

SHORT REPORT: MOLECULAR EVALUATION OF THE EFFICACY OF CHLOROQUINE TREATMENT OF UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA IN EAST TIMOR

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Abstract. The efficacy of chloroquine treatment of uncomplicated *Plasmodium falciparum* malaria in East Timor was investigated via molecular tools. Genotyping of the polymorphic markers *msp1* and *msp2* was performed to investigate the number and type of parasite alleles in pre- and posttreatment blood samples collected from 48 patients. Patients were infected with a minimum of 8 *msp1* and 14 *msp2* allelic types of parasite, and 43% of the patients had more than one allelic type before treatment. The genotyping also revealed that 66.7% of the patients were infected with at least one identical allelic type of parasite before and after treatment and therefore were likely to have experienced recrudescence. All parasites in pre- and posttreatment blood samples carried the K76T mutation in *pfcr*, regardless of the clinical response to chloroquine. The sequence polymorphism patterns in *pfcr* in the majority of parasites examined were identical to those observed in Bougainville, Papua New Guinea.

INTRODUCTION

Chloroquine has been used as first-line therapy for uncomplicated falciparum malaria in East Timor. However, until recently, reliable data on the efficacy of chloroquine in the country were unavailable. In mid-2000, MERLIN (Medical Emergency Relief International) conducted an *in vivo* chloroquine efficacy study in Lautem district, Los Palos, a hyperendemic area in the eastern sector of East Timor (Ezard N and others, unpublished data). Forty-eight East Timorese patients with uncomplicated *Plasmodium falciparum* malaria, who were aged between 8 months and 29 years and who lived within a 1-hour car journey, were enrolled. Results of a questionnaire on chloroquine usage before the initiation of the trial revealed that 9 of the 48 patients had taken chloroquine within a 1-month period preceding the trial. Overall, of 48 patients, two-thirds (32 of 48) experienced recurrent parasitemia after treatment with 1,500 mg chloroquine over 3 days, all but one patient failed to respond to late treatment (recurrent parasitemia occurred 7–28 days after treatment) (Ezard N and others, unpublished data).

After clinical investigation, molecular analysis was performed on pre- and posttreatment blood samples collected from these patients to investigate whether the recurrent parasitemia resulted from parasites recrudescing under chloroquine treatment or from new infections and whether sequence polymorphism in the *pfcr* gene can be used as a molecular marker to predict clinical outcomes after chloroquine treatment in this region.^{1,2} Blood samples were collected and preserved either on filter paper or in 6 M guanidine hydrochloride on days 0, 14, and 28 after commencing chloroquine treatment, or on the day when symptoms recurred. We extracted DNA via the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI) according to the manufacturer's instructions. *Msp1* and *msp2* were amplified by polymerase chain reaction (PCR) and analyzed by agarose gel electrophoresis.³

Nested PCR was performed to amplify the *pfcr* gene. Briefly, the first round of PCR was carried out in a 50- μ L reaction mixture containing 1.5 μ g/mL of each primer 5'-GTGGAGGTTCTTGCTTGGT-3' and 5'-TCCAGTAGT-

TCTTGTAAGACC-3', 200 μ M of dNTPs, buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂), and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT). The samples were denatured at 94°C for 10 minutes before 40 cycles of amplification (94°C for 50 seconds, 51°C for 50 seconds, and 70°C for 1 minute). The second round PCR were performed under the same conditions by use of the following primers: 5'-TGTGCTCATGTGTT-TAAACTT-3' and 5'-AAAGCTTCGGTGTCGTTTC-3'.⁴ The nested PCR product was purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), then sequenced in an ABI PRISM 370 sequencer (Applied Biosystems, Inc., Foster City, CA).

Genotyping of 2 polymorphic markers, *msp1* and *msp2*, revealed a minimum of 8 (410–580 bp) and 14 (380–800 bp) allelic types, respectively, indicating that many allelic types of *P. falciparum* were transmitted in the area. Multiple allelic type infections of *P. falciparum* were detected in 45.8% (22 of 48) of the patients before treatment, indicating a high level of malaria transmission in the area. The genotyping also revealed that 66.7% (32 of 48) of patients had at least one allelic type of parasite in posttreatment blood samples identical to those in pretreatment samples (Table 1, original types). Twenty-eight of these patients came from the *in vivo* resistant group and 4 from the *in vivo* susceptible group. Given the number of allelic types in the area, the chance of reinfecting with the same allelic type is low. Therefore, these patients are likely to have experienced recrudescence resulting from parasites that grew in the presence of chloroquine. Approximately 10% (5 of 48) of patients had different allelic types of parasites in their pre- and posttreatment blood samples, indicating new infections (Table 1, new types) during the 28 day follow-up period. Of the 5 patients with new infections, 4 were in the *in vivo* resistant group and 1 in the susceptible group. In the 16 patients classified as *in vivo* susceptible to chloroquine, genetic analysis revealed 4 patients to have recrudescing parasites and 1 patient to have a new infection. This finding suggests that these patients were infected with very low number of parasites that were below the limit of microscopic detection.

