Chloroquine-resistant *P. falciparum* parasites and severe malaria in Orissa

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Malaria is one of the major causes of morbidity and mortality in Orissa. The present study was undertaken to measure the contribution of chloroquine (CQ) drug-resistant parasites to the risk of severe malaria and their biological advantage in this part of the country. Totally 79 uncomplicated and 93 severe malaria cases were selected according to the WHO criteria. The drug-resistance property was assessed using molecular markers (*pfcrt* K76T and *pfmdr1* N86Y) and multiplication rates of the parasites were measured in the first cycle of *in vitro* culture. The study reveals that a significant number of severe malaria cases in our study population harbour CQ drug-resistant parasites, indicating treatment failure. Further, the association of fatal severe malaria with wild-type parasites and no difference in *in vitro* multiplication potential between wild and mutant-type parasites causing severe malaria have been discussed in the light of the virulence of the parasite populations found in this epidemiological setting.

Keywords: Drug resistance, parasite multiplication, *Plasmodium falciparum*, virulence.

MALARIA is a major public-health problem associated with high morbidity and mortality in India. The National Vector Borne Diseases Control Programme (NVBDCP) reports 2.5–3.2 million parasite-positive cases and more than 1000 deaths every year in the subcontinent, with an estimated 0.95 million disability adjusted life years (DALYs)[1]. Orissa, an eastern Indian state with 3.47% of the country's population, contributes 23% of the malaria cases, 40% of *Plasmodium falciparum* cases and 50% of malaria attributed deaths in the country[2]. Retrospective analysis[3] of the epidemiological data of Orissa reveals that there is a steady rise in the number of malaria-attributed deaths from 131 in 1995 to 465 in 2002. In sub-Saharan Africa the increasing prevalence of resistance to chloroquine (CQ) during the 1980s and 1990s has been associated with a measurably higher burden of severe disease and mortality[4]. But, it is not known whether this is due to the process of selection under drug pressure and increase in infection reservoir[5] or to any intrinsic parasite characteristics. Indeed, high multiplication rate with greater invasiveness of blood-stage parasites and shorter duration of blood schizogony with high merozoite yield have been observed in CQ-resistant strains of *P. falciparum* than in CQ-sensitive strains in some geographical regions of the world[6,7]. The therapeutic efficacy study conducted by the drug monitoring unit of NVBDCP during 2003–06 in six different places of Orissa, has reported 36–95% of CQ treatment failure in *P. falciparum* and a three-fold increase in the incidence of CQ resistance during 1995–2002 (NVBDCP unpublished data). Hence it is likely that a significant proportion of patients develop severe malaria in Orissa as a result of persistence of drug-resistant parasites after treatment for uncomplicated malaria with ineffective antimalarials. But, there have been no controlled studies that accurately measure the contribution of resistant parasites to the risk of severe malaria in Orissa and their biological advantage. This study is an attempt to find: (i) the association of disease severity with parasites harbouring CQ-resistance mutations, and (ii) the *in vitro* multiplication rate, indicative of biological advantage of *P. falciparum* drug-resistant mutant isolates causing severe malaria in Orissa.

The study was carried out in SCB Medical College and Hospital, Cuttack, from June 2005 to March 2007. Patients with fever and slide positive for *P. falciparum* attending the out-patient clinic and severe cases satisfying the WHO criteria[8] admitted as in-patients were recruited for the study. Malaria patients with other acute infections or prior hospitalization for any other reason and/or having other chronic infections such as tuberculosis and leprosy were excluded from the study. CQ was given as the first line of treatment and all severe cases were treated with appropriate dose of quinine in the hospital. Both the groups of patients came from Keonjhar, Anugul and Jajpur districts, considered as perennial malaria-transmission areas with seasonal peaks (June to October). The ethical committee of the Regional Medical Research Centre, Bhubaneswar has approved the study.

DNA was isolated from 100 µl of blood following the standard protocol, with slight modification[9]. In brief, erythrocytes were lysed with lysis buffer (10 mM Tris-HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 20 µg/ml RNAase; 0.5% SDS and 10,020 µg/ml proteinase K) at 55°C for 16 h. DNA was obtained by phenol–chloroform and ethanol precipitation and resuspended in 50 µl of DNAase-free water.

