PLASMODIUM FALCIPARUM GENOTYPES ASSOCIATED WITH CHLOROQUINE AND AMODIAQUINE RESISTANCE IN GUINEA-BISSAU

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Abstract. Chloroquine is the most commonly used antimalarial in Guinea-Bissau and high doses are routinely prescribed. Blood from 497 patients treated with different doses of chloroquine or amodiaquine were genotyped. Pfcr and pfmdr1 polymorphisms were identified. Pfmsp2 analysis identified recrudescent infections. The pfcr 72–76 haplotypes were CVIET and CVMNK. The pfcr 76T prevalence was 23% at day 0 and 96%, 83% and 100% at recrudescence following treatment with 25mg/kg and 50mg/kg of chloroquine and 15mg/kg of amodiaquine respectively. When treating pfcr 76T carrying P. falciparum the efficacy of 50 mg/kg and 25mg/kg of chloroquine was 78% and 34% respectively (P = 0.007). The genetic basis of chloroquine resistance is probably the same in Guinea-Bissau as in the rest of Africa. The low pfcr 76T prevalence suggests that resistance to normal dose chloroquine does not confer a major advantage to falciparum in Bissau and could be a result of treatment with high-dose chloroquine.

INTRODUCTION

Chloroquine-resistant (CQR) Plasmodium falciparum was first reported in Guinea-Bissau in 1990. Since then, we have conducted nearly annual in vitro drug resistance assays and several randomized clinical trials. The results indicate that the level of CQR is stable and low despite continued widespread use of chloroquine. In 1990, a survey of children 2–9 years old found a P. falciparum prevalence of 44–79%, and estimated the number of infective bites to be four per child during the rainy season. Recently there has been a perceived decrease of P. falciparum infections, possibly because of repeated campaigns for the use of impregnated bed nets since 2003.

The chloroquine resistance transporter gene (pfcr) and the multidrug resistance gene (pfmdr1) have both been associated with CQR and amodiaquine resistance (AQR). Specifically, the pfcr 76T allele has been causally associated with CQR and has spread through the continent within the pfcr 72–76 haplotype, CVIET. This association seems to be modulated by the pfcr 163R and possibly 152A alleles. Pfmdr1 86Y is the other single nucleotide polymorphism (SNP) most commonly associated with CQR, and it has been suggested to modify the level of CQR in pfcr 76T carrying parasites. Both pfcr 76T and pfmdr1 86Y have also been linked to AQR.

The pfmdr1 S1034C, N1042D, and D1246Y SNPs have also been associated with CQR in vitro. They seem to vary little in West Africa, generally coding for the amino acids S, N, and D, respectively. The pfmdr1 F184Y is the only other known SNP in the gene, but it has not been conclusively associated with CQR.

To study why the CQR prevalence has not changed in Guinea-Bissau, we analyzed the above-mentioned haplotype and SNPs to determine the genotypes associated with resistance to normal and double dose CQ and to AQ.

MATERIALS AND METHODS

The clinical part of this study is described elsewhere. In summary, a randomized clinical trial was conducted between 2001 and 2004 in Guinea-Bissau. A total of 729 children ≤15 years of age were randomly assigned to treatment with 25 or 50 mg/kg of CQ or 15 or 30 mg/kg of AQ. The drugs were given once daily for 3 days with the exception of 50 mg/kg of chloroquine that was given twice daily for 3 days. The children were followed for 35 days. Finger prick blood samples for parasite counts and genotyping were drawn on a weekly basis and whenever a child reappeared at the clinic with a possible malaria infection.

Blood collection. Blood was collected on filter papers (3MM Whitmann, Brentford, UK), dried, and stored in individual plastic bags until DNA extraction. For logistic reasons, filter papers were not available at the beginning of the study, and therefore, blood was not collected. This study is therefore based on blood samples from 497 children collected at Day 0 and whenever parasites reappeared. Day 0 filter papers were available from 127, 125, 121, and 124 children in the groups treated with 25 and 50 mg/kg of CQ and 15 and 30 mg/kg of AQ, respectively (Figure 1). Age, sex, and parasite density for each group were similar (Table 1). These variables were also similar when all patients included in the clinical study were compared with the patients included in this molecular analysis (Table 1).

