RESEARCH PAPER | Medical Science | Volume : 5 | Issue : 9 | September 2015 | ISSN - 2249-555X

Interallelic Polymorhisms of Three Different Blocks of the C- and N-Terminal of PfMSP-1 (Plasmodium falciparum Merozoit Surface Protein 1) 2, 4 &17 from Field Isolates in South-East of Iran

KEYWORDS: Interallelic polymorphism, Merozoite Surface Protein-1, Plasmodium falciparum, Nested-PCR, C-terminal Region, Iran

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ABSTRACT *Malaria is still a public health problem in Iran as an endemic area. Many malarial proteins have been proposed for use as a vaccine candidate antigen, but merozoite surface protein-1, Plasmodium falciparum (PfMSP-1) is the most used. It is a 180-200 kDa glycoprotein with 17 blocks from N- to C-terminal, some variable, some semi and some constant. The present study was aimed at analysing interallelic polymorphisms of MSP-1, blocks 2 , 4 (N-terminal) and 17 (C-terminal) of three different regions, of MSP-1 gene among Plasmodium falciparum field isolates in southeast Iran. A total of 94 P. falciparum patients , were included in this descriptive cross-sectional survey. Blood samples were collected from March 2011 to September 2013. Eighty-nine cases (94.7%) of the 94 samples were successfully amplified. Genomic DNA was extracted and genetic polymorphism of PfMSP-1 blocks 2 , 4 and 17 was investigated using nested polymerase chain reactions (PCR). Two alleles of MSP-1, block 2 (K1 and MAD20) were observed and all four alleles of MSP1- block 4 genotypes and both MAD20 and K1 types of MSP-1, block 17, respectively. The block17 is one of the most important candidate region for Malaria Vaccine which obtaining MSP1-19 KDa. This area has a specific antibody epitope which plays as an inhibitor roll in invitro and invivo assay. These data are useful for malaria prevention and control in Iran.*

Introduction

Malaria is the most common infectious disease in tropical regions, according to the World Health Organization status (WHO 2011). [1, 2]. The *Plasmodium falciparum*, it is one of the most threatening parasitic diseases in man [4]. 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 [3, 4, 5]. The most malignant form of malaria is caused by *Plasmodium falciparum* [6]. 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% [7]. A declining trend has been noted in the number of malaria cases in Iran in the past few years; based on the 2009 World Health Organization (WHO) malaria report [8]. The elimination programs are often set up in areas where a really low rate of malaria transmission is reported and therefore such a goal would be achievable, so Iran is among countries where implementing appropriate plans could lead to the elimination of the disease [9, 10, 11, 12]. 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 [13]. *Pf*MSP-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 [14]. 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 [13-16]. Iran is one of the countries placed in the Eastern Mediterranean Region which malaria endemicity is low in some of its regions [17, 18]. 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 [19]. In this study, we have analysed the genetic diversity in MSP-1 blocks 2, 4 and 17 using nested PCR. [20, 21]. This province has a subtropical climate, bordering Pakistan and Afghanistan from the east and Oman Sea from the south [20]. 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 [22]. Thus, *Plasmodium malariae* has been existed in the study area for many years, but during three to four decayes has not exhibited except few cases which Ebrahimzadeh *et al., 2008,* reported using nested PCR [23]

2. Materials and Methods

2.1 Ethical issue

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 or parents of the younger ones. However, finally during two peaks serial randomly.

2.2 Study area and Sampling

This survey was carried out on the population seeking care at the malaria health centers in Chabahar, Iranshahr, Nikshahr and Sarbaz from March 2011 to September 2013. These districts are the endemic foci of malaria in Iran. In all, 94 patients were selected in this descriptive cross-sectional study, 67 percent of them were male. Five patients were excluded from the study due to negative

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PCR outcome.

2.3 DNA Extraction

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. Genomic DNA was extracted and genetic polymorphism of *Pf*MSP-1 gene was investigated by nested polymerase chain reactions (PCR).

