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A point mutation in codon 76 of \textit{pfcrt} of \textit{P. falciparum} is positively selected for by Chloroquine treatment in Tanzania

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Abstract

This study was undertaken to validate the relevance of Chloroquine (CQ) resistance markers \textit{pfcrt}\textsuperscript{76} and \textit{pfmdr1}\textsuperscript{86} in an endemic area in Tanzania. After treatment with CQ, recrudescence was distinguished from new infection by \textit{msp2} genotyping, and the number of concurrent infections was also determined. The rate of children with recrudescent parasites at day 7 and/or day 14 amounted to a parasitological failure rate of 22.4% using PCR. The mean multiplicity of infection at day 0 was 3.2 (\(n = 71\)). The allelic frequencies of the mutated \textit{pfcrt}\textsuperscript{76} and \textit{pfmdr1}\textsuperscript{86} were estimated to be 92 and 77\%, respectively. Both values exceeded by far the observed frequency of 14\% of recrudescent parasites as calculated on the whole analysed parasite population taking multiple infections into account. Although neither mutant allele is of predictive value for parasitological resistance, there is evidence for a role of \textit{pfcrt}\textsuperscript{76} in CQ resistance in the natural parasite population. All wild-type \textit{pfcrt}\textsuperscript{76} alleles were eliminated before day 3, after the onset of CQ treatment and no recrudescent parasite with the wild-type allele was observed at later time points. The discrepancy between the rate of resistant parasites (14\%) and the frequency of the mutant \textit{pfcrt}\textsuperscript{76} allele (92\%) however, indicates that other polymorphisms and other factors must be involved in CQ resistance. No selective elimination of the \textit{pfmdr1}\textsuperscript{86} wild-type allele was observed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Drug resistance; Chloroquine; Multiplicity of infection; \textit{P. falciparum}; Malaria tropica; Genotyping; \textit{pfcrt}; \textit{pfmdr}; \textit{msp2}

1. Introduction

Chloroquine (CQ) has long been used as first line drug for the treatment of uncomplicated malaria tropica in Africa for the last decades due to its affordability, efficacy and safety. The rapid spread of CQ resistant parasites, however, has limited its use for malaria treatment (White, 1992) and there is an urgent need for alternatives.

The mechanism of CQ action and resistance is still a matter of investigation. CQ accumulates in the digestive vacuole of the parasite (Yayon et al., 1984) where it is supposed to interfere with haem detoxification (Slater and Cerami, 1992), or modifying aspartic and cysteine protease activity (Goldberg et al., 1991), or intravesicular pH (Krogstad et al., 1985). Resistant parasites are characterised by a reduced amount of CQ accumulated in the food vacuole as compared to sensitive parasites (Fitch, 1970). Two genes (\textit{pfmdr1} and \textit{pfmdr2}) homologous to the mammalian multidrug resistance gene (\textit{mdr}) have been identified in \textit{P. falciparum} (Foote and Kemp, 1989). \textit{mdr} codes for an ATP driven P–glycoprotein pump which confers drug resistance in mammalian cancer cell lines (Roninson, 1987). Point mutations leading to amino acid exchanges in \textit{pfmdr1} have been related to drug resistance in \textit{P. falciparum} (Ponnudurai et al., 1981; Foote et al., 1990; Freen et al., 1992; Cox-Singh et al., 1995; von Seidlein et al., 1997). Several field studies, however, failed to show an association between \textit{pfmdr1} polymorphism and drug resistance (Haruki et al., 1994; Bhattacharya et al., 1997; Bhattacharya and Pillai, 1999). Recently, allelic replacement experiments have shown that polymorphisms in \textit{pfmdr1} modify the sensitivity to CQ in parasites already resistant to CQ (Reed et al., 2000). These findings indicate that other genes might be crucial for CQ resistance. Genetic cross experiments mapped CQ resistance to a 36 kb region in chromosome 7 (Su et al., 1997). In this region, the genes \textit{cg2} and \textit{cg3} displayed codon exchanges which associated well with CQ resistance in several strains (Su et al., 1997). Allelic replacement with \textit{cg2} and \textit{cg3} sequences from CQ sensitive parasites, however, did not modify the degree of resistance in a CQ resistant strain (Fidock et al., 2000a).
Recent evidence points to an essential role of pfcrt for CQ resistance, another gene within this 36 kb region on chromosome 7 (Fidock et al., 2000b). This gene codes for a transmembrane protein which is located in the digestive vacuole of the parasite. A mutation in codon 76 leading to a change from lysine to threonine has been found exclusively in CQ resistant strains of P. falciparum. This mutation is associated with an increased acidification of the digestive vacuole, the compartment of CQ action (Fidock et al., 2000b).

