1.1 Malaria

Malaria is one of the most widespread health problems in tropical and subtropical countries and a major cause of mortality throughout the world. Malaria in humans is mainly caused by four parasite species, viz., *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. *Plasmodium knowlesi*, a fifth malaria parasite species that causes malaria in monkeys, has been recently documented to cause human infection in forested areas of South-East Asia (Chin et al., 1965; Cox-Singh et al., 2008; Lee et al., 2011). Among all species, *P. falciparum* and *P. vivax* poses the greatest public health challenges all over the world. *P. falciparum* is the most virulent parasite responsible for the high rate of disease related morbidity and mortality while *P. vivax* has a wider geographic distribution but less dangerous while other species are found less frequently (Perkins et al., 2011; WHO, 2011).

Globally 97 countries and territories has ongoing malaria transmission, an estimated 3.2 billion people, nearly half of the world’s populations are at risk of being infected with malaria and developing disease as shown in Figure 1 and ~ 1.2 billion people are at risk (WHO, 2014a). According to WHO report 2014, 198 million cases of malaria occurred globally and the disease led to 584000 deaths. In high transmission areas, children under 5 years age are particularly more susceptible to infection, illness and death and 70% of all malaria deaths occur in this age group. In African region the burden is heaviest where an estimated 80% of all malaria cases and 90% of malaria death were found while 13 % contributed by South East Asia Region (SEAR) countries (WHO, 2013). However, Latin America and the Middle East are also at risk but to a lesser extent. According to the latest estimates, in 2013 the global total of microscopic examinations was 197 million which were dominated by India accounted for over 120 million slide examinations (WHO, 2014a). The incidence of malaria in India accounted for 58% of malaria cases and 41% of malaria deaths in SEAR countries. In 2013, 0.88 million malaria cases have been reported with *P. falciparum* causing 53% while *P. vivax* causing 47% of total infections (WHO, 2014a). According to latest estimate, India accounted for 58% of total malaria cases reported in the South East Asia Region with 95% of the population residing in malaria endemic regions, each year 0.7-1.6 million people get affected from malaria and 400-1000 deaths occurs annually (Kumar et al., 2007; NVBDCP website). However, a recent study contradicts
NVBDCP and WHO estimates which reported that these official numbers could be far underestimated as reported in the national survey conducted by Dhingra et al., 2010. According to this study, around 0.2 million malaria deaths have been estimated per year and 90% of the deaths were recorded in rural areas belong mainly to Orissa followed by Chhattisgarh, Jharkhand and Assam, which is enormously high from the official estimates (Dhingra et al., 2010; Hay et al., 2010). One another study estimated the malaria mortality in India at 46,800 in 2010 (Murray et al., 2012). According to the latest estimates of National Vector Borne Disease Control Programme (NVBDCP) reported that the total annual number of cases in India may be about 9.7 million, with about 30,014 – 48,660 which assess India’s actual malaria death burden (Sinha, 2012).

![Figure 1- Global Malaria Distribution showing ongoing transmission of malaria, (World Malaria Report, 2014, World Health Organisation)](image-url)

*Plasmodium falciparum* is responsible for the majority of malaria deaths globally; nearly 85% of cases in Africa are caused by *P. falciparum*. *Plasmodium vivax*, is the second most
significant species and most geographically widespread of the human malarias occurring in major part of Asia, Central and South America, and also prevalent in South East Asia and Latin America (Carter et al., 2002; Rich et al., 2006). *P. ovale* cause infections in Africa, New Guinea and the Philippines while *Plasmodium malariae* is restricted to some parts of India, western Pacific and South America (Carter et al., 2002). A fifth species *Plasmodium knowlesi* has also been reported from South East Asian countries (Singh et al., 2009; Daneshvar et al., 2009; Putaporntip et al., 2009).

