

Genetic Diversity in the Circumsporozoite Protein Gene of *Plasmodium falciparum* from Major Endemic Regions of Iran

*A Heidari¹, H Keshavarz¹, M Assmar², M Razavi², M Nateghpour¹, S Dittrich³, T Jelinek³

¹Dept. of Medical Parasitology, School of Public Health, Tehran University of Medical Sciences, Iran

²Dept. of Parasitology, Pasteur Institute of Iran, Tehran, Iran

³Berlin Institute of Tropical Medicine, Berlin-Germany

(Received 4 Jul 2005; revised 13 Dec 2005; accepted 11 Apr 2006)

Abstract

Circumsporozoite protein (CSP) is one of the stage specific antigens, which is used for the development of vaccines and to investigate the genetic diversity in *Plasmodium falciparum* malaria parasite. Polymerase chain reaction was used for typing of CSP genes on 67 positive *falciparum* malaria patients from Sistan and Baluchistan Province of Iran. Three fragments were detected for CSP gene. Twenty, 38 and 4 samples showed 700, 750 and 800 bp fragments, respectively. Sequences of some samples were aligned and compared with *P. falciparum* csp gene in gene bank. While the *falciparum* malaria endemic region of Iran is classified in low to moderate group but, extensive polymorphism was observed in the samples that could be taken into account in designing malaria vaccine.

Keywords: *Circumsporozoite protein*, *Plasmodium falciparum*, *Malaria*, *Iran*

Introduction

Malaria is one of the most important infectious diseases in the world. It causes 300-500 million clinical cases and 1.1-2.7 million deaths annually (1).

Genetic diversity presented by *P. falciparum* field isolates and the occurrence of variant from of the parasite in different geographic area constitute one of the main obstacle to the design of a malaria vaccine (2, 3).

The invasive stage of the vertebrate host consists of sporozoites, which are injected by the mosquito vector during its blood meal (4). Circum Sporozoite Protein (CSP) encodes predominant protein found in the structure of the sporozoite (5). This protein causes immune response in human and is important candidate for development of malaria vaccine (6, 7). A vaccine against sporozoites would, if effective, prime the human immune system to kill sporozoites injected by the mosquito and prevent the

subsequent stages responsible for the disease and transmission of the infection to others (4).

The gene for the (CSP) of *P. falciparum* is comprised of two terminal regions that are not repetitive (5' NR and 3' NR), which embrace a central region (CR) of tandemly repeats (mostly between 40 and 50) which vary in size and relative position from one strain to another strain of *P. falciparum* (8). It encodes predominant protein that contains approximately 420 amino acids as deduced from the nucleotide sequence (9). PCR that detect low numbers of parasite in small sample volume has been used for detection of genetic diversity (10) and the geographical distribution of the various alleles of polymorphic genes of the *P. falciparum* (11, 12). The tribulations encountered in Sistan and Baluchistan Province are resistance of *P. falciparum* to drugs (13, 14) and that of vectors to insecticides, likewise importation of malaria mostly of *P. falciparum* kind originating from Afghanistan

immigrants and to some lesser extent, from Pakistani. Hence designing of efficient malaria vaccine is useful for control of *falciparum* malaria in this area.

Since there is no information about polymorphism of the gene encoding CSP vaccine candidate in south east of Iran, we investigated the diversity of the gene using PCR and some partial sequences from field isolates from Sistan and Baluchistan province.

Materials and Methods

Study area and blood collection *falciparum* malaria is endemic in south and southeast of Iran. Sistan and Baluchistan province is located in the southeast endemic area of the country. A total of 12885 malaria cases were reported in 2004, 56% of these cases were reported from Sistan and Baluchistan that consisted 85% of total *falciparum* malaria (15).