Blood samples from children with reappearing P. falciparum. In the groups treated with 25 and 50 mg/kg of CQ and 15 and 30 mg/kg of AQ, there were 32, 13, 16, and 6 patients with reappearing parasites, respectively. Blood samples for genotyping were available from 29, 9, 13, and 4 patients, respectively. One polymerase chain reaction (PCR) amplification failed in the group treated with 25 mg/kg of CQ. In the group treated with 15 mg/kg AQ, three PCR amplifications failed from children with parasitemias of 40, 40, and 160 parasites/μL. In the group treated with 30 mg/kg of AQ, PCRs from three children failed, of whom two only had 40 and 80 parasites/μL. We also failed to amplify pfmsp2 in one sample previously treated with 15 mg/kg of AQ and two samples tested with 25 mg/kg of CQ.
Extraction of DNA. Approximately 25 μL of blood was cut from the filter papers and extracted on an ABI Prism 6100 Nucleic Acid Prepstation (Applied Biosystems, Fresno, CA). Extraction was performed according to the manufacturer’s protocol for isolation of DNA from whole blood with minor modifications. Extracted DNA was frozen in aliquots at −20 °C until amplification by PCR.

Genotyping. Nested PCR followed by mutation-specific restriction was used to detect *pfcrt* SNPs K76T, T152A, and S163R and *pfmdr1* SNPs N86Y, F184Y, S1034C, N1042D, and D1246Y. The SNPs were distinguished according to previously described methods with minor modifications.11,15,16 *pfmsp2* was amplified as described previously.17

Except for *pfmsp2* amplifications, PCR reactions included 0.2 mmol/L dNTPs, 1 μmol/L of each primer, 1 × *Taq* polymerase reaction buffer, 2.5 mmol/L magnesium chloride, and 1.25 units of *Taq* DNA polymerase (Promega Corp., Madison, WI).

PCR and restriction products were resolved on 2% agarose gels (Amresco, Solon, OH). All gels were stained with ethidium bromide and visualized under UV transillumination (BioRad GelDoc System, BioRad, Hercules, CA).

Sequencing. The exon containing the *pfcr77–76* haplotype was amplified by nested PCR and resolved as described above.15 Purification followed by sequencing using the for-

**Figure 1.** Study structure. ETF was defined as development of severe malaria or appearance of danger signs on Days 1–3 or fever ≥ 37.5 on Day 2 together with parasitemia greater than at Day 0. LCF was defined as 1) fever and malaria positive film or 2) a history of fever and clinical symptoms of malaria and a positive malaria film or 3) a malaria smear containing ≥ 20 parasites per 200 leukocytes during follow-up. LPF was defined as reappearing parasitemia on Day 7 or later for children without ETF or LCF. As reported previously,5 the PCR-adjusted adequate clinical and parasitologic response rates were 80%, 92%, 94%, and 94% on Day 28, respectively.

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>All patients</th>
<th>Patients with blood samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median age</td>
<td>Sex M:F</td>
</tr>
<tr>
<td>All</td>
<td>5.1 (2.8–8.9)</td>
<td>373:357</td>
</tr>
<tr>
<td>CQ 25 mg/kg</td>
<td>5.0 (3.1–8.2)</td>
<td>91:91</td>
</tr>
<tr>
<td>CQ 50 mg/kg</td>
<td>5.0 (2.5–8.0)</td>
<td>100:84</td>
</tr>
<tr>
<td>AQ 15 mg/kg</td>
<td>5.1 (2.7–9.0)</td>
<td>91:90</td>
</tr>
<tr>
<td>AQ 30 mg/kg</td>
<td>6.0 (2.9–9.5)</td>
<td>91:91</td>
</tr>
</tbody>
</table>

Age is given in years with the interquartile range in parentheses.

* Mean number of *P. falciparum* per microliter. The 95% confidence interval is given in parentheses.
ward nest primer was performed at Macrogen (Seoul, South Korea).  

Statistics. The prevalence of children infected with *P. falciparum* containing *pfcr* 76T, *pfmdr* 86Y, and *pfmdr* 184F alleles were calculated at Day 0. The allele prevalences were also calculated for recrudescent infections as defined by *pfmsp2* analysis. Recrudescence was defined as the occurrence of at least one identical band on Day 0 and during re-parasitemia. The recrudescent allele prevalences were compared with Day 0 using Fisher exact two-tailed tests. *P* < 0.05 was considered to be significant. Samples with both genotypes at a locus were considered to be mixed infections and therefore contributed to the prevalence of both genotypes. A logistic regression model was used to assess linkage disequilibrium.