Alleles of *pfcrt* and *pfmdr1* were examined among all the enrolled cases. The *pfcrt* gene was analysed by nested PCR using the primers and the protocol as described by Djimide *et al.*[10]. The 145-bp nested PCR product was then digested with *Apo*I-restriction enzyme. This enzyme cuts the *pfcrt*-K76 (wild type), but not the *pfcrt*-76T (resistant type). Analysis of the *pfmdr1* gene was carried out by PCR–RFLP, as described by Vathsala *et al.*[11]. The *pfmdr1* alleles were then identified by digesting the 603-bp PCR product with the *Afl*III restriction enzyme, which

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does not cut the coding sequence of the allele *pfmdr1*-86N (wild type), but cuts the *pfmdr1*-86Y (resistant type).

The *in vitro* culture was set up as described by Chotivanich *et al.*[7] to determine the parasitized erythrocyte multiplication rate (PEMR) within 12 h of collection of blood. Briefly, 1 ml of venous blood sample collected in heparin from patients with ≥2% parasitaemia was centrifuged at 400 *g* for 5 min to remove plasma and buffy coat. Packed red blood cells were washed three times with incomplete RPMI 1640 and resuspended in malaria culture medium supplemented with 10% of human AB-positive serum. The parasites were cultured using the candle-jar technique until they developed to the mature schizont stage (38–40 h). The schizonts (1.8–15% parasitaemia) were then enriched with a 60% Percoll gradient. The enriched schizonts (90–95% parasitaemia) were washed twice before being resuspended and adjusted with normal O-group red blood cells from a single healthy donor to a ratio of 1% schizonts to uninfected red blood cells with a 3% haematocrit. They were then incubated in a candle-jar. The genotypes (*pfcrt* 76 K or T) of the schizonts of the wild and mutant parasite groups were checked to ensure the growth of the respective parasite isolates. After 4 h of incubation, thin blood films were made and were stained with Giemsa. For each isolate, the number of infected red blood cells per 1000 red blood cells was counted. Multiplication rate (PEMR) was assessed as the number of ring-infected red blood cells after schizogony per 1000 red blood cells/number of infected red blood cells before schizogony per 1000 red blood cells. A red blood cell containing ≥ two parasites was counted as a single infected red blood cell.

Difference in proportions was tested by X^2 or Fisher's exact test and the continuous variables were compared using Student's *t*-test.

Seventy-nine patients (mean age: 33.6 yrs) with uncomplicated malaria signs/symptoms and 93 patients (mean age: 33.9 yrs) with severe complications of malaria were selected for the study based on successful typing of *pfcrt* and *pfmdr1* genes. In the severe group, all the cases were of cerebral malaria. Among these, 4 (4.5%) had hyperparasitaemia (>250,000 parasites/μl of blood), 42 (47.8%) had scizontaemia, 36 (40.9%) had generalized convulsions, 12 (13.6%) had severe anaemia (Hb < 5 g/dl), 9 (10.2%) had hypotension, 81 (87.1%) had jaundice, 39 (41.9 %) had acute renal failure and 4 (4.3%) had acute respiratory distress syndrome. None of the patients with uncomplicated malaria developed features of severe disease and all of them were parasitologically and clinically cured after treatment with CQ/primaquine.

The overall frequency of the resistance-associated allele *pfcrt*-76T/*pfmdr1*-86Y among uncomplicated and severe malaria cases was 30.4%/24.1% and 65.6%/55.9% respectively. Excluding the mixed infections (mutant + wild), the frequency of *P. falciparum* isolates harbouring *pfcrt*-76T and *pfmdr*-86Y alone or in combination was observed to be significantly high ($P < 0.001$) among the severe malaria cases than the uncomplicated ones. Further, there was a tendency of higher mixed infections in both *pfcrt*-76T and *pfmdr*-86Y alleles ($P < 0.05$) among the uncomplicated cases (Table 1).

