I by F generating the 173**F** allele - was detected in Amazonas, Acre, Amapá and Pará states in this study and also in *P. vivax* parasites from French Guiana (34,37) and Amazonas, Brazil (15). Conversely, the non-synonymous mutation at position F57**L** – not recorded in this study - was exclusively reported in Southeast Asian samples, findings that could reflect different selection in old and new worlds alleles. In fact, the genetic similarity of this specific SNP recorded in *P. vivax* parasites from two neighboring South-American countries could reflect the existence of a geographic subdivision of different *P. vivax* parasites in samples from the old and new worlds (38,34).

Concerning the *pvdhps* gene, previous data indicated that mutations were mainly detected at codons A383**G** and A553**G** (25,35,39,40,41) and suggested that these mutations alone could be responsible for reduced sensitivity to sulfa and sulfones (42,43). In the present work, the wild type (52%) and the mutated codon 383**G** (48%) were detected at similar frequencies among *P. vivax* isolates, as well as reported in Tai-Myanmar border (47%; 44) and Indonesia (50%; 16). However, in South American (Colombian) in a study investigating polymorphisms in the *pvdhps*, the wild type was the more frequently detected (71.6%; 35); the same was true in Pakistan (98%; 31), India (79%; 26), Papua (67%; 45) and in Thai/Cambodia border (74%; 44). Therefore, the *pvdhps* wild-type allele seems to be still common in malaria endemic areas along the world, probably due to a lower SP drug selection on the sympatric *P. vivax* populations of these countries.

In conclusion, no molecular evidence of *P. vivax* SP resistance was observed in Brazilian samples. Since mutations in *P. vivax dhps* and *dhfr* genes provide a valuable tool for epidemiological surveillance of SP resistance, the prevalence of point mutations on these genetic markers of SP resistance should be assessed for providing information for future treatment policy with alternative antifolate drugs due to the appearance and dispersion of resistance in malaria endemic areas.

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4.3. Manuscrito 3: Absence of *k13* polymorphism in *Plasmodium falciparum* parasites from Brazilian endemic areas.

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Conhecedores de que resistência do *P. falciparum* à artemisinina representa um grande problema para o controle e eliminação da malária no mundo porque ainda não existe nenhum tratamento eficaz para substituir esta droga, aliado à detecção de parasitos resistentes no sudoeste Asiático, Suriname e Guiana Francesa, nos voltamos para o rastreamento de mutações no gene *k13* conhecidamente associadas a resistência à ART, em isolados da Amazônia Legal.

Absence of *K13* Polymorphism in *Plasmodium falciparum* Parasites from Brazilian Endemic Areas.

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Running Head: No K13 mutations in Brazilian *P.falciparum* isolates.

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Abstract

The World Health Organization (WHO) recommends efficacy monitoring for ACTs every 2 to 3 years in all endemic countries. *P. falciparum* ART-resistant parasites can be evaluated examining polymorphisms in the Kelch (PfK13) propeller domain. A total of 69 blood samples from patients diagnosed for *falciparum* malaria by microscopic and/or molecular tests were analyzed. All samples were from Brazilian endemic areas of the

following states: Acre (n=14), Amapá (n=15), Amazonas (n=30) and Pará (n=10). *P. falciparum* DNA was successfully sequenced in all 69 isolates and after alignment with the 3D7 reference sequence, all samples were found to be wild-type. The present data contributes to the ongoing surveillance of ART resistance parasites by providing baseline data on K13-propeller mutations and reinforce the pertinence of the use of ACTs in Brazilian endemic areas.

Keywords: artemisinin; Kelch domain; malaria; *P.falciparum*; Brazil

Introduction

Globally, 216 million clinical cases of malaria were estimated in 2016 with 445,000 deaths (range 235,000-639,000), mainly in Sub Saharan Africa (1). *Plasmodium falciparum* is the most pathogenic and lethal species causing human malaria worldwide (2). *P. falciparum* resistance to different anti-malarial drugs, is a serious obstacle to malaria elimination. For this reason, the WHO´s (World Health Organization) recommended the use of artemisinin (ART) combined therapies (ACTs) as the first line therapeutic schemes for falciparum malaria treatment (3) and here after the burden of malaria declined substantially (4).

However, within a few years, the first clinical cases of artemisinin resistance were reported in western Cambodia (5), followed by the detection of *P. falciparum* parasites with reduced *in vivo* susceptibility to artesunate (6, 7). Since then, resistance to artemisinin (ART) derivatives has locally emerged or spread throughout Southeast Asian countries (Myanmar, Thailand, Cambodia, Vietnam and Laos) (8, 9, 10, 11, 12, 13). A delayed clearance of *P. falciparum* parasites was also noted recently in Equatorial Guinea (14).

In South America, C580Y mutant parasites of independent origin of those observed in South East Asia was recently reported in Guyana (15). In fact, ACTs systematic self-medication – including incomplete courses of ART monotherapy – by gold miners in French Guiana together with illegal miner movement through the Brazil / French-Guiana borders, represent a serious risk for the emergence of ART-resistance in Guiana Shield (16) and its consequent spread to the Brazilian endemic areas.

Brazil has achieved the goal of significantly reducing the number of falciparum malaria cases (17) and now intends the *P. falciparum* pre-elimination stage. In such a low transmission area prone to emergence of resistance, it is, therefore, urgent to closely monitor the ACT therapeutic efficacy and identify early warning signs of ART-resistant *P. falciparum* parasites. Genome association studies strongly linked a *locus* on *P. falciparum* chromosome 13 to artemisinin resistance in the kelch propeller domain (K13-propeller) (18). To date, almost 200 K13 mutations have been described worldwide but only 6 non synonymous single nucleotide polymorphisms (SNPs) have been associated with artemisinin resistance (19).

The purpose of this work was to assess polymorphisms in Pf*k13* gene in Brazilian isolates from patients who attended the National Reference Centre for Diagnostics and Training in the extra-Amazon Region (*Centro de Pesquisa, Diagnóstico e Treinamento em Malária – CPD-Mal*) at Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil.

Results

A total of 69 patients were diagnosed for *falciparum* malaria by microscopic and/or molecular tests. Although the study was not designed as a clinical drug efficacy study, all treated patients were followed up both clinically and through parasitological and molecular examinations (*P. falciparum* samples were diagnosed by Giemsa-stained thick and thin smears and by polymerase chain reaction (PCR) (23), as mentioned

earlier. No recrudescence was recorded after the treatment period. All samples were from Brazilian endemic areas of the following states: Acre (n=14), Amapá (n=15), Amazonas (n=30) and Pará (n=10).

P. falciparum DNA was successfully sequenced in all 69 isolates. After alignment with the 3D7 reference sequence, all samples were found to be wild-type.

Discussion

The *PfK13* gene has been identified as a molecular marker of ART resistance and some mutations were found to be associated with delayed parasite clearance, opening new possibilities for tracking ART resistance (18, 20). The existence of mobile populations engaged in gold mining, logging or illegal activities with risk of malaria transmission in the Brazil / French-Guiana borders, raise the potentiality of spreading of ART resistance alleles with serious implications for *P. falciparum* surveillance, malaria control and elimination efforts in Brazil. Indeed, a study conducted in illegal gold miners in French Guiana, showed that the majority of recruited participants were from Brazil (93.8%) and that PCR-based methods revealed *P. falciparum* in 47.9% of the cases (21).

In this work, all *P. falciparum* isolates collected before ACT treatment (Day 0) exhibited the 3D7 wild-type allele in the propeller region of *Pf K13* gene, including those of Brazil-Guiana Shield border (Amapá state). In an analogous study, only with samples from Acre state, no polymorphism was also found in patients with parasitological and clinical cure (22).

Clinical ART resistance is defined as a reduced parasite clearance rate, expressed as an increased parasite clearance half-life or a persistence of microscopically detectable parasites on the third day of ACT (6, 9). Here, all patients were followed up to assess the parasite clearance in the first three days and, wherever possible, for 42 days. No parasite was observed on day 3 and no treatment failure was detected. The lack of

mutations in the K13 gene of *P. falciparum* parasites from Brazilian endemic areas is in agreement with the adequate clinical and parasitological responses, supported by PCR results, showing the efficacy of the ACT. Although *in vitro* ring-stage survival assay (RSA) was not performed, it is well known that the parasite clearance half-life parameter correlates strongly with the RSA results. Consequently, the present data contributes to the ongoing surveillance of ART resistance parasites by providing baseline data on K13-propeller mutations and reinforce the pertinence of the use of ACTs in Brazilian endemic areas.

Notwithstanding, similar studies with a larger number of samples will be helpful to ascertain the emergence of ART resistance, if any, and routine monitoring must continue to ensure that the ACTs are effective in the treatment of *falciparum* malaria in Brazilian endemic areas.

