V. Discussion:

5.1 Malaria epidemiology:

India’s northeastern part of is strategically very important area. This area is characterized by long International border with neighbouring countries, difficult terrain and poor communication and health infrastructure. Favourable topographic and demographic factors of this region contribute in very high incidence of malaria. *P. falciparum* is the dominant infection throughout these states causing considerable mortality. 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) (Fig 1).

Fig 1: Malaria situation in northeastern states of India.

Although entire northeastern region is prone to malaria, foothill areas and places which lie close to interstate/international borders (Dhiman *et al.*, 2010a; Dhiman *et al.*, 2011) are more affected. 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 (Table 1).

**Table 1: Slide positivity rate (SPR) in northeastern states**

<table>
<thead>
<tr>
<th>States</th>
<th>Arunachal Pradesh</th>
<th>Assam</th>
<th>Meghalaya</th>
<th>Manipur</th>
<th>Mizoram</th>
<th>Nagaland</th>
<th>Tripura</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2007</td>
<td>13.06</td>
<td>3.92</td>
<td>11.00</td>
<td>0.99</td>
<td>0.77</td>
<td>3.95</td>
<td>6.56</td>
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<tr>
<td>2008</td>
<td>11.62</td>
<td>3.12</td>
<td>11.22</td>
<td>0.53</td>
<td>0.62</td>
<td>4.45</td>
<td>7.59</td>
</tr>
<tr>
<td>2009</td>
<td>10.32</td>
<td>3.02</td>
<td>15.31</td>
<td>0.93</td>
<td>0.63</td>
<td>5.43</td>
<td>6.75</td>
</tr>
<tr>
<td>2010</td>
<td>9.44</td>
<td>1.59</td>
<td>9.53</td>
<td>0.80</td>
<td>0.75</td>
<td>2.71</td>
<td>7.24</td>
</tr>
<tr>
<td>2011</td>
<td>6.83</td>
<td>1.14</td>
<td>6.71</td>
<td>0.66</td>
<td>0.84</td>
<td>1.76</td>
<td>5.25</td>
</tr>
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</table>

Number of fatal malaria species *P. falciparum* were higher in Tripura, Meghalaya and Assam, whereas malaria attributable death rates were higher in Nagaland, Meghalaya and Mizoram.

**Fig 2: Prevalence of *P. falciparum* infection in Northeastern India**

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).
Epidemiological survey in the South Tripura District indicate that the rate of malaria incidence along the indo Bangladesh border areas varied to a considerable extent between the age groups but not over the years. *P. falciparum* infection is the predominant one in all the seasons however; percent parasitaemia is highest in the premonsoon season in comparison to the monsoon season.

Malaria transmission is perennial and persistent throughout the northeastern India with peak transmission occurring during May to September i.e. the entire rainy season (Dev et al., 2003). Major malaria vectors *An. minimus, An. dirus* and *An. fluvialis* causes the maximum transmission of malaria. Moreover vectors of secondary importance like *An. phillipinensis/nivipes, An. annularis, An. culicifacies, An. maculates* may also playing important role in transmisssion of malaria. Monthly malaria incidence in the study area reflects a typical malaria incidence scenario prevalent in northeastern India i.e. peak transmission during June and July. The biting rate of major malaria vector *An. minimus* also found to attain a sharp peak during May to June (Dev et al., 2004).

In the present study, most of the malaria cases reported fro the regions were attributed to *P. falciparum* (88.3%). More than 60% of the patients were children below 15 years of age and children of the age groups 1-4 were found to be highly susceptible. In Assam there is significant difference among age groups with regard to incidence of malaria (Dev et al., 2004). There was an increasing trend in the proportion of malaria with age in males up to 15 years of age. In central India prevalence of malaria is highest among children of age group 4-8 years followed by those in 1-4 years age group (Singh et al., 2006). Again in Gujarat children of age group 11-14 years are most susceptible to malaria.

There was no gender bias in the incidence of malaria although the males were generally exposed to the vectors due to their outdoor activity and poor clothing. No appreciable difference was observed in the proportion of malaria cases between the sexes in Assam except in 5-15 years
olds in whom the proportion of females was lower (Dev et al., 2004) unlike Central India were both the sexes were equally affected by malaria irrespective of their age group.

