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Genotyping of *Plasmodium falciparum* Field Isolates in Major Endemic Region of Iran and Potential Uses in Identification of Field Strains

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A number of the stage specific antigens were used to develop the vaccines and to differentiate the strains of *Plasmodium falciparum*. Polymerase chain reaction amplification was used to determine polymorphisms of the genes of Merozoite Surface Protein 1 (MSP-1) and merozoite surface protein 2 (MSP-2) and as well as Circum Sporozoite Protein (CSP). A total of 67-microscopically positive *Plasmodium falciparum* infected individuals from a major endemic region, Southeast Iran, were included in this trial. Five fragments of MSP-1, 8 of MSP-2 and 3 of CSP were identified. The results showed that amplified products from these surface antigens varied in size and there was a specific pattern for each strain, besides, regarding this pattern 10 multiple infections with at least two clones were observed. Fragment 320 bp MSP-1 loci demonstrated high frequency in patients of more severe clinical manifestations. While the malaria endemic region of Iran is classified as low to moderate group but extensive polymorphisms was observed for each marker and the MSP-2 central repeat was the most diverse one.

Key words: *Plasmodium falciparum*, Polymorphism, MSP-1, MSP-2, CSP, Iran

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INTRODUCTION

Malaria is one of the most prevalent parasitic diseases in the world. It is endemic in about 100 countries or territories (Escalante *et al*., 1998). *Plasmodium falciparum*, the agent of malaria, is a matter of great clinical and epidemiological importance (Durand *et al*., 2003).

Genetic diversity presented by *P. falciparum* field isolates, the occurrence of variant forms of the parasite in different geographic areas and inoccultation of multiple genotypes during a single mosquito constitutes one of the main obstacles to the design of a malaria vaccine (Raj *et al*., 2004; Moore *et al*., 2002).

The genes of a number of malaria proteins such as merozoite surface proteinl (MSP-1), MSP-2 and Circum Sporozoite Protein (CSP) have been shown extensive polymorphism. The mentioned proteins are causing immune response in human (Aubouy *et al*., 2003). The PCR which detect low numbers of parasite in small sample volume has been used for the detection of genetic diversity, the search for marker of parasitic virulence (Babiker and Wallilker, 1997), the geographical distribution of the various alleles of the polymorphic genes of the parasite (Aubouy *et al*., 2003; Jordan *et al*., 2001; Arez *et al*., 1999) the numbers and types of parasite subpopulations present in a given sample (Farnert *et al*., 1997).

The N-and-C terminals of the genes that encode these proteins are conserved but the central regions are highly polymorphic in different strains.

There is limited information on the polymorphic nature of these genes among field isolates in southeast of Iran. Hence we investigated the genetic diversity and complexity of *P. falciparum* infection in this region.

MATERIALS AND METHODS

Study area and blood collection: Iran is located in the Eastern Mediterranean region and grouped as low-moderate endemic region (Rakhshani *et al*., 2003).

The *falciparum* malaria endemic area is located in the southeast of Iran and is considered as the oriental eco-epidemiological region of malaria (Sadrizadeh, 1999). The Sistan and Baluchistan Province is located in southeast endemic area of the country. It is bordered by Pakistan and Afghanistan. The tribulations encountered here are resistance of *P. falciparum* to drugs (Edrissian, 1989; Edrissian *et al*., 1993; Eskandarian *et al*., 2002) and that of vectors to insecticides, likewise importation of malaria, mostly of *P. falciparum* originating from Afghanistan immigrants and to some lesser extent, from Pakistani. Prevalence of malaria in Iran in the past 5 years has been from 13000 to 24000 (Mean: 18500) individuals. Fifty-five percent of these cases reported in Sistan and Baluchistan Province (WHO, 2005). In this area malaria transmission occurs year-round with two peaks in May to June as well as October to November. This study involved 67 resident subjects aged from 2 to 45 years. Sample collection was carried out from May 2003 to December 2004. *P. falciparum* malaria patients attending local malaria clinics and health centers were enrolled in the study. Residence in the region for over 6 months, no history of anti malarial treatment for the last monthand written informed consent were required for inclusion in the 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 smear. Origin, clinical manifestations, age, sex and parasitemia (number of asexual parasites per micro liter of blood) of each patient were recorded. Blood was collected in tubes containing anti coagulant solution, stored at -20°C and then transported in dry ice to the Institute of Tropical Medicine of Berlin- Germany for genotyping.

