Plasmodium vivax Chloroquine Resistance and Anemia in the Western Brazilian Amazon

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Data on chloroquine (CQ)-resistant *Plasmodium vivax* in Latin America is limited, even with the current research efforts to sustain an efficient malaria control program in all these countries where *P. vivax* is endemic and where malaria still is a major public health issue. This study estimated *in vivo* CQ resistance in patients with uncomplicated *P. vivax* malaria, with use of CQ and primaquine simultaneously, in the Brazilian Amazon. Of a total of 135 enrolled subjects who accomplished the 28-day follow-up, parasitological failure was observed in 7 (5.2%) patients, in whom plasma CQ and desethylchloroquine (DCQ) concentrations were above 100 ng/dl. Univariate analysis showed that previous exposure to malaria and a higher initial mean parasitemia were associated with resistance but not with age or gender. In the multivariate analysis, only high initial parasitemia remained significant. Hemoglobin levels were similar at the beginning of the follow-up and were not associated with parasitemia. However, at day 3 and day 7, hemoglobin levels were significantly lower in patients presenting CQ resistance. The *P. vivax dhfr* (*pvdhfr*), *pvmrp1*, *pvmdr1*, and *pvdhps* gene mutations were not related to resistance in this small sample. *P. vivax* CQ resistance is already a problem in the Brazilian Amazon, which could be to some extent associated with the simultaneous report of anemia triggered by this parasite, a common complication of the disease in most of the areas of endemicity.

Plasmodium vivax is the most geographically spread malaria-related species (1) and was responsible in 2010 for 363,948 cases of malaria in the Americas (231,618 cases were reported in Brazil) (2). Chloroquine (CQ) has been the well-tolerated therapy of choice for the treatment of acute vivax malaria since 1946 (3). The drug clears fever and parasitemia within 72 h of the first dose and is rapidly absorbed and slowly eliminated, principally as the parent drug and as the metabolite desethylchloroquine (DCQ) in a proportion of roughly 3:1 (4). The plasma half-life is about 50 h, and therapeutic levels against vivax malaria persist in blood until days 21 to 35 after the start of treatment (5). CQ-resistant *P. vivax* favors recurrence due to recrudescence, which means the reappearance of parasitemia from asexual blood-stage parasites following blood schizonticidal therapy (6). Ideally the confirmation of *in vivo* CQ resistance (CR) requires the determination of the levels of the drug and its major active metabolite in the blood.

P. vivax CR was first described in 1989, when Australians expatriated from Papua New Guinea failed routine treatment (7). Since that pioneer report, *P. vivax* CR has been demonstrated especially in East Asia (Indonesia, Malaysia, Myanmar, Thailand, Vietnam, and Philippines) (6). In South America, there is already evidence of the phenomenon, but not many data are available.

After the first evidence of failure of combined CQ and primaquine (PQ) therapy for *P. vivax* malaria acquired by Canadian travelers in Guyana (8), some studies carried out in different regions did not detected recurrent parasitemia within 28 days (9–11) or 30 days (12). In Brazil, the first clinical evidence was reported in 2000 in Manaus (13). In Colombia, *P. vivax* CR was described in three cases among 27 subjects (14), and in Peru four cases were confirmed among 177 subjects (15). In 2007, the proper 28-day follow-up of 109 patients with *P. vivax* prescribed

only CQ (PQ prescription was postponed to day 28) led to the confirmation of 10.1% resistance after plasmatic CQ dosage (16).

PQ is routinely used as an hypnozoiticidal drug despite having schizonticidal activity against *P. vivax* by itself (17). There is some *in vitro* evidence of synergy between primaquine and CQ against *P. falciparum* schizonts (18). However, there is no evidence of synergy between these two drugs against *P. vivax* asexual blood stages. Actually, the available evidence shows that treatment efficacy was not significantly different at the 28-day follow-up between the CQ monotherapy group and the group receiving CQ with PQ for 14 days in patients with uncomplicated vivax malaria (19). However, these data refer only to strains from Asia and cannot be easily extrapolated to Latin America.