The percent parasitemia among the village population found to vary between the seasons and years. More than 5% of the malaria positive cases recorded in the study area were detected to be having P. falciparum / P. vivax mixed infections although they are generally considered to be as P. falciparum infection and treated accordingly. P. falciparum was clearly the dominant parasite species as the percent parasitemia of P. falciparum in mixed infection was higher than that of P. vivax.

5.2 Molecular epidemiology of drug resistance:

The areas along Indo-Bangladesh international border have been under tremendous antimalarial resistance due to various reasons (Dhiman et al., 2010a). The present study suggested that K76T and N86Y mutations are prevalent in the region in quite a high number (Fig 3).

**Fig 3: Distribution of Pfcrtrt and Pfmdr1 wild and mutant genotypes**

![Graph showing distribution of Pfcrtrt and Pfmdr1 wild and mutant genotypes](image)

Most of the chloroquine treatments failed during early treatment stage only indicating that the chloroquine should no longer be used as drug of choice against falciparum malaria. Previous studies conducted in the same area found that the chloroquine ETF cases were predominant as
compared to the LTF cases (Dhiman et al., 2010a). However some studies report that the LTF cases are considerably higher than the ETF cases (Lemnge et al., 2006; Saha et al., 2011). The treatment failure has been highest among the children. Studies have suggested that parasite density, anemia, age, body temperature and immunity influence the treatment outcome among the children (Dhiman et al., 2010; Dorsey et al., 2001; Lemnge et al., 2006).

In the recent years, in addition to the conventional in vivo and in vitro methods, molecular markers based approach to study and elucidate the antimalarial drug resistance has proved useful (Menard et al., 2006). Currently, we have described the distribution of pfmdr1 N86Y mutation in the study area and attempt has been made to evaluate the correlation of these mutations with in vivo clinical outcome. The association of pfmdr mutation with chloroquine resistance has suggested that the K76T mutation has been most reliable molecular marker in chloroquine resistance (Djimde et al., 2001a; Djimde et al., 2001b, Mallick et al., 2012).

Pfmdr1 N86Y mutations were 39.4% and 44.44% respectively in the study population; however distribution of these mutations in both chloroquine susceptible and resistant cases varies considerably. K76T mutation was found in all the in vivo resistant cases; however its presence was not exclusive to resistant cases and also found in 6 ACPR cases. On the other hand, N86Y mutation, though more frequent, was found in 10 in vivo resistant cases only. Both wild and mutant types of Pfmdr1 were present in CQ susceptible cases also (Fig 4).
Fig 4: Distribution of \textit{Pfcr}t and \textit{Pfmdr} 1 wild and mutant genotypes in resistant and susceptible cases

![Graph showing distribution of \textit{Pfcr}t and \textit{Pfmdr} 1 genotypes]

The previous study has suggested the distribution of K76T mutation appeared with more than one \textit{pfcr}t haplotypes and therefore not much reliable in detecting chloroquine resistance (Vieira \textit{et al.}, 2004). Different types of haplotype of \textit{Pfcr}t gene had been described based on the amino acid residues present from position 72\textsuperscript{nd} to 76\textsuperscript{th} (Awasthi \textit{et al.}, 2011; Vathsala \textit{et al.}, 2004). \textit{Pfcr}t haplotyping of the samples showed four type of haplotype viz- CVIEK, CVMNK, CVIET and CVMNT. The wild type haplotypes CVIEK and CVMNK were limited to ACPR and could not be recorded in treatment failure cases. On the other hand, the mutant type haplotypes CVIET and CVMNT were found in all the treatment failure cases, of which CVIET was more frequently distributed (Fig 5). Presence of CVMNT haplotype may be linked to more severe form of the disease prevalent in these areas as it is linked to complicated infections (Awasthi \textit{et al.}, 2011).
Study evidences that merely presence of mutant haplotype may not necessarily confer to the chloroquine resistance. The presence of haplotypes as molecular markers may correspond to malaria parasites intrinsic characteristics but may not be conclude to the treatment failure (Djimde et al., 2003; Dorsey et al., 2001; Pillai et al., 2001; Vieira et al., 2004). The treatment failure may depend upon the host immunity and interactions of host with parasite and drug (Pillai et al., 2001). Mutant haplotype CVIET is more endemic in this region; however few studies have suggested that multiple mutant pfcrt haplotypes were observed in high malaria transmission regions (Mitra et al., 2006; Sa et al., 2009; Vieira et al., 2004.). CVIET haplotype is expected to be observed in the northeastern states and might have been spread due to inbreeding of *P. falciparum* in the study area. The areas which have low level of complex and multiclonal malaria infections, the inbreeding of malaria parasite having mutant genotype could spread the antimalarial drug resistance at an extraordinary rate (Paul et al., 1996). The prevalence of SVMNT haplotype in highly malaria endemic study area indicates the widespread of chloroquine resistant *P. falciparum*, which might have evolved due to prolonged use of antimalarial amodiaquine in malaria chemotherapy (Alifrangis et al., 2006; Dittrich et al., 2005; Pandya et al., 1994). Studies have evidenced that *P. falciparum* mutant Pfcrt haplotypes
have selective advantage in competitive mosquito infections by protecting immature gametocytes from chloroquine (Ecker et al., 2011).

