PLASMODIUM FALCIPARUM mdr1 MUTATIONS AND IN VIVO CHLOROQUINE RESISTANCE IN INDONESIA

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Abstract. Mutations in the Pfmdr1 gene are reported to be associated with chloroquine resistance in some Plasmodium falciparum isolates. A polymerase chain reaction/restriction fragment length polymorphism method was used for the detection of Pfmdr1 mutations in chloroquine-resistant field isolates of P. falciparum collected in Irian Jaya. The frequency of Pfmdr1 mutations was significantly higher in chloroquine-resistant P. falciparum parasites than background frequencies observed in the same location. The 7G8 mutation was identified in some parasites although always in a mixed genotype status. Chloroquine-resistant P. falciparum specimens were characterized using the World Health Organization 28-day criteria, supplemented by demonstrating adequate chloroquine absorption and genetic analysis.

Resistance of Plasmodium falciparum to chloroquine (CQ) and other antimalarial drugs is a widespread phenomenon that challenges current control efforts. Since early reports in the late 1950s, CQ resistance has been identified in most regions of the world where malaria is endemic. This problem has been investigated in several islands of the Indonesian archipelago such as Lombok, Java, and Irian Jaya, where the first Indonesian case of CQ-resistant P. falciparum was described. To understand the mechanism of CQ resistance in malaria parasites, a search for the responsible gene(s) has been launched. In 1989 two P. falciparum genes homologous to the mammalian multiple drug resistance (MDR) gene were identified, mapped to chromosome VII, and named Pfmdr1 and Pfmdr2. This discovery, coupled with the fact that the calcium channel blocker verapamil reverses CQ resistance, gave support to the theory that a mechanism similar to multiple drug resistance in mammalian tumor cells, in which overexpression and amplification of the MDR gene confers drug resistance to tumor cells via an efflux mechanism, operates in P. falciparum. The product of the Pfmdr1 gene, P-glycoprotein homolog 1 (Pgh1) has been localized to the membrane of the digestive vacuole of mature blood stage parasites. This model predicted that the Pfmdr1 gene would be amplified and/or over expressed in CQ-resistant isolates but several studies obtained mixed results.

Considerable controversy has been generated by conflicting reports that tested a second model: that mutations in the Pfmdr1 gene confer CQ resistance. DNA sequencing of the Pfmdr1 gene from reference strains and field isolates has revealed several point mutations that correlated with CQ resistance. These mutations were classed into two genotypes: the K1 genotype, resulting in an asparagine (Asn) to tyrosine (Tyr) change at position 86 and the 7G8 genotype, with four point mutations resulting in amino acid substitutions at positions 184, 1034, 1042, and 1246. Shortly after a rapid polymerase chain reaction (PCR) and restriction digest method for the detection of these two genotypes in field isolates was developed; field studies applied this technique and confirmed the presence of these mutations in CQ-resistant isolates from Malaysia, Guinea-Bissau, Nigeria, and sub-Saharan Africa. No association, however, was found in isolates from Sudan, Thailand, Cambodia, or 17 cultured strains. Moreover, genetic studies found no linkage between CQ resistance and the Pfmdr1 gene, and a CQ-resistance trait has been localized to chromosome VII, indicating that there are other mechanisms involved in CQ resistance in P. falciparum. It should be noted that expression of the wild type Pfmdr1 product (Pgh1) in Chinese hamster ovary cells confers a CQ-sensitive phenotype, whereas expression of the 7G8 mutant Pgh1 confers a CQ-resistant phenotype in the same cells, corroborating the involvement of this gene in CQ resistance. The conflicting evidence seems to indicate that CQ resistance in P. falciparum involves multiple mechanisms.

This study reports the frequencies of the K1 and 7G8 Pfmdr1 mutations in P. falciparum specimens collected from patients failing chloroquine treatment during a 28-day in vivo test conducted in Arso PIR V, Irian Jaya, Indonesia and compares them with the background frequencies of Pfmdr1 mutations in the same location.

