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Confirmed *Plasmodium vivax* Resistance to Chloroquine in Central Vietnam

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***Plasmodium vivax* resistance to chloroquine (CQ) is currently reported in almost all countries where *P. vivax* is endemic. In Vietnam, despite a first report on *P. vivax* resistance to chloroquine published in the early 2000s, *P. vivax* was still considered sensitive to CQ. Between May 2009 and December 2011, a 2-year cohort study was conducted in central Vietnam to assess the recommended radical cure regimen based on a 10-day course of primaquine (0.5 mg/kg/day) together with 3 days of CQ (25 mg/kg). Here we report the results of the first 28-day follow-up estimating the cumulative risk of *P. vivax* recurrences together with the corresponding CQ blood concentrations, among other endpoints. Out of 260 recruited *P. vivax* patients, 240 completed treatment and were followed up to day 28 according to the WHO guidelines. Eight patients (3.45%) had a recurrent *P. vivax* infection, at day 14 ($n = 2$), day 21 ($n = 1$), and day 28 ($n = 5$). Chloroquine blood concentrations, available for 3/8 recurrent infections (days 14, 21, and 28), were above the MIC (>100 ng/ml whole blood) in all of these cases. Fever and parasitemia (both sexual and asexual stages) were cleared by day 3. Anemia was common at day 0 (35.8%), especially in children under 10 years (50%), and hemoglobin (Hb) recovery at day 28 was substantial among anemic patients (median change from day 0 to 28, +1.7 g/dl; interquartile range [IQR], +0.7 to +3.2). This report, based on CQ blood levels measured at the time of recurrences, confirms for the first time *P. vivax* CQ resistance in central Vietnam and calls for further studies using standardized protocols for accurately monitoring the extent and evolution of *P. vivax* resistance to chloroquine in Vietnam. These results, together with the mounting evidence of artemisinin resistance in central Vietnam, further highlight the increasing threat of antimalarial drug resistance to malaria elimination in Vietnam.**

Plasmodium vivax is the most widely distributed malaria parasite species; an estimated 2.85 billion people were at risk of infection in 2009, the vast majority (2.59 billion [91.0%]) living in central and southeast Asia (1). Moreover, since malaria elimination has been on the global health agenda (2), the public health importance of vivax malaria has been increasingly reassessed, since it is more difficult to control than *Plasmodium falciparum* malaria, and severe clinical syndromes as well as new foci of chloroquine resistance are increasingly reported (3–5). Chloroquine (CQ) is the first-line treatment for *P. vivax* in most countries where it is endemic. *P. vivax* resistance to CQ was first reported in 1989 from Papua New Guinea (PNG) (6), rapidly followed by reports from Indonesia in 1991 (7, 8), Myanmar in 1993 and 1995 (9, 10), India in 1995 (11, 12), Malaysian Borneo in 1996 (13), and several South American countries (Guyana, Brazil, and Columbia) from 1996 onwards (14–16). In Vietnam, little evidence of *P. vivax* susceptibility to CQ has been published so far; one study in Binh Thuan province (southeastern coast region) in the early 2000s reported *P. vivax* resistance to chloroquine (17), while this was absent in the neighboring Khanh Hoa province (18). The National Malaria Control Program (NMCP) has been closely monitoring antimalarial drug resistance, mainly focused on *P. falciparum* resistance (19–21), since 1995 in several sentinel sites across the country. Since 2003, *P. vivax* susceptibility to CQ has been assessed in six sentinel sites, and a rate of between 0 and 5.7% of late parasitological failures has been reported (22).

Vietnam is currently engaged in malaria elimination (23, 24), and the issue of drug resistance is a priority, as *P. falciparum* resistance to artemisinins has been already reported in five (Tier I) provinces of central Vietnam (25, 26). Moreover, the control of *P. vivax* is another challenge, as this species is becoming increasingly prevalent (27–30). The main difficulty in controlling vivax malaria lies in the need to radically treat not only blood forms but also the hepatic dormant forms (hypnozoites) that cause relapses for months to years after the initial infection. The World Health Organization (WHO) currently recommends for radical cure a 3-day course of CQ (total of 25 mg/kg) together with a 14-day course of primaquine (PQ) (0.25 mg/kg/day), the recommended treatment in Vietnam since 2009. Nevertheless, between 2007 and 2009, in-

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stead of the 14-day course, PQ was given for 10 days at a higher dose (0.5 mg/kg/day) (31). The efficacy of such treatment on liver stages was assessed by following up a cohort of treated *P. vivax* patients in central Vietnam for 2 years. We report here the results of the first 28-day follow-up done according to the WHO guidelines (32).

