Original Article

Detection of Amino Acid Mutations in Dihydrofolate Reductase & Dihydro Pteroate Synthase Gene in Plasmodium Vivax Isolates

Rizwana Yasmin, Musarrat Jehan, Mehwish Kalam, Syeda Mariam Siddiqa, Mahadev Harani, Mustafa Kamal

ABSTRACT

OBJECTIVE: To assess the frequency and patterns of mutations and distribution of alleles in Plasmodium. Vivax Dihydrofolate Reductase (DHFR) & Dihydro Pteroate Synthase (DHPS) genes in Karachi.

METHODOLOGY: The descriptive cross-sectional study was carried out at the Department of Biotechnology, University of Karachi from June 2014 to February 2016. The sampling technique used was Non-probability convenient sampling method.

A total of n=200 malaria cases irrespective of age and gender from Jinnah Medical College Hospital Korangi Karachi were selected. Around 200 µL of blood was collected in micro centrifuge tubes. Among them, 36 cases of P. vivax were detected by microscopy. DNA was extracted from these 36 samples followed by polymerase chain reaction (PCR) and sequencing of amplified genes of Plasmodium vivax Dihydrofolate reductase (PVDHFR) & Plasmodium vivax Dihydro pteroate synthase (PVDHP).

RESULTS: The analysis of 36 amplified P. vivax isolates revealed presence of various alleles of pvdhfr and pvdhps genes producing both wild type (W) and mutant amino acids having single, double & multiple mutations at multiple positions. In pvdhfr gene, common mutations were observed in 'S' amino acids at codons 58 &117 and 'D' at codons 105 &157. Regarding pvdhps gene, common mutations were observed in 'V' amino acids at codon 398 and 'K' at codon 590.

CONCLUSION: The frequency and variety of mutations we detected in pvdhfr and pvdhps genes signify the drug resistance in plasmodium vivax so there is need of some measures to control drug resistance by better formulation using combination of drugs.

KEYWORDS: Plasmodium vivax, pvdhfr gene, pvdhps gene, Antifolate drug resistance markers.

This article may be cited as: Yasmin R, Jehan M, Kalam M, Siddiqa SM, Harani M, Kamal M. Detection of Amino Acid Mutations in Dihydrofolate Reductase & Dihydro Pteroate Synthase Gene in Plasmodium Vivax Isolates. J Liaquat Uni Med Health Sci. 2020;19(02):102-8. doi: 10.22442/jlumhs.201920671

INTRODUCTION

Malaria is highly endemic in developing countries like Pakistan[1]. According to the World malaria report 2018, malaria accounted for an estimated 219 million cases worldwide as reported in 2017, and there were an estimated 435,000 deaths from malaria globally[2].

Plasmodium vivax is the most widely distributed malarial species. Control of P. vivax infection is more challenging because of its unique biological and epidemiological characteristics. The level of parasitemia is low but gametocytes emerge before illness manifests and before control measures are taken. Also, hypnozoite (dormant liver stage) infection occurs out of season and so can extend the geographical area affected[3].

P. vivax infection has high morbidity and some mortality[4]. This can be attributed to many factors, mainly treatment failure caused by emergence of drug resistant strains including antifolate drugs such as Sulphadoxine-Pyrimethamine (SP). Persistent use of SP produces resistant strains, which develop sequential mutations in dhfr and dhps genes[5,6]. Unicellular eukaryotes and prokaryotes synthesize their own folic acid catalyzed by DHPS and DHFR enzymes. Pyrimethamine inhibits the dhfr enzyme and sulfadoxine targets the dhps enzyme in the folate biosynthetic pathway of the parasite[7,8]. SP is a cheap anti malarial drug commonly prescribed against P. vivax in Asian countries[9]. SP resistance in P. vivax is reported in many regions of Pakistan[10-12]. There are several factors which are responsible for the emergence of resistance like microscopic misdiagnosis and irrational use of anti malarial drugs especially Sulphadoxine, which is also used for the treatment of GIT & respiratory tract infections as well as other causes of fever and chill[12]. Molecular markers for SP resistance, mutations in dhfr and dhps genes are valuable tools for evaluating SP resistance patterns in P. vivax populations[9].

