

Pyrimethamine and Proguanil Resistance-Confering Key Mutations in Dihydrofolate Reductase Gene of *Plasmodium falciparum* Isolates from Iran

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Abstract

Using mutation specific PCR assay and analysis of position 108 amino acid in dihydrofolate reductase-thymidilate synthase gene in 70 *Plasmodium falciparum* isolates of malarial patients from southern areas of Iran, showed 4 different situations: i-wild type (108-Ser) ii-mutant type (108-Thr) iii-mutant type (108-Asn) and iv-mixed wild and mutant type (108-Ser+Thr) with frequency of 58(82.8%), 2(2.9%), 8(11.4%) and 2(2.9%) respectively. Many studies have demonstrated that mutations in position 108 have key role for emerging resistance to anti-folate drugs in *falciparum* malaria so, the resistance to pyrimethamine and proguanil, presents in *Plasmodium falciparum* isolates from malarious endemic regions of Iran.

Keywords: *Plasmodium falciparum*, Drug resistance, Pyrimethamine, Proguanil, Iran

Introduction

Malaria is still a major health problem in many countries which has ever more complicated with emerging resistance to many effective anti-malarial drugs in some strains of parasite (1-4). Malaria has been prevalent in Iran for centuries. Due to prevention and control attempts, malaria has decreased in many parts of country but it is endemic in three south and southeastern provinces as Hormozgan, Sistan Va Bluchistan and Kerman (5). In these areas, three species of *Plasmodium* (*P.*) *vivax*, *falciparum* and *malariae*, are prevalent regarding their frequency respectively (6). The number of malaria patients is varying between about 25000 and 60000 year to year (4, 6). Several kinds of anti-malarial drugs have been prescribed for fourty last years, so the resistance to some drugs has been emerged. The first cases of *P.falciparum* resistance to chloroquine in isolates from Sistan va Bluchistan province were reported in 1985(5,7), using in vivo and in vitro (micro test) methods

(7). So far, resistance to some other drugs as fansidar, mefloquin and quinine has been reported (4). The current methods for drug resistance studies in malaria have their limitations, so molecular methods are used in new surveys in Iran. We reported the first molecular drug resistance assessment results recently (8). This is second paper which is published in this sense. Pyrimethamine (PYR) inhibits dihydrofolate reductase-thymidilate synthase(DHFR-TS) enzyme and is usually used in combination with sulfadoxine which acts through dihydropteroate synthase (DHPS) enzyme that catalizes the earlier step in the folate pathway of parasite (3,9). Proguanil (PRO) also inhibits DHFR-TS and is used for malaria prophylaxy, often in combination with chloroquine (10).

Point mutations which confer resistance to PYR and cycloguanil (CYC) - the active form of PRO-arise in the parasite gene encoding DHFR-TS, leading to amino acid changes in the active site pocket of the enzyme (9). A 108-Ser

genotype is linked to sensitivity to both PYR and CYC, a mutation to asparagine at position 108 confers resistance to PYR and a moderate loss of response to CYC and a threonine at position 108 is associated with resistance to CYC with another mutation (Ala 6Val). There are some other mutations in positions 16, 51, 59 and 164 in DHFR gene which modulate level of resistance but mutations in position 108 has basic role, so analysis of this position was performed in present study (11-13).

Materials and Methods

Sample Collection and DNA extraction

Seventy pure *falciparum* malaria patient blood samples were collected during two malaria peaks of patients at Bandar Abbas, Minab and Kahnooj districts in 2002. Presence of unique *falciparum* malaria and determination of parasitemia was performed by microscopic examination of Giemsa-stained thin and thick blood films. Suitable cases were selected for sampling. About 20-40 μ l of Patients' blood was taken on the bottom of filter paper (3 MM; Whatman, Hillsboro, OR) strips with size of 0.5 cm x 3cm by finger pricking (9,14). After air-dried and stored in separate envelopes, there were in ambient or 4°C temperature until DNA extraction (even months later).

DNA extraction Parasite's genomic DNA was extracted by saponin RBC lysis method (14). Briefly, about 10mm² of impregnate filter paper was soaked in 500 μ l of 0.05% saponin in phosphate buffer saline (PBS;137 mM NaCl, 2.7 mM KCl, 8mM Na H₂Po₄, 1.5mM KH₂po₄) for 30 min at 4°C, then washed once in 500 μ l PBS. One hundred micro liters 1X Taq DNA polymerase buffer (Roche) was added to the paper and the mixture cycled 3-5 times between 50 and 95°C. After centrifugation of 10000xg for 4 min, 4 μ l of supernatant was added to a 25 μ l (final volume) PCR reaction (8, 12, 14).

Mutation specific PCR Since up to date a wild type and two point mutations were seen in position 108 of DHFR gene, so amplification was performed using specific primers: DIA-3, DIA-9 and DIA-12 each paired with a common primer SP1 which are shown in table 1. They design to determine serine, threonine and asparagine in the position 108 respectively (9, 14, 15). If matching occurred between the specific primer and relate amino acid codon in position 108, amplification would occurred and a specific 337 bp bond observed on the line of same primer on 2% agarose gel stained with ethidium bromide. DNA amplification with all three primers performed for each isolates (see Fig.1as an example shows wild type).

