Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage:www.elsevier.com/locate/apjtd

Document heading doi:10.1016/S2222-1808(11)60062-5

Prevalence of severe chloroquine resistance associates the point mutation in *pfcrt* and *pfmdrI* gene in eastern India

Santanu Kar Mahapatra[1], Subhankari Prasad Chakraborty[1], Sabyasachi Das[1], AmiyaKumar Hati[2], Somenath Roy[1*]

[1]*Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore-721 102, West Bengal, India*

[2]*Former Director, Head of the Department of Medical Entomology and Chairman, Division of Parasitology, Calcutta School of Tropical Medicine, Kolkata-700 073, West Bengal, India*

ARTICLE INFO

Article history:
Received 11 September 2011
Received in revised form 8 October 2011
Accepted 28 October 2011
Available online 28 December 2011

Keywords:
Plasmodium falciparum
Chloroquine resistance
pfmdrI
pfcrt
Point mutation

ABSTRACT

Objective: To evaluate the molecular mechanism of chloroquine resistant *Plasmodium falciparum* malaria in West Bengal, eastern part, India. **Methods:** Parasitic bloods were collected from patients in Kolkata and Purulia, *in vitro* drug susceptibility test were performed in those 179 isolates. Now parasitic DNA was isolated by phenol chloroform extraction method and then polymerase chain reaction and restriction fragment length polymorphism analysis of different codons of *pfcrt* gene (76) and the *pfmdrI* gene (86, 1246) were assessed. **Results:** The response of 179 patients to chloroquine was determined. The prevalence of both *pfcrt* K76T (68.72%) and *pfmdrI* N86Y (43.1%) mutation was found here. But most importantly severe *in vitro* chloroquine resistance has been found (53.07%) here, with either double 76T + 86Y mutation or triple 76T + 86Y + 1246Y mutation. **Conclusions:** Our present findings implicate that due to enormous drug (chloroquine) pressure, double mutation with *pfcrt* 76T + *pfmdrI* 86Y and triple mutation with *pfcrt* 76T + *pfmdrI* 86Y + *pfmdrI* 1246Y was highly correlated (P = 0.001) with *in vitro* chloroquine resistance for the first time in eastern India.

1. Introduction

Malaria is a devastating infection caused by protozoa of the genus *Plasmodium*. Drug resistant is widespread. No new chemical class of anti-malarial drug has been introduced into clinical practice since 1996, and there is a recent rise of parasite strains with reduced sensitivity to the newest drugs. With approximately 243 million cases and 863 000 attributed deaths reported globally in 2009[1], malaria is one of the most severe infectious diseases; primarily affecting the world's most disadvantaged populations. Of the four typically recognized *Plasmodium* species causing disease in humans, *Plasmodium falciparum* (*P. falciparum*) causes most mortality, mainly in children below the age of 5, additionally representing a reservoir of latent infection that hampers current control and future elimination efforts[2]. No new class of antimalarials has been introduced into clinical practice since 1996[3], owing to the intrinsic difficulties in discovering and developing new antimicrobials, as well as a relative lack of public and private resource commitment towards antimalarial research. Moreover, treatment of malaria is becoming problematical due to resistance in *P. falciparum* to the commonly used drug, chloroquine. Thus there is an urgent need to diagnose the chloroquine resistant pattern.

The clinical outcome of malaria infection depends on parasite factor (drug resistance, multiplication rate, cytoadherence, rosetting, and antigenic polymorphism), host factors (immunity, pro-inflammatory cytokines, genetics, and age), geographic and social factors. The most important factor is the emergence of drug resistant malaria in endemic areas. Early detection of drug [commonly used chloroquine, sulfadoxine-pyrimethamine (SA-PY)] resistant and virulent form of parasite is very much important for therapy. Generally such decision making relies on clinical studies, supported by *in-vitro* sensitive testing. In this situation molecular analysis of parasite genes essential for assessing resistance to the chloroquine (*crt* gene and *MDR*

*Corresponding author: Dr. Somenath Roy, Professor, Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore -721 102 West Bengal, India.
Tele-fax: 91-3222 - 275329
E-mail: roysomenath@hotmail.com

Foundation Project: This work was financially supported by Department of Science and Technology, Government of India (grant No. SR/SO/HS-106/2005).

gene) classes of drugs are very much appropriate. It is now known that chloroquine (4-aminoquinoline) resistant *in vitro* and *in vivo* is associated with point mutations in *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) and *P. falciparum* chloroquine resistance transporter gene (*pfcrt*), a putative transporter that modulates intra-parasitic drug concentrations[4]. Sometimes chloroquine resistance is likely to be a consequence of multi-factors and enzymes in glutathione (GSH) system[5].

The prevalence of multi drug resistant malaria is now reaching alarming levels in India, increasing the need for regular monitoring of drug resistance and changes in drug prophylactics[6]. The *pfcrt* gene, which encodes a trans-membrane protein located in the *P. falciparum* digestive vacuole, was recently characterized[7]. A perfect correlation was found between *in vitro* response to chloroquine and sequence polymorphism at the *pfcrt* locus[4,7]. In particular, *in vitro* chloroquine resistance was associated with the substitution of lysine for threonine at position 76 in field isolates and laboratory strains. In addition, haplotypes with specific combinations of mutations showed a specific geographical distribution, with distinct haplotypes in Malawi African and Asian isolates compared to South American isolates, suggesting that *pfcrt* polymorphism is a useful tool for the public health surveillance of chloroquine resistance[4,7-9]. In our present study, we want to investigate the genetic diversity in *pfcrt* and *pfmdrI* gene to find out the resistant pattern of the *P. falciparum* clinical isolates in eastern India.