The aim of this study was to evaluate the extent of antifolate-resistance in P.vivax species by detecting mutations in DHFR and DHPS and to outline the changing genetic diversity.

METHODOLOGY

The descriptive cross-sectional study was carried out

at the Department of Biotechnology, University of Karachi. The duration of project carried out was from June 2014 to February 2016. The sampling technique used was Non-probability convenient sampling method.

A total n= 200 malaria cases irrespective of age and gender were selected from various hospitals & clinics of Karachi. Around 200 µL of blood was collected in micro centrifuge tubes. Among them, 36 cases of P. vivax were detected by microscopic examination of Giemsa stained thick and thin smears as described by WHO guidelines[14].

DNA was extracted from blood samples of these P. vivax cases using the QIAGEN DNA extraction kit (Germany) According to manufacturer's protocol and instructions.

The PCR was performed by using nested PCR for confirmation and identification of new genes. The confirmation of P. vivax was done by targeting the 18S rRNA, as reported by Iqbal et al in 2013[15]. Further pvdhf and dhps genes were amplified and sequencing was performed for detecting mutation or polymorphism in these genes. The extracted samples of all isolates were then stored at -20°C for further use. Quality control of each lot of the QLAamp DNA Mini kit is already tested against predetermined specification to ensure consistent product quality.

Amplification of Pvdhfr & Pvdhps Gene

A total of 36 P. vivax positive cases were selected and nested PCR was performed on dhfr and dhps genes. The primer sequences are shown in Table I.

TABLE I: PRIMER SEQUENCES USED FOR AMPLIFICATION OF PVDHFR AND PVDHPS GENES

Gene	Primer No	Primer Sequence
Pvdhfr (P)	107 108	F: 5'-CACCGCACCAGTTGATTCCT-3' R: 5'-CCTCGGCGTTGTTCTTCT-3'
Pvdhfr (S)	109 110	F:5'-CCCCACCACATAACG AAG-3' R:5'-CCCCACCTTGCTGTAAACC-3'
Pvdhps (P)	111 112	F: 5'-GATGGCGGTTTATTTGTCG-3' R: 5'-GCTGATCTTTGTCTTGACG-3'
Pvdhps (S)	113 114	F: 5'-GCTGTGGAGAGGATGTTC-3' R: 5'-CCGCTCATCAGTCTGCAC-3'

The cycling conditions were used with slight modifications as previously reported by Huang et al in 2015[16]. The PCR was carried out in a tube containing 50 µl of a reaction mixture made up of the following components: 15 pmol of each forward and reverse primers for pvdhfr & pvdhps genes as shown in **Table I**. The 500 µM of four deoxynucleotides, 2 U of Taq polymerase (Promega), 10 x PCR buffer and 1.5 mM $MgCl_2$. The thermal cycler (Master Gradient PCR System, Eppendorf AG, Germany) was programmed to first incubate the sample for 10 minute at 94°C followed by 40 cycles at 94°C for 1 minute, at 45°C for 2 minutes and at 72°C for 1 minute with final extension for 7 minute at 72°C.PCR amplification of pvdhfr gene was done at codons 50, 52, 58, 93, 105, 106, 117,135, 157 and 185 while for pvdhps gene was done at positions 389, 391, 397, 398, 399, 400, 401, 426, 459, 528, 549, 577, 582, 586, 588, 589 and 590.

The products were identified by electrophoresis on a 2% agarose gel stained with ethidium bromide, and evaluated under transilluminator as described by Ding S et al[17]. The sizes of PCR amplified product were estimated according to the migration pattern of a 1 kb DNA ladder (Gibco BRL Life Technologies). The amplified products were visualized and photographed using DOC gel documentation system (Vilber System). The amplified fragment of pvdhfr gene was 755 bp size shown in Figure I and of pvdhps gene was 731 bp shown in Figure III. The samples were then sent for sequencing. Mutation analysis was done by aligning the sequence with the reference sequence.

