

## Association between Prevalence of Chloroquine Resistance and Unusual Mutation in *pfmdr-I* and *pfcr* Genes in India

Sabyasachi Das, Subhankari Prasad Chakraborty, Amiya Kumar Hati, and Somenath Roy\*

Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore, West Bengal, India; Department of Medical Entomology, Division of Parasitology, Calcutta School of Tropical Medicine, Kolkata, West Bengal, India

**Abstract.** This study deals with the underlying causes of failure of chloroquine in the treatment of *Plasmodium falciparum* infection in some malaria-endemic regions of India. Samples were collected from 141 patients in Purulia from March of 2007 to April of 2008. *In vitro* drug susceptibility tests, parasitic DNA isolation followed by polymerase chain reaction, and restriction fragment-length polymorphisms of different codons of the *pfcr* gene (76) and *pfmdr-I* genes (86, 1042, and 1246) were assessed. The responses of 141 patients to chloroquine were determined. Prevalence of double *pfmdr-I* (58.16%) mutation (86Y+1246Y) and some (14.89%) single *pfcr* mutations with triple *pfmdr-I* mutation (76T+86Y+1042D+1246Y) were found. Interestingly, double *pfmdr-I* mutation (86Y and 1246Y codons) was observed with the early treatment failure cases. These results show, for the first time in India that *in vitro* chloroquine resistance and *in vivo* chloroquine treatment failure were caused by double *pfmdr-I* ( $P < 0.001$ ) mutation.

### INTRODUCTION

Malaria is one of the major public health problems in our country. Around 1.5 million confirmed cases are reported per annum by the National Vector Borne Disease Control Program (NVBDCP), of which 40–50% of cases are caused by *Plasmodium falciparum*.<sup>1</sup> Drug resistance to *P. falciparum* is the major factor for death of malaria.<sup>2</sup> The spread of multidrug-resistant *P. falciparum* is a serious worldwide problem considering the limited number of drugs available, the lack of vaccine, and the morbidity and mortality impact of malaria.<sup>3</sup> In India, extensive and haphazard use of chloroquine (CQ) for more than five decades in treatment of *P. falciparum* malaria has resulted in the emergence of CQ-resistant *P. falciparum* malaria.<sup>4</sup> Understanding the molecular mechanisms in drug-resistant malaria is of utmost importance for both designing new drugs and providing molecular markers to monitor drug activity and treatment efficacy.<sup>3</sup> Current molecular studies of *P. falciparum* isolates suggest that few gene loci are associated with CQ resistance to *P. falciparum*. These genes have been named as *pfcr* and *pfmdr-I*. Point mutations in the *pfcr* gene were found to be associated with *in vitro* CQ resistance in *P. falciparum* isolates from Africa, South America, and South East Asia.<sup>5,6</sup> In addition, the *pfmdr-I* gene located on chromosome 5 has mutations that can play a significant role in *P. falciparum* resistance to various antimalarials, such as mefloquine, quinine, and artemisinin derivatives.<sup>7</sup> Specific combinations of *pfcr* and *pfmdr-I* alleles, resulting in varying responses to CQ, seems geographically restricted,<sup>8</sup> which may explain why some field studies reported an association between *pfmdr-I* polymorphisms and CQ resistance,<sup>9,10</sup> whereas other studies did not.<sup>11</sup> Studies from different geographical areas of the world suggest that the point mutation of asparagine to tyrosine in codon 86 (N86 to 86Y) of the *pfmdr-I* gene is associated with CQ resistance.<sup>9</sup> Several other *pfmdr-I* polymorphisms, like 1042D and 1246Y, were implicated to varying degrees in CQ

resistance.<sup>10</sup> In India, it has been reported that the *pfcr* 76T mutation of *P. falciparum* causes CQ resistance but not the multidrug-resistant gene of *P. falciparum* malaria.<sup>12,13</sup>

In India, one of the malaria-endemic zones is situated in the most remote corner of Purulia district (Bandwan Block; geographical coordinates are 22°47'0" N and 86°30'0" E) surrounded by deep forest, where the largest number of poor tribal people resides. CQ and sometimes quinine have been used here for more than five decades against *P. falciparum* malaria. Therefore, the drug pressure of CQ has gone up, and the drug efficacy has declined. Hence, the present investigation was conducted to determine the cause of *in vivo* CQ clinical failure and *in vitro* CQ resistance in this part of India.

### MATERIALS AND METHODS

**Selection of subjects.** The criteria needed to conduct the experiments included history of fever during the past 24 hours and mono-infection with *P. falciparum* based on the microscopic examination of Giemsa-stained thin and thick blood smears. Additionally, a rapid diagnostic test based on the detection of *Plasmodium*-specific lactate dehydrogenase (pLDH; OptiMAL-DT) was used with a range of parasite density of 1,000–200,000 asexual parasites/μL blood, and subjects could not have a recent history of self-medication with antimalarial drugs. Patients with signs and symptoms of severe and complicated malaria, as defined by the World Health Organization (WHO), were excluded.

**Collection of sample.** The study was carried out from March of 2007 to April of 2008 before the launch of artemisinin combination therapy (ACT) by the NVBDCP. Parasite-infected blood was taken from 184 patients using ethylene diamine tetra acetic acid (EDTA)-coated vacutainer tubes; the majority of patients come from a tribal race. Giemsa-stained blood smears were examined to check for the mono-infection with *P. falciparum*. Finally, 141 patients were enrolled in this study. The samples were processed for *in vitro* assays, and aliquots were stored at –20°C before genomic DNA extraction. Informed consent was obtained from the respective patient or the patient's guardians for adult and child patients. The experimental protocol of this study was followed as per

\*Address correspondence to Somenath Roy, Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore 721 102, West Bengal, India. E-mail: roysomenath@hotmail.com

WHO guidelines and was duly approved by the Institutional Ethical Committee.