MATERIALS AND METHODS

Study site and participants. The study was carried out between April 2009 and December 2011 at the Tra Leng Commune Health Center (CHC), located in a remote forested area in the southwestern part of Quang Nam province, central Vietnam. A detailed description of the study area and population has been reported elsewhere (33). The study was designed as a 28-day follow-up after treatment of *Plasmodium vivax* cases with CQ and PQ (32). Male and female patients, aged between 3 and 60 years, presenting at the CHC (or identified through active case detection by the study team) with suspected malaria were screened for eligibility. Inclusion criteria were as follows: axillary temperature of ≥37.5°C and/or history of fever in the previous 48 h, *P. vivax* monoinfection with asexual parasites confirmed by light microscopy (LM), residency in the study area, and written informed consent from all participants aged 18 years or older (or from parents/guardians for minors). Patients were excluded if they presented general danger signs with severe or complicated malaria, had any acute or chronic concomitant illness, or had already been treated with PQ within the past 30 days. Pregnant or lactating women, patients with known glucose-6-phosphate dehydrogenase (G6PD) deficiency (or history of "black urine" following PQ treatment), or patients with any history of intolerance to the study drugs were excluded. According to the national guidelines, patients were not tested for G6PD deficiency prior to PQ treatment. The prevalence of G6PD genetic polymorphism (*Viangchan* mutation) was estimated to be below 1.5% in both males and females (33), with no difference between ethnic groups.

Procedures. Study drugs were provided by the national malaria control program and consisted of CQ tablets of 300 mg chloroquine base (lot no. 08001CN; registration no. VNB-4144-05) and PQ tablets containing 15 mg primaquine base (lot no. 010109; registration no. VD-0877-06).

A general physical examination was performed at inclusion (day 0) and daily during treatment (days 1 to 9); subsequently, patients were examined weekly at days 14, 21, and 28 and during any unscheduled visit. Patients were asked to return daily to the CHC for direct observed therapy with CQ (25 mg base/kg) and PQ (0.5 mg/kg/day) during the first 3 days (days 0 to 2) and then with PQ alone for the remaining 7 days (days 3 to 9). More specifically, signs and symptoms of acute hemolysis (jaundice, black urine, fatigue, tachycardia, shock, etc.) were systematically checked at each visit by the study clinician; adverse drug reactions and concomitant medications were recorded. Patients not attending scheduled visits were visited at home. Any recurrent *P. vivax* or *P. falciparum* infection detected by LM during the 28-day follow-up was treated with dihydroartemisinin-piperaquine (DHA-PPQ) for 3 days following national guidelines.

Blood samples (finger prick) were collected at days 0, 1, 2, 3, 7, 14, 21, and 28 for LM (blood films) and later molecular analysis (2 blood spots dried on filter paper). Additional blood samples were taken at days 0, 14, and 28 for hemoglobin (Hb) concentration; at day 7 and any day of recurrent *P. vivax* infection, 100 μl of blood was taken on a separate filter paper for later measurement of CQ blood level.

Thick and thin films were stained with 3% Giemsa solution for 45 min; parasite density was estimated by counting the number of parasites per 200 white blood cells (WBCs) and assuming 8,000 WBCs/μl. A slide was declared negative if no parasite was found after counting 1,000 WBCs. All slides were read independently by two expert technicians who in case of discrepancy reread the slide until reaching agreement. A later and systematic quality control examination of all blood slides was done by a senior technician at the central level (NIMPE, Hanoi); in case of disagreement, a second senior technician would reread the slide until agreement was reached. The hemoglobin concentration was measured with the HemoCue Hb 301 device (HemoCue AB, Angelholm, Sweden) following the manufacturer's instructions (34). Filter paper blood samples (FPBS) were dried outside direct sunlight, kept in individual sealed plastic bags, and stored at −20°C (NIMPE, Hanoi) until they were processed.

The concentrations of CQ and desethylchloroquine (DEC) in dried blood filter paper samples were determined using a validated high-pressure liquid chromatography (HPLC) method with a fluorescence detector at excitation and emission wavelengths of 250 and 400 nm, respectively, a modification of the previous published method (35). Following mincing of the filter paper (Whatman grade 3), extraction was performed using 3 ml of 25% ammonia and 3 ml of ethyl acetate-hexane (1:9). The solution was vortexed for 30 s and centrifuged to separate the organic phase, which was then transferred to another tube and evaporated to dryness. The sample was reconstituted with HPLC mobile phase, and 20 μl was injected into the HPLC system (Waters, USA). We used an X-Bridge Phenyl 5-μm (4.6- by 150-mm) column as the stationary phase. The mobile phase used was diethylamine (0.05%)-acetonitrile (55:45), pumped isocratically at flow rate of 1.0 ml/min and temperature of 30°C. Pyrimethamine was used as an internal standard.