PVDHFR and PVDHPS Sequence Analysis

The nested-PCR products of pvdhfr and pvdhps genes were subjected to sequence analysis. The forward primer was purified and sequenced commercially by Macrogen Incorporation in Seoul Korea. DNA sequences were translated into amino acid sequences and compared with the published sequences of pvdhfr and pvdhps genes[18]. Multiple sequence alignment of P.vivax dhfrgene is shown in Figure II, and multiple sequence alignment (MSA) of P.vivax dhps gene is shown in Figure VI, V and VI.

FIGURE I: PVDHFRGEL ELECTROPHORESIS:
showing fragment size.
Lanes 1 and 19: band on sample 7 not seen.100 bp DNA molecular weight marker
Lanes 2 – 20 and 26 – band on sample 21 and 26 not seen: Amplified pvdhfrgenomic DNA

FIGURE II: MULTIPLE SEQUENCE ALIGNMENT OF PVDHFR GENE

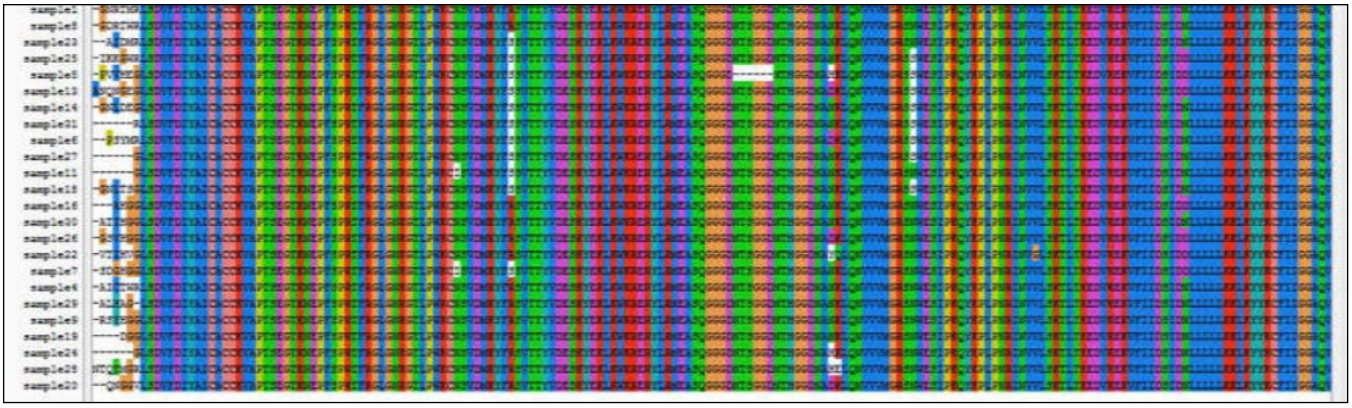

FIGURE III: PVDPS GELECTOPHORESIS: Showing fragment size.
Lanes 1 and 25: 100bp DNA molecular weight markers
Lanes 2-26 and 34: Amplified pvdps genomic DNA