Materials and Methods

Collection of blood samples and DNA isolation

Blood samples were collected between 2010 and 2017 from febrile patients that attended the clinic for diagnosis and treatment for malaria at the *Ambulatório de Doenças Febris Agudas*, INI-IPEC, Fiocruz. The study was performed at the Laboratório de Pesquisa em Malária, Headquarter office of the *Centro de Pesquisa, Diagnóstico e Treinamento em Malária* (CPD-Mal) of Fiocruz, Rio de Janeiro - Brazil. The inclusion criterion comprised patients with microscopic and/or molecular diagnosis of *falciparum* malaria. After obtaining informed consent, venous blood collection was performed according to protocols previously approved by the Ethical Research Committees of Fiocruz (32839013.6.00005248), before starting malaria therapy. *P. falciparum* samples were diagnosed by Giemsa-stained thick and thin smears and by polymerase chain reaction (PCR) (23). Genomic DNA was isolated from whole blood using QIAamp midi

columns, as described by the manufacturer (Qiagen). All patients were treated with fixed combination Artesunate+Mefloquine for 3 days according to Brazilian National Malaria Program guideline (Guia prático de tratamento de malaria no Brasil, 2010) and followed at least until parasite clearance or, whenever is possible, up for 42 days.

Detection of SNPs in K13 gene by nested - PCR and Sanger sequencing

PfK13 gene fragment on chromosome 13 was amplified by nested-PCR using previously published primers (Ariey et al, 2014). Quality controls were performed in each PCR run with Cambodian isolates with known K13 mutations (provided by DM). DNA sequencing was carried out after purification using the Wizard SV Gel and PCR Clean-Up System (Promega). Briefly, the amplified fragments were sequenced using Big Dye® Terminator Cycle Sequencing Ready Reaction version 3.1 (Applied Biosystems) and ABI PRISM DNA Analyzer 3730 (Applied Biosystems) (24) at the Genomic Platform/PDTIS/Fiocruz. Polymorphisms in the *PfK13* gene were analyzed by direct DNA sequencing of amplicons and the obtained sequences (from codon 443 to 666, i.e. 720 bp) were aligned with the *PfK13-propeller* region (GenBank accession nº XM_001350122.1) of 3D7 reference sequence (PF3D7_1343700), using the free software Bioedit Sequence Alignment Editor version 7.2.5.

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5. Discussão

Atualmente, a vigilância da resistência às drogas antimaláricas reside em três diferentes e complementares abordagens: estudos *in vivo* para a detecção de falhas terapêuticas, avaliação *ex-vivo*/*in vitro* da sensibilidade do parasito às drogas e a detecção de assinaturas moleculares do parasito associadas com à QR. Essas abordagens apresentam vantagens e desafios.

Os ensaios *in vivo* fornecem informação sobre a eficácia da droga estudada em pacientes, tem um protocolo estabelecido pela OMS (WHO, 2009), e permanece o padrão ouro para avaliar a eficácia terapêutica das drogas antimaláricas. Entretanto, são difíceis de conduzir devido a logística árdua e aos altos custos e requer microscopia de alta qualidade. Em áreas de baixa transmissão, como as brasileiras, esta abordagem é particularmente desafiadora, pela necessidade da triagem de centenas ou milhares de pacientes. Ademais, o desfecho do tratamento pode ser confundido com muitos fatores, como a imunidade adquirida, a adesão ao tratamento, o estado de nutrição, gravidez e farmacogenética, fatores esses que poderão levar a superestimação da QR. Outra limitação se refere a interpretação dos resultados de genotipagem para diferenciar recrudescência de reinfecção o que pode levar ao erro de classificação do desfecho do tratamento devido a não detecção dos clones minoritários (Messerli et al, 2017).

As técnicas ex-vivo/*in vitro* provem informação sobre a susceptibilidade do parasito e requer infraestrutura substancial para o cultivo do parasito e pessoal muito bem treinado. Cinco métodos laboratoriais podem ser utilizados para avaliar a susceptibilidade do *P. falciparum*: microtest OMS (WHO, 2001), ensaio isotópico (Chulay et al, 1983), ELISA (HRP2) (Makler et al, 1993), Citometria de Fluxo (Woodrow et al, 2015), *Sybr Green* (Johnson et al, 2007) e *Ring Stage Survival* (RSA) (Witkowski et al, 2013). Cada um deles é de difícil padronização e sempre existe, independente do método utilizado, variabilidade no crescimento do parasito intra e

entre ensaios. No caso do *P.vivax,* que não pode ser continuamente cultivado *in vitro*, os estudos de QR foram consideravelmente retardados, mas a aplicação de abordagens moleculares e a conclusão do genoma deste parasito vêm permitindo um rápido progresso.

Os marcadores moleculares validados são extremamente relevantes para detectar e monitorar em tempo real a distribuição geoespacial de parasitos resistentes e, a prevalência dessa população parasitária é um bom indicador do nível de resistência clínica. Embora a análise de marcadores moleculares também requeira pessoal bem treinado e infraestrutura compatível, os resultados não dependem do observador o que minimiza a variabilidade de resultados. Os custos dos ensaios moleculares de PCR vêm decrescendo e o uso de consórcios para o sequenciamento visando reunir dados de diferentes localidades numa plataforma comum como os Laboratórios de Referência podem ser estabelecidos para compartilhar os custos.

Em vista do exposto, é inegável que os métodos moleculares têm permitindo a melhor compreensão da emergência e disseminação da resistência das drogas antimaláricas. Nas últimas duas décadas, os mecanismos de resistência para as drogas mais largamente utilizadas tem sido revelados em parte usando técnicas moleculares, e foi verificado que a resistência as drogas está frequentemente associada com a existência de SNPs ou com a amplificação de genes codificando proteínas ou transportadores alvos das drogas.

A seleção e a disseminação de parasitos da malária resistentes a maioria das drogas utilizadas no combate à doença, como já reconhecido, é uma ameaça global para o controle e eliminação da endemia e é de particular interesse no caso do *P.*

falciparum, a mais mortal das espécies que infecta o homem. Mas a QR as drogas antimaláricas também têm sido relatada no *P.vivax* e vem se tornando uma crescente preocupação nas regiões endêmicas e um sério problema de saúde pública, mesmo que sua extensão seja de menor magnitude quando comparado àquela do *P. falciparum*.

De fato, o *P. vivax* continua sendo o segundo agente mais comum de malária no mundo e o mais amplamente presente em diferentes regiões do globo (Thongdee et al, 2013; WHO, 2016).

Na maioria das áreas endêmicas, incluindo o Brasil, CQ e PQ correspondem a primeira linha para o tratamento dos casos de *P. vivax*, porém este esquema vem sendo cada vez mais ameaçado devido ao surgimento e disseminação de parasitos de malária *vivax* resistentes à CQ. De fato, o primeiro caso de resistência à CQ em isolados de *P. vivax* foi observado em 1989 no lado oriental do arquipélago de Papua (anteriormente Irian Jaya) (Baird et al, 1991). Posteriormente, diferentes estudos confirmaram, numa prevalência significativa, parasitos resistentes a CQ também no norte de Papua (Baird et al, 1997; Murphy et al, 1993; Tjitra et al, 2002) e na Indonésia (Baird et al, 1996; Fryauff et al,1998). Mais recentemente, em áreas da Tailândia até então sem evidências de QR a CQ, foi constatado um número elevado de parasitos resistentes na fronteira Tai-Mianmar (Rungsihirunrat et al, 2015).

Estudos de monitoramento para avaliar a eficácia do atual tratamento, através do uso de marcadores moleculares são vantajosos para essa espécie em particular em decorrência de problemas inerentes a detecção da quimiorresistência *in vivo* e da ausência de métodos exequíveis para o cultivo contínuo de isolados *in vitro*. No Brasil, estudos de genotipagem molecular em isolados de *P. vivax* são escassos e devido a isso nos direcionamos para avaliar a ocorrência e a prevalência de

mutações em genes potencialmente associados à CQ como o *pvmdr1* (Lu et al, 2011).

Nessa conjuntura, no nosso primeiro artigo caracterizamos as principais mutações do gene *pvmdr1* em isolados das regiões Amazônica (Acre, Amazonas, Pará e Rondônia) e Extra-Amazônica (Rio de Janeiro) e nossos achados sugerem que pelo menos a mutação única no códon 976, previamente proposta como um marcador de resistência à CQ na Ásia (Suwanarusk et al, 2008; Fernández-Becerra at al, 2009), não seria válida para o monitoramento da QR à CQ no Brasil, porque todos os pacientes cujos isolados apresentaram tal mutação tiveram boa resposta ao tratamento. Tais achados sugerem que o polimorfismo no códon 976 não parece ter relação com a resistência à CQ. Aliado aos nossos resultados, na África e em outros países da América do Sul, os polimorfismos identificados no códon 976 também foram encontrados em isolados de pacientes com *P.vivax* sem relatos de recrudescência a CQ (Vargas-Rodriguez et al, 2012). Por isso, mutações no códon 976 poderiam estar relacionadas mais as características geográficas do que a resistência à CQ. Com efeito, em que pese o n amostral, a mutação 976F não foi constada nas amostras de pacientes com malária vivax de Mata Atlântica por nós estudados, onde as características de transmissão (aí incluindo as climáticas e vetoriais) são bem diferentes das de regiões da Amazônia.

