Characterization of Indian *Plasmodium falciparum* isolates with special reference to *in vitro* susceptibilities and molecular markers for antimalarials resistance

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By

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STATEMENT

As required under the University ordinance, I hereby state that the present thesis for Ph.D. degree entitled **"Characterization of Indian *Plasmodium falciparum* isolates with special reference to *in vitro* susceptibilities and molecular markers for antimalarials resistance"** is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area. The literature related to the problem investigated has been cited. Due acknowledgement has been made whenever facilities and suggestions have been availed of.

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Date:

CERTIFICATE

This is to certify that the thesis entitled **"Characterization of Indian *Plasmodium falciparum* isolates with special reference to *in vitro* susceptibilities and molecular markers for antimalarials resistance"** submitted by **Ms. Supriya Sharma**, for the award of the Degree of Doctor of Philosophy in Biotechnology is based on original studies carried out by her under our supervision.

The thesis or any part thereof has not been submitted for any other degree or diploma in any university or institution.

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As suggested by the External Examiners, appropriate correction are incorporated into the thesis on relevant pages.

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Date

Place: New Delhi ***Supriya Sharma***

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ABBREVIATIONS

%	Percentage
µl	Microliter
Cm	Centimeter
^{0}C	Degree centigrade
ACTs	Artemisinin Combination based therapies
AQ	Amodiaquine
ART	Artemisinin
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
CI	Confidence Interval
CQ	Chloroquine
CMM	Cell medium mixture
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid disodium salt
ELISA	Enzyme Linked Immunosorbent Assay
HRP II	Histidine Rich Protein II
IC_{50}	Concentration of an inhibitor where the response is reduced by half
JSB	Jaswant Singh Bhattacharya
hrs	Hours
kg	Kilogram
K13	Kelch 13
mg	Milligram
ml	Milliliter
MQ	Mefloquine

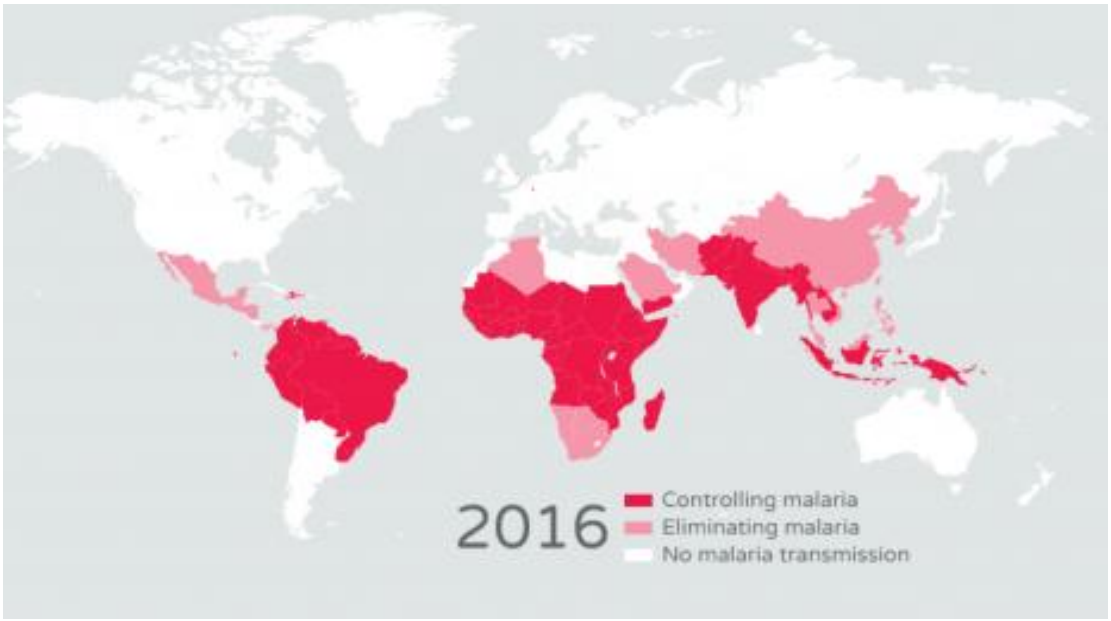
N	Number
ng	Nanogram
nM	Nanomolar
NVBDCP	National Vector Borne Disease Control Programme
OD	Optical density
p	Calculated probability
P	Plasmodium
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
pfmdr-1	*Plasmodium falciparum* multidrug resistance 1
pfdhps	*Plasmodium falciparum* dihydropteroate synthase
pfdhfr	*Plasmodium falciparum* dihydrofolate reductase
pfATPase6	*Plasmodium falciparum* ATPase ortholog
QN	Quinine
r	Coefficient of correlation
r^2	Coefficient of determination
RBCs	Red blood cells
RPMI 1640	Roswell Park Memorial Institute
TMB	3, 3′, 5, 5′-Tetramethylbenzidine
WHO	World Health Organization

Chapter 1

Introduction and Review of Literature

1.1 Malaria: Introduction and epidemiology

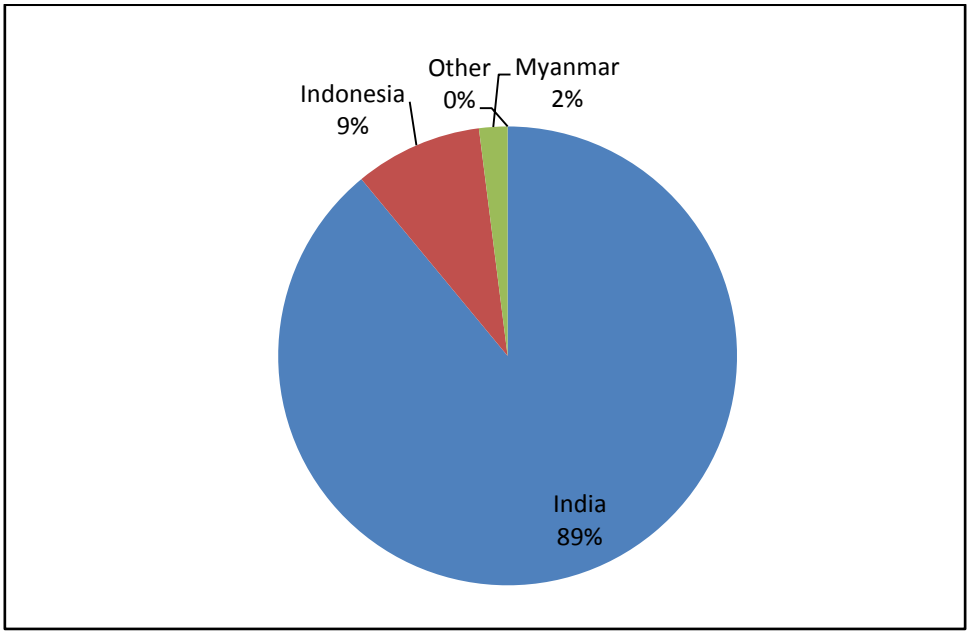
Malaria is a parasitic disease caused by the protozoan of genus Plasmodium. As per World Health Organization (WHO) report there were 212 million cases of malaria in 2015 with approximately 0.42 million deaths in children under five year age in Africa (WHO report. 2016). Worldwide, malaria is the fifth cause of death amongst infectious diseases and the second in Africa, after HIV/AIDS (World Health Organisation. 2013). Huge burden of malaria remains inexplicably in Sub-Saharan Africa region. In the year 2015, thirteen countries from Sub Saharan Africa accounted for 76% and 75% of malaria cases and death respectively. Approximately 70% of deaths occurs due to malaria in children under 5 years with low immunity in the areas of high transmission of malaria. The number of endemic countries for malaria have reduced to 91 in 2016 (Fig. 1.1), owing to the wide-scale organization for malaria control interventions.