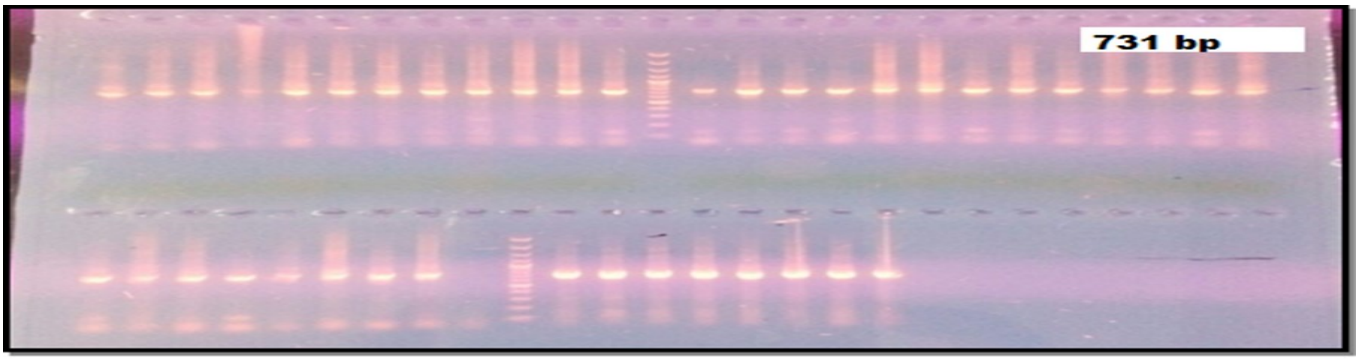

FIGURE IV: MULTIPLE SEQUENCE ALIGNMENT OF PVDHPS GENE

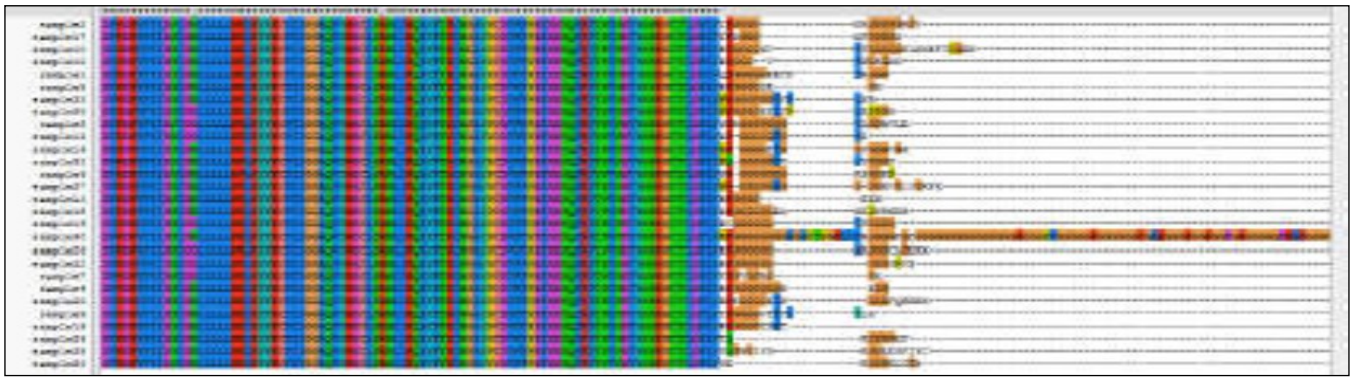

FIGURE V: MULTIPLE SEQUENCE ALIGNMENT OF PVDHPS GENE

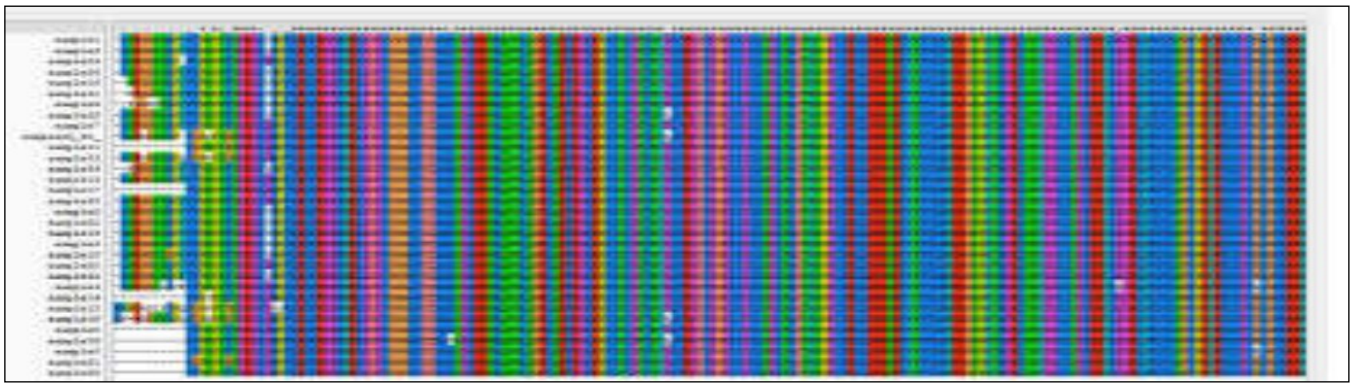

FIGURE VI: MULTIPLE SEQUENCE ALIGNMENT OF PVDHPS GENE

Bioinformatics tools to observe the pattern of resistance in Pvdhfr and Pvdhps Genes

