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Original Article

Partial Sequence Analysis of Merozoite Surface Proteine-3α Gene in *Plasmodium vivax* Isolates from Malarious Areas of Iran

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Abstract

Background: Approximately 85-90% of malaria infections in Iran are attributed to *Plasmodium vivax*, while little is known about the genetic of the parasite and its strain types in this region. This study was designed and performed for describing genetic characteristics of *Plasmodium vivax* population of Iran based on the merozoite surface protein-3α gene sequence.

Methods: Through a descriptive study we analyzed partial *P. vivax* merozoite surface protein-3α gene sequences from 17 clinical *P. vivax* isolates collected from malarious areas of Iran. Genomic DNA was extracted by Q1Aamp® DNA blood mini kit, amplified through nested PCR for a partial nucleotide sequence of *PvMSP-3α* gene in *P. vivax*. PCR-amplified products were sequenced with an ABI Prism Perkin-Elmer 310 sequencer machine and the data were analyzed with clustal W software.

Results: Analysis of *PvMSP-3α* gene sequences demonstrated extensive polymorphisms, but the sequence identity between isolates of same types was relatively high. We identified specific insertions and deletions for the types A, B and C variants of *P. vivax* in our isolates. In phylogenetic comparison of geographically separated isolates, there was not a significant geographical branching of the parasite populations.

Conclusion: The highly polymorphic nature of isolates suggests that more investigations of the *PvMSP-3α* gene are needed to explore its vaccine potential.

Keywords: *Plasmodium vivax*, Merozoite surface protein-3α, Iran

Introduction

Malaria is a major health threat in many areas of the world, particularly in tropical and subtropical countries. The disease affects 300-500 million people worldwide (1).

Although among the four human malaria parasites (*Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*), *P. falciparum* causes the most sever forms of malaria, in the recent years, the number of reported cases of *P. vivax* has been increasing in many regions of the world. At present, *P. vivax* has become the most prevalent of the four human malaria species (2). Human malaria caused by *P. vivax* causes a debilitating febrile illness in approximately 90 million people each year. Sever and widespread morbidity associated with endemic *P. vivax* malaria in Asia and the Americas imposes a heavy social and economic burden (3).

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In Iran, in recent years about 85-90% of malaria cases caused by *P. vivax* and its transmission is almost located in southern and east-southern provinces of the country (59%, 25%, 6% and 1% of malaria cases in the country reported from Sistan-Baluchistan, Hormozgan, Kerman and Bushehr provinces, respectively) (4). The majority of publications on *Plasmodium* genetic structure using polymorphic markers such as merozoite surface protein-1 (*MSP-1*), *MSP-2*, glutamate- rich protein, and microsatellites focused on *P. falciparum*,. In case of *P. vivax* only the dimorphic circumsporozoite protein (CSP) gene and the *MSP-1* gene have been widely used for genotyping (3). The *PvCSP* gene has a central repeat domain that differs in sequence and number of repeat units (5, 6). The *P. vivax MSP-1* gene is used to assess the genotypic variety of isolates from different geographical regions and to determine whether a malaria infection is a result of a new infection or a relapse (7-9). The *P. vivax* merozoite surface protein-3α (*PvMSP-3α*) is a genetic marker and a potential vaccine candidate that has been recently validated and used for studies on population genetic structure (3, 10, 11). *PvMSP-3α* gene is highly polymorphic, and three major types of the gene (A, B, and C) are distinguishable (2, 11, 12). As, immune responses targeting one form of an antigen may not be effective against parasite strains expressing other forms of the antigen, overcoming genetic diversity is a great challenge in designing MSP-based vaccines, and the extent of genetic diversity of candidate antigens must be thoroughly evaluated before an effective vaccine is developed (2).

In spite of several investigations on genetic structure of *P. vivax* and large number malaria cases in Iran, there is not adequate information on genetic structure of *P. vivax* population in the country and little is known about its strain types in malarious areas of Iran. Therefore, characterization of field population of *P. vivax* in Iran based on the partial sequences of *PvMSP-3α* gene was the main objective of this study.

