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Genetic diversity in human malarial parasites of Khyber Agency Pakistan

Lubna Khatoon[1]*, Muhammad Ishtiaq Jan[2], Inam Ullah Khan[2], Farhat Ullah[3] and Salman A. Malik[2]

[1]Faculty of Health and Medical Sciences, University of Copenhagen, Denmark;
[2]Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan;
[3]Kohat University of Science and Technology, Kohat, Pakistan

Abstract

Malaria is wide spread in poor world and its burden has been assessed by the enumeration of malarial parasites in blood of patients. This study was designed to find a relationship between social structure, and spread of malaria in Khyber agency. The average parasite density was 2050 parasite/µl in Khyber Agency. Due to economic and social setup most of the people have habit of sleeping in open air thus playing role in high malaria prevalence and *Plasmodium vivax* remains the prevalent species. Genetic study performed on 110 Blood samples showed less genetic diversity for both *Plasmodium vivax* and *Plasmodium falciparum*. Eight alleles were distinguished both for *Pvmsp* 3α and *Pvmsp* 3β in total of 20 and 39 amplified samples of *P. vivax* respectively. Out of 17 samples amplified for *P. falciparum* 11 showed genotype K1 and 10 for MAD at *Pfmsp*-1 while 14 alleles were identified for 3D7/1C and two for FC27 of corresponding families of *Pfmsp*-2 gene. This shows that *Plasmodium* parasites are not genetically diverse in Khyber agency.

Keywords

Malaria, human, Khyber, genetic diversity

Introduction

Malaria a vector-borne infectious disease caused by protozoan parasites (*P. falciparum* and *P. vivax*) still remains widespread in most of the tropical and subtropical regions of the world (Snow *et al.* 2005). Co-circulation of more than one species of human malaria parasites is a common feature of most areas where malaria is endemic including Pakistan (Bruce *et al*. 2000; Khatoon *et al.* 2009). The extensive diversity of malarial surface antigens is one the main reasons why clinical immunity develops after repeated infections with the same species over several years (Day and Marsh 1991). Parasite biology has been studied extensively after the introduction of PCR genotyping. Different genetic markers have been studied both for *P. falciparum* and *P. vivax* as potential vaccine candidates. One of the most common genetic markers is *merozoite surface proteins.*

The genetic diversity among *Plasmodium* population is an important indicator of the malaria transmission intensity in an area (Babiker *et al.* 1997). An endemic area is attributed to higher parasite diversity and multiple genotypes of parasites. While, in low transmission areas parasites have a limited genetic diversity with monoclonal infections (Ghanchi *et al.* 2010).

With the increase in global traveling and immigration of people from malaria endemic areas the incidence of imported cases of malaria has risen. The free movement of Afghan refugee across the border facilitates transmission of malaria in Pakistan (Leslie *et al*. 2009).

While studying *P. vivax* isolated from Pakistan, Zakeri and co-workers observed three major size alleles for *Pvmsp-3α* gene: type A (1.8 kb), type B (1.5 kb) and type C (1.2 kb) with type A (72.7%, 139/187) as the most frequent allele size showing 40 (*AluI*) distinct variants (Zakeri *et al.* 2010).

In another study from Bannu district of Pakistan four distinct allele groups: A (1.9 kb), B (1.5 kb), C (1.2 kb), and D (0.3 kb) were detected for *Pvmsp-3α*, type A being the most prevalent (82%). Conversely, amplification of the *P. vivax msp-3β* locus produced two allele groups: A (1.7–2.2 kb, 62%) and B (1.4–1.5 kb, 33%), with 5% mixed-strain infections (Khatoon *et al.* 2010). Out of 130 studied isolates from Kohat region of Khyber Pakhtoonkhwa (KPK) 37 Type A, 4 Type B,

*Corresponding author: lukh@sund.ku.dk

9 Type C, 2 Type D (0.75 kb) and 2 mixed genotypes were investigated using PCR amplification (Khatoon *et al.* 2012).

In their study Ghanchi *et al*. (2010) from Pakistan demonstrated that a majority of patients had monoclonal infections. 24% and 22% carried multiple *P. falciparum* genotypes in *msp-1* and *msp-2*, respectively. They observed 25 genotypes *msp-1* (12 KI; 8 MAD20; 5 RO33) and 33 for *msp-2* (14 FC27; 19 3D7/IC) among 238 studied isolates (Ghanchi *et al*. 2010). From Bannu, Pakistan, all three known genotypes of *Pfmsp-1* and two of *Pfmsp-2* were observed, with MAD20 occurring in 67% and 3D7/IC in 65% of the isolates, respectively while, 24% of the isolates exhibited mixed-strain infections (Khatoon *et al*. 2010). From Kohat, Pakistan *P. falciparum* showed that K1+MAD20 mixed genotype was dominant in msp-1 and FC27 in msp-2 (Khatoon *et al*. 2012).

