Allelic Variation of Polymorphic Vaccine Candidates Merozoite Surface Protein-2 in Plasmodium falciparum Isolates from South-East of Iran

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ABSTRACT

Background: Genetic diversity of Plasmodium falciparum—the main causative agent of malaria—provides the parasite with the potential of escaping the immune response and results in the selection of vaccine and drug-resistant species. Study the allelic variation of different vaccine candidate genes in regions of malaria territory could be used to design and introduce new therapeutic methods. Therefore, Merozoite surface protein 2 (MSP-2) was selected for the purpose of evaluating allelic variation in the southeastern region of Iran.

Materials and Methods: In this study Nested Polymerase Chain Reaction (Nested-PCR) amplification was used to determine different allelic forms of MSP-2 gene using specific oligonucleotides. A total of 94 microscopically positive P. falciparum specimens from South-East of Iran were included.

Findings: Of all 94 Plasmodium falciparum specimens, 85 were confirmed for the presence of MSP-2 alleles. The frequency of MSP-2 different allelic classes was considerably high and calculated to be 50.5% and 34.2% for 3D7 and FC27 respectively.

Conclusion: Both dimorphic alleles of MSP-2 gene were detected where the frequency of 3D7 was the highest among the regions. The frequency of the alleles does not differ much from the results of studies in other regions of the world. However, this information can be beneficial to have a new vaccine designed on the basis of studies on the candidate antigens.

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INTRODUCTION

Malaria is still one of the most life-threatening parasitic diseases worldwide [1]. Malaria results in approximately 300-500 million clinical cases and 1-3 million deaths each year worldwide mainly of young children [2]. Amongst the four species of Plasmodium that transmit human malaria, Plasmodium falciparum is responsible for the most severe clinical manifestations of disease and causes most malaria morbidity and almost all malaria mortality [3]. Annually, a big proportion of funding and staffing in the world is devoted to malaria problems [4]. However, a variety of factors, including insecticide resistance in vectors, the lack of efficient vaccine, and the emergence and rapid spread of drug-resistant strains are contributing to the deterioration of global malaria situation [5]. Therefore, there is an urgent need to develop effective malaria vaccines [6].

However, extensive genetic diversity in natural parasite populations is the major barrier for the development of an effective vaccine against human malaria parasites [7], since antigenic diversity limits the efficacy of acquired protective immunity to malaria [8]. Such extreme antigenic diversity increases the ability of the parasite to evade the host immune responses [9]. A true understanding about frequency of vaccine candidate antigens and changes in natural parasite populations is important to design a successful and effective malaria vaccine and also provides useful facts to interpret immunological responses to vaccination [7]. A limited number of stage-specific antigens of the Plasmodium falciparum vaccine candidates have been identified using novel molecular techniques [10]. We have analyzed the genetic diversity of Merozoite surfaceprotein-2 (MSP-2) antigens as potential vaccine candidate [11]. Plasmodium falciparum MSP-2 antigen is a 45 to 55 KDa glycoprotein that is produced during the early stages of schizogony during the parasite life cycle and appears on the surface of the merozoites [12]. MSP-2 gene which is located on chromosome 2 encodes a glycoprotein on the surface of Merozoites that is widely used in designing new malaria vaccines [7,13]. DNA sequencing has revealed that each copy of the MSP-2 gene is conserved by C-terminal and N-terminal regions (blocks 1 and 5).
and two regions of repeated sequences (blocks 2 and 4). There is only a central polymorphic area known as Block 3 [14]. In the central polymorphic area of MSP-2 gene, FC27 and 3D7 dimorphic alleles show the greatest diversity [15]. In comparison to FC27 family, 3D7 alleles are much more variable in length and sequence [16]. Furthermore, this antigen is capable of inducing an effective immune response during blood stage [6,11]. MSP-2 is therefore a strong vaccine candidate with limited epidemiologic data; data that are needed to support continued development along the proposed malaria vaccine roadmap [6,11,15,17]. Iran is located in the Eastern Mediterranean Region, and grouped as low-moderate endemic region [18]. Sistan and Baluchistan Province, South-East of Iran, is the endemic area of *falciparum* malaria and is considered as the oriental epidemiological region of malaria [19].

