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CIRCUMSPOROZOITE PROTEIN (CSP) GENETIC DIVERSITY IN INDIAN *PLASMODIUM VIVAX*

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Abstract: India is one of the *Plasmodium vivax* endemic countries and contributes the highest number of malaria cases worldwide. *P. vivax* CSP (*PvCSP*) is one of the most extensively studied antigen and is considered to be a leading malaria vaccine candidate gene. VK210 and VK247 are the two variants described in the central repeat region of *P. vivax* CSP gene. VK210 (98%) is found to be more prevalent as compared to VK247 (2%) in Indian context. It was noticed that different geographical regions show diversity in nucleotides as well as in haplotype with respect to *PvCSP* gene of Indian isolates. As natural selection always plays an important role in shaping genomic diversity in Indian *P. vivax*, the central repeat region of *PvCSP* gene of all the five-populations found to be under strong purifying selection. In this region, a number of synonymous mutations were found to be higher in number as compared to non-synonymous mutation, which clearly indicated that *PvCSP* gene is involved in invasion of hepatocytes in the host body and the parasite can't afford non-synonymous changes in this region. The present study helps us to understand the nature of *P. vivax* population in India, and also that might be helpful in planning the development of *PvCSP*-based vaccine in India.

Keywords: CSP gene, India, Natural selection, *Plasmodium vivax*, Sequences, Tajima D.

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INTRODUCTION

Protozoans are single celled organisms found worldwide in most of the habitats. Most species are free living, but some are parasitic and pathogenic (Verma and Prakash, 2020). These are microscopic and unicellular with a relatively complex internal structure and carry out complex metabolic activities (Verma, 2021). *Plasmodium* is a notorious malaria-causing protozoan that belongs to the group Sporozoa (Ashok, 2017).

According to WHO (2021) malaria Report, the cases reported from South East Asia, India contributed 83% cases out of the total cases and

among them also two-third cases are due to *P. vivax* only. It causes benign and non lethal malaria, however, in recent year's reports on the severe *P. vivax* malaria have surfaced, initially in India (Gupta *et al.,* 2016) and then in several other *P. vivax* endemic countries of the world. It is therefore highly probable that *P. vivax* has somehow changed its clinical manifestation and malaria outcome due to its infection might not be considered 'benign'.

In a malaria epidemiological setting like India where both *P. vivax* and *P. falciparum* are present and co-infect a single human host, this type of competition increases the parasite load, then in places where single parasite infection rate is higher than co-infection (Paul *et al.,* 2003). There is a clear-cut probability that in order to increase its population size in a single host setting, *P. vivax* becomes more aggressive in a competition to outperform *P. falciparum*. Such an increase in the parasite load in a single individual setting not only increases the transmission rate but also causes mortality to the affected individuals because of high density of parasites infecting a greater number of RBC's resulting in multiple organ failure (Mehndiratta *et al.,* 2013). Apart from causing severity, some of the unique characteristic features of *P. vivax*, include early appearance of gametocytes before clinical symptoms (Bousema *et al.,* 2011), ability to form latent hypnozoites responsible for relapse fever (Arévalo-Herrera *et al.,* 2010) and shorter development cycle in the vector as compared to other parasites, makes eradication of *P. vivax* more difficult (Mueller *et al.,* 2015).

In the present scenario, an effective vaccine against *P. vivax* is the only valuable tool, which can help to eliminate this parasite (Mueller *et al.,* 2015). The genetic diversity in *P. vivax* antigens is a major hurdle in developing an effective malaria vaccine. Some of the previous studies revealed that *P. falciparum* RTS, S vaccine, which is based on Circumsporozoite protein (CSP) provides partial protection against malaria (Mueller *et al.,* 2015). So, *PvCSP* is a potential vaccine candidate gene in *P. vivax* homologous to *P. falciparum* CSP gene.

Author studied the *PvCSP* molecular variants in Indian *P. vivax* samples and tried to i) compare the SNPs present in the different populations of Indian *P. vivax* samples, ii) compare the nucleotide diversity among the different populations of Indian *P. vivax,* iii) detect the SNPs under any selection (positive or purifying) and, iv) explore the fixation index among the populations to understand the pattern of haplotype sharing and other aspects. This research will also help us to understand the malaria epidemiology in India and simultaneously to understand the antigenic diversity among the different Indian *P. vivax* populations.