Materials and Methods

Study population

Through a descriptive study, in year 2006, blood samples were collected from patients with clinical symptoms of malaria attending to malaria clinics in the malarious areas of Sistan - Baluchistan, Hormozgan, Kerman, and Bushehr provinces. Sample collection was approved by the Ethical Committee of Tehran University of Medical Sciences and performed after obtaining informed consent from each subject.

Blood specimens were taken by experienced technicians using venipuncture or finger prick. Blood films were prepared, stained with Giemsa and examined microscopically by experienced microscopists. Treatment was administrated to those positive for malaria according to guideline provided by Iranian Ministry of Health. In positive cases approximately 1000 µl of venous blood was collected in EDTA and stored in -20 ºC for further tests.

DNA extraction and PCR amplification

DNA was extracted by Q1Aamp® DNA blood mini kit 50 (Qiagen, Germany) according to the instruction. Reconfirmation of primary microscopy diagnosis of the parasite in all samples were checked by nested-PCR using plasmodium genus specific (primary PCR) and *P. vivax* and *P. falciparum* species-specific primers (nested PCR) (13). The target sequensem was amplified through nested PCR by primers bind at positions 111-131 and 2286-2305 (primary PCR),

P1-5/ CAGCAGACACCATTTAAGG3;P2-5/CCGTTTGTT GATTAGTTGC3/,

and positions 205-227 and 2078-2100 (nested) N1-5/GACCAGT GTGATACCATTAACC3/; N2-5/ATACTGGTTCTTCGTCTTCAGG3/ of the Belem reference laboratory strain coding sequence (9). PCR was performed, based on

previously introduced protocol (9), with an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 56 °C for 30 sec and 68 °C for 2.5 min. Nested PCR was performed with 30 cycles of 94 °C for 30 sec, 30 sec at 57 °C, 68 °C for 2.5 min. DNA sequences of polymorphic region, that was performed trough CinnaGen Company (Tehran, Iran) were obtained from 17 isolates. Pair wise sequence alignment and comparison were performed using BLAST program in the NCBI databases http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Nucleotides (14). Multiple sequence alignment was constructed with ClustalW version 1.83 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Sequence data were compared with published sequences of *P .vivax* including Thailand isolates (Accession number: AY833025) and Belem reference strain (Accession number: AF093584). A phylogenetic tree was derived from the aligned nucleotide to determine any geographical branching and relationships.

Results

Seventeen PCR products corresponding to nucleotides about 1950-2368 of the Belem reference strain (15) (779–847 bp) were sequenced (Figs. 1-3). Sequence analysis showed that the nucleotide sequences in all three biotypes were accompanied with a large number of insertions and deletions compared between together and with the well-known Belem strain (Accession number AF093584) and a strain from Thailand (Accession number AY833025.1) that some of them are biotype-specific (Fig. 1-3).

Aligned nucleotides were used for phylogenetic analysis. Five sequences, 39, 23, 415, 414 and 312, were assigned to a separate group (common branch) (Fig. 4) in the tree. We found that no isolate grouping was based on the geographic origin (Fig. 4). Phylogenetic analysis showed that only two isolates [64 and 617] from similar geographical origin (Boushehr province) were grouped together. Instead, many of the closely related sequences were isolates from different geographical locations (e.g. clustering of isolate51 from Hormozgan and isolate 610 from Boushehr in Fig. 4).

The homology between the isolates grouped in type A (isolates 213, 77, 67, 64, 617, 37, and 34) was 82/8, type B (isolates 51, 111, 610 and 614) was 88/2 and type C (isolates 312, 23, 415, 414, 13 and 39) was 95%. The mean of the sequence homology score in ClustalW between the isolates of type A and Belem reference strain (83/9), and type B and Belem reference strain [76] and type C and Belem reference strain (87/3) were less than the mean of the sequence homology between isolates of the types A, B and C and Thailand strain (86/9, 74 and 84/7 respectively).

