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Brief Communication

Genetic diversity of *Plasmodium vivax* and *Plasmodium falciparum* in Kohat District, Pakistan

Lubna Khatoon*, Inam Ullah Khan, Shahid Ali Shah, Muhammad Ishtiaq Jan, Farhat Ullah, Salman Akbar Malik

Quaid-i-Azam University, Islamabad, Pakistan

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Malaria is one of the serious diseases threatening human health in Pakistan and contributes to a large proportion of the total malaria deaths in South Asia. However, little is known about the nature and extent of genetic diversity of the malarial parasites circulating in Pakistan. This study was designed to assess the infection status of *Plasmodium* and the genetic diversity of *Plasmodium vivax* and *Plasmodium falciparum* by analyzing *msp-3 α* , *msp-3 β* and *msp-1*, *msp-2* genes respectively using allele specific nested PCR and RFLP assays. For this purpose, 130 field isolates were collected from the individuals who exhibited clinical symptoms associated with malaria in the Kohat region of Khyber Pakhtoonkhwa (KPK), Pakistan. Among 130 blood samples collected, *P. vivax* was detected in 105/130 (80.8%) and *P. falciparum* in 21/130 (16.2%). Mixed infections with both parasites were detected in 4/130 (3%) of the isolates. A large number of distinguishable alleles were found for *msp* genetic markers: 10 alleles for *msp-3 α* and seven for *msp-3 β* with one mixed infection in case of *msp-3 β* . The genotyping of *P. falciparum* showed that K1+MAD20 mixed genotype was dominant in *msp-1* and FC27 in *msp-2*. The results collectively suggest that *P. vivax* and *P. falciparum* populations in this region are highly polymorphic and mixed infections are prevalent.

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Malaria is one of the most serious communicable diseases in the world. Despite enormous control efforts over many decades, malaria still remains a significant health problem.¹ It is hyperendemic in Pakistan and is characterized by the presence of two *Plasmodium* species, *Plasmodium vivax* and *Plasmodium falciparum*, and mixed infections are very common.² To fight against malaria, an effective vaccine is urgently required, but the genetic diversity of *Plasmodium* field isolates, the occurrence of variant forms of the parasites in different geographic regions, and multiple

genotypes constitute obstacles to the development of a malaria vaccine.³

A number of antigens – such as merozoite surface proteins (MSPs), expressed at different stages of the parasite's life cycle – have been characterized with respect to their use in vaccine development.⁴ However, the genetic diversity of the malarial parasites circulating in Pakistan is poorly understood. Recent study^{5,6} has shed some light on genetic diversity of malarial parasites in Pakistan, but since these studies were from small areas they might not represent the entire country.

* Corresponding author at: Department of Biochemistry, Quaid-i-Azam University, Islamabad, 45320, Pakistan
E-mail address: lubnakhatoonqau@gmail.com (Lubna Khatoon)

In this study, we investigated the population diversity of *P. falciparum* and *P. vivax* by analyzing polymorphic genetic markers, Pfm_{sp}-1, Pfm_{sp}-2, Pvm_{sp}-3 α , and Pvm_{sp}-3 β in isolates from the Kohat region of N.W.F.P., Pakistan, to contribute to the global understanding of genetic diversity of *Plasmodium* population, in order to design an efficient antimalarial vaccine. The study area, Kohat, is a medium sized town in the Khyber Pakhtoonkhwa (KPK) province of Pakistan, located at 33°35'13" N 71°26'29" E, at an altitude of 489 meters. Mountains and hills dominate the topography of the district, and the intervening valleys between the hills are seldom over eight kilometers in width. The climate of the district is hot from May to September, and June is the hottest month. Rainfall is received throughout the year, and the monsoon rainy season prevails from May to October, and August is the rainiest month,⁷ thus providing favorable conditions for the growth of mosquitoes.

After the signing of informed consents, blood samples were collected in EDTA coated tubes from 130 infected patients attending the main hospital of the Kohat district during the summer of 2008. Earlier, thin blood smears had been prepared for microscopic detection of parasites. This study was approved by the Ethical Committee of the Quaid-i-Azam University, Islamabad.

A QIAamp DNA mini kit (Cat#51306) was used for extraction of genomic DNA following the manufacturer's instructions and was kept at -20°C. Using species-specific oligonucleotide primers, the samples were subjected to nested PCR to confirm *P. vivax* or *P. falciparum* infection.⁸ For the amplification of msp-1 and msp-2 genes of *P. falciparum*, the primers and PCR protocol of Snounou et al. was followed.⁹

The allelic diversity of the *P. vivax* msp-3 α gene was studied using primers and PCR-RFLP protocol, as described earlier.¹⁰ Similarly, for the amplification of *P. vivax* msp-3 β gene, the primers and nested PCR-RFLP protocol were followed.¹¹ All amplifications were performed on a T-personal (Biometra) thermocycler using 2 μ L of genomic DNA for nest 1, and 2 μ L of its product for second amplification reactions.