Since people living in FATA region are poor and there are less facilities available to them thus making area endemic to malaria. This study was aimed at finding a relationship between sleeping habits and several parameters of a symptomatic malaria disease such as parasite load and hemoglobin level of people of Khyber agency. To add to our understanding of molecular surveillance of *Plasmodium* in this region genotyping studies were also carried out on selected isolates.

Materials and Methods

Khyber Agency is a tribal area in FATA region of Pakistan with extreme climate. It ranges from the Tirah valley down till Peshawar bordering Afghanistan to its East, Orakzai agency to its North and Peshawar to its West (http://www.fata.gov.pk).

A total of 264 symptomatic malaria patients (161 males; 103 females) of age ranging from 1 to 70 years attending Civil Hospital Jamrud, Khyber Agency, Pakistan, during August and September 2009 were selected for this study. For inclusion in study, days to illness, body temperature of patients and periodic fall and rise in body temperature after an interval were use as a guideline for symptomatic malaria before microscopy. Patients included in this study were ill for at least 1 day and maximum of 7 days illness was observed. Average body temperature was found to be 102 F irrespective of the age or gender of the patients. Other parameters like sleeping habits of patients, their hemoglobin (Hb) level and parasite index (PI) were also calculated (Table I).

After taking informed consent and patient history whole blood samples were collected in EDTA coated vacutainer tubes. This study was approved by ethical review committee of Quaid-i-Azam University, Islamabad.

Thick slides were prepared by using 4 µl blood and were stained immediately using Giemsa/ stain and parasite density was calculated according to the formula:parasite index = parasite count WBC count × WBC count per µl of blood (O'meara *et al*. 2006). Hb level was determined by the use of cyanmethemoglobin method using Spectrophotometer (Microlab, Merck, Model No. 200).

Extraction of DNA and PCR based *Plasmodium* species identification

Using whole blood samples parasitic DNA was extracted by QIAamp® DNA mini kit (CAT#51306) according to the instructions of the Manual provided.

The nested PCR amplification procedures described by (Snounou *et al.* 1999; Singh *et al.* 1999), was applied for the detection and characterization of the infecting *Plasmodium* species. An initial outer amplification, Nest 1 reaction, involved the use of genus-specific oligonucleotide primer pair, rPLU5 (5'-CCTGTTGTTGCCTTAAACTTC-3') and rPLU6 (5'-TTAAAATTGTTGCAGTTAAAACG-3'). The products of the Nest 1 reactions were then used as the template for the second inner, Nest 2 amplification. The species specific oligonucleotide primer pairs were used to detect each *Plasmodium* species; rFAL1 (5'-TTAAACTGGTTTGGGAAAA CCAAATATATT-3') and rFAL2 (5'-ACACAATGAACT-CAATCATGACTACCCGTC-3') for *P. falciparum*, and rVIV1 (5'-CGCTTCTAGCTTAATCCACATAACTGATAC-3') and rVIV2 (5'-ACTTCCAAGCCGAAGCAAAGAAA GTCCTTA-3') for *P. vivax*.

The amplifications were performed on T personal, thermocycler (Biometra), the total volume of the reaction was 20 µl, containing 0.4 µl of 10 mM of each dNTPs of each, and 2.5 units of Taq DNA polymerase, 20 µM of each primer and 3 µl, of product DNA was used for the Nest 1 and 2.0 µl of Nest 1 PCR product was used as the template for the Nest 2 reaction. PCR amplification was performed on the following conditions initial melting at 94°C for 3 minutes followed by 34 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 60 seconds with an extension at 72°C for 5 minutes.

PCR amplification of *P. falciparum msp*-1 and *msp*-2 genes

The family specific analysis of *P. falciparum msp*-1 and *msp*-2 genes, the primer and PCR protocol described by Snounou *et al*. (1999) was followed. Briefly, in first reaction oligonucleotide primers pairs M1-OF (5'-CTAGAAGCTTTAGAA-GATGCAGTATTG-3'), M1-OR (5'-CTTAAATAGTATTC TAATTCAAGTGGATCA-3') corresponding to conserved sequences spanning the polymorphic regions of *msp*-1 gene and M2-OF (5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3') and M2-OR (5'-CTTTGTTACCATCGGTACATTCTT-3'), corresponding to conversed sequences spanning the polymorphic regions of *msp*-2 gene were included, using as a template the product generated in the first reaction, five separate nested reactions were then performed in order to determined the presence allelic variation from the MAD20, KI and RO33 families of *msp*-1 block 2, the FC27 and 3D7/IC families of *msp*-2 block 3. Three specific nested reactions were performed for amplification of *msp*-1 gene using three sets of primers:

