Reemergence of Chloroquine-Sensitive
Plasmodium falciparum Malaria after Cessation of Chloroquine Use in Malawi

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In 1993, Malawi became the first African country to replace chloroquine with sulfadoxine-pyrimethamine nationwide in response to high rates of chloroquine-resistant falciparum malaria. To determine whether withdrawal of chloroquine can lead to the reemergence of chloroquine sensitivity, the prevalence of the pfcrT76T molecular marker for chloroquine-resistant Plasmodium falciparum malaria was retrospectively measured in Blantyre, Malawi. The prevalence of the chloroquine-resistant pfcrT genotype decreased from 85% in 1992 to 13% in 2000. In 2001, chloroquine cleared 100% of 63 asymptomatic P. falciparum infections, no isolates were resistant to chloroquine in vitro, and no infections with the chloroquine-resistant pfcrT genotype were detected. A concerted national effort to withdraw chloroquine from use has been followed by a return of chloroquine-sensitive falciparum malaria in Malawi. The reintroduction of chloroquine, ideally in combination with another antimalarial drug, should be considered in areas where chloroquine resistance has declined and safe and affordable alternatives remain unavailable.

In 1993, Malawi became the first sub-Saharan African country to discontinue the routine use of chloroquine against Plasmodium falciparum malaria and to elevate the antifolate combination sulfadoxine-pyrimethamine (SP) to the antimalarial of first choice nationwide. The decision was based on chloroquine’s increasing failure to produce adequate clinical and hematological recovery. High-level parasitological resistance was observed in >80% of Malawian children treated with chloroquine in 1990 [1]. Since 1993, SP has been the only available treatment for uncomplicated malaria in all government health facilities and is dispensed without prescription. Although chloroquine has remained available by prescription and through unauthorized private sources, a national information campaign was largely successful in convincing health practitioners and the public to accept SP as the treatment of choice for children with malaria. Other African countries, including Kenya in 1999 [2], have instituted similar changes in national drug policy in the face of increasing chloroquine resistance.

Chloroquine resistance in P. falciparum is conferred by mutations in the parasite pfcrT, which encodes a putative transporter localized to the digestive vacuole...
One mutation, K76T, was perfectly associated with in vitro resistance in all progeny of a genetic cross between chloroquine-sensitive and -resistant parental clones and among a set of geographically diverse parasite isolates [3]. Several field studies summarized in a recent review have since confirmed the absolute specificity of the p~f~rl K76T to clinical chloroquine resistance [5]. Polymorphisms in pfmdrl, encoding the P. falciparum P glycoprotein homologue 1, modulate chloroquine resistance in mutant p~f~r~r~l-harboring parasites in vitro [6], although their role in vivo has yet to be substantiated [7]. SP resistance is conferred by mutations in 2 genes encoding enzymes in the parasite’s folate synthesis pathway, dihydrofolate reductase (dhfr) and dihydropteroate synthase [8].

It is not known whether years of reliance on antimalarials other than chloroquine can lead to the reemergence of chloroquine-sensitive P. falciparum and permit the reintroduction of this safe and affordable drug. Using polymerase chain reaction (PCR)–based assays, we measured the prevalence of resistance-conferring mutations in malaria-infected human blood samples obtained from southern Malawi before and after the 1993 change in drug policy. We demonstrate a progressive decline in the frequency of the pf~r~l K76T mutation over the decade after the proscriptior of chloroquine and a much lower prevalence of 76T in Malawi than in neighboring Zambia, where chloroquine remained in use. We confirm these molecular findings by demonstrating chloroquine sensitivity in vitro and in vivo in contemporary infections from this same region of Malawi.

