Malaria is one of the major health problems in tropical and subtropical countries where transmission occurs regularly. According to the latest report of WHO, 198 million cases of malaria occurred globally and this disease led to 584,000 deaths. Development and spread of drug resistance in parasites especially in *Plasmodium falciparum* has become an issue of utmost concern throughout the world. Drug resistance is one of the most important factors in malaria control which implicated in enhanced mortality and morbidity. Malaria is caused by protozoan parasite *Plasmodium* that affects humans and five species of *Plasmodium* are responsible for the malaria throughout the world. Malaria is carried by *Anopheles* mosquitoes which are transmitted from one person to another by biting of female mosquitoes. Of the over 400 *Anopheles* species, only 58 anophelines species have been reported from India, out of which 10 have been recognized as a malaria vector.

Control of vector population and malaria transmission, using the vector control strategies and killing of malaria parasite by treatment with anti-malarials are the main strategies to control malaria as no effective vaccine is made available till date. Vector control strategies include indoor residual spraying (IRS) with insecticides, use of insecticide treated mosquito nets (ITN or LLIN) and antilarval measures. Anti-malarials are important in order to kill the parasite and therefore, keystone of malaria control efforts. At present, insecticide resistance and drug resistance is the biggest obstacle in malaria control programmes. Anti-malarial drug resistance has evolved as a greatest challenge and parasite has developed resistance against all commonly used drugs i.e. chloroquine, sulfadoxine-pyrimethamine, mefloquine, halofantrine, and also in artemisinin derivatives in the entire world. Thus, prevention of drug resistance and routine surveillance of anti-malarials has become important to combat this problem.

Malaria remains a major health problem in India with *P. falciparum* being the predominant species accounts 50% of total malaria cases. Previous studies revealed that, India has resistant parasite especially in *P. falciparum* against all currently used anti-malarial drugs like chloroquine (CQ) and sulfadoxine-pyrimethamine (SP). Since 2005, Indian anti-malarial drug policy used SP as a long acting partner drug in artemisinin-based combination therapy (ACT) in high endemic areas. Later in 2010, this treatment ACT+SP became recommended.
first line treatment all over India. At present ACT+SP is used throughout India which is a promising drug policy, but a recent study revealed that resistance to SP had been well documented from northeastern part of India, which replaced AS + lumefantrine as first-line malaria treatment in these parts of country since year 2013. This widespread resistance against SP raised a threat about long term effectiveness of ACT in India. Chloroquine is still recommended as anti-malarial treatment against *P. vivax* malaria in India. In vivax prevalent areas, sometimes due to improper-or-mis-diagnosis, *Plasmodium falciparum* infections are often exposed to this treatment in mixed infection cases which could provide selection pressure on gene responsible for chloroquine resistance in *P. falciparum* and may retain CQ resistant *Plasmodium falciparum* in population. Almost a decade before, CQ was discontinued in high transmission areas so that monitoring of CQ resistance is also important to check that there is any reversal or decrease in mutations trends against chloroquine resistance in these areas. Thus, routine molecular monitoring of partner drug SP and chloroquine is essential in malaria endemic regions for better management of anti-malarial policy and this will help in making an effective malaria treatment policy.

In this context, we attempted to determine the changes in the frequencies of *dhfr* and *dhps* mutations correlated with SP resistance and *crt* and *mdr-1* mutations associated with CQ resistance in *P. falciparum* isolates collected from four states of India i.e. Jharkhand, Odisha, Andhra Pradesh and Uttar Pradesh, to assess the level of SP and chloroquine resistance. Therefore, this prompted us to carry out this study with following objectives:

- To study the sulfadoxine-pyrimethamine drug resistance status in India against *P. falciparum*.
- To study the chloroquine drug resistance status in India against *P. falciparum*.

In this study, malaria patients from four different epidemiological regions of India were genotyped for *pf dhfr*, *pf dhps*, *pf crt* and *pf mdr-1* gene polymorphisms using DNA sequencing methods. A total 217 *Plasmodium falciparum* isolates were collected from two states of India with high malaria incidence i.e., Jharkhand and Odisha and two states with low malaria
incidence i.e., Andhra Pradesh and Uttar Pradesh between years 2006 to 2012. Part of sulfadoxine-pyrimethamine (SP) drugs resistance genes i.e., *pfdhfr* and *pfdhps* and parts of chloroquine drugs resistance genes i.e., *pf crt* and *pf mdr-1* were PCR-amplified, sequenced and analyzed.

The results obtained from this study are summarized as follows:

1. In case of SP drug, all 217 isolates were successfully sequenced for *pf dhfr* and *pf dhps* genes covering codon positions A16V, N51I, C59R, S108N and I164L of *pf dhfr* gene and codons S436A, A437G, K540E, A581G and A613S of *pf dhps* gene. No mutant alleles were detected at codon 16 in *pf dhfr* gene and at codon 613 in *pf dhps* gene but remaining all codon positions of both the genes showed pure mutant and mixed mutant alleles at different rate.