Outcomes. Efficacy outcomes were classified into early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR), following the WHO criteria (32). For all efficacy outcomes, no distinction was made between relapse, recrudescence, and reinfection, and any new microscopically detected *P. vivax* infection after initial parasite clearance was defined as "*P. vivax* recurrence." The primary endpoints were the proportion of patients with ACPR by day 28 and the parasite clearance time (PCT). Secondary endpoints included fever and gametocyte clearance times, the proportion of confirmed CQ-resistant *P. vivax* recurrences (CQ plus DEC concentration of >100 ng/ml), and hematological changes between days 0 and 28.

Data analysis. The sample size was calculated on the basis of retrospective data (2003 to 2007) reporting LPF ranging from 0% to 5.7% among *P. vivax* patients treated with CQ (22). Assuming a minimum treatment failure rate of 5% and a loss to follow-up of 10%, a sample size of 204 *P. vivax* patients would be needed for estimation with a 3% precision and at 5% significance level ("CSample" command/Epi Info 6). The sample size was further increased to comply with the requirements of the cohort evaluation, details of which will be published separately. Data were double entered and cleaned using Epidata version 3.1. The data set was analyzed using STATA version 11 (Stata Corp., College Station, TX). The survey design (survey data set) was taken into account using the *svy* command in STATA, with villages as strata and household as sampling unit. Descriptive statistics were used to compute baseline sociodemographic characteristics. Ownership of livestock (pigs, buffaloes, and cows) was used as a proxy for the economic status of the household, using a principal-component analysis (33). The PCT was estimated using the daily proportion of patients still parasitemic from day 0 until the day of complete parasite clearance. The proportion of recurrence-free patients by day 28 was assessed by Kaplan-Meier survival analysis. Patients were censored on the day they had last been seen in follow-up. Fever clearance time was estimated by determining the proportion of febrile patients during follow-up among febrile patients at day 0. Similarly, gametocyte clearance was expressed as the proportion of patients with gametocytes during follow-up among gametocyte-positive patients at day 0. Hematological recovery was estimated by computing the median Hb concentration at days 0, 14, and 28 as well as the median of individual Hb differences between day 0 and day 28. Anemia was defined as an Hb concentration of <11 g/dl, for both sexes and all ages (36). The Wilcoxon rank sum test and sign rank test were applied as required to compare Hb medians. A survey logistic regression ("*svy*" command in STATA) was used to carry out a multivariate adjusted analysis for the risk of anemia before and after treatment (adjusting for all potential confounders, such as sex, age, baseline parasitemia, splenomegaly, and ethnicity). Similarly, survey logistic regression was also used to assess if baseline parasite density (day 0) or age was

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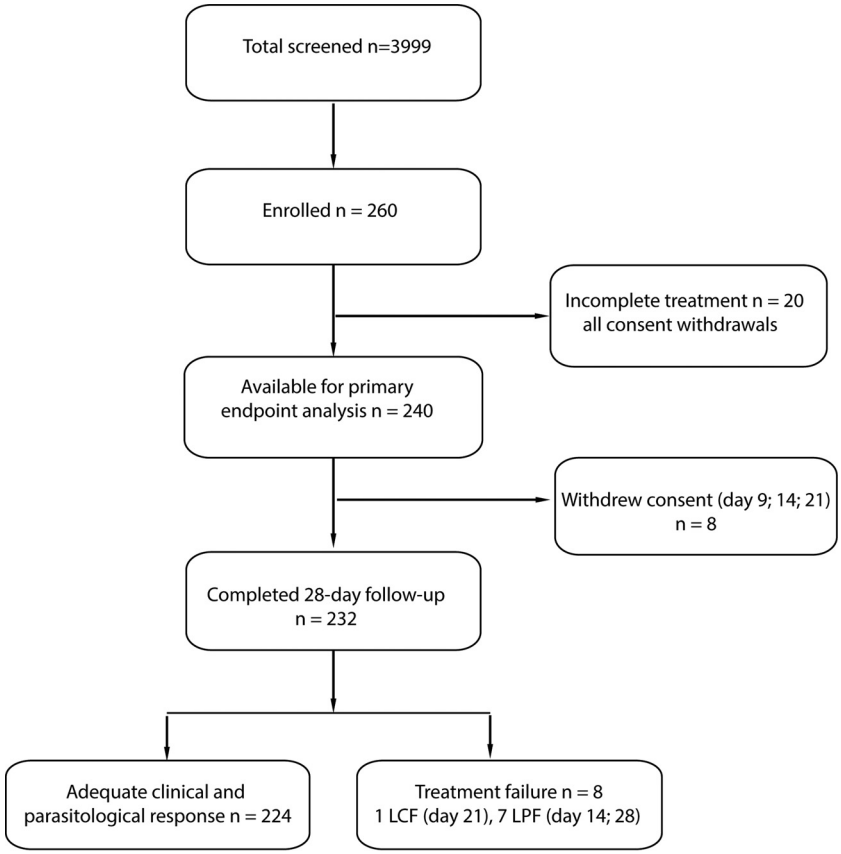