**In vivo drug testing.** The new standard 28-day test of therapeutic efficacy developed by the WHO was used in this study.<sup>14</sup> Patients with positive rapid diagnostic test results and microscopically confirmed uncomplicated *P. falciparum* malaria were randomized with CQ (Resochin; Bayer) initially at 10 mg/kg body weight on day 0 followed by a double dose of 5 mg/kg body weight on day 1. On day 2, a single dose of 5 mg/kg body weight was administered. After pLDH confirmation at day 0, patients were given as a single dose of 10 mg/kg body weight; therefore, day 0 is day 1 for drug treatment. Additionally, day 1 can be treated as day 2, and day 2 can be treated as day 3. Large numbers of patients (31%) were below the age of 6 years, and some patients were pregnant women. Therefore, the doctors preferred a double dose of 5 mg/kg body weight instead of a single dose of 10 mg/kg body weight on day 2. The clinical conditions and parasite density were monitored on days 0, 1, 2, 3, 7, 14, 21, and 28. Blood samples were obtained by finger pricking or intravenous blood draw on enrollment and all follow-up days, including any unscheduled day to use for analysis of thick and thin blood smears. Aliquots were stored at  $-20^{\circ}\text{C}$  for additional molecular analysis. The therapeutic responses were classified as adequate clinical and parasitological response (ACPR), early treatment failure (ETF), late treatment failure (LTF), and late parasitological failure (LPF) according to the criteria adopted by the WHO. The patients not responding to CQ treatment were treated with ACT (artesunate + sulfadoxine-pyrimethamine [SP]).

**In vitro drug sensitivity assay.** *In vitro* drug sensitivity assays were performed according to the method in the work by Trager and Jensen<sup>15</sup> on the clinical isolates, with prior adaptation to the *in vitro* culture conditions for 5–7 days until the parasitemia reaches  $> 0.8$ – $1.0\%$ . Infected erythrocytes were suspended in the complete folate and *p*-amino benzoic acid free Roswell Park Memorial Institute (RPMI) 1640 medium consisting of 0.5% Albumax II, 25 mM N-2-hydroxy ethylpiperazine-N-2 ethane sulphonic acid (HEPES), 25 mM  $\text{NaHCO}_3$ , 25  $\mu\text{g}/\text{mL}$  gentamicin, and 0.2% hypoxanthine at a hematocrit of 1.5% and an initial parasitemia of 0.2– $1.0\%$ . If the blood sample contained a parasitemia  $> 1.0\%$ , fresh uninfected  $\text{O}^+$  erythrocytes were added to adjust the parasitemia from 0.8% to 1.0%. The one-half maximal inhibitory concentration ( $\text{IC}_{50}$ ) is a mea-

sure of the effectiveness of a compound in inhibiting biological function. Sterile RPMI 1640 was used to prepare stock solutions and dilutions of CQ (CQ phosphate; Sigma) for detecting the  $\text{IC}_{50}$ . The final concentrations ranged from 10 to 10,000 nM for CQ. Finally, 200  $\mu\text{L}$ /well suspension of parasitized erythrocytes were distributed in microculture plates (WHO plate), and 25  $\mu\text{L}$  each concentration of CQ were distributed in each well. Two wells (without drug) were used as controls for the experiment, and each concentration was studied in duplicate or triplicate. Plates were incubated for 48 h at  $37^{\circ}\text{C}$  in an atmosphere of 5%  $\text{O}_2$ , 5%  $\text{CO}_2$ , and 90%  $\text{N}_2$  and a relative humidity of 95%. Plates were then frozen and kept at  $-20^{\circ}\text{C}$ ;  $\text{IC}_{50}$  was determined using tritiated hypoxanthine uptake assay.<sup>16</sup> The calculation was based on non-linear regression analysis of the logarithm of concentrations plotted against the percentage of growth inhibition. Two culture-adapted cloned strains of *P. falciparum* (CQ-sensitive strain 3D7 and CQ-resistant Dd2 strains) were used for quality controls. Reference strains were cryopreserved and thawed before each measurement.

**Isolation of parasitic DNA.** Erythrocytes were separated from the patients' blood, and the parasitic DNA was extracted as described.<sup>16</sup> The extracted DNA was air dried, resuspended in Tris EDTA (TE) buffer (10 mM Tris, 1 mM EDTA), and stored at  $-20^{\circ}\text{C}$  until use. The DNA was quantified by agarose gel electrophoresis and spectrophotometrically by calculating the  $A_{260}/A_{280}$  ratios and the  $A_{260}$  values to determine protein impurities and DNA concentrations.

**Polymerase chain reaction/restriction fragment-length polymorphism analysis of *pfert* and *pfmdr-1*.** The regions of the *pfert* and *pfmdr-1* genes surrounding the polymorphisms of interest were amplified by polymerase chain reaction (PCR) using the Eppendorf thermal cycler under the following conditions: approximately 200 ng genomic DNA, 15 pmol primers, reaction buffer (10 mM Tris, 50 mM KCl, pH 8.3), 2.5 mM  $\text{MgCl}_2$ , 250  $\mu\text{M}$  2'-deoxynucleoside 5'-triphosphate (dNTP), and 1 unit Taq DNA polymerase (Roche Applied Science) in a 25- $\mu\text{L}$  reaction mixture at different reaction conditions for different genes. Primers were designed (Table 1) on the basis of the complete *P. falciparum* Dd2/Indochina strain sequence (accession number AF030694) available in GenBank.<sup>16,17</sup> Single nucleotide polymorphisms of the *pfert* and *pfmdr-1* genes at their specific codons were determined by enzymatic digestion of specific restriction enzymes. In the *pfert* gene, the *ApoI* (New England Biolabs) enzyme digested

