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Research Article

In Vitro Chloroquine Resistance in *Plasmodium falciparum* Isolates from Tertiary Care Hospital

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Chloroquine (CQ) has been the mainstay of treatment of malaria for decades. This cost-effective and safe drug has become ineffective for treatment of *falciparum* malaria in many parts of the world due to development of resistance by the parasite. In addition CQ is not gametocytocidal for *P. falciparum* and thus cannot block transmission. The extent of problem of chloroquine resistance in *P. falciparum* is increasing every year. The study was done in period of 2 years. A total of 5653 specimens were examined for malarial infection by employing different diagnostic modalities. Four hundred and thirty-five were found to be positive for *P. falciparum* by using different diagnostic techniques. All positive specimens were cultured on RPMI 1640 medium; only 108 were found to be culture positive. Sensitivity of isolates to chloroquine was done using Mark III WHO sensitivity plates. The prevalence of malaria infection was found 9.54% in 2010. There were schizont formation at 8 pmol/liter or more of chloroquine concentration in 26 isolates. The emergence of chloroquine (CQ) resistance pattern in Aligarh isolates increases. Antimalarial agents should be used with caution; monotherapies should be avoided.

1. Introduction

Chloroquine has for decades been the primary chemotherapeutic means of malaria treatment and control. This safe and inexpensive 4-aminoquinoline compound accumulates inside the digestive vacuole of the infected red blood cell. Chloroquine resistance (CQR) was first reported in Southeast Asia and South America and has now spread to the vast majority of malaria-endemic countries [1]. Antimalarial drug resistance is a major public health problem which hinders the control of malaria. In India resistance of *Plasmodium falciparum* to chloroquine was first reported in the year 1973 from Diphu of the Karbi Anglong district in Assam state.

Various *in vitro* sensitivity test systems have been developed and applied to sensitivity monitoring of *P. falciparum* in endemic areas. The most commonly used methods are *in vitro* tests based on the measurement of the effect of drugs on the growth and development of malaria parasites, that is, schizont maturation or growth inhibition [2, 3], incorporation of radiolabeled precursors [4], enzymatic activity of parasite lactate dehydrogenase (pLDH) [5], or histidine-rich protein II (HRP II) [6]. The *in vitro* sensitivity test based on the standard micro-technique recommended by the World Health Organization [2] using the schizont maturation inhibition test has been applied successfully in most of the highly multi-drug-resistant areas worldwide. The study was done to determine the prevalence of *P. falciparum* infection in Aligarh, a north Indian district and to test the chloroquine sensitivity using Mark III WHO sensitivity plates.

2. Material and Method

2.1. Place of Study and Study Cohort. The study was conducted in Aligarh, a district of north India, where there is high prevalence of *P. falciparum* species. The study was conducted from February 2010 to December 2011, but majority of cases (>90%) were detected in months of July to October in both years. A total of 5653 patients, who presented with fever, chills, rigors, or any pyrexia of unknown origin were screened for malarial infection by using different diagnostic tests

like peripheral blood smear examination and quantitative buffy coat assay and by various antigen detection assays. The blood was cultured from every positive case of *P. falciparum*. Parasite density was not taken into account. The drug sensitivity was done against chloroquine as it is the most commonly used antimalarial agent in out-patient departments for uncomplicated malarial infection. It is also prescribed empirically even before the laboratory diagnosis is confirmed. We also want to study chloroquine sensitivity because of its improper prescription and dosing in fever of unknown origin in this area by unqualified medical practitioners.

2.2. Specimen Collection. 5 mL of blood was collected by venipuncture taking all sterile precaution in heparinized tube. Both thick and thin smears were examined by staining with Giemsa stained using standard protocols. Parasitemia was determined by counting 200 WBCs on thick films and expressed as parasites per microliter of blood. A total of 200 oil emergence fields were examined before the smear is labeled negative or positive.

2.3. In Vitro Cultivation of Plasmodium falciparum Isolates and Drug Sensitivity Test. The *in vitro* cultivation of *P. falciparum* isolates followed a modification of the standard culture techniques [7, 8]. The culture medium consisted of RPMI 1640 (Sigma Aldrich), 2 g glucose, and 40 μg/mL gentamycin sulphate with supplemented 10% AB+ serum. Culture medium was sterilised by filtration through a Millipore filter of 0.22 μm porosity and pH was adjusted to 7.4 by the addition of 4.2 mL of sterile 5% sodium bicarbonate.