MATERIALS AND METHODS

Study sites and sample sources. Blantyre is a city of ~700,000 residents in southern Malawi with moderate-to-intense levels of seasonal P. falciparum transmission that peak during December through March. Archived, anonymized microscope slides with Field’s-stained thick smears were provided by the Malaria Project, Blantyre, Malawi. These slides had been collected in the January–June malaria seasons in 1992–1996 from children hospitalized for severe malaria. The 1998–2000 Malawian samples were collected at the Ndirande Health Centre in Blantyre from children with uncomplicated malaria, according to standard definitions [9], and were stored as dried blood spots on filter paper, as described elsewhere [7]. The Zambian samples were collected in 1999 as filter papers and blood smears from symptomatic children in the outpatient department of the Isoka district hospital in the northern province. All available samples from 1992–1996 and randomly selected samples from the Malawi 1998–2000 and Zambia 1999 sample sets were analyzed. All samples were obtained before initiation of malaria treatment.

Sample analysis. DNA extraction techniques and mutation-specific assays are detailed on the Internet: http://medschool.umd.umd.edu/CVD/powe.html. In brief, the dried thick smear was detached from the slide with a new razor blade, and the debris were collected in a microcentrifuge tube. DNA was prepared for PCR by a methanol-fixation/heat extraction method, as described elsewhere [7], for preparation of DNA from dried blood spots. Nested PCR and subsequent allele-specific restriction analysis were performed to identify polymorphic codons of interest. Infections presenting with mixed alleles in any assay were defined as polyclonal and were characterized as mutant for that assay. Direct DNA sequencing was performed at the University of Maryland Baltimore Biopolymer Facility. The χ2 test for linear trend using Epi Info (version 6.04c; CDC) was used to test for significance.

In vitro chloroquine assays. P. falciparum isolates were collected in February 2001 at the Ndirande Health Centre from consenting individuals presenting with uncomplicated malaria. Drug-sensitivity assays were performed essentially, as described elsewhere [10], using the World Health Organization (WHO) microtest method, a field test that estimates drug susceptibility by culturing fresh parasites for 1 48-h life cycle in the presence of increasing drug concentrations and by determining parasite survival by light microscopy. In brief, 0.1 mL of blood was collected in a sterile, 100-μL heparin-treated capillary tube and immediately was added to 0.9 mL of RPMI 1640 culture medium. Blood-medium mixture (50 μL) was added to each well of tissue culture plates predosed with chloroquine diphosphate salt (Sigma) at 1, 2, 4, 8, 16, 32, and 64 pmol/well. The plates were incubated at 37.5°C for 30 h, according to standard methodology. After incubation, Field’s-stained thick blood films were prepared, and the number of mature schizonts was counted per 200 asexual parasites. According to the WHO protocol, chloroquine susceptibility was defined as complete schizont inhibition at ≤4 pmol of chloroquine, and chloroquine resistance was defined as schizont formation at ≥8 pmol of chloroquine. Isolates that formed schizonts at 4 pmol of chloroquine but had complete schizont inhibition at 8 pmol were considered to be intermediate.

In vivo chloroquine efficacy. Because the last available in vivo chloroquine efficacy studies showed unacceptably high rates of treatment failure, it was deemed unethical to assess chloroquine efficacy in persons with symptomatic malaria or in children, who are at higher risk of becoming symptomatic. Therefore, we conducted a preliminary in vivo chloroquine efficacy study in infected adults with no symptoms. Consenting asymptomatic adults accompanying sick children to the Ndirande Health Centre in January–September 2001 were screened for occult P. falciparum infection by standard microscopy. Those with positive blood smears were treated by directly observed therapy with standard doses of two 300-mg tablets of chloroquine phosphate on the treatment day and on day 1 after treatment and one 300-mg tablet on day 2 after treatment.
Chloroquine efficacy was measured after slight modifications of standard protocols, as described elsewhere [11], with active microscopic and clinical follow-up on days 3, 7, and 14 after treatment and passive surveillance by continuous availability of medical care throughout the follow-up period. Because study participants were asymptomatic, parasitologic rather than therapeutic outcome definitions were used; efficacy results were recorded as sensitive or as resistant at the RI, RII, or RIII levels [11]. An additional follow-up assessment was conducted on day 28 for study participants who could be located at this time.

