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In vivo and in vitro analysis of chloroquine resistance in *Plasmodium falciparum* isolates from Senegal

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Abstract

To determine the predictive value of chloroquine (CQ) resistance markers in Senegal, *Plasmodium falciparum* DNA polymorphisms in *pfmdr1* and *pfcrt* were examined in relation to clinical outcome. Despite CQ treatment, 17% of patients had parasitemia after 28 days. Examination of molecular markers of CQ resistance revealed that 64% of all isolates had the T76 resistant allele at the *pfcrt* locus, while 30% carried the Y86 resistant allele at the *pfmdr1* locus. The *pfcrt* T76 allele was present not only in all in vivo resistant isolates, 89% of in vitro resistant isolates, but also in 35% of in vitro sensitive isolates. The *pfmdr1* N86Y polymorphism did not correlate with in vitro or in vivo CQ resistance. Our data suggest that the *pfcrt* T76 allele alone is required but not a sufficient predictor for in vivo CQ resistance.

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Introduction

Morbidity and mortality due to malaria have a significant impact on the healthcare infrastructure of Senegal: 35% of patients attending outpatient clinics are infected and 8,000 deaths are attributable to malaria every year (Republique du Senegal 2001). The risk of childhood mortality due to *Plasmodium falciparum* has increased 5.5-fold since the emergence of chloroquine (CQ) resistant malaria in Senegal (Trape et al. 1998).

Genetic polymorphisms in two parasite genes have been implicated in CQ resistance. The *P. falciparum* transporter gene *pfcrt*, encoding a vacuolar transmembrane protein, has been linked to in vivo and in vitro CQ resistance (Djimde et al. 2001a; Fidock et al. 2000). Overall, in vivo resistance is correlated with *pfcrt* T76 allele, however, in vivo clearance of parasites carrying this allele has also been reported from many field sites (Mayor et al. 2001; Dorsey et al. 2001; Pillai et al. 2001). This result is attributed to host immunity in adults. In vitro studies, which minimize an immune effect, have reported that the *pfcrt* T76 allele correlates highly to in vitro resistance, though this genotype has been seen in a small number of in vitro sensitive isolates as well (Lim et al. 2003; Basco and Ringwald 2001). The in vitro test can be confounded by poor growth and isolates can only be tested once directly, unless they undergo culture adaptation to confirm the in vitro result. A second polymorphism associated with CQ resistance is in the multidrug resistant gene, *pfmdr1*, which is a member of the ATP-binding cassette superfamily (Volkman et al. 1993). The *pfmdr1* Y86 allele has been linked to in vivo CQ resistance (Duraisingh et al. 1997). Thus, polymorphisms in both these genes have been correlated with in vitro and in vivo CQ resistance, but with different levels of association.

Thomas et al. found that these molecular markers were not sufficient predictors of in vitro CQ resistance in a study of 44 malaria patients in Senegal (Thomas et al. 2002). The present study confirms and extends these results by examining the association of *pfcrt* and *pfmdr1* polymorphisms to in vivo as well as in vitro CQ resistance.

Materials and methods

Study site and population

Samples were collected during the 2001 transmission season at a health clinic in Pikine, a suburb of Dakar with a population of over one million. Malaria transmission in this hypoendemic area occurs mainly from September to November (Robert et al. 2000). There are many marshes around Pikine that breed mosquitoes long after the rainy season and are a source of continued transmission beyond November. Patients aged 18 or higher, infected with *P. falciparum*, as determined by blood smear, were eligible to participate. Patients were excluded if another *Plasmodium* species in addition to *P. falciparum* was detected in their blood smear, if they were recently treated with antimalarial therapy (using detection of CQ metabolites via Saker–Solomon urine test), or if they manifested clinical symptoms of chronic disease such as tuberculosis, meningitis, pneumonia, or chronic diarrhea. The Human Subjects Committee of Harvard School of Public Health in Boston and the Ethics Committee of Cheikh Anta Diop University in Dakar approved the protocol used in this study.

Collection of blood samples

After informed consent was obtained from all adult participants, 10 ml of blood was drawn on the day of enrollment. Participants were followed-up by collecting finger prick blood samples daily from days 1 to 7, at days 14 and 28. For each follow-up sample, a drop of blood was routinely placed onto filter paper (ISOCODE, Schleicher & Schuell), air-dried at room temperature and stored in a plastic bag with silica gel for subsequent DNA extraction.

