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Progressive increase in point mutations associates chloroquine resistance: Even after withdrawal of chloroquine use in India

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ABSTRACT

Chloroquine (CQ) is highly effective against *P. vivax*, due to the rapid spread of CQ resistance in *P. falciparum* parasites; it is no longer the drug of choice against *P. falciparum*. This study elucidates the scenario of chloroquine efficacy at times that coincided with a new drug policy and especially assessed the chloroquine resistant molecular markers after withdrawal of chloroquine in Kolkata and Purulia, two malaria endemic zones of West Bengal, India. *In vitro* CQ susceptibility was tested in 781 patients with *P. falciparum* mono infections between 2008 and 2013, of which 338 patients had received CQ in 2008–2009. Genotyping of the *pfcrt* and the *pfmdr1* gene was carried out in all isolates. Early treatment failure was detected in 114 patients {43 (31·39%) from Kolkata and 71 (35·32%) from Purulia} while recrudescence was identified in 13 (9.49%) and 17 (8.46%) patients from Kolkata and Purulia respectively. *In vivo* chloroquine resistance was strongly associated with CVMN**T**-**Y**YSN**Y** ($p < 0.01$) and **S**VMN**T**-**Y**YSNY ($p < 0.05$) allele in Kolkata. In Purulia chloroquine resistance was associated with CVMNK-**Y**YSN**Y** ($P < 0.005$), **S**VMN**T**-**Y**YSNY ($P < 0.01$) allele. The proportion of *in vitro* chloroquine resistance increased in subsequent years to 87.23% and 93·10% in 2013, in Kolkata and Purulia, respectively. Isolates with **S**VMN**T**-**YF**SND, **S**VMN**T**-**YF**SN**Y**, CV**IET**-**YF**SND and CV**IET**-**Y**YSN**Y** haplotypes increased gradually ($p < 0.05$) from 2010 to 2013, leading to a rise in IC_{50} ($p < 0.05$) of chloroquine. An increase in *in vitro* chloroquine resistance and candidate gene mutations even after five years of chloroquine withdrawal against *P. falciparum* calls for synchronized research surveillance and proper containment strategies.

1. Introduction

Chloroquine (CQ) is cheap, well tolerated and easily administered and has been used as an antimalarial against *P. falciparum* for more than five decades (Sehgal et al., 1973), but the progressive development of resistance has led to the replacement of CQ by the combination of Artesunate + Sulfadoxine-Pyrimethamine {Artemisinin Combination Therapy (ACT)} in mid 2009 (Government of India, 2009). CQ efficacy was found to be declining in different states of India around 2000. In 1990 only 5·2% of isolates possessed CQ resistance in Madhya Pradesh, which increased to 53% at neighboring state Uttar Pradesh in 2005 (Singh and Shukla, 1990; Wijeyaratne et al., 2005). High rates of CQ treatment failure were also reported in other parts of India such as Orissa and Assam (Satpathy et al., 1997; Dua et al., 2003). The genetic basis of CQ-resistance has now been well studied in *P. falciparum*. *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene located on chromosome 5 encodes a transporter for importing solutes, including different drugs (CQ, quinine, mefloquine) into the food vacuole. Polymorphisms' leading to the substitution of asparagine with tyrosine at codon 86 of pfmdr1 gene reduces CQ influx into the food vacuole, resulting in CQ resistance (Foote et al., 1990). Some other *pfmdr1* polymorphisms, like Y184F, S1034N, N1042D and D1246Y, were implicated in varying degrees to CQ resistance (Duraisingh et al., 2000; Andriantsoanirina et al., 2010). *P. falciparum* CQ resistance transporter (*pfcrt*), a key gene for CQ

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resistance, was identified on chromosome 7. Specific point mutation in 72–76 codon of PFCRT protein promoted CQ resistance by efflux out the CQ from food vacuole (Fidock et al., 2000; Djimdé et al., 2001; Mehlotra et al., 2001; Sharma, 2005).

In 1993, Malawi became the first country in Africa to replace CQ by sulfadoxine-pyrimethamine (SP) (Bloland et al., 1993), and subsequently CQ-resistant mutant *pfcrt* and *pfmdr1* alleles decreased till they became undetectable in 2001, suggesting that CQ might once again be effective. Finally, CQ was reintroduced after 12 years of withdrawal (Laufer et al., 2006) in Malawi. Thus withdrawing the use of CQ in CQ resistant region could result in the re-emergence of CQ-sensitive parasite. In such settings, the molecular markers of CQ-resistance needed to be determined after the introduction of ACT in India. Therefore, the present investigation was conducted to evaluate CQ efficacy prior to ACT treatment and also to assess the CQ resistant molecular markers after withdrawal of CQ in Eastern India.

2. Materials and method

2.1. Study site

This study deals with the clinical assessment of chloroquine resistance (*in vivo* as well as *in vitro*) in India from February 2008 to December 2013, before and after introduction of ACT. Blood samples were collected from Kolkata {Goutam Laboratory (NABL accredited laboratory, ISO 15189:2007-M-0423)}, and Purulia (Purulia district hospital) two highly malaria endemic regions of India. In 2010, there were 134795 cases of malaria in West Bengal. The Kolkata Metropolitan Corporation contributes 96693 (71.73%) malaria cases (both *P. falciparum* and *P. vivax*) whereas Purulia contributed more than 75% of *P. falciparum* infection (Fig. 1) (Annual District wise Epidemiological Report of Malaria of West Bengal, 2006, 2010). The experimental design and protocol of this study were duly approved by Vidyasagar University Ethical Committee. Written informed consent was obtained from parents or guardians for child patients.

2.2. Patients' selection and in vivo CQ treatment

A standard 28-day test of therapeutic efficacy was used (WHO, 2003) to assess treatment of *P. falciparum* infection in 2008–2009. Clinical isolates collected from 2010 to 2013 were only tested for *in vitro* CQ susceptibility and polymorphisms in different candidate genes. Patients suffering from fever (body temperature >37.5 °C) with headache, shivering, and vomiting tendency during previous 24 h s were tested for malaria. Two ml of intravenous blood was collected from each of 5210 suspected patients, in an anticoagulant coated (EDTA) vacutainer. Patients with positive rapid diagnostic test results and microscopically confirmed *P. falciparum* malaria with a parasite density of 1000–200000 asexual parasites/μl blood and no recent history of self-medication with antimalarial drugs received the standard dose of 10 mg/kg CQ on day 1 and day 2, 5 mg/kg CQ on day 3. Study drugs were purchased from standard commercial sources (Resochin; Bayer) and administered under direct observation by trained study nurses. The clinical conditions, hemoglobin and parasite density were monitored on days 0, 1, 2, 3, 7, 14 and 28 (WHO, 2003). Unscheduled follow-up visits were performed at any time between scheduled visits when symptoms of malaria recurred. Patients who vomited the drug (CQ) twice were withdrawn from the study and transferred to Kolkata National Medical College hospital for further care. Patients with signs and symptoms of severe and complicated malaria, pregnant women, lactating mothers, children below the age of 3 years and those with hematocrit <20% were excluded from the study. Therapeutic responses were classified as adequate clinical parasitological response (ACPR) (absence of parasitaemia after day 28 of drug administration; symptoms of malaria subside by day 3, parasitaemia gradually declines and disappears by day 3), early treatment failure (ETF) (Irrespective of axillary temperature, patients having parasitaemia on day 2 higher than from day 0; or parasitaemia on day 3 > 25% of count on day 0 with axillary temperature >37.5 °C) and late treatment failure (LTF) (Recrudescence of parasite within 4–28 days) according to WHO guideline (WHO, 2003). Patients who did not respond to CQ treatment were treated with Artesunate + SP combination.

