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<th>Polymorphism of the Plasmodium falciparum multidrug resistance and chloroquine resistance transporter genes and in vitro susceptibility to aminoquinolines in isolates from the Peruvian Amazon.</th>
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<td>Author(s)</td>
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POLYMORPHISM OF THE \textit{PLASMODIUM FALCIPARUM} MULTIDRUG RESISTANCE AND CHLOROQUINE RESISTANCE TRANSPORTER GENES AND \textit{IN VITRO} SUSCEPTIBILITY TO AMINOQUINOLINES IN ISOLATES FROM THE PERUVIAN AMAZON

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\textbf{Abstract.} \textit{In vitro} drug sensitivity to chloroquine (CQ), mefloquine (MQ) and quinine was investigated in 60 culture-adapted \textit{Plasmodium falciparum} isolates from malaria patients in Padrecocha, a village in the Amazonian Department of Loreto, Peru. All isolates showed resistance to CQ, decreased susceptibility to quinine, and sensitivity to MQ. These isolates were examined for mutations in the \textit{P. falciparum} multidrug resistance 1 (\textit{pfmdr1}) and chloroquine resistance transporter (\textit{pfcrt}) genes previously linked to CQ resistance. The mutations N86Y and D1246Y, two of the five mutations commonly observed in the \textit{pfmdr1} gene of CQ-resistant clones, were not found. The \textit{pfcrt} mutation K76T, associated with CQ resistance, was identified in all the isolates tested. Sequence analysis of codons 72-76 in the \textit{pfcrt} gene showed the haplotypes SVMNT and CVMNT.

\textbf{INTRODUCTION}

Malaria is increasingly a serious burden in most tropical countries and a major cause of death in children in sub-Saharan Africa. The situation became more difficult since resistant \textit{Plasmodium falciparum} strains appeared in late 1950s.\textsuperscript{1–3} In South America, the first \textit{P. falciparum} strains resistant to chloroquine (CQ) were reported in 1961 in Colombia\textsuperscript{4} and Brazil.\textsuperscript{5} Perú, the country with the second highest number of malaria cases in South America,\textsuperscript{6,7} used CQ extensively until 1998. In 1999, the National Malaria Control Program of the National Institutes of Health of Peru\textsuperscript{8} reported 30–70% therapeutic failures to CQ. Subsequently, the combination of sulfadoxine/pyrimethamine (S/P) was introduced for the treatment of uncomplicated \textit{P. falciparum} malaria. Single-dose primaquine was also given concurrently as a gametocytocidal agent.

Since CQ resistance was first described, great efforts have been made to understand the mechanism and, two relevant proteins, Pgh1\textsuperscript{9} and PICRT,\textsuperscript{10} have been identified as candidates that participate in CQ resistance. The \textit{P. falciparum} multidrug resistance gene 1 (\textit{pfmdr1}) analog codes for Pgh1 and the \textit{P. falciparum} chloroquine resistance transporter gene (\textit{pfcrt}) codes for PICRT. These genes are located on chromosomes 5 and 7, respectively. Both proteins are localized in the food vacuole membrane and may modulate CQ uptake and/or pH regulation.\textsuperscript{11}

It has been proposed that point mutations in the \textit{pfmdr1} gene producing amino acid changes at positions 86, 184, 1034, 1042, and 1246 are associated with CQ and quinine resistance, as well as increased levels of susceptibility to mefloquine (MQ).\textsuperscript{9,12} In other studies, \textit{pfcrt} mutations at codons 74, 75, 76, 220, 271, 326, 356, and 371, have been related to CQ resistance.\textsuperscript{13} Notably, mutation K76T is consistently found in CQ-resistant strains\textsuperscript{11,13} and its contribution to CQ resistance has been recently elucidated by transfection experiments.\textsuperscript{14} Results from experiments conducted with laboratory strains need to be corroborated by those obtained from field isolate studies. Based on field studies, geographic variation in the parasite line due to regional differences has been observed.\textsuperscript{11,15}

There is little information in Peru about molecular characterization of \textit{P. falciparum} strains. To determine the prevalence of CQ resistance-associated markers, haplotype analysis of the \textit{pfcrt} and \textit{pfmdr1} genes was performed in Peruvian \textit{P. falciparum} isolates from the Amazonian Department of Loreto.