In India, two *Plasmodium* species are prevalent *Plasmodium falciparum*, *Plasmodium vivax* while *Plasmodium malariae* has been reported from Odisha (Kumar et al., 2007; Sharma et al., 2006; Das et al., 2012). Although *Plasmodium falciparum* and *Plasmodium vivax* are unevenly distributed across India, the central, eastern and northeastern regions of India report the most malaria cases. Orissa contributes about 25% of the total annual malaria cases having more than 40% of *Plasmodium falciparum* cases and about 30% of deaths caused by malaria in India, followed by Meghalaya, Jharkhand, West Bengal, Assam, Chhattisgarh, the western states of Gujarat, Madhya Pradesh and Rajasthan (Joshi et al., 2008). Previously, *Plasmodium vivax* has been the major infecting species in India, but over the past several years *P. vivax* cases have decreased. Recent estimates have revealed that even though there is a decline in the incidence of malaria infection caused by *Plasmodium falciparum* from 1.14 million to 0.77 million per year since 1995. However, the percentage of *Plasmodium falciparum* malaria cases has increased from 13% in 1978 to >50% in 2009 and this change is continuous as shown in Figure 2 (NVBDCP website). Advent of drug resistance in *P. falciparum* has led to an increase in number of this parasite which is a possible cause for the changing scenario in India (Valecha et al., 2009b; Shah et al., 2010).
1.2 History of malaria

Malaria is probably one of the oldest diseases in medical literature with its mention in the ancient Roman and Chinese manuscripts, more than 4000 years ago. The term malaria originated when Italians referred to intermittent fever as mal’aria (mala – bad, aria – air) caused by exposure to the swamps and marshland. Hippocrates was probably the first malariologist who noted the principal symptoms of malaria and described about the various malaria fevers of human being. In Susruta Samhita (an ancient medical treatise of India), the symptoms of malaria fever were described and associated with the bites of certain insects while Charaka Samhita classified the fevers into different categories. The first breakthrough discovery in malaria was made by Charles Louis Alphonse Laveran in 1888, a French physician, found a moving object while examining a fresh blood film from a patient of malaria. He identified the malaria parasite and called this parasite Oscillaria malariae. In 1889, Plasmodium term was given by Italian scientists, Marchiafava and Celli. The second important discovery was made by Sir Ronald Ross in 1898. Ronald Ross discovered oocysts
in the gut of anopheline mosquito at Secunderabad, India, and proved that mosquito was the vector for malaria. He also identified the sporozoites in the salivary glands of the mosquito and transmitted malaria through infected mosquitoes which in case of humans is *Anopheles spp.* Later on life cycles of *Plasmodium vivax* and *Plasmodium falciparum* were illustrated (Grassi *et al.*, 1899).

### 1.3 The vector – *Anopheles spp.*

Malaria is mosquito-borne disease transmitted from one person to another person by female mosquitoes of the genus *Anopheles*. There are about 460 different species of *Anopheles* mosquitoes, across the world but only 100 of these are important vectors which transmit malaria. In India, approximately 60 morphologically distinct anopheline species have been described out of which six are recognized as primary malaria vectors i.e. *Anopheles culicifacies*, *Anopheles stephensi*, *Anopheles fluviatilis*, *Anopheles minimus*, *Anopheles dirus* and *Anopheles sundicus*. There are some other vectors of secondary importance viz. *Anopheles varuna*, *Anopheles annularis* s.l., *Anopheles philippinensis-nivipes* and *Anopheles jeyporensis* (Rao, 1984; Dash *et al.*, 2007). The distribution pattern of the important malaria vectors across the world shown in Figure 3.

1.4 Disease - symptoms

Malaria is a fatal disease characterized by fever and related symptoms. The clinical symptoms of the disease first appear during the erythrocytic schizogony in the blood. The incubation period is the time period between the inoculation of the sporozoites in the human host and the appearance of the first clinical symptoms. Incubation period is different for all malaria species and depending on species, host immunity and climatic conditions i.e. it last for 9-14 days for Plasmodium falciparum, 12-17 days for Plasmodium vivax, 16-18 days for Plasmodium ovale and 18-40 days for Plasmodium malariae. The clinical symptoms include headaches, fever, vomiting, dizziness, splenomegaly, anxiety and restlessness. The classical symptom of malaria is the sudden rise and fall in body temperature, with cyclical occurrence of sudden chills followed by shivering after regular intervals of 48hrs to 72hrs depending on the Plasmodium species. This rise and fall in body temperature of a malaria patient starts when malaria parasites enter the blood stream to infect and destroy red blood cells (RBCs) which leads to fever. Uncomplicated malaria can be caused by all Plasmodium species. But only Plasmodium falciparum malaria can progress to severe disease complications like cerebral malaria, severe anaemia, jaundice, renal failure, impaired consciousness, convulsions etc. If the patient does not take proper treatment in time or the treatment is inadequate or the parasite is resistant to the used drug, these all leads to severe complications. In case of P. vivax and P. ovale, clinical relapse may occur with in few weeks to months after the first infection. This relapse of malaria arises due to dormant liver forms called hypnozoites which is not found in P. falciparum and P. malariae.

