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Association of *pfcrt* But Not *pfmdr1* Alleles with Chloroquine Resistance in Iranian Isolates of *Plasmodium falciparum*

Sedigheh Zakeri,* Mandana Afsharpad, Tahmineh Kazemzadeh, Kambiz Mehdizadeh, Ashraf Shabani, and Navid Dinparast Djadid

Malaria and Vector Research Group (MVRG), Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran; Biochemistry Department, Al-Zahra University, Tehran, Iran; Chabahar Public Health Department, Zahedan University of Medical Sciences, Chabahar, Iran

Abstract. This study was designed to analyze the *Plasmodium falciparum chloroquine* resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance 1 (*pfmdr1*) mutations as markers of chloroquine (CQ) resistance in 200 blood samples collected from malaria patients in south-eastern Iran during 2002–2005. Among these, 25 (post-treatment) fulfilled the 28-day follow-up study. A high number of Iranian *P. falciparum* (97%) strains harbored quadruple mutations at codons 76T, 220S, 326D, and 356L. All post-treatment isolates harbored the mutant allele 76T, but low rates of the mutant allele 86Y (44%) of the *pfmdr1* gene were detected. No wild haplotype of *pfcrt* (72-CVMNKAQNIR-371) was found in post-treatment samples; however, 56% of clinical "failure" samples carried the wild type of *pfmdr1* (NYSND). The present results suggest a strong association between *pfcrt* 76T, but not *pfmdr1* 86Y mutation and *in vivo* CQ resistance. Furthermore, we found the CQ resistance-associated SVMNT haplotype, which previously had been seen in South American isolates. Although Iran is located more proximally to Southeast Asia than to South America, no CQ resistance-associated CVIET haplotye has been observed in this region. Therefore, these results were not consistent with the earlier presumed spread of CQR parasites from Southeast Asia to Africa via the Indian subcontinent. In conclusion, *P. falciparum* mutations associated with resistance to CQ are abundant in south-eastern Iran and this finding strongly supports that CQ as the first line drug is inadequate for treatment of uncomplicated falciparum malaria in Iran.

INTRODUCTION

Malaria is an infectious disease that continues to be associated with considerable morbidity and mortality and has significant social and economic impact on developing societies. Today, shortages of resources and drug resistance have been significant obstacles to malaria control in many developing countries. For the past 60 years, chloroquine (CQ) has been the gold standard in prevention and treatment of uncomplicated malaria. Since the early 1960s, sensitivity of the parasites to CQ, the best, cheapest, and most widely used drug for treating malaria, has been on the decline.[1] Newer antimalarials have been discovered to overcome this problem, but all of these drugs are either expensive or have undesirable side effects. In addition, the parasites, especially the falciparum species, have started showing resistance to these drugs. The first report of CQ resistance (CQR) was documented from Thailand (Southeast Asia) and Columbia (South America) in early 1960s. Since then, resistance has been spreading worldwide and reached the Indian state of Assam in 1973.[2,3] In Iran, the first cases of resistance were reported from Sistan and Baluchistan province in 1983 and later in Hormozgan province in 1986.[4] Further, *in vivo* and *in vitro* studies on response of *P. falciparum* to CQ during 1990–1996 showed rather high resistance of *P. falciparum* at RI and RII and lower numbers of RIII levels.[5] Although resistance has been reported since 1983, CQ is still used as antimalaria treatment in Iran. An *in vivo* study carried out by Raeisi and co-workers during 2002–2004 also showed that the frequency of treatment failure by day 28 was 78.5% in south-eastern provinces of Iran.[6]

