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ASSESSING DRUG SENSITIVITY OF *PLASMODIUM VIVAX* TO HALOFANTRINE OR CHOROQUINE IN SOUTHERN, CENTRAL VIETNAM USING AN EXTENDED 28-DAY *IN VIVO* TEST AND POLYMERASE CHAIN REACTION GENOTYPING

W. R. J. TAYLOR, DOAN HANH NHAN, NGUYEN DIEU THUONG, TRAN THI UYEN, D. J. FRYAUFF, E. GÓMEZ-SALADÍN, K. C. KAIN, LE DINH CONG, AND J. K. BAIRD

US NAMRU-2, Jakarta, Indonesia; The Department of Tropical Medicine, Tulane University School of Public Health, New Orleans; The Institute of Malariology, Parasitology, and Entomology, Hanoi, Vietnam; The Tropical Disease Unit, The Toronto Hospital and the University of Toronto, Canada

Abstract. Chloroquine-resistant *Plasmodium vivax* malaria is emerging in Oceania, Asia, and Latin America. We assessed the drug sensitivity of *P. vivax* to chloroquine or halofantrine in two villages in southern, central Vietnam. This area has chloroquine-resistant *Plasmodium falciparum* but no documented chloroquine-resistant *P. vivax.* Standard dose chloroquine (25 mg/kg, over 48 hours) or halofantrine (8 mg/kg, 3 doses) was administered to 29 and 25 patients, respectively. End points were parasite sensitivity or resistance determined at 28 days. Of the evaluable patients, 23/23 100% (95% confidence interval [CI] 85.1–100) chloroquine and 21/24 (87.5%) (95% CI 67.6–97.3) halofantrine-treated patients were sensitive. Three halofantrine recipients had initial clearance but subsequent recurrence of their parasitemias. Genotyping of the recurrent and Day 0 parasitemias differed, suggesting either new infections or relapses of liver hypnozoites from antecedent infections. Among these Vietnamese patients, *P. vivax* was sensitive to chloroquine and halofantrine. Genotyping was useful for differentiating the recurrent vivax parasitemias.

INTRODUCTION

Multidrug-resistant *Plasmodium falciparum* malaria is well established in many malaria-endemic countries.[1–3] Chloroquine-resistant *Plasmodium vivax* (CRPV) is now emerging and has been reported in Papua New Guinea,[4] India,[5] Burma,[6] Guyana,[7] and Indonesia.[8–13] Chloroquine-resistant *Plasmodium vivax* is currently a significant public health problem in Irian Jaya, where documented treatment failure rates are as high as 78% at 28 days.[12] Alternative drugs to chloroquine are needed to treat chloroquine-resistant *P. vivax.*

In vivo data from different malaria-endemic areas are of prime importance for determining optimal treatment and prophylaxis recommendations.[14,15] The 28-day *in vivo* test is a good method for defining parasite sensitivity but interpretation of recurrent parasitemia during follow-up is an inherent problem because recrudescence, relapse of liver hypnozoites, or a new infection cannot be clinically differentiated with certainty. This difficulty can be alleviated by genotyping the recurrent and Day 0 parasitemias. Previous studies indicate that genotyping of *Plasmodium* species-specific polymorphic genes by the polymerase chain reaction (PCR) utilizing single-strand conformational polymorphism (SSCP) lysis techniques can distinguish recrudescent from new infections with a probability of $>$ 90% for *P. falciparum,*[16,17] and $>$ 80% for *P. vivax.*[13,18]

Measuring drug levels, e.g., by high performance liquid chromatography (HPLC), adds further weight to the 28-day *in vivo* test because it confirms whether adequate drug absorption has taken place—a prerequisite for meeting the World Health Organization definition of drug-resistant malaria.[19]

We report the results of treating patients with *P. vivax* malaria in southern Vietnam to determine the efficacy of chloroquine and assess halofantrine as a potential alternative drug.

