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In Vivo Therapeutic Efficacy of Chloroquine Alone or in Combination with Primaquine against Vivax Malaria in Kolkata, West Bengal, India, and Polymorphism in *pvmdr1* and *pvcrt-o* Genes

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Plasmodium vivax* malaria, though benign, has now become a matter of concern due to recent reports of life-threatening severity and development of parasite resistance to different antimalarial drugs. The magnitude of the problem is still undetermined. The present study was undertaken to determine the *in vivo* efficacy of chloroquine (CQ) and chloroquine plus primaquine in *P. vivax* malaria in Kolkata and polymorphisms in the *pvmdr1* and *pvcrt-o* genes. A total of 250 patients with *P. vivax* monoinfection were recruited and randomized into two groups, A and B; treated with chloroquine and chloroquine plus primaquine, respectively; and followed up for 42 days according to the WHO protocol of 2009. Data were analyzed using per-protocol analyses. We assessed polymorphisms of the *pvmdr1 and pvcrt-o* genes by a DNA-sequencing method. Out of the 250 patients recruited, 204 completed a 42-day follow-up period, 101 in group A and 103 in group B. In group A, the non-PCR-corrected efficacy of CQ was 99% (95% confidence interval [CI], 0.944 to 1.00), and in group B, all cases were classified as adequate clinical and parasitological response (ACPR). Day 3 positivity was observed in 11 (5.3%) cases. No specific mutation pattern was recorded in the *pvcrt-o* gene. Eight nonsynonymous mutations were found in the *pvmdr1* gene, three of which were new. The Y976F mutation was not detected in any isolate. Chloroquine, either alone or in combination with primaquine, is still effective against *P. vivax* malaria in the study area. (The study protocol was registered in CTRI [Clinical Trial Registry-India] of the Indian council of Medical Research under registration no. CTRI/2011/09/002031.)

The burden of malaria caused by *Plasmodium vivax* has been greatly underappreciated in terms of both its clinical spectrum and incidence of disease (1, 2). *P. vivax* is the most widely distributed cause of malaria in the world; approximately 2.6 billion people are at risk, and 10 countries, including India, are at the highest risk of infection (3, 4, 5, 6).

P. vivax infections have been associated with mild symptoms, such as fever, headache, fatigue, chills, and musculoskeletal pain, and, in particular, paroxysms. Recently, however, severe complications, including renal failure, jaundice, acute respiratory distress syndrome, cerebral malaria, seizures, anemia, hyperparasitemia, thrombocytopenia, pulmonary edema, splenic rupture, and death, have been reported in exclusive association with *P. vivax* (7, 8). The situation is further complicated by the emergence of resistance of the parasite to chloroquine (CQ).

In most of the world, CQ remains the first-line treatment for patients with vivax malaria. In India, CQ was replaced by artemisinin combination therapy (ACT), a combination of artesunate and sulfadoxine-pyrimethamine, in 2010 for *Plasmodium falciparum* malaria, but for *P. vivax* malaria, CQ remains the first-line agent, along with primaquine (PQ) (0.25-mg/kg base) for 14 days under supervision or by detecting the glucose-6-phosphate dehydrogenase (G6PD) level. The first case of *P. vivax* resistance to CQ was reported in 1989 from Papua New Guinea (9). A higher rate of CQ-resistant *P. vivax* malaria, which exceeded 50%, was reported from different regions of Indonesia (10, 11, 12, 13, 14). Further sporadic cases were subsequently observed in the Philippines, Myanmar, Vietnam, Colombia, Guyana, and Turkey (15). Similar reports are also available from Madagascar (16) and Ethiopia (17). Despite these reports, it remains difficult to estimate the worldwide prevalence of *P. vivax* resistance to CQ. In India, the first case of CQ-resistant *P. vivax* malaria was reported from Assam in 1995 (18) and then in Mumbai (19) and Gujarat (20).

The molecular mechanisms underlying CQ resistance in *P. vivax* malaria remain unknown and may involve multigenic loci, but two genes orthologous to the *pfmdr1* and *pfcrt* genes that encode putative transporters, *pvmdr1* (21) and *pvcrt-o* (22), have been suspected as possible genetic markers of CQ resistance.

Information about the prevalence of CQ resistance in *P. vivax* malaria and the distribution of possible genetic markers of CQ resistance in India is very sparse, particularly from northeast India. The present study (CTRI/2011/09/002031) was designed to investigate the therapeutic efficacy of chloroquine and chloroquine plus primaquine in *P. vivax* malaria and the polymorphisms of the *pvmdr1* and *pvcrt-o* genes associated with it.

