Mutations in the *pfmdr1*, *cg2*, and *pfcrt* genes in *Plasmodium falciparum* samples from endemic malaria areas in Rondonia and Pará State, Brazilian Amazon Region

Mutações nos genes *pfmdr1*, *cg2* e *pfcrt* em isolados de *Plasmodium falciparum* provenientes de localidades malarígenas dos Estados de Rondônia e Pará, Amazônia Legal Brasileira

Giselle Maria Rachid Viana [1]
Ricardo Luís Dantas Machado [2]
Vanja Sueli Pachiano Calvosa [1]
Marinete Marins Póvoa [1]

[1] *Seção de Parasitologia, Instituto Evandro Chagas, Belém, Brasil.*
[2] *Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto, São Paulo, Brasil.*

Correspondence
M. M. Póvoa
Laboratório de Pesquisas em Malária, Seção de Parasitologia, Instituto Evandro Chagas.
Rod. BR-316, km 7, s/n, Ananindeua, PA
67030-000, Brasil.
marinetepovoa@iec.pa.gov.br

Abstract

The objectives of this study were to investigate the molecular basis for Plasmodium falciparum resistance to chloroquine in isolates from the Brazilian Amazon and to identify polymorphisms in the pfmdr1 *gene, codons 184, 1042, and 1246, the kappa and gamma regions of the cg2 gene, and the* K76T *mutation of the* pfcrt *gene, in order to calculate the distribution of polymorphism within each target gene, comparing samples from distinct geographic areas, using allele-specific polymerase chain reaction (PCR) for the* pfmdr *gene and PCR plus restriction fragment length polymorphism (RFLP) for the cg2 and* pfcrt *genes. The sample consisted of 40 human blood isolates, already collected and morphologically diagnosed as carriers of* P. falciparum *parasites, from four localities: Porto Velho in Rondonia State and Maraba, Itaituba, and Tailandia in Pará State. Distribution of* P. falciparum *in vitro chloroquine resistance in the isolates was 100% for* pfmdr1, cg2 *gamma region, and* pfcrt, *except for the polymorphism in the* cg2 *kappa region, which was not found.*

Plasmodium falciparum*; Genetic Polymorphism; Chloroquine*

Introduction

The first references to malaria cases from chloroquine-resistant *Plasmodium falciparum* date to 1960 in South America and Southeast Asia, and since then drug resistance has been viewed as a public health problem in various countries of the world [1].

In Africa, chloroquine-resistant *P. falciparum* malaria has been expanding since the 1970s. Currently, resistance to this drug is found in nearly all the endemic countries, especially in East Africa [2,3]. In Brazil, the first reports of resistance to anti-malarials date to the early 20th century, when it was observed that quinine was losing its efficacy [4]. In relation to chloroquine, clinical failure is already reaching nearly 100% in the majority of endemic areas in Brazil [1,5,6,7].

The molecular basis for chloroquine resistance has been studied, but various aspects remain to be elucidated. In 1989, two homologues of the multiple drug resistant gene (*mdr*) were identified and denominated *pfmdr1* and *pfmdr2*, located respectively in chromosomes 5 and 14 of *P. falciparum*. Polymorphisms in the *pfmdr1* homologue have been associated with the chloroquine resistance phenotype [8]. Although some studies have indicated a lack of association between the chloroquine resistance phenotype and point mutations in this gene [9,10], there is growing evidence of the role of the *pfmdr1* gene in *P. falciparum* chloroquine resistance [5,6,11,12,13].

Polymorphism in the $^{Asn}86^{Tyr}$ codon has been related to chloroquine resistance in *P. falciparum* isolates in Nigeria [11], Guinea-Bissau [12], Gambia [14], and Malaysia [15]. An association has also been demonstrated between polymorphism in the $^{Asn}1042^{Asp}$ and $^{Asp}1246^{Tyr}$ codons in the *pfmdr1* gene and *in vitro* chloroquine resistance and absence of mutation in the $^{Asn}86^{Tyr}$ codon in samples in Brazil [5].

