Iranian Journal of Clinical Infectious Diseases
*2010;5(3):126-132*


**ORIGINAL ARTICLE**

# Genetic structure of *Plasmodium vivax* population assessed by sequence analysis of the merozoite surface protein 3*β* gene

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## ABSTRACT

**Background**: The endemicity and transmission intensity levels of malaria are related to genetic diversity of the parasites. Merozoite surface protein 3β (MSP3β) is an important marker for assessing the polymorphic nature of *Plasmodium vivax* while it is also a vaccine candidate against the parasite.

**Patients and methods**: In this study we investigated the genetic structure of *P. vivax* population by sequence analysis of a polymorphic region of the *P. vivax* MSP3β gene in isolates from Iran. Blood samples were collected from 100 patients with clinical symptoms. DNA was extracted and the target gene was amplified by polymerase chain reaction (PCR). The sequences of 17 samples were used for sequence analysis using nucleotide Blast search and ClustalW multiple alignment. Phylogenetic tree was derived to describe the geographical branching and relationships.

**Results**: A large number of nucleotide insertions and deletions were observed in the sequences of polymorphic region of PvMSP3β gene that were not specific in each biotype. Single nucleotide polymorphism (SNP) was found extensively in the sequences. The phylogenetic analysis did not show any significant geographical branching.

**Conclusion**: The lack of any geographical branching and extensive polymorphism in MSP3β gene of *P. vivax* isolates suggests that more investigations are needed to find a more suitable gene in order to develop a vaccine.

**Keywords**: *Plasmodium vivax, Merozoite surface protein 3β, Malaria, Vaccine.*
***(Iranian Journal of Clinical Infectious Diseases 2010;5(3):126-132).***

## INTRODUCTION

Although it is usually a non-lethal disease, *Vivax* malaria has major injurious effects on human well-being growth and on the economic progress at individual, family, community, and national levels because of its extended and recurrent infection (1). At present, *Plasmodium vivax* causes an estimated 80-90 million cases each year and is the most widely distributed human malaria species in the world. In the Americas and Asia, *P. vivax* is the most prevalent malaria species (2). Lately, re-emerging of *vivax* malaria has occurred in many regions where it had been largely eradicated during the malaria control programs such as Korea, temperate provinces of China, and some former Soviet republics (3-6). Although, there are rare and

*Received*: 15 November 2009 *Accepted*: 20 June 2010
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sporadic reports of severe and fatal *P. vivax* infections (7), as resources for malaria control are currently often directed against the more lethal *P. falciparum* malaria, the hidden burden of *P. vivax* malaria is likely to become increasingly apparent.

For monitoring the distribution of drug resistance and for evaluation the performance of the vaccines under development, understanding of population structures of the parasite is important (8). It has been shown that different *P. vivax* isolates exhibit distinctive biological characteristics (e.g., relapse patterns or transmissibility to mosquitoes). This is a matter of fact even within a local population, which are often used to specify geographical strains and subspecies (9). Such a biological diversity is demonstrated by genotyping approach using various polymorphic genetic markers including the genes coding for circum sporozoite protein (CSP) (10), the apical membrane antigen 1 (AMA1) (11), the Duffy binding protein (DBP) (12), the merozoite surface protein 1 (MSP1) (13), and the merozoite surface protein 3α (MSP3α) (14-16) and merozoite surface protein 3 β (MSP3 β) (17). The *P. vivax* MSP3 α, β and γ constitute a protein family with the central Ala-rich region with an extensive coiled-coil structure (18, 19). The genes encoding these proteins are highly polymorphic (17-23). Sequences of MSP3β in *P. vivax* isolates from various geographical regions of the world are radically divergent with the majority bearing large insertion/deletion mutations in the central Ala-rich region (21).

In regions where *P. vivax* and *P. falciparum* are coexistent, transmission of *P. vivax* has been become more prevalent. In Iran, more than 80% of all malaria cases causes by *P. vivax* (24). Today, transmission of *P. vivax* is almost located in southern and east-southern provinces of the country including Sistan and Bluchestan, Hormozgan, Kerman and Boushehr (24). As malaria studies are of practical importance in the development and utilization of control programs (8), and because of the great importance of MSP3β gene as a genetic polymorphic marker and a vaccine candidate (20, 21,17), we have described the polymorphic nature of MSP3β gene based on the partial sequence analysis of the gene in *P. vivax* isolated from malarious areas of Iran. These data can enrich the databases obtained from other malarious countries.

