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A Preliminary Study of Genetic Diversity of MSP-1 Types in *Plasmodium falciparum* in Southern Province of Sistan Baluchistan of Iran

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Abstract: *Plasmodium falciparum* merozoite surface protein-1 (MSP-1) shows extensive antigenic diversity. This is due to the presence of seven variable blocks, five semi-conserved and also five conserved blocks. The variable blocks in the MSP-1 gene are principally dimorphic, displaying either K1 or MAD20 type; except for the block 2 region which is represented by three alleles, an RO33 type in addition to the other two. Allelic diversity is reported to be generated by intra-genic recombination between the variable blocks. A study of allelic variation of MSP-1 gene in *Plasmodium falciparum* was carried out in the southern province of Sistan Baluchistan in Iran in 2001-2003. Samples were obtained from 30 febrile patients and DNA was extracted and association types between blocks 2 and 6 was identified on each block using specific primers and compared with those from Vietnam, Brazil and Africa. The association types obtained, were similar though less in number than the ones from Vietnam, but more than those from Africa and Brazil.

Key words: Merozoite surface protein, association types, *Plasmodium falciparum*

INTRODUCTION

Malaria causes over 300 million cases and 2 million deaths annually world wide (WHO, 1993) *Plasmodium falciparum* is one of the main causes of cerebral malaria and the most pathogenic protozoan parasite (Girard *et al.*, 1988). Increased insecticide resistance by the mosquito and drug resistance by the parasite itself has made vaccine preparation an urgency, (Cowman *et al.*, 2000). One of the vaccine candidates for the blood stage parasites is the 190-200 kD major glycoprotein merozoite surface protein-1 (MSP-1) which after proteolytic processing, leaves only a 19 kD C-terminal fragment remains anchored on the merozoite surface during invasion (Blackman *et al.*, 1992). An immune response to MSP1 is expected to stop merozoite invasion of erythrocytes, the developmental stage of the parasite that causes clinical malaria (Cooper *et al.*, 1992; Pirson and Perkin, 1985; Riley *et al.*, 1992; Riley, 1996). The protein however, shows extensive polymorphism, but is very strongly associated with immunity to the disease (Diggs *et al.*, 1993).

An alternative approach could be a multivalent vaccine based on polymorphic antigens whose

composition can be changed regularly to match locally prevalent antigenic variants. This could be an alternative to those made of highly conserved but poorly immunogenic antigens (Anderson *et al.*, 1997).

Tanabe *et al.* (1987) compared different sequences and described 7 variable regions in the MSP1 gene interspersed with conserved or semi-conserved regions. The 19 kD C-terminus region corresponds approximately to three conserved block 17 (Fig. 1) which is a major target of naturally acquired anti-malarial immunity (Tanabe *et al.*, 1987). There are two basic versions of each block named after the representative isolates K1 and MAD20 (Certa *et al.*, 1987). The only known exception to the allelic dimorphism occurs in block 2 which has a third version named RO33 (Peterson *et al.*, 1988). Most allelic diversity is generated by recombination near the 5' end of the gene and the variations in the tripeptide repeats found in the MAD20 and K1 version of block 2. This block of MSP1 has been defined as a principal target of antibodies associated with clinical immunity in Amazonian patients (Da Silveira *et al.*, 1999).

Almost all possible combinations of PfMSP1 variable blocks, are found in clinical isolates (Da Silveira *et al.*, 1999). A similar study has been carried out in Brazil,

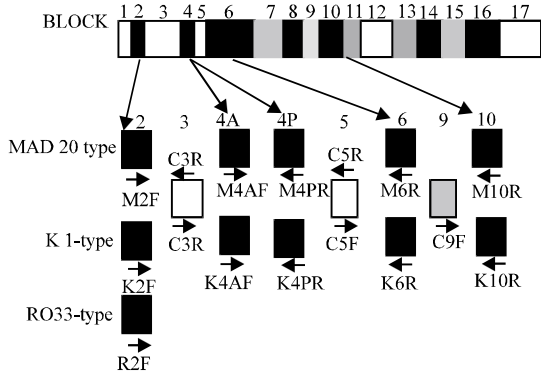


Fig. 1: Primers for determination of allelic types in variable blocks of the *Plasmodium falciparum* MSP1 gene. Conserved, semiconserved and variable blocks of the gene are shown as white, gray and black boxes, respectively. Block numbers are after Tanabe *et al.* (1987). Location and directions of primers are indicated by arrows