1.5 Malaria species diagnosis

Apart from the clinical symptoms, rapid and accurate diagnosis of malaria is integral part of appropriate malaria treatment. Microscopic blood examination is conventional and necessary for the confirmation of the disease. In this method, thick and thin blood smears are examined under the microscope after staining with Giemsa or JSB stain (Singh, 1956) to confirm malaria infection with either of the Plasmodium species. Microscopy is the most economic, preferred and reliable technique for identification of the malaria parasite because all four malaria parasite species have distinguishing characteristics. It has been used for hundreds of
years and still remains the gold standard for malaria diagnosis. However, it also depends on the quality of the reagents, microscope, and on the experience of the laboratory technician otherwise may lead to misdiagnosis.

Rapid Diagnostic Tests (RDTs) use the mechanism of antigen-antibody interaction most often use a dipstick and provide results in about 20 minutes. RDTs can detect 2 types of malaria antigens; one is specific only for *P. falciparum* and the other are found in all four human species of malaria. Histidine-rich protein II (HRP II) antigen is specific for *Plasmodium falciparum*, aldolase or lactate dehydrogenase (pLDH) enzyme part of the glycolytic pathway, specific for different *Plasmodium* species are utilized for parasite detection in this method (Beadle et al., 1994; Moody, 2002). The other method of *Plasmodium* diagnosis is using PCR based assays but these are more applicable to large-scale surveys than to clinical diagnosis (Singh et al., 1998). This method is more accurate than microscopy and can detect the infection at a very low parasitemia levels. However, this method is expensive and requires a specialized laboratory so it cannot be used in the field.

### 1.6 Malaria control

Malaria control is carried out by control of the vector population which prevent malaria transmission using vector control strategies and killing of the malaria parasite by treatment with anti-malarials because no effective vaccine is made available till date. Vector control strategies are the main way to reduce malaria transmission includes Indoor residual spraying (IRS) with insecticide, Insecticide treated mosquito nets (ITNs) or long lasting Insecticide nets (LLINs), anti-larval measures and source reduction. Synthetic pyrethroids, organochlorines, organophosphates and carbamates are commonly used insecticides. A long lasting insecticidal net (LLIN) is preferred for protection against mosquito bites and acts as the first line of defense for malaria prevention. Indoor residual spraying (IRS) with insecticides is also a popular and powerful method to reduce malaria transmission in rural areas while anti-larval operations are used in urban areas. In India DDT (Dichlorodiphenyltrichloroethane), HCH (hexachlorocyclohexane or Lindane), Malathion and pyrethroids are used for LLINs and IRS strategies while temephos, baytex and larvivorous fishes used for larval control (NIMEP, 1985). Source reduction i.e. removal or
permanent destruction of mosquito breeding sites, and biological controls are also used in vector control strategies (Kumar et al., 1998). However, emergence of insecticide resistance in malaria vectors against DDT, HCH, Malathion as well as the commonly used synthetic pyrethroids has been reported from different part of India (Mittal et al., 2002; Singh et al., 2002). Therefore, the development of new alternative insecticides is on high priority and regular monitoring of mosquito populations should be an essential for effective vector control management.