2. In *pf dhfr* gene, majority of isolates were observed with pure mutant alleles 59R (n= 117, 54%) and 108N (n= 139, 64%) and mixed mutant allele 59R* was observed in 23.04 % (n = 50) and 108 N* in 18.43 % (n = 40) isolates. However, pure mutant alleles 51I (n = 2, 0.92 %) and 164 L (n = 7, 3.22 %) were less prominent at codons 51 and 164 respectively. Mixed mutant alleles were observed in 0.46 % (n = 1) and 2.30 % (n=5) at codon 51 and 164 respectively.

3. In *pf dhfr* gene, pure wild genotype ANCSI was found in 17.51 % (n = 38) isolates. Prevalence of single mutant (ANCNI), double mutant (ANRNI) genotypes were observed in 2.76 (n = 6) and 46.08 % (n = 100) isolates while mixed single mutant genotype (ANCN*I) and mixed double mutant genotypes (ANR*N*I) were observed in 2.76 (n = 6) and 23.50 % (n = 51) isolates respectively. Pure triple mutant genotypes were two type ANRNL (3.22) and AIRNI (0.92 %) while mixed triple mutant genotypes ANRNL* (2.30) and AIR NI* (0.46 %). No quadruple mutant genotypes were found in *pf dhfr* gene.
4. While in *pfldhps* gene, wild-type alleles were predominant (79.26 %, n = 172) at all the codons. Pure mutant alleles at codons 436A, 437G, 540E and 581G were observed in 7.37% (n = 16), 16.58% (n = 36), 7.83% (n= 17) and 7.83 % (n = 17) isolates respectively. However, mixed mutant alleles were also prevailed in 1.84%, 2.30%, 2.30% and 1.38 % at codons 436A*, 437G*, 540E* and 581G* respectively.

5. In case of *pfldhps* gene, majority of isolates (79.26 %) were found pure wild genotype i.e., SAKAA, SGKAA (1.84 %), AAKAA (0.46 %) and SAEAA genotype (0.46 %) are different pure single mutant genotypes, while SAE*AA (0.92 %) is mixed single mutant genotype. Prevalence of pure double mutant genotype (SGKGA) and triple mutant genotype (AGEAA) were (7.83%) and (6.91%) respectively, while mixed double mutant (SG*KG*A) and triple mutant (A*GEAA) were (0.92%) and (1.38%) respectively. Only one isolate showed mixed quadruple mutant genotype i.e. A*GEGA.

6. This study found that combination of *pfldhfr* and *pfldhps* mutations are categorized in 13 (GEN 1-13) two locus genotypes which showed different level of clinical resistance and observed at varying rates in different study sites. Wild-type two locus genotypes were observed in 17.51 % (n = 38) isolates while mutant two locus genotypes were observed in 82.48 % (n = 179) isolates. Out of that, 62.6 % (n = 136) isolates showed double mutant (ANRNI) in *pfldhfr* gene and wild-type *pfldhps* gene in the two-locus combination all the study sites. However, single mutant ANCNI, triple mutants (ANRNL or AIRNI) in *pfldhfr* gene and single mutants (SGKAA, AAKAA and SAEAA), double mutant SGKGA, triple mutant AGEAA, quadruple mutant AGEA in *pfldhps* gene were also observed. In this study, isolates from high transmission areas also showed quintuple (Jharkhand=5.95%, Odisha=11.43%) and sextuple (Jharkhand=4.76%, Odisha=4.29%) mutant genotype which are associated with higher level of resistance to SP suggests selective drug pressure due to its use over a long period. In contrast, the low transmission areas (Uttar Pradesh and Andhra Pradesh) showed only double mutant genotype, which infers that *P. falciparum* population in these regions were susceptible to SP treatment and resistance development is in progress.
7. In case of chloroquine drug, only 206 isolates gave successful result for pfcr\textsubscript{T} gene covers various single nucleotide polymorphisms (SNPs) C72\textsubscript{S}, M74I, N75E/D, K76T, H97L (codon 44-177) and A220S (codon 181-222). While for pfmdr-1 gene, all 217 isolates were successfully sequenced for single nucleotide polymorphism (SNP) N86Y associated with chloroquine resistance.

8. In pfcr\textsubscript{T} gene, isolates were prevalent with pure mutant alleles 220S (n=152, 73.78\%), 76T (n=148, 71.84 \%), 75E (n=84, 40.77\%), 74I (n=82, 39.8\%) and 72S (n=51, 24.75\%) and mixed alleles was found in lower proportion which were 2.91\%, 1.45\%, 3.39\%, 3.39\%, and 8.25\% respectively for above corresponding codons. H97L mutation was found only in Odisha isolates with a prevalence of pure mutant allele 97L (n=18, 25.71\%) and mixed allele 97L* (n=2, 2.85\%). In pfmdr-1 gene, wild allele N86 was significantly prevalent in 56.22\% (n=122) isolates while mutant allele 86Y and mixed mutant allele 86Y* was observed in 33.64\% (n=73) and 10.13\% (n=22) isolates respectively. Mutant allele 86Y was most prevalent in high transmission areas i.e. Jharkhand and Odisha while less prevalent in Andhra Pradesh and only wild allele N86 was found in Uttar Pradesh.