From the total of 15 μ L reaction volumes, 7 μ L of PCR product was digested individually with 0.3 μ L of AluI and PstI restriction enzyme for Pvm_{sp}-3 α and Pvm_{sp}-3 β , respectively,

as described by manufacturer (Fermentas — USA). The digested products were later electrophoresed on 2% agarose gel in a TBE buffer and DNA fragments were visualized under UV light. Samples were classified into different allele groups according to particular restriction patterns, as suggested earlier.⁵

Overall, the results of both diagnostic methods, i.e. microscopic and PCR, were in agreement for all 130 isolates; however, there was discrepancy between the results of these two assays when species-specific diagnosis is taken into account. By microscopy, 128 (98.5%) were identified as having only *P. vivax* and 2 (1.5%) as *P. falciparum* infections. No isolate was found to have mixed infection. However, PCR revealed 105 (80.8%) isolates to be infected with *P. vivax*, 21 (16.2%) with *P. falciparum* and four (3%) isolates as mixed infections. 54 out of 105 samples of *P. vivax* were successfully amplified for the msp-3 α gene, resulting in four major allelic variants.

As a whole, 37 Type A (1.9 kb), four Type B (1.5 kb), nine Type C (1.1 kb), two Type D (0.75 kb) and two mixed genotypes were investigated using PCR amplification. The RFLP analysis of msp-3 α with AluI resulted in a band nearly equal to 600 bp, but the smaller bands showed great variation among different samples. The sum of fragment sizes was not always equal to the size of the intact PCR product, indicating some non-resolvable small size fragments of digested products. Samples with identical RFLP patterns were grouped together as a separate allele group.

At least 10 allele groups (A1-D) were identified, five for type A (A1-A5), two for type B (B1-B2), two for type C (C1-C2) and one for type D. Allele A3 was the most abundant found in 32% (17/54) of the isolates (Fig. 1). Out of 23 amplified *P. vivax* positive samples for msp-3 β gene, nine type A (1.7– 2.2 kb), 10 type B (1.4–1.6 kb) and four mixed infections were found. Samples with multiple fragments of variant sizes were designated as mixed infection. RFLP of PCR product by PstI resulted in seven allele groups, thus showing even more diversity. In total, four alleles were identified for type A (A1-A4) and three for type B (B1-B3). None of the samples was found to have mixed infection when RFLP analysis was performed, as depicted in Fig. 1.

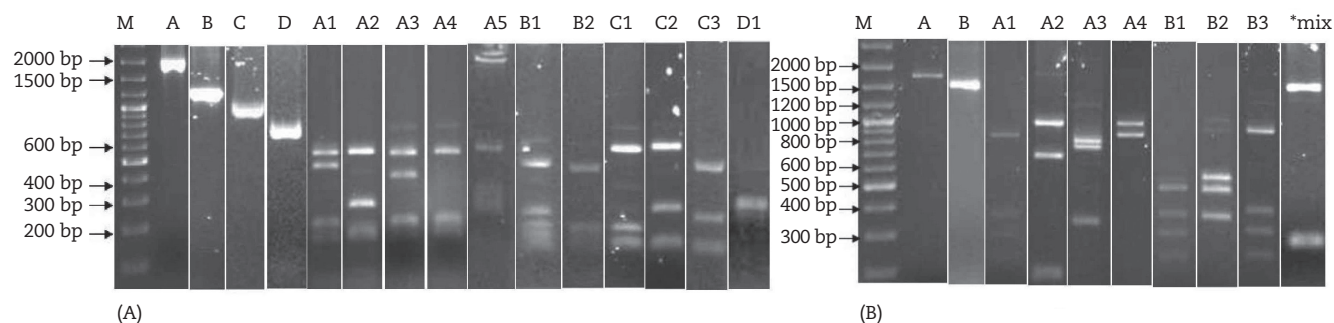


Fig. 1 - Major alleles of merozoite surface proteins MSP-3 α (a) and MSP-3 β (b) as identified by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) in the *P. vivax* population from Kohat, Pakistan.

Lane M = 2 kilobase (Kb) DNA marker. A, B, C and D are the genotypes obtained by PCR only while the alleles of these types obtained by RFLP are labeled with suffixes. Frequencies of the alleles were as follows: (A) A1 = 11%; A2 = 11%; A3 = 32%; A4 = 13%, A5 = 4%, B1 = 4%, B2 = 4%, C1 = 5%, C2 = 9%, D1 = 4%; (B) A1 = 11%; A2 = 4%; A3 = 4%; A4 = 26%; B1 = 22%; B2 = 9%; B3 = 26%. Obtained by PCR but RFLP resulted in only one allele type.