2. Materials and methods

2.1. Chemicals and reagents

Phenol, chloroform, Isoamyl alcohol, gentamycin, folate, agarose, *p*-amino benzoic acid-free RPMI 1640, HEPES, cell culture grade DMSO were purchased from Himedia, India. Tris-HCl, Tris buffer, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), ethylene diamine tetra acetate (EDTA), sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), sodium acetate, ammonium acetate, isopropanol, ethanol, boric acid were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. Albumax-II, hypoxanthine, proteinase K, RNase A, ethidium bromide, chloroquine phosphate were purchased from Sigma Chemical Co., USA. Oligonucleotide primers, restriction enzymes were purchased from New England Biolab, Baverly, USA. PCR grade nucleotide mixture, $MgCl_2$, dNTPs and Taq DNA polymerase were purchased from Roche applied science, USA. pLDH kit was purchased from Diatek, Kolkata, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest grade available.

2.2. Selection of subjects

Inclusion criteria for this experiment includes fever at consultation or history of fever within the past 24 h, a mono infection with *P. falciparum* based on the microscopic examination of Giemsa-stained thin and thick blood smears, a parasite density of 40-40000 asexual parasites/μL of blood, and no recent history of self-medication with antimalarial drugs. Patients with signs and symptoms of severe and complicated malaria, as defined by the World Health Organization, were excluded (WHO, 2003).

2.3. Collection of sample

The study was carried out from May 2008 to January 2010. Respectively One hundred thirty two and forty seven clinical isolates of *P. falciparum* were enrolled in this study from the Kolkata, and Primary Health Center, Purulia, two malaria endemic zone of West Bengal, India. Informed consent was obtained from the respective patient and the patient's guardian both in case of adult and child patients. The experimental protocol of this study was followed the World Health Organization (WHO) and duly approved by the Institutional Ethical Committee.

2.4. Transport of sample

After collection of the sample from Kolkata, and Purulia of West Bengal, samples are transported to the laboratory as early as possible to carry out the different experiments.

2.5. Separation of red blood cell (RBC)

5-10 mL of venous blood samples were collected in a vacutainer (BD falcon) coated with an anticoagulant (EDTA) and washed in folate and *p*-amino benzoic acid-free RPMI 1640 medium for several times; followed by centrifugation at 2000 × g for 10 min at 4 ℃, an aliquot of 1.5-2 mL of the RBC pellet was obtained[10].

2.6. In vitro drug sensitivity assay

In vitro drug sensitivity assays were performed on the clinical isolates with prior adaptation to the *in vitro* culture conditions[11]. Infected erythrocytes were suspended in the complete folate and *p*-amino benzoic acid free RPMI 1640 medium consisting of 0.5% Albumax II, 25 mM HEPES, 25 mM $NaHCO_3$, 25 μg/mL gentamycin and 0.2% hypoxanthine at a hematocrit of 1.5% and an initial parasitemia of 0.2-1.0%. If the blood sample had a parasitemia >1.0%, fresh uninfected, type O^+ erythrocytes were added to adjust the parasitemia to 0.6% to 1% and cultured at 37 ℃ in 5% CO_2.

2.7. IC_{50} value

The 50% inhibitory concentration or IC_{50} value means the inhibition of growth up to 50%. Sterile RPMI 1640 were used to prepare stock solutions and dilutions of chloroquine. The final concentrations ranged from 1 to 1000 nM for chloroquine. Twenty five micro liters of each concentration were distributed in micro culture plate and IC_{50} was determined using microscopic examination, detection of pLDH and hypoxanthine uptake assay[10]. The calculation

was based on non linear regression analysis of the logarithm of concentrations plotted against the percentage growth inhibition. Isolates were defined as susceptible to chloroquine when IC_{50} values were <60 nM, intermediate susceptible when 60- 100 nM and resistant when >100 nM.

2.8. Isolation of parasitic DNA

Erythrocytes (infected and uninfected) were suspended in 15 mL of ice-cold NET buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5) and lysed with 0.015% saponin (Sigma). The lysate was centrifuged at 2000 × g for 10 min at 4 ℃ and the pellet was transferred to a 1.5-mL micro centrifuge tube and suspended in 500 μL of NET buffer. The mixture was treated with 1% N-lauroylsarcosine (Sigma) and RNase A (100 μg/mL) at 37 ℃ for 1 h and proteinase K (200 μg/mL) at 50 ℃ for 1 h. Parasite DNA was extracted three times in equilibrated phenol (pH = 8), phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1) and precipitated by the addition of 3 M sodium acetate and cold absolute ethanol. The extracted DNA was air dried and re-suspended in TE buffer (10 mM Tris, 1 mM EDTA). Parasite DNA was stored at -20 ℃ until use[10].