2.4 Nested PCR Amplifications

The variable block 2, 4 and 17 regions of MSP-1 were amplified using a previously described nested PCR protocol [22]. An initial amplification of the outer regions of block 2 was carried out using a pair of oligonucleotides M1-OF: M1-OR and allelic family specific primers (MAD20, K1 and RO33) in second nested PCR were used as it showed in Table 1.

Table 1. Representive spicific sequences of primers used in first reaction of nested PCR for genotyping of block 2, 4 and 17 (19 KDa) of *PfMSP-1* gene, MAD20 and K1 alleles (22, 35, 42).

Primer	Sequence 5′→3′	Features of primer	Specificity
M1-OF	CTAGAAGCTTTAGAAGATGCAGTATTG	Nested 1	Common block 2
M1-OR	CTTAAATAGTATTCTAATTCAAGTGGATCA	Nested 1	Common block 2
C3F	5′-TTCGTGCAAATGAATTAGACGTAC-3′	Nested 1	A member of block 3
C5R	5′-GGATCAGTAAATAAACTATCAATGT-3′	Nested 1	A member of block 5
MAD20F	5′-GCAATATCTGTCACAATGG-3′	Nested 1	block 17(19KDa)-MAD20
K1F	5′-GCAG TAACTCCTTCCGTAATTG-3′	Nested 1	block 17(19KDa)- K1
UR	5′-TTAGAGGAACTGCAGAAAATACCA-3	Nested 1	Universal Reverse

The semivariable block 4 located in the N-terminal region of gene and in 83-kDa polypeptide [24-26]. Block 4 of the *PfMSP-1* gene was reproduced with the same method and based on Kaneko and colleagues guidelines [26]. The first stage PCR using C3F and C5R olygonucleotide (Table 1). And as the same for the 3 chosen blocks the second stage was performed using specific primers K1 and MAD20 allelic (Table 2)

For amplification block 17 of MSP-1 and final segment of proteolytic procceses as a C-terminus which obtaining *PfMSP-1* – 19 kDa using same PCR methods and according to Sutton & colleagues instruction were duplicated [26]. Primer sequences are presented for the first stage of nested PCR in Table 1 and for the second step you can check in Table 2.

Table 2 : Sequences of specific primers used for genotyping of *PfMSP-1* genes, for block 2,4 and 17 in the second step of nested PCR (22, 35, 42)

Primer	Sequence 5′→3′	Features of primer	Specificity
M1-MF	AAATGAAGGAACAAGTGGAACAGCTGTTAC	Nested 2	block 2-MAD20
M1-MR	ATCTGAAGGATTTGTACGTCTTGAATTACC	Nested 2	block 2-MAD20
M1-KF	AAATGAAGAAGAAATTACTACAAAAGGTGC	Nested 2	block 2-K1
M1-KR	GCTTGCATCAGCTGGAGGGCTTGCACCAGA	Nested 2	block 2-K1
M1-RF	TAAAGGATGGAGCAAATACTCAAGTTGTTG	Nested 2	block 2-RO33
M1-RR	CATCTGAAGGATTTGCAGCACCTGGAGATC	Nested 2	block 2-RO33
K4AF	5′-AATGAAATTAAAAATCCCCCACCGG-3′	Nested 2	block 4-K1
M4AF	5′-TTGAAGATATAGATAAAATTAAAACAGATG-3	Nested 2	block 4-MAD20
K4PR	5′-TCCTCGATTTTTTTGTTCTTATCAAG-3′	Nested 2	block 4-K1
M4PR	5′-TCGACTTCTTTTTTCTTATTCTCAG-3′	Nested 2	block 4-M AD20
MAD20F	5′-CCATAACGACTTCGAAGC-3′	Nested 2	block 17(19KDa)-MAD20
K1F	5′-CGTTGGAATTGCTGATTTATCAACAG-3′	Nested 2	block 17(19KDa)-K1
UR	5′-TTAGAGGAACTGCAGAAAATACCA-3′	Nested 2	Universal Reverse

Results and discussion

3.1 Results of Block two *of PfMSP-1*

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 3). 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

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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 (Figures 1, 2, 3) .