This study involved 67 native individual aged between 2 and 45 yr. Fifty-six blood samples were collected from the Pishine area main region for endemic *falciparum* malaria of Iran and 11 samples from Iranshar area. Sample collection was carried out from May 2003 to December 2004. Patients with *P.falciparum* malaria attending the local malaria clinics and health centers were selected for the study. Native individuals without history of anti malaria treatment for the last month and written consent were required for inclusion in the study. Diagnosis of *P.falciparum* was confirmed by light microscopy on thick blood smear. Blood samples were collected in tubes containing anti coagulant solution, stored at 20 °C.

Isolation of DNA and sequencing DNA was extracted from the blood sample by a modification in Claude-Leclerc method (16). Two hundred µl of blood was washed 3 times in phosphate-buffered saline (PBS). The red blood cells were lysed by suspension in 100 mM NaCl, 10 mM EDTA, 45 µl of 10% SDS and 8 µl of Rnase at 500 µg/ml. The mixture was incubated for 2 h at 37 °C. Then 10 µl of proteinase K at 10 mg/ml was added and the mixture was incu-

bated for 2 h at 55 °C. Then DNA was isolated in 20 µl of sterile water.

We used a set of primers including 5' (5' ATAGTAGATCACTTGGAGA 3') and 3' (5' GCATATTGTGACCTTGTCCA3') described by Wooden (17), to amplify the region corresponding to base pairs 203-861 of the sequence from the Sal1 strain (U20969). For amplification, two µl of DNA was used in total reaction volume of 26 µl containing 50 mM KCl; 1.5mM MgCl₂; 125 µM of each dNTP; 1u of Taq and a pair of primers (0.33 µM 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 were electrophoresed on 1% agarose gels, stained with ethidium bromide and visualized under ultraviolet light.

The PCR products of 5 samples were purified using Qiaquick PCR purification Kit (Qiagen Company). Sequences of DNA were performed by MWG Biotech (Germany). The DNA and amino acid sequences were aligned using Blast of pubmed. Chi-square test was applied to calculate significance of the results.

Results

PCR was performed on 67 positive *falciparum* malaria patients. The sizes of the PCR product ranged from 700 to 800 bp (Fig.1). Three fragments (700, 750 and 800 bp) were detected for the CSP gene. Twenty-five (37.3%) samples showed 700 bp fragment, 38 (56.7%) and 4 (5.9%) samples 750 and 800 bp fragments, respectively. In the Pishin area, 36% of the fragments illustrated as 700 bp, 57% as 750 bp and 7% as 800 bp. In the Iranshar area, 45.5% of fragments were detected as 700 bp and 54.5% as 750 bp (Fig. 2). There was no significant difference in the prevalence of these fragments between the two areas. There was no significant association between sex, age, and distribution of fragments in the CSP gene.

Sequences of CSP gene from blood samples of 5 *falciparum* malaria patients were aligned and compared with *P.falciparum* CSP gene sequences from published data and gene bank.

The similarity in nucleotides within the CSP isolates varied from 83% to 97%. The similarity in nucleotides with SalI allele (U20969) was 85%, 98%, 88%, 94%, and 92% for IRI1, IRI2, IRI3, IRI4, and IRI5, respectively.

The homology within CSP isolates with *P.falciparum* clone Ken 612 CSP gene (AF540457) was 86%,

99%, 88%, 95% and 92% for IRI1, IRI2, IRI3, IRI4, and IRI5, respectively. The homology in CSP isolates with *P.falciparum* isolate 96 M320 (AB11607) was 86%, 99%, 88%, 95% and 92% for IRI1, IRI2, IRI3, IRI4, and IRI5, respectively.