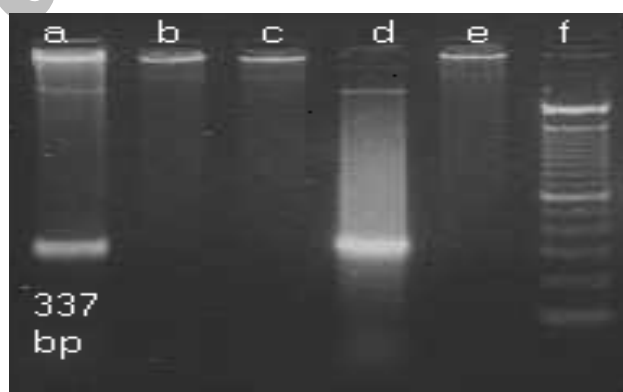


Fig.1: PCR result of position 108 DHFR gene of *P.falciparum* isolate of a malarial patient from Minab, 2002. The lines are: a-DNA sample with DIA-3, b-DAN sample with DIA-9, c-DNA sample with DIA-12, d- positive control (mixed DHFR-108-Ser-Thr-Asn inserted in plasmid), e-negative control (normal human blood DNA), f-100 bp ladder size marker

Table 1: Sequence and bonding site of primers which were used

Mutation specific PCR primers	Gene	Amino acid residues	Strand	Code
5' – GAATGCTTTCCCAGC – 3'	<i>dhfr</i>	108 – 113	anti-sense	DIA-3
5' – GAATGCTTTCCCAGG – 3'	<i>dhfr</i>	108 – 113	anti-sense	DIA-9
5' – GGAATGCTTTCCCAGT – 3'	<i>dhfr</i>	108 – 113	anti-sense	DIA-12
-ATGATGGAACAAGTCTGCGAC – 3'	<i>dhfr</i>	1 – 7	Commo sense	SP1

DNA amplification Amplification of DNA was performed in 40 cycles by thermal cycler (Genius, Techne). Amplification program consisted of a first denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 50 sec and extension at 72°C for 60 sec, with a final extension at 72°C for 5 min yielding a product of 337 bp when match occurred between the parasite DNA and the diagnostic primer. Taq DNA polymerase (1U) was used in each 25- μ l reaction. Magnesium chloride was present in the reaction mixtures at a concentration of 1.5 mM, and a 200 μ M concentration of each dNTP and 1 μ M of concentration of each primer was used in all reactions. Separate areas of the laboratory were designated for setting up and analysis PCRs. Positive and negative control was used with each PCR.

DNA sequencing Sequence analysis was performed on 8 samples, which confirmed the PCR results (data not shown).

Results

The results revealed 4 different genotypic situations in DHFR-TS gene of *P. falciparum* isolates. Out of 70 isolates 58(82.8%) had serine at the position of 108 and were wild type and sensitive to PYR and PRO, 2(2.9%) had threonine which paired with another mutation in position of 16(Ala 16 Val) which show resistance to CYC, 8(11.4%) with mutation. The Serine to asparagines change-the most important mutation-which causes resistance to PYR. In 2(2.9%) of isolates observed both serine and asparagine at position 108 which means presence of two different isolates of parasites in these patients' blood or heterogeneity at this position on DHFR gene (Table 2). We concluded that the genotype of resistance to anti-folate drugs as PYR and PRO presents in *P.falciparum* in southern Iran. Although its rate is low now, but it could increase in endemic areas rapidly so it would be considered by physicians and malaria manager.

Table 2: Response to pyrimethamine /proguanil determines by mutation specific PCR in 70 *P. falciparum* isolates from southern Iran in 2002

Amino acid in position-108 in <i>dhfr</i> gene of <i>P.f</i>	No (%)	Response to PYR/PRO
Ser-108	58 (82.8%)	Sensitive/sensitive
Thr-108*	2 (2.9%)	Sensitive/resistance
Asn-108*	8 (11.4%)	Resistance/~resistance
Ser/Thr-108* (mixed)	2 (2.9%)	Sensitive/sensitive
Total	70 (100%)	

* Mutations

Discussion

There are considerable recently publications which linked the resistance to anti-folate drugs as PYR and PRO in *P. falciparum* to point mutations in DHFR-TS coding gene of this parasite (1, 2). The mutations almost occur in the positions: 16, 51, 59, 108 and 116, among them, mutations in position 108 have the most importance for emerging the resistance (2, 11, 13). Regarding this fact, we assay the genotypic construction of position 108 at this gene in field isolates of *P. falciparum* from Kahnooj and Minab districts of Iran; however the PCR products of some isolates were analyzed by sequencing method. Although, in most countries in Middle East, malaria is prevalent but, there is little molecular studies for comparison. DHFR-TS mutations which identified in Iranian isolates were similar to those found in African isolates in which amino-acid residues 51, 59 and 108 (Ser to Asn substitution) exhibit polymorphism most frequently (13, 15, 16, 17). The Ser-to-Thr substitution in codon 108, which rarely has been reported from field isolates originating from Africa, was seen in 4 Iranian isolates. Our study revealed that, the development of molecular techniques as a complementary approach to define the epidemiology of drug resistant malaria is of major importance and genetic analysis of resistance genes can be incorporated into drug-resistance monitoring as an integral component of a malaria control program. Low parasitemia in some samples was a problem in DNA extraction, and amplification, so it is better to use the samples with average or high parasitemia, otherwise the Nested PCR will be necessary.

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