Observamos ainda que o códon 958 foi o mais prevalente entre as amostras estudadas e esses resultados foram similares aos já publicados em trabalhos realizados na Amazônia brasileira (Sá et al, 2005; Chehuan et al, 2013). Além do Brasil, em Madagascar (Barnadas et al, 2008), Nepal (Ranjitkar et al, 2011) e Tailândia (Rungsihirunrat et al, 2015) a mutação na posição 958 foi a mais frequente, embora todos os indivíduos (assim como os do Brasil) tenham mostrado uma resposta terapêutica bem sucedida à CQ. Para corroborar com esses resultados,

uma análise realizada com amostras de diferentes regiões geográficas identificou também a mutação no códon 958 como a mais prevalente (Schousbe et al, 2015), o que também sugere a falta de associação dessa variante alélica com a resistência a CQ e que, possivelmente, este alelo está distribuído em isolados da Ásia da África e da América do Sul.

As infecções por *P. vivax* nunca foram tratadas com a combinação SP no Brasil, apesar da sensibilidade dos parasitos dessa espécie plasmodial a essa combinação terapêutica. Conhecedores da existência de marcadores moleculares para avaliar a QR frente a essa combinação nos lançamos, num trabalho inédito, na investigação do perfil das mutações desses genes em áreas endêmicas de nosso país porque os padrões de mutações nos genes *pvdhfr* e *pvdhps* parecem estar relacionados à intensidade com que parasitos de *P. vivax* foram expostos à pressão seletiva exercida pela SP (Hawkins et al, 2007), o que presumidamente ocorreu em nosso país, no caso de infecções mistas por *P. vivax* e *P. falciparum* subdiagnosticadas, ou ainda pelo amplo uso de antifolatos para o tratamento de infecções bacterianas e fúngicas.

Assim, como exposto no manuscrito 2, não encontramos pacientes infectados com parasitos que carreavam simultaneamente polimorfismos nos dois genes, os denominados quádruplo ou quíntuplo mutantes *pvdhfr*/*pvdhps*, cuja presença está fortemente associada à falha ao tratamento (Imwong et al, 2001). Não obstante, identificamos haplótipos de *pvdhfr* portando 2 ou 3 SNPs (58**R** + 117**N** ou 58**R** + 117**N** + 173**L**), corroborando com os achados do Brasil em estudos prévios de nosso grupo (Gama et al, 2009) ou de áreas endêmicas de outros países como Iran (Sharifi-Sarasiabi et al, 2016), Índia (Ganguly et al, 2014), Papua Nova Guiné (Marfurt et al. 2008), Pakistão (Khatoon et al, 2013), Afeganistão (Zakeri et al, 2010), China (Huang

et al, 2014), Nepal (Ranjitkar et al, 2011), Tailândia (Brega et al, 2004), Indonésia (Hastings et al, 2004), Colômbia (Saralamba et al, 2016; Hawkins et al, 2007) e Guiana Francesa (Brega et al, 2004).

Com relação ao gene *pvdhps* observamos que a frequência entre o alelo do tipo selvagem e o alelo apresentando SNP no códon 383**G** foram similares nas amostras de *P. vivax*, tal qual na fronteira Tailândia-Mianmar (Thongdee et al, 2013) e na Indonésia (Asih et al, 2015). Recentemente, num estudo de caracterização do gene *pvdhps,* incluindo isolados da Colômbia, a maioria das amostras apresentou o alelo do tipo selvagem (Saralamba et al, 2016). É notável que em amostras de *P. vivax* o tipo selvagem ainda esteja amplamente distribuído no globo, provavelmente devido a baixa pressão seletiva da droga SP nas populações simpátricas de *P. vivax* desses países.

Desse modo, considerando a emergência de quimiorresistência à CQ, é fundamental conhecer a diversidade e a prevalência de haplótipos associados, caso se cogite utilizar a SP para o tratamento da malária vivax, como droga alternativa.

Baseando-se agora no *P. falciparum,* o primeiro relato de QR na América do Sul aos antimaláricos surgiu na década de 1960. Estudos detectaram inicialmente a resistência a CQ em isolados de *P. falciparum* na Colômbia (Moore e Lanier, 1961) e 20 anos após já ocorria QRCQ em todos os países sul-americanos endêmicos de malária (Wongsrichanalai et al, 2002). Esta droga foi então substituída pela SP como primeira linha de tratamento, mas a QR a SP emergiu em diferentes locais do sudoeste Asiático, África e América do Sul (Lumb et al, 2011). Depois da falha da SP, a MQ a substituiu mas, como previsível, após 5 anos de uso difundido foram identificados parasitos resistentes (Smithuis et al, 1993). Após a falha da MQ foi

proposto o uso combinado de drogas porque a probabilidade do parasito desenvolver simultaneamente resistência a duas drogas é considerado menor do que para uma única droga (White, 1998).

Os derivados de ART foram descobertos nos anos 70 (White, 1998), e correspondem as drogas antimaláricas mais potentes disponíveis até o momento, capazes de promover um rápido e acentuado declínio na densidade parasitária, apesar da sua curta vida-média. Quando combinada com drogas de meia vida mais longa, a droga parceira pode clarear os poucos parasitos restantes, tornando essa combinação de tratamento altamente eficaz e menos propensa a resistência.

Atualmente, os ACTs representam o principal esteio para o tratamento da malária mundialmente. Inicialmente, se mostraram eficazes em todos os países endêmicos durante os últimos 15 anos. Entretanto, parasitos com susceptibilidade reduzida aos derivados de ART, assim como à droga parceira, foram detectados no sudoeste Asiático (Ashley et al, 2014), e em regiões da China e Guiana (Menard et al, 2016; Chenet et al, 2016).

É fácil de compreender que a resistência do *P. falciparum* à ART represente um grande problema para o controle e eliminação da malária no mundo, tendo em vista que além de ter sido documentado a QR deste parasito a praticamente todos os antimaláricos já utilizados, ainda não existe outro tratamento eficaz para substituir os ACTs (Dondrop et al, 2009; Amaratunga et al, 2012; Flegg et al, 2013).

A QR a ART tem sido associada a várias mutações no domínio em forma de hélice (propeller) do gene kelch, localizado no cromossomo 13 do *P. falciparum* (Straimer et al, 2015), mas o mecanismo exato de resistência ainda não foi

totalmente elucidado até a atualidade. Embora as mutações no gene *k13* tenham surgido de forma independente, já foi observado que genótipos bem sucedidos evolutivamente de parasitos multirresistentes a outras drogas se disseminaram para a Índia e a África (Mita et al, 2009).

Frente a este problema é necessário o rastreamento da QR dos ACTS de maneira a se tentar impedir a emergência global da doença. A identificação do gene *pfk13* como o marcador molecular da resistência à ART abriu novas possibilidades para o rastreamento da resistência deste fármaco. Desde então estudos de monitoramento de alelos mutantes na propeller *k13* têm sido realizados com o intuito de evitar ou conter a introdução de parasitos ART-resistentes em regiões onde as ACTs são eficazes.

No Brasil, em 2006, o Programa Nacional de Controle da Malária (PNCM) investiu no uso dos ACTs como primeira linha de tratamento para *P. falciparum* em todas as regiões da Amazônia Legal e da Extra-Amazônia. Até o presente momento não foi notificado nenhum caso de resistência aos ACTs no Brasil, porém existe a preocupação de que parasitos de *P. falciparum* que circulam em áreas fronteiriças do Brasil com a Guiana Francesa venham a ser introduzidos em nosso país. Por certo, as populações migrantes que estão envolvidas em atividades de mineração, exploração e ouro e outras atividades ilegais em áreas de transmissão da malária, aumentam o potencial de propagação de parasitos ART-resistentes, podendo assim, causar sérias implicações para a vigilância, controle e eliminação da malária por *P. falciparum*, como no caso do Brasil.

Com efeito, um estudo realizado em garimpos ilegais na Guiana Francesa mostrou que 93,8% dos trabalhadores recrutados eram brasileiros e que entre eles 47,9% estavam infectados por *P. falciparum* (Douine et al, 2016). Esse cenário só reforça a importância do monitoramento contínuo da resistência à ART para

detecção precoce de parasitos ART-resistentes, possibilitando, assim, mudanças oportunas nas políticas de tratamento.