Isolation of DNA: DNA was extracted from the blood sample by modified *Leclerc* method (Leclerc *et al*., 2002). Two hundred microliters of blood was washed 3 times in Phosphate-Buffer Saline (PBS) and the red blood cells were lysed by suspension in 100 mM NaCl, 10 mM EDTA and 45 μL of 10% SDS and 8 μL of Rnase at 500 μg mL^{-1}. The mixture was incubated for 2 h at 37°C. Then 10 μL of proteinase k at 10 mg mL^{-1} was added and the mixture was incubated for 2 h at 55°C, after wards, Then DNA was isolated in 20 μL of sterile water.

The DNA product was either used immediately or stored at 4°C for later exploit. PCR was performed for MSP-1, MSP-2 and CSP. The primers used in the amplification reaction were conserved among all isolates and were purchased from Roch Company.

The sequences of the primers were as follows:

- MSP-1 5'primer Al596→ 5' gAA gAT gCA gTA TTg ACA gg 3'
- MSP-1 3'primer Al597→ 5'gAg TTC TTT AAT AgT gAA CAA g 3'
- MSP2 5'primer Al598→ 5'gAg TAT AAg gAg AAg TAT gg 3'
- SP2 3'primer AL599→ 5'CCT gTA CCT TTA TTC TCT gg 3'

- SP 5'primer AL600→ 5'ATA gTA gAT CAC TTg gAg A 3'
- SP 3' primer AL601→ 5'gCA TAT TgT gAC CTT gTC CA 3'

For amplification, two µL of DNA samples was used in total reaction volume of 26 µL containing 50 mM KCl; 1.5 mM MgCl 2; 125 µM of each dNTP (Invitrogen Company); 1 u of Taq and a pair of 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. The PCR amplified gene fragments of MSP-1; MSP-2 and CSP were electrophoresed on 3% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

χ^2 tests were applied to confirm significance of the results.

RESULTS

Sixty percent of subjects studied were male. Parasitemia in patients ranged from 1000 to 30000 asexual parasites mm^{-3} (mean; 11700 parasite mm^{-3}). Among the fragments of 67 samples at the MSP-1 locus, 5 variants ranged from 280 to 360 bp, for the MSP-2 locus, 8 variants from 440 to 600 bp and at the CSP locus 3 variants from 700 to 800 bp (Table 1).

However, variant 300 MSP-1, 500 MSP-2 and variant 750 bp CSP demonstrated the most frequency. Seven (10.4%) and 8 (12%) samples illustrated multiclonal infection at least with two clones at MSP-1 and MSP-2 genes, respectively. The varied size bands and multiple infection for MSP-1, MSP-2 and CSP genes are shown in 9 samples in Fig. 1 (lane 2-10). The number of strains was determined via combination of 3 gene fragments. Overall, 44 strains or clones were identified in southeast of Iran. Twenty patients had more sever clinical signs than others. These patients mention of fever (above 40°C) experienced repeated vomiting and loss of conscious. The variants 320 of MSP-1, 520 of MSP-2 and 700 bp of CSP were showed high frequency in these patients. There was significant correlation between variants 320 bp MSP-1 and severity of manifestation ($p<0.001$). There was no significant association between age, sex and origin of patients with variants in three mentioned genes.

Table 1: Genetic diversity of MSP-1, MSP-2 and CSP genes in field isolates of major endemic region of Iran

MSP-1 variants (bp)	Number (%)	MSP2 variants (bp)	Number (%)	CSP variants (bp)	Number (%)
280	9 (12.1)	440	3 (4)	700	25 (37.3)
300	37 (50)	460	10 (13.3)	750	38 (56.7)
320	13 (17.5)	480	11 (14.7)	800	4 (6)
340	14 (19)	500	21 (28)	Total	67
360	1 (1.4)	520	9 (12)	-	-
Total	74	540	14 (18.6)	-	-
-	-	580	5 (6.7)	-	-
-	-	600	2 (2.7)	-	-
-	-	Total	75	-	-

