

SHORT REPORT: ASSOCIATION BETWEEN CHLOROQUINE AND AMODIAQUINE RESISTANCE AND ALLELIC VARIATION IN THE *PLASMODIUM FALCIPARUM* MULTIPLE DRUG RESISTANCE 1 GENE AND THE CHLOROQUINE RESISTANCE TRANSPORTER GENE IN ISOLATES FROM THE UPPER NILE IN SOUTHERN SUDAN

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Abstract. Amodiaquine, a 4-aminoquinoline compound, is being considered as an alternative to chloroquine and pyrimethamine/sulfadoxine where resistance in *Plasmodium falciparum* to both drugs has been selected. Although amodiaquine is more potent than chloroquine, its effectiveness is reduced in areas where chloroquine resistance is high. We report an association of the *P. falciparum* chloroquine resistance transporter (*pfcr*) gene and the *P. falciparum* multiple drug resistance 1 (*pfmdr1*) gene, two chloroquine resistance markers, with chloroquine and amodiaquine efficacy *in vivo* in southern Sudan. The data show that the allele of the *pfcr* gene with a lysine to threonine change at codon 76 is strongly associated with both chloroquine and amodiaquine resistance. No such association was observed with the *pfmdr1* gene.

In response to the spread of resistance to chloroquine (CQ) and pyrimethamine/sulfadoxine (Fansidar[®]; F. Hoffmann-La Roche, Basel, Switzerland), amodiaquine (AQ) is now being considered as an alternative option for the management of uncomplicated *Plasmodium falciparum* malaria in Africa.^{1,2} Although this drug remains effective in areas of substantial CQ resistance,^{3–6} the two drugs are chemically related and several clinical^{1,2,7} and *in vitro*^{8,9} reports have shown cross-resistance between CQ and AQ or the active metabolite of AQ.

Many studies have been devoted to understanding the mechanism of CQ resistance.¹⁰ Point mutations in the *P. falciparum* chloroquine resistance transporter (*pfcr*) gene and, to a lesser extent, in the *P. falciparum* multiple drug resistance 1 (*pfmdr1*) gene are associated with CQ resistance. Polymorphism in the *pfcr* gene has been reported to correlate with CQ resistance.^{11,12} Among the amino acid changes in this protein, the lysine to threonine change at position 76 (*pfcr* 76T) is the most strongly associated with CQ resistance both *in vivo* and *in vitro*.^{11,12} Recently, transfection of the *pfcr* gene has clearly demonstrated the role of this mutant allele in CQ resistance *in vitro*.^{13,14} However, in semi-immune populations, the value of this mutation for predicting clinical outcomes after CQ treatment has not been consistent.^{15,16} The point mutation of asparagine to tyrosine at codon 86 in the *pfmdr1* gene (*pfmdr1* 86Y) has been associated with CQ resistance in some studies,^{17,18} but not in others.^{19,20}

The molecular mechanisms of CQ and AQ cross-resistance have not yet been addressed, but the similarity of their chemical structures, their likely common mode of action,^{21,22} and some apparent cross-resistance suggest that molecular markers selected as a function of CQ use might also compromise effectiveness of AQ. We report the impact of mutant alleles *pfcr*76T and *pfmdr1* 86Y on the clinical efficacy of AQ and CQ in southern Sudan, an area where CQ efficacy is still at high levels.

We analyzed samples collected during a clinical trial of efficacy of antimalarial agents in southern Sudan between June and December 2001. The study was reviewed and approved by the Ethical Committee of Médecins sans Frontières-Holland (Amsterdam, The Netherlands). Local authorities

and the Sudanese People's Democratic Front/counterpart agreed with the study and helped to notify the population. Blood collected by finger prick (50 μ L) was spotted onto filter paper, air-dried, and stored in plastic bags with silica gel at ambient temperature. Parasite genomic material was prepared using the methanol procedure described elsewhere.²³ To detect a single base change at codon 76 of *pfcr* and codon 86 of *pfmdr1*, we used the polymerase chain reaction (PCR)–restriction enzyme protocol described in detail by Professor Christopher Plowe (University of Maryland, Baltimore, MD; Web site: http://medschool.umaryland.edu/cvd/2002_pcr_asra.htm).