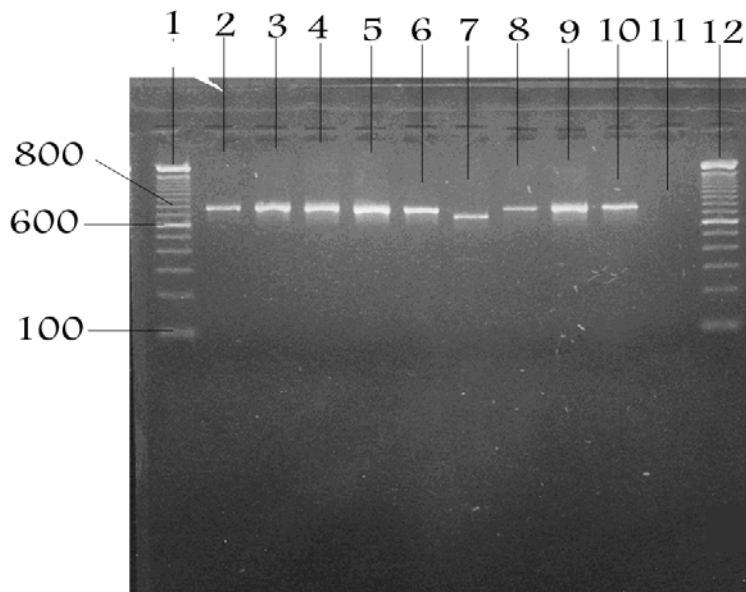


Fig. 1: Gel photograph showing PCR amplified products of CSP from different *P.falciparum* infected isolates in Southeast of Iran. The DNA size marker is a 100 bp ladder shown on the left and right sides. Lane 11 is negative control

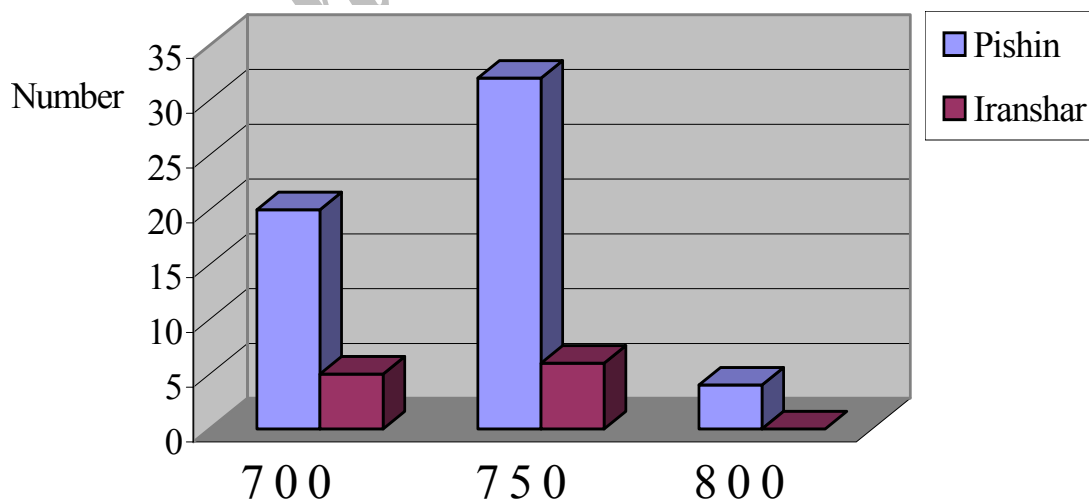


Fig. 2: Distribution of variants of CSP in two regions of Iran

	Sal1-
KDEDKRDGNNEDGNNEDNEKLRKPKHKLLKQPADGNPDNANPNVDPNANPNVDPNANPNVDPNANPNAN	PNANP
IRI1-	
KDENKRDGNNETGNNEDNEKLRKPKLKKLKH	HPGVINPDNANPNVDPNANPNVDPNANPNVDPNANPNANP
NANP	
IRI2-	
KDEDKRDGNNEDGNNEDNEKLRKPKHKLLKQPGDGNPDNANPNVDPNANPNVDPNANPNVDPNANPNAN	PNANP
IRI3-	
KDEDKRDGNNEDGNNEDNEKLRKPKHKLLKQPGGINPDNANPNVDPNANPNVDPNANPNVDPNANPNANP	NANP
IRI4-	
KDEDKRDGNNEDGNNEDNEKLRKPKHKLLKQPGVINPDNANPNVDPNANPNVDPNANPNVDPNANPNANP	NANP
IRI5-	
KDEDKRDGNNEDGNNEDNEKLRKPKHKLLKQPGVGNDPDNANPNVDPNANPNVDPNANPNVDPNANPNAN	PNANP