MATERIALS AND METHODS

This study was conducted in accordance with U.S. Navy and Republic of Indonesia regulations governing the protection of human subjects in medical research. Committees for the protection of human subjects from the U.S. Navy Medical Research Unit No. 2 and the Indonesian Communicable Diseases Research Center reviewed and approved the procedures followed in this project. Chloroquine treatment group. Specimens were collected from Javanese volunteers living in Arso PIR V, a transmigration settlement in Irian Jaya, Indonesia in September 1995 following an evaluation of rapid diagnostic methodologies for malaria. After obtaining signed informed consent, volunteers were enrolled in the study and screened for malaria. Giemsa-stained blood smears were examined by standard microscopic procedures.

Subjects positive for malaria were then invited to participate in the 28-day in vivo test and treated with standard dose CQ by provincial health care providers according to national standards. Blots for high-performance liquid chromatography (HPLC) measurement of CQ levels were obtained on treatment days 0, 2, and 28 or on the day parasitemia recurred. Blots for DNA were obtained on the day of
recurrent parasitemia. Blood smears were obtained on the day of screening, daily during the first weeks after treatment, and on days 11, 14, 18, 21, and 28 after treatment except in the case of recurrent parasitemia, in which case the last smear was taken when the attending physician provided alternate treatment for malaria and the subject was dropped from the study. A resistant case was classified as RI if the parasitemia cleared before day 7 but recurred within 28 days, RII if parasitemia was markedly reduced (> 75%) by day two of treatment but did not clear by day 7, or RIII if parasitemia was not markedly reduced by day two.

Control group. Blood blots were collected from participants in a clinical trial assessing the prophylactic efficacy of desethylchloroquine (DCQ) of 200 ng/ml in whole blood and processed as previously described. Temperature. Blood blots were cut, macerated, placed in vials, Kingdom) #1 filter paper, air-dried, and stored at room temperature. Fingerprick, blotted on Whatman (Maidstone, United Kingdom) #1 filter paper, air-dried, and stored at room temperature. Blood blots were cut, macerated, placed in vials, and processed as previously described. The sum of CQ plus desethylchloroquine (DCQ) of 200 ng/ml in whole blood was considered to be the minimum effective concentration (MEC) for killing CQ sensitive Plasmodium falciparum parasites.

Reference strains. Eight cultured strains of P. falciparum were obtained from the American Type Culture Collection (Rockville, MD) (as controls: 30194 Cambodian-1, 30930 FCR-1/FVO, 30932 FCR-3/FMG, 30950 Hondurans-1/CDC, 30993 FCC-2/HAINAN, 50005 FCR-3/F-86, 50028 FCR-8 W.AFR, and 50072 FCR-3/A-2). Three additional strains were used: strain K1 isolated from Thailand in 1979 (resistant to CQ, quinine, and sulfadoxine/pyrimethamine), strain NF54 from west Africa (CQ sensitive), and strain 7G8 collected in Brazil (CQ resistant). The DNA was extracted directly from the original vials without cultivation of the parasites.

Measurement of whole blood CQ levels. For normal phase HPLC, a 100-μl aliquot of whole blood was collected by fingerprick, blotted on Whatman (Maidstone, United Kingdom) #1 filter paper, air-dried, and stored at room temperature. Blood blots were cut, macerated, placed in vials, and processed as previously described. The sum of CQ plus desethylchloroquine (DCQ) of 200 ng/ml in whole blood was considered to be the minimum effective concentration (MEC) for killing CQ sensitive Plasmodium falciparum parasites.