TABLE 1

PCR primer sequences and reaction conditions were used for the amplification of sequences encoding <i>P. falciparum</i> <i>pfert</i> and <i>pfmdr-1</i> gene			
Primer name	Primer sequence	PCR product size (bp)	PCR cycling conditions
<i>pfert</i>			
K76T F (sense)	5'-TGTGCTCATGTGTTTAAACTT-3'	134	95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute; 72°C for 4 minutes
K76T R (antisense)	5'-CAAACTATAGTTACCAATTTTG-3'	134	
<i>pfmdr-1</i>			
N86Y F (sense)	5'-TTTACCGTTTAAATGTTTACCTGC-3'	310	95°C for 5 minutes; 40 cycles of 95°C for 30 seconds, 56°C for 35 seconds, and 72°C for 1 minute; 72°C for 5 minutes
N86Y R (antisense)	5'-CCATCTTGATAAAAAACACTTCTT-3'	310	
N1042D F (sense)	5'-TATGTCAAGCGGAGTTTTTGC-3'	337	95°C for 5 minutes; 42 cycles of 95°C for 30 seconds, 56°C for 40 seconds, and 72°C for 1 minute; 72°C for 5 minutes
N1042D R (antisense)	5'-TCTGAATCTCCTTTTAAAGGAC-3'	337	
D1246Y F (sense)	5'-GTGAAAATCAACTTTTATGA-3'	499	95°C for 5 minutes; 44 cycles of 95°C for 30 seconds, 58°C for 40 seconds, and 72°C for 1 minute; 72°C for 8 minutes
D1246Y R (antisense)	5'-TTAGGTTCTCTTAATAATGCT-3'	499	

the amplicon (10  $\mu$ L) at 50°C for 1 hour and identified the wild-type 76 lysine. Digestion with *AflIII* enzyme at 37°C for 1 hour recognized the mutant tyrosine amino acid at codon 86 of the *pfmdr-I* gene, whereas the *AsnI* (New England Biolabs) enzyme identified the asparagine amino acid at codon 1042 of the *pfmdr-I* gene. The *EcoRV* (New England Biolabs) enzyme detected the mutant tyrosine at 1246 codon after 1 hour of digestion at 37°C.<sup>17</sup> DNA fragments were separated by 1.2% agarose gel electrophoresis and visualized under an ultraviolet transilluminator after staining with ethidium bromide. In this experiment, 3D7 and Dd2 served as control strains.

**Multiplicity of infection and prevalence of monoclonal infections.** The multiplicity of infection, defined as the highest number of alleles detected at either of the two loci, was estimated by using an allelic family-specific nested PCR (MAD20 and K1 for *pfmsp-1* and 3D7 Africa and FC27 for *pfmsp-2*).<sup>18</sup> Clonality was defined as the highest number of alleles detected at either of the two loci, and it used to classify isolates as monoclonal or polyclonal infection and distinguish recrudescence from new infection for all patients failing therapy after the seventh day (isolates from day 0 and day of recurrence). All PCR amplifications contained a positive control (genomic DNA from strains FCM29 and 3D7 Africa) and a negative control (no target DNA).

**Assessment of antimalarial drug pressure and population mobility.** Cross-sectional surveys were carried out from May of 2007 to July of 2007 in Purulia. Thirty households from the four blocks were randomly selected. Three randomly selected individuals in each household were interviewed about their recent travel and antimalarial drug consumption as previously described.<sup>19</sup> Data collected from the individuals and the household were secondarily aggregated to characterize each site.

**Statistical analysis.** The data were expressed as mean  $\pm$  SEM. Fisher exact and Mann–Whitney *U* tests were used to study the relation between IC<sub>50</sub> values and genotypes. The relationship between *in vivo* and *in vitro* phenotypes with molecular genotypes was studied by Fisher exact tests. All the statistical analyses were performed using a statistical package (Origin 6.1; Northampton, MA) with Fisher exact or Mann–Whitney *U* test; *P* < 0.05 was the limit of significance (GraphPad InStat software 3.0).

## RESULTS

**Clinical feature of CQ.** The clinical data were available for 184 suspected cases with high fever, headache, and other symptoms compatible with this disease. A total of 141 (76.63%) isolates from 184 suspected cases were enrolled, and they were carried with *P. falciparum* mono-infection. Monoclonal *P. falciparum* isolates were confirmed by using allelic family-specific nested PCR. This group consisted of 41% females and 31% children below the age of 6 years. CQ treatment exerted ACPR in 54 subjects (38.29%). ETF was found in 76 (53.90%) isolates, with a median of day 2 (range = 1–3 days), whereas LTF was observed in 11 (7.80%) isolates, with a median of day 9 (range = 4–14 days).

***In vitro* susceptibility to CQ.** *In vitro* drug susceptibility tests were performed after clonal selection of *P. falciparum* isolates. *In vitro* assay for CQ yielded interpretable results on all 141 monoclonal *P. falciparum* isolates. It was found, through *in vitro* testing, that only 12 (8.51%) isolates were CQ-sensitive (geometric mean IC<sub>50</sub> = 45 nM, range = 15–

TABLE 2

Distribution of *pfprt* and *pfmdr-I* genotypes in 141 blood samples from Purulia with *P. falciparum* infection

Codons	<i>n</i>	Wild	Mutant	Mixed
<i>pfprt</i> 76	141	88 (62.41%)	45 (31.91%)	8 (5.67%)
<i>pfmdr-I</i> 86	141	12 (8.51%)	123 (87.23%)	6 (4.26%)
<i>pfmdr-I</i> 1042	141	108 (76.60%)	24 (17.02%)	9 (6.38%)
<i>pfmdr-I</i> 1246	141	24 (17.02%)	95 (67.38%)	22 (15.60%)

60 nM), 17 (12.06%) of 141 isolates were intermediately susceptible to CQ (mean IC<sub>50</sub> = 90 nM, range = 61–100 nM) and 112 (79.43%) isolates were highly resistant to CQ (mean IC<sub>50</sub> = 521.22 nM, range = 110–2,250 nM). 98 (87.5%) of 112 resistant isolates were found with double *pfmdr-I* mutation (86Y+1246Y). Mixed isolates were taken as mutant isolates.

