The rapid spread of anti-malarial drug resistance is the biggest concern all over the world which is augmented by use of ineffective anti-malarials and responsible for the malaria morbidity and mortality. The increasing failure rate of several anti-malarial drugs has emphasized the need for systematic monitoring of anti-malarial drug resistance to suggest that, where malaria treatment drug policies should be revised and to make an effective drug policy. Molecular markers for drug resistance are great tools for close monitoring of the epidemiology and dynamics of drug resistance and greatly facilitate this process to overcome difficulties in using traditional methods for assaying drug sensitivity. Rapid molecular markers have been developed for the detection of gene amplifications or mutations of the parasite genes associated with resistance to a number of anti-malarials. In this study, polymorphisms in the *pfcrt*, *pfmdr-1*, *pfdhfr* and *pfdhps* genes were analyzed in *Plasmodium falciparum* isolates collected from six different sites of India to find out the extent of resistance to chloroquine (CQ) and antifolates (SP), both these drugs are still used as a part of malaria treatment in national drug policy. Sulfadoxine-pyrimethamine (SP) combination drug is currently being used for the treatment of *Plasmodium falciparum* as longer acting partner drug in artemisinin-based combination therapy (ACT) all over the India except northeastern states. According to national anti-malarial drug policy, CQ is still a primary choice for *Plasmodium vivax* infection due to its effectiveness against vivax malaria (National Drug Policy, 2013). Currently, combination of fast and long acting anti-malarial drugs is recommended as an ideal approach over the use of single anti-malarial drug (Lin et al., 2010). Optimizing the choice of long acting partner anti-malarial drug in ACT is important challenge to be addressed in successful malaria treatment programme (Mallick et al., 2012). Presence of resistant parasites against the long acting anti-malarial used in ACT can hamper the treatment efficacy and can also lead to emergence of artemisinin resistant parasite (Mishra et al., 2014). However, some recent studies reported that parasite resistance to artemisinins has also been detected in Thailand, Cambodia, Myanmar and Viet Nam (Dondorp et al., 2009; Ashley et al., 2014; WHO, 2014c). All malaria endemic parts of the India experienced mutant parasites conferring resistance to all conventional anti-malarial drugs like CQ, SP and thus there was country wide adoption of AS+SP as ACT in year 2010 (Shah et al., 2011). However, resistance to SP had been well documented from northeastern
part of the India, which led to use of AS+lumefantrine (AS+AL) as first-line malaria treatment in these parts of country since year 2013 (Mishra et al., 2014). Northeast region of India has already been documented as important route for invasion of parasite bearing resistant genotypes against many anti-malarials and proved its potential to be a focus for spread of resistant parasite to other parts of country (Mohapatra et al., 2014). In vivax prevalent areas, sometimes due to improper-or-mis-diagnosis, Plasmodium falciparum infections are often exposed to CQ treatment in mixed infection cases and this chloroquine pressure may retain CQ resistant Plasmodium falciparum in population. Thus, monitoring of mutation status of partner drug SP and chloroquine are important for better management of anti-malarial policy. Here, mutation status of pf dhfr and pf dhps gene responsible for resistance against SP and mutation status of pf crrt and pf mdr-1 gene responsible for chloroquine resistance was evaluated for isolates from four different geographic areas.