Table 3: Distribution of MSP-1 block 2 genotypes in Iran

Family	No. of samples	PCR product size (bp)	Frequency (%)
MAD20	41	170-210	46.1
K1	32	160-200	35.9
RO33	7	160	7.9
K1+MAD20 7 - 7.9			
MAD 20+RO33	2	-	2.2
Total	89	-	100

Figure 1: Schamatic presentation of PfMSP1 block 2, MAD20 allele in 1.8% Agarose gel
(lane M, 100bp ladder Marker, L1 Positive Co, 190 bp, L2 Negative Co, L3-7 Positive cases in study area)

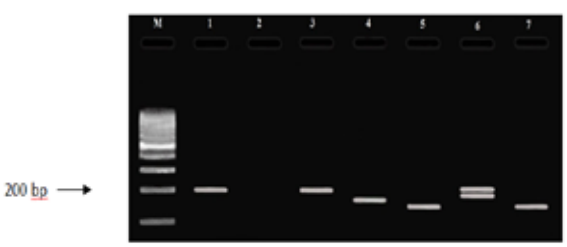

Figure 2: Schamatic presentation of PfMSP1 block 2, K1 allele in 1.8% Agarose gel(lane M, 100bp ladder Marker, L1 Positive Co, 200 bp, L2 Negative Co, L3-7 Positive cases in study area)

Figure 3: Schamatic presentation of PfMSP1 block 2, RO33 allele in 1.8% Agarose gel
(lane M, 100bp ladder Marker, L1 Positive Co, 160 bp, L2 Negative Co, L3-7 Positive cases in study area)

3.2 Results of Block Four of PfMSP-1

In the block four allelic forms were observed as: MM, KK, MK and KM. The size fragments and frequency for MM, 97 bp (21.3%), KK 80 bp (24.8%), MK 94 bp (25.8) and KM 83 bp (16.9%) were observed, respectively (Table 3). Multi-clonal infections in 11.2% of the samples were found. Therefore, despite the total number of samples was 89, But the frequency of fragments in these patients was 103, which indicate that some patients have infection with two or more genotypes (Table 4, Figures 4, 5, 6) .

Table 4 : Distribution of genotype frequencies in Block 4 of *PfMSP-1* gene in Sistan and Baluchestan Province

Genotype	Frequency	%
MK	23	25.8
KK	22	24.8
MM	19	21.3
KM	15	16.9
MK+ KM	6	6.8
MM+ MK+ KK	3	3.3
MM+ KM+ KK	1	1.1
Total	89	100

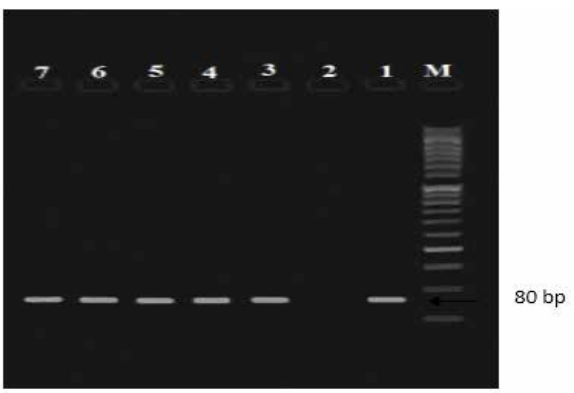

Figure 4: Schamatic presentation of *PfMSP1* block 4, KK allele in 1.8% Agarose gel
(lane M, A 50bp ladder Marker, L1 Positive Co, 80 bp, L2 Negative Co, L3-7 Positive cases in study area)