Although resistance to CQ has been well documented, this anti-malarial drug is still used as the standard treatment of uncomplicated malaria in many geographic regions. Some progress has been made in understanding the mechanism of action of CQ, but it has not yet been fully understood. Although the molecular basis of CQ resistance is not fully elucidated, several genes encoding a candidate protein involved in the transport of CQ into or out of the digestive vacuole have been proposed. The genes considered for CQ resistance are *pfcrt* (*P. falciparum* chloroquine resistance transporter), *cg2* (encoding a ~330-kDa protein), and *pfmdr1 (P. falciparum* multidrug resistance).[7–11] Based on transfection studies, *cg2* was not the CQ resistance determinant.[12] Also, association of *pfmdr1* gene with CQ resistance remains unclear,[13] and genetic crosses showed no linkage to CQ resistance.[14] In several studies, the *pfmdr1* gene was known to undergo mutations leading to the substitution of amino acids at codons 86, 184, 1034, 1042, and 1246.[7,11,14–16] In other studies, no association of these mutations with CQR was found,[17–20] but most recent investigations showed that polymorphisms in *pfmdr1* in some geographical areas were associated with increased *in vitro* mefloquine sensitivity.[21,22] In contrast, the *crt* gene showed absolute association with CQ resistance in parasite lines[23] and clinical isolates.[24,25] In addition, CQ resistance is associated with a K76T mutation of the *pfcrt* gene,[7] while a multidrug resistance analogue (*pfmdr1*) N86Y variation may modulate its degree.[26] In eastern Sudan, Babiker and co-workers[27] demonstrated a significant association between high levels of CQR and *pfcrt* 76T and *pfmdr1* 86Y alleles. Moreover, a commonly strong association between *pfcrt* 76T and *pfmdr1* 86Y alleles, especially in CQR parasites, suggests a joint role in development of resistance.[28] However, the implication of *pfmdr1* 86Y in CQR was challenged in other studies.[29]

In several studies, point mutations were observed in more than 20 codons in the *pfcrt* gene from various regions, which were found to be associated with the CQR phenotype.[30–32] These include mutations at amino acid positions 72, 74, 75, 76,

* Address correspondence to Sedigheh Zakeri, Malaria and Vector Research Group (MVRG), Biotechnology Research Center, Pasteur Institute of Iran, Pasteur Avenue, PO Box 1316943, Tehran, Iran. E-mail: zakeris@yahoo.com, azad@institute.pasteur.ac.ir

97, 144, 148, 160, 194, 220, 271, 326, 356, and 371.[7,30,33–35] In particular, the CQR parasite isolates from Southeast Asia and Africa have *pfcrt* genes with 7–9 mutated codons, 72-CIETH(L)SEST(I)I-371.[7,30] The CQR parasites from South America and Papua New Guinea also possess 4–5 mutated codons, 72-S(C)MNTHSQDLR-371, in *pfcrt* genes.[7,30,36] The mutation K76T was found in all CQR parasites and A220S was found in all but 2 CQR isolates sequenced thus far,[7,30,31] indicating their essential role in CQR. In another study, Chen and co-workers[33] reported that mutation in codon A220S in *pfcrt* is associated with CQR in African countries but not in the Philippines.

There is little published molecular data available on characterization of resistant *P. falciparum* in Iran,[37–41] and to our knowledge, no such study on large samples examining the *pfcrt* and *pfmdr1* haplotypes in parasites has been conducted in this region before. Thus the current study has been designed to investigate the prevalence of CQR-associated markers in Iran. This aim was achieved by genetic analysis of *pfcrt* and *pfmdr1* genes, and detection of their haplotypes in 200 Iranian *P. falciparum* strains collected during 2002–2005. In addition, the association between *in vivo* CQR and *pfcrt* polymorphisms in clinical *P. falciparum* isolates from malaria hypoendemic regions of Iran was evaluated in where CQ has been used as the first-line treatment and CQR was first reported in 1983. In addition, to compare and determine the association of *pfmdr1* alleles with CQR, this gene was also characterized on the same isolates.

MATERIALS AND METHODS

Study site and patients. *P. falciparum* field isolates (N = 175) were obtained from patients (ages 1–70 years old) with uncomplicated malaria before treatment during the years 2002–2005. All patients with the average age of 27 years attended malaria clinics at primary health care centers in Chabahar district in Sistan and Baluchistan, Iran (Figure 1). The majorities were male (80%) and were nationals of three countries: Iran (60%), Afghanistan (5%), and Pakistan (22%); those of the remainder were not recorded. In addition, a large fraction of the patients had traveled to Pakistan (36%) 2–3 weeks prior to blood sampling. Details of study areas in Chabahar district are described elsewhere.[39]

FIGURE 1. Map of Iran indicating the location of the study area in Chabahar district situated in the south-eastern corner of Sistan and Baluchistan province where the *P. falciparum* isolates were collected (Ch, Chabahar).