Curiously, the same areas reporting *P. vivax* CR also have identified severe disease attributed to this species (20). The rationale of a more prolonged parasitemia due to drug resistance explains especially severe anemia; however, to date no evidence of individual patients well characterized for *P. vivax* CR evolving with anemia exists, because in most of these areas, CQ is no longer used.

In this study, we have estimated *in vivo* CQ resistance in patients with uncomplicated *P. vivax* from the western Brazilian Amazon with use of CQ (standard dose of 25 mg/kg over the first 3 days) and PQ (0.5 mg/kg/day over the first 7 days), as well as the

TABLE 1 Oligonucleotide primers used for PCR amplification and DNA sequencing of *P. vivax* genes

Gene	Chromosome	Primer	Sequence (5′ → 3′)	bp	Mutations sought	Reference(s)
pvdhfr	5	PV1F	CAGTGAAGGGACAAAGAATGAACC	560	S58R/S117N/I173L	42, 43
		PV1R	ACTCGGGGAAGAAGACGTCAC			
pvmrp1	2	PV5F	CATATCGGGAAAAAGCGTAATTAACG	523	L1282I/Y1393D/G1419A/V1478I/H1586Y	44
		PV5R	CTTCGATTGGTCTATGGCTGGTG			
		PV6F	TCGAGAACGTATTCGTCAGTTATAAG	497		
		PV6R	GTTGCTCGAAAGGTTAGCCTTTC			
pvmdr1	10	PV7F	GCCATGTTCATTTCTGAGACGCTG	337	M908L/L958M	36, 45
		PV7R	TCGCTCTGATGGCAAACACTC			
pvdhps	14	PV8F	TTTTAAAGTACATTGAGCAAATCGTG	200	S382C/A383G	42, 43
		PV8R	CTGATCACTTGTGTGGTTTATGTG			
		PV9F	GCGGTTTATTTGTCGATCCTGTG	244		
		PV9R	TTTTTCCTGGCATCACTTGCTG			

hemoglobin level dynamics over the follow-up period in both resistant and sensitive groups.

MATERIALS AND METHODS

Study site and patients. The study was performed from December 2007 to July 2008 at the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), which reports 20% of all the malaria cases in Manaus (03°06′S, 60°01′W). Patients living in the urban or periurban areas of this city with uncomplicated *P. vivax* malaria confirmed by a thick blood smear (TBS) and PCR *a posteriori* (21) were randomly selected in the outpatient clinics, and epidemiological and clinical history was fully obtained. Parasite densities were estimated by experienced microscopists by counting the number of parasites in 100 leukocytes in high-magnification fields and using the individual number of leukocytes/mm³ from the full blood count. The study included patients of both sexes, aged 12 to 60 years, presenting a blood parasite density of 250 to 100,000 parasites/ml and an axillary temperature of ≥37.5°C or history of fever in the last 48 h. Exclusion criteria were the use of antimalarials in the previous 60 days, refusal to be followed up for 28 days, and any clinical complication.

Treatment. Patients received daily supervised treatment with 25 mg/kg of CQ phosphate (Farmanguinhos) over a 3-day period (10 mg/kg on day 0 and 7.5 mg/kg on days 1 and 2) associated with PQ (Farmanguinhos) over a 7-day period at the dosage of 0.5 mg/kg per day. The same batches of drugs were used in the study. Patients who vomited the first dose within 30 min after drug ingestion under observation were retreated with a similar dose.

Patient follow-up and resistance definition. Participants were evaluated on days 0, 1, 2, 3, 7, 14, and 28 (active surveillance) and, if they felt ill, at any time during the follow-up period (passive surveillance). A full blood count was performed only on day 0, and in every evaluation, determination of hemoglobin in venous blood (using a portable HemoCue photometer [Anglholm, Sweden]), TBS, and PCR were performed. CQ and DCQ plasma levels were determined only in cases of parasitological failure (22). Three aliquots of 100 μl of whole blood from day-of-recurrence (DR) samples were spotted onto filter paper for later analysis by high-performance liquid chromatography (HPLC) to determine the levels of CQ and DCQ as previously described (23, 24). Resistance was defined as peripheral parasitemia in the presence of a CQ and DCQ blood level sum exceeding the minimal effective concentration of 100 ng/ml. Resistant cases were further treated with oral arthemeter/lumefantrine for 3 days.