2.3. In vitro CQ susceptibility

In vitro drug sensitivity assay was performed in all clinical isolates after culture adaptation as a part of the antimalarial susceptibility surveillance during 2008–2013, as described earlier (Trager and Jensen, 1976; Basco and Ringwald, 2000). A synchronized parasite culture was maintained over at least three life cycles prior to drug (CQ) exposure. RPMI1640 was used to prepare stock solutions and dilutions of CQ (Sigma). The IC_{50} was evaluated using hypoxanthine incorporation assay following our established laboratory protocol (KarMahapatra et al., 2011; Das et al., 2014). Isolates were defined as CQ susceptible when IC_{50} values were $\leq$100 nM, and CQ-resistant when IC was >100 nM. The CQ-sensitive strain 3D7 and CQ-resistant strain Dd2 were used as controls.

2.4. DNA extraction, genotyping of candidate gene

Parasite DNA was extracted from 1 ml of infected blood using the phenol-chloroform extraction method as described elsewhere (Basco and Ringwald, 2000). Regions of the *pfcrt* and *pfmdr1* genes surrounding the polymorphism of interest were amplified by polymerase chain reaction using an Eppendorf thermal cycler. Primers were designed as described in our previous laboratory work (Das et al., 2014). Different polymorphisms of *pfcrt* and *pfmdr1* gene were analyzed as described earlier (Lopes et al., 2002; Das et al., 2013, 2014). 3D7 and Dd2 strains served as controls. Sequencing was carried out using an ABI Prism Big Dye Terminator cycle sequencing ready reaction kit and run on a model 3730 xl genetic analyzer (Applied Biosystems) (Das et al., 2014). Sequencing was performed at IIT, Kharagpur, and SciGenome Company (Kochi) for cross validation. Sequences were translated using an online translation tool (http://www.expasy.org) and aligned using the multiple sequence alignment tool ClustalW2 (http://www.ebi.ac.uk/clustalw). Mutations were confirmed by reading forward and reverse strands.

2.5. Assessment of isolate clonality

An allelic family-specific nested PCR was used to identify the multiplicity of infection i.e. the highest number of alleles detected in either of the two loci (MAD20 and K1 for *pfmsp1* and 3D7 Africa and FC27 for *pfmsp-2*) (Snounou et al., 1993). It was used to classify the isolates as monoclonal or polyclonal infection and distinguish recrudescence from new infection for all patients failing therapy after day seven (isolates from day 0 and day of recurrence). All amplifications contained a positive control (genomic DNA from strain 3D7) and a negative control (no target DNA).

2.6. Evaluation of antimalarial drug pressure

Cross-sectional surveys were carried out from June 2008 to August 2008 and April 2012 to September 2012 in Kolkata and Purulia. A total of 1440 individuals were interviewed (720

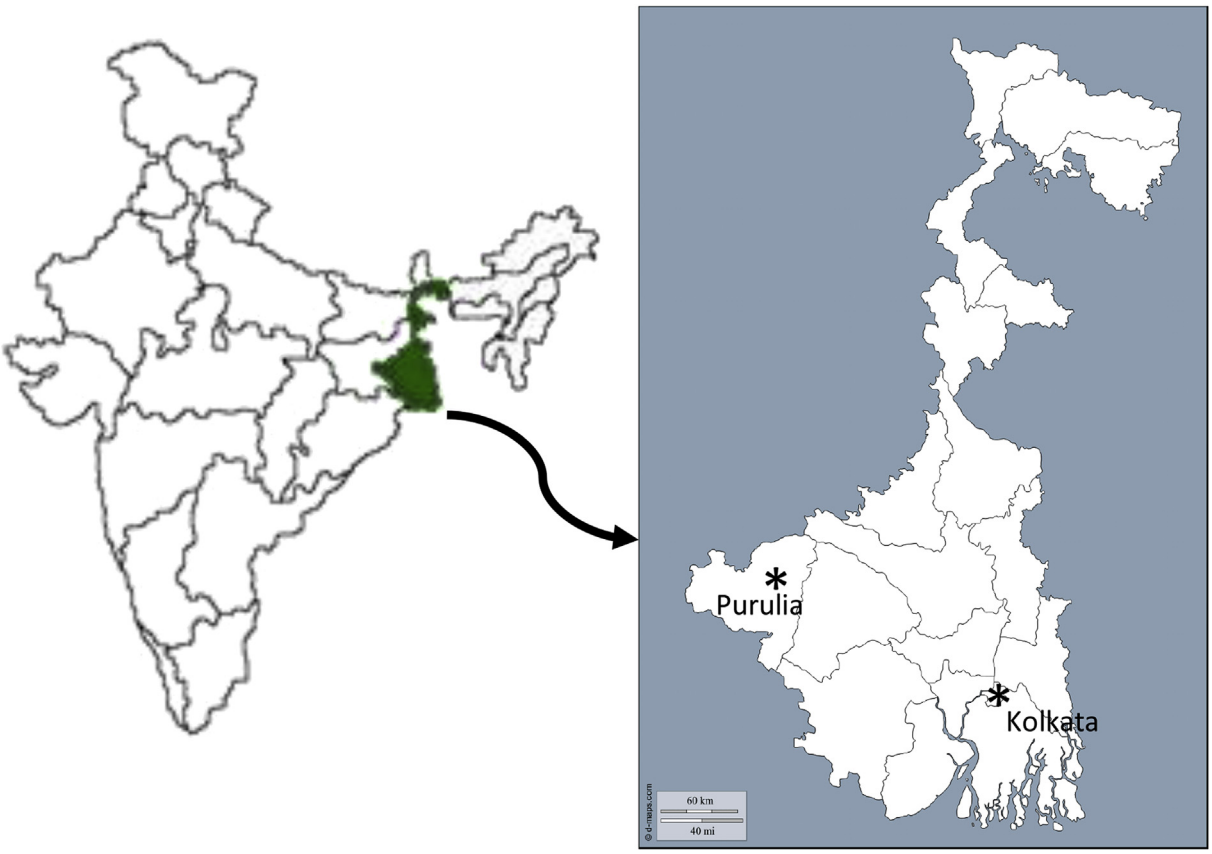

Fig. 1. Geographical map of the study sites. Kolkata and Purulia are shown as asterisks.

individual each from Kolkata and Purulia). Forty households were randomly selected from three zonal blocks (rural, semi rural and urban division) in each districts, with different socio-economical status. Three randomly selected individuals (age range 19–75) from each household were interviewed about their recent travel and antimalarial drug consumption, as described elsewhere (Gardella et al., 2008).

2.7. Statistical analysis

IC_{50} values of CQ were expressed as mean ± standard deviation (SD). The relation between treatment efficacies and molecular genotypes was studied by Fisher's exact test and regression analysis. IC_{50}s were expressed as the geometric mean (95% confidence intervals). The Mann-Whitney *U* test was used to compare between two groups, and the Kruskal-Wallis-test (H-test) was used to compare between more than two groups. Differences were considered statistically significant when $p < 0{\cdot}05$. Analyses were performed using the statistical package Origin 6.1, and GraphPad In Stat software 3.0.