The study showed 17.51% wild-type pf dhfr gene and 79.26% wild-type pf dhps gene. Higher number of mutant pf dhfr gene was observed in comparison to pf dhps gene at all the study sites infer development of mutations occurred first in pf dhfr gene and then in pf dhps gene under selective drug (SP) pressure. The prevalence of double mutant (ANRNIRNI) in pf dhfr gene and wild-type pf dhps gene at all the study sites corroborated earlier reports of predominant presence for the same (Sharma, 2012). However, single mutant ANCNI, triple mutants (ANRNL or AIRNI) in pf dhfr gene and single mutants (SGKAA, AAKAA and SAEEAA), double mutant SGKGA, triple mutant AGEAA, quadruple mutant AEGGA in pf dhps gene were also observed. Single or double pf dhfr mutations alone cannot cause sulfadoxine-pyrimethamine treatment failure but the double pf dhfr mutations with a single or multiple mutations in in pf dhps gene can cause various level of SP resistance (Wang et al., 1997). In addition, triple mutant pf dhfr alone can cause various level of SP resistance. The DHFR-DHPS two locus mutations have importance to monitor as it can infer the clinical susceptibility of SP (Wang et al., 1997; Ahmed et al., 2004). This study observed 13 such two locus genotypes (GEN 1-13) within 217 isolates (Table 15). Out of all isolates, only 17.51% were wild-type (GEN1). In total, double mutant genotype (GEN3) was observed in 52.99% isolates and its predominance indicates continuous emergence of SP resistance in all study sites. The study sites include both high malaria transmission area (Odisha and Jharkhand) and low malaria transmission areas (Andhra Pradesh and Uttar Pradesh). Triple
mutant genotype (GEN 4-8) that can confer high SP resistance were observed in Odisha and Jharkhand with prevalence of 12.85% and 5.95% respectively, while quadruple mutant (GEN9) was found only in Jharkhand with 21.42% prevalence. Isolates from high transmission areas also showed quintuple (Jharkhand=5.95%, Odisha=11.43%) and sextuple (Jharkhand=4.76%, Odisha=4.29%) mutant genotype. Quintuple and sextuple mutant genotypes associated with higher level of resistance to SP suggests selective drug pressure due to its use over a long period. Here, high transmission areas showed higher number of mixed mutations (both wild and mutant alleles) in both \textit{pf}dhfr and \textit{pf}dhps genes, which were possibly due to multi-clonal infection, since high recombination events are expected here, which in turn would add to allelic variation. In these high transmission areas, high genetic diversity of both gene under selection and neutral microsatellite markers were reported when compared to low transmission region of India (Mallick et al., 2012, 2013a). Higher genetic diversity and more probability of mutation fixation in genes, responsible for various antimalarial resistances were observed earlier and suggested the role of malaria transmission intensity and drug exposure in emergence of drug resistance (Lumb et al., 2009; Mallick et al., 2013b). The mutations 164\textit{L} in \textit{pf}dhfr and 437\textit{G} and 540\textit{E} mutations in \textit{pf}dhps gene were reported to be responsible for therapeutic failure of SP (Mohapatra et al., 2014; Saha et al., 2012), and these mutations were observed in 9.52% and 34.52% of isolates respectively in Jharkhand. In Odisha, 4.28% isolates showed another mutation 51\textit{I} in \textit{pf}dhfr gene which was responsible to accelerate the SP resistance (Wang et al., 1997; Kublin et al., 2002). In addition, these mutations are also part of two-locus genotypes (GEN 7-13) which would be involved in clinical resistance against SP. In \textit{pf}dhps gene, triple mutant AG\textit{E}AA was found in 15.71% of Odisha isolates, while double mutant SG\textit{K}GA was found only in 23.80% of Jharkhand isolates. The prevalence of mutants found here in high transmission areas are similar to those reported earlier from northeastern region (Mohapatra et al., 2014; Saha et al., 2012), however the prevalence of mutant two locus genotypes were not similar. Mutations like S436\textit{F}, A613T/S was not observed in this study.

In contrast, the low transmission areas (Uttar Pradesh and Andhra Pradesh) showed single mutation (11.11%) at codon positions 108, double mutations (66.67%) at codon positions 59 and 108 in \textit{pf}dhfr gene, while no isolate showed the N51I and I164L mutations associated with SP treatment failure. Thus, the triple and quadruple mutations were not observed in
pf dhfr gene. In case of pf dhp s all isolates were wild-types, which infers that P. falciparum population in these regions was susceptible to SP treatment and resistance development is in progress. The low transmission areas showed mutations similar to the earlier reports of similar single and double mutations from Uttar Pradesh and Delhi in year 2001, which suggested higher susceptibility for SP, was maintained due to higher clonal populations in these regions (Ahmed et al., 2004). In addition, P. vivax is prevalent in Uttar Pradesh and chloroquine is still effectively used as anti-malarial treatment against P. vivax in India, which could provide selection pressure on gene responsible for chloroquine resistance in P. falciparum (Mallick et al., 2012). Thus, no or low selection pressure of antifolate drugs in P. falciparum was predicted in these P. vivax prevalent areas.

