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Original Article

Cloning and Sequence Analysis of Recombinant *Plasmodium vivax* Merozoite Surface Protein 1 (*PvMSP-1$_{42}$ kDa*) In pTZ$_{57}$R/T Vector

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Abstract

Background: Carboxy-terminal 42 kDa region of *Plasmodium vivax* merozoite surface protein-1 is considered as an important antigen in blood stage. Since, this region has been observed to be polymorphic among isolates of *P. vivax*, it is significant to survey on different regions of this antigen in various areas of the world.

Methods: In the present study, the genetic diversity of cloned *PvMSP-1$_{42}$ kDa* gene from an Iranian patient is analyzed. Parasite DNA was extracted from a *P. vivax* - infected patient in Iran. The region of *PvMSP-1$_{42}$ kDa* was amplified by PCR, cloned into pTZ$_{57}$R/T vector and then sequenced.

Results: Sequencing of cloned *PvMSP-1$_{42}$ kDa* gene clearly has a high degree of homology (95%) with reference Sal-I sequence and also with the homogeneous sequences from some studied countries (97%). Thirty eight SNPs (single nucleotide polymorphism) were identified in cloned *PvMSP-1$_{42}$ kDa* gene which the mutations had localized in the 33 kDa fragment (*PvMSP-1$_{33}$ kDa*), while there was nearly no variation in the 19 kDa fragment (*PvMSP-1$_{19}$ kDa*). 2 out of 38 mutations were found as to be novel haplotypes.

Conclusion: High similarity of cloned *PvMSP-1$_{42}$ kDa* gene in comparison to reference sequence and other sequences could be beneficial as a remarkable molecular marker for serological diagnostic kits of *P. vivax* in malarious neighboring countries of Iran and around the world.

Introduction

Plasmodium vivax (*P. vivax*) is the second widespread malaria species that causes disease in human and imposes sizeable socioeconomic burden and public health difficulty on many countries, particularly in Asia and South and Central America (1). Although, *P. vivax* is responsible for more than 50% of malaria morbidity outside Sub-Saharan Africa, but little consideration for control and research has been dedicated to this parasite (2). At present, about 2.8 billion people universally are at risk of *P. vivax* infection and occurrence of the disease is nearly 132-391 million cases each year (3). In a nutshell, in malaria life cycle**,** being bit by female anopheles mosquitoes, sporozoites are transferred to human and infection is initiated. Within hepatocytes, the sporozoites reproduce and increase in quantity to thousands of merozoites, which attack the red blood cells (RBCs) in the next stage. In the infected RBCs, the small ring shape changes through trophozoite to schizont, in which it bursts and liberates more merozoites (4).

Up to now, some problems such as non–cultivable nature of *P. vivax,* caused limitations in the study of its molecular biology, but genetic diversity and population organization of this parasite have become more clear by sequencing and genetic engineering including cloning methods (5). Several *P. vivax* antigens have been proposed as detectable potential candidates (6), among which C-terminal region of *PvMSP-1* has high expression on the mature merozoites surface as well as mass protein production, that it has been proven to take part in the parasite invasion to the erythrocyte. Specific antibodies against *MSP-1*, have been particularly shown react to the C-terminal region (33-kDa and 19-kDa sub fragments) (7- 9). Antigenic variation is one of the limitative agents in antibody detection that could be due to haplotype variations on target gene. It can ultimately affect the protein conformation, affinity of antibody-antigen and serological test results. Little information is available regarding the genetic polymorphism of *MSP-1*$_{42}$ among Iranian *P. vivax* population.

In this study, in order to assess the nature of Iranian *P. vivax* isolate, molecular diversity of cloned *PvMSP-1*$_{42}$ *kDa* gene was analyzed.