The detailed clinical results of CQ and AQ efficacy in Sudan have been presented elsewhere.²⁴ Briefly, 104 and 101 patients were treated with CQ (10 mg/kg on day 0, 10 mg/kg on day 1, and 5 mg/kg on day 2) and AQ (10 mg/kg on day 0, 10 mg/kg on day 1, and 5 mg/kg on day 2), respectively. Of these, 14 (13.5%) of 104 and 7 (6.9%) of 101 had positive parasitemias within 14 days after treatment and were scored as CQ resistant and AQ resistant, respectively: these are parasitologic failures. Those whose blood samples were negative 14 days after treatment were scored as an adequate parasitologic response or harboring sensitive isolates. To test whether polymorphisms in *pfcr* and *pfmdr1* are associated with the CQ and AQ resistance response, we genotyped *pfcr* at codon 76 and *pfmdr1* at codon 86 in isolates from four groups of patients: those showing an adequate parasitologic response to CQ ($n = 28$) and AQ ($n = 39$), and those who had *P. falciparum* parasites in their blood within 14 days after treatment with CQ ($n = 13$) and AQ ($n = 6$). All of these isolates were collected on the day of admission into the study before treatment was given.

The *pfcr* and *pfmdr1* genes were successfully amplified in all isolates except for one from the AQ-resistant group (for *pfcr*) and two from the CQ-sensitive group (for *pfmdr1*). The analysis of *pfcr* showed that 93% (26 of 28) of the isolates from patients treated with CQ with an adequate parasitologic response were wild type (*pfcr* 76K) and all 13 CQ-resistant isolates carried the mutant allele (*pfcr* 76T). The same trend was observed in patients treated with AQ: 85% (33 of 39)

TABLE 1
Allelic polymorphisms of the *pfprt* gene at codon 76 in samples collected before chloroquine and amodiaquine treatments*

	Chloroquine (CQ) treatment		Amodiaquine (AQ) treatment	
	CQ sensitive (n = 28)	CQ resistant (n = 13)	AQ sensitive (n = 39)	AQ resistant (n = 5)
<i>pfprt</i> 76K (wild type)	26/28 (93%)	0	33/39 (85%)	0
<i>pfprt</i> 76T (mutant)	2/28 (7%)	13/13 (100%)	6/39 (15%)	5/5 (100%)

* *pfprt* = *Plasmodium falciparum* chloroquine resistance transporter (gene).

were wild type and 100% (5 of 5) carried the mutant allele (*pfprt* 76T) in the sensitive and resistant groups, respectively (Table 1). The analysis of *pfmdr1* showed that 92% (24 of 26) of the CQ-sensitive isolates, as well as 92% (36/39) of the AQ-sensitive isolates, were wild type (*pfmdr1* 86N). In contrast, the *pfmdr1* 86Y mutant allele was only found in 62% (8 of 13) and 50% (3 of 6) of the isolates that failed to respond to CQ and AQ, respectively (Table 2). When *pfprt* and *pfmdr1* were analyzed together, more than 80% of the CQ- and AQ-sensitive isolates carried the wild type *pfprt* 76K-*pfmdr1* 86N genotype (Figure 1). However, the combination of mutant genotypes *pfprt* 76T-*pfmdr1* 86Y was observed only in approximately 60% of both AQ- and CQ-resistant isolates.

This is the first report of the assessment of the impact of *pfprt* markers on *in vivo* efficacy of AQ. Our data clearly demonstrate that the *pfprt* allele at codon 76, the most common marker for CQ resistance,¹¹⁻¹⁴ is also associated with AQ resistance *in vivo*. Both CQ and AQ are 4-aminoquinoline agents, and several reports^{21,22} have shown that these two drugs act in a similar manner against *P. falciparum* by inhibiting the polymerization of heme, the toxic by-product of hemoglobin degradation. It has been suggested that AQ is more potent than CQ because of a higher accumulation of AQ in the digestive vacuole of the parasite.^{21,22,25} Therefore, since their chemical structures and their mode of action are similar, one would expect that the selection of CQ resistance would impact on the efficacy of AQ. As a consequence, selection of markers for CQ resistance would have a bearing on AQ resistance; this is what our data clearly show. Overall, CQ and AQ both retain excellent efficacy in our study site, and under these circumstances, the *pfprt* mutant could be used as a predictor of both CQ and AQ resistance.