MATERIALS AND METHODS

The study was a randomized, double-arm, open-label, interventional trial for evaluation of the clinical and parasitological responses of CQ and CQ plus PQ for treatment of uncomplicated *P. vivax* malaria based on the 2009 WHO therapeutic-efficacy protocols (23).

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Study site. The study was conducted in the malaria clinic attached to the Protozoology Unit, Calcutta School of Tropical Medicine, from December 2011 to August 2012. In Kolkata, vivax malaria is perennial and falciparum malaria is seasonal, with a peak from August to December each year. *Anopheles stephensi* is the principal vector. *P. falciparum* and *P. vivax* are the two predominant species, with an incidence of almost 1:1. The study was approved by the Institutional Ethics Committee of the Calcutta School of Tropical Medicine.

Patient recruitment and follow-up. All patients attending the clinic were screened for the presence of the malaria parasite by examining thick and thin smears of peripheral blood, followed by Giemsa staining, to identify patients who might meet the inclusion criteria according to the WHO protocol (23). A total of 250 patients with *P. vivax* monoinfection, confirmed by the presence of a negative histidine-rich protein II (HRP-II) test, over 6 months of age were included in the study. The inclusion criteria included parasitemia of 1,000 to 100,000 parasites/μl blood, absence of severe disease, no antimalarial treatment during the preceding 4 weeks, and no history of hepatic or kidney diseases. Written informed consent for study participation was obtained from all patients or their guardians.

On enrollment, each patient was thoroughly clinically examined. A questionnaire documenting fever, body weight, history of malaria, etc., was also completed. Three to 5 ml of blood was collected from each patient for biochemical and molecular biological studies.

All recruited patients were requested to return for follow-up on days 1, 2, 3, 7, 14, 21, 28, 35, and 42 after initiation of treatment and were examined both clinically and parasitologically. Members of the study team paid home visits to patients who missed the scheduled clinic visits for clinical examination and collection of blood samples for parasitological assessment.

Treatment. Before commencement of the treatment, the G6PD levels of all recruited patients were determined qualitatively using a G6PDH Hemopak kit (Apin Diagnostics & Chemicals, Vadodara, India). All the recruited patients were treated with a standard dose of CQ (25 mg/kg of base over 3 days) in group A and CQ (25 mg/kg of base over 3 days) plus PQ (0.25 mg/kg daily for 14 days) in group B and followed up for 42 days. The patients were directly observed for 30 min after treatment, and the dose was readministered if vomiting occurred. Patients who repeatedly vomited their first dose of study medication were excluded from the study. In the present study, PQ therapy was supervised for the first 7 days and then again on day 14 by examining the empty blister packs.

Study endpoints and statistical analysis. Therapeutic outcomes were classified according to WHO guidelines (23) into early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), and adequate clinical and parasitological response (ACPR). Therapeutic efficacy was determined by the per-protocol method using WHO software. The data were entered into a standard data entry program designed by the Global Malaria Programme and analyzed with a Kaplan-Meier survival curve according to WHO standard procedures (http://www.who.int/malaria/resistance). The 95% confidence interval (CI) was calculated with the Dimension Research calculator (http://www.dimensionresearch.com/resources/resourcesoverview.html).

Laboratory examination. (i) Blood smear for malaria diagnosis and parasite count. Giemsa-stained thick-smear slides were read independently by two microscopists and diagnosed as negative on initial review if no parasites were seen in 100 oil immersion fields. The number of parasites per 200 white blood cells (WBC) was determined. Assuming the WBC count to be 8,000/μl, parasitemia was calculated and expressed per μl of blood.

(ii) Rapid diagnostic test. All microscopically positive *P. vivax* cases were screened for *P. vivax*-specific plasmodial lactate dehydrogenase (pLDH) and HRP-II (SD Bio Standard Diagnostics Pvt. Ltd., Gurgaon, India) to detect any mixed infection.

(iii) Preparation of DNA templates from blood samples. Genomic DNA of *P. vivax* from all blood samples collected in EDTA-coated vials was extracted by using a QIAamp DNA blood kit (Qiagen, Hilden, Germany), following the manufacturer's instructions with minor modifications (the incubation time with proteinase K was increased to 20 min at 56°C to improve the yield of the extraction). The extracted parasite genomic DNAs from all the samples were preserved at −20°C, and an aliquot was used as the DNA source for further study.