Nesse panorama, no manuscrito 3 realizamos a investigação de alelos mutantes do gene *pfK13* em 69 isolados plasmodiais provenientes de áreas endêmicas brasileiras coletados antes do início do tratamento com o ACT (dia 0). Em nossas análises, todas as amostras investigadas exibiram alelos do tipo selvagem, incluindo àquelas da fronteira Brasil-Guiana (Amapá), ou seja, não encontramos nenhum dos 20 códons mutantes associados à resistência à ART e durante o *follow up* de 42 dias dos pacientes pós tratamento não foram constados nenhum caso de recrudescência.

Esses dados corroboram com os resultados de outros países endêmicos onde os ACTs também são utilizados como primeira linha de tratamento para malária *falciparum*. Como por exemplo, em países da África Subsaariana (Quênia, Uganda, Benin e Províncias Angolanas) (Muwanguzi et al, 2016; Cooper et al, 2015; Ogouyèmi-Hounto et al, 2016; Plucinsky et al, 2015) e na Índia (Mishra et al, 2015) também não foram evidenciados haplótipos associados a QR à ART. No Haiti, onde os ACTs não são prescritos como primeira linha de terapia, também não foram encontrados SNPs no gene *pfk13*, acontecimento que não foi surpreendente posto que, diferente dos países que adotam ACTs com primeira linha, os isolados haitianos de *P. falciparum* não estão submetidos a uma pressão seletiva por esta droga (Carter et al, 2015).

Considerando que a eficácia da ART é particularmente importante na África aonde a infecção pelo *P. falciparum* é predominante em praticamente todo o continente, é digno de nota o relato de um haplótipo mutante tipo M579**I** no domínio *propeller k13* em paciente chinês infectado pelo *P. falciparum* na Guiné Equatorial (Lu et al, 2017). Os parasitos que este paciente portava mostraram um aumento na

taxa de sobrevida *in vitro* associado ao clareamento parasitário *in vivo* somente após o dia 3. Embora, de acordo com os critérios da OMS, seja prematuro concluir que M579**I** seja um marcador de resistência à ART (Rasmussen et al, 2017), esse achado ressalta a necessidade de monitoramento contínuo da resistência à ART e a propriedade de se melhor explorar o papel do mutante M579**I** como marcador desta QR.

6. Conclusões

Os estudos aqui exibidos proveram dados inéditos de caracterização molecular de mutações do gene *pvmdr1* em isolados *P. vivax* provenientes de zonas de Mata Atlântica Fluminense que mostraram que a diversidade dos SNPs observada no Mundo Novo foi semelhante àquela encontrada no Velho Mundo, parecendo refletir a grande capacidade de variação deste parasito.

As mutações 976**F** e 1076**L** do gene *pvmdr1* não parecem ser bons marcadores moleculares de QR à CQ do *P. vivax*, sendo assim mais estudos se fazem necessários para se definir e validar um marcador molecular para monitorar a QR do *P. vivax* à CQ em populações brasileiras. Por outro lado, foi digno de nota a ausência da mutação 976*F* nas amostras de pacientes com malária *vivax* de Mata Atlântica, onde as características de transmissão (aí incluindo as climáticas e vetoriais) são bem diferentes das de regiões da Amazônia.

Nenhuma evidência molecular de QR à SP foi identificada em amostras brasileiras de *P.vivax* recentemente coletadas, sugerindo que a SP possa ser uma droga alternativa devido a emergência e dispersão de QR à CQ em áreas endêmicas de malária.

Da mesma forma não foi constada nenhuma evidência de QR aos ACTs em pacientes que acorreram ao diagnóstico e tratamento no Centro Pesquisa de Diagnóstico e Treinamento em Malária (CPDMal), provenientes de diferentes estados da Amazônia Legal. Esses dados forneceram uma linha de base das mutações do *k13* e reforçam a pertinência do uso de ACTs em áreas endêmicas brasileiras.

Em suma, existe uma necessidade urgente de fortalecer os sistemas de vigilância voltados para a resistência às drogas antimaláricas com estratégias que possam prover sinais precoces de alerta antes que a resistência aos ACTs tenha se disseminado além da região do Grande Mekong.

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8. ARTIGOS ADICIONAIS

8.1. Artigo 1: A world map of *Plasmodium falciparum* K13-propeller polymorphisms.

The New England of Medicine, 2017; 374:2453-64

No decorrer do desenvolvimento desta tese fomos convidados para participar num estudo da rede internacional dos Institutos Pasteur sobre a investigação dos polimorfismos no gene *k13* de *P. falciparum*, cujos resultados serão sumarizados a seguir.

ORIGINAL ARTICLE

A Worldwide Map of *Plasmodium falciparum* K13-Propeller Polymorphisms

D. Ménard, N. Khim, J. Beghain, A.A. Adegnika, M. Shafiul-Alam, O. Amodu, G. Rahim-Awab, C. Barnadas, A. Berry, Y. Boum, M.D. Bustos, J. Cao, J.-H. Chen, L. Collet, L. Cui, G.-D. Thakur, A. Dieye, D. Djallé, M.A. Dorkenoo, C.E. Eboumbou-Moukoko, F.-E.-C.J. Espino, T. Fandeur, M.-F. Ferreira-da-Cruz, A.A. Fola, H.-P. Fuehrer, A.M. Hassan, S. Herrera, B. Hongvanthong, S. Houzé, M.L. Ibrahim, M. Jahirul-Karim, L. Jiang, S. Kano, W. Ali-Khan, M. Khanthavong, P.G. Kremsner, M. Lacerda, R. Leang, M. Leelawong, M. Li, K. Lin, J.-B. Mazarati, S. Ménard, I. Morlais, H. Muhindo-Mavoko, L. Musset, K. Na-Bangchang, M. Nambozi, K. Niaré, H. Noedl, J.-B. Ouédraogo, D.R. Pillai, B. Pradines, B. Quang-Phuc, M. Ramharter, M. Randrianarivelojosia, J. Sattabongkot, A. Sheikh-Omar, K.D. Silué, S.B. Sirima, C. Sutherland, D. Syafruddin, R. Tahar, L.-H. Tang, O.A. Touré, P. Tshibangu-wa-Tshibangu, I. Vigan-Womas, M. Warsame, L. Wini, S. Zakeri, S. Kim, R. Eam, L. Berne, C. Khean, S. Chy, M. Ken, K. Loch, L. Canier, V. Duru, E. Legrand, J.-C. Barale, B. Stokes, J. Straimer, B. Witkowski, D.A. Fidock, C. Rogier, P. Ringwald, F. Ariey, and O. Mercereau-Puijalon, for the KARMA Consortium*

ABSTRACT

BACKGROUND

Recent gains in reducing the global burden of malaria are threatened by the emergence of *Plasmodium falciparum* resistance to artemisinins. The discovery that mutations in portions of a *P. falciparum* gene encoding kelch (K13)–propeller domains are the major determinant of resistance has provided opportunities for monitoring such resistance on a global scale.

METHODS

We analyzed the K13-propeller sequence polymorphism in 14,037 samples collected in 59 countries in which malaria is endemic. Most of the samples (84.5%) were obtained from patients who were treated at sentinel sites used for nationwide surveillance of antimalarial resistance. We evaluated the emergence and dissemination of mutations by haplotyping neighboring loci.

RESULTS

We identified 108 nonsynonymous K13 mutations, which showed marked geographic disparity in their frequency and distribution. In Asia, 36.5% of the K13 mutations were distributed within two areas — one in Cambodia, Vietnam, and Laos and the other in western Thailand, Myanmar, and China — with no overlap. In Africa, we observed a broad array of rare nonsynonymous mutations that were not associated with delayed parasite clearance. The gene-edited Dd2 transgenic line with the A578S mutation, which expresses the most frequently observed African allele, was found to be susceptible to artemisinin in vitro on a ring-stage survival assay.

CONCLUSIONS

No evidence of artemisinin resistance was found outside Southeast Asia and China, where resistance-associated K13 mutations were confined. The common African A578S allele was not associated with clinical or in vitro resistance to artemisinin, and many African mutations appear to be neutral. (Funded by Institut Pasteur Paris and others.)

The authors' full names and academic degrees are listed in the Appendix. The authors' affiliations are listed in the Supplementary Appendix, available at NEJM.org. Address reprint requests to Dr. Ménard at the Institut Pasteur in Cambodia, 5 Monivong Blvd., P.O. Box 983, Phnom Penh, Cambodia, or at dmenard@pasteur-kh.org.

*A complete list of the investigators in the K13 Artemisinin Resistance Multicenter Assessment (KARMA) Consortium is provided in the Supplementary Appendix, available at NEJM.org.