After genetic crossing between a resistant strain (W2) and one sensitive to chloroquine (HB3), Su et al. [16] characterized the *cg2* gene (region 36-kb of chromosome 7) as responsible for chloroquine resistance. The *cg2* genotype is characterized by twelve point mutations and three length polymorphisms (kappa, gamma, and omega), which are associated with clones having chloroquine resistance phenotypes [16].

Currently, the study of molecular markers for resistance in malaria epidemiology focuses on polymorphism in the protein coded by the *pfcrt* gene (chloroquine-resistant *P. falciparum* related to the transporter), which contains 13 exons and is located in chromosome 7 of the parasite [17,18]. Point mutations in this gene have been associated with *in vitro* chloroquine resistance in *P. falciparum* isolates from Africa, Southeast Asia, and South America, in which a mutation, substitution of lysine (K) with treonine (T) in position 76 (K76T), was present in all of the chloroquine-resistant samples and absent in all the isolates that were sensitive to this drug according to *in vitro* and *in vivo* analysis [2,19,20,21,22,23].

Despite the results of studies reporting flaws in the association between the K76T mutation of the *pfcrt* gene and chloroquine resistance [24,25], genetic transformation experiments with plasmids expressing mutant forms of *pfcrt* conferred resistance in the three different chloroquine-sensitive clones tested, thus demonstrating this gene's important role for *in vitro* chloroquine resistance [26].

The high rate of chloroquine-resistant *P. falciparum* cases in Brazil requires studies on the molecular basis of this resistance through the identification and characterization of genes with *pfmdr1*, *cg2*, and *pfcrt*, given that the studies conducted to date in the region (localities in the States of Amapá [5], Amazonas [7,22], Mato Grosso [6,7,22], and Rondônia [7,22]) are scarce in light of the problem's geographic extension. It is equally important to produce knowledge allowing the development of new drugs and the application of anti-malarial prophylactic measures.

The objective of the current study was to investigate the molecular basis for *P. falciparum* chloroquine resistance in isolates from the Brazilian Amazon, by identifying polymorphisms in codons $^{Asn}1042^{Asp}$, $^{Asp}1246^{Tyr}$, and $^{Tyr}184^{Phe}$ of the *pfmdr1* gene, polymorphisms in κ (kappa) and γ (gamma) in the *cg2* gene, and the K76T mutation in the *pfcrt* gene.

Material and methods

Study area

The selected study localities were Porto Velho (longitude 63° 55' 00"; latitude: 8° 40' 00"), Rondônia; Marabá (longitude 49° 07' 44.2"; latitude: 5° 23' 27.5"), Itaituba (longitude 55° 59' 27"; latitude: 4° 16' 33.3"), and Tailândia (longitude 48° 57' 07.2"; latitude: 2° 56' 06.6"), Pará, all located in the Brazilian Amazon.

In each study area, ten human blood samples were used that were already available in the blood samples bank at the Malaria Research Laboratory of the Evandro Chagas Institute (Instituto Evandro Chagas), diagnosed microscopically as *P. falciparum* malaria, totaling an overall sample of forty isolates (n = 40). The study was approved by the Research Ethics Committee of the Instituto Evandro Chagas.

Laboratory methods

- **Preparation and washing of samples**

From each total blood sample, five 20μL drops were added to a fiberglass membrane measuring 2.5cm in diameter (Whatman International, Maidstone, UK), placed on Whatman filter paper measuring 5.5cm in diameter (Whatman International, Maidstone, UK). After drying the droplets at room temperature and properly identifying the samples, the membranes were stored in plastic recipients (BHL Limited, Poole, UK) at -20°C until submitting to washing according to Warhurst et al. [27].