***In vivo* assay.** In this study, patients were selected according to inclusion criteria suggested by the standard WHO 28-day *in vivo* test.[42] Thin and thick blood films were stained with Giemsa and examined microscopically for detection of *P. falciparum* asexual stages. Approximately 1 mL of venous blood was obtained pre-treatment in a tube containing EDTA from 175 patients who were confirmed to be positive for the presence of *P. falciparum* parasites. CQ (25 mg/kg body weight over 3 days) was then administrated orally under supervision, and patients were observed for eventual vomiting for at least 30 minutes. The 25/175 consenting patients fulfilled a 28-day follow-up. Therefore, in total, 200 *P. falciparum* clinical isolates were obtained from malaria patients pre- (175) and post-treatment (25) in this study. The clinical condition, body temperature, and parasite density were assessed at each visit. Parasite clearance was monitored by thick-film microscopy each day after treatment, and follow-up blood samples were collected not only before treatment (D0) but also at D1, D2, D3, D7, D14, D21, and D28 following treatment (post-treatment). The clinical response to treatment was classified as sensitive (S) or resistant (R) based on the asexual stage parasite clearance time and recurrence of blood-stage parasites as follows: parasite clearance within 7 days of treatment which was sustained for 28 days (S); parasite clearance by D7 but reappearance before D28 (RI); parasitemia was markedly reduced (> 75%) by D2 of treatment but not cleared by D7 (RII); and parasitemia not reduced by D2 (RIII). Patients' or parents' informed consent was obtained before inclusion in the study. The study was reviewed by, and received Ethical Clearance from, Pasteur Institute of Iran. All pre- and post-treatment blood samples were collected in tubes containing EDTA, stored at 4°C, and then transported to the main laboratory in Tehran.

Genotyping of *P. falciparum* parasites. To determine whether the patients had recrudescent parasites or new infections, *P. falciparum* genetic diversity was compared between the pre- and post-treatment samples by using *msp-1* and *msp-2* encoding highly polymorphic loci from merozoite surface protein genes.[43] These genes were genotyped by using allelic type-specific primers in nested PCR for *msp-1* (with K1, MAD20, and RO33 allelic families) and *msp-2* genes (for 3D7 or FC27 allelic family).

Nested PCR and mutation-specific amplifications of *pfcrt* and *pfmdr1* genes. The parasite genomic DNA was extracted from infected red blood cells using phenol/phenol–chloroform followed by ethanol precipitation as described previously.[44] Nested PCR was performed for *crt* and *mdr1* genes on pre- and post-treatment samples. All reactions were carried out in 25-μL reaction mixtures containing 1.5–3 mM $MgCl_2$, 200 μM dNTP mixture (Invitrogen, Carlsbad, CA), 1 unit of *Taq* polymerase (Invitrogen), and a pair of primers (10 pM each). For both *crt* and *mdr1,* 1–2 μL of DNA was used as template in the first reaction and 1 μL of samples (if no band was seen) was used, whereas 1 μL of a 1/100 to 1/500 dilution of the samples with an intense band from the first-round PCR product was used as template for the secondary

PCR. The PCR primers and conditions for both genes were those published previously[45,46] with modification. PCR products were resolved by electrophoresis on 1–2% agarose gels and visualized by staining with ethidium bromide.

Detection of *pfcrt* and *pfmdr1* mutations by restriction digestion of PCR products (PCR/RFLP). Following amplification of the fragments concerned, polymorphisms in the *pfcrt* and *pfmdr1* genes were assessed by the mutation-specific restriction endonuclease digestion to detect single nucleotide polymorphisms (SNPs) in *pfcrt* at positions K76T, A220S, Q271E, N326S/D, I356T/L, and R371I/T and in *pfmdr1* at positions N86Y/H, Y184F, S1034C, N1042D, and D1246Y.[45,46] A number of restriction enzymes were used for RFLP of PCR products.[45,46] For *pfcrt*, the PCR products were digested with *Apo*I, *Bgl*I, *Xmn*I, *Mse*I, *Cai*I, and *AFl*II to determine the polymorphisms at codons 76, 220, 271, 326, 356, and 371, respectively (detailed information is available on the internet at http://medschool.umaryland.edu/cvd/nejm2001djimde.asp). For *pfmdr1,* 3 enzymes, *Apo*I, *Nsi*I, and *AFl*III were used to identify codon 86, while *Tas*I, *Dde*I, *Ase*I, and *Eco*RV were used to determine the polymorphisms at codons 184, 1034, 1042, and 1246, respectively. Digestions were done in 20-μL reactions containing 10 μL of PCR products according to the manufacturer's instructions (New England Biolabs, Beverly, MA, Fermentase, Vilnius, Lithuania, and/or Invitrogen). If there was doubt about a complete digestion, reactions were repeated overnight. Digested products were subjected to electrophoresis on 1.5–2% agarose or 2–3% Metaphor agarose gels and visualized by ultraviolet (UV) transillumination.

