

## Genetic Diversity of Variable Region Block 2 in the Merozoite Surface Protein-1 (MSP1) in *Plasmodium falciparum* Field Isolates from South-East of Iran

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### Abstract

Merozoite surface protein 1 of *Plasmodium falciparum* (PfMSP-1) is a leading malaria vaccine candidate. However, extensive genetic diversity of this gene in field isolates of *P. falciparum* represents a major obstacle for the development of an effective vaccine against malaria. The present study was aimed at analysing genetic polymorphisms of K1, MAD20 and RO33 allelic types of MSP-1 block 2 among *P. falciparum* isolates from Sistan and Baluchestan, Iran.

In this study a total of 94 *P. falciparum* infected persons from Sistan and Baluchistan Province of Iran, were included. Blood samples were collected from March 2011 to September 2012. Block 2 of the MSP-1 gene was genotyped by allele-specific nested polymerase chain reaction (PCR) after DNA extraction. Eighty-nine (94.7%) of the 94 samples were successfully amplified; 7 distinct MSP-1 genotypes were identified by size differences on agarose gels. MAD20 was the predominant MSP-1 allelic family identified in 46.1% (41/89) of the samples while RO33 family had the least frequency (7.9%). A total of 9/89 (10.1%) samples exhibited multiple infections with two alleles at PfMSP-1. The present study shows that the level of genetic diversity is relatively low in south-east of Iran and most of infections are composed of one clone, which is consistent with an area of low malaria transmission. These data are useful for malaria prevention and control in Iran.

### Key words

Genetic diversity; Merozoite Surface Protein -1; *Plasmodium falciparum*; Iran.

### Introduction

Malaria is caused by four species of the genus *Plasmodium* protozoa. It is one of the most threatening parasitic diseases in man [1]. Although many efforts have been made to control this infectious disease, the global burden is still estimated at 216 million clinical cases every year resulting into 655,000 deaths [2]. The most malignant form of malaria is caused by *Plasmodium falciparum* [1]. In Iran, the majority of the clinical cases of malaria are caused by *Plasmodium vivax* (86%), while *P. falciparum* is responsible for the remaining 14% [3].

One of the most commonly used markers for genotyping of *P. falciparum* is merozoite surface protein 1 (MSP-1). Genetic polymorphism of this gene is thought to be a major obstacle for malaria vaccine design [4]. PfMSP-1 is synthesized during schizogony as a 190-200 kDa glycoprotein, located at the surface of *P. falciparum* merozoite and is a target of the host's immune response, thus this is considered as a strong vaccine candidate [5].

Based on the degree of amino acid substitution, MSP-1 gene has been divided into 17 sequence blocks that are either conserved (semi-conserved) or variable. The semi-conserved and variable regions are generally dimorphic for either K1 or MAD20; except for the block 2

which has an additional allele RO33, and block 4 which has one of four possibilities: MM, KK, MK, or KM [6]. Block 2 is a highly variable region near to the N-terminus of the gene and is under the strongest selection to maintain alleles within populations [4,7].

Iran is one of the countries placed in the Eastern Mediterranean Region which malaria endemicity is low in some of its regions [8]. Sistan and Baluchistan Province, in the South-East of Iran, is the *falciparum* malaria endemic area and considered as the oriental eco-epidemiological region of malaria [9].

In this study, we have analysed the genetic diversity in MSP-1 block 2 using nested PCR in Sistan and Baluchestan, Iran.

### Materials and Methods

Sistan and Baluchistan Province lies on the south-east of Iran and the largest province in the country, with an area of 181,785 Km<sup>2</sup> and a population of around 2.5 million, at the time of study [10,11]. This province has a subtropical climate, bordering Pakistan and Afghanistan from the east and Oman Sea from the south [10]. Malaria transmission occurs during the whole year with two peaks of May to August and October to November. *P. vivax* is the dominant species during the first peak; in second peak both *P. vivax* and *P. falciparum* infections are usually reported [12]. This survey was carried out on the population seeking care at the malaria health centers of Sistan and Baluchestan Province from March 2011 to September 2012.

Selection criteria of the cases were as follows: no history of treatment with anti-malarial drugs within the last month, residence in considered regions for over 6 months, patient satisfaction and signed consent form by older cases and parents of the younger ones. However 94 *P. falciparum* infected individuals who lived in four main districts of the province (Chabahar, Iranshahr, Nikshahr and Sarbaz) were included in the study.

About 2 ml of venous blood was taken from each patient. In order to confirm the presence of *Plasmodium falciparum* parasite, four drops of each sample was used for preparation of thick and thin blood smears. Then smears were stained by Giemsa method and evaluated by light microscopy. The remaining of each sample was collected in tubes containing EDTA and stored at -20°C until use.

Parasite genomic DNA was prepared using Fermentas Genomic DNA Purification Kit (Thermo Fisher Scientific Inc), according to the manufacturer's instructions and stored at -20°C until use.

The variable block 2 region of MSP-1 was amplified using a previously described nested PCR protocol [13]. An initial amplification of the outer regions of block 2 was carried out using a pair of oligonucleotides M1-OF: M1-OR. Allelic family specific primers (MAD20, K1 and RO33) were used in second amplification reaction. The sequence of the primers is presented in Table 1.

