

CHLOROQUINE-RESISTANT *PLASMODIUM VIVAX* MALARIA IN PERU

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Abstract. Reports from several sites in South America suggest the presence of isolated cases of chloroquine-resistant *Plasmodium vivax* malaria. To investigate the possibility of chloroquine-resistant *P. vivax* in Peru, we conducted 28-day *in vivo* drug efficacy trials at three sites in the Amazon region and one site on the northern Pacific Coast between 1998 and 2001. A total of 242 patients between the ages of 2 and 60 years were enrolled (177 from the Amazon region and 65 from the northern coast). All subjects received directly observed therapy with chloroquine, 25 mg/kg, over a three-day period. On enrollment, 49% had a documented fever and 96% had a history of fever; their geometric mean parasite density was 5,129 parasites/ μ L. A total of 212 (88%) of the 242 subjects completed their 28-day follow-up. Four of the 177 patients from the Amazon region had a recurrence of *P. vivax* parasitemia on days 21 and 28 after treatment was initiated. Two of these patients had chloroquine-resistant infections, based on polymerase chain reaction–single-stranded conformational polymorphism genotyping and chloroquine-desethylchloroquine blood levels, which were ≥ 97 ng/mL at the time of the reappearance of parasitemia. None of the subjects studied on the northern Pacific Coast had recurrent parasitemia.

INTRODUCTION

Since chloroquine (CQ)–resistant *Plasmodium vivax* infections were first identified in Papua New Guinea in 1989, resistant strains have been reported from various sites in Indonesia, as well as Myanmar and India.^{1–5} More recent reports from Indonesia indicate therapeutic failures within 14 days of starting therapy in up to 44% of patients.⁶ Although several reports of CQ-resistant *P. vivax* infections in the Americas have appeared in the literature,^{7–9} the best documented is that from Guyana.⁷

In 1998, because of concerns about the spread of antimalarial drug resistance in Peru, the Ministry of Health began a series of *in vivo* therapeutic efficacy trials. Initial studies showed high levels of resistance in *P. falciparum* to both CQ and sulfadoxine-pyrimethamine at several sites in the Amazon Basin and high levels of resistance to CQ, but not to sulfadoxine-pyrimethamine, on the northern Pacific Coast.¹⁰ (G. Stennies, personal communication) As part of this effort to assess the efficacy of current and potential future first-line antimalarial drugs in Peru, we conducted 28-day *in vivo* efficacy trials of CQ for the treatment of *P. vivax* infections at three sites in the Amazon region and one on the northern Pacific Coast.

MATERIALS AND METHODS

Study sites. The studies were conducted at the Padre Cocha, Moronacocho, and Caballococha Health Centers and the Hospital de Apoyo Iquitos (Department of Loreto) in the Peruvian Amazon region and the Bellavista Health Center (Department of Piura) on the northern coastal plain of Peru during the 1998 and 2000–2001 malaria transmission seasons (Figure 1). The Hospital de Apoyo and Moronacocho Health Center are located in the city of Iquitos and draw their patients from the city's periurban and surrounding rural areas. The Padre Cocha Health Center is located in a riverine village (population = 1,400) approximately 5 km from Iquitos. The Caballococha Health Center serves a town with a population of 3,300 in the northeastern Amazon region, within 30 km of

the borders with Colombia and Brazil. The Bellavista Health Center on the northern coast draws its patients from the periurban areas of the city of Sullana, which has a population of approximately 100,000.

Malaria transmission in Peru is seasonal with a peak from March to August. *Plasmodium vivax* makes up approximately 70–90% of all infections, with *P. falciparum* accounting for the remainder. *Anopheles darlingi* is the major vector in the Amazon Basin and *A. albimanus* is the major vector on the northern coast. Although CQ can be purchased without prescription in local stores and pharmacies at all four study sites, most patients with suspected malaria in Peru tend to seek treatment at Ministry of Health facilities, and self-treatment does not appear to be as common as it is in many other Latin American countries.¹¹ The studies were reviewed and approved by the Ethical Review Committees of the Instituto Nacional de Salud and the U.S. Navy.

Patient enrollment and follow-up. The methods used followed the recommendations of the Pan American Health Organization for *in vivo* antimalarial drug efficacy testing of *P. falciparum* in the Americas and draft recommendations for *P. vivax in vivo* drug efficacy testing¹² (Gusmão R, unpublished data). Patients between 2 and 60 years of age with suspected malaria attending the three health centers were screened for parasitemia by thick blood smears. Those with *P. vivax* mono-infections between 250 and 50,000 parasites/ μ L of blood and an elevated axillary temperature ($\geq 37.5^{\circ}\text{C}$) and/or a history of fever within the previous 48 hours who gave informed consent were enrolled in the study. Subjects were excluded if they had symptoms or signs of severe malaria, had another obvious cause for their fever, or had a history of allergy to CQ.