DNA sequence analysis of *pfcrt* gene. In the present study, the *pfcrt* haplotypes (amino acids at codons 72–76, 326, and 356) were preformed by PCR amplification, followed by both restriction enzyme digestion and DNA sequencing in 175 (pre-) and 25 post-treatment field samples whose *in vivo* patterns of CQ response were unknown and known, respectively. The following forward and reverse primers (7G8 type: AF233067) were designed and used for amplification:

crt1F: 5′ TATTTTCATTGTCTTCCAC 3′ (codons 72–76)
crt1R: 5′AGGAATAAACAATAAAGAAC 3′
crt4F: 5′ CCTTTTTAGTTCATTTACC 3′ (codon 326)
crt4R: 5′ AGTAATAAGCAATTGCTA 3′
crt5F: 5′ TATCGACAAATTTTCTACCATG 3′ (codon 356)
crt5R: 5′ TAATTGAATCGACGTTGG 3′

Amplified fragments were gel-purified using the Qiagen DNA purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Direct sequencing of the DNA fragments was performed in both directions for each PCR product using an ABI-3100 sequencer (Primm Company, Milan, Italy). Nucleotide and amino acid sequences were aligned and compared with the 7G8 sequence (accession no. AF233067) using CLUSTALX.

RESULTS

***In vivo* CQ response study.** In this study, a total of 200 *P. falciparum*-positive blood samples were collected; and among these, 25 were recruited for *in vivo* study and had a complete 28-day follow-up. Based on WHO standard protocols, the parasitological responses were classified as 2 (8%), 16 (64%), 4 (16%), and 3 (12%) that were sensitive (S), RI, RII, and RIII, respectively. The 2 sensitive parasites were isolated from patients who showed recurrent infection at D30 and D58 of treatment with CQ. The important issue to consider is that genotyping of all 25 *P. falciparum* isolates with both *msp-1* and *msp-2* markers showed the same allelic families and lengths of the PCR products (data not shown).

Analysis of *pfcrt* mutations by the PCR/RFLP method. In this study, 6 loci of *pfcrt,* the gene involved in CQ resistance were analyzed in 200 *P. falciparum* clinical samples, including 175 pre- and 25 post-treatment samples. No mutations were detected at codons 271 and 371 among all samples examined. However, analysis of pre- and post-treatment samples showed that 97.7% and 100% of the samples harbored the pure mutant allele of *pfcrt* 76T, respectively. For the other codons—220S, 326S/D, and I356L—the prevalence of the mutations was 97%, 97.7%, and 100% among pre-treatment and 96%, 100%, and 100% among post- treatment samples. Regarding these 6 SNPs in the *pfcrt* gene, no significant difference in prevalence was observed in the pre- and post-treatment samples after treatment with CQ, although the number of post-treatment samples was small. A high number of examined Iranian *P. falciparum* (97% of all 200) isolates harbored quadruple mutations at codons 76T, 220S, 326S/D, and I356L. The SNPs results of the *pfcrt* in pre- and post-treatment *P. falciparum* isolates are shown in Table 1.

Sequence analysis of *pfcrt* gene. Sequence analysis of DNA from 200 *P. falciparum* isolates showed that the codon 72-SVMNT-76 haplotype associated with CQR isolate was found in 98% of sequenced isolates and accounted for the vast majority of *P. falciparum* infections. However, the remaining 4 (2%) pre-treatment isolates carrying the K76 allele had CVMNK wild haplotype. No CVIET sequence was found in any of these samples. Sequence analyses of codons N326S/D

TABLE 1
Distribution of *pfcrt* polymorphisms in pre- and post-treatment samples from Iran based on PCR/RFLP*