Twenty-one samples detected as *P. falciparum*-positive by PCR were analyzed to differentiate parasite populations with respect to the *msp-1* gene. Six isolates could not amplify for *msp-1* and therefore could not be assigned a specific *msp-1* allelic family, and the remaining 15 isolates showed high diversity of this genetic marker. As a whole, eight different allelic variants for the *msp-1* gene were detected; three length variants of approximately 180 bp, 200 bp and 250 bp were detected for K1 family, four for MAD20 family with size range between 120-250 bp, and only one allele for RO33 family of approximately 150 bp. The samples exhibiting amplification for more than one family were regarded as mixed infection. 20% (3/15) of the isolates exhibited K1 genotype, 26% (4/15) MAD20, mixed genotypes K1+MAD20 and K1+MAD20+RO33 were observed in 7% (1/15); while the predominant genotype family found in 40% (6/15) of the isolates remained K1+MAD20.

Out of 21 samples, 11 isolates did not show amplification for the *msp-2* gene, while the remaining 10 showed both known genotypes for *msp-2* gene, i.e., 3D7/IC and FC27. As a whole, eight different alleles were identified for *msp-2*, four for 3D7/IC family with a size range of 400-580 bp, and four for FC27 family with a range of 300-400 bp. Samples that amplified for both families showed mixed infection. 50% (5/10) of the isolates exhibited genotype FC27, 3% (3/10) 3D7/IC, while 20% (2/10) were identified as carrying mixed genotype.

In this study, the overall incidence of *P. vivax*, *P. falciparum*, and mixed infections suggests the supremacy of molecular diagnosis over conventional microscopic methods, as previously reported.¹² Mixed infections are common in Pakistan, as shown by different researchers,^{5,13} which supports our findings. High levels of genetic variability at *msp-3α* and *msp-3β* loci suggest that the *P. vivax* population is more diverse here as compared to the hyperendemic area of Papua New Guinea, where 24 alleles were investigated by Bruce et al.¹⁰ using two restriction enzymes, Thailand, and Korea; but less diverse than the Indian *P. vivax* population, where 37 alleles have been investigated by Kim et al.¹

The frequency of the three *msp-3α* types was consistent with those found for India, Papua New Guinea, Thailand,¹⁴ and Pakistan.⁵ Diversity of merozoite surface protein-3β is in close agreement with the investigations made in Thailand¹¹ and other Asian isolates,¹⁵ where 10 and 12 alleles have been detected for *msp-3β*, respectively.

This high degree of diversity and the presence of mixed infections in KPK is an indication of the complexity of the disease in this region. This may be attributed to the free movement of people through tribal areas between Kohat and Afghanistan. Moreover, the presence of alleles in common with studies from other regions indicates a global distribution of some alleles, while others might be regionally biased.

The presence of eight different variants at the *msp-1* locus is close to that reported by Heidari et al. from Iran.³ A common feature of this work and those reported from India,¹⁶ Iran,³ and Thailand¹⁷ is the existence of only one fragment for RO33. However, these results are different from those reported from Gabon¹⁸ and West Uganda,¹⁹ where the RO33 family was polymorphic with three and four fragments, respectively. A relatively higher polymorphism has been reported from Gabon and India, with 25 and 13 different allelic variants at the

msp-1 locus, respectively. The *msp-2* gene has demonstrated limited diversity in this region as compared to Iran,³ India,¹⁶ and Karachi (Pakistan).^{5,20}

A high degree of multi-strain infection in *P. falciparum* patients with more than one gene type shows the complexity of the disease in this region. The movement of Afghan refugees across the border to Kohat through the neighboring tribal areas, might have introduced different alleles of *Plasmodium* species into this area of Pakistan. The presence of more than one parasitic gene type in a single human host may lead to cross fertilization, meiotic recombination, and generation of new strains during the developmental stages of the mosquito vector.⁷

Although the pattern observed in our study can not be defined as the nationwide epidemic situation of malaria in Pakistan, due to the limited numbers of samples obtained from a restricted study area, the results suggest that *P. vivax* is highly polymorphic. In order to determine the nationwide epidemic pattern of malaria in Pakistan, further detailed studies with a large number of samples collected from different geographic areas of the country would be required.

Conclusions

The present study reported a variation in the selected vaccine candidate antigens in isolates from the Kohat region that could be taken into account in developing a malaria vaccine. Further population-based studies of *msp-3α*, *msp-3β*, *msp-1*, *msp-2*, and other candidate antigens of *Plasmodium* species, especially from mixed infections, will provide more information for the development of a malaria vaccine.

Conflict of interest

All authors declare to have no conflict of interest.

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