Source: WHO database

Fig. 1.1: Situation of countries across the world for malaria in 2016

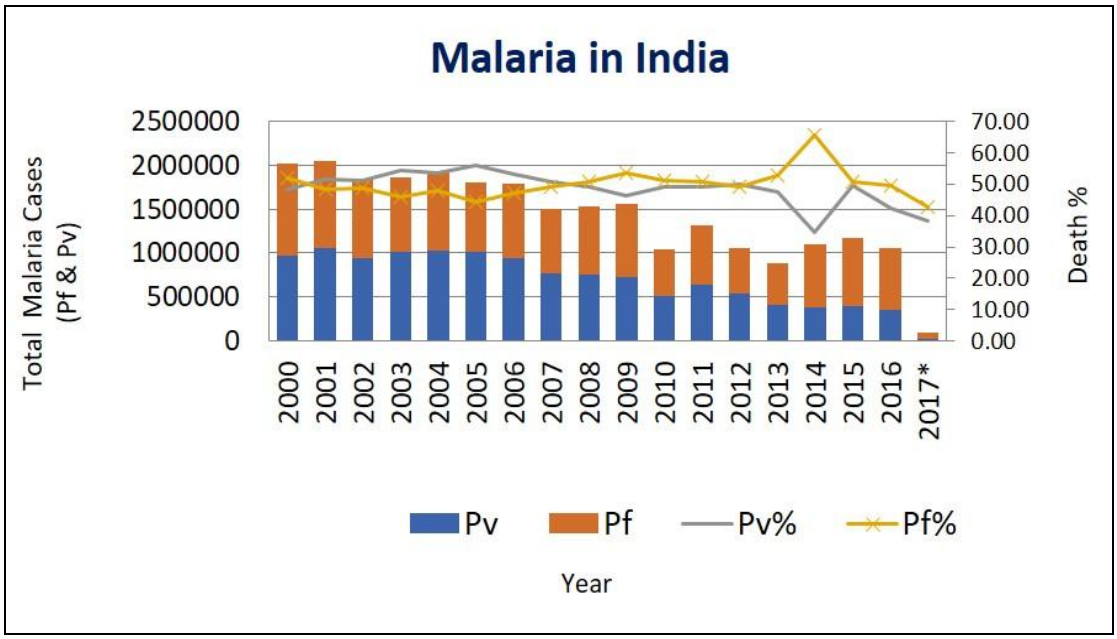
Regardless of dynamic measures taken to tackle malaria, it remains a significant health problem in the South-East Asia (SEA). WHO is working with the member countries to reduce the cases of malaria leading to its elimination in SEA region combating the artemisinin drug resistant parasite. As per WHO report 2011-2012, SEA region bears the second largest burden of malaria after Africa with approximately 1.4 billion people at risk for malaria and 237 million at high risk. Among SEA region, India shares 89% (Fig. 1.2) followed by Indonesia (9%) and Myanmar (2%) and others (WMR-2016-annexes)



Source: WMR-2016-annexes

Fig. 1.2: Share of malaria cases in SEA region during the year 2015

National Vector Borne Disease Control Programme (NVBDCP) has shown decline in the rate of total malaria cases along with *P. falciparum* cases over the years (Fig. 1.3).



Source: http://nvbdcp.gov.in/malaria3.html

Fig. 1.3: During the year 2001 to 2014 the cases have steadily deteriorated from 2.08 million to 1.10 million. Similarly, *Pf* cases have debilitated from 1.0 to 0.72 million cases during the same period. Less than 2000 deaths were reported during all the years within this period with a peak in 2006 and 2014, when epidemic was reported in NE states. The data for the year 2017 is provisional*.

More than 91% of cases of malaria and 99% of death due to malaria are reported from the states having the high burden of malaria disease, these included Northeastern (NE) states, Andhra Pradesh, Chhattisgarh, Gujarat, Jharkhand, Karnataka, Madhya Pradesh, Maharashtra, Odisha, Rajasthan and West Bengal. Though, other states have also reported malaria but the distribution is not even (NVBDCP. 2015). The ratio of *P. vivax* and *P. falciparum* fluctuates in India; the number of *P. falciparum* range from 30–90% of the total cases in the forest areas and <10% of malaria cases are reported often from Indo-Gangetic plains and northern hilly states, north-western India, and southern Tamil Nadu (Kumar et al. 2007). However, a recent report suggests that there is an increase in the incidence of *P. falciparum* over *P. vivax* in past 30 years in India by approximately 60% (Kumar et al. 2013).

1.2 Parasite

There are five species of parasite namely *Plasmodium falciparum (P. falciparum), Plasmodium vivax (P. vivax), Plasmodium ovale (P. ovale), Plasmodium malariae (P. malariae)* and *Plasmodium knowlesi (P. knowlesi)* that cause malaria in humans. In India, the most common and life threatening species is *P. falciparum* which has contributed to 75.89% of the total malaria cases followed by 24.19 % of *P. vivax* cases in the year 2017 till January (Provisional data from NVBDCP). Rare cases of *P. malariae* are also reported from certain areas of Odisha and infrequent reports of *P. ovale* are there from the country (NVBDCP. 2013a).

