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In vitro susceptibility of Indian *Plasmodium falciparum* isolates to different antimalarial drugs & antibiotics

Pooja Agarwal[1,2], A.R. Anvikar[3], C.R. Pillai[3] & Kumkum Srivastava[1,2]

[1]Division of Parasitology, CSIR-Central Drug Research Institute, Lucknow, [2]Academy of Scientific & Innovative Research & [3]ICMR-National Institute of Malaria Research, New Delhi, India

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***Background & objectives*: The *in vitro* assays for susceptibility of *Plasmodium falciparum* to antimalarial drugs are important tools for monitoring drug resistance. During the present study, efforts were made to establish long-term continuous *in vitro* culture of Indian field isolates of *P. falciparum* and to determine their sensitivity to standard antimalarial drugs and antibiotics.**

***Methods*: Four (MZR-I, -II, -III and -IV) *P. falciparum* isolates were obtained from four patients who showed artemisinin-based combination therapy (ACT) from Mizoram, a north-eastern State of India, and characterized for their *in vitro* susceptibility to chloroquine diphosphate (CQ), quinine hydrochloride dehydrate, mefloquine, piperaquine, artemether, arteether, dihydro-artemisinin (DHA), lumefantrine and atovaquone and antibiotics, azithromycin and doxycycline. These patients showed ACT treatment failure. Two-fold serial dilutions of each drug were tested and the effect was evaluated using the malaria SYBR Green I fluorescence assay. K1 (chloroquine-resistant) and 3D7 (chloroquine-sensitive) reference strains were used as controls.**

***Results*: Growth profile of all field isolates was identical to that of reference parasites. The IC_{50} values of all the drugs were also similar against field isolates and reference parasite strains, except K1, exhibited high IC_{50} value (275±12.5 nM) of CQ for which it was resistant. All field isolates exhibited higher IC_{50} values of CQ, quinine hydrochloride dihydrate and DHA compared to reference strains. The resistance index of field isolates with respect to 3D7 ranged between 260.55 and 403.78 to CQ, 39.83 and 46.42 to quinine, and 2.98 and 4.16 to DHA, and with respect to K1 strain ranged between 6.51 and 10.08, 39.26 and 45.75, and 2.65 and 3.71. MZR-I isolate exhibited highest resistance index.**

***Interpretation & conclusions*: As the increase in IC_{50} and IC_{90} values of DHA against field isolates of *P. falciparum* was not significant, the tolerance to DHA-piperaquine (PPQ) combination might be because of PPQ only. Further study is required on more number of such isolates to generate data for a meaningful conclusion.**

Key words Antibiotics - antimalarials - field isolates - *in vitro* culture - Mizoram - *Plasmodium falciparum*

Malaria remains one of the most widespread infectious diseases of the world. More than 85 per cent of malaria cases and 90 per cent of malaria deaths occur in Sub-Saharan Africa, mainly in