(iv) Genus- and species-specific PCR. To ensure *P. vivax* monoinfection, a genus- and species-specific PCR method was applied as described elsewhere (24).

(v) Sequencing of *pvmdr1* and *pvcrt-o* genes. The complete DNA sequence of the *pvmdr1* gene is 4,606 bp, and the gene is located on chromosome 10. The full *pvmdr1* gene was amplified by PCR. Six different pairs of primers, as described previously (25), were used for PCR amplification. All the PCRs were performed in a total volume of 35 μl. The reaction mixture consisted of 3 μl of genomic DNA, 0.3 μM each primer pair, 0.2 mM each deoxynucleotide triphosphate (dATP, dTTP, dGTP, and dCTP), 2.5 mM $MgCl_2$, PCR buffer, and 2 units of *Taq* DNA polymerase (PerkinElmer, Branchburg, NJ). Amplification was performed using a Veriti 96-well thermal cycler (PerkinElmer, Branchburg, NJ) under the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 55°C (57°C for primers 1 and 2) for 1 min, and extension at 72°C for 2 min, and a final extension period at 72°C for 10 min.

The complete DNA sequence of the *pvcrt-o* gene is 4,281 bp, and the gene is located on chromosome 1. The gene contains 14 exons and 13 introns. Amplification of the full-length *pvcrt-o* gene by PCR was performed with a PCR mixture similar to that for *pvmdr1* and five pairs of synthetic oligonucleotides, as described previously (25). PCR was performed under the following conditions: 95°C for 10 min and 45 cycles of 95°C for 40 s, 55°C (57°C for primer 3 and 4 and 59°C for primer 1) for 1 min, and 72°C for 2 min, and final extension at 72°C for 10 min.

The quality and concentrations of the PCR products were ascertained by agarose gel electrophoresis. They were subsequently purified using a commercial kit (QIAquick PCR Purification Kit; Qiagen) and used as templates for sequencing. Sequencing reactions were carried out with the ABI Prism BigDye Terminator cycle-sequencing ready-reaction kit on a 3730 XL genetic analyzer (PerkinElmer, Branchburg, NJ).

The sequences were analyzed using the free software Bioedit Sequence Alignment Editor version 7.0.5.2. The sequences were then aligned with the *pvmdr1* (GenBank accession no. AY618622) and *pvcrt-o* (GenBank accession no. AF314649) genes of the *P. vivax* Sal I strain using the online sequence alignment tool ClustalW (http://www.ebi.ac.uk/clustalw).

RESULTS

Study population. During the study period, 7,439 patients attending the malaria clinic and presenting with fever were examined for the presence of the malaria parasite, 1,695 of whom were positive (148 *P. falciparum*, 1,544 *P. vivax*, and 3 mixed infections with both *P. falciparum* and *P. vivax*). Of the 1,544 *P. vivax*-positive cases, 250 patients were enrolled in the study. All the recruited patients had normal G6PD test results. Their baseline characteristics are described in Table 1.

***In vivo* drug efficacy.** The total of 250 recruited patients were divided into 2 study groups, A and B, each group comprising 125 patients. The patients in study group A received only chloroquine, and those in study group B received both chloroquine and primaquine.

In study group A, 24 patients were lost to follow-up, and 101 patients completed the CQ treatment course, as well as the 42-day follow-up schedule, and reached one of the study endpoints. In the study group, there was one case of LCF on day 42, and the remaining 100 (99%; 95% CI, 0.96 to 1.00) patients were classified as ACPR (Fig. 1). Subsequently, we could not classify the LCF case as either relapse or reinfection, as we were unable to amplify the

TABLE 1 Baseline characteristics of study patients

Characteristic	Value	
	CQ (n = 125)	CQ + PQ (n = 125)
Sex [no. (%)]		
Male	110 (88)	113 (90.4)
Female	15 (12)	12 (9.6)
Wt (kg)		
Mean	49.3	49.38
Range	11–87	16–95
SD	±12.703	±13.56
95% CI	47.07–51.53	41–51.76
Age category [no. (%)]		
5–15	26 (20.8)	24 (19.2)
Adult	99 (79.2)	101 (80.8)
Age (yr)		
Mean	25.21	25.16
Range	5–65	6–60
SD	±12.86	±12.82
95% CI	22.96–27.47	22.91–27.41
Temp (°C)		
Mean	37.79	37.72
Range	37.5–39.0	37.5–38.5
SD	±0.28	±0.17
95% CI	37.74–37.84	37.69–37.75
Hemoglobin (g/dl)		
Mean	12.61	12.60
Range	6.8–20	7.6–20.0
SD	±1.95	± 2.07
95% CI	12.27–12.95	12.24–12.97
Parasite count (no/μl)		
Mean	4,744	4,285
Range	1,000–31,680	1,000–25,600
SD	±4595	±4214
95% CI	3,938–5,549	3,543–5,027

parasite DNA of the day 42 sample for the *pvcsp* and *pvmsp-3*α genotypes.