Extraction of DNA and the PCR. A 20-μl aliquot of whole blood was collected onto filter paper, air-dried, and stored at room temperature in separate plastic sample bags. The DNA was extracted using the Ready-Amp Genomic DNA Purification System (Promega, Madison, WI). A 5-μl aliquot of extract was used as template for the PCR using published primers that flank nucleotide # 754 (positions 499 to 853) of the Pfmdr1 gene in a 50 μl mixture containing 20 mM Tris-Cl, 50 mM KCl, 2 mM MgCl2, 0.2 mM dNTP, 2 ng/μl of each primer, and 1.25 unit of Thermus aquaticus DNA polymerase. Samples were then incubated for denaturation at 94°C for 15 sec, annealing at 51°C for 15 sec, and extension at 72°C for 15 sec for 35 cycles in a Perkin-Elmer (Norwalk, CT) 9600 thermal cycler. A second set of primers (MDR5 5’AATTTTCAAACCAAT CTGGAT3’ and MDR6, 5’GGTTCTCTTAAATGCTCTA3’) was used to amplify a 355-basepair (bp) fragment (positions 4133–4488) flanking nucleotide position 4234. The reaction conditions were the same except the annealing temperature was 50°C and the MgCl2 concentration was 3 mM. A 10-μl aliquot of each PCR mixture was then mixed with loading buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA, 50% glycerol, 0.25% bromophenol blue) and subjected to electrophoresis on 2% agarose gels, which were then examined under ultraviolet light with a Bio-Rad (Hercules, CA) Gel Doc 1000 imaging system after staining with ethidium bromide.

Restriction fragment length polymorphism. A master mixture consisting of enzyme, buffer, and sterile distilled water was made, and a 20-μl aliquot of this mixture was added to each 0.2-ml tube. A 5-μl aliquot of the amplicons was then added to each sample tube. Nsp I digests were incubated for 16 hr at 37°C in a Perkin-Elmer 9600 Thermal Cycler in the presence of 1 unit of the restriction enzyme, 50 mM Tris-HCl, pH 8.0, and 10 mM MgCl2 in a 25-μl reaction volume. Eco RV digests were performed as above except that 2 units of restriction enzyme were used in 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, and 1 mM DTT, pH 7.9, in a 50-μl reaction. Digest mixtures were then loaded onto 2% agarose gels, subjected to electrophoresis, stained with ethidium bromide, and viewed with a Bio-Rad Gel Doc 1000 imaging system. Nsp I was used to detect the K1 mutation (A to T) resulting in Asp1246 to Tyr1246, whereas Eco RV was used to detect the 7G8 mutation (G to T) resulting in Asp1246 to Tyr1246.

RESULTS

Malaria cases. Of 97 malaria cases identified in the CQ group (by microscopy and PCR) during screening, 53 (55%) were P. falciparum, 31 (32%) were P. vivax, and 13 (15%) were mixed infections; these latter infections were counted as P. falciparum cases. Recurrent post-treatment parasitemias appeared between days 4 and 20. Of the 66 P. falciparum cases, 65 (98.5%) were resistant: 30 (46%) RI, 16 (25%) RII, and 19 (29%) RIII. There was only one P. falciparum case that did not recur and was considered CQ sensitive. All total CQ levels on the day of recurrence were above the MEC of 200 ng/ml of whole blood. The mean CQ plus DCQ level was 913 ng/ml, and the minimum level was 215 ng/ml. Data on CQ were missing for three cases that were removed from statistical analysis. In the control group, there were 87 cases of malaria: 55 (63.2%) were P. falciparum, 29 (33.3%) were P. vivax, and 3 (9.2%) were mixed infections. These infections occurred in all three study arms, azithromycin (28), doxycycline (3), or placebo (56), between the third and the 17th week after radical cure.

Polymerase chain reaction. The 354-bp fragment flanking nucleotide position 754 (amino acid 86) and 355-bp fragment flanking nucleotide position 4234 (amino acid 1246) of the Pfmdr1 gene were amplified in 65 blood specimens obtained upon recurrence of P. falciparum malaria in the CQ group. In the control group, 59 P. falciparum specimens were amplified using the same Pfmdr1 primers. Amplicons were also obtained from 11 reference strains.

