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Pfmdr1 $^{Asn}1042^{Asp}$ and $^{Asp}1246^{Tyr}$ Polymorphisms, Thought to Be Associated with Chloroquine Resistance, Are Present in Chloroquine-Resistant and -Sensitive Brazilian Field Isolates of *Plasmodium falciparum*

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Póvoa, M. M., Adagu, I. S., Oliveira, S. G., Machado, R. L. D., Miles, M. A., and Warhurst, D. C. 1998. *Pfmdr1* $^{Asn}1042^{Asp}$ and $^{Asp}1246^{Tyr}$ polymorphisms, thought to be associated with chloroquine resistance, are present in chloroquine-resistant and -sensitive Brazilian field isolates of *Plasmodium falciparum. Experimental Parasitology* **88,** 64–68. Parasite resistance to antimalarial drugs, particularly chloroquine, is the most disturbing problem of malaria chemotherapy. There is evidence that the codon 86^{Tyr} polymorphism of the *Pfmdr1* gene is associated with chloroquine resistance in West African *Plasmodium falciparum.* The association of this and four other coding alterations of the *Pfmdr1* gene with chloroquine resistance has not been extensively investigated in South American isolates. In this study, we examined 51 Brazilian *P. falciparum* isolates for the presence or absence of $^{Asn}86^{Tyr}$, $^{Asn}1042^{Asp}$, and $^{Asp}1246^{Tyr}$ polymorphisms. While these isolates were all sensitive *in vitro* to mefloquine, amodiaquine, and quinine, only 2 (4%) were chloroquine-sensitive. The findings reported here provide the first observations of this kind on a large number of field parasite samples from South America. We show that *in vitro* chloroquine-resistant and -sensitive strains carry the $^{Asn}1042^{Asp}$ and $^{Asp}1246^{Tyr}$ polymorphisms and provide support for earlier suggestions that $^{Asn}86^{Tyr}$ may be rare or absent in South American *P. falciparum.*

Index Descriptors and Abbreviations: malaria; *Pfmdr1* gene; point mutations; chloroquine resistance; Brazil; PCR, polymerase chain reaction; GFM, glass fiber membrane; CQS, chloroquine-sensitive; CQR, chloroquine resistance; CQ, chloroquine; MQ, mefloquine; AMQ, amodiaquine; QN, quinine.

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INTRODUCTION

The emergence of CQR coupled with its spread and persistence continues to pose problems in malaria chemotherapy. Observations on the parasites studied so far show that resistant parasites accumulate less chloroquine than sensitive strains, which is thought to indicate changes in drug import or export. Although this observation led to a number of biochemical and genetic explanations of CQR in *Plasmodium falciparum,* the exact mechanism of resistance remains unclear. A point mutation in codon 86 (Asn → Tyr) of the *Pfmdr1* gene (chromosome 5) has been strongly associated with CQR in African and some Southeast Asian isolates (Foote *et al.* 1990; Adagu *et al.* 1995, 1996, 1997; Basco *et al.* 1995; Cox-Singh *et al.* 1995). Amino acid substitutions in four other positions of the *Pfmdr1* gene have been found in a CQR Brazilian *P. falciparum,* 7G8 (Foote *et al.* 1990). However, other studies (Wellems *et al.* 1990, 1991) linked CQR to changes in a 400-kb region of chromosome 7. Recent reports (van Es *et al.* 1994a,b; Peel *et al.* 1994) provide compelling evidence implicating the *Pfmdr1* gene in CQR. The transfection study of Volkman *et al.* (1995) shows that *Pfmdr1* will functionally replace the *Ste6* gene for the mating pheromone a- factor export molecule in yeast. While evidence is accumulating for the involvement of codon 86^{Tyr} polymorphism in African *P. falciparum* resistant to CQ, the association of CQR with *Pfmdr1* polymorphisms in South

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American isolates has not been extensively investigated. In this study, therefore, we examined *P. falciparum* isolates from two areas (Macapá and Serra do Navio) of Amapá, Brazil. In the Brazilian isolates studied, two of the amino acid changes ($^{Asn}1042^{Asp}$ and $^{Asp}1246^{Tyr}$) examined here are found in the majority of the isolates.

MATERIALS AND METHODS

Parasite samples. Parasite samples were obtained from patients presenting uncomplicated falciparum infections in malaria clinics of Macapá and Serra do Navio Amapá, Brazil. The parasites were characterized for sensitivity to CQ, MQ, AMQ, and QN using the WHO mark II *in vitro* microtest (WHO 1990). Blood samples were spotted onto GFM and were prepared for PCR using the method of Warhurst *et al.* (1991). In all, 51 parasite samples were collected and examined for the presence or absence of the putative polymorphisms associated with CQR.

Polymerase chain reaction. The regions of the *Pfmdr1* gene amplified include nucleotide stretches containing the codons of interest with the expected changes as follows: A754T, A3622G, and G4234T. The sequences and concentrations of primers flanking A745T and G4234T were as described previously (Awad-El-Kariem *et al.* 1992; Frean *et al.* 1992). Oligonucleotides 5′-GCG TGT ATT TGC TGT AAG AG-3′ (forward) and 5′-CAG CAT AAC TAC CAG TAA AT-3′ (reverse) (1 μ*M* final concentration) were selected to flank A3662G. See Frean *et al.* (1992) for the PCR conditions. All primers were obtained from Pharmacia (Biotech Ltd).