MATERIALS AND METHODS

To study the genetic diversity and variants of *PvCSP* gene in Indian *P. vivax,* the DNA sequences of the same were already available in Genbank from India (accession no. FJ491064.1 - FJ491141.1 and MG981128 - MG981156) were used. To understand the genetic diversity of the Indian *PvCSP* gene several bioinformatic analyses were performed on my personal laptop from the month of April to July 2022, and these statistical tests were performed on the SNP data of *PvCSP* gene. SNPs were detected using the DnaSPv5 software (Librado and Rozas, 2009). Haplotype diversity for Indian *PvCSP* gene sequences was calculated with the help of DnaSP5 software (Librado and Rozas, 2009). In order to determine nucleotide diversity in the CSP gene of Indian *P. vivax* population, several statistical tests were performed including a number of haplotypes (Nei, 1987).

Furthermore, the DNA sequence polymorphism of *PvCSP* gene was estimated using the two parameters of nucleotide diversity (π and θ_w). The estimation of π (Nei, 1987) is based on the average number of pairwise differences per site, whereas, θw (Watterson, 1975) is based on the number of segregating sites in the sample. The nucleotide diversity was determined using various tools available in two different softwares, such as, DnaSP (Librado and Rozas, 2009) and MEGA (Tamura *et al.,* 2013).

Tajima's D (Tajima *et al.,* 1989) was used to distinguish a DNA sequence evolving randomly (neutrally) and the one evolving under a non-random process, like directional or balancing

selection or it may be under demographic expansion or contraction, genetic hitchhiking, or introgression. DnaSP computes the D, D*, F and F* test statistics proposed by Fu and Li (1993) that test the various predictions made by the neutral theory of molecular evolution (Kimura, 1983). In this study, CSP gene of *P. cynomolgi* was used as an outgroup that was downloaded from NCBI site with an accession no. AB524342.1. DnaSP software uses the critical values obtained by Fu and Li (1993) to determine the statistical significance of D, F, D* and F* test. McDonald and Kreitman (1991) test is used to determine the positive or purifying selection, which detects and measures the amount of adaptive evolution within a species, by determining whether the adaptive evolution has occurred, and the proportion of substitutions that resulted from positive or purifying selection (also known as directional selection).

For data sets containing more than two sequences, the average number of synonymous substitutions and the average number of non-synonymous substitutions were computed using Z-test, which is similar to the test mentioned above (MK test). The variance of these two quantities is estimated by the bootstrap method (Nei and Kumar, 2000). Datamonkey is a web version of HyPhy (HYpothesis testing using PHYlogenies), an open-source software package for the analysis of genetic sequences using various techniques in phylogenetics, molecular evolution, and machine learning. Four different codon-based maximum likelihood methods, SLAC, FEL, REL, and FUBAR, estimate the dN/dS ratio (also known as Ka/Ks or ω) at every codon in the alignment. The codon-based maximum likelihood method, IFEL, can investigate whether the sequences sampled from a population (*e.g.,* viral sequences from different hosts) and subject to selective pressure at the population level (*i.e.,* along internal branches).

To study the recombination event in Indian *PvCSP* gene, recombination tests were computed using the DnaSP module. DnaSP module has also included the algorithm (the four-gametic test) described in Hudson and Kaplan (1995) to estimate the RM, the minimum number of recombination events in the history of the sample. For further inference, Datamonkey.org (web version of hyphy), GARD (Genetic Algorithm for Recombination Detection) were performed to find all the recombination breakpoints. Fixation index (FST) is a measure to differentiate the population based on the genetic structure. This parameter was calculated using the Arlequin V3.5.1.3 computer program (Excoffier and Lischer, 2010).

To understand the genetic relationship among different populations, the population pair-wise values were used to construct the neighbour-joining population phylogenetic tree (Das *et al.,* 2004; Chittoria *et al.,* 2012) using the Mega v7.08 computer program (Tamura *et al.,* 2013). Haplotype networking was used to reconstruct the phylogenetic networks and trees. Two independent methods were used for deriving the optimal networks, *namely*: the Reduced Median Networks (Bandelt *et al.*, 1995) for binary data, and the Median Joining Networks (Bandelt *et al.*, 1999) for all types of data.