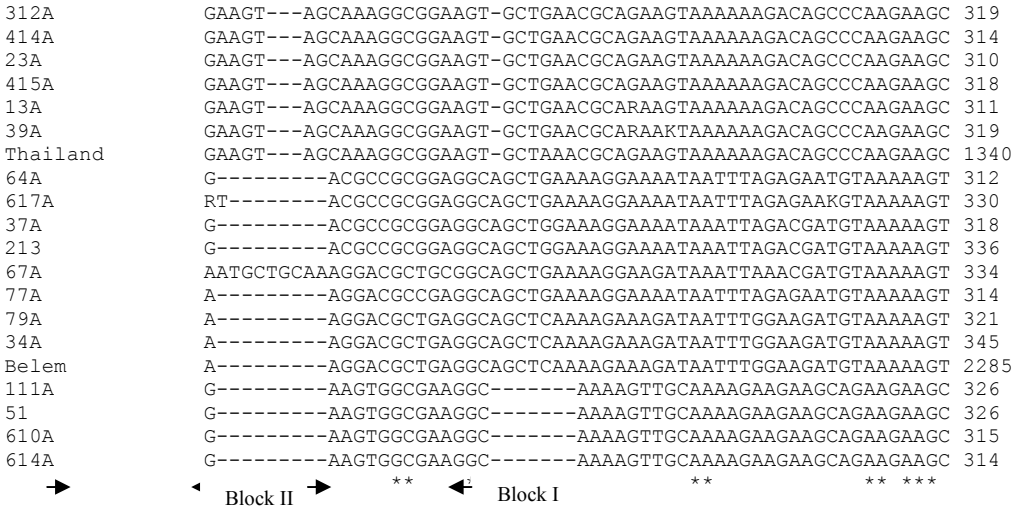

Fig. 1: Partial nucleotide sequences alignment of *PvMSP-3α* gene in *P. vivax* isolated from malarious areas of Iran and corresponded part of the gene in one isolate from Thailand (Accession number AY833025) and Belem reference strain (Accession number AF093584). Specific deletions for the type B and A variants (block II for type B and block III for types A and B) is shown

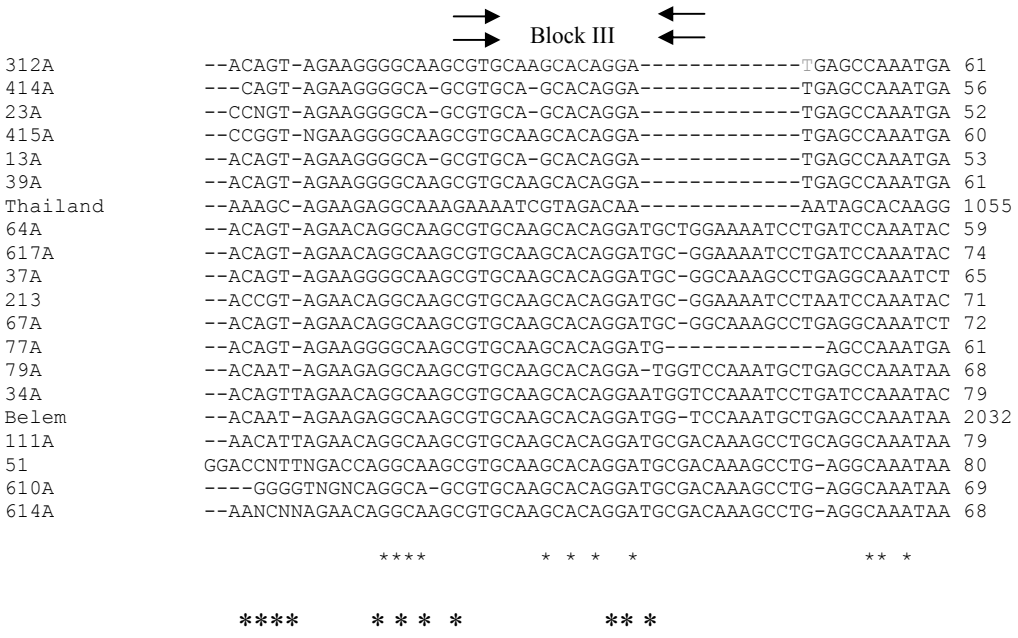

Fig. 2: Partial nucleotide sequences alignment of *PvMSP-3α* gene in *P. vivax* isolated from malarious areas of Iran and corresponded part of the gene in one isolate from Thailand (Accession number AY833025) and Belem reference strain (Accession number AF093584). Block I indicates deletions specific to type C of the gene and Thai isolate