Molecular characterization. Samples from all patients with *P. vivax* CR and a from a subsample (the same number selected randomly) of patients without *P. vivax* CR were subjected to molecular characterization of a few candidate genes, mostly nonexplored in the literature, which

could be putative markers of CQ resistance. Extraction of whole DNA was carried out using a QIAamp DNA Minikit (Qiagen, Germany) according to the manufacturer's protocol. The PCR primers and different reaction conditions used to amplify *P. vivax dhfr* (*pvdhfr*), *pvmrp1*, *pvmdr1*, and *pvdhps* gene sequences were as previously described (Table 1). Briefly, after initial denaturation at 94°C for 2 min, the samples were subjected to 35 cycles (94°C for 1 min more, 58°C for 30 s, and 72°C for 1 min), with a final extension at 72°C for 10 min. For each fragment, PCR products were visualized by 1% agarose gel electrophoresis and staining with ethidium bromide to confirm a single band. The DNA concentration was measured with a NanoDrop 2000 instrument (Thermo Scientific). Sequencing reactions were carried out using an ABI 3130xl Genetic Analyzer (Applied Biosystems, USA) as specified by the manufacturer's protocol.

Statistical analysis. The estimation of possible factors associated with *P. vivax* CR was assessed in both univariate and multivariate (logistic regression) analyses. The interaction term between resistance status and day of follow-up was assessed by random-effects linear regression. Mean parasitemia levels throughout the follow-up were compared by using the Mann-Whitney U test. Fisher's test was employed to evaluate the association between point mutations and CQ resistance. Correlation between hemoglobin and parasitemia on day 0 was evaluated through the Spearman test. A P value of <0.05 was considered significant for all the analyses. Analysis was performed using SPSS software for Windows (version 16; SPSS, Inc., Chicago, IL).

Ethics. This study was approved by the ethics committee of the FMT-HVD, and informed consent was obtained from all patients.

RESULTS

Of a total of 154 patients included, 135 fulfilled the follow-up. Thirteen subjects voluntarily withdrew from the 28-day follow-up, and six were excluded because they were diagnosed with mixed *P. vivax-P. falciparum* malaria on day 0 (confirmed by PCR performed *a posteriori*). Demographic and clinical characteristics of the enrolled patients are presented in Table 2.

Parasitological failure was observed in 7/135 patients (5.2%; 95% confidence interval, 0.2 to 10.5). Blood CQ/DCQ concentrations in these seven patients were all above the minimal effective concentration (>100 ng/ml).

On day 3, mean blood levels of CQ/DCQ were lower in patients carrying resistant parasites (1,069.8 ng/ml) than in patients carrying sensitive ones (3,311.1 ng/ml) ($P = 0.049$), even when the drug plasma concentration was more than 10 times higher than the effective levels in the first group. On days 7, 14, and 28, drug levels

TABLE 2 Demographic and clinical features of the study participants on admittance and before treatment

Parameter	Value
Sample size	135
Mean age, yr (SD)	35.9 (12.5)
No. (%) male	102 (75.5)
Wt, kg (SD)	72.1 (14.1)
Mean body temp, °C (SD)	36.7 (1.1)
No. (%) with axillary temp > 37.5°C	33 (24.4)
Parasite geometric mean per mm^3 (SD)	3.720 (4.136)
No. (%) with presence of gametocytes at microscopy	132 (97.8)
Mean hemoglobin, g/dl (SD)	13.4 (1.7)
No. (%) with anemia[a]	27 (20.0)

[a] Hemoglobin level of <12 g/dl for females and <13 g/dl for males (according to WHO criteria).