3. Results

3.1. Study population

Malaria infection was screened in 5210 suspected cases between 2008 and 2013, of which 1721 patients (33.03%) found malaria positive cases. A total of 870 patients (50.55%) were identified as *P. falciparum* positive isolates. 746 patients (43.35%) were excluded, as they contained *P. vivax* infection. Another 105 patients were eliminated due to co-infection with *P. vivax*. These co-infections were only found in Kolkata. Multiplicity of infection was analyzed for a subset of all 870 *P. falciparum* positive patients. The proportion of the monoclonal infections was very high. Of the 870 cases, 819 patients (94·14%) contained a single allelic form i.e. either *mspI* or *mspII*, and these isolates were enrolled in this study. Finally 781 (95·36%) patients had completed the 28 days follow up treatment (Fig. 2). 63 patients were found to be under the age of five. The characteristics of participants in two study sites during enrolment were quite similar (Table 1) and year wise enrollment information was presented in Supplemental Table 1.

3.2. Clinical efficacy of chloroquine

P. falciparum positive patients were received standard dose of CQ in 2008–2009. 338 patients had completed the 28 day follow up treatment (Table 2). Early treatment failure (ETF) was detected in 114 patients {43 patients (31·39%) from Kolkata and 71 (35·32%) patients from Purulia} (33·73%) while 38 patients (11·24%) were identified as late treatment failure (16 patients from Kolkata and 22 patients from Purulia) cases. *msp1*, *msp2* and glurp were analyzed in all 38 apparent LTF cases to differentiate between 'new infection' and 'recrudescence'. Of the 38 cases, 30 patients (13 from Kolkata and 17 from Purulia) were finally identified as true 'recrudescence' case. Three LTF cases (2.19%) from Kolkata and five LTF cases (2.49%) from Purulia were classified as a new infection. CQ treatment failure (ETF + recrudescence) increased in subsequent year from 37.09% to 44.0% in 2008–2009, in Kolkata whereas in Purulia treatment failure was observed in 45·33% of patients in 2008, which slightly decreased to 42.96% in 2009 (Table 2).

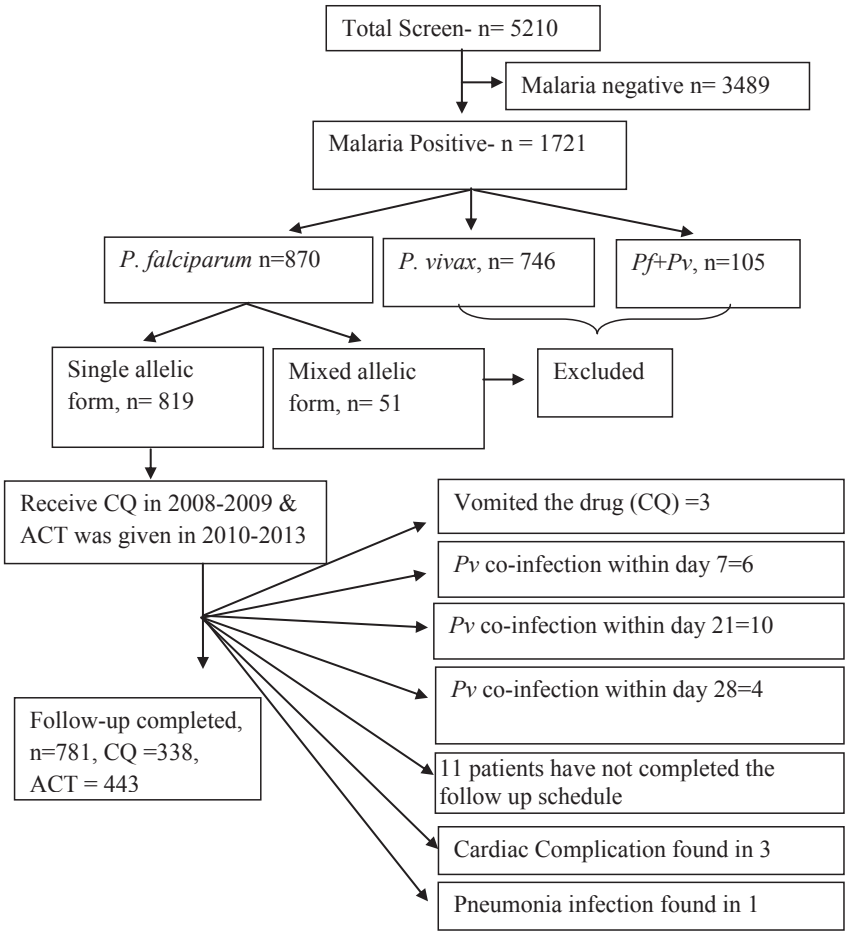

Fig. 2. Schematic presentation of patients' selection during the study period.

Table 1
Patient characteristics at enrolment of the study in different study area.

Patient Characteristics	Kolkata	Purulia
Age (year)	28.76 (95%CI, 3–72)	26.31(95%CI, 3–73)
Sex ratio (Women/Men)	177/168	191/245
Axillary Temperature on day 0 (°C)	39.38 °C (95%CI, 37.71–40.67)	38.44 °C (95%CI, 37.33–39.89)
Parasitaemia (parasite/μl)	86188 (95%CI, 11500–181000)	77452 (95%CI, 11000–156000)
Mean haemoglobin (g/dl)	9.6 ± 2.1	10.2 ± 1.7

Table 2
Distribution of CQ treatment efficacy in Kolkata and Purulia during 2008–2009.

Year	ACPR		ETF		LTF			
	Kolkata	Purulia	Kolkata	Purulia	Kolkata		Purulia	
					Recrud-escence	New infection	Recrud-escence	New infection
2008	38 (61.29%)	41 (54.67%)	18 (29.03%)	28 (37.33%)	5 (8.06%)	1 (1.62%)	6 (8.0%)	0 (0.00%)
2009	40 (53.33%)	67 (53.17%)	25 (33.33%)	43 (34.13%)	8 (10.67%)	2 (2.67%)	11 (8.73%)	5 (3.97%)
Total	78 (56.93%)	108 (53.73%)	43 (31.39%)	71 (35.32%)	13 (9.49%)	3 (2.19%)	17 (8.46%)	5 (2.49%)

3.3. *In vitro* CQ susceptibility

In vitro CQ susceptibility assay was successfully performed in 738 isolates out of 781 cases. Culture adaptations were failed in 12 isolates from Kolkata and 31 isolates from Purulia. The proportion of *in vitro* CQ resistance (mean IC_{50} = 146.5 nM, 95%CI, 110–210 nM) in Kolkata was 58.06% in 2008 which was gradually increased in subsequent years to 87.23% in 2013 (mean IC_{50} = 238·60 nM; 95% CI, 121–321 nM). 76% of isolates in Purulia were highly resistant to CQ (mean IC_{50} = 162·25 nM; 95%CI, 112–254 nM) in 2008 which was exceedingly increased in consequent years to 93·10% in 2013 (mean IC_{50} = 247·42 nM; 95%CI, 126–316 nM) (Fig. 3).

