High prevalence of *pfprt* K76T and *mdr1* N86Y mutations in Sonitpur district of Assam, India

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Abstract Present study reports the prevalence and distribution of *pfprt* K76T and *mdr1* N86Y mutations in malaria endemic areas of Sonitpur district of Assam. Out of 163 individuals tested for malaria, 67 (SPR = 41.1 %) were detected positive for malaria infection using rapid detection kit in the field and PCR assay in the laboratory. Nested PCR–RFLP assay was used to amplify *pfprt* K76T and *mdr1* N86Y genes flanking the K76T and N86Y mutations. *P. falciparum* was most abundant (91.04 %) among the three *Plasmodium* species reported and its prevalence was significantly higher as compared to *P. vivax* and *P. malariae* ($\chi^2 = 150.76$; $p \leq 0.0001$; $df = 2$). Malaria was equally distributed among all the age groups

and both the sexes. Hemoglobin contents in severe anaemic patients had a significant linear decreasing trend among patients with the increase in age ($\chi^2 = 4.33$; $p = 0.03$), whereas non severe anaemic patients exhibited significant linear increasing trend among the patients with the increase in age ($\chi^2 = 18.38$; $p \leq 0.0001$). *Pfprt* K76T mutation was recorded in 44 (72.13 %) isolates, whereas *mdr1* N86Y mutation could be detected in 28 (41.79 %) isolates only. Only 32.7 % of the samples had both *pfprt* K76T and *mdr1* N86Y mutations. Number of *pfprt* K76T mutant isolates was significantly higher than the wild type. However no significant difference was observed among the number of isolates with *mdr1* N86Y mutant and wild isolates.

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Introduction

Malaria is endemic throughout the northeastern states of India and majority of the cases are contributed by *Plasmodium falciparum*. Despite of sincere control efforts, the disease appears and disappears at irregular intervals and the intervention appears to be futile in many places mainly due to spread of insecticide resistance, antimalarial drug resistance, population migration and growth and globalization (Greenwood and Mutabingwa 2002; Dhiman et al. 2010a). In addition to this, conducive climatic conditions for the proliferation of potential vectors namely, *Anopheles dirus*, *An. minimus*, *An. fluviatilis* and *An. annularis* support the annual transmission in the northeastern states (Dev et al. 2003a; Dhiman et al. 2012). Malaria control in India largely relies upon the active case detection and treatment using chemoprophylaxis. However in northeastern region,

frequent movement of susceptible population involved in various developmental work and security complicate the malaria control (Dhiman et al. 2010a). Chloroquine has been the drug of choice in malaria treatment until recently, however increase in chloroquine resistance has posed a serious challenge to the world health. In India, chloroquine resistance in *P. falciparum* was first reported in Assam in 1973 and subsequently reported from several parts of the country (Sehgal et al. 1973; Dev et al. 2003b; Valecha et al. 2009). Multiple studies conducted in *falciparum* malaria endemic areas have suggested that *pfprt* K76T and *mdr1* N86Y mutations have been associated in chloroquine resistance (Happi et al. 2006). Therefore, in addition to assessing the therapeutic efficacy of antimalarial drugs, it is important to study the prevalence of antimalarial drug associated mutations in *P. falciparum*, so as to adopt suitable strategies for malaria treatment. In the present study, prevalence and distribution of *pfprt* K76T and *mdr1* N86Y mutations has been studied among *P. falciparum* malaria patients in malaria endemic Sonitpur district of Assam.

Materials and methods

Study sites

The study was carried out in three different malaria endemic places namely, Missamari (92°30′57″E, 26°51′47″N), North Jamuguri (92°55′46″E, 26°45′59″N) and Behali (93°24′19″E, 26°52′35″N) of Sonitpur district of Assam. All three areas have hot and humid climate and are located near Assam Arunachal border. These areas are inhabited by ethnic tribes, tea tribes and Nepali communities and fringed by dense forest & tea gardens (Rabha et al. 2012). All the samples were collected in mobile malaria camps organized by local medical authorities from 2007 to 2010. Patients diagnosed with malaria in the camps received treatment as per prevailing antimalarial drug policy.

Diagnosis and collection of blood samples

Suspected malaria patients were diagnosed in the field using rapid detection kit (Optimal-IT®; M/S Diamed AG, Switzerland) and paired blood smears were collected by trained technicians for microscopic examination of malaria parasite. Nearly 200 µl of peripheral blood was collected on FTA™ papers for molecular study. Papers were air dried and stored in desiccators and subsequently in −20 °C deep freezer until DNA extraction. Hemoglobin level of blood was also determined in the field using HemoCue™ Hb201+ system (Hemocue AB, Sweden).