2.9. Polymerase chain reaction

The regions of the *pfcrt* and *pfmdr1* genes surrounding the polymorphisms of interest were amplified by the polymerase chain reaction (PCR) using the Eppendorf thermal cycler under the following conditions; approximately 200 ng of genomic DNA, 15 pmol of primers, reaction buffer (10 mM Tris, 50 mM KCl, pH = 8.3), 2.5 mM $MgCl_2$, 250 μM dNTPs, and 1 unit of Taq DNA polymerase (Roche applied science) in a 25 μL reaction mixture at 95 ℃ for 5 min for the first cycle and 30 sec in subsequent cycles, 50 ℃ for 30 sec in all cycles, and 72 ℃ for 1 min in all cycles, for a total of 30 cycles. The primers were designed on the basis of the complete *P. falciparum Dd2* strain sequence available in the genomic data bank. Five micro liters of the amplification product was loaded on a 1.2% agarose gel, subjected to electrophoresis, stained with ethidium bromide, and visualized under ultraviolet trans-illumination to confirm the presence of the particular DNA fragment according to base pair (bp) size[10,12].

The *pfcrt* K76T mutation analysis was performed using the primers 5′-TGTGCTCATGTGTTTAAACTT-3′ (sense) and 5′-CAAAACTATAGTTACCAATTTTG-3′ (anti-sense) and the PCR procedures described above. For all amplification reactions, only 1 μL of the DNA template was necessary.

A310-bp region surrounding the *pfmdr1* N86Y mutation was amplified using the 5′-TTTACCGTTTAAATGTTTACCTGC-3′ forward primer and the 5′-CCATCTTGATAAAAAACACTTCTT-3′ reverse primer and PCR conditions described above for 40 cycles.

The primer pair, 5′-GTGGAAAATCAACTTTTATGA-3′ (forward) and 5′-TTAGGTTCTCTTAATAATGCT-3′ (reverse), was used for amplification and analysis of the polymorphism at amino acid position 1246 in *pfmdr1*. PCR conditions were as described above for 35 cycles.

2.10. RFLP analysis of pfcrt and pfmdrI gene

Single nucleotide polymorphism of the *pfcrt* and *pfmdrI* genes at their specific codons was determined by enzymatic digestion of specific restriction enzymes. In *pfcrt* gene *ApoI* (New England Biolabs) enzyme digests the PCR products (10 μL) at 50 ℃ for one hour and identifies 76 lys which is wild type of allele.

Ten micro liters of amplification product was digested with the restriction enzyme *AflIII* (New England Biolabs) at 37 ℃ for one hour to detect the presence of asparagine amino acid at 86 codon of *pfmdrI* gene. In *pfmdrI* gene, digestion with *EcoRV* (New England Biolabs) at 37 ℃ for one hour to identify the tyrosine at 1246 codon.

15 μL of the restriction enzyme treated product was mixed with 2 μL of bromophenol blue and finally loaded on a 1.2 % agarose gel; subjected to electrophoresis and stained with ethidium bromide, and visualized under ultraviolet trans illuminator to confirm the presence of the particular DNA fragment according to bp size[12].

2.11. Statistical analysis

The data were expressed as Mean ± SEM. Fisher's exact tests, Mann-Whitney U-test were used to study the relation between IC_{50} values and genotypes. The relation between *in vitro* phenotype with molecular genotypes was studied by Fisher's exact tests and Spearman correlation tests. All the data analysis was performed using a statistical package, Origin 6.1, (Northampton, MA 01060 USA) and GraphPad InStat software 3.0.

3. Results

3. 1. In vitro susceptibility to chloroquine

In vitro assay for chloroquine yield interpretable result on all 132 isolates from Kolkata and 47 isolates from Purulia respectively. Using the *in vitro* responses to chloroquine drug 57 (43.18%) isolates of Kolkata and 6 (12.76%) isolates from Purulia were chloroquine sensitive (geometric mean IC_{50}= 45 nM, range= 8-60 nM) (Figure 1). Out of 132 isolates from Kolkata, 15 (11.36%) and 6 (12.76%) isolates from Purulia were intermediately susceptible to chloroquine (mean IC_{50} = 90 nM, range = 61-100 nM). 60 (45.45%) isolates were highly resistant to chloroquine (mean IC_{50} = 280.2 nM, range = 110-480 nM) in Kolkata, whereas 35 (74.66%) isolates from Purulia were also highly resistant.

3.2. pfcrt and pfmdrI genotypes

The region of *pfcrt* and *pfmdrI* genes flanking the polymorphism of interest were amplified by PCR (multiplex PCR), followed by digestion with specific restriction enzyme to detect each variant (Figure 2).

3.3. PCR/ RFLP – test for pfcrt gene

During the SNP analysis of *pfcrt*, amplification with the primer gives a 134 bp PCR product that run in 1.2% agarose gel and visualized under UV trans-illuminator. Now restriction fragment length polymorphism (RFLP) analysis of *pfcrt* gene has been done and the presence of the K76T mutation was detected using the ApoI restriction enzyme. ApoI cuts out 34 bp from the 134-bp *pfcrt* PCR product in wild-type alleles but does not cut the mutant allele (Figure 2a). The number of bp present in DNA fragments was detected, using Bio-Rad Quantity one (Version 4.6.7) software. From Table 1, the frequency of samples composed of *pfcrt* mutant is only 82 (62.12%) isolates, in Kolkata except these, all other 50 (37.88%) isolates contain wild type of allele. Here mixed alleles are taken as mutant one. While in Table 2, we found that most of isolates (87.23%) obtained from Purulia contain mutant *pfcrt* K76T allele.