Treatment

Patients received the standard care regimen as determined by the Senegalese Ministry of Health guidelines. Uncomplicated malaria was treated with CQ sulfate (100 mg/pill) at a dose of 25 mg/kg for 3 days. Patients who received CQ had to remain at the clinic for at least 30 min, allowing the medical team to monitor for adverse reactions. Treatment was repeated in case of vomiting, and if persistent, patients were excluded from the study. Every pill was administered under medical supervision to ensure that treatment failure was not due to low drug dose.

Determination of parasite densities and subject definitions

Parasitemia was measured by counting the number of asexual parasites against a number of leucocytes in the thick blood film, based on a putative mean count of 8,000 leucocytes per microliter. The number of asexual parasites is counted against 200 leucocytes. The parasitemia per microliter is calculated by using the formula: Parasitemia (per microliter) = number of parasites × 8,000/number of leucocytes.

Any of the following conditions observed during the first 3 days of follow-up constitute early treatment failure: (1) development of danger signs or severe malaria on day 1, 2, or 3, in the presence of parasitemia; (2) axillary temperature ≥ 37.5°C on day 2 with parasitemia greater than day 0 count; (3) Axillary temperature ≥ 37.5°C on day 3 in the presence of parasitemia; (4) parasitemia on day 3 ≥25% of count on day 0.

Any of the following conditions observed during the follow-up period from days 4 to 14 constitute late treatment failure: (1) development of danger signs or severe malaria in the presence of parasitemia on any day from days 4 to 14, without previously meeting any of the criteria of early treatment failure; (2) axillary temperature ≥ 37.5°C in the presence of parasitemia on any day from days 4 to 14, without previously meeting any of the criteria of early treatment failure (WHO 1996).

In vitro susceptibility of *P. falciparum* isolates to chloroquine

Blood samples were collected in EDTA-coated Vacutainer tubes (Becton Dickinson & Co., Franklin Lakes, NJ, USA). From each sample, 5 ml was retained for in vitro CQ susceptibility testing while the remainder was used for DNA extraction. Samples were washed to remove serum and white blood cells. In vitro CQ sensitivity assays were performed in duplicate on all the isolates using the DELI assay (Moreno et al. 2001). Infected erythrocytes were diluted in RPMI medium supplemented with Albumax (Gibco BRL, Grand Rapids, NY, USA) and hypoxanthine (Sigma, St Louis, MO, USA) to obtain a hematocrit of 2% and a parasitemia of 0.5–1%. For CQ sensitivity testing, 200 μl of the suspension of erythrocytes was added to each well of a 96-well plate containing different concentrations of CQ sulfate (Rhone Poulenc Rorer, Vitry, France). Parasites were allowed to grow at 37°C in a candle jar for 48 h and then frozen at −20°C. Parasites were lysed by freezing and thawing the plates three times. One hundred microliters of each erythrocyte suspension was added to a 96-well plate coated with the primary anti-pLDH monoclonal antibody, 17E4. Plates were incubated at 37°C for 1 h, washed with 1% PBS-BSA (BSA-Sigma, St Louis, MO, USA), and subsequently incubated at 37°C for 1 h with a second biotinylated anti-pLDH monoclonal antibody, 19G7. Plates were again washed with 1% PBS-BSA to remove unbound antibody and then incubated with a streptavidin–peroxidase solution (Boehringer Mannheim, Indianapolis, IN, USA) at room temperature for 30 min. Plates were washed as before with 1% PBS-BSA. A solution of peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD, USA) was then added. The reaction was halted with the addition of 1 M 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 average of test wells compared to drug-free control wells. Isolates with IC_{50} values > 100 nM were considered resistant. The test was repeated if the optical density measurements had a large variability between duplicate samples or were not conclusive.

DNA extraction and DNA amplification

Parasite DNA was extracted using the phenol chloroform isoamyl alcohol method as described previously (Thomas et al. 2002). The DNA from filter papers was extracted using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The regions surrounding codon 76 for the *pfcrt* and codon 86 for the *pfmdr1* genes were amplified by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) with specific restriction enzymes. We used the same procedures as those described (Thomas et al. 2002). In order to determine if a single or multiple *P. falciparum* strains caused the infection, parasite isolates were genotyped using *msp1*. Primer sequences and PCR conditions were as described (Snounou et al. 1999). Isolates yielding a single fragment corresponded to a mono-infection, while those with more than one fragment represented multiple infections.

