

# Genetic Characterization of C-Terminal Region of SERA5 Gene in Isolates of *Plasmodium vivax* in Southwestern Iran

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## ABSTRACT

**Backgrounds:** *Plasmodium vivax* is one of the leading causes of malaria as a severe and death disease. Malaria has always been a major challenge for human health. The study of the genetic diversity of genes in malaria-causing agents has always been a concern for researchers. One of these genes is SERA, which plays a key role in parasite escape from the immune system.

**Materials & Methods:** DNA was extracted from 40 blood samples taken from symptomatic malaria patients infected with *P. vivax* in southern and southwestern Iran using a DNA extraction kit. Then PCR was performed with specific primers, and the data were analyzed by sequencing and recording genes.

**Findings:** In this study, 14 different isolates were identified among all samples, which were recorded in the World Gene Bank. The number of haplotypes among the 14 samples was 12. Also, there were 25 polymorphic nucleotide positions out of about 400 nucleotide sites. The ratio of non-synonymous to synonymous mutations (1.87094) and the amount of Tajima's D (-0.57671) indicated the positive effect of natural selection on the genetic diversity of C-terminal region in the SERA5 gene.

**Conclusions:** Considering the safety and relative immunogenicity of vaccines, in addition to performing clinical trials, a regional vaccine should be developed to overcome genetic variation and antigenic changes in proteins.

**Keywords:** *Plasmodium vivax*, Malaria, Genetics, Iran

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## Introduction

Malaria is a major global health problem caused by intracellular protozoan parasites belonging to the *Plasmodium* species located in the phylum Apicomplexa. The five species of *Plasmodium* that could cause malaria disease in humans are as follows: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* [1-3]. The main vector of these parasites is *Anopheles* mosquito, which infects humans by biting. According to the latest WHO report in 2021, 241 million malaria cases and 627 thousand deaths due to malaria were reported worldwide [4]. Iran is located in the eastern Mediterranean region (EMR) as one of the countries where malaria has existed since ancient times. About 400,000 Iranians live in high-risk areas of the disease. Also, according to the latest reports, indigenous malaria cases in Iran were zero for three consecutive years from 2018 to 2020 [4]. More than 90% of malaria cases reported in Iran are related to southern provinces such as Sistan and Baluchestan, Hormozgan, and the tropical region of Kerman province. Studies have shown that of all the malaria cases reported in Iran in 2017, about 1.26% were associated with *P. falciparum*, and about 38.88% were associated with *P. vivax*. Challenges to fight against malaria have led to extensive research on the development of vaccines against this disease [5]. Due to the complexity of the parasite life cycle and the presence of co-infections with *P. falciparum* and *P. vivax* in endemic areas, the need for multi-antigens and multi-strain vaccines is felt. The surface proteins of the parasite in the intruder stages are considered suitable candidates for vaccine development [6-8]. Research on the development of effective vaccines against *P. vivax* has faced many challenges due to the lack of funding and technical problems, although many vaccine candidates have been introduced [9, 10]. Recent studies have shown that one of the vaccine candidates is the PV

SERA multigene family, given its pivotal role in the evolutionary process and various stages of the protozoa life. Serine repeat antigen (SERA5) is one of the genes observed in the final stages of the development of schizont and pleuritic thrombosis. In all *Plasmodium* species including *P. vivax*, this gene plays a major role in the evolution and escape of the parasite from the immune system [11-13].

**Objectives:** The current study aimed to investigate the genetic diversity of this gene in *P. vivax* strains isolated from malaria patients in contaminated cities of Kerman and Shiraz provinces.

## Materials and Methods

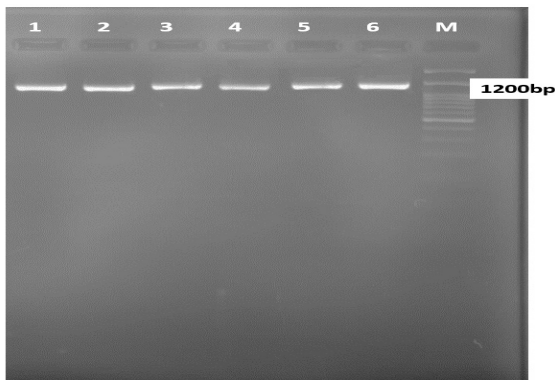
This study was performed on patients who referred to health centers in Shiraz and Kerman during 2017 to 2020 after obtaining patients' consent. A total of 40 blood samples were collected from malaria patients. The samples were confirmed to be infected with *P. vivax* based on positive microscopy and molecular methods of contamination detection. Following diagnosis, the blood samples taken from patients were treated with EDTA and stored at 70°C. To extract DNA from the samples and slides in this study, QIAquick PCR purification kit (Qiagen, Germany) was used according to the manufacturer's instructions. After extraction, DNA was kept at -20°C until used for molecular studies. PCR reaction was performed using lyophilized PCR master mix (PCR PreMix, Bioneer Co., Korea) with a final volume of 25 µL consisting of 5 µL of PreMix, 16 µL of distilled water, 2 µL of DNA, 1 µL of forward primer, and 1 µL of reverse primer. PCR amplification was performed using a forward primer with a length of 21 bp and a sequence of NRSERA5PV6F-5GC-GCGGAAGAAGGTGCAAAG3 and a reverse primer with a length of 21 bp and a sequence of NRSERA5PV6F-5GCGCGGAAGAAGGTGCAAAG3. PCR thermal cycling conditions were adjusted following Rahul et al. (2015)

