

Genetic diversity in merozoite surface protein (MSP)-1 and MSP-2 genes of *Plasmodium falciparum* in a major endemic region of Iran

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Abstract: Merozoite surface protein-1 (MSP-1) and merozoite surface protein-2 (MSP-2) were used to develop vaccines and to investigate the genetic diversity in *Plasmodium falciparum* malaria in Iran. Nested polymerase chain reaction amplification was used to determine polymorphisms of block 2 of the MSP-1 and the central domain of MSP-2 genes. A total of 67 microscopically positive *P. falciparum* infected individuals from a major endemic region, south-east Iran, were included in this trial. Nine alleles of MSP-1 and 11 alleles of MSP-2 were identified. The results showed that amplified product from these surface antigen genes varied in size and there was specific pattern for each isolate. Besides, regarding this pattern, 23 multiple infections with at least 2 alleles were observed. While the endemic regions of malaria in Iran is classified in low to moderate group, but extensive polymorphism was observed for each marker and the MSP-2 central repeat was the most diverse that could be considered in designing malaria vaccine.

Key words: *Plasmodium falciparum*, merozoite surface protein-1 (MSP-1), MSP-2, malaria, Iran

Malaria is a major public health problem and is associated with 300-500 million clinical cases worldwide as well as 0.5-3 million deaths annually, almost all of them are caused by *P. falciparum* (Ferreira et al., 2004; Phillips, 2000). Genetic diversity presented by *P. falciparum* field isolates, the occurrence of variant forms of the parasite in different geographic areas, and occultation of multiple genotypes during a single mosquito, constitute one of the main obstacles to the design of a malaria vaccine (Raj et al., 2004; Moor et

al., 2002).

Merozoite surface protein (MSP)-1 and merozoite surface protein-2 (MSP-2) are 2 proteins causing immune responses in humans (Taylor et al., 1995; Aubouy et al., 2003) and are important candidates for development of blood stage malaria vaccines (Moorthy and Hill, 2002). The block 2 MSP-1 is particularly polymorphic and 3 distinct allelic families have been described as Mad 20, K1 and Ro33 (Contamin et al., 1996). The polymorphic central domain of the gene encoding MSP-2 belongs to 2 distinct families; Ic and Fc27 (Sallenave et al., 2000). Allelic forms of these antigen genes have been reported from different parts of the world (Babiker and Waliker, 1997; Jordan et al., 2001) and further characterization of the degree of polymorphism in these antigens will be of interest for

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appropriate design of malaria vaccine.

The tribulations encountered in Sistan and Baluchistan Provinces, Iran, are resistance of *P. falciparum* to drugs (Edrissian et al., 1993; Eskandarian et al., 2002; Jafari et al., 2003), and that of vectors to insecticides, likewise importation of malaria mostly of *P. falciparum* originating from Afghani and to some lesser extent, from Pakistani immigrants. Hence, designing efficient malaria vaccine is useful for control of falciparum malaria in this area. We investigated the polymorphic nature of these vaccine candidate antigen genes and complexity of *P. falciparum* infection among field isolates in an endemic area of Iran. In this study, nested polymerase chain reaction (PCR) was used for genotyping *P. falciparum* isolates.

Iran is located in the Eastern Mediterranean Region, and grouped as low-moderate endemic region (Rakhshani, 2003). Sistan and Baluchistan Province, southeast Iran, is the endemic area of falciparum malaria and is considered as the oriental eco-epidemiological region of malaria (Sadrizadeh, 1999). It is bordered by Pakistan and Afghanistan. Prevalence of malaria has been 1,382 cases in Iran in 2004 (WHO, 2005).

This study involved 67 resident subjects aged from 2 to 45 years. Sample collection was carried out in 2004. *P. falciparum* malaria patients attending randomly local malaria clinics and health centers were enrolled in this study. Residence in the regions for over 6 mo, no history of anti-malarial treatment for the last month, and written informed consent were required for inclusion in this study. This study was approved by the Ethical Review Committee of Research in Tehran University of Medical Sciences, Iran.

Diagnosis of *P. falciparum* was confirmed by light microscopy on thick blood smears. Origin, clinical signs, age, sex and parasitemia (number of asexual parasites per μ l of blood) of each patient were recorded. Blood was collected in tubes containing anti-coagulant solution and stored at -20°C until used. Isolation of DNA and genotyping were performed in the Institute of Tropical Medicine of Berlin, Germany, in 2005.

DNA was extracted from the blood sample by QIAamp DNA mini Kit (Qiagen kit, Germany). PCR was performed following a 2-step amplification scheme, in which the product of the first reaction (outer PCR) was used as the template for the second reaction (nested PCR) for both MSP-1 and MSP-2, the primers used in the first amplification reaction were conserved among all isolates. Allelic family-specific primers were used in second amplification reaction for block 2 of MSP-1 corresponding to Mad 20, K1 and Ro33 allelic families, and Fc27 and Ic for the central region of MSP-2. The sequences of the primers are listed elsewhere (Arez et al., 1999). For amplification, 2 μ l DNA was used in outer PCR in total reaction volume of 26 μ l containing 50 mM KCl; 1.5 mM MgCl_2 ; 125 μ M of each dNTP (Invitrogen); 1 unit of Taq and pair primers (160 nM each). This reaction was amplified for 35 cycles at 94°C for 1 min, 58°C for 2 min and 72°C for 2 min. Two μ l of DNA product from the outer PCR reaction was used in nested PCR in a total reaction volume of 50 μ l containing 50 mM KCl; 1.5 mM MgCl_2 ; 175 μ M of each dNTP; 1.5 unit of Taq and the 3 and 2 pairs of primer (160 nM each) separately for MSP-1 and MSP-2, respectively.