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8.2. Low-grade sulfadoxine-pyrimethamine resistance in *Plasmodium falciparum* parasites from Lubango, Angola

Malaria Journal, 2016; 15:309

Conhecedores da importância de se monitorar a quimiorresistência do *P. falciparum à* combinação sulfadoxina pirimetamina, notadamente, na África aonde tal combinação é utilizada no tratamento presuntivo preventivo de grávidas por recomendação da OMS, nos lançamos na análise dos polimorfismos dos genes *pvdhpr* e *pvdhps* em isolados de Angola.

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Malaria Journal

RESEARCH Open Access

Low-grade sulfadoxine–pyrimethamine resistance in *Plasmodium falciparum* parasites from Lubango, Angola

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Abstract

Background: Malaria is a major parasitic disease, affecting millions of people in endemic areas. *Plasmodium falciparum* parasites are responsible for the most severe cases and its resistance to anti-malarial drugs is notorious. This is a possible obstacle to the effectiveness of intermittent preventive treatment (IPT) based on sulfadoxine–pyrimethamine (SP) cures administrated to pregnant women (IPTp) during their pregnancy. As this intervention is recommended in Angola since 2006, it has assessed, in this country, the molecular profiles in *P. falciparum dhfr* and *dhps*, two polymorphic genes associated to pyrimethamine and sulfadoxine resistance, respectively.

Methods: Blood samples from 52 falciparum patients were collected in Lubango, Angola and *pfdhfr* and *pfdhps* polymorphisms were analysed using nested-PCR and DNA sequencing.

Results: In the *pfdhfr* gene, the 108**N** mutation was almost fixed (98 %), followed by 59**R** (63 %), 51**I** (46 %), 50**R** and 164**L** (2 %, respectively). No 16**V**/**S** mutations were found. The most common double mutant genotype was CN**RN** (59 + 108; 46 %), followed by C**I**C**N** (51 + 108; 29 %) whereas **IRN** (51 + 59 + 108; 15 %), CN**RN**VL (59 + 108 + 164; 2 %) and **RI**C**N** (50 + 51 + 108; 2 %) triple mutant genotypes were detected. Investigations of the *pfdhps* gene showed that the 437**G** mutation was the most prevalent (97 %). Only two and one samples disclosed the 540**E** (7 %) and the 436**A** (3 %), respectively. Single mutant S**G**KAA (437; 86 %) was higher than S**GE**AA (437 + 540; 7 %) or **AG**KAA (436 + 437; 3 %) double mutants genotypes. No polymorphism was detected at codons 581**G** and 613**T**/**S**. Combining *pfdhfr* and *pfdhps* alleles two triple mutant haplotypes (double mutant in *dhfr* and single mutant in *dhps*) were observed: the AC**I**C**N**VI/S**G**KAA in 14 (56 %) samples and the ACN**RN**VI/S**G**KAA in five (20 %) samples. One quadruple mutant haplotype was detected (AC**IRN**VI/S**G**KAA) in six (24 %) *P. falciparum* samples. No quintuple *pfdhfr*–*pfdhps* mutant was noted.

Conclusion: *pfdhfr* and *pfdhps* gene mutations in isolates from Lubango are suggestive of a low-grade SP resistance and IPT for pregnant women and infant based on SP treatment could be effective. Routine molecular studies targeting polymorphism in these two genes need to be routinely conducted at country level.

Keywords: Malaria, *P. falciparum*, *pfdhfr*, *pfdhps*, Sulfadoxine, Pyrimethamine , Angola

Background

Plasmodium falciparum malaria is the one of the major cause of morbidity and mortality in sub-Saharan Africa, including Angola, where three million clinical cases and 6000 deaths occurred in 2015. The deaths reported in Angola territory, accounting for 35 % in children under five years old and 25 % of the maternal deaths (Angolan National Malaria Control Programme, 2015).

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In endemic malaria communities pregnant women and infants are more vulnerable to malaria episodes [1]. The World Health Organization strategy to protect mothers during their pregnancy and consequences in newborns such as low weight includes the implementation of intermittent preventive treatment in pregnant women (IPTp) [2]. This treatment was introduced in Angola in 2006, using the sulfadoxine–pyrimethamine (SP) at the second trimester of pregnancy. However, the emergence of *P. falciparum* resistances to both sulfadoxine and pyrimethamine can jeopardize these strategies.

Sulfadoxine–pyrimethamine resistant parasites are frequent in Southeast Asia and are becoming more and more prevalent in several East African countries [3]. SP acts by inhibiting the *P. falciparum* dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*), two fundamental enzymes involved in the folate biosynthesis pathway [4]. Single nucleotide polymorphisms (SNPs) leading non-synonymous mutations in these two genes have been shown to be associated with pyrimethamine (*dhfr*) and sulfadoxine (*dhps*) resistances. The Angolan National Malaria Control Programme intends to introduce IPT in infants, as has already been done in other sub-Saharan Africa countries. To inform current policy makers when recommending the use of SP in IPT, *dhfr* and *dhps* molecular markers are used to differentiate high and low grade SP resistance areas and to define its geographical distribution [5].

Malaria is endemic throughout Angola, but the transmission patterns is heterogenous varying from intense transmission and hyperendemicity in northern, mesoendemic stable in the centre, to seasonal or epidemic unstable malaria in the southern part of the country [6]. No study for investigating *pfdhfr* and *pfdhps* mutations was performed in southern part of Angola where Lubango is located. The great majority of previous studies were conducted at the northern in Luanda, [7, 8]; Uige [9, 10], Cabinda, Kwanza Norte and Malanje [10], followed by those at the Central Angola in Benguela [11] and Huambo [10].

The objective of this study was to investigate the polymorphism at codons A16**V**/**S**, C50**R**, N51**I**, C59**R**, S108**N**, V140**L** and I164**L** in the *pfdhfr* gene and at codons S436**A**/**F**/**C**, A437**G**, K540**E**, A581**G** and A613**T**/**S** in the *pfdhps* gene, in order to provide baseline data regarding the proportion of *P. falciparum pfdhfr* and *pfdhps* mutations, before SP-IPTi introduction in Lubango, an Angolan malaria stable transmission region.

Methods

Study site, blood samples and DNA extraction

Blood samples were collected in 2011 from 52 patients presenting falciparum malaria at the Central Hospital in Lubango, located in Huila Province, South of Luanda, Angola capital. Lubango is located in southern Angola, with an area of 79,022 Km^2, 1790 m above sea level, with an estimated population of 1,414,115. Malaria transmission occurs throughout the year, with peaks during rainy season, between March and May. Malaria is mesoendemic and *P. falciparum* is the predominant malaria species. Inclusion criteria comprised individuals with *P. falciparum* aging over 12 years and no evidence of complicated malaria. The Central Hospital is the main health facility in Lubango and it is a reference for malaria in the area. The malaria diagnosis was performed by thick blood smear and nested-PCR, as previous described [12]. All malaria cases were treated following the Angolan malaria treatment policy. After obtaining informed consent, venous blood collection was performed according to protocols approved by the National Institute of Public Health and the Ethical Committee of Angola and Fiocruz, Brazil (#372/07). The samples were cryopreserved and stored at −20 °C until DNA extraction. Genomic DNA was extracted from 1 mL whole blood using the Q1Aamp Midi columns (Qiagen), as described by the manufacturer.

Nested polymerase chain reaction (PCR) and electrophoresis

pfdhfr and *pfdhps* genes were amplified by nested-PCR approach using two gene-specific primers (external and internal) as already reported [13]. The nested-PCRs were performed to detect the presence of mutations at codons: A16**V**/**S**, C50**R**, N51**I**, C59**R**, S108**N**, V140**L** and I164**L** in the *pfdhfr* gene and at codons S436**A**/**F**/**C**, A437**G**, K540**E**, A581**G** and A613**T**/**S** in the *pfdhps* gene. The choice of these codons was based on the pioneer study of Pearson and colleagues [13].

DNA sequencing and SNP polymorphisms detection

After purification of the amplicons using the Wizard SV Gel and PCR Clean-Up System (Promega), PCR products were sequenced using Big Dye® Terminator Cycle Sequencing Ready Reaction version 3.1 (Applied Biosystems) and ABI PRISM 3730 DNA Analyzer [14] at the Genomic Platform/PDTIS/Fiocruz. The sequences of the amplicons were aligned with the wild-type 3D7 strain for *pfdhfr* (GenBank accession number XM_001351443) and for *pfdhfr* (GenBank accession number Z30654). The presence of SNPs was confirmed by reading both the forward and the reverse strands using the free software Bioedit Sequence Alignment Editor Version 7.2.5. Parasites with mixed alleles (in which both wild-type and mutant alleles were present) were considered mutants for estimation of the prevalence of the SNPs. Haplotypes for drug resistance markers were reconstructed from the full

sequence presenting an unambiguous single allele signal at all positions. Statistical significance of differences between the haplotypes was assessed using Fisher's tests and a p value <0.05 was considered significant.

