MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON. XIII. ANALYSIS OF PFCRT MUTATIONS AND IN VITRO CHLOROQUINE RESISTANCE

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Abstract The key Lys76Thr amino-acid substitution in Plasmodium falciparum chloroquine-resistance transporter (PfCRT) has been shown to be a reliable marker associated with chloroquine-resistant phenotype in reference clones, but few discordant results have been observed in field isolates. To further examine the relationship between in vitro chloroquine response and PfCRT alleles, the entire exon 2 of the pfcrt gene of 157 Cameroonian isolates was sequenced. All isolates were characterized as having either Cys-72, Met-74, Asn-75, and Lys-76 (wild-type alleles), Cys-72, Ile-74, Glu-75, and Thr-76 (mutant alleles), or mixed alleles. The hypothetical threshold 50% inhibitory concentration (IC50) set at 100 nM distinguished between isolates carrying the wild-type alleles and those with mutant alleles in a large majority of cases (135 of 139 isolates with unmixed pfcrt alleles). Isolates presenting discordant results generally had IC50s within an intermediate range. In vitro chloroquine response of isolates with mixed pfcrt alleles was highly variable. Although discordant results between chloroquine-resistant phenotype and pfcrt alleles were not explained by the immediate adjacent codons, the key Lys76Thr codon may prove to be a highly reliable genetic marker for the epidemiologic monitoring of chloroquine resistance by means of molecular techniques.

INTRODUCTION

Studies on genetic mechanisms underlying chloroquine resistance in Plasmodium falciparum had not yielded consistent data over more than a decade, during which at least two major candidate genes, P. falciparum multidrug resistance gene 1 (pfmdr1) and cg2, have drawn the attention of malaria investigators but have subsequently been considered to play, at best, a secondary role. Recent experimental studies on laboratory-adapted P. falciparum clones and transfected parasites have suggested that P. falciparum chloroquine-resistance transporter (PICRT) may play a major role in determining chloroquine-resistant phenotype. In vitro chloroquine response of a panel of reference clones and strains from various geographic origins was perfectly correlated with the key Lys-to-Thr substitution at position 76. Subsequent in vitro studies using field isolates showed high but imperfect correlation between chloroquine response and the key pfcrt mutation. In a Brazilian study, all 30 isolates were resistant in vitro to chloroquine and characterized to have the key Thr-76 pfcrt mutation, but the study did not include any chloroquine-sensitive isolates for comparison.

pfcrt is a 13-exon gene in which eight codons differ between the chloroquine-sensitive HB3/Honduras and the chloroquine-resistant Dd2/Indochina reference clones. Of these codons, Lys76Thr and Ala220Ser substitutions have been consistently observed in all chloroquine-resistant laboratory-adapted strains. pfcrt polymorphisms found in laboratory-adapted strains suggest that the other amino-acid substitutions at positions 74, 75, 271, 326, 356, and 371 are associated with chloroquine-resistant phenotype to a varying degree, possibly in relation to the geographic origin of the parasites. To further evaluate the relationship between in vitro chloroquine resistance and pfcrt polymorphism, DNA sequence of exon 2, containing three of eight codon differences between resistant and sensitive reference clones (amino-acid residues 74, 75, and 76), was determined and compared with the in vitro response of clinical isolates in this study. An additional codon (amino-acid residue 72), which may undergo mutation in South American strains and clones, lies within this exon. The other codons that lie outside exon 2 (220, 271, 326, 356, and 371) were not examined in this study.

MATERIALS AND METHODS

P. falciparum isolates. Venous blood samples were obtained after consent from old children aged > 12 years old and adults spontaneously consulting the Nlongkak Catholic missionary dispensary, Yaoundé, in 2000–2001 if the following criteria were met: signs and symptoms of acute uncomplicated malaria, presence of P. falciparum at a parasitemia > 0.1% without other Plasmodium species, and denial of recent self-medication with an antimalarial drug as confirmed by the Saker-Solomon's urine test. Young children (age < 12 years old), pregnant women, and patients with signs and symptoms of severe and complicated malaria were excluded. A large majority of patients were treated with oral amodiaquine, which is officially recommended as an alternative first-line drug by the Cameroonian Ministry of Public Health. Patients with repeated vomiting were given quinine by parenteral route, followed by sulfadoxine-pyrimethamine. The patients were followed by the dispensary staff to ensure parasite and fever clearance on or before day 4. This study was approved by the Cameroonian National Ethics Committee and Cameroonian Ministry of Public Health.