- **Polymerase chain reaction (PCR)**

One eighth of one drop of blood soaked into the membrane (the equivalent of 5μL of extracted DNA) and obtained with a sterile scalpel was used as the source of DNA. The following reagents were added to this part of the membrane for analysis of the *pfmdr1*, *cg2*, and *pfcrt* genes: 5μL of 10X buffer [tris-HCl 10mM, pH 8.3; gelatin 0.01%(p/v)]; KCl 10M; and MgCl2 15mM) (Bioline, Massachusetts, USA – cat. M95801B); 2 to 2,5μL of each specific initiator for each region of the target gene; 0.75 to 1.0μL of each dNTP (2.5μmol – Pharmacia Biotech) (final concentration 200μM); 0.25μL of Taq polymerase (Biotaq

5U/µL: 550 units – Bioline M95801B); and sterile distilled water q.s.p. 50µL/tube. The initiator sequences and PCR conditions for codons 184, 1042, and 1246 of the *pfmdr1* gene were conducted according to Grobusch et al. [13] and Adagu & Warhurst [28], for the kappa and gamma of the *cg2* gene according to Adagu & Warhurst [28], and for the K76T mutation of the *pfcrt* gene according to the protocol by Christopher Plowe (University of Maryland, USA) [26].

In addition to the test samples, size 50 or 100bp markers were used, and as controls, types HB3 (standard sensitive clone, Honduras), 7G8 (standard resistant clone, Brazil), DD2 (standard resistant clone, Southeast Asia), and K1 (standard resistant clone, Thailand).

The tubes were centrifuged rapidly at 14,000rpm and placed in a thermal cycler (Hybaid OmniGene® Thermal Cycler) to be submitted to the specific amplification conditions for each region of the target gene.

The PCR products were fractionated electrophoretically in agarose gel at 2% for codons 184, 1042, and 1246 on the *pfmdr1* gene, at 3% for the kappa and gamma regions of the *cg2* gene, and at 1% for the K76T mutation of the *pfcrt* gene (Ultra pure agarose, BRL 155517-014), at 100 volts for one hour. Later, the agarose gel was stained with ethidium bromide (5µL/mL in TBE) for 30 minutes and the resulting bands were viewed under ultraviolet light (Fluo-Link, Flowgen) and photographed in a photodocumentation system (Kodak® Edas 290).

- **Restricted fragment length polymorphism (RFLP)**

For the kappa region of the *cg2* gene, the endonuclease *Tse I* was used. The PCR products with a pattern similar to DD2 or K1 produced three fragments with 558bp, 90bp, and 68bp, while the isolates with a pattern similar to HB3 produced two fragments with 632bp and 68bp. For the gamma region of this same gene, the restriction enzyme was *Rca 1*. The PCR products with a pattern similar to 7G8 produced two bands with 194bp and 97bp, while the isolates with a pattern similar to HB3 produced three bands with 119bp, 106bp, and 45bP. For the *pfcrt* gene, the *APO 1* endonuclease was used, and the samples with a pattern similar to 7G8 produced a fragment with 134bp, and those with a pattern similar to the HB3 type produced two fragments, with 100bp and 34bp. Digestions were conducted at 65ºC in 10µL volume, using 0.2µL of restriction endonuclease (*Tse I*, *Rca 1*, or *APO 1*, according to the target gene), 1µL of buffer solution, 4.8µL of sterile distilled water, and 4µL of the PCR products in each Eppendorf tube. The restriction fragments had the sizes fractionated in agarose gel at 2% at 100 volts for one hour. Later, the agarose gel was stained with ethidium bromide (5µL/mL in TBE) for 30 minutes and the bands were viewed under ultraviolet light (Fluo-Link, Flowgen) and photographed in a photodocumentation system (Kodak® Edas 290).

Results

In relation to codons 184 and 1042 on the *pfmdr1* gene, all 40 isolates showed a profile similar to type 7G8 (the standard resistant clone for Brazil) according to the allele-specific PCR technique (Figures 1 and 2). For codon 1246 on the same gene, the study samples showed a similar profile to 7G8, except for samples 808/98, 809/98, 810/98, 813/98, 815/98, 816/98, and 817/98 from the municipality of Porto Velho, which showed a profile similar to K1 (standard resistant clone for Thailand). For the kappa region of the *cg2* gene, all 40 isolates showed a profile similar to HB3 (standard sensitive clone for Honduras) in both tests (PCR and RFLP) (Figure 3), while for the gamma region of this same gene, all 40 samples showed a profile similar to type 7G8 (standard resistant clone for Brazil) in both tests (Figure 4).