Anti-malarials are important in order to kill the parasite and therefore, keystone of malaria control efforts. The effectiveness of early diagnosis and prompt treatment through effective anti-malarials not only reduces the mortality and morbidity of malaria, but also reduces the risk of resistance to anti-malarial drugs. After confirmation of malaria by blood smear examination/ RDT, patient is provided with proper medication following the National Drug Policy on Malaria issued by the National Vector Borne Disease Control Programme (NVBDCP), Government of India. In the earlier days, quinine was the choice of drug for malaria treatment but due to its toxicity it was replaced with various new anti-malarial drugs. Chloroquine, first developed in the 1930s, and became the most widely used anti-malarial against malaria infection during the 1960s and 1970s. Due to its cost and safety, chloroquine became first choice of treatment of falciparum malaria. Some other drugs like sulphadoxine-pyrimethamine (anti-folates), mefloquine and primaquine are also used for malaria treatment. However, emergence of anti-malarial drugs resistance has become a major obstacle in the successful malaria treatment. Chloroquine resistance was first appeared in Thailand in 1957 and spread in Southeast Asia, it was seen in Africa and South America by the 1970s (Young and Moore 1961; Wernsdorfer and Payne 1991; Pickard and Wernsdorfer 2002). In India, chloroquine (CQ) resistance was first time reported in 1973 in Assam district (Sehgal et al., 1973). Chloroquine resistance has led to the urgency in deployment of alternative anti-malarial drugs i.e. sulfadoxine–pyrimethamine and mefloquine which are readily absorbed with a long half-life and can permit effective single dose treatment of malaria. When sulfadoxine-pyrimethamine (SP) was introduced as a drug of choice for the treatment of CQ resistant malaria, resistance has appeared in same year of introduction and spread quickly throughout South-East Asia by late 1990 (Bjorkman and Phillips-Howard, 1990; Bloland,
2001). In India, resistance against SP drugs have also been reported from *P. falciparum* predominated areas like eastern, northeastern and central states (Sharma, 2012). Artemisinin and its derivatives i.e. artesunate, artemether and arteether are the new and most effective anti-malarial drugs derived from the Chinese herb, *Artemisia annua*, which are used in combination with other anti-malarial drugs and used for treatment of *P. falciparum* infection (Rosenthal, 2008). Artemisinin has a very short high life but is very effective. Combining of an artemisinin drug with a long acting partner drug is done in order to get better drug efficacy and delay the development of resistant parasite and it is called combination therapy. Artemisinin based combination therapies (ACTs) were introduced in 1990s and currently ACTs are the first-line treatment for uncomplicated *P. falciparum* malaria recommended by the WHO. The ACT therapy recommended in the country is artesunate plus SP (AS+SP) for uncomplicated falciparum malaria all over the India except northeastern states. In north east India, where resistance to SP has been documented, the National Drug Policy recommends the use of Artemether plus Lumefantrine (AS+AL) for the treatment of *P. falciparum* malaria instead of AS+SP (National Drug Policy, 2013). Artemisinin-based therapies are currently the most effective treatment for malaria but in 2009 artemisinin resistance was observed in the Thai-Cambodia border region and now increasingly reported in South East Asia (Dondorp et al., 2009; Ashley et al., 2014). *P. vivax* remains sensitive to chloroquine and the drug is used all across the country. At present malaria patients are treated following the National Drug Policy on Malaria, 2013 as shown in Table 1.

Lots of research work has been undertaken to develop an effective vaccine but without any success. Currently, there are no available licensed vaccines against malaria parasite. However, recently an effective vaccine known as RTS, S/AS01, against *P. falciparum* is the most advanced and has shown promising results in imparting protection against *Plasmodium falciparum* in Phase III Clinical Trials (Agnandji et al., 2011). However, there is still time before it becomes readily available to the malaria exposed populations.
<table>
<thead>
<tr>
<th>Plasmodium vivax infection</th>
<th>Uncomplicated Plasmodium falciparum infection</th>
<th>Mixed infection (P. falciparum + P. vivax)</th>
<th>Severe Plasmodium falciparum infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine-25mg/kg</td>
<td>In North-Eastern States</td>
<td>In North-Eastern States</td>
<td>Artesunate-2.4mg/kg</td>
</tr>
<tr>
<td>over 3 days +</td>
<td>Artemether(20 mg)-</td>
<td>Age-specific ACT-AL for</td>
<td>(IV) at 0,12.24 hr and then once a day</td>
</tr>
<tr>
<td>Primaquine-0.25mg/kg</td>
<td>Lumefantrine (120mg) +</td>
<td>3 days + Primaquine</td>
<td>Or</td>
</tr>
<tr>
<td>for 14 days.</td>
<td>Primaquine- 0.75mg/kg on day 2</td>
<td>0.25mg/kg for 14 days.</td>
<td>Arteether-150mg daily for 3 days (only for adults)</td>
</tr>
<tr>
<td></td>
<td>In Other States</td>
<td>In Other States</td>
<td>Or</td>
</tr>
<tr>
<td></td>
<td>Artesunate- 4mg/kg for 3 days +</td>
<td>Artesunate- 4mg/kg for 3 days +</td>
<td>Artemether-3.2mg/kg (IM) at day 0 and then 1.6mg/kg per day</td>
</tr>
<tr>
<td></td>
<td>Sulfadoxine(25mg/kg) +</td>
<td>Sulfadoxine(25mg/kg) +</td>
<td>Or</td>
</tr>
<tr>
<td></td>
<td>Pyrimethamine(1.25mg/kg) on day 1 +</td>
<td>Pyrimethamine(1.25mg/kg) on day 1 +</td>
<td>Quinine-20mg/kg (IV or IM) followed by 10mg/kg every 8hrs</td>
</tr>
<tr>
<td></td>
<td>Primaquine- 0.75mg/kg on day 2</td>
<td>Primaquine- 0.25mg/kg for 14 days.</td>
<td></td>
</tr>
</tbody>
</table>

