MOLECULAR EPIDEMIOLOGY OF MALARIA IN YAOUNDÉ, CAMEROON. III.
ANALYSIS OF CHLOROQUINE RESISTANCE AND POINT MUTATIONS IN THE
MULTIDRUG RESISTANCE 1 (pfmdr 1) GENE OF PLASMODIUM FALCIPARUM

LEONARDO K. BASCO AND PASCAL RINGWALD

Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM) and Laboratoire de Recherches sur le Paludisme, Laboratoire Associé Francophone 302, Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC), Yaoundé, Cameroon

Abstract. It has been postulated that chloroquine resistance may be associated with a single point mutation at codon 86 of the Plasmodium falciparum multidrug resistance 1 (pfmdr 1) gene. Using a simple and rapid molecular technique involving polymerase chain reaction and restriction fragment length polymorphism, the frequency of the Asn-to-Tyr mutation associated with chloroquine resistance was established among 129 clinical isolates obtained from indigenous patients in Yaoundé, Cameroon. The results showed that 110 of 129 isolates display a mutant codon. The other clinical isolates had either a pure wild-type Asn-86 codon (n = 12) or mixed Asn/Tyr alleles (n = 7). In vitro drug assays were performed to compare the genotype and phenotype in 102 clinical isolates. Of these isolates, 86 displayed pure Tyr-86 mutant codon; 48 (56%) mutant isolates were chloroquine-resistant (50% inhibitory concentration [IC50] > 100 nM), as expected, but 38 (44%) mutant isolates were chloroquine-sensitive (IC50 < 100 nM). Three chloroquine-resistant isolates and seven chloroquine-sensitive parasites carried a wild-type Asn-86 codon. Mixed alleles were found in six isolates (four chloroquine-sensitive and two chloroquine-resistant isolates). Our results did not confirm previous observations on the possible association between chloroquine resistance phenotype and genotype based on the pfmdr 1 gene.

Malaria is a major public health problem associated with high mortality and morbidity rates in Cameroon. Yaoundé, the capital city situated in southern Cameroon, is one of the study sites where in vitro and in vivo antimalarial drug surveillance has been conducted regularly.1-4 At present, the first-line drug for the treatment of acute uncomplicated Plasmodium falciparum malaria is still chloroquine (or amodiaquine) for the indigenous population in Cameroon. Second- and third-line drugs for resistant cases are sulfadoxine-pyrimethamine and quinine, respectively.

In our recent in vitro study, it was found that 62% of the fresh clinical isolates of P. falciparum obtained from symptomatic Cameroonian patients residing in Yaoundé are resistant to chloroquine.5 The proportion of chloroquine-resistant isolates is in agreement with in vivo studies conducted in Yaoundé.6-8 A similar proportion (50–60%) of chloroquine-resistant P. falciparum isolates has been reported from other localities in southern Cameroon.6-8 Since chloroquine plays a pivotal role in malaria control programs in central and West Africa, epidemiologic surveillance of chloroquine resistance is an important means to assure continued efficacy of the drug.

Although the genetic basis of chloroquine resistance is still debated, previous studies have shown an association between chloroquine resistance in vitro and a single Asn to Tyr-86 mutation in the P. falciparum multidrug resistance 1 (pfmdr 1) gene in parasites originating from various geographic areas in Asia and Africa.9-12 Chloroquine-resistant P. falciparum strains in South America were reported to be characterized by distinct triple mutations in amino acid residues 1034, 1042, and 1246 of the pfmdr 1 gene.13 Since most previous studies have been based on few clinical isolates from diverse geographic origin, we have evaluated the correlation between mutation in codon 86 of the pfmdr 1 gene and in vitro response to chloroquine in 129 Cameroonian isolates using a simple and rapid molecular technique that is adapted for field use in Africa. The main objective of this study was to determine whether an alternative molecular technique is amenable to replace in vitro assays to follow the evolution of chloroquine resistance.

MATERIALS AND METHODS

Parasite DNA. The study was part of a clinical trial comparing the efficacy of chloroquine and pyronaridine at the Nlongkak Catholic missionary dispensary in Yaoundé.4 One hundred twenty-nine clinical isolates of P. falciparum were obtained by venipuncture before treatment from symptomatic Cameroonian patients residing in Yaoundé. The patients included both young children and adults of both sex. An aliquot of 1–1.5 ml of red blood cell pellet was used to extract parasite DNA. Informed consent was obtained from either the patients or a guardian accompanying the sick children. The study was approved by the Cameroonian National Ethics Committee.