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                    ➔ Block IV ➔
312A                GCGGTGGACGC----------------------------ATTGGAAGAAGCGTATGCA 527
414A                GCGGTGGACGC----------------------------ATTGGAAGAAGCGTATGCA 522
23A                 GCGGTGGACGC----------------------------ATTGGAAGAAGCGTATGCA 518
415A                GCGGTGGACGC----------------------------ATTGGAAGAAGCGTATGCA 526
13A                 GCGGTGGACGC----------------------------ATTGGAARAAGCGTATGCA 519
39A                 GCGGTGGACGC----------------------------ATTGGAAGAAGCGTATGCA 527
Thailand            GCGGTGGACGC----------------------------ATTGGAAGAAGTGTATGCA 1548
64A                 ----------------------------------------TTCTGATACAACAAAAACG 507
617A                ----------------------------------------TTCTGATACAACAAAAACG 525
37A                 ----------------------------------------TTCTGATACAACAAAAACG 513
213                 ----------------------------------------TTCTGATACAACAAAAACG 531
67A                 ----------------------------------------ATTTAAGACAACAAAAACG 508
77A                 TCAGATGAGGCCCAGAAGGCACACGCGAATGTGCAACAGGCTTCTAAGACAAAAGAAGCT 551
79A                 CCCCCAAARCGGCAAAGGGC----------------------CTCTAAACCAACAAAACCG 516
34A                 CCAAAAAAGGGGCAAAAAGCAAACCCGAAARACCAACRGGGTTTTAARACAGCAAARGGG 582
Belem               ----------------------------------------TTCTGATACAACAAAAACG 2480
111A                ------------------------------------------CAGCAACGTCAGACGCG 516
51                  ------------------------------------------CAGCAACGTCAGACGCG 516
610A                ------------------------------------------CAGCAACGTCAGACGCG 505
614A                ------------------------------------------CAGAAACGTCAGACGCG 504
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Fig. 3: Partial nucleotide sequences alignment of *PvMSP-3α* gene in *P. vivax* isolated from malarious areas of Iran and corresponded part of the gene in one isolate from Thailand (Accession number AY833025) and Belem reference strain (Accession number AF093584). An insertion of block IV specific to type C of the gene and Thai isolate is shown

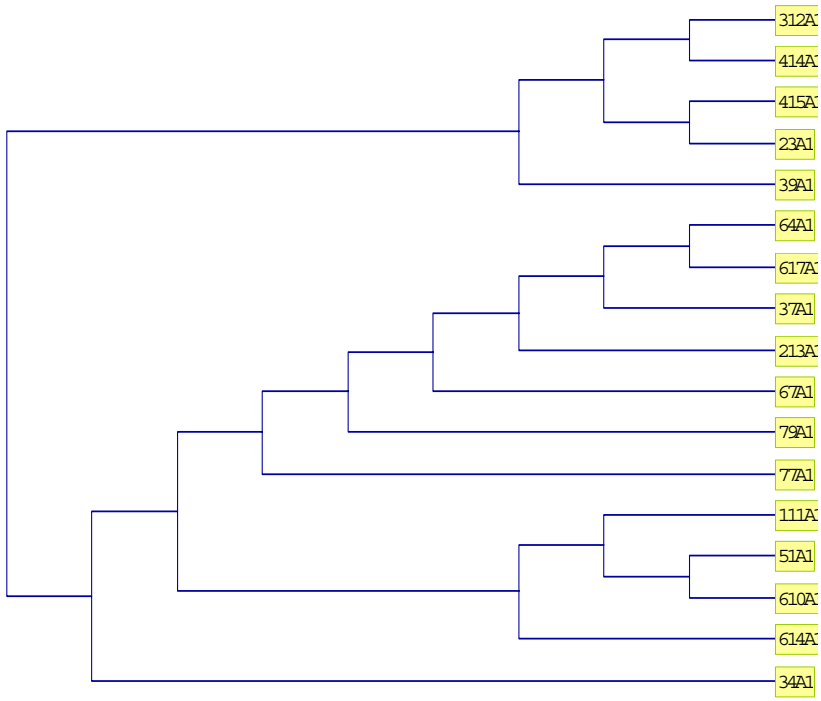

Fig. 4: Phylogenetic tree of *PvMSP-3α* gene alleles constructed using 17 available sequences from malarious areas of Iran., A significant geographical branching of the parasite populations is not seen in phylogenetic comparison of geographically separated isolates