Materials and Methods

Preparation of insert DNA

In this study, 11 isolates of Chabahar district (Sistan and Balouchestan Province) that had *P. vivax* infection symptoms were chosen and sequenced in order to surveying their similarities. One out of 11 mentioned isolates which had highest homology was selected to cloning process. . The parasite genomic DNA was extracted from the whole blood by Genomic DNA Extraction Kit, DNG–plus (Cinna Gene, Iran, DN8118C) using the kit manufacturer's guidelines. Continuously, Genomic DNA quantity and quality was controlled by electrophoresis on 0.8% agarose gel and a biophotometer (Ependorf) at 260 and 280 nm. Amplification from 42 kDa partial regions of *PvMSP-1* gene was done which included 19 kDa and 33kDa fragments. The primers were designed on the basis of the sequence of *PvMSP-1*$_{42}$ *kDa* gene, (Genbank: ACCESSION NO: DQ907673). The following primers were used for sequencing:
MSP1.42F (25mer) (5'-GGATCCGACCAAGTAACAACGGGAG-3'),
MSP1.42R (25mer) (5'-GAATTCCAAAGAGTGGCTCAGAACC-3').

The PCR was performed in a PCR tube containing: 200 ng (1.5µl) extracted DNA as template, 20 pmol (0.25 µl) of each primer, 7.5 µl of PCR master mix 2X that contained *Taq* and 5.5 µl of ddH_2O. The objective gene was amplified for 30 cycles (Initial denaturation at 96 ˚C for 5 min, 96 ˚C for 30s, 58 ˚C for 30s and 72 ˚C for 1 min and final extension for

20 min) subsequently, the PCR product was controlled on 1% agarose gel against a standard DNA ladder (Fermentase Co.). PCR products that consisted of a single specific band can be directly ligated to T-vectors. However, the removal of the impurities such as primers and nonspecific products by gel separation causes increase in the percentage of colonies containing the correct inserts. Thermo-stable DNA polymerase which does not preferentially add a 3'-A at the ends of the PCR products is essential to removal of that enzyme by gel separation. Purification and recovery of the DNA from agarose gel will be explained in the next sections. Furthermore, the concentration of the purified DNA was estimated as described. Purified DNA fragment was inserted into pTZ$_{57}$R/T that provided by the following protocol.

T-Vector preparation

Any plasmid could be selected to gratifies our requirements including the plasmid contains a unique blunt-end restriction site in the multiple cloning site (in this study, the *E*coR V site of the pTZ$_{57}$R plasmid was used that has blue/white color selection).

Digesting plasmids with Blunt-End restriction enzyme

Digesting plasmids were performed in a 0.5 ml microcentrifuge tube containing: 25 µl plasmid DNA (2µg), 4 µl of 10X *E*coR V buffer, (10 unit) 1 µl of EcoR V (Fermentase Co.) and ddH_2O up to 40µl. Then it was mixed by gentle vortex, centrifuged and incubated at 37 °C for 2 hours. In the next step, 2 µl of the mixture was taken and run on a 1% agarose gel to make sure the digestion was completed. Thereafter it was incubated to 65 °C by water bath for 10 min at the end of digestion (Fig. 1A). Blunt-ended plasmid DNA was purified by electrophoresis on 1% agarose gel.

Making T-overhangs from the Blunt-Ended plasmid vector

Taq polymerase and dTTP were used to add a 3'-T to the blunt-ended plasmid. Briefly, the making T-overhangs was performed in a 200 µl PCR tube containing: 40 µl blunt-ended plasmid DNA, 5 µl of 10 X PCR buffer ($MgCl_2$ free), 2.5 µl of $MgCl_2$ (250 mM), 0.5 µl of dTTP (100 mM), 2 µl of Taq polymerase (5 U/µl). It was mixed by gentle vortex, centrifuged shortly and incubated at 72 °C for 2 h.

Separation, purification and recovery of T-vectors on agarose gel

T-vectors were separated from the self-ligated and concatemerized plasmid DNA on a 1% agarose gel by 0.5 µg/ml of ethidium bromide. The gel was run at 5 volts/cm in TAE buffer. The DNA bands were visualized by a hand-held long wave (365 nm) UV light. The T-vector bands were excised by a sterile scalpel. The gel slice transferred to a 2 ml micro centrifuge tube, and then T-vectors were purified and recovered by Silica Bead DNA Gel Extraction # K0513 (Fermentas Co.) via the kit manufacturer's guidelines.