The DNA sequences were aligned by using various web tools. Six frame translation of nucleotide sequence into amino acid sequence was done using EMBOSS transeq tool of EBI. The suitable frame was selected with the help of Blast suite of NCBI. Clustal X 2.1 was used to perform multiple sequence alignment and phylogenetic analysis. Secondary structure prediction was done by PSIPRED method, and a 3D model was made using Swiss model. Energy calculation and Ramachandran plot analysis was done by PROSA web server and RAMPAGE, respectively.

RESULTS

Of the total 200 cases, only 36 were processed for amplification and sequencing. The samples showed wild type (W) as well as mutated haplotype of DHFR genes when at least one amino acid substitution in the related residue was seen. The mutation patterns and wild type haplotypes of DHFR gene is summarized in Table II.

TABLE II:
MUTATION ANALYSIS OF PVDHFR GENE

CODON	WILD TYPE	MUTANT TYPE	
		Mutant Type	No. of Mutants
50	N	I	2
52	V	A	1
58	S	R	17
93	S	H	1
105	D	N	18
		Y	1
		H	4
106	K	E	1
		Q	1
117	S	N	20
135	V	G	1
157	D	N	21
185	R	K	3

The most common W type amino acids detected in pvdhfr gene were S at codons 58, 93 & 117, D at codons 105 & 157 and V at codons 52 & 135. Common mutations detected in pvdhfr gene were S58R (17/36, 47% of cases), S117N (20/36, 56% of cases), D105N (18/36, 50% of cases) and D157N (21/36, 58 % of cases).

In dhfr gene,2 types of mutations were seen at position 106 (K106E in 1/36, 2.8 % of cases) (K106Q in 1/36, 2.8 % of cases) and 3 types of mutations at position 105 (D105N in 18/36, 50 of cases %), (D105Y in 1/36, 2.8 % of cases) and (D105H in 4/36, 11.1% of cases).

The mutation patterns and wild type haplotypes observed in pvdhps gene are mentioned in Table III.

TABLE III:
MUTATION ANALYSIS OF PVDHPS GENE

CODON	WILDTYPE	MUTANT TYPE	
		Mutant Type	No. of Mutants
389	N	R	3
		S	1
391	S	T	1
397	L	V	1
398	V	D	18
399	M	R	1
400	P	S	1
401	V	A	1
426	A	S	1
459	D	A	4

528	E	K	1
549	G	R	2
577	G	A	1
582	R	S	1
586	H	R	1
588	M	N	1
589	G	E	1
590	K	E	15

Regarding pvdhps gene, the most common wild type amino acids were Gat codons 549, 577 & 589, V at codons 398 & 401 and M at codons 399 & 588. In pvdhps gene, common mutations were V398D (18/36, 50% of cases) and K590E (15/36, 42% of cases). Codon 389 had 2 types of mutations (N389R in 3/36, 8.3% of cases) (N389S in 1/36, 2.8% of cases).

DISCUSSION

The recent trend of resistance of P. vivax isolates to various antimalarials including antifolate drugs like Sulphadoxine-Pyrimethamine (SP) is alarming and need to be monitored carefully to avoid losing this important drug option. Molecular drug resistance markers for SP resistance include genes encoding DHFR and DHPS enzymes which can be valuable tools to serve this purpose as suggested by many studies[10,12,18,19].

In this study, we examined the distribution and pattern of mutant alleles and polymorphisms in pvdhfr and pvdhps genes of P. vivax by sequence analysis. The established data on P. vivax resistance profile is scarce in Pakistan especially in Karachi. Our study revealed both distinct polymorphisms of wild type allele and also high prevalence of mutant alleles with double triple as well as novel mutations in both pvdhfr and pfdhps genes. These mutations have shown to confer antifolate drug resistance[5,6].

