The prevalence and severity of drug-resistant malaria is emerging rapidly in the Amazon basin of Brazil. In support of clinical trials using the new antimalarial drug combination of atovaquone and proguanil, we performed in vitro drug sensitivities, molecular characterization of parasite populations using the circumsporozoite protein, merozoite surface antigen-1 (MSA-1), and MSA-2 markers, and an analysis of the Plasmodium falciparum multidrug resistance (pfmdr1) gene sequence and copy number in 26 isolates of P. falciparum obtained in a gold-mining endemic area in Peixoto de Azevedo, Mato Grosso State. All 26 isolates were found to be resistant to chloroquine (50% inhibitory concentration [IC₅₀] = 100–620 nM) and sensitive to mefloquine (IC₅₀ < 23 nM) and halofantrine (IC₅₀ < 6 nM). The isolates also showed reduced susceptibility to quinine (IC₅₀ = 48–280 nM). Sequence analysis of the pfmdr1 gene revealed Asn, Phe, Cys, Asp, and Tyr in positions 86, 184, 1034, 1042, and 1246, respectively. These point mutations were similar to those previously described in other Brazilian isolates. Southern blot analysis revealed no amplification of the pfmdr1 gene. These results suggest that three different mechanisms for drug resistance exist for chloroquine, mefloquine, and quinine.

Malaria has increased 300–400% over the past decade in Brazil. Annually, there are 450,000 cases reported, approximately 50% Plasmodium vivax and 50% P. falciparum, with an estimated 10,000 deaths. The Amazon region accounts for more than 95% of the cases in Brazil. This increased incidence is due at least in part to changes in land use and occupational exposure. During the 1970s and 1980s, there was increased migration of population from many states of Brazil, attracted by government colonization projects and mining activities.

Compounding the existing problem is the emergence and spread of P. falciparum resistant to chloroquine, the most commonly used antimalarial in Brazil. Several factors are associated with the emergence of drug resistance, but certainly a major contributing factor is the drug pressure in the region. Today, in many malaria-endemic areas of Brazil clinical failures of chloroquine are reaching 100% and the drug is no longer recommended for treatment of uncomplicated P. falciparum malaria (Pang L, Walter Reed Army Institute of Research [WRAIR], unpublished data). With the advent of chloroquine resistance, other antimalarials were introduced. The most commonly used was Fansidar® (F. Hoffmann-La Roche, Basel, Switzerland), a combination of pyrimethamine and sulfadoxine. Resistance to this drug emerged rapidly and recent genetic analysis has demonstrated the widespread prevalence of a mutation in dihydrofolate reductase–thymidylate synthase (DHFR-TS) gene, which renders it insensitive to pyrimethamine. As a result of this reduced efficacy of chloroquine and Fansidar®, the standard treatment in Brazil for uncomplicated P. falciparum malaria has become quinine-tetracycline.

Our study site, Peixoto de Azevedo, is a municipality of Mato Grosso State located in the southern part of the Amazon region. In the past several years, the population has increased to its present population of 70,000, largely due to the influx of gold miners from neighboring states. During 1993, there were 4,800 parasitologically confirmed cases of malaria from Peixoto de Azevedo and approximately half of these were P. falciparum. Standard treatment for P. falciparum is a combination of quinine and tetracycline and recent clinical data demonstrate an increase in the average of parasite clearance time in the last two years (average = 55 hr) with some clinical failures. During 1995, approximately 10% of the confirmed P. falciparum infections did not respond to the quinine regimen and were treated with mefloquine. The use of mefloquine in this population is limited, but is increasing with both its availability and with the increasing numbers of quinine-tetracycline failures. This allows the investigation of the parasite populations during a period of increasing drug use.