Chemoprophylaxis (For Travellers)

<table>
<thead>
<tr>
<th></th>
<th>Doxycycline- 100mg once daily from 2 days before travel to 4 weeks after returning from malaria endemic regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short Term</td>
<td></td>
</tr>
<tr>
<td>(upto 6 weeks)</td>
<td></td>
</tr>
<tr>
<td>Long Term</td>
<td>Melfoquine- 250mg weekly from 2 weeks before, during and 4 weeks after returning from malaria endemic regions</td>
</tr>
<tr>
<td>(&gt; 6 weeks)</td>
<td></td>
</tr>
</tbody>
</table>
1.7 Anti-malarial drug resistance

Drug resistance in malaria against all currently available anti-malarial drugs has become one of the major problems in malaria control programme. In India, *Plasmodium vivax* and *Plasmodium falciparum* are the two most common species causing malaria but *P. falciparum* is more fatal and this parasite has developed resistance towards most of the commonly used anti-malarial drugs (Kshirsagar, 2006; Hyde, 2007; Shah et al., 2011). In India alone, *P. falciparum* accounts for more than 50% of all malaria attacks instead of previously reported 23% since the advent of drug resistance and this proportion of *P. falciparum* has been attributed to arise in chloroquine resistance across India (Shah et al., 2011). Chloroquine was once considered to be a gold standard drug used to treat malaria due to its safety and cost effectiveness. In India chloroquine resistance was first reported in 1973 in Karbi-Anglong district and in 1974 in Nowgong district of state Assam (Sehgal et al., 1973). Gradually it has spread towards the eastern part of country, west and including south covering almost the entire country (Clyde, 1987; Sharma, 2007). Chloroquine resistance is associated with points mutations in a transporter gene located on chromosome 7, named *P. falciparum* chloroquine resistance transporter gene (*pfCRT* gene) (Fidock et al., 2000; Djimde et al., 2001a). A point mutation causing non-synonymous amino acid change from Lysine (K) to Threonine (T) at codon 76 of the *pfCRT* gene seems to be hallmark of CQR and strongly correlated with both *in vitro* and *in vivo* CQ resistance (Djimde et al., 2001b; Sharma, 2005). Large numbers of field isolates and laboratory studies have revealed that approximately 20 additional single nucleotide polymorphisms (SNP’s) i.e. C72S/R, M74I/T, N75E/D/K/I, K76T/I/N, I77H, N86Y, A144F/T, L148I, L160Y, I194T and A220S (mutations represented in bold letters) have been identified in the *pfCRT* gene, which may arise due to high CQ drug pressure (Foote et al., 1990; Cooper et al., 2005). In addition to *pfCRT* gene, the *P. falciparum* multidrug-resistance 1 (*pfMDR-1*) gene located on chromosome 5 has a point mutation N86Y that also plays an important role in *P. falciparum* resistance to various anti-malarial such as mefloquine, quinine and artemisinin derivatives (Duraisingham et al., 2005). Specifically, a non-synonymous point mutation at amino acid position 86 which replaced wild type Asparagine (N) with resistant type Tyrosine (Y) and some other mutations at codons Y184F, N1042D and D1246Y has been associated with reduced susceptibility to CQ treatment (Adagu and...
Warhurst, 2001; Than et al., 2003; Sharma, 2005). The studies from different geographical areas of the world including the India has observed a weak association between pfmdr-1 mutations and CQ resistance (Basco et al., 1996; Vinayak et al., 2003; Vathsala et al., 2004). But a recent study from India implies that pfmdr-1 mutation are highly associated (p value > 0.05) with In vitro CQ resistance and In vivo treatment failure (Das et al, 2014). Hence, both pfcrt haplotype (mutations at amino acid positions 72-76 and codon 220) and pfmdr-1 mutation 86Y are used as a genetic marker to determine chloroquine resistance (CQR) in P. falciparum isolates. CVIET and SVMNT, two major haplotypes defined by combination at specific point mutations at amino acid positions 72-76 of pfcrt gene have been linked to drug pressure and have been identified in different geographical locations (Wootton et al., 2002). SVMNT haplotype is predominantly found in South America (SA), Papua New Guinea (PNG) and Philippines (Mehlotra et al., 2001; Chen et al., 2005) while CVIET haplotype is characteristic of South East Asia (SEA) and Africa (Wellens et al., 2001; Nagesha et al., 2003). CMNKA, wild type haplotype are considered to be fully susceptible to CQ. Earlier studies showed that, in India SVMNT haplotype is found throughout the country but most prevalent in low transmission area i.e. central India while CVIET haplotype has been observed in high endemic areas with high transmission rate of malaria (Vathsala et al., 2004; Mittra et al., 2006; Keen et al., 2007; Mixson-Hayden et al., 2010).