Taxonomy of Plasmodium (Parasite)

Kingdom: Animalia

Phylum: Protozoa

Class: Sporozoa

Order: Coccidiida

Family: Plasmodiidae

Genus: *Plasmodium*

1.3 Malaria vectors in India

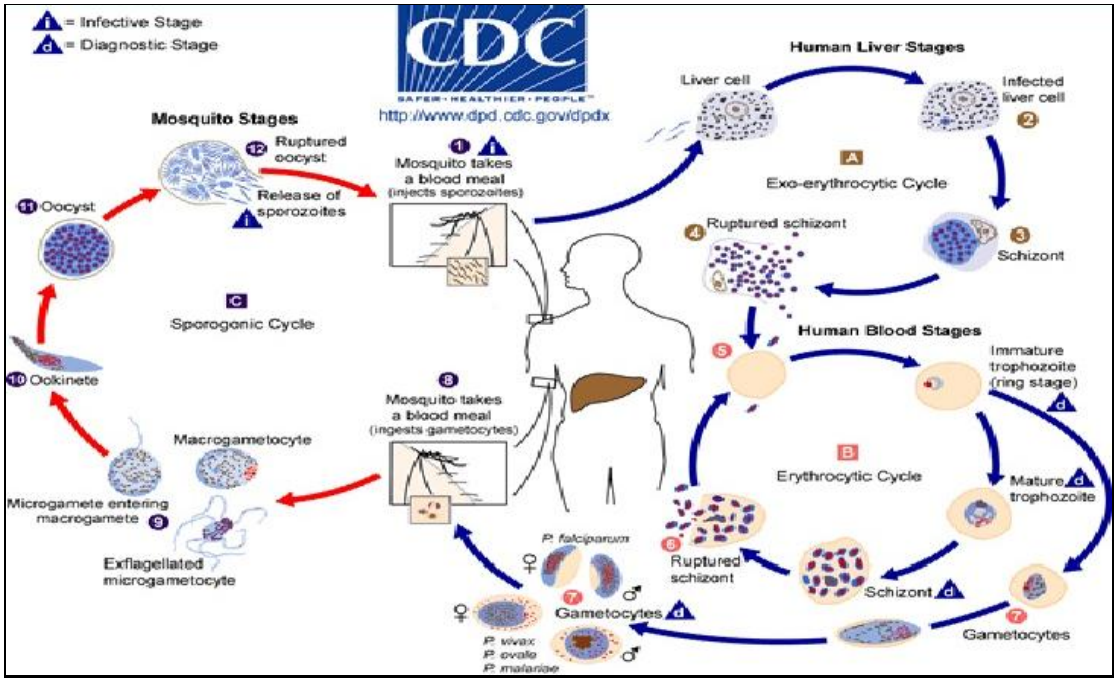
The disease carriers are known as vectors and they spread infection by carrying pathogen from one host to another. Vector for malaria is female Anopheles mosquitos which breed in clean water and feed during night.

There are 480 species of anopheles, only 50 species are responsible for transmission of malaria. The six recognized primary vectors of malaria in India are *Anopheles culicifacies, Anopheles stephensi, Anopheles dirus, Anopheles fluviatilis, Anopheles*

minimus and *Anopheles sundaicus.* Vectors of secondary importance are *Anopheles annularis, Anopheles varuna, Anopheles philippinensis, Anopheles culicifaciesis* which are the vector of rural and urban malaria in India (Sharma et al. 2015).

1.4 Life cycle of malaria parasite

Plasmodium displays a complex life cycle which includes an insect vector (mosquito) and a vertebrate host (human). All four (*P. falciparum, P. vivax, P. malariae* and *P. ovale)* species exhibit a more or less same life cycle with minor differences. Human is the intermediate host for asexual reproduction of malaria parasite which occurs in liver and red blood cells (RBCs).



Source: Centers for Disease Control and Prevention (CDC)

Fig. 1.4: Life cycle of malaria parasite

1.5 Life cycle in the mosquito

The sexual phase of malaria parasites starts when a female Anopheles mosquito feed on person infected malaria. The sporozoite of parasite present in the blood of infected

person blood, sucked into the mosquito's stomach, produce four to eight flagella. Each flagellum separate from the parent body and swims through the coagulating blood in the mosquito's stomach; when it finds a female malaria parasite (macrogametocytes), it enters and fertilizes it. After fertilization, a zygote is formed, which travel to the wall of the mosquito's stomach, where it squeezes between the cell of the stomach wall, settles under the outer lining. In this oocyst, the malaria parasites multiply until the oocyst contain many thousands of new parasites. Now oocysts rupture and releases the spindle shaped sporozoites which further moves to the salivary gland of mosquito. The time needed for completion of the parasite life cycle in the mosquito is between the time the female mosquito ingests an infected blood meal and the time it can transmit malaria, varies according to the species and the ambient temperature and humidity, but is usually 7-21 days (Fig. 1.4).

1.6 Malaria in humans

1.6.1 Liver stage

While the infected female *Anopheles mosquito* bites a human being, sporozoites are introduced with the saliva that the mosquito uses as an anticoagulant. Inside the human being, the sporozoites move quickly to the liver, where they try to invade liver cells. In infected liver cells, a single parasite divides and generates many thousands of new parasites over 7-21 days forming schizonts. This finally bursts, releasing thousands of merozoites into the bloodstream, which quickly adhere and enter in red blood cells. When it eats food, it becomes a trophozoite

P.vivax and *P.ovale* have a little changed life cycle, as a number of the parasites that initially make an entry to liver cells do not directly become liver schizonts but enter a

kind of dormant phase called hypnozoites, these dormant parasites are responsible for the relapses that occur at intervals after the first malaria attack.

1.7 Incubation period

The period between infection with the parasites and the beginning of malaria symptoms is called the malaria incubation period. Malaria incubation period is between 10 days to four weeks. The incubation period varies in species to species of plasmodium parasite.

1.8 Transmission

When a mosquito bites an infected individual, it sucks the gametocyte, the sexual form of the parasite, along with the blood. These gametocytes continue the sexual phase of the cycle within the mosquito gut and the sporozoites that develop then fill the salivary gland of mosquito. The female mosquito infected with malaria parasite when bites another human it injects the sporozoites into the blood stream of the fresh victim, this way Anopheles mosquito spreads the infection.

1.9 Symptoms

The symptom of malaria includes the following phases:

- The cold phase (shivering, feeling cold)
- The host phase (vomiting, fever, headache, convulsions in children)
- The sweating phase (sweating, normal temperature, sleepiness)
- Body aches

1.10 Diagnosis

Diagnosis of malaria includes it identification in blood smear or the presence of any antigen in the blood of infected patient. Prompt and accurate diagnosis is critical for the effective management of malaria

There are two methods for the malaria diagnosis

- Clinical diagnosis of malaria.
- Laboratory diagnosis of malaria.

1.10.1 Clinical diagnosis of malaria

Clinical diagnosis includes physical examination of patient for any sign and symptoms such as fever, vomiting etc. The earliest symptoms of malaria are very nonspecific and inconstant, it includes headache, fever, weakness, vomiting, myalgia, dizziness, abdominal pain, chills, diarrhea, nausea, anorexia.

1.10.2 Laboratory diagnosis of malaria

The general nature of the clinical signs and symptoms of malaria may lead to either no treatment or over-treatment of malaria in malaria-endemic areas, and misdiagnosis in non-endemic areas. In the laboratory, malaria is diagnosed by different technique such as microscopic diagnosis by staining thin and thick peripheral blood smear, using rapid diagnostic test kits and various molecular diagnostic techniques.

1.10.2.1 Microscopy

Microscopy is the gold standard method; malaria is diagnosed by observing stained thick and thin blood smear on a glass slide through binocular microscope(100X), to visualize malaria parasites. Microscopy is time consuming and ill-suited for high-through put use as well as species determination at low parasite density is still challenging. Therefore, in remote area, e.g. peripheral medical clinics with no electricity and no health-facility resources, microscopy is often unavailable (Ndao et al. 2009).