RESULTS AND DISCUSSION

A sum of 106 sequences downloaded from NCBI site, were first aligned together and trimmed to the same size. During the alignment, there was a number of deletions were reported from all the five population in India. A large number of nucleotide deletions were observed during the alignment, including single nucleotide deletion to maximum 27 nucleotide. Due to a large number of nucleotide deletion around 8 sequences from already published Indian *PvCSP* gene were not included in the analysis; total 98 sequences were used for analysis excluding Gaps and Insertions.

Further author also tried to study the SNPs, nucleotide diversity and haplotype diversity in the C-terminal part of the gene, which was always considered as highly conserved as compared to central repeat where large number of changes do occur. The occurrence of a higher number of nucleotide substitutions in the C-terminal region of *PvCSP* gene in Indian *P. vivax,* offered an opportunity to estimate the genetic diversity. It is noted that all the five populations contained a higher number of haplotypes; ASM and MDP

populations had 6 haplotypes each, higher in number followed by DEL and TMN with 5 and 4 haplotypes respectively (Table 1). DEL had lower haplotype diversity (0.550) as compared to other four populations. ASM had high haplotype diversity as compared to other four populations followed by TMN (0.618), whereas; GUJ and TMN had almost similar and lower haplotype diversity (Table 1).

Table 1: Details of mutation and summary statistics of *PvCSP* gene in Indian *P. vivax.*

Population	Length (bp)	No. of isolates	Mutations			Haplo-type	Haplotype Diversity	Nucleotide Diversity		Neutrality Test		
			S	NS	Total			π	θ^w	Tajima D	Li and Fu D*	Li and Fu F*
Assam (ASM)	251 bp	21	3	7	10	6	0.724	0.00785	0.01107	-1.00351	-0.88736	-1.15935
DELHI (DEL)	251 bp	25	2	2	4	5	0.550	0.00276	0.00422	-0.93359	-1.00506	-1.16547
Madhya Pradesh (MDP)	251 bp	21	1	5	6	6	0.557	0.00391	0.00664	-1.29346	-0.87697	-1.03359
Gujarat (GUJ)	251 bp	14	1	1	2	3	0.582	0.00267	0.00251	0.17874	-0.44573	-0.32441
Tamil Nadu (TMN)	251 bp	17	2	1	3	4	0.618	0.00328	0.00354	-0.20380	-0.06265	-0.11496

Author used two different parameters to estimate the nucleotide diversity and recorded highest nucleotide diversity (θw and π) in *P. vivax* isolates from ASM (θw = 0.00785 and π = 0.01107) and lowest among the GUJ population (θw = 0.00267 and π = 0.00251). Among other three populations, MDP has slightly higher nucleotide diversity as compared to TMN and DEL, which had almost similar values (Table 1). In all the five-populations, number of segregation sites (θw) were found to be higher as compared to pairwise differences (π) except GUJ population. Higher estimated values of nucleotide diversity in all the five-population samples of *P. vivax* reflect the fact that the *PvCSP* gene is highly variable in Indian *P. vivax.*

The evolutionary pattern of the *PvCSP* gene follows the neutral equilibrium model of molecular evolution, the Tajima' D, Fu Li D* and F* statistic was also calculated, which displayed high values in all the five different Indian population samples but in opposite directions except Tajima D positive value in GUJ population. In all the *P. vivax* populations studied, the departure from neutrality was observed (except GUJ Tajima D value) towards the negative side of value (Table 1). The negative value of the entire neutrality estimator in all the five Indian populations was the outcome of the presence of greater number of segregation sites than the number of pairwise difference. The value for the entire neutrality test in all the five-populations found to be insignificant.

With a view to gain further in-depth insights into the possible role of natural selection on molecular evolution at the *PvCSP* gene in Indian *P. vivax*, we followed several other statistical approaches. For C-terminal region of *PvCSP* gene, author performed McDonald-Krietmann Test, taking *P. cynomolgi* as an out-group for all the five populations. As a result, author got statistically insignificant values for all the five populations.

Furthermore, when Z-test for selection was performed, author found that this region is not under any selection. Similarly, the Z-test for selection was performed for central repeat region in all the five *P. vivax* Indian populations. Fascinatingly, statistically highly significant value ($p<0.001$) could be obtained in *P. vivax* isolates sampled from ASM (3.15), whereas other three populations also shown statistically significant value ($p<0.05$) for MDP, GUJ (1.831) and TMN (2.109) respectively and DEL (2.651) value was about to be statistically significant, signifying evidences of purifying selection in the

PvCSP gene in all the five populations. Furthermore, with *P. cynomolgi* as an out-group, McDonald-Krietmann Test was performed for all the five populations but due to higher number of deletions and additions, author was unable to perform this test properly. But on the basis of Z-test, it appeared that the *PvCSP* gene was evolved under a model of purifying natural selection in all the five presently studied population samples of Indian *P. vivax*.