3.4. Antimalarial drug pressure assessment

A total of 720 individuals were interviewed (360 individual each from Kolkata and Purulia) in 2008 (mean age = 26·82 years, age range = 21–75 years). An antimalarial treatment was consumed by 17.24% and 21.37% of individual from Kolkata and Purulia respectively in previous 30 days at primary health center or hospital. CQ was the most commonly prescribed antimalarial drug by the clinicians (79%) followed by SP (12%), quinine (QU; 7%) and ACT (2%) in Kolkata. Similarly, CQ (74·5%) was frequently used antimalarial drug after SP (11%) and quinine (QU; 14·5%) in Purulia. A total of 36·45% individuals had taken self medication at home; of them 62% declared that they had used CQ, remaining 28% and 10% of individuals had used QU and SP respectively. In previous 30 days 26.11% of individuals from Purulia travelled to Kolkata whereas only 5.83% of individuals from Kolkata travelled to Purulia. The proportion of individuals who had travelled outside the site in previous 30 days was estimated very high in Purulia (41.11%) than Kolkata (24.72%).

A total of 360 individuals (mean age = 23·41 years, age range = 19–72 years) from each study sites were interviewed again in 2012. ACT was the most commonly prescribed antimalarial drug (86%) by the clinicians followed by chloroquine (9%) and artesunate monotherapy (5%) in Kolkata. Like Kolkata, ACT (72·6%) was also the most preferred antimalarial after artesunate monotherapy (11%), chloroquine (15·4%) and quinine (1%) in Purulia. Self medication (22·5% of total individuals) with CQ, artesunate monotherapy, and ACT had taken by 46%, 31%, and 23% of individuals respectively. The proportion of individuals who travelled outside the site in the previous 30 days was estimated very high in Purulia (46.38%) than Kolkata (29.16%).

3.5. Genotyping of pfcrt and pfmdr1 before new national drug policy

The key polymorphisms leading to the substitution of lysine with threonine at codon 76 of the *pfcrt* gene was 66.13% in 2008 which increased to 72% in 2009, in Kolkata while K76T polymorphism increased from 44% to 57·94% in 2009, in Purulia (Supplementary Fig. 1). Mutation was absent in 73, 74 and 75 codon of *pfcrt* gene in 2008. Polymorphisms leading to the substitution of asparagine with tyrosine at codon 86 of *pfmdr1*gene (N86Y) was recorded 41·94% and 46·67% in 2008 and 2009 respectively from Kolkata while this mutation was identified in 66·97% and 64·28% of isolates from Purulia in 2008 and 2009 respectively. The rates of isolates consisting of mutant D1246Y alleles were very high in 2008 (53·33%) and in 2009 (46·03%) (Supplementary Fig. 2). In Purulia, wild-type *pfcrt* (CVMNK) haplotype was prevalent (56%), whereas in Kolkata single mutant CVMN**T** haplotype (40·32%) was more common than wild type CVMNK (33·87%) haplotype in 2008. Interestingly *pfcrt* CVMN**T** (34·67%) haplotype was prevalent, followed by **S**VMN**T** (33·33%) and CVMNK (28%) haplotype in Kolkata, while in Purulia, **S**VMN**T** (31·75%) haplotype was the most common *pfcrt* allele, followed by wild type CVMNK (29.37%), single mutant CVMN**T** (23·02%) haplotype, in 2009 (Fig. 4).

In case of *pfmdr1* gene, wild type NYSND haplotype (46·77%) was prevalent followed by single mutant **Y**YSND (25·81%) and double mutant **Y**YSN**Y** (16·13%) haplotype in Kolkata whereas in Purulia **Y**YSN**Y** haplotype (36%) was frequently found after wild type NYSND (29.33%) and **Y**YSND (17·33%) haplotype in 2008. Like 2008, wild type NYSND haplotype (37·33%) was prevalent, followed by **Y**YSND (26·67%) and **Y**YSN**Y** (20%) haplotype in 2009. Just like 2008, *pfmdr1* **Y**YSN**Y** haplotype (40.48%) was commonly found after NYSND (30·16%) and **Y**YSND (20·63%) haplotype in 2009 in Purulia (Table 3) (Fig. 5).

3.6. pfcrt and pfmdr1 polymorphisms after new national drug policy

CQ is no longer the drug of choice against *P. falciparum* after 2009 in India, but the genes (*pfcrt,* and *pfmdr1*) which are responsible for CQ resistance continued to increase in number. The proportion of *pfcrt* K76T polymorphism increased to 94·64% and 96·15% in 2012, in Kolkata and Purulia respectively (Table 4). Like

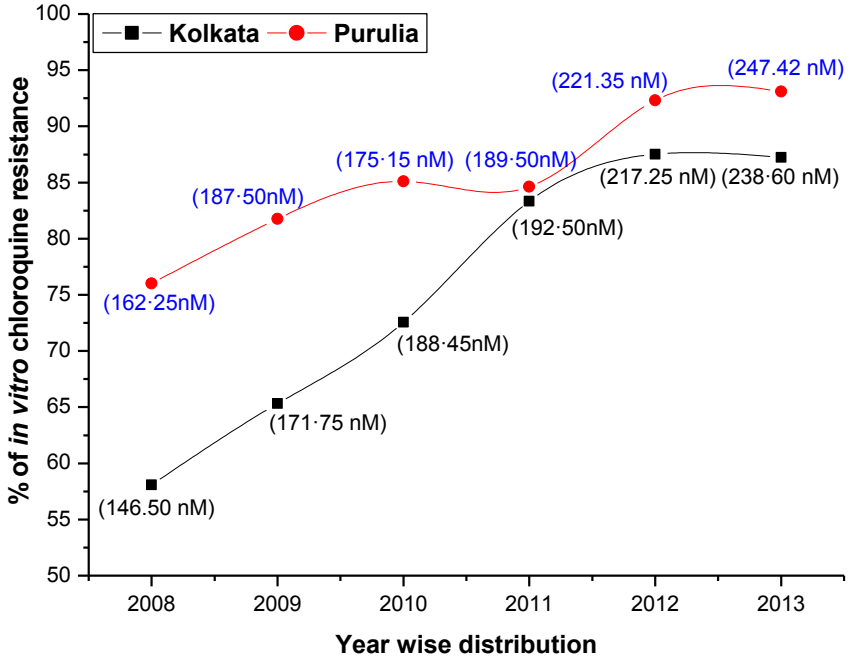

Fig. 3. Proportion of *in vitro* CQ resistance before and after new national drug policy. Mean IC_{50} values of CQ in successive years were presented.