	Pfcrt						
	K76T	**A220**S	**Q271**E	**N326**S/D	**I356**T/L	**R371**I/T	Total (%)
Pre-treatment	**K**	**A**	**Q**	**N**	**I**/L	**R**	**4 (2.3%)**
	T	**A**	**Q**	S/D	**I**/L	**R**	**1 (0.6%)**
	T	S	**Q**	S/D	**I**/L	**R**	**170 (97.1%)**
	T **= 97.7%**	S **= 97%**	E **= 0**	S/D **= 97.7%**	**I**/L **= 100%**	I/T **= 0**	**175 (100)**
Post-treatment	**K**	**A**	**Q**	**N**	**I**/L	**R**	—
	T	**A**	**Q**	S/D	**I**/L	**R**	**1 (4%)**
	T	S	**Q**	S/D	**I**/L	**R**	**24 (96%)**
	T **= 100%**	S **= 96%**	E **= 0**	S/D **= 100%**	**I**/L **= 100%**	I/T **= 0**	**25 (100%)**

* Analysis of codon N326S/D by *Mse*I restriction enzyme digestion showed that 97.7% of our samples had either amino acid serine (S) or aspartic acid (D). Analysis of codon I356T/L, using the *Alw*NI restriction enzyme, which digests the resistant allele 357T, showed no digestion in the presence of *Alw*NI in any of our samples, which means that in this position there was either amino acid isoleucine (I) or amino acid leucine (L).

and I356T/L in *pfcrt* gene showed that 98% of sequence samples harbored aspartic acid (D) at codon 326 and leucine (L) at codon 356.

Analysis of *pfmdr1* mutations. Similarly, we investigated 5 loci of *pfmdr1*, the gene that might be involved in CQR. The frequency of the mutant *pfmdr1* 86Y allele was 36.6% and 44% among pre- and post-treatment isolates, respectively. No 86H mutation was detected in all Iranian *P. falciparum* isolates examined.[47] All pre- and post-treatment isolates with 86Y mutation also carried the mutant *pfcrt* 76T allele. Only 3 samples harbored mixed *pfmdr1* N86Y alleles. Furthermore, for other 2 codons, 184F and 1042D, the prevalence of the mutations was 6.3%, and 0.6%, respectively in pre-treatment samples; however, for post-treatment, this prevalence was 0% and 12%, respectively. In addition, no mutation has been detected at codon S1034C in pre-treatment but one (4%) in post-treatment samples; and also D1246Y in any of the isolates examined. The SNPs results of the *pfmdr1* in pre-treatment and post-treatment *P. falciparum* isolates are shown in Table 2.

Distribution of *pfcrt* and *pfmdr1* haplotypes from Iran. Three different profiles of the *pfcrt* 72-371 codons were identified by analysis of nucleotide sequences obtained from both pre- and post-treatment samples. The 72-SVMNTSQDLR-371 (97%) was the highest prevalent haplotype among both samples. The distribution of the *pfcrt* haplotypes is shown in Table 1 and Figure 2.

The *pfmdr1* haplotype with amino acid NYSND at positions 86, 1034, 1042, and 1246 in the 3D7 laboratory clone was also defined wild type. A total of six distinct haplotypes were identified in all examined isolates in this study (Figure 3). Haplotypes NYSND (58.8% to 56%) with no mutation and YYSND (34.3% to 32%) with a single mutation were the most common among both pre- and post-treatment samples, respectively (Figure 3). Haplotype YYCDD with mutations at 3 positions was detected in 1 (4%), but YYSDD with double mutations was identified in 2 (8%) of the post-treatment samples with RI parasitological response. Two haplotypes, NFSND (4.6%) and YFSND (1.7%), were identified only in pre-treatment samples (Figure 3).

Combination of *pfcrt* and *pfmdr1* haplotypes among all 200 samples in this study demonstrated 9 distinct haplotypes (Figure 4). The 2 most prevalent haplotypes among both pre- and post-treatment samples were SVMNTSQDLR/NYSND (55% to 52%) and SVMNTSQDLR/YYSND (33% to 28%). Haplotype SVMNTSQDLR/YYCDD with 6 mutations at different positions on both *pfcrt* and *pfmdr1* was isolated from a Pakistani patient (post-treatment) who came to Iran to seek treatment at a Chabahar clinic.