FIG 1 Study profile.

independently associated with parasite clearance at day 2. A multivariate linear regression model was used to determine the independent effect of the baseline Hb values (day 0 = Hb0) on the relative Hb changes at day 14. Potential risk factors (age, ethnicity, etc.) with a *P* value of <0.05 in the univariate analysis were included in the multivariate model and retained if the *P* value was <0.05. Interactions were systematically checked for up to order two. The 5% cutoff was defined as a significant *P* value for all statistical tests.

Ethical clearance. Ethical clearance was obtained from both the Ethical Committee of the NIMPE in Hanoi and that of the University of Antwerp. The fundamental principles of ethics in research on human participants were upheld throughout the project. The study objectives and methods were explained to the people's committee, the village's leader, and the local people. All study participants had given their informed consent after the study objectives and procedures, as well as their right to withdraw without prejudice for themselves or their families, were explained. Written informed consent was obtained from parents or guardians of children below 18 years; children between the ages of 12 and 18 years were asked to provide a written assent.

RESULTS

Trial profile and baseline characteristics. Between April 2009 and December 2010, 260 *P. vivax*-infected patients were enrolled and given the 10-day radical treatment of PQ (0.50 mg/kg/day) associated with CQ for the first 3 days (total, 25 mg/kg); 240 patients (92.3%; 240/260) completed the treatment and were included in the analysis, and 232 patients completed the 28-day follow-up. All incomplete follow-ups were due to consent withdrawal (Fig. 1) following prolonged absence, mainly because of work requirement in forest fields. Patients were recruited in all four study villages, and the vast majority (78.5%; 204/260) belonged to the M'nong group (Table 1). Males (61.1%; 159/260) slightly outnumbered females, and almost half of the participants (43.1%; 112/260) were children aged 3 to 9 years. The majority of participants had no bed net at home (70.8%; 184/260) and very low socioeconomic status, and all adults were farmers.

More than half of the study patients (59.2%; 154/260) had measurable fever at enrollment; headache (36.1%; 94/260) and fatigue (33.1%; 86/260) were the most common symptoms, and about 6% (16/260) had an enlarged spleen. The mean parasite density at enrollment was 2,754.1/μl (geometric mean [GM]), and gametocytes were found in most of the patients (86.1%; 224/260), though at much lower densities (GM = 387.7/μl). The median hemoglobin concentration at enrollment was 11.7 g/dl, and more than one-third of the patients (35.8%; 93/260) were anemic (Hb, <11g/dl). The treatment was well tolerated, no clinical sign or symptoms of acute hemolysis were observed (despite the occurrence of transient acute hemolysis [see below]), and only few patients (12.3%; 32/260) complained of nausea following PQ administration, though none of them vomited their dose of CQ or PQ.

Primary endpoints. No ETF was observed; there were eight late treatment failures, i.e., 2 LPFs at day 14, 1 LCF at day 21, and 5 LPFs at day 28 (Table 2). The rate of ACPR at day 28 was 96.6% (95% confidence interval [CI], 93.7 to 98.2). *P. vivax* recurrence was not associated with delayed parasite clearance, as five of the

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TABLE 1 Baseline demographic, clinical, and parasitological characteristics at enrollment ($n = 260$)

Parameter	*n*	%	95% CI
Village			
1	101	38.85	34.83–43.02
2	64	24.62	21.25–28.33
3	39	15.0	12.84–17.45
4	56	21.54	17.63–26.04
Gender			
Male	159	61.15	55.76–66.82
Female	101	38.85	33.72–44.24
Ethnic group			
M'nong	204	78.46	73.96–82.37
Cadong	56	21.54	17.63–26.04
Age (yr)			
3–9	112	43.08	37.45–48.89
10–19	71	27.31	21.84–33.55
20–29	44	16.92	12.75–22.11
30–60	33	12.69	9.36–17.0
Occupation			
None (children <6 yr)	70	26.92	22.02–32.47
Farmer	85	32.69	27.51–38.34
Pupil	105	40.38	34.34–46.74
Bed net in house			
None	184	70.77	61.81–78.37
At least one	76	29.23	21.63–38.19
Economic status[a]			
Lowest	147	56.54	47.20–65.43
Low	26	10.0	5.70–16.96
Higher	87	33.46	25.18–42.9
Clinical symptoms (most frequently reported)			
Fever (axillary temp ≥37.5°C)	154	59.23	53.06–65.12
Headache	94	36,15	30.42–42.32
Fatigue	86	33.08	27.23–39.5
Dizziness	28	10.77	7.51–15.22
Nausea	32	12.31	8.83–16.90
Enlarged spleen	16	6,15	3.58–10.39
Laboratory data			
Asexual parasites/μl, GM (95% CI)	2754.07 (2271.87–3338.61)		
Gametocytes/μl, GM (95% CI)	387.72 (324.84–462.80)		
Patients with gametocytes	224	86.15	81.37–89.86
Hemoglobin (g/dl), median (IQR)	11.7 (10.4–13.1)		
Patients with anemia (Hb < 11 g/dl)	93	35.77	29.8–42.21