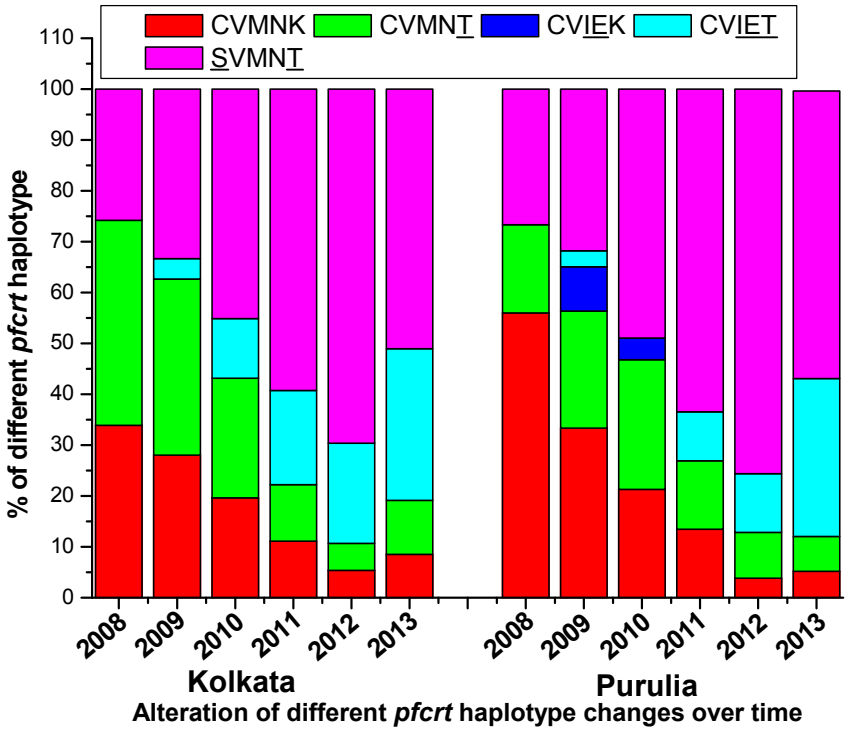

Fig. 4. Frequency of different *pfcrt* haplotype in Kolkata and Purulia in 2008–2013.

Table 3
Distribution of different *pfcrt* and *pfmdr1* haplotype in relation to *in vivo* CQ treatment efficacy and *in vitro* CQ susceptibility in Kolkata and Purulia before new national drug policy.

Year	*pfcrt* haplotype	*Pfmdr1* haplotype	No of isolates		CQ treatment efficacy						*In vitro* CQ response			
	(72–76)	86,184, 10 34, 1042, 1246	K	P	ACPR K	ACPR P	ETFK	ETFP	LTFK	LTFP	S K	S P	R K	R P
2008	CVMNK	NYSND	19	13	19	13	–	–	–	–	19	13	–	–
	CVMNK	**Y**YSND	2	6	2	6	–	–	–	–	1	2	1	4
	SVMN**T**	NYSND	6	3	4	2	1		1	1	1	–	5	3
	CVMN**T**	NYSND	4	6	4	6	–	–	–		2	1	2	5
	CVMN**T**	**Y**YSND	7	3	2	2	4	1	1	–	1	–	6	3
	CVMN**T**	NYS**D**D	4	–	3	–	1	–	–	–	1	–	3	–
	CVMN**T**	**Y**YSN**Y**	10	4	2	1	7	3	1	–	1	–	9	4
	SVMN**T**	NYSN**Y**	3	3	1	3	1	–	1	–	–	–	3	3
	SVMN**T**	**Y**YSND	7	4	1	3	4	–	2	1	–	–	7	4
	CVMNK	**Y**YSN**Y**	–	23	–	4	–	17	–	2	–	2	–	21
	SVMN**T**	**Y**YSN**Y**	–	10	–	1	–	7	–	2	–	–	–	10
2009	CVMNK	NYSND	21	5	21	5	–	–	–	–	21	5	–	–
	SVMN**T**	NYSND	7	21	4	17	1	–	2	4	2	1	5	20
	CVMNK	**Y**YSN**Y**	–	37	–	5	–	26	–	6	–	4	–	33
	CVIEK	**Y**YSN**Y**	–	11	–	1	–	8	–	2	–	1	–	10
	CVIET	**Y**YSND	3	4	–	2	3	1	–	1	–	–	3	4
	CVMN**T**	**Y**YSND	11	12	2	11	6	1	3	–	1	4	10	8
	SVMN**T**	NYS**D**D	5	–	5	–	–	–	–	–	–	–	5	–
	CVMN**T**	**Y**YSN**Y**	15	3	3	–	11	3	1	–	–	–	15	3
	SVMN**T**	NYSN**Y**	5	5	4	5			1		1	1	4	4
	SVMN**T**	**Y**YSND	6	14	1	9	3	3	2	2	1	–	5	14
	SVMN**T**	N**F**SND	2	–	–	–	1	–	1	–	–	–	2	–
	CVMN**T**	NYSND	–	12	–	10	–	1	–	1	–	5	–	7
	CVMN**T**	NYSN**Y**	–	2	–	2	–	–	–	–	–	2	–	–
	Total		**137**	**201**	**78**	**108**	**43**	**71**	**16**	**22**	**52**	**41**	**85**	**160**

Bold and underline amino acid are the mutant codon. Here *in vitro* test responses are classified as sensitive (S) (IC_{50} value < 100 nM) and Resistant (R) (IC_{50} value > 100 nM). Here K denotes for Kolkata and P denotes Purulia.

K76T polymorphism, *pfcrt* C72S mutation also reached to its maximal level of 69·64% and 75·4% in 2012, in Kolkata and Purulia respectively. The rate of isolates consisting of *pfmdr1* N86Y and Y184F polymorphisms were markedly increased from 2009 (46·67% and 2·67% respectively) to 2013 (87.23% and 53.19% respectively) in Kolkata. Similarly, polymorphism at N86Y and Y184F allele were found to 87·93% and 62·07% in 2013, in Purulia respectively (Supplementary Fig. 1, Supplementary Fig. 2).

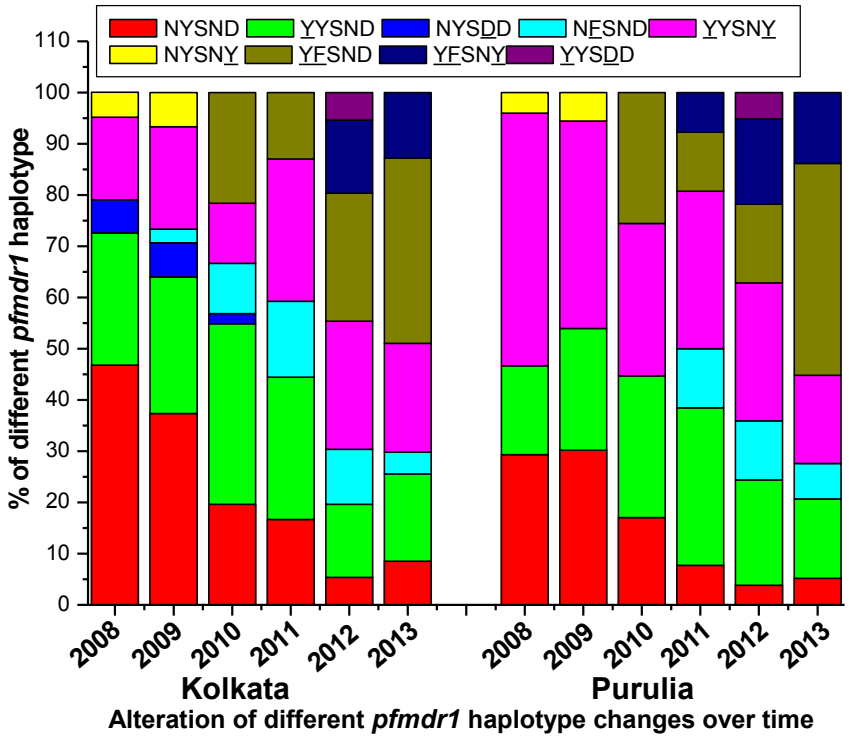

Fig. 5. Allele frequency of different *pfmdr1* haplotype in Kolkata and Purulia in 2008–2013.