Results

Among the 52 successfully sequenced samples for *dhfr*, 51 (98 %) showed non-synonymous mutations. The most frequent mutation was 108**N** (51/52; 98 %, 95 % CI) followed by 59**R** (33/52; 63 %, 95 % CI) and 51**I** (24/52; 46 %, 95 % CI). The 50**R** and 164**L** mutations were detected at the same frequencies (1/52; 2 %, 95 % CI) and no mutations were found at codons 16**V**/**S** and 140**L** (Table 1).

Double mutants (51**I**/108**N**, 59**R**/108N) were observed in 39 isolates (76.5 %; 39/51) and triple mutants (51**I**/59**R**/108**N**, 50**R**/51**I**/108**N**, 59**R**/108N/164**L**) in ten (19.5 %; 10/51) *P. falciparum* samples. The 108**N** single mutant was found in only two (4 %; 2/51) isolates (Table 1). In samples presenting *dhfr* double mutation, the CN**RN** (59 + 108) allele was the most frequent (24/46 %) followed by C**I**C**N** (51 + 108) (15/29 %). This contrasts with the frequency of the *dhfr* triple mutant alleles which were detected at lower rates: **IRN** (51 + 59 + 108), CN**RN**V**L** (59 + 108 + 164) and **RI**C**N** (50 + 51 + 108) (8 (15 %), 1 (2 %) and 1 (2 %), respectively). The wild-type ACNCSVI was presented in only one isolate (Tables 2 and 3).

Of the 52 samples tested, 29 (56 %) were successfully amplified for the *dhps* gene. Among them 28 (97 %) presented a mutation at codons S436**A**, A437**G** and K540**E**. The 437**G** mutant allele was highly prevalent (97 %), while the 540**E** (7 %) and 436**A** (3 %) were observed in lower frequencies. No 581**G** and 613**T**/**S** alleles were observed (Table 1). The frequency of the single mutant S**G**KAA (437) (25/86 %) was higher than double mutants S**GE**AA (437 + 540) (2/7 %) or **AG**KAA (436 + 437) (1/3 %) ($p < 0.05$). The *dhps* wild-type SAKAA was rare and observed in only one (3 %) *P. falciparum* sample (Tables 2 and 3).

Table 1 *Plasmodium falciparum dhfr* and *dhps* mutated codons in parasites from Lubango, Angola

Gene	SNPs	Prevalence N (%)
dhfr (n = 52) 95 % CI	50**R**	1 (2)
	51**I**	24 (46)
	59**R**	33 (63)
	108**N**	51 (98)
	164**L**	1 (2)
dhps (n = 29) 95 % CI	436**A**	1 (3)
	437**G**	28 (97)
	540**E**	2 (7)

Table 2 Prevalence of *P. falciparum dhfr* and *dhps* mutants in parasites from Lubango, Angola

Gene	Mutation	SNPs	Prevalence N (%)
dhfr n = 51	Single	S108**N**	2 (4)
	Double	N51**I**/S108**N**	15 (29)
		C59**R**/S108**N**	24 (47)
	Triple	N51**I**/C59**R**/S108**N**	8 (16)
		C50**R**/N51**I**/S108**N**	1 (2)
		C59**R**/S108**N**/I164**L**	1 (2)
dhps n = 28	Single	A437**G**	25 (89)
	Double	A437**G**/K540**E**	2 (7)
		S436**A**/A437**G**	1 (4)

Table 3 Deduced haplotype profiles for *dhfr* and *dhps P. falciparum* parasites from Lubango, Angola

Gene	Haplotypes	N	%
dhfr (n = 52)	CN**RN**	24	46
	C**I**C**N**	15	29
	C**IRN**	8	15
	CNC**N**	2	4
	CN**RN**V**L**	1	2
	RIC**N**	1	2
	ACNCSVI	1	2
dhps (n = 29)	S**G**KAA	25	86
	S**GE**AA	2	7
	AGKAA	1	3
	SAKAA	1	3

Combining *pfdhfr* and *pfdhps* alleles, not all isolates had an interpretable haplotype (Table 4). The PCR amplification failure rate of the *dhps* gene in Lubango samples might be somehow attributed to primer mismatched due to unknown polymorphisms in target sequences, because in all the 52 samples amplification was achieved when primers strictly developed to amplify conserved sequences for diagnosis purposes were tested [12]. The number of samples herein considered was based on amplification of both PCRs just to avoid the identification of incomplete haplotypes.

Two triple mutant haplotypes (double mutant in *dhfr* and single mutant in *dhps*) were observed: the AC**I**C-N**V**I/S**G**KAA in 14 (56 %) samples and the ACN**RN**VI/S**G**KAA in five (20 %) samples. One quadruple mutant haplotype was detected (AC**IRN**VI/S**G**KAA) in six (24 %) *P. falciparum* samples (Table 4). The high-grade SP resistant haplotypes (quintuple, sextuple and septuple *pfdhfr/pfdhps*) [3] were not seen among these isolates.

Discussion

At present, SP continues to be used in IPTp and IPTi strategies, which are likely to provide sub-optimal effect. Therefore, monitoring the spread of SP resistance using *dhfr* and *dhps* as molecular markers are strongly recommended [15]. This study was performed with *P. falciparum* samples from Lubango, an Angola region with unstable meso-endemic malaria transmission. The vast majority of tested isolates (98 %) presented at least one mutation in the gene encoding enzymes associated with pyrimethamine resistance.

High prevalence of 108**N** mutation (considered as the initial mutation occurring in *dhfr* multiple mutants) was detected, similar to observations already done in several Angolan regions [8, 10, 11] and in other African countries [16–26]. However, contrary to various Angolan localities, the 59**R** was the second more frequent *dhfr* mutation in Lubango, instead of the 51**I** [8–11]. In addition, it is worth to noting that the 164**L** *pfdhfr* SNP was also detected for the first time in Angola. This mutation is rare in Africa [5] but its presence in association with 108**N** + 51**I** and/or 59**R** [27] as well as with the *dhfr* + *dhps* quintuple mutant [5], confers high levels of in vivo and in vitro resistance that could render SP totally ineffective. The 164**L** mutation was also combined to cycloguanil resistance [28] and is commonly found in *P. falciparum* samples from Southwest Asia [29, 30] and South America [31]. The possible reason for the detection of mutant 164**L** in Lubango (north of Angola) and not in other correlate Angolan studies (North and Central Angola) is, probably, due to the greater proximity of Lubango with Zambia, where the presence of quintuple mutants is greater than 50 % [3].

From previous reports, it was known that the triple African mutant (N51**I** + C59**R** + S108**N**) shares a common ancestry with resistant *dhfr* from Southeast Asia [32, 33]; this mutant emerged in Asia and was introduced later in Africa, including Angola [19, 20, 33–36]. With respect to *dhfr* mutation 50**R**, another non-frequent *dhfr* mutation in Africa, it was also identified in this study. This low-grade pyrimethamine resistant mutant [27] was first described as specific of South American isolates [27] but, besides South America, it has already been described in Africa in isolates from Kenya [35], Central African Republic [37] and Angola [8].

In relation to *dhps* gene, the 437**G** mutation was the most common, reaching almost fixation, similarly to other Angolan localities [8, 10, 11] and other African countries [16, 20, 23, 26], especially in western and Central Africa.

The *dhps* 540**E** mutation, less commonly observed in western Africa, was also identified in this study, confirming previous data from Luanda [7, 8], Cabinda, Huige, Kwanza Norte, Malange and Huambo [10]. However, the spatial distribution of this allele in Angolan provinces seems heterogeneous and even in the same province its frequency can vary overtime [9, 10]. Thus, screening for *dhps* 540**E**, a surrogate for the quintuple mutant, is a priority in West Africa where it is comparatively rare [3].

The combination of 437**G** with the triple mutant 51**I** + 59**R** + 108**N** which is considered to be associated with SP treatment failure [38–40], was detected in 33 % of Lubango samples. However, in vitro studies showed that the single 437**G** mutant has a lesser degree of tolerance to sulfadoxine than the 437**G** + 540**E** double mutant. Thus, triple mutant 51**I** + 59**R** + 108**N** associated with single 437**G** mutant could have a less detrimental effect on SP-IPT than the associated 437**G** + 540**E** mutations—the sustained protective efficacy of SP-IPTi in western and Central Africa reinforce these points [41].