1.10.2.2 Rapid diagnostic test

The World Health Organization acknowledged serious requirement for innovative, simple, rapid, precise, and low cost diagnostic tests for determining the presence of malaria parasites; owing to this, many new malaria-diagnostic techniques have been developed (Tangpukdee et al. 2009). is a device that detects Malaria antigen is detected by a device known as rapid diagnostic test in a small amount of blood, usually 5-15µl, by Immunochromatographic assay with monoclonal antibodies directed against the target parasite antigen. Most products target a *P. falciparum*-specific protein, e.g. Histidine Rich Protein II (HRP II) or lactate dehydrogenase (LDH).

1.10.2.3 Molecular diagnosis

Molecular diagnostic tools are the most accurate and sensitive method for the detection of Plasmodium species, and for the detection of subclinical infections. There is various diagnostic method used in molecular biological technologies: Polymerase Chain Reaction (PCR), Loop mediated isothermal amplification (LAMP), Microarray, Mass spectrometry, Flow cytometery. The regular molecular diagnosis of malaria parasite is done by nested PCR method which has an advantage of parasite detection up to 10 parasite/µl of blood but it takes a longer time and also requires equipments. Recently, LAMP technique has been developed and now researchers are working to make it user friendly point of care technique.

1.11 Control and prevention of malaria

The malaria control programme aims to reduce the burden of disease following the prevention methods and control strategies. It is a complex chain of events that frequently balance one another. Early diagnosis of disease is new goal for prevention

and control of disease. The prevention methods include self-prevention by applying and burning mosquito repellent, use of bed nets etc. while public protection method includes spraying of insecticidal chemicals and larvicidal chemicals on stagnant water.

1.12 Treatment

There are limited number of drugs to treat or prevent malaria. The most widely used are ACTs, quinine and its derivatives and antifolate combination drugs. The national guideline for diagnosis and treatment of malaria in India 2014 gives clear algorithm for its diagnosis and treatment. The guide line recommends treatment for uncomplicated *P. falciparum, P. vivax* malaria, malaria in pregnancy, severe malaria in the country. According to the guidelines, the uncomplicated *pf* malaria should be treated with ACT for 3 days and a single dose of PQ (primaquine) on day 2. In northeast, the recommended ACT is artemether + lumefantrine (AL) and for rest of India it is artesunate plus sulphadoxine-pyrimethamine (AS + SP). The treatment of uncomplicated malaria caused by *P. vivax* include 3 days dose of CQ (chloroquine) with 14 days dose of PQ.

1.13 Mode of action of antimalarials and drug resistance

The antimalarials are broadly classified on the basis of class compounds as follows:

- Quinolines - Chloroquine, Mefloquine, Quinine, Amodiaquine, Primaquine
- Antifolates - Pyrimethamine, Proguanil and Sulphadoxine
- Artemisinin derivatives - Artemisinin, Artesunate, Artemether and
- Hydroxynaphthoquinones - Atovaquine

1.13.1 Chloroquine

In the late 1940s, chloroquine, a 4-aminoquinoline family drug was introduced for treatment of malaria and prevention. Its efficiency, affordability and safety, even during

pregnancy, made it the gold standard treatment of malaria for many years (Alkadi et al. 2007). Approximately 10 years after its introduction, the resistance to chloroquine emerged, initially along the Thai–Cambodian border and then later in 1950s in Colombia (Mita et al. 2009). *P. falciparum* resistance to chloroquine in India, was first reported from Diphu of Karbi-Anglong district in Assam (1973) and by the end of the 1980s, CQ resistance had spread widely (Valecha et al. 2009). The mode of action of chloroquine has been an area of research for decades and many evidence supports that the principal target is the heme detoxification pathway in the digestive vacuole, where the parasite degrades erythrocytic hemoglobin and polymerizes the liberated toxic heme monomers to inert biocrystals of hemozoin (Fidock et al. 2000).

1.13.2 Amodiaquine

Amodiaquine, a 4-aminoquinoline, has similar structure to chloroquine related structure and remained in use for >70 years (Sá et al. 2009). Amodiaquine is hypothesized to act by inhibiting heme detoxification as it has the structural similarity to chloroquine, and it also accumulate within the digestive vacuole to bind to heme *in vitro* (Mukanganyama et al. 2008). Cross-resistance between chloroquine and amodiaquine has been reported and mutations in *pfcrt* and *pfmdr1* are associated with decreased susceptibility to both drugs (Petersen et al. 2011).

1.13.3 Mefloquine

Mefloquine was introduced in the 1970s (Trenholme et al. 1975) is a 4-methanolquinoline with a long half-life of 14–18 days (White et al 2008). An increase in the copy number of *P. falciparum* multidrug resistant gene 1 (*pfmdr1*) is linked to mefloquine resistance (Preechapornkul et al. 2009). Although the exact mechanism of action remains unclear,

in vitro experiments demonstrate that mefloquine can bind to heme and exert some antimalarial activity by inhibiting heme detoxification. Mefloquine is used for the oral treatment of uncomplicated multi-drug-resistant falciparum malaria. It is being prescribed increasingly in South East Asia (SEA) and some parts of South America. Mefloquine resistance is easier than chloroquine resistance to induce experimentally. The multi-drug-resistant parasites prevalent in South-East Asia show reduced sensitivity to all the aryl amino alcohols and also to other structurally unrelated drugs. Mefloquine, although effective to treat multidrug resistant falciparum malaria, has a long duration of action which makes it vulnerable for development of resistance. Cross resistance between quinine and mefloquine is also reported (Rathod et al. 1997). The polymorphism in the *P. falciparum* transporter gene (*pfcrt*) particularly at codon 76 where threonine (K) changes to tyrosine (T) in resistant parasite is widely accepted as a marker for CQ resistance (CQR). Also in *P. falciparum* multidrug-resistant protein (*pfmdr1*) a change in asparagine at 86 position to tyrosine is linked with CQR (Vathsala et al. 2004, Chaijaroenkul et al. 2011)

1.13.4 Quinine

In Europe, during the 17th century, use of extracts from the bark of the cinchona tree was the first effective chemotherapy available. In 19th century its principle active compound quinine (QN) was isolated. Its structure is based on the quinoline ring system. A key role in the treatment of severe malaria was played by quinine; when there was increased resistance to chloroquine (Yakoub et al. 1995). Quinine is known as gametocytocidal for *P. vivax* and *P. malariae* with an exception for *P. falciparum* also, it has rapid action against intra erythrocytic malaria parasites as scizonticidal. Quinine is not antipyretic but has analgesic properties. One of the postulate regarding the

mechanism of action is the inhibition of heme detoxification in the parasite digestive vacuole (Hawley et al. 1998). The anti-malarial mode of action of quinine is still unknown (Achan et al. 2011). However, there are reports where decreased sensitivity of QN in the *in vitro* assays has been strongly linked to a protein on the food vacuolar membrane of *pfmdr1* and polymorphisms in multiple genes *pfcrt*, *pfmdr1*, and the sodium/ hydrogen exchanger gene (*pfnhe1*) is also linked to its resistance (Bohórquez et al. 2012, Cheruiyot et al. 2014).