Mechanisms of drug resistance in P. falciparum is an area of active research interest and has important practical applications both in diagnosis and surveillance and with regard to the design and implementation of new antimalarial drugs. In the case of pyrimethamine, the target enzyme, DHFR-TS, has been identified as having mutations that confer resistance. For quinine, chloroquine, and mefloquine, the molecular targets have yet to be definitively identified; however, at least in the case of chloroquine, the polymerization of heme appears the most likely candidate. Resistance appears not to be linked to the molecular target, but instead is related to a reduced accumulation of drug in the target organelle, the digestive vacuole. Biochemical, molecular, and genetic studies have identified at least two candidates that may play...
DRUG-RESISTANT \textit{P. falciparum} FROM THE BRAZILIAN AMAZON

<table>
<thead>
<tr>
<th></th>
<th>Chloroquine</th>
<th>Quinine</th>
<th>Mefloquine</th>
<th>Halofantrine</th>
<th>Artheeter</th>
<th>Atovaquone</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textbf{IC}_{50} (nanomolar)

Isolates
a role in this resistance, a gene on chromosome 7 that segregates with chloroquine resistance in a genetic cross (cg2)\(^9\) and a member of the ATP-binding cassette transporter family\(^10\) with a significant homology with the multidrug resistant gene family, the \(pfmdr1\) gene.\(^10\)\(^{-}17\)

Much of the work is based on laboratory experiments with parasites that have been in culture for an extended period or on cross-sectional studies of parasites. Field isolates such as those generated in this study allow us to examine laboratory-based drug resistance mechanisms as compared with those in the field. The major goal of this initial study is to investigate the \(P. falciparum\) parasite population in the study site with regard to drug resistance to establish the baseline characteristics of parasite populations. This was done at the level of clinical drug response, in vitro drug sensitivity of isolated parasites, molecular characterization of parasite populations using the circumsporozoite protein (CSP), merozoite surface antigen-1 (MSA-1), and MSA-2 markers and an analysis of the \(pfmdr1\) gene sequence and copy number in isolated parasites.

**Materials and Methods**

**Parasites.** All patients in this study were part of the quinine/tetracycline clinical trial performed by WRAIR and the Fundação Nacional da Saúde/Brazilian Ministry of Health. The patients that participated were informed about all procedures and signed a document of agreement. The study was reviewed and approved by the Ethical Committee of the Fundação Oswaldo Cruz (Rio de Janeiro, Brazil) and the Federal University of Rio de Janeiro. Study samples were obtained from patients with slide-confirmed \(P. falciparum\) infection (parasite densities ranging from 1,000 to 16,000 parasites/ml). Five milliliters of blood were collected by venipuncture using vacutainers with EDTA (Becton Dickinson, Lincoln Park, NJ). The samples were centrifuged and the pellet containing packed blood cells was mixed with equal volume of cryopreservation solution (0.9% NaCl 4.2% sorbitol 28% glycerol), frozen, and transported in liquid nitrogen containers to Rio de Janeiro. Of the 46 samples collected, 26 were successfully grown in vitro by the modified method of Trager and Jensen\(^18\) and used for the analysis in this study. Clones W2, a chloroquine-resistant clone and D6, a mefloquine-resistant but chloroquine-sensitive clone,\(^19\) were used as controls.

**Drug sensitivity assay.** The drugs used for the in vitro assays were atovaquone,\(^20\) \(\beta\)-arteether,\(^21\) chloroquine diphosphate salt,\(^22\) halofantrine,\(^23\) mefloquine,\(^24\) and quinine sulfate\(^25\) (Division of Experimental Therapeutics, WRAIR). In vitro drug sensitivity assays were performed according to the modified method of Desjardins and others\(^26\) and Lebras and Deloron.\(^27\) Each parasite isolate was tested in the presence of increasing concentrations of drug. Plates (96-well) containing three-fold dilutions of the test drug were prepared and the patient isolates were tested in duplicates for growth. The final drug concentrations used in the test ranged from 0.09 to 68 \(\text{nM}\) for atovaquone, 0.08–61 \(\text{nM}\) for arteether, 0.068–50 \(\text{nM}\) for halofantrine, 0.28–200 \(\text{nM}\) for mefloquine, 10–4,860 \(\text{nM}\) for chloroquine, and 4.4–3,240 \(\text{nM}\) for quinine. The incorporation data were analyzed by nonlinear regression software (NFIT; University of Texas Medical Branch, Galveston, TX) to generate the 50% inhibitory concentration (IC\(_{50}\)).