**PCR/restriction fragment-length polymorphism: test for *pfprt* and *pfmdr-I* genes.** The region of *pfprt* and *pfmdr-I* genes flanking the polymorphism of interest was amplified by PCR (multiplex PCR) followed by digestion with specific restriction enzymes to detect each variant. The total numbers of *pfprt* mutant isolates at codon 76 were 31.91%. We found that 5.67% of isolates contained mixed K76T allele and 62.41% of isolates were wild K76 allele. The two alternative forms of codon 76 (i.e., threonine, and lysine) were discriminated by digestion with *ApoI* enzyme. The frequencies composed of *pfmdr-I* mutant isolates at codons 86, 1042, and 1246 were, respectively, 87.23%, 17.02%, and 67.38% (Table 2). Double *pfmdr-I* (86Y+1246Y) mutation was predominantly found in 58.16% of isolates, and 14.89% of isolates were observed with single *pfprt* mutation and triple *pfmdr-I* (76T+86Y+1042D+1246Y) mutation. Additionally, some triple mutations were found with single *pfprt* mutations and double *pfmdr-I* mutations (76T+86Y+1246Y). Some triple *pfmdr-I* mutations (86Y+1042D+1246Y) were also found here.

**Relationship between *pfprt* and *pfmdr-I* genotypes in *in vitro* and *in vivo* data.** It was observed that the presence of *pfmdr-I* point mutations was linked to *in vitro* resistance to CQ, whereas there was no such correlation observed with *pfprt* mutation, because there was no single 76T mutation. Here, the 76T mutation was always associated with other *pfmdr-I* mutations like 86Y, 1246Y, or both (86Y+1246Y). All of these mutant alleles possessed very high IC<sub>50</sub> values for CQ and showed *in vitro* resistance to CQ (Fisher test: CQ, *P* < 0.05 for 76T+86Y; CQ, *P* < 0.05 for 76T+86Y+1246Y). The phenotype of *in vitro* susceptibility to CQ was associated with *pfmdr-I* genotypes at positions 86 and 1246 but not 1042 (Fisher test: CQ, *P* < 0.001 for codon 86 and *P* < 0.01 for codon 1246; *P* was not significant at the level of 0.05 for the 1042 codon) (Table 3). Double *pfmdr-I* (86Y+1246Y) mutations were found in 82 isolates; 75 (91.46%) of 82 isolates were *in vitro* CQ-resistant. Very high IC<sub>50</sub> values were observed with wild-type *pfprt* genotypes (more specifically with K76 alleles than mutant 76T alleles, which was very unlikely) (Figure 1). Double *pfmdr-I* (86Y+1246Y) mutations were also associated with very high IC<sub>50</sub> values for CQ and resulted in *in vitro* resistance to CQ (Figure 2) (Fisher test: *P* < 0.001). Therefore, we compared the whole haplotype sequence of *pfprt* and *pfmdr-I* genes with IC<sub>50</sub> values. Most interestingly, it was observed that 82 (93.18%) of 88 wild-type K76 alleles were found with double *pfmdr-I* (86Y+1246Y) mutations (Figure 3). Because the wild-type K76 allele was found with the double *pfmdr-I* (86Y+1246Y) mutation, it



TABLE 3  
Distribution of *pfprt* and *pfmdr-1* genotypes in relation to CQ treatment efficacy and *in vitro* susceptibility to CQ in Purulia

Number	<i>pfprt</i> genotype 76	<i>pfmdr-1</i> genotype			<i>In vivo</i> response to CQ			<i>In vitro</i> susceptibility to CQ		
		86	1042	1246	ACPR	ETF	LTF	S	I	R
4	K	N	N	D	4/4	-	-	4	-	-
3	T	N	N	Y	3/3	-	-	1	2	-
11	T	Y	N	D	10/11	-	1/11	3	3	5
2	K	Y	N	D	2/2	-	-	-	1	1
7	T	Y	D	D	5/7	1/7	1/7	1	1	5
77	K	Y	N	Y	13/77	61/77	3/77	2	4	71
4	T	Y	D	Y	1/4	2/4	1/4	-	-	4
5	K	Y	N	Y+D	2/5	3/5	-	-	1	4
5	T	N	D	Y+D	5/5	-	-	1	2	2
6	T	N+Y	N	Y+D	4/6	1/6	1/6	-	1	5
6	K+T	Y	D	Y+D	3/6	3/6	-	1	1	4
9	T	Y	N+D	Y	2/9	3/9	4/9	-	1	8
2	K+T	Y	D	Y	-	2/2	-	-	-	2

CQ treatment responses are classified as ACPR, ETF, and LTF. Here, *in vitro* test responses are classified as susceptible (S), intermediate (I), and resistant (R).

possessed higher  $IC_{50}$  values for CQ. From Figure 3, it was observed that the wild-type K76+N86+N1042+D1246 (KNNND) haplotype possessed lower  $IC_{50}$  values for CQ.