1.13.5 Primaquine

Primaquine (PQ), an 8-aminoquinoline, has been known for its gametocidal activity against *P. falciparum, P. vivax, P. malariae*, and *P. ovale* and hence it plays a central role in malaria treatment. It is the only compound currently available that prevents relapsing malaria and hence act as a prophylactic agent. It is also used as gametocidal and sporonticidal agents. In spite of its clinical importance, the worth of this drug has been restricted by its side effects such as methemoglobinemia, gastrointestinal disturbances, and hemolytic anemia, particularly in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Basso et al. 2011).

1.13.6 Sulphadoxine-Pyrimethamine

Pyrimethamine, proguanil and sulfonamides belongs to the class of antimalarial compounds known as the antimetabolites. The mode of action for these drugs are well established. Antimetabolites inhibit the folate synthesis pathway enzymes dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*). The folate pathway is necessary for pyrimidine synthesis by the parasite. Mutations in the genes encoding the drug target, *dhps* or *dhfr* gene, alter the binding affinity and reduce parasite sensitivity to the drug

(Daily et al. 2006).The mutations are used as molecular markers for decreased parasite susceptibility to antimetabolites. Increased CQ resistance has forced many countries to switch to sulphadoxine-pyrimethamine as first line of drug (Muller et al. 1996). However, there are reports of resistance to this drug combination also from Africa, Asia, Indonesia, and South America (Basco et al. 1998). In the year 2001, WHO has recommended the use of artemisinin-based combination therapies (ACTs) in countries where *P.falciparum* malaria has developed resistance to chloroquine, sulphadoxine-pyrimethamine and amodiaquine. ACTs ensure the highest cure rates and have the potential to reduce the spread of drug resistance (Olumese et al. 2012). There are reports which suggests that with decreasing *in vitro P. falciparum* susceptibility the rate of mutation in both the gene increases .These mutations have been linked to treatment failure in the *in vivo* studies. Mutation in *dhfr* appear to be more important in causing treatment failure than *dhps* mutations (Muller et al. 1996). The factors causing the development and spread of these mutants are not clear even though, the molecular basis for SP resistance is understood (Gatton et al. 2004).

These drugs are not now used alone to treat malaria, although proguanil is still used in antimalarial prophylaxis. Combinations of pyrimethamine with sulphadoxine or sulphalene, or of chlorproguanil with dapsone are used to treat chloroquine-resistant falciparum malaria. Proguanil is an oral pro-drug for the active triazine metabolite cycloguanil, and chlorproguanil is the corresponding precursor of chlorcycloguanil, Pyrimethamine, trimethoprim, cycloguanil, and chlorcy-cloguanil are all competitive inhibitors of plasmodial dihydrofolate reductase (*dhfr*). The sulphonamides and sulphones inhibit dihydropteroate synthase (Wang et al. 1997). Combinations of the two classes therefore provide sequential inhibition of folate biosynthesis, and show marked

synergy in antimalarial activity. This synergy is very important for the efficacy of the drug. Following a standard dose, plasma concentrations provide effective antimalarial synergy against fully sensitive *P. falciparum* for about 50 days (Watkins et al. 1988). For the *dhfr* inhibitors, the initial mutation conferring resistance is usually at position 108 (SER-ASN) in the gene encoding *dhfr* gene (Peterson et al. 1988). Parasites with multiple mutations for *dhfr* at positions 51, 59, and 108 are resistant to pyrimethamine-sulphadoxine, but can still be treated with this drug as the plasma concentrations achieved in most patients are still in the range giving antimalarial synergy. Such parasites are now increasingly prevalent in East Africa (Watkins et al. 1988). Acquisition of a mutation at position 164 (found in many South-East Asian isolates) reduces sensitivity such antimalarial synergy cannot be obtained at current doses, and the infections are highly resistant in *vivo* to pyrimethamine and cycloguanil, alone or in combination. Resistant *P. falciparum* often contain mutations in the genes encoding both *dhfr* and *dhps*. Point mutations in *dhps* confer variable reductions in susceptibility to sulphadoxine, although their precise contribution to synergy and thus pyrimethamine - sulphadoxine resistance remains to be characterized (Wang et al. 1997)

1.13.7 Artemisinin

Artemisinin and its derivatives are the most rapidly acting antimalarials. They are sesquiterpene lactone peroxides derived from the plant Artemesia annua, and they are effective even against multi-drug-resistant falciparum malaria. These drugs are being used increasingly throughout the tropics. The artemisinin compounds act by the haem-catalysed intraparasitic production of highly reactive carbon-centered free radicals (Meshnick et al. 1996). Artemisinin resistance extends across much of Myanmar. *P*

falciparum have been known to carry Kelch13-propeller region mutations more in occurrence in the areas next to northeastern border of India

1.14 Drug resistance

A major hurdle in the successful treatment of malaria and related mortality is the emergence of resistance to antimalarial drugs. Till date, the drug resistance has been reported in *P. falciparum, P. vivax,* and *P. malariae.* The resistance for all antimalarial has been developed in *P. falciparum. P. vivax* has been found to be resistant to chloroquine and primaquine; and *P. malariae* has been reported to be resistant to chloroquine and pyrimethamine in some areas (World Health Organization . 2015).The parasite *P. falciparum* has been found accountable for majority of mortality and morbidity linked with malaria infection. This work is focused on *P. falciparum* drug resistance. The two defined roles of antimalarials could be first as for effective and timely treatment of malaria which further could lead to severe disease and preventing the gametocyte transmission (Gosling et al. 2011). Secondly, antimalarial can be used as preventive drugs in endemic populations, with many policies for chemoprophylaxis, irregular preventive measure and mass drug administration (Greenwood et al. 2010). WHO well-defined drug resistance in 1967, as the capacity of the parasite strain to live or multiply in spite of the administration and absorption of a drug given in doses equal to or higher than those frequently recommended but within the acceptance of the subject. This definition was advanced to contain the sentence: "The form of the drug active against the parasite must be able to gain access to the parasite or the infected erythrocyte for the period of the time essential for its normal action". Resistance to all known anti-malarial drugs, including the introduced artemisinin based combination

therapy (World Health Organisation. 2015), has developed to various degrees in several countries.