Among the 93 severe malaria cases enrolled in the study, 84 (90.3%) survived and 9 (9.7%) patients died. Excluding mixed infections, the frequency of *P. falciparum* isolates harbouring wild-type *pfcrt* and wild-type *pfmdr1* alleles was found to be significantly associated (Fisher's exact test, two-tailed $P = 0.008$ and 0.040) with severe fatal malaria (Table 2).

The PEMR of wild-type *P. falciparum* parasites collected from severe malaria cases ranged from 1 to 3.2 (*n*: 22, mean: 2.03, 95% CI: 1.8–2.9) and mutant parasites from 1 to 4.0 (*n*: 20, mean: 1.99, 95% CI: 1.7–2.3). The statistical comparison of PEMR between these two group, of parasites did not show any significant difference ($t = 0.23$, $P > 0.05$). Similarly, the PEMR of *P. falciparum* isolates (wild and mutant combined) associated with

Table 1. Distribution of chloroquine resistance marker genes among *Plasmodium falciparum* cases with uncomplicated and severe clinical manifestations

	pfcrt			*pfmdr1*			
Clinical cases	Wild (%)	Mutant (%)	Mixed (%)	Wild (%)	Mutant (%)	Mixed (%)	*pfcrt* + *pfmdr1* (%)
Uncomplicated	55/79 (69.6)	9/79 (11.4)	8/79 (10.1)	59/79 (74.7)	5/79 (6.3)	7/79 (8.9)	7/79 (8.9)
Severe	32/93 (34.4)	32/93 (34.4)	4/93 (4.3)	41/93 (44.1)	24/93 (25.8)	3/93 (3.2)	25/93 (26.9)

Table 2. *pfcrt* and *pfmdr1* alleles in *P. falciparum* infections with severe clinical manifestations

	pfcrt			*pfmdr1*			
Clinical cases	Wild (%)	Mutant (%)	Mixed (%)	Wild (%)	Mutant (%)	Mixed (%)	*pfcrt* + *pfmdr1* (%)
Severe malaria deaths	7/9 (77.8)	1/9 (11.1)	0/9 (0.00)	7/9 (77.8)	1/9 (0.00)	0/9 (0.00)	1/9 (11.1)
Severe malaria, no deaths	25/84 (29.8)	31/84 (36.9)	4/84 (4.8)	34/84(40.5)	23/84 (27.4)	3/84 (3.6)	24/84 (28.6)

uncomplicated malaria ranged from 1 to 3 (*n*: 32, mean: 1.94, 95% CI: 1.8–2.1) and those causing severe malaria ranged from 1 to 4 (*n*: 42, mean: 2.01, 95% CI: 1.8–2.2) without any statistical difference ($t = 0.59$, $P > 0.05$).

The association of CQ resistance with *pfcrt* (K76T) and to some extent *pfmdr1* (N86Y) alleles in *P. falciparum* isolates has recently been demonstrated in different geographical regions, including Orissa[12–16]. Therefore, *pfcrt* and *pfmdr1* (though not in perfect association) have become reliable markers of CQ resistance in *P. falciparum* parasites, despite the fact that CQ resistance molecular markers are not absolute indicators of clinical response as other human factors such as immune response may influence treatment outcome[17]. During this study the use of molecular markers has allowed us to identify CQ-resistant *P. falciparum* isolates among patients with severe malaria. This would not have been possible using the *in vivo* test for ethical reasons as patients with severe malaria must be treated promptly with more efficacious drugs like quinine and not CQ. In the present study both *pfcrt*-76T and *pfmdr1*-86Y alone or in combination have been observed to be associated with development of severe complications. This finding largely confirms the observations made by Meerman *et al.*[18] among Gambian children, indicating that maximum cases of severe complications in this area is due to the CQ drug resistance. Secondly, we have found that wild-type *P. falciparum* parasites are more associated with severe fatal disease despite the high frequency of mutant alleles of both genes among the severe non-fatal cases as observed in Sudan[19]. Although the sample size was not enough to confirm the increased virulence in wild-type CQ-sensitive parasites, these data at least affirm the disparity between virulence and parasite mutations that are associated with CQ resistance. Further, the PMER under standard conditions in the first cycle of *in vitro* culture was also not significantly different between wild and drug-resistant mutant-type *P. falciparum* isolates causing severe malaria. Studies in rodent malaria parasites have demonstrated a potential fitness burden of drug resistance. A pyremethamine-resistant mutant *P. chabaudi* was found to grow slower than its progenitor and in a mixture the sensitive clone outgrew the mutant form[20], although in another study the resistant parasite was found to grow as well as its sensitive parent[21], similar to our observation. Therefore, no difference in PMER between isolates associated with uncomplicated malaria and severe disease in the present study might be due to the development of some compensatory mutations, as proposed by Bjorkman *et al.*[22]. However, the large parasite burden in severe cases compared to uncomplicated ones in our study population might be due to longer period of unrestrained multiplication because of treatment failures and not due to any extra intrinsic factor.