The mutant allele K76T of the *pfcrt* gene was found in all 40 samples. Amplification of a 134bp region containing codon 76 was obtained in these 40 isolates by nested PCR and confirmed by RFLP using the *APO 1* endonuclease, presenting a profile similar to 7G8 (standard resistant clone, Brazil) (Table 1) (Figure 5).

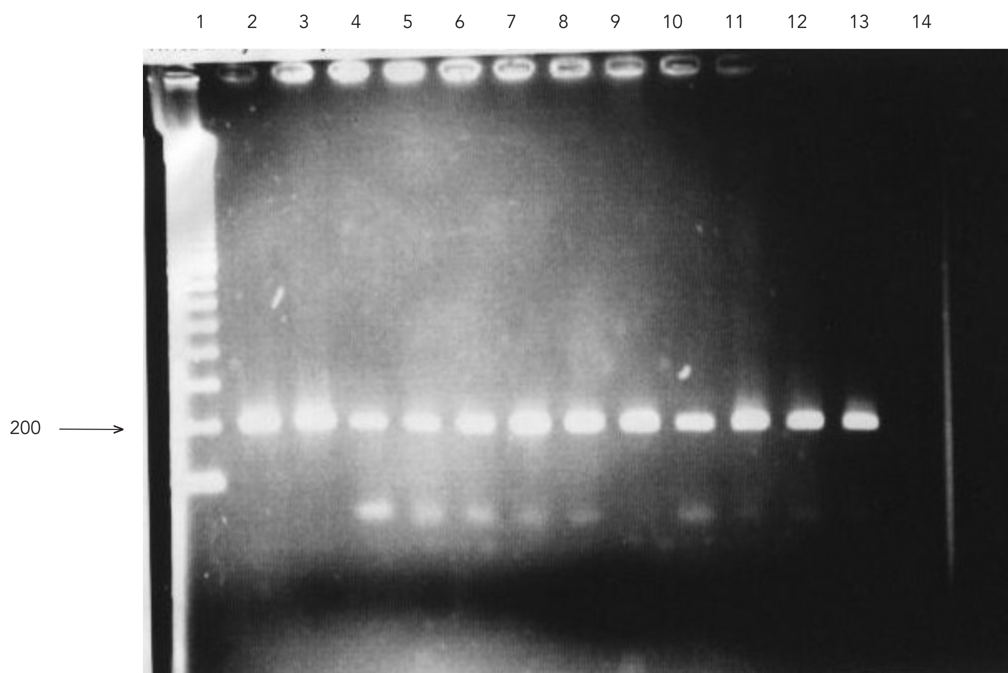
Discussion

The molecular mechanisms for the development of chloroquine resistance by the parasite are still not completely clear, but many candidate molecular markers have been identified [20,21,29] and there is evidence that *P. falciparum* chloroquine resistance is a multigenic event [2,10,16].

In relation to the *pfmdr1* gene, the 40 isolates were tested for the $^{TYR}184^{PHE}$, $^{ASN}1042^{ASP}$, and $^{ASP}1246^{TYR}$ mutations associated with the chloroquine resistance phenotype from samples in Africa, Asia, and South America [5,11,12,13,30,31]. Although all the isolates tested for the $^{TYR}184^{PHE}$ mutation presented a resistance pattern similar to 7G8 (standard Brazilian resistant clone), changes in this codon appear not to be correlated with chloroquine resistance [29]. Meanwhile the presence of a profile similar to clone 7G8 in the $^{ASN}1042^{ASP}$ and $^{ASP}1246^{TYR}$ mutations in the

