Literature Review

2.1.1 The burden of malaria

Malaria is one of the most important infectious diseases in the world, affecting mainly the tropics and the sub-tropics. At present, about 100 countries or territories are considered malarious, with nearly 50% of them in Sub-Saharan Africa. Globally, approximately 3 billion people corresponding to 40% of the world’s population are at risk of infection (Hay et al., 2004). Malaria is a vector-borne parasitic disease caused by intracellular protozoan parasites of the genus Plasmodium. Four species, *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, infect humans. The parasites multiply asexually in the human host and go through sexual reproduction in the anopheline mosquito vector. Each type of infection causes debilitating febrile illness, but approximately 90% of clinically manifest infections are caused by *P. falciparum*. *P. vivax* accounts for nearly 10% of the global malaria incidence. The main causes of mortality are severe anaemia and cerebral malaria caused by *P. falciparum*. Recent estimates suggest that between 500 million and 5 billion clinical episodes and up to 3 million deaths occur each year due to malaria, with Sub-Saharan Africa having 90% of this mortality burden. Moreover, the devastating consequences of malaria are a major obstacle to social and economic development in affected regions (Breman et al., 2004; Mendis et al., 2001; Snow et al., 2005).

The South East Asian countries reports >100 million cases (WHO, 2010), whereas Indian records about 2 million cases annually (NVBDCP, 2010). Among the four malaria parasite species, *P. falciparum* has been reported to contribute about 50% of the total cases. Northeastern states of India are highly endemic to malaria and reports
high number of malaria cases every year. As the region has uneven malaria distribution and frequent localized focal out breaks, controlling has been a daunting task.

In the 1950s and early 1960s, elimination of malaria seemed possible and the World Health Organization (WHO) launched the Global Malaria Eradication campaign with a main focus on indoor residual spraying (IRS) with dichlorodiphenyltrichloroethane (DDT) and mass drug administration (MDA) with chloroquine (CQ) or pyrimethamine (PYR). The eradication strategy was not only abandoned due to logistical, social and political reasons, but mainly because of the occurrence of chemoresistance in both, the vector and the parasite (D’Alessandro and Buttiens, 2001). Thereafter, the world was facing a rapid resurgence of the disease. This has been attributed to several factors, such as the change of agricultural practices creating new vector breeding sites, political crises leading to a weakening of public health systems, and long-term climate changes favouring malaria transmission (Sachs and Malaney, 2002). In the absence of an effective vaccine, current control efforts of the global partnership program Roll Back Malaria (RBM) clearly focus on reducing malaria morbidity and mortality. Methods include the reduction of transmission by either lowering vector densities using insecticides or biological measures and reducing their contact with humans by the use of insecticide-treated mosquito-nets. A further element is the early detection or forecasting of malaria epidemics and rapid application of appropriate control measures. But the cornerstone in the control of the disease is the reduction of malaria cases by early diagnosis followed by prompt and effective treatment and prophylaxis of people at greatest risk (i.e., infants and pregnant women) (WHO, 2005a; RBM, 2006).
However, the efficacy of this control strategy is hampered by the emergence and spread of drug resistant malaria which is the major challenge in the control of the disease at present. Therefore, research efforts into the design and development of new antimalarial drugs, which are safe, effective and affordable, have to be sustained. Important measures to prevent or delay the spread of resistance include the protection of currently used and newly introduced drugs by combination therapy (White, 1999) and improvement of access to prompt and effective treatment (Panosian, 2005). Further critical elements to detain resistance include the adoption of adequate methods to assess and monitor drug resistance in order to deploy evidence-based drug policies (Olliaro and Taylor, 2003).

Emerging trends of antimalarial resistance is a major concern in malaria control programs (Parija et al, 2011). Resistance to chloroquine is wide spread in this region and contributes to many epidemic out breaks regularly (Shiv et al. 1997). National vector borne disease control programme (NVBDCP) of India in 2008 changed the drug policy of the region and replaced chloroquine by artemisinin base combined therapy (ACT) for treatment of uncomplicated malaria.

2.1.2 Malaria situation in northeastern India

Northeastern part of India which comprise of eight states is a very strategically important area. These states shares long International border with the countries like with Bhutan, China, Myanmar and Bangladesh and also interstate border among themselves. All the international borders are mostly hills and foothills covered by thick forest cover and lack proper communication and health infrastructure. Due to congenial climate, difficult terrain, unstable population, human migration and other malarialogenetic factors these states are highly endemic to malaria (Mahanta et al., 1998). Although, the
population of north eastern region is only 4% of the country, but record about 10% of total malaria cases in India annually. *P. falciparum* is the major infection throughout these states causing considerable mortality. About 11% of the *P. falciparum* cases of India reported from these states (Mahanta *et al.*, 1998). Among all the Northeastern states Assam is the most populated state and contribute majority of the malaria cases (~50%) followed by Arunachal Pradesh, Meghalaya and Tripura (NVBDCP data).

Malaria cases are reported across the region, there is greater concentration of cases in the foothill areas and places which lie close to interstate/international borders (Dhiman *et al.*, 2010a; Dhiman *et al.*, 2011). The available malaria data of seven states of the region (excluding Sikkim) for the years 2007-2011 revealed that the slide positivity rates (SPR) have been higher consistently in Arunachal Pradesh, Meghalaya and Tripura. Percent *falciparum* cases were higher in Tripura, Meghalaya and Assam, whereas malaria attributable death rates were higher in Nagaland, Meghalaya and Mizoram. Meghalaya is comparatively a small state of northeastern region sharing border with adjacent lowlands of Bangladesh. Malaria incidence are commonly reported from these lowland areas and consequently increasing the malaria burden in the state of Meghalaya (Dev *et al.*, 2010).

In India, 58 species of *Anopheles* mosquitoes have been recorded, out of which six species namely, *Anopheles culicifacies*, *An. dirus*, *An. minimus*, *An. fluviatilis*, *An. sundaicus* and *An. stephensi* are regarded as malaria vector of major importance (Dhiman *et al.*, 2010b). In northeastern region 45 species of *anopheles* mosquitoes have been recorded so far (Malhotra *et al*. 1994), of which *An. minimus*, *An. baimai* and *An. fluviatilis* play crucial role in malaria transmission in this region. *An. minimus* is a perennial species where as *An. baimai* (formerly *An. dirus* D) a monsoon species and
An. fluviatilis mostly occurs in the winter months. Due to their anthropogenic behaviour and exophagic characteristics they are highly efficient in transmission of malaria (Dev, 1996, Dev et al., 2001). These vectors are susceptible to DDT and Deltamethrin which are mainly used in the vector control program but because of their exophilic and exophagic behaviour they avoid resting on the sprayed walls and thus avoid killing action of these insecticides (Sharma et al., 2006).