DNA sequence analysis

Nine isolates were sequenced to confirm RFLP results. The PCR products were cloned into the pCR2.1 Topo vector (TOPO TA Cloning system, Invitrogen, Carlsbad, CA, USA). Plasmid DNA was isolated using the Wizard Plus SV mini-prep kit (Promega, Madison, WI, USA). After restriction digest with *Eco*RI (New England Biolabs, Beverly, MA, USA), insert-containing plasmids were sequenced.

Statistical analysis

Data were analyzed using STATA Statistics/Data Analysis 7.0 (College Station, TX, USA). The ranksum test and the *T*-test were used to assess statistical associations between the *pfcrt* and *pfmdr1* polymorphisms and treatment outcome. When P value < 0.05, result was considered as significant.

Results

Sixty adult patients with fever (mean 38°C) and uncomplicated malaria were enrolled. At enrollment, patients were mildly ill, with a body mass index mean of 16.9, and an average hemoglobin count of 12.8 g/dl. Headaches were the most common symptom (95%), followed by chills (75%). Thirty-six patients (60%) were male with a mean age of 24 years (range 18–42 years). The mean parasite density was 18,617/μl (range 2,080–56,263).

Analysis of *pfcrt* and *pfmdr1* polymorphisms

Using methods described in Thomas et al., 36 isolates with positive PCR for both *pfcrt* and *pfmdr1* were further analyzed (Thomas et al. 2002). *Msp1* (Mad20, K1, and RO33) genotyping for these isolates was used to distinguish between infections with single and multiple strains. The amplification products for five out of 31 isolates (17%) indicated multiple infections. This is consistent with other studies using multiple microsatellite markers for clonailty which found < 20% multiple clones in this region (Leclerc et al. 2002).

The RFLP was used to differentiate between wild type and mutant alleles. In total, 25 isolates contained the *pfmdr1* N86 wild type allele, 11 the Y86 mutant allele. Similar analysis of the *pfcrt* locus showed that the K76 wild type allele was present in 13 isolates, and the T76 mutant

allele was found in 23 isolates. RFLP data for *pfcrt* was confirmed by sequence analysis in nine selected isolates.

Analysis of in vivo resistant isolates

There were a total of four treatment failures in our substudy population of 36 isolates from patients. All early treatment failures were defined by axillary temperature ≥ 37.5°C on day 2 or 3 with parasitemia greater than day 0 count and parasitemia on day 3 ≥ 25% of Day 0 count. All these isolates possessed the *pfcrt* T76 allele, but none of them possessed *the pfmdr1* Y86 allele. While 3/4 of the isolates were also in vitro CQ resistant (IC_{50} = 112, 129, and 396 nM), one isolate was susceptible to CQ (IC_{50} = 22 nM).

Analysis of in vitro resistant isolates

The correlation between in vitro CQ susceptibility and *pfcrt* and *pfmdr1* polymorphisms were analyzed for a total of 36 isolates (Table 1). While we had data on 45 isolates, the samples with multiple clones were eliminated from analyses described in this report. Nineteen out of 36 isolates were resistant to CQ, defined as an IC_{50} > 100 nM. Eighty-nine percent (17 out of 19) of in vitro resistant isolates contained the mutant *pfcrt* T76 allele, and 37% (7 out of 19) contained the *pfmdr1* Y86 allele. The two in vitro resistant isolates with sensitive RFLP profiles were confirmed to be K76 by sequence analysis. Using the ranksum test (STATA 7.0 College Station, TX, USA), we found significant association between the *pfcrt* polymorphism and in vitro CQ resistance (P = 0.03). The sensitivity of the *pfcrt* T76 mutation to detect in vitro CQ resistance is 89%, however, this marker was only 64% specific. No significant association was found between the *pfmdr1* polymorphisms and in vitro CQ resistance (P = 0.6). There was no significant association between the *pfcrt* and the *pfmdr1* polymorphisms and parasite density.