## PATIENTS and METHODS

For this descriptive study, a total of 100 positive-blood samples were collected from patients with clinical symptoms of malaria attending to Malaria clinics in the malarious areas of Sistan and Baluchestan, Hormozgan, Kerman, and Boushehr provinces. Under the supervision of Ethical Committee of Tehran University of Medical Sciences, sample collection was achieved after obtaining informed consent from each subject. Blood specimens were taken by experienced laboratory technicians with use of venipuncture or finger prick. Blood slides were stained with Giemsa and examined by well-experienced microscopists. When revealed positive, approximately 1000µl of venous blood was mixed with EDTA and stored in -20ºC for further studies.

For DNA extraction and PCR amplification, Q1Aamp® DNA blood mini kit 50 (Qiagen, Germany) was used. All samples were rechecked by nested-PCR using plasmodium genus specific (primary PCR) and *P. vivax* and *P. falciparum* species-specific primers (nested PCR) (25) for reconfirming of primary microscopy diagnosis of the parasite.

The fragment was amplified through PCR by forward (5`- AACTTGAGAAACGGATG-3') and reverse (5'-TGCGAGTGTTTTATGCG-3') specific primers (21), which amplify a small fragment of *P. vivax* MSP3ß (PvMSP3ß) by binding at positions 102-118 and 1943-1961 of corresponding region of PvMSP3ß in the Belem strain (GenBank accession number AF099662). The PCR was performed with an initial denaturation of 2.5min at 95°C, followed by 35

cycles of 95°C for 30sec, 56°C for 30sec and 72°C for 2.5min (26).

Seventeen samples selected according to the previous study (17), were subjected to DNA sequencing. DNA sequencing of the target polymorphic DNA region was performed through Cinnagen Company (Tehran, Iran). The program BLAST (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) was used for pair wise sequence alignment and comparison with all similar sequences deposited in GenBank. The version 1.83 of ClustalW program *(http://www.ebi.ac.uk/Tools/clustalw2/index.html)* was used for multiple sequence alignment and comparison between obtained sequences and published relevant sequences of *P. vivax* including Bengal isolates (Accession number AY454084), Belem reference strain (Accession number AF099662) and SalI strain (Accession number AY454095). A phylogenetic tree was derived from the aligned nucleotides to describe geographical branching and relationships.

## RESULTS

Totally, 17 PCR products corresponded to nucleotides 1780-2800 of the Belem reference strain (Accession number AF093584), 1-1030 of the Bangal strain (Accession number AY454084) and SalI strain (Accession number AY454095) were selected for sequencing. In our setting, associated gene sequenced part contained approximately 830-1025 bp (Figure 1A). A large number of nucleotide insertions and deletions were observed, of which none matched with previously described biotypes (17) (Figures 1B and 1C). In some cases the blocks of nucleotide insertions and deletions were respondent to the strains of other countries such as Bangal strain (Figure 1C). Single nucleotide polymorphism (SNP) was seen extensively in the sequences (Figures 1B and 1C). The phylogenetic analysis did not show any significant biotype or geographical specific branching (Figure 2). Only four sequences (isolates 619, 69, 63, and 604 from Boushehr province) were assigned to a separate group or specific branch in the phylogenetic tree (Figure 2). The range of homology score among the isolates through ClustalW program was between 41-97%. The average of the sequence homology score through ClustalW program was 72% (in the range of 45-82) between the isolates of Iran and Belem reference strain and 71% (in the range of 48-82) between Iran and SalI strain.

