

## 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*

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**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 [ $IC_{50}$ ] > 100 nM), as expected, but 38 (44%) mutant isolates were chloroquine-sensitive ( $IC_{50} < 100$  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.<sup>1-4</sup> 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.<sup>3</sup> The proportion of chloroquine-resistant isolates is in agreement with *in vivo* studies conducted in Yaoundé.<sup>2,4</sup> A similar proportion (50-60%) of chloroquine-resistant *P. falciparum* isolates has been reported from other localities in southern Cameroon.<sup>5-8</sup> 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.<sup>9-12</sup> 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.<sup>9</sup> 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.<sup>4</sup> 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  $2,000 \times g$  for 10 min and the pellet was transferred to a 1.5-ml microfuge tube and suspended in 500  $\mu$ l of NET buffer. The mixture was treated with 1% Sarkosyl and RNase A (100  $\mu$ g/ml) at 37°C for 1 hr and proteinase K (200  $\mu$ g/ml) at 50°C for 1 hr. Parasite DNA was extracted three times in equilibrated phenol (pH 8), phenol-chloroform-isoamyl alcohol (v/v/v/ 25:24:1) and chloroform-isoamyl alcohol (v/v 24:1) 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*



*l* gene was first described by Freaux and others.<sup>13</sup> A 609-basepair DNA fragment spanning codon 86 of the *pfmdr 1* gene was amplified by the polymerase chain reaction using the PTC-100 thermal cycler (MJ Research, Watertown, MA) under the following conditions: approximately 200 ng of DNA, 15 pmol of primers 5'-AGAGAAAAAGA-TGGTAACCTCAG-3' (sense) and 5'-ACCACAAACA-TAAATTAACGG-3' (anti-sense), buffer (50 mM KCl, 10 mM Tris, pH 8.4, 1.5 mM MgCl<sub>2</sub>, 200 μM dNTP, and one unit of *Taq* DNA polymerase in a 50-μl reaction at 94°C for 5 min for the first cycle and 1 min in subsequent cycles, 50°C for 5 min for the first cycle and 1 min in subsequent cycles, and 72°C for 5 min for the first cycle and 2 min in subsequent cycles, for a total of 30 cycles. The synthetic oligonucleotide primers were designed based on the complete sequence of the gene published by Foote and others.<sup>14</sup>

The quantity of the polymerase chain reaction products was adjusted to 2–3 μg based on optical density reading by spectrophotometer to ensure complete enzymatic digestion. The amplified fragment was treated with restriction endonuclease *Nsp I* for 3 hr at 37°C. At the end of the incubation period, the restriction enzyme was heat inactivated. The digested DNA fragments were resolved by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet transillumination.

The presence of a mutant Tyr-86 codon is indicated by digestion of the polymerase chain reaction products into two fragments (238 and 371 basepairs). Since the Asn-86 codon is not a restriction site, the 609-basepair fragment remains undigested if wild-type codon is present. If an isolate carries mixed alleles, three bands (one undigested fragment plus two digested fragments) are observed after agarose gel electrophoresis. In the latter case, enzymatic digestion was repeated with increased quantity of restriction enzyme for a longer incubation period to ensure that the undigested fragment originated from mixed alleles. The wild type Asn-86 residue in the *pfmdr 1* gene is associated with chloroquine sensitivity, while the mutant Tyr-86 is associated with chloroquine resistance.<sup>9</sup>

***In vitro* drug sensitivity assay.** *In vitro* assay was performed for 102 of 129 isolates. Twenty-seven blood samples were not tested for drug sensitivity assay due to positive urine test indicating recent intake of antimalarial drugs.<sup>15</sup> Infected erythrocytes were washed three times in RPMI 1640 medium and suspended in the complete RPMI 1640 medium consisting of 10% human serum (obtained from European blood donors without previous history of malaria), 25 mM HEPES, and 25 mM NaHCO<sub>3</sub>, at a hematocrit of 1.5% and an initial parasitemia ranging between 0.1% and 1.0%. If the blood sample had a parasitemia > 1.0%, fresh, uninfected, type A<sup>+</sup> erythrocytes were added to adjust the parasitemia to 0.6%.

The isotopic semi-microtest used in this study was described in our previous study.<sup>3</sup> Seven hundred microliters of the suspension of infected erythrocytes were distributed in each well of the 24-well tissue culture plates. The parasites were incubated at 37°C in 5% CO<sub>2</sub> for 18 hr. <sup>3</sup>H-hypoxanthine (specific activity = 5 Ci/mmol, 1 μCi/well; Amersham International, Plc., Buckinghamshire, United Kingdom) was added to assess parasite growth. After an additional 24 hr of incubation, the plates were frozen to terminate the *in vitro*

drug sensitivity assay. The plates were thawed, and the contents of each well were collected on glass-fiber filter papers, washed, and dried using a cell harvester. The filter disks were transferred into scintillation tubes, and 2 ml of scintillation cocktail (Organic Counting Scintillant®; Amersham International PLC) were added. The incorporation of <sup>3</sup>H-hypoxanthine was quantitated using a liquid scintillation counter (Wallac 1410; Pharmacia, Uppsala, Sweden).