To analyze the results on natural selection in central repeat region of *PvCSP* gene in Indian *P. vivax* we several other analytical tests were also performed. Interestingly, test for selection in Indian *PvCSP* gene (using the Datamonkey computer program) revealed the action of purifying selection on number of sites under different models. The overall value of dn/ds (SLAC (dn/ds= 0.31), REL (dn/ds= 0.7), FEL model analysis found to be smaller than one and ω value (FUBER model analysis) found to be -7.28, again greater lower than one, which clearly demonstrated that the gene found to be under purifying selection. Most of the sites were found to be under purifying selection and FUBER analysis also had shown 4 sites were under strongly pervasively diversifying selection.

The minimum number of recombination event (Rm) was calculated in all the 5 populations individually. The estimation of recombination parameter Rm reveals 12 and 1 recombination sites in population from ASM and TMN whereas, DEL and MDP populations had equal number of potential recombination sites (Rm=5), followed by GUJ population estimating recombination parameter with rm=2 value (Table 2). The recombination event was found very high in central repeat region, all the five populations studied, found to under the process of recombination and all the potential recombination sites has given in table 2. The event of recombination was also supported by GARD model of Hyphy package in all the five populations of Indian *P. vivax* samples.

Table 2: Details of recombination in 5 different Indian *P. vivax* populations with recombination sites.

Population	Rm	Rm sites
ASM	12	(62,69) (69,105) (116,143) (143,170) (170,197) (213,240) (258,278) (278,305) (321,332) (332,348) (386,467) (467,484)
DEL	5	(5,8) (62,89) (89,105) (105,170) (231,359)
MDP	5	(89,105) (105,116) (116,143) (143,186) (186,554)
GUJ	2	(89,105) (213,240)
TMN	1	(159,294)

In order to determine the genetic inter-relationship among the Indian *P. vivax* populations, the population pair-wise Fst was calculated. Most of the values found to be insignificant, but MDP has shown statistically significant value with all the four-populations. Similarly, ASM Fst values also showed statistically significant with DEL populations. Further the GUJ showed lowest Fst value with ASM and DEL (Table 3).

The genetic distance matrix (Fst values obtained; Table 3), was used to construct the neighbour joining (NJ) population phylogenetic tree. GUJ and TMN were placed together while DEL, MDP and ASM populations were placed distantly (Fig. 1). Surprisingly, the Nm values were found to vary among all population pairs, with a minimum value of 1.23751 (between MDP and TMN population samples) and a maximum value of infinity (between GUJ and TMN and between GUJ and DEL) (Table 3). Interestingly, the GUJ showed highest Nm value with two different population pairs like DEL vs. GUJ were very distantly located, with a distance of approximately 930.1 km. Similarly distance between TMN and GUJ is approximately 11,185 km. Another interesting feature was that the

lowest value of Nm was seen between MDP and TMN population samples, which are also very much distantly located around 1, 299.6 km. In neighbor joining tree, author found that all the populations are joined together as all these share the same haplotype somewhere (H_16) (Fig. 2).

Table 3: Pair wise FST estimates for 5 population of Indian *P. vivax* using *PvCSP* gene.

Population	TMN	DEL	GUJ	ASM	MDP
TMN		8.94522	inf	7.43054	1.23751
DEL	0.05294		inf	5.28392	4.75657
GUJ	0	0		11.23142	1.60099
ASM	0.06305	0.08645*	0.04262		1.80702
MDP	0.28777*	0.09521*	0.23798*	0.21673*	