MATERIALS AND METHODS

Study design and study site. This was a 28-day *in vivo* test to assess the drug sensitivity of *P. vivax* to chloroquine or halofantrine. The study was conducted from May to August 1995 in Khanh Nam and Khanh Vinh (Khang Hoa province), two villages situated in an area of high malaria transmission about 40 miles inland from the coastal town of Nha Trang in southern, central Vietnam. Previous malariometric surveys had shown malaria prevalence rates of between 25–28% in adults and 30–40% in children $<$ 9 years (Nhan DH, unpublished data). Chloroquine-resistant *P. falciparum*[20] is the dominant species at 60%; *P. vivax* and *P. malariae* account for 32% and 5%, respectively. Malaria incidence rates, measured as infections per person-year, are 1.9 for *P. falciparum,* 1.1 for *P. vivax,* and 0.03 for *P. malariae.*

Written, informed consent was obtained from all participating patients or their parents/guardians. The study was conducted according to the Vietnamese Ministry of Health and the United States Navy regulations regarding the protection of human subjects in medical research.

Pre-study assessment. Potential volunteers were identified during village-wide screening or were self-referred. Team physicians made medical assessments that consisted of a history, a drug history (previous drug use within one month, drug allergies), a physical examination, a malaria film, a urine pregnancy test, and an electrocardiogram (ECG) rhythm strip (lead II), supplemented by a 12 lead strip, if clinically indicated. Eligible patients were healthy males or non-pregnant females, aged $\geq$ 5 years, with slide-proven vivax parasitemia, and a normal QT_C interval on the ECG. Specific exclusions included mixed malaria infections, age $<$ 5 years, pregnant or breast-feeding women, known hypersensitivity to chloroquine or halofantrine, ingestion of mefloquine in the previous two weeks, concurrent use of drugs which prolong the QT interval, clinical cardiac disease, and a QT_C of $>$ 0.44 ms.

Conduct of study. Enrolled patients were classified as

TABLE 1
Patient characteristics at enrollment

	Halofantrine (n = 25)	Chloroquine (n = 29)
No. (%) males	12 (48)	14 (48.3)
Median (range) age, years	15 (5-55)	14 (5-40)
Prestudy antimalarial drug use*	7 (28)	6 (20.7)
No. (%) symptomatic†	24 (96)	23 (79.3)
No. (%) febrile‡	16 (64)	14 (48.3)
No. (%) with splenomegaly	6 (25)	9 (31)
Median parasitemia/μl (range)§	140 (40–6,360)	440 (40–34,920)

* Number (%) consuming any antimalarial drug within one month of enrollment.
† Defined as fever or chills or headache or myalgia.
‡ Defined as an oral temperature >37.0°C.
st $P = 0.086$, by Mann-Whitney U test.

symptomatic if complaining of at least one of the following symptoms: fever, chills, headache, or myalgia. Because this was not a comparative clinical trial, there were no predetermined criteria for patient randomization/study drug administration. Standard dose chloroquine (Avloclor, Zeneca) was administered in the clinic and at home: 10 mg chloroquine base/kg on Days 0 and 1, and 5 mg/kg on Day 2. Subjects who received halofantrine (SmithKline Beecham, UK) were admitted into hospital for one day. Halofantrine, at a dose of 8 mg/kg, was administered three times at 6 hourly intervals on an empty stomach or at least one hour before, or two hours after, a meal. All drug doses were administered and documented by a team member.

Follow-up thick and thin film blood smears were Giemsa-stained and examined for malaria parasites by experienced microscopists on Days 1, 2, 4, 7, 11, 14, 18, 21, and 28, or the day of recurrent parasitemia. A positive smear was defined as one or more asexual forms seen after examining 200 thick film fields under 1000 × magnification. The parasite count was quantified as the number of asexual parasites per 200 white blood cells on the thick film, multiplied by 40, and expressed as parasites/μL.[21]

Whole blood specimens (100 μl) were collected by finger stick with a capillary tube and blotted on filter paper (No. 1 Whatman, Fairfield, NJ) for chloroquine drug levels, and fiber glass filter paper (Titetek, UK) for PCR analysis, on Days 0 (predrug administration), 2, and 28, or the day of recurrent parasitemia. Blots were air dried and stored in individual plastic bags at ambient temperature until analysed. Chloroquine and desethylchloroquine (the main active metabolite of chloroquine) were measured by HPLC, according to published methods.[22] Chloroquine concentrations (ng/mL) are reported as whole blood total chloroquine (TCQ) i.e., the sum of chloroquine (CQ) and desethylchloroquine (desethylCQ).