Major factors could be cause of antimalarial drug resistance are:

- The unknown genetic structure of malaria parasites in areas where resistance for antimalarials has emerged
- Use of fake or inferior antimalarials for treatment
- Not regulated or not administered as directed use of antimalarial drug
- Monotherapy use of artemisinin

1.14.1 Drug resistance in SEA region

The increase in the drug resistant parasites are a result of widespread use of antimalarial drugs and have significant contribution to the present scenario of drug resistance in SEA region. This region has been known as a productive ground for the spread of drug resistant *P. falciparum*. Already, this region has been identified for harboring parasites resistant to chloroquine, sulphadoxine, pyrimethamine, quinine, and mefloquine, now it has parasites against the most potent antimalarial drug artemisinin and hence, causing a major threat for the elimination target. The use of CQ and its resistance pattern are known in this region with the prevalence of CQR parasite remains more than 90% (Phompradit et al. 2014).Similarly, higher prevalence of mutation in *dhps* gene are emerging in the SEA region. Microsatellites flanking region examination has revealed that N51I/C59R/S108N which appears to have emerged in Southeast Asia. Apt therapeutic regimens should be tested immediately and applied comprehensively if emergence of artemisinin resistance to other regions is to be avoided (Dhorda et al. 2015). The resistance towards artemisinin, as of March 2017 has been well-known in 5 countries of the Greater Mekong Subregion

(GMS): Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Vietnam. However, along the Cambodia-Thailand border, to almost all available antimalarial drugs *P. falciparum* has become resistant. Multidrug resistance may emerge in the near future in the sub region as other parts in the near future.

1.15 Tools for monitoring antimalarial drug resistance

The knowledge of various tools to measure antimalarial resistance is important not only in monitoring the drug resistance, but also in assessing the burden of the malaria and the economic cost it causes. The elementary process for antimalarial drug efficacy and drug resistance includes ***in vivo* therapeutic efficacy studies, *in vitro* tests, use of molecular markers and measurement of drug concentrations**. These methods have their merits and limitations; however, the optimum information can be obtained by using two or more methods simultaneously.

1.15.1 *In vivo* study

In vivo therapeutic efficacy study is defined as "treatment of symptomatic patients infected only with *P. falciparum* with a standard dose of an antimalarial drug and subsequent follow-up of parasitaemia and clinical signs and symptoms over a defined period (response of the host parasite system to the drug)" (Otsuji et al. 2010). It is a simple method with minimal training required (except microscopy) and minimal equipment and supplies required. However, it has certain limitations as it involves the interference of immunity, previous drug intake, variation of drug absorption or metabolism. Also, the misclassification of reinfection and recrudescence remains a major issue. The treatment failures do not necessarily reflect the level of true drug resistance (Otsuji et al. 2010).

1.15.2 *In vitro* tests

In vitro tests do not come across many of the confusing factors which result *in vivo* tests as it removes parasites from the host and placing them into a controlled experimental environment. The micro-technique is the most frequently used procedure where finger-prick blood sample parasites are obtained and are exposed in microtiter plates to precisely known quantities of drug and observed for inhibition of maturation into schizonts. This test more accurately reflects "pure" antimalarial drug resistance. Multiple tests can be performed on isolates, several drugs can be assessed simultaneously, and experimental drugs can be tested. However, the test has certain significant disadvantages such as the lack of a universal standardized protocol which can have a high impact on the level of drug response (Basco . 2007).There are four major types of field based assays which has been evaluated so far as *in vitro* assay for antimalarials:

- Morphological assays (e.g. WHO Mark III),
- Radio- isotope assays (e.g. H^3 hypoxanthine),
- ELISA-based assays (e.g. HRP II based) and
- Fluorometric assays (e.g. SYBR green assay).

Few novel assays such as flow cytometer and others requiring the sophisticated equipments are unsuitable for field application. All assays have different end points and they measure parasite growth metabolism and maturation, necessarily they do not yield direct comparable results.

Testing parasite susceptibility to antimalarial drugs using *in vitro* is not routinely required by all malaria control programs. It is, however, a very useful tool to investigate specific issues, such as temporal and geographical trends in parasite susceptibility,

which may be an indication of future changes of drug efficacy which we measure through *in vivo* procedure, patterns of cross-resistance of *P. falciparum* to different drugs; assessment of the parasite baseline susceptibility to new drugs, before their use in a certain area. Considering these opportunities attempt has been made to standardized and study two *in vitro* test procedures viz WHO Micro Mark III Test and HRP II ELISA Test in this study.

The *in vitro* micro (Mark III) gives information on the quantitative drug response of *P. falciparum* regardless of the patient's immune status. For assessing baseline sensitivity and monitoring the drug response *in vitro* assay acts an epidemiological tool and hence can be a major source for related information on development and evaluation of drug policies. An indicator of forthcoming therapeutic failure in parasite could be a change in the drug sensitivity through *in vitro* tests. The significances of an *in vitro* test are not troubled by ongoing malaria transmission which is contrasting with respect to the *in vivo* tests. The *in vitro* test does not depend on the patient's clinical condition. Another feature is that it can be used with several drugs simultaneously.

A histidine and alanine rich protein named Histidine Rich Protein II (HRP II) is a naturally occurring protein which is localized in cell compartments like the cytoplasm of *P. falciparum*. Currently studies have shown HRP II as an important factor involved in the heme detoxification. In all *P. falciparum* strains regardless of knob phenotype, HRP II protein is present as water-soluble protein which is secreted and found in plasma and culture supernatants (Wernsdorfer et al. 2002). It might be an indicator of parasite growth and its inhibition by antimalarial drugs. The availability of commercial HRP II enzyme linked immuno sorbent assay (ELISA) kits make application of this

assay faster for the quantification of *P. falciparum* HRP II than any other malaria *in vitro* drug sensitivity test (Noedl et al. 2005).

1.15.3 Molecular techniques

Molecular techniques are very promising for malaria diagnosis; they can be assessed under laboratory conditions, reliable and highly sensitivity. PCR is used to detect the mutations involved in the resistance to antimalarials drugs (Bloland 2001).Sample of parasites obtained from patients from a given area could theoretically give frequency of occurrence of specific gene mutations showing drug resistance. Drug resistance studies involving genetic markers are relatively fast, quantitative and less expensive compared with clinical studies where patient care and follow-up is essential (Fidock et al. 2008). In addition to these, collection, storage and transport of specimens for subsequent molecular analysis are far easier than for *in vitro* tests (World Health Organization . 2003).Molecular markers for drug resistance malaria are based on non-synonymous changes in the genes that make parasite resistant to drugs. The genetic mechanism in P. falciparum for antimalarial drug resistance is yet to be completely elucidated. Molecular markers plays a significant role in monitoring and assessment of drug resistance as they predict the emerging resistance pattern and is required thus as help in designing an effective antimalarial drug policy. A lot of research studies are ongoing in this area. *P. falciparum* resistance to pyrimethamine and proguanil is associated with point mutations in the *dhfr* gene, while sulphadoxine resistance is linked to point mutations in *dhps.* However, molecular surveillance of resistance to sulphadoxine/ pyrimethamine still requires the identification of a few genetic markers (specific combinations of *dhfr* and *dhps* gene mutations) that are highly predictive of treatment failure. The precise molecular mechanism of resistance to chloroquine and other antimalarial drugs, such as

mefloquine, halofantrine and quinine, is still not clear. In addition, for the validation of the genetic markers will require comparative pre and post-treatment observations on the diversity of parasite isolates (to exclude reinfections).