In vitro drug sensitivity assay. Stock solution and two-fold dilutions of chloroquine sulfate (Aventis; Antony, France) were prepared in sterile distilled water. Culture plates were precoated with dilutions (final concentrations ranging from 25–1,600 nM) in triplicate and dried. Samples were washed with RPMI 1640 culture medium three times by centrifugation within 2 h after blood extraction. The technical procedures of the in vitro isotopic microtest and calculation of 50% inhibitory concentrations (IC50) were described previously.

Polymerase chain reaction and DNA sequencing. The nested polymerase chain reaction protocol for amplifying the entire exon 2 of pfcrt gene was described in previous studies. The secondary amplified product was purified by using the High Pure PCR Purification kit (Roche Diagnostics; Meylan, France). The 145-base pair product was marked with
fluorescent nucleotides in the following mixture: 200 ng of amplified product, 4 μL of Terminator Ready Reaction Mix (Perkin Elmer Corp.; Les Ulis, France), 80 mM Tris-HCl (pH 9.0), 2 mM MgCl₂, 3.2 pmol of primer 5'-TGTGCTCATGTTTTAACTT-3', in a final volume of 20 μL. The PTC-100 thermal cycler was programmed as follows: 96°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min, for 25 cycles. Residual dye terminators were removed by ethanol precipitation method. The extension product was sequenced by using the ABI Prism automated DNA sequencer (Perkin Elmer).

**Data interpretation.** Based on the determination of the *in vitro* chloroquine response of isolates obtained from young Cameroonian children aged < 5 years failing to respond to chloroquine treatment, the threshold IC₅₀ value for chloroquine resistance was fixed at ≥ 100 nM. The following alternative amino-acid residues have been found in reference clones and strains: Cys or Ser at position 72, Met or Ile at position 74, Asn or Glu at position 75, and Lys or Thr at the key position 76. Wild-type *pfcrtd* was defined as the presence of Cys-72, Met-74, Asn-75, and Lys-76, as in the chloroquine-sensitive reference clone HB3/Honduras. Mutant *pfcrtd* was defined as the substitution with the following amino acids present in the chloroquine-resistant reference clone Dd2/Indochina: Cys-72, Ile-74, Glu-75, and Thr-76. Some reference strains have been characterized with combinations of amino acid residues at positions 72, 74, 75, and 76 that are different from those described above.

**RESULTS**

A total of 157 consecutive samples obtained in 2000–2001 were analyzed. Based on the threshold of chloroquine IC₅₀ ≥ 100 nM, 62 (39%) isolates were chloroquine sensitive (geometric mean IC₅₀, 34.7 nM; range, 14.6—93.1 nM), and 95 (61%) were chloroquine resistant (geometric mean IC₅₀, 216 nM; range, 102—714 nM). DNA sequencing showed that all isolates had either the wild-type allelic combination Cys-72, Met-74, Asn-75, and Lys-76 or the mutant allelic combination Cys-72, Ile-74, Glu-75, and Thr-76. No new mutation or combination of mutations was found in exon 2. Among isolates with IC₅₀ < 100 nM, 46 had the characteristic wild-type Cys-72, Met-74, Asn-75, and Lys-76 combination, three (IC₅₀ 73.0, 83.0, and 93.0 nM) had the mutant Cys-72, Ile-74, Glu-75, and Thr-76 combination, and 13 were mixed alleles (Figure 1). The three highest IC₅₀ values among these isolates with IC₅₀ < 100 nM and the wild-type *pfcrtd* alleles were 61.1, 63.7, and 87.7 nM. For isolates with chloroquine IC₅₀ ≥ 100 nM, 89 of 95 (94%) were mutants (Ile-74, Glu-75, and Thr-76), only one isolate (IC₅₀ 143 nM) was wild-type, and five were mixed. It

![Fig. 1](https://example.com)
was evident by visual inspection of trace peaks of electropherograms that mixed alleles consisted of two alternative nucleotide bases in all three codons 74, 75, and 76.