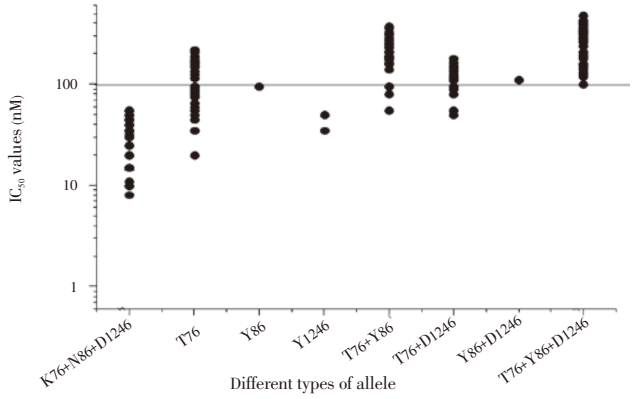

Figure 1. Relationship between the phenotype determined by *in vitro* drug sensitivity assays and expressed as IC_{50} of chloroquine and the *pfcrt* and *pfmdrI* genotype, defined by either the absence of mutations (wild-type) or presence of point mutations. The solid line (corresponding to 100 nM, highly resistant) hypothetically shows the sever resistance levels for *in vitro* chloroquine resistance.

3.4. PCR/ RFLP – test for pfmdrI gene

Figure 2b and 2c shows the polymorphism in the *pfmdrI* gene. Amplification with N86Y primer gives a PCR product 310 bp. The tyr variant of codon 86 are discriminated by *AflIII* enzyme. Restriction digest with *AflIII* results in two fragments of approximately 190 bp and 120 bp in mutant alleles (Lane 3 & 4). Wild-type alleles yield an uncut PCR fragment of 310 bp (Lane 2). Mixed isolate is also found here (lane 5) where 310bp, 190bp and 120bp fragments were present (Figure 2b). The number of bp present in DNA fragments was detected by Bio-Rad Quantity one (Version 4.6.7) software. Out of 132 isolates from Kolkata only 40 (30.30%) isolates having mutant type 86 Y allele, whereas a large number 84 (63.64%) isolates having wild type of allele rest 8 (6.06%) are mixed allele (Table 1). In Table 2 it is observed that out 47 isolate from Purulia only 18 (38.30%) isolates having wild type N86 allele, rest 29 (61.70%) isolates are mutant alleles, of which 21 (44.68%) isolates are pure mutant allele and left 8 (17.02%) are mixed allele.

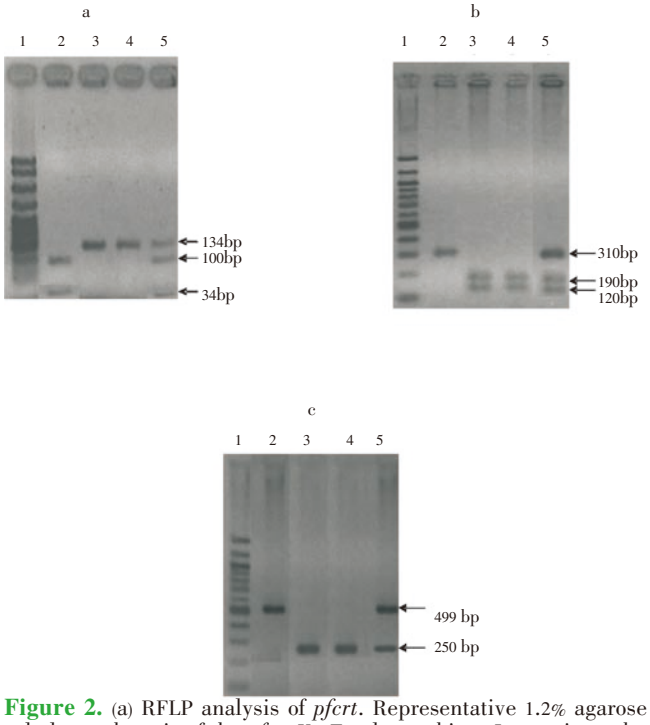

Figure 2. (a) RFLP analysis of *pfcrt*. Representative 1.2% agarose gel electrophoresis of the *pfcrt* K76T polymorphism. Lane 1 is 100 bp ladders. Presence of the K76T mutation was detected using the ApoI restriction enzyme. ApoI cuts out 34 bp from the 134-bp *pfcrt* PCR product in wild-type alleles (lane 2) but does not cut the mutant allele (Lanes 3 and 4). A mixed isolate is shown in lane 5. The number of bp present in DNA fragments was detected using Bio-Rad Quantity one (Version 4.6.7) software.
(b) RFLP analysis of *pfmdr1*. Representative 1.2% agarose gel electrophoresis of the *pfmdr1* N86Y polymorphism. Lane 1 is 100 bp ladders. Presence of the N86Y mutation was detected using the *AflIII* restriction enzyme digestion. Wild-type alleles yield an uncut PCR fragment of 310 bp (Lane 2). Restriction digest with *AflIII* results in two fragments of approximately 190 bp and 120 bp in mutant alleles (Lane 3 & 4). A mixed isolate is shown in Lane 5, where 310 bp, 190 bp and 120 bp fragments were found. The number of bp present in DNA fragments was detected by Bio-Rad Quantity one (Version 4.6.7) software.
(c) RFLP analysis of *pfmdr1* D1246Y polymorphism. Lane 1 is 100 bp ladders. Presence of the D1246Y mutation was detected using the *EcoRV* restriction enzyme digestion. Wild-type alleles yield an uncut PCR fragment of 499 bp (Lane 2). Restriction digest with *EcoRV* results in two fragments of approximately 250 bp each in mutant alleles (Lane 3 & 4). A mixed isolate is shown in Lane 5, where 499 and 250bp fragments were found. The number of bp present in DNA fragments was detected in Bio-Rad Quantity one (Version 4.6.7) software.