TABLE 3 Average blood levels of chloroquine plus desethylchloroquine in patients carrying resistant and susceptible *P. vivax* parasites

Day of follow-up	CQ-DCQ level(ng/ml, mean ± SD) in blood of patients carrying:		P value
	Resistant isolates	Susceptible isolates	
3	1,069.8 ± 810.6	3,311.1 ± 2,361.8	0.049
7	1,321.5 ± 922.6	1,914.2 ± 1,700.0	0.463
14	1,044.3 ± 667.6	1,351.4 ± 1,763.0	0.679
28	647.4 ± 227.2	783.8 ± 530.0	0.543

were not significantly different between patients carrying resistant and susceptible *P. vivax* parasites (Table 3).

In the univariate analysis, previous exposure to malaria and a higher initial parasitemia mean were associated with treatment failure resistance (Table 4). In the multivariate analysis, only high initial parasitemia remained associated with CQ resistance, independently of age and gender.

Baseline parasitemia on day 0 was significantly higher in resistant than in sensitive cases. In the following days, however, parasitemia was approximately the same in both groups, until day 28, when recrudescences were observed in all seven resistant cases (late parasitological failures) (Fig. 1A). Hemoglobin levels were similar in the beginning of the follow-up and were not correlated with parasitemia (Fig. 1B). From this point, hemoglobin remained relatively constant for the sensitive group but decreased in the resistant patients. On days 3 and 7, hemoglobin levels were significantly lower in patients presenting CQ resistance. After day 14, however, hemoglobin levels improved in this group, reaching levels similar to those in the sensitive group (Fig. 2).

The *pvdhfr*, *pvmrp1*, *pvmdr1*, and *pvdhps* gene mutations studied were not related to CQ resistance (Table 5).

DISCUSSION

CQ and PQ are still the drugs of choice to treat vivax malaria in many areas of endemicity, including Brazil. A previous report from a study in the Brazilian Amazon demonstrated that CQ failure in *P. vivax* infection was around 10.1% (16). In this study, the *in vivo* CQ resistance rate in patients with uncomplicated *P. vivax* malaria using CQ and PQ simultaneously was 5.2%, with a proper dosage of blood levels of CQ and DCQ. It has been claimed that the concomitant use of PQ and CQ could result in a synergistic effect against asexual forms of *P. vivax*, as has been shown for *P. falciparum* parasites (18). However, the *in vivo* data available for *P. vivax* show that the parasitological responses to CQ and to CQ plus PQ are quite similar (19). Consequently, the possibility that such synergism results in the underreporting of CQ resistance in areas where PQ is systematically used should be examined. The findings presented here suggest that treatment efficacy was not substantially improved by the concurrent administration of PQ in terms of reducing therapeutic failure (the confidence interval ranged from 0.2 to 10.5% and therefore included the 10.1% frequency found in a study performed in Manaus years ago, in which CQ was used by itself and PQ was postponed until day 28).

There is a paucity of available data regarding risk factors associated with *P. vivax* CR. In a clinical trial carried out in Thailand, children <5 years old were at greater risk of recurrent *P. vivax* infection than older patients, probably due to less natural immunity against malaria in this age group (25). The present study included patients aged 12 to 60 years, following World Health Organization guidelines (22), and no association with mean age was found. In this work, clearance occurred with a similar time profile in both sensitive and resistant groups. Gender and baseline values of weight, temperature, hemoglobin levels, and platelet counts also were not associated with resistance. In Afghanistan, a lower initial hemoglobin concentration was independently associated with recurrence on day 56 (26). There is a previous report of body temperature of ≥38°C as a factor contributing to delay in parasite clearance in uncomplicated falciparum malaria in children (27).

In this work, only higher parasitemia on day 0 was an independent risk factor for *P. vivax* CR. Some authors found that high baseline parasitemia density may be a predictor of CQ resistance in patients presenting falciparum malaria (27, 28). It is known that laboratory strains of *P. falciparum* (29) and *P. vivax* (30) have a marked variability in growth rates, with CQ-resistant isolates growing faster than CQ-susceptible isolates.