In study group B, 21 patients were lost to follow-up and 104 patients completed the CQ treatment course, as well as the 42-day follow-up schedule, reaching one of the study endpoints. In this study group, one case was classified as ETF but subsequently, after parasite DNA amplification by PCR, was found to be a case of mixed infection by both *P. vivax* and *P. falciparum*. Hence, this case was excluded from the study. The remaining 103 (100%; 95% CI, 0.96 to 1.00) patients were classified as ACPR. There was no case of therapeutic failure (Fig. 1).

In all subjects, fever subsided by day 2. In group A, the parasite was cleared in 63 (57.8%), 34 (31.2%), and 6 (5.5%) cases by days 1, 2, and 3, respectively. In group B, it was 66 (60%), 24 (21.8%), and 5 (4.5%) cases by days 1, 2, and 3, respectively. No significant difference was observed in day 1 (Z = 1.74; P = 0.08), day 2 (Z = 0.465; P = 0.641), or day 3 (Z = 0.337; P = 0.735) positivity in both study groups, but in 11 cases (6 in group A and 5 in group B), the parasite was detected in peripheral blood smears on day 4, which was cleared on day 5 without any additional antimalarial drug. Ten patients with long parasite clearance times were adults, with ages ranging from 20 to 60 years, and 1 patient belonged to the age group 5 to 15 years.

During the entire study, no significant adverse side effects were recorded, apart from headache (n = 55), nausea (n = 34), vomiting (n = 4), abdominal pain (n = 8), diarrhea (n = 6), and pruritis (n = 15) during the course of medication, which were treated symptomatically.

Polymorphism of *pvcrt-o* and *pvmdr1* genes. As we did not find any significant proportion of therapeutic-failure cases, we attempted to sequence 10% of the total isolates, including the 11 cases with long parasite clearance times, for the *pvcrt-o* and *pvmdr1* genes.

The sequences of the *pvcrt-o* genes of all 25 isolates were of mutant type, but no specific pattern of polymorphism was detected. Four synonymous (L122L, P565P, Q782Q, and K807K) and four nonsynonymous (N706P, P1201L, R1224P, and R1278K) mutations were detected. Among the synonymous mutations, L122L, P565P, and K807K were located in exons 1, 4, and 7, respectively, and Q782Q in intron 6. Among the nonsynonymous mutations, N706P and P1201L were located in introns 5 and 10, respectively, and R1224P and R1278K were located in exons 11 and 12, respectively (Table 2).

The *pvmdr1* gene was fully sequenced in 25 isolates; among them, 5 (20%) isolates were wild type. Four synonymous (K68K, G172G, L310L, and L697L) and eight nonsynonymous (R88Q, A296V, E478G, S513R, G698S, M908L, T958M, and F1076L) point mutations were recorded. However, the Y976F substitution, thought to be involved in CQ resistance in *P. vivax*, was not recorded in the present study. Five different haplotypes with two ($G_{478}S_{698}$ [2; 8%]), three ($S_{698}L_{908}M_{598}$ [7; 28.0%] and

