

## Genetic Diversity Block 2 of Surface Protein-1 in Plasmodium Falciparum Merozoite by Nested-PCR Method in Southeastern Iran

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**ABSTRACT**

**Background and Objectives:** Plasmodium falciparum merozoite surface protein-1 (PfMSP-1) is a promising vaccine against malaria during its blood stages which play an important role in immunity to this disease. Polymorphic nature of this gene is a major obstacle in making an effective vaccine against malaria. In this study, the genetic diversity of Plasmodium falciparum isolates was investigated in Sistan-Baluchestan Province using allelic families of the MSP-1.

**Methods:** From March/April 2011 to August/September 2012, 94 blood samples were collected from patients with falciparum malaria who were living in four districts of Sistan-Baluchestan Province. The extracted genomic DNA and genetic diversity of MSP-1 block 2 were evaluated by nested polymerase chain reaction.

**Results:** From a total of 94 patients, 89 patients (94.7%) had positive PCR results and the remaining five patients were excluded. Seven different alleles of MSP-1 were identified through size difference on agarose gel. Comprising 46.1% of the samples, MAD20 was identified as the predominant MSP-1 allelic family, while the R033 family had the lowest frequency (with 7.9%). In 10% of samples infection with two alleles was observed.

**Conclusion:** The results of this study suggest that genetic diversity of PfMSP-1 in Southeastern Iran is relatively low and most infections originate from a clone that is consistent with an area of low malaria transmission. This information is useful for the prevention and control of malaria in Iran.

**Keywords:** Merozoite Surface Protein 1, Plasmodium Falciparum, Polymerase Chain Reaction.

## INTRODUCTION

Malaria is caused by four species of Plasmodium protozoans (1). The disease is one of the most serious parasitic diseases in humans with about 207 million people affected in the world (according to the World Health Organization in 2012) and a mortality rate of about 627 thousand people (1, 2). The most serious malaria is caused by Plasmodium falciparum (1). In Iran, 90% of clinical cases are caused by Plasmodium vivax and Plasmodium falciparum is responsible for the remaining 10% (2). Merozoite surface protein-1 is one of the most common markers for genotyping of Plasmodium falciparum (3). PfMSP-1 is a glycoprotein with a molecular weight of 190-200 kDa that is synthesized during schizogony and is placed on Plasmodium falciparum merozoite surface. As this protein is the target of host immune responses, it is considered as a promising vaccine candidate (4). Based on polymorphism of the amino acids, MSP-1 is divided into 17 blocks that are protected, partially protected or variable. Each block has an allelic dimorphism K1 or MAD20, except block 2 which has additional allele of RO33 (5). Block 2 is a variable area near the amine end of this gene, and is affected by the most powerful choices to maintain alleles in the population (3, 6,). Iran is among low endemic countries for malaria in the Eastern Mediterranean region (7). Sistan-Baluchestan Province is an endemic area of falciparum malaria which is considered as eastern eco-epidemiological zone of malaria (8). The province has the largest border with Afghanistan and Pakistan (9). In this study, genetic diversity in MSP-1 block 2 was evaluated by nested-PCR in malaria endemic area of Iran, Sistan-Baluchestan Province. The results of this study can be used to control the disease and design an effective vaccine against Plasmodium falciparum.

## MATERIAL AND METHODS

In Sistan-Baluchestan Province, which has a subtropical climate, malaria transmission occurs throughout the year and its first peak is from April/May to August/September and the second peak is from September/October to November/December (10-12). In this cross-sectional study conducted from March/April 2011 to August/September 2012, 94 patients with falciparum malaria were selected and evaluated from among those attending health centers in Chabahar, Iranshahr, Nikshahr and Sarbaz. Inclusion criteria included no history of

treatment with antimalarial medications in the past month, living in the target areas for more than 6 months, patient's consent, and for children, parent's consent. Two ml venous blood was taken from each patient. Four drops of each sample was used for thick and thin smear preparation to confirm the development of Plasmodium falciparum, after smear preparation, they were stained with Giemsa and then evaluated by optical microscopy. The rest of each sample was collected in tubes containing ethylene diamine tetra-acetic acid anticoagulant (EDTA) and kept at -20 °C until the time of DNA extraction. Parasite DNA was extracted by genomic DNA extraction kit (Fermentas, Lithuania) according to kit instructions, and kept at -20 °C until the time of PCR amplification. Variable block 2 was amplified from PfMSP-1 by nested-PCR according to the guideline of Snounou et al. (13). The first stage of PCR was performed by oligonucleotides of M1-OF and M1-OR and the second stage by allele-specific primers of MAD20, K1 and RO33 families. Primer sequences are shown in Table 1. Reactions were performed in the thermal cycler (Biometra, Germany) with the final volume of 20 µl by AccuPower TLA PCR Premix (Bioneer, Korea) which included the following ingredients: the enzyme TLA DNA polymerase (1 U), Deoxynucleotide Triphosphate (250 µM), buffer 10X (2µl), stabilizer and dye tracer. After 1.5 µl DNA extracted from the samples and 0.75 µl of each primer concentration of 10 picomolar were added to a tube containing premix, the final volume was increased to 20 µl by sterile distilled water. To reduce transmission of the first stage PCR primers to the second stage (nested-PCR), only 1 µl of the first PCR product was used as template in the second reaction. The thermal cycler's temperature – time program is presented in Table 2. Plasmodium falciparum purified DNA was used as a positive control. Standard strains were purchased from Malaria Research Resources Center in Manassas, United States. Negative control also contained all PCR reaction materials except DNA. Electrophoresis of PCR products was performed in agarose gel 2% containing ethidium bromide and then was observed with a trans-luminator. Positive and negative controls and a 100 bp DNA Ladder (Fermentas, Lithuania) were used to interpret the size of bands.