(temperature at 94°C for 3 minutes then at 94°C for 36 cycles) [14].

**Sequence analysis:** Using Chromas sequencing software (Ver.2.33), sequences were detected and edited. Phylogenetic tree was drawn using DnaSP (Ver. 5.10.0) and MEGA (Ver. 5.0) sequence analysis software. Phylogenetic analysis of the haplotypes reported in this study was carried out using a neighbor-joining (NJ) phylogenetic tree in MEGA (Ver.5.0). The reliability and reliability of the phylogenetic tree plotted by 1000 replications were evaluated by bootstrap method.

### Findings

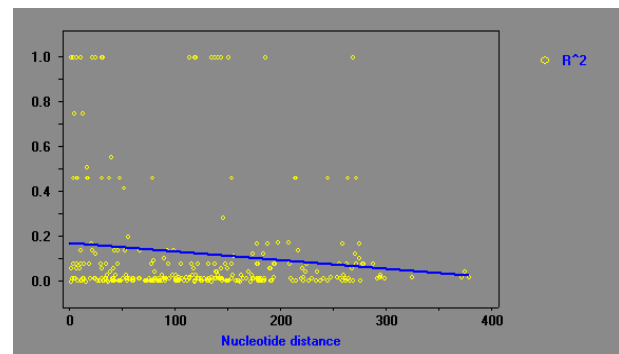
Image of PCR amplification product was visualized on 1.5 % agarose gel. In this study, a 100 bp marker was used to attach and confirm the desired bond (Fig. 1).



**Figure 1)** Gel electrophoresis of a nearly 1200 bp fragment of *P. vivax* SERA5's C-terminal region, M: 100 bp marker; Lane 1: positive control, Lane 2–6: *P. vivax* samples

In this study, after removing completely identical sequences, 14 different isolates were identified among all samples, which were recorded in the World Gene Bank for genetic diversity analysis. Among the 14 specimens, the number of polymorphic sites with a haplotype diversity (Hd) of 0.967 was determined to be 25 positions. The ratio of non-synonymous site mutations (Tajima's D: -0.57671) to synonymous site mutations (Tajima's D: -0.57671) was 1.87094. Also, there

were 12 haplotypes with a haplotype (gene) diversity (Hd) of 0.967 (Table1). The number of observed recombinations was very low, and at least four recombination sites (116,238) (277,278) (278,289) (293,393) occurred. DnaSP software (Ver. 5.10.0) was used in this study to analyze DNA polymorphisms. Linkage disequilibrium (LD) pattern was plotted using DnaSP software (ver. 5) (Fig. 2). The mild gradient of this graph shows the effect of the recombination, although small, on the desired gene confirmed by our observations.