Molecular analyses of haplotypes associated with chloroquine drug resistance in *P. falciparum* isolates suggest that mutant type haplotypes of *pfcrt* are widely distributed in the study area. *Pfcr* K76T mutant haplotypes, though detected in all the treatment failure cases, could not be sufficient in deciding the chloroquine resistance. Similarly, *Pfmndr1* N86Y marker has very limited role in corresponding the chloroquine resistance. The study emphasizes that the *Pfcr* mutant haplotypes are spreading diligently, which needs to be taken care while devising effective malaria treatment policy.

Again in Sonitpur District the selected study areas for the present study are one of the worst affected areas by malaria in the entire district. Due to new settlement of human population and rapid deforestation these areas experienced frequent and uneven focal outbreak of malaria. (Nath et al., 2012). Adding to the owes, antimalarial drug resistance is also wide spread in this area further complicating the control scenario. Very high to moderate resistance against CQ and SP resistance has been reported from these areas in the recent past (Baruah et al., 2005; Campbell et al., 2006; Gogoi et al., 1995). In Paneri an area near Indo Bhutan border, Gogoi et al., 1995 have reported RI, RII and RIII level of resistance from tea garden population. Baruah et al., 2005; have reported RI and RII level of resistance from Assam Arunachal Border areas in Sonitpur District. In another study, more than 90% cases of treatment failure were reported from different places of Assam including Sonitpur district (Campbell et al., 2006).

In the present study prevalence of *Pfcr* K76T and *Pfmndr1* N86Y mutation was estimated in local population. High prevalence of *Pfcr* K76T mutation (72.13%) was observed in the local population compared to *Pfmndr1* N86Y mutation (41.79%) (Fig 6).
Mutant Pfcr\textit{t} has found to be associated with \textit{in vivo} CQ resistant and considered as a good marker for indicating CQ resistance in the area (Djimde A \textit{et al}., 2001b). However mere presence of the mutations may not necessarily indicate treatment failure \textit{in vivo} (Vinayak \textit{et al}., 2003). High prevalence of these mutations in the study area may be an intrinsic property of the parasite may not correspond to treatment outcome moreover it is in indicative of increasing trend of CQ resistance in the study area. Transmission intensity of \textit{P. falciparum} infection and spread of drug resistance show complex relation. However areas with low transmission intensity favours increased rates of drug resistance (Campbell \textit{et al}., 2006; Dura\textit{isingh} \textit{et al}., 2005b; Hastings \textit{et al}., 2002; Wongsrichanalai \textit{et al}., 2002). Though association of \textit{Pfmdr1}N86Y mutation with chloroquine resistance is not very clear but earlier studies have reported its presence along with Pfcr\textit{t} K76T mutation can cause higher level of CQ resistance (Babiker \textit{et al}., 2001; Duraisingh \textit{et al}., 2000). In the present study 32.8% sample were found to contain both the mutation. This may be a warning signal for occurrence of high level of CQ resistance in the study area.