Figure 1

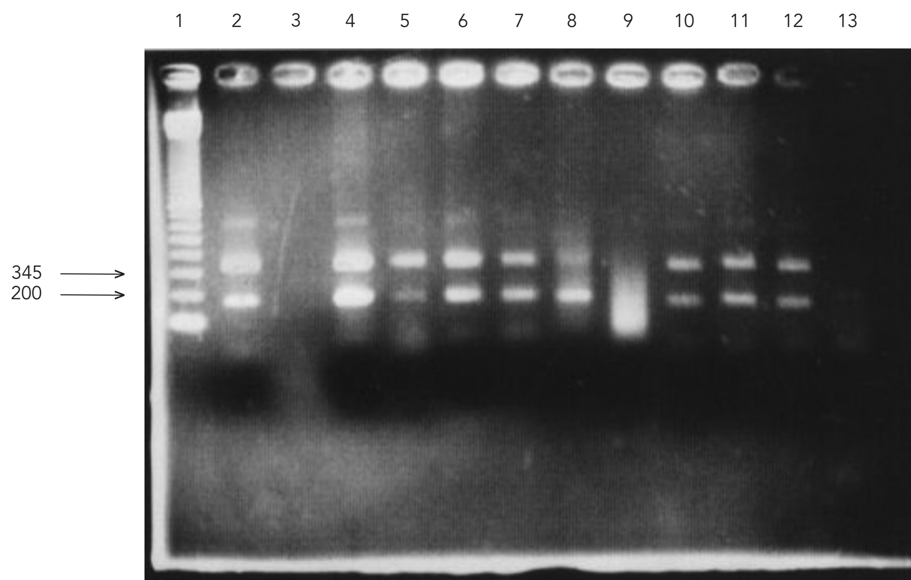
PCR electrophoresis pattern for codon 184 of the *pfmdr1* gene.



1 = 100bp size marker; 2 = 7G8 (standard resistant clone, Brazil); 3 to 13 = test samples; and 14 = sterile distilled water.

Figure 2

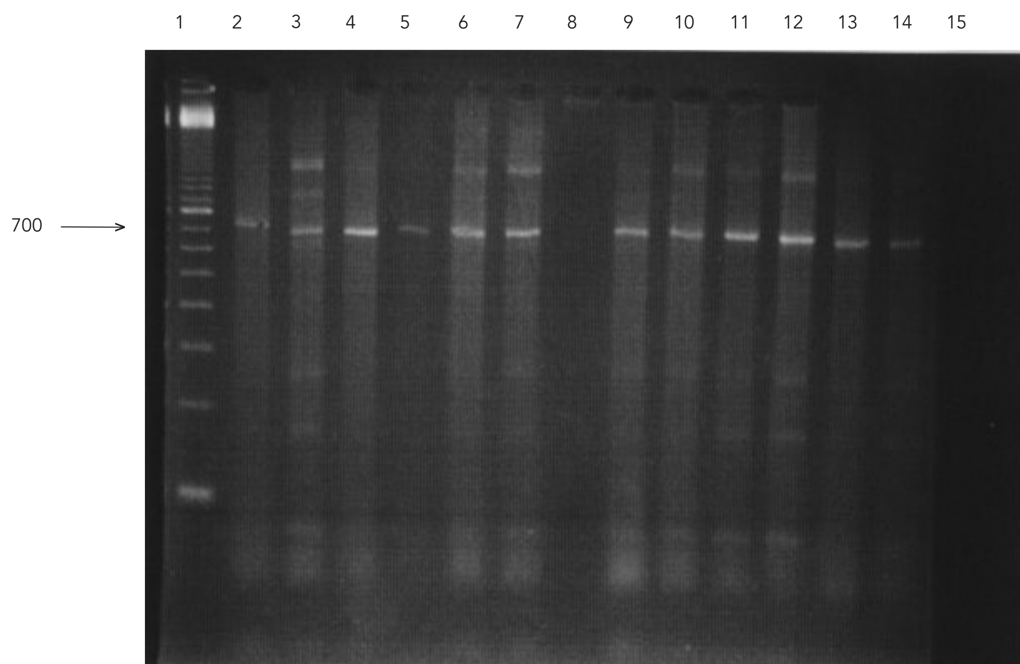
PCR electrophoresis pattern for codon 1042 of the *pfmdr1* gene.



1 = 100bp size marker; 2 = 7G8 (standard resistant clone, Brazil); 3 = blank; 4 to 12 = test samples; and 13 = sterile distilled water.