Blue colour: Fst value; Pink Colour: Nm value

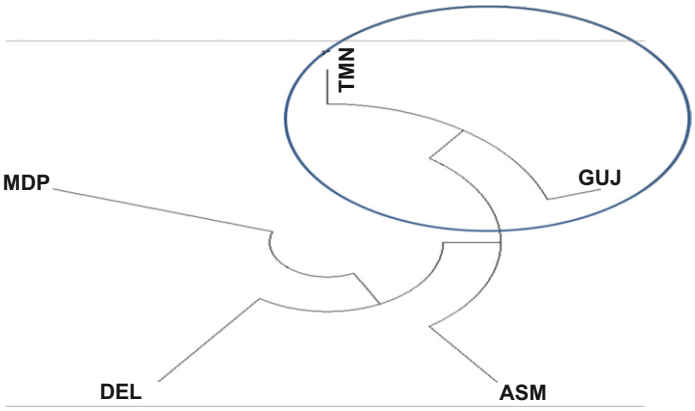

Fig. 1: Neighbour-joining population phylogenetic tree describing genetic inter-relationship among population samples of Indian *Plasmodium vivax* using *PvCSP* gene.

On the basis of haplotypes, author tried to analyze the genetic interrelationship among the Indian *PvCSP* populations. A total of 65 haplotypes were identified out of 98 sequences (27 sequenced +71 from NCBI database) and haplotype diversity (0.920) of *PvCSP* gene across the Indian population found to be very high. Out of 65 haplotypes, interestingly only 5 haplotypes found to be shared between different locations, rest 60 haplotypes were singleton in nature (Table 4; Fig. 2). Interestingly, one high frequency haplotypes seem to be unique to ASM (H_59) and DEL (H_54) respectively. H_16 haplotype was found to be with high frequency (37%) and shared between all the five populations, similarly H_3 found to be second high frequency haplotype (12%) shared between four populations (GUJ, DEL, TMN and ASM). H_39 found to be shared with only two populations of north India (ASM and DEL) (Table 4; Fig. 2).

Table 4: Details of haplotypes with their frequency and sharing population.

Haplotype	No. of Haplotype	Haplotype Frequency (%)	Shared populations
H_3	8	12	TMN, ASM, GUJ, DEL
H_16	24	37	MDP, ASM, GUJ, DEL & TMN
H_39	2	3	ASM & DEL
H_54	2	3	DEL
H_59	2	3	ASM

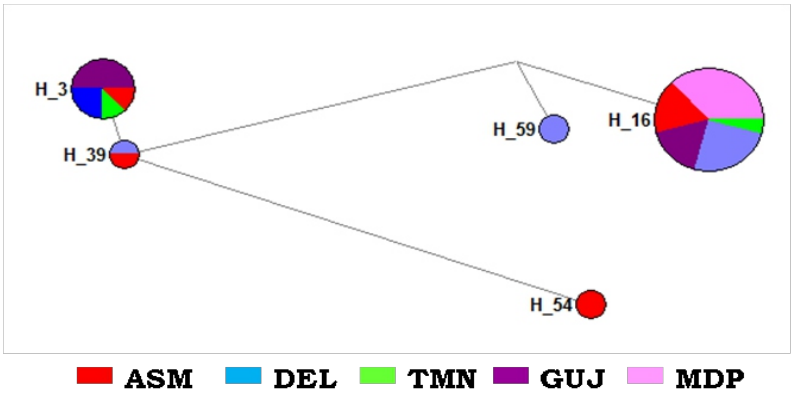

Fig. 2: Haplotype network showing genetic inter-relationship among Indian *P. vivax* population. The size of the pie refers to haplotype frequency; colours represent the different locations.

The sporozoite stage of malaria parasite (*Plasmodium* spp.) secretes a protein commonly known as Circumsporozoite protein (CSP) that is an antigenic target of RTS, S, a pre-erythrocytic malaria vaccine undergoing clinical trials in the African countries (Porter *et al.,* 2013). The structure of CSP consists of an immunodominant central repeat region flanked by conserved motifs at the N and C termini, and this protein helps the parasite to travel from the mosquito to the mammalian vector (Aldrich *et al.,* 2012). CSP protein is involved in motility and invasion of hepatocytes in the human host, and hence considered as a primary vaccine candidate gene among the other antigenic genes (Hernández-Martínez *et al.,* 2011).

Nucleotide sequences from the central repeat domain and C terminal domain of *PvCSP* were determined. An 891 bp sequence was obtained from 27 isolates. In this study VK210 variant is highly prevalent (98%) as compared to VK247 (2%), even the previous studies also states that VK247 sequence have been established less prevalent as compared to VK210, which show high prevalence in several countries: Thailand (Wirtz *et al.,* 1990), Peru (Franke *et al.,* 1992), Brazil (Cochrane *et al.,* 1990) and many more. Interestingly, the pattern of genetic diversity was studied in C-terminal of the Indian *PvCSP* gene does not seem to be uniform across the five different sample populations studied.