The drug sensitivity (based on the M-III methods) can be determined *in vitro* in *P. falciparum* culture by using standard 96-well microtitre plates (WHO plates), predosed of the test drug. The test plates were predosed with ascending concentrations of chloroquine A–H 0, 1, 2, 4, 8, 16, 32, and 64 pmol. Blood medium mixture (BMM) was prepared by gently shaking the tube to mix the blood and medium. Preculture thick and thin films were prepared. All the wells of appropriate column were dosed with 50 μL of the blood medium mixture (1 : 9).

The plate was put in a candle jar and placed in the incubator set at 37.5°C for 24–30 hrs, depending upon development stage of the parasite. After 24-hour incubation, a thin smear was prepared from the control well to see the mature schizonts, and if more than 10% schizonts were seen, it was considered to be valid; thick smears were prepared from each well by discarding the excess media with a micropipette.

2.4. Determination of In Vitro Effective Concentration (EC) Values of the Chloroquine. The mean number of schizonts counts per well was fed directly into nonlinear regression software, H N-Non-Lin V1.1, specific for malaria *in vitro* drug sensitivity test. Individual dose response curves were generated and their EC 50, EC 90, and EC 99 values determined.

Table 1: Clinical features in 435 patients of *P. falciparum* infection.

S. no.	Clinical features	No. of patients
(1)	Fever	435 (100%)
(2)	Chills	372 (85.51%)
(3)	Rigors	310 (71.26%)
(4)	Convulsions	156 (35.86%)
(5)	Neck rigidity	103 (23.67%)
(6)	Other signs of meningeal irritation	79 (18.16%)
(7)	Yellowish discoloration of sclera	45 (10.34%)
(8)	Renal complaints	39 (8.96%)
(9)	Altered consciousness	325 (60.74%)

2.5. Data Analysis. The geometric means and 95% confidence intervals (CIs) of EC values were estimated in SPSS (SPSS Inc., USA).

2.6. Observations. Out of 5653 patients who presented with pyrexia of unknown origin or pyrexia associated with symptoms suggestive of malarial infection, only 435 patients had *P. falciparum* infection. Fever was present in all patients (100%); chills (85.51), rigors (71.26%), and altered level of consciousness (60.74%) were the common features with which *P. falciparum* positive cases presented (Table 1). Four hundred and thirty-five *P. falciparum*-positive blood samples were cultured; 108 cultures were positive; from all these isolates when subjected to chloroquine sensitivity, 26 isolates were found to be resistant to chloroquine, that is, schizont maturation at 8 pmols or more. Isolates showed schizont maturation up to 32 pmols or more (Figure 1). The mean effective concentrations of chloroquine EC 50 = 6.07 nm/liter, EC 90 = 33.33 nm/liter, EC 95 = 43.44 nm/liter, and EC 99 = 47.40 nm/liter, which shows decreased sensitivity of *P. falciparum* isolates to chloroquine (Table 2).

3. Discussion

The major obstacle in malaria treatment and control is impeded by the drug-resistant parasites, particularly *Plasmodium falciparum*, which have disseminated and enhanced mortality [9]. Chloroquine drug pressure remains high in India as it has been used since decades as first-line drug in malaria therapy both as self-treatment at home and in health-care facilities [10] Shah et al. [11] analyzed 337 studies and investigated chloroquine efficacy in 17189 patients. The number of studies and proportion of failures varied between regions and in states within a region. The median proportion of chloroquine failure was 35.1% (IQR 13.0–58.2) in studies with a 28-day followup. Studies with 7-day followup, which largely detected early treatment failures, were phased out from 2000 and the last 7-day study was done in 2003. The proportion of failures detected is higher in 28-day follow-up studies than in 7-day follow-up studies done in the same areas, because late treatment failures are detected in 28-day follow-up studies. Studies done between 1978 and 2007 show an increasing proportion of failures to chloroquine over time.

Table 2: Geometric mean EC 50, EC 90, and EC 99, 95% confidence interval (CI) of chloroquine against *Plasmodium falciparum* isolates.