The 50% inhibitory concentration (IC<sub>50</sub>) values, defined as the drug concentration corresponding to 50% of the uptake of <sup>3</sup>H-hypoxanthine measured in the drug-free control wells, were determined by linear regression analysis of logarithm of concentrations plotted against the logit of growth inhibition. The threshold IC<sub>50</sub> value for *in vitro* resistance to chloroquine was estimated to be > 100 nM.<sup>16</sup>

## 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 *pfmdr 1* 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 IC<sub>50</sub> values and a point mutation in codon 86. *In vitro* chloroquine sensitivity and the amino acid residue 86 of the *pfmdr 1* 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*.<sup>17–20</sup> 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.<sup>21–24</sup> 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

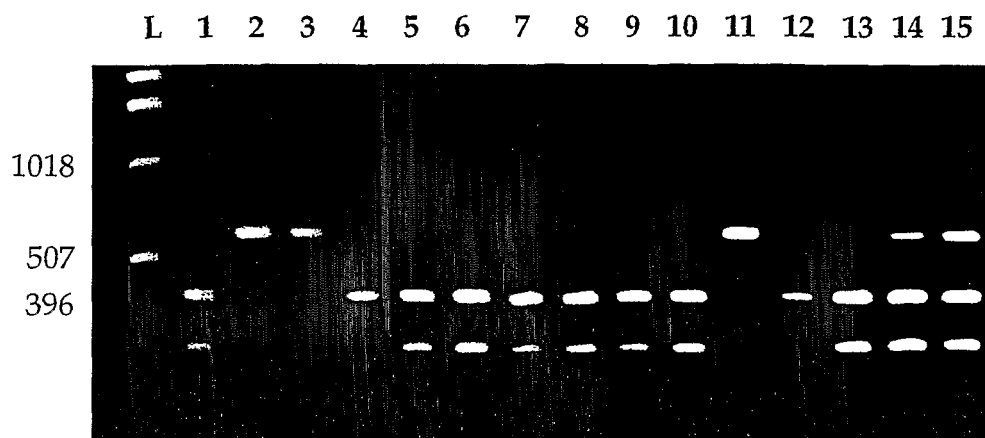


FIGURE 1. Typical example of a polymerase chain reaction–restriction fragment length polymorphism profile of the *Plasmodium falciparum* multidrug resistance (*pfmdr 1*) gene obtained using *Nsp I* endonuclease. Agarose gel electrophoresis followed by staining with ethidium bromide and ultraviolet transillumination reveals two cleaved fragments of the *pfmdr 1* gene (238 and 371 basepairs) if a given *P. falciparum* isolate is characterized by the presence of the mutant Tyr-86 codon previously associated with chloroquine resistance.<sup>9</sup> The 609-basepair bands represent undigested DNA fragment in isolates characterized by wild-type Asn-86 codon associated with chloroquine sensitivity. Numbers over each lane correspond to different clinical isolates. Lane L, 1-kilobase DNA ladder. Values on the left are in basepairs.

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 *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.<sup>9</sup> A further argument that tends to substantiate this possibility is provided by studies using *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.<sup>25-30</sup> If this hypothesis is plausible, it may be argued that the majority of clinical isolates in Yaoundé are characterized by a mutant-type *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.<sup>9,28,30</sup> 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.<sup>9,10</sup> Third, another genetic mechanism involving amplification of the *pfmdr 1* gene and overexpression of its protein product may underlie chloroquine and/or mefloquine resistance.<sup>14,25,26,31-39</sup> 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 *pfmdr 1* gene has been shown to be involved in substrate transport process,<sup>40-43</sup> Wellems and others have provided evidence that another chloroquine resistance gene may be present.<sup>44,45</sup> This gene, designated *cg2*, has been isolated, cloned, and sequenced recently.<sup>46</sup> The expression product of this gene may or may not interact with the *pfmdr 1* protein. Further studies are needed to determine whether the *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 *in vivo* chloroquine resistance has not yet been established. Two recent clinical studies have attempted to correlate phenotype (*in vivo* chloroquine sensitivity) and genotype (Tyr-86 allele of the *pfmdr 1* gene).<sup>47,48</sup> The results of the present paper suggest that the determination of *pfmdr 1* sequence does not accurately predict *in vitro* chloroquine sensitivity in clinical isolates obtained in Yaoundé. Our *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 *in vivo* response to chloroquine treatment in Cameroonian patients.<sup>47</sup> Although Duraisingh and others have suggested that the Tyr-86 allele may

TABLE I

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

Chloroquine sensitivity†	Amino acid residue 86 of the <i>pfmdr 1</i> gene*		
	Wild-type allele (Asn)	Mixed allele (Asn + Tyr)	Mutant allele (Tyr)
Sensitive	7	4	38
Resistant	3	2	48

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

† *In vitro* chloroquine sensitivity and chloroquine resistance were associated with wild-type Asn-86 allele and mutant Tyr-86 allele of the *pfmdr 1* gene by Foote and others, respectively.<sup>9</sup>

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.<sup>48</sup> 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 recrudescence) blood samples from patients who are cured and those who present recrudescence parasitemia after chloroquine therapy.

At present, contradictory findings reported in other studies seem to preclude the use of molecular techniques with the *pfmdr 1* gene as the molecular target to monitor chloroquine sensitivity in an epidemiologic survey.<sup>9-12,25-30</sup> While we expect to replace *in vitro* assays with molecular techniques for monitoring pyrimethamine and sulfadoxine resistance in *P. falciparum*,<sup>20,21</sup> the determination of chloroquine resistance by simple and rapid molecular techniques needs to await further studies to clarify the eventual role of the *pfmdr 1* gene in drug resistance and identify the chloroquine resistance gene.

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