Quantification and storage of the purified T-vector

The density of the purified T-vectors was determined by running 1 µl of the T-vector in compared to standard DNA ladder in a 1% agarose gel at 5 volts/cm for 45 min and then compared the comparative brilliantness of the bands. T-vectors were aliquoted to several tubes and stored them at -20 °C. Long period storage may reduce the cloning efficiency and intensify the background.

Ligation

Usually 50-60 ng of T-vector is enough for each ligation. 1:3 molar ratio of T-vector to insert DNA was recommended, but the amount of A-tailed DNA solution in a 10 µl ligation should not be more than 2 µl. In a sterile 0.5 ml micro centrifuge tube, added: 2 µl T-vector, 2 µl of A-tailed DNA , 1 µl of 10

X ligation buffer, 1 µl of T4 DNA ligase (2-3 U/µl) and 4 µl of ddH_2O. It was mixed gently, centrifuged briefly and incubated at 14 °C for overnight (Fig. 1B).

Transformation of the ligated vector

Before transformation, Luria-Bertani (LB) agar plates contained 100 µg/ml of ampicillin, were prepared and spreaded with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and isopropylthio-β-D-galactoside (IPTG). For each ligation reaction, 50 µl aliquoted of frozen competent cells (Top 10) was thawed on ice and added 2 µl of the ligation reaction to the competent cells and mixed gently by stirring with the pipette tip. The cells were incubated on ice for 30 min then heats shocked for 30 s in the 42 °C water bath, and then were immediately placed on ice for 2 min. The transformed cells were spreaded on each labeled LB-ampicillin plate with X-Gal and IPTG. The plates were inverted and placed at 37 °C incubator for overnight. The positive colonies that had white color were identified either by restriction enzyme and screening by PCR method after transformation. PCR screening was carried out with same primers that be used for PCR amplification for insert production. For screening by restriction enzyme method, the insert was released by digestion with two unique restriction enzymes (with cutting sites, *Eco*R1: GAA↓TTC and *BamH*1: GGA↓TCC) from the multiple cloning sites, and the insert size was confirmed by agarose gel electrophoresis (Fig. 1C).

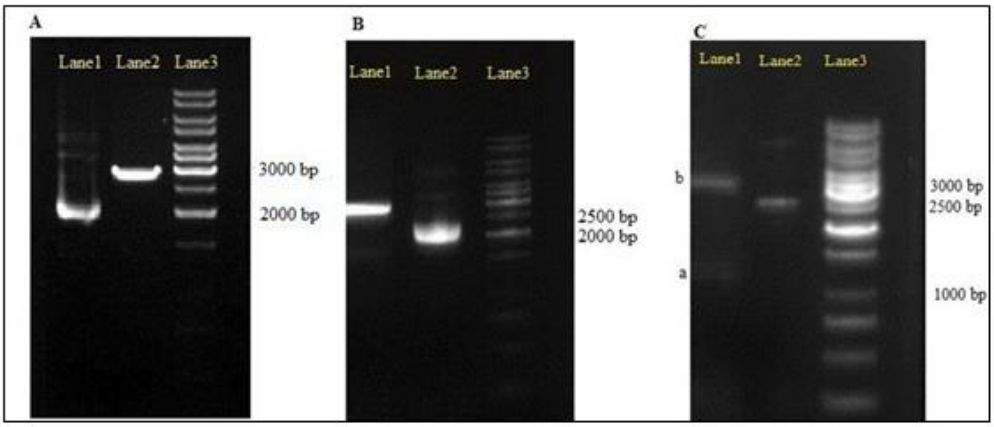

Fig. 1: A: Lane1: Intact plasmid $pTZ_{57}R$, Lane2: Digested plasmid $pTZ_{57}R$ by *EcoRV*, Lane3: Size marker: 1kb. B: Lane1: Recombinant plasmid, Lane2: Intact Plasmid, Lane3: Size marker1kb. C: Lane1: (a: Separated PCR product from digested recombinant plasmid), Lane2: Intact recombinant plasmid, Lane3: Size marker1kb

DNA sequencing and phylogenetic analysis

PCR product of *PvMSP-1 42 kDa* was directly analyzed on ABI PRISMTM 310 automated sequencer to determine *P. vivax* variation (strain/haplotype) in the infected patient. Both directions of our sequences were aligned and edited using Sequencher Tm 4.0.4 software for PC (Gene Codes Corporation) and MEGA 5 software for phylogenetic analysis and then were compared with some GenBank sequences for their similarity (10). A phylogenetic hypothesis of *P. vivax* was generated with *PvMSP-1 42 kDa* using the nonparametric bootstrapping, neighbor joining method.

