INTRODUCTION

After its introduction to malaria therapy in the 1940s, chloroquine very soon became the drug of choice for therapy and prevention. It combined low toxicity and cheap production costs with high efficacy against malaria parasites, and seemed to be a crucial tool for malaria eradication. However, resistance developed almost simultaneously in the late 1950s in Southeast Asia and South America and spread rapidly to other endemic areas. Specifically, Africa has been affected severely by chloroquine resistance: In Senegal, its emergence to be a crucial tool for malaria eradication. However, resistance developed almost simultaneously in the late 1950s in Southeast Asia and South America and spread rapidly to other endemic areas. Specifically, Africa has been affected severely by chloroquine resistance: In Senegal, its emergence over a 12-year period was associated with at least a doubling of the risk of death from malaria in children younger than 10. But despite its diminishing efficacy, chloroquine remains the primary antimalarial agent in many endemic areas because no affordable alternatives are available.

The efficacy of chloroquine on the parasite is attributed to interference with the plasmoidal heme metabolism in the digestive vacuole. Resistant parasites manage to diminish the digestive vacuole. Resistant parasites manage to diminish the accumulation of chloroquine. Resistance to chloroquine has been linked to a form of multidrug resistance (mdr) found in some mammalian tumor cell lines. Plasmodium falciparum mdr1-gene, which is homologue to the mdr-gene of cancer cells, has been identified on chromosome 5 of chloroquine-resistant laboratory clones of P. falciparum. Investigations point to a central role of the specific mutation Lys76Thr (K76T). Recently, point mutations on the Pfmdr1-76- and Pfmdr1-1246 genes are conferring plasmodial resistance to chloroquine. Whether the described molecular changes have been associated with the exchange of amino acids in some chloroquine-resistant isolates and may play a role in the determination of drug resistance. Single base polymorphisms detected in codons 1042 and 1246 cause amino acid alterations Asn→Tyr and Asp→Tyr. Because of inconsistent results, a straightforward association between the mentioned polymorphisms and chloroquine resistance has been questioned by various groups. Wellens et al. (1989) demonstrated by genetic crossing between sensitive and resistant clones that polymorphisms in the pfmdr1 did not necessarily determine resistant phenotypes in the offspring of these clones.

Chloroquine resistance also has been linked to changes in a 36kb segment of chromosome7 and a number of complex variations in the gene cg2. However, a decisive role of this gene in determining resistance has been ruled out by allelic modification experiments. Recently, point mutations on the gene pfert, which is located on chromosome 7 near cg2, have been associated with chloroquine resistance in several laboratory clones of P. falciparum.3 Investigations point to a central role of the specific mutation Lys76Thr (K76T). Recently, correlations between the Lys76Thr mutation on pfert and, to a lesser extent, Asp→Tyr on pfmdr1 and in vivo resistance to chloroquine have been demonstrated in field isolates of P. falciparum in Mali and Laos.

Evidence is mounting that the described molecular changes are conferring plasmodial resistance to chloroquine. Whether there are genetic differences between the modes of resistance in various regions has yet to be determined. In addition, the suitability of the proposed genetic polymorphisms for predicting true phenotypic resistance in diagnostic or epidemiologic settings has to be assessed. Plasmodial drug resistance has not been investigated in Mauritania, where malaria transmission is normally low and seasonal. When atypically strong rainfalls in 1998 led to an outbreak of an epidemic of falciparum malaria that affected non-endemic regions bordering the Saharan desert, it became possible to compare samples from two areas with different malaria endemics.

PATIENTS, MATERIALS, AND METHODS

Sample collection and DNA preparation. Blood samples were collected from 386 febrile patients recruited during a study of in vivo drug resistance in Hodh El Gharbi in southeast Mauritania at the end of 1998 (Figure 1). Two medical centers were involved, those of Aioun, the capital city of Hodh El Gharbi in a mostly urban and arid area, and of Kobeni, a small city on the border of Mali in a rural and...
humid area. While the former area is classified as non-endemic under normal conditions, latter has a holoendemic malaria situation. Altogether, 165 samples from patients seeking medical care in Aioun and 221 samples from patients treated in Kobeni were analyzed. Inclusion criteria were monoinfection with *P. falciparum*, a parasitemia of at least 300 parasites/μL, fever or history of fever within 24 hours, no intake of antimalarials in the 4 weeks before presentation, no complicated malaria, and informed consent of the parents. All patients enrolled were treated with chloroquine tablets at a dose of 25 mg/kg over 3 days. Therapy was supervised. If change of treatment became necessary during follow-up, a standard oral dose of sulphadoxine-pyrimethamine was given.