Infected erythrocytes were suspended in 15 ml of ice-cold NET buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5) and lysed with 0.015% saponin. The lysate was centrifuged at 2,000 × g for 10 min and the pellet was transferred to a 1.5-ml microfuge tube and suspended in 500 μl of NET buffer. The mixture was treated with 1% Sarkosyl and RNase A (100 μg/ml) at 37°C for 1 hr and proteinase K (200 μg/ml) at 50°C for 1 hr. Parasite DNA was extracted three times in equilibrated phenol (pH 8), phenol-chloroform-isoamyl alcohol (1/1/1) and chloroform-isoamyl alcohol (1/1/1) and precipitated by the addition of 0.3 M sodium acetate and cold absolute ethanol. The extracted DNA was air-dried under a hood and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). Parasite DNA was stored at −20°C until use.

Polymerase chain reaction-restriction fragment length polymorphism. The technique combining the polymerase chain reaction followed by enzymatic digestion with a restriction endonuclease to detect point mutations in the pfmdr
**RESULTS**

Figure 1 presents a typical result of the polymerase chain reaction–restriction fragment length polymorphism technique. Of the 129 isolates of *P. falciparum*, 110 were characterized and found to have a mutant *pfmdr1* gene. Twelve isolates were of wild-type (Asn-86), and seven isolates displayed mixed alleles (Asn-86 plus Tyr-86). An *in vitro* drug assay was carried out with 102 isolates to determine the correlation between chloroquine IC50 values and a point mutation in codon 86. *In vitro* chloroquine sensitivity and the amino acid residue 86 of the *pfmdr1* gene from these clinical isolates are summarized in Table 1. Of the 102 isolates, 86 were carriers of pure mutant-type Tyr-86 codon. Forty-eight mutant isolates (56%) were resistant to chloroquine, as predicted by our criteria, while 38 (44%) mutant isolates were sensitive to chloroquine. Ten isolates (seven chloroquine-sensitive and three chloroquine-resistant) had a wild-type Asn-86 codon. Six isolates (four chloroquine-sensitive and two chloroquine-resistant) had mixed alleles at position 86.

**DISCUSSION**

In our previous studies, we have shown a very high correlation between *in vitro* antifolate drug sensitivity and point mutations in the dihydrofolate reductase–thymidylate synthase gene determined either by DNA sequencing or polymerase chain reaction–restriction fragment length polymorphism, which was similar to the technique used in the present study, in African isolates of *P. falciparum*. Our results suggest that molecular techniques based on polymerase chain reaction and enzymatic digestion can replace *in vitro* assays for antifolate drugs, which are second-line drugs for chloroquine-resistant infections. Similar molecular techniques may also be helpful to determine sulfadoxine sensitivity by analyzing point mutations in the dihydropteroate synthase gene. Although drug assays have been useful, there are several disadvantages for field application, including the use of radiolabeled compounds, the need for a liquid scintillation counter for interpretation of results, other costly equipment (incubator, hood), and well-trained personnel. Thus, one of the objectives of the present study in comparing the *in vitro* chloroquine sensitivity and polymerase chain reaction–restriction fragment length polymorphism was to
evaluate the utility of the latter technique to replace isotopic drug assay.