In case of chloroquine resistance, the present study deals with the distribution of the pf c r t haplotypes (concatenating mutations at codon 72-76, codon 97 and codon 220) and the pf m d r -1 mutation N86Y across six different parts of India and evaluated the prevalence of chloroquine mutations in parasite isolates collected from these sites. The genetic basis of CQ resistance has been attributed to both the pf c r t and pf m d r -1 genes, the pf c r t gene mutation K76T is directly implicated in CQ resistance whereas pf m d r -1 N86Y mutation modulates the level of chloroquine resistance to a higher degree when present with pf c r t mutations (Fidock et al., 2000; Sidhu et al., 2002; Mita et al., 2006; Valecha et al., 2009a). However, some recent studies from India reported that in high CQ resistant areas, double mutations (86Y +1246Y) in pf m d r -1 gene were highly correlated with In vitro chloroquine resistance and show early treatment failure in In vivo studies (Das et al., 2013, 2014) which was odd with previous studies from India (Vahsala et al., 2004). Considering these findings, this study enforced that monitoring of pf m d r -1 genotypes is also very much essential as monitoring of pf c r t haplotypes for detecting the CQ resistance pattern in a given area. In pf c r t gene, wild type CVMNKA haplotype was found in 15.53% (n=32) isolates covering all sites except Uttar Pradesh where no wild type haplotypes were found. SVMNTS haplotype, which is characteristic of CQ resistant isolates of Papua New Guinea (PNG) or South America was observed in all study sites with exceptionally high proportion in Uttar Pradesh (low transmission areas) i.e. 96.77% isolates. In Andhra Pradesh, the SVMNTS haplotype was also prevalent in 40.63% isolates and this finding supports the earlier studies, which reported that the SVMNTS haplotype is predominantly found in low transmission areas (Vahsala et
Another haplotype CVIETS, characteristics of South East Asia (SEA) CQ resistance, it is a highly mutant haplotype as compared to other haplotypes and is predominantly found in Jharkhand and Odisha isolates (High transmission areas). Predominance of CVIETS in high transmission areas might be due to high incidence of CQ resistance in these areas. The CVIETS haplotype is associated with high level of CQ resistance because they could persist under high drug pressure of In vitro studies and showed higher IC$_{50}$ for CQ and also the increased prevalence of CVIETS haplotype in high endemic areas (Mitra et al., 2006).

Earlier studies reported that the prevalence CVIETS haplotype in eastern and northeastern region of India, because these parts of country share international borders Bangladesh, Myanmar, China and others from where mutant parasite strain may migrate to India (Valecha et al., 2009a; Mallick et al., 2012; Sharma, 2012; Mohapatra et al., 2014). In this study, we found that SVMNTS haplotype was observed across all the study sites. The observation may be correlated with use of amodiaquine, as monotherapy or in combination with other drug, in chloroquine resistant areas (Dittrich et al., 2005; Alifrangis et al., 2006). In 1980’s, amodiaquine was used as a presumptive drug in India for CQ resistant parasites (Barkakaty et al., 1980; Ghosh et al., 1992; Pandya et al., 1994) and this might have resulted in the introduction and spread of SVMNTS haplotype.