Despite the presence of parasites with triple *dhfr* mutant at positions 51**I**, 59**R** and 108**N** (15 %) and double *dhps* mutant at positions 437**G** + 540**E** (7 %), the quintuple mutant (51**I** + 59**R** + 108**N** + 437**G** + 540**E**), considered a significant predictor of SP treatment failure [42] was not detected in the studied isolates from Lubango. To date, this quintuple mutant was not observed in central and northern areas of Angola [7–11]. Besides Lubango, similar low frequencies of double 437**G** + 540**E** *pfdhps* mutant were described in Huambo (12.5 %) [10], Benguela (8 %) [10], Cabinda (5.3 %) [10], Malanje (3.1 %) [10], Luanda (3 %) [8], Kwanza Norte (2.1 %) [10] and Uige (1.9 %) [10], contrasting with the higher frequencies of this mutant detected in Eastern Africa: Kenya (86 %), Tanzania (90 %), Malawi (95 %) and Uganda (95 %). With the exception of Malanje (3.9 %), the frequencies of the triple 51**I** +59**R** + 108**N** *dhfr* mutant in Angolan provinces, including Lubango, were higher than the double 437**G** + 540**E** *pfdhps* mutant but, East African countries presented higher prevalence rates [43].

These findings are expected because East African countries presented a high rate of SP treatment failure of falciparum malaria together with the increased presence of the quintuple mutant considered the most specific molecular marker for SP [44, 45].

Table 4 Combined molecular *dhfr* and *dhps* gene profiles in 25 samples of *P. falciparum* from Lubango, Angola

Genes	Haplotypes	Mutated	
dhfr + *dhps*		N/ %	Type
51I/108 N + 437G	AC**I**C**N**I + S**G**KAA	14/56	Double and single
59R/108 N + 437G	ACN**R**N**N**I + S**G**KAA	5/20	Double and single
51I/59R/108 N + 437G	AC**IRN**I + S**G**KAA	6/24	Triple and single

Besides quintuple mutants, the concept of "super resistant genotypes" is emerging which further raise the threshold of drug tolerance in parasites comprising additional mutations in combination with N51**I** + C59**R** + S108**N** and A437**G** + K540**E** [3]. The additional mutations are *dhfr* I164**L** (found in one sample; 2 %) and *dhps* A581**G** and A613**T/S**, which were not found in this study. Although, these mutations are rare, their impact on SP-IPTi or SP-IPTp efficacy is considered highly detrimental [5]. When the quadruple mutant N51**I** + C59**R** + S108**N** + A437**G** occur in the absence of 540**E**, these parasite populations are classified as partially resistant genotypes, and its resistance levels are not expected to be as high [3]. The efficacy of IPTp in western Africa, where N51**I** + C59**R** + S108**N** and A437**G** exist in combination with 581**G** but not with 540**E** supports the view that these parasites should be regarded differently [3]. In this case, molecular markers of super resistance may be an important warning about failing efficacy of IPTp.

Many studies suggested that IPTp with SP remains effective, or at least it is not associated with harm, in areas with high prevalence of *P. falciparum* quintuple mutant parasites [46, 47]. There is not enough evidence yet to establish a threshold prevalence of the classical *dhfr* + *dfps* quintuple mutant, or even of the *dhps* 581, *dhps* 540 or *dhfr* 164 point mutations above which there is a clear loss of IPTp-SP effectiveness. Therefore, the significance of the additional mutation at codon 540 needs further investigation.

Conclusion

In view of the data reported here and elsewhere, it was conclude that, although the number of samples available for the present study was limited, *pfdhfr* and *pfdhps* gene mutations in isolates from Lubango are suggestive of a low-grade SP resistance and their presences highlight the importance of molecular markers screening to monitor the evolution of *P. falciparum* SP resistance in Angola.

Authors' contributions

MFFC carried out the study and the manuscript. EPSKD performed and analysed DNA sequencing and drafted the manuscript. LRG performed statistical analysis and edited the manuscript. BEG reviewed the DNA sequences. DM and CTDR participated in the discussions and reviewed the final manuscript. NKAO performed DNA extraction and PCRs. FF recruited the patients. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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8.3. Frozen blood clots can be used for the diagnosis of *Plasmodium* species in human and non-human primates from Brazilian Atlantic Forest.

Submetido Malaria Journal MALJ-D-18-00148

Zoonoses como a febre amarela e a malária simiana ocorrem em áreas endêmicas superponíveis, infectando símios de baixa volemia. Assim, otimizamos o diagnóstico dessas patologias em pequenos volumes de sangue, utilizando coágulos de primatas humanos e não humanos para a detecção de plasmódios em condições de baixa parasitemia.

Malaria Journal

Frozen blood clots can be used for the diagnosis of distinct Plasmodium species in human and non-human primates from brazilian Atlantic Forest

--Manuscript Draft--

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Abstract:	Abstract Background: Zoonotic infections with epidemic potential as simian malaria and yellow fever can overlap geographically. Optimizing a small blood sample for diagnosis and surveillance is of great importance. Therefore we tested the usually discarded blood clots from human and non-human-primates for detection of Plasmodia DNA, even in low parasitaemia conditions. Results: Malaria diagnoses results with DNA extracted from blood clots were in agreement with those obtained with whole blood, including mixed Plasmodium vivax/simium and P. malariae/brasilianum infection. Conclusion: Blood clots from human and non-human primates may be an important and low cost source of DNA for malaria surveillance in Atlantic Forest. Key words; Blood clot, Plasmodium, Alouatta guariba clamitans, Non-human-primates, Yellow fever, Atlantic forest.	
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Is this study a clinical trial?<hr>A clinical trial is defined by the Word Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes'.	No

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Frozen blood clots can be used for the diagnosis of distinct Plasmodium species in human and non-human primates from brazilian Atlantic Forest

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Abstract

Background: Zoonotic infections with epidemic potential as simian malaria and yellow fever can overlap geographically. Optimizing a small blood sample for diagnosis and surveillance is of great importance. Therefore we tested the usually discarded blood clots from human and non-human-primates for detection of *Plasmodia* DNA, even in low parasitaemia conditions.

Results: Malaria diagnoses results with DNA extracted from blood clots were in agreement with those obtained with whole blood, including mixed *Plasmodium vivax/simium* and *P. malariae/brasilianum* infection.

Conclusion: Blood clots from human and non-human primates may be an important and low cost source of DNA for malaria surveillance in Atlantic Forest.

Key words; Blood clot, *Plasmodium*, *Alouatta guariba clamitans*, Non-human-primates, Yellow fever, Atlantic forest.

Backgroud

Some zoonotic infections with epidemic potential may overlap geographically. Their surveillance and follow-up during epizooties and epidemics depends on the screening for distinct infectious agents often in a single blood sampling from animal reservoirs. Frequently, reservoirs are small weight animals with low blood volume and sometimes in hypovolemia at the sampling moment preventing the collection of ideal amount of blood. Therefore, optimizing a small blood sample may be critical for diagnosis by different methods at a time.

Non-human-primates (NHPs) are among the reservoirs of main zoonotic diseases. For instance, in South America and Africa, the areas of occurrence of simian plasmodia infective to humans and the sylvatic transmission cycle of the yellow fever virus (YFV), in which NHP

are the reservoir, frequently overlap [1–3]. Thus, simultaneous surveillance of these zoonotic infections in a single blood sampling of NHP is of great importance.

Recently, Southeast Brazil has reported outbreaks of human malaria of simian origin and yellow fever acquired in sylvatic transmission cycle. The states of Espírito Santo (ES) and Rio de Janeiro (RJ) were the most affected by both outbreaks [4–8]. Accordingly, the most severe yellow fever outbreak of the last seven decades occurred in areas under the influence of the Atlantic Forest with 274 humans and 642 NHPs confirmed deaths [7]. The YFV circulated in the same localities where human malaria of simian origin has been recorded for decades, with recent reports of outbreaks due to infection by *Plasmodium simium*, a simian parasite close related to *Plasmodium vivax* [1, 5, 9–13]. The other New World simian malaria parasite - *P. brazilianum* - may also infect humans and is highly similar to *P. malariae* [1]. Conventional PCR protocols for malaria diagnosis used worldwide [e.g., 14, 15] do not distinguish *P.vivax* and *P.malariae* from *P.simium* and *P. brazilianum*, respectively [5, 11, 16]. *P. vivax* can confidently distinguished from *P. simium* only by the presence of specific SNPs in mitochondrial DNA usually searched in red blood cells or whole blood of human and NHPs [5, 6, 17]. Parasitaemia by *P. simium* is usually low to scanty, both in simian and human hosts. Concerning YFV, the Brazilian Ministry of Health preconizes the active surveillance of NHPs, which requires their capture with traps or anesthetic darts and blood sampling for serological and molecular assays. Thus, blood is regularly collected in tubes without anticoagulant to obtain serum [18]. Usually, the blood clot is discarded after serum collection.

