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Original Article

Polymorphism of Merozoite Surface Protein-3α Gene of *Plasmodium vivax* in Isolates of Iran

*A Shahbazi [1,2], A Raeisi [3], *M Nateghpour [2], H Mirhendi [2], M Mohebali [2], M Asmar [4]*

[1]*Dept. of Parasitology, Tabriz University of Medical Sciences, Iran*
[2] *Dept. of Medical Parasitology and Mycology, Tehran University of Medical Science, Iran*
[3] *National Malaria Control Programme, Disease Management Center of Ministry of Health, Iran*
[4] *Dept. of Parasitology, Pasteur Institute, Iran*,

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Abstract

Background: The worldwide distribution of *P. vivax* has expanded significantly and the number of reported cases has been on the rise. Approximately 88% of malaria cases in Iran are caused by *Plasmodium vivax*, and in order to management of the disease, understanding the population genetic structure of the parasite is necessary for designing and applying drugs and vaccines. Among many potential candidates, merozoite surface protein-3α gene (*PvMSP-3α*) is promising target to develop an effective vaccine. This study was carried out to determine the variation of this gene, as a genetic marker, in *Plasmodium vivax* isolates in malarious areas of Iran.

Methods: Diversity in *PvMSP-3α* gene was assessed in 85 *Plasmodium vivax* isolated from four southern and east-southern provinces of the country by PCR/RFLP method. Amplification was performed with two primer pair sets in a nested PCR format and the products were digested by the enzyme *Hha*I in RFLP method.

Results: Based on the size of the PCR products, we observed three biotypes A (about 1900bp), B (about 1400bp) and C (about 1100bp) of *PvMSP-3α* gene. Biotype A was predominant. According to RFLP patterns, 10 allelic groups of the gene were observed, that, 7, 2 and 1 groups correspond to the biotype A, B and C, respectively. Mixed genotype and multiple infections were not seen.

Conclusion: RFLP method with *HhaI* enzyme is a useful method for determining the polymorphism of biotype A of *PvMSP-3α* gene.

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

Introduction

Among the four human malaria parasites (*Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*), *P. falciparum* causes the most sever forms of malaria and is responsible for most malaria deaths. In the recent years, the worldwide distribution of *P. vivax* has expanded significantly and the number of reported cases has been on the rise and even in many regions of the world, *P. vivax* has become the most prevalent of the four human malaria species (1). *P. vivax* causes most of the malaria morbidity in endemic regions of central and South America, North Africa, and Asia (2).The majority of publications on *Plasmodium* genetic structure focus on *P. falciparum*, using polymorphic markers such as merozoite surface protein-1 (*MSP-1*), *MSP-2*, glutamate- rich protein, and microsatellites. For *P. vivax* only the dimorphic circumsporozoite protein (CSP) gene and the *MSP-1* gene have been widely used for genotyping (2). The *PvCSP* gene has a central repeat domain that differs in sequence and number of repeat units (3-5). The *P. vivax MSP-1* gene has been used to determine whether a malaria infec-

***Corresponding author:** Tel: +98-21-88989130,
E-mail: nateghpourm@sina.tums.ac.ir

tion is a result of a new infection or a relapse (6), and to assess the genotypic variety of isolates from different geographical regions (7-10).

The *P. vivax* merozoite surface protein-3α (*PvMSP-3α*) is another genetic marker has been recently validated and used for studies on population genetic structure and is a potential vaccine candidate (2, 11-17). Studies on *PvMSP-3α* have revealed that this gene is highly polymorphic, and three major types of the gene (A, B, and C) are distinguishable (1, 16, 17).

Despite of the extensive investigations on genetic structure of *P. vivax* and large number malaria cases in Iran, there is less information on genetic structure of *P. vivax* population in the country. Total malaria cases in Iran in 2005, were 18966, which 91% of all cases were reported from four southern and east southern provinces of the country (59 %, 25 %,6 % and 1 % of malaria cases in the country reported from Sistan and Baluchistan , Hormozgan, Kerman and Bushehr provinces, respectively), and about 88% of them caused by *P. vivax* (18).

Considering that malaria imposes an intense burden to the economy of the affected provinces and the whole country (18) and the importance of genetic information for producing effective drugs and vaccine against the parasite, this study was conducted to determine the diversity of a highly polymorphic and potential vaccine candidate marker, *PvMSP-3α* gene, in field *P. vivax* population in malarious areas of southern and east-southern provinces of Iran by PCR/RFLP method.