After 2010 wild type *pfcrt* CVMNK haplotype rapidly reduced to only 8·51% and 5·17% of isolates in 2013, in Kolkata and Purulia respectively. The frequency of CVMN**T** haplotype decreased while **S**VMN**T** haplotype highly increased and reached its highest pick in 2012. The most vulnerable *pfcrt* CV**IET** haplotype was rare in 2008–2009, but this haplotype gradually increased in subsequent years to 29.78% and 31.03% in 2013, in Kolkata and Purulia respectively (Fig. 4). Predominance of *pfmdr1* **YF**SND allele was observed both in Kolkata (36·17%) and in Purulia (41·38%) in 2013. Wild type NYSND and **Y**YSN**Y** haplotypes were gradually decreased in subsequent years while triple mutant **YF**SN**Y** allele progressively increased (Fig. 5).

3.7. Genotypic correlation of CQ efficacy in vivo and in vitro

CQ resistant pattern was observed much different in Purulia from Kolkata. CQ treatment failure was strongly correlated to *pfmdr1* 86Y+1246Y mutation ($r^2 = 0.9990$, $p < 0.0011$) but not with the *pfcrt* gene in Purulia, while *in vivo* CQ treatment efficacy was strongly correlated to *pfcrt*76T + *pfmdr1*86Y polymorphism or *pfcrt* 76T + *pfmdr1*86Y+1246Y mutation in Kolkata (Fisher test: CQ, $p < 0.01$ for *pfcrt* 76T, *pfmdr1* 86Y codon; $p < 0.05$ for 1246Y codon) (Table 3). The phenotype of *in vitro* CQ sensitivity was also highly correlated to codon 76 and 72 of *pfcrt* gene and codon 86, and 1246 of the *pfmdr1* gene (CQ, $p < 0.05$ for codon 76 and 72 and $p < 0.01$ for codon 86, and 1246) in 2008–2009.

Patients with wild type CVMNK-NYSND haplotype always produced ACPR after CQ treatment. Isolates with CVMN**T**-NYSND, **S**VMN**T**-NYSND and CVMN**T**-NYSN**Y** haplotype presented low to moderate IC_{50} values of CQ and were not associated with an ETF ($p = 0·74$, Kruskal-Wallis test). Triple mutant **S**VMN**T**-NYSN**Y**, **S**VMN**T**-NYS**D**D haplotype were found to have moderate to high IC_{50} of CQ but these haplotypes were not correlated with an ETF ($p < 0·01$, Mann-Whitney *U* test). Isolates with CVMN**T**-**Y**YSN**Y** haplotype were highly correlated with ETF in Kolkata in 2008–2009 ($p < 0·005$). Three out of five LTF were observed to have this triple mutant haplotype in Kolkata. Isolates containing CVMNK-**Y**YSN**Y** and **S**VMN**T**-**Y**YSN**Y** haplotype was found to have association with ETF in 2008 as well as in 2009 ($p < 0·001$). Isolates with CVMN**T**-**Y**YSND, and **S**VMN**T**-**Y**YSND haplotype represented very high IC_{50} of CQ ($p < 0·001$) and found to have less susceptible to CQ *in vivo* ($p < 0·05$). Isolates with quadruple mutant CV**IET**-**Y**YSND haplotype was observed to have highest IC_{50} of CQ and proved to correlated with CQ treatment failure ($p < 0·05$) (Fig. 6A). Alteration of molecular genotyping, CQ treatment efficacy and *in vitro* CQ resistance were found to have strongly correlated ($r^2 = 0·9993$, $p < 0·015$).

3.8. Haplotype diversity in relation to in vitro CQ susceptibility in 2010–2013

The frequency of wild type CVMNK-NYSND allele was found to have decreased after 2010. Most of the haplotype was observed to have $IC_{50} > 100$ nM of CQ proving *in vitro* CQ resistance. Isolates with **S**VMN**T**-**Y**YSND, **S**VMN**T**-N**F**SND, CVMN**T**-**Y**YSN**Y** and **S**VMN**T**-**YF**SND haplotype increased gradually from 2010 to 2013, leading to a rise in IC_{50} of CQ ($p < 0·05$) in Kolkata as well as in Purulia (Fig. 6B). Isolates with quintuple mutant **S**VMN**T**-**YF**SN**Y** and CV**IET**-**Y**YSN**Y** haplotype were found to have exceedingly high IC_{50} of CQ ($p < 0·005$) while quintuple mutant CV**IET**-**YF**SND haplotype represented the highest IC_{50} of CQ in Kolkata as well as in Purulia (Supplementary Fig. 3, Table 4).

3.9. Regional bias and chronological diversity in pfcrt and pfmdr1 polymorphism

In Kolkata, 30·65% and 28% of patients were found to have the wild type CVMNK-NYSND haplotype combination in 2008 and 2009 respectively while only 17·33% and 3·96% of isolates represented this wild haplotype in Purulia. The proportion of this wild

Table 4
Distribution of different *pfcrt* and *pfmdr1* haplotype in relation to *in vitro* CQ susceptibility after implementation of ACT.

Year	*pfcrt* haplotype	*Pfmdr1* haplotype	No of isolates		*In vitro* CQ response			
	(72–76)	86,184,1034,1042, 1246	K	P	S K	S P	R K	R P
2010	CVMNK	NYSND	10	2	10	2	–	–
	CVIET	**Y**YSND	6	-	–	–	6	–
	CVIEK	**Y**YSN**Y**	–	2	–	–	–	2
	CVMN**T**	NYSND	–	6	–	3	–	3
	CVMN**T**	**Y**YSND	6	2	1	–	5	2
	SVMN**T**	NYS**D**D	1	–	–	–	1	–
	CVMN**T**	**Y**YSN**Y**	6	4	1	–	5	4
	SVMN**T**	**NFSND**	5	–	–	–	5	–
	SVMN**T**	**Y**YSND	6	11	1		5	11
	CVMNK	**Y**YSN**Y**	–	8	–	2	–	6
	SVMN**T**	**YF**SND	11	12	–	–	11	12
2011	CVMNK	NYSND	6	4	6	4	–	–
	SVMN**T**	NYSND	3	–	1	–	2	–
	CVMNK	**Y**YSN**Y**	–	3	–	1	–	2
	SVMN**T**	**YF**SND	7	6	–	–	7	6
	CVIET	**Y**YSND	4	–	–	–	–	4
	CVIET	**Y**YSN**Y**	6	5			6	5
	CVMN**T**	**Y**YSND	6	7	2	2	4	5
	SVMN**T**	**YF**SN**Y**	–	4	–	–	–	4
	SVMN**T**	**Y**YSND	5	9	–	1	5	8
	SVMN**T**	**NF**SND	8	6	–	–	8	6
	SVMN**T**	**Y**YSN**Y**	9	8	–	–	9	8
2012	CVMNK	NYSND	3	3	3	3	–	–
	CVMN**T**	**Y**YSND	3	7	2	3	1	4
	SVMN**T**	**Y**YSN**Y**	7	12	1	–	6	12
	SVMN**T**	**Y**YSND	5	9	–	–	5	9
	SVMN**T**	NFSND	6	9	1	–	5	9
	SVMN**T**	**Y**YS**D**D	3	4	–	–	3	4
	CVIET	**Y**YSN**Y**	7	9	–	–	7	9
	CVIET	**YF**SND	4	–	–	–	4	–
	SVMN**T**	**YF**SND	10	12	–	–	10	12
	SVMN**T**	**YF**SN**Y**	8	13	–	–	8	13
2013	CVMNK	NYSND	4	3	4	3	–	–
	CVMN**T**	**Y**YSND	5	4	2	1	3	3
	SVMN**T**	**Y**YSND	3	5	–	–	3	5
	SVMN**T**	**YF**SND	9	13	–	–	9	13
	SVMN**T**	**YF**SN**Y**	6	8	–	–	6	8
	SVMN**T**	**NF**SND	2	4	–	–	2	4
	CVIET	**Y**YSN**Y**	6	7	–	–	6	7
	CVIET	**YF**SND	8	11	–	–	8	11
	SVMN**T**	**Y**YSN**Y**	4	3	–	–	4	3