young children below five years of age[1]. Incidences of chloroquine (CQ) resistance were first reported in the late 1950s from South-East Asia and South America[2]. In India, the first CQ-resistant *Plasmodium falciparum* case was documented in 1973 from the North-East Karbi - Anglong district of Assam[3]. In 1982, the National Antimalarial Drug Policy was introduced to improve malaria case management and sulphadoxine-pyrimethamine (SP) combination was recommended as the treatment for CQ-resistant malaria[4]. In November 1984, mefloquine (MQ) was introduced as the first-line treatment for uncomplicated falciparum malaria in Thailand, but despite careful regulation of its use, substantial resistance was developed within five years and emerged in adjacent countries, such as Burma and Cambodia[5]. Western Cambodia and Thailand-Myanmar border were the regions of greatest concern because resistance to both MQ and SP emerged in this area[6]. To overcome the problem associated with resistant falciparum malaria, artemisinin-based combination therapy (ACT), which included artesunate+MQ, artemether (ARTM)+lumefantrine (LUME) and artesunate+amodiaquine, was recommended as a first-line antimalarial treatment[7-10]. However, the artemisinin class of drugs have also shown signs of reduced efficacy[11-14] providing a potential threat to ACT. In 1994, two randomized trials were carried out in Western Cambodia (Pailin) and North-western Thailand (Wang Pha) by Dondorp *et al*[11], who gave two regimens of artesunate treatment *i.e.* 2 mg/kg/day for seven days and 4 mg/kg/day for three days, followed by 25 mg/kg of MQ given in two doses. The results revealed that median parasite clearance times were 84 and 48 h in Pailin and Wang Pha, respectively, and recrudescence occurred in 30 per cent patients receiving artesunate monotherapy and in five per cent patients receiving artesunate-MQ combination therapy in Pailin, as compared with 10 and 5 per cent, respectively in Wang Pha. However, no reduction in *in vitro* susceptibility was observed by Dondorp *et al*[11]. Another long-term study[13] was carried out on 3202 patients in north-western border of Thailand. During this study, the patients received various artesunate-containing therapies between 2001 and 2010. The findings revealed that 'genetically determined artemisinin resistance in *P. falciparum* emerged along the Thailand-Myanmar border at least eight years ago and has increased substantially and may reach in Western Cambodia in 2-6 yr[13]. In 2012, Amaratunga *et al*[14] explored the involvement of malaria parasite genetics and host factors towards the development of artemisinin resistance in six districts of Pursat, Western Cambodia. They gave participants 4 mg/kg artesunate at 0, 24 and 48 h, 15 mg/kg mefloquine at 72 h and 10 mg/kg mefloquine at 96 h and assessed parasite density on thick blood films every six hours until undetectable. The parasite clearance half-life was calculated. The findings revealed artemisinin resistance in Pursat. Thus, the area in Western Cambodia, especially along the border with Thailand, is considered the world's hot spot for *P. falciparum* multidrug resistance[15-18]. A molecular marker (K13-propeller) has been developed as a tool to monitor the spread of artemisinin-resistant mutations[19,20]. Tun *et al*[20] observed that in Homalin, Sagaing Region in Myanmar, 25 km from the Indian border, 21 (47%) of 45 parasite samples exhibited K13-propeller mutations. The present study was undertaken to explore *in vitro* chemosensitivity of four Indian field isolates of *P. falciparum* collected from Mizoram, a north-eastern State of India, against standard antimalarial drugs and antibiotics.

Material & Methods

This study was conducted in the division of Parasitology, CSIR-Central Drug Research Institute (CDRI), Lucknow, India.

Parasites

Field isolates: Cryopreserved vials of four field isolates (MZR-I, MZR-II, MZR-III and MZR-IV) of *P. falciparum,* obtained from Mizoram in 2008, were procured from ICMR-National Institute of Malaria Research (NIMR), New Delhi. The four isolates were collected from four patients who exhibited dihydro-artemisinin (DHA)-piperaquine (PPQ) combination treatment failure.

Reference strains: (*i*) 3D7 chloroquine (CQ) sensitive strain - This parasite strain is maintained in CSIR-CDRI. It is a clone derived from NF54 strain; the original isolate was obtained from a patient living near Schiphol Airport, Amsterdam[21]; (*ii*) K1 strain (MRA159) CQ-resistant strain - This strain was obtained from MR4, American Type Cell Culture (ATCC), Manassas, Virginia, USA.

In vitro cultivation of parasite: Four field isolates (erythrocytic stages) were revived and maintained in 5 ml culture medium (RPMI-1640; Sigma, USA, R4130-1L) at 3 per cent haematocrit suspension [human red blood cells (RBCs)]. The culture medium was prepared

as described elsewhere[22]. AlbuMAX-II (GIBCO, New Zealand; 11021/045) 0.5 and 10 per cent foetal bovine serum (FBS, Sigma, F2442/HYCLONE, SH30396.03HI) were used as serum supplements. The cultures were maintained stationary at 37°C in a CO_2 incubator (Thermo Electron Corporation, USA; water jacketed, model no. 3131) in the atmosphere of five per cent CO_2 and air mixture. The per cent parasitaemia (%P) was monitored daily. The *in vitro* culture of both reference strains was also maintained in the laboratory.

Assessment of per cent parasitaemia (%P): The Giemsa-stained blood smears were examined under the light microscope (100×, oil immersion, Nikon, Japan). Approximately 10,000 RBCs per smear were scanned and %P was calculated as number of parasitized RBCs (PRBCs)/total number of RBCs × 100.

Assessment of growth profile of field isolates: %P of all the parasites was monitored daily to determine the comparative growth profile of field isolates (MZR-I, MZR-II, MZR-III and MZR-IV) and reference parasite strains (3D7 and K1). Three replicates were carried out for each parasite isolate.

Assessment of chemosensitivity of field isolates: Fifty and 90 per cent inhibitory concentration (IC_{50} and IC_{90}) values of standard antimalarials and antibiotics were determined using malaria SYBR Green I fluorescence assay[23] and compared with those obtained against 3D7 and K1 parasites.