**Analysis of DNA.** For DNA extraction, cultivated parasites were centrifuged and suspended in NET buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5) and treated with 1% sarcosyl and proteinase K (20 mg/ml for 2 hr at 50°C). The DNA was then extracted using phenol, phenol/chloroform, and chloroform, precipitated with 0.3 M sodium acetate and ethanol, and resuspended in distilled water. These were used for Southern blot analysis and as template for a polymerase chain reaction (PCR)–based DNA sequencing analysis. For Southern analysis, DNA was digested (1 mg/lane) with Eco RI, resolved by electrophoresis on 1% agarose gels, and transferred to derivatized membranes. The hybridization was performed using two PCR-generated fragments, one corresponding to bases 3073–3749 of the \(pfmdr1\) gene coding region and the other derived from the CSP gene\(^14\). The DNA fragments were radiolabeled using the random oligonucleotide priming technique.\(^16\) The autoradiographies were scanned in a Hewlett Packard (Greeley, CO) ScanJet 3C and the bands were analyzed with the National Institutes of Health (Bethesda, MD) Image Software.

The sequence analysis of \(pfmdr1\) was performed using a PCR-based method by direct sequencing of single-stranded PCR products generated by \(\lambda\)-exonuclease digestion. Commercial oligonucleotide primer pairs were obtained based on the published full-length sequence\(^13\) or analysis of the regions previously described as presenting the following point mutations: Tyr86Asn, Tyr184Phe, Ser1034Cys, Asn1042Asp, and Asp1246Tyr. The primers pairs used for each reaction were amino acids 86 and 184: 5’-TM630, 5’-ACGGTTTAAATGTTTACCTG-3’, 3’-TM11280, 5’-GA-CACCACAAACATAAAATTAAGG-3’; amino acids 1034

**Figure 2.** Drug susceptibility test on the response of three less susceptible Brazilian isolates of \(Plasmodium falciparum\) to quinine. In vitro drug sensitivity assays of the Brazilian isolates Px101 (●), Px105 (■), and Px109 (▲) were performed according to the modified method of Desjardins and others\(^26\) and Lebras and Deloron.\(^27\) D6 (▼) was used as a quinine-sensitive control.
and 1042: 5’-TM3073, 5’-GTCGAAAAAGACTATGAGACGTTGAGA-ACGTTAGA-TAG-3’, 3’-TM3759R, 5’-CTCCTTTAAAGGACAT-TAATTTCGGC-3’; and amino acid 1246: 5’-TM4139, 5’-TTTCAACCATCTGATCGAGAT-3’, 3’-TM4700R, 5’-TCGTGTGTGCATGACTGAC-3’.

One of the primers from each pair was phosphorylated using T4 polynucleotide kinase and used in the PCR (50 pmol of each primer, 200 mM dNTPs, 100–200 ng of genomic DNA, 1× reaction buffer, 2 mM MgCl₂, 2.5 U of AmpliTaq® DNA polymerase [Perkin-Elmer, Branchburg, NJ] in a 100-μl reaction for 30 cycles at 94°C for min, 47°C for 2 min, and 74°C for 3 min).

The PCR product was directly purified with the Gene CleanII Kit (Bio101, Inc., La Jolla, CA). It was then resuspended in water (44 μl), 5 μl of λ-exonuclease buffer (670 mM glycine-NaOH, pH 9.4, 25 mM MgCl₂, 500 mg/ml of bovine serum albumin) and 1 μl of λ-exonuclease (5 U) was added, and digestion was carried out for 10 min at 37°C. It was then extracted with phenol-chloroform and chloroform, precipitated with ethanol, and resuspended in 10 μl of water for deoxynucleotide chain termination sequencing using the Taq Track Sequencing System (Promega, Madison, WI).

**Multiplex PCR fingerprint.** Multiplex PCR analysis was performed according to the method of Wooden and others. The technique uses primers specific for genes encoding CSP, MSA-1, and MSA 2. Genomic DNA (extracted by the same
method used for DNA sequencing) was added to an amplification mixture (50 pmol of each primer, 200 mM dNTPs, 100–200 ng of genomic DNA, 1× reaction buffer, 2 mM MgCl$_2$, 2.5 U of AmpliTaq® DNA polymerase [Perkin-Elmer] in a 100-μl reaction for 30 cycles at 94°C for 1 min, 47°C for 2 min, and 74°C for 3 min) containing the three sets of oligonucleotide primers. The PCR products were analyzed by electrophoresis on a 2% agarose gel and staining with ethidium bromide.