2.2. Antimalarial chemotherapy

The elimination of malaria from most of the regions in Europe and North America lead to a loss of interest in malaria for more than 25 years. Between 1975 and 1999, only 4 of 1393 newly developed drugs were antimalarials (Trouiller et al., 2002). Because of the limited armoury of drugs in endemic countries and a lack of affordable new drugs, malaria control has heavily relied on a restricted number of medicaments mainly belonging to the quinolines and the antifolates. It has been only recently that the artemisinin-based compounds have been introduced widely. Because the useful therapeutic life (UTL) of many of the currently used drugs is severely compromised by drug resistance and newly introduced drugs have to be protected, combination therapy using compounds belonging to different drug classes is strongly recommended (Kremsner and Krishna, 2004; WHO 2001; WHO, 2006). The most common antimalarials used in malaria control programmes, either as mono- or combination therapy and their mode of action are summarised in
Table 1: Mode of action of current antimalarial drug classes

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Members</th>
<th>Target location</th>
<th>Target molecule</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antifolates</td>
<td>PYR, PG SDX, DAP</td>
<td>Cytosol</td>
<td>DHFR, DHPS</td>
<td>Blood- stage scizonticide</td>
</tr>
<tr>
<td>Quinolines</td>
<td>CQ, AQ, QUIN, MEF, HAL, LUM, PRIM*</td>
<td>Food vacuole</td>
<td>Heam, Others?</td>
<td>Blood- stage scizonticide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gametocytocide*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tissue stage scizonticide*</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>Dihydroartemisinin and derivates</td>
<td>Food vacuole</td>
<td>PfATP6 Others?</td>
<td>Blood- stage scizonticide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gametocytocide</td>
</tr>
<tr>
<td>Naphthoquinones</td>
<td>ATQ</td>
<td>Mitochondrion</td>
<td>Cytochrome bc1</td>
<td>Blood- stage scizonticide</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>DOX, TET</td>
<td>Apicoplast</td>
<td>Apicoplast ribosome</td>
<td>Blood- stage scizonticide</td>
</tr>
</tbody>
</table>

2.2.1 Quinolines

The 4-aminoquinolines chloroquine (CQ) and amodiaquine (AQ) and the related quinoline methanols quinine (QUIN) and mefloquine (MEF) have been the mainstay of malaria chemotherapy during much of the past 40 years. Halofantrine (HAL), another related phenanthrene methanol, is no longer recommended due to the occurrence of fatal cardiotoxicity, low bioavailability and its high cost (Nosten et al., 1993). The new analogue lumefantrine (LUM) was developed and is now a component of the combination regimen coartemether (Riamet®, Coartem®; van Vugt et al., 2000). Primaquine (PRIM) is an 8- aminoquinoline which is highly active against gametocytes of all malaria species in humans and the hypnozoites of the relapsing species *P. vivax* and *P. ovale*. It is recommended as antirelapse treatment and gametocytocidal drug
against *P. falciparum* in low or nontransmission areas only (WHO, 2006). Despite extensive studies and the proposition of various mechanisms (reviewed in O’Neill *et al.*, 1998), the mode of action of these drugs is not completely understood. However, the commonly accepted hypothesis is that quinoline containing drugs accumulate in the digestive vacuole (DV) of the intra-erythrocytic parasite and primarily interfere with haemoglobin metabolism. Degradation of haemoglobin by the parasite produces toxic byproducts, the most important being ferriprotoporphyrin IX (FPIX or haem) and free oxygen radicals, which have to be detoxified by a series of parasite-specific processes (Francis *et al.*, 1997). The major mechanisms postulated to be involved in CQ accumulation are,

1) trapping of the weak base CQ in the acidic DV through passive diffusion down a pH gradient,

2) active uptake of CQ by specific transporters, and

3) binding of CQ to the intravacuolar receptor haem (reviewed in Foley and Tilley, 1998).

The build-up of CQ haemcomplexes subsequently interferes with DV functions eventually leading to parasite death through the following mechanisms. CQ interferes with haem detoxification by the inhibition of its polymerization to b-haematin and sequestration as malaria pigment, haemozoin (Bray *et al.*, 1999; Egan *et al.*, 1994; Slater, 1993). The inhibition of peroxidase degradation (Loria *et al.*, 1999) and glutathione-dependent degradation of unpolymerized haem (Ginsburg *et al.*, 1998; Zhang *et al.*, 1999) leads to peroxidative damage of parasite proteins and lipids and membrane disruption. The closely related AQ has been suggested to exert its activity by
a similar mechanism (Foley and Tilley, 1998) because efficiency of haembinding and inhibition of haem-polymerization have been shown to be comparable to CQ (O’Neill et al., 1997; Slater, 1993). Data are conflicting as to whether the mode of action of quinoline/phenantrene methanols is similar to that of CQ (Foley and Tilley, 1997). However, there are several lines of evidence that the interaction with haem is also central to the activity of QUIN, MEF and HAL (Bray et al., 1999; Mungthin et al., 1998; Slater et al., 1993). Drug action of 8-aminoquinolines seems to be different from that of 4-aminoquinolines (Meshnick & Marr, 1992) and PRIM has been proposed to exert its activity by interfering with mitochondrial function (Beaudoin & Aikawa, 1968; Boulard et al., 1983).

2.2.2 Antifolates

In contrast, the primary targets of another important drug class, the antifolates, have long been established. Antifolates exert their antimalarial action by disruption of the de novo biosynthesis of folate, an important co-factor in the amino acid, purine and pyrimidine pathways, which eventually leads to blockage of DNA synthesis and lowered production of glycine and methionine (Krungkrai et al., 1989). There are two important groups of antifolates: 1) the sulpha drugs, such as sulphadoxine (SDX) and dapsone (DAP), which are structural analogues of para-aminobenzoic acid (pABA) and inhibit dihydropteroate synthetase as part of a bifunctional protein with hydroxymethylpterin pyrophosphokinase (PPPK-DHPS), and 2) pyrimethamine (PYR) and proguanil (PG), which is metabolised in vivo to the active form cycloguanil (CG), both inhibiting dihydrofolate reductase as part of the bifunctional enzyme with thymidylate synthetase (DHFR-TS) (Yuthavong, 2002). Due to their marked synergistic effect (Chulay et al., 1984), DHFR and DHPS antagonists are mainly used as
combination regimens, the most common being SDX plus PYR (SP, Fansidar™) and PG plus DAP (Lap-Dap™) (Watkins et al., 1997).