TABLE 1

Allelic types and sequence polymorphisms detected in pre- and posttreatment blood samples and their correlations with *in vivo* response to chloroquine treatment

In vivo chloroquine susceptibility	No. of patients (n = 48)	MSP1/MSP2 in posttreatment samples		Polymorphism in PfCRT protein								
				Pretreatment				Posttreatment				
				MSP1/M SP2 types	No. of patients	T76			T76			
K76	SVMNT*	CVIET*	Negative			K76	SVMNT*	CVIET*	Negative			
Susceptible	16	Original type New type Undetected	4 1 11	0	16	0	0	0	0	2	1	13
									0	1	0	3
									0	0	1	0
Resistant	32	Original type New type Undetected	28 4 0	0	32	0	0	0	0	32	0	0
									0	28	0	0
									0	4	0	0
								–	–	–	–	

* SVMNT and CVIET represent amino acids at positions 72 to 76 in PfCRT.

The pre- and posttreatment blood samples were also examined for sequence polymorphisms in the *pfcr* gene at positions corresponding to amino acids 72, 74, 75, and 76. Mutation K76T in *pfcr* has been demonstrated to correlate with decreased susceptibility to chloroquine *in vitro*, and sequence polymorphisms at amino acids 72, 74, and 75 are associated with geographical origins of parasite samples.^{1,2,5} K76T was detected in all parasites in both pre- and posttreatment blood samples, regardless of the clinical response to chloroquine (Table 1). The patients with new infections were all reinfected with parasites carrying the K76T mutation (Table 1). The results are in accord with a recent study conducted in Laos, which showed that although K76T was perfectly associated with *in vivo* chloroquine resistance, the mutation was present in all amplifiable *in vivo* susceptible isolates.⁶ In the 16 patients cured of malaria, the elimination of mutation carrying parasites may be due to a stronger antiparasitic immune response in these individuals. A correlation in patient age and predictive value of K76T mutation reported recently supports this explanation.⁷ However, in this group of patients, there was no correlation (chi-square test, $P > 0.5$) between the recrudescence rate and patient's age when patients were grouped as age < 5 (n = 11), 5–10 (n = 16) and > 10 (n = 21), or < 5 (n = 11) and > 5 (n = 37), or < 1 (n = 2), 2–3 (n = 2), 4–5 (n = 7) and > 5 (n = 37) years. This could have resulted from relatively small number of volunteers in each age group. There is also a possibility that the parasites in these individuals have lower levels of chloroquine resistance compared with those in patients not responding to the treatment. Therefore, the presence of mutation K76T in *pfcr* is necessary, but not sufficient, to predict clinical outcome of chloroquine treatment. In accord with the *in vivo* findings, our molecular analysis also indicated that chloroquine-resistant *P. falciparum* is widespread in this region of East Timor (Ezard N and others, unpublished data).

As to the sequence polymorphisms at other positions in *pfcr*, S72, M74, and N75 were detected in all but one sample (C72, I74, and E75) (Table 1). In previous studies, it was found that polymorphisms at codon positions 74, 75, 271, 326, 356, and 371 in *pfcr* were linked into blocks in parasites with the K76T mutation.^{1,2,5} So far, M74/N75/Q271/D326/L356/R371 has been identified in *pfcr* of all analyzed chloroquine resistant samples from the mainland Papua New Guinea

(PNG), the island of Bougainville, PNG, and Brazil, whereas I74/E75/E271/S326/T(I)356/I371 has been found mainly in isolates from Southeast Asia and Africa.^{1,2,5} At amino acid 72, however, a S or C has been observed in the South American/PNG isolates.^{1,2,5} A synonymous DNA sequence polymorphism of S_{AGT} or S_{TCT} was also observed in the isolates from mainland PNG.⁵ However, in our set of samples from East Timor and Bougainville, PNG, AGT was the only codon type identified in chloroquine-resistant parasites for S72. These findings suggest that majority of the chloroquine-resistant parasites from East Timor and Bougainville, PNG, may be derived from a common origin as a result of the geographical proximity of the 2 islands.

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