**RESULTS**

DNA was recovered, and assays for *pfcrt* 76T were performed successfully on 39/40 thick smears from 1992, 23/24 from 1993–1994, and 24/27 from 1995–1996. Similar results were obtained for the other assays (sample sizes are shown in figure 1). Success rates for 1998–2000 filter-paper samples were even higher, ranging from 93% to 100% (data not shown). The number of 1998–1999 samples analyzed for *dhfr* mutations was higher, because a large set of samples had been analyzed previously for a separate study.

The chloroquine resistance mutation *pfcrt* 76T was detected in 30 (85%) of the 39 isolates obtained in 1992 (figure 1A), and its prevalence diminished progressively and significantly \((P < .0001)\) over the subsequent 8 years, from 11 (50%) of 22 isolates obtained in 1993–1994 to 10 (13%) of 75 isolates in 2000. No infections with *pfcrt* 76T were found among 25 samples collected in 2001 that were analyzed for both in vivo chloroquine resistance and the presence of this mutation. The current low prevalence of the *pfcrt* 76T in Malawi is in sharp contrast to its frequency in neighboring Zambia, where chloroquine has remained the first-line antimalarial drug. We analyzed isolates obtained in 1999 from the Isoka region of northern Zambia, 80 km from the western border of Malawi, and found a 92% prevalence of the mutation (46/50 infections). These findings in Zambia are similar to those of a recent study conducted in southern Mozambique, where a 91% prevalence of the mutant *pfcrt* was detected [12].

The institution of SP was followed by an increased prevalence of 2 mutations in the parasite *dhfr* that confer pyrimethamine resistance and are common throughout East and Central Africa [13]. In 1992, the *dhfr* N51I and C59R were present in only 16 (6%) of 38 and 7 (18%) of 38 Malawi infections, respectively (figure 1B). By 1999, nearly all infections were mutant at these codons: N51I, 96% (144/150); and C59R, 94% (217/231). The significant increase in *dhfr* mutations \((P < .0001)\) is consistent with the increasing rates of antifolate resistance evident in Malawi during the 1990s [14].

We found less dramatic trends for 2 mutations in the *pfmdr1* (figure 1C), N86Y and D1246Y, which have been associated in some studies with chloroquine resistance [6, 15] and for which a role in antifolate resistance has not been described. Both mutations exhibited a modest decrease in frequency over the decade, with the decline in D1246Y, from one-half of the infections in 1993 to one-fourth in 2000, attaining statistical significance \((P = .0231)\).

The 76T has invariably been found to be accompanied by sets of additional *pfcrt* mutations [3]. We analyzed a subset of the infections from 1993 (8 with 76K infections and 4 with 76T) and 1998–2000 (10 with 76K infections and 2 with 76T) for the resistance-associated mutations at *pfcrt* codons 74, 75,
220, 271, and 371 and found that the full array of mutations always cosegregated with 76T, whereas none of the infections that were exclusively 76K were mutant at these codons (data not shown). Thus the pfcrt in Malawi appears to have maintained the integrity of its fully mutant and wild alleles, and the reemerging 76T pfcrt are not mutants that simply reverted to K76, which alone can confer the in vitro chloroquine-sensitive phenotype [3]. Variation was detected at pfcrt codons 326 and 356, which are polymorphic but not associated with resistance [7], among both the sensitive and the resistant infections (data not shown). This heterogeneity at the pfcrt locus, along with our finding that 55% of pfcrt 76T-harboring infections over the time course were mutant at dhfr codons 51 and/or 59 (data not shown), are indicative of the polyclonal nature of malaria infections in this setting and the high recombination rates of P. falciparum in Africa [16]. Malaria harboring the sensitive pfcrt appears to have arisen from low levels in the indigenous parasite populations and/or to have emigrated from bordering countries where chloroquine pressure persists. But we can exclude as an explanation for the diminished prevalence of the mutant pfcrt a selective clonal replacement of antifolate-sensitive/chloroquine-resistant parasites by antifolate-resistant parasites that coincidently harbor a sensitive pfcrt.

Of the 11 isolates successfully assayed for in vitro drug susceptibility, 9 (82%) were determined to be fully chloroquine-sensitive, whereas 2 isolates were of intermediate chloroquine susceptibility. No isolates exhibited in vitro chloroquine resistance.