[a] Score in tertiles defined as "high," "medium," and "low" economic status, following principal-component analysis (33).

eight patients with recurrence had cleared parasitemia before 24 h. The mean parasite density at day of recurrence was very low (GM = 41.1/μl; interquartile range [IQR], 23.3 to 855.8).

At day 1, more than half of the patients (57.9%) were still parasitemic, at day 2 only 7.1% were, and at day 3 none of them had detectable parasitemia. Parasite clearance at day 2 was significantly associated with a higher asexual parasite density at day 0 (odds ratio [OR] = 1.79; 95% CI, 1.14 to 2.82; $P = 0.012$) but not with age.

Secondary endpoints. All patients were afebrile and without gametocytemia by day 3 (Table 2). Dried blood samples for measuring CQ blood concentrations were available (at day 7 and the day of recurrence) for 5 of the 8 patients with vivax malaria recurrence, and among these, three had interpretable results. The CQ blood concentrations at day 7 ranged from 365.1 to 1,347.1 ng/ml, confirming adequate drug absorption. The three CQ blood concentrations at time of recurrence were 114.7 ng/ml (day 14), 133.1 ng/ml (day 21), and 125.9 ng/ml (day 28); all of them were above the 100-ng/ml threshold, confirming CQ resistance.

The median Hb value at day 0 among patients with ACPR ($n = 224$) was 11.7 g/dl (IQR = 10.5 to 13.1), and children were significantly more at risk of anemia (50.0%; 49/98) than older patients

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TABLE 2 Primary and secondary endpoints

Endpoint	*n* (%)	95% CI
Primary ($n = 240$)		
Adequate clinical and parasitological response (KM[a])	224 (96.55)	93.67–98.15
Cumulative incidence of treatment failures (KM)	8 (3.45)	1.85–6.33
Late clinical failure (day 21)	1	
Late parasitological failure ($n = 7$) at day:		
14	2	
28	5	
Patients with asexual parasitemia at day:		
1	139 (57.92)	51.44–64.13
2	17 (7.08)	4.39–11.23
3	0	
Secondary		
Fever clearance ($n = 139 = 100\%$ at day 0) at day:		
1	34 (24.46)	18.7–31.32
2	5 (3.59)	1.49–8.41
3	0	
Gametocyte clearance ($n = 207 = 100\%$ at day 0) at day:		
1	83 (40.1)	33.96–46.56
2	11 (5.31)	3.0–9.23
3	0	
CQ blood concn at day of failure > 100 ng/ml at day:	3/3	
14 (LPF)	114.66	
21 (LCF)	133.09	
28 (LPF)	125.87	
Hemoglobin recovery, median individual Hb change from day 0–28, g/dl (IQR) for patients:		
All ($n = 224$)	+0.7 (−0.2–+1.6)	
Anemic at day 0 ($n = 78$)	+1.7 (+0.7–+3.2)	
Nonanemic at day 0 ($n = 146$)	+0.25 (−0.4–+1.0)	

[a] KM, Kaplan-Meier estimate.

(23.0%; 29/126) even after adjusting for baseline parasitemia (adjusted OR [AOR] = 3.60; 95% CI, 1.88 to 6.88; $P < 0.001$). By day 28, the median Hb increased to 12.3 g/dl (IQR = 11.3 to 13.4), and the median value of individual Hb changes between day 0 and day 28 was +0.7 g/dl (IQR = −0.2 to +1.6). Among anemic patients at day 0 ($n = 78$), the median Hb was 9.9 g/dl (IQR = 8.3 to 10.5), and it significantly increased to 11.5 g/dl (IQR = 10.8 to 12.1) by day 28 (sign rank test, $P < 0.001$), with a median change of +1.7 g/dl (IQR = +0.7 to +3.2) (Fig. 2A). This change was slightly lower in children (median = 1.5 g/dl; IQR, 0.7 to 2.5) than in adults (median = 1.9; IQR, 1.1 to 4.2) (Wilcoxon rank sum test, $P = 0.08$). After treatment, 24.5% (24/98) of children and 8.7% (11/126) of adults were still anemic (AOR = 3.5; 95% CI, 1.7 to 7.0; $P = 0.001$). Patients who were still anemic by day 28 were treated with hematinic drugs (ferrous sulfate and folic acid).