Results

In Fig. 1, intact plasmid $pTZ_{57}R$, digested plasmid $pTZ_{57}R$ by *EcoRV* and digested recombinant $pTZ_{57}R$/ MSP by *BamHI* and *EcoRI* restriction enzymes and expected insert

band are presented. As Fig. 2 shows an 1124 bp band has been demonstrated from extracted genomic DNA by PCR.

The yield of PCR was cloned in $pTZ_{57}R$ and then sequenced. In the Iranian *P. vivax* isolate, as compared to the reference Sal I sequence, 38 SNP of *PvMSP-1$_{42}$ kDa*, were identified.

As Fig. 4 shows, *MSP-1 $_{42}$ kDa* phylogenetic tree based on bootstrap-neighbor joining method in Iranian sequenced isolate (MSP 42-YYXXXXXX-Iran 01) and other recorded sequences in GenBank. All of the mutations were localized in the *PvMSP-1$_{33}$kDa*, while there was almost no variation in the *MSP-1$_{19}$ kDa*. 2 out of 38 mutations (haplotype) were novel in the 545 and 573 positions as compared to the homogeneous sequences from other surveyed countries (Fig. 3, 4).

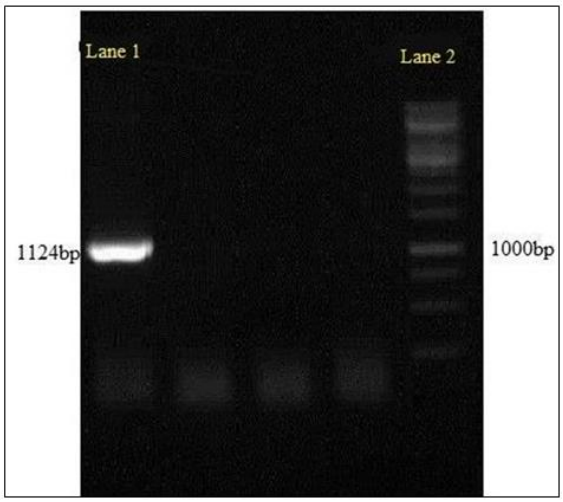

Fig. 2: Gel electrophoresis of PCR product from a *P. vivax* – infected patient. Lane 1: Pv MSP-1$_{42}$ kDa gene was nearly 1124bp long, Lane 2: Size marker 1kb

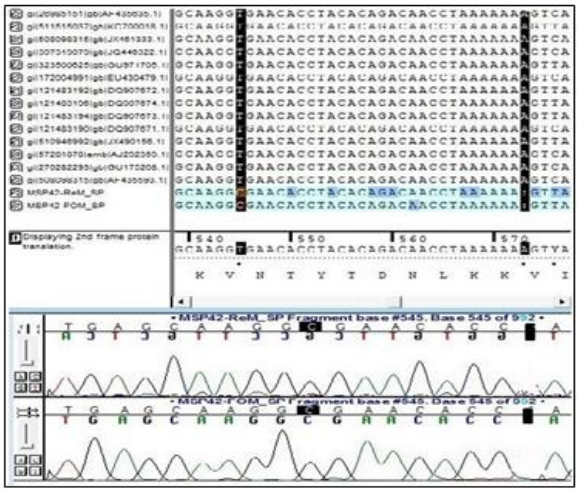

Fig. 3: Sequence alignment of Pv MSP-1$_{42}$ kDa gene in *P. vivax* isolated from Iran and correlated part of the isolated genes from other countries

