IN VITRO CHLOROQUINE SUSCEPTIBILITY AND PCR ANALYSIS OF PFCRT AND PFMDDR1 POLYMORPHISMS IN PLASMODIUM FALCIparUM ISOLATES FROM SENEGAL

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Abstract. Chloroquine resistance has been linked to mutations in the pfcrt and pfmdr1 genes of Plasmodium falciparum. To estimate the prevalence of the pfcrt K76T, pfmdr1 N86Y, and pfmdr1 D1246Y polymorphisms, isolates of P. falciparum from Senegal, West Africa, were analyzed, and the results were compared to in vitro chloroquine susceptibility. By the in vitro DELI test, 31% of these samples were resistant to chloroquine. Polymerase chain reaction-based assays and confirmatory sequencing demonstrated the pfcrt T76, pfmdr1 Y86, and pfmdr1 Y1246 alleles in 79%, 31%, and 2% of the isolates, respectively. All three mutant alleles were present in both in vitro susceptible and resistant isolates. On the basis of these findings, it appears that these molecular markers are not consistently predictive of in vitro chloroquine resistance in Senegal.

INTRODUCTION

Plasmodium falciparum accounts for a significant portion of all-cause morbidity and mortality in sub-Saharan Africa. The rapid spread of chloroquine resistant strains of P. falciparum has hampered efforts to reduce mortality. In a 12-year prospective study in Senegal, deaths caused by malaria among children increased by as much as 5.5-fold after the arrival of chloroquine resistance.1

Chloroquine-resistant P. falciparum in Senegal was first reported in Dakar in 1988, and in vivo resistance was reported in other regions of the country shortly afterward.2,3 In 1997 Sokhna et al.4 reported in vivo chloroquine resistance in 14% to 50% of isolates from various regions of Senegal.

Because chloroquine remains the first-line antimalarial agent in much of Africa, rapid and field-applicable methods for monitoring rates of resistance are essential for morbidity and mortality control. In vivo methods of detecting resistance are logistically difficult to conduct on a population basis, and host immunity can falsely lower estimates of resistance.5 In vitro methods such as the isotopic microtest and the double-site enzyme-linked lactate dehydrogenase enzyme immuno-detection (DELI) test provide alternative means of assessing the prevalence of drug resistance in field settings.6,7 Another rapid and feasible approach to monitoring the emergence and progress of antimalarial drug resistance is through the use of molecular markers of resistance.

The utility of such molecular markers is exemplified by the use of polymorphisms in the dihydrofolate reductase (dhfr) and dihydropteroate synthetase (dhps) genes as correlates of in vitro and in vivo resistance to the combination of pyrimethamine and sulfadoxine (Fansidar).8,9 Pyrimethamine and sulfadoxine act by binding and inhibiting dhfr and dhps, respectively.10,11 Resistance to each drug is conferred by point mutations at amino acid positions 86 and 1246 of the dhps gene, belonging to the adenosine triphosphate-binding cassette family, are possibly associated with this mechanism of resistance.12,13 How-

MATERIALS AND METHODS

Patients. This study was approved by the Institutional Review Boards of the Cheikh Anta Diop University in Dakar and the Harvard School of Public Health in Boston. Patients were recruited from the local outpatient health clinic in Pikine, a suburb of Dakar. Malaria in this region is hypoen-

demic with intense seasonal transmission between August and December. Sample collection was performed during August and September 2000. Criteria for participation in the study were age of 5 years or more; symptoms suggestive of malaria, including fever, chills, and headache; and a positive blood smear for P. falciparum. Exclusion criteria were pregnancy, severe or complicated malaria as defined by the World
Health Organization, or history of recent treatment with antimalarials.27

The number of parasites per microliter of blood was calculated by comparing the number of infected erythrocytes per 1,000 leukocytes with the average white blood cell count (8,000/µL for children younger than 14 years of age and 6,000/µL for adults). Samples were considered negative if no parasites were detected after complete examination of the Giemsa-stained thick smear.