\textbf{MATERIALS AND METHODS}

\textbf{Study site.} Padrecocha is a village of 1,400 inhabitants on the Nanay River, 5 km northwest of Iquitos, the departmental capital of Loreto, Peru. An epidemiologic study\textsuperscript{16} was carried out in Padrecocha from August 1997 to July 1998, and of 4,046 blood smears obtained, 36% were positive for malaria parasites. \textit{Plasmodium falciparum} was found in 17% of all positive cases and \textit{P. vivax} was found in 83%. Due to widespread and frequent therapeutic failures of CQ for \textit{P. falciparum} malaria in this area, the currently recommended treatment is S/P, 25 mg and 1.25 mg/kg, respectively, and primaquine, 0.75 mg/kg administered as a single dose. Therapeutic failures with S/P are treated with quinine and tetracycline in adults or quinine and chloroquine in children. \textit{Plasmodium vivax} malaria is treated with a standard regimen of CQ for three days.

\textbf{Isolates of \textit{P. falciparum.}} \textit{Plasmodium falciparum} isolates were collected from 64 patients with uncomplicated acute \textit{P. falciparum} malaria in Padrecocha from March to May 1999. Patients enrolled in the therapeutic efficacy trial were asked to donate 5 mL of venous blood. The trial was conducted with a modified version of the Pan American Health Organization template protocol for conducting therapeutic efficacy trials in the Americas.\textsuperscript{17} Individual, written, informed consent was obtained from all participants. The trial was conducted under the Walter Reed Army Institute of Research protocol No. 719 approved by the U. S. Army Surgeon General Human Subjects Research Review Board and the Universidad Peruana Cayetano Heredia Institutional Review Board. Each sample was collected into two microtubes and cryopreserved in liquid
nitrogen as previously described\textsuperscript{18} in Iquitos, and later transported to the Naval Medical Research Center Detachment Laboratory in Lima. One microtube of each sample was used for the drug resistance test after being successfully culture adapted. The second tube was used for DNA extraction and gene analysis.

**Laboratory strains.** The CQ-sensitive *P. falciparum* strain FCRC\textsubscript{3} from The Gambia and the CQ-resistant strain K1 from Thailand were used as controls for the detection of *pfmdr1* polymorphism and direct sequencing analysis of *pfcr* gene. These strains were propagated in vitro in the Department of Protozoology, Institute of Tropical Medicine, Nagasaki University (Nagasaki, Japan).

**Drug sensitivity testing.** In vitro susceptilities to antimalarials were examined by the infection test of \textsuperscript{3}H-labeled hypoxanthine uptake in cultures of field-collected parasites, as previously described.\textsuperscript{19} Briefly, a microtube sample was thawed for culture adaptation of 2–6 weeks. Sixty of the 64 collected samples successfully adapted to culture were tested for susceptibility to CQ diphosphate, MQ hydrochloride, and quinine citrate. Drugs were dispensed in 25-μL aliquots of two-fold serial dilutions into 96-well, flat bottom microplates to achieve the following final concentrations: CQ = CQ: 1,000 ng/mL (0.98 ng/mL), quinine = 1,000 ng/mL (0.98 ng/mL), and MQ = 250 ng/mL (0.24 ng/mL).

Two strains of *P. falciparum* were used as controls for the *in vitro* assays: strain D6, which is MQ resistant but otherwise drug sensitive and strain W2, which is CQ and quinine resistant, but MQ sensitive.\textsuperscript{20,21} Both strains were obtained from Dr. Dennis Kyle (Walter Reed Army Institute of Research, Washington DC).

The results of these assays are reported as the 50% inhibitory concentration (IC\textsubscript{50}) of the respective drug, that is, the concentration of drug, usually in ng/mL, added to the culture that inhibits 50% of growth of the parasites.

In vitro \textsuperscript{3}H-labeling of hypoxanthine can be defined as the ability of *P. falciparum* isolates to grow at a CQ concentration of 100 nM or 33 ng/mL in culture.\textsuperscript{3,22} The cut-off value nicely separates CQ sensitivity, which is well below 33 ng/mL, and CQ resistance, which is above the cut-off value. Clear, reproducible, and unambiguous results can be obtained with carefully controlled assay conditions that avoid pH variations, variable serum activity, inaccurate gas conditions, inoculum effects, and other variations such as poorly growing parasites.

**Extraction of DNA.** Two hundred microliters each from 60 samples was subjected to DNA extraction using QIAamp DNA Blood kit (Qiagen, Valencia, CA) to yield 200 μL of genomic DNA. The DNA was then eluted and stored in elution buffer, according to the manufacturer’s instructions and used for a polymerase chain reaction (PCR) immediately or stored at −20°C.

