

## Frequency of *Pfcr* T76 and *Pfdhfr* Asn-108 Drug Resistance Mutations in *falciparum* Malaria in Southeastern Malaria Endemic Area of Iran

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### Abstract

**Background:** Point mutations in the genes, *Pfcr* and *Pfdhfr*, have been identified elsewhere as strongly associated with resistance to chloroquine (CQ) and pyrimethamine, respectively.

**Methods:** Using a restriction digestion method, we report the frequency of two important mutations, T76 and Asn108, responsible for conferring resistance to chloroquine and pyrimethamine, respectively, in patients residing in malarious areas of southeastern Iran.

**Results:** Ninety eight and 84% of the patients were found to harbor chloroquine resistant (threonine-76) and pyrimethamine resistant (asparagin-108) mutants, respectively.

**Conclusions:** Despite the high frequency of T76 and Asn 108 mutations in Iranian patients, combination of CQ and pyrimethamine-sulfadoxine (SP) was shown to be quite successful with 100% adequate clinical and parasitological responses.

**Keywords:** Malaria, Drug resistance, Chloroquine, Pyrimethamine/sulfadoxine, Iran

### Introduction

Malaria endemic areas of Iran are located in the south-eastern part of the country, bordered in south by the Persian Gulf and the Oman Sea, and to the east by Pakistan and Afghanistan. These areas include provinces of Sistan va Baluchistan, Hormozgan and Kerman (mainly the Kahnuj district). Development of resistance to chloroquine by *Plasmodium falciparum* in these areas has become a major public health concern (1), however the molecular basis of chloroquine resistance (CQR) is not totally elucidated. Point mutations in the gene, *Pfcr*, have been identified elsewhere as strongly associated with in vitro CQR in parasite lines and in natural isolates (2, 3). In particular, the *Pfcr* K76T mutation has always been detected in CQR parasites. As the result of spread of CQR *falciparum* the antifolate combination of

pyrimethamine and sulfadoxine (SP) has increasingly become the drug of choice for treatment of *falciparum* malaria. There are some concerns over fast developing of resistance to this drug. In some part of the world such as Africa, Asia, and South America resistance to this medication has led to treatment failure (4). There are also some reports of resistance to SP in some patients from Iran with CQR *falciparum* who were either treated with a combination of SP and amodiaquine or with SP alone during implementing some drug efficacy studies (1). Pyrimethamine and sulfadoxine act synergistically to inhibit two enzyme important enzymes in the parasite's folate biosynthetic pathway, dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*). Point mutations in the *dhfr* and *dhps* genes confer resistance to pyrimethamine and sulfadoxine, respectively.

However, the mutations in *dhfr* appear to be more important in causing treatment failure than *dhps*. For *dhfr*, a point mutation causing a serotonin to asparagin change at position 108 has shown to be associated with resistant to pyrimethamine. This paper reports the frequency of the two important mutations, T76 and Asn108, in malarious areas of south-east Iran. Such studies will help us to foresee, to some extent, the possible treatment failures and adopt the strategies to reduce the pace of selection for the available drugs.

## Materials and Methods

**Study area and sample collection** This is part of a larger study on detecting polymorphisms in the *Pfcr* and *Pfmdr-1* genes in individuals residing in malaria endemic areas of the country. The study was carried out between November and December 2003, in the five sites of Iranshahr and Minab of Sistan va Baluchistan and Hormozgan provinces, respectively in southeastern Iran. Blood samples were collected from febrile patients, who were seeking treatment from public health centers after their verbal consent. An oral temperature  $\geq 37$  or prior fever within 48 h and a *P. falciparum* positive Giemsa-stained slide were inclusion criteria. All the blood samples were reconfirmed to harbor *P. falciparum* by rDNA-polymerase chain reaction. The patients' age ranged from 2 to 55 yr (mean 15.7 yr) with 4/3 male/female ratio. As their history showed 30% of patients had contracted the disease in Pakistan. The whole blood samples were frozen at  $-70^{\circ}\text{C}$  until used.

**DNA extraction** DNA extraction was accomplished by proteinase K digestion and phenol/chloroform extraction (5). Simply, one ml of cold water was added to 300  $\mu\text{l}$  of citrated blood, mixed gently, kept on ice for 10 min and centrifuged at 8000 rpm for 7 min. The supernatant was gently discarded and the pellet was resuspended in 1 ml of cold water and treated as before. The pellet was then resuspended in 200  $\mu\text{l}$  of extraction buffer and 10  $\mu\text{l}$  of 20 mg/ml proteinase K. The suspension was incubated at  $60^{\circ}\text{C}$  overnight and then at  $90^{\circ}\text{C}$  for 10 min. The sus-

pension was then treated with RNase at  $37^{\circ}\text{C}$  and subjected to two rounds of phenol/chloroform extraction. DNA was precipitated with two volumes of ethanol and resuspended in 50 of double distilled  $\text{H}_2\text{O}$ .