Significantly lower hemoglobin levels were observed in patients harboring CQ-resistant *P. vivax* on days 3 and 7, suggesting that CQ resistance could be an important anemia-triggering factor during malarial episodes. However, after a temporary parasite clearance, hemoglobin increases to a level similar to that observed for sensitive parasite carriers. This short effect of resistant parasites upon hemoglobin levels could be explained by the late parasitological failures seen in all patients from the study. In areas with more severe and widespread *P. vivax* CR, one could hypothesize that the impact could be more evident. Although higher parasitemia was demonstrated for resistant patients on day 0, the parasite loads became similar on day 3, showing that parasitemia alone should not explain the hemoglobin decrease from day 0 to day 7 in the resistant group, unless initial parasitemia in the bone marrow milieu plays a role in worsening of anemia (31).

The simultaneous occurrence of severe vivax disease and CQ resistance in some countries has raised the question of a possible association between severity and resistance (32). CQ resistance actually has been reported from Brazil almost at the same time as clinical severity. Our data showed a link between CQ resistance and a malaria complication represented here by hemoglobin decrease, but it is difficult to predict how this phenomenon contributes to severity and anemia in areas where malaria is endemic from a long time perspective. Previous evidence showed that a lower initial hemoglobin concentration was independently asso-

TABLE 4 Univariate and multivariate (logistic regression) analyses of factors associated with treatment resistance in patients with uncomplicated *Plasmodium vivax* malaria under supervised treatment with chloroquine plus primaquine

	Univariate analysis				Logistic regression analysis			
		95% Confidence interval				95% Confidence interval		
Factor	Odds ratio	Lower	Upper	P value[a]	Adjusted odds ratio	Lower	Upper	P value
Anemia[b]	0.61	0.11	3.35	0.568	1.47	0.24	8.99	0.674
History of 2–5 previous malaria episodes	6.15	1.14	33.12	**0.016**	4.83	0.86	27.23	0.074
Asexual parasitemia > 5,000	7.50	1.39	40.56	**0.008**	5.83	1.04	32.77	**0.045**
Constant					0.01			0.000

[a] Boldface indicates significance.
[b] Hemoglobin level of <12 g/dl for females and <13 g/dl for males.

ciated with recurrence of vivax malaria treated with CQ ([26]), suggesting a similar relationship between drug resistance and decreased hemoglobin during malaria episodes. As we found that high parasite densities were concurrent with *P. vivax* CQ resistance, the possibility arises that the highly CQ-resistant isolates found in Papua may be more pathogenic to the host, in agreement with previous observations for falciparum malaria in Papua ([33]). When Fernandez-Becerra et al. ([34]) compared a patient with se-

vere vivax malaria to another patient with uncomplicated malaria, they found higher levels of both *pvmdr1* and *pvcrt-o* gene expression in the former patient.

With respect to molecular genotyping, we did not find any molecular marker related to CQ resistance. Actually, no trustworthy molecular marker for *P. vivax* CR has been identified so far ([1], [6]). Molecular characterization of *P. vivax* isolates carried out previously in Brazil has shown that the sequences of the *pvcrt-o* and *pvmdr1* genes were not associated with CQ resistance ([35], [36]).

Evidence is growing that *P. vivax* may infect many more people than has been appreciated ([37]). Evidence also points to this supposedly benign parasite causing a spectrum of severe and life-threatening syndromes ([38], [39]). *P. vivax* CR may be rising in the Brazilian Amazon, and probably this fact is contributing to the increase in complicated vivax malaria. To maintain an efficient malaria control program, drug resistance surveillance assays must be conducted on a regular basis to assess antimalarial efficacy and to ensure that the information is available to policy makers. The Amazon Network for the Surveillance of Antimalarial Drug Resistance (RAVREDA), funded in 2001, was definitely a pivotal initiative that contributed to expanding the knowledge on *P. vivax* CR in Latin America.