9. Wild type CVMNKA (n=46, 22.33\%) haplotype occurred in all sites except Uttar Pradesh. Triple mutant SVMNTS haplotype (characteristics of Papua New Guinea (PNG) and South America) was observed in all states but was highly prevalent in Uttar Pradesh (n=30, 96.77\%) and Andhra Pradesh (n=13, 40.63\%) isolates, mainly in low transmission areas. Jharkhand and Odisha (high transmission areas) isolates were shown considerable variation in haplotypes i.e. in Jharkhand quadruple mutant haplotype CVIETS (characteristics of South East Asia) was highly prevalent i.e. 40(54.8\%) and various mutant types SVMNTS (n=8, 10.9\%), CVIETS* (n=6, 8.2\%), S*VI*E*T*S* (n=4, 5.47\%) and one each isolate (1.36\%) of following haplotypes CVMNKS, SVMNTA was also found. While in Odisha quadruple mutant haplotype CVIETS was found in (n=31, 44.28\%) isolates along with various haplotype i.e.
SVMNTS (n=5, 7.15%), CVIETS* (n=4, 5.71%), S*VI*E*T*S* (n=4, 5.71%), SVMNKS (n=2, 2.86%) and CVMNKS (n=3, 4.29%).

10. In case of CQ resistance, combination of pfcr* haplotypes and Pfmdr-1 mutations revealed 12 (GEN 1-12) haplotypes distributed at varying rates within different field sites. The haplotype SVMNTS-N was detected in all sites predominantly in Uttar Pradesh (96.77%) and Andhra Pradesh (40.63%), this finding also supports the earlier studies, which revealed that the SVMNTS haplotype is predominantly found in low transmission areas. The other mutant haplotypes CVIETS-N, CVIETS-Y, SVMNTS-Y, CVMNKA-Y and CVMNKA-N were observed with considerable variation from Jharkhnad and Odisha isolates and indicates the prevalence of CQ high resistance in these malaria transmission areas. Jharkhand and Odisha also showed higher level of CQ resistance (RII/RIII and RIII) with the prevalence of 68.5% and 55.7% respectively. However, isolates from UP and AP were predominantly found to contain lower level of CQ resistance i.e. RI/RII.

This study revealed that in SP drug resistance, only double mutants of pfdhfr was present in low transmission area (Uttar Pradesh and Andhra Pradesh) with total absence of pfdhps mutants and up to sextuple mutations were present in high transmission areas (Odisha and Jharkhand) for both the genes combined. Presence of multiple mutations in pfdhfr and pfdhps genes linked to SP resistance in high transmission area may lead to fixation of multiple mutations in presence of high drug pressure and high recombination rate. In conclusion, we suggest that SP can be effective for the treatment of uncomplicated falciparum malaria as a partner drug of ACT in Andhra Pradesh and Uttar Pradesh (low transmission areas). In Jharkhand and Odisha (high transmission area), present results suggest that mutation rate will increase continuously due to continued drug pressure and malaria transmission, which in turn will ultimately lead to SP treatment failure in near future, as was reported in northeastern parts of India. In case of CQ, we conclude that isolates with the pfcr-pfmdr-1 two loci mutations, which confer high level of CQ resistance was predominantly found in Jharkhand and Odisha where CQ has been replaced by SP as a first line treatment for falciparum malaria since 2007. This indicates that pfcr* and pfmdr-1 genes mutations are now fixed in this area. Wild type parasites were also detected but in less proportion which is indicative of a situation
where no reversal of wild parasite could occur even in absence of chloroquine drug pressure. This study also observed a fixed pattern of mutant SVMNTS pfcr\textit{t} haplotype mainly prevalent in low transmission areas, which raise concern about rapid spread of chloroquine resistance in these areas. While increased prevalence of CVIETS haplotype in high transmission areas showed that mutations conferring CQ resistant parasite population are fixed in this areas.

In conclusion, we found significant proportion of mutant alleles for both SP and chloroquine drug resistance. The study reflects the presence of resistant strains in the country which can be considered as alarming signals for drug policy makers. We, therefore, strongly recommend continuous molecular surveillance of various long lasting partner drugs of artemisinin (including SP) and CQ to understand dynamics of the parasite resistance development. The real-time drug-resistance status would help the malaria policy makers to maintain an effective drug policy for delaying drug-resistance development in malaria parasite.