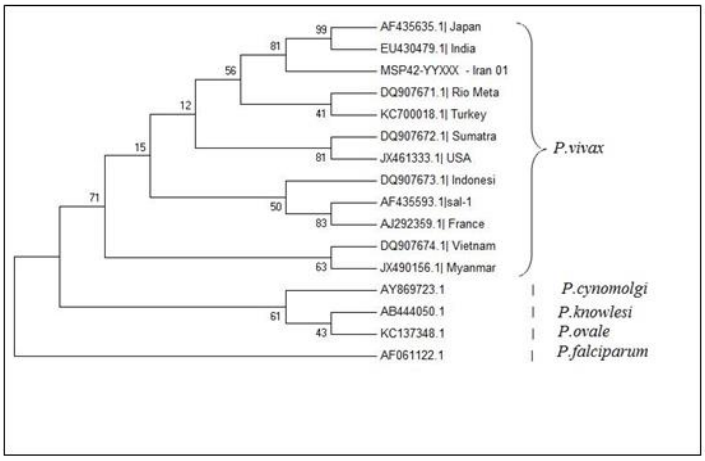

Fig. 4: Bootstrap neighbor-joining consensus tree of Pv-MSP-1$_{42}$ kDa data set by MEGA5. Sequence of Iranian isolate is shown for MSP 42-YYXXXXXX-Iran 01 in clad of Indian and Japonica sequences (Accession Nos: EU 430479.1, AF435635.1)

Discussion

In research on diagnostic potential for *P. vivax* malaria parasite subunits, the focus is on the C-terminal region of *P. vivax MSP-1*. As Fig.5 depicts, generally, *MSP1* is manufactured as a big protein (~200 kDa) which is connected to the cell membrane by a glycophosphatidylinositol (GPI) anchor in the carboxyl terminus region (11). MSP1 undergoes a series of proteolytic maturation changes at the same time as the merozoite unleashed from (RBC) and generates four polypeptide fragments of nearly 83, 30, 38, and 42 kDa from the N-terminus to C-terminus (12), which stays as interconnected parts on the cell surface by the anchored C-terminal segment (p42) (13). New RBCs are invaded rapidly by attachment of free merozoites induces second in a series cleavage of the p42 peptide to generate p33, which is shed together with the previous fragments and p19, abides by anchoring to the membrane of parasite as it invades the cell (14,15). High plentifulness and important function on the cell surface, probably caused *MSP1* to be a principal object of the host immune system and antibodies are identified on different regions of this protein (16). Antibodies that recognize the C-terminal region of *Plasmodium falciparum MSP-1*, inhibit the invasion of merozoites into the host erythrocytes in vitro and immunization of experimental animals with *MSP-1$_{19}$ kDa* also confers protective immunity (17, 18). These findings demonstrate that *MSP-1$_{42}$ kDa* is a promising candidate antigen for blood stage vaccine development and diagnostic kits.

In this study, the *Pv MSP-1$_{42}$ kDa* was cloned, sequenced, and subsequently compared with homogeneous genes that previously had been recorded in GenBank. 38 SNP of *PvMSP-1$_{42}$ kDa*, were recognized using the Sequencher 4.0.4 and MEGA 5 programs. Most of the mutations were concentrated in the *PvMSP-1$_{33}$ kDa*. Pacheco et al. in Indonesia tried to show the genetic diversity of the 42 kDa fragment of *MSP-1* antigen in *P. vivax*.

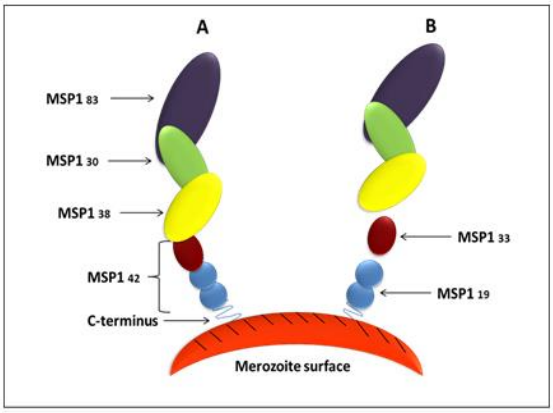

Fig. 5: Schematic showing processing of merozoite surface protein 1 (MSP1). Panel (A) shows primary processing, and (B) shows secondary processing