Materials and Methods

The blood samples were collected from 85 patients attending to malaria clinics in the Sistan and 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. Approximately 1000 µl of venous blood was collected in EDTA. DNA was extracted by Q1Aamp® DNA blood mini kit (5o). All samples were rechecked by nested polymerase chain reaction (PCR) using plasmodium genus specific (primary PCR) and *P. vivax* and *P. falciparum* species specific primers (nested) (19). Then, the DNA was amplified by nested PCR by primers bind at positions 111-131 and 2286-2305 (primary PCR) and positions 205-227 and 2078-2100 (nested) of the Belem laboratory strain coding sequence (10): P1-5/ CAGCAGACACCATTTAAGG3; P2-5/ CCGTTTGTTGATTAGT TGC3/;N1-5/GACCAGTGTGATACCATTAAC C3/;N2-5/ATACTGGTTCTTCGTCTTCAGG 3/. PCR was performed, based on previously introduced protocol (10), 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.

The PCR products digested by *HhaI* enzyme in RFLP procedure, in which approximately 5 µl of the PCR product was digested with *HhaI* and analyzed by electrophoresis on 1.8% agarose gel (10). Major alleles were classified based on the differences in restriction banding patterns. RFLP/PCR products visualization was performed under UV illumination after electrophoresis on 1.5% agarose gels containing 0.25 µg/ml of ethidium bromide. Marker 6 (Roche) was used for sizing of products.

Results

Based on the size of PCR products three biotypes of the parasite A (about 1900bp), B (about 1400bp) and C (about 1100bp) were observed (Fig.1). Biotype A of *MSP-3α* that corresponded to the expected size of the published sequence of the Belem laboratory strain was predominant (about 78%). Type B and C accounted for 6% and 16% of the parasite genotype, respectively. We observed 10 allelic groups

based on restriction patterns from digestion of PCR products with *HhaI* (Fig. 2), that, seven of them (73.9% of isolates) correspond to biotype A, two allelic groups (24.7% of isolates) correspond to biotype C, and one allelic group (1.1% of isolates) correspond to biotype B. The largest fragments about 1000 bp in all samples were not applied for distinguishing different alleles, as it was difficult to analyze and resolve. Mixed genotype and multiple infections were not seen in this study.