Fig. 3: Amino acid partial sequences of 5 Iranian *P.falciparum* isolates in comparison with Sal1 isolate

Discussion

Determination of polymorphism of the gene encoding the polymorphic loci CSP of *P.falciparum* would help us evaluate the potential of this protein as anti malarial vaccine. (18).

Our genotyping results are less than those found in Orrisa, India are (5 alleles) (19), but more than those found in New Delhi in the same country (1 allele) (20). It seems that different number of alleles is found in various geographical regions. However, the values obtained are slightly inferior to those observed in Senegal (4 alleles) with a high rate of malaria transmission (16). However, in general, Iran is a hypo endemic country for malaria (21), but our finding shows polymorphism in major *falciparum* endemic region of Iran. It seems movement and migrating of people between the mentioned regions and neighboring countries (Pakistan and Afghanistan) may introduce different alleles of *P.falciparum* into in these regions of Iran.

Multiple infections in these loci have not been shown, this finding agrees with studies in India and Senegal (16, 20). Of course, we have shown multiple infections in other genes such as Merozoites surface protein (MSP) 1 and MSP2 in these regions (22).

There was not significant association between distribution of fragments and two regions of study. It seems frequent travels between the mentioned regions is a reason for it. Sequencing results indicated that 5 distinct alleles for *P.falciparum* CSP were detected among these 5 randomly sequenced isolates. 83% to 97% homologies in the alignment between isolates of the study have indicated presence of synonymous and non-synonymous substitutions.

Several (one to ten) nucleotide substitutions occurred before the region 5 of the central repeat between pairs of alleles, this finding agrees with a study on African isolates (23). Substitutions in the partial sequenced as compared to the previously published sequences of Sal1 isolate some amino acids in the region of 90 to 165 CSP genes (Fig. 3). The present study reported the variation in the selected vaccine candidate antigens in Iranian isolates of *P.falciparum* that could be taken into account in developing malaria vaccine.

Further population-based studies of sequences of other candidate antigen genes of *P.falciparum* will provide more information in this area for designing of malaria vaccine.

Acknowledgements

We would like to thank those individuals from the malaria endemic region of Iran, who kindly contributed to this study and are grateful to Mr Satvat, Mr Akbarzadeh, and Mr Seidzadeh for the assistance to sampling, Dr Edrissian, Dr Rezaii for their invaluable guidance's and all staff of Institute of Tropical Medicine of Berlin. This study was supported financially by the Iranian Ministry of Health and the Institute of Tropical Medicine Berlin-Germany.