RESULTS

Drug susceptibility testing. Forty-six blood samples (collected at the Peixoto de Azevedo Clinic) with parasitemias greater than 10 parasites per thick film field (World Health Organization WHO plus system)$^{29}$) were selected for this study. Of the 46 original samples, 26 isolates were successfully adapted in continuous culture. The isolates were first characterized for their drug resistance pattern by the in vitro drug test incorporation using $^3$H-hypoxanthine. All parasites were tested with standard drugs (chloroquine and quinine), drugs recently introduced in the field (halofantrine, mefloquine, and artether) and atovaquone, a drug that is being evaluated in a phase III clinical trial conducted by WRAIR in Peixoto de Azevedo. In vitro susceptibility to the drugs was assessed by measuring the ability of different drug concentrations to inhibit incorporation of radio-labeled hypoxanthine into the nucleic acid of the parasite.$^{27}$ Parasites were grown in duplicate under three-fold increasing concentrations of the tested drug. The IC$_{50}$ of each isolate for each drug was determined using a nonlinear curve fitting program. The results of these assays are shown in Figure 1.

All isolates tested with chloroquine were scored as chloroquine resistant (IC$_{50}$ > 100 nM, ranging from 103 to 620 nM (Figure 1A). Two of the isolates (Px109 and Px112) were highly resistant to chloroquine, with IC$_{50}$ values of 620 nM for both isolates. These results are consistent with the low clinical efficacy of chloroquine in Brazil.$^4$ The chloroquine IC$_{50}$ values for the control clones W2 (chloroquine resistant) and D6 (chloroquine sensitive) were 223 and 12.6 nM, respectively.

When the parasites were tested with quinine, the 26 isolates had reduced susceptibility to quinine (IC$_{50}$ > 30 nM [range = 74–280 nM]) when compared with control parasites. We also observed three different groups of parasites (Figure 1B): one group with IC$_{50}$ levels ranging from 48 to 79 nM (Px110, Px118, Px123, and Px124), a second group ranging from 107 to 157 nM (Px111, P.112, Px114, Px117, Px119, Px121, Px122, Px125, and Px126) and a third and less quinine-susceptible group ranging from 204 to 280 nM (Px101, Px102, Px103, Px104, Px105, Px106, Px107, Px108, Px109, Px113, Px115, Px116, and Px120). The in vitro test curve of the three less susceptible quinine isolates (Px101, Px105, and Px109) showed a shift of more than one log in comparison with the sensitive strain D6 (Figure 2). In addition, the isolates tested were susceptible to mefloquine (IC$_{50}$ < 23 nM [5.8–14 nM]) and halofantrine (IC$_{50}$ < 6 nM [0.71–2.5 nM]) (Figure 1C and D). The IC$_{50}$ levels for artether and atovaquone for the samples were in a low nanomolar range (2.9–9.6 nM and 1.8–2.7 nM, respectively) (Figure 1E and F).

Multiplex PCR fingerprint. To analyze the genomic variation of the Peixoto parasite population we performed a multiplex PCR.$^{28}$ The fingerprint of the isolates using primers specific for CSP, MSP-1 and MSP-2 is shown in Figure 3. We observed several alleles of both the MSA-1 and MSA-2 genes (second and third bands, respectively) but little allelic variation in the CSP gene. This is consistent with the presence of several different strains of P. falciparum coexisting in this population. The fingerprint patterns differentiated Peixoto strains from each other and the reference strains W2 and D6.

Identification of the pfmdr1 point mutations. The sequence of regions of the pfmdr1 gene at positions 86, 1034, 1042, and 1246 was determined in all the isolates to explore the possibility of intragenic alleles being associated with the drug resistance pattern found in this study (chloroquine resistance and mefloquine sensitivity). All isolates from Peixoto had Asn, Phe, Cys, Asp and Tyr at positions 86, 1034, 1042, and 1246, respectively, and the sequence autoradiography did not show any nucleotide mixture in the point mutation sites. In comparison with the previously described sequences reported by Foote and others,$^{13}$ these five point mutations were identical in other South American strains resistant to chloroquine. The mutations Cys1034 and Asp1042, previously related with chloroquine resistance, were also found in all isolates analyzed in this study. The variation in amino acid 184, which was previously linked with mefloquine resistance by Wilson and others,$^{14}$ did not show any correlation in our study. The isolates with higher quinine IC$_{50}$ levels did not differ in sequence.