Drugs used: Chloroquine diphosphate (CQ), quinine hydrochloride dihydrate (QUIN), mefloquine hydrochloride (MQ), PPQ, ARTM, arteether (ARTE), DHA, LUME and atovaquone (ATQ) and antibiotics, azithromycin (AZI) and doxycycline (DOXY) were used for the assessment of chemosensitivity. CQ (C6628), QUIN (Q1125), MQ (M2319) and ATQ (A7986) were purchased from Sigma while PPQ, ARTM, ARTE, DHA and LUME were obtained (as gift) from IPCA Laboratories Ltd., Mumbai. The antibiotics were purchased from Biogene, USA.

Preparation of drugs: Stock solutions (10 mM) of all the drugs was prepared in dimethyl sulphoxide (DMSO) except CQ and PPQ which were prepared in sterile water. The subsequent dilutions were prepared in CRPMI (RPMI-1640 with 10% FBS and 9.2 µM hypoxanthine).

Table I. Highest concentration of test samples used for assessment of chemosensitivity against *Plasmodium falciparum*

Antimalarials + antibiotics	Highest concentrations (nM) used against		
	3D7	K1	MZR parasites
CQ	50	2000	20,000
MQ	100	100	100
QUIN	200	200	20,000
PPQ	50	50	50
ARTM	10	10	10
ARTE	10	10	10
DHA	4	4	10
ATQ	10	10	10
LUME	50	50	50
AZI	50,000	50,000	50,000
DOXY	50,000	50,000	50,000

CQ, chloroquine diphosphate; MQ, mefloquine hydrochloride; QUIN, quinine hydrochloride dehydrate; PPQ, piperaquine; ARTM, artemether; ARTE, arteether; DHA, dihydro-artemisinin; ATQ, atovaquone; LUME, lumefantrine; AZI, azithromycin; DOXY, doxycycline

Evaluation of IC_{50} and IC_{90} values: CRPMI (50 µl) was dispensed in each well of 96-well flat bottom plate, followed by the addition of 50 µl test sample constituting 4× highest concentration in duplicate wells in row 'B' and 2-fold serial dilutions were made up to row 'H'. To obtain best-fit curve, highest concentrations of the same drug varied amongst parasite strains as depicted in Table I. The 50 µl volume from wells of row H was discarded. Subsequently, 50 µl of 2.0 per cent PRBCs suspension containing 1.0 per cent parasitaemia (with >90% ring stages) was added to each well, except four wells in row 'A' (A9-A12) which received two per cent non-parasitized cell suspension. The plates were incubated at 37°C in CO_2 incubator for 72 h. After which, 100 µl of lysis buffer containing 2× concentration of SYBR Green I was added to each well and incubated for one hour at 37°C. The plates were examined at 485±20 nm of excitation and 530±20 nm of emission for relative fluorescence units per well using the fluorescence plate reader (FLX 800 BIOTEK). The IC_{50} and IC_{90} values (in nM) were determined using non-linear regression analysis of dose-response curves using pre-programmed excel spreadsheet. The resistance factor of MZR-I, -II, -III and -IV parasites in respect to 3D7 and K1 strains was calculated as IC_{50} values obtained against MZR parasites/IC_{50} values obtained against 3D7 or K1 parasites.

Statistical analysis: The data were analyzed by two-way ANOVA followed by multiple comparisons using Newman Keuls test (STATISTICA 7.0, StatSoft, USA) to establish significance between IC_{50} and IC_{90} values obtained against reference strains and MZR isolates.

Results

The continuous *in vitro* cultures of all the field isolates were successfully maintained in laboratory. Growth profile is depicted in Table II. It was evident that the initial %P of all the parasite lines ranged between 1.1 and 1.4 which rose to 8.4 and 9.0 per cent after seven days of cultivation exhibiting identical growth profile of field isolates as well as reference parasite (3D7 and K1) strains.

Subsequent efforts were made to determine the sensitivity of all four field isolates to nine standard antimalarials and two antibiotics. The IC_{50} and IC_{90} values (in nM) of all the antimalarials and antibiotics obtained against MZR field isolates and 3D7 and K1 parasites are depicted in Tables III and IV, respectively. The IC_{50} and IC_{90} values of all the standard antimalarials and antibiotics against MZR field isolates and reference parasite strains were almost identical, except K1 strain which exhibited high IC_{50} and IC_{90} values of CQ, for which it was resistant and all the field isolates exhibited higher IC_{50} and IC_{90} values of CQ, quinine and DHA.