2.2.3 Artemisinins

An important new and entirely different class of compounds originates from the Chinese herb qinghao (*Artemisia annua*) from which the parent compound artemisinin was first isolated in the 1970s. Since then, several analogues, such as dihydroartemisinin, arteether, artemether and artesunate, with better bioavailability have been developed. Artemisinins are endoperoxide-containing sesquiterpene lactones. A number of studies have shown that the endoperoxide-bridge can be cleaved by reductive interaction with iron, yielding free radicals that lead to parasite death, possibly by alkylation of different plasmodial targets (Meshnick *et al*., 1996; Oliaro *et al*., 2001). The formation of covalent adducts between artemisinins and haem and several plasmodial and host proteins have been described, but the precise mechanisms involved in antimalarial activity are still to be resolved (Krishna *et al*., 2004; Meshnick, 2002). More recently, an alternative hypothesis for the mode of action has been proposed, based on structural similarities between artemisinin and thapsigargin, a potent inhibitor of sarcoendoplasmic reticulum Ca2+-ATPases (SERCA s) in a variety of organisms (Eckstein-Ludwig *et al*., 2003). Evidence in favour of this hypothesis included the specific inhibition of the SERCA of *P. falciparum* (*PfATPase6*) by artemisinins, the interference of thapsigargin with the action of artemisinins, the iron-dependent inhibition of *PfATPase6*, and the strong positive correlation between inhibition of *PfATPase6* and death in cultured parasites. The artemisinins have considerable advantage over other antimalarials because they kill parasites more rapidly and affect a broader range of asexual blood stages (Hien & White, 1993). Unlike 4-aminoquinolines
and antifolates, which exert their antiparasitic action on schizonts, artemisinins also impede gametocytes, which limits transmission to new hosts (Price et al., 1996; Targett et al., 2001). Moreover, they inhibit important pathophysiological processes, such as cytoadherence and rosetting, more effectively than other drug classes (Udomsangpetch et al., 1996).

2.2.4 Other drug classes

Atovaquone (ATQ) is a naphthoquinone derivate and a structural analogue of coenzyme Q (ubiquinone). ATQ acts by specifically binding to the ubiquinone oxidation site in the cytochrome bc1 complex (CYT bc1) in the electron transport chain and collapsing mitochondrial membrane potential in the parasite (Srivastava et al., 1999a). Though inappropriate as monotherapy due to rapid selection of resistant parasites, ATQ is clinically successful when used in combination with the synergistically acting partner drug PG (Malarone™) for both, chemoprophylaxis and therapy of P. falciparum malaria (Hogh et al., 2000; Looareesuwan et al., 1999; Srivastava et al., 1999b). A number of antibiotics, such as tetracycline (TET) and doxycycline (DOX), are effective, though slow-acting, antimalarial compounds. They are suggested to inhibit different steps of prokaryote-like protein synthesis in the apicoplast of the parasite (Ralph et al., 2001). However, they are currently used in combination with other drugs or as chemoprophylactic agents in non-immune travellers only (WHO, 2005b).

2.2.5 Chemotherapy In India

In India NVBDCP has laid down the guide lines for treatment of malaria cases. NBVDCP in 2007 has provided new malaria drug policy guidelines for entire India, some of the guidelines are as follows;
1. All the fever cases should preferably be investigated for malaria by microscopy or rapid detection test.

2. The first line of treatment is chloroquine and the second line is ACT (Artesunate + Sulfadoxine/ pyrimethamine) combination. In case resistant to these formulations and to treat severe and complicated malaria, Quinine will be the drug of choice.

3. Microscopically confirmed cases of *P. falciparum* cases should be treated with Chloroquine in therapeutic dose of 25mg/kg body weight over three days and a single dose of primaquine0.75 mg/kg body weight on the first day. The practice is to be followed at all levels including Voluntary Health workers like drug distribution centers, fever treatment depots and accredited social and health activist as well.

According to drug policy all *P. vivax* cases, undiagnosed fever cases and clinical malaria cases should be treated with chloroquine in full therapeutic doses. ACT is the first line of anti malarial drug for treatment of *P. falciparum* in chloroquine resistant areas. According to NBVDCP guide lines all the Northeastern states has switched over to ACT from chloroquine as the first line of treatment since 2008.

2.3 Drug resistant malaria

2.3.1 Epidemiology

The parasite’s ability to develop resistance affects all currently available drugs except the artemisinin derivates, although the degree of resistance varies depending on different drugs and regions (Bloland, 2001). After the introduction in 1943, CQ came into universal use as therapeutic and prophylactic agent against malaria. The success has
been based on high clinical efficacy, good safety and tolerability, ease of use and cost-effective production. However, resistance to CQ was first described at the Thai-Cambodian border in the late 1950s (Harinasuta et al., 1965) and in Colombia and Venezuela in the 1960s (Payne, 1987). A further focus emerged in the 1970s in Papua New Guinea (PNG) (Grimmond et al., 1976). In Africa, CQ resistance was first documented in the late 1970s in Kenya (Fogh et al., 1979) and Tanzania (Campbell et al., 1979), and spread first to the central and southern parts before arriving in West Africa in 1983. By 1989, CQ resistance was widespread in Sub-Saharan Africa (Wernsdorfer and Payne, 1991). Today, \textit{P. falciparum} resistance to CQ occurs everywhere except in Central America, the island of Hispaniola and some regions of Southwest Asia (WHO, 2005b). Recent data from population genetic surveys suggest that CQ resistance emerged independently at a limited number of sites: two in South America (Cortese et al., 2002; Wootton et al., 2002), one in PNG (Mehlotra et al., 2001), and one on the Philippines (Chen et al., 2003). These data had shown similarities of parasites from Asian and African origin, but differences from those from South America and PNG, supporting the hypothesis that parasite migration played a critical role in the spread of CQ resistance (Wellems and Plowe, 2001). Despite the widespread use of CQ, resistance of \textit{P. vivax} has been very limited, apparently having originated in PNG (Rieckmann et al., 1989; Schuurkamp et al., 1992). CQ resistant \textit{P. vivax} malaria may be characterized as endemic to the Indonesian archipelago, sporadic in the rest of Asia, and rare in South America (Baird, 2004). The spread of CQ resistant malaria has led to increasing use of the combination regimen sulphadoxine-pyrimethamine (SP) as standard first-line regimen in many countries. Antifolate resistance emerged almost instantaneously and independently from several areas where the drug had been
introduced on national level. First reported at the Thai-Cambodian border in the late 1960s (Björkman and Phillips-Howard, 1990), high-level SP resistance was rapidly spreading in southeast Asia and the Amazon Basin and moderate frequencies were observed on the Pacific coast of South America, in southern Asia and Oceania (Bloland, 2001). In Africa, sensitivity started to decrease in the late 1980s, with the highest levels reported from the eastern part of the continent (Wongsrichanalai et al., 2002). Similar to CQ resistance, molecular data suggest that resistance to antifolates has arisen at only a few independent foci and was followed by inter- and intracontinental spread of resistant parasites (Cortese et al., 2002; Nair et al., 2003; Roper et al., 2004). Reports of clinical resistance to QUIN have been started to accumulate since the mid-1960s, especially from the Thai-Cambodian border. High levels of resistance were described in Thailand in the 1980s, where the introduction of QUIN monotherapy as interim therapy against SP resistant malaria has led to a rapid decrease in sensitivity to the drug (Wernsdorfer, 1994). Therefore, QUIN has been used in combination with other drugs during the following decades and is currently recommended as second-line regimen against uncomplicated malaria and treatment of severe cases only (WHO, 2006).