In Table 3, different genotypes of all isolates with complete genotyping in relation to *in vivo* and *in vitro* results were described. Because mixed and mutant genotypes showed similar *in vitro* phenotypes, mixed genotypes were considered as mutant for this analysis. Most of the samples (87.5%) exhibited *in vitro* resistance to CQ, and they were mainly associated with double *pfmdr-1* (86Y+1246Y), triple *pfmdr-1* (86Y+1042D+1246Y), or single *pfprt* and triple *pfmdr-1* (76T+86Y+1042D+1246Y) mutations (Table 3).

No such correlation was observed with CQ treatment outcome and *pfprt* mutation. Here, 76 (53.90%) ETF and 11 (7.80%) LTF cases were found. Whatever might be the other mutation in the *pfprt* and *pfmdr-1* genes, most interestingly, 72 of 76 ETF isolates were found with double *pfmdr-1* (86Y+1246Y) mutation ( $P < 0.001$ ). In concise view, however, 64 (84.21%) of 76 ETF cases were predominantly found with the 86Y+1246Y mutation (KYYNY haplotype). Three (2.13%)

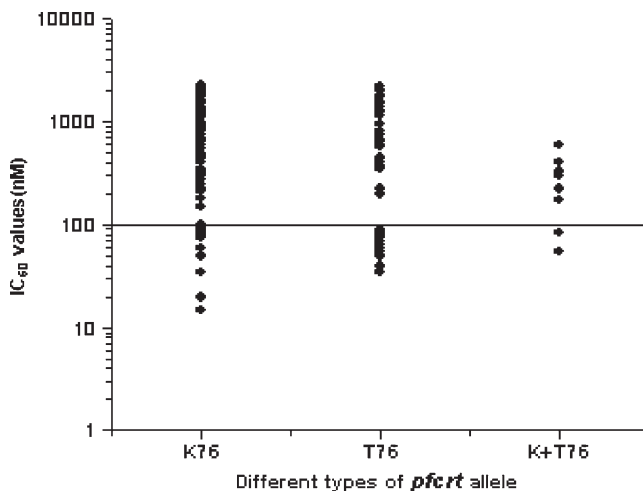


FIGURE 1. Relationship between the phenotype determined by *in vitro* drug sensitivity assays, expressed as  $IC_{50}$  of CQ, and the *pfprt* genotype, defined by either the absence of mutations (wild type) or presence of point mutations. Here, different genotypes are classified as K76, T76, and K+T76. The solid line (corresponding to 100 nM CQ; highly resistant) hypothetically shows the resistance levels for *in vitro* CQ resistance.

LTF cases were also found with this double *pfmdr-1* mutation, and 18 (12.77%) *in vitro* CQ-resistant isolates and 10 (7.09%) ETF cases were found with single *pfprt* and triple *pfmdr-1* (76T+86Y+1042D+1246Y) mutations. Five (3.55%) LTF cases were also observed with this mutation. One (0.71%) LTF case was associated with a single 76T mutation and a single 86Y mutation (76T+86Y), whereas another LTF was found because of an isolate presenting with a single *pfprt* mutation and a double *pfmdr-1* (76T+86Y+1246Y) mutation. One LTF case was associated with a single *pfprt* mutation and a double *pfmdr-1* mutation (76T+86Y+1042D) (Table 3).

**Prevalence of monoclonal infections.** Multiplicity of infection was analyzed for a subset of 184 isolates, and it showed that the proportion of monoclonal infections was very high.

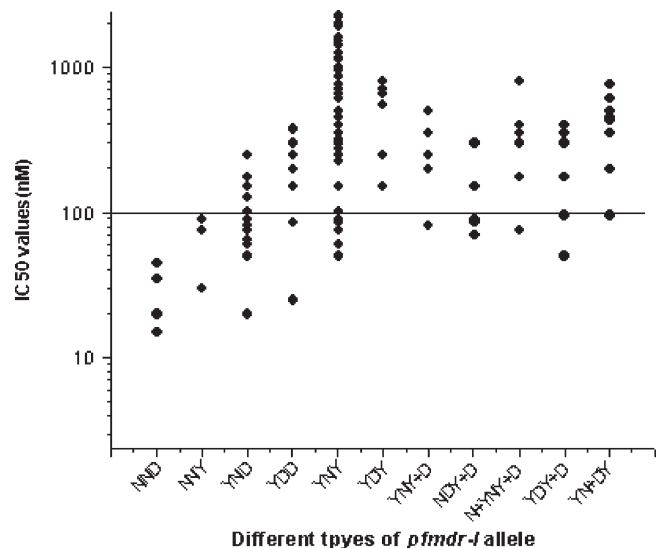


FIGURE 2. Relationship between *in vitro*  $IC_{50}$  values of CQ and *pfmdr-1* genotypes, defined by either the absence of mutations (wild type) or presence of point mutations. Here, different genotype are classified as NND (N86, N1042, D1246), NNY (N86, N1042, Y1246), YND (Y86, N1042, D1246), YDD (Y86, D1042, D1246), YNY (Y86, N1042, Y1246), YDY (Y86, D1042, Y1246), YN+D (Y86, N1042, Y+D1246), NDY+D (N86, D1042, Y+D1246), N+YNY+D (N+Y86, N1042, Y+D1246), YDY+D (Y86, D1042, Y+D1246), and YN+DY (Y86, N+D1042, Y1246). The solid line (corresponding to 100 nM; highly resistant) hypothetically shows the resistance levels for *in vitro* CQ resistance.

