IN VITRO CHLOROQUINE SUSCEPTIBILITY AND PCR ANALYSIS OF PFCRT AND PFMDR1 POLYMORPHISMS IN PLASMODIUM FALCIPARUM ISOLATES FROM SENEGAL

SUSAN M. THOMAS, OMAR NDIR, THERESE DIENG, SOULEYMANE MBOUP, DAVID WYPIJ, JAMES H. MAGUIRE, AND DYANN F. WIRTH

Department of Immunology and Infectious Diseases and Department of Biostatistics, Harvard School of Public Health, Boston, Massachusetts; Faculty of Medicine and Pharmacy, Cheikh Anta Diop University, Dakar, Senegal; Laboratory of Bacteriology and Virology, Hospital A. Le Dantec, Dakar, Senegal

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 in the respective target enzyme.9,11,12 In contrast, the molecular mechanism by which chloroquine resistance occurs appears more complicated and remains largely unclear. Chloroquine exerts its antimalarial activity by interfering with heme metabolism in the digestive vacuole of P. falciparum.13,14 However, the precise targets and mechanism of action of chloroquine are not well understood. Research to date has implicated various genetic loci in conferring chloroquine resistance to the parasite. Chloroquine-resistant parasites have diminished accumulation of the drug in the digestive vacuole, and mutations at amino acid positions 86 and 1246 of the pfmdr1 gene, belonging to the adenosine triphosphate-binding cassette family, are possibly associated with this mechanism of resistance.15,16 Both mutations have been implicated in in vitro and in vivo chloroquine resistance in some studies, whereas no association has been found in others.16–21

More recently, multiple polymorphisms in the pfcrt gene, which encodes a transmembrane protein in the digestive vacuole of the parasite, were shown to be associated with a chloroquine-resistant phenotype.22 In particular, the K76T mutation was highly associated with both in vitro and in vivo chloroquine resistance, although most in vivo studies identified the presence of this mutation in both chloroquine-sensitive and chloroquine-resistant isolates.23–26

The relatively low rates of chloroquine resistance in parts of Senegal provide an opportunity to follow the progress of chloroquine resistance in a setting where this drug continues to be used as the first-line antimalarial treatment. Djimde et al.,23 in their study conducted in Mali, West Africa, reported the use of pfcrt in monitoring chloroquine resistance in this region. Here we report the findings from a pilot study that was undertaken to determine the utility of the molecular markers, pfcrt and pfmdr1, in monitoring chloroquine resistance in Senegal, West Africa.

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
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%. Chloroquine susceptibility testing, 200 µL of the suspension of erythrocytes was added to each well of a 96-well plate containing different concentrations of chloroquine sulfate (Rhone 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, 17E4.28 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 (IC₅₀) 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 IC₅₀ values less than 70 nmol were classified as sensitive, and those with IC₅₀ values greater than 100 nmol were considered resistant.7,26

**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) transcription after staining with ethidium bromide.

The regions of the *pfcr* 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 *pfcr* K76T mutation analysis was performed using the primers 5′-TGTGCTCATGTGTGTTAAAATT-3′ and 5′-CAAAACTATAGTTACCAATTTTG-3′ and the PCR procedures described by Djimde et al.25 (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 5′-TTTACGTATTTTACCAATATACCTGC-3′ forward primer and the 5′-CCATCTTGATAAAAACACTCTT-3′ reverse primer (GenBank accession no. X56851) and PCR conditions described by Djimde et al.25 Five microliters of each amplification product was digested with the restriction enzyme *AluI* (New England Biolabs, Beverly, MA) at 37°C.

The primer pair, 5′-GTGGGAATCAATTGTTGTTATGA-3′ and 5′-TAGGGTTCTTTTATAATGCT-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 microliters 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-
products could be identified when DNA from the seven parasites of independent origins (Table 1). No PCR positive msp-2 alleles that differed in length, indicating the presence of negative patients were amplified with msp-1 families showed the existence of several distinct PCR product for any of the loci tested and had an inconclusive in vitro DELI test was excluded from the analysis. 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 t and pfmdr1 polymorphisms. Pfcrt 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 t 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 pfcrt were also examined in the seven isolates from which the 134-bp pfcrt 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 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 pfcrt fragment and has the following results: 18 (41%) isolates were wild-type (lanes 3 and 4) and 22 (59%) were mutant (lanes 1 and 2). The remaining 9 (21%) isolates were classified as mixed, yielding a RFLP fragment for both the K76 and T76 alleles.
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 pfcr1 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 pfcr T76 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. 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.

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 pfcr T76 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 ($P = .60$).
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 allele and *pfmdr1* Y86 alleles 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 IC50 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 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 IC50 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.37,38 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 IC50 levels indicating in vitro sen-
sitivity to chloroquine usually carry the pfcr T76 allele. Nevertheless, the elevated frequency of the pfcr 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 pfcr 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.

Acknowledgments: We are grateful to Dr. Phyllis Kanki and Dr. Jean-Louis Sankale for assistance with the implementation of this study. We thank Dr. Leonardo Basco for essential advice on sample collection and DNA extraction protocols, Dr. Christian Hippi for input on the RFLP analysis, and the staff at Fann Hospital, Dantec Hospital, and the Cheikh Anta Diop University Parasitology Laboratory in Dakar for assistance with this study.

Financial support: This study was supported by the Fogarty International Center, the Exxon Mobile Corporation, National Institutes of Health grant 1 RO1 GM61351-01, and the Harvard Malaria Initiative.

Authors’ addresses: Susan M. Thomas and Dyann F. Wirth, Harvard School of Public Health, Department of Immunology and Infectious Diseases, 665 Huntington Avenue, Boston, MA 02115, Telephone: 617-432-4906, Fax: 617-739-3857; Department of Immunology and Infectious Diseases, 665 Huntington Avenue, Boston, Ma 02115, Telephone: 617-432-4906, Fax: 617-739-1781.}

**REFERENCES**