Further evidence was also given in a epidemiological study in Malawi by Djimdé et al. (2001), where it was shown that the pfcrt mutation was selected after CQ treatment.

The objective of this study was to assess the association of the pfcrt76 and the pfmdr186 polymorphisms with CQ resistance in a field trial in Tanzania. The samples derived from a standard in vivo test series carried out in Massai, one of the sentinel sites for monitoring drug resistance in Tanzania. To distinguish between recrudescence and new infection, all samples were PCR amplified for the locus of the merozoite surface protein 2 (msp2) (Snounou and Beck, 1998).

2. Materials and methods

2.1. Study site and study population

The study was conducted at Nagaga Health Centre in the Massai District of Tanzania between January and March of 1999. This district is located in the southernmost area bordering Mozambique. The area under study is uniform comprising coastal plains with sandy soils and occasional rocky outcrops with hilly ridges. There are numerous marshy areas which may be associated with rivers or water courses where rice is cultivated during the long rains (March–June).

Malaria transmission is intense and perennial. Nagaga Health Centre was randomly selected from a list of all health centres in the district. The WHO protocol for assessment of therapeutic efficacy of antimalarial drugs for uncomplicated P. falciparum malaria in areas with intense transmission was followed (World Health Organization, 1996). Children attending the outpatient clinic between the ages of 6 and 60 months were recruited and clinically examined to rule out other causes of fever. Axillary temperatures, weights and blood slides for parasitaemia were taken and packed cell volumes (PCV) were also determined. Those children meeting all inclusion criteria set by World Health Organization (1996) were enrolled and standard oral CQ treatment was administered under supervision. All children were seen at the hospital again on days 1 and 2 for clinical examination and CQ administration. They were again seen on days 3 and 7 for blood slides and those with treatment failure, were given a single dose of pyrimethamine/sulfadoxine (Fansidar) (Hoffmann-La Roche, Basel, Switzerland). They were again seen on day 14, when in addition to the clinical examination, a blood slide was made and PCV was determined.

All children were treated with CQ tablets (150 mg base of CQ phosphate tablets, Helm Pharmaceuticals GMBH Hamburg) with a dose of 10 mg/kg body weight on days 0 and 1 and 5 mg/kg body weight on day 2. Fansidar was given in case of treatment failure as a single dose (1.25 mg pyrimethamine/kg body weight). In addition, Paracetamol tablets were also given for the first 2 days and during the follow-up if fever was documented.

2.2. Laboratory examination

Thick and thin blood smears were made on the same slide and stained with 5% Giemsa stain at pH 7.2 for 20 min. Parasite density was assessed by counting the number of asexual parasites per 200 leucocytes in the thick smear. Parasite numbers were converted to a count per microlitre by assuming a standard leucocyte count of 8000/µl. Species confirmation was done by examining the thin film.

2.3. Classification of therapeutic and parasitological responses

Using the clinical and parasitological criteria set by WHO the therapeutic responses to CQ treatment were classified into early treatment failure (ETF), late treatment failure (LTF) and adequate clinical response (ACR) (World Health Organization, 1996). The sum of the rate of ETF and LTF was referred to as the clinical treatment failure rate. Parasites present at day 7 and/or day 14 after CQ treatment were classified as recrudescent if their msp2 genotype had been previously detected. Genotypes which were not seen before day 7 were classified as new infections. The parasitological resistance rate was defined as the percentage of children harbouring true recrudescent parasites, a more stringent indicator for drug resistance than the clinical treatment failure rate.

2.4. Molecular analysis

For PCR analysis blood was collected at days 0, 3, 7 and 14 on Whatman 3MM filter paper and DNA was extracted using the Chelex method. A piece of filter paper of about 10 mm² was soaked overnight in 1 ml 0.5% saponin in PBS and subsequently washed in 1 ml PBS for 30 min at 4 °C. It was transferred to 200 µl of a preheated (100 °C) 5% Chelex-100 resin in sterile water, vortexed for 30 s and boiled for 10 min at 100 °C. The reaction tube was centrifuged twice at 10,000 g for 2 min and the supernatant was transferred to a new tube after each centrifugation step.

msp2 analysis was carried out as described previously (Felger et al., 1999) with 5 µl of the above DNA preparation. The baseline multiplicity of infection was determined as the number of msp2 genotypes, which were detected at day 0. For the analysis of pfmdr1 codon 86 polymorphism 2 µl of DNA were amplified in a volume of 50 µl using a PTC-100 thermocycler (MJ Research). PCR reactions were done with 1 µM of each primer (Frean et al., 1992),
1.5 mM MgCl₂, 100 μM dNTPs, 0.75 units Taq-Polymerase (Gibco). The thermal profile was as follows: 94 °C for 30 s (4 min 30 s at cycle 1), 48 °C for 1 min with an increment of +0.2 °C per cycle, and 72 °C for 2 min (7 min at cycle 40) for 40 cycles. Aliquots of 10 μl from each PCR product were cleaved by 1 unit Apo I (which cleaves the wild-type) and 1 unit Afl III (which cleaves the mutant).