Forty-four patients with *P. falciparum* infection were invited to participate in the study. In addition, seven patients with negative blood smears were randomly selected to participate in the study as controls. Control samples were processed in the same manner as *P. falciparum*-positive samples for in vitro drug susceptibility testing and DNA extraction and subsequent molecular analyses.

After obtaining informed consent, 10 mL of venous blood were drawn from each patient. All *P. falciparum*-infected persons received quinine, and controls received appropriate treatment for the conditions diagnosed.

**In vitro chloroquine susceptibility testing.** Blood samples were collected in edetic acid (EDTA)-coated Vacutainer tubes (Becton Dickinson & Co., Franklin Lakes, NJ). From each sample, 5 mL were retained for in vitro chloroquine susceptibility testing, and the remainder was used for DNA extraction. Samples were washed to remove serum and white blood cells. In vitro chloroquine sensitivity assays were performed in duplicate on all the isolates using the DELI assay.7

Infected erythrocytes were diluted in RPMI medium supplemented with Albumax (Gibco BRL, Grand Island, NY) and 1 mg/L hypoxanthine (Sigma, St. Louis, MO) to obtain a hematocrit of 2% and a parasitemia of 0.5% to 1%. For chloroquine sensitivity testing, 200 μL of the suspension of erythrocytes was added to each well of a 96-well plate containing different concentrations of chloroquine sulfate (Rhône Poulenc Rorer, Vitry, France). Parasites were allowed to grow at 37°C in a candle jar for 48 hours and then frozen at −20°C.

To determine in vitro susceptibility to chloroquine, the DELI assay, as previously described by Moreno et al.7 was used. Parasites were lysed by freezing and thawing the plates three times. One hundred microliters of each erythrocyte suspension were added to a 96-well plate coated with the primary anti-pLDH monoclonal antibody, 17E4.25 Plates were incubated at 37°C for 1 hour, washed with 1% phosphate-buffered saline–bovine serum albumin (PBS-BSA; Sigma, St. Louis, MO), and subsequently incubated at 37°C for 1 hour with a second biotinylated anti-pLDH monoclonal antibody, 19G7.28 Plates were again washed with 1% PBS-BSA to remove unbound antibody and then incubated with a streptavidin-peroxidase solution (Boehringer Mannheim, Indianapolis, IN) at room temperature for 30 minutes. Plates were washed as before with 1% PBS-BSA. A solution of peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD) was then added. The reaction was halted with the addition of 1 mol phosphoric acid, and color development was measured at 450 nm using a microplate reader (Avantec, Rungis, France).

The 50% inhibitory concentration (IC$_{50}$) was calculated from the maximum optical density measured in the test wells compared with drug-free control wells. On the basis of the literature, isolates with IC$_{50}$ values less than 70 nmol were classified as sensitive, and those with IC$_{50}$ values greater than 100 nmol were considered resistant.2,26

**DNA extraction.** Whole blood samples were centrifuged at 2,000g for 10 minutes to remove plasma and washed three times with RPMI medium to remove white blood cells. A pellet of 1.0 mL to 1.5 mL of red blood cells was used for DNA extraction. Parasite genomic DNA was extracted as described elsewhere.30 Briefly, red blood cells were lysed with 0.015% saponin in NET buffer (150 mmol sodium chloride, 10 mmol EDTA, 50 mmol Tris, pH 7.5). After centrifugation, the pellets were resuspended in NET buffer and treated with 1% N-lauroyl-sarcosine (Sarkosyl) and RNase A (100 µg/mL) at 37°C for 1 hour, and then incubated with proteinase K (200 µg/mL) for 1 hour at 50°C. Parasite DNA was extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated with 0.3 mol sodium acetate and absolute ethanol. DNA pellets were air dried and resuspended in 20 μL TE buffer (10 mmol Tris, 1 mmol EDTA) and stored at −20°C until ready to use.