Figure 3

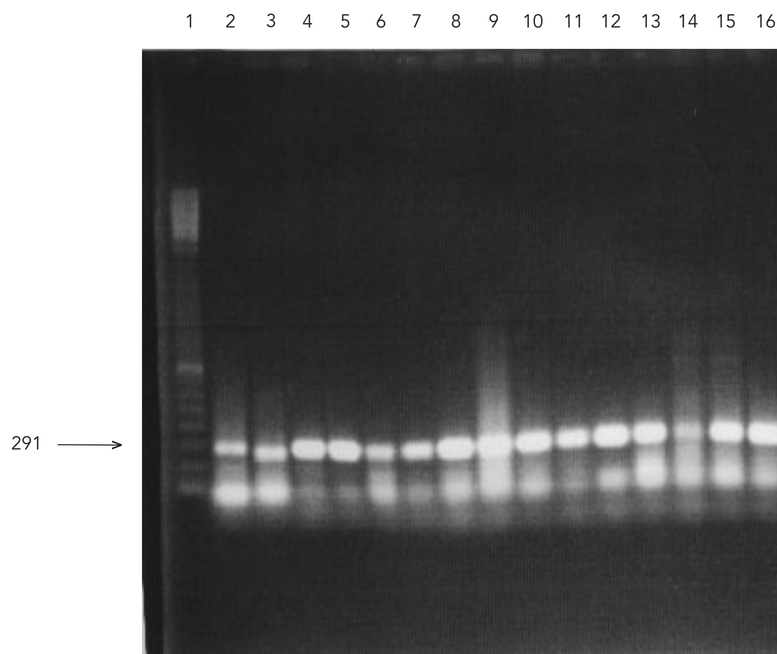
PCR electrophoresis pattern for the kappa region of the *cg2* gene.



1 = 100bp size marker; 2 = HB3 (standard sensitive clone, Honduras); 3 to 7 = test samples; 8 = blank; 9 to 14 = test samples; and 15 = sterile distilled water.

Figure 4

PCR electrophoresis pattern for gamma region of the *cg2* gene.



1 = 100bp size marker; 2 = 7G8 (standard resistant clone, Brazil); 3 = HB3 (standard sensitive clone, Honduras); and 4 to 16 = test samples.

Table 1

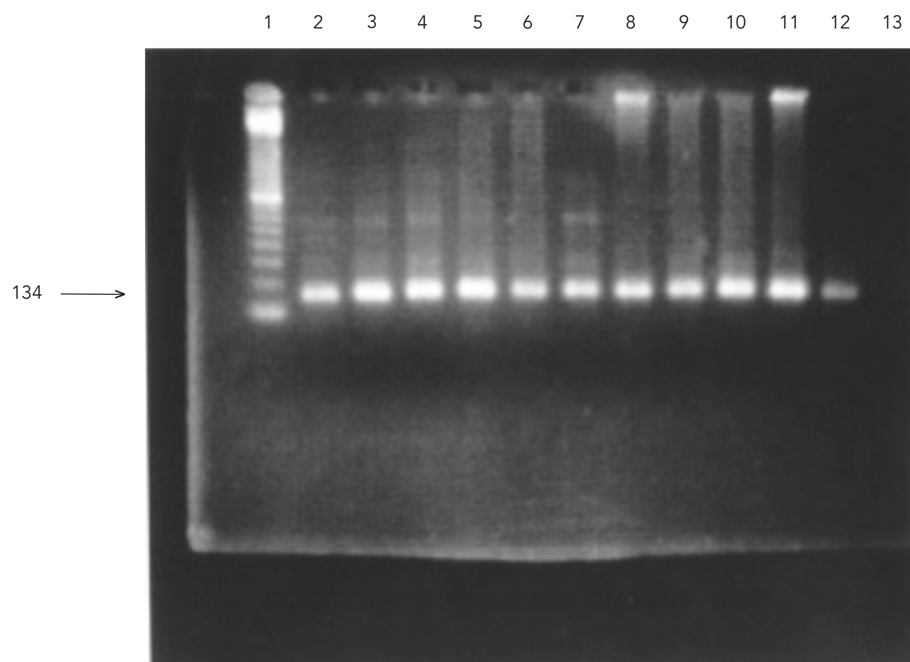
Percentage distribution of resistance associated with *pfmdr1*, *cg2*, and *pfcrt* genes in 40 samples from the Brazilian Amazon.