Pfmdr1 mutations in reference strains. Strains Cam-
bodian 1, FCR-1, FCR-3 (3 clones), and K1 were found to contain the K1 mutation whereas strains Honduras-1, FCC-2, FCR-8, and 7G8 lack this mutation. The Pfmdr1 genotype corresponded with published CQ sensitivity for nine (81%) of 11 strains (Table 1). The CQ status of strain Camb1 is not known and our culture of strain NF54, reported to be CQ sensitive, has a mixed genotype for Pfmdr1 (Table 1). As expected, the only control strain that contained the 7G8 mutation was BRZL 7G8 (Table 1).

### Pfmdr1 mutations in field isolates

After excluding specimens for which no HPLC data was available in the CQ group, 49 (79%) of 62 CQ-resistant, PCR-positive, *P. falciparum* specimens contained an Nsp I restriction site at nucleotide 754 indicating the presence of the K1 mutation in the Pfmdr1 gene (Figure 1). In the control group, 30 (50.8%) of 59 samples contained the K1 mutation. There was no statistical difference in the frequency of the K1 mutation in the antibiotic (azithromycin/doxycycline) group versus the placebo group ($P = 0.237$); thus, these study subjects were grouped together as the control group. When we compared the proportion of K1 mutants by $\chi^2$ distribution in the CQ group versus the control group, we found statistical association between the presence of the K1 mutation and exposure to CQ ($P = 0.001$; Table 2). Data obtained from *in vivo* testing was used to evaluate the level of resistance for each *P. falciparum* specimen. A $\chi^2$ analysis showed that there was no statistical association between K1 genotype and level of resistance ($P = 0.795$).

The 7G8 mutation was found in 14 (23%) isolates in the CQ group and 2 isolates (3%) in the control group. We found a statistical association between the presence of the 7G8 mutation and exposure to CQ ($P = 0.00018$; Table 2). However, in all cases, including the positive control strain 7G8 (Brazil), this mutation was present only as a mixed genotype and often as a very faint band (Figure 2). There was a tendency for the K1 and 7G8 mutations to occur exclusively of each other (Fisher exact $P = 0.005$), although in the CQ group there were 6 of 62 isolates that harbored both mutations (either as mixed parasite populations or double mutants). There were seven CQ-resistant isolates that lacked either mutation, including two which were RIII resistant cases.

### DISCUSSION

We have found in this study that the K1 mutation was present in the majority of *P. falciparum* isolates that demonstrated *in vivo* resistance to CQ therapy. The frequencies of the K1 and 7G8 mutations in CQ-resistant specimens were significantly higher than background frequencies in the same area. This data set associates the presence of CQ in subjects’ whole blood by HPLC, *in vivo* CQ resistance, and genetic analysis of the Pfmdr1 gene for the first time in Indonesia, adding to the growing database on genetics of drug resistance in Southeast Asia. When we compared the parasite population that survived CQ treatment, and a control population not exposed to CQ, we found that the K1 mutation of the Pfmdr1 gene was statistically more likely to be found among *P. falciparum* specimens in the CQ group. Although this data set suggests that the K1 mutation of the Pfmdr1 gene is involved in CQ resistance, it also suggests that it is not the only factor, especially since two isolates with RIII type resistance lacked either Pfmdr1 mutation. Interestingly, some CQ-resistant strains that lacked the K1 mutations possessed instead the 7G8 mutation, although only

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**Table 1**

<table>
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<tr>
<th>ATCC#</th>
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<th>7G8</th>
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<td>?</td>
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<td>Asp$^{1246}$</td>
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<td>FCR-1/FVO</td>
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<td>R</td>
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<td>Asp$^{1246}$</td>
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<td>Asp$^{1246}$</td>
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<tr>
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<td>S</td>
<td>Asp$^{86}$</td>
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<td>Brazil</td>
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**Table 2**