The main host of both *P. simium* and YFV in Southeastern and Southern Brazil are howler monkeys, genus *Alouatta* [1, 18]. These animals hardly enter traps and their capture in the forest canopy is difficult and expensive. As the opportunity of assembling blood samples from free-living howler monkeys and other neotropical NHPs is relatively rare, it is important

to optimize the collected materials. Therefore, we adapted the methodology proposed by Lundblom et al. (2011) [19] for the detection of *Plasmodia* DNA in blood clots from human and NHPs, even in the presence of low parasitaemia conditions frequently reported in these infections.

Methodology

Non-human primates samples. During a YFV surveillance in 2016, one *Alouatta guariba clamitans* (sample M1) and one *Callithrix* hybrid. (M2) were captured in Atlantic Forest areas of the Rio de Janeiro state, Brazil, following the technical guidelines of sampling and security measures [18]. A total of 5 and 3mL of venal blood were respectively collected from femoral puncture of M1 and M2, and then the blood was equally split in two tubes, one without anticoagulant and another containing EDTA. Following centrifugation (2,000 g x 10 min), sera, clot, plasma and whole blood samples were stored at -80°C until DNA extraction.

Human samples. Five mL blood samples collected in January 2017 from *P. vivax* patients (H1 and H2) infected in Atlantic Forest area of Rio de Janeiro state, Brazil, and from a clinically healthy donor (H3) were used. Both whole blood and blood clots were obtained and stored, as described for non-human samples.

DNA extraction and PCR. After thawing, 600-800µL of lysis buffer (AL lysis buffer provided in QIamp® DNA mini kit, Qiagen) was added to 300-400µL (2:1) of each blood clot in tubes containing or not glass beads and a high-speed shaking (6000 rpm / 40 sec; Bertin Precellys 24) was performed for clot disruption. After that, DNA was extracted using the QIAamp® DNA mini kit, according to the manufacturer's instructions, except for the elution step that was made in a final volume of 50µl instead of 100 µl. DNA from whole blood was performed using the same QIamp® DNA mini kit, Qiagen, according manufactures. To check DNA extraction efficiency 3 sets of extractions were realized with the same blood clot sample

as already described. The presence of gDNA was evaluated after electrophoresis (110 A / 1 h) on ethidium bromide agarose gel, under UV light. To check PCR assay precision, PCR were tested in replicates (intra-assays) and in three different days (between assays).

For conventional PCR assays, DNA samples were tested for *P. vivax* using the cysteine proteinase gene (GenBank number L26362) [20], for *P. malariae* using nested PCR [14] and for *Plasmodium* genus [21]. PCR products were visualized under UV light after electrophoresis on 2% agarose gels. DNA from patients' blood diagnosed with *P.vivax* or *P.malariae* were used as positive control in each round of amplification. DNA from non-infected human and monkey as well as blank samples (no DNA) were employed as negative controls.

Parasitological diagnosis. Giemsa stained thick and thin blood films of human and NHP samples were examined before PCR assays. Samples H1, H2 and M1 were positive, displaying 208, 368 and 300 malaria parasites / µL. The other two samples, a human (H3) and a NHP (M2) were negative (Table 1).

Results

The results of PCR performed with DNA extracted from clots were in agreement with those obtained with DNA extracted from whole blood, using or not glass beads. Concerning extraction without beads, small residual fragments were observed but they were dissolved in subsequent incubations and gDNA was strongly detectable under UV light on both conditions. Samples H1 and H2 were positive in PCR for genus *Plasmodium* and *P. vivax* and negative for *P. malariae* whereas M1 (*A. clamitans*) was positive for genus, *P. vivax* and *P. malariae*. Despite the low parasitaemia, samples were consistently positive intra-assays (repeatibility) and between assays (reproducibility) in 3 different days (Table 1).

Inversely, clot disruption using glass beads, besides required centrifugation steps, generated a weak and not sharped gDNA band under UV light and PCR reactions were negative, independently of PCR target.

Table 1: PCR results from whole blood and blood clots according to malaria species and microscopy examination (thick and thin blood film).

(TABLE 1 SHOULD BE HERE)

Discussion

This is the first report where blood clot was used for the detection of *Plasmodium* DNA in NHP. It is also described for the first time that *P. vivax / simium* and *P. malariae / P. brasilianum* can be detected in clot samples even under low parasitemia and small blood volume conditions. In fact, blood clot disrupted with high speed shaking was only reported for the detection of *P. falciparum* in larger samples of human blood clots [19] with no information of precision (repeatability and reproducibility) parameter.

Therefore, the use of blood clots, that are often discarded, allow the investigation of malaria parasites in the same blood sampling from which serum is extracted for other use, such as the surveillance of YFV and other arboviruses in NHPs occurring in sympatry.

Interestingly, the high-speed shaking technique without the use of beads showed best blood clot disruption performance. This corresponds to an advantage because of the reduced risk of accidents and contaminations due to handling, as described for other disruption techniques [22–24].

In addition to diagnosing malaria, PCR templates of quality obtained from NHPs blood can be used for several epidemiological, ecological and evolutionary purposes. In fact, trypanosomes of NHPs infecting man, such as *Trypanosoma cruzi*, can be even more

sensitively detected in blood clots when compared to whole blood and leukocytes samples [25] or to serum in the case of detecting fungi causing aspergillosis in man [26].

Furthermore, human blood clots stored at -20ºC from 1 to 2.5 years were still useful for the investigation of single-nucleotide polymorphisms [27]. PCR results were equivalent to whole blood, as demonstrated in this paper with blood clots stored at -70ºC for around 2 years, revealing the quality of DNA template. Therefore, blood clot storage may be a stable source of primate's genetic sample for several phylogeny, dispersion and gene flow studies. Whole blood tissues, hair or even feces were already used as DNA sources for several researchers [28–32] (non-exhaustive list). Along with increasing deforestation, studies of habitat fragmentation effects, especially in small populations of endangered NHP, are necessary to support management strategies [33] and clotted blood could be an stable genetic bank for several populations.

In conclusion, blood clots can be used as valuable sources of information [27]. Its use may optimize the costs, handling time and to reduce the amount of blood collected from each individual [25]. Thereby, the creation of a blood clot bank could help in answering several ecological and evolutionary issues, besides the epidemiological ones.

Finally, in view of the results reported here, we suggest that the Brazilian Ministry of Health surveillance protocols recommend the storage of blood clots resulting from NHP blood collection and examination. It corresponds, indeed, to a low-cost initiative that can optimize the results of capture and safety of NHP, contributing to knowledge expansion in different fields of primatology and epidemiology, and protection of human against zoonotic diseases.

Conclusion

We conclude that blood clots of humans and non-human primates are important sources of DNA that can be used for *Plasmodia* detection even in low parasitemias.

Declarations

Ethics approval and consent to participate

The protocols for handling and blood collection of NHP were approved by the Institutional Ethics Committee of Animal use at IOC (CEUA licenses LW-34/2014 and L037/2016, respectively). Capture of wild NHPs and mosquitoes were agreed by the Brazilian environmental authorities: SISBIO-MMA licenses 54707-137362-2 and 52472-1, and INEA license 012/2016012/2016. Protocol for human blood collection was approved by INI-Fiocruz Ethical Board (#0062.0.009.000-11) and by Ethical Research Committees of Fiocruz (# 32839013.6.00005248). All participants provided informed written consent.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

FVSA and LRG carry out blood clot DNA extractions and PCR assays; DST, ES and FVSA captured and collected NHPs samples; APC, CBJ and PB provided human samples; MFFC diagnosed by PCR malaria patients; MFFC and RLO conceived the study; RLO examined PNHs microscopic slide; CTDR, MFFC and RLO raised grants for funding the work; FVSA, MFFC and RLO drafted and finalised the manuscript. All authors read and approved the final version of the manuscript.

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Code	Specie	Thick and thin blood films	Parasitsemia (p/µl)	PCR: Whole Blood *Plasmodium*	PCR: 1st Blood Clot DNA extraction	PCR: 2nd Blood Clot DNA extraction	PCR: 3rd Blood Clot DNA extraction
H1	*Homo sapiens*	+	208 *P. vivax*	+	*Plasmodium; P. vivax/P. simium*	*Plasmodium; P. viva /P. simium*	*Plasmodium; P. vivax/P. simium*
H2	*Homo sapiens*	+	368 *P. vivax*	+	*Plasmodium; P. vivax/P. simium*	*Plasmodium; P. vivax/P. simium*	*Plasmodium; P.vivax/P. simium*
H3	*Homo sapiens*	-	-	-	-	-	-
M1	*Alouatta g. clamitans*	+	300 *P. vivax*	+	*Plasmodium; P. vivax/P. simium and P. malariae/ P. brasilianum*	*Plasmodium; P. vivax/P. simium and P. malariae/ P. brasilianum*	*Plasmodium; P. vivax/P. simium and P. malariae/ P. brasilianum*
M2	*Callithrix* hybrid	-	-	-	-	-	

P: parasite; +: Positive results; -: Negative results