CONCISE COMMUNICATION

Prevalence of the K76T Mutation in the Putative *Plasmodium falciparum* Chloroquine Resistance Transporter (*pfcrt*) Gene and Its Relation to Chloroquine Resistance in Mozambique

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K76T, a mutation in the *Plasmodium falciparum* chloroquine (CQ) resistance transporter protein, has been implicated in resistance to CQ. A modified 14-day in vivo test to estimate the CQ resistance level was done in southern Mozambique: 21 (42%) of 50 subjects who completed the follow-up were CQ susceptible. Use of *msa2*–restriction fragment length polymorphism (RFLP) genotyping to differentiate new from recrudescent infections made little difference in the estimated prevalence of resistance. The K76T mutation prevalence was estimated by RFLP–polymerase chain reaction and sequencing, and its relation to parasitological CQ resistance was explored on day 0 samples: 51 of 56 pretreatment samples presented the T76 codon, and it was present in 100% of children with parasitological resistance. T76 also was present in 18 of 23 subjects in whom the infection resolved after CQ treatment. These findings show a high prevalence of the K76T mutation among wild isolates but also suggest additional factors responsible for CQ resistance.

Malaria, especially that due to *Plasmodium falciparum*, is a leading cause of disease and death worldwide. The widespread availability of cheap and effective antimalarial drugs, particularly chloroquine (CQ) and pyrimethamine-sulfadoxine, has probably decreased both morbidity and mortality, but it also likely has encouraged the development and spread of drug resistance [1].

Despite widespread resistance, CQ remains the most widely used antimalarial drug in the world. CQ resistance results from a reduced accumulation of the drug by parasites, although the precise molecular mechanisms responsible have not been elucidated fully. The intra-erythrocytic malaria parasite consumes hemoglobin, detoxifying heme by polymerization to hemozoin (i.e., pigment produced by malarial parasites), a process that is inhibited by CQ [2]. It has been thought that CQ resistance is a multigenic process [3] associated with polymorphisms in a 36-kb segment of the parasite’s chromosome 7. This segment contains *cg2*, a polymorphic gene encoding a unique 330-kDa protein, which may have a transporter function [4]. Mutations in a novel predicted integral transmembrane protein, *P. falciparum* CQ resistance transporter (*PFCRT*) [5], have recently been linked to the verapamil-reversible CQ resistance phenotype of *P. falciparum*. K76T, a mutation in the first transmembrane segment of the protein, has been proposed to be implicated in the resistance to CQ.

We have carried out an in vivo study, to assess parasitological resistance and therapeutic efficacy of CQ in southern Mozambique. To that end, we used *msa2* typing to distinguish recrudescent from newly acquired *P. falciparum* infections. We also assessed the prevalence of the K76T mutation and its relation to resistance.

Materials and Methods

*Study area and population.* The study was done in Manhiça (Maputo Province), a rural town in southern Mozambique. Malaria (mainly due to *P. falciparum*) is endemic in the area and has perennial transmission but marked seasonality. The entomologic inoculation rate is estimated to be 15 infectious bites per person per year. CQ is the first-line treatment for uncomplicated malaria. Patients attending the Manhiça District Hospital outpatient clinic were recruited over a 2-month period from May through July 1999.

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The study was reviewed and approved by the Ethical Committee, Ministry of Health, Mozambique. Written and witnessed informed consent was obtained from parents or guardians of study children.

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Inclusion criteria were (1) age between 6 and 59 months; (2) mono-infection with *P. falciparum*, with parasitemia of 1000–150,000 asexual parasites/μL; (3) axillary temperature ≥37.5°C or history of fever during the previous 24 h; (4) hematocrit >15%; (5) absence of general signs of danger or signs of severe and complicated falciparum malaria, according to the World Health Organization (WHO) definition; (6) absence of other diseases; and (7) absence of allergy to CQ.

**Treatment, follow-up, and definition of outcomes.** Children whose parents agreed to participate were seen in a separate but adjacent clinic, where CQ was administered directly by study staff at doses of 10 mg/kg on days 0 and 1 and 5 mg/kg on day 2. Subjects were observed after each dose, and a full dose was repeated if vomiting occurred within 30 min. Follow-up was based on a modified 28-day in-vivo test. In brief, patients were monitored clinically, and blood samples for parasite determinations were obtained every 24 h from day 0 until day 3 and then routinely on days 7, 14, 21, and 28. Herein, we report results based on a day 14 analysis. Parasitological resistance was classified as resistance levels RI, RII, and RIII, and clinical response was classified as adequate or as early or late treatment failure, according to a WHO protocol, on the basis of analysis on day 14 [6].