Tanzania and Southern Vietnam and it was seen that some combinations and gene types from different continents (Kaneko *et al.*, 1997) are significantly more prevalent than expected by random assortment of allelic types (Ferreira *et al.*, 2003). Using the PCR strategy developed by Kaneko *et al.* (1997), we carried out a study of the patterns of allelic diversity in Iranian patients infected by *P. falciparum* between the years 2000-2002 from Sistan Baluchistan State.

MATERIALS AND METHODS

Sample preparation: 2 ml of blood was obtained from 30 willing febrile patients confirmed as being infected with *Plasmodium falciparum* by microscopic examination of Gimesa stained thick blood smears. The patients were residing in nearby cities in the Sistan Baluchistan province in south-east Iran. Information revealing their personal data, details of drug therapy and recent travels to neighboring countries were noted in a questionnaire.

DNA extraction: 1.5 mL blood was centrifuged (1200 rpm for 5 min) at 4°C. The precipitate was washed with RPMI or PBS. Erythrocytes were initially lysed in 700 µL PBS buffer containing saponin on ice for 5 min. Cells were then centrifuged at 10,000 g for 5 min and washed twice with 1 mL PBS. Lysis was carried out in lysis buffer (50 mM tris (pH 7.8), 50 mM EDTA, 10 mM NaCl, 1% triton X-100) and 15 µL proteinase K overnight at 60°C. Phenol chloroform extraction was done according to the method of Sambrook *et al.* (1989). DNA was dissolved in 120 µL deionized DW. The entire experiment was carried out on ice.

PCR amplification: 35 PCR cycles (each one 3 min at 94°C, 45 sec at 91°C, 45 sec at 50°C and 45 sec at 70°C and 7 min at 70°C) in a thermal cycler (Eppendorf). PCR amplification was performed in a 25 µL reaction mixture containing 1 µL each of forward and reverse primers designed by Tanabe *et al.* (1987), 1 µM of dNTP, 1 unit of DNA polymerase (Roche), 2.5 µL of PCR buffer and 1 µL of DNA were prepared from the infected erythrocytes. Every PCR assay included a tube without template as negative control. PCR products were electrophoresed on 0.8 to 2% agarose gel (depending on predicted size of PCR products), in 0.5X Tris-borate EDTA buffer and documented with gel doc (Mitsubishi).

Determination of MSP-1 association type by PCR: Sequences of oligonucleotide primers and PCR protocols was used to type the blocks as described by Tanabe *et al.* (1989).

RESULTS

The common primer between block 3 and 5 was detected in all samples (Fig. 2a) as a heavy band of 500 bp. Allelic family typing of block 2 with one of the 3 allele specific primers (K2F, M2F, R2F) and a conserved primer (C3R) (Tanabe *et al.*, 1987) yielded specific bands of predicted sizes (Fig. 2a). Specific amplification was also observed for typing of block 6 with a conserved primer C5F and either of the two allele specific primers (K6R and M6R) (Fig. 2c). All the three variable types of block 2, K1, MAD20 and RO33 type were detected (Fig. 2b). Nested PCR of block 4 once again demonstrated the presence of both K1 and MAD20 types (Fig. 2d).

In total, 98 MSP-1 association type clones were determined from 30 blood samples. Twelve out of 30 samples exhibited two different association type clones and more than 3 association type clones were seen in another 12 isolates including those containing as many as 8 clones. Thus, 24/30 isolates (80%) contained multiple association type clones (Fig. 3). The average number of MSP-1 association type clones per patient was 3.26 in Sistan Baluchistan in 2001-2003.