For pfcrt amplification (Djimdé et al., 2001), a primary PCR was done with 1 μM of primer CCGTTAATTAAATACACGCAG (CRTP1) and CCGATGTACAACAC-TAAGTTACC (CRTP2), 2.5 mM MgCl₂, 200 μM dNTPs and 0.75 units Taq-Polymerase (Gibco). The thermal profile was 94 °C for 30 s (3 min at cycle 1), 56 °C for 30 s, and 60 °C for 1 min (3 min at cycle 43) for 45 cycles. A volume 2 μl of primary PCR product were used in a 50 μl nested PCR reaction with 1 μM of primer TGTGCTCATGTGTATTAAACTT (CRTD1) and CAAAACTATTAGTTAACCAATTTTG (CRTD2), 2.5 mM MgCl₂, 200 μM dNTPs and 0.75 units Taq-Polymerase (Gibco). The thermal profile was 92 °C for 30 s (3 min at cycle 1), 48 °C for 30 s, and 65 °C for 30 s (3 min at cycle 30) for 30 cycles yielding a 145 bp PCR product. Aliquots were digested with 1 unit Apo I, which cleaves the wild-type into 111 and 34 bp fragments. Genomic DNA from strain 3D7 was amplified and digested in the same way serving as control for complete digestion. Restriction digests of both pfmdr1 and pfcrt were analysed on 10% polyacrylamide gels.

2.5. Statistical analysis

To allow for the effects of varying multiplicity in estimates of resistance rates, we used a non-linear statistical model to estimate the allele frequencies of resistance markers prior to treatment. Assuming resistant parasite clones to be transmitted independently of sensitive clones, the likelihood for a sample containing no resistant clones is \((1 - p^n)\), where \(p\) is the allele frequency for the resistance marker and \(n\) is the multiplicity of infection of the sample. Similarly, the likelihood for a sample with no wild-type clones is \(p^n\) and for a mixture of wild-type and mutant clones is \(1 - p^n - (1 - p^n)\). The likelihood over the whole data set for \(p\) is computed as the product of these likelihood over all samples, using values of \(n\) derived from the msp2 typing. A Markov Chain Monte Carlo algorithm (Program Winbugs 1.3) was used to obtain estimates and credible intervals (Bayesian confidence intervals (CI)) for \(p\), making use of this likelihood, and assuming a uniform (0.1) prior distribution for \(p\).

3. Results

Among 79 children enrolled, one was lost during follow-up. According to the clinical and parasitological criteria set by WHO, 54 children (69.2%) had an adequate clinical response (ACR), 17 (21.8%) and 7 children (9.0%) showed early and late treatment failures (ETF, LTF), respectively. Thus, the clinical treatment failure rate in this study was 31%. Parasites on day 7 post-treatment were detected in 20 children by microscopy, amounting to a parasitological resistance rate in the host of 25.6% (Table 1). Among them, three had asymptomatic parasitaemia at day 7 and were classified as ACR, 10 as ETF and 7 as LTF (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Multiplicity of infection</th>
<th>D0 pfcrt&lt;sup&gt;76&lt;/sup&gt;</th>
<th>Clinical response</th>
<th>Parasite response by msp2&lt;sup&gt;77&lt;/sup&gt;</th>
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<tr>
<td></td>
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<td>D0 + 3</td>
<td>Rec.</td>
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<tr>
<td>137</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>

<sup>a</sup>D0: day 0; D0+3: day 0 and 3; Rec: recrudesc; new: new at day 7 and/or day 14; ND: not determined.
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Fig. 1. Tracing of parasites by PCR–RFLP of msp2 alleles in two patients (A and B) at days 0, 3 and 7. Restriction fragments after Hinfl I digest were analysed by polyacrylamide electrophoresis (10%) and ethidium bromide staining. Patient A harboured several recrudescent and one new genotype at day 7, patient B harboured one recrudescent and one new genotype.

msp2 analysis revealed that 12 children harboured recrudescent infections, two only new infections and two a mixture of recrudescent and new infections (Fig. 1, Table 1). In four children, parasite genotypes at day 7 and/or day 14 could not be analysed. Thus, the parasitological resistance rate in the host population after the subtraction of new infections determined by msp2 genotyping was 22.4% (14/16 × 25.6%).