Ethics. Children and/or their parents were informed of the study and consented to participate verbally because the literacy rate is low. The information was standardized and in accordance with the principles of the Helsinki Declaration. Ethical approval was given by the Ministério da Saúde Pública in Guinea-Bissau (019/DHE/2004), Karolinska Institute in Stockholm, Sweden (2005/111-31/1), and the Central Ethical Committee in Denmark (624-01-0042). The clinical part of this study was registered at ClinicalTrials.gov (https://register.clinicaltrials.gov/; study ID: PSB-2001-chl-am0).

**RESULTS**

**Prevalence of *pfcr* K76T, *pfmdr* 86Y, and *pfmdr* 184Y genotypes at Day 0.** The genotype at position 76 of *pfcr* was successfully identified in 478 of 497 possible samples. *pfcr* 76T was found in 23.2% (111/478) and *pfcr* 76K in 82.4% (394/478). *pfmdr* N86Y and F184Y alleles were identified in 477 samples. The prevalences were as follows: *pfmdr* 184Y 47.6% (227/477); *pfmdr* 184F, 80% (382/477); *pfmdr* 184Y, 37.3% (178/477).

**Table 2.** Prevalence before and after treatment of *pfcr* K76T, *pfmdr* 86Y, and *pfmdr* 184F genotypes

<table>
<thead>
<tr>
<th>Group</th>
<th>CQ 25 mg/kg</th>
<th>CQ 50 mg/kg</th>
<th>AQ 15 mg/kg</th>
<th>AQ 30 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pfcr</em> 76T genotypes</td>
<td>T TK K</td>
<td>T TK K</td>
<td>T TK K</td>
<td>T TK K</td>
</tr>
<tr>
<td>Nr at Day 0</td>
<td>28 6 87</td>
<td>21 2 99</td>
<td>22 5 87</td>
<td>22 4 95</td>
</tr>
<tr>
<td>Nr recrudescent 21 – 1</td>
<td>53.7 (7.8–224.7)* 5 – 1 21.5 (2.2–1027.7)† 7 – – Undefined* 1 – –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nr re-infections 3 – 1</td>
<td>7.7 (0.6–405.7) 2 – 1 8.6 (0.4–512.8) 1 – 1 3.2 (0–255.8) – – –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 24 – 3†</td>
<td>20.5 (5.6–110.6)* 7 – 2 15.1 (2.6–153.4)* 9† – 1 29 (3.6–1285)* – – –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pfmdr</em> 86Y genotypes</td>
<td>Y YN N</td>
<td>Y YN N</td>
<td>Y YN N</td>
<td>Y YN N</td>
</tr>
<tr>
<td>Nr at Day 0</td>
<td>33 25 63</td>
<td>35 18 68</td>
<td>33 23 58</td>
<td>40 20 61</td>
</tr>
<tr>
<td>Nr recrudescent 7 1 12</td>
<td>0.7 (0.2–2.1) 3 – 3 1.3 (0.2–10) 7 – – Undefined 1 – –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nr re-infections 1 1 2</td>
<td>1.1 (0.1–15.4) 1 1 1 2.6 (0.1–153.6) 1 – 1 1 (0–82.7) – – –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 8 2 15†</td>
<td>0.7 (0.3–1.9) 4 1 4 1.6 (0.3–8.5) 8 – 1 8.3 (1–373.9)† 1 – –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pfmdr</em> 184Y genotypes</td>
<td>F FY Y</td>
<td>F FY Y</td>
<td>F FY Y</td>
<td>F FY Y</td>
</tr>
<tr>
<td>Nr at Day 0</td>
<td>76 16 28</td>
<td>79 17 25</td>
<td>69 23 22</td>
<td>76 16 28</td>
</tr>
<tr>
<td>Nr recrudescent 17 – 4</td>
<td>1.3 (0.4–5.7) 5 – 1 1.5 (0.2–71.5) 7 – 0§ Undefined 1 – –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nr re-infections 3 – 1</td>
<td>– – Undefined 2 – 1 0.6 (0.357) 2 – – Undefined – – –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 21 1 4†</td>
<td>1.7 (0.5–7.2) 7 – 2 1 (0.2–10.6) 9 – 0§ Undefined 1 – –</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* SNP undefined by *pfmsp2* analysis.
* *P* < 0.001.
†* P < 0.01.
‡* P < 0.05.
§* De-selection of *pfmdr* 184Y.