**Detection of *pfmdr1* gene polymorphism.** To determine the presence of sequence polymorphisms in *pfmdr1*, a PCR–restriction fragment length polymorphism method was used. For this purpose, a nested PCR procedure (nest 1, nest 2) was carried out. The region encompassing the polymorphic codons 86 and 184 was amplified using primers dr2 (5’-AGATGGTAACCTCAGTAT-3’) and dr3 (5’-AGTC-TTTTCTCCACAATA-3’) and region encompassing the polymorphic codons 1034, 1042, and 1246 was obtained using primers dr5 (5’-GGAATTTGTTAAAGATCCAGA-3’) and dr6 (5’-CAGCACAACCTTACTAACA-3’) in nest 1. The design of primers was based on the complete nucleotide sequence of the previously published GH2 strain (National Center for Biotechnology Information Gene Bank, accession number S53996). Figure 1 shows the amplification strategy based on a previously reported protocol.\textsuperscript{22} Nest 1 amplification conditions were one cycle at 94°C for two minutes, an amplification of 35 cycles (94°C for 30 seconds, 45°C for one minute, and 72°C for one minute), and a final extension at 72°C for five minutes. Two microliters of PCR products obtained in nest 1 were used for the second amplification, nest 2, by using primers A4, A2, 1034f, 1042r, 1246f, and dr6. The amplification conditions for nest 2 were as previously reported and the products were subjected to enzyme digestion.\textsuperscript{22} This technique produces digestion patterns corresponding to alternative polymorphisms following resolution by electrophoresis on 1–3% agarose gels (Nusieve 3:1; BioWhittaker Molecular Applications, Rockland, ME). The reaction conditions for enzyme digestion were as previously described.\textsuperscript{22} The PCR reagents were obtained from the TaKaRa Shuzo Co. (Kyoto, Japan). Restriction enzyme digestions were done with Aco I (New England Biolabs, Inc., Beverly, MA) Dra I (Takara Shuzo Co.), Dde I (Sigma-Aldrich, Inc., St. Louis, MO), Vsp I (Gibco-BRL, Gaithersburg, MD), and Eco RV (Takara Shuzo Co.) for the characterization of codons 86, 184, 1034, 1042, and 1246, respectively.

**Amplification of the *pfcr* gene.** To amplify the *pfcr* gene, a PCR was carried out using primers cr5 (5’-TATA-GATTATTTTCATTGTCACAT-3’) and cr6 (5’-TTCTTTATATAAGTGATGCG-zAAG-3’) in nest 1. For the second amplification, nest 2, the previously reported primers 23402up and 24011dn\textsuperscript{15} were used to amplify the region encompassing the polymorphic codons 72-76 and 97 in exon 2. Nest 1 amplification conditions were one cycle at 94°C for two minutes, an amplification of 35 cycles (94°C for 30 seconds, 50°C for one minute, and 60°C for two minutes), and a final extension at 60°C for 10 minutes. Nest 2 amplification was carried out according to previously reported conditions.\textsuperscript{15}

**Direct DNA sequence analysis.** The PCR amplification products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced directly on an ABI310 automated sequencer using the ABI PRISM Big Dye Terminator Cycle kit (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions.

**Data and statistical analysis.** Data for the *in vitro* drug sensitivity test were analyzed by non-linear regression. The IC\textsubscript{50}
of tested isolates was compared with that of the D6 and W2 strains, and the mean IC<sub>50</sub>s were compared using the Student’s t-test. SPSS software for Windows (SPSS Inc., Chicago, IL) was used.

RESULTS

Susceptibility of *P. falciparum* isolates to CQ, MQ, and quinine. The *in vitro* susceptibility data for CQ, MQ, and quinine for 60 Peruvian isolates, and the profile of control strains D6 and W2 are shown in Table 1. The IC<sub>50</sub>s of the isolates ranged from 29 to 35 ng/mL and were much higher than the one showed by the CQ-sensitive control strain, which ranged from 1 to 5 ng/mL. The Peruvian isolates were at the lower level of IC<sub>50</sub> values usually seen in CQ-resistant isolates. All isolates showed high IC<sub>50</sub> values for quinine, ranging from 45 to 56 ng/mL, consistent with decreased susceptibility. They also showed low IC<sub>50</sub> values for MQ, which ranged from 2.06 to 2.41 ng/mL, consistent with sensitivity to this drug.