The mean multiplicity of infections per child prior to CQ treatment was 3.2 with a total number of 229 infections in 71 children. The mean multiplicity amounted to 3.5, if genotypes newly identified at day 3 post-treatment were included in the baseline, resulting in a total number of 248 individual infections on day 0 and/or day 3. Among those, 35 parasite infections were found to be recrudescent on day 7 and/or day 14 by msp2 genotyping (Table 1), amounting to a resistance rate of 14% within the parasite population.

Baseline frequencies of both resistance markers pfcrt76 (Fig. 2) and pfmdr186 (Fig. 3) were analysed. About 79% of children carried only infections with the pfcrt76 mutation, 18% had mixed infections, and only 3% had the wild-type lysine genotype (Table 2). Both children with wild-type pfcrt76 genotype infections at day 0 cleared their parasitaemia by day 3, one had a recrudescence on day 7 after CQ treatment, which was identified as a mutant pfcrt76 infection already present on day 3. At days 0, 13 children (45%) harboured parasites with only the Tyrosine pfmdr186 mutation, 12 children (41%) carried both mutant and wild-type infections, and 4 children (14%) carried only asparagine wild-type infections (Table 2).

Table 2
Day 0 frequencies of patients carrying wild-type, mutant and mixed pfcrt76 and pfmdr186 genotypes

<table>
<thead>
<tr>
<th></th>
<th>pfcrt76</th>
<th>pfmdr186</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>Mutation</td>
<td>78.9% (56)</td>
<td>44.8% (13)</td>
</tr>
<tr>
<td>Mutation and wild-type</td>
<td>18.3% (13)</td>
<td>41.4% (12)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>2.8% (2)</td>
<td>13.8% (4)</td>
</tr>
<tr>
<td>Estimated allelic frequency</td>
<td>0.918 (95% CI: 0.876; 0.951)</td>
<td>0.766 (95% CI: 0.665; 0.850)</td>
</tr>
</tbody>
</table>

Fig. 2. Representative polyacrylamide gel of Apo I restriction digests of amplified regions of pfcrt containing the codon 76 polymorphism. The wild-type codon is cleaved into a 111 and a 34bp fragment.

Fig. 3. Representative polyacrylamide gel of amplified regions from pfmdr1 containing the codon 86 polymorphism. Each PCR product was analysed by Apo I which cleaves the wild-type, and Afl III which cleaves the mutant.

Table 2
Day 0 frequencies of patients carrying wild-type, mutant and mixed pfcrt76 and pfmdr186 genotypes
Cies of pfcrt population were estimated to be 92% (CI 95%: 88, 95%) and pfcrt should not increase as compared to day 0. At a wild-type not positively selected for its frequency after CQ treatment. The widespread use of CQ in the parasite populations. In this study, a parasitological prediction of resistance will thus lead to apparently higher resistance rates of studies in which CQ resistance was not associated with mutations in this gene (Haruki et al., 1994; Bhattacharya and Pillai, 1999). Our results confirm the hypothesis that pfmdr1 is at most a modulator of CQ resistance (Reed et al., 2000).

In conclusion, the complete elimination of the pfcrt wild-type after CQ treatment gives strong evidence for the relevance of this gene in CQ resistance in a natural parasite population.

In our study, the mean baseline multiplicity of infection prior to treatment was 3.2 which is in the range of other regions in Tanzania known for their high endemicity of malaria (Smith et al., 1999). Many studies have shown that multiplicity varies with the season, between closely related areas and also by age (Smith et al., 1999). But the effect of multiple infections on resistance rates has not generally been considered. With increasing multiplicity of infections the proportion of patients bearing either pure mutant or pure wild-type infections decreases and the proportion of patients bearing mixed infections increases assuming allelic frequencies to be independent of multiplicity (Fig. 4). The predicted trends of an increasing multiplicity are supported by our experimental data, however, they are less prominent (Fig. 4). At a given gene frequency a parasite population in individuals harboring many parasite clones is more likely to contain resistant clones than in individuals with only one or a few concurrent infections (Fig. 4), in vivo tests of resistance will thus lead to apparently higher resistance rates in highly endemic areas, even if there is no difference in the parasite populations. In this study, a parasitological resistance rate of 22.4% within the host population was determined, whereas the frequency of resistant parasites was only 14%. In order to make resistance rates of studies with different multiplicities of parasites fully comparable allelic frequencies within the parasite population have to be determined for each mutation of a given resistance marker.

Hence, it is necessary to establish a genetic resistance index (GRI), which would link the observed frequency of
point mutations at the community level with the observed clinical or parasitological failure rate at the health facility level. Such GRI would allow quantification of resistance and would provide support in decisions concerning drug policies.

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