Table 1- Sequences of primers used for genotyping PfMSP-1 (13) block 2

Primer	Sequences of primer	Primer characteristics
Common PCR block 2 - first stage	5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3'	M1-OF
Common PCR block 2 - first stage	5'-CTTAAATAGTATTCTAATTC AAGTGGATCA-3'	M1-OR
MAD20 family-specific – second stage of PCR	5'-AAATGAAGGAACAAGTGGAAACAGCTGTTAC-3'	M1-MF
MAD20 family-specific – second stage of PCR	5'-ATCTGAAGGATTTGTACGTCTTGAATTACC-3'	M1-MR
K1 family-specific – second stage of PCR	5'-AAATGAAGAAGAAATTA CTACAAAAGGTGC-3'	M1-KF
K1 family-specific – second stage of PCR	5'-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3'	M1-KR
RO33 family-specific – second stage of PCR	5'-TAAAGGATGGAGCAAATACTCAAGTTGTTG-3'	M1-RF
RO33 family-specific – second stage of PCR	5'-CATCTGAAGGATTTGCAGCACCTGGAGATC-3'	M1-RR

Table 2- Thermal cycler's temperature – time program

Thermal cycler's program	First stage of PCR			Second stage of PCR		
	Time	Temperature	Number of cycles	Time	Temperature	Number of cycles
Initial Denaturation	5 minutes	90° C	1	5 minutes	95° C	1
1. Denaturation	1 minute	94° C	25	1 minute	94° C	30
2. Annealing	2 minutes	58° C		2 minutes	61° C	
3. Extension	2 minutes	72° C		2 minutes	72° C	
Final Extension	5 minutes	72° C	1	5 minutes	72° C	1

## RESULTS

This cross-sectional study was conducted on 94 patients with *Plasmodium falciparum* in Sistan-Baluchestan Province. Five patients were excluded due to the negative PCR results. Overall, 98 bands and seven different variants of MSP-1 were detected in 89 patients, in which three allelic variants in MAD20 family, three allelic variants in K1 family and only one allelic variant in RO33 family were observed. The size of PCR products was 170-210 bp in the allelic family of MAD20 and 160-200 bp in the allelic family of K1. The variant 190 bp in the allelic f

amily of MAD20 and the variant 200 bp in the allelic family of K1 were the most frequent variants. Unlike two other families, no genetic diversity was observed in the RO33 allelic family and only one variant of 160 bp was detected. Among samples, 46.1% of samples belonged to the allelic family of MAD20, 35.9% to the allelic family of K1 and 7.9% to the allelic family of RO33 (Table 3). Multi-clonal infection with two allelic families (MAD20 +K1, MAD20 + RO33) was observed in 10.1% of samples, while three allelic families of MSP-1 were not observed in any sample.

Table 3- The frequency distribution of MSP-1 block 2 genotypes in Sistan-Baluchestan Province

Family	Number of samples	Size of PCR product (Bp)	Frequency (%)
MAD20	41	170-210	46.1
K1	32	160-200	35.9
RO33	7	160	7.9
Multi-clonal infection	9	-	10.1
Total	89	-	100

## DISCUSSION

The genetic structure of *Plasmodium falciparum* populations plays an important role in naturally acquired immunity against malaria (14). In this study, nested-PCR was used to evaluate the genetic diversity of PfMSP-1 in Southeastern Iran. Studies show that the sensitivity and specificity of this method are at most 94% (15), that produces more products compared to other PCR methods in our subject field, and is very affordable unlike sequencing method. Malaria transmission is low and seasonal in Southeastern Iran and infections with clinical signs occur most often in adults (16). In this area, a limited genetic diversity of PfMSP-1 was detected. Seven different variants were observed at this locus, which is less than those in hypoendemic areas of Pakistan with 25 genotypes and Myanmar with 14 genotypes. In contrast, our findings are similar to a study conducted in Peru, in which seven different alleles were identified at MSP-1 locus (6, 17, 18). The difference in the number of genotypes in two neighboring countries, Iran and Pakistan, shows the fact that *falciparum* malaria in these countries has separate origins and this species of malaria is endemic in the region. In this study, all three allelic families of MSP-1 (K1, MAD20 and RO33) were observed; however, MAD20 was the predominant allelic family. In similar studies in Iran, Pakistan, Myanmar, Bangladesh and Colombia, MAD20 family also had the highest frequency (6, 16, 17, 19, 20), but K1 was the predominant allelic family in studies conducted in Laos, Zambia, Gabon, Congo and other African countries (21-24). In this study, monomorphic bands in electrophoresis of the RO33 allele family were observed on agarose gel. This finding is in contrast to studies conducted in Bangladesh and Togo, where the

RO33 family was polymorphic with four variants (19, 25). RO33 monomorphic bands were also observed in sub-Saharan African countries, Haiti, East and Northeast India (26-28). In the study of Heidari et al, nine alleles and a relatively large polymorphism were observed at the PfMSP-1 locus in Southeastern Iran, whereas in the study of Zakeri, the vast genetic diversity and a large percentage of multi-clonal infection were reported in block 2 of this locus in this region (8,16). It seems that reduced parasite transmission can be attributed to drought and reduced immigration from neighboring countries, especially Afghanistan, in recent years. Therefore, it is suggested that the genetic diversity in block 2 of this gene be evaluated in the coming years and at different intervals to obtain an acceptable reason for different results of researchers in this area by following up the process of genetic changes in this block.

## CONCLUSION

Genetic diversity in block 2 of this gene in Southeastern Iran is relatively low and most infections are composed of a clone that is compatible with a low transmission malaria zone.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest between them.

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