RECOVERY OF CHLOROQUINE SENSITIVITY AND LOW PREVALENCE OF THE
PLASMODIUM FALCIPARUM CHLOROQUINE RESISTANCE TRANSPORTER GENE
MUTATION K76T FOLLOWING THE DISCONTINUANCE OF CHLOROQUINE USE
IN MALAWI

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Abstract. In 1993, Malawi stopped treating patients with chloroquine for Plasmodium falciparum malaria because of a high treatment failure rate (58%). In 1998, the in vitro resistance rate to chloroquine was 3% in the Salima District of Malawi; in 2000, the in vivo resistance rate was 9%. We assayed two genetic mutations implicated in chloroquine resistance (N86Y in the P. falciparum multiple drug resistance gene and K76T in the P. falciparum chloroquine resistance transporter gene) in 82 P. falciparum isolates collected during studies in 1998 and 2000. The prevalence of N86Y remained similar to that in neighboring African countries that continued to use chloroquine. In contrast, the prevalence of K76T was substantially lower than in neighboring countries, decreasing significantly from 17% in 1998 to 2% in 2000 (P < 0.02). However, neither mutation was significantly associated with in vivo or in vitro resistance (P > 0.29). Withdrawal of the use of chloroquine appears to have resulted in the recovery of chloroquine efficacy and a reduction in the prevalence of K76T. However, other polymorphisms are also expected to contribute to resistance.

INTRODUCTION

Chloroquine resistance in Plasmodium falciparum has been increasing in Africa since the late 1970s, and has been associated with an increase in malaria mortality and morbidity.1,2 Although the mechanisms of resistance to chloroquine have not been fully elucidated, several putative molecular markers of resistance have been identified. The P. falciparum multiple drug resistance 1 (pfmdr1) gene, a homolog of the mammalian multiple drug resistance gene encoding a P glycoprotein, has been identified on chromosome 5 of P. falciparum.3 A point mutation (N86Y) resulting in an amino acid change from asparagine to tyrosine at position 86 in the pfmdr1 gene has been reported to be associated with in vitro chloroquine resistance in isolates originating from various regions of Asia and Africa.4,5 However, other reports have questioned the contribution of N86Y, suggesting that other mutations are also necessary for the acquisition of chloroquine resistance.6,7

Recently, the P. falciparum chloroquine resistance transporter (pfert) gene product localized to the parasite digestive vacuole has also been implicated in chloroquine resistance. One of the 10 point mutations initially identified results in a lysine to threonine change at position 76 (K76T) that increases acidification in the digestive vacuole and the efflux of large amounts of chloroquine.8 In early field studies, pfert K76T was found in all in vivo chloroquine-resistant isolates from malaria-endemic regions of Africa and Southeast Asia.9–12

In Malawi, chloroquine treatment of uncomplicated malaria was discontinued in 1993 due to an unacceptable treatment failure rate (58%).13 Since then, the ban on chloroquine has been strictly enforced. Our previous study in the Salima District of Malawi in 1998 revealed a low prevalence of in vitro chloroquine resistance (3%).14 To investigate the effect of this ban on chloroquine sensitivity, we conducted in vivo chloroquine studies in 2000 in the same community and genotyped the N86Y and K76T polymorphisms of the P. falciparum isolates from both the 1998 in vitro and 2000 in vivo studies.

MATERIALS AND METHODS

Study site. Two studies were conducted in the Salima District of Malawi along Lake Malawi, where malaria is holoendemic. The highest rates of infection occur in this region between February and April.

In vitro test. The in vitro chloroquine efficacy study was conducted in July and August 1998 as previously described.14 This study included P. falciparum isolates from 60 asymptomatic children less than five years old who had been brought to the district hospital with parasite densities >2,000/μL of blood. An in vitro micro-test kit (Mark III; World Health Organization, Manila, The Philippines) was used to assess susceptibility of P. falciparum to chloroquine and the level of in vitro responses were classified as chloroquine sensitive, borderline, and resistant according to the criteria of the World Health Organization (Table 1).