Table II. Per cent parasitaemia of MZR-I, MZR-II, MZR-III and MZR-IV field isolates, and 3D7 and K1 strains of *Plasmodium falciparum* as observed on eight consecutive days

Days of observation	Per cent parasitaemia (mean±SD), n=3					
	MZR-I	MZR-II	MZR-III	MZR-IV	3D7	K1
0	1.4±0.22	1.3±0.13	1.2±0.06	1.3±0.08	1.1±0.31	1.2±0.09
1	2.5±0.02	2.4±0.05	2.2±0.21	2.3±0.07	2.1±0.08	2.2±0.20
2	3.3±0.06	3.1±0.19	3.2±0.18	3.2±0.26	2.9±0.16	3.0±0.17
3	4.8±0.14	4.7±0.09	4.5±0.15	4.6±0.08	4.3±0.26	4.4±0.16
4	6.7±0.04	6.6±0.24	6.4±0.19	6.5±0.20	6.0±0.08	6.3±0.17
5	7.5±0.12	7.4±0.09	7.2±0.22	7.3±0.08	6.9±0.09	7.3±0.18
6	8.2±0.04	7.9±0.13	7.7±0.09	7.8±0.06	7.5±0.07	7.6±0.14
7	9.0±0.02	8.8±0.05	8.7±0.07	8.6±0.12	8.4±0.22	8.5±0.18

SD, standard deviation

Table III. IC_{50} values of standard antimalarials and antibiotics against laboratory maintained 3D7, K1 strains and field isolates of *Plasmodium falciparum*

Antimalarial drugs	IC_{50} in nM (mean±SD), n=3					
	3D7	K1	MZR-I	MZR-II	MZR-III	MZR-IV
CQ	6.87±1.05	275±12.50	2774±89.75	1840±20.00	1790±25.06	1860±13.23
MQ	8.89±1.0	9.45±0.15	9.18±0.03	8.06±0.03	8.69±0.10	8.73±0.16
QUIN	41.17±1.08	41.77±2.87	1911±19.86	1640±9.17	1740±7.76	1890±8.54
PPQ	3.15±0.10	2.98±0.12	2.54±0.07	2.63±0.11	2.61±0.15	2.81±0.13
ARTM	1.37±0.06	1.32±0.11	1.54±0.12	1.34±0.05	1.42±0.11	1.45±0.08
ARTE	1.41±0.08	1.38±0.06	1.50±0.06	1.30±0.04	1.41±0.06	1.47±0.03
DHA	0.49±0.03	0.55±0.04	2.04±0.04	1.55±0.05	1.52±0.11	1.46±0.04
ATQ	1.15±0.02	1.22±0.01	1.12±0.05	1.11±0.05	1.15±0.07	1.25±0.06
LUME	6.16±0.10	6.03±0.21	5.96±0.14	5.86±0.05	5.85±0.16	5.85±0.15
AZI	4856±13.42	4986±12.34	4566±34.04	4331±12.66	4500±11.06	4550±8.89
DOXY	3613±10.0	3312±9.29	3240±14.19	3506±6.56	3400±2.65	3600±7.09

SD, standard deviation
Abbreviations for drugs are as given in Table I

Table IV. IC_{90} values of standard antimalarials and antibiotics against laboratory maintained 3D7, K1 strains and field isolates of *Plasmodium falciparum*

Antimalarial drugs	IC_{90} in nM (mean±SD), n=3					
	3D7	K1	MZR-I	MZR-II	MZR-III	MZR-IV
CQ	15.11±0.95	9240±9.29	9604±4.16	5432±9.17	5562±22.23	5234±10.58
MQ	27.28±1.12	33.24±0.32	29.17±0.08	30.77±0.11	14.53±0.56	13.89±0.09
QUIN	64.66±1.24	69.92±0.92	8758±574.81	6975±279.62	17,462±11.59	16,988±5.13
PPQ	14.88±2.08	13.98±0.06	14.16±0.02	14.96±0.05	14.76±0.12	15.11±0.05
ARTM	5.21±0.86	5.46±0.09	5.41±0.09	5.23±0.11	4.98±0.12	4.67±0.19
ARTE	5.36±0.40	5.35±0.05	5.74±0.09	5.43±0.12	5.22±0.03	5.11±0.10
DHA	2.11±0.12	2.46±0.03	6.15±0.04	3.16±0.15	3.15±0.05	3.21±0.03
ATQ	2.11±0.15	3.05±0.07	3.06±0.06	2.13±0.06	2.16±0.05	2.43±0.09
LUME	16.63±0.16	15.73±0.29	18.39±0.12	15.63±0.32	15.43±0.11	14.98±0.09
AZI	9252±87.75	10,519±17.62	9952±8.02	10,514±10.58	9450±1998.0	9845±12.22
DOXY	9343±70.40	8428±18.01	10,418±3.51	10,303±83.16	10,282±10.02	10,112±11.14