Chloroquine (Ciba-Geigy, S.A., Basel, Switzerland), 25 mg/kg (base), was administered over a three-day period (10 mg/kg on days 0 and 1 and 5 mg/kg on day 2). All drug doses were administered under the supervision of a member of the study staff. Subjects were observed for vomiting for 30 minutes after ingesting the drugs; those who vomited the first dose were re-treated with an identical dose. Subjects who vomited twice



FIGURE 1. Sites of *Plasmodium vivax* *in vivo* drug efficacy trials in Peru, 1998–2001.

were dropped from the study. Patients with axillary temperatures $\geq 37.5^{\circ}\text{C}$ were treated with paracetamol.

Patients were asked to return for follow-up evaluations and temperature measurements on days 1, 2, 3, 7, 14, 21, and 28 (days 1, 2, 4, 7, 11, 18, 21, and 28 for the Padre Cocha study). Thick blood smears were prepared at the time of all follow-up visits except day 1. Patients who did not return were traced to their homes. All patients were treated with primaquine, 0.5 mg/kg/day for seven days, at the end of their follow-up to prevent relapses, as recommended by the Peruvian National Malaria Control Program. Patients who failed to respond to CQ were re-treated with quinine plus tetracycline or clindamycin.

Standard World Health Organization definitions of parasitologic response were used.¹³ The patient's therapeutic response was classified according to the guidelines of the Pan American Health Organization for *in vivo* antimalarial drug efficacy testing.¹²

Laboratory analyses. Thick blood smears were stained with Giemsa and the parasite density was calculated by counting the number of asexual parasites per 300 white blood cells, based on 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, a difference in species diagnosis, or $> 50\%$ difference in parasite density), the blood smear was re-examined by a third microscopist. The final parasite density was an average of the densities of the two concordant microscopists. A minimum of

200 oil-immersion fields were examined before a blood smear was considered negative.

Blood levels of CQ and its major metabolite, desethylchloroquine (DCQ), were measured by high-performance liquid chromatography (HPLC) analysis according to the method described by Patchen and others.¹⁴ Venous blood was collected in EDTA at the time of recurrence of parasitemia and refrigerated at 4°C until testing. Chloroquine and DCQ were extracted by adding 0.05 mL of an internal standard (isopropyl analog of CQ), 0.5 mL of 20% $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$, and 0.5 mL methyl-tertbutylether to 0.1 mL whole blood. The solution was vortexed for 30 seconds and centrifuged to separate the organic phase. The organic phase was then transferred to another tube and dried with air. The sample was reconstituted with an HPLC mobile phase and a portion was injected into the HPLC system.

To confirm that treatment failure was due to a recrudescence, rather than a reinfection, in the two patients who had therapeutic blood levels (CQ-DCQ > 90 ng/mL), parasite DNA was extracted from blood on day 0 and the day of failure using the QIAamp kit (Qiagen, Chatsworth, CA). The DNA was amplified by polymerase chain reaction (PCR) for the *Pv200* gene using gene-specific primers.¹⁵ Amplified DNA was subjected to electrophoresis on a 10% Tris-borate-EDTA agarose gel, stained with silver, and assessed for genotypic patterns of the *Pv200* gene based on single-stranded conformational polymorphism (SSCP) analysis, as described.¹⁵

Statistical analysis. Data were double-entered. Statistical analyses were carried out using SPSS (SPSS Inc., Chicago, IL). Dichotomous variables were compared with the chi-square or Fisher's exact tests. The Shapiro-Wilk test was used to test for normality of continuous variables and the Student *t*-test or Mann-Whitney U test was used to compare means.

RESULTS

A total of 242 patients were enrolled at the four sites. The characteristics of these subjects are shown in Table 1. Their median age was 19.0 years; 59.1% were males. Their mean \pm SD duration of illness before enrollment was 4.1 ± 3.2 days. Ninety-six percent had a history of fever and 48.8% had a documented fever (axillary temperature $\geq 37.5^{\circ}\text{C}$). Their geometric mean parasite density was 5,129 parasites/ μL .

Thirty subjects (12.4%) were excluded from the analysis: 11 (14.3%) in Iquitos, 9 (17.3%) in Caballococha, 6 (9.2%) in Sullana, and 4 (8.3%) in Padre Cocha. Twenty-three of the subjects were lost to follow-up (8 on or before day 7, 10 on day 14, 2 on day 21, and 3 on day 28), three had mixed infections diagnosed during follow-up, three voluntarily withdrew from the study, and one received an incorrect dosage of CQ. All remaining subjects completed their 28-day follow-up. Four patients vomited their first dose of CQ within 30 minutes and were re-treated.