We found the most common wild type amino acids in pvdhfr gene are S at codons 58, 93 &117, D at codons 105 &157 and V at codons 52 & 135. Zakeri S 2009[20] in his study observed prevalence of both wild type alleles as well as mutants in pvdhfr genes. Auliff A et al[18]. examined genetic mutations in dhfr and dhps in P. vivax samples from six Asian-Pacific countries. They observed wild-type dhfr alleles to be present at a relatively high proportion in P. vivax parasites from China, East Timor, the Philippines, and Vietnam while less so from Papua New Guinea and Vanuatu.

We found high level of mutations in Pvdhfr gene. Among mutant strains we found amino acid S and D had multiple mutations. Common mutations found in Pvdhfr genes were S58R (17/36, 47% cases), S117N (20/36, 56% cases), D105N (18/36, 50% cases) and D157N (21/36, 58 % cases). Amino acid D showed 3 types of mutations at codon105 (D105N, D105Y & D105H).

Many other studies also reported mutations S58R and S117NinPvdhfr gene similar to our observations. Gresty et al[19] reported high prevalence of mutant alleles (58R/117N) in pvdhfr genes. Also studies by Raza A 2013[11], Kuesap J 2011[21] and Khatoon L 2009[10] reported similar mutations in pvdhfr gene. Recent studies of Nyunt et al.reported variant of wild type and mutant allele withdouble mutations (S58R and S117T/N) or quadruple mutations (F57L/I, S58R, T61M, and S117T) of pvdhfr gene in Thailand, Myanmar, Korea, Cambodia, and India[22]. Studies by Huang B et al[16] found mutations at codons F57I/L, S58R, T61M, H99R/S, and S117N/T.

In contrast to our findings, Khattak AA 2013[12] observed low prevalence of SP resistance associated mutations in P.vivax in Pakistan. However, there were pvdhfr S117N single and S58R plusS117N double mutants with highest prevalence in Sindh and Khyber Pakhtunkhwa provinces.

Also studies of Sharifi-Sarasiabi K 2016[23] from Southern Iran and Lekweiry KM et al[24] from Mauritania reported low prevalence of pvdhfr gene mutation reflecting reduced antifolate drug resistance and these results are contrary to our study.

Regarding pvdhps gene, our study showed common wild type amino acids were Gat codons 549, 577 & 589, V at codons 398 & 401 and M at codons 399 & 588. In our study, we observed that most common mutations in pvdhps gene were in amino acids V and K. Common mutations were V398D (18/36, 50% of cases) and K590E (15/36, 42% of cases). A similar pattern of significantly higher mutant rate of pvdhps, was observed by Nyunt MH et al[22] with triple or quadruple mutant alleles which accounted for more than 50% of all samples.

Kuesap J 2011[21] described mutations at codons 383 and 553 of Pvdhps gene. Their results suggest that all P. vivax isolates in Thailand carry some combination of mutations in Pvdhfr and Pvdhps genes. Similar results were seen by Pornthanakasem W et al[8] who confirmed the role of key mutations in dhps gene in sulfa drug resistance in P. vivax. Enzyme inhibition studies with sulfa drugs revealed that differences in sulfa drug sensitivity between the wild-type and mutant enzymes are responsible for the sulfa drug resistance observed for P. vivax.

In contrast to our study, Gretsy KJ et al[19] and Zakeri S 2009[20] observed that all of P.vivax isolates possessed a wild type dhps gene and no mutation was seen. Similarly, Khattak AA 2013[12] and Lekweiry KM et al[24] reported that majority of samples contained the wild-type haplotype in pvdhps gene.

CONCLUSION

The obtained results after multiple sequence alignment of pvdhfr gene and changing of amino acid

sequence on various positions revealed a large variety of mutations in pvdhfr and pvdhps genes. This signifies that urgent measures needed for control of drug resistance and development of effective, affordable new anti malarial drugs that should be used judiciously. The future of effectual malaria control will depend heavily upon appropriate data collection and more precise evidence-based decision on changing pattern of mutant strain of P. vivax as there is no anti malarial vaccine available so far.

Ethical permission: Jinnah Medical & Dental College Karachi letter No. 00023, Dated 17-9-2017.

Conflict of Interest: There is no conflict of interest.

Funding: There was no any funding agency.