In the present study, author found that the *PvCSP* gene has high genetic diversity, which is similar to the reports of high genetic diversity in another *P. vivax* vaccine candidate gene *P. vivax* apical membrane antigen-1 (*PvAMA-1*) analysis from India (Rajesh *et al.,* 2007). Around 66% (65/98) haplotypes were found in all the Indian *P. vivax* population. H_16 haplotype was the common haplotype shared by all the five populations with maximum sharing by MDP, followed by DEL, GUJ and ASM equally and TMN had only one sharing, which inferred that all the five populations share haplotype with each other.

Being an antigenic gene, *PvCSP* might be under high selective pressure, and thus evolved to the extent to provide efficient liver-stage invasion of P. *vivax*. In *P. cynomolgi,* CSP central repeat region, the VK210 and VK247 are two distinct repeats that showed the evidence of selection (Hughes, 1991). The VK210 subtype was found to be highly dominant in present study almost similar to the worldwide scenario (Bonilla *et al.,* 2006; Zakeri *et al.,* 2010). Previous studies from the Asian and American subcontinent have shown less prevalence of VK247 subtype (Qari *et al.,* 1993; Bonilla *et al.,* 2006), which was also observed from present study with only two sequences of VK247 subtype from DEL and ASO. Author also found some deletions in the central repeat region of *PvCSP* gene, which could be due to various genetic mechanisms like recombination, slipped strand mis-pairing (Levinson and Gutman, 1987) and gene conversion. In present study, author found there is no selection pressure on the conserved region of *PvCSP* gene almost similar to Cambodian

population (Parobek *et al.,* 2014) and in central repeat region there were a greater number of synonymous changes as compared to non-synonymous population.

In Z-test, all the five-populations showed statistically significant values to be under purifying selection, and same finding was found for all 98 sequences ds>dn (2.701, $p<0.05$), which again indicated that the *PvCSP* gene central repeat region is under purifying selection. This analysis is in accordance with the result of Datamonkey that revealed the role of purifying selection in this region. TheVK247 central repeat were also found to be under purifying selection from all over the world (Dias *et al.,* 2013; Parobek *et al.,* 2014; Talha *et al.,* 2014). Similarly, the signature of purifying selection was reported consistently from *P. falciparum* CSP gene (Escalante *et al.,* 1998; Hartl, 2004), that suggest the limited number of amino acid changes allowed in this region as this gene plays very important role in gliding and motility inside the host body. Mosquito vector is one of the environmental factors which can explain both the purifying and directional selection of parasite *PvCSP* central repeat region. Further, it was ascertained that the population of Indian *P. vivax* is less genetically structured than the Indian *P. falciparum* population (Tyagi *et al.,* 2015).The circumsporozoite protein also expressed in the mosquito during various stages of their life cycle (Golenda *et al.,* 1990) and there is no overlapping in the distribution of *Anopheles* species between Asian and other continents (Yoo *et al.,* 2013).

CONCLUSION

The present study helps us to understand the nature of *P. vivax* population in India, that might be helpful in planning the development of *PvCSP*-based vaccine in India. Since malaria parasite harbours enormous diversity in the field isolates and modulate them to successfully invade the host body, so to understand the antigenic diversity in field samples, these types of study basically provide a baseline information by which it can easily be ascertained a particular vaccine in the whole population.