Genotyping by PCR. Parasite DNA was extracted from whole blood from the three paired specimens (the primary and recurrent isolates). A portion of the MSP1 and CS genes were amplified by PCR using oligonucleotide primers matching the conserved sequences flanking the variable region ICB5–ICB6 of the MSP1 gene and region I and II of the CS gene as previously described.[18] The paired primary and recurrent MSP1 PCR products were analysed by SSCP. MSP1 PCR products were analysed with and without Rsa 1 enzyme digestion (New England Biolabs, Mississauga, ON, Canada). Following digestion, chloroform extraction, and ethanol precipitation, each sample was resuspended in 9 μl of sterile water. PCR products were denatured prior to SSCP analysis by adding 1 μL of 0.5 mM NaOH/10 mM EDTA to 100–200 ng of amplified DNA in 9 μl of sterile water, followed by heating at 42°C for 5 mins. Loading buffer (0.5% bromophenol blue, formamide, and glycerol) was added and each sample was placed on ice before electrophoresis on a nondenaturing 10% TBE polyacrylamide minigel (1 mm; Novex, San Diego CA). Lanes containing 1 kilobyte (kb) DNA markers or reference strains that had been similarly treated, were used to standardize SSCP patterns. Samples were run under constant voltage with 0.8X glycerol tolerant running buffer (USB, Cleveland, OH), and 5% glycerol for 2250 volt-hr (MSP1 undigested samples), 1500 volt-hr (digested MSP1), or 2625 volt-hr (CS). SSCP banding patterns were detected by silver staining (Bio-Rad, Hercules CA). Digested MSP1 DNA fragment SSCP patterns were scanned, digitalized, and analysed using the BioImage System with Whole Band Analysis, Version 3 (Millipore Corp., Ann Arbor, MI). The SSCP patterns of the recurrent isolates were compared to each of the primary isolates.

Our criterion for identity was that the mobility of the SSCP band of the recurrent isolate had to match the corresponding band of the primary (Day 0) isolate; this would indicate a treatment failure or a relapse of the current infection.[13,18,23] Non-identity would indicate a new infection or relapse of liver hypnozoites of an antecedent infection.

End points. The parasitological end points were classified as either sensitive (S) or resistant (R). Sensitive = the complete clearance of asexual parasitemia to Day 28. Resistant = failure to clear parasitemia or recurrence of the original parasitemia (assessed by PCR) following initial clearance.

Data analysis. Data were double-entered, validated, and analysed using Epi Info 6.04 (Centers for Disease Control and Prevention, Atlanta, GA). Continuous data were compared using the student'st (normal distribution) or the Mann-Whitney U tests (skewed distribution). A two-sided P value ≤ 0.05 was considered statistically significant.

RESULTS

A total of 54 patients were treated, 29 with chloroquine and 25 with halofantrine (H); their enrollment characteristics are detailed in Table 1. A minority of patients, 13/54 (24.1%), gave a history of consuming an antimalarial drug in the previous month. During follow-up, 3 patients default-