type haplotype decreased gradually in subsequent years to 8.51% and 5.17% in 2013, in Kolkata and Purulia respectively. Isolates with double mutant CVMNK-**Y**YSN**Y** haplotype was frequently found in 2008 (30·67%) and in 2009 (29·37%), although it decreased in subsequent years to 17.02% and 5.76% in 2010 and 2011 respectively but it was never observed after 2012 in Purulia. Surprisingly, this haplotype combination was never found in Kolkata. Quadruple mutant CV**IE**K-**Y**YSN**Y** haplotype (8·73%) was found in Purulia but it was absent in Kolkata. Isolates with double mutant CVMN**T**-**Y**YSND and triple mutant CVMN**T**-**Y**YSN**Y** haplotypes were the most common mutant haplotype found in Kolkata whereas quite unlike **S**VMN**T**-NYSND and **S**VMN**T**-**Y**YSND mutant allele was most frequently found haplotype in 2008–2009, in Purulia. Isolates with quintuple mutant **S**VMN**T**-**YF**SN**Y** haplotype was observed initially in 2011, in Purulia (7.69%), further it spread in subsequent years to Kolkata (14.29% and 12.76% in 2012 and 2013 respectively). Similarly, isolates containing CV**IET**-**YF**SND haplotype was observed primarily in 2012, in Kolkata (7.14%), which spread rapidly in 2013, in Kolkata (17.02%) as well as in Purulia (18.97%). Isolates with quadruple mutant CV**IET**-**Y**YSND haplotype was commonly found haplotype up to 2011, in Kolkata but after 2009 this haplotype had never observed in Purulia. (Table 3, Table 4).

4. Discussion

The findings of this study have provided the evidence of high proportion of CQ treatment failure (*in vivo*) in both the study sites which supported the change of previous national guidelines for the treatment of uncomplicated *P. falciparum* malaria (Government of India, 2009). The high rates of CQ treatment failure indicated the enormous CQ pressure over this parasite population, as more than 40% of cases were produced in *vivo* CQ resistance (Das et al., 2014). The second major finding of this study have provided the evidence of increased *in vitro* chloroquine resistance as well as rapid rise in candidate gene mutations (*pfcrt* and *pfmdr1*) in subsequent years, after withdrawal of chloroquine. These major findings were not corroborated with the previous findings from Malawi (Laufer et al., 2006). The rationale provided for this approach was to evaluate the chloroquine efficacy at times that coincided with the new national drug policy and especially assessed the chloroquine resistant molecular markers even after withdrawal of chloroquine. In our study, we have used multiple approaches, like *in vivo* CQ treatment efficacy, *in vitro* drug susceptibility assessment and also molecular genotyping of *pfcrt* and *pfmdr1* genes to confirm this hypothesis.

Our study provides a comprehensive evidence of distinct

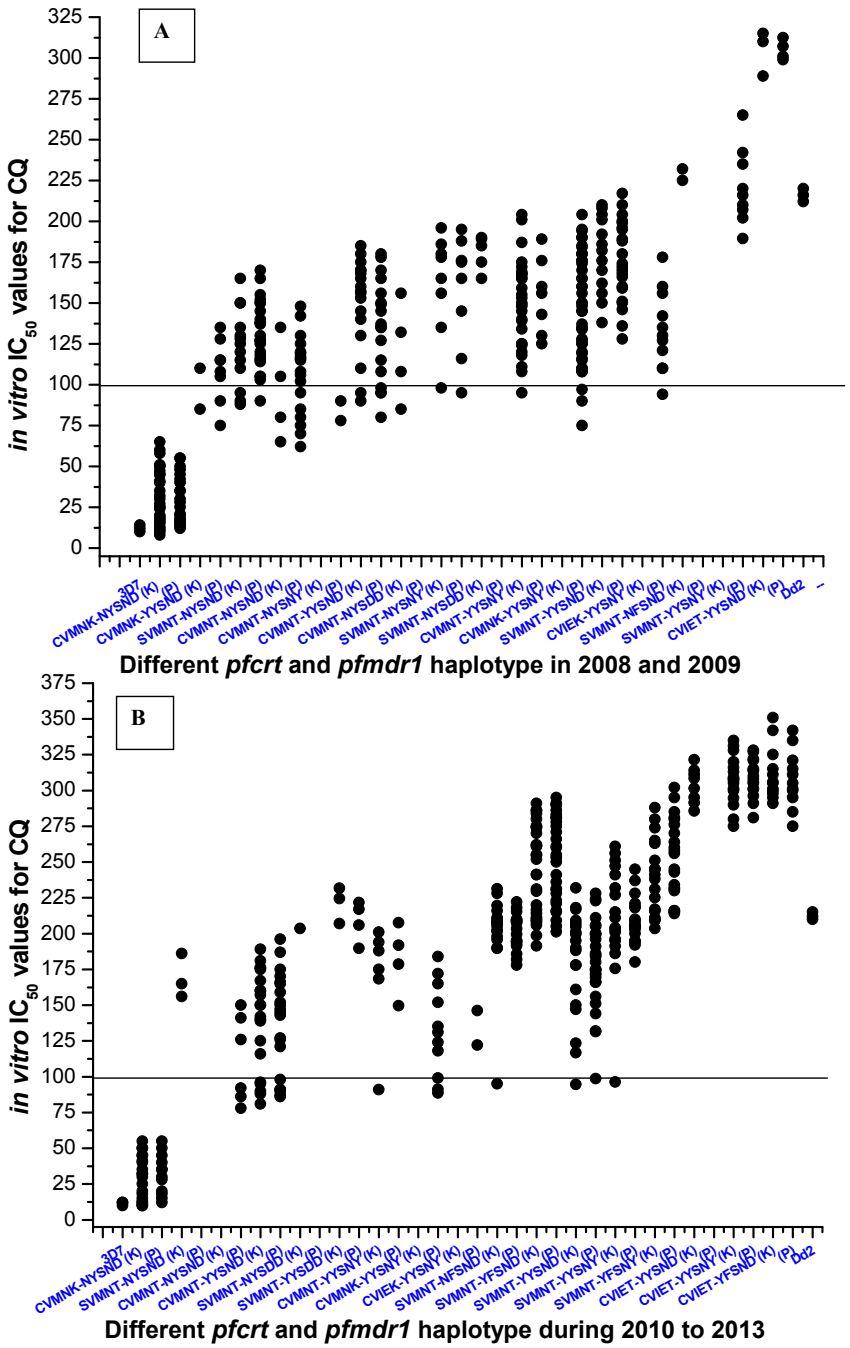

Fig. 6. AAlteration of *in vitro* IC_{50} values for CQ against different *pfcrt and pfmdr1* haplotype in Kolkata and Purulia in 2008–2009. The solid line (corresponding to 100 nM of CQ) is hypothetical and shows the level of chloroquine resistance *in vitro*. 'K' represents for Kolkata and 'P' represents the haplotypes of Purulia. B: Variations of *in vitro CQ,* IC_{50} values in relation to different *pfcrt and pfmdr1* haplotype in Kolkata and Purulia during 2010–2013. The solid line is hypothetical and shows the level of chloroquine resistance *in vitro*. 'K' represents for Kolkata and 'P' represents the haplotypes of Purulia.

