

Original Article

## PCR-RFLP Based genetic diversity of *Plasmodium vivax* genotypes in district Mardan, Pakistan

Diversidade genética baseada em PCR-RFLP de genótipos de *Plasmodium vivax* no distrito de Mardan, Paquistão

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### Abstract

*Plasmodium vivax* is the most common human malaria parasite in Asian countries including Pakistan. Present study was designed to explore the genetic diversity of *plasmodium vivax* genotypes based on *Pvmsp-3α* and *Pvmsp-3β* genes using allelic specific nested PCR and RFLP assays markers from field isolates in district Mardan, Pakistan. Blood samples of 200 *P. vivax* malarial patients were collected after taking their written informed consent. Genetic diversity in nested PCR products was determined by Restriction Fragment Length Polymorphism (RFLP) utilizing *AluI* and *PstI* restriction enzymes for alpha and beta gene products digestion, respectively. For analysis the genetic diversity of the sub allelic variants of *Pvmsp3α* and *Pvmsp3β* genes, Chi-Square test was performed by utilizing Minitab programming software 18. The P value 0.05 was considered as statistically significant. For *Pvmsp-3α* genes after gel electrophoresis of digested products, four distinct genotypes were obtained from total of 50 samples; type A: 35 (70%) (1.5-2.0 kb), 12 of type B (24%) (1.5-1.7 kb), 2 of type C (4%) (0.5-1.5) and one for type D (2%) (0.5-0.65 kb) which could be characterized into 9 allelic pattern (A1-A4, B1-B3, C1, D), in which A3 remained the most predominant. For *Pvmsp-3β* genes, three distinct genotypes were obtained from 50 samples; 40(80%) of type A (1.5-2.5 kb), 9 (18%) of type B (1.0-1.5kb) and 1(2%) of type C (0.65 kb) which could be characterized into 6 allelic patterns (A1-A3, B1-B2, and C1). Most dominant one in Type A was A1 alleles which were noted (46%), while in Type B, the most dominant were B1 (10%). This study is the first ever report of molecular epidemiology and genetic variation in *Pvmsp-3α* and *Pvmsp-3β* genes of *P. vivax* isolates by using PCR/RFLP from District Mardan and showed a remarkable level of genetic diversity in the studied genes of circulating parasites in the study area. The results of this study will contribute in future studies about the genetic structure of parasite and vaccine development against the malaria.

**Keywords:** *P. vivax*, Merozoite surface proteins (MSP), RFLP analysis, Mardan.

### Resumo

O *Plasmodium vivax* é o parasita da malária humana mais comum nos países asiáticos, incluindo o Paquistão. O presente estudo foi desenhado para explorar a diversidade genética de genótipos de *Plasmodium vivax* baseados nos genes *Pvmsp-3α* e *Pvmsp-3β*, usando marcadores de ensaios alélicos nested PCR e RFLP de isolados de campo no distrito de Mardan, Paquistão. Amostras de sangue de 200 pacientes com malária por *P. vivax* foram coletadas após assinatura do termo de consentimento livre e esclarecido. A diversidade genética em produtos de PCR nested foi determinada por polimorfismo de fragmento de restrição (RFLP) utilizando as enzimas de restrição *AluI* e *PstI* para a digestão dos produtos dos genes alfa e beta, respectivamente. Para análise da diversidade genética das variantes subalélicas dos genes *Pvmsp3α* e *Pvmsp3β*, o teste Qui-quadrado foi realizado utilizando o software de programação Minitab 18. O valor P = 0,05 foi considerado estatisticamente significativo. Para os genes *Pvmsp-3α*, após eletroforese em gel de produtos digeridos, quatro genótipos distintos foram obtidos de um total de 50 amostras; tipo A: 35 (70%) (1,5-2,0 kb), 12 do tipo B (24%) (1,5-1,7 kb), 2 do tipo C (4%) (0,5-1,5) e um para o tipo D (2%) (0,5-0,65 kb), que podem ser caracterizados em nove padrões alélicos (A1-A4, B1-B3, C1, D), em que