In vivo test. The in vivo study was conducted in June and July 2000 in two primary schools. Informed consent was obtained from parents of the children who participated in the study and their teachers, and the study was reviewed and approved by the Malawi Ministry of Health. Fifty-four schoolchildren (age range = 6–15 years) with mono-infections of P. falciparum, but who did not show any clinical symptoms of malaria and had not ingested any antimalarials in the preceding two weeks, were included in this study. They were treated with 25 mg of chloroquine base per kilogram of body weight over a three-day period, and followed up for 28 days. The parasitologic responses were classified according to criteria of the World Health Organization (Table 1).

Extraction of DNA. Finger prick blood (75 μL) was drawn into heparinized capillary tubes (Drummond Scientific Company, Broomall, PA) and transferred onto to chromatography filter paper (ET31CHR; Whatman, Ltd., Maidstone, United Kingdom). Each piece of filter paper was dried at ambient temperature, sealed in a separate plastic bag, and stored at 4°C. Plasmodium falciparum DNA was extracted and purified from a one-fourth of the dried filter spot, using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) accord-
ing to the manufacturer’s instruction with some modifications as previously described. 15

**Molecular analysis.** A polymerase chain reaction (PCR) and restriction fragment length polymorphism protocol for the *pfmdr1* gene was modified from the methods previously described. 59 Amplification by a nested PCR for the *pfmdr1* gene was done in a 50 μL reaction mixture containing 2 μL of template DNA, 200 μM of each dNTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM MgCl2, 1.25 units of Taq polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA), and 0.5 μM of each primer. In the initial PCR, the forward primer used for *pfmdr1* codon 86 amplification was MDROUT-F: 5'-TTGTGTTGAAGATGGGTA-3' and the reverse primer used was MDROUT-R: 5'-TACTTCTTATACATATGACACCA-3'. The initial amplification conditions were one cycle at 94°C for 12 minutes; 45 cycles at 94°C for one minute, 45°C for 45 seconds, and 65°C for one minute; and one cycle at 65°C for five minutes. In the nested PCR, the forward primer was MDRIN-F: 5'-TGAACAAAAAGAGTAACCTCTG-3' and the reverse primer was MDRIN-R: 5'-ATTACGGAAAAACCGCAAGT-3'. The nested amplification conditions were one cycle at 94°C for 12 minutes; 25 cycles at 94°C for one minute, 45°C for 30 seconds, and 65°C for 45 seconds; and one cycle at 65°C for five minutes. A 550-basepair (bp) nested PCR product that included codon 86 in the *pfmdr1* gene was digested with the restriction enzymes Apo I and Afl III when the N86 and N86Y alleles, respectively, were present.

The K76T polymorphism was also assessed by a nested PCR, restriction digestion, and allele-specific PCR as described by Djimdé and others. 9 Briefly, a 145-bp nested PCR product including codon 76 in the *pfcrt* gene was digested with the restriction enzyme Apo I. This enzyme digests K76 but not K76T. Thus, the cleavage of the amplicon into two fragments (34 and 111 bp) indicates the presence of the wild-type codon K76. If the 145-bp fragment remained undigested, a nested mutation-specific PCR was used to detect the mutant codon K76T.

**Statistical analysis.** Statistical significance was assessed using the G-test for independence with Williams’s adjustment for small sample sizes. 16

**RESULTS**

The results of the *in vitro* and *in vivo* studies are shown in Table 1. Sixty *P. falciparum* isolates were tested *in vitro*. Twenty-nine (48%) showed valid tests results for chloroquine sensitivity. Of these, 66% (19 of 29), 31% (9 of 29), and 3% (1 of 29) were classified as sensitive, borderline, and resistant, respectively. Detailed information on the *in vitro* results has been previously reported. 14 Of 54 children tested *in vivo*, one withdrew from follow up. Of those remaining, 91% (48 of 53), 8% (4 of 53), and 1% (1 of 53) were classified as sensitive, RI, and RII, respectively. The day 0 geometric mean parasite density was 1,729/μL of blood (range = 400-16,080)/μL.