**Molecular analyses.** The highly polymorphic loci, msp-1 and msp-2, were used to genotype the parasite isolates. High yields of genomic DNA for all isolates obviated the need for nested polymerase chain reaction (PCR). The K1 and MAD20 allelic families of msp-1 and the ICI allelic family of msp-2 were analyzed. Primer sequences and PCR conditions were as described by Snounou et al.31 Ten microliters of each of the PCR products were electrophoresed on a 1% agarose gel and visualized under ultraviolet (UV) transillumination after staining with ethidium bromide.

The regions of the *pfcrt* and *pfmdr1* genes surrounding the polymorphisms of interest were amplified by PCR and analyzed by restriction fragment length polymorphism (RFLP). For each locus, RFLP products were electrophoresed on 2% agarose gels and visualized under UV transillumination after staining with ethidium bromide.

The *pfcrt* K76T mutation analysis was performed using the primers 5′-TGTGCTCATGTGTTTAACCTT-3′ and 5′-CAAAACTATAGTTACCAATTTTG-3′ and the PCR procedures described by Djimde et al.23 (GenBank accession no. AF030694). For all amplification reactions, only 0.5 μL of the DNA template was necessary. RFLP analysis of the resulting PCR products was conducted by digesting 5 μL of the PCR product with *ApoI* (New England Biolabs, Beverly, MA) at 50°C.

A 310-base pair (bp) region surrounding the *pfmdr1* N86Y mutation was amplified using the primers 5′-TTTACGTGTGTTTACCTGC-3′ forward primer and the 5′-CCATCTTGATAAAAACACTCTT-3′ reverse primer (GenBank accession no. X56851) and PCR conditions described by Djimde et al.23 Five microelitors of each amplification product was digested with the restriction enzyme *AluI* (New England Biolabs, Beverly, MA) at 37°C.

The primer pair, 5′-TGCGAGATCAATCCTTTTGA-3′ and 5′-TTAGGTCTCTTTTAAATGTCT-3′, was used for amplification and analysis of the polymorphism at amino acid position 1246 in *pfmdr1*. PCR conditions were as described by Frean et al.32 Five microelitors of each PCR product was digested with *EcoRV* (New England Biolabs, Beverly, MA) at 37°C.

**DNA sequence analysis.** For each locus, RFLP results were confirmed by sequencing a subset of the samples. Individual PCR products were cloned using the TOPO TA Cloning sys-
tem (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Plasmid DNA was isolated using the Wizard Plus SV mini-prep kit (Promega, Madison, WI) and tested for presence of a PCR insert by restriction digest with EcoRI (New England Biolabs, Beverly, MA). Plasmids containing inserts of interest were sequenced (Seqwright, Houston, TX), and sequences were aligned using the SeqMan and MegAlign programs (DNASTAR, Madison, WI).

Statistical analysis. Prevalence of each of the three mutant alleles was determined without prior knowledge of the in vitro chloroquine susceptibility status of the isolates. Fisher’s exact test was used to assess statistical associations between point mutations and in vitro resistance. The nonparametric Mann-Whitney \( U \) test was used to compare the distribution of IC\( _{50} \) values according to \( pfcr \) and \( pfmdr1 \) genotypes. All tests were two sided, and \( P \) values less than .05 were considered statistically significant.

RESULTS

A total of 51 patients were enrolled in the study, 44 of whom were slide positive for \( P. falciparum \). The mean age of the falciparum-infected persons was 19 years (range, 5–45 years). Of these, 55\% were male. The geometric mean parasitemia was 13,692 parasites/\( \mu \)L of blood (range 1,416–96,888 parasites/\( \mu \)L of blood). The mean age of the seven controls was 23 years (range, 17–32 years), and 57\% were female.

