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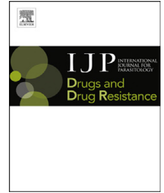

Progressive increase in point mutations associates chloroquine resistance: Even after withdrawal of chloroquine use in India

Sabyasachi Das [a], Satyajit Tripathy [a], Sourav Chattopadhayay [a], Balaram Das [a], Santanu Kar Mahapatra [c], Amiya Kumar Hati [b], Somenath Roy [a, b, c, *]

[a] Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore, 721 102, West Bengal, India
[b] Division of Parasitology, Calcutta School of Tropical Medicine, Kolkata, 700073, West Bengal, India
[c] Department of Biotechnology, School of Chemical and Biotechnology, SASTRA University, Thanjavur, 613402, Tamil Nadu, 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.
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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

* Corresponding author. Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore, 721 102 West Bengal, India.
E-mail addresses: roysomenath@hotmail.com, roysomenath1954@yahoo.in (S. Roy).

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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 reemergence 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 ≤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

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