The current study population was recruited from a malaria endemic area with previous reports of CQ resistance which may not sufficient represent the entire population of the region but presence of the both the mutation at high level may be reason for caution. Chloroquine has been
the drug of choice as first line of treatment until recently, Government of India revised the drug policy and ACT replaced CQ as first line of treatment (NVBDCP, 2010). However CQ still in use as presumptive treatment which may further complicates the situation resulting in emergence of highly resistance strains. Spread of mutations found to be associated with drug resistance is a sign of worry which may complicate the entire treatment regime thus affecting the national malaria control programme as a whole in the near future.

5.3 Molecular Epidemiology of genetic diversity of \textit{P. falciparum}.

In our present investigation allelic diversity of \textit{Plasmodium falciparum} was studied in two geographically isolated areas. Most commonly used marker for genetic diversity studies namely MSP1, MSP2 and GLURP of \textit{Plasmodium falciparum} were used. Attempts have been made to understand the pattern of allelic diversity in two different epidemiological settings and area of different transmission intensity.

Extensive diversity was observed in both the study areas with respect to all the three genetic markers i.e. MSP1, MSP2 and GLURP. In South Tripura District high diversity was observed with respect to all the markers and MSP1 diversity was found to be the highest. Again multiplicity of infection is also found very high among the samples especially with respect to MSP1 which is higher among the other two. The \textit{P. falciparum} diversity seen in the area was comparable to hyper endemic Gabon and Tanzania (Aubouy \textit{et al.}, 2003) but much higher than that reported for most mesoendemic areas (Zwetyenga \textit{et al.}, 1999) other than Ghana (Franks \textit{et al.}, 2001). The extent of diversity seen in the present study is in agreement with the diversity reported from other parts of Assam (Baruah \textit{et al.}, 2009, Joshi \textit{et al.}, 2007). Higher diversity than that expected in isolates from areas of intermediate and low transmission intensity has been reported by earlier investigators (Ferreira \textit{et al.}, 1998; Paul \textit{et al.}, 1998) while some other authors (Peyerl-Hoffmann \textit{et al.}, 2001) found no relationship between transmission intensity and parasite diversity. Nonetheless, the observed higher frequency than that expected for our mesoendemic study areas may be attributed in part high malaria incidence during the time of
sample collection. Since the study was conducted during peak transmission season, which may be a reason for higher diversity. On the other hand In Sonitpur District diversity found was lower than the South Tripura District. Diversity was calculated as total number of alleles present per individual. So complexity of the parasite is less in Sonitpur in comparison to the South Tripura District. This may be due to the time of collection of the sample as because the Sonitpur District samples were collected over a period of time regardless of the transmission pattern (Fig 7).

In some other studies conducted in different regions of Africa with a markedly seasonal transmission, such as in Sudan, it has been observed that the diversity of genotypes and multiplicity of infection reduces significantly with the transmission (Babiker et al., 1995).

**Fig 7: Comparision of MOI and Diversity of P. falciparum in study areas**

![Bar chart showing MOI and diversity comparison between South Tripura District and Sonitpur District.]

In the contrary, in Benin, a country where the transmission is perennial, the decrease in transmission does not have a substantial influence on the diversity of *msp-2* alleles or on the multiplicity of infection (Issifou et al., 2001). Moreover, the difference in multiplicity of
infection between areas with seasonal transmission and those with stable transmission, the MOI being higher in the later due to genetic recombination (Babiker et al., 1999)

In South Tripura District alleles from all the allelic families of MSP1 and MSP2 and different alleles of GLURP were detected. There were 21, 17 and 7 alleles of MSP1, MSP2 and GLURP were found. On the other hand in Sonitpur District less number of MSP1 allele i.e 17 were detected but in case of MSP2 and GLURP alleles not much difference was observed. High numbers of alleles were also reported from Assam in separate study. Baruah et al., 2009; reported high diversity in msp1. Maximum 33 different alleles were reported by them in a spatio-temporal variation study of different areas from Assam.

Frequency distribution of allelic families showed that MAD20 family has the highest distribution followed by K1 and RO33 in both the study areas (Fig 8).