Genes	Porto Velho (%)	Marabá (%)	Itaiatuba (%)	Tailândia (%)
pfmdr1	100	100	100	100
cg2κ	0	0	0	0
cg2 γ	100	100	100	100
pfcrt	100	100	100	100

pfmdr1: gene 1 for multiple drug resistance in *Plasmodium falciparum*; *cg2κ*: kappa region of gene 2 for chloroquine resistance; *cg2γ*: gamma region of gene 2 for chloroquine resistance; *pfcrt*: chloroquine-resistant *Plasmodium falciparum* related to transporter.

Figure 5

RFLP electrophoresis pattern for mutation K76T on the *pfcrt* gene.



1 = 100bp size marker; 2 = 7G8 (standard resistant clone, Brazil); 3 to 12 = test samples; and 13 = sterile distilled water.

samples corroborates previous studies reporting the association between polymorphisms in these codons and *in vitro P. falciparum* chloroquine resistance in South American samples [2,5,6]. The seven samples from the municipality of Porto Velho (808/98, 809/98, 810/98, 813/98, 815/98, 816/98, and 817/98) with profile similar to K1 (standard resistant clone, Thailand), indicate that previously rare mutations in South America are now emerging on this continent [23,29].

In transfection studies, Reed et al. [32] reported that the switch from aspargine to tyrosine on codon 1246 of *pfmdr1* increased the chloroquine resistance in a clone known to be resistant and carrying the *pfcrt* mutant. The association between *pfmdr1* and *in vitro* chloroquine resistance has not been confirmed by all the studies [9,10], indicating that there are diverse chloroquine resistance mechanisms [10] and that the *pfmdr1* gene does not play a primary

role in conferring chloroquine resistance in *P. falciparum* [2,26].

In the κ (kappa) region of *cg2*, the second candidate gene for the resistance phenotype to this 4-aminoquinoline in *P. falciparum*, a 100% sensitivity pattern was found in the isolates, although studies report an association between repetitions in the kappa region and failures in sensitivity to chloroquine in samples from Africa [28,29]. These findings suggest that kappa repetitions in the *cg2* gene are not important genetic markers in isolates from the Brazilian Amazon, unlike the γ (gamma) region of this same gene, which presented a pattern similar to the 7G8 clone in the 40 study samples. Thus, this result together with that of Calvosa et al. [33], who found 100% *in vitro* chloroquine resistance in the same samples in the municipality of Marabá, suggest a significant correlation between gamma repetitions in *cg2* and *in vitro* chloroquine resistance in samples from the Brazilian Amazon.

The presence of mutations in the *pfmdr1* and *cg2* genes in the same samples suggests a selective pressure for its installation and maintenance in a given geographic area, in addition to other factors favoring this drug pressure, like the malaria transmission patterns, the degree of population immunity, population migration, pharmacokinetic factors, and indiscriminate use and/or sub-therapeutic dosage of antimalarials [20,21].

Mutations in the *pfcrt* gene are associated with *in vivo* chloroquine resistance in Africa and *in vitro* resistance in South America [2,17,21,22,23,34] and allele transfection studies with this gene, like the K76T mutation, have shown that the latter confers *in vitro* resistance to chloroquine [26,35].