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<tr>
<td></td>
<td>A</td>
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</tr>
<tr>
<td>Tyr$^{66}$</td>
<td>49 (79%)</td>
<td>30 (51%)</td>
</tr>
<tr>
<td>Asn$^{86}$</td>
<td>13 (21%)</td>
<td>29 (49%)</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>59</td>
</tr>
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</table>

* A. A statistical association was found between the presence of the K1 mutation (Tyr$^{66}$) and exposure to CQ (uncorrected $\chi^2 = 10.6, P = 0.0011$). B, a statistical association was found between presence of the 7G8 mutation (Tyr$^{1246}$) and exposure to CQ (uncorrected $\chi^2 = 9.70, P = 0.0018$). For definition of abbreviations, see Table 1.
as a fraction of the amplicon. The relevance of this finding for parasite survival in the presence of CQ is not clear. Because nearly all of the *P. falciparum* cases in this study were CQ resistant, statistical comparisons between resistant and sensitive strains were not possible. To represent the background frequency of *Pfmdr1* mutations in Irian Jaya we used *P. falciparum* isolates that survived in the presence of antibiotics or placebo (but in the absence of CQ) in the control group.

Our results are consistent with other studies of *Pfmdr1* mutations among *P. falciparum* CQ-resistant strains conducted in Malaysia,\(^18\) Guinea-Bissau,\(^19\) Nigeria,\(^20\) and sub-Saharan Africa.\(^21\) These data, however, are in conflict with reports of lack of association between *Pfmdr1* mutations and CQ resistance in *P. falciparum* using field isolates from Sudan,\(^22\) Thailand,\(^23\) and several laboratory strains.\(^25\) The majority of *Pfmdr1* studies completed to date have characterized the CQ sensitivity of their specimens in *vitro*. Because our study characterized the resistant isolates in vivo following World Health Organization guidelines, substantiated by measurement of CQ in the patients blood, we believe this data set adds strength to the theory of involvement of the *Pfmdr1* mutations in CQ resistance.

The exact role of the *Pgh1* encoded by the *Pfmdr1* gene in CQ resistance still remains to be demonstrated, although significant evidence suggests that it is one of the factors. Studies with Chinese hamster ovary cells have suggested that *Pgh1* is involved in movement of chloride ions, and thus pH regulation in the food vacuole of the parasite.\(^28\) In addition, *Pgh1* expression in yeast cells confers cellular resistance to mefloquine, quinine, halofantrine, and quinacrine.\(^33\)

If one considers that CQ resistance originated almost simultaneously in Southeast Asia and South America, it is possible that this phenomenon involves multiple transport mechanisms and multiple genes. Recently, field studies\(^43\) have identified a series of mutations of the *cg2* gene in CQ resistant *P. falciparum*, suggesting that this gene is also involved. Moreover, we have detected the presence of the 7G8 mutation (first identified in Brazil) in several cases, although always in a mixed genotype situation. Although rare, this is not the first report of such an occurrence in Southeast Asia: at least one other strain, from Papua New Guinea, was determined to have the 7G8 mutation.\(^16\)

Chloroquine-resistant *P. falciparum* has been reported from each of Indonesia’s 27 provinces,\(^15\) but CQ remains the first-line drug for treatment of malaria. Clearly, alternative antimalarials are needed to control *P. falciparum* infection in this archipelago.

In summary, we have found that a significant majority of CQ-resistant *P. falciparum* isolates in this study carried the K1 mutation in their *Pfmdr1* gene. When comparing this frequency with that of our control population, we found a statistical difference. These observations provide evidence in support of a link between in *vitro* sensitivity to CQ and mutations in the *Pfmdr1* gene. Further studies are needed to investigate the function of *Pgh1* in CQ resistance in *P. falciparum* as well as other mechanisms involved.

Acknowledgments: We express our gratitude to the transmigrant community of Arso PIR V, Irian Jaya for participation in this study. The technical assistance of A. F. Rusdi is gratefully acknowledged. Financial support: This study was supported in part by U.S. Navy Medical Research and Development Command work units 2408 and 2601.

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