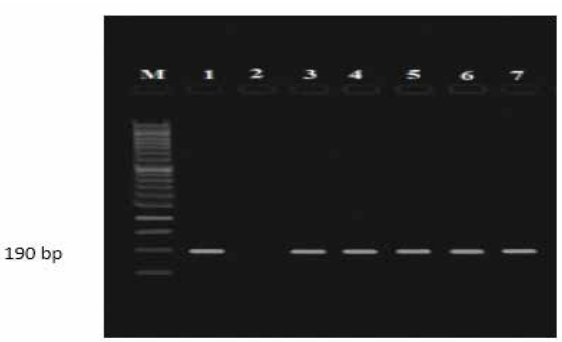

Figure 5 : Schamatic presentation of *PfMSP1* block 4, MM allele in 1.8% Agarose gel
(lane M, A 50 bp ladder Marker, L1 Positive Co, 190 bp, L2 Negative Co, L3-7 Positive cases in study area)

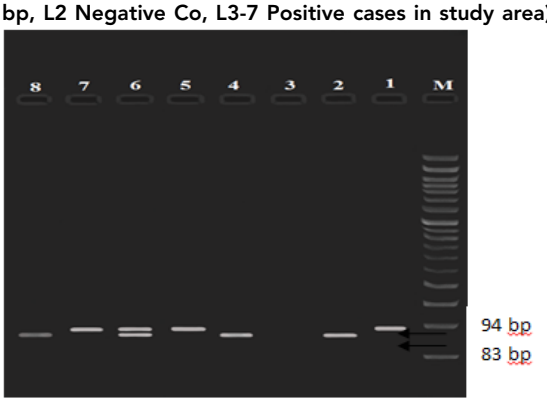

Figure 6 : Schamatic presentation of *PfMSP*1 block 4,

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MK and KM alleles in 1.8% Agarose gel
(lane M, A 50bp ladder Marker, L1 Positive Co, for MK allele 94 bp, , L2 positive Co, for KM allele 83bp, L3 Negative Co, L4-8 Positive cases in study area)

3.3 Results of Block17 *of PfMSP-1*

PCR amplification showed alleles and variants in the block 17, were two cases, Both K1 and MAD20 alleles. Overall, 69 patients (77.5%) belonged to the MAD20 family allele, And 20 patients (22.5%) belonged to the K1 allelic family (Table 5 and Figures 7, 8).

Table 5: Distribution of allele frequency and variants in C-terminal *P fMSP-1* region, in terms of in sampling in different regions of Sistan and Baluchestan

Region / Allele	Province		Sarbaz		Nikshahr		Iranshahr		Chabahar	
	%	frequency	%	Frequency	%	Frequency	%	frequency	%	frequency
MAD20	77.5	69	13.5	12	21.3	19	19.1	17	23.6	21
K1	22.5	20	4.5	4	4.5	4	5.6	5	7.9	7
Total	100	89	18	16	25.8	23	24.7	22	31.5	28

Figure 7: Schamatic presentation of *PfMSP1* block 17, MAD20 allele in 1.8% Agarose gel
(lane M, 50 bp ladder Marker, L2 Positive Co, 450 bp, L1 Negative Co, L6-7 Positive cases in study area

Figure 8: Schamatic presentation of *PfMSP1* block 17, K1 allele in 1.8% Agarose gel
(lane M, 50 bp ladder Marker, L1 Positive Co, 450 bp, L2 Negative Co, L3-6 Positive cases in study area)