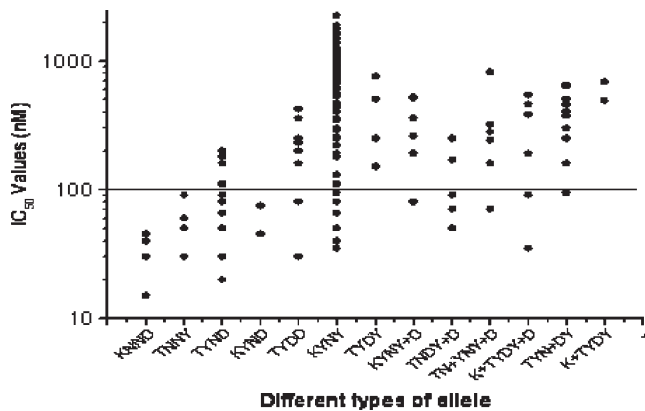


FIGURE 3. Relationship between the phenotype determined by *in vitro* drug sensitivity assays, expressed as  $IC_{50}$  of CQ, and the *pfcr* and *pfmdr-I* genotypes, defined by either the absence of mutations (wild type) or presence of point mutations. Here, different genotype are classified as KNND (K76, N86, N1042, D1246), TNNY (T76, N86, N1042, Y1246), TYND (T76, Y86, N1042, D1246), KYND (K76, Y86, N1042, D1246), TYDD (T76, Y86, D1042, D1246), KYNY (K76, Y86, N1042, Y1246), TYDY (T76, Y86, D1042, Y1246), KYN+D (K76, Y86, N1042, Y+D1246), TND+D (T76, N86, D1042, Y+D1246), TN+YNY+D (T76, N+Y86, N1042, Y+D1246), K+TYDY+D (K+T76, Y86, D1042, Y+D1246), TYN+DY (T76, Y86, N+D1042, Y1246), and K+TYDY (K+T76, Y86, D1042, Y1246). The solid line (corresponding to 100 nM; highly resistant) hypothetically shows the resistance levels for *in vitro* CQ resistance.

The results of multilocus genotype analysis of 141 isolates had a single allelic form and were considered as monoclonal infection. These monoclonal infection isolates were considered in the study.

**Antimalarial drug pressure and population mobility.** Three hundred sixty individuals were interviewed (mean age = 22.17 years, age range = 3–78 years), and 24% declared that they had been prescribed an antimalarial treatment in the previous 30 days at the health center or hospital. Surprisingly, CQ was the most commonly prescribed antimalarial drug by clinicians (73%) followed by SP (18%) and quinine (QU; 9%). The combination of artesunate plus amodiaquine or artesunate plus SP was not prescribed. Among the 38% of individuals who had taken an antimalarial drug at home, 62% declared that they had used CQ, 28% declared that they had used QU, and 10% declared that they had used SP. A population mobility survey showed that one-seventh of the individuals lived at another site 1 year before the interview. The proportion of individuals who traveled outside the site in the previous 30 days was estimated as 3.14%.

## DISCUSSION

The present study deals with the association of *pfmdr-I* genotype with *in vivo* CQ clinical treatment failure and *in vitro* CQ resistance in Purulia, West Bengal, India. CQ was efficient in treating malaria attacks in different parts of India, although in recent times, most of the isolates were *in vitro*-resistant to CQ. The NVBDCP has introduced ACT as the first-line option to keep abreast of treating all *P. falciparum* cases in the country.<sup>1</sup> This study was carried out to evaluate the efficacy of CQ before the launch of ACT by the NVBDCP in eastern India.

Several factors may explain the dissimilarity between *in vitro*- and *in vivo*-resistant patterns of isolates. Sometimes,

this dissimilarity pattern was increased. Treatment failures reflect a combination of several parameters, including parasite resistance to the drug, drug level achieved in the host, and action of the host immune response. The increasing failure rate of several antimalarial drugs in the majority of the malaria-affected areas indicates the essentiality of close monitoring of the epidemiology and dynamics of drug resistance. The identification and validation of easy and rapid molecular markers of drug resistance would greatly facilitate this process and allow us to overcome difficulties in using of traditional methods for assaying drug sensitivity.

The former accepted view revealed that the major role of CQ resistance was determined by polymorphisms in the *pfcr* gene that encoded a transporter, which promoted drug efflux from the parasite digestive vacuole in its mutated forms,<sup>8</sup> whereas *pfmdr-I* mutation modulates the level of *in vitro* CQ resistance.<sup>20</sup>

In our study, we combined *in vitro* and *in vivo* tests as well as molecular genotyping at *pfcr* and *pfmdr-I* loci. CQ treatments failed in 87 (61.70%) patients, of which 76 (53.90%) patients were ETFs and 11 (7.80%) patients were LTFs. 112 (79.43%) *in vitro* CQ-resistant isolates and 12 (8.57%) CQ-sensitive isolates were also found here. 58.16% of isolates presented the double *pfmdr-I* (86Y+1246Y) mutation, and 14.89% of isolates showed the single *pfcr* and triple *pfmdr-I* (76T+86Y+1042D+1246Y) mutation. However, this result indicated a very high rate of *in vitro* CQ resistance as well as enormous drug pressure of CQ over this population, because more than 60% of cases belonged to *in vivo* CQ treatment failure cases. This enormous drug pressure of CQ might be because of the extensive and haphazard use of CQ for more than five decades. This molecular genotype and *in vitro* resistance as well as *in vivo* CQ treatment failure were strongly related.