**PCR and allele specific restriction analysis of *Pfcr* codon 76** A nested PCR and digestion were performed as noted by Plowe et al. (6) for all the samples. The first round of nested PCR was performed by the following primers: CRTP1 (5'-CCGTTAATAATAAATACACGCA-G-3') and CRTP2 (5' CGGATGTTACAAAAC-TATAGTT-ACC-3'). The second nested PCR was performed with the internal primers CRTD1 (5'-TGTGCT-CATGTGTTTAAACTT-3') and CRTD2 (5'-CA-AACTATAGTATCCAATTTG-3'). 10  $\mu\text{l}$  of PCR product containing the approximately 145 bp amplicon was digested with 0.5 U of ACSI according to manufacturer's protocol (Roche, Germany) and run on a 3% agarose gel for 80 min under 65 V. ACSI digested the wild type allele, making it about 30 bp shorter, but the mutant one remained intact.

**PCR and allele specific restriction analysis of *Pfdhfr* codon 108** The following primers of: FR100-A (5'-GGGGGGCAGTTACAACATAT-GTGA-3') and FR100-B (5'-GGGGGCACATT-CATATGTACTATTT-3') were used for amplification of first round of nested PCR. Forty pM of each primer were used in a 25  $\mu\text{l}$  reactions containing 100 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  of each dNTP, 30 ng of DNA (1.5  $\mu\text{l}$ ) and 0.7 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Inc). The thermal cycler (Techne USA) programmed for  $95^{\circ}\text{C}$  for 5 min followed by 45 cycles of  $92^{\circ}\text{C}$  for 30 sec,  $45^{\circ}\text{C}$  for 30 sec and  $65^{\circ}\text{C}$  for 45 sec and a final extension step at  $72^{\circ}\text{C}$  for 7 min. For the second round, 2  $\mu\text{l}$  of the first round PCR product was added to each PCR mixture containing the primers FR108-D (5'-CTAATTCT-AAAAAATTACAA-AATG T-3') and FR164-D3 (5'-TTTCTTTTCT-AAAAATTCTTGATAAACAACGGAACCTC-TTA-3'). The PCR mixture and amplification condition were as before except for annealing tem-

perature and number of cycles which reduced to 42° C and 25 cycles respectively. 10 µl of PCR product was subjected to digestion with *AluI* enzyme according to manufacturer's protocol (Roche, Germany). Cleavage of the 250 bp PCR product into two bands of about 40 and 210 bp on a 3% agarose gel indicated the wild (serine) type while the intact PCR product represented the mutant ones (asparagin).

## Results

Ninety eight out of 99 PCR reactions for *Pfcr*t were successful and 97 samples were shown to be mutant (threonine at position 76) and only one sample was wild type (lysine at position 76). The number of successful PCR reactions for *Pfdhfr* was 93 comprising 79 mutant type (asparagin at position 108) and 14 wild type (serine at position 108).

## Discussion

The effective treatment of malaria by chloroquine in Iran is increasingly facing the major problem of drug resistance. In vivo studies on 1301 patients during 1990-1996 showed that 890 cases (68.4%) were CQR (1). We could detect the *Pfcr*t K76T mutation in 98% of patients residing in malaria endemic areas. However, an in-vivo study on antimalarial drugs efficacy carried out around the same period of time revealed 78.5% chloroquine resistance with 17.4% of early treatment failure, 34.7% of late clinical failure and 26.4% of late parasitological failure (7). Our findings are supported by some previous reports suggesting that the K76T mutation is not always linked to CQR. In a similar study by Durand et al. (2001) the agreement between *Pfcr*t genotype and *in vitro* susceptibility test was shown to be 82%. A report (8) showed that the prevalence of K76T mutation in some sites of Mali was 2-3 times higher than the prevalence of clinical chloroquine resistance. Finally, the study by Dorsey et al. (8) in Kampala failed to identify any sequence that could predict a sensitive or resistant response to chloroquine therapy. It seems that K76T mutation could be necessary but not sufficient to ac-

quire CQ resistance. It has been shown that some other point mutations, confined to some geographical areas, in *Pfcr*t were associated with CQ resistance. Attempts to detect further mutations in *pfcr*t gene in Iranian patients may yield a clearer picture of association of CQR and molecular markers (8). Monitoring the therapeutic efficacy of CQ and SP combination selected as the first line regimen for treatment of *falciparum* cases from the first peak of malaria transmission in 2005 turned out to be quite successful with 100% adequate clinical and parasitological response during the recent nine months of 2005. However, the exceptional fast pace of selection for resistance to pyrimethamine-sulfadoxine which has reduced its useful therapeutic life (UTL) in some countries in South Asia and South America (9, 10) has raised some concerns over the possibility of this medication becoming ineffective in near future in Iran. Therefore, it is necessary to understand the forces that affect the selection for resistance to these drugs and adopt strategies to delay it as long as possible.

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The authors declare that they have no Conflict of Interests.

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