Presence of the D1246Y mutation was detected using the *EcoRV* restriction enzyme digestion. Wild-type alleles yield an uncut PCR fragment of 499 bp (Lane 2). Restriction digest with *EcoRV* results in two fragments of approximately 250 bp each in mutant alleles (Lane 3 & 4). A mixed isolate is shown in Lane 5, where 499 and 250 bp fragments were found (Figure 2c). Only 33 (25%) isolates shows the mutant 1246Y allele, large number of (88 isolates, 66.66%) wild D1246 allele are also found. 11 (8.33%) isolates shows mixed type of allele in D1246Y codon in Kolkata (Table 1). Whereas 20 (42.55%) mutant 1246Y allele and 3 (6.38%) mixed D+Y1246 allele have been found in Purulia. Only 51.06% (24) isolate have been found with wild D1246 allele. Here all mixed type of allele is taken as mutant allele (Table 2).

Table1
Distribution of *pfcrt* and *pfmdrI* genotype in 132 blood samples form Kolkata, with *P. falciparum* infection.

Codons	*n*	Wild	Mutant	Mixed
pfcrt 76	132	50 (37.88%)	59 (44.70%)	23 (14.42%)
pfmdrI 86	132	84 (63.64%)	40 (30.30%)	8 (6.06%)
pfmdrI 1246	132	88 (66.66%)	33 (25.00%)	11 (8.33%)

Table2
Distribution of *pfcrt* and *pfmdrI* genotype in 47 blood samples form Purulia, with *P. falciparum* infection.

Codons	*n*	Wild	Mutant	Mixed
pfcrt 76	47	6 (12.76%)	41 (87.23%)	0
pfmdrI 86	47	18 (38.30%)	21 (44.68%)	8 (17.02%)
pfmdrI 1246	47	24 (51.06%)	20 (42.55%)	3 (6.38%)

Table3
Distribution of *pfcrt* and *pfmdrI* genotype in relation to chloroquine *in vitro* susceptibility to chloroquine in Kolkata (K) and Purulia (P).

	No	*pfcrt* genotype	*pfmdrI* genotype		*In vitro* susceptibility to CQ		
K		76	86	1246	S	I	R
K	48	K	N	D	48	–	–
K	24	T	N	D	5	9	10
K	1	K	Y	D	–	1	–
K	1	K	N	Y	1	–	–
K	15	T	Y	D	1	1	13
K	11	T	N	Y	2	2	7
K	32	T	Y	Y	–	2	30
P	4	K	N	D	4	–	–
P	6	T	N	D	1	2	3
P	1	K	N	Y	1	–	–
P	1	K	Y	Y	–	1	–
P	14	T	Y	D	–	1	13
P	7	T	N	Y	–	2	5
P	14	T	Y	Y	–	–	14

CQ– Chloroquine.

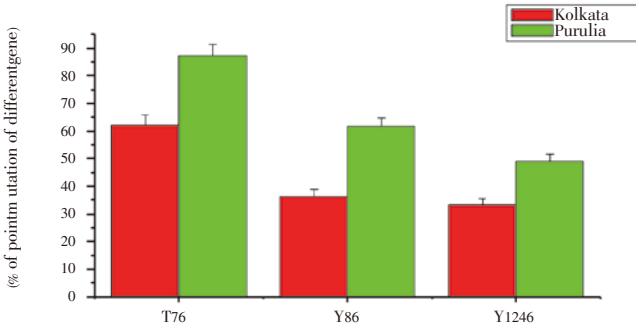

Figure 3. The percentage of point mutation of different genes in Kolkata and Purulia.

In short, out of 132 samples of Kolkata region, 76T in *pfcrt* gene, 86Y and 1246Y in *pfmdr1* gene mutation were present in 82 (62.12%), 48 (36.36%) and 44 (33.33%) isolates, that indicates the increasing pattern of *pfmdrI* mutation in this region.

In addition, out of 47 samples of Purulia region, 76T in *pfcrt* gene, 86Y and 1246Y in *pfmdr1* mutation were present in 41 (87.23%), 29 (61.70%) and 23 (48.93%) isolates, indicating the prevalence of both *pfcrt* and *pfmdrI* mutation (Figure 3).

Single 76T mutation has been found predominantly in Kolkata region instead of Purulia, whereas wild type alleles (*i.e.* no mutation) more frequently found in Kolkata. Out of these 132 isolates from Kolkata double mutation (both *pfcrt* and *pfmdrI*) were found in 43.93% isolates but most interestingly 76.6% cases of double mutation have been found in isolates from Purulia respectively. Out of this 76.6% cases of double mutation in Purulia, 29.79% cases have been found with mutation of K76T allele of *pfcrt* gene and both N86Y and D1246Y allele of *pfmdrI* gene (Figure 4).