The prevalences of *pfmdr1* N86Y and *pfcrt* K76T gene mutations are also shown in Table 1. The N86Y mutation in the *pfmdr1* gene and the K76T mutation in the *pfcrt* gene were detected in 45% (37 of 82) and 7% (6 of 82) of all isolates, respectively. Nearly all N86Y and K76T polymorphisms were detected in isolates with mixed infections (i.e., N86 plus N86Y and K76 plus K76T). The prevalence of K76T decreased significantly from 17% in 1998 to 2% in 2000 (P < 0.02), whereas the prevalences of N86Y (58% and 38%) did not differ significantly (P > 0.07). No statistically significant associations were observed between either *in vitro* or *in vivo* chloroquine responses and N86Y or K76T (*P* > 0.29). None of the three isolates with both K76T and N86Y was resistant to chloroquine either *in vivo* (*n* = 1) or *in vitro* (*n* = 2).

**DISCUSSION**

In our *in vitro* study, 1998, only 3% of the isolates were chloroquine resistant. This was substantially less than the 47% observed in a neighboring area along Lake Malawi surveyed in 1988. 14,17 In *in vivo* chloroquine resistance was 2% and 4% in 7- and 14-day assessments, respectively, in the asymptomatic schoolchildren in our study compared with 20% and 32% in Tanzania, a neighboring country that continues to use chloroquine. 18

The prevalence of K76T in our study population was substantially lower than that recently observed in other African countries that continue to use chloroquine (e.g., 41% in Mali 9 and 83% in Mozambique 11). In contrast to K76T, the prevalence of N86Y in Malawi is comparable with that in other African countries that continue to use chloroquine (e.g., 50% in Mali 9 and 56% in Uganda 10). Since the *pfcrt* and *pfmdr1* genes are on different chromosomes, they segregate independently. Discontinuing the use of chloroquine appears to have resulted in selection against the *pfcrt* K76T mutation, but has had little or no effect on the *pfmdr1* N86Y mutation. Al-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Susceptibility classification</th>
<th>n</th>
<th>Wild type (N86)</th>
<th>Mixed (N86 + N86Y)</th>
<th>Mutant (N86Y)</th>
<th>Wild type (K76)</th>
<th>Mixed (K76 + K76T)</th>
<th>Mutant (K76T)</th>
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<tr>
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<td></td>
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</table>

* pfmdr1 = *Plasmodium falciparum* multiple drug resistance 1; *pfcrt* = *P. falciparum* chloroquine resistance transporter.
† Resistant = minimum inhibitory concentration (MIC) > 1.6 × 10−7 M; borderline = MIC = 1.6 × 10−7 M.
‡ RII = parasite level decreased but not cleared by day 7; RI = no parasites on day 7 followed by recrudescence by day 28.
though the observed decrease in the prevalence of K76T from 17% in 1998 to 2% in 2000 supports this hypothesis, samples taken in or prior to 1993 would be required to confirm it.

The quantitative ratio of parasites with sensitive and resistant alleles may influence both in vitro and in vivo responses to drug challenges. This may explain the chloroquine sensitivity profiles of some infections that contain both the K76 and K76T alleles. However, the absence of a statistically significant association between K76T and chloroquine resistance suggests that other alleles or loci have a role in chloroquine resistance. This is consistent with the recent observation that all 24 in vitro chloroquine-resistant P. falciparum isolates collected in Madagascar contained the wild-type K76 allele.

Different levels of drug pressure are expected to result in different levels of gene stability. Therefore, alleles of genes contributing to chloroquine resistance in areas of reduced levels of drug pressure ought to be different from those in areas with ongoing drug pressure. The apparently substantial recovery of chloroquine sensitivity observed in Malawi predicted that some of the mutations contributing to chloroquine resistance might be difficult to maintain in the absence of drug pressure. The interplay among various mutations in the pfcr gene and the roles of as yet unidentified alleles and genes should be considered in the attempt to understand the molecular mechanisms that contribute to chloroquine resistance of P. falciparum under different levels of drug pressure.

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