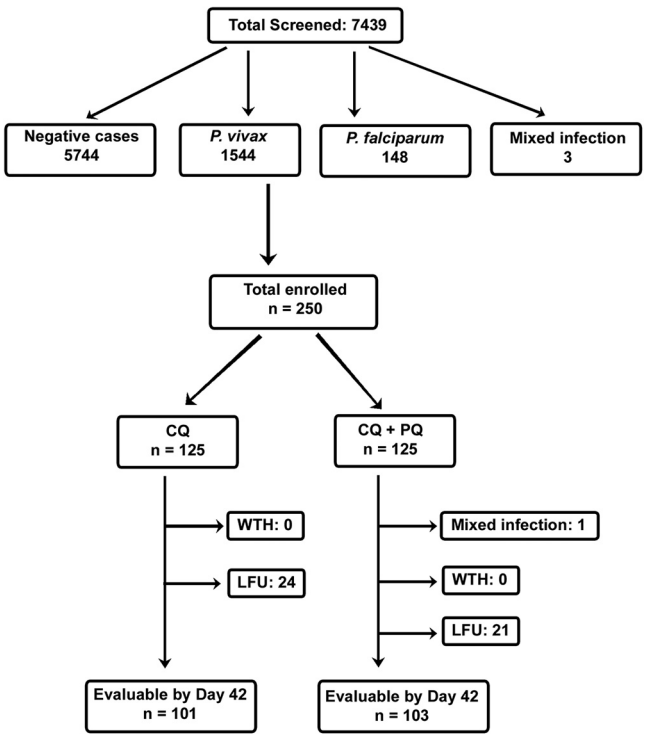

FIG 1 Consort flow chart. WTH, withdrawn; LFU, lost to follow-up.

TABLE 2 Mutation profiles of *pvmdr1* and *pvcrt-o* genes in study isolates

Candidate gene and mutation[a]	Occurrence of mutation in study population		
	n	%	95% CI
pvmdr1 ($n = 25$)			
Synonymous mutations			
K68K	2	8.0	0–18.63
G172G	4	16.0	1.63–30.37
L310L	3	12.0	0–24.74
L697L	5	20.0	4.32–35.68
Nonsynonymous mutations			
R88**Q**	7	28·0	10.4–45.6
A296**V**	3	12.0	0–24.74
E478**G**	2	8.0	0–18.63
S513**R**	4	16.0	1.63–30.37
G698**S**	9	36.0	17.18–54.82
M908**L**	18	72.0	54.4–89.6
T958**M**	18	72.0	54.4–89.6
F1076**L**	4	16.0	1.63–30.37
Haplotypes			
Wild type	5	20.0	10.4–45.6
Double mutant			
$\mathbf{G}_{478}\mathbf{S}_{698}$	2	8.0	0–18.63
Triple mutants			
$\mathbf{S}_{698}\mathbf{L}_{908}\mathbf{M}_{958}$	7	28.0	10.4–45.6
$\mathbf{L}_{908}\mathbf{M}_{958}\mathbf{L}_{1076}$	4	16.0	1.63–30.37
Quadruple mutants			
$\mathbf{Q}_{88}\mathbf{V}_{296}\mathbf{L}_{908}\mathbf{M}_{958}$	3	12.0	0–24.74
$\mathbf{Q}_{88}\mathbf{R}_{513}\mathbf{L}_{908}\mathbf{M}_{958}$	4	16.0	1.63–30.37
pvcrt-o ($n = 25$)			
Synonymous mutations			
L122L	4	16.0	1.63–30.37
P565P	12	48.0	28.42–67.58
Q782Q	3	12.0	0–24.74
K807K	4	16.0	1.63–30.37
Nonsynonymous mutations			
N706**P**	23	92.0	81.37–102.63
P1201**L**	3	12.0	0–24.74
R1224**P**	3	12.0	0–24.74
R1278**K**	24	96.0	88.32–103.68

[a] Nonsynonymous mutations are shown in boldface.

$L_{908}M_{958}L_{1076}$ [4; 16.0%]), and four ($Q_{88}V_{296}L_{908}M_{958}$ [3; 12.0%] and $Q_{88}R_{513}L_{908}M_{958}$ [4; 16.0%]) amino acid substitutions were identified (Table 2). The distribution of point mutations in *pvcrt-o* and *pvmdr1* and haplotypes of *pvmdr1* were equally prevalent among the cases with long parasite clearance times and other cases.

DISCUSSION

In vivo studies remain the gold standard for assessment of the efficacies of different antimalarial drugs. However, there are some operational difficulties associated with the follow-up of recruited patients for 42 days and differentiation of recrudescence and reinfection in areas of endemicity. In the case of *P. falciparum*, *msp 1*, *msp 2*, and *glurp* genotyping can address the problem (26). Relapse in *P. vivax* malaria caused by hypnozoite reactivation makes interpretation of recurrences in drug efficacy trials complicated. Genotyping cannot distinguish relapse from reinfection when the parasites causing the relapse arise from a dormant subset of the inoculated sporozoites that caused the primary infection. *P. vivax* parasites causing primary infections, as well as relapses, have been compared using the molecular markers *pvcs* and *pvmsp1*, and *pvmsp1* alone, respectively (27, 28).