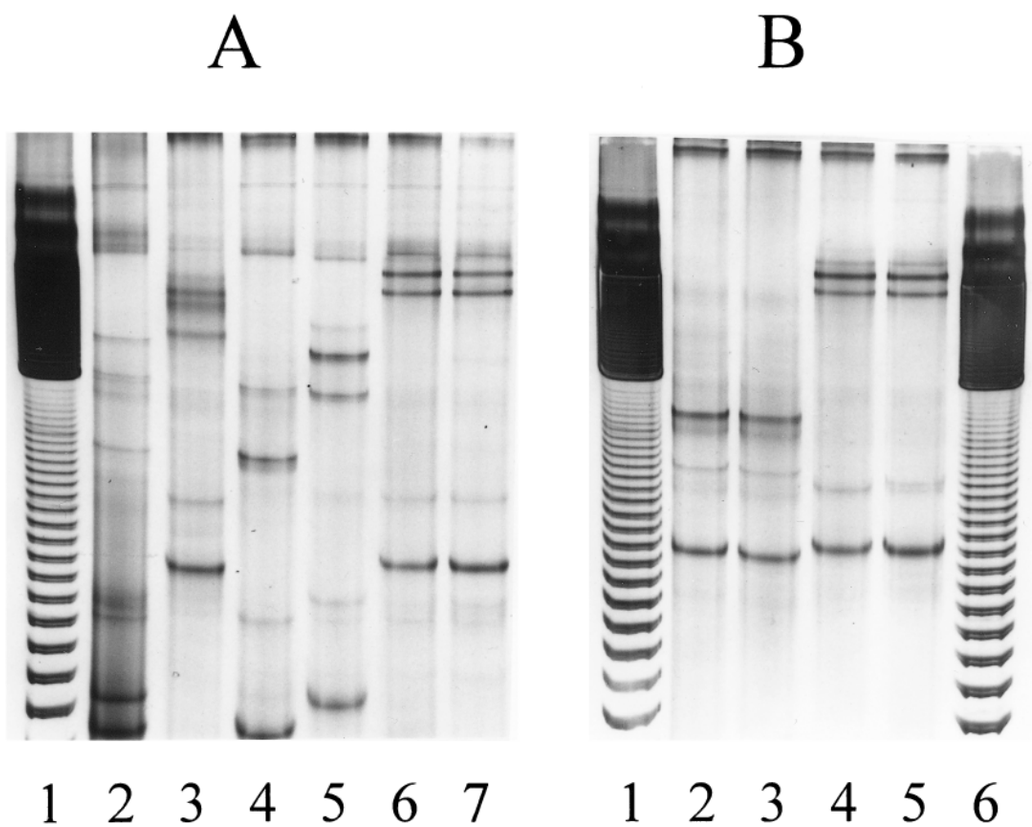

FIGURE 1. Polymerase chain reaction utilizing single-strand conformational polymorphism (SSCP) analysis of the MSP-1 gene from the Day 0 and recurrent vivax isolates showing a mismatch between the SSCP patterns (Panel A: Lanes 2–3 and 4–5). Previously confirmed primary and resistant *Plasmodiium vivax* pairs are shown as controls (Panel A: Lanes 6–7, Panel B: Lanes 2–3, and 4–5).

ed (CQ arm), 4 developed falciparum malaria (3 in CQ arm and 1 in H arm), and 3 had recurrent parasitemia (H arm). These recurrences occurred on Days 14, 25, and 28 in patients aged 5, 23, and 24 years, respectively. Of the remaining patients, 23/23 (100%) (95% CI 85.1–100) chloroquine recipients and 21/24 (87.5%) (95% CI 67.6–97.3) halofantrine recipients cleared their parasitemias. On Day 0, 4 samples had high levels of chloroquine (170–466 ng/mL) but undetectable desethylCQ, consistent with contamination. These four values were excluded from further CQ analyses. The median Day 0 TCQ in 25 CQ-treated patients was 36 (range 0–1038) ng/mL. Sixteen had detectable CQ and twelve had TCQ concentrations $\geq$ 100 ng/mL, median 511.5 (range 115–1038) ng/mL. The median parasite counts of these 12 patients on Day 0 were higher (1240μl) than patients with a TCQ $<$ 100 ng/mL (320μl) but this difference was not statistically significant ($P = 0.34$). TCQ concentrations measured on Day 2 showed marked inter-individual variation, ranging from 517–7020 ng/ml (median 1132 ng/ml). There was no correlation between the Day 2 and Day 0 TCQ levels: $r^2 = 0.0$ (95% CI, −0.42–0.43). The day 28 median TCQ concentration (n = 22) was 139.5 (24–530) ng/mL; 14 patients (63.6%) had a TCQ $\geq$ 100g/mL. No correlation existed between the Day 28 and Day 2 TCQ levels: $r^2 = 0.0$ (95% CI, −0.44–0.44).