In order to better understand the relationship between Hb changes, age and baseline Hb values (Hb0), we plotted the individual changes at day 14 relative to day 0 (%) (Fig. 2B) as a function of Hb0 and carried out a multivariate linear regression analysis adjusting for the potential confounding effect of age. The final model showed that relative Hb changes at day 14 were independently (and negatively) associated with Hb0 ($P < 0.001$) and that age was not a confounder, since it was associated only with the exposure and not with the outcome variable. Moreover, the linear regression model showed that the effect of Hb0 on relative Hb changes at day 14 was significantly different between anemic and nonanemic patients at day 0 (interaction term coefficient $\beta = -5.99$; $P < 0.001$). Indeed, while in nonanemic patients the Hb decreased by 6.5% for every increase in Hb0 unit ($\beta = -6.45$; $P < 0.001$), in the anemic group, the Hb increased by 10% for every decrease in Hb0 unit ($\beta = -10.00$; $P < 0.001$). Interestingly, 9 patients experienced more than a 25% reduction in Hb by day 14, ranging from −58.2% to −32.8%, without any sign or symptom of hemolysis detected during the 28-day follow-up. All but one of these patients had normal Hb values at day 0, and the majority (6/9) of them had recovered a normal Hb value by day 28.

Similar results were found for the association between Hb0 and relative Hb changes by day 28, with a significant interaction (interaction term $\beta = P < 0.001$) and a slightly stronger effect of Hb0 among anemic patients ($\beta = -14.00$; $P < 0.001$) and a smaller effect ($\beta = -3.38\%$; $P < 0.001$) in the nonanemic group (data not shown).

DISCUSSION

This study confirms for the first time *P. vivax* CQ resistance in Vietnam, as three patients with recurrent *P. vivax* infections were found to have CQ blood concentrations above the MIC (100 ng/ml of whole blood). Suspected *P. vivax* resistance to chloroquine was observed in Binh Thuan province in southern Vietnam in the late 1990s, with 16% treatment failure after a 3-day course of CQ (25 mg/kg) (17), but could not be confirmed because CQ blood concentrations were not available. Indeed, the latter is necessary (37), as recurrent infections could be the consequence of

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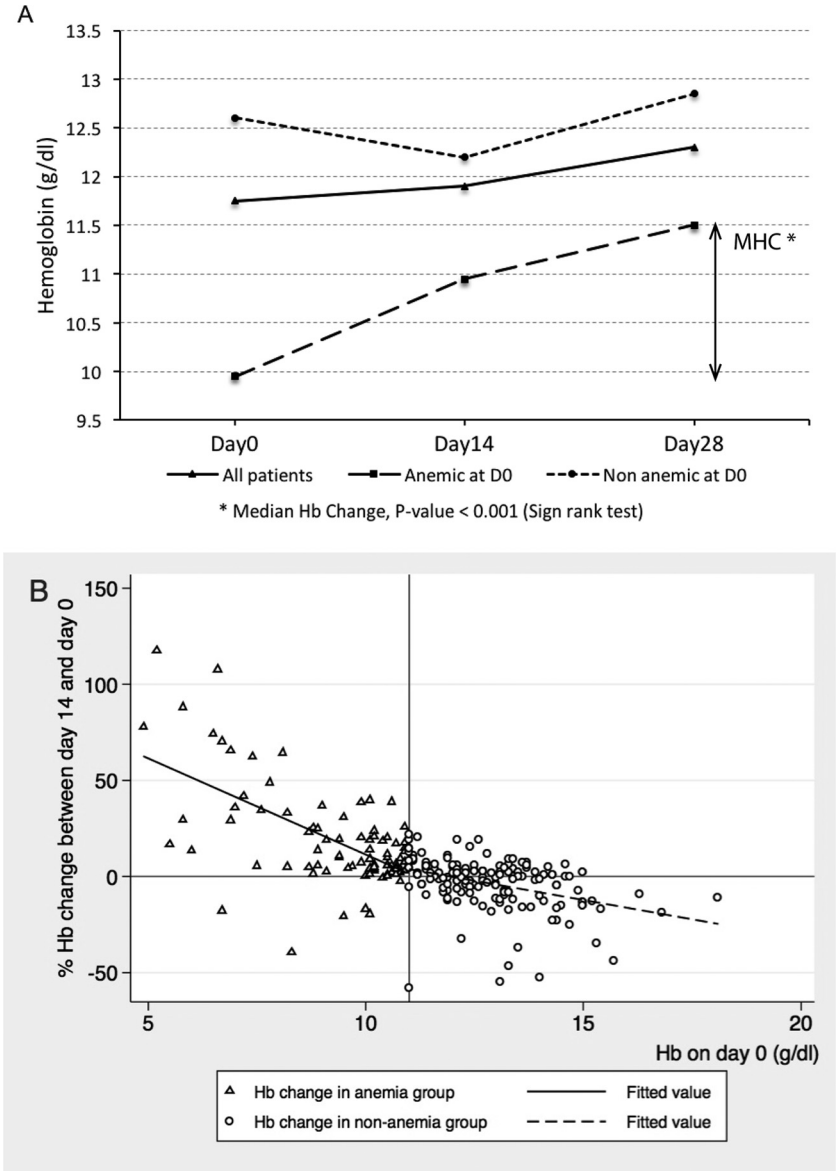