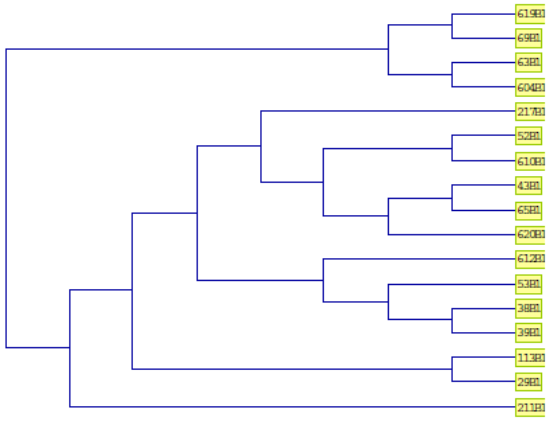

**Figure 2.** Phylogenetic tree of PvMSP-3β gene sequences using 17 isolates from malarious areas of Iran. A significant geographical branching of the parasite populations is not seen in geographically separated isolates. Only four isolates (619, 69, 63, and 604) from similar geographical origin (Kangan district in Boushehr province) were grouped together in a separate branch

## DISCUSSION

The endemicity and transmission intensity levels of malaria may have some relationship with genetic diversity of the malaria parasites (27). Diversity and multiplicity of *P. vivax* in hyper-endemic areas such as Papua New Guinea is high (14). In hypo-endemic areas such as Thailand and Iran, diversity and polymorphism of the parasite and its complex genetic structures has been reported (6,16,17,23,28).

In the present study we report high level of single nucleotide polymorphism (SNP) and several blocks of insertions and deletions among