In this study, 100% of the study isolates in the four municipalities in the Brazilian Amazon presented the K76T genotype, similar to the 7G8 type (standard resistant clone for Brazil). This finding agrees with the work of Vieira et al. [22], who found a high prevalence of this mutation in samples from the Brazilian Amazon, although this mutation is not absolute, since it can be found both in chloroquine-resistant samples and those sensitive to this drug, suggesting that other factors control the expression of the resistance phenotype [24,25,36]. Possible reasons for this fact may be associated with the involvement of the host response, like the immune status, which can cause negative conversion of parasitemia, regardless of whether the sample is resistant to chloroquine. In addition, the drug's absorption and individual metabolic rates can also influence the type of response to chloroquine treatment. Another potential factor is the possibility that other *pfcrt* mutations, like those in codons 72, 74, 75, 97, 144, 148, 160, 194, 220, 271, 326, 333, 356, and 371, are also associated with the resistance phenotype [34,35,37], in addition to the fact that *P. falciparum* has more transport proteins than those already known to influence the pathogen's physiology [30].

There is evidence that chloroquine resistance is a multigenic phenomenon and that the K76T mutation in the *pfcrt* gene is necessary, but not sufficient, to confer resistance [20]. Therefore, other studies are needed to evaluate the role of *pfcrt* as a reliable genetic marker for *in vivo* response to chloroquine [21].

The presence of polymorphisms in the *pfmdr1*, *cg2*, and *pfcrt* genes in the same samples indicates that these loci are important genetic markers for *in vitro* chloroquine resistance [38] and that this connection is maintained by the drug's selective pressure [19].

Conclusions

The presence of the $^{ASN}1042^{ASP}$ and $^{ASP}1246^{TYR}$ mutations in the *pfmdr1* gene in the samples from the Brazilian Amazon suggests that these codons are important markers for *in vitro* chloroquine resistance in *P. falciparum* isolates from South America; however, the κ (kappa) region of the *cg2* gene is not a relevant marker for *P. falciparum* resistance to 4-aminoquinoline in samples from the Brazilian Amazon. Meanwhile, the presence of polymorphism in the γ (gamma) region of the *cg2* gene in the study isolates suggests an association between *in vitro P. falciparum* chloroquine resistance and this mutation. The presence of the K76T mutation in the *pfcrt* gene in all the study samples suggests that this mutation is an important genetic marker for *in vitro P. falciparum* resistance to chloroquine. The identification of the mutations in the *pfmdr1*, *cg2*, and *pfcrt* loci in samples from the Brazilian Amazon suggests that they are important genetic markers for *in vitro P. falciparum* chloroquine resistance and that the chloroquine resistance phenotype in *P. falciparum* is a multigenic process, indicating a possible local clonal expansion of *P. falciparum* chloroquine resistance, selected by the indiscriminate or inadequate use of this drug in the Brazilian Amazon.

Resumo

O estudo foi desenvolvido para investigar a base molecular da resistência do Plasmodium falciparum à cloroquina em isolados da região Amazônica brasileira e identificar os polimorfismos nos códons $^{TYR}184^{PHE}$, $^{ASN}1042^{ASP}$ *e* $^{ASP}1246^{TYR}$ *do gene* pfmdr1, *as regiões* kappa *e* gamma *do gene* cg2 *e a mutação* K76T *do gene* pfcrt, *a fim de determinar a distribuição percentual dos alelos de cada gene estudado, comparando amostras de áreas geográficas distintas, utilizando a reação em cadeia da polimerase (PCR) alelo-específica para o* pfmdr1 *e a PCR e o polimorfismo do comprimento do fragmento de restrição (RFLP) para os genes* cg2 *e* pfcrt. *A amostra foi constituída de quarenta isolados de sangue humano já coletados e microscopicamente diagnosticados com malária por* P. falciparum *das localidades de Porto Velho (Rondônia) e Marabá, Itaituba e Tailândia (Pará). A distribuição percentual da resistência* in vitro *do* P. falciparum *à cloroquina nas amostras estudadas foi de 100% de resistência para os genes* pfmdr1, *região* gamma *do* cg2 *e* pfcrt. *O polimorfismo na região* kappa *do gene* cg2 *não foi encontrado nas amostras estudadas.*

Plasmodium falciparum*; Polimorfismo Genético; Cloroquina*

Contributors

G. M. R. Viana, R. L. D. Machado, and V. S. P. Calvosa collected the samples. M. M. Póvoa, G. M. R. Viana, and R. L. D. Machado participated in the tests, data analysis, discussion of the results, and drafting of the article.

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