**DISCUSSION**

In our initial study on *pfcrt* characterized in 111 isolates collected during 1994–1998, the key *pfcrt* codon at position 76 was highly correlated with chloroquine IC\(_{50}\) but 10 isolates (9%) presented discordance between *in vitro* chloroquine response and *pfcrt*. Most of these discordant isolates had chloroquine IC\(_{50}\) that were within the intermediate range (48–125 nM) and/or were multiple infections, as determined by the number of detectable polymorphic merozoite surface antigen (*msa*) genes 1 and 2. Mixed parasite populations within a given isolate, with possibly different levels of chloroquine sensitivity, occur commonly in malaria endemic regions where transmission is intense and continuous. In our study site, 57% of symptomatic patients are infected with multiple parasite populations. The *in vitro* response of mixed parasites may be sensitive, resistant, or intermediate, and based on the chloroquine IC\(_{50}\) value alone, it cannot be deduced whether a given isolate consisted of heterogeneous parasite populations. This is one of the methodologic limits of studies using naturally occurring *P. falciparum* isolates without prior adaptation to continuous *in vitro* culture and parasite cloning.

Another possible explanation for discordance observed in few isolates between the key *pfcrt* Lys76Thr allele and *in vitro* chloroquine response may lie in the influence of other *pfcrt* alleles on the chloroquine-resistant phenotype. This hypothesis was based on the observation that some reference strains and clones of *P. falciparum* have been characterized by allelic combinations that differ from those of the reference clones HB3/Honduras and Dd2/Indochina. This possibility has been partially ruled out by our results, which show that three neighboring codons (72, 74, and 75) of the key codon 76 are of one of two possible sets in naturally occurring clinical isolates in Yaoundé, with Cys-72 being invariable, as in the reference clones HB3/Honduras and Dd2/Indochina. This observation may be extended to *P. falciparum* isolates from other parts of Cameroon, which also display either one of the two possible allelic combinations at positions 74, 75, and 76. However, the exact role of other codons lying outside exon 2 was not examined in the present study.

Our results revealed the high accuracy (97%) with which the key Lys76Thr amino-acid substitution can predict the *in vitro* chloroquine response. Of 139 consecutive samples (excluding those with mixed *pfcrt* alleles), only 4 had discordant IC\(_{50}\) values as compared with *pfcrt* alleles. Three of these discordant values were within the gray area, i.e., within the range of 70–100 nM. The possible origins of an intermediate response include mixed parasite populations, presence of trace amounts of antimalarial drugs (or antibiotics) in the blood sample, inadequate *in vitro* parasite growth, transformation into gametocytes, or laboratory artifact. Likewise, undetected mixed *pfcrt* alleles and/or an amplification or sequencing error may account for the single discordant case among chloroquine-resistant isolates, presenting chloroquine IC\(_{50}\) of 143 nM and wild-type *pfcrt* allele.

In another study, it has been suggested that the threshold IC\(_{50}\) value for chloroquine resistance may be 60 nM, instead of 80 nM, using the isotopic semimicrotest. In that study, *pfcrt* alleles and *in vitro* response to chloroquine, with the threshold IC\(_{50}\) set at 80 nM, were associated in 87% of isolates originating from various geographic areas and imported by nonimmune travelers. However, in their study infected blood samples were stored up to three days before the *in vitro* assay was performed, which is expected to kill part of the parasites, favoring a selection of the most biologically fit parasite populations. Furthermore, many nonimmune individuals traveling to malaria endemic areas take chemoprophylactic measures, and many of those who return to Europe with malaria represent cases of prophylactic failure. Even a trace amount of residual antimalarial drugs will influence the *in vitro* sensitivity pattern, introducing an important bias in *in vitro* results. Babiker et al. and Chen et al. used the World Health Organization microtest to correlate *pfcrt* mutations and *in vitro* response, the results of which cannot be compared with isotopic *in vitro* assays.

Despite minor discrepancies between the *in vitro* chloroquine response and *pfcrt* alleles, this molecular marker seems to be a promising tool for the epidemiologic surveillance of chloroquine resistance. Although it cannot be ruled out definitively at present that there may be a possible influence on chloroquine-resistant phenotype exerted by five additional codons that differ between the reference clones HB3/Honduras and Dd2/Indochina and that were not examined in our study, our data suggest that, at least in Cameroon, the determination of a single nucleotide base in codon 76, either by allele-specific nested polymerase chain reaction or restriction endonuclease, provides useful data. This approach would facilitate the technical aspect of monitoring drug resistance by molecular techniques.

The potential importance of *pfcrt* to explain, at least partially, chloroquine resistance has been further emphasized by clinical studies that have suggested selection of parasites with mutant *pfcrt* alleles after chloroquine treatment. While more investigations are required to find a reliable means to predict therapeutic failure by analyzing molecular markers of pretreatment isolates, *pfcrt* seems to be an important component, or one of the components, involved in chloroquine-resistant phenotype of *P. falciparum*.

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