REFERENCES

1. **Aldrich C., Magini A., Emiliani C., Dottorini T., Bistoni F., Crisanti A. and Spaccapelo R.** (2012). Roles of the amino terminal region and repeat region of the *Plasmodium berghei* circumsporozoite protein in parasite infectivity. PLoS One. 7:e32524. http://doi.org/10.1371/journal.pone.0032524
2. **Arévalo-Herrera M., Chitnis C. and Herrera S.** (2010). Current status of *Plasmodium vivax* vaccine. *Human Vaccines*. 6:124-132.
3. **Ashok K.V.** (2017). A Handbook of Zoology. Shri Balaji Publications, Muzaffarnagar. 5th edn. 648p.
4. **Bandelt H.J., Forster P. and Röhl A.** (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*. 16:37-48.
5. **Bandelt H.J., Forster P., Sykes B.C. and Richards M.B**. (1995). Mitochondrial portraits of human populations using median networks. *Genetics*. 141:743-753.
6. **Bonilla J.A., Validum L., Cumming R. and Palmer C.J.** (2006). Genetic diversity of *Plasmodium vivax* Pvcsp and Pvmsp1 in Guyana, South America. *The American Journal of Tropical Medicine and Hygiene*. 75:830-835.
7. **Bousema T. and Drakeley C.** (2011). Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *Clinical Microbiology Reviews*. 24:377-410.
8. **Chittoria A., Mohanty S., Jaiswal Y.K. and Das A**. (2012). Natural selection mediated association of the Duffy (FY) gene polymorphisms with *Plasmodium vivax* malaria in India. *PloS One*. 7(9): e45219. 10.1371/journal.pone.0045219.
9. **Cochrane A.H., Nardin E.H., de Arruda M., Maracic M., Clavijo P., Collins W.E. and Nussenzweig R.S.** (1990). Widespread reactivity of human sera with a variant repeat of the circumsporozoite protein of *Plasmodium vivax*. *The American Journal of Tropical Medicine and Hygiene*. 43:446-451.
10. **Das A., Mohanty S. and Stephan W.** (2004). Inferring the population structure and demography of *Drosophila ananassae* from multilocus data. *Genetics*. 168:1975-1985.

11. **Dias S., Wickramarachchi T., Sahabandu I., Escalante A.A. and Udagama P.V.** (2013). Population genetic structure of the *Plasmodium vivax* circumsporozoite protein (Pvcsp) in Sri Lanka. *Gene*. 518:381-387.
12. **Escalante A.A., Lal A.A. and Ayala F.J.** (1998). Genetic polymorphism and natural selection in the malaria parasite *Plasmodium falciparum*. *Genetics*. 149:189-202.
13. **Excoffier L. and Lischer H.E.** (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*. 10:564-567
14. **Franke E.D., Lucas C.M., San Roman E. and Wirtz R.A.** (1992). Prevalence of antibody to the variant repeat of the circumsporozoite protein of *Plasmodium vivax* in Peru. *The American Journal of Tropical Medicine and Hygiene*. 46:708-710.
15. **Fu Y.X. and Li W.H.** (1993). Statistical tests of neutrality of mutations. *Genetics*. 133:693-709.
16. **Golenda C.F., Starkweather W.H. and Wirtz R.A.** (1990). The distribution of circumsporozoite protein (CS) in *Anopheles stephensi* mosquitoes infected with *Plasmodium falciparum* malaria. *Journal of Histochemistry and Cytochemistry*.38:475-481.
17. **Gupta P., Sharma R., Chandra J. and Singh V.** (2016). Severe Malaria due to *Plasmodium vivax*: Case Report. *Current Pediatric Reserach*. 20:24-28.
18. **Hartl D.L**. (2004). The origin of malaria: mixed messages from genetic diversity. *Nat Rev Microbiol*. 2(1): 15-22.
19. **Hernández-Martínez M.Á., Escalante A.A., Arévalo-Herrera M. and Herrera S.** (2011). Antigenic diversity of the *Plasmodium vivax* circumsporozoite protein in parasite isolates of Western Colombia. *The American Journal of Tropical Medicine and Hygiene*. 84:51-57.
20. **Hudson R.R. and Kaplan N.L.** (1995). The coalescent process and background selection. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*. 349(1327):19-23.
21. **Hughes A.L.** (1991). Circumsporozoite protein genes of malaria parasites (*Plasmodium* spp.): evidence for positive selection on immunogenic regions. *Genetics*. 127: 345-353.
22. **Kimura M.** (1983). The neutral theory of molecular evolution. Cambridge University Press.
23. **Levinson G. and Gutman G.A.** (1987). Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Molecular *Biology and Evolution*. 4:203-221.
24. **Librado P. and Rozas J.** (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*. 25:1451-1452.
25. **McDonald J.H. and Kreitman M.** (1991). Adaptive evolution at the Adh locus in *Drosophila*. *Nature*. 351:652-654.
26. **Mehndiratta S., Rajeshwari K. and Dubey A.P.** (2013). Multiple-organ dysfunction in a case of *Plasmodium vivax* malaria. *Journal of Vector Borne Diseases*. *50*(1): 71-73.
27. **Mueller I., Shakri A.R. and Chitnis C.E.** (2015). Development of vaccines for *Plasmodium vivax* malaria. *Vaccine*. 33:7489-7495.
28. **Nei M. and Kumar S.** (2000). Molecular evolution and phylogenetics. Oxford University Press.
29. **Nei M.** (1987). Molecular Evolutionary Genetics, Columbia University Press, New York, 512pp.
30. **Parobek C.M., Bailey J.A., Hathaway N.J., Socheat D., Rogers W.O. and Juliano J.J.** (2014). Differing patterns of selection and geospatial genetic diversity within two leading *Plasmodium vivax* candidate vaccine antigens. *Plos Neglected Tropical Diseases*. 8:e2796. https://doi.org/ 10.1371/ journal.pntd.0002796.
31. **Paul R.E.L., Ariey F. and Robert V.** (2003). The evolutionary ecology of Plasmodium. *Ecology Letters*. 6(9): 866-880.