DNA extraction and molecular typing

DNA was extracted from FTA™ cards using Qiagen blood DNA mini kit as per manufacturer guidelines with minor modifications in buffers volume. Nested PCR assay was used to amplify *pfprt* and *mdr1* gene flanking the mutation using primers described elsewhere (Djimde et al. 2001). PCR was performed in 25 µl reaction volume containing 5 µl of sample DNA, 2.5 mM MgCl₂, 200 mM of dNTPs, 1 mM each forward & reverse primers and 0.5 U of *Taq* polymerase (Roche). Reaction conditions were essentially similar to those described previously (Djimde et al. 2001). Nested PCR products of *pfprt* and *mdr1* were further restriction digested using *ApoI* and *AfIII* enzyme (New England Biolab) for allelic identification.

Data analysis

The percent malaria positive cases were recorded as slide positivity rate (SPR), whereas the proportion of blood smears found positive for *P. falciparum*, *P. vivax* and *P. malariae*, were recorded as slide *P. falciparum* rate (SfR), slide *P. vivax* rate (SvR) and slide *P. malariae* rate (SmR) respectively. Chi-square test with Yates correction was used to analyse the difference in malaria incidences, hemoglobin levels and among wild and mutant isolates. The confidence interval was determined using Katz approximation whereas odds ratios were determined following Woolf approximation. The study has been approved by the institutional ethical committee and consents were obtained from the patients before collecting the blood samples.

Results

Malaria epidemiology

Of the total 163 individuals tested for malaria, 67 were detected positive for malaria infection using rapid detection kit in the field and PCR in the laboratory. The age wise and sex wise distribution of malaria among the individuals has been depicted in Tables 1 and 2 respectively. *P. falciparum* was most abundant (91.04 %) and its prevalence was significantly higher as compared to *P. vivax* and *P. malariae* ($\chi^2 = 150.76$; $p \leq 0.0001$; $df = 2$). SPR was higher among the 5–11 years age group however it was not statistically significant as compared to other age groups ($\chi^2 = 0.67$, $p = 0.72$) (Table 1). Both male and females were equally affected by malaria as no difference in malaria incidence could be observed among both the sexes ($\chi^2 = 0.64$, $p = 0.423$) (Table 2). Hemoglobin contents were <7 g/dl (severe anaemic) in 19.4 % of malaria

Table 1 Age wise distribution of malaria among the study population

Age group	BSE/M/SPR	Pf/Pv/Pm	SfR/SvR/SmR	χ^2	<i>p</i> (df)
<5	40/17/42.5	16/1/0	94.1/5.9/0	42.5	<0.0001 (2)
5–11	37/17/45.9	14/3/0	82.4/17.6/0	28.8	<0.0001 (2)
>11	86/33/38.4	31/0/2	93.9/0/6.1	82.1	<0.0001 (2)

BSE, Blood smear examined; M, Malaria positive cases; Pf, *P. falciparum*; Pv, *P. vivax*; Pm, *P. malariae*; SPR/SfR/SvR/SmR, Slide positive/*falciparum*/*vivax*/mixed rate

Table 2 Sex-wise distribution of malaria among the study population

Sex	BSE/M/SPR	Pf/Pv/Pm	SfR/SvR/SmR	χ^2	<i>p</i> (df)
Male	90/40/44.4	36/2/2	90/5/5	86.7	<0.0001 (2)
Female	73/27/37.0	25/2/0	92.6/7.4/0	64.3	<0.0001 (2)

BSE, Blood smear examined; M, Malaria positive cases; Pf, *P. falciparum*; Pv, *P. vivax*; Pm, *P. malariae*; SPR/SfR/SvR/SmR, Slide positive/*falciparum*/*vivax*/mixed rate

patients, of which 53.8 % children of <5 years ($\chi^2 = 4.39$, $p = 0.11$). Hemoglobin contents in severe anaemic patients had a significant linear decreasing trend among patients with the increase in age ($\chi^2 = 4.33$, $p = 0.03$). Among the non severe anaemic patients, where hemoglobin contents were >7 g/dl, 57.41 % were from >11 years age group ($\chi^2 = 21.5$; $p \leq 0.0001$). The hemoglobin contents in non severe anaemic patients exhibited significant linear increasing trend among the patients with the increase in age ($\chi^2 = 18.38$; $p \leq 0.0001$).