Resistance to MEF was first reported from the Thai-Cambodian border in the late 1980s, five years after it has been introduced (Wongsrichanalai et al., 2001). The high level of MEF resistance in Thailand was most probably due to the heavy use of the chemically related drug QUIN (Brasseur et al., 1992). Though there have been sporadic reports of clinical failure from the Amazon Basin, Bangladesh and India (Wernsdorfer, 1994), and reduced in vitro sensitivity of *P. falciparum* strains in Africa has been observed (Jelinek et al., 2001), MEF resistance is rare outside Southeast Asia. Resistance to ATQ developed in 1996, the same year when the drug was introduced
(Looareesuwan et al., 1996) and is currently used as fixed-dose combination with PG (Malarone™) only. Foci of established multidrug resistant malaria, defined as resistance to more than three operational antimalarial compounds, are found at the border region of Thailand and Cambodia and Myanmar, respectively, and some focal areas in the Amazon Basin (Wongsrichanalai et al., 2002).

2.3.2 Drug Resistance In Northeastern India:

Northeastern India is highly endemic to malaria with very high prevalence of P. falciparum infection also recorded antimalarial resistance in last three decades. First case of Chloroquine resistance in India was reported from Karbi Anglong District of Assam in 1973 (Sehgal et al., 1973). Subsequently it has been reported in different part of Northeastern India viz- Arunachal Pradesh, Nagaland, Mizoram and Meghalaya during 1979-81(Pattanayak et al., 1979). Chloroquine resistance strains multiplied fast in this region under influence of very efficient vectors. P. falciparum gradually developed multiple resistances particularly in border areas. Presently entire Northeastern region considered to be nidus of spread of drug resistance (Dev et al., 2003a). Upsurge of malaria cases in 1970s and subsequent reports of treatment failure with antimalarials led the policy makers to review the entire situation and a new programme called P. falciparum containment Programme (PfCP) was launched in 1978 with the help of Swedish International Development Agency (SIDA). The main objective of this programme is to contain the spread of P. falciparum malaria specially the drug resistant ones. In its 10 years of operation drug resistant foci was diluted to a greater extent (Ray et al., 1998).
There have been few studies conducted on antimalarial efficacy from these states. Resistance to chloroquine is wide spread in this region which has been proved by different authors from time to time. Sehgal et al. (1973) reported RI resistance in 52.5% and RII in 22.5% cases. In another study Sehgal et al., (1974) reported 24% RI resistance in some tea garden population of Assam. Gogoi et al (1995) also reported RI, RII and RIII resistance in some Tea garden population in Assam. Baruah et al (2005) reported RI and RII level of resistance in different age groups. Altogether 37% of Chloroquine resistance was observed but no resistance cases were reported for SP treatment. However, most of the studies involve limited number of samples and are of localized nature. In 1997 national level compilation of antimalarial drug resistance was done which included 12863 cases of P. falciparum. Study revealed about 24% cases of chloroquine resistance of varying level. Overall resistance at RI level is common in most of the parts of India except Northeastern States whereas spread of RII and RIII resistance was limited and was restricted to selected areas (Shiv. et al 1997). High level of resistance was generally confined to Northeastern states particularly along international border areas along Bangladesh and Myanmar (Dev et al 2003b). Recently chloroquine resistance of varying level was also reported from different places of Tripura. Dhiman et al., (2010) reported 35% treatment failure in Chloroquine treatment from South Tripura and Majumdar et al., (2011) reported 67.5% Chloroquine resistance in Dhalai District of Tripura. In one of the largest drug sensitivity study conducted in this region Campbell et al., (2006) reported very high treatment failure in case of Chloroquine (95.8%) and SP (57.1%). However resistance to Mefloquine and Mefloquine Artesunate combination is not very much prevalent. Resistance to SP has been recorded in many places mostly in forest fringe areas of Northeastern states.
Resistance to Artemisinin group drugs has not been reported from this area making it drug of choice for treatment of uncomplicated malaria (Campbell et al., 2006 and Mohapatra et al., 1996). Multi drug resistance has also been reported from this area though these are confined to some places only (Dua et al., 2003; Dev et al., 2003). This may be a warning call for policy makers to take appropriate step so that spread of multidrug resistance strain can be stopped. It can be concluded that resistance to chloroquine and other antimalarials is wide spread in this region. Increasing development activities and high human migration in this region may result in spread of resistant strains to new areas and can create new threat to malaria control.

2.3.3 Molecular basis of parasite resistance to antimalarial drugs

Advances in the understanding of the mechanisms of drug action during the last two decades have led to the identification of the putative molecular targets and the genetic basis responsible for parasite resistance to antimalarial drugs. Genetic events conferring resistance include single point mutations in or changes of copy numbers of genes encoding drug targets, such as important enzymes or transporters regulating intraparasitic drug concentrations.

2.3.3.1 Resistance to quinolines

Recent molecular analyses strongly argue for multiple genes and epistasis, rather than a single genetic determinant, to be involved in CQ resistance (Anderson et al., 2005; Duraisingh & Refour, 2005b). The two main characteristics that distinguish CQ resistant from CQ sensitive prasites are diminished accumulation of CQ in the parasite’s digestive vacuole (DV) and reversal of resistance through chemosensitization by verapamil (VP) or other Ca2+-channel blockers (Krogstad et al., 1987; Martin et al.,
1987). These observations suggested that CQ resistance is most probably associated with altered drug transport processes into the DV and several genes encoding candidate proteins involved in the transport of CQ into or out of the DV have been proposed: *P. falciparum* multidrug resistance gene 1 (*pfmdr1*), candidate gene 2 (*cg2*), and *P. falciparum* chloroquine resistance transporter (*pfcrt* or *cg10*).