Primer	Sequence 5'→3'	Specificity
M1-OF	CTAGAAGCTTTAGAAGATGCAGTATTG	Common block 2 – Nest 1
M1-OR	CTTAAATAGTATTCTAATTC AAGTGGATCA	Common block 2 – Nest 1
M1-MF	AAATGAAGGAACAAGTGG AACAGCTGTTAC	MAD20 family-specific – Nest 2
M1-MR	ATCTGAAGGATTTGTACGCTCTTGAATTACC	MAD20 family-specific – Nest 2
M1-KF	AAATGAAGAAGAAATTACTACAAAAGGTGC	K1 family-specific – Nest 2
M1-KR	GCTTGCATCAGCTGGAGGGCTTGCACCAGA	K1 family-specific – Nest 2
M1-RF	TAAAGGATGGAGCAAATACTCAAGTTGTTG	RO33 family-specific – Nest 2
M1-RR	CATCTGAAGGATTTGCAGCACCTGGAGATC	RO33 family-specific – Nest 2

**Table 1:** Sequences of the oligonucleotide primers used to genotype block 2 of PfMSP-1[13].

Amplifications were performed on a thermal cycler (Biometra GmbH, Germany). The purified DNA from *Plasmodium falciparum* 3D7, 7G8 and Dd2 strains was provided by the Malaria Research and Reference Reagent Resource Center, American Type Culture Collection (Manassas, VA) and used as positive controls during all amplification reactions.

PCR products were electrophoresed on 2% agarose gels, and DNA visualized by UV transillumination after ethidium bromide staining. Positive controls and a 100bp Ladder Marker (Fermentas, Lithuania) were used to interpret the fragments sizes.

## Results

In all, 94 patients with *P. falciparum* infection were selected in this descriptive cross-sectional study, 67 percent of them were male. Five patients were excluded from the study due to negative PCR outcome.

A total of 98 distinct fragments and seven different MSP-1 variants were detected among 89 patients, representing MAD20 (three variants), K1 (three variants) and RO33 (one variant) allelic families. The length variants of the PCR product were 170-210 base pairs (bp) for MAD20 and 160-200 bp for K1 (Table 2).

Family	No. of samples	PCR product size (bp)	Frequency (%)	Family	No. of samples	PCR product size (bp)
MAD20	41	170-210	46.1	MAD21	41	170-211
K1	32	160-200	35.9	K2	32	160-201
RO33	7	160	7.9	RO34	59.2	59.65
K1+MAD20	7	-	7.9	K1+MAD21	7	-
MAD20+RO33	2	-	2.2	MAD20+RO34	2	-
Total	89	-	100	Total	89	-

**Table 2:** Distribution of MSP-1 block 2 genotypes in Iran

Unlike the other two family types, the RO33 family did not show any polymorphism, only one variant (160 bp) was observed. The

variant 190 bp of MAD20 and 200 bp of K1 allelic family MSP-1 demonstrated the most frequency.

The proportions of MAD20, K1 and RO33 types were 46.1%, 35.9% and 7.9%, respectively. Two allelic types (K1/MAD20, MAD20/RO33) were observed in 10.1% of infections, whereas no sample contained all three allelic types of MSP-1.

## Discussion

Genetic structure of *P. falciparum* populations has an important role in the natural acquisition of immunity in malarial infections [14]. Therefore, survey on genetic structure of parasite population in clinical isolates from different endemic areas is necessary to control the disease and design of effective vaccines against *P. falciparum*.

In this study, nested PCR were used to screen allelic variations within the malaria vaccine candidate PfMSP-1 in south-east of Iran. Because nested PCR has been shown to exhibit a sensitivity and specificity of up to 94% in some trials [15], possesses a high-throughput capacity in comparison to other PCR modifications in this field of study, and is considerably more cost-efficient versus sequencing, we decided to adapt it to screen for *P. falciparum* MSP-1 N-terminal region variations.

Malaria transmission in south-east of Iran is low and seasonal with mainly symptomatic infections in adults [16]. A limited genetic diversity of PfMSP-1 block 2 was identified in this area. Seven different genotypes were identified at MSP-1 locus, which showed lower rates than that of a similar report in hypoendemic regions of Pakistan (25 genotypes) and Myanmar (14 different alleles); in contrast our findings was similar to Peru's trials, where seven different alleles of MSP-1 were detected [7,17,18]. In the present study all three families of MSP-1 (K1, MAD20 and RO33) were observed. The predominant family was MAD20. These findings are similar to previous studies in Iran, Pakistan, Myanmar, Bangladesh and Colombia which demonstrated the predominance of the MAD20 [7,16,17,19,20]. By contrast, previous studies in Lao PDR, Zambia, Gabon, Congo and other African countries showed that K1 was the predominant allelic family [21-24]. Monomorphic band of RO33 allelic types in Iran were observed on agarose gel electrophoresis. This result differs from that of Bangladesh and Togo, where the RO33 family was polymorphic with four fragments [19,25]. Monomorphic band of RO33 was also identified in sub-Saharan Africa, Haiti, eastern and north eastern India [26-28].

In the study of Heidari, nine different variants of MSP-1 and relatively high polymorphisms were observed in *P. falciparum* isolates collected in south-east of Iran, whereas in the study conducted by Zakeri, high frequency of multiple genotypes and extensive genetic diversity were reported in the same location [9,16,29]. It seems that decrease in the rate of parasite transmission because of drought, decrease in immigration from neighboring countries (especially Afghanistan) that occurred in recent years are reasons for this finding.

This study provides information on the genetic diversity of MSP-1 and the prevalence of MSP-1 alleles in *P. falciparum* field isolates from Iran. Comparison of this information with reports from other geographical areas can be useful in designing an effective malaria vaccine.

## Conclusion

A major finding of this study was that natural populations of *P. falciparum* in isolates from Iran exhibited relatively low genetic

diversity in MSP-1 block 2. Moreover, the majority of infections were monoclonal with predominance of MAD20 allelic family of MSP-1.

Our findings are compatible with the general belief that Iran represents a low malaria transmission setting. These data are useful for malaria prevention and control in this area.

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