FIG 2 (A) Median hemoglobin (Hb) concentration at days 0, 14, and 28 ($n = 224$ patients with ACPR); (B) relative Hb change (between day 0 and day 14) according to baseline Hb values (cutoff for anemia, Hb concentration of <11.0 g/dl) ($n = 240$). Relative Hb change on day 14 (%) by linear regression: (i) anemia group, coefficient $\beta = -10.00$; 95% CI, −12.81 to −7.19; $P < 0.001$; (ii) nonanemia group, $\beta = -6.46$; 95% CI, −7.41 to −5.51; $P < 0.001$. A significant interaction was found between Hb change at day 14 and anemia status at day 0 (interaction term $\beta = -5.99$; 95% CI, −8.87 to −3.11; $P < 0.001$).

inadequate drug concentration due to suboptimal drug quality and dosage or low intestinal absorption rather than CQ resistance. For this study, these factors can be excluded, as the day 7 CQ concentrations were within the optimal range, at least for the five patients with available results at day 7.

Since the first reports from Papua New Guinea (PNG) in 1989 (6, 8, 38, 39), *P. vivax* resistance to chloroquine has rapidly reached unacceptably high levels in Indonesia and PNG, prompting the WHO to recommend artemisinin-based combination therapies for *P. vivax* (40). Moreover, a recent systematic review showed that *P. vivax* resistance to chloroquine can be found in most countries where vivax malaria is endemic, across continents (41). The apparently low cumulative risk of recurrence by day 28 estimated in our study together with the absence of ETF suggest a low grade of resistance compared to that in other Southeast Asian countries, particularly Indonesia, where ETFs ranged from 6% to 24% and 28-day recurrence rates from 18% to 100% (41). Similarly, the recurrence rate may be considered negligible compared to that (16%) observed in Binh Thuan province about 15 years ago (17). Nevertheless, when considering that CQ was coadministered with high-dose (0.5-mg/kg/day) PQ, which has also an effect on *P. vivax* asexual blood stages (42, 43), the estimation of *P. vivax* resistance to chloroquine provided here is probably much lower than its true prevalence. Indeed, in Indonesia adding PQ to CQ decreased the day 28 treatment failure from 78% to 15% (39). Therefore, our seemingly low-grade resistance is the CQ failure when combined with high-dose PQ, while the true failure related to CQ resistance is probably higher, possibly up to 5-fold higher

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(39). Therefore, *P. vivax* resistance to chloroquine in Quang Nam province is probably similar to that reported 15 years ago from Binh Thuan province (17). As CQ (monotherapy) efficacy measured in 6 sentinel sites in central and southern Vietnam between 2006 and 2011 has been consistently at 100% (24), it is possible that *P. vivax* resistance to chloroquine in Vietnam has not reached the high levels observed in PNG and Indonesia. Indeed, despite the lack of power, with sample sizes between 25 and 65 patients (24), which are far below the minimum of 75 recommended by the WHO (32), it is unlikely that high-grade resistance would have been missed. Therefore, *P. vivax* resistance to chloroquine was present in central and southern Vietnam since at least the late 1990s, and unlike in PNG and Indonesia, it did not evolve to high-grade levels. The most likely explanation for such a difference could be the much lower CQ pressure, as artemisinin derivatives have been used since the early 1990s for the treatment of multidrug-resistant *P. falciparum*.

When considering the timing of the observed recurrent infections, the two LPFs at day 14 are probably recrudescences, as *P. vivax* infections recurring before day 16 are almost certainly due to a recrudescence from the primary infection (37). Infections recurring later may be either recrudescences or relapses, with CQ-resistant parasites if the CQ blood level is above the MICs (37). As this is an area of extremely low transmission, more than one infectious bite within 1 month is unlikely, though it cannot be excluded as farmers often stay overnight in their forest fields, where they are at higher risk of exposure to the main vector, *Anopheles dirus* (44, 45). Genotyping alone is usually of limited help to distinguish between recrudescence and relapse/new infection, since relapses can occur with either the same or different clones (46).