This study investigates allelic variation in the *P. falciparum* MSP-2 gene among samples collected from four different endemic regions in South-East of Iran. Such data are important because the increased frequency of simple infections in such a setting enables us to look at changes in allele frequency over time, which might provide evidence for or against the presence of allele-specific and variant-specific immune responses.

**MATERIALS AND METHODS**

In this cross-sectional study, 94 individualssuffering from *falciparum* malaria referring to Malaria Centers of Chabahar, Iranshahr, Nikshahr and Sarbaz were selected from April 2011 to September 2012 to characterize allelic variation within *P. falciparum* MSP-2 in this endemic area. Residence in these regions for over 6 months, no history of consuming anti-malarial drugs during the last month, and written informed consent were required for inclusion in this study. The presence of *P. falciparum* infections in the samples were confirmed microscopically using thick and thin Giemsa-stained slides in Department of Parasitology, Zahedan University of Medical Sciences. Venous whole blood (2 ml) was collected from each consenting patient. The samples were stored at -20 °C until using for DNA extraction. DNA was extracted using Fermentas Genomic DNA Purification Kit (Thermo Fisher Scientific Inc.) from the whole blood samples. All DNA samples were stored at -20°C before genotyping with a polymerase chain reaction.

**Table 1: List of Primers and sequences**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence Length 5′→3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-2-OF</td>
<td>GAG TAT AAGGAG AAG TAT GG</td>
</tr>
<tr>
<td>MSP-2-OR</td>
<td>CTT GTA CCT TTA TTC TCT GG</td>
</tr>
<tr>
<td>M1-FC27-FCF</td>
<td>AAT ACT AAG AGT GTA GGT GCA ATG CTC CA</td>
</tr>
<tr>
<td>M1-FC27-FCR</td>
<td>TTT TAT TTGGTG CAT TGC CAG AAC TTG AAC</td>
</tr>
<tr>
<td>M1-IC-ICF</td>
<td>AGA AGT ATGGCA GAA AGT AAG CCT CTC ACT</td>
</tr>
<tr>
<td>M1-IC-ICR</td>
<td>GAT TGT AAT TCQGGG GAT TCA GTT TGT TCQ</td>
</tr>
</tbody>
</table>

The first and second round PCR amplifications were performed in a final volume of 20 μl using AccuPower TLA PCR Premix (Bioneer, Korea Rep). Cycling conditions for the first PCR cycle were 94°C for 5 minutes (Initial Denaturation), 94°C for 1 minutes (Denaturation), 58°C for 1 minutes (Annealing), and 72°C for 1 minutes (Extension), followed by a final extension at 72°C for 5 minutes, for a total of 24 cycles. The second PCR cycles conditions were the same whereas the annealing temperature was considered 61°C for a total of 30 cycles. Purified DNA from *P. falciparum* 3D7 (MRA-102G) and FC27 strains were provided by the Malaria Research and Reference Reagent Resource Center, American Type Culture Collection (Manassas, VA) and used as positive control during the amplification reactions. The second amplification products were directly separated by electrophoresis on a 2.0% ethidium bromide agarose gel and visualized on a Tranillumination Imaging System. Positive controls and a 1000bp Ladder Marker (Bioneer, Korea Rep) were used to interpret the fragments sizes. Total number of gene variants observed in the central region of MSP-2 was 8. Four fragments (280, 300, 380, 400bp) were observed in FC27 alleles and 4 of which (400, 470, 500, and 600bp) were relevant to 3D7 alleles. Multi-clonal infections were defined by the presence of FC27 and 3D7 MSP-2 alleles simultaneously.

**RESULTS AND DISCUSSION**

**Results:**

The aim of this study was to analyze the polymorphic antigen MSP-2 gene across South-East of Iran among four different districts to identify differences in allele frequency and genetic diversity. Among 94 *P. falciparum* samples obtained from the four districts, 85 samples were successfully scored for the presence of MSP-2 gene. Nested PCR on MSP-2 confirmed samples revealed that both 3D7 and FC27 allele classes of *P. falciparum* MSP-2 were present in the districts of study. The MSP-2 allele classes (FC27 and 3D7 types)
showed reasonable prevalence in all districts (Table 2). The frequency of MSP-2 genes in the districts of Chabahar, Iranshahr, Nikshahr and Sarbaz were 34.2%, 23.6%, 23.3% and 3.53% whereas the overall frequencies of FC27 and 3D7 allele classes were 34.2% and 50.5% respectively for each. Among the samples, 13 (15.3%) cases showed multi-clonal infections. The frequency of variants in different areas did not show significant differences.