Discussion

The prevalence of in vitro CQ resistant parasites in adult patients exhibiting uncomplicated malaria in this study is consistent with our previous report (Thomas et al. 2002). We determined that the *pfcrt* T76 and the *pfmdr1* Y86 mutations were present, respectively, in 89 and 37% of resistant isolates (compared to 91 and 44% from the previous year), and 35 and 24% of sensitive isolates (compared to 76 and 25% from the previous year). The prevalence of the *pfcrt* T76 mutation in resistant isolates is consistent with other field studies (Mayor et al. 2001; Dorsey et al. 2001; Vieira et al. 2001). Two independent studies have also shown a strong association between presence of the *pfcrt* T76 allele and CQ resistance in vivo (Djimde et al. 2001a; Chen et al. 2001). In Cameroon, *pfcrt* T76 mutation had also a significant association (P < 0.001) with both in vitro and in vivo CQ response (Basco and Ringwald 2001). However, Kyosiimire-Lugemwa et al. in Uganda were unable to demonstrate a correlation between *pfcrt* mutation and in vivo outcome in individual samples (Kyosiimire-Lugemwa et al. 2002). Thus, it appears that the *pfcrt* T76 mutation cannot be used as a stand-alone predictor of in vivo CQ resistance in certain geographical locations (Vinayak et al. 2003). These results suggest that additional factors, which may be regional, determine the strength of the T76 mutation in mediating CQ resistance.

This study documents longitudinal data on CQ resistance, which is important for the decision-making aspects of drug treatment recommendations in Senegal, and extends the analysis to parasites isolated from in vivo failures. The discrepancy that we observed between the large number of isolates with the *pfcrt* T76 allele and small number of isolates exhibiting in vivo resistance may be due to multiple factors. One of these is host immunity. Carlton and others have shown that the ability of individuals to clear infections by parasites carrying the T76 mutation in a highly endemic area was strongly associated with increasing age and immunity (Carlton et al. 2001). We suggest that this correlation can be extended to a hypoendemic area,

and predict that the adult populations in our study have a higher rate of acquired immunity compared to the general population. This observation has been confirmed by data gathered from 2002 and 2003 by Sarr and others (unpublished data) who have shown that the T76 allele in patients under 10 years of age is associated with clinical failure ($P = 0.01$) this association is not observed for those over 10 ($P = 0.2$).

The prevalence of the *pfcrt* T76 allele (64%) in the population correlates with published reports showing a strong association between this mutation and clinical treatment failure (Djimde et al. 2001a; Schneider et al. 2002). Overall, our data indicate that the *pfcrt* T76 allele is statistically associated with in vitro CQ resistance in Senegal, however, the low specificity of the test lessens its predictive value as noted elsewhere (Jelinek et al. 2002). The *pfcrt* T76 allele also appears to be correlated to in vivo CQ resistance, but its predictive value could not be established due to the small number of clinical failures. The samples isolated from patients with in vivo failure that underwent in vitro testing in general gave consistent results. However, one of these isolates demonstrated in vitro sensitivity. This discrepancy could be due to the presence of a second, sensitive clone that was not detected through the initial *msp1* typing or to the limitation of the in vitro assay. Field isolates tested directly are prone to growth differences and variability resulting at times in erroneous test results. We are developing long-term culturing of parasites derived from isolates with discrepancies between in vitro or in vivo data to further examine these correlations under ideal experimental conditions. The *pfmdr1* Y86 allele is associated with CQ resistance in some field studies, but not in others (Ochong et al. 2003; Vieira et al. 2001; Chen et al. 2001; Tinto et al. 2003). Our study suggests that *pfmdr1* Y86 is not a predictor of in vivo or in vitro CQ resistance in this population in Senegal. Identification of additional single nucleotide polymorphisms in novel genes may provide more accurate diagnostic standards to predict which isolates will progress to clinical resistance.

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Table 1

Prevalence of *pfcrt* and *pfmdr1* polymorphisms in relation to in vitro CQ susceptibility

Polymorphism	CQ susceptible# isolates (proportion)	CQ resistant# isolates (proportion)	*P* value
Pfcrt K76	11/17 (0.65)	2/19 (0.11)	< 0.05
Pfcrt T76	6/17 (0.35)	17/19 (0.89)	
Pfmdr1 N86	13/17 (0.76)	12/19 (0.63)	0.6
Pfmdr1 Y86	4/17 (0.24)	7/19 (0.37)	

Note: In vitro CQ susceptibility is defined as IC_{50} values less or equal to 100 nM

[a] Average 51, range 11–102 nM, CI 37–64

[b] Average 474; range 120–2,153 nM, CI 273–577