In vitro chloroquine susceptibility testing. Chloroquine susceptibility could be determined for 36 of the 44 samples obtained from individuals who were slide positive for \( P. falciparum \). The IC\( _{50} \) values segregated into two groups (< 70 nmol and > 100 nmol). None of the observed IC\( _{50} \) values fell between 70 and 100 nmol. Parasites from 11 samples (31\%) were classified as chloroquine resistant (geometric mean IC\( _{50} \), 942 nmol; range, 588–1,965 nmol). Chloroquine sensitivity could not be determined for eight samples because of lack of growth under the culture conditions used. Parasite densities of these eight samples ranged from 1,416 to 55,792 parasites/\( \mu \)L of blood compared with 1,544 to 96,888 parasites/\( \mu \)L of blood for the 36 samples successfully analyzed, indicating that failure to grow was not the result of a low parasite count. Venous samples drawn from the seven patients who were slide negative for \( P. falciparum \) were also assayed; as expected, none of these gave a positive signal in the DELI test.

Genotype analysis. \( Msp-1 \) and \( msp-2 \) alleles were detected in 30 of the 44 samples from persons with \( P. falciparum \) infection. Amplification products of regions surrounding the highly polymorphic blocks of the \( msp-1 \) and \( msp-2 \) allelic families showed the existence of several distinct \( msp-1 \) and \( msp-2 \) alleles that differed in length, indicating the presence of parasites of independent origins (Table 1). No PCR positive products could be identified when DNA from the seven \( P. falciparum \) negative patients were amplified with \( msp-1 \) or \( msp-2 \)-specific primers.

Prevalence of \( pfcr \) and \( pfmdr1 \) polymorphisms. \( Pfcr \) and \( pfmdr1 \) polymorphic markers were examined in 43 of the 44 \( P. falciparum \)-positive samples. One isolate that did not yield PCR product for any of the loci tested and had an inconclusive in vitro DELI test was excluded from the analysis.

A 134-bp region surrounding the \( pfcr \) K76T mutation was amplified by PCR, and the mutation was detected using the \( Apol \) restriction enzyme. \( Apol \) digestion produces two fragments of 34 bp and 100 bp in wild-type alleles, whereas the mutant allele remains undigested (Figure 1). RFLP analysis revealed the presence of the mutant T76 allele in 34 of 43 (79\%) isolates. One of these samples was mixed, yielding an RFLP fragment for both the K76 and T76 alleles. The remaining 9 (21\%) isolates carried the wild-type K76 allele. The results of the RFLP analysis were confirmed by sequencing the 134-bp fragment from one wild-type and six mutant isolates. All six isolates classified as mutant by RFLP encoded threonine at position 76, whereas the one wild-type isolate encoded amino acid lysine at that position. Two other polymorphisms at codons 74 and 75 of \( pfcr \) were also examined in the seven isolates from which the 134-bp \( pfcr \) fragment was sequenced. All six isolates carrying the T76 allele also possessed the I74 and E75 polymorphisms. The isolate with the K76 allele carried the wild-type alleles, M74 and N75, at these two positions.

The \( pfmdr1 \) N86Y mutation was similarly detected by RFLP analysis. A 310-bp region surrounding the mutation at position 86 was amplified by PCR and digested with the restriction enzyme \( AflIII \). \( AflIII \) cuts out 34 bp from the 134-bp \( pfcr \) fragment and into two fragments corresponding to approximately 120 and 190 bp, whereas the wild-type allele remains undigested (Figure 2A).

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. with positive PCR (%) (n = 44)</th>
<th>No. of distinct alleles</th>
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<tr>
<td>( msp-1 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>18 (41%) 5</td>
<td></td>
</tr>
<tr>
<td>MAD20</td>
<td>2 (5%) 2</td>
<td></td>
</tr>
<tr>
<td>( msp-2 )</td>
<td></td>
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</tr>
<tr>
<td>IC1</td>
<td>19 (43%) 1</td>
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*PCR = polymerase chain reaction.*

**FIGURE 1.** Restriction fragment length polymorphism analysis of \( pfcr \). Representative agarose gel electrophoresis of the \( pfcr \) K76T polymorphism. Presence of the K76T mutation was detected using the \( Apol \) restriction enzyme. \( Apol \) cuts out 34 bp from the 134-bp \( pfcr \) polymerase chain reaction product in wild-type alleles (lane 2) but does not cut the mutant allele (lanes 3 and 4). A mixed isolate is shown in lane 5. Lane 1, 100 bp ladder.
Eight of the 43 isolates did not yield interpretable results. Of the remaining 35 isolates, 11 (31%) carried the mutant allele, whereas 24 (69%) had the wild-type allele at position 86.