The PCR-SSCP analysis of the MSP-1 gene from primary and recurrent isolates from patients treated with halofantrine demonstrated that in all three paired isolates, the SSCP pattern of the recurrent vivax isolate did not match the primary isolate. The results of two of the halofantrine patients are shown in Figure 1 (Panel A, Lanes 2–3 and 4–5). Previously confirmed, paired primary and resistant *P. vivax* isolates are included as controls (Panel A: Lanes 6–7, Panel B: Lanes 2–3 and 4–5).

DISCUSSION

This 28-day *in vivo* study has shown that in this group of patients from southern, central Vietnam, *P. vivax* was sensitive to chloroquine and halofantrine. However, three halofantrine-treated patients had recurrent parasitemia that, on the basis of the PCR results, were unlikely to represent resistant infections.

Our study was a non-comparative, 28-day *in vivo* test to determine the sensitivity of *P. vivax* to chloroquine, an ineffective drug against *P. vivax* in some endemic countries, and halofantrine, an effective drug against multidrug-resistant *P. falciparum*,[24] and chloroquine-resistant *P. vivax*.[7,12] While the 28-day *in vivo* test provides useful data on parasite drug sensitivity, a vivax parasitemia occurring during follow-up could be due to a new infection, a recrudescence of the original infection, or a relapse of liver hypnozoites of the current or an antecedent infection. With an incidence rate of 1 vivax infection/person-year in the study villages, the

expected number of new vivax infections in 54 patients followed for one month is 3.25. This figure is consistent with our three cases of recurrent parasitemia, demonstrating that reinfections were a minor confounding factor in this study. PCR genotyping showed these three recurrent parasitemias to be different from their respective Day 0 parasitemias and is strong supporting evidence that they were not resistant infections but were either new infections or relapses of antecedent infections. In studies of *P. vivax* in Indonesia,[13] and of returned expatriates to Canada,[7,18] matching fingerprints of recurrent and Day 0 parasitemias had a > 80% probability of indicating either resistance or relapse (Kain K, unpublished data). The recurrent parasitemias in our patients occurred on Days 14, 25, and 28 of follow-up. At these times the concentrations of halofantrine and N-desbutylhalofantrine (its major metabolite) would have been low or nonexistent given that their measured half lives are in the range of 1–6 days in patients with *P. falciparum* malaria.[26,27]

The measurement of drug levels to document adequate absorption (in this field study only chloroquine levels were measured) provides the pharmacological basis for interpreting the 28-day *in vivo* test. Failure to completely clear parasites after documenting adequate drug absorption is evidence of resistance[19] and distinguishes it from treatment failure due to other causes (i.e., poor compliance).

Determining the therapeutic range of chloroquine against *P. vivax* is problematic because of the changing sensitivity of *P. vivax* to chloroquine over time. In 1948, the chloroquine concentration required to cure patients with *P. vivax* malaria was the equivalent of a mean, whole blood, total chloroquine concentration of 100 ng/mL over 4 days.[28] This level was achieved with chloroquine doses of 275–650 mg (~4–10 mg/kg for a 70kg adult). In Sweden in 1987, standard dose chloroquine (25 mg/kg) cured all 15 adults with non *P. falciparum* malaria (*P. vivax, P. ovale,* and *P. malariae*). The minimum whole blood TCQs were 1.76 μmol/L (~528.5 ng/mL) at 48 hours, 1.48 μmol/L (~444.4 ng/mL) on Day 4, and 1.09 μmol/L (~327.3 ng/ml) on Day 7.[29]