**Table 2: Merozoite surface protein-2 allele prevalence in South-East of Iran**

<table>
<thead>
<tr>
<th>District</th>
<th>Chabahar</th>
<th>Iranshahr</th>
<th>Nikshahr</th>
<th>Sarbaz</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td><strong>FC27</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>280bp</td>
<td>2</td>
<td>2.36</td>
<td>2</td>
<td>2.36</td>
<td>2</td>
</tr>
<tr>
<td>500bp</td>
<td>3</td>
<td>3.53</td>
<td>2</td>
<td>2.36</td>
<td>2</td>
</tr>
<tr>
<td>380bp</td>
<td>3</td>
<td>3.23</td>
<td>1</td>
<td>1.20</td>
<td>1</td>
</tr>
<tr>
<td>400bp</td>
<td>1</td>
<td>1.20</td>
<td>3</td>
<td>3.53</td>
<td>2</td>
</tr>
<tr>
<td><strong>3D7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>470bp</td>
<td>10</td>
<td>11.80</td>
<td>5</td>
<td>5.90</td>
<td>4</td>
</tr>
<tr>
<td>500bp</td>
<td>4</td>
<td>4.70</td>
<td>2</td>
<td>2.36</td>
<td>3</td>
</tr>
<tr>
<td>600bp</td>
<td>0</td>
<td>0.00</td>
<td>1</td>
<td>1.20</td>
<td>2</td>
</tr>
<tr>
<td>FC27 + 3D7</td>
<td>5</td>
<td>5.90</td>
<td>2</td>
<td>2.36</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>34.20</td>
<td>20</td>
<td>23.60</td>
<td>20</td>
</tr>
</tbody>
</table>

**Discussion:**

We used Nested PCR to evaluate allelic variations within the malaria vaccine candidate *P. falciparum* MSP-2 in South-East of Iran. Nested PCR has been shown to possess high sensitivity and specificity of up to 94% in some tests [20] and exhibits a high-throughput capacity in comparison to other PCR modifications in the field studies, and is considerably more cost-efficient versus sequencing [18,19]. We allele typed 85 individual *P. falciparum* infections containing *P. falciparum* MSP-2 genes using Nested PCR and realized that both 3D7 and FC27 allele classes of MSP-2 gene were present in the region. In the present study seasonal frequencies of each allele classes were ignored. It was observed that the MSP-2 gene in this region consists of 8 fragments that shows a higher rate in comparison to a similar study in Columbia [21] and Senegal [22] in which 3 and 7 allele classes were ignored. It was observed that the MSP-2 gene in this region consists of 8 fragments that shows higher rate in comparison to a similar study in Columbia [21] and Senegal [22] in which 3 and 7 fragments were demonstrated respectively. Although this extent of allelic diversity is not comparable to 17 fragments for MSP-2 gene in Thailand [23]. It seems as if variations distribution is highly affected by geographical region and the gene status and number of fragments can vary from area to area and act as a major contributor to allele variation in small populations. It should be kept in mind that the population structure of *Plasmodium Falciparum* different studies has some limitations because of using different molecular methods and differences in experimental conditions [16]. But the comparison of this study with similar studies especially in areas with low endemicity shows that the genetic diversity of these parasites is at higher grade (24). Therefore, in the major endemic foci of *Plasmodium Falciparum* in Iran we are not dealing with a homogeneous population of parasites and it is likely that patients become contaminated with more than two parasite clones or strains simultaneously (Multi-clonal Infection) [25].

Due to the heterogeneous population in the region multi-stage vaccines should be designed to control *P. falciparum* different stages of evolution. Therefore, these data are needed to support development of a vaccine based on MSP-2 antigen along the malaria vaccine road map. Also, the results show no remarkable predominance of any allele in the studied area. There should be a comparative analysis in different seasonal peaks to indicate the allelic polymorphism of the MSP-2 over a period of time. These data support the hypothesis of a biologically important role for MSP-2 in parasite development and highlight the importance of evaluating the distribution of MSP-2 allelic forms in different geographical regions to provide valuable genetic information to design an effective malaria vaccine despite the extensive present genetic diversity.

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**REFERENCES**