PCR-based methods did not detect minor clones in a mixed population; although a wild-type clone might remain undetected, this finding was unlikely for *in vitro* susceptibility, because  $IC_{50}$  mainly reflects the susceptibility of the major clones present in the blood sample. The correlation between *pfmdr-I* genotypes and quinoline resistance has often generated conflicting results. It has been suggested that *pfmdr-I* 86Y can be correlated with increased CQ resistance in parasites that originated from different areas of the world,<sup>7,8</sup> but in India, field studies had not corroborated these findings, and the results of the *P. falciparum* genetic cross indicated that CQ resistance did not depend on the *pfmdr-I* gene.<sup>12,21</sup> Different groups of workers showed that, in India, CQ-resistant *P. falciparum* and the CQ treatment failure mainly occurred because of the mutation in the *pfcr* and not the *pfmdr-I* gene.<sup>13</sup>

Our present findings implicated that double *pfmdr-I* mutations (86Y+1246Y) were highly correlated ( $P < 0.001$ ) with *in vitro* CQ resistance in Purulia. Because the presence of both N86Y and D1246Y in our samples was largely dependent on their CQ response, it indicated that CQ seemed to exert a selective pressure on this area, which was at odds with previous findings from India.<sup>13</sup>

Our results confirmed that *pfcr* 76T was a key mutation; it can cause *in vitro* resistance to CQ, with association with 86Y or 86Y+1246Y mutation ( $P < 0.05$ ), but it cannot cause early treatment failure. No single 76T mutation was found here; instead, of the 14 isolates, isolates were found either with the

76T+86Y or 76T+1246Y mutation. They showed moderate to high IC<sub>50</sub> values for *in vitro* CQ response but exerted only a single *in vivo* LTF case. In six isolates, 76T mutation was found along with double *pfmdr-1* mutation (86Y+1246Y), and five isolates showed very high IC<sub>50</sub> values for CQ but possessed only one ETF case and one LTF case. Similarly, very high IC<sub>50</sub> value was also found with single *pfcr*t and triple *pfmdr-1* mutation (76T+86Y+1042D+1246Y). Importantly, 10 ETF and 5 LTF cases were found with these mutations. The impact of the *pfcr*t/*pfmdr-1* combination mutation depends on the genetic background of the strain, which was shown by studies with genetically manipulated lines or recombinant progeny of experimental crosses,<sup>22</sup> and the history of using antimalarial drugs (CQ and QU being the two main drugs used in West Bengal, India.).

Finally, these findings aroused some most important questions. (1) What was the cause of this large number of ETF cases? (2) What would be the possible cause of the less prevalent *pfcr*t mutant parasite population despite huge drug pressure of CQ, which was largely different from other parts of India? (3) Was monitoring of isolates with *pfcr*t mutant alleles the best way to assess the incidence of CQ resistance in West Bengal, India, as previously described?<sup>12,13,21</sup>

Our results provided the answer for the question of the large number of treatment failures. Prevalence of double *pfmdr-1* mutation (86Y+1246Y) was highly associated ( $P < 0.001$ ) with *in vitro* CQ resistance as well as ETF ( $P < 0.001$ ). All the ETF isolates were found with this double *pfmdr-1* mutation, whereas the 76T and 1042D mutations increased the degree of resistance indicated by very high IC<sub>50</sub> values.

Here, we observed that CQ was the most frequently prescribed drug in the public sector for nearly five decades (starting in late 1950s), and this use might be the cause of the specific selective pressures exerted on the parasite population over CQ drug in Purulia, West Bengal, India. Additional factors contributing to the uncommon drug resistance situation in West Bengal might be its geographical position, the broad range of malaria epidemiological strata, in which the three *Plasmodium* species (*P. falciparum*, *P. vivax*, and *P. malariae*) were present, and the admixture of inhabitants with multiple tribal ethnic origins. We also confirmed from our study that monitoring only the *pfcr*t mutant allele was not the best way to assess the incidence of CQ resistance in this part of India. Monitoring of *pfmdr-1* genotype was also very much essential for detecting the CQ-resistant pattern.

Similar types of results were found in Madagascar as previously described.<sup>15</sup> In Madagascar, single nucleotide polymorphisms at codons 86, 184, and 1246 in *pfmdr-1* were highly prevalent, likely reflecting the widespread use of quinolines, such as CQ and QU. The elevated frequency of *pfmdr-1* mutant alleles (YYD, NFD, YFD, and YFY) associated with CQ resistance and the low frequency of *pfcr*t mutant genotypes might account for the high rate of late clinical failure of CQ treatment.<sup>8,23</sup>

*pfcr*t mutations were known to have an effect on CQ resistance, which was proven by genetic cross-breeding between sensitive and resistant parasites.<sup>13</sup> However, the unbalanced numbers of genotypes here did not allow us to draw any conclusion on the impact of *pfcr*t mutations.

It was concluded that *in vitro* CQ resistance and failure of CQ treatment in this area of India was caused mainly by the combination of two *pfmdr-1* (86Y and 1246Y) mutations but

not the *pfcr*t (76T) mutation, which was a very new finding in the field of drug-resistant *P. falciparum* in India. The increase in the number of *pfmdr-1* mutations was strongly correlated to CQ resistance. Additional studies are needed to determine the precise incidence of the combination of *pfcr*t and *pfmdr-1* gene mutations and the role of double *pfmdr-1* mutation on CQ and other antimalarial drug efficacy. New cheap anti-malarial combinations (except ACTs) should be tested for treatment of the drug-resistant *P. falciparum*.

Received December 21, 2011. Accepted for publication June 11, 2012.

Published online March 18, 2013.

Acknowledgments: The authors thank Vidyasagar University, Midnapore, for providing the facilities to execute these studies. The American Society of Tropical Medicine and Hygiene (ASTMH) assisted with publication expenses.

Disclaimer: We declare that we have no conflict of interest.

Authors' addresses: Sabyasachi Das, Subhankari Prasad Chakraborty, and Somenath Roy, Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore, West Bengal, India, E-mails: sdas.vu@gmail.com, subhankariprasad@gmail.com, and roysomenath@hotmail.com. Amiya Kumar Hati, Department of Medical Entomology, Calcutta School of Tropical Medicine, Kolkata, West Bengal, India, E-mail: amiya\_hati@rediffmail.com.