Recently, transfection studies of *pfprt* have shown that isolates expressing the mutant *pfprt* 76T allele retain sensitivity to AQ while showing a reduced susceptibility to monodesethylamodiaquine (MDAQ), the active metabolite of AQ.¹³ Therefore, the association between the mutant allele *pfprt* 76T and AQ resistance we have found *in vivo* may reflect an association of this allele with the active metabolite MDAQ.

The *pfmdr1* 86Y allele is not as strongly associated with resistance as *pfprt* 76T. Indeed, we have found that only 62% of the CQ-resistant isolates and 50% of the AQ-resistant isolates harbor the mutant *pfmdr1* 86Y allele. The lack of association between this allele and CQ resistance has been reported in different malaria-endemic areas.^{15,19,20} We have confirmed these observations in southern Sudan with CQ and also report the lack of association of this marker with AQ resistance.

In conclusion, our study shows that the mutant *pfprt* 76T allele is correlated with CQ resistance as previously reported.^{11,12} We also provide evidence that the selection of this allele could explain, at least partly, the cross-resistance observed between CQ and AQ *in vivo*. However, this study was carried out in an area where CQ is still very effective. Therefore, it remains to be seen if this pattern will also be observed in the many areas of Africa where CQ resistance is already at high levels.

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TABLE 2
Allelic polymorphisms of the *pfmdr1* gene at codon 86 in samples collected before chloroquine and amodiaquine treatments*

	Chloroquine (CQ) treatment		Amodiaquine (AQ) treatment	
	CQ sensitive (n = 26)	CQ resistant (n = 13)	AQ sensitive (n = 39)	AQ resistant (n = 6)
<i>pfmdr1</i> N86 (wild type)	24/26 (92%)	5/13 (38%)	36/39 (92%)	3/6 (50.00%)
<i>pfmdr1</i> Y86 (mutant)	2/26 (8%)	8/13 (62%)	3/39 (8%)	3/6 (50.00%)

* *pfmdr1* = *Plasmodium falciparum* multiple drug-resistance (gene).

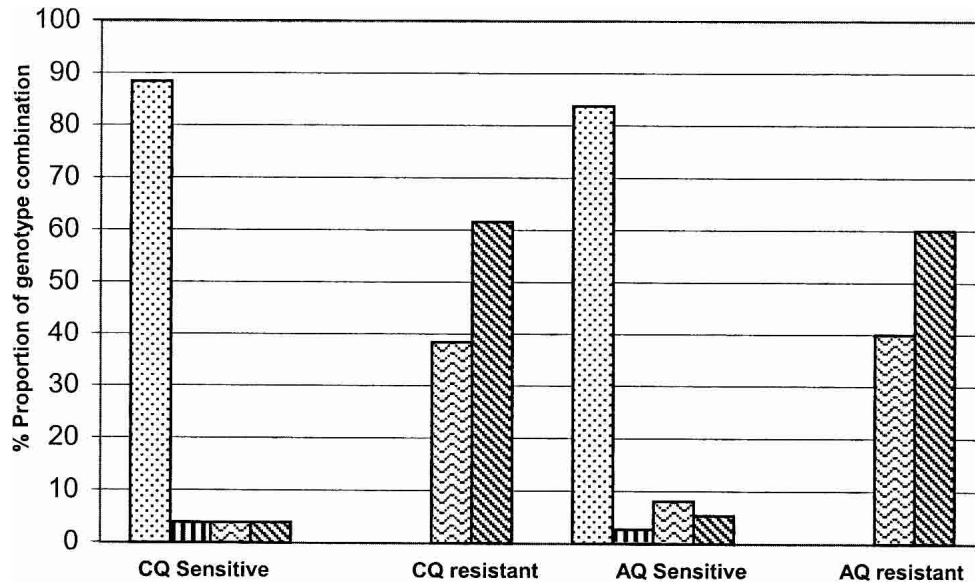


FIGURE 1. Allelic frequency of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcr1*) gene at codon 76 and the *P. falciparum* multiple drug resistance 1 (*pfmdr1*) gene at codon 86 in isolates collected before treatment with chloroquine (CQ) and amodiaquine (AQ) in southern Sudan. The allelic combinations analyzed were wild type *pfcr1* 76K and wild type *pfmdr1* 86N; (dotted bars); wild type *pfcr1* 76K and mutant *pfmdr1* 86Y; (vertically striped bars); mutant *pfcr1* 76T and wild type *pfmdr1* 86N (wavy-patterned bars); mutant *pfcr1* 76T and mutant *pfmdr1* 86Y (diagonally striped bars).

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