UNUSUAL PATTERN OF PLASMODIUM FALCIPARUM DRUG RESISTANCE IN THE NORTHWESTERN PERUVIAN AMAZON REGION

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Abstract. High levels of Plasmodium falciparum resistance to both chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) have been documented throughout the Amazon Basin of South America. Because of reports about the persistent efficacy of both of these drugs in the northwestern Peruvian Amazon region, we carried out an evaluation of the therapeutic efficacy of chloroquine (25 mg/kg) and SP (25 mg/kg of the sulfadoxine component) for the treatment of uncomplicated P. falciparum infections at two sites: Ullpayacu and Pampa Hermoza/Alianza. A total of 111 patients were enrolled. Only 5 (14.3%) of the 35 patients who received CQ had an adequate clinical and parasitologic response (ACPR). Six subjects (17%) had early treatment failure, 1 (2.9%) had late clinical failure, and 23 (65.7%) had late parasitologic failure (LPF). Of the subjects treated with SP, 92.3% had ACPR and 7.7% had LPF. Based on these findings, it is clear that there are at least limited areas within the Peruvian Amazon region where P. falciparum strains continue to be sensitive to SP.

INTRODUCTION

The emergence of antimalarial drug resistance is a major public health problem in Peru. On the northern Pacific Coast, strains of Plasmodium falciparum are resistant to chloroquine (CQ) but continue to respond well to sulfadoxine-pyrimethamine (SP) therapy.1 In contrast, very high levels of resistance to both CQ and SP have been documented in the central and eastern Peruvian Amazon region bordering Colombia and Brazil.2 Despite this, CQ continued to be used as the first-line treatment of uncomplicated P. falciparum malaria in the northwestern Amazon region until 2002, and local physicians remained convinced of its efficacy. To determine if there really was an area in the northwestern Amazon region of Peru where CQ and SP efficacy remained high, we carried out in vivo efficacy trials of both drugs at two sites in the northwestern Amazon region of Peru.

MATERIALS AND METHODS

Study sites. The first study was conducted in 2000 in two small, contiguous towns, Alianza and Pampa Hermoza (total population of 4,000 inhabitants), which are located along the highway that connects Tarapoto and Yurimaguas. The second study was conducted in 2002 at Ullpayacu, a town with a population of 900 inhabitants on the Pastaza River, to confirm the findings of the earlier study (Figure 1).

This study protocol was approved by the US Naval Medical Research Center Institutional Review Board (NMRCD.2001.0007 [DoD 31519]) and the National Institutes of Health of Peru (Protocol 0005-2000) in compliance with all Federal regulations governing the protection of human subjects.

Malaria transmission in these areas is unstable and seasonal, with a peak between the months of March and August. About 30% of cases are caused by P. falciparum; the rest are caused by P. vivax. All age groups are affected, and most infections are asymptomatic,3 although severe malaria and deaths caused by malaria are rare.4 The major vector of malaria in this area is believed to be Anopheles benarrochi5 rather than An. darlingi, the principal vector in the remainder of the Amazon region.

Patients and procedures. The methods used followed the recommendations of the World Health Organization (WHO) for in vivo antimalarial drug efficacy testing,6 with modifications made by the Pan American Health Organization (PAHO).7 Sample size was determined using the lot quality assurance sampling technique.8 This method is intended to identify areas in which the prevalence of drug resistance is above a pre-determined critical level with much smaller sample sizes than is required using more traditional methods for determining the proportion of treatment failures within narrow confidence limits.8 For the purposes of these two studies, a level of RII/RIII resistance > 30% was considered to be unacceptably high for a first- or second-line antimalarial drug in Peru.

Patients were evaluated on their suitability for the study based on specific inclusion criteria: age ≥ 2 years; axillary temperature ≥ 37.5°C and/or a history of fever within the previous 48 hours; and monoinfection with P. falciparum between 500 and 50,000 parasites/µL. Patients meeting the inclusion criteria were assigned to treatment with CQ or SP using a table of random numbers. CQ and SP were administered as recommended by the Ministry of Health of Peru.9 CQ (150 mg base; Ciba-Geigy, Basel, Switzerland) at 25 mg/kg was administered over 3 days (10 mg/kg on Day 0 and Day 1 and 5 mg/kg on Day 2. SP (Roche, Basel, Switzerland) was administered in a single oral dose of 25 mg/kg of the sulfadoxine component on Day 0. All drugs were given under the supervision of a member of the study staff. After drug ingestion, subjects were observed for 30 minutes for signs of vomiting. Those who vomited once were re-treated with an identical dose, and those who vomited after the second drug dose were withdrawn from the study. Patients with axillary temperatures ≥ 37.5°C were treated with paracetamol.