Frequency of MSP-1 association types: The analysis of 98 MSP-1 association type revealed 19 distinct types. The frequency distribution of the expected 24 association types was shown in the histogram (Fig. 4). As frequency is expressed only for MSP-1 association type clones and not parasite clones, it is probable that the same MSP-1 association type clone is shared by different parasite clones. Particular association types were predominant in Sistan Baluchistan like type number 17 (with MAD20

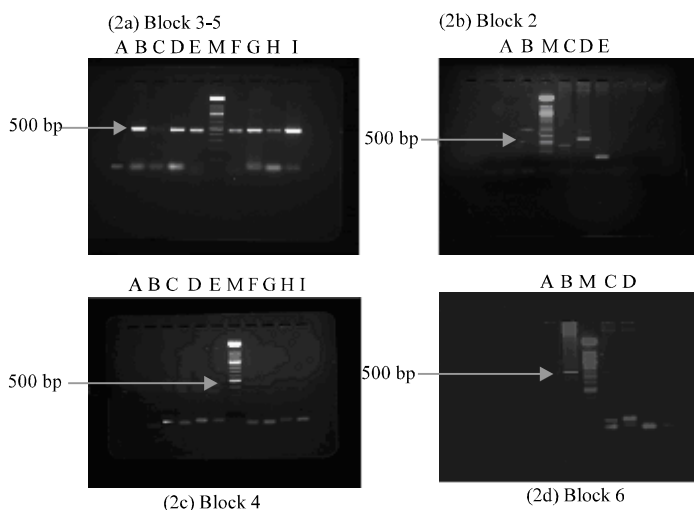


Fig. 2: PCR amplified fragment of the *Plasmodium falciparum* MSP-1 gene variable blocks, 2, 3-5, 6 and nested PCR of block 4 using specific primers analyzed by 2% agarose gel electrophoresis. MW markers are 100 bp ladders containing a mixture of 15 double stranded DNA ranging from 100 to 1500 bp containing an additional 2642 bp band (Roche). (2a) Lane A is without template and lane M contains MW markers. And the rest show common primers for analysis of blocks 3-5; 500 bp fragment amplified by common primers (2b) shows variable block 2-3; Lane A, without template, lane B positive control, lane C RO33 at 270 bp, lane D, K1 at 264- 269 bp and lane E shows MAD 20 at 154 bp and lane M, molecular weight marker (2c) Nested PCR using amplified fragment from block 2-5, lane A without template, lane B positive control, lane M molecular weight markers, lane I MAD20/MAD20 97 bp, lane H MAD20/K1 at 94 bp, lane G K1/K1 at 80 bp and lane F K1/MAD20 at 84 bp (2d) lane A positive control, lane B MAD20 at 111 bp, lane C K1 at 119 bp, lane D without template and lane M molecular weight markers

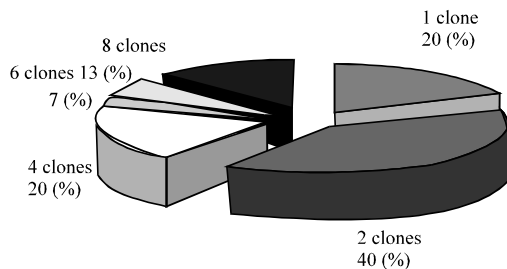


Fig. 3: Percentage of MSP-I genotype clones

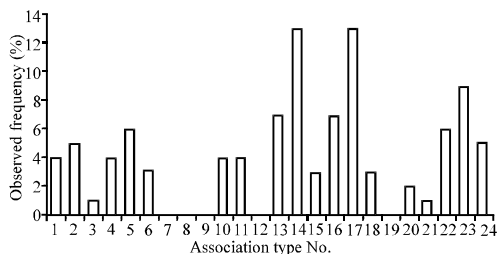


Fig. 4: Histogram depicting frequency percentage of MSP1 association types. Frequency distributions of MSP1 association types were compared by χ^2 test on 2 x R contingency tables; 5% was employed as the level of significance

Different kinds of association type numbers combinations of K1, MAD20, RO33 in blocks 2, 4a, 4P and 6.