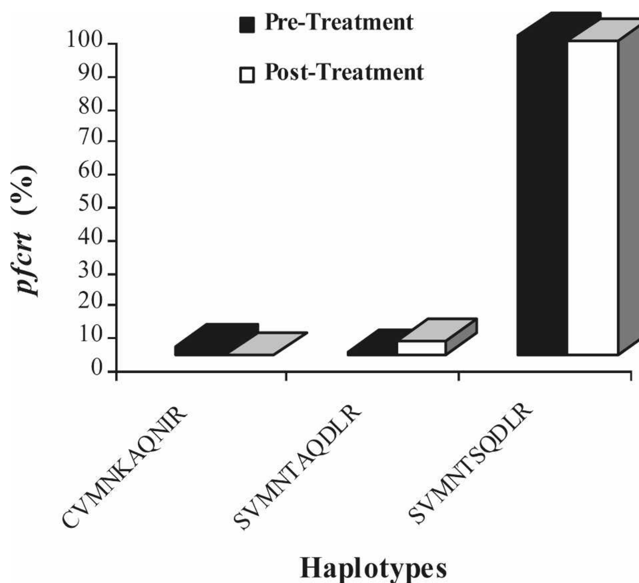

FIGURE 2. Frequency distribution of the different *pfcrt* haplotypes was obtained from 175 pre-treatment and 25 post-treatment samples collected from Iran. Based on sequencing analysis, a high prevalence of quadruple mutations at codons 76T, 220S, 326D, and 356L has been found among both samples. Complete absence of parasites with wild mutation K76 in post-treatment samples indicates *in vivo* selection for the mutant allele 76T.

DISCUSSION

Identification of *pfcrt* as the central determinant of chloroquine-resistant *P. falciparum* malaria provides a molecular marker that can be used for surveillance of resistance and to evaluate drug treatment and prophylaxis policies. The present results further support this role of the *pfcrt* gene. In the current study, 200 *P. falciparum* clinical isolates were collected during 2002–2005 when CQ was used as the first-line anti-

TABLE 2
Distribution of *pfmdr1* polymorphisms of *P. falciparum* in pre- and post-treatment samples from Iran based on PCR/RFLP analysis

	pfmdr1					
	N86Y	Y184F	S1034C	N1042D	D1246Y	Total (%)
Pre-treatment	N	Y	S	N	D	103 (58.8%)
	N	F	S	N	D	8 (4.6%)
	Y	F	S	N	D	3 (1.7%)
	Y	Y	S	D	D	1 (0.6%)
	Y	Y	S	N	D	60 (34.3%)
	Y	Y	C	D	D	—
	Y = 36.6%	F = 6.3%	C = 0	D = 0.6%	Y = 0	175 (100%)
Post-treatment	N	Y	S	N	D	14 (56%)
	N	F	S	N	D	—
	Y	F	S	N	D	—
	Y	Y	S	D	D	2 (8%)
	Y	Y	S	N	D	8 (32%)
	Y	Y	C	D	D	1 (4%)
	Y = 44%	F = 0	C = 4%	D = 12%	Y = 0	25 (100%)

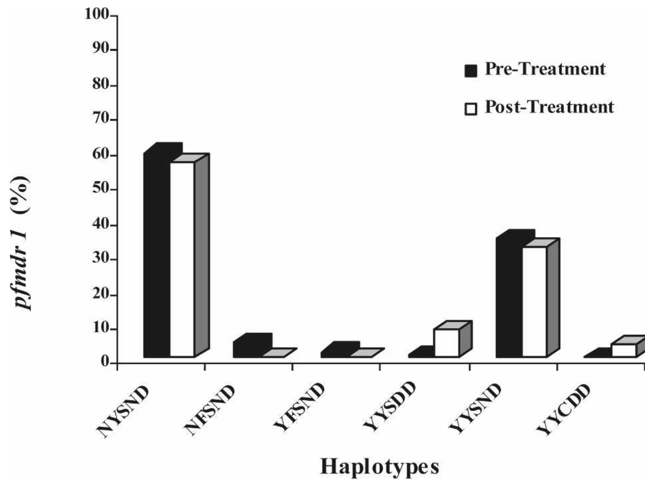

FIGURE 3. Frequency distribution of the different *pfmdr1* haplotypes was obtained from 175 pre-treatment and 25 post-treatment samples collected from Iran. Wild haplotype NYSND was the most prevalent haplotype among both pre- and post-treatment samples.

malaria treatment of uncomplicated falciparum patients in south-eastern Iran. Genotyping of all 25 recrudescent isolates with both *msp-1* and *msp-2* showed no re-infection. Two follow-up patients recruited at D30 and D58 after treatment with CQ and based on the parasitological response were classified as sensitive (S). However, *msp-1* and *msp-2* genotyping of those samples at D0, D30, and D58 showed the same allelic families and length of PCR products, and therefore, these 2 samples might have been classified as RI. Hence, this data suggested that the standard WHO 28-day *in vivo* test might need to be revised and extended to 60 days after treatment.