The clinical implication of this study is that cases of severe malaria in Orissa are less likely to respond to CQ. Since CQ is still the first line of treatment of uncomplicated malaria here, delay in changing this drug to which increased resistance has been developed, could increase the incidence of severe disease by selecting resistant parasites. Further, field surveys have demonstrated that even though selection under drug pressure is the primary force for the evolution of drug resistance[12], when the drug pressure is reduced, the frequency of resistant parasites also decreases[23,24]. Therefore, the current antimalarial drug policy needs urgent evaluation.

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Development and performance of single-axis shake table for earthquake simulation

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The exact simulation of earthquake motion has been a serious challenge to researchers and engineers. Shake table testing is being increasingly used in earthquake engineering research centres worldwide, as it is the only available means of nearly truly reproducing the dynamic effects that earthquakes impose on structures. To upgrade the dynamic testing facility at IIT Kanpur, a uniaxial shake table has been installed which is servo-hydraulic-operated and supported on low-friction ball bushing bearings. A relatively simple system has been assembled with care to ensure an adequate replication of input motion by the shake-table system. Subjective comparisons of input signal vs shake-table response, in both time and frequency domain have been utilized to provide a measure of the capabilities of the simulator to reproduce earthquake motions scaled according to similitude laws. This communication discusses briefly various components of the shake table, its assembly and the investigations that were carried out to provide specific insights into its response characteristics.

Keywords: Assembly, earthquakes, performance verification, shake table.

THE shake table is an indispensable testing facility for development of earthquake-resistant techniques. A shaking table is a platform excited with servo-hydraulic actuators to simulate different types of periodic and random motions, such as artificial earthquakes and other dynamic testing signals of interest in the laboratory. This is the only experimental technique for direct simulation of inertia forces, which can be used to simulate different types of motion such as recorded earthquake ground motions, sine sweeps, etc. Shake table test results enhance further the understanding of the behaviour of structures and calibration of various numerical tools used for analysis. This facility can be utilized for verification of earthquake-resistant design of buildings, other structures, mechanical components, devices, etc.

The ground motion is multidirectional in reality and its simulation in the laboratory with multi-axial shake-table system is complex and costly[1]. A single-axis table is the simplest form of earthquake simulator which is not only useful for many investigations when it is only desirable to excite the specimen in one axis, but also simplifies subsequent interpretation of the results. Further, the current trends suggest that structural laboratories worldwide, are finding uniaxial shake tables easy to operate and maintain. For example, at the EUCENTRE, priority was given to platform size and driving power rather than number of directions of shaking in order to perform tests on full-scale or large-scale models of test structures and foundations. Consequently, a large, powerful, uniaxial shake-table platform was chosen instead of a small, six degree-of-freedom table with limited performance capabilities[2]. For similar reasons, a uni-directional shake table was chosen for the large outdoor facility developed under the NEES program at University of California, San Diego[3].

Smaller-sized shake tables are also better suited for small-scale model analysis. In addition, they avoid high operational and development costs, but are versatile enough in the case of dynamic experiments for instructional and research purposes. However, like every system, the small-sized uniaxial shake-table also has certain limitations. In spite of advanced hydraulic actuators and servo control system, and low-friction high-rigidity ball bushing bearings, the shake-table system may possess certain imperfections. The resulting distortions in table

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