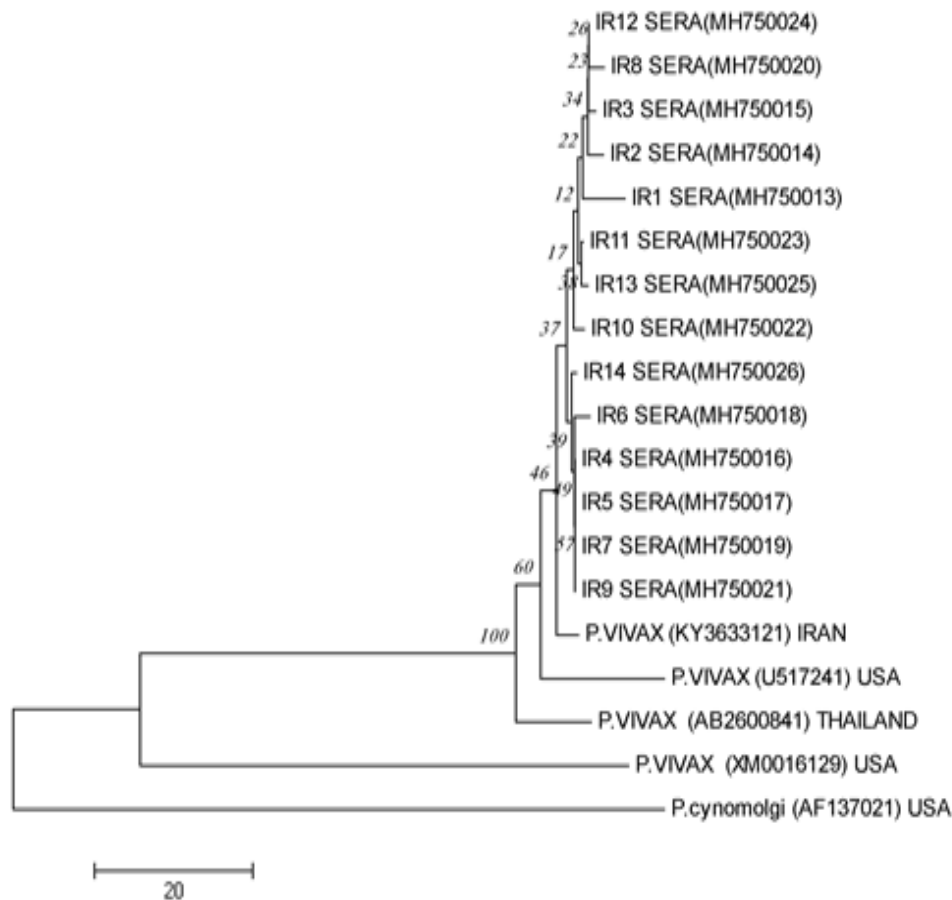


**Figure 2)** Linkage disequilibrium (LD) index obtained from sequence analysis of the C-terminal region of Iranian isolates of PvSERA5

**Observation of nucleosides:** In this study, 400 nucleotide positions were investigated among the samples. Of the 400 sites, 375 cases were invariable (monomorphic sites), 375 cases were polymorphic sites, and 25 cases were variable. Of the 25 mutated sites among existing isolates, the number of parsimony informative sites was 11, and the number of singleton variable sites was 14. In this study, 12 haplotypes were identified among 14 strains isolated. The phylogenetic tree of the samples was drawn using neighbor-joining (NJ) method by MEGA software Ver.5.0 (Fig. 3).

### Discussion

In countries that are struggling with malaria and are in the final phases of eliminating and



**Figure 3)** The phylogenetic tree, derived from the samples with the accession numbers MH750013 to MH750026, is located next to samples from Iran, the United States, and Thailand, and is considered as “OUT GROUP” in relation to the phylogenetic tree of *P. cynomolgi*.

eradicating malaria, such as Iran, it is important to find new ways to prevent epidemics and the sudden onset of malaria and to treat malaria cases [15, 16]. One of the most effective ways to prevent and treat the disease is to find an effective vaccine. This requires a thorough understanding of the genetic diversity of the genome of the disease-causing agents, namely *Plasmodium*, as a vaccine candidate. Accordingly, considering the genetic variation of these genes seems necessary to develop a local or global vaccine [17, 18]. Compared to *P. falciparum*, few vaccine candidates against *P. vivax* have been introduced, including *msp-1*, *csp*, *ama*, and SERA genes. Serin repeat antigens (SERAs) are genes expressed in the bloodstream during

the parasite life cycle. This gene (SERA) is the main motive for the attack of merozoites on target cells that control the invasion and exit of merozoites [19, 20]. *P. vivax* has the highest number of known SERA genes among *Plasmodium* species, with 12 different types of SERA gene. Previous studies have suggested that only PvSERA5 is transcribed in these homologs [11]. Following this study, other studies have shown that the highest transcription rates were related to PvSERA4, followed by PvSERA2, PvSERA10, and PvSERA11 [14]. The differences in these studies results and the high expression levels of both PvSERA4 and PvSERA5 segments indicate their importance as vaccine candidates. Therefore, the study of the amount

of polymorphism and the role of natural selection in the genetic diversity of this gene (SERA) has been considered by various researchers. On the other hand, other researchers have reported the highest variation in amino acids derived from the C-terminal parts of these two genes<sup>[13,14]</sup>. Therefore, evaluating the diversity of this part of the gene in different populations of the parasite could be helpful. Thus, in this study, the genetic diversity of the C-terminal region of the SERA5 gene was evaluated in *P. vivax* strains isolated from patients in Kerman and Shiraz. After removing completely identical sequences, 14 different isolates were identified among all samples, which were recorded in the World Gene Bank for genetic diversity analysis. In total, 25 mutation types and 25 polymorphism sites were detected in these 14 isolates, indicating that there were variations in mutation at some nucleotide sites, and that more than one mutation type occurred at some sites. The nucleotide diversity of  $P_i$  was estimated to be 0.01511, which indicates the genetic variation in this section. Abolghazi et al. (2018) in Sistan and Baluchestan, Iran, reported genetic diversity in the C-terminal of SERA5 gene in this area, and that this diversity, although small, was more than expected, which is exactly the same as our findings in this study. Aoki et al. (2002) in Japan and Miller et al. (2002) in the United States have reported more genetic variation in the SERA5 gene than in the rest of the SERA genes<sup>[21,22]</sup>. In a 2011 study in Japan, Nobuko Arisue et al. also stated that SERA genes had genetic diversity<sup>[19]</sup>. Rahul et al. (2013) in India investigated the genetic variation of C-terminal sections of PvSERA4 and PvSERA5 in *P. vivax* isolates and reported the highest nucleotide variation rate of 0.17 in PvSERA5<sup>[14,23]</sup>, which is far more than the nucleotide diversity among Iranian isolates. The differences in the prevalence and severity of transmission of malaria in India and Iran are related to the fact that Iran is in the final stages of malaria eradication; thus,