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A3 permaneceu como o mais predominante. Para *Pvmsp-3* genes, três genótipos distintos foram obtidos a partir de 50 amostras; 40 (80%) do tipo A (1,5-2,5 kb), 9 (18%) do tipo B (1,0-1,5 kb) e 1 (2%) do tipo C (0,65 kb), que podem ser caracterizados em seis padrões alélicos (A1-A3, B1-B2 e C1). Os mais dominantes no tipo A foram o alelo A1, observados em 46%, enquanto, no tipo B, os mais dominantes foram B1 (10%). Este estudo é o primeiro relato de epidemiologia molecular e variação genética em *Pvmsp-3*. Os genes *Pvmsp-3* de isolados de *P. vivax* utilizando PCR/RFLP do Distrito Mardan mostraram um nível notável de diversidade genética nos genes estudados de parasitas circulantes na área de estudo. Os resultados desse estudo contribuirão em estudos futuros sobre a estrutura genética do parasita e o desenvolvimento de vacinas contra a malária.

**Palavras-chave:** *P. vivax*, proteínas de superfície de merozoíta (MSP), análise de RFLP, Mardan.

## 1. Introduction

Malaria is the main threat to public health and it also affects the economic developmental status of many countries (Carlton et al., 2008). Millions of deaths occur every year due to malarial infection and approximately more than 40% world population living in the malaria infected countries (Tsuboi et al., 2010). Among five species of *Plasmodium* there are two common species, *P. vivax* and *P. falciparum*, in which *P. vivax* is highly prevalent species of human malaria parasite globally. *Plasmodium vivax* is the highly widespread human malaria parasite throughout the world except sub-Saharan region, Africa. In Africa its prevalence is low because of fixation in Duffy negativity trait in Negro population of African peoples (Miller et al., 1976). In Pakistan the malaria burden of *P. vivax* accounts for 92.4% while *P. falciparum* accounts for 4.7%, so *P. vivax* is the common prevailing species of malaria parasite. It affects a large proportion of people throughout the country (WHO, 2019). The climate of the district Mardan is hot from May to September and June-July are the hottest months. Precipitation is received often in the year, and the rainstorm season continues from May to October, and August is the rainiest month, thus giving perfect conditions to the advancement of mosquitoes. In 2010, major cases of *P. vivax* and *P. falciparum* were recorded in Sindh district of Pakistan where *P. falciparum* was 18.36% and *P. vivax* was 81.63% (Khatoun et al., 2010). Study the population structure is very important to know about the evolution of malaria parasite virulence and the role of parasite genetic diversity in malaria transmission which help in vaccine designing and also helps in impact of malaria control measures (Thakur et al., 2008). Genetic diversity among *Plasmodium* population is an important sign of the malaria transmission intensity in an area (Babiker et al., 1997). Many number of genes encoding for *P. vivax* as MSPs (merozoite surface proteins), in which *Pvmsp-3* and *Pvmsp-3* are highly polymorphic and having huge diversity. *Pvmsp-3* and *Pvmsp-3* have also been used as markers in population genetic analysis globally (Mueller et al., 2002; Smith et al., 2012). High level of global traveling and immigration of people from that area where malaria is endemic having incidence of imported cases of malaria has risen. The free traveling and movement of Afghan refugees across the border increase the transmission of malaria in Pakistan (Leslie et al., 2009). The aim of the present study was to investigate the genetic polymorphism and genetic diversity of *P. vivax* population from malaria endemic area of Mardan, by analyzing the polymorphic molecular markers *Pvmsp-3* and *Pvmsp-3*. In this study of molecular investigation the transmission of *P. vivax* has

been explored by looking at polymorphic genetic markers of *Pvmsp-3* alpha and beta genes from the Mardan region of Khyber Pakhtunkhwa, Pakistan.

## 2. Material and Methods

### 2.1. Study site and Samples collection

This research work was done in District Mardan, Pakistan from May 2017 to December 2018. The present study was based on molecular characterization of human *P. vivax* isolates in district Mardan approved by the Board of study (BOS) and advanced studies research board (ASRB) committee of the Department of Biochemistry, Abdul Wali Khan University Mardan. In this research work the patients with the symptoms of malaria in accordance to their response on the pretested questionnaire were selected. Blood collection was done from subjects in Mardan Medical Complex and District Head Quarter Hospital and private diagnostic labs. Both males and females of all ages were the target of this study. Total 200 blood samples were taken from positive patients with the help of lab technician cooperation. After obtaining their written informed consent 5 ml of blood was collected from all malarial patients in EDTA tubes. These samples were confirmed by Giemsa stained based thick and thin layer microscopy. These blood samples were shifted to molecular Biology lab in the Department of Biochemistry, AWKUM (Abdul Wali Khan University Mardan) and stored in the refrigerator at -20 °C till the DNA extraction. In this research work fifty samples were amplified for gene *MSP3* (*Pvmsp-3*) and *MSP3* (*Pvmsp-3*) alleles.