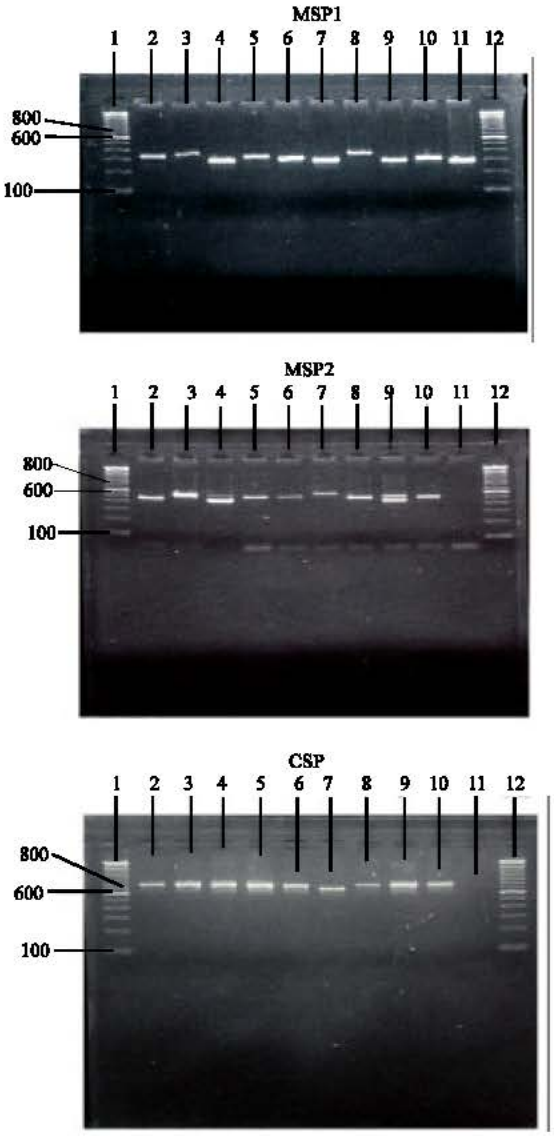

Fig. 1: Gel photographs showing PCR amplified products of MSP1, MSP-2 and CSP from different *P. falciparum* infected isolates in Southeast of Iran. The DNA size marker is a 100 bp ladder shown on the left and right sides. Lane 11 is negative control. Lane 9 MSP-2 shows 2 variants

DISCUSSION

Determination of polymorphisms of the genes encoding the highly polymorphic loci MSP-1, MSP-2 and CSP of *P. falciparum* can help us evaluate the potential

for long term effectiveness of these proteins as anti malarial vaccine, strain identification and developing new drugs (Tanabe *et al*., 2004; Jordan *et al*., 2001; Wooden *et al*., 1999).

This study illustrated 5 and 8 different fragments at MSP-1 and MSP2 loci respectively, which shows higher rate than that of a similar report in hypo endemic region in Colombia (Montoya *et al*., 2003), where one alleles 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 while in Gabon (Aubouy *et al*., 2003) 25 alleles of MSP-1 and 12 alleles for MSP-2 were reported. Our genotyping finding of CSP is lower than found in India (5 Variants) (Ranjit *et al*., 1999), but higher than reported in another study in the same country (1 variant) (Sidhue *et al*., 2000). This might indicate a restricted geographical localization of the gene.

Although Iran, in general, is low endemic country for malaria (Rakhshani *et al*., 2003), but our findings showed high polymorphisms in major *falciparum* malaria endemic region of it. 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 in this region of Iran.

The MSP-2 gene has been reported to be very polymorphic, (Wooden *et al*., 1999a; Peyerl-Hoffmann *et al*., 2001). Present finding showed agreement with this pattern and MSP-2 was polymorphic than MSP-1 and CSP genes. A PCR approach using primer pairs for MSP-1, MSP-2 and CSP with different *P. falciparum* infected isolates showed different fragments, so we could distinguish 44 strains in 67 patients of southeast of Iran. This approach already was able to characterize strains in the laboratory (Wooden *et al*., 1999b).

Since patients with more severity manifestations are not reported in Iran, it seems, a newly *P. falciparum* strain introduced to this region. In this study, thirteen of the samples from the patients with severity manifestations showed the MSP-1 320 bp fragment. This clone however was present in more than half of the patients with more severity manifestations. A new strain can be imported from other areas through travelers or can be the result of a genetic recombination in the vector. In addition, importation of new parasite clones into a community means introduction of new mixed parasite populations to the vector. This may generate new strains due to genetic recombination in the mosquitoes. The coexistence of two or more colonal population from one gene within one host has been constantly reported (Virikyakosol *et al*., 1995; Farnert *et al*., 1997). Finding of 15% of the patients with more than one gene types in Systan and Baluchistan Province showed, lower degree of multi-strain infection in comparison to isolates from India (50.3%) (Raj *et al*., 2004), Vietnam (44%) (Ferreira *et al*., 1998a) and Northern Tanzania (60%) (Ferreira *et al*., 1998b).

It is concluded that there is extensive polymorphisms in the malaria endemic region of Iranand that the applications of genotyping could be regarded as useful tool for determination the strains in the field isolates.

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