## REFERENCES

1. Government of India, 2009. *Guidelines for Diagnosis and Treatment of Malaria in India*. Government of India. NIMR/TRS-06/APR-2009.
2. Peters W, 1987. Resistance of human malaria I, III and IV. *Chemotherapy and Drug Resistance in Malaria*, 2nd Ed. London, United Kingdom: Academic Press.
3. Aubouy A, Jafari S, Huart V, Florence M, Mayombo J, Durand R, Mohamed B, Jacques Le B, Deloron P, 2003. *DHFR* and *DHPS* genotypes of *Plasmodium falciparum* isolates from Gabon correlate with *in vitro* activity of pyrimethamine and cycloguanil, but not with sulfadoxine-pyrimethamine treatment efficacy. *J Antimicrob Chemother* 52: 43–49.
4. Sehgal PN, Sharma MID, Sharma SI, Gopal S, 1973. Resistance to chloroquine in *falciparum* malaria in Assam state, India. *J Commun Dis* 5: 175–180.
5. Durand R, Jafari S, Vauzelle J, Delabre JF, Jesic ZZ, Le Bras J, 2001. Analysis of *pfcr*t point mutations and chloroquine susceptibility in isolates of *Plasmodium falciparum*. *Mol Biochem Parasitol* 114: 95–102.
6. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV, Coulibaly D, 2001. A molecular marker for chloroquine resistant *falciparum* malaria. *N Engl J Med* 344: 257–263.
7. Duraisingh MT, Cowman AF, 2005. Contribution of the *pfmdr-1* gene to antimalarial drug-resistance. *Acta Trop* 94: 181–190.
8. Andriantsoaririna V, Ratsimbaoa A, Bouchier C, Tichit M, Jahevitra M, Rabearimanana S, Raherinjafy R, Mercereau-Pujalon O, Durand R, Menard D, 2010. Chloroquine clinical failures in *P. falciparum* malaria are associated with mutant *Pfmdr-1*, not *Pfcr*t in Madagascar. *PLoS One* 5: e13281.
9. Sharma YD, 2005. Genetic alteration in drug resistance markers of *Plasmodium falciparum*. *Indian J Med Res* 121: 13–22.
10. Than N, Manoj D, Michael R, Alan CF, 2003. Analysis of *Pfcr*t, *pfmdr-1*, *dhfr* and *dhps* mutation and drug sensitivities in *Plasmodium falciparum* isolates from patients in Vietnam before and after treatment with artemisinin. *Am J Trop Med Hyg* 68: 350–356.
11. Mehlotra RK, Mattera G, Bockarie MJ, Maguire JD, Baird JK, 2008. Discordant patterns of genetic variation at two chloroquine resistance loci in worldwide populations of the malaria

- parasite *Plasmodium falciparum*. *Antimicrob Agents Chemother* 52: 2212–2222.
12. Vathsala PG, Pramanik A, Dhanasekaran S, Rangarajan PN, Padmanaban G, 2004. Widespread occurrence of the *Plasmodium falciparum* chloroquine resistance transporter (*Pfcr*) gene haplotype SVMNT in *P. falciparum* malaria in India. *Am J Trop Med Hyg* 70: 256–259.
  13. Valecha N, Joshi H, Mallick PK, Sharma SK, Kumar A, Tyagi PK, Shahi B, Das MK, Nagpal BN, Dash AP, 2009. Low efficacy of chloroquine: time to switchover to artemisinin-based combination therapy for *falciparum* malaria in India. *Acta Trop* 111: 21–28.
  14. World Health Organization (WHO), 2003. *Assessment and Monitoring of Antimalarial Drug Efficacy for the Treatment of Uncomplicated Falciparum Malaria*. Geneva, Switzerland: WHO.
  15. Trager W, Jensen JB, 1976. Human malaria parasites in continuous culture. *Science* 193: 673–675.
  16. Basco KL, Ringwald P, 2000. Molecular epidemiology of malaria in Yaounde, Cameroon VI. Sequence variations in the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene and *in vitro* resistance to pyrimethamine and cycloguanil. *Am J Trop Med Hyg* 62: 271–276.
  17. Lopes D, Rungsihirunrat K, Nogueira F, Seugorn A, Gil JP, do Rosário VE, Cravo P, 2002. Molecular characterizations of drug-resistant *Plasmodium falciparum* from Thailand. *Malar J* 1: 12.
  18. Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosol S, 1999. Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans R Soc Trop Med Hyg* 93: 369–374.
  19. Gardella F, Assi S, Simon F, Bogreau H, Eggelte T, Ba F, Foumane V, Henry MC, Kientega PT, Basco L, Trape JF, Lalou R, Martelloni M, Desbordes M, Baragatti M, Briolant S, Almeras L, Pradines B, Fusai T, Rogier C, 2008. Antimalarial drug use in general populations of tropical Africa. *Malar J* 7: 124.
  20. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein *PfCRT* and evidence for their role in chloroquine resistance. *Mol Cell* 6: 861–871.
  21. Vinayak S, Biswas S, Dev V, Kumar A, Ansari MA, Sharma YD, 2003. Prevalence of the K76T mutation in the *pfcr* gene of *Plasmodium falciparum* among chloroquine responders in India. *Acta Trop* 87: 287–293.
  22. Sa JM, Twu O, Hayton K, Reyes S, Fay MP, 2009. Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Proc Natl Acad Sci USA* 106: 18883–18889.
  23. Valerie A, Arsene R, Christiane B, Martial J, Stephane R, Rogelin R, Voahangy A, Tantely R, Ange RM, Magali T, Leon RP, Odile M-P, Remy D, Didier M, 2009. *Plasmodium falciparum* drug resistance in Madagascar: facing the spread of unusual *pfdhfr* and *pfmdr-1* haplotypes and the decrease of dihydroartemisinin susceptibility. *Antimicrob Agents Chemother* 53: 4588–4597.