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

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

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 pfcr 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 pfcr 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 pfcr, 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. 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. 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. In relation to chloroquine, clinical failure is already reaching nearly 100% in the majority of endemic areas in Brazil.

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. Although some studies have indicated a lack of association between the chloroquine resistance phenotype and point mutations in this gene, there is growing evidence of the role of the pfmdr1 gene in P. falciparum chloroquine resistance.
Polymorphism in the Asn86Tyr codon has been related to chloroquine resistance in *P. falciparum* isolates in Nigeria, Guinea-Bissau, Gambia, and Malaysia. An association has also been demonstrated between polymorphism in the Asn1042Asp, Asp1246Tyr, and Tyr184Phe 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 Taillândia (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 20 µL drops were added to a fiberglass membrane measuring 2.5 cm in diameter (Whatman International, Maidstone, UK), placed on Whatman filter paper measuring 5.5 cm 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 5 µL 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: 5 µL 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 2.5 µL of each specific initiator for each region of the target gene; 0.75 to 1.0 µL of each dNTP (2.5 µmol – Pharmacia Biotech) (final concentration 200µM); 0.25 µL of Taq polymerase (Biotaq
of sterile distilled water, and 4
µL of restriction endonuclease
(Kodak® Edas 290). Photographed in a photodocumentation system
under ultraviolet light (Fluo-Link, Flowgen) and
stained with ethidium bromide (5µL/mL in TBE) for 30
minutes and the bands were viewed
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 (stan-
dard 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
cloned for Brazil) in both tests (Figure 4).

The mutant allele K76T of the pfcr1 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 molec-
ular 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 TYR184PHE, ASN1042ASP,
and ASP1246TYR 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 TYR184PHE
mutation presented a resistance pattern simi-
lar to 7G8 (standard Brazilian resistant clone),
changes in this codon appear not to be corre-
lated with chloroquine resistance 29. Meanwhile
the presence of a profile similar to clone 7G8 in the
ASN1042ASP and ASP1246TYR mutations in the

For the kappa region of the cg2 gene, the endo-
nuclease 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 restric-
tion enzyme was Rca I. The PCR products with a
pattern similar to HB3 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 pfcr1 gene, the
APO 1 endonuclease was used, and the samples
with a pattern similar to 7G8 produced a frag-
ment with 134bp, and those with a pattern simi-
lar to the HB3 type produced two fragments, with
100bp and 34bp. Digestions were conducted at
65°C 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 prod-
ucts in each Eppendorf tube. The restriction frag-
ments 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).

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ASN1042ASP and ASP1246TYR 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 = blank; 4 to 12 = test samples; and 13 = 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 to 13 = test samples; and 14 = 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 \textit{pfmdr}1, cg2, and \textit{pfcrt} genes in 40 samples from the Brazilian Amazon.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Porto Velho (%)</th>
<th>Marabá (%)</th>
<th>Itaiatuba (%)</th>
<th>Tailândia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{pfmdr}1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>\textit{cg2}κ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{cg2}γ</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>\textit{pfcrt}</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>


Figure 5

RFLP electrophoresis pattern for mutation K76T on the \textit{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 \textit{in vitro} \textit{P. falciparum} chloroquine resistance in South American samples \cite{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 \cite{23,29}.

In transfection studies, Reed et al. \cite{32} reported that the switch from asparagine to tyrosine on codon 1246 of \textit{pfmdr1} increased the chloroquine resistance in a clone known to be resistant and carrying the \textit{pfcrt} mutant. The association between \textit{pfmdr1} and \textit{in vitro} chloroquine resistance has not been confirmed by all the studies \cite{9,10}, indicating that there are diverse chloroquine resistance mechanisms \cite{10} and that the \textit{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 pfcr 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 pfcr 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 pfcr gene is necessary, but not sufficient, to confer resistance 20. Therefore, other studies are needed to evaluate the role of pfcr as a reliable genetic marker for in vivo response to chloroquine 21.

The presence of polymorphisms in the pfmdr1, cg2, and pfcr 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 ASN1042ASP and ASP1246TYR 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-aminoquinolines 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 pfcr 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 pfcr 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 **TYR184PHE**, **ASN1042ASP** e **ASP1246TYR** do gene **pfmdr1**, as regiões kappa e gamma do gene cg2 e a mutação K76T do gene pfcr, 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 pfcr. 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 pfcr. O polimorfismo na região kappa do gene cg2 não foi encontrado nas amostras estudadas.

*Plasmodium falciparum; Polimorfismo Genético; Clo-roquina*

Contributors


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