In accordance with WHO recommendations for in vivo drug efficacy studies in effect in 2000, patients enrolled in Pampa Hermoza/Alianza were asked to return for follow-up

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medical histories, temperature measurements, and thick blood smears on Days 1, 2, 3, 7, and 14. To increase the possibility of detecting late treatment failures in the study in Ullpayacu, a small and isolated village where follow-up was also easier, patients were asked to return on Days 1, 2, 3, 7, 14, 21, and 28. Patients who did not return were traced to their homes. Patients who failed to respond to CQ were treated with SP; SP failures were treated with quinine plus clindamycin.

Thick blood smears were stained with Giemsa and examined at ×1,000 to identify the parasite species and determine the level of parasitemia. Parasite density was calculated by counting the number of asexual parasites per 200 white blood cells in thick smear, assuming a mean white blood cell count of 6,000/μL. Each blood smear was independently examined by two microscopists. In the case of a difference in results (positive/negative; species diagnosis; or > 2-fold difference in parasite density), the blood smear was re-examined by a third independent microscopist. The final parasite density was an average of the densities of the two concordant microscopists.

Approximately 10–20 μL of blood were collected on Iso- code stix collection matrix (Schleicher and Schuell, Keene, NH) on the day of enrollment (Day 0), and total parasitic DNA was extracted following the instructions from the manufacturers. Five microliters of the genomic *P. falciparum* DNA was amplified in a 50-μL reaction using mutation-specific nested polymerase chain reactions (PCRs) to detect the mutations S108T/N, C50R, N51I, C59R, and H164L in the DHFR gene, as previously described.\(^{10,11}\) Detection of the known DHPS mutations K540E, A581G, and A613T/S was performed using mutation-specific nested PCRs, whereas enzymatic digestion was used to detect mutations S436A and A437G, as previously described.\(^{10,11}\) Amplification products were analyzed by electrophoresis on 1% or 2% agarose and run in ethidium bromide-free gel and stained afterward in 0.5 μg/mL ethidium bromide gel for UV visualization.

Standard definitions of clinical and parasitologic response (as well as the earlier classification RI, RII, RIII, and S) recommended by WHO were used. An early treatment failure (ETF) was defined as the development of danger signs or severe malaria with parasitemia on Day 4 or subsequently, without meeting the ETF criteria, or as the appearance of parasitemia with axillary temperature > 37.5°C between Days 4 and 14 (or 28 in 28-day studies), without having previously met ETF criteria. LCF was defined as the absence of parasitemia on Day 14, and (21 and 28 for 28-day trials), without having previously met ETF criteria. Isolates collected on Day 0 and day of failure from the SP component of the study were genotyped using the nested PCR method previously described.\(^{10}\) The nested PCR products were DNA sequenced using Big dye sequencing mix and an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Proportions were compared by \(\chi^2\) test; continuous variables were analyzed by the Kruskal-Wallis test or one-way analysis of variance.\(^{12}\) Risk factors for parasitologic failure were assessed in a univariate analysis. \(P < 0.05\) was considered significant. Risk of recrudescence of parasitemia was estimated with the Kaplan-Meier technique.\(^{13}\)

### RESULTS

At the Pampa Hermoza/Alianza site, 916 febrile patients were screened for parasitemia, 73 had *P. falciparum* parasitemia, and 49 were enrolled over a 4-month period. At Ullpayacu, 1,029 febrile patients were screened, 112 had *P. falciparum* parasitemia, and 62 were enrolled over a 6-month period. The main reasons for non-enrollment were that the subjects were planning to leave the community during the follow-up period for work purposes, had a low parasite density, or had signs of severe malaria.