REFERENCES

1. Zubairi ABS, Nizami S, Raza A, Mehraj V, Rasheed AF, Ghanchi NK et al. Severe plasmodium vivax malaria in Pakistan. Emerg Infect Dis. 2013 Nov; 19(11): 1851–1854. doi: 10.3201/eid1911.130495
2. Organization WH. Malaria. World Health Organization. Available from: https://www.who.int/news-room/fact-sheets/detail/malaria.
3. Howes RE, Battle KE, Mendis KN, Smith DL, Cibulskis RE, Baird JK, et al. Global Epidemiology of Plasmodium vivax. Am J Trop Med Hyg. 2016; (6):15-34. doi:10.4269/ajtmh.16-0141
4. Baird JK. Evidence and implications of mortality associated with acute Plasmodium vivax malaria. Clin Microbiol Rev. 2013; 26(1): 36-57.
5. Imwong M, Pukrittayakamee S, Rénia L, Letourneur F, Charlieu JP, Leartsakulpanich U, et al. Novel point mutations in the dihydrofolate reductase gene of Plasmodium vivax: evidence for sequential selection by drug pressure. Antimicrob Agents Chemother. 2003; 47(5): 1514 –21. doi:10.1128/aac.47.5.1514-1521.2003.
6. Hastings MD, Porter KM, Maguire JD, Susanti I, Kania W, Bangs MJ, et al. Dihydrofolate reductase mutations in Plasmodium vivax from Indonesia and therapeutic response to sulphadoxinee plus pyrimethamine. J Infect Dic. 2004; 189 (4): 744-50.
7. Capasso C, Supuran CT. Sulfa and trimethoprim-like drugs – antimetabolites acting as carbonic anhydrase, dihydropteroate synthase and dihydrofolate reductase inhibitors. J Enzyme Inhib Med Chem. 2014; 29(3): 379-87. doi:10.3109/14756366.2013.787422
8. Pornthanakasem W, Riangrungroj P, Chitnumsub P, Ittarat W, Kongkasuriyachai D, Uthaipibull C, et al. Role of Plasmodium vivax dihydropteroate synthase polymorphisms in sulfa drug resistance. Antimicrob Agents Chemother. 2016; 60(8): 4453-63. doi:10.1128/AAC.01835-15
9. Saralamba N, Nakeesathit S, Mayxay M, Newton PN, Osorio L, Kim JR, et al. Geographic distribution of amino acid mutations in DHFR and DHPS in Plasmodium vivax isolates from Lao PDR, India and Colombia. Malar J. 2016; 15: 484.
10. Khatoon L, Baliraine FN, Bonizzoni M, Malik SA, Yan G. Short Report: Prevalence of Antimalarial Drug Resistance Mutations in Plasmodium vivax and P. falciparum from a Malaria-Endemic Area of Pakistan. Am J Trop Med Hyg. 2009; 81(3):525-8.
11. Raza A, Ghanchi NK, Khan MS, Asim Beg MA. Prevalence of drug resistance associated mutations in Plasmodium vivax against sulphadoxine- pyrimethamine in Southern Pakistan. Malar J. 2013; 12: 261. doi: 10.1186/1475-2875-12-261
12. Khattak AA, Venkatesan M, Khatoon L, Ouattara A, Kenefic LJ, Nadeem MF. Prevalence and patterns of antifolate and chloroquine drug resistance markers in Plasmodium vivax across Pakistan. Malar J. 2013; 12: 310. doi: 10.1186/1475-2875-12-310
13. Khan SY, Khan A, Arshad M, Tahir HM, Mukhtar MK, Ahmad KR, et al. Irrational use of antimalarial drugs in rural areas of eastern Pakistan: a random field study. BMC Pub Health. 2012; 12: 941. doi: 10.1186/1471-2458-12-941
14. Organization WH. Malaria Microscopy Quality Assurance Manual – Ver 2. World Health Organization. 2016; Available from: https://www.who.int/malaria/publications/atoz/ 9789241549394/en/.
15. Iqbal A, Mushtaq R, Hussain F, Saleem M, Naz M, Maqsood B. Comparison of Conventional and PCR based detection of Plasmodium falciparum and Plasmodium vivax infections in human blood. J Adv Biol. 2013; 3(3): 248-51. doi:10.24297/jab.v3i3. 1930
16. Huang B, Huang S, Su X, Tong X, Yan J, Li H, et al. Molecular surveillance of Pvdhfr, Pvdhps, and Pvmdr-1 mutations in Plasmodium vivax isolates from Yunnan and Anhui provinces of China. Malar J. 2014; 13: 346. doi:10.1186/1475-2875-13-346
17. Ding S, Ye R, Zhang D, Sun X, Zhou H, McCutchan TF, et al. Anti-folate combination therapies and their effect on the development of drug resistance in Plasmodium vivax. Sci Rep. 2013; 3: 1008. doi: 10.1038/srep01008
18. Auliff A, Wilson DW, Russell B, Gao Q, Chen N, Anh L, et al. Amino acid mutations in Plasmodium vivax DHFR and DHPS from several geographical regions and susceptibility to antifolate drugs. Am J Trop Med Hyg. 2006; 75(4): 617-21.
19. Gresty KJ, Gray KA, Bobogare A, Wini L, Taleo G, Hii J, et al. Genetic mutations in Plasmodium falciparum and Plasmodium vivax dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) in Vanuatuand Solomon Islands prior to the introduction of artemisinin combination therapy. Malar J. 2014; 13: 402. doi:10.1186/