In this study, 6 codons of *pfcrt* were monitored by the RFLP method; however, for identification of the *pfcrt* haplotype, DNA sequencing was applied. Both pre- (97%) and post-treatment (96%) *P. falciparum* isolates harbored mutations at positions 76T, 220S, 326D, and 356L, but other codons (271 and 371) showed no mutation associated to CQR. Although only 25/175 consenting patients fulfilled the 28-day follow-up, most of the patients showed recurrent infection in different days after treatment with CQ without fulfilling the 28-day follow-up (such post-treatment samples were not available for molecular work). The high prevalence of CQ treatment failure by D28 (78.5%) in south-eastern province of Iran was also reported in another study.[6] Therefore, the present data suggest that these 4 mutations in the *pfcrt* gene might have been selected by treatment and therefore can be used as molecular markers to monitor CQR in this region of the world.

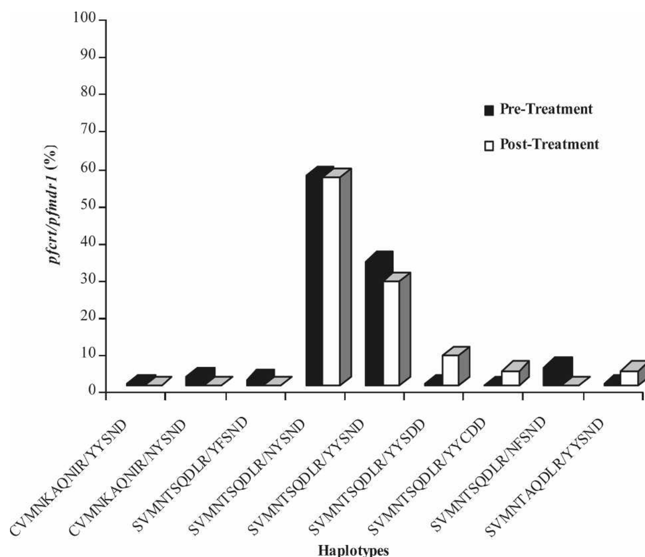

FIGURE 4. Frequency distribution of the combination *pfcrt/pfmdr1* haplotypes was obtained from 200 isolates collected from Iran. The haplotype SVMNTSQDLR/NYSND was the most prevalent among both pre- (N = 175) and post- (N = 25) treatment samples. There was no association between the *pfmdr1* 86Y mutation and *in vivo* CQ resistance observed in this study.

We also observed predominance of wild haplotype N86, Y184, S1034, N1042, and D1245 (NYSND) of the *pfmdr1* gene in our samples. The presence of this predominant haplotype among Iranian *P. falciparum* strains was in concordance with findings in Thai isolates[48]; however, it was distinct from African isolates.[14] Although all parasite isolates examined in this study harbored 86Y also the 76T mutation in both pre- and post-treatment isolates, there was no association between the *pfmdr1* 86Y mutation and *in vivo* CQ resistance observed in this study. This result was also in concordance with other studies[24,28,49] and confirms that the N86Y position in the *pfmdr1* gene is not a reliable molecular marker to monitor CQR in an epidemiologic survey in Iran. A low number of *P. falciparum* isolates showed mutations at positions 184F and 1034C; these samples had been isolated from patients who had taken a trip to Pakistan or from Pakistani patients who came to Iran for seeking treatment with antimalarial drugs. This issue must be considered for control of the disease because there is a risk of invasion of resistant parasites with different mutations, and its spread might occur either by local transmission and/or migration of reservoirs from the Indian subcontinent to Iran and from there may be distributed to other malaria settings.