**Collection of blood samples.** Fingerprick blood samples were obtained, blood slides were prepared, and blood was placed onto filter paper (no. 903; Schleicher & Schuell). The samples were air-dried and were stored in plastic bags with silica gel at room temperature until DNA extraction. Blood slides were processed and read according to quality-controlled procedures, as described elsewhere [7].

**Typing of *P. falciparum* by msa2 restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR).** RFLP-PCR was done on all paired samples (i.e., samples from day 0 and from the day when resistance was established) from patients with resistant parasites. A section corresponding to ~1/6 of the blot on the filter paper, corresponding to ~6 μL of blood, was cut with a sterile blade. This piece then was washed with distilled water and was directly placed in a PCR reaction tube. A nested PCR was done, as described elsewhere [8]. The filter paper was used for the primary reaction (S2 and S3 primers), and 2 μL of PCR product was used for the nested reaction (S1 and S4 primers) in a 100-μL reaction [9]. To discriminate between false microscopic positives and msa2 amplification failures, we tried to amplify the cg2 locus of samples that did not amplify by use of the above protocol.

The different msa2 alleles were genotyped, as described elsewhere [10]. Nested PCR product (10 μL) was digested with the restriction enzymes *DdeI*, *RsaI*, *HinfI*, and *ScrFI*, respectively, and was run on a 10% polyacrylamide gel. All digests of 1 restriction enzyme were loaded side by side for all samples of an individual patient, and sizes were calculated by use of a 1-kb DNA ladder. RFLP patterns were visualized by ethidium bromide and were documented by using a Polaroid camera. A parasite infection with the same msa2 genotype before and after treatment was considered a recrudescence. Posttreatment infections that differed from pretreatment infections were considered new infections.

**Determination of the encoded amino acid sequence in position 76 of pfcrt.** After a piece of the blood filter paper was fixated on methanol, 50 μL of water was added and was heated for 15 min at 95–100°C. A 5-μL aliquot of extract was used as template for a nested PCR analysis, using primers TCRP1 and TCRP2 for the primary PCR and TCRD1 and TCRD2 for the nested PCR in a 100-μL volume. Both PCR reactions were performed in a Perkin Elmer Thermocycler 480 with the following profiles: the primary PCR consisted of 45 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 60°C, with a final extension at 60°C for 3 min. The nested PCR consisted of 30 cycles of 30 s at 92°C, 30 s at 48°C, and 30 s at 65°C, with a final extension at 65°C for 3 min. Negative and positive control samples from children infected with CQ-susceptible and -resistant parasites, which had been digested previously to confirm the codon 76 sequence, were included with each set of PCR reactions. After the 134-bp fragment around codon 76 was amplified, alleles carrying the K76 or T76 codon were discriminated by ApoI restriction. A 5-μL aliquot of the ampiclons was added to each sample tube. ApoI digests were incubated for 6 h at 50°C. Digest mixtures were then loaded onto 10% polyacrylamide gels. The samples not digested by ApoI were sequenced on both strands by use of a sequencing kit (Rhodamine Terminator Cycle; Perkin Elmer Applied Biosystem) to confirm the sequence in codon 76. Detailed information on these techniques is available at [http://medschool.umaryland.edu/CVD/plowe.html](http://medschool.umaryland.edu/CVD/plowe.html).

**Results**

Fifty-six 6–59-month-old children were enrolled in the study. Six subjects were withdrawn before an outcome was determined on day 14 (2 were not brought back for follow-up visits, 2 migrated, 1 repeatedly vomited, and 1 was found to have a packed cell volume of <15% on day 1). On the basis of light microscopy blood slide readings, 21 (42%) of the 50 subjects who completed the 14-day follow-up were fully susceptible to CQ, 7 (14%) were RI, 7 (14%) were RII, and 15 (30%) were RIII. Seventy-three percent of the patients were classified as having an adequate clinical response.

Complete sample pairs from the 29 children with parasite resistance were analyzed by msa2 typing. Twenty-three children (79%) had exactly the same parasites in their pre- and post-treatment samples, and therefore their infections were termed recrudescences. Four of the children (14%) in whom CQ failed to kill parasites showed evidence of both new infections and recrudescent parasites. A posttreatment sample reported to be positive on the basis of light microscopy results on day 7 did not amplify msa2 and cg2, suggesting an incorrect microscope reading. On day 14, however, a new infection was identified. In summary, the genetic analysis of recrudescent parasites gave a parasitological resistance outcome of 6 RI (12%), 6 RII (12%), and 15 RIII (30%); the analysis had little effect on our overall resistance estimate, changing it from 58% to 54%.