In early 1980s, emergence of CQ resistance in P. falciparum led to introduction of SP as a second line anti-malarial drug in CQ resistant area for the treatment of uncomplicated falciparum malaria. Synergistically combination of sulphadoxine and pyrimethamine inhibits dihydropteroate synthase (dhps) and dihydrofolate reductase (dhfr) genes, both coding for essential enzyme in folate pathway of parasite (Chulay et al., 1998). SNPs in Plasmodium falciparum dhps (pfdhps) and Plasmodium falciparum dhfr (pfdhfr) genes of P. falciparum confer resistance to SP (Peterson et al., 1988; Triglia et al., 1997). The resistance against SP occurs in a stepwise fashion and starts with single point mutation with an increase in number of mutations in pfdhfr and pfdhps contributed to an increased risk of treatment failure (Lozovsky et al., 2009). Primarily point mutation at codon108 in pfdhfr gene initiates resistance against pyrimethamine and followed by mutations at codon 16, 51, 59 and 164, which are considered for augmentation of pyrimethamine resistance (Peterson et al., 1988; Lozovsky et al., 2009; Plowe et al., 1997). Similarly, resistance against sulphadoxine is
started by point mutation at codon 436 or 437 and augmented by mutations at codon 540, 581 and 613 of *P. falciparum* *dhps* gene (Triglia et al., 1997; Plowe et al., 1997; Gregson et al., 2005). *P. falciparum* infection carrying multiple mutations either in single gene or in combinations in both genes was associated with treatment failure of SP while single mutation in either *pf dhfr* or *pf dhps* gene is not enough. Triple mutant *pf dhfr* (**ANRL, AIRNL**) alone or combinations of *pf dhfr*-*pf dhps* quintuple mutants are more likely to fail the SP treatment while only the few *pf dhfr* mutations can cause only infection in the malaria patients (Wang et al., 1997; Kublin et al., 2002). In India, double mutation at codon 59 and 108 in *pf dhfr* gene was predominantly found along with reported single, double, triple and quadruple mutant from various different part of country (Ahmed et al., 2004; Ahmed et al., 2006; Saha et al., 2012; Mohapatra et al., 2014). Triple mutant and quadruple mutant *pf dhfr* gene indicating high level of antifolate resistance was prevalent in high and low frequencies from Car Nicobar Island and northeastern parts of India respectively (Das et al., 2010; Sharma et al., 2012; Mohapatra et al., 2014).

Wild-type allele in *pf dhps* gene was predominant in all geographic regions of India except Andaman and Nicobar Islands where lower frequency of mutations in *pf dhps* gene was observed in comparison to *pf dhfr* gene, which supports that mutations first emerged in *pf dhfr* and then occurs in *pf dhps* gene (Sharma et al., 2012). Double and triple mutations was observed in low frequencies from Madhya Pradesh, northeast and Odisha (Lumb et al., 2011; Sutar et al., 2013; Mohapatra et al., 2014). While, single mutation at codon 437 was observed in low frequency from Assam, Odisha, Madhya Pradesh and Uttar Pradesh (Sharma et al., 2012; Pathak et al., 2014). However, recent studies from northeastern part of the India showed increased number of triple and quadruple mutations in *pf dhps* gene including the key mutation at codon 437 (Mishra et al., 2014; Mohapatra et al., 2014).