*Pfmdr1*, which is localized on chromosome 5, encodes a P-glycoprotein homolog (Pgh1) and has been localized to the parasite DV (Foote *et al.*, 1989; Cowman *et al.*, 1991). Pgh1 has a typical structure shared by members of the ATP binding cassette (ABC) transporter family (Endicott and Ling, 1989). Initial sequence analysis of the full-length *pfmdr1* revealed five polymorphic residues that appear to be dimorphic: N86Y, Y184F, S1034C, N1042D, and D1246Y (Foote *et al.*, 1990a). In the same study, *pfmdr1* mutations were strongly linked to the CQ resistant phenotype, but several subsequent studies failed to confirm the association (Basco and Ringwald, 2002). Moreover, analysis of the progeny of a genetic cross between a CQ resistant and a CQ sensitive parasite line found no association between inheritance of the CQ resistant phenotype and the *pfmdr1* locus (Wellems *et al.*, 1990). However, more recent experiments utilizing newly available transfection methods have shown that *pfmdr1* mutations can increase resistance levels to CQ (Reed *et al.*, 2000). In field studies, most attention has been given to the investigation of the *pfmdr1* N86Y allelic variant which is widespread in Africa and Asia. Several studies have demonstrated the selection of the mutant allele following treatment with CQ or AQ (Duraisingh *et al.*, 1997; Sutherland *et al.*, 2002). A positive, though incomplete, association has also been found between *pfmdr1* N86Y and *in vivo* CQ resistance by several authors (Basco *et al.*, 1995; Nagesha *et al.*, 2001; von Seidlein *et al.*, 1997). Nevertheless, numerous other studies have
demonstrated contradictory results (Bhattacharya et al., 1997; Basco and Ringwald, 1997; Haruki et al., 1994; Pillai et al., 2001). The allelic variant pfmdrl N86Y has not been observed in a large number of South American strains. In contrast, the triple mutation S1034C, N1042D, plus D1246Y is more prevalent on this continent, but was seen in CQ resistant and CQ sensitive parasites (Foote et al., 1990a; Huaman et al., 2004; Povoa et al., 1998). Variation in copy number of the pfmdrl gene was observed in a number of CQ resistant isolates (Barnes et al., 1992; Foote et al., 1989), but this correlation could not be confirmed in a wide variety of field and laboratory strains (Basco et al., 1995; Wellemes et al., 1990).

A series of highly systematic experiments using the progeny of the genetic CQ resistant-CQ sensitive cross led to the discovery of the cg2 gene family on chromosome 7. Different members were screened for polymorphisms that might correlate with the CQ resistant phenotype in a large array of laboratory-adapted P. falciparum strains from around the world. An initially promising candidate, cg2, showed a complex pattern of polymorphism that was tightly, but not perfectly, linked with CQ resistance (Su et al., 1997). Though the weak association between allelic variants of cg2 and CQ resistance was confirmed by some studies (Basco and Ringwald, 1999), others found no correlation (Sharma et al., 2001). Moreover, transfection experiments, where the cg2 gene from CQ resistant parasites was replaced with the variant from their CQ sensitive counterparts, showed no effect on the level of CQ resistance in the transformed parasites (Fidock et al., 2000a). Further analysis of the progeny of the genetic cross of Wellemes et al. (1990) localized the CQ resistance determinant to a 36kb segment on chromosome 7 (Su et al., 1997; Wellemes et al., 1991). Subsequent studies of the segment identified a highly interrupted gene with 13 exons, termed pfcrter, encoding a putative transporter.
protein that was localized to the DV membrane of the parasite. Several polymorphisms in \textit{pfcrt} showed linkage to the CQ resistant phenotype in a large set of laboratory-adapted \textit{P. falciparum} lines from Africa, South America, and Southeast Asia, but with considerable variations depending on the geographical region (Cooper \textit{et al.}, 2002; Fidock \textit{et al.}, 2000b). Furthermore, an allelic exchange approach replacing the endogenous \textit{pfcrt} allele of a CQ sensitive strain with \textit{pfcrt} from CQ resistant lines from different origins provided conclusive evidence that mutant haplotypes of the \textit{pfcrt} gene product confer CQ resistance with characteristic VP-reversibility and reduced CQ accumulation (Sidhu \textit{et al.}, 2002). The mutation K76T seems to play a major role in determining the CQ resistant phenotype since it was invariably found in all CQ resistant strains so far. The mutation is usually not isolated, but associated with other single nucleotide polymorphisms (SNP) at other codons, C72S, M74I, N75E, H97Q, A220S, Q271E, N326S/D, I356T/L and R371T/I, the role of which is not very well defined. It was suggested that these mutations might play a critical role in maintaining important functional properties of the protein in CQ resistant parasites (Wellems and Plowe, 2001). The importance of the K76T mutation has been further corroborated by several clinical studies which have shown a higher prevalence of the K76T mutation in post-treatment than in pre-treatment samples, which alludes to a strong selection towards the mutant allele under CQ treatment (Djimde \textit{et al.}, 2001a; Schneider \textit{et al.}, 2002). Moreover, the presence of the mutant allele has been shown to be present in all \textit{P. falciparum} isolates which failed treatment with CQ (Basco \textit{et al.}, 2002; Djimde \textit{et al.}, 2001a; Thomas \textit{et al.}, 2002). However, the mutation has also been observed in CQ sensitive isolates (Kyosiimire-Lugemwa \textit{et al.}, 2002) which suggest that either additional mutations in \textit{pfcrt} or other genes may be involved in the determination of the
CQ resistant phenotype. Interestingly, it has recently been shown that \textit{pfcr} K76T mediated parasite resistance was reversed by concomitant carriage of the \textit{pfcr} mutation S163R, and further modified by the additional mutation in \textit{pfcr} T152A (Johnson \textit{et al.}, 2004). Currently, there is no evidence that alterations in gene copy number or expression levels of \textit{pfcr} are involved in CQ resistance (Durrand \textit{et al.}, 2004). Regarding the proposed similar, though not equal, mode of action of the related quinoline drugs MEF, QUIN, HAL and LUM, molecular studies on parasite resistance to these drugs have mainly focused on the two membrane transporter genes \textit{pfcr} and \textit{pfmdrl}. Mutations in \textit{pfcr} have been shown to be associated with resistance to QUIN (Mu \textit{et al.}, 2003). In addition, several \textit{in vitro} studies could demonstrate that point mutations in \textit{pfmdrl} modulate resistance to MEF, QUIN and HAL (Reed \textit{et al.}, 2000; Duraisingh \textit{et al.}, 2000). However, more recent \textit{in vivo} studies conducted in Peru and Gabon did not provide evidence for an association between \textit{pfmdrl} mutations and MEF resistance (Mawili-Mboumba \textit{et al.}, 2002). Interestingly, the presence of the \textit{pfmdrl} wild-type allele N86 has been found to be associated with \textit{in vitro} resistance to MEF (Duraisingh \textit{et al.}, 2000; Price \textit{et al.}, 1999) and more recently, with \textit{in vivo} resistance to LUM (Sisowath \textit{et al.}, 2005). Amplification of the \textit{pfmdrl} gene copy number has been found to be associated with resistance to MEF and HAL in both, laboratory (Cowman \textit{et al.}, 1994, Peel \textit{et al.}, 1994) and field (Price \textit{et al.}, 1999) isolates. Though amplification of \textit{pfmdrl} seems not to be a prerequisite for increased MEF resistance (Lim \textit{et al.}, 1996; Chaiyaroj \textit{et al.}, 1999), its important role in predicting \textit{in vitro} and \textit{in vivo} MEF failure has been shown in Thailand (Price \textit{et al.}, 2004). Moreover, recent experiments using \textit{pfmdrl} knockdown clones of the parasite could provide further evidence for this gene modification to be important in mediating resistance to MEF, QUIN, and HAL (Sidhu \textit{et}
al., 2006). More recent studies have demonstrated that other genes, such as *pfmrp* (multidrug resistance protein) or *pfinhe1* (sodium hydrogen exchanger), and as yet not fully characterised loci encoding other transporter molecules, are involved in conferring resistance to quinolines (Ferdig *et al*., 2004; Klokouzas *et al*., 2004; Mu *et al*., 2003). These findings further underscore the current hypothesis that phenotypic resistance to this drug class requires the involvement and interaction of many different genes (Bray *et al*., 2005; Duraisingh and Cowman, 2005b).