In the present study, one patient treated with chloroquine returned with fever, and his peripheral blood smear showed *P. vivax* infection on day 42. We could not classify it as either reinfection, recrudescence, or relapse, as we were unable to amplify the parasite DNA for *pvcsp* and *pvmsp-3*α genotyping. In the other study group, patients treated with chloroquine plus primaquine, all cases were categorized as ACPR. Thus, in Kolkata, CQ alone was 99% and CQ plus PQ was 100% effective against *P. vivax* malaria. Similar observations were also made from 1998 to 2001 (29) and 2003 to 2004 (30). Therefore, during the past 15 years, the efficacy of CQ remained unchanged in *P. vivax* malaria, but in *P. falciparum* malaria, the CQ resistance level reached 76.3% (31). However, day 3 parasite positivity was noted in 11 (5.3%) patients. Ten of them were in the adult group and one in the 5- to 15-year age group. The mean day 0 parasite count of those 11 patients was 10,825 (range, 2,200 to 31,680; standard deviation [SD], ±7,946.7), which was higher than those of patients whose parasites were cleared on day 3, with a mean day 0 parasite count of 4,210 (range, 1,000 to 25,600; SD, ±3,961.9). All 11 cases were classified as ACPR according to the 2009 WHO protocol (23). Perhaps a long parasite clearance time is an indication of diminished sensitivity of the parasite to chloroquine alone, as well as chloroquine plus primaquine. G6PD is an important issue related to *P. vivax* malaria. A Mediterranean type of G6PD deficiency significantly protects against *P. vivax* infection among Afghan refugees in Pakistan (32). A similar report is also available for the G6PD Mahidol variant from Southeast Asia (33).

Molecular markers seem to be useful for monitoring the drug resistance of malarial parasites. Two genes, *pfcrt* and *pfmdr1*, were found to be associated with the efficacy of CQ in *P. falciparum* malaria. The K76T mutation in the *pfcrt* gene is known to be involved in CQ resistance, but little information is available regarding the possible relationship between the *pvcrt-o* and *pvmdr1* genes and CQ resistance. Only a few studies have been carried out, and associations between treatment failure and nonsynonymous mutations in isolates obtained before treatment have not yet been clearly established (21, 34, 35). However, the Y976F substitution in the *pvmdr1* gene is thought to be involved in CQ resistance in *P. vivax* (25, 36). Suwanarusk et al. (25) observed that the geometric mean 50% inhibitory concentration of CQ was significantly higher in *P. vivax* isolates carrying the Y976F mutation than in isolates with the wild-type allele.

In the present study, no definite mutation pattern was noticed in the *pvmdr1* gene. Out of 25 isolates studied, 13 were wild type. Four synonymous and eight nonsynonymous mutations were observed in the *pvmdr1* gene, three of which (R88Q, A296V, and E478G) were not identified previously. However, no mutation at codon 976 was observed. In *pvcrt-o*, four nonsynonymous mutations were found, two (N706P and P1201L) in introns 5 and 10 and two (R1224P and R1278K) in exons 11 and 12.

Frequent multiple mutant *pvmdr1* haplotypes (quadruple, sextuple, and septuple) were reported from Madagascar, but none of the mutant haplotypes was found to be associated with CQ-resistant *P. vivax* malaria (16). In the present study, only double ($G_{478}S_{698}$ [8.0%]), triple ($S_{698}L_{908}M_{598}$ [28.0%] and $L_{908}M_{958}L_{1076}$ [16.0%]) and quadruple ($Q_{88}V_{296}L_{908}M_{958}$ [12.0%] and

$Q_{88}R_{513}L_{908}M_{958}$ [16.0%]) mutant haplotypes, along with the wild-type allele (20.0%), were observed. Though the sample size was small, the absence of a Y976F point mutation and multiple mutant haplotypes (sextuple or septuple) of *pvmdr1*, along with the persistence of a significant proportion of the wild-type gene, justified our *in vivo* therapeutic outcomes.

In the study area, CQ alone and CQ plus PQ were effective (99% and 100%) against *P. vivax* malaria. The day 3 parasite positivity in 11 (5.3%) cases needs further evaluation for the development by the parasite of resistance to the drugs. Neither a definite pattern of polymorphisms of *pvcrt-o* and *pvmdr1* nor mutation at *pvmdr1* Y976F was observed. Hence, it is difficult to correlate any association of genetic markers with therapeutic outcomes. Periodic monitoring may elucidate the changing pattern of the susceptibility of the parasite to CQ and CQ plus PQ.

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