Since 2007, ACT was introduced as the first-line treatment in high endemic areas of India (East and Northeast region) with identified resistance. However, in 2009, evidence of resistance to artemisinin-based combination therapy (ACT) was reported initially in the Thai-Cambodia border region and now increasingly in Southeast Asia. Some recent studies revealed that the single point mutations in the “propeller” region of the *P. falciparum* kelch protein gene on chromosome 13 (*kelch13*) strongly associated with slowly clearing infections
were detected throughout mainland Southeast Asia from southern Vietnam to central Myanmar (Ariey et al., 2014; Ashley et al., 2014). However, resistance to partner drug SP has already been reported in the country, which threatens the useful life of the ACT therapy (Ahmed et al., 2006; Sharma et al., 2012; Mohapatra et al., 2014). The Indian northeastern region shares international boundaries with Myanmar, Bangladesh and China and parasites where much slower clearance against ACT have also been identified in these regions recently, which is a alarming sign for ACT therapy in India (White et al., 2011; Amaratunga et al., 2012; Hien et al., 2012; Phyo et al., 2012). In this context, continuous monitoring of recommended anti-malarials should be done nationwide to control malaria by making effective drug policy.

1.8 Rationale of the study

In India, *P. falciparum* being the predominant species accounts 50% of total malaria cases and major health problem in rural/tribal areas of the central, eastern and north-eastern states of India (Das et al., 2012). Anti-malarial drug resistance, mainly in *P. falciparum* was a major factor in resurgence of malaria throughout the world including India. South East Asia has been considered as the centre for origin of drug resistance against all conventional anti-malarials i.e. chloroquine, SP, mefloquine etc. Chloroquine resistance in *Plasmodium falciparum* was first reported in 1957 at Thai-Cambodian border (SE Asian region) and after a decade followed by sulfadoxine-pyrimethamine resistance at same region (Wernsdorfer et al., 1980; Boland et al., 2001). India shares international boundaries with Bangladesh, Myanmar and China which are also known to be epicenter for drug resistance. India also experienced increased proportion of falciparum malaria cases due to advent of chloroquine (CQ) resistance in 1973 (Sehgal et al., 1973; Farooq et al., 2004; WHO, 2010a; Shah et al., 2011). The reports of chloroquine resistance in *P. falciparum* in early 1980s led to introduction of SP as a second line anti-malarial drug in CQ-resistant areas of India (Anvikar et al., 2014). India has evidenced resistant parasite especially *P. falciparum* against all available conventional anti-malarials like chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) in 1979 in northeast states (Dash et al., 2008; Farooq et al., 2004; WHO, 2010a; Shah et al., 2011). A decade long use of artemisinin-based combination therapy (ACT) had been proved a hallmark anti-malarial therapy for all the malaria endemic countries (Lin et al.,
Sulfadoxine and pyrimethamine act as a synergistic combination and are used as a long acting partner anti-malarial drug in ACT in South Asia, Middle East and South America (Lin et al., 2010). Since 2005, Indian anti-malarial drug policy has introduced artemesunate with SP as ACT in place of SP in high malaria endemic areas, and later in 2010, this treatment became the recommended first line treatment throughout India (National Drug Policy, 2010; Shah et al., 2011). Further, since 2013, prevalence of resistant genotype of falciparum against this partner drug SP, led to introduction of artemether-lumefantrine as anti-malarial therapy for northeastern part of India (National Drug Policy, 2013; Mishra et al., 2014).

Resistance to sulfadoxine and pyrimethamine in *P. falciparum* is linked with mutations in dihydropteroate synthase (*pfdhps*) and dihydrofolate reductase (*pfdhfr*) genes respectively. SP acts synergistically and inhibits folate-pathway of parasite (Chulay et al., 1998). Chloroquine resistance in *P. falciparum* is associated with mutations in *pfcrt* and *pfmdr-1* genes. Mutations at codon positions 72-76 and at codon 220 of *pfcrt* gene defined as *pfcrt* haplotype and N86Y *pfmdr-1* mutation are associated with the CQ resistant parasites (Foote et al., 1990; Fidock et al., 2000; Adagu and Warhurst, 2001; Babiker et al., 2001).