### 2.2. Genomic DNA Extraction

All positive blood samples of *P. vivax* were subjected to DNA extraction by using Phenol-Chloroform method (Renshaw et al., 2015). The isolated Genomic DNA of all samples was subjected to gel electrophoresis using Ultraviolet (UV) gel documentation (Gel Doc) system. Extracted DNA products were analyzed by 2% agarose gel stained with ethidium Bromide. Electrophoresis was performed in TAE buffer.

### 2.3. Amplification of *P. vivax* *MSP-3* and *MSP-3*

DNA samples with good quality were further analyzed by PCR using *Pvmsp-3* alpha and beta genes specific primers. Two sets of *Pvmsp-3* primers (N1) forward+

(N1) reverse were used for initial PCR and for Nested PCR *Pvmsp-3α* (N2) forward, (N2) *Pvmsp-3α* reverse primers were used. Similarly, for *Pvmsp-3β* genes confirmation two sets of primers (N1) forward (N1) reverse were used for initial PCR and for Nested PCR *Pvmsp-3β* (N2) forward (N2) reverse primers were used. For PCR 12 μL of PCR water, 10 μL of Solis Biodyne Master-mix were taken in PCR tubes then 1 μL of forward and reverse primers of each *Pvmsp-3α*, *Pvmsp-3β* gene were taken in these tubes were mixed and 2 μL of particular individual extracted DNA was taken for initial PCR. The initial denaturation was done at 95 °C for 5 minutes, annealing was done for alpha gene at 55 °C for 30 seconds and for beta gene annealing was done at 53-54 °C for 40 seconds 35 cycles for denaturation was done for 5 minutes and extension was done at 72 °C for 1 minute.

#### 2.4. Visualization of PCR products by Gel Electrophoresis

To observe amplified DNA PCR fragments were resolved by 2% agarose gel visualized with ethidium bromide and 1 kb DNA ladder was utilized to affirm the exact size of amplified fragments. The amplified PCR bands were captured at a wavelength of 254 nm using UV gel documentation system.

#### 2.5. PCR/RFLP Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism of Nested PCR was done by this method for the fragments digestion to show polymorphism by using different digestive enzymes. For *Pvmsp-3α* genes digestion, *Alu1* restriction enzymes were used and for *Pvmsp-3β* genes digestion, *PstI* restriction enzymes were used. In this method 18 μL PCR water was taken and 10 μL Nested PCR products were mixed in PCR tubes. In this mixture 2 μL *Alu1* restriction enzymes buffer were added for alpha products digestion and 2 μL *PstI* restriction enzymes were added for beta gene products digestion. These tubes were incubated at 37 °C for 4 hours. After reaction RFLP products were run in 2% agarose gel and were visualized under UV Trans-illuminator using ethidium bromide.

#### 2.6. Statistical analysis

Statistical data and frequencies of *P. vivax* segregates were analyzed by Chi square test at a measurable centrality dimension of  $P < 0.05$  utilizing Minitab Programming Software 18.

**Table 1.** Characteristics summary of total study.

S. No.	Total infected blood samples	Female	Male	Region
1	110	48	62	Katlang
2	56	35	21	MMC*
3	34	18	16	DHQ**
Total	200	101	99	

\*MMC (Mardan Medical Complex); \*\*DHQ (District Health Officer).

### 3. Results

Out of 200 malaria infected patients, 101 were female patients while 99 were male patients in which 48 female patients and 62 male patients belong to Katlang region. Samples collected from Mardan Medical Complex (MMC) were 56 in which 35 were female and 21 were male patients. Another 34 samples collected from District Headquarter (DHQ) Mardan included 18 female and 16 male patients. The frequency of female patients was more than male. Among 200 samples, 150 samples were found to be positive for *P. vivax* by both microscopy and PCR. The *Pvmsp-3α* allele was successfully amplified for 50 *P. vivax* samples while *Pvmsp-3β* was also amplified for 50 samples in this study (Table 1 and 2).