In case of pfmdr-1 gene, wild type allele (N86) was the most prevalent in low transmission areas i.e. Uttar Pradesh isolates showed 100% wild type allele while in AP, 78.12% isolates showed wild type allele which is in agreement with the previous finding in these areas (Mitra et al., 2006; Sharma, 2012; Mallick et al., 2012; Pathak et al., 2014). This specific observation and expansion of triple mutant SVMNTS pfcrt haplotype and pfmdr-1 N86 wild type allele in low transmission areas may be due to higher inbreeding potential of resistant parasites which is due to low frequency of multiclonal infections (Schmidt, 1995; Paul et al., 1995). This low genetic diversity is also supported by our results that in UP and AP, mixed pfcrt haplotypes and pfmdr-1 mixed allele i.e. S*VI*E*T*S*-Y* was found only in few isolates. Continued exposure of chloroquine, in P. vivax predominant areas are the other reason for the expansion of this combined two locus haplotype of pfcrt-pfmdr-1 (SVMNTS-N). The significant occurrence (P < 0.001) of wild type N86 allele in low Plasmodium
Prevalent areas seems to be fixed in this region and this fixation could lead to the rapid spread of resistance in these areas.

The mutant allele 86Y and also mixed allele 86Y* were predominantly observed in Jharkhand and Odisha isolates that coincides with higher level of resistance to CQ in this region. In Jharkhand and Odisha, wild type allele was found in 47.62% and 37.14% isolates respectively. According to a recent study, prevalence of the wild type N86 allele of pfmdr-1 gene could be a cause for concern because this allele is associated with a decreased sensitivity to lumefantrine in In vitro studies (Duraisingh et al., 2000; Sisowath et al., 2007) and also has been associated with resistance to one of the artemisinin-based combination therapy (ACT-Coartem®) disseminated in East Africa (Sisowath et al., 2005). However, a recent study reported that the therapeutic efficacy of Coartem® gave successful result without any selection of N86 allele in high P. falciparum transmission areas (Valecha et al., 2009b).

Two loci mutations (pfcrtpfmdr-1) combined haplotypes were highly prevalent in high endemic areas (Mittra et al., 2006). Parasite isolates from Jharkhand were found to contain different type of two loci haplotype with the prevalence CVIETS-N in 27.40% and CVIETS-Y in 27.40% isolates while in Odisha isolates there was prevalence of CVIETS-Y haplotype i.e. 35.71%. Mixed mutant haplotypes were also observed in Jharkhand isolates i.e. CVIETS*-Y* in 8.21% isolates and S*V*I*E*T*S*-Y* in 5.47% isolates while in case of Odisha, CVIETS*-Y* and S*V*I*E*T*S*-Y* haplotypes carrying isolates were 5.71% each. In high transmission area, CVMNKA-Y* and CVMNKS-Y* haplotypes was found in 9.79% and 2.79% isolates respectively. Increased prevalence of mixed two loci haplotypes in these areas as compared to UP and AP also suggested high transmission potential of resistant parasite along with high genetic diversity in high transmission areas (Mittra et al., 2006; Sa’ et al., 2009). An additional mutation H97L in pfcr gene were also observed in an appreciable number of isolates i.e. 28.57% (n=20) only from Odisha isolates. However, this mutation does not have any strong association with other pfcr and pfmdr-1 mutation, which is described in an earlier study, which first time reported this mutation from India including Odisha isolates (Fidock et al., 2000; Durrand et al., 2004; Sutar et al., 2011). The combinations of pfcr haplotypes and pfmdr-1 mutations are categorized in 12 different
haplotypes (GEN1-GEN12) distributed at varying rates and also showed various levels of clinical resistance against chloroquine treatment in various *In vivo* and *In vitro* studies (Misra, 1996; Dev et al., 2003; Biswas et al., 2003; Mittra et al., 2006). Our categorization indicates that large number of isolates with a higher level of CQ resistance (belongs to categories RII/RIII and RIII) was predominantly observed in high transmission areas whereas number of isolates with lower level of CQ resistance was significantly found in low transmission areas. Jharkhand and Odisha 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.

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 *pfcrtpfmdr-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 *pfcrtpfmdr-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 *pfcrtpfmdr-1* 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.