variation of *in vivo* CQ resistant pattern in two study sites, however it was quite different from that of previously reported in India. Generally in India, *in vivo* CQ resistance was solely associated with polymorphisms of *pfcrt*, CVMN**T** or **S**VMN**T** or CV**IET** haplotype, by promoting drug efflux from the parasite digestive vacuole (Duraisingh et al., 2000; Vinayak et al., 2003; Vathsala et al., 2004; Mittra et al., 2006), while *pfmdr1* gene mutation at N86Y codon (**Y**YSND) modulated the degree of resistance, i.e. increase in IC_{50} of CQ (Fidock et al., 2000; Duraisingh and Cowman, 2005). Our study confirmed that, CQ resistance was highly associated with CVMN**T**-**Y**YSND and CVMN**T**-**Y**YSN**Y** haplotype in Kolkata, which was identical with the previous finding reported from different parts of India (Vinayak et al., 2003; Sharma, 2005; Das et al., 2014). In Purulia, CQ treatment failure (60.71% ETF and, 33.33% LTF, in 2008; 60.47% ETF and 37.5% LTF in 2009) was found to associated with CVMNK-**Y**YSN**Y** allele, which was quite uncommon in India. CQ non responding patients (33.33%) with wild type *pfcrt* CVMNK allele was previously observed in 2005, in Madhya Pradesh, India (Bharti et al., 2010). These findings suggested that *pfmdr1* polymorphism at N86Y and D1246Y codon possessed a pivotal role in CQ resistance in

2008–2009, in Kolkata as well as in Purulia. The association flanked by the *pfmdr1* genotypes and CQ resistance habitually generated convincing results in Africa and Southeast Asia (Duraisingh et al., 2000; Sa et al., 2009; Andriantsoanirina et al., 2010), but in India, most of the studies did not support these findings (Vinayak et al., 2003; Mittra et al., 2006). On the basis of our finding, it is postulated that the assessment of *pfcrt-pfmdr1* combination mutation was very important to unfold the CQ resistant pattern, in this part of India, as this combination mutation depended on the genetic background of the strain as well as variation of antimalarial drug pressure (Sharma, 2005; Mittra et al., 2006). Polymorphisms of *pfmdr1* gene at codon 86, 184, and 1246 were quite uncommon in India, although it was frequently found in Madagascar (Andriantsoanirina et al., 2009, 2010). The extensive and haphazard use of CQ and quinine over the parasite population might employ some specific selective pressure over this parasite population. Therefore, detection of *pfcrt* genotype alone would not be sufficient to predict the CQ resistant scenario in this part of India.

CQ is no longer the drug of choice against *P. falciparum* after 2009 but *in vitro* CQ resistance increased in subsequent years to 87.23% and 93.10% in 2013 in Kolkata and Purulia respectively (Fig. 3). Isolates containing wild type *pfcrt* CVMNK and *pfmdr1* NYSND haplotype were drastically reduced in both the place. Isolates with **S**VMN**T**-**YF**SND, **S**VMN**T**-**YF**SN**Y**, CV**IET**-**YF**SND and CV**IET**-**Y**YSN**Y** haplotypes increased gradually ($p < 0{\cdot}05$) from 2010 to 2013, leading to a rise in IC_{50} of CQ ($p < 0{\cdot}05$) (Table 4). High malaria transmission and rapid population mix up, might helps to outspread of these vulnerable parasite haplotype in Kolkata (Gardella et al., 2008). Previous study suggested that higher the number of *pfcrt* mutations, higher will be the levels of CQ resistance (Lim et al., 2003; Durrand et al., 2004). It was reported from different parts of the world, including North-eastern India, that *P. falciparum* isolates with CV**IETS** haplotype showed higher levels of *in vitro* CQ resistance rather than the isolates presenting **S**VMN**TS** and CVMN**TS** haplotype (Fidock et al., 2000; Durrand et al., 2004; Mittra et al., 2006). We had found that isolates with **S**VMN**T**-**YF**SND, **S**VMN**T**-**YF**SN**Y**, CV**IET**-**YF**SND and CV**IET**-**Y**YSN**Y** haplotype seemed to have an advantage over those with the CVMN**T**-NYSND, **S**VMN**T**-NYSND, CVMN**T**-**Y**YSND and **S**VMN**T**-**Y**YSND haplotype, as they could able to survive in the high drug pressure (Supplementary Fig. 3) (Mittra et al., 2006; Das et al., 2014). We implied from our study that, acquisition of CQ resistance is a stepwise process, where CVMNT haplotype of pfcrt gene might occur first, followed by the SVMNT haplotype, whereas CVIET haplotype might take place independently with an increase in drug pressure. In case of *pfmdr1* gene, **Y**YSND haplotype might be the first mutation to occur followed by **Y**YSN**Y** and **YF**SND, whereas **YF**SN**Y** haplotype seemed to be independent with an increase drug pressure. Additional mutations in *pfcrt-pfmdr1* gene would give rise to a higher level of CQ resistance.

Finally the most important question would be raised regarding the deciding factors that promoted or somehow increased the spreading of these vulnerable *pfcrt-pfmdr1* combination haplotypes, since AS + SP was recommended as a first line of drug against uncomplicated *falciparum* malaria. It was reported that *P. vivax* is more prevalent than *P. falciparum* while large numbers of isolates were found to have *Pv* + *Pf* mixed infection in India (Annual District wise Epidemiological Report of Malaria of West Bengal, 2010). CQ was the principle drug of choice against *P. vivax*. Genetic cross breeding of mixed *Pf* + *pv* infection, leading to a rise in CQ pressure over *P. falciparum*. On the contrary, in absence of quinoline derivatives only artemisinin derivatives might elicit partial pressure on *pfmdr1* gene. It was reported previously that polymorphism in 86Y and 184F codon in re-infecting parasites after artemether + lumifantrine treatment might constitute a first step toward resistance (Sisowath et al., 2005). Finally drug pressure assessment confirmed the irresponsible use of CQ by the private practitioners as well as haphazard and random self medication (CQ) by the civilians made the situation worse day by day.

In conclusion, proper knowledge in treating malaria (both Government and private practitioners) and awareness of common civilians is much crucial to cope up with this drug resistant scenario. Further exploration of whole genome sequencing would provide greater knowledge of parasite genome, which might elucidate the evolutionary history and consequent spreading of a resistant parasite in this part of India. Government surveillance in treatment of malaria, awareness of pharmaceutical shops (stop selling antimalarial without proper prescription of doctor) and synchronized thorough research, surveillance, as well as containment strategies would be very essential to cope up with this drug resistance burden.

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Transparency declaration

All authors declare that we have no conflict of interest.

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Appendix A. Supplementary data

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