Vivax malaria-associated anemia was common, and hematological recovery at day 28 depended on baseline Hb. Indeed, the more pronounced hematological recovery was observed among patients who were anemic before treatment. This observation is similar to a recent report from PNG (47). In addition, young children were at a much higher risk of anemia than older patients, and this risk remained high after treatment, illustrating the importance of an efficacious radical treatment for *P. vivax* in children (48). The linear regression model showed that age was indirectly associated with Hb changes only through its significant association with Hb0. Moreover, in anemic patients, the lowest Hb0 values corresponded to the more marked Hb increase during follow-up; for the other patients, the higher the Hb0, the more marked was the Hb decrease during follow-up. This inverse relation could be partly explained by the increased hemolytic risk in older red blood cells (49) and by the suppressive activity of hemozoin (digested Hb) on erythropoiesis (50). It is possible that anemic patients were infected for longer periods and at day 0 had already reached their lowest Hb value. This would have resulted in a more robust bone marrow response than in nonanemic, recently infected patients (47). Transient asymptomatic Hb reductions (≥50%) after PQ treatment, as either radical cure or single gametocytocidal dose, have been observed among G6PD-deficient and nondeficient African children (51–53).

A quick *post hoc* genotyping (54) was carried out to screen for the four most commonly reported G6PD mutations in Vietnam (*Vieng Chang*, *Canton*, *Union*, and *Kaiping*) among the 9 patients who experienced a >25% reduction in Hb by day 14 (together with 9 randomly selected control patients [having no change in Hb]). Only one patient was found positive for the *Vieng Chan* mutation, i.e., a 26-year-old male of Cadong ethnicity with a transient Hb decrease of 52.9% by day 14 (Hb0 = 14.0 g/dl) followed by a full recovery at day 28 (Hb28 = 13.6 g/dl). It is not possible to exclude, among these 9 patients, the presence of other G6PD variants (i.e., *Vietnam I*, *Vietnam II*, *Gaohe Gaozhou*, *Coimbra*, etc.) also reported in different ethnic groups from central Vietnam (55, 56). This will be further investigated by carrying out an in-depth analysis of the G6PD genetic polymorphism in all 240 study patients in relation to their Hb changes. Moreover, the observed transient but potentially life-threatening hemolysis (>50% Hb change at day 14) questions the national policy, which currently does not recommend G6PD testing prior to radical PQ treatment. To better estimate the risk of hemolysis linked to PQ use, there is the urgent need of determining the prevalence of the G6PD-deficient phenotype together with the G6PD genetic polymorphisms among different ethnic minorities living in areas of residual malaria endemicity.

The main limitation of our study is the concomitant use of PQ and CQ, which most likely resulted in a substantial underestimation of the true CQ-related cumulative risk of recurrence by day 28. As per WHO recommendations (32), *P. vivax* resistance to chloroquine can be accurately estimated only by standard 28-day *in vivo* studies with CQ monotherapy, with PQ being withheld until day 28. Strictly speaking, resistance could not be confirmed in all eight vivax malaria recurrences, as CQ blood levels results were available for only three patients. However, given the pharmacokinetics of CQ (37), it is likely that the other patients also had adequate CQ blood levels. In addition, the concomitant administration of PQ and its synergistic effect on asexual blood stages could also explain why no association was found between the PCT and the risk of recurrence, unlike that reported in a recent meta-analysis by Price and colleagues (41). For all these reasons, a new study has been initiated using CQ monotherapy to accurately determine its *in vivo* and *in vitro* efficacy for treating *P. vivax* infections.

Another limitation of our study is the 24-h sampling schedule, which was not optimal for an accurate determination of the PCT. For future studies, 8- to 12-h sampling and a baseline parasite density of at least 250/μl are needed to accurately determine parasite clearance (32, 57).

Conclusion. In conclusion, this is the first confirmed evidence of *P. vivax* resistance to chloroquine in central Vietnam, an area where we recently reported *P. falciparum* resistance to artemisinins (25). *P. vivax* resistance to chloroquine should continue to be monitored in different sentinel sites of central Vietnam, using standardized and sufficiently powered *in vivo* protocols with CQ monotherapy and with PQ therapy delayed to day 28. Vietnam has committed to malaria elimination by 2030, and within this context, antimalarial drug resistance, not only *P. falciparum* resistance to arteminins but also *P. vivax* resistance to CQ, is as a major threat. New treatment guidelines based on short and highly effective drug regimens as well as regional and *Plasmodium* genus-wide integrated strategies for the containment of antimalarial drug resistance in the Greater Mekong Subregion need to be urgently developed.

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