Restriction fragment length polymorphisms (*RFLPs*). Restriction enzymes *Afl*III (Boehringer Mannheim), *Vsp*1 (Strategene), and *Eco*RV (GIBCO BRL), which recognize the A754T, A3622G, and G4234T changes, respectively (see Adagu *et al.* 1995), were used to digest the PCR products. Digestions were performed in a 10-μl volume at 37°C [5.75 μl of ddH_2O, 4 μl of PCR product, 1 μl of 10× reaction buffer, and 0.25 μl of endonuclease (0.5 U)].

RESULTS

In vitro test. A total of 51 parasite samples were characterized for sensitivity to CQ, MQ, AMQ, and QN. Parasitemia varied between 1500 and 70,000/mm^3 of blood. These isolates were sensitive to MQ, AMQ, and QN. Only 2 of the 51 parasites were sensitive to CQ; the remaining 49 were CQ-resistant. In all the resistant isolates, schizont maturation was inhibited only with chloroquine concentrations equal to or more than 16 pmole/50 μl well. This level of resistance corresponds in general to RII or RIII *in vivo* CQ resistance (see Wernsdorfer and Kouznetsov 1980).

Polymerase chain reaction. The amplified regions of the *Pfmdr1* gene containing codon 86, 1042, and 1246 mutations produced amplicons of 355, 400, and 500 bp, respectively (not shown). Standard laboratory isolate, K1, has been shown to have the codon 86^{Tyr} polymorphism while 7G8 contains the remaining two polymorphisms examined in this study. DNA samples from K1 and 7G8 were therefore used in the positive control PCR amplification of DNA from the 51 field samples.

*Restriction fragment length polymorphisms. Afl*III digestion of the amplified product which contains codon 86 produced a restriction pattern of 100 and 255 bp in the positive control sample, indicating the presence of the 86^{Tyr} polymorphism (Fig. 1). However, none of the field samples examined shows this restriction pattern, indicating the absence of the polymorphism. Interestingly, a *Vsp*I restriction pattern compatible with codon 1042^{Asp} was detected in 50 of the 51 samples tested. The presence of this nucleotide change results in the loss of one of the two *Vsp*I restriction sites contained in the amplified region of the *Pfmdr1* gene: a 160 and 240-bp restriction pattern indicates the presence of the polymorphism in these 50 field samples (see Fig. 2). Figure 3 presents *Eco*RV digestion products. The 500-bp PCR products for all samples were digested producing two fragments of 250 bp each—revealed as one fragment. This restriction pattern shows the presence of the codon 1246^{Tyr} polymorphism in all these isolates. Codon 86^{Tyr} polymorphism was not detected in 7G8. Similarly, codon 1042^{Asp} and 1246^{Tyr} polymorphisms were not detected in K1; these standard laboratory isolates served as internal controls. The internal control (7G8) for *Afl*III digests (Fig. 1, lane 14) was not digested, indicating the absence of the 86^{Tyr} polymorphism. In Fig. 2, lane 14, K1 PCR product (internal control for *Vsp*I digests) was digested, giving a 160, 130, and 110-bp restriction pattern indicative of the absence of codon 1042^{Asp} polymorphism. The K1 PCR product (internal control for *Eco*RV digests) in lane 14 of Fig. 3 was not digested, indicating the absence of codon 1246^{Tyr} polymorphism.

DISCUSSION

In this study, the *in vitro* sensitivity test shows that only 2/51 (4%) parasites examined were sensitive to CQ. The remaining 49 (96%) were CQ-resistant. This is in agreement with previous studies (di Santi *et al.* 1987, 1988; Rosario 1983) reporting high level of CQR in Brazilian *P. falciparum.* Chloroquinized salt has been used extensively in our study areas, particularly in Serra do Navio where the mine workers used the salt for prophylaxis.

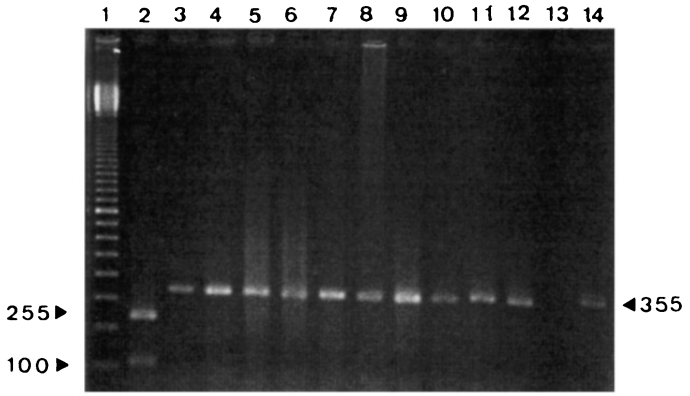

FIG. 1. Representative agarose gel (2%) electrophoresis of *Afl*III restriction digests of amplified regions of the *Pfmdr1* gene containing the codon 86 polymorphism. Lane 1, 100-bp ladder, lane 2, positive control for 86^{Tyr}, 1042^{Asp}, or 1246^{Tyr}; lanes 3–12, field samples; lane 13, negative control; lane 14, internal control (see text). Band size is indicated in base pairs (bp).