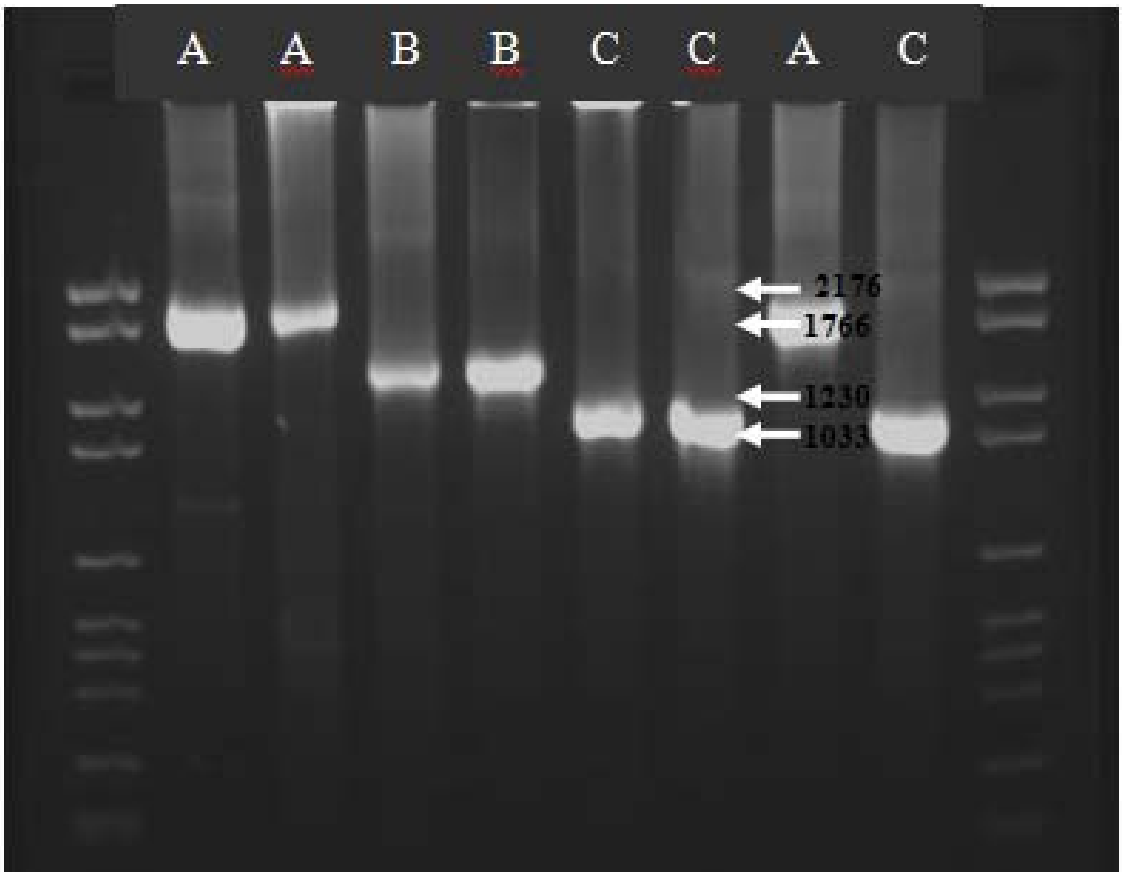

Fig. 1: Major 3 biotypes of *Plasmodium vivax* isolate from southern and east-southern of Iran using Polymerase chain reaction of the msp-3α gene. Lane 1 and 10 contains DNA marker 6 (Roche).

Fig.2: Major 10 allelic groups of Plasmodium vivax merozoite surface protein -3 α gene in isolates from southern and east-southern Iran using RFLP/PCR. Lane 1 and 20 contains DNA marker 6 (Roche). PCR products were digested with *Hha1* enzyme. Capital letters indicate the biotype of the allelic group. Biotype A of the gene has more variation after digesting with *Hha1* enzyme than others.

Discussion

In the recent years the economy of the four malaria endemic provinces of Iran (Sistan-Baluchistan, Hormozgan, Kerman and Bushehr) has sustained heavy losses during the malaria epidemics, and malaria control activities imposes a grave disbursement to the socio-economic development programs (18). Therefore, more investigations on parasite genetic structure for vaccine and drug development against the parasite seem to be necessary.

Previous studies addressing antigenic diversity of merozoite stage vaccine candidates in *P. falciparum* have demonstrated the existence of significant levels of genetic polymorphisms in parasite population, and recent studies assessing the genetic diversity of *P. vivax* merozoite antigens (e.g. *Pv msp-1*, *Pv AMA 1* and *Pv MSP-3α*) revealed similar findings (1). Genotyping the extremely polymorphic *P. vivax MSP-1* relies almost entirely on sequencing. Therefore, there is a need for developing the molecular markers that are more suitable for rapid genotyping.

One such marker is the *MSP-3α* gene, because its polymorphism can be readily evaluated by polymerase chain reaction/ restriction fragment length polymorphism (PCR/PFLP) analysis (16). The *PvMSP-3α* gene encodes a merozoite surface protein with an alanine-rich central domain that is predicted to form a coiled–coil tertiary structure (20), and nested PCR amplification of the gene from field isolates demonstrates size polymorphism in PCR products and many allelic patterns based on RFLP (2, 11-13, 16,17). Our study indicated that *P. vivax* isolates from malarious areas of Iran were highly diverse, and the results are almost similar to the results of previous studies carried out in other countries (2, 13), but this variety is higher than that of India (11) and Papua New Guinea (20), although the sample sizes were different. The frequencies of biotypes based on the size of PCR products of the *PvMSP-3α* gene are similar to that of previous study in Sistan and Baluchistan Province (17).

Based on restriction patterns from digestion of PCR products with *HhaI* (small fragments from 50-500bp) we detected 10 distinct alleles (Fig. 2), that was two allele less than the previous study. We found that seven of the ten RFLP allelic groups corresponded to biotype A of *PvMSP-3α* gene*,* biotype C had two and biotype B had only one RFLP allelic pattern. Therefore, RFLP method is more suitable for polymorphism studies of biotype A of *PvMSP-3α* gene than other two biotypes (B and C).

This investigation will serve as the foundation for future more detailed studies on population genetic structure of *P. vivax* especially on structure and function of *PvMSP-3* gene family and their products in order to providing effective vaccine and drugs against the parasite. We are continuing the study in order to reveal genetic structural differences of this gene based on sequencing.

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