Seventy-two adults were enrolled in the study of in vivo chloroquine resistance. As expected, initial parasitemias were low, with a geometric mean of 600 asexual forms/mm³ and a range of 50–12,250 asexual forms/mm³. Nine adults were lost to follow-up before day 3, precluding any determination of outcome. Each of 63 remaining asymptomatic P. falciparum infections in adults was successfully cleared within 3 days after chloroquine treatment without development of fever or other symptoms of malaria. Fifty-five adults completed the 14-day follow-up, and 42 were followed-up for 28 days. Five participants were lost to follow-up after treatment day 3, and 3 were lost to follow-up after day 7. No recurrent infections were detected during the follow-up period; thus, 100% had sensitive outcomes.

**DISCUSSION**

Our findings suggest that chloroquine-sensitive P. falciparum parasites have reemerged and are now predominant at a site in Africa where chloroquine was withdrawn in 1993, whereas high levels of chloroquine resistance have persisted unabated in nearby areas where the drug has remained in use. The withdrawal of antimicrobial drug pressure does not always compromise the fitness of resistant microorganisms and result in selection of drug-sensitive phenotypes [17, 18]. Incomplete withdrawal of the drug, cross-resistance to agents still in use, and compensatory mutations that reduce the burden of resistance without jeopardizing the resistance itself [19] may allow for resistant organisms to persist, despite restricted availability of an established antimicrobial agent. In P. falciparum malaria in Malawi, the in vivo fitness burden of harboring the mutant pfcrt appears to be substantial in the absence of selective drug pressure, and with the mechanisms of antifolate resistance distinct from those of the aminoquinolines, there appears to have been a strong selection of chloroquine-sensitive parasites after the nationwide replacement of chloroquine with SP.

The slower decline in prevalence of mutations in pfmdr1 suggests that these mutations may be less deleterious to parasite fitness than are pfcrt mutations. Although genetic transformation experiments have found that pfmdr1 mutations can modulate the level of chloroquine resistance once it has been conferred by mutations in pfcrt, mutations in pfmdr1 are by themselves insufficient to confer chloroquine resistance [20]. Recent studies have reported that pfmdr1 mutations, in addition to pfcrt mutations, are no more strongly associated with chloroquine treatment failure than pfcrt mutations alone [7] and that pfmdr1 mutations do not add to the predictive value of pfcrt mutations for chloroquine treatment failure [21]. Even if pfmdr1 mutations do contribute to treatment failure, they require pfcrt mutations to exert an effect on the response to chloroquine. Therefore, persistence of pfmdr1 mutations in a population with a very low prevalence of pfcrt mutations would not be expected to reduce chloroquine efficacy in that population.

The increase in mutations in dhfr is consistent with what is known about SP efficacy in this area during the 1990s. Before SP was introduced in Malawi as the first-line drug, efficacy rates as high as 100% were reported [22], and parasitological cure rates of 90.5% were found in southern Malawi 1 year after the introduction of SP [23]. Our own studies from the site of the current study have found that, by 2000, SP’s parasitological cure rate had decreased to <70% and its clinical efficacy to ~80% (authors’ unpublished data), corresponding to the increasing prevalence of dhfr mutations.

Standard oral doses of chloroquine cleared asymptomatic P. falciparum infections in adults with no exceptions, and there was no evidence of recurrent infection among the 53 subjects monitored for at least 14 days, thus meeting the criteria for sensitive parasitological outcome in high transmission areas. Some loss to follow-up was expected in this asymptomatic urban adult population, and we cannot rule out the possibility of recurrent infections among subjects who were lost to follow-up between 3 and 14 days after treatment. However, the complete lack of recurrent infections in the 76% of subjects who were followed for ≥14 days is strong evidence of chloroquine’s ability to clear P. falciparum infections in this setting.
It is possible that some of these semi-immune adults would have spontaneously cleared their malaria infections even without drug treatment, but it was felt to be unethical to include a placebo or nontreatment arm in this first in vivo assessment of chloroquine in Malawi since the drug was withdrawn. On the basis of the preliminary evidence of chloroquine efficacy presented here, a controlled trial of chloroquine alone or in combination with other drugs may now be ethically justifiable in Malawi.