#### 3.1. Amplification of *Pvmsp-3α* genes by PCR

Fifty (50) malaria samples were amplified for *Pvmsp-3α* genes and were identified for different genotypes. In this study we observed four PCR length variants A, B, C, D. Thirty five of type A (70%), 12 of type B (24%), 2 of type C (4%) and one for type D (2%) (Figure 1).

#### 3.2. PCR-RFLP analysis of *Pvmsp-3α*

From RFLP analysis of *Pvmsp-3α*, 9 types of alleles were detected in current study including Type A with 4 subtypes (A1, A2, A3, A4) fragment size ranged 1.5-2.0 kb showing frequency of 70%, three Type B (B1, B2, B3) alleles with 24% frequency, one Type C (0.5-1.5) 4% and one Type D (0.5-0.65 kb) 2%. Most dominating one genotype was Type A3 (28%) followed by A1 (18%) and then A4 (14%) (Figure 2).

#### 3.3. Amplification of *Pvmsp-3β* genes by PCR

Fifty Malaria samples were amplified for *Pvmsp-3β* genes and were identified for different genotypes. In this study we observed three PCR length variants A, B, C. Forty (40) of type A (80%), nine of type B (18%) and one of type C (2%) (Figure 3).

#### 3.4. PCR-RFLP analysis of *Pvmsp-3β*

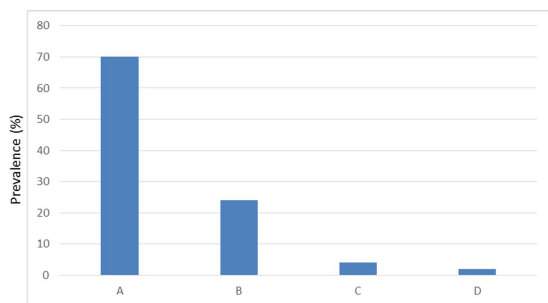
From RFLP analysis of *Pvmsp-3β*, 6 types of alleles were detected in current study including Type A with

**Table 2.** Location wise incidence of malaria in District Mardan.

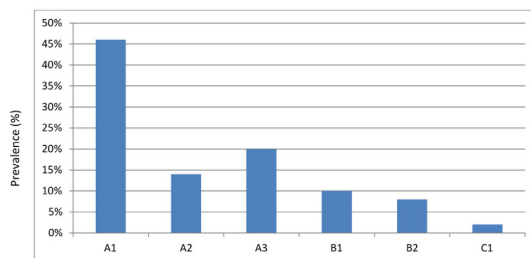
S. No.	Source	P. vivax cases	Percentage
1	Hospital	111	55.5%
2	Laboratories	89	44.5%
Total		200	100%

**Table 3.** Alleles size of *Pvmsp-3α* and *Pvmsp-3β* genes in District mardan.

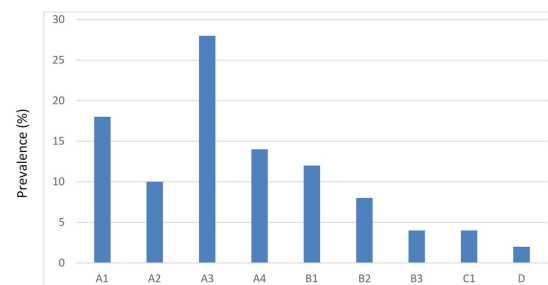
Allele <i>m</i> sp-3α genes	Size	Allele of <i>m</i> sp-3β genes	Size
Type A	1.5- 2.0 kb	Type A	1.5- 2.5kb
Type B	1.5- 1.7kb	Type B	1.0-1.5kb
Type C	0.5-1.5kb	Type C	0.65kb
Type D	0.65kb		



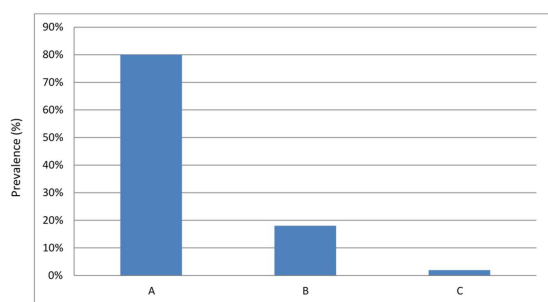
**Figure 1.** Prevalence of four different alleles of *Pvmsp-3α* (A, B, C and D) from PCR-RFLP



**Figure 4.** Prevalence of six different sub-allele types of *Pvmsp-3β* (A1-A3, B1-B2 and C1) based on PCR-RFLP.



**Figure 2.** Prevalence of nine different sub-allele types of *Pvmsp-3α*(A1-A4), (B1-B3), C1 and D are the nine different alleles from PCR-RFLP.