Two major haplotypes CVIET and SVMNT are mostly found to be associated with Chloroquine resistance in malaria endemic regions (Awasthi *et al*., 2011, Vathsala *et al*., 2004). These data can provide valuable information of spread and evolutionary history of chloroquine resistance in *P. falciparum*. Four different chloroquine resistant haplotypes along with two mother haplotypes CVIET and SVMNT of *pfcrt* has been reported from this area. SVMNT haplotype is present in isolates from majority in India. Whereas CVIET haplotype is more prevalent in highly endemic areas with high transmission rate such as Northeastern states. Association of this allele with higher Chloroquine resistance is also observed. Isolates with single mutant allele were few and are reported from Assam and Arunachal Pradesh. Chloroquine sensitive haplotype CVMNK was also found in Northeastern states but at a lower frequency (2-3%) (Mitra *et al*., 2006). Evolutionary studies suggested that India seems to have received chloroquine resistant *P. falciparum* from Southeast Asian countries. Observing distribution of different haplotypes it is inferred that CVIET haplotype might have entered India through Myanmar from Southeast Asia. Again SVMNT haplotype which was originated from Papua New Guinea seems to be reached India through Southeast Asia via Andaman and Nicober Islands (Awasthi *et al*., 2011).
2.3.3.2 Resistance to antifolates

Though the detailed molecular basis of parasite resistance to antifolates is not yet completely clear, a variety of studies, including genetic analyses, biochemical assays and transfection experiments, have contributed to a better understanding of the molecular events involved in resistance to the individual antifolate compounds.

Resistance to PYR is caused by mutations in the \( pf\text{d}hfr \) gene, which lead to weaker drug binding, but maintain enzyme activity (Chen et al., 1987; Cortese & Plowe, 1998; Sirawaraporn et al., 1997). Several studies have shown the key role of the S108N mutation in conferring the PYR resistant phenotype in \( P.\ falciparum \) (Cowman et al., 1988; Peterson et al., 1988). Additional mutations at codons N51I, C59R and I164L progressively enhance resistance to the drug (Basco and Ringwald, 2000; Wang et al., 1997a). Triple mutants S108N+N51I+C59R are mainly seen in Africa and Southeast Asia, where they are responsible for high level PYR resistance (Hyde, 1990; Sibley et al., 2001). Though relatively uncommon in Southeast Asia and South America (Beren et al., 2003; Biswas et al., 2000; Plowe et al., 1997), and only sporadically reported from single foci in Africa (Alker et al., 2005; Hastings et al., 2002; Staedke et al., 2004), quadruple mutants (plus I164L) represent the severest form of resistance and are responsible for high level resistance to the DHFR inhibitors PYR and CG. The allelic variation A16V coupled with an alternative change at position 108 (S108T) is involved in resistance to CG, with only moderate loss of sensitivity to PYR (Foote et al., 1990b; Peterson et al., 1990). Amino acid changes C50R/I and V140L in \( pf\text{d}hfr \) are rare and were only observed in isolates originating from single foci in South America (Vasconcelos et al., 2000). \( Pf\text{d}hfr \) mutations have been shown to segregate with the drug resistant phenotypes in a genetic cross (Peterson et al., 1988) and final proof for
their role in PYR resistance has been obtained by parasite transfection experiments (Wu et al., 1996). Though never been demonstrated in vivo, chromosomal rearrangement and gene amplification were demonstrated under drug pressure in vitro (Thaithong et al., 2001) and can not yet be ruled out as contributory factors to clinical resistance.

Similarly to pfldhfr, resistance to SDX and other sulpham drugs is associated with decreased drug binding and has been linked to mutations in pfldhps (Triglia et al., 1999). Amino acid changes at five different sites, S436A/F, A437G, K540E, A581G and A613S/T, have as yet been reported (Brooks et al., 1994; Triglia and Cowman, 1994; Triglia et al., 1997; Wang et al., 1997a). As done for pfldhfr, the role of these mutations in conferring resistance to sulpham drugs has been demonstrated in cross-mating and allelic exchange experiments (Triglia et al., 1998; Wang et al., 1997b). Whereas the S108N change in pfldhfr seems to be a prerequisite for further accumulation of mutations which results in a progressive increase in PYR resistance, a similar, but less clear-cut, situation is assumed for pfldhps, since the A437G mutation, alone or in combination with additional mutations in the gene, predominated in field isolates (Sibley et al., 2001).

An important aspect of antifolate resistance is the rapid selection of resistant parasites due to pharmacologically sub-optimal amounts of drugs persisting in the body after treatment, the reason being that PYR and SDX have long elimination half-lives of 116 h and 81 h, respectively (Diourte et al., 1999; Watkins et al., 1997). Selection pressure exerted by the short-acting antifolates CG and DAP has been shown to be lower (Curtis et al., 1998; Nzila et al., 2000a).