In case of SP resistance, various parts of India reported single, double, triple and quadruple mutant *pfdhfr* genes (Ahmed et al., 2004, 2006; Saha et al., 2012; Mohapatra et al., 2014; pathak et al., 2014). However, double mutation at codon 59 and 108 in *pfdhfr* gene was predominant throughout India (Ahmed et al., 2004, 2006; Srivastava et al., 2013; Mohapatra et al., 2014; Pathak et al., 2014). Triple mutant *pfdhfr* gene indicating high level of antifolate resistance was observed in India from northeast states, Car Nicobar Island and Odisha (Wang et al., 1997; Saha et al., 2012; Sharma, 2012; Mishra et al., 2014; Mohapatra et al., 2014). Highly resistant quadruple mutant allele was observed in high and low frequencies from Car Nicobar Island and northeastern parts of India respectively (Ahmed et al., 2006a; Ahmed et al., 2006b; Das et al., 2010; Saha et al., 2012; Mohapatra et al., 2014). Wild-type allele in *pfdhps* gene was predominant in all geographic regions of India except Andaman and Nicobar Islands, where lower frequency of mutations in *pfdhps* gene was observed in comparison to *pfdhfr* gene, which supports that mutations first emerged in *pfdhfr* and then occurs in *pfdhps* gene (Sharma, 2012). Single mutation at codon 437 was observed in low frequency from Assam, Odisha, Madhya Pradesh and Uttar Pradesh (Lumb et al., 2011;
Further, double and triple mutation including mutation at codon 437 was also observed in low frequencies from Madhya Pradesh, northeast and Odisha (Lumb et al., 2011; Sutar et al., 2013; Mohapatra et al., 2014). However, recent studies from northeastern part of India showed increased number of key mutation at codon 437 included in triple and quadruple mutations in pfdhps gene (Saha et al., 2012; Mishra et al., 2014; Mohapatra et al., 2014).

In case of chloroquine resistance, wild type CVMNK-A haplotype of pfcrtn gene found in all regions of India also including high Plasmodium falciparum predominant areas (Orissa, Chhattisgarh, Jharkhand) and high P. falciparum prevalent areas (Orissa, Jharkhand, Chhattisgarh) and supports earlier reports on acquisition of immunity in high endemic areas (Djimde et al., 2003; Klein et al., 2008). SVMNT-S, the most prevalent haplotype of pfcrtn gene found in all regions of India but predominantly found in low transmission areas (Vathsala et al., 2004; Mittra et al., 2006; Keen et al., 2007). While CVIET-S haplotype prevalent in high transmission areas (Northeastern India and South India) along with the prevalence mutant 86Y allele of pfmdr-1 and might be indicate the high level of drug pressure in these parts of India (Mittra et al., 2006; Sa’ et al., 2009). Similarly, in case of pfmdr-1 wild type allele N86 was prevalent throughout India while mutant type allele 86Y mainly observed in high transmission areas.

Development of resistance against ACT is currently a major threat and P. falciparum bearing resistance against Artemisinin’s partner drug (here, SP) may lead to ACT failure (Lin et al., 2010). The reports of widespread resistance against SP generate concern about long-term effectiveness of ACT in India (Shah et al., 2011; Mohapatra et al., 2014). A recent study reported significant reduction in efficacy of SP treatment from northeastern areas of India (Saha et al., 2012; Mishra et al., 2012; Mohapatra et al., 2014). Northeastern India, which is considered as a gateway for invasion of drug resistant parasite from Southeast Asia, this widespread resistance in this area against SP raised a major threat about long term effectiveness of ACT in India (Shah et al., 2011; Saha et al., 2012; Mishra et al., 2014; Mohapatra et al., 2014). Thus, routine molecular monitoring SP resistance markers is important in malaria endemic regions for better management of anti-malarial policy and this will help in making an effective malaria treatment policy. In addition to ACT, Chloroquine is
still recommended as anti-malarial treatment against *P. vivax* in India, so that *P. falciparum* population are often exposed to this treatment in mixed infection cases due to improper or misdiagnosis of type of infection, which could provide selection pressure on gene responsible for chloroquine resistance in *P. falciparum* (Mallick et al., 2012). Many years ago, CQ was discontinued in high transmission areas so that monitoring of CQ resistance is important to check any reversal of wild type parasite population or decrease in rate of mutations in chloroquine resistant parasite population in absence of CQ pressure.

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 the India (Jharkhand, Odisha, Andhra Pradesh and Uttar Pradesh) to assess the level of SP and CQ resistance. All conventional anti-malarials have become ineffective due to advent of drug resistant parasite. Thus, routine monitoring of anti-malarial drugs is essential for effective malaria control strategies. This study will provide valuable information on drug resistance status against partner drug SP and chloroquine which may help in modification of future drug policy.

Therefore, continuous monitoring of anti-malarials is essential in malaria endemic regions, so 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*