Regarding the prevalence of *pfmdr1* haplotypes, 2 haplotypes predominated among our samples (NYSND, N = 58.5% and YYSND, N = 34%). No wild haplotype of *pfcrt* (72-CVMNKAQNIR-371) was found in post-treatment samples; however, 56% of clinically failed samples carried the wild-type *pfmdr1* (NYSND). Therefore, our study does not support the role of *pfmdr1* in conferring CQR in *P. falciparum* strains, as has been shown in studies from Uganda, Laos, Cameroon, South Africa, Brazil, and Peruvian Amazon, verifying that this mutation is not predictive of treatment outcome in their regions.[9,50–52]

One of the interesting results of this study was genetic investigation of the codons 72–76 haplotypes among Iranian isolates. CQ-sensitive *P. falciparum* strains have the haplotype CVMNK, regardless of geographic origin. However, so far, 8 distinct *pfcrt* allelic patterns for codons 72–76, including SVMNT, CVMNT, CVMET, CVIET, SVIET, CVIKT, SVMIT, and RVMNT, have been associated with *P. falciparum* CQR phenotype.[7,53,54] In general, CQR *P. falciparum* strains from South America have most often the haplotype SVMNT, CVMNT, and CVMET, whereas CQR parasites from Africa/Southeast Asia carry an allele encoding the CVIET haplotype, suggesting two main independent origins of CQR[7,35] and an apparently separate site in Papua New Guinea (PNG),[30,35] Philippines,[55] and India.[56] Sequence

analysis of our samples revealed that 72-SVMNT-76 was the most prevalent haplotype among examined samples, which was in agreement with similar findings in India and PNG.[35,53,56] In addition, parasite isolates from other malaria setting, such as Laos and Tanzania (possibly, after large-scale use of amodiquine), share this haplotype with the South American isolates.[57,58] Remarkably, haplotype 72-CVIET-76, which was characteristic of CQR parasites from Southeast Asia,[24,30] has not been found among our examined isolates, and surprisingly, we found that there is a closer relationship between the Iranian and South American 7G8 line than Asian/African CQR *P. falciparum*. Therefore, the high prevalence of 72-SVMNT-76 haplotype that accompanies other mutations at codons 220S, 326D, and 356L of *pfcrt* gene (72-SVMNTSQDLR-371 haplotype) and the absence of CQR CVIET haplotype (Asia/Africa) among examined isolates in this study were not consistent with the earlier presumed spread of CQR parasites from Southeast Asia to Africa via the Indian subcontinent. In addition, CQR isolates carrying the 72-SVMNT-76 haplotype from the present study were detected in Iran, India,[55,59] Philippines,[55] PNG,[30,35] and Laos[57] and share the same *pfcrt* haplotype as the Brazilian line 7G8, indicating their origin as apparently independent mutational events.

Nevertheless, genetic analysis of a large number of samples from different parts of malaria setting in Iran and similar studies on samples from Pakistan and Afghanistan, which serve as bridges between India and Iran, could help our understanding of how CQR evolved in this region of the world. In addition, the high prevalence of mutations at codons 220S, 326D, and 356L (97%) that accompanies 72-SVMNT-76 in Iranian *P. falciparum* isolates suggests that the population of *P. falciparum* that circulates in south-eastern Iran has been selected by the long use of CQ. The overall picture emerging from this study is that resistance to this drug is abundant in south-eastern Iran, and this finding strongly supports withdrawal of CQ as the first-line drug for treatment of falciparum malaria in Iran.

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Authors' addresses: Sedigheh Zakeri, Mandana Afsharpad, and Navid Dinparast Djadid, Malaria and Vector Research Group (MVRG), Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran, Pasteur Avenue, PO Box 1316943, Tehran, Iran, Tel: +98 21 66480780, Fax: +98 21 66465132, E-mail: zakeris@yahoo.com, azad@institute.pasteur.ac.ir. Tahmineh Kazemzadeh and Ashraf Shabani, Al-Zahra University, Tehran, Iran. Kambiz Mehdizadeh, Chabahar Public Health Department, Zahedan University of Medical Sciences, Chabahar, Iran.

Reprint requests: Sedigheh Zakeri, Malaria and Vector Research Group (MVRG), Biotechnology Research Center, Pasteur Institute of Iran, Pasteur Avenue, PO Box 1316943, Tehran, Iran, Tel: +98 21 66480780, Fax: +98 21 66465132, E-mail: zakeris@yahoo.com; azad@institite.pasteur.ac.ir.

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