Despite the association shown, it is important to notice that no causal relationship could be definitely established between *P. vivax* CR and anemia in the present work, especially due to the limitation of the small sample size. Moreover, both phenomena still have unclear pathogenesis. Anemia, for instance, seems to present multifactor determinants ([40]), with rosetting being one of
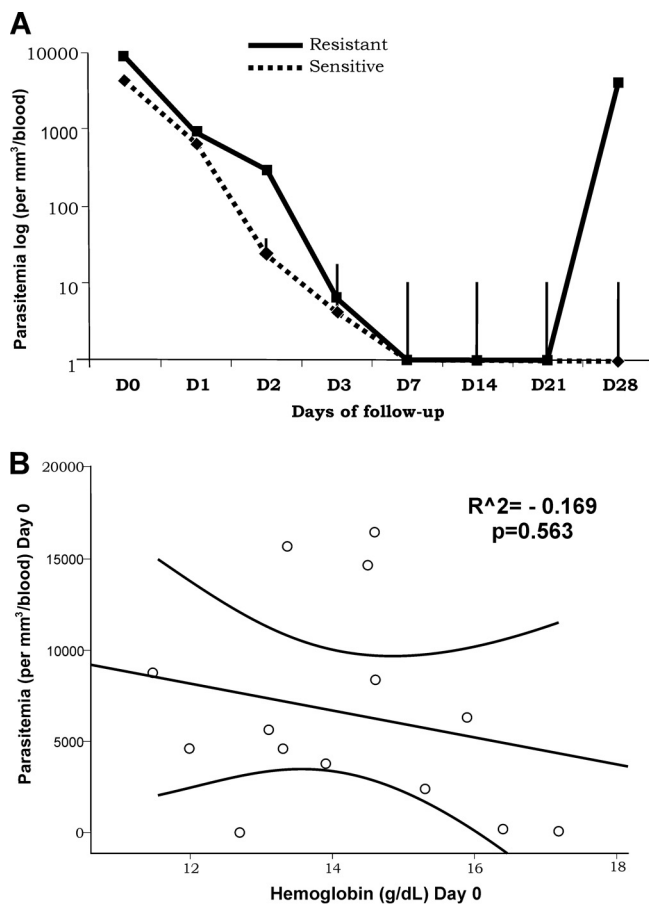

FIG 1 Parasitemia levels (presented as means with error bars) throughout the follow-up according to resistance status (A) and correlation between hemoglobin and parasitemia on day 0 (B). *, statistically significant.
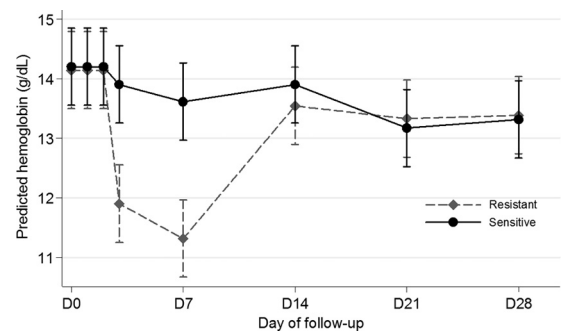

FIG 2 Hemoglobin levels during the follow-up according to resistance status.

TABLE 5 Frequency of polymorphisms in the *pvdhfr*, *pvmrp1*, *pvmdr1*, and *pvdhps* genes for sensitive and resistant samples

Gene and polymorphism	No. of isolates/total (%)		*P*
	Resistant	Susceptible	
pvdhfr			
58	6/7 (85.7)	3/7 (42.8)	0.266
117	6/7 (85.7)	7/7 (100)	1.000
173	3/7 (42.8)	2/7 (28.5)	1.000
pvmrp1			
1282	7/7 (100)	7/7 (100)	
1393	7/7 (100)	7/7 (100)	
1419	4/7 (57.1)	2/7 (28.5)	0.592
1478	7/7 (100)	7/7 (100)	
1586	1/7 (14.2)	2/7 (28.5)	1.000
pvmdr1			
908	0/7 (0)	2/7 (28.5)	0.067
958	7/7 (100)	7/7 (100)	
pvdhps			
205	5/7 (71.4)	6/7 (85.7)	1.000
382	5/7 (71.4)	5/7 (71.4)	1.000
383	2/7 (28.5)	5/7 (71.4)	0.286

the recently described mechanisms (41). Further studies from other areas of endemicity in which other causes of anemia are well characterized may contribute to the better understanding of the association between *P. vivax* CR and anemia.

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