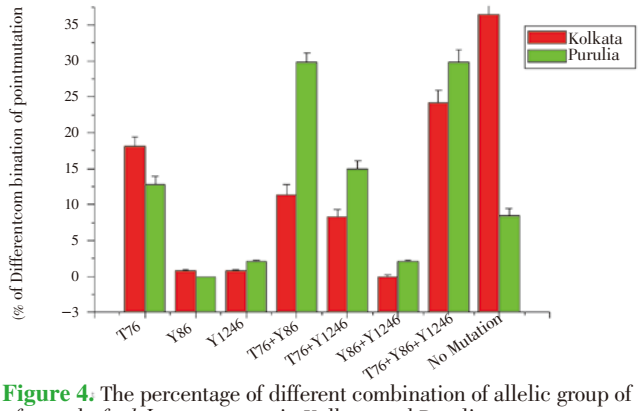

Figure 4. The percentage of different combination of allelic group of *pfcrt* and *pfmdrI* gene present in Kolkata and Purulia.

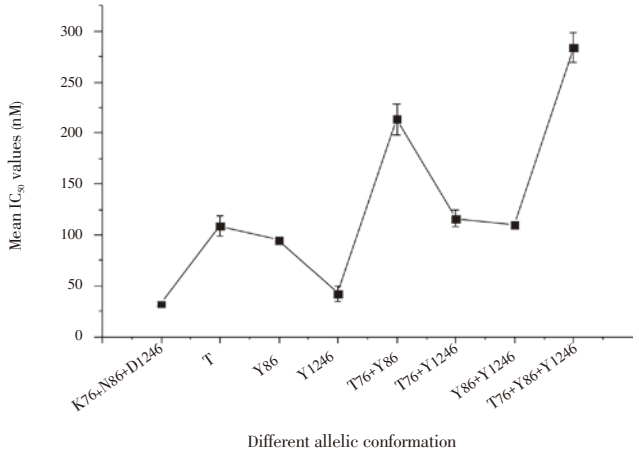

Figure 5. The variation of mean IC_{50} values in different allelic group present in the samples.

3.5. Relation between pfcrt and pfmdrI genotypes, in vitro data

The presence of *pfcrt* and *pfmdrI* point mutations was linked to *in vitro* resistance to chloroquine.

The phenotype of *in vitro* susceptibility to chloroquine was associated with *pfcrt* genotype at positions 76, and also with *pfmdrI* genotypes at positions 86, but not with 1246 (Fisher's exact test, chloroquine, $P < 0.05$ for codons 76 and $P < 0.05$ for codon 86; and P was not significant at the level of 0.05 for 1246 codon) (Table 3), but most interestingly double mutation with 76T + 86Y was found in 29 isolates and triple

mutation with 76T + 86Y+ 1246Y was found in 46 isolates. Out of these 29 and 46 isolates, 26 (89.65%) and 44 (95.65%) isolates respectively were severe *in vitro* chloroquine resistance. Figure 5 shows that low IC_{50} values were associated with wild *pfcrt* genotypes more specificialy in K76 allele. Single mutation with 1246Y also possesses very low IC_{50} value. Mutation with single 76T allele and 86Y allele possess high (border line resistant) IC_{50} values, Whereas double mutation associated with 76T + 86Y and triple mutation with 76T + 86Y +1246Y having very high IC_{50} value for chloroquine and also resistant to chloroquine (Fisher′s exact test, $P<0.001$) Whereas no such relation was observed with double mutation with 76T+1246Y and 86Y+1246Y.

Table 3 shows the different genotypes of all isolates with complete genotyping in relation to *in vitro* results. As mixed and mutant genotypes showed similar *in vitro* phenotypes, mixed genotypes were considered as mutant for this analysis

4. Discussion

The emergence of drug resistant malaria is a serious problem in tropical countries and an early detection is very important for providing proper medical treatment. Recently molecular genotyping of parasites has been proved an useful tool in assessing to drug resistant in *P. falciparum* malaria particularly the point mutations in *pfmdr1*, *pfcrt* (for chloroquine resistance)[12-21]. Several factors may explain the cause of dissimilarity between *in vitro* and *in vivo* resistant pattern of isolates. Sometimes this dissimilarity pattern is increases, treatment failures reflect the combination of several parameters, including parasite resistance to the drug, drug level achieved in the host, and action of the host immune response.

The increasing failure rates of several anti-malarial drugs in the majority of malaria affected areas means that close monitoring of the epidemiology and dynamics of drug resistance are necessary if we are to implement measures to circumvent the problem. The identification and validation of easy, rapid molecular markers of drug resistance would greatly facilitate this process, and would allow us to overcome difficulties in the use of traditional methods for assaying drug sensitivity.