1. M1-KF (5'-AAATGAAGAAGAAATTACTACAAAAG-GTGC-3'),
   M1-KR (5'-GCTTGCATTCAGCTGGAGGGCTTGCAC-CAAGA-3'),
2. M1-MF (5'-AAATGAAGGATTTGTACGTCTTGATTA CC-3'),
   M1-MR (5'-ATCTGAAGGATTTGTCGTCTTGAATTA CC-3')
3. M1-RF (5'-TAAAGGATGGAGCAAATACTCAAGTTGT TG-3'),
   M1-RR (5'-CATCTGAAGGATTTGCAGCACCTGGA-GATC-3').

Two separate nested reactions were performed using following two primers pair's for *msp*-2 gene in order to determine the allelic variation from each other.

1. M2-FCF (5'-AATACTAAGAGTGTAGGTGCARATGCT CCA-3'),
   M2-FCR (5'-TTTTATTTGGTGCATTGCCAGAACTTGA AC-3') and
2. M2-ICF (5'-AGAAGTATGGCAGAAAGTAAKCCTYC-TACT-3'),
   M2-ICR (5'-GATTGTAATTCGGGGGATTCAGTTTGTT CG-3').

PCR reaction the total volume of the reaction was kept 20 µl, containing 0. 4 µl of dNTPs of 10 mM each primer, 2.0 µl of each 10X PCR Buffer and 25mM $MgCl_2$ and 5U/µl of Taq DNA polymerase and 3.0 µl of DNA template. Nested PCR amplification was performed on the following condition. Initial melting at 95°C for 5 minutes, followed by 34 cycles of 94°C for 30 seconds, 58°C for 30 secs, 72°C for 60 secs and final extension at 72°C for 5 min. 2.0 µl of Nest 1 PCR product was used as the template for the Nest 2 PCR amplification. The amplification conditions were same as in Nest 1 reaction with a difference being only in the DNA template used (2.0 µl).

The products were run on 2% Agarose gel and amplified fragments of different families of *msp*-1 and *msp*-2 genes were distinguish according to their base pair presented by Snounou *et al.* (1999).

PCR-RFLP of *P. vivax msp*-3α and *P. vivax msp*-3β genes

Allelic diversity at the *P. vivax* merozoite surface protein-3a (*pvmsp-3*a) locus was investigated using a combined polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) protocol described by Khatoon *et al.* 2010. In Nest 1 reaction one pair of oligonucleotide primer pair P1 (5'-CAGCAGACACCATTTAAGG-3') and P2 (5'-CCGTTTGTTGATTAGTTGC-3') was used for amplification of *pvmsp*-3α gene. The total volume of the reaction was 20 µl, containing 0. 4 µl of dNTPs of 10 mM each, 2.5 units of Taq DNA polymerase, 200 nM of each primer and 3 µl of product DNA was used for the Nest 1 PCR reaction.

Nested PCR amplification was performed on the following condition. 95°C for 3 min followed by 34 cycles of 94°C for 30 sec, 58°C for 30 sec, 68°C for 2.5 min and 72°C for 5 min. 2.0 µl of Nest 1 PCR product was used as the template for the Nest 2 PCR amplification; using primer N1 (5'-GACCAGT-GTGATACCATTAACC-3') and N2 (5'-ATACTGGTTCTTC GTCTTCAGG-3'). The amplification conditions were same as in Nest 1 reaction with a difference being only in the DNA template used.

For RFLP analysis the product of *pvmsp*-3α gene was incubated with the restriction enzyme Alu1 at 37° C for a period of 4 hours.

Table I. Comparison of age and sleeping habits of malaria patients in relation to their blood Hb (Hemoglobin) and PI (Parasite Index)

Age (years)	Sleeping habits					Hb level (mg/100ml)	PI = Parasite/ µL of blood
	Open air (%)	Bed nets (%)	Near fire (%)	Use of spray (%)	Use of mat (%)		
01–10 (n=132)	28	48	06	13	05	9.572 ± 1.124	2337 ± 1066
11–20 (n = 66)	49	18	18	13	02	10.525 ± 0.700	1758 ± 593
21–30 (n = 48)	51	23	14	12	00	11.300 ± 1.650	1618± 1107
31–40 (n=13)	61	25	7	7	00	10.555 ± 0.864	1506 ± 580
41–60 (n=7)	75	13	12	00	00	–	1106 ± 356
61–70 (n=2)	50	50	00	00	00	–	890 ± 268