32. **Porter M.D., Nicki J., Pool C.D., DeBot M., Illam R.M., Brando C., Bozick B., De La Vega P., Angra D., Spaccapelo R. and Crisanti A.** (2013). Transgenic parasites stably expressing full-length *Plasmodium falciparum* circumsporozoite protein as a model for vaccine down-selection in mice using sterile protection as an endpoint. *Clinical and Vaccine Immunology.*20:803-810.

33. **Qari S.H., Shi Y.P., Goldman I.F., Udhaykumar V., Collins W.E., Lal A.A. and Alpers M.P.** (1993). Identification of *Plasmodium vivax*-like human malaria parasite. *The Lancet.* 341:780-783.

34. **Rajesh V., Elamaran M., Vidya S., Gowrishankar M., Kochar D. and Das A.** (2007). *Plasmodium vivax*: genetic diversity of the apical membrane antigen-1 (AMA-1) in isolates from India. *Experimental Parasitology.* 116:252-256.

35. **Tajima F.** (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics.* 123:585-595.

36. **Talha A.A., Pirahmadi S., Elgaily E.M., Zakeri S. and Nour B.Y.** (2014). Low Prevalence of *Plasmodium vivax-Plasmodium falciparum* Mixed-Infection in Patients from Central and Eastern Part of Sudan: Implication for Case Management in Sudan. *International Journal of Tropical Disease and Health.* 4:887-895. 10.9734/IJTDH/2014/10916

37. **Tamura K., Stecher G., Peterson D., Filipski A. and Kumar S.** (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution.* 30:2725-2729.

38. **Tyagi S. and Das A.** (2015). Mitochondrial population genomic analyses reveal population structure and demography of Indian *Plasmodium falciparum*. *Mitochondrion.* 24:9-21.

39. **Verma A.K.** (2021). Protozoans: Animals or Protists? *International Journal of Life Sciences.* 9(1): 41-44.

40. **Verma A.K. and Prakash S.** (2020). Status of Animal Phyla in different Kingdom Systems of Biological Classification. *International Journal of Biological Innovations.* 2 (2): 149-154. https://doi.org/10.46505/IJBI.2020.2211.

41. **Watterson G.A.** (1975). On the number of segregating sites in genetical models without recombination. *Theoretical Population Biology.* 7:256-276.

42. **WHO** (2021). World malaria report 2021. World Health Organization.https://apps.who.int/iris/handle/10665/350147.

43. **Wirtz R.A., Rosenberg R., Sattabongkot J. and Webster H.K.** (1990). Prevalence of antibody to heterologous circumsporozoite protein of *Plasmodium vivax* in Thailand. *The Lancet.* 336: 593-595.

44. **Yoo D.H., Shin E.H., Park M.Y., Kim H.C., Lee D.K., Lee H.H., Kim H.K. and Chang K.S.** (2013). Mosquito species composition and *Plasmodium vivax* infection rates for Korean army bases near the demilitarized zone in the Republic of Korea, 2011. *The American Journal of Tropical Medicine and Hygiene*. 88(1):24-28. 10.4269/ajtmh.2012.11-0755

45. **Zakeri S., Kakar Q., Ghasemi F., Raeisi A., Butt W., Safi N., Afsharpad M., Memon M.S., Gholizadeh S., Salehi M. and Atta H.** (2010). Detection of mixed *Plasmodium falciparum* and *P. vivax* infections by nested-PCR in Pakistan, Iran and Afghanistan. *Indian Journal of Medical Research.* 13:31-35.