Thirty-five patients were enrolled in the CQ arms and 76 in the SP arms. The characteristics of these subjects are shown in Table 1. No significant differences were observed at the time of enrollment in terms of age, sex, presence of documented fever on Day 0 (axillary temperature \(\geq 37.5°C\)), history of fever, or the geometric mean parasite density between patients who received CQ and those who received SP. Of the 111 enrolled patients, 11 (9.9%), all from the SP arm, were excluded from the analysis (finally 65 remained in the arm

### Table 1

Characteristics of patients enrolled in *in vivo* CQ and SP efficacy studies in the northwestern Peruvian Amazon region, 2000–2002

<table>
<thead>
<tr>
<th>Variable</th>
<th>CO ((N = 35))</th>
<th>SP ((N = 76))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age</td>
<td>20</td>
<td>21</td>
<td>NS*</td>
</tr>
<tr>
<td>Range (years)</td>
<td>5–48</td>
<td>2–60</td>
<td></td>
</tr>
<tr>
<td>Sex (% men)</td>
<td>51.4%</td>
<td>44.7%</td>
<td>NS</td>
</tr>
<tr>
<td>History of fever in previous 48 hours</td>
<td>91.4%</td>
<td>98.7%</td>
<td>NS</td>
</tr>
<tr>
<td>Axillary temperature (\geq 37.5°C) (Day 0)</td>
<td>37.1%</td>
<td>43.4%</td>
<td>NS</td>
</tr>
<tr>
<td>Geometric mean parasite density (/μL) (Day 0)</td>
<td>4,578</td>
<td>4,245</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NS, \(> 0.05\).
SP). Six patients were excluded for having mixed parasitemia, four were lost to follow-up, and one had a density < 250 asexual parasites/μL on re-examination of the Day 0 blood smear. All remaining subjects completed their 28-day follow-up at Ullpayacu and 14-day follow-up at Alianza/Pampa Hermoza.

Only 5 (14.3%) of the 35 patients who received CQ had an ACPR; 6 (17.1%) had ETF, 1 (2.9%) had LCF, and 23 (65.7%) had LPF. Among the 65 patients who received SP, 60 (92.3%) had ACPR, and 5 (7.7%) had LPF. All five treatment failures were from Ullpayacu (Table 2). PCR amplification and DNA sequencing of the \( \text{Pfmsp-2} \) gene from isolates collected on Day 0 and day of failure from the four of five patients that failed treatment with SP showed identical DNA sequences, signifying that the recurrence of the infections was caused by recrudescence and not reinfection. Regrettably, the sample collected on day of failure from the fifth patient was lost.

The cumulative risk of recurrence of parasitemia by Day 14 in patients who had received CQ was 82.9%, whereas it was only 3.0% in those who had received SP. Figure 2 shows survival curves, taking into account that follow-up in Alianza/Pampa Hermoza was 14 days, whereas in Ullpayacu, it was 28 days. No association was found between subject’s parasitologic outcome and age, parasite density on enrollment, or a history of vomiting.

Samples from all 5 patients who failed SP treatment and 50 patients infected with sensitive strains were chosen for PCR. Of the 55 isolates tested, 31 contained the \( S108N \) mutant allele in the \( \text{DHFR} \) gene and wild-type alleles at codons 50, 51, 59, and 164. No mutations were found in the \( \text{DHPS} \) alleles analyzed in any of the 55 samples: 436, 437, 540, 581, and 613. At Ullpayacu, 100% of the 29 analyzed samples presented one mutation in allele \( S108N \). At Alianza/Pampa Hermoza, only 3 (11.5%) of the 26 analyzed samples contained the mutation in allele \( S108N \).

### DISCUSSION

Since \( P. falciparum \) resistance to SP was first reported from the Amazon Basin in the 1960s,\textsuperscript{14} it has become widespread. Because of the uniformly high levels of resistance to both CQ and SP, most countries making up the Amazon Basin have changed their malaria treatment policies in recent years to combination therapy with an artemisinin derivative\textsuperscript{15,16} (K. Carter, personal communication).

The results of this study are the first to provide evidence of an area within the Amazon Basin where the therapeutic efficacy of SP remains high. RI/RIII resistance to CQ at both sites was high (22.9% and 34.3%). In contrast, in a 14-day study in 2000 in Pampa Hermoza/Alianza, we found no treatment failures with SP. Two years later, at the Ullpayacu site, 3 (4.6%) patients failed to respond at 14 days and 5 (7.7%) at 28 days. This pattern of high-level resistance to CQ and low-level resistance to SP is very similar to that observed on the northern Pacific Coast of Peru. The apparent increase in SP resistance in the 2002 study in Ullpayacu could be caused by emerging resistance or simply reflect the small differences in patterns of resistance sometimes seen in different study sites.

Several possible explanations for this pattern of resistance in the western Peruvian Amazon region exist. SP has been widely used as an alternative to CQ since the late 1990s in the areas surrounding the city of Iquitos and eastward toward the borders with Colombia and Brazil.\textsuperscript{17} In contrast, in the areas around Pampa Hermoza/Alianza and Ullpayacu, SP use was minimal, and consequently, drug pressure has been much lower, because CQ continued as the first-line treatment of \( P. falciparum \) infections until late 2002. Population movements could also play a role in the spread of resistant strains.