Southern blot analysis. Determination of the pfmdr1 gene copy number by Southern blot analysis is summarized on Table 1. Eleven of the 26 samples were analyzed and contained only one copy of the pfmdr1 gene when compared with the W2 clone. The lack of gene amplification in the Brazilian isolates is consistent with the mefloquine susceptibility of the samples.

DISCUSSION

The purpose of this study was to analyze P. falciparum samples isolated from the Amazon region in Brazil in relation to their drug resistance profiles and the possible mechanisms involving resistance.

The in vitro test demonstrated that 26 of 26 (100%) isolates were resistant to chloroquine in a region were chloroquine is no longer used as a standard treatment. Two previous surveillance studies of drug-resistant malaria in the Amazon region demonstrated a high level of chloroquine resistance.$^3,30$ Thus, in this case the in vitro data correlates with the in vivo clinical resistance. An attempt to treat malaria in the Amazon region using a high-dose regimen of chloroquine may have delayed the development of clinical resistance, but it did not prevent it.

A surveillance study performed between 1980 and 1990 showed that 75 (100%) of the isolates from the Amapa state in the northern part of the Amazon, when tested by the microtest of Reickmann and others,$^{31}$ were sensitive to me-
One of the overall goals of this work was to explore the possibility of intragenic alleles and amplification of the pfmdr1 gene being associated with the drug resistance pattern. The sequence data from the Brazilian isolates showed that all have the same allelic variation (Asn, Phe, Cys, Asp, and Tyr at positions 86, 184, 1034, 1042, and 1246, respectively). These five polymorphisms were found in other strains from a different location in the Amazon region in Brazil where the Cys1034 and Asp1042 were correlated with chloroquine resistance.\(^\text{13}\) Complementation experiments showed that transmembrane transport of an a-factor mating pheromone of Saccharomyces cerevisiae is functional when pfmdr1 includes the mutations Ser1034 and Asn1042 but remains dysfunctional when the mutations are Cys1034 and Asp1042.\(^\text{34}\) This could indicate that modifications at these two sites are critical for the transport function of the pfmdr1 protein. We could not find any specific mutation that could be related with the reduced susceptibility to quinoline. The lack of gene amplification and specific point mutations at positions 86, 184, 1034, 1042, and 1246 in the isolates with high quinine IC\(_{50}\) levels indicates...
that quinine resistance probably has a different molecular mechanism than chloroquine and mefloquine resistance.

Previous molecular studies of the pfmdr1 gene in isolates from Thailand showed the potential role of these gene in mefloquine resistance.\(^{14}\) All the isolates had an increased copy number of the pfmdr1 gene. On the other hand, the recent work of Basco and others showed that not all mefloquine-resistant strains had an increased copy number.\(^ {35}\)

In our study, all the isolates that showed susceptibility to mefloquine had a single copy. Many groups have also attempted to correlate the pfmdr1 point mutations with chloroquine and mefloquine resistance. A remarkable degree of genetic heterogeneity was noted in the isolates analyzed from Thailand\(^ {14}\) and Africa.\(^ {36,37}\)

Although the isolates showed allelic polymorphism for CSP, MSA-1, and MSA-2, we could not find a isolate showing a unique genotypic variation. In comparison with African isolates described by Basco and others,\(^ {35}\) the Peixoto samples showed less allelic variation.

In conclusion, our data confirm an increase of quinine IC\(_{50}\) levels in conjunction with a delay in parasite clearance observed in the region. Although we found a strong correlation between pfmdr1 point mutations and chloroquine resistance, none of the molecular markers were related to the quinine-resistant parasites. These results could indicate that there probably exists three different mechanisms for drug resistance for chloroquine, mefloquine, and quinine.

Acknowledgments: We thank Dr. Crespim Cerruti and The Fundação Nacional da Saúde for kind support.

Financial support: This work was supported by National Institutes of Health International Centers for Tropical Disease Research grant (#AI-16305).

Authors’ addresses: Mariano G. Zalis and Mariana S. Silveira, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil. Lorrin Pang, United States Army Institute of Research, Washington, DC 20307-5100. Dyann Wirth, Harvard School of Public Health, Boston, MA 02115.

REFERENCES