References

1. WHO (2000). WHO expert committee on malaria, twentieth report. *WHO technical report series*, No. 892. Geneva: 3-6.
2. Moore SA, Surgey EG (2002). Malaria vaccines: where are we and where are we going? *The lancet Infect Dis*, 2: 737-43.
3. Escalante AA, Grebert HM, Isea R, Goldman IF, Basco L, Magris M, Biswas S, Kariuki S, Lal AA (2002). A study of genetic diversity in the gene encoding the circumsporozoite protein (CSP) of *P. falciparum* from different transmission area. *Mol Biochem Parasitol*, 125: 83-90.
4. Waters AP, Mota MM van Dijk MR, Janse CJ (2005). Malaria Vaccines: Back to the future. *Science*, 307: 528-30.
5. Dame JB, Willimas J, McCutchan TF, Weber JL, Wirtz RA, Hockmeyer WT, Maloy WL, Haynes JD, Schneider I, Roberts D (1984). Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *P. falciparum*. *Science*, 225: 593-99.
6. Hviid L (2005). Naturally acquired immunity to *P. falciparum* malaria in Africa. *Acta Trop*, 95(3): 270-55.
7. Shiy P, Hasnain SE, John B Sacci (1999). Immunogenicity and in vitro protective efficacy of a recombinant multi stage *P. falciparum* candidate vaccine. *Proc Natl Acad Sci USA*, 96:1615-1620.
8. Rich SM, Ayala FJ (2000). Population structure and recent evolution of *P. falciparum*. *Proc Natl Acad Sci USA*, 20(97): 6994-7001.
9. McCutchan TF, Cruz VF, Good MF, Wellem TE (1988). Antigenic diversity in *P. falciparum*. *Prog Allergy*, 41:173-92.
10. Moody Anthony (2002). Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev*, 15(1): 66-78.
11. Aubouy A, Migot-Nabias F, Deleron P (2003). Polymorphism in two merozoite surface proteins of *P. falciparum* isolates from Gabon. *Malar J*, 2:12.
12. Arez AP, Snounou G, Pinto J, Sousa CA, Modiano D, Ribeiro H, Franco AS, Alves J, do Rosario VE (1999). A clonal *Plasmodium falciparum* population in isolated out break of malaria in the Republic of Cabo Verde. *Parasitology*, 118: 347-55.
13. Edrissian G H, Afshar A, Sayedzadeh A, Mohsseni G, Satvat MT (1993). Assessment of the response in vivo and in vitro of *Plasmodium falciparum* to sulphadoxine-pyrimethamine in the malarious areas of Iran. *J Trop Med Hyg*, 96 (4): 237-40.
14. Eskandarian AA, Keshavarz H (2002). Do mutations in *Plasmodium falciparum* dihydropteroate synthase and dihydrofolate reductase confer resistance to sulphadoxine-pyrimethamine in Iran? *Trans R Soc Trop Med Hyg*, 96:96-8.
15. The work of WHO in the Eastern Mediterranean region (2005). *Annual report of regional director- WHO/ Regional office for the Eastern Mediterranean*. Available from: www.emro.who.int
16. Leclerc CM, Durand P, de Meeus T, Robert V, Renaud F (2002). Genetic diversity and population structure of *P. falciparum* isolates from Dakar, Senegal, investigated from microsatellite and antigen determinant loci. *Microbes Infect*, 4: 685-92.

17. Wooden J, Gould EE, Paull AT, Sibley CH (1992). *Plasmodium falciparum*: A simple polymerase chain reaction method for differentiating strains. *Exp Parasitol*, 75: 207-12.
18. Tanab K, sakihama N, Kaneko A (2004). Stable SNPs in malaria antigen genes in isolated populations. *Science*, 303(23): 493.
19. Ranjit MR, Sharma YD (1999). Genetic polymorphism of *falciparum* malaria vaccine candidate antigen genes among field isolates in India. *Am J Trop Med Hyg*, 61(1): 103-8.
20. Sidhu AB, Madhubala (2000). *Plasmodium falciparum*: Detection and strain identification of Indian isolates by Polymerase chain reaction. *Southeast Asian J Trop Med Public Health*, 31(2): 213-18.
21. Rakhsani F, Ansari AR, Alemi R, Moradi A (2003). Knowledge, perceptions and prevention of malaria among women in Sistan Va Baluchestan, Islamic Republic of Iran. *East Mediterr Health J*, 9(3): 248-56.
22. Heidari A, Keshavarz H, Dittrich S, Jelinek T (2006). Genetic Diversity in The Merozoite Surface Protein 1 (MSP-1) and MSP-2 Genes of *Plasmodium falciparum* in Major Endemic Regions of Iran. *J M Sciences*, (in press).
23. Cruze V, McCutchan TF (1986). Heterogeneity at the 5 end of the circumsporozoite protein gene of *Plasmodium falciparum* is due to previously undescribed repeat sequence. *Nucleic Acids Res*, 14 (11): 4695.