They found that the *MSP-1$_{33}$ kDa* fragment exhibits greater genetic diversity than the *MSP-1$_{19}$ kDa* generally. Previous observations confirmed that the *MSP-1$_{19}$ kDa* fragment is more conserved than the *MSP-1$_{33}$ kDa* fragment (19). In present study, polymorphism of *P. vivax MSP-1$_{33}$ kDa* is not evenly spreaded, indeed there is a region of 70 bp in *MSP-1$_{33}$ kDa* where a clear excess of non-synonymous is observed in the overall *MSP-1$_{33}$ kDa* results, while there is closely no significant variation in the *MSP-1$_{19}$ kDa.*

Two out of 38 mutations were new haplotypes (545 and 573 positions) in the 33 kDa fragment as compared to the homogeneous sequences from other countries including Japan, Turkey, USA, India, Sumatra, Vietnam, Indonesia, RioMeta, China, France and reference sequence from Sal-I (20) (Fig. 3). Due to the differentiation of *MSP1* amino acid sequences between *Plasmodium* spp., it is necessary to use recombinant *MSP-1* to detection antibody from *P. vivax* in Iranian cases (21). Shahbazi et al. compared the *P. vivax* MSP-3ß gene with ssrRNA gene as genetic markers for the parasite detection. According to their results the sensitivity of ssrRNA gene was high-

er than the PvMSP-3ß gene (100% vs. 95%). They concluded that the PvMSP-3ß gene cannot be a suitable marker for detection of *P. vivax* in blood sample by PCR (22). One of the prominent problems in diagnostic kits is genetic polymorphisms that encoding on this region, within and between the *P. vivax* population. Therefore, polymorphism examination of this gene on disparate territories could be helpful in improving diagnostic kits based on antibody detection in malarious areas of Iran. Shahbazi and colleagues in Iran assessed the genetic structure of *P. vivax* population by sequence analysis of the merozoite surface protein 3β (MSP-3ß) gene. They found the single nucleotide polymorphism (SNP) extensively in the sequences. Moreover, their phylogenetic analysis did not show any significant geographical branching (23). Zaman et al. studied the *P. vivax* dihydrofolate reductase (Pvdhfr) mutations among 50 blood samples of symptomatic patients from 4 separated geographical regions of south-east Iran. They reported point mutations at residues 57, 58, 61, and 117 by using the PCR-RFLP method. Polymorphism at positions 58R, 117N, and 117T of Pvdhfr gene has been found in 12%, 34%, and 2% of isolates, respectively. They demonstrated five distinct haplotypes of the Pvdhfr gene (24).One of the endemic zones for *P. vivax* throughout the world is Iran from which there are reported cases annually (25). Asymptomatic carriers of *P. vivax* among treated patients have been shown as a major reservoir of parasites and maintenance of high levels of transmission in malarious areas of Iran (26). Although genetic polymorphisms in the regions of *MSP-1* in Iranian *P. vivax* isolates has been previously analyzed, but little information is available regarding the genetic polymorphism of *MSP-1_{42} kDa* among Iranian *P. vivax* isolates. In this study, the genetic polymorphism of *MSP-1_{42} kDa* in an Iranian isolate was analyzed for a better understanding of the nature of Iranian *P. vivax*. Thus, it is significant to investigate the molecular character of the parasite placed within Iran and comparing with the same species in other regions.

Predicted protein sequence of cloned *PvMSP-1_{42} kDa* gene obviously has a high degree of identity (97%) with strong homology to the *Pv MSP-1_{42} kDa* gene of *P. vivax*. Asian isolates specially Japan and India also have sequence similarity (95%) *Pv MSP-1_{42}kDa* gene from Sal-I isolate (as reference sequence). In general, diversity of anopheles mosquitoes, presence of several *plasmodium* species, biotic interactions and hybrid / mixed infections can affect the genotyping variation of *plasmodium* in low to high degree. This might result in parasite pathogenicity, host specificity, establish and persist of infection, different clinical manifestation, transmission dynamics and antigenicity of infection.

Conclusion

The cloned *PvMSP-1_{42} kDa* gene can be used as a strong candidate for diagnostic kits based on antibody, antigen detection and vaccine development.

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