The polymorphism at codon 1246 was detected by amplification of a 499-bp fragment surrounding this position, followed by digestion with EcoRV, which produces a fragment of approximately 250 bp (corresponding to two fragments of 243 and 256 bp) in mutant alleles (see Figure 2B). Only one of the 43 isolates (2%) carried a mutant allele; the remaining 42 isolates (98%) exhibited a wild-type allele at this locus.

**Correlation between polymorphisms in pfcrt and pfmdr1 and in vitro chloroquine susceptibility.** Correlations between the three polymorphic loci and chloroquine susceptibility were analyzed for the 36 isolates for which the results of in vitro drug testing were available (Table 2). The pfcrT76 allele was present in 10 of 11 (91%) chloroquine-resistant isolates but also in 19 of the 25 (76%) sensitive isolates ($P = .40$).

Analysis of the range of IC$_{50}$ values corresponding to each allele of pfcr shows the K76 allele to be restricted, except in one case, to IC$_{50}$ values less than 40 nmol (Figure 3A) with a geometric mean IC$_{50}$ of 20.5 nmol. In contrast, the T76 allele is present in isolates with a broad range of IC$_{50}$ values and a geometric mean IC$_{50}$ of 50.3 nmol. The difference in distribution of IC$_{50}$ values for the two alleles was not statistically different ($P = .18$).

Two other polymorphisms in the pfcr gene, at amino acid positions 74 and 75 and previously shown to be associated with chloroquine resistance, were also analyzed in a subset of the isolates for which the pfcr amplification product was sequenced.$^{22,23}$ The I74 and E75 polymorphisms were present in all the isolates carrying the T76 mutation; therefore, like the T76 mutation, these additional polymorphisms were also present in both chloroquine-sensitive and chloroquine-resistant isolates. The close correlation between the polymorphisms at codons 74 and 75 and the T76 allele is consistent with previously published reports.$^{23}$

The pfmdr1 Y86 allele was detected in four of nine (44%) chloroquine-resistant isolates and five of 20 (25%) susceptible isolates ($P = .40$). Interestingly, the pfcrT76 and pfmdr1 Y86 alleles occurred together in four of nine (44%) of the resistant isolates but in only two of 20 (10%) of the sensitive isolates ($P = .06$).

Comparison of the range of IC$_{50}$ values associated with the pfmdr1 N86Y alleles shows presence of both alleles in isolates with a broad range of IC$_{50}$ values (see Figure 3B). The geometric mean IC$_{50}$ corresponding to the N86 and Y86 alleles were 37.3 nmol and 56.6 nmol, respectively, and distribution of IC$_{50}$ values in the two groups was not statistically significant ($P = .60$).
of Dakar, Senegal. By the in vitro DELI assay 31% of the *P. falciparum* isolates were chloroquine resistant, although by molecular analysis 79% of the isolates carried the mutant *pfcr* T76 allele that has been associated with chloroquine resistance in other studies.\(^{23,24}\) The *pfmdr1* Y86 and Y1246 alleles were present in 31% and 2% of the isolates, respectively. Comparison of the molecular markers and the in vitro results shows that the *pfcr* T76 allele was present in 91% of the resistant parasites, whereas *pfmdr1* Y86 and Y1246 were present in 44% and 9%, respectively. Interestingly, the single isolate with all three resistance alleles had one of the highest IC\(_{50}\) levels. Previous data have suggested a linkage disequilibrium between the *pfcr* T76 and *pfmdr1* Y86 alleles in chloroquine-resistant isolates, and a similar analysis in this study indicates a possible association between these two polymorphic alleles and in vitro chloroquine resistance. Technical issues, including the choice of the DELI assay for testing in vitro drug susceptibility and presence of trace amounts of antimalarial drugs, also may have influenced the outcome of this study.