We measured the Day 2 (48 hr) TCQ concentration to indicate adequate chloroquine absorption because this represents the approximate time of peak absorption (Tmax) of standard-dose chloroquine.[29] The lowest Day 2 total chloroquine concentration was 517 ng/mL, but this value was sufficient to produce a satisfactory pharmacokinetic profile of chloroquine to kill vivax parasites in this group of Vietnamese patients for up to 28 days. However, this value may not be applicable to patients in other geographical areas.[30] In Indonesia, adequately absorbed standard-dose chloroquine, documented by Day 2 TCQ levels similar to our study, failed to cure all patients with vivax malaria.[8,10–12,31,32] In these studies, a recurrent parasitemia, whether a recrudescence, relapse, or new infection that occurred in the presence of a whole blood TCQ ≥ 100 ng/mL, was considered resistant.

On Day 0, 12 chloroquine-treated patients had total chloroquine levels of ≥ 100 ng/mL (median 511.5, range 115–1038). Some of these parasites might have been resistant to chloroquine at doses used by Berliner or to CQ prophylaxis; 300 mg of chloroquine weekly produced median TCQ levels of 710–263 ng/mL over 7 days.[33] However, all 12 patients had sustained parasite clearance to 28 days, confirming sensitivity to standard dose chloroquine (25 mg/kg). On Day 28, approximately two-thirds of patients had TCQ levels of ≥ 100 ng/mL, high enough to exert a prophylactic effect against sensitive vivax parasites.

In Vietnam, artemisinin[34] and artesunate combined with mefloquine[35] have been used to treat vivax malaria. These drugs produced very rapid fever and parasite clearance times but recurrent parasitemia occurred in some patients treated with shorter courses of artemisinin—an inherent problem with this class of antimalarial drug.[36] The optimal dose of oral artesunate monotherapy for vivax malaria is unknown but 7 days is likely to be necessary, as for falciparum malaria.[36] Use of such a valuable class of antimalarial drug is not justified for the treatment of chloroquine-sensitive vivax malaria.[36] The artemisinins, as monotherapy or in combinations, have become the treatment of choice in some Southeast Asian countries where multidrug resistant *P. falciparum* malaria exists. In the absence of a microscopical diagnosis, blind treatment with an artemisinin would be satisfactory for both falciparum and vivax malaria. In areas where microscopy is available and the patterns of drug resistance are known, treatment can be tailored accordingly. The optimal treatment for chloroquine-resistant *P. vivax* is unknown. In Vietnam, the artemisinins would be an obvious choice because of their high efficacy, safety, and low cost (~US$1 per adult course). Halofantrine is efficacious against chloroquine-resistant *P. vivax*[7,12] but can cause potentially serious cardiac arrhythmias, and is expensive (UK retail price ~US$10 for three 500 mg tablets).[24] Halofantrine may have a role against chloroquine-resistant *P. vivax* in countries where the artemisinins are not yet available.

To summarize, this 28-day *in vivo* test conducted in two villages of southern, central Vietnam found evidence of reduced sensitivity of *P. vivax* to chloroquine but no clinical resistance to therapeutic doses of chloroquine or halofantrine. Continuing surveillance is required to see whether this area becomes a focus of chloroquine-resistant *P. vivax* in the future.

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Authors' addresses: Walter R.J. Taylor, US NAMRU-2, Box 3, Unit 8132, APO, AP 96520-8132 and the Department of Tropical Medicine, Tulane University School of Public Health, Canal Street, New Orleans; Doan Hanh Nhan, Nguyen Dieu Thuong, Tran Thi Uyen, and Le Dinh Cong, Institute of Malariology, Parasitology, and Entomology, Hanoi, Vietnam. Eduardo Gómez-Saladín and J. Kevin Baird, US NAMRU-2, Box 3, Unit 8132, APO, AP 96520-8132; and Kevin C. Kain, Tropical Disease Unit, The Toronto Hospital and the University of Toronto, Canada.

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