3.4 Discussion

Genetic structure of *P. falciparum* populations has an important role in the natural acquisition of immunity in malarial infections [21]. 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 *Pf*MSP-1 in south-east of Iran. The nested PCR has been shown to exhibit a sensitivity and specificity of up to 94% in some trials [22], possesses a high-throughput capacity in comparison to other PCR modifications in this field of study, and is considerably more cost-efficient versus sequencing. This survey aimed to adapt and screen interallelic polymorphisms of three different region of MSP-1 for *P. falciparum*. Malaria transmission in south-east of Iran is low and seasonal with mainly symptomatic infections in adults [22]. A limited genetic diversity of *Pf*MSP-1 block 2 and semi-variable block4 and C-terminus block 17 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 [29-31]. In the present study all three families of MSP-1 (K1, MAD20 and RO33) were observed. The predominant family was MAD20. The best candidates for malaria vaccine , which are against immunogen and conserved protein region and have limited genetic diversity. Section 19 kDa C-terminal region of *PfMSP-1* candidate for vaccine. In *invitro* and *invivo* Studies have shown, antibodies against the 19 kDa polypeptide inhibit the invasion of merrozoits to red blood cells and block the parasite life cycle [32]. Therefore, to control malaria and designing vaccine for *Plasmodium falciparum*, the genetic diversity of this part of the protein in clinical isolates from different endemic areas should be studied. The transmission of malaria in south eastern Iran, is low and seasonal, and clinical signs of infection usually occurs in adults [33]. To study the C-terminal region of MSP-1 19 kDa polypeptide that is located in this area, using specific primers of MAD20 and K1 allelic family.

These findings are similar to previous studies in Iran, Pakistan, Myanmar, Bangladesh and Colombia which demonstrated the predominance of the MAD20 [24, 29 ,30 , 34, 35]. By contrast, previous studies in Lao PDR, Zambia, Gabon, Congo and other African countries showed that K1 was the predominant allelic family [36-37]. 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 [34-36]. Monomorphic band of RO33 was also identified in sub-Saharan Africa, Haiti, eastern and north eastern India [35].

In the study of Heidari *et al.,* (2007), 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 *et al.,* (2005), high frequency of multiple genotypes and extensive genetic diversity were reported in the same location [22, 35]. 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.

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In this study, 11.2% of the samples contain multi-clonal infections, this value compared to the multi-clonal infections were observed in Iran, 80% in 5 'region of the *PfMSP-1* gene, Philippines with 33% , Colombia with 5.79% , Thailand with 5.60% and Vietnam with 43% showing less value. But towards the island of Vanuatu with 7.0%, indicating a higher rate [26, 27, 38, 39, 40, 41]. Differences in the epidemiology of malaria among that countries and Iran can be one of the reasons, for low multi-clonal infections in this region. This study confirms the occurrence of recombination in Block 4. As we know recombination cause increasing in diversity in *PfMSP-1*. This study provides information on the genetic diversity of MSP-1 and the prevalence of MSP-1 alleles in *P. falciparum* field isolates from south east Iran. Comparison of this information with reports from other geographical areas can be useful in designing an effective malaria vaccine.

3.5 Conclusion

This research provide an important information about genetic polymorphisms of *PfMSP-1* in Iran. A major finding of this study was that natural populations of *P. falciparum* in field isolates from Iran exhibited relatively low genetic diversity in MSP-1 block 2. The Genetic variation in the MSP-1 block 4 is relatively low in the southeast, of Iran. Moreover, the majority of infections were monoclonal with predominance of MAD20 allelic family of MSP-1. And about genetic polymorphisms in the C-terminal PfMSP-1 this study showed that the genetic diversity of C-terminal region of this gene is limited in southeastern of Iran and as it said before ,The best candidates for malaria vaccine , which are against immunogen and conserved protein region and have limited genetic diversity. Section 19 kDa C-terminal region of *PfMSP-1* is a potential candidate for vaccine because antibodies against the 19 kDa polypeptide inhibit the invasion of merrozoits to red blood cells and block the parasite life cycle.

ACKNOWLEDEMENTS

This study was supported from Zahedan University of Medical Sciences with Thesis NO: 2501.

We are specifically grateful to Dr Maliheh Metanat, Managing Director of Research Center for Infectious Diseases and Tropical Medicine, Zahedan University of Medical Sciences, for her unending assistance during the study and also of Mr Gharayi for their sincere cooperation in sampling.

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