Nine of 11 fresh *P. falciparum* isolates were sensitive to chloroquine in vitro, consistent with a recent report of a high prevalence of in vitro chloroquine-sensitive *P. falciparum* in central Malawi [24]. The finding that 2 infections yielded microtest values in the intermediate range is most likely explained by inaccuracies of the in vitro test, or by mixed resistant and sensitive parasites infections. Microtest assays are well known to be subject to variation and artifact, and, although they are useful as general measures of in vitro resistance in field surveys, they lack the reliability to definitively characterize individual infections [25–27]. *P. falciparum* infections in settings like Malawi also are frequently polyclonal, so that discrepant results could arise if in vitro survival of a minor number of resistant parasites is detected among a predominantly sensitive population. Such parasites would have had to be too few to be detected by the molecular assays, which may not detect subpopulations comprising <5%–10% of an infection. A more careful evaluation of the chloroquine in vitro response using labeled nutrient uptake assays and assessing the verapamil-reversal phenotype associated with chloroquine resistance would have been more definitive but was not possible on these fresh isolates collected under field conditions.

Earlier reports from China [28] and Gabon [29] showed less dramatic declines in measures of chloroquine resistance after reductions in chloroquine use. However, regions of Columbia and Venezuela, where chloroquine use is reportedly minimized, continue to sustain mutant *pfcrt* 76T–harboring parasites [30], which suggests that the trends observed in Malawi may not be replicated in settings with different epidemiological and transmission characteristics. Nonetheless, in malaria-endemic countries with semi-immune host populations, such as Malawi, even a partial resumption of chloroquine sensitivity may positively impact public health. In Mali, up to two-thirds of infections carrying the mutant *pfcrt* are cured by chloroquine treatment, an outcome due, at least in part, to the contribution of host immunity to parasite clearance [31, 32]. By inference, the 13% prevalence of mutant *pfcrt* seen in 2000 in Malawi predicts a chloroquine failure rate <5%, although chloroquine failure rates >50% have recently been reported in both Zambia [33] and Mozambique [12].

Our results support the possibility of reintroducing chloroquine to areas where it has been withdrawn and replaced by other drugs that will eventually fail. Many African countries are delaying decisions to change antimalarial therapies, despite high rates of chloroquine resistance that are associated with increasing malaria-attributable disease and death, fearing a slippery slope of increasingly expensive antimalarial drugs with limited useful therapeutic lives. The preliminary in vivo data reported here justify the conduct of controlled trials of chloroquine efficacy in areas where chloroquine use has been substantially reduced for a period of years. If these trials confirm a return of chloroquine’s clinical efficacy, governments can consider withdrawing chloroquine and switching to other drugs on an interim basis, knowing that they may be able to later reintroduce chloroquine, which is unparalleled in its safety and low cost. However, it must be emphasized that Malawi made a concerted and sustained effort to withdraw chloroquine from use and that less rigorous efforts to reduce chloroquine drug pressure may not result in the same reemergence of chloroquine sensitivity.

Chloroquine’s useful therapeutic life span in Malawi is almost certain to be short-lived if it is reintroduced as monotherapy. There is growing consensus that combination chemotherapy should be the rule in malaria treatment, to deter the development of drug resistance, and that the artemisinin derivatives, because of their rapid reduction of parasite biomass and continued efficacy against multidrug resistant *P. falciparum*, should constitute a component of most such regimens [34]. As SP failure rates increase, the reintroduction of chloroquine in Malawi in combination with artesunate or with other short-acting drugs, such as chlorproguanil-dapsone, should be considered and would likely extend the useful life span of each of the drugs. This study reinforces the need for rational antimalarial drug policies at both the national and regional levels and demonstrates the utility of surveillance using molecular assays to monitor the effects of these drugs on drug resistance.

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**References**


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