Samples were amplified for 30 cycles at 94°C for 1 min, 61°C for 2 min and 72°C for 2 min. The PCR amplified gene fragments of MSP-1 and MSP-2 were electrophoresed on 3% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. Chi-square test was applied to analyze the results.

Sixty percent of studied subjects were males. Parasitemia in patients ranged from 500 to 30,000 parasites/ mm^3 (Mean 11,700). All samples were analyzed for polymorphisms on MSP-1 and MSP-2 genes. Among the fragment of 67 samples at the MSP-1 locus 40, 31 and 9 fragments belonged to K1, Mad 20 and Ro33 families, respectively. For the MSP-2 locus, 37 and 46 fragments were identified as FC27 and IC families, respectively (Table 1). However, the variant 160 bp of Ro33, 180 bp of K1 and 190 bp of Mad 20 allelic family MSP-1, and variant 400 bp of Fc27 and 470 bp of Ic for MSP-2 demonstrated the most frequency. Thirteen (19.4%) samples of MSP-1 and 15 (44.6%) of

Table 1. Distribution of different allele types of merozoite surface protein (MSP)-1 and MSP-2 genes of *Plasmodium falciparum* in Southeast of Iran

	MSP-1			MSP-2		Total
	K1	Mad 20	Ro33	Fc27	Ic/3D7	
No. of distinct bands	4	4	1	6	5	20
No. (%) of samples containing alleles of the corresponding family	40(59/7)	31(46/2)	9(13/4)	37(68/6)	46(55/2)	-
Length of variants PCR product (bp)	160-220	150-210	160	200-400	400-600	-

MSP-2 contained multiclonal infection at least with 2 clones and in total 23 samples (34.3%) illustrated multiclonal infection. There was no significant association between sex, age, parasitemia, and origin of patients with alleles for the 2 mentioned genes.

This study illustrated 9 and 11 different variants at MSP-1 and MSP-2 loci, respectively, which showed higher rates than that of a similar report in a hypoendemic region in Colombia (Montoya et al., 2003), where 1 allele of MSP-1 and 3 alleles of MSP-2 were detected. A common factor of this work and the present study is the low malaria endemicity compared to Thailand or African countries. In Thailand (Snounou et al., 1999), 10 alleles of MSP-1 and 17 alleles of MSP-2 were observed, whereas in Gabon (Aubouy et al., 2003), 25 alleles of MSP-1 and 19 alleles of MSP-2, and in West Uganda (Peyerl et al., 2001), 22 alleles of MSP-1 and 12 alleles for MSP-2 were reported. However, the Ro33 family of MSP-1 did not show any polymorphism, with only 1 variant (160 bp) detected. This result differs from that of Gabon and West Uganda, where the Ro33 family was polymorphic with 3 and 4 fragments, respectively (Aubouy et al., 2003, Peyerl et al., 2001), but was close to that in Senegal (Zwetyena et al., 1998) and Brazil (Sallenave et al., 2000), where the Ro33 family was poorly polymorphic. At the MSP-1 locus alleles belonging to the K1 family were more frequent, whereas at the MSP-2 locus alleles belonging to the IC alleles were more frequent; our results demonstrated discrepancy with a previous study (Zakeri et al., 2005), in which all patients referred to a clinic were selected. Disparity in the number of clinics and geographical area may justify this finding. Although Iran, in general, is a low endemic country

for malaria (Rakhshani et al., 2003), our findings showed high polymorphisms in a major falciparum malaria endemic region. It seems that movement and migrating of people between the mentioned region and neighboring countries (especially Afghanistan) may introduce different alleles of *P. falciparum* into this area of Iran. The coexistence of 2 or more clonal population from 1 gene within a host has been constantly reported (Virikyakoso et al., 1995). Finding of 34.3% of patients with more than 1 gene type in Sistan and Baluchistan Provinces showed lower degree of multi-strain infection in comparison to isolates from previous study in this area that showed a 88% multi-infection. It seems that the decrease of immigration from neighboring countries that occurred in recent years is a reason for this finding.

The presence of more than 1 parasitic gene type in a single human host may lead to cross fertilization, meiotic recombination and generation of new strains during the developmental stage in the mosquito vector (Raj et al., 2004; Snounou et al., 1999). This might be a reason that Iranian isolates showed high polymorphisms in each gene.

In this study, nested-PCR showed a suitable method to study MSP-1 and MSP-2 polymorphisms. The present study reported variations in the selected vaccine candidate antigens in Iranian isolates of *P. falciparum* that could be taken into accounts in developing malaria vaccines. Further population-based studies of sequences of MSP-1 and MSP-2 and genotyping of other candidate antigen genes of *P. falciparum* will provide more information in this area for designing of malaria vaccines.

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