the lower incidence of malaria in Iran could be clearly justified. In this study, there were 12 haplotypes among 14 sequenced samples, which were found to be the first samples reported by BLAST in Iran and the world. Compared to other studies, Rahul et al. (2013) in India observed 15 different haplotypes in 18 sequences<sup>[14]</sup>, this result is consistent with the present study result, indicating genetic variation among the samples. The researchers considered the positive effect of natural selection on the variation of this gene in Indian specimens. In general, the two evolutionary factors of natural selection and intragenic recombination could play a role in the genetic variation of the C-terminal portion of the SERA5 gene. In the present study, the ratio of nonsynonymous mutations to synonymous mutations indicates that natural selection plays a fundamental role in the observed genetic diversity in Iranian isolates. Additionally, the amount of the TajimaD index also reflects the significant role of natural selection in genetic variation, which is consistent with the results of researchers in India. This means that mutations in this gene are generally favorable to the parasite, and it seems that the human immune system pressure elicits genetic variation in this gene. It should be noted that sexual outcrossing and population recombination play a decisive role in intragenic recombination, and both are strongly associated with malaria endemicity and transmission severity. Since the diploid phase of the *P. vivax* life cycle occurs in the midgut of *Anopheles* mosquito, it seems that intragenic recombination also plays a role in the genetic diversity of the gene<sup>[24]</sup>. In this study, a gradual decrease in LD ( $R^2$ ) index with increasing distance between nucleotide sites suggests the occurrence of intragenic recombination in *P. vivax* isolates. A phylogenetic tree is, in fact, a diagram that depicts the evolutionary lines or relationships or relative evolution of various species and genes from a common ancestor and represents the events through which

genes evolve. The phylogenetic tree derived from the 14 isolates in this study showed that the specimens were included in two separate branches or clades. Comparison of the alignments of the samples showed that only one sample (IR2 P.VIVAX SERA MH750014) was located in the lower branch, and the rest of the specimens were located in the upper branch. The phylogenetic tree drawn in similar studies is based on samples from the World Gene Bank, OUT GROUP, and *P. cynomolgi*. In this study, it was shown that the isolates from Iran, Thailand, and the United States were located alongside our samples, and sal1 was located in the sorority branch. Malaria-rich regions of Iran are among malaria hypoendemic regions, and genetic variation and the number of haplotypes are expected to be very low. However, due to the proximity of Sistan and Baluchestan provinces to Pakistan and Afghanistan and the migration and transit of hosts between these areas, the genetic diversity of this gene and the number of haplotypes in this hypoendemic region are more than expected. Therefore, it seems that a regional vaccine should be considered to control malaria in Iran; in addition, it is necessary to study the condition of this gene in adjacent countries with high malaria transmission. *Plasmodium* parasite SERA5 is a bloodstream antigen, and the production of a vaccine against it could prevent the penetration and invasion of merozoites into the red blood cells [25,26]. Phase Ia and Ib vaccines, SE36 or SERA5, have been reported in human clinical trials and tested in various countries. In a study, out of 66 patients, patients aged 6 to 20 years received a vaccine and were monitored for a period of 130 to 365 days after the second dose of the vaccine. In this study, the level of parasitic infection and fever in the vaccine recipient group was significantly lower than in the control group [27,28].

## Conclusion

Considering the safety and relative immuno-

genicity of vaccines, in addition to performing clinical studies, a regional vaccine should be developed to overcome genetic variation and antigenic changes in proteins. Therefore, the information obtained in this study could be useful for the design and manufacture of local and even global vaccines.

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**Authors' contribution:** Conceptualization: AA and GSH; data curation and formal analysis: LA; funding acquisition: AA, GSH, and AS; investigation: AA, GSH, and AS; methodology and project administration: AA; resources: AA and AS; supervision: AA; writing of original draft: AA; writing, reviewing, and editing: AA, GSH, and AS.

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**Consent to participate:** Authors declare that all patients signed the participation form.

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