1475-2875-13-402
20. Zakeri S, Motmaen SR, Afsharpad M, Djadid ND. Molecular characterization of antifolates resistance associated genes, (dhfr and dhps) in Plasmodium vivax isolates from the Middle East. Malar J. 2009; 8: 20.
21. Kuesap J, Rungsrihirunrat K, Thongdee P, Ruangweerayut R, Na-Bangchang K. Change in mutation patterns of Plasmodium vivax dihydrofolate reductase (Pvdhfr) and dihydropteroate synthase (Pvdhps) in P. vivax isolates from malaria endemic areas of Thailand. Mem Inst Oswaldo Cruz. 2011; 106(Suppl 1): 130-3. doi: 10.1590/ s0074-02762011000900017
22. Nyunt MH, Han JH, Wang B, Myo AK, Hla AK, Lee S, et al. Clinical and molecular surveillance of drug resistant vivax malaria in Myanmar (2009-2016). Malar J. 2017; 16(1): 117. doi:10.1186/ s12936-017-1770-7.
23. Sharifi-Sarasiabi K, Haghighi A, Kazemi B, Taghipour N, Mojard EN, Gachkar L. Molecular surveillance of Plasmodium vivax and Plasmodium falciparum DHFR mutations in isolates from Southern Iran. Rev Inst Med Trop Sao Paulo. 2016; 58: 16. doi: 10.1590/S1678-9946201658016
24. Lekweiry KM, Boukhary AO, Gaillard T, Wurtz N, Bogreau H, Hafid JE, et al. Molecular surveillance of drug- resistant Plasmodium vivax using Pvdhfr, Pvdhps and pvmdr1 markers in Nouakchott, Mauritania. J Antimicro Chemother. 2012; 67(2): 367-74. doi: 10.1093/jac/dkr464

AUTHOR AFFILIATION:

Dr. Rizwana Yasmin *(Corresponding Author)*
Professor of Microbiology, Department of Pathology
Jinnah Medical & Dental College
Karachi, Sindh-Pakistan.
Email: dr.rizwana@live.com

Dr. Musarrat Jehan
Assistant Professor, Department of Pathology
Jinnah Medical & Dental College
Karachi, Sindh-Pakistan.

Ms. Mehwish Kalam
Lecturer, Department of Biotechnology
Jinnah University for Women
Karachi, Sindh-Pakistan.

Ms. Syeda Mariam Siddiqa
Associate Professor, Department of Biotechnology
University of Karachi, Karachi, Sindh-Pakistan.

Prof. Mahadev Harani
Department of Pathology
Jinnah Medical & Dental College
Karachi, Sindh-Pakistan.

Prof. Mustafa Kamal
Professor, Department of Biotechnology
University of Karachi, Karachi, Sindh-Pakistan.