Patients were seen again on days 3, 7, and 14 and on any other day in case of symptoms. Thick blood films were prepared from finger-prick blood, stained with Field's stain, immediately read by an experienced laboratory assistant, and later re-read at the central laboratory. Outcome was assessed according to WHO guidelines in sensitive and RI-III resistant. While RI classifies initial response to the drug and recrudescence at a later stage, RII is defined as incomplete parasitologic cure during treatment and RII as complete lack of response. During standard malaria testing by thick and thin blood film, 10 μL of full blood from each patient were dotted on Whatman 3MM chromatography paper and air-dried at room temperature before initiation of treatment. DNA was prepared from the dried blood spots as previously described. Half of the bloodspot (corresponding to approximately 5 μL of blood) was cut from the filter, transferred to a tube containing 180 μL of 5% Chelex-100 (Bio-Rad Laboratories, Munich, Germany) and mixed intensively. After incubation in boiling water for 5 minutes, the tube was vortexed for 30 seconds and incubated in boiling water for 10 minutes more. The Chelex was separated by centrifugation (12,000×g for 2 min, repeated once) and the supernatant containing the isolated DNA was transferred to a fresh tube.

**PCR amplification and product analysis.** For detection of the single base change at codon 86, a 330-basepair DNA fragment was amplified under the following conditions: 5 μL of DNA-Template, 0.5 μmol of primers pfmdr1-1 AGA TGG TAA CCT CAG TAT CA and pfmdr1-2 TTA CAT CCA TAC AAT AAC TTG, reaction buffer, 200 μM dNTP and one unit of *Taq* DNA polymerase in a 20-μL reaction. Denaturation at 94°C was performed for 2.5 min, followed by 38 cycles of denaturation at 94°C for 45 sec, annealing at 47°C for 2 min, and extension at 72°C for another 1 min, with final extension at 72°C for 10 min. Similarly, the primers pfmdr1-3 GCG TGT ATT TGC TGT AAG AG and pfmdr1-4 CAG CAT AAC TAC CAG TTA AT flank a predicted PCR product of 400 bp necessary for detection of polymorphism at codon 1042. Primers pfmdr1-5 GTG GAA AAT CAA CTT TTA TGA and pfmdr1-6 TTA GGT TCT CTT AAT AAT GCT flank codon 1246 and span a fragment of 500 bp.

The outcome of the amplification was controlled by electrophoresis in an ethidium bromide–stained 1–2% agarose gel. A band size of about 330 bp indicated successful amplification of the DNA portion at codon 86, 400 bp for codon 1042, and 500 bp for codon 1246. Laboratory strains K1 and 7G8 were included in the amplification procedures as controls. PCR amplification was followed by restriction-
fragment-length-polymorphism analysis and carried out as reported earlier.\(^{18}\) Depending on the quality of the bands in the control gel, 7 μL or more of polymerase chain reaction (PCR) product were incubated with restriction enzymes according to the manufacturer’s instructions and analyzed on an ethidium bromide-stained 2% agarose gel. The putative resistant mutant 86\(^{Ty}S\) is defined by the appearance of an AflIII restriction site, the amplified region containing codon 1042 was incubated with AseI, and EcoRV was used to identify the polymorphism at codon 1246. AflIII digestion of amplificates from K1-type strains produced digested products of approximately 110 and 220 base pairs. Since the 86\(^{Asn}\) codon is not a restriction site, there was no digestion if the wild-type codon was present and a band size of 330 bp was maintained. AseI digestion of amplificates from 7G8 type strains revealed a digest product of 160 and 240 bp while wild-type 1042\(^{Asn}\) showed a three-band pattern of 160, 130, and 110 bp. Two bands of each 250 bp indicated successful digestion and mutation 86\(^{Ty}S\), whereas the wild-type pattern was not digested. K1 served as positive control for AflIII digestion and as negative control for codons 1042 and 1246. Strain 7G8 was used as positive control for codons 1042 and 1246.