Normal level of Hb
Female = 11.0–16.0 g/100ml
Male = 12.0–18.0 g/100ml

Calculation for determination of parasite index
PI= parasite count/WBC count×WBC count per µL of blood
n = sample size

Allelic diversity at the *P. vivax* merozoite surface protein-3β (*pvmsp-3*β) locus was amplified using pair of oligonucleotide primer BF1 (5'-GGTATTCTTCGCAACACTC-3') and BR1 (5'-GCTTCTGATGTTATTTCCAG-3') for Nest 1 and using 2.0 µl of Nest 1 PCR product as template for the Nest 2 PCR amplification; employing primers BF2 (5'-CGAGGGGCGAAATTGTAAACC-3') and BR2 (5'-GCTGCTTCTTTTGCAAAGG-3'). The amplification conditions were same as in Nest 1 reaction.

The RFLP analysis of *pvmsp*-3β gene was performed by incubating the PCR product with the restriction enzyme *PestI* at 3°C for a period of 4 hours.

Results

According to microscopic analysis, 53% of the studied subjects were infected with *P. vivax* and 47 % with *P. falciparum*. Both of these parasitic species were observed to be in different stages of their life cycle like tropozoite, tropozoite end gametocytes and gametocyte.

The Hb level of malarial patients was considerably low from the normal (standard Hb level but healthy individuals were not tested for this study). Parasite density level was high in males as compared to females. The average parasite density among males and females was observed as 2050 parasite/micro litter of blood (Table I). It was observed that Hb level decreased with increase parasite index particularly in those patients who slept in open air because they were more prone to be infected from malaria. The maximum patients among age group 01–10 years sleep in bed nets, but because of some other activities like playing near marsh etc showed highest parasite index and lowest Hb level among both males and females. Hb level for females and males was observed as 9.6g/100 ml and 10.9g/100 ml, respectively. Parasite density/Parasite index is the level of parasetemia in the patient suffering from malaria. In this study the parasite density level was high in males as compared to females (data not shown). The average parasetemia level among the males was observed high in comparison to females as 2190 and 1937 parasite/micro litter of blood.

Out of 110 PCR positive samples that were infected with *Plasmodium*, 81 were identified as *P. vivax* and 17 as having mixed infection (*P. vivax* + *P. falciparum*), 12 samples were not infected with any species hence were not taken into further consideration. 20 *P. vivax* samples amplified out of 81 for *pvmsp*-3α gene resulted in two allele types 20% (4/20) A (2.2 kb) and 70 % (14/20), B (2.0 kb) while 10% (2/20) were mixed types as shown in Fig. 1. RFLP pattern resulted in nine allele types with B3 as the most prevalent genotype (20%) as given in Table II.

Thirty nine samples amplified for *pvmsp*-3β gene could be differentiated in to two allele sizes i.e. for 49% (19/39) Type A (2.1–2.5 kb), 36% (14/39) Type B (1.7–2.0 kb), and 15% (6/39) mixed types. Restriction analysis subdivided them in to further genotypes while 6 lacked restriction sites as shown in Fig. 1 and Table II. A sample was regarded as mixed genotype when it had more than one fragment after amplification or sum of RFLP fragments was higher than the size of PCR