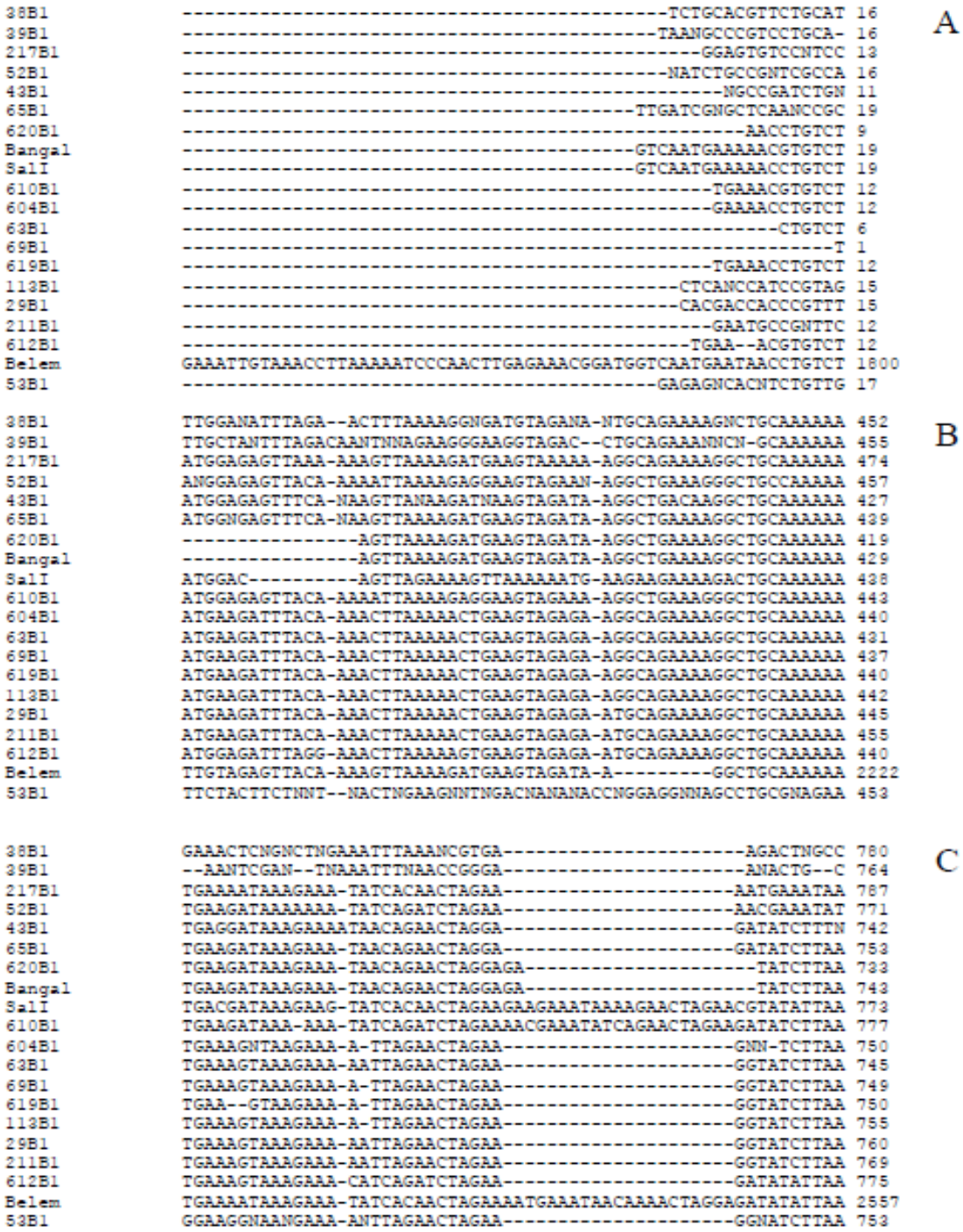


**Figure 1.** Partial nucleotide sequences alignment of *PvMSP-3β* gene in *P. vivax* isolated from malarious areas of Iran and corresponded part of the gene in Belem reference strain (Accession number AF093584), Bangal strain (Accession number AY454084) and SalI strain (Accession number AY454095). High level of single nucleotide polymorphism among the isolates and a block of deletion in sequence of the isolate 620 is noticeable in part B and large block of insertion is noticeable in isolate 610 correspond to nucleotide 2526-2547 of Belem strain and to nucleotide 742-763 of SalI strain in part C. None of the other isolates entailed this block.

nucleotide sequences of the *PvMSP3β* locus in the Iranian isolates (Fig. 2). Such diversity has been reported previously in only two reports (21,22); therefore this is the first sequence-based study for the gene in Iran and the third one all over the world.

Malaria in Iran is hypo-endemic and transmission is seasonal and experiences a break during the winter (24), hence, generation and maintenance of considerable genetic diversity at the nucleotide sequences of the *PvMSP3β* locus in malarious areas of the country is partially due to increased travel followed by the introductions of diverse parasite strains from neighboring endemic countries (6,24,29).

In our study the most homology in the *PvMSP3β* locus was found between the isolates 619 and 63 (98%) both from Kangan district, and minimum homology was found between isolate 43 and 52 both from Minab district. Therefore, in case of this parasite, belonging to the same geographical origin can not necessarily cause a dramatic homology in genetic structure. A considerable number of insertions, deletions and SNP in the sequences was observed, but was locality or biotype specific variety (17) (Fig. 1B). Such a variety has been formerly reported in other studies (21,27). Existence of blocks of insertion and deletions means that the diversity of *PvMSP3β* locus can be easily assessed by size polymorphism or restriction fragment length polymorphism (RFLP) (2), as we have already shown the utility of these methods for diversity assessment of this locus (17).

We compared our sequences with the sequences of Belem reference strain, Bangal strain and SalI strain. Maximum and minimum identity of the sequences with Belem reference strain were 85% and 45% corresponding to isolates number 619 and 53 from Kangan in Boushehr province and Minab in Hormozgan province, respectively. Comparison of sequences with Bangal strain and SalI strain showed the maximum identity score of 95% and 84% (both from Kangan), respectively.

According to phylogenetic dendrogram, we found that there is not a significant geographical branching of the parasite populations (Fig. 2) and only four isolates (619,69,63 and 604) from similar geographical origin (all from Kangan district in Boushehr province) were grouped together in a separate branch. Up to now, attempts to reveal phylogenetic branching and relationships among the global *P. vivax* isolates failed to demonstrate any geographical structure of the parasite populations, and sequences obtained from distinct geographical areas cluster together in the phylogenetic trees (22). In the present study isolates 612 from Bousher, 53 from Hormozgan and 38 from Sistan and Baluchestan with distance of more than 1000Km have clustered together (Fig. 2). However, it cannot be ignored that the similarities between our isolates and the isolates from other localities of the world suggests that some of geographic isolation may exist.

In conclusion, based on the high diversity and lack of adequate genetic data about the parasite, more extensive and comprehensive studies are needed to demonstrate polymorphic and population genetic aspects of the *P. vivax* in Iran and other geographical countries.

## ACKNOWLEDGEMENT

This study was supported by Tehran University of Medical Sciences and the Center for Diseases Management in Iranian Ministry of Health. We thank the Malaria clinic health care workers in Sistan and Baluchestan, Hormozgan, Boushehr and Kerman provinces for their kind help during sample collection.

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