The results of the present study did not confirm the association between in vitro chloroquine resistance and Asn-to-Tyr mutation in amino acid residue 86 of the \textit{pfmdr 1} gene. There are several possible explanations. First, the direct correlation between point mutation and chloroquine resistance may be dependent on the geographic origin of the parasites. The presence of a distinct genetic profile in South American isolates supports this hypothesis.9 A further argument that tends to substantiate this possibility is provided by studies using \textit{P. falciparum} isolates from Sudan, The Gambia, and India, and multidrug-resistant strains from southeast Asia that have also questioned the direct correlation between point mutations and chloroquine sensitivity.25–30 If this hypothesis is plausible, it may be argued that the majority of clinical isolates in Yaoundé are characterized by a mutant-type \textit{pfmdr 1} gene, possibly without any relation to chloroquine resistance. Second, a number of previous studies that have reported an association between Tyr-86 codon and chloroquine resistance have not included a comparable proportion of chloroquine-sensitive and chloroquine-resistant parasites for statistical comparison.9,28,30 Analysis of such data may lead to a biased conclusion. In the present study, 49 chloroquine-sensitive isolates were compared with 53 chloroquine-resistant isolates, which equilibrates the proportion of sensitive and resistant parasites. Moreover, the clinical isolates were representative of a single urban site, rather than collected from widely separated geographic areas. The sampling distribution of the parasites used in our study may possibly have corrected some of the biases in the previous studies.9,10 Third, another genetic mechanism involving amplification of the \textit{pfmdr 1} gene and overexpression of its protein product may underlie chloroquine and/or mefloquine resistance.14,25,31–39 So far, experimental demonstration has been mostly limited to a few laboratory strains and field isolates adapted to culture, and there are insufficient data on fresh clinical isolates to support the third hypothesis. Fourth, although the expression product of the \textit{pfmdr 1} gene has been shown to be involved in substrate transport process,40–43 Wellems and others have provided evidence that another chloroquine resistance gene may be present.44,45 This gene, designated cg2, has been isolated, cloned, and sequenced recently.46 The expression product of this gene may or may not interact with the \textit{pfmdr 1} protein. Further studies are needed to determine whether the \textit{pfmdr 1} gene is directly or indirectly involved in chloroquine resistance.

The molecular technique described in this paper is relatively easy and rapid to perform. However, the relevance of Tyr-86 allele as a reliable genetic marker of \textit{in vivo} chloroquine resistance has not yet been established. Two recent clinical studies have attempted to correlate phenotype (\textit{in vivo} chloroquine sensitivity) and genotype (Tyr-86 allele of the \textit{pfmdr 1} gene).47,48 The results of the present paper suggest that the determination of \textit{pfmdr 1} sequence does not accurately predict \textit{in vitro} chloroquine sensitivity in clinical isolates obtained in Yaoundé. Our \textit{in vitro} observation extends to discordant results between chloroquine sensitivity predicted by the Asn-86 allele (or chloroquine resistance predicted by the Tyr-86 allele) and \textit{in vivo} response to chloroquine treatment in Cameroonian patients.47 Although Duraisingh and others have suggested that the Tyr-86 allele may

### Table 1

Comparison between in vitro chloroquine sensitivity of Cameroonian \textit{Plasmodium falciparum} isolates and point mutation in the \textit{P. falciparum} multidrug resistance 1 (\textit{pfmdr 1}) gene

<table>
<thead>
<tr>
<th>Chloroquine sensitivity</th>
<th>Wild-type allele (Asn)</th>
<th>Mixed allele (Asn + Tyr)</th>
<th>Mutant allele (Tyr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>7</td>
<td>4</td>
<td>38</td>
</tr>
<tr>
<td>Resistant</td>
<td>3</td>
<td>2</td>
<td>48</td>
</tr>
</tbody>
</table>

* Number of isolates that are chloroquine-sensitive (50% inhibitory concentration [IC\textsubscript{50}] < 100 nM) or chloroquine-resistant (IC\textsubscript{50} > 100 nm).

"In vitro" chloroquine sensitivity and chloroquine resistance were associated with wild-type Asn-86 allele and mutant Tyr-86 allele of the \textit{pfmdr 1} gene by Foote and others, respectively.9
be associated with RII/RIII chloroquine resistance and may select chloroquine-resistant parasites in RI resistance, their conclusion was based on a limited number of cases that were not matched with patients cured with chloroquine therapy, who would have been expected to be infected with parasites carrying Asn-86 allele if their conclusion were correct.48 In their study, selection of parasites with the Tyr-86 allele was inferred, rather than demonstrated. Thus, the clinical relevance of the Tyr-86 allele needs further evaluation using pretreatment (and recrudescent) blood samples from patients who are cured and those who present recrudescent parasitaemia after chloroquine therapy.

At present, contradictory findings reported in other studies seem to preclude the use of molecular techniques with the pfmdr1 gene in drug resistance and identify the chloroquine resistance gene. By simple and rapid molecular techniques needs to await the future studies to clarify the eventual role of the pfmdr1 gene in drug resistance and identify the chloroquine resistance gene.

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Authors’ address: Leonardo K. Basco and Pascal Ringwald, Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM) and Laboratoire de Recherches sur le Paludisme, Laboratoire Associé Francophone 302, Organisation de Coopération pour la lutte contre les Endémies en Afrique Centrale (OCEAC), BP 288, Yaoundé, Cameroon.

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