Discussion

Recently, the economy of the malaria endemic provinces of Iran (Sistan-Baluchistan, Hormozgan, Kerman and Bushehr) has suffered from heavy losses during the malaria epidemics, and malaria control program imposes a brutal burden to any development programs (4), therefore, more investigations on parasite genetic structure for vaccine and drug development against the parasite will be vital. *Plasmodium* merozoite surface proteins, which interact with the red blood cells, are first vaccine candidates (2). One of these proteins that is seems to be a useful marker for genetic polymorphism of *P. vivax* in endemic areas is merozoite surface protein-3α, that's why, this marker has been the subject of many studies in the world (3, 12, 16). Besides its epidemiologic importance, this marker is also known to be a potential candidate for vaccine development (12). The potential vaccines should focus on the C-terminus (the nucleotide sequence positions 1,300–2,058) of the alanine-rich domain and the acidic C-terminal region, because this region is highly conserved over a range of geographically separate *P. vivax* isolates (12). Until today, no information was available on the sequence–based genetic characteristics of *PvMSP-3α* gene of the Iranian isolates, thus, studies at this antigenic site were required.

We have already reported the size polymorphism and RFLP patterns of *PvMSP-3α* gene. (17). As we presented there, three biotypes of the parasite A (about 1900bp), B (about 1400bp) and C (about 1100bp) in 78%, 6% and 16%, respectively were observed. According to our previous study as well as the results of Zakeri *et al*. (10), Iranian isolates was classified into three allelic types, A, B and C, based on the size of *PvMSP-3α* gene. Theses groups are almost similar to the results of previous studies carried out in other countries (3, 17). Although the sample sizes were different, this variety was higher than that of India (10) and Papua New Guinea (18). In the present study we have obtained partial *PvMSP-3α* sequences from 17 *P. vivax* isolates from malarious areas of Iran and assessed the sequence diversity of the gene in the isolates. Analysis of *PvMSP-3α* sequences in this study demonstrated extensive polymorphisms, comparable with other studies (2, 3, 12) albeit the sequence identity between isolates of the same types was relatively high. By sequence analysis we identified specific deletions in type B and A variants (block I in type B and block II in types A and B in Fig. 1). Since both variant types have been found in isolates from other malaria areas, it is rational to assume that these deletions are not fundamental for the surviving of the parasite (3). However, it may have reduced their eligibility because the two variants are present in less than 22 % of parasite genotypes (based on the size of PCR product) in our study. Comparing with type A and type B, type C has specific blocks of deletion and insertions that are similar to Thailand isolate. For example, deletions of block III corresponding to nucleotides 2008–2020 of the Belem reference strain (Fig. 2) and insertions corresponding to nucleotides 1286-1290 of the Thai strain (Fig. 1) and insertions of block IV (Fig.3) are specific to type C. It is noteworthy that the regions with deletions are the most polymorphic, which suggest that this region of the molecule might be selected against by the host immune system (3). The result of this study suggested the lack of significant geographical branching of the parasite populations (Fig.4) and only two isolates [64 and 617] from similar geographical origin (Boushehr province) were grouped together, although there are limited number of *PvMSP-3α* gene sequence available to perform a phylogenetic comparison of geographically separated isolates. Attempts to recognize phylogenetic relationships among the global *P. vivax* isolates failed to show any geographical structure of the parasite populations, and sequences that cluster together in the phylogenetic trees are often

from distinct geographical areas (2), (e.g. clustering of isolate 51 from Hormozgan and isolate 610 from Boushehr in Fig.4). In the other word, there is no indication of clear allelic families that are present only in certain geographical samples (2), Although, the fact that isolates from Iran have different similarities to the isolates from different regions of the world (Fig. 1-3) suggests that some of geographic isolation may exist. However specific studies need to be performed to address this issue.

In conclusion this preliminary study will serve as a basis for future detailed studies about the population genetic of *P. vivax* in different geographic regions. High parasite heterogeneity and inadequate detailed knowledge of the parasite genetic in Iran deserve further study.

Acknowledgments

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