Chloroquine	Geometric mean EC 50, (95% CI) nanomolar (nm/liter)	
	Sensitive isolates	Resistant isolates
EC 50	1.30 (1.99–1.0)	6.07 (02.74–13.42)
EC 90	3.94 (5.53–1.88)	33.33 (16.94–61.09)
EC 95	5.56 (7.22–2.48)	43.44 (19.15–65.78)
EC 99	6.17 (1.0–0.92)	47.40 (21.39–66.98)

EC: effective concentration.

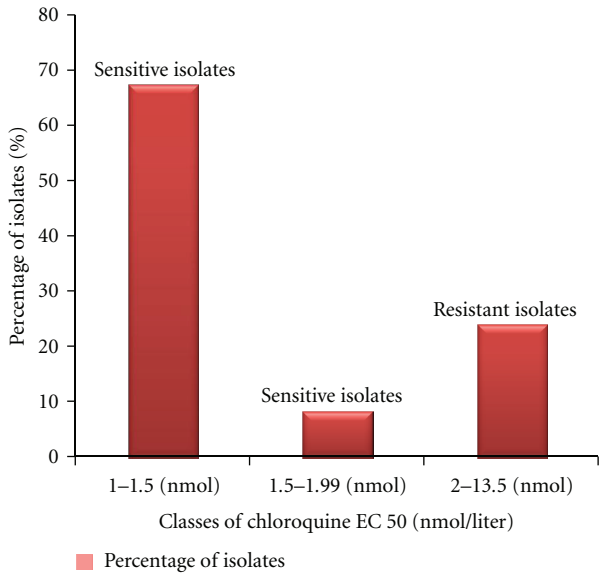

Figure 1: Relative EC 50 (nmol/liter). Distribution pattern of chloroquine among *P. falciparum* isolates.

When routine monitoring of drug resistance began in 1978-1979, two of 17 studies exceeded the 10% threshold used in India to switch an area to the second-line treatment. In 2006-2007, the proportion of chloroquine studies exceeding 10% treatment failure increased to 35 of 40 studies. Drug-efficacy studies of chloroquine, with at least 30 patients in any follow-up period, have exceeded 10% treatment failure in 115 districts. These districts represent 20 of 28 states and two of five union territories. The remaining states and territories have low incidence of *P. falciparum*, or have not done any trial of antimalarial drug resistance [11]. Culture was positive in 108 patients with confirmed malaria diagnosis. Only 31% cases have given adequate culture results due to low parasitemia, previous history of inadequate treatment by quacks. This is a tertiary care hospital where most of the patients come after getting treatment from one or more sources.

Most of the studies done in India mainly determined the *in vivo* resistance patterns of chloroquine [12]. There is paucity of data from India regarding *in vitro* drug resistance pattern in India. In our study when chloroquine was tested against *P. falciparum* isolates, high level of resistance was noticed by *in vitro* Mark III sensitivity assay 53.76% of isolates resistant to 8 pmols of chloroquine which is the WHO cut-off value for sensitivity. The level of resistance in this part of India is so high that 10.75% strains were resistant to even 32 pmols of drug. One of the major objectives of the study is to determine effective concentration values (EC values) of chloroquine. This result is needed to estimate the effective therapeutic levels in human body. Our results showed that mean EC5O value of sensitive strains was 1.30 and for resistant 6.07 (nmol/liter), EC 90, EC 95, and EC 99 were found to be 3.94 nm/liter and 33.33 nm/liter, 5.56 nm/liter and 43.44 nm/liter, 6.17 nm/liter, and 47.40 nm/liter for sensitive and resistant isolates, respectively. These results indicate high level of resistance of *P. falciparum* to chloroquine among the isolates tested, and this is confirmed by high EC values which are excessively higher than effective therapeutic concentration. These values are significantly higher than those reported from India and other countries [13, 14]. This high-level resistance to chloroquine in our patients may be due to previous exposure to improper therapeutic regimes, over-the-counter availability of drug, and high drug pressure by improper prescribing habits of private practitioners. So this high level of resistance should be monitored regularly to prevent further spread of resistant strains. The use of combination therapy should be advocated to prevent the drug resistance in malaria-endemic areas.

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