### TABLE 2

<table>
<thead>
<tr>
<th>Drug site</th>
<th>( N )</th>
<th>ETF*</th>
<th>LCF†</th>
<th>LPF‡</th>
<th>ACPR§</th>
<th>RIII</th>
<th>RII</th>
<th>RI (early)</th>
<th>RI (late)¶</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pampa Hermoza/Alianza</td>
<td>15</td>
<td>3</td>
<td>0</td>
<td>9</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>Ullpayacu</td>
<td>20</td>
<td>3</td>
<td>1</td>
<td>14</td>
<td>2</td>
<td>3</td>
<td>7</td>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>6</td>
<td>1</td>
<td>23</td>
<td>5</td>
<td>14</td>
<td>12</td>
<td>8</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>SP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pampa Hermoza/Alianza</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>33</td>
</tr>
<tr>
<td>Ullpayacu</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>27</td>
<td>0</td>
<td>1</td>
<td>2</td>
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<td>27</td>
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<tr>
<td>Total</td>
<td>65</td>
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<td>0</td>
<td>5</td>
<td>60</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>60</td>
</tr>
</tbody>
</table>

\* Early treatment failure.  
† Late clinical failure.  
‡ Late parasitologic failure.  
§ Adequate clinical and parasitologic response.  
¶ Because follow-up in the study in Pampa Hermoza/Alianza was limited to 14 days, it was not possible to detect late RI failures.

**Figure 2.** Cumulative incidence of cure in \( P. falciparum \) infections treated with SP in the western Peruvian Amazon region.
P. falciparum is quite isolated from Iquitos, and areas to the east can be accessed only by river. In contrast, communication by road with the Pacific Coast of Peru, where P. falciparum is still susceptible to SP, is simpler. Another possible reason for the different patterns of resistance could be related to the malaria vectors. The major vector in the western Amazon region is An. benarrochi, which is not considered as efficient a vector as An. darlingi, the principal vector in the central and eastern Amazon.18

Previous studies have shown that the S108N mutation in DHP5 usually occurs first, but does not consistently confer in vivo resistance to SP.19 The presence of at least three mutations in DHFR and two mutations in DHP520 is reliably related to in vivo resistance. In areas of the Peruvian Amazon, where resistance rates > 25% have been found, Kulin and others20 found up to four mutations in DHFR (DHFR 108-51-164-30) and three in DHP5 (DHP5 437-540-581). Mutations in DHFR 108-51-64/DHP5 437-540-581 were associated with RII/RIII resistance. The findings of these two in vivo trials in the western Peruvian Amazon region correlate well with those of the molecular analyses. A single S108N mutation was found in the DHFR gene in 13% of the samples obtained in 2000 at Alianza/Pampa Hermoza and in 100% of the samples obtained in 2002 at Ullpayauc. This pattern is similar to the one in the north coast of Peru, where SP is still efficacious. P. falciparum isolates from two sites on the north coast of Peru revealed rates > 70% of the resistant allele S108N, but no other mutations were found (C. Salas, personal communication). Current literature supports a low level of SP resistance with just the S108N mutation.21 Resistance increases significantly if N51I is also present.21 Because exposure to P. falciparum is seasonal, and cases are sporadic, the circulating population of parasites is not exposed to constant drug pressure that could result in rapid selection of a resistant phenotype. Also, the circulating genotypes could be quite homogeneous because the population is quite isolated, and the risk of importing SP-resistant malaria into this area is minimal.

After these studies, the Peruvian Ministry of Health considered the option of recommending SP as the first-line treatment of P. falciparum malaria in the western Amazon region, but it was felt that implementation of two different malaria treatment policies in the same region would be extremely difficult logistically. For that reason, it was decided to recommend a uniform policy for the treatment of falciparum malaria throughout the region. Combination therapy with mefloquine-artesunate was first implemented in Iquitos and surrounding areas in 2001, but by January 2003, this policy had been extended to the entire Peruvian Amazon. SP plus artesunate had already been implemented as the first-line treatment on the northern Pacific Coast of Peru in 2001, and the Ministry of Health did not want to make a further change in treatment policy. To monitor changes in antimalarial drug resistance, the National Malaria Control Program plans to carry out in vivo testing in both regions every 2–3 years.

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