In a separate study (Póvoa *et al.*, in preparation), more than one clone was detected by genotyping in only about 30% of these 51 isolates, although individual isolates were genetically distinct. This could indicate a relatively low transmission rate compared with African isolates and/or the presence of a strongly selective drug background pressure. It is intriguing that these isolates were all sensitive to MQ, AMQ, and QN. This finding therefore provides support for the use of the current therapy of QN plus tetracycline in the treatment of *P. falciparum* infections in the study areas.

These studies report the first observations of *Pfmdr1* made on a large number of parasite samples from defined geographical areas of Brazil. In all, the results indicate the absence of the codon 86^{Tyr} polymorphism in the isolates examined. This is consistent with previous reports associating this with African and Southeast Asian parasites but not South American parasites. It is possible, based on results presented here, that the 86^{Tyr} could be used as a marker for the detection of African or Southeast Asian strains of *P. falciparum* imported into Brazil.

Of the 51 isolates, only 1 (CQ resistant) did not show the codon 1042^{Asp} polymorphism. However, PCR products from all 51 isolates revealed a restriction pattern indicative of the presence of codon 1246^{Tyr}. The detection of the two polymorphisms, 1042^{Asp} and 1246^{Tyr} polymorphisms, in chloroquine-sensitive and -resistant isolates supports the proposed multigenic nature of the CQR phenomenon. An alternative interpretation is that 1042^{Asp} and 1246^{Tyr} are "wild

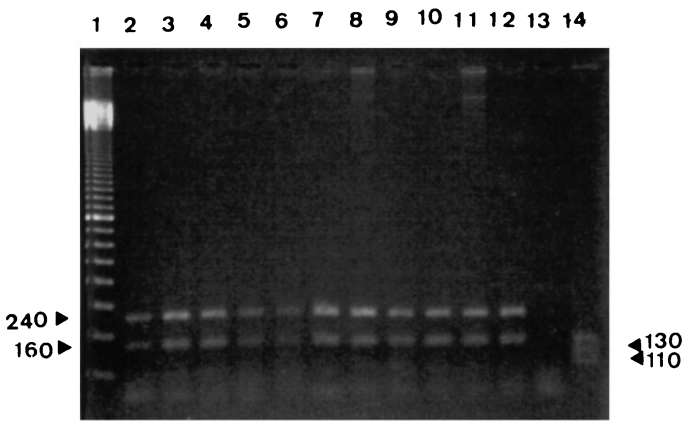

FIG. 2. Representative agarose gel (2%) electrophoresis of *Vsp*I restriction digests of amplified regions of the *Pfmdr1* gene containing the codon 1042 polymorphism. Lane 1, 100-bp ladder; lane 2, positive control for 86^{Tyr}, 1042^{Asp}, or 1246^{Tyr}; lanes 3–12, field samples; lane 13, negative control; lane 14, internal control (see text). Band size is indicated in base pairs (bp).

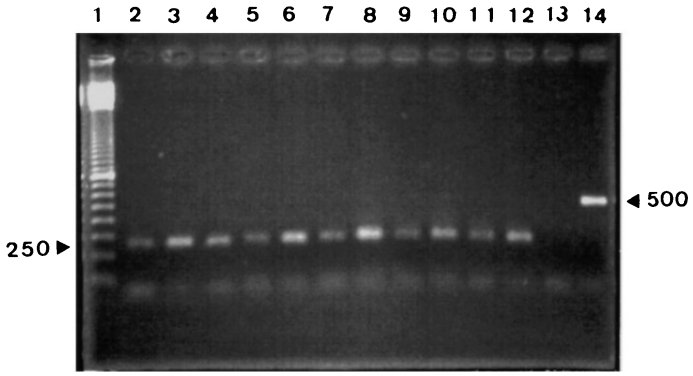

FIG. 3. Representative agarose gel (2%) electrophoresis of *Eco*RV restriction digests of amplified regions of the *Pfmdr1* gene containing the codon 1246 polymorphism. Lane 1, 100-bp ladder; lane 2, positive control for 86^{Tyr}, 1042^{Asp}, or 1246^{Tyr}; lanes 3–12, field samples; lane 13, negative control; lane 14, internal control (see text). Band size is indicated in base pairs (bp).

type" for *Pfmdr1* in Amapá. Where there is an incomplete association of allele with resistance, it is difficult to obtain a sufficient number of CQ-sensitive strains to be able to draw meaningful statistical conclusions.

It is still not clear what other genetic factors are involved in CQR. The findings of Wellems *et al.* (1990, 1991) are of major importance, and perhaps the unknown changes in chromosome 7 may regulate functions such as the baseline lysosomal pH, which, if lowered, may cancel out the resistance-conferring effect of mutations in the 5′ or 3′ halves of the *Pfmdr1* gene.

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