Most of the pretreatment samples (24 [89%] of 27) and 10 (38%) of 26 posttreatment samples contained >1 msp2 allele and were therefore mixed infections with >1 parasite clone. The median multiplicity was 2.23 (SD, 1.15) on the pretreatment samples and 1.9 (SD, 1.1) on the posttreatment samples.

Restriction enzyme digestion with ApoI confirms that there is a 5'-A/GAATTC/T-3' sequence. If ApoI does not cut, it means...
that there is a nucleotide change on either the second or third nucleotide of codon 76 or 77 or on the first nucleotide of codon 78. The undigested samples were sequenced to confirm the presence of the K76T mutation and of no other change that modified the ApoI restriction sequence. After sequencing, it was shown that there was not a unique nucleotide mutation but rather a more complex change. Fifty-one of the 56 pretreatment samples presented the T76 codon, and 4 of them consisted of a mixed infection with parasites with both alleles, giving an 85% prevalence of the mutated allele. For the rest of the analysis, mixed infections were considered to be a single infection with the mutated allele.

Analysis of the pfcrt gene showed that the K76T pfcrt mutant allele was present in 100% of children with parasitological resistance (table 1). However, the mutation was also present in 18 of the 23 subjects in whom the infection resolved after CQ treatment. The presence of the K76T mutation was significantly associated with the risk of not clearing the P. falciparum infection after CQ treatment (P = .016, Fisher’s exact test). Among these children, however, there was no correlation between age and parasitological outcome after treatment.

Discussion

In Mozambique, as in much of Africa, CQ is the first-line drug treatment for uncomplicated malaria. In this rural area, 58% of sick children brought to hospitals had resistant parasites, and there was an adequate clinical response to CQ in 73% of the patients. Our estimates of the prevalence of parasite resistance to CQ, as determined by the 14-day test, were not substantially modified after differentiating new from recrudescent infections by msa2-RFLP typing (58% vs. 54%).

The number of clones per patient that we detected on day 0 was high (2.2 clones) but tended to be lower after antimalarial drug pressure (1.9 clones). These data support the finding of previous studies that primary infections are frequently mixed in nature. In addition, it extends these observations by demonstrating that infections frequently remain mixed after treatment failure.

PFCRT is a novel predicted integral transmembrane protein recently linked to CQ resistance [5]. It has been proposed that the K76T mutation is implicated in the resistance phenotype. In our study, 85% of the pretreatment samples showed the mutated sequence. This high prevalence can be due to heavy drug pressure and/or to exposing the parasite to subinhibitory drug concentrations, which would provide the selective pressure to resistance. The mutation was present in all CQ-resistant samples and in 78% of the sensitive parasites (table 1). This would indicate that only the mutated parasites grow under drug pressure in vivo but also that CQ remains effective in clearing parasites that have that mutation.

What are the possible explanations? First, these results could be due to a silent change that modified the ApoI restriction sequence without implicating the K76T mutation. After sequencing, it was shown that the mutation is not a simple nucleotide change but rather a more complex mutation, implying a deletion and insertion of a T, which changes the 2 amino acids M74 and N75 into I74 and E75, plus a transversion from A to C, which changes the K76 to T76. We cannot determine whether the correlation between CQ resistance and genotype is due to the K76T mutations or to the M74I-N75E change.

Second, the clearing of parasites with the mutation could be explained by a synergistic effect of CQ and partial acquired immunity in children after natural exposure. However, in our study, all the children were symptomatic and thus were eligible for study inclusion, which suggests that their clinical immunity was not sufficient to prevent disease or infection. Furthermore, there was no significant correlation between age and the parasitological outcome after treatment, which suggests that the immune status was not a key element in the clearing of parasites.

Third, host differences in the ability to absorb or metabolize drugs may contribute to therapeutic efficacy. Fourth, K76T could be a first step in a multigenic mutation process, whereby changes might tend to occur in a progressive, step-wise fashion, with higher levels of resistance occurring in the presence of multiple mutations. Last, the gene underlying the CQ resistance effect may be closely linked to the pfcrt gene but not be pfcrt itself, or resistance could depend on the level of expression of pfcrt and not just on its primary sequence.

This study reports the high frequency of the K76T mutation of pfcrt in symptomatic children and suggests that this mutation appears to be a necessary but not sufficient condition for CQ resistance. It does not appear to be a good predictor of CQ resistance, as clinical response to CQ is likely to be mediated both by the immune response of the host and by parasite resistance.

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