**Sequencing.** Codons 59–85 of *pfcr* were successfully sequenced from 45 recrudescent infections, 9 re-infections, and 3 undetermined re-parasitemias. Thirteen successfully treated samples containing *pfcr* 76T and 15 containing *pfcr* 76K according to the restriction results were randomly selected and sequenced. The *pfcr* 76–76 haplotypes identified were CVIET, CVMNK, or a combination of the two. For all samples sequenced, the *pfcr* 76 genotype identified was the same as that found by restriction.

**Selection of *pfcr* 76T by CQ and AQ.** Treatment with 25 and 50 mg/kg of CQ resulted in a *pfcr* 76T prevalence of 96% (21/22) and 83% (5/6) among recrudescent infections, respectively. Compared with the Day 0 prevalences of 28% (34/121) and 19% (23/122), respectively, this represented a significant selection of the 76T allele (*P* < 0.001 and *P* = 0.002; Table 2). The efficacy of 25 and 50 mg/kg of CQ against *P. falciparum* with *pfcr* 76T was 38.2% (13/34) and 78.3% (18/23), respectively (odds ratio [OR], 5.8; 95% confidence interval [CI], 1.5–24.3; *P* = 0.007). In the groups treated with 25 and 50 mg/kg of CQ, children infected with *pfcr* 76T carrying *P. falciparum* had a mean parasitemia of 12,148 and 11,937/µL and a median age of 5.1 and 5.6 years, respectively.

After treatment with 15 mg/kg of AQ, the *pfcr* 76T prevalence was 100% (7/7) in the recrudescent group (*P* < 0.001; Table 2).

We were only able to identify the genotype from one sample after treatment with 30 mg/kg AQ. The alleles identified were *pfcr* 76T, *pfmdr* 86Y, and *pfmdr* 184F.

**Selection of *pfmdr* 86Y.** After treating with 25 and 50 mg/kg CQ, *pfmdr* 86Y was identified in 40% (820) and 50% (3/6) of recrudescent infections, respectively. Compared with the Day 0 prevalence of 48% (58/121) and 44% (53/121), we found no significant selection of *pfmdr* 86Y by CQ.

After treatment with 15 mg/kg AQ, *pfmdr* 86Y was found in 100% (7/7) of recrudescent infections and was thus selected for by treatment (*P* = 0.01).
Selection of pfmdr1 184F. No significant selection was seen irrespective of treatment used; however, when treated with 15 mg/kg AQ, the pfmdr1 184F allele was found in all the recrudescent parasites (77%) compared with the Day 0 prevalence of 80% (92/114). Although pfmdr1 184F was not significantly selected, pfmdr1 184Y was deselected. The pfmdr1 184Y prevalence decreased from 39% (45/114) at Day 0 to 0% (0/7) after treatment with 15 mg/kg AQ ($P = 0.045$).

**Linkage disequilibrium and selection of SNP combinations.** Associations between different SNPs were assessed using a logistic regression model. Only data from infections with a single genotype are presented, because inclusion of the mixed genotypes did not change the associations significantly. We observed associations between carriage of pfcr 76T and pfmdr1 86Y adjusted (OR, 3.1; 95% CI, 1.7–5.5; $P < 0.001$) in the Day 0 samples. Similarly carriage of pfmdr1 184F (adjusted OR, 11.6; 95% CI, 5.2–26.3; $P < 0.001$) was associated with pfmdr1 184F, but this disappeared when it was adjusted for the association between pfcr 76T and pfmdr1 86Y (OR, 1.7; 95% CI, 0.78–3.7; $P = 0.18$). Despite these associations, no significant selection of the combination pfcr 76T and pfmdr1 86Y or pfcr 76T and pfmdr1 184F or pfmdr1 86Y and 184F were seen in any of the groups.