1	2	3	4	5	6	7	8	9	10	11	12
K1	MA	RO3	K1	MA	RO3	K1	MA	RO3	K1	MA	RO3
K1	D20	3	MA	D20	3	K1	D20	3	MA	D20	3
K1	K1	K1	D20	MA	MA	MA	K1	K1	D20	MA	MA
K1	K1	K1	K1	D20	D20	D20	MA	MA	MA	D20	D20
	K1	K1	K1	K1	K1	K1	D20	D20	D20	MA	MA
				K1	K1		K1	K1	K1	D20	D20
										K1	K1

13	14	15	16	17	18	19	20	21	22	23	24
K1	MA	RO3	K1	MA	RO3	K1	MA	RO3	K1	MA	RO3
K1	D20	3	MA	D20	3	K1	D20	3	MA	D20	3
K1	K1	K1	D20	MA	MA	MA	K1	K1	D20	MA	MA
MA	K1	K1	K1	D20	D20	D20	MA	K1	MA	D20	D20
D20	MA	MA	MA	K1	K1	MA	D20	MA	D20	MA	MA
	D20	D20	D20	MA	MA	D20	MA	D20	MA	D20	D20
				D20	D20		D20		D20	MA	MA
										D20	D20

type in blocks 2 and 4A, K1 type in block 4P and MAD20 type in block 6) and association type 14 (with MAD20 type in block 2 and block 6 and K1 type in 4A and 4P). Association types having MAD20 type in block 6 existed at a high rate in all isolates (right half of histogram, Fig. 4).

DISCUSSION

During the period of our study from 2001-2003, the total number of malaria cases in Iran dropped from 19,303 to 15,558 cases. In 2001 17,145 *P. vivax* cases were reported, whereas in 2003, it had dropped to 13,1318 and the *P. falciparum* cases from 1875 had gone to 2,219 whereas mixed infection dropped to 195 (Communications from the Center for Prevention of Communicable Diseases, Tehran, Iran, 2004 (unpublished)). On the whole, malaria cases had reduced and the downward trend is fortunately continuing.

Allelic variations arise due to different associations in the variable blocks which could be either of K1, MAD20 or RO33 types. The total number of association types reported so far is 24 (Kaneko *et al.*, 1997; Da Silveira *et al.*, 1999; Ferreira *et al.*, 2003) though it is assumed to be much more, as the conserved blocks also show sequential variations. This is demonstrated by 6 different sizes of the K1 allelic types in block 2 which could be due to size polymorphism of the tripeptide repeats, (Jonguwities *et al.*, 1992; Miller *et al.*, 1993; Daubersies *et al.*, 1996; Kaneko *et al.*, 1997), which was also observed in this study.

Earlier studies in Vietnam have reported 21 (n = 186), Brazil have shown 15 (n = 620) and Tanzania 13 (n = 60), whereas we have observed 19 of the 24 reported association types. In the different association types observed in Sistan Baluchistan province, number 14 (MAD20-K1-K1-MAD20) and number 17 (MAD-20-K1-K1-MAD20) were seen in the highest frequency (about 13%). The Vietnam study also had number 17 as the highest frequency (42%) but number 14 was only 12% of total associations observed.

Predominance of MAD20 type alleles have also been reported in Africa as early as 1991 by Conway *et al.* (1991) who used monoclonal antibodies recognizing polymorphic epitopes of MSP1 have shown the prevalence of MAD20 type parasites in block 6/18. As

can be seen in our histogram, types 7, 8, 9,12 and 19 were not detected at all, in the Vietnam study too, these were represented poorly as 1, 0, 1, 1 and 6% of total frequencies observed.

The number of samples in this preliminary study were statistically significant though many more would be required to reach a final conclusion as to the reason for this variation including samples from Kerman province and Bandar Abbas, the two southern states from which *P.falciparum* is reported. This would require a much longer period of time as malaria is fortunately following a downward curve in our country.

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