The high prevalence of the *pfcr* T76 allele observed in this study is consistent with rates of 65% to 100% reported previously in various geographical locations.\(^{33–35}\) The fact that the T76 allele was present in all but one chloroquine-resistant isolate suggests that this polymorphism is important for chloroquine resistance. It is also likely that changes in expression levels or additional mutations in other genes are necessary for conferring chloroquine resistance. Such a multigenic mechanism of resistance may explain the relatively slow development and spread of chloroquine resistance compared with other drugs such as pyrimethamine.\(^{36}\) The presence of the K76 allele in one in vitro resistant isolate needs further confirmation because this has not been previously reported for *pfcr*. However, *msp-2* data available on this isolate revealed a single band indicating this to be a single infection.

For both the *pfmdr1* N86Y and D1246Y polymorphisms, no correlation was observed with in vitro chloroquine susceptibility, because the wild-type and mutant alleles for each locus were present in both sensitive and resistant isolates. Analysis of altered gene expression and other mechanisms that may contribute to a resistant phenotype is needed before a role for the *pfmdr1* gene can be excluded.

The low prevalence of the *pfmdr1* Y1246 allele is consistent with prior data from Africa.\(^{37–39}\) Studies to date show this polymorphism to be found predominantly in isolates originating from South America.\(^{16,39}\) The presence of a Y1246 mutant allele in one of the isolates from Senegal, however, may indicate the introduction of new strains into this region.

The genotype profile of the parasite population was determined using the standard *msp-1* and *msp-2* markers. Results of this analysis in combination with data from the molecular marker analyses indicate substantial genotypic diversity in the parasites from this study population. This suggests that the majority of the clinical attacks observed during this study period were caused by distinct *P. falciparum* strains.

The molecular markers examined in this pilot study do not, by themselves, provide a reliable estimate of the prevalence of chloroquine resistance. It is clear from the data that parasite isolates with very low IC\(_{50}\) levels indicating in vitro sen-

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Distribution of 50% inhibitory concentration (IC\(_{50}\)) values by *pfcr* K76T and *pfmdr1* N86Y alleles. A, *Pfcr*. The K76 allele of *pfcr* is associated with chloroquine sensitivity and the T76 allele with chloroquine resistance. B, *Pfmdr1*. The N86 allele of *pfmdr1* is associated with chloroquine sensitivity and the Y86 allele with chloroquine resistance. *P* values were obtained using the Mann-Whitney *U* test to compare IC\(_{50}\) distributions among the groups.

The *pfmdr1* mutation at position 1246 was found in only one of 11 (9%) chloroquine-resistant isolates and was not present in any chloroquine-sensitive isolates. This one isolate harboring the Y1246 allele also carried the mutant alleles for both *pfcr* K76T and *pfmdr1* N86Y. However, other chloroquine-resistant isolates with the *pfcr* T76 and *pfmdr1* Y86 polymorphisms carried the wild-type allele at position 1246, suggesting no correlation between the 1246 mutation and chloroquine resistance. The isolate carrying the mutant 1246 allele had an IC\(_{50}\) value of 569 nmol, the second highest value observed in this study, and was also isolated from the patient with the highest parasite density (96,888 parasites/µL blood).

**DISCUSSION**

The goal of this pilot study was to evaluate the utility of molecular markers as indicators of chloroquine resistance in isolates of *P. falciparum* obtained from residents of a suburb...
sitivity to chloroquine usually carry the pfcrt T76 allele. Nevertheless, the elevated frequency of the pfcrt T76 polymorphism raises the intriguing possibility that the T76 allele is a harbinger of emerging chloroquine resistance. Alternatively, it may indicate that this polymorphism occurs at a high frequency for reasons unrelated to drug pressure. Although the identification of the polymorphisms in the pfcrt and pfmdr1 genes represents a significant advance in the understanding of the mechanisms underlying chloroquine resistance, more extensive studies are necessary to determine the role of these polymorphisms in the in vitro and in vivo responses to drug treatment.

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