Prevalence of *pfprt* K76T and *mdr1* N86Y mutations

Pfprt K76T and *mdr1* N86Y mutation detected in all the 61 *P. falciparum* isolates in the study area has been depicted in Fig. 1. *Pfprt* K76T mutation was recorded in 44 (72.13 %) isolates whereas *mdr1* N86Y mutation could be detected in 28 (41.79 %) isolates only. The total number of isolates with *pfprt* K76T mutation were significantly higher

Fig. 1 Prevalence and distribution of *pfprt* K76T and *mdr1* N86Y mutations among the study population

than the wild type ($\chi^2 = 22.16$; $p \leq 0.0001$; OR = 0.149 (95 % CI = 0.07–0.33). On the other hand, no significant difference was observed among *mdr1* N86Y mutant and wild isolates ($\chi^2 = 0.52$; $p = 0.47$; OR = 1.39 (95 % CI = 0.68–2.83). There was no sex biasing in the distribution of *Pfprt* K76T ($p = 0.98$) and *mdr1* N86Y ($p = 0.59$) mutations and both the mutations were equally distributed among both the sexes. Only 32.7 % of the samples had both *Pfprt* K76T and *mdr* N86Y mutations.

Discussion

The present study areas are most endemic for malaria in Sonitpur district of Assam. In addition to countless reported malaria cases in these areas many cases remains unreported due to various logistic and geopolitical problems (Dhiman et al. 2011). Human re-settlements and rapid deforestation have led to frequent and uneven focal malaria outbreaks annually (Nath et al. 2012). *P. falciparum* still remains the major malaria parasite transmitted by many efficient *Anopheles* vectors throughout the year (Dhiman et al. 2012; Rabha et al. 2012). Children <5 year age had hemoglobin comparable to that severe malaria patients. The children are thought to have high level of malaria susceptibility due to lower immunity, poor clothing and high outdoor activity, which may lead to rise in parasitemia level and subsequently decrease in hemoglobin contents (Singh et al. 2006; Owusu-Agyei et al. 2009; Dhiman et al. 2010b).

Wide spread drug resistance to common antimalarials has further complicated the control interventions (Gogoi et al. 1995; Baruah et al. 2005; Campbell et al. 2006). The present study highlights the high prevalence of *pfprt* K76T and *mdr1* N86Y mutation among the local population. High prevalence of *pfprt* K76T mutation (72.13 %) as compared to *mdr1* N86Y mutation (41.79 %) has been observed in the study area. *Pfprt* mutation has been found associated with in vivo chloroquine resistant and considered as a good marker for indicating chloroquine resistance in many areas (Picot et al. 2009). A recent study conducted in India has suggested that mutant haplotypes occurred irrespective of clinical outcome whereas, wild haplotypes occurred only in adequate clinical and parasitological response patients (Mallick et al. 2012). In Assam, both *pfprt* K76T and *mdr1* N86Y mutation have been found to be prevalent, however, unlike the present study, *mdr1* N86Y mutation has been predominantly observed as compared to the wild (Mallick et al. 2012). Further, similar to the present study, the prevalence the two loci mutation (*pfprt*-*pfmdr*-1) has also been reported in high number in Assam, indicating the increased transmission potential of these resistant malaria parasites (Mallick et al. 2012).

However, studies have suggested that the presence of *pfcr* mutations may not necessarily indicate treatment failure and chloroquine drug resistance in vivo (Vinayak et al. 2003; Mallick et al. 2012). High prevalence of these mutations in the study area may be an intrinsic property of the parasite may not always correspond to the treatment outcome, however it is an indication of the increasing trend of chloroquine resistance. Transmission intensity of *P. falciparum* infection and spread of drug resistance shows complex relation as the areas with low transmission intensity favours increased rates of drug resistance (Hastings 2002; Wongsrichanalai et al. 2002). Around one-third isolates in the present study showed the presence of *pfcr* K76T mutation along with *mdr1* N86Y mutation in the same patient. Although, the association of *mdr1* N86Y mutation with chloroquine resistance is not clearly understood but previous studies have reported the co-appearance of *mdr1* N86Y mutation along with *pfcr* K76T mutation could elevate the chloroquine resistance to a higher level (Babiker et al. 2001).

Chloroquine has been most acceptable antimalarial as first line of malaria treatment until recent, when the antimalarial drug policy was revised and artemisinin based combination therapy replaced chloroquine as first line of treatment (NVBDP 2010). However, chloroquine is used widely in presumptive treatment which may further complicate the situation resulting in emergence of highly resistance strains. Spread of mutations found associated with drug resistance is a serious concern, which might complicate the entire malaria treatment regime. The present study, however limited in scope, indicates that the study area may have occurrence of high level of chloroquine resistance. Accelerated acquisition of multi drug resistance marker indicates that the *P. falciparum* is rapidly mutating toward the higher level of fitness against antimalarials.

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Conflict of interests None.

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