Polymorphism of the *pfmdr1* and *pfcrt* genes. Analysis of the *pfmdr1* gene showed wild type codons at positions 86 (Asn) and 1246 (Asp). However, at codons 184, 1034, and 1042, the substitutions Y184F, S1034C, and N1042D were found in all samples. The *pfmdr1* polymorphisms at codons 86 and 184 of representative Peruvian *P. falciparum* isolates are shown in Figure 2. The *pfmdr1* haplotype for all 60 Peruvian isolates was NFCDD for positions 86, 184, 1034, 1042, and 1246, respectively.

Analysis of the *pfcrt* gene showed that the K76T substitution was present in all Peruvian *P. falciparum* isolates evaluated. The DNA sequences at polymorphic codons 72, 74, 75, and 97 were also analyzed. At position 72, cysteine and serine (encoded by TCT, Ser<sub>c</sub>) were found. The deduced haplotypes of the *pfcrt* gene at positions 72-76 and 97 are shown in Table 2.

Genotyping of *P. falciparum*. Codons 72-76 of the Peruvian isolates were sequenced and the haplotypes SVMNT (30 of 60, 50%) and CVMNT were found. CVMNT and SVMNT are haplotypes previously reported for laboratory strains from Ecuador and Brazil, respectively, and in field samples from Peru. These two haplotypes showed the same *in vitro* susceptibility pattern to CQ (*P* = 0.21), MQ (*P* = 0.69), and quinine (*P* = 0.11). Figure 3 shows scatter plots of the data. No polyclonal infections were found in any of the tested alleles.

DISCUSSION

In this study, we report the prevalence of known genetic polymorphisms of the *pfmdr1* and *pfcrt* genes and the *in vitro*

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**TABLE 1**

<table>
<thead>
<tr>
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<th>Chloroquine</th>
<th>Mefloquine</th>
<th>Quinine</th>
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<tr>
<td><strong>Peruvian isolates (n = 60)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geometric mean</td>
<td>29.24</td>
<td>2.13</td>
<td>45.74</td>
</tr>
<tr>
<td>Mean</td>
<td>31.83</td>
<td>2.23</td>
<td>50.1</td>
</tr>
<tr>
<td>95% CI</td>
<td>28.36–35.30</td>
<td>2.06–2.41</td>
<td>44.53–55.66</td>
</tr>
<tr>
<td><strong>Control strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D6 (MQ-R, otherwise S)</td>
<td>3.72</td>
<td>6.65</td>
<td>13.17</td>
</tr>
<tr>
<td>Mean</td>
<td>0.29</td>
<td>1.63</td>
<td>0.64</td>
</tr>
<tr>
<td>Profile</td>
<td>1–5 (S)</td>
<td>8–15 (R)</td>
<td>8–15 (S)</td>
</tr>
<tr>
<td>W2 (CQ-R and quinine-R, MQ-S)</td>
<td>43.19</td>
<td>3.14</td>
<td>75.43</td>
</tr>
<tr>
<td>Mean</td>
<td>4.09</td>
<td>0.45</td>
<td>4.26</td>
</tr>
<tr>
<td>Profile</td>
<td>35–100 (R)</td>
<td>0.5–3 (S)</td>
<td>35–100 (R)</td>
</tr>
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</table>

* Mean and SD values are for triplicate determinations in each drug assay. IC<sub>50</sub> = 50% inhibitory concentration; CI = confidence interval; R = resistant; S = sensitive.

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**TABLE 2**

<table>
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<tr>
<th>Amino acid position</th>
<th>72</th>
<th>74</th>
<th>75</th>
<th>76</th>
<th>97</th>
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</thead>
<tbody>
<tr>
<td><strong>CQ-sensitive strains</strong></td>
<td>C</td>
<td>M</td>
<td>N</td>
<td>K</td>
<td>H</td>
</tr>
<tr>
<td><strong>CQ-resistant strains</strong></td>
<td>C</td>
<td>I</td>
<td>E</td>
<td>T</td>
<td>H</td>
</tr>
<tr>
<td>Southeast Asia and Africa</td>
<td>S</td>
<td>M</td>
<td>N</td>
<td>T</td>
<td>–</td>
</tr>
<tr>
<td>Papua New Guinea</td>
<td>S/C</td>
<td>M</td>
<td>N</td>
<td>T</td>
<td>H</td>
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</table>

* CQ = chloroquine.
drug susceptibility profiles against the aminooquinoline-based antimalarial drugs in *P. falciparum* isolates from the Peruvian Amazon.