SD, standard deviation
Abbreviations for drugs are as given in Table I

Table V. Resistance index of MZR field isolates in respect to 3D7 (chloroquine sensitive) strain of *Plasmodium falciparum*

Antimalarials	Mean IC_{50} (nM) values					Resistance index			
	3D7	MZR-I	MZR-II	MZR-III	MZR-IV	MZR-I	MZR-II	MZR-III	MZR-IV
CQ	6.87	2774	1840	1790	1860	403.78	267.83	260.55	270.74
QUIN	41.17	1911	1640	1740	1890	46.42	39.83	42.26	45.91
DHA	0.49	2.04	1.55	1.52	1.46	4.16	3.16	3.10	2.98

Abbreviations for drugs are as given in Table I

Table VI. Resistance index of field isolates in respect to chloroquine-resistant (K1) strain of *Plasmodium falciparum*

Antimalarials	Mean IC_{50} (nM) values					Resistance index			
	K1	MZR-I	MZR-II	MZR-III	MZR-IV	MZR-I	MZR-II	MZR-III	MZR-IV
CQ	275	2774	1840	1790	1860	10.08	6.69	6.51	6.76
QUIN	41.77	1911	1640	1740	1890	45.75	39.26	41.66	45.25
DHA	0.55	2.04	1.55	1.52	1.46	3.71	2.82	2.76	2.65

Abbreviations for drugs are as given in Table I

The IC_{50} and IC_{90} values of CQ and quinine against field isolates were significantly higher (P<0.001) when compared with 3D7 and K1 strains. The resistance index values of four field isolates to CQ, QUIN and DHA with respect to 3D7 and K1 strains are shown in Tables V and VI. It was observed that resistant index of all four field isolates, with respect to 3D7 strain, ranged between 260.55 and 403.78 to CQ, 39.83 and 46.42 to quinine and 2.98 and 4.16 to DHA, whereas the respective values to K1 strain ranged between 6.51 and 10.08, 39.26 and 45.75 and 2.65 and 3.71. MZR-I isolate exhibited highest resistance index with respect to 3D7 as well as K1 strain. The high *in vitro* IC_{50} and IC_{90} values of DHA (which is a metabolite of artemisinins[24]) correlated well with ACT treatment failure.

Discussion

A large number of studies have been carried out to observe *in vitro* susceptibility of both *P. falciparum* and *P. vivax* isolates to monitor drug resistance[25-28] from different parts of the world, especially where CQ resistance is prevalent[29]. Wongsrichanalai *et al*[30] have observed the prevalence of high resistance to MQ in Thai-Myanmar border region. There are many

consecutive reports claiming spread of Artemisinin ART resistance in Western Cambodia[11,12,14] and a recent report has provided evidence for the presence of artemisinin-resistant falciparum malaria across much of Upper Myanmar, close to the Indian border[20]. Artemisinins are the backbone of malaria therapy as these form the basis of ACTs where artesunate replaces quinine in the treatment of severe malaria[31,32]. As these isolates showed tolerance to DHA-PPQ combination therapy, we planned to explore the *in vitro* susceptibility of these against existing antimalarials and commonly used antibiotics and observed significantly higher IC_{50} (nM) and IC_{90} (nM) values of CQ and quinine compared to reference strains. The increase in IC_{50} and IC_{90} values of DHA was not significant.

In conclusion, our findings showed that these field isolates of *P. falciparum* developed resistant to CQ and quinine but not to DHA as increase in IC_{50} and IC_{90} values of DHA were not significant. These findings indicated that the tolerance to DHA-PPQ combination might be because of PPQ only. Further study is required to establish a genetic correlation with high IC_{50} and IC_{90} values of CQ and QUIN.

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Conflicts of Interest: None.

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Reprint requests: Dr Kumkum Srivastava, Division of Parasitology, CSIR-Central Drug Research Institute, Sector-10, Jankipuram Extension, Sitapur Road, Lucknow 226 031, Uttar Pradesh, India
e-mail: kumkum1110@yahoo.com