The molecular basis of chloroquine resistance in malaria parasites is not well understood. In case of falciparum, polymorphism in *mdrI* (multidrug resistant) gene and *crt* (chloquine resistance transporter) gene are studied to correlate anti-malarial drug (chloroquine, mefloquine, halofantrine) resistance. The tyrosine allele of *pfmdr1* gene (N86Y mutation) had been reported to associate with anti-malarial chloroquine and mefloquine resistance. Increased *pfmdr1* copy number is also attributed for mefloquine drug resistance in falciparum[12]. The function of *pfcrt* is not clear yet. And it is felt that K76T mutation in *pfcrt* and N86Y mutation in *pfmdr1* can serve as molecular markers for chloroquine resistance *in vivo* and *in vitro* [21]. In recent studies in Cambodian sample, it was reported that the MNK/A/Q haplotype correspond to susceptible, IDT/S/E haplotype for intermediate response and IET/S/EW for highest IC_{50} for chloroquine and the expression level of *pfcrt* had no effect on the response of the parasite to the drug *in vitro*[22].

In our study we combined *in vitro* tests, as well as molecular genotyping at *pfcrt* and *pfmdrI* loci. Here 63 (35.15%) isolates were chloroquine sensitive and 21 (11.73%) isolates were intermediate resistant to chloroquine in *in vitro* culture. All the sensitive isolate consist of wild type K76+N86+D1246 allele. Whereas intermediate resistant isolates are composed of either single Y86, Y1246, 76T mutation or some time double mutation with 76T+Y1246 and 86Y+1246Y allele. Whereas in Kolkata out of 132 isolates 60 (45.45%) isolates found *in vitro* chloroquine resistant, while in Purulia 35 (74.46%) isolates found *in vitro* chloroquine resistance. So out of 95 chloroquine resistant isolates 70 (73.68%) isolates have either double 76T+86Y ($P<0.01$) or triple 76T+86Y+1246Y ($P<0.001$) mutation, and left 25 chloroquine resistant isolates have either single 76T mutation or 76T+1246Y mutation.

Molecular and *in vitro* data were strongly related. PCR-based methods do not detect minor clones in a mixed population, but although a wild-type clone may remain undetected, this is unlikely for *in vitro* susceptibility, as IC_{50} mainly reflects the susceptibility of the major clone(s) present in the blood sample.

The correlation between *pfmdr1* genotypes and quinoline resistance has often generated conflicting results; although it has been suggested that *pfmdr1* 86Y can be correlated with increased chloroquine resistance in parasites which originated from different areas of the world, but in India chloroquine resistance is mainly correlated with *pfcrt* gene not with *pfmdrI* gene. The threonine allele of *pfcrt*1 gene (K76T mutation) had been reported to associate with anti-malarial chloroquine resistance[12,21]. Several groups of scientists showed that in India *pfmdrI* mutation was a key mutation but it can not cause severe *in vitro* resistance to chloroquine, instead of this it causes intermediate resistance to chloroquine. They concluded that *P. falciparum* genetic cross indicated that chloroquine resistant did not depend on the *pfmdr1* gene.

Our present findings implicate that double mutation with *pfcrt* 76T + *pfmdrI* 86Y and triple mutation with *pfcrt* 76T + *pfmdrI* 86Y + *pfmdrI* 1246Y was highly correlated ($P = 0.001$) with *in vitro* chloroquine resistance here, since the presence of both 76T and 86Y in our samples was largely dependent of their chloroquine response, indicating that chloroquine appear to exert selective pressure on this area of the gene.

Our results confirmed that *pfcrt* K76T and *pfmdrI* D1246Y is a key mutation but it may or may not cause severe *in vitro* resistance to chloroquine with single mutation, instead of this it causes intermediate resistance to chloroquine. Single *pfcrt* K76T and *pfmdrI* D1246Y mutations are known to have an effect on chloroquine resistance, as proven by genetic crossbreeding between sensitive and resistant parasites. However the unbalanced numbers of genotypes here does not allow us to draw conclusions on the impact of these mutations.

We think that this *Plasmodium* species might be a new serotype of *P. falciparum*, because of its alteration of genetic marker.

From this study it was concluded that severe chloroquine resistance in the eastern part of India is associated mainly with the combination of both *pfcrt* and *pfmdrI* (either double mutation with 76 T + 86Y or triple 76T+ 86Y+ 1246Y) mutation. The increase in the number of both *pfcrt* and *pfmdrI* mutations was strongly correlated to chloroquine resistance as chloroquine exerts a numerous drug pressure in this region of India. Further studies are needed to determine the total genomics of *pfcrt* and *pfmdrI* gene, precise incidence of the combination of *pfcrt* and *pfmdrI* gene mutations and the role of double *pfcrt* and *pfmdrI* mutation on chloroquine treatment outcome.

So changes in antimalarial policies in favor of the use of chloroquine in these areas of India are likely to increase chloroquine drug pressure, and the clinical efficacy of chloroquine may rapidly fade. New cheap antimalarial combinations (as treatment with ACT is much expensive to third world country) should be tested for treating the drug resistant *P. falciparum*.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgements

The authors express gratefulness to the Department of Science and Technology, Government of India for funding having D.O. No: SR/SO/HS-106/2005. The authors also express gratefulness to Vidyasagar University, Midnapore for providing the facilities to execute these studies

References

[1] World Health Organization. World malaria report. Geneva: WHO; 2009. [Onling] Available from: http://www.who.int/malaria/publications/atoz/9789241563901/en/index.html. [Accessed on 3rd April, 2010]

[2] Anstey NM, Russell B, Yeo TW, Price RN. The pathophysiology of vivaxmalaria. *Trends Parasitol* 2009; **25**: 220-227.