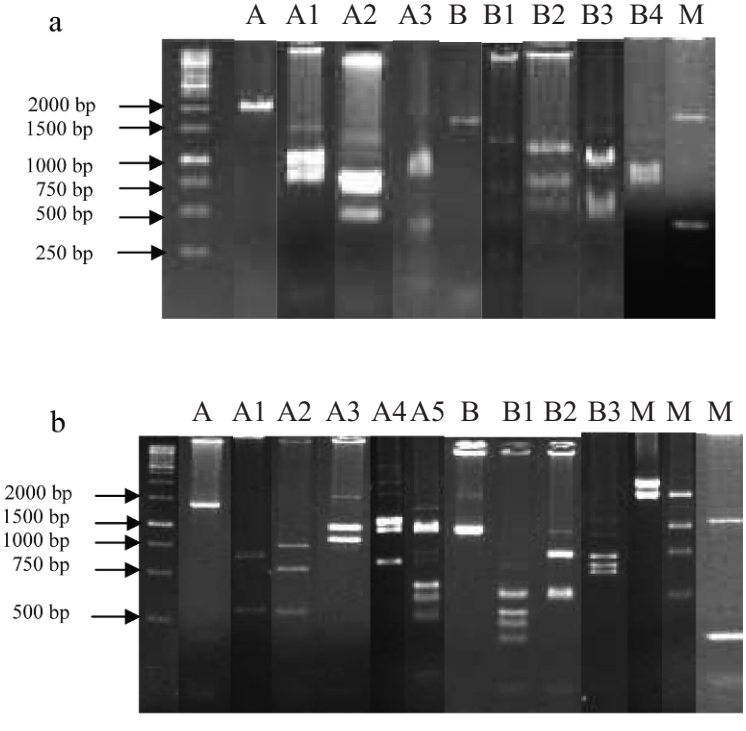

Fig 1. PCR-RFLP analysis of P. vivax msp-3α (a) and msp-3β (b) Where A, B = Undigested PCR products and A1-A5 and B1-B4= PCR-products digested with AluI (Pvmsp-3α) and PestI (Pvmsp-3β) M= mixed genotypes

Table II. Distribution of merozoit surface protein (msp) genes of *P. vivax* and *P. falciparum*

P. vivax			***P. falciparum***	
***Pvmsp*-3α (n=20)**		***Pvmsp*-3β (n=39)**	***Pfmsp*-1**	**(n=14)**
A1	2	5	K1	4
A2	1	5	MAD20	3
A3	1	11	K1+MAD20	7
A4	–	1	***Pfmsp*-2**	**(n=14)**
A5	–	3	FC27	0
B1	4	3	3D7/IC	12
B2	4	2	FC27+3D7/IC	2
B3	6	2		
B4	2	–		
M	–	1		
U	–	6		

M = Mixed, U = uncut.

product as depicted in Fig. 1. Out of 17 *P. falciparum* isolates 11 amplified for *pfmsp*-1 and 16 for *pfmsp*-2 gene. *Pfmsp*-1 showed both known genotypes i.e., KI and MAD20, similarly *pfmsp*-2 gene resulted in two genotypes FC27 and 3D7/1C; while lethal RO33 was not found in any of the isolates as shown in Table II.

Discussion

Pakistan is one of the moderately endemic countries for malaria in Asia. There is a great variation in prevalence of malarial parasite among the provinces and areas (Ghulam *et al*. 2009). It could be attributed to different living conditions in different areas. In rural and less developed areas people sleep in open air and hence more exposed to mosquitoes.

This study is in consistent with the previous about higher incidence of *P. vivax* (Yasinzai and Kakarsulemankhel 2008; Khatoon *et al*. 2009). The parasite density is strongly associated with a clinical diagnosis of malaria for all species of malaria. In our study the average parasite density was 2050 parasites/µl which are comparable with the study observed from Punjab, Pakistan (Pryblski *et al*. 1999) and Thailand (O'maera *et al*. 2006) though quite less as compared to 10,000 parasites/µl was observed in Africa (Rogier *et al*. 1996). *Msp*-3α was found to have two alleles A and B of which B was more prevalent and polymorphic contradicting previous studies from Iran (Zakeri *et al*. 2006a, b), India and Pakistan (Khatoon *et al*. 2010) where three alleles were observed with type A being the most prevalent. The *pvmsp*-3β gene resulted in amplification of two major alleles of which 15% of type B remains uncut which mirrors previous findings from Pakistan [Khayber Pakhtoonkhaw] (Khatoon *et al*. 2010 and 2012) but contradicting observations from China and Thailand (Yang *et al*. 2006).

P. falciparum msp-1 and *msp*-2 candidates for malaria vaccine development (Moorthy and Hill 2002) have been found to have 11 genotypes for former and 16 for later, which is comparable to 10 and 17 alleles of Thailand (Snounou *et al*. 1999) respectively but somewhat less diverse than 25 alleles of *msp*-1 and 19 alleles of *msp*-2 of West Uganda as described by Aubuoy (Aubouy *et al*. (2003). We observed total 4 alleles for K1 family and 3 for MAD20 and 7 mixed while no allele was seen for RO33 family in corresponding area. At *msp*-2 locus 3D7/IC was major allele while 2 were mixed infections and none of the samples was carrying allele type FC27 resulting in less diversity at msp locus in this region. A study from Gabon during analysis of *msp-1* locus, 14 alleles of K1 family 8 MAD20 alleles and 3 of the RO33 family were identified while for *msp-2* locus, 11 FC27 alleles and 8 3D7/1C alleles were documented. There were only two mixed infection were identified belonging to K1 family of *msp*-1 and to FC27 and 3D7/1C family of *msp*-2 (Aubouy *et al*. 2003).

Conclusion

It can be concluded from this study that though malaria is prevalent in this region but the parasites lack genetic diversity.

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