**Other pfcr and pfmdr1 alleles identified.** The pfcr T152A and S163R and pfmdr1 S1034C, N1042D, and D1246Y alleles were identified in blood samples collected at Day 0 and during reappearing parasitemia from 177 and 43 patients, respectively. In a sample collected from a successfully treated patient, we found a pfcr 163R allele together with a pfcr 76K allele. A pfmdr1 1246Y allele was found in a sample from another successfully treated patient. All the other parasites carried alleles pfcr 152T, pfcr 163S, pfmdr1 1034S, pfmdr1 1042N, and pfmdr1 1246D. Further analyses of samples collected at Day 0 were not done as we found such little allelic variation.

**Recurrent pfcr 76K carrying parasites.** One pfcr 76K parasite from the group treated with 25 mg/kg AQ and one pfcr 76K parasite from the group treated with 50 mg/kg AQ recrudesced at Days 28 and 21, respectively. The CQ concentration at Day 7 was 355 nmol in the patient receiving 50 mg/kg CQ and was not determined in the other patient. This CQ concentration was lower than in the group treated with 25 mg/kg but still higher than that found in several successfully treated patients. The parasitemias at Day 0 were 14,000 and 1,200/µL, respectively. Gametocytes were not detected in the recrudescent samples.

**Re-infections occurred after day 14. pfmsp2 genotypes were identified from 48 children with reappearing parasitemias.** Thirty-six were recrudescent infections, nine were re-infections, and three were not determined because pfmsp2 analyses failed. Re-infections were only identified from Day 21 onward. The undetermined parasitemias were also identified after at least 21 days of follow-up.

**DISCUSSION**

Finding the pfcr 72-76 haplotype CVIET and linking pfcr 76T with CQ resistance in Guinea-Bissau suggests that the genetic basis of CQR is the same in Guinea-Bissau as in the rest of Africa. This is as expected considering the likely spread of this resistance associated pfcr haplotype from Asia and through Africa. Chloroquine resistance was first reported in Guinea-Bissau in 1990, and CQ is still the most commonly used antimalarial. We would therefore expect pfcr 76T to be the most common genotype as it is in neighboring Senegal. However, we only detected this resistance-associated genotype in 23% of patients with uncomplicated *P. falciparum* infection. The low pfcr 76T prevalence is in agreement with *in vivo* data showing relatively low and constant levels of CQR.

It is not clear why the prevalence of CQR *P. falciparum* is low in Guinea-Bissau. However, we show that 50 mg/kg CQ is at least twice as efficacious as 25 mg/kg CQ (78% versus 34%) when treating *P. falciparum* with pfcr 76T ($P = 0.007$). This explains why 50 mg/kg CQ is more effective than 25 mg/kg, and is of considerable interest, because an average CQ dose of 76 mg/kg is reportedly used in Guinea-Bissau. Furthermore, it has previously been suggested that parasite resistance to high doses of CQ comes at such a cost to fitness that resistant parasites cannot compete with other strains and consequently will not thrive. Our results are by no means conclusive but raise the question of whether high-dose CQ, as used in Guinea-Bissau, delays the spread of *P. falciparum* resistant to 25 mg/kg CQ. In view of these data, the apparently safe and well-tolerated CQ dose of 50 mg/kg is an option to consider when discussing the re-introduction of chloroquine as part of a combination therapy.

Another aspect of our findings is that monitoring of pfcr 76T can be used to estimate *in vivo* CQR in Guinea-Bissau by calculating the genotype failure index (GFI) as suggested by Djimdé and others. When treating with 25 and 50 mg/kg CQ, we found PCR-adjusted adequate clinical and parasitologic treatment outcomes at Day 28 of 76% and 92%, respectively. Using the respective 28% and 19% pfcr 163R allele together with a pfcr 76T prevalence is potentially a cause of another successfully treated patient. All the other parasites carried alleles pfcr 152T, pfcr 163S, pfmdr1 1034S, pfmdr1 1042N, and pfmdr1 1246D. Further analyses of samples collected at Day 0 were not done as we found such little allelic variation.

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pfcr76T prevalence is only 23%. *P. falciparum* with pfcr76T are significantly more sensitive to treatment with 50 mg/kg than to treatment with 25 mg/kg CQ. Together with the report that high doses of CQ are commonly prescribed in Guinea-Bissau, this raises the important issue of whether an increased CQ dose regimen can delay the development of resistance.

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