**Fig 8: Frequency distribution of different MSP1 alleles in study areas.**

![Bar graph showing frequency distribution of different MSP1 alleles](image)

However frequency of the alleles were more in South Tripura District compared to Sonitpur. Similar findings were observed by Joshi et al., 2007; in different places of Assam. However, Baruah et al., 2009 have reported high diversity in all the families in different seasons and different places with out much difference in the frequency in a study conducted in different
locations of Assam. Recently, Yuan et al., 2013 have also reported higher frequency of MAD20 than the other two from north eastern Myanmar. Others have also reported similar type of trends from different parts of Asia i.e. Zhu et al., 1999 from Yunnan province China, Snounou et al., 1999 from Thailand. Kang et al 2010 in a recently reported different finding where RO33 allele was not present at al from Central Myanmar. In Malaysia frequency of the RO33 family was found higher than the other two (Atroosh et al., 2011). These findings are in agreement with previous studies in Brazil and Gabon which demonstrated the predominance of the RO33 allelic family (Kimura et al., 1990, Kun et al., 1998).

In our present study diversity of MSP2 reveal high diversity in both the study area. MOI in South Tripura District was found 1.98 where as in Sonitpur District it was found 3.0. Frequency distribution of the allelic families showed that 3D7 allele was more than FC27 (Fig 9).

**Fig 9: Frequency distribution of different MSP2 alleles in study areas**

Joshi et al., 2007; had also reported higher frequency of 3D7 allelic family from Assam isolate whereas isolates from Odissa and West Bengal were had reported more of FC27 family. Studies conducted in other parts of India also revealed the presence of higher frequency of FC27 family (Farooq et al., 2009).Similarly Bhattacharya et al., 1998 reported only FC27 allele in some limited number of Indian isolates. These types of findings were in accordance with the studies
conducted in Africa and Papua New Guinea Felger et al., 1994, Felger et al., 1999. Other studies conducted in Thailand (Snounou et al., 1999), Laos (Khaminsou et al., 2011) and Myanmar (Yuan et al., 2013) has reported almost equal prevalence of both the allelic families. However Atroosh et al., 2011; have reported higher prevalence of 3D7 family. This type of pattern of parasite diversity may have arised due to unique geographical position of northeastern states and their relation to the neighbouring areas.

In case of GLURP gene MOI in South Tripura District was found to be 1.21 and in the Sonitpur District was 1.35. However Number of allele in South Tripura District was more. These findings are in accordance with the study carried out in Bangladesh (Akter et al., 2012).

In the present study polyclonal infection was found in about two third of the population from the South Tripura District. However monoclonal frequency of GLURP is much higher than the other two. On the other hand in the Sonitpur District monoclonal frequency of MSP1 and GLURP is more than MSP2. In FC27 family monoclonal allele frequency was lower than the 3D7. Presence of high number of polyclonal frequency in the study area represents the complexity of the infections and population structure of the parasite. Higher polyclonal frequency of the alleles were reported from areas with high malaria transmission (Babiker et al., 1998, Paul et al., 1998., Legrand,et al., 2005). Polyclonal infection and asymptomatic malaria was found to be associated, in a study carried out in Congo a highly malaria endemic country (Ekala et al., 2002). In our present study high proportion of multiclonal infection and high MOI were recorded among the samples of both the study sites. Higher malaria endemicity may be one of the reasons for such high diversity. Moreover diversity in the South Tripura District was found higher than the Sonitpur District which may be due to the transmission intensity of South Tripura as the sampling time was the peak malaria season or the monsoon time. Other workers had also reported identical trend of observation from different parts of Assam viz- Karbi Anglong and Kamrup (Joshi et al., 2007) and Guabari, Kondoli and Dimakusi ( Baruah et al., 2009). Similar observation that the extent of diversity and multiplicity of infection (MOI) in an
area is related to the malaria endemicity of the area was made by Ranjit et al., 1999 and Babiker et al., 1997.

The present study for the first time depicted the population structure of the study area. Observation made in the study revealed that population structure is similar to some of the highly malaria endemic areas on the other hand it showed some similarity to both Southeast Asian countries as well as Indian population. This may be due to northeastern states act as a corridor for communication of Indian and Southeast Asian countries. Higher diversity in these areas may have arisen due to different factors such as high endemicity and variable transmission intensity at local level. Other factors such as drug susceptibility status of the parasite, vector population and host genetic factors further add to the diversity and creating a unique type of population structure in these areas.