The question how much mutations in pfldhfr and pfldhps contribute to the level of in vivo antifolate resistance has been and still is a matter of debate. Laboratory and field studies
dealing with this question are difficult for many reasons. Systematic investigations are hampered by the fact that antifolate drugs are frequently used in combination and act synergistically. Furthermore, numerous different pf dhfr/pfdhps haplotypes are observed in field samples (Plowe et al., 1997). However, there is ample evidence for a positive correlation between the number of mutations in both genes and the level of prior SP usage. In the Middle East for instance, where little SP has been used, all isolates had wild type pf dhfr and pfdhps, whereas most isolates from Southeast Asia were highly mutated in both genes (Wang et al., 1997a). Also a number of sites in Africa, where SP has been widely used within the last decade, reported high prevalence rates of triple-pf dhfr plus double-pfdhps genotypes, such as in Northern Tanzania where rates up to 60% were measured in community surveys (Pearce et al., 2003).

The overall tendency is to consider that the triple-pf dhfr mutation could be a useful genetic marker for in vivo resistance to SP and that point mutations in pfdhps play a secondary role in determining treatment failure (Basco et al., 1998; Mockenhaupt et al., 2005; Mugittu et al., 2004). However, there are several other authors who claim mutations in pfdhps to be equally or even more important in predicting treatment response to SP (Berens et al., 2003; Dorsey et al., 2004). Reports are conflicting because host factors confound the association between molecular resistance markers and in vivo drug response. In addition, the investigation of a relationship is further complicated by the fact that many, but not all, Plasmodium strains have the ability to use exogenous folate from the host. This salvage pathway (i.e., exogenous folate utilization via a pathway that obviates the need for DHPS), which is believed to provide only a minority of folate production in the parasite, the majority being produced by de novo biosynthesis, can be blocked by PYR (Wang et al., 1997b; Wang et al., 1999).
This might not only be a possible explanation for the observed synergy of drug action between PYR and SDX, it could also be a putative explanation for the asymmetric selection of mutations in *pf dhfr* and *pf dhps*, which has been demonstrated in many *in vitro* and field studies (Mberu *et al.*, 2000; Nzila *et al.*, 2000b; Plowe *et al.*, 1997). Selection for mutations in *pf dhfr* occurs first and mutations in *pf dhps* are only selected if parasites carry at least a double mutation in *pf dhfr*. It therefore seems that mutations in *pf dhps* become important once resistance in *pf dhfr* has reached a degree where therapeutic levels of PYR are not sufficient anymore to kill the parasite by the inhibition of DHFR alone (Sims *et al.*, 1999). Though the exact genetic basis for this ‘folate effect’ is not fully elucidated at this time, current molecular hypotheses assume the locus for the differences in folate utilisation to be closely linked to *pf dhfr* (Wang *et al.*, 1997b; Wang *et al.*, 2004). However, data about the prevalence of this capacity in natural parasite populations and its contribution to a SP resistant *in vivo* phenotype are still scarce (Dzinjalamala *et al.*, 2005). More recently, the conversion of SDX by DHPS to sulpha-pterin adducts, which have inhibitory effects further downstream the folate biosynthesis pathway, has been shown (Mberu *et al.*, 2002). The effect was independent of mutations in *pf dhfr* or *pf dhps* and led to the hypothesis that sulpha drugs could inhibit the parasite by mechanisms other than the blockage of DHPS and therefore, resistance could be mediated by other genes (Patel *et al.*, 2004). Moreover, the assumption that these drug adducts have detrimental effect on folate synthesis led to the speculation that sulph drugs resistant parasites may be selected on the basis of lower production of these toxic adducts rather than reduced competition for binding to DHPS (Hyde, 2005b).

It has been proven that higher number of mutation gives rise to higher level of resistance (Ahmed *et al.*, 2004). In Northeastern states frequency of triple mutation is
present in higher number than North India which indicate higher level of resistance to Pyrimethamine (Ahmed et al., 2006). Again mutations at sulfadoxine binding enzyme \textit{P. falciparum} dihydropteroate synthetase (\textit{Pfdhps}) have shown its reduction in binding capacity rendering it to become resistant (Triglia et al., 1997). There are five different mutations at amino acid position 436,437,540,580 and 613 in \textit{pfdhps} which are associated with Sulfadoxine resistance. Wild type allele of \textit{pfdhps} are mostly prevalent throughout India except some places like Andaman & Nicobar Island .In Assam four types of mutant alleles were found indicating higher resistance level. Isolates from Northeastern states showed higher number of multi-locus mutation and thus could be having higher level of SP resistance than other parts of India (Ahmed et al., 2004; Lumb et al., 2009).

\textbf{2.4. Genetic diversity of \textit{Plasmodium falciparum}:}

\textit{Plasmodium falciparum} is the most virulent species among all the human malaria parasite causing malaria associated complications leading to death. This malaria parasite show great diversity in epidemiological characteristics as well as polymorphism in genes coding antigenic proteins which have profound implications in development of natural immunity against the parasite. Different parasite antigen e.g.- merozoite surface antigens, circumspozoite protein, apical membrane antigen etc. show considerable genetic variation in the natural population. Many of these antigens are targeted for development of vaccine against malaria. Polymorphism in these surface antigens helps the parasite to develop immune evasion mechanism. The genetic complexity and in particular its ability to generate mutant variant, makes it a successful pathogen. Information on vaccine candidate gene polymorphism has direct relevance on successful implementation of vaccine using that candidate (Kiwanuka., 2009).
Merozoite surface protein 1 (msp1) is divided into 17 blocks, based on the analysis of sequence diversity: seven highly variable blocks are interspersed with five conserved and five semiconserved regions. Block 2 of the msp1 gene appears to be subjected to rapid intragenic recombination and so is highly polymorphic (Snounou et al., 1993). The Block 2 region of the N-terminal of the msp1 gene is trimorphic and three allelic families: MAD20, KI and RO33 have been identified (Tanabe et al., 1987; Ferreira et al., 1998) and contain antigenically unique tripeptide repeats with extensive diversity in the number of repeats (Happi et al., 2004; Tetteh et al., 2005).