Plasmodium vivax parasitemia decreased slowly during the first 3–5 days after treatment was initiated. A total of 28.5% of the patients still had positive blood smears on day 3, 15.0% on day 4, and 10.0% on day 5. It was not until day 7 that all patients had negative blood smears.

Four subjects from the Amazon region had a recurrence of parasitemia during the 28-day follow-up period, two each in Iquitos and Caballococha (Table 2). No recurrences of para-

TABLE 1

Characteristics of patients enrolled in 28-day *in vivo* efficacy trials of chloroquine for the treatment of *Plasmodium vivax*, malaria in Peru, 1998–2001

Characteristic	Padre Cocha (n = 48)	Iquitos (n = 77)	Caballococha (n = 52)	Sullana (n = 65)
Median age (years)	18.5	25.0	18.0	17.0
Sex (male)	64.6%	66.2%	59.6%	46.2%
History of fever	93.8%	94.8%	94.2%	100.0%
Mean \pm SD duration of illness (days)	2.4 \pm 1.0	4.9 \pm 3.8	3.7 \pm 2.8	5.0 \pm 3.1
Axillary temperature \geq 37.5°C (day 0)	34.0%	45.5%	57.7%	56.9%
Geometric mean parasite density μ L (day 0)	4,869	4,592	5,413	5,817

sitemia were observed in the 59 patients followed for 28 days on the northern Pacific Coast. The recurrences occurred in two patients on day 21 and in the other two on day 28 (Table 3). These four subjects were 7, 7, 13, and 15 years old; three were females. Their parasite densities on day 0 ranged from 1,013 to 24,268 parasites/ μ L. None had a history of vomiting or diarrhea during the course of their three-day treatment and none had a history of fever or a documented fever at the time of the recurrence of their parasitemia.

Blood was collected at the time of the recurrence of parasitemia from three of the four subjects for determining CQ-DCQ blood levels and genotyping. In two patients, H032 and H071, the levels of CQ-DCQ were \geq 97 ng/mL, indicating that the parasites were resistant to CQ. Although the third patient had every dose of her CQ treatment supervised by a member of the study staff and could have been infected with a resistant strain, her CQ-DCQ blood levels were only 79 ng/mL.

The two strains from patients H032 and H071 were evaluated by PCR and both day 0 and recurrent parasites were shown to be *P. vivax*. When tested by PCR-SSCP, these two strains were identical genetically at their *Pv200* locus to the parasites found in the same patients on day 0, strongly suggesting that the recurrence of parasitemia was due to a recrudescence of infection or relapse, rather a new infection (Figure 2).

DISCUSSION

The potential emergence of CQ-resistant *P. vivax* in the Americas represents a serious public health problem. *Plasmodium vivax* makes up 82% of all malaria infections reported from the Americas.¹⁶ Even in the Amazon Basin of Peru, where the incidence of *P. falciparum* is traditionally

much higher, *P. vivax* still accounted for 76% of all infections during 2000.¹⁷ Although CQ has been widely available for many years in stores and pharmacies throughout the region and self-treatment is commonplace in most countries,^{18–20} until recently, CQ efficacy has remained high. For that reason, together with its safety, low cost, and the lack of suitable alternatives, CQ continues to be the first-line drug for *P. vivax* infections in all national malaria control programs in the region.

Studies conducted in Indonesia by Baird and others have helped to define *P. vivax* resistance to CQ in the context of *in vivo* drug efficacy trials. The minimal effective concentration of CQ against *P. vivax* is 90–100 ng/mL of whole blood.²¹ In patients treated with a standard 25 mg/kg dose of CQ, mean whole blood levels of CQ and its major metabolite, DCQ, were 141 ng/mL (95% confidence interval = 115–167 ng/mL) at 28 days after initiation of therapy.²² Consequently, the presence of CQ in the blood up to 28 days after therapy should be sufficient to eliminate or suppress CQ-sensitive parasites. Based on these data, Baird and others concluded that *P. vivax* parasitemia within 28 days after a standard 25 mg/kg course of CQ represents strong evidence of resistance, regardless of whether the parasites originated from a recrudescence, a relapse, or even a new infection.²² It is likely that the parasites studied by Baird and others and those in our study are both of the “tropical” variety, which characteristically give rise to a series of relapses beginning one to two months after the primary attack.²³

Because of the extremely serious public health implications that identification of CQ-resistant *P. vivax* has for the Americas, we believe that the criteria for accepting a *P. vivax* infection as CQ resistant should be even more rigorous. First, the patient should be treated with the standard 25 mg/kg CQ regimen and all drug doses should administered under supervision. Second, the recurrence of parasitemia should occur within the first 28 days after therapy is initiated. Finally, CQ-DCQ blood levels at the time of the recrudescence should be \geq 90–100 ng/mL. As additional evidence, it is helpful if genotyping can confirm that the infecting parasite is *P. vivax* and that recurrent parasites are genetically identical to those from the day 0 samples at at least one locus.