A total of 60 culture-adapted isolates were subjected to the drug sensitivity test for CQ, quinine, and MQ. All isolates were moderately CQ resistant. This result was not unexpected since CQ resistance have been observed in Peru since 1992, and more than 30% of the reported cases of malaria in 1998 showed therapeutic failure to CQ treatment. The fact that all isolates showed a narrow range of IC₅₀s against the three aminooquinoline-based drugs tested might reflect the presence of a uniform *P. falciparum* population in Padrecocha village. Therefore, imported clones rather than indigenous parasites, which had acquired drug resistance, might have spread in the area. Conversely, we should consider that these isolates do not entirely represent the *P. falciparum* population because they were once selected by a culture condition.

The mutation Y184F was identified in the *pfmdr1* gene of all CQ-resistant Peruvian strains analyzed in this study. It had been previously reported for CQ-resistant laboratory strains and field isolates. However, the mutation N86Y was not found in the same CQ-resistant strains, in contrast to studies carried out in Malaysia, Indonesia, Guinea-Bissau, Nigeria, and sub-Saharan Africa, but in agreement with a study carried out in Brazil. Other clinical studies in Uganda, Laos, Cameroon, and southern Africa reported that the N86Y mutation in *pfmdr1* was not predictive of treatment outcome. In addition to the mutations Y184F and N86Y, other three-point mutations in *pfmdr1* gene, previously related to CQ resistance, quinine resistance, and MQ sensitivity, were studied. The point mutations S1034C and N1042D were identified in our isolates, but D1246Y was not detected. Reed and others investigated the role of Cys 1034, Asp 1042, and Tyr 1246 in aminoquinoline-based drug resistance by using transfection technology. They reported that the three mutations play a role in resistance to quinine, as well as in the sensitivity to MQ, and suggested that Tyr 1246 is a pivotal player in MQ sensitivity. This suggestion is contradictory to the results with our MQ-sensitive isolates, in which such a mutation was absent.

Previous studies evaluated *pfmdr1* and *pfcrt* genes, and suggested that mutations in *pfmdr1* may confer some advantage to the parasite in the presence of CQ, increasing the level of CQ resistance. When mutations are present in both genes, IC₅₀s to CQ are higher than when only mutations in the *pfcrt* gene are found. In our study, two of five mutations studied in *pfmdr1* were not present in the Peruvian isolates. This may explain the low levels of CQ resistance observed in our isolates. The detection of only one pattern of *pfmdr1* mutations in this area again supports the idea that imported clones with the same drug-resistant characteristics might spread.

With regard to the *pfcrt* gene, all Peruvian isolates showed the CQ-resistant phenotype and the K76T mutation. Our results are in agreement with those reported by Fidock and others, Djimde and others, and Vieira and others on *in vitro* susceptibility profiles. In addition, previous reports suggested that this mutation is the major determinant for CQ resistance, but the clinical outcome might involve other factors.

Sequencing of the *pfcrt* gene identified two haplotypes,
CVMNT and SVMNT, at codons 72-76. The SVMNT haplotype was previously found in laboratory strains of *P. falciparum* from Brazil,1,3 and the CVMNT haplotype was related to Ecuadorian and Colombian strains.1,5 Recently, Cortese and others23 studied drug resistance mutations in South American isolates, including 16 Peruvian samples, and identified these two haplotypes.

Aramburu and others4 speculated that three types of drug-resistant *P. falciparum* isolates converged in the Iquitos region of Peru. These are S/P-resistant CQ-resistant parasites from Brazil, variably S/P-resistant CQ-resistant parasites from the Loreto region, and S/P-sensitive CQ-sensitive parasites from coastal Peru. With regard to this hypothesis, our study suggests that the *P. falciparum* population in Padrecocha village in Iquitos includes Brazilian strains with the SVMNT haplotype, which correlates well with CQ and S/P resistance (Huaman MC and others, unpublished data), and Loretana strains with the CVMNT haplotype, which correlates with CQ resistance and S/P variable resistance. This finding suggests that SVMNT and CVMNT haplotypes might be useful markers of strain origin that would be complementary to merozoite surface protein-1 (MSP-1), MSP-2, glutamate-rich protein, and microsatellite markers.

In conclusion, we suggest that the two types of *P. falciparum* strains with low-grade resistance to aminoquinolines were recently introduced into Iquitos in the Peruvian Amazon and spread without further mutations.

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