Merozoite surface protein 2 (msp2) is a surface glycoprotein and is widely studied as a major vaccine candidate. Gene sequence of the gene reveals that it has conserved N and C terminal domains (Block 1 & 5), two non repetitive variable regions (Block 2& 4) and polymorphic central region (Block 3) containing variable number of tandem repeats, which also vary in sequence and length (Basco et al., 2004; Eisen et al., 1998; Joshi et al., 2007 and Roussilhon et al., 2007). MSP2 alleles differ in number and sequence of intragenic repeats can be grouped into two allelic families FC27 and 3D7/IC, according to central dimorphic domain.

Glutamate Rich Protein (GLURP) is located on Chromosome 10 and has a high degree of polymorphism. It is present in all parasite stages and is expressed in both the pre-erythrocytic and erythrocytic stages of the parasite including on the surface of newly released merozoites (Borre et al., 1991). It contain 1271 amino acids including an amino terminal non repetitive region (R0), a central repeat region (R1) and an immunodominant C-terminal repeat region (R2). GLURP is highly antigenic and there are few polymorphisms in the gene encoding GLURP in P. falciparum isolates from different geographic regions (Theisen et al., 1995; de-Stricker et al., 2000). Polymorphisms in
GLURP mainly involve variations in the numbers of repeats of certain genomic sequences that therefore affect the size of the gene and its protein product.

Generation of antigenic diversity can be mainly attributed to sexual reproduction in mosquito host, which is believed to be dependent on transmission intensity. It is suggested that frequent recombination event between surface antigens intermittently generate novel alleles in high transmission areas (Tanabe et al., 2007). Diversity in the above discussed genes had been reported from different parts of world which show variation according different parameters like time and place, transmission intensity, disease profile and epidemiological characteristics. *Plasmodium falciparum* diversity is thought to reflect endemicity (Kiwanuka N., 2009). It is suggested that in endemic areas, the number of clones of malaria parasite co infecting a single host can be a useful indicator of the level of transmission and/ or the immune status of the host (Owusu-Agyei et al., 2002; Raj et al., 2004; ). Increase in the transmission level is generally associated with progressive increase in the average number of malaria parasite clones per host (Appawu et al., 2004). Higher diversity than that expected in isolates from areas of intermediate and low transmission intensity has been reported by earlier investigators (Paul et al., 1998; Ferreira et al., 1998) while some other authors (Peyerl-Hoffmann et al., 2001) found no relationship between transmission intensity and parasite diversity. It has also been proposed that allelic diversity of *P. falciparum* may not be entirely dependent on frequency of transmission but also on multiplicity of infection, number of alleles prevalent in the local parasite population, immune status of the population and drug pressure among other factors (Ferreira et al., 1998; Hastings, 2002; Takala et al., 2006).
Parameters describing the infection dynamics of *P. falciparum* are important
determinant of the potential intervention Parameters describing the infection dynamics
of *P. falciparum* are important determinants of the potential impact of interventions and
are potential outcome measurements for malaria intervention trials. Low parasite
densities, periodic sequestration of parasites, and the presence of multiple concurrent
infections make it essential to use molecular techniques to estimate the force of
infection and duration of infections in endemic areas. To date, a high degree of
polymorphism has been demonstrated at both the *msp*-1 and *msp*-2 loci in parasites
from areas of stable malaria transmission. As a consequence, in such areas it is rare to
find parasites of the same two-locus genotype in more than one subject. Some surveys
have documented *P. falciparum* multiplicity of up to nine different parasite clones at a
given time in a single asymptomatic host (Ranjit *et al.*, 2005). In the last decade, several
studies has been carried out to investigate if any relationship exists between multiplicity
of infection and variables like parasite density, age and infection outcomes. Studies
conducted in Tanzania60 and in Papua New Guinea61 suggested that, individuals
having substantial previous exposure to malaria, co-infection with multiple clones of *P.
falciparum* can protect against subsequent clinical malaria attacks (Al-Yaman *et al*.,
1997; Beck *et al*., 1997). However, other studies, mainly of individuals with little
previous exposure such as in infants, multiplicity was positively associated with parasite
density and risk of clinical morbidity (Felger *et al*., 1999; Mayor *et al*., 2003) contrary
to the suggestion from many studies that high multiplicity is protective against clinical
malaria. As with parasite density, the relationship between multiplicity and age is still
unclear—some studies have reported decrease in multiplicity with age (Lindblade *et al*.,
1999), others have observed a positive correlation in infants and children but not in
older individuals (Engelbrecht et al., 2000, Smith et al., 1993). Some reports have indicated decrease of MOI during adulthood to the levels found in infants (Mayor et al., 2003) while others did not observe any relationship between the two parameters (Felger et al., 1999; Zwetyenga et al., 1998). This suggests that the mechanisms controlling multiplicity of infection and parasite densities follow different profiles and so are different.

Genetic diversity of the Plasmodium falciparum vary considerably in different parts of the world even in nearby areas. It is important to study the diversity at micro level as well as in large scale to get an idea of the parasite population structure, which may answer numerous complicated questions like epidemiological pattern, disease complexity, role of transmission intensity etc.

In India limited numbers of studies have been carried out to study the genetic diversity of P. falciparum. Joshi et al., 2007; have reported msp1 and msp2 diversity in different areas of India. They have recorded all the prevalent alleles of both the genes with varying proportion. Presence of higher proportion of multiclonal infection as well as high MOI in highly malarious areas of Karbi Anglong District (Assam) and Sundergarh (Oddisa) indicate pattern of infection in high transmission areas. They have also found considerable similarity in sequence and allele pattern indicating considerable amount of gene flow between states. Baruah et al., 2009 have also reported high degree of diversity and high MOI of msp1 in different parts of Assam. They have also related presence or absence of certain allele in different transmission season. Other workers have also reported high multiclonal infection among Indian isolates and correlated multiplicity of infection with malaria endemicity (Raj et al., 2004; Ranjit et al., 1999). Pattern of diversity in severe malaria cases revealed certain alleles are mostly found in
severe cases and MOI of msp2 is higher in severe cases (Ranjit et al., 2005). Bhattachrya, 1999 have reported only FC27 allele of msp2 in Oddisa and West Bengal isolates. Pattern of msp1 and msp2 diversity also show similarity with other countries of Southeast Asia. Again diversity of GLURP correlated with malaria endemicity of the area i.e more the endemicity higher the diversity. Previous studies of GLURP in low malaria endemicity areas have reported two alleles in Honduras (Haddad et al. 1999), three alleles in French Guyana (Arley et al. 1999) and four alleles in Colombia (Montoya et al. 2003). In the highly endemic areas of Africa and Asia, anywhere from eight GLURP alleles in India to 20 alleles in Sudan have been reported (A- Elbasit et al., 2007; Mlambo et al. 2007, Mwingira et al., 2011; Ranjit et al., 2005; Snounou et al. 1999).