Based on these criteria, many of the reported cases of CQ-resistant *P. vivax* from South America would not qualify as resistant. In an early report from Brazil, the recurrences of parasitemia occurred three and seven months after the initial infection and were almost certainly relapses.²⁴ Chloroquine blood levels were not measured in either the later report from Brazil or a study in Colombia.^{8,9} In fact, the only well-

TABLE 2

Response to chloroquine treatment of subjects with *Plasmodium vivax* malaria in Peru, 1998–2001

Site	No.	Parasitologic response (%) [*]				No.	Therapeutic response (%) [†]		
		RIII	RII	RI	S		ETF	LTF	ACR
Padre Cocha	44	0	0	0	100.0	44	0	0	100.0
Iquitos	66	0	0	3.0	97.0	66	0	3.0	97.0
Caballococha	43	0	0	4.6	95.4	43	0	4.6	95.4
Sullana	59	0	0	0	100.0	59	0	0	100.0
Total	212	0	0	1.9	98.1	212	0	1.9	98.1

^{*} S, RI, RII, and RIII refer to World Health Organization resistance levels.¹³

[†] ETF = early treatment failure; LTF = late treatment failure; ACR = adequate clinical response.¹²

TABLE 3
Patients with recurrences of *Plasmodium vivax* parasitemia during follow-up in Peru, 2000

Site	Subject no.	Age (years)	Sex	Day 0 parasite density μL	Day of recurrent parasitemia	Blood levels (ng/mL)		
						Chloroquine	Desethylchloroquine	Total
Iquitos	032	7	F	4,259	28	81	16	97
Iquitos	071	13	M	24,268	21	115	31	146
Caballococha	361	15	F	1,013	28	Not done	Not done	–
Caballococha	493	7	F	1,645	21	65	14	79

documented case of CQ-resistant *P. vivax* in South America is in one of three travelers from Guyana reported by Phillips and others.⁷

Our finding of two patients with CQ-resistant *P. vivax* malaria in the Peruvian Amazon region represents only the second confirmed report of this infection in South America. Both patients were residents of a relatively circumscribed area, 10–20 km to the west of the city of Iquitos. While residents of this area may have more ready access to antimalarial drugs than residents of more isolated regions of the Amazon Basin, there are no obvious reasons why a focus of CQ resistance should appear in this area rather than another. Similar to the studies conducted in Indonesia, in which CQ resistance was seen primarily in children,²¹ our two patients were only 7 and 13 years old.

Although we treated the patients who had recurrences of parasitemia with quinine plus tetracycline or clindamycin, there is no consensus on the treatment of choice for CQ-resistant *P. vivax* infections. Both quinine and mefloquine have activity against *P. vivax*, and amodiaquine and halofantrine have been shown to be more active than CQ against CQ-resistant strains of *P. vivax*.²⁵ Although primaquine has

only limited blood schizonticidal activity in the doses normally used to eliminate hypnozoites or *P. falciparum* gametocytes, in combination with CQ it is also more efficacious than CQ alone for the treatment of CQ-resistant *P. vivax*.⁶ In Peru, as in all other countries in the Americas, patients infected with *P. vivax* who are treated in Ministry of Health facilities receive both CQ and primaquine (usually 0.25 mg/kg/day for 14 days or 0.5 mg/kg/day for 7 days), and this may have played some role in suppressing the appearance and/or extension of CQ-resistant parasites in the region.

The existence of these two confirmed cases of CQ-resistant *P. vivax* in the Amazon region of Peru is of obvious concern to the Peruvian Ministry of Health. Plans have been made to conduct a larger survey in the same area to try to define better the extent of this problem. Since CQ continues to cure blood-stage *P. vivax* parasitemia in the great majority of patients, no changes have been made in national malaria treatment policy.

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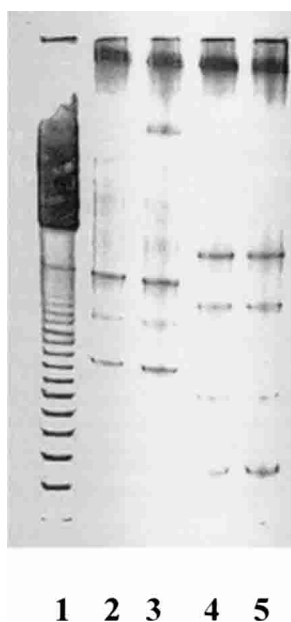


FIGURE 2. Genotypic patterns for the *Pv200* gene following polymerase chain reaction–single-stranded conformational polymorphism analysis of *Plasmodium vivax* DNA on day 0 and day of recrudescence. Lane 1, 123-basepair ladder; lane 2, day 0 patient H-032; lane 3, day 28 patient H-032; lane 4, day 0 patient H-071; lane 5, day 21 patient H-071.

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