A nested PCR protocol was used to identify the K76T in the Pfcrt gene.\(^{12,14}\) A 5-μL aliquot of extract was used as template for a nested PCR using primers TCRP1 (5′ CGG TTA ATA ATA AAT ACA CGC AG 3′) and TCRP2 (5′ CGG ATG TTA CAA AAC TAG TAC C 3′) for the primary PCR, and TCRD1 (5′ TGT GCT CAT GTG TTT AAA CTT 3′) and TCRD2 (5′ CAA AAC TAG TAT CAC CAA TTT TG 3′) for the nested PCR, in a 50-μL mixture containing 20 mM Tris pH 8.8, 50 mM KCl, 2.5 mM MgCl\(_2\), 0.2 mM each dNTP, 1 mM each primer and 1.25 U Taq polymerase. Both PCR reactions were performed with the following profiles: The primary PCR consisted of initial denaturation at 94°C for 2.5 min followed by 45 cycles of 30 s at 94°C, 30 s at 56°C, and 1 minute at 60°C, with a final extension at 60°C for 3 min. The nested PCR consisted of initial denaturation at 92°C for 2.5 min followed by 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 controls were included with each set of PCR reactions. After amplification of a 145-bp fragment around the 76 codon, alleles carrying the K76 or T76 codon were discriminated by ApoI-restriction. A 5-μL aliquot of the amplicons was added to each sample tube. ApoI digests were incubated for 6 hr at 50°C. Digest mixtures were then loaded onto 2–3% polyacrylamide gels, subjected to electrophoresis, visualized by ethidium bromide, and documented using a camera. Data were entered and analyzed with Epinfo 2000 software (Centers for Disease Control and Prevention, Atlanta, Ga.)

## RESULTS

Blood samples of 85 patients were analyzed for this study. Median parasite density before treatment was 2,782 parasites/μL (range 910–98,000). Chloroquine resistance was detected in vivo in 33 samples (38.8%). Table 1 presents the prevalence of genetic polymorphisms in isolates obtained from patients before initiation of treatment and their association with parasitologic outcome. No mutations were detected in the loci 1042 and 1246 of Pfmdr1. The prevalence of the resistance association mutation at Pfmdr-86 was clearly increased in isolates with RI-resistance (P = 0.032; Mantel-Haenszel test). However, isolates identified with RII or RIII resistance in vivo showed no significant association with the mutation (P = 0.129). Investigation of the K76T mutation at the Pfcrt-gene provided evidence of a complete absence of wild-type strains among resistant isolates (Table 1). Although the K76T mutation also was present among chloroquine-sensitive samples, its presence was significantly associated with resistance (P < 0.001).

The value of the detection of resistance-associated mutation for diagnostic and epidemiologic purposes was judged by calculating the odds ratio for in vivo resistance in isolates that were tested for mutations at day 0 before treatment (Table 2). In this detection of Pfmdr-76-tyrosine showed an increased odds ratio (2.91) for resistance (P = 0.0195). However, by use of this codon alone, sensitivity for detection of resistance was 60.6%, and specificity was 65.3%. In comparison, detection of the K76T mutation at Pfcrt showed a very high sensitivity (100%), while specificity remained relatively low (65.4%). For the combination of mutations on both genes, the odds ratio for detection of resistance increased to 5.31 (P = 0.0005). Here, sensitivity was again decreased to 60.6%, while specificity increased to 76.9%.