In this study, the molecular markers for genes *P. falciparum* chloroquine resistance transporter *(pfcrt*), *P. falciparum multidrug resistance1 (pfmdr-1*), dihydropteroate synthase *(pfdhps*), dihydrofolate reductase *(pfdhfr* , Plasmodium ATPase6 *(pfATPase6*) gene and Kelch13 propeller region were studied. The numerous mutations in the *pfcrt* gene has been known to cause resistance to chloroquine (CQ) and it plays vital role in therapeutic failure and *in vitro* resistance (Johnson et al. 2004 , Sidhu et al. 2005). Resistance of *P. falciparum* to chloroquine has been associated with lower drug accumulation. Mutations in the *pfcrt* gene have been strongly linked to the mechanism of chloroquine resistance (Fidock et al. 2000)The presence of Tyr-76 residue within the *pfcrt* protein is linked to chloroquine resistance, suggesting that it plays an important role in the mechanism of resistance to this antimalarial. Polymorphisms and/or amplification of *pfmdr1*gene have also been shown to affect the susceptibility to structurally non related antimalarial drugs, including artesunate, mefloquine, lumefantrine and quinine (Price et al. 1999). Additionally, mutations within the *pfmdr1* gene have been shown to confer increased resistance to chloroquine, suggesting that they play a role in modulating higher levels of chloroquine resistance. The same mutations also have been shown to confer quinine resistance and alter the level of resistance and sensitivity to mefloquine and artemisinin (Cheruiyot et al. 2014). Resistance against sulphadoxine and pyrimethamine (SP) is conferred by single or multiple mutations in *dhps* and *dhfr* genes, respectively (Wang et al. 1997) (Watkins et al. 1988). A mutation in the *dhfr* enzyme, changing Ser-108 to Asn-108 is known for resistance to pyrimethamine, and subsequent mutations can

greatly increase the level of resistance to this drug. Ten mutant genotypes for *dhfr* have been reported from a large number of field samples. Resistance to sulfonamide and sulfones has been shown to result from mutations within *dhps*. The amino acid changes at four positions (Ser-436, Gly-437, Ala-581, and Ala-613) have been shown to confer resistance to sulphadoxine and also cross-resistance to sulfones and sulfonamides (Shrivastava et al. 2014, Sharma et al. 2015).

Mutation in the *pfATPase6* gene is important determinants of artemisinin resistance (Jambou et al. 2005). In addition, recently Kelch13 propeller polymorphism is a useful molecular marker for large-scale surveillance efforts to detect artemisinin resistance as confirmed through *in vitro* and *in vivo* studies (Ariey et al. 2014).

This study was designed with an aim to assess different *in vitro* and molecular tools to monitor antimalarial drug resistance. The purpose of *in vitro* studies is to quantify the sensitivity of parasites to antimalarial drugs whereas the molecular markers are used to predict the status of partner antimalarial drug resistance in the parasite target genes.

Keeping in view of the above facts the main objective of this work was to characterize the drug susceptibilities in Indian *P. falciparum* isolates from field and culture method via *in vitro* and molecular markers. To achieve these objectives following studies were undertaken:

1. To study the *in vitro* sensitivity of *P. falciparum* isolates to antimalarials by
    - WHO Mark III micro test
    - HRP II ELISA

2. To study the molecular markers of drug resistance in Indian *P. falciparum* field samples and culture isolates and to correlate the findings with *in vitro* sensitivity data. The markers for studies are:

   - *pfcrt* (codon K76T)
   - *pfmdr* (codon N86Y)
   - *pfdhfr* (codon S108N, C59R, N51I, I164L and A16V)
   - *pfdhps* (codon S436F and A437G)
   - *pfatp6* (codon D639G and E431K)

Chapter 2

Comparison of WHO Mark III and HRP II ELISA for in vitro sensitivity of Plasmodium falciparum

2.1 Introduction

The antimalarial drug resistance in *P. falciparum* has become a topic of paramount importance for the control of malaria worldwide. As per the National Vector Borne Disease Control Programme official provisional data for 2017, India has 0.13 million cases of confirmed malaria which includes 0.09 million of *Plasmodium falciparum* cases (NVBDCP. 2017). Specific treatment programs as well as malaria control policies are in needs which are based on drug sensitivity. Fundamentally, drug susceptibility of antimalarials is assessed primarily via two methods namely *in vitro* and *in vivo*. Advantages of *in vitro* tests include the estimation of the underlying sensitivity of malaria parasites. Also, it does not relate to host immunity and other host associated factors such as drug failure. It gives us a better understanding of the intrinsic drug sensitivity than *in vivo* tests. Besides, pricking for blood collection, *in vitro* test does not involve any intervention of patient and involve no risk for patient (Noedl et al. 2003). Principally, all *in vitro* tests are based on the measure of drug effect on the parasite maturation and growth. The widely used *in vitro* assays are World Health Organisation (WHO Mark III) assay; methods based on lactate dehydrogenase (LDH), (H^3) Hypoxanthine incorporation (Desjardins et al. 1979) SYBR Green I-based fluoroassay (Rason et al. 2008) and [3H] ethanolamine incorporation assay and histidine rich proteins (HRP II) (Noedl et al. 2002). These methods have their own merits and demerits. Based on the available expertise and infrastructure, the limit of detection can be chosen accordingly (World Health Organisation 2001, Basco et al. 2007, Wernsdorfer et al. 2002).

Modified WHO Mark III and HRP II ELISA test are based on the schizoint maturation as quantum for the parasite growth. The macro test (Trager et al. 1976) was first method

wherein schizont maturation was used as a measure of parasite growth, which has numerous drawbacks, primarily due to the minimal culture conditions used in this test. The micro test, which shadowed the macro test, surpasses many of these shortcomings by modifying a lot of elements from the continuous culture. Micro test involving the schizont maturation assays is comparatively easy to perform (World Health Organisation. 2001). It need little technical tools, useful for samples with low parasite densities, and generally requires only 24 hours of incubation. The total numbers of parasites are counted on thick films prepared from the cultured samples and schizonts are counted against it. Schizont maturation assay is not commonly used with microscopists as this is a labor-demanding technique and also the acknowledged low-cost of the assay disappears as technicians are paid by Western standards (Basco et al. 1995). Adding to this, schizont maturation tests necessitate highly skilled persons to identify and limit the single inconsistency in counting and determining the different stages of the parasites.

The cytoplasm of *P. falciparum* together with several cell compartments contains Histidine rich protein II (HRP II), a naturally occurring histidine and alanine-rich protein. The stability of this protein is major advantage for *in vitro* drug susceptibility assays (Noedl et al. 2002).The amount of HRP II produced varies between parasite strains. As *in vitro* drug sensitivity tests are internally measured, the amount of HRP II found in culture samples is directly related with parasite growth and development (Desakornl et al. 1997). The HRP II assay practices a longer culture incubation time (72 h rather 48 h) than most other assays which gives advantage of testing for slow metabolizing drugs with no changes in the procedure. A simple, commercially available double-site sandwich, ELISA test kit (or basically any ELISA that is specific to HRP II protein) can be used for the assessment of parasite growth and development by

measuring the production of HRP II (Noedl et al. 2003). The simplicity of use and less need for laboratory equipment and skilled person has made ELISA based *in vitro* test a successful tool for antimalarial susceptibility testing (Noedl et al. 2005).