**Figure 3.** Prevalence of three different alleles of *Pvmsp-3β* (A, B, C) from PCR-RFLP.

3 sub types alleles (A1, A2 and A3) with fragment size ranged 1.5-2.5 kb and frequency of 80%, two allele for Type B (B1 and B2) with fragment size ranged 1.0-1.5kb and frequency of 18% and one allele for Type C (0.65 kb) 2% were observed (Table 3). Genotype A1 (46%) was the most abundant followed by A3 (20%) (Figure 4)

#### 4. Discussion

Molecular investigation revealed that malaria is one of the most important protozoan diseases after *M. tuberculosis* infection. Each year approximately 400-600 million malaria cases are reported worldwide with the death rate of 1.5-2.8 million. According to world malaria report 2017, 12% of world malaria cases occur in Pakistan. *P. vivax* population studies have become possible through identification of merozoite surface proteins-3 alpha and beta locus (Verma et al., 2013). In present study total 200 malaria patients were explored including males, females and children were analyzed from May, 2018 to December, 2018. In the present study only fifty samples were detected with four different sizes of PCR products (A, B, C and D) for *P. vivax msp-3α*. Nine 9 different types of sub-allelic families of *Pvmsp-3α* were noted in which Type A alleles of *P. vivax msp-3α* genes were dominant having four different sub allelic families of Type A (A1, A2, A3 and A4) detected after PCR-RFLP. The band size of Type A for *Pvmsp-3α* was (1.7- 2.0 kb) with frequency of 70% (1.5- 1.7 kb), 24% Type C was (0.5- 1.5 kb) 4% and Type D was (0.65 kb) 2%. *Pvmsp-3β* genes in our results showed three different allele types (A, B, C) in which Type A was dominant having three different sub allelic families of Type A (A1, A2, A3) detected after PCR-RFLP. In our results, 6 different types of sub-allelic families of *Pvmsp-3β* were noted. The band size of Type A was 1.5- 2.5 kb with frequency of 80%, Type B size was 1.0- 1.5 kb (18%) and Type C was 0.65kb (2%). Our results showed similarities with (Sinka et al., 2013) that recognized four alleles sizes dependent on the length variations of the PCR results of *Pvmsp-3α* and three alleles of *Pvmsp-3β* genes. Highly genetic polymorphism at *Pvmsp3α* has also been reported from Korea, Iran, Thailand, South America and Papua New Guinea, thus shows similarities with the present study (Cui et al., 2016, Yasinzai et al., 2004, Bruce et al., 1999, Rayner et al., 2002). Our study differs from hypo endemic northern Iran where type C of the *Pvmsp-3α* genes was predominant (Han et al., 2004; Zakeri et al., 2006). In India

3 different alleles were noted in *Pvmsp-3α*, Type A (1.8 kb), Type B (1.5 kb), and Type C (1.2 kb). Among these the most predominant one was Type A (75.4%) followed by Type B (14.3%) and Type C (10.0%) (Zakeri et al., 2009). The basic purpose behind indicating a moderate polymorphism is that, Mardan has a huge water system framework. District Mardan has vast irrigation system, after heavy rainfall in monsoon a lot of standing water remains for long which provides ideal environmental condition for mosquito breeding. In the monsoon season from the month of July to November has reported great prevalence of malaria infection. *Plasmodium vivax* is extensively distributed across Khyber Pakhtunkhwa due to wide agricultural practices, massive irrigation system, and monsoon rainfall. No use of anti-malarial sprays, poor hygienic system, sharing of the housing with livestock, improper diagnosis, poor drainage system, lack of awareness, and load shedding also play a major role in the spread of malaria in district Mardan, Pakistan.

## 5. Conclusion

It is concluded from present study that malaria still remains prevalence in District Mardan. Out of 200 samples, nine types of variants size of alleles were identified in alpha genes and six types of alleles in beta genes. Type A showed high frequency than alleles of type B and C, it means that the parasites show the genetic diversity in district Mardan. Acute attention is needed to carry out further study regarding *Pvmsp-3* genes along with other recently explored important vaccine candidate genes diversity to be conducted on mass level with respect to size of samples and regions of infection.

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