[3] Ekland EH, Fidock DA. *In vitro* evaluations of antimalarial drugs and their relevance to clinical outcomes. *Int J Parasitol* 2008; **38**: 743-747.

[4] Fidock DA, T Nomura AK, Talley RA, Cooper SM, Dzekunov MT, Ferdig LM. et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein *pfcrt* and evidence of their role in chloroquine resistance. *Mol Cell* 2000; **6**: 861-871.

[5] Wisedpanichkij Raewadee, Chaicharoenkul Wanna, Mahamad Poonuch, Prompradit Prapichaya, Na-Bangchang Kesara. Polymorphisms of the oxidant enzymes glutathione S-transferase and glutathione reductase and their association with resistance of *Plasmodium falciparum* isolates to antimalarial drugs. *Asian Pac J Trop Med* 2010, **3** (9); 673-677.

[6] NIMR. *Guidelines for diagnosis and treatment of malaria in India 2009*, Government of India. Dwarka, New Delhi: Director, National Institute of Malaria Research Sector 8; 2009.

[7] Omer AO, Tahir EL Ahmed, Abdelwahid O Mohamed, Gasmelseed Nagla, Mergani Adil, Nasr Eldin MA Elwali. Co-existence of dihydrofolate reductase (*dhfr*108) Gene with *Plasmodium falciparum* chloroquine resistance transporter gene (*Pfcrt* T76) in *P. falciparum* Isolates from Gezira State, Central Sudan. *Open Trop Med J* 2010, **3**; 15-17.

[8] Nkhoma Standwell, Nair Shalini, Mukaka Mavuto, Molyneux E Malcolm A, Ward Stephen, et al. Parasites bearing a single copy of the multi-drug resistance gene (*pfmdr*-1) with wild-type SNPs predominate amongst *Plasmodium falciparum* isolates from Malawi. *Acta Trop* 2009; **111**(1): 78-81.

[9] Nkhoma S, Molyneux M, Ward S. *In vitro* antimalarial susceptibility profile and *prcrt/pfmdr*-1genotypes of *Plasmodium falciparum* field isolates from Malawi. *Am J Trop Med Hyg* 2007; **76**(6): 1107-112.

[10] Basco KL, Ringwald P. Molecular epidemiology of malaria in Yaounde, Cameroon. VI. Sequence variations in the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene and *in vitro* resistance to pyrimethamine and cycloguanil. *Am J Trop Med Hyg* 2000; **62**(2): 271-276.

[11] Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; **193**: 673-675.

[12] Lopes D, Rungsihirunrat K, Nogueira F, Seugorn A, Gil PJ, do Rosário VE, et al. Molecular characterisation of drug-resistant *Plasmodium falciparum* from Thailand. *Malar J* 2002; **1**: 12.

[13] Bouyou-Akotet MK, Mawili-Mboumba DP, Tchantchou Tde D, Kombila M. High prevalence of sulfadoxine/pyrimethamine-resistant alleles of *Plasmodium falciparum* isolates in pregnant women at the time of introduction of intermittent preventive treatment with sulfadoxine/pyrimethamine in Gabon. *J Antimicrob Chemother* 2010; **65** (6): 438-441.

[14] Reza YM, Taghi RM. Prevalence of malaria infection in Sarbaz, Sistan and Bluchistan province. *Asian Pac J Trop Biomed* 2011; **1**(6): 491-492.

[15] Zerihun T, Degarege A, Erko B. Association of ABO blood group and *Plasmodium falciparum* malaria in Dore Bafeno Area, Southern Ethiopia. *Asian Pac J Trop Biomed* 2011; **1**(4): 289-294.

[16] Wiwanitkit V. Concurrent malaria and dengue infection: a brief summary and comment. *Asian Pac J Trop Biomed* 2011; **1**(4): 326-327.

[17] Ahmad N, Fazal H, Ayaz M, Abbasi BH, Mohammad I, Fazal L. Dengue fever treatment with *Carica papaya* leaves extracts. *Asian Pac J Trop Biomed* 2011; **1**(4): 330-333.

[18] Krungkrai SR, Krungkrai J. Malaria parasite carbonic anhydrase: inhibition of aromatic/heterocyclic sulfonamides and its therapeutic potential. *Asian Pac J Trop Biomed* 2011; **1**(3): 233-242.

[19] Kumar S, Wahab N, Warikoo R. Bioefficacy of *Mentha piperita* essential oil against dengue fever mosquito *Aedes aegypti* L. *Asian Pac J Trop Biomed* 2011; **1**(2): 85-88.

[20] Lorenz V, Karanis P. Malaria vaccines: looking back and lessons learnt. *Asian Pac J Trop Biomed* 2011; **1**(1): 74-78.

[21] Veiga IM, Ferreira EP, Bjorkman A, Gil Pedro J. Multiplex PCR- RFLP methods for *pfcrt*, *pfmdrI* and *pfdhfr* mutation in *Plasmodium falciparum*. *Mol Cell Probes* 2006; **20**: 100-104.

[22] Durrand V, Berry A, Sem R, Glaziou P, Beaudou J, Fandeur T. Variations in the sequence and expression of the *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) and their relationship to chloroquine resistance *in vitro*. *Mol Biochem Parasitol* 2004; **136**(2); 273-285.