## DISCUSSION

In this chloroquine treatment trial in southern Mauritania, only a minority of samples showed in vivo resistance (38.8%).

| TABLE 1 | Prevalence of genetic polymorphisms in isolates obtained from patients before initiation of treatment with chloroquine and association with outcome (n = 85) |
| --- | --- | --- | --- |
| Gene/codon | Sensitive (%) (n = 52) | RI (%) (n = 19) | RII/III (%) (n = 14) | All (%) (n = 85) |
| Pfcrt-76-mutation | 23 (44.2) | 19 (100) | 14 (100) | 56 (65.9) |
| Pfcrt-76-wild-type | 29 (55.8) | 0 | 0 | 29 (34.1) |
| Pfmdr1-86-mutation | 18 (34.6) | 12 (63) | 8 (57) | 38 (44.7) |
| Pfmdr1-86-wild type | 34 (65.4) | 7 (37) | 6 (43) | 47 (55.3) |

| TABLE 2 | Association between the presence of genetic polymorphisms before treatment and the likelihood of in vivo resistance (n = 85) during an outbreak of falciparum malaria in southern Mauritania |
| --- | --- | --- | --- |
| Genetic polymorphism | Prevalence in chloroquine-sensitive isolates % (n/total n) | Prevalence in chloroquine-resistant isolates % (n/total n) | Odds ratio (95% CI)* | P value† |
| Pfcrt-76-mutation | 44.2 (23/52) | 100 (33/33) | undefined | <0.0001 |
| Pfmdr-86-mutation | 34.6 (18/52) | 60.6 (20/33) | 2.91 (1.08–7.95) | 0.0195 |
| Pfcrt-76-mutation + Pfmdr-86-mutation | 23.1 (12/52) | 57.6 (20/33) | 5.31 (18–14.96) | 0.0005 |

* Mantel-Haenszel test
† CI = Confidence interval
This finding is clearly in contrast with observations from neighboring countries, where chloroquine-resistant malaria is highly prevalent. A possible reason is the generally low prevalence of malaria, which has been confined to the southern areas in most years. Thus, selection pressure on the parasites by self-treatment of the population with chloroquine may be limited. An interesting feature of the investigated population was the near absence of multiple-parasite genotypes, a finding confirmed by genotyping studies. Thus, the usual limitations of studies in highly endemic areas outside of outbreak situations (high prevalence of multiple infections, limited predictive value of molecular methods for the development of in vivo resistance) did not apply. It should be mentioned, however, that the method of restriction digestion applied here generally performs with a lower sensitivity than sequencing methods. Some mixed infections may have been missed because of this limitation. An effect of age or hospital site on the results was not seen in this study, suggesting a lack of immunity to the malaria strains, most probably imported, that caused the outbreak. This allows for a clearer picture of the effect of resistance-related mutations on in vivo resistance. However, since populations examined in this study were relatively small, the observed effect of lacking immunity may be due to chance.

The Pfcr7 point mutation K76T was present in all the chloroquine-resistant isolates. Parasites presenting with K76T, the wild-type genotype that is associated with sensitivity in laboratory strains, were not present in a single isolate found in patients with in vivo resistance. This indicates an absolute selection of this mutation in parasites under chloroquine drug pressure. Detection of T76 in P. falciparum isolates at the time of malaria diagnosis provided a 100% sensitivity for in vivo resistance (Tables 1 and 2). However, it was not highly predictive of treatment outcome (positive predictive value [PPV] 58.9%), since it was also present in susceptible isolates. These data support the theory that T76 of Pfcr7 is a necessary determinant for chloroquine resistance. The effect of this mutation on the parasite metabolism may be overcome by other factors (e.g., host immunity), thus leading to in vivo sensitivity despite fulfilled genetic requirements for resistance on the site of the parasite.

Detection of mutations on the Pfmdr1-86 codon (tyrosine) had an odds ratio of 2.91 for site of the parasite. Despite fulfilled genetic requirements for resistance on the development of in vivo resistance (Tables 1 and 2). However, it was not highly predictive of treatment outcome (positive predictive value [PPV] 58.9%), since it was also present in susceptible isolates. These data support the theory that T76 of Pfcr7 is a necessary determinant for chloroquine resistance. The effect of this mutation on the parasite metabolism may be overcome by other factors (e.g., host immunity), thus leading to in vivo sensitivity despite fulfilled genetic requirements for resistance on the site of the parasite.