The aim of this study was to compare the two widely used *in vitro* assay viz; modified WHO Mark III test and HRP II ELISA tests on the field isolates and culture isolates and to find out which one is more suitable for assessment of antimalarial susceptibilities of *P. falciparum* in India. Besides the report (Anvikar et al. 2012) on *in vitro* susceptibilities of antimalarials in India, very few reports have been published on the susceptibilities of parasites since 2010 after the introduction of ACT as first line of treatment. These *in vitro* studies may be very helpful in evaluating the national guideline for malaria treatment.

2.2 Materials and Methods

Total fifty *P. falciparum* isolates were collected from five states (Chhattisgarh (n=10), Meghalaya, Mizoram, and Tripura (n=20), Odisha (n=10) of India from December 2011 to September 2014. These samples also included culture adapted samples from the MPB (n=10) of National Institute of Malaria Research (NIMR).

2.2.1 Pre-evaluation of test subjects

Pre evaluation of test subjects were done in case the characterization of fresh isolates was taken. All subjects were questioned with respect to their recent history of antimalarial drug use. Those persons were excluded from the study who had received quinine, artemisinin or artemisinin derivatives within the last 7 days, 4-aminoquinolines within the last 14 days, pyrimethamine and/or sulfonamides within the last 28 days, or mefloquine within the last 56 days.

Thick and thin blood films were taken from screened subject and stained with Giemsa or stained with Jaswant Singh Bhattacharji (JSB) stain. Patients, who had mono-infections with *P falciparum* and asexual parasitaemia in excess of 1000 parasites, but less than 80000 parasites per µl blood, were considered suitable for testing.

2.2.2 Informed consent

The study was approved by institutional ethics committee of NIMR New Delhi, India. Written consent was obtained from all the patients. Ascent from parents/guardians was obtained in case of children.

2.2.3 Treatment

All the diagnosed cases of malaria were treated as per the national drug policy in the study area. Antimalarials were given only after collection of specimens. Antipyretics and antiemetic were given as per need.

2.2.4 Sample collection

About 2 ml blood was collected by venipuncture using a heparinized vacutainer by taking aseptic precautions. Thick and thin blood films were prepared, thoroughly dried, fixed with methanol and stained with Giemsa (10%, 30 mins). Slides were examined microscopically (immersion oil, 1000x magnification) and parasitaemia assessed.

2.2.5 *In vitro* cultivation of malarial parasites

2.2.5.1 Preparation of culture medium

- Took one packet of RPMI 1640 (contains 25 mM of HEPES buffer) and dissolved in 960 ml of triple distilled water.
- Added 2 gm of glucose and dissolved

- To avoid contamination 40 µg/ml of Gentamycin sulfate was added (1.2 ml of Gentamycin/L).
- The solution was sterilized by passing through a filter of 0.22 µm porosity (Millipore) and stored at 4 ^{o}C as 96 ml aliquots in glass media bottle.

2.2.5.2 Preparation of 5% sodium bicarbonate

- Sodium bicarbonate (5 gm in 100 ml) was dissolved in double distilled water and filtered through 0.22 µm porosity (Millipore) filter and stored at 4 ^{o}C.

2.2.5.3 Preparation of washing media (incomplete media)

- RPMI-1640 media stock (96 ml) was added to 4.2 ml of 5% Sodium bicarbonate.
- The freshly prepared sodium bicarbonate should be added to the RPMI-1640 media only at the time of requirement.

2.2.5.4 Preparation of complete media

- For complete media, 10 ml normal inactivated AB^{+} human serum was added to 90 ml of incomplete media & stored at 4 ^{o}C.

2.2.5.5 Preparation of erythrocytes (RBCs) for culture

- A^{+} blood was collected in anticoagulant.
- The whole blood was dispensed into aliquots in glass bottles
- The required quantity of blood from the glass bottles was poured into centrifuge tubes.
- Centrifuged at 1500 rpm for 10 min.
- Removed the plasma & buffy coat with sterile Pasteur pipette.
- Added washing media and centrifuged at 1500 rpm for 10 min.
- Repeated the process thrice.
- Equal amount of complete media was added to the pallet. Stored it at 4^{o}C till further use.

2.2.5.6 Collection of serum

- AB^+ blood was collected without anticoagulant and kept overnight at 4 ^oC.
- Separated the serum aseptically and kept in aliquots.
- The serum was inactivated by keeping it at 56 ^oC water bath for half an hour.
- After inactivation, the serum was stored in deep freezer at – 20 ^oC or -70 ^oC.

2.2.5.7 Revival of cryopreserved cell lines

- All *P. falciparum* field isolates along with reference strains 3D7 and Dd2 were revived by following standard *in vitro* techniques as below:
- From the liquid nitrogen container, the vial was taken out for thawing quickly in a 37 ^oC water bath.
- The content from vial was transferred to centrifuge tube and centrifuged at 1500 rpm for 10 min.
- After removing the supernatant an equal volume of 3.5 % NaCl was added.
- Again, this suspension was centrifuged to remove the supernatant.
- Complete medium supplemented with 15 % serum was used to wash the pellet twice.
- The cryopreserved isolates were used after washing cells and culture was initiated by adding fresh washed erythrocytes.

2.2.5.8 Collection of *P. falciparum* positive blood samples from the field

- Patients with no previous history of intake of antimalarials were checked for the presence of healthy asexual parasites in the peripheral smear of patients.
- 2-5 ml blood was taken with a sterile disposable syringe and transferred to a heparinized centrifuge tube.
- The blood was stored at 4 ^oC and transported in ice to the laboratory.

2.2.5.9 Initiation of culture

- The infected blood was centrifuged and plasma was removed.
- Cells were washed following the same procedure for preparing normal cells.
- 50% suspension with complete media (use complete media with 15% serum) was prepared.
- Appropriate numbers of uninfected cells (washed) were added to get an initial parasitaemia of 0.5 to 1.0%.
- Complete media was diluted with blood pellet to get 8% cell suspension. Dispensed in vials.
- The vials were incubated at 37°C in a CO_2 incubator with gas mixture of 90% N, 5% CO_2 and 5% O_2 for 25-30 hours.

2.2.5.10 Checking of culture for growth

- The vials taken out from the CO_2 incubator.
- The media was removed using a sterile Pasteur pipette without disturbing the cells settled down.
- Mixed the cells without frothing and place a small drop of blood on the slide and made a thin film.
- Added fresh complete media mix and incubated in the CO_2 incubator.
- Each thin film was stained with JSB stain and examined for parasitaemia. The counts were compared with the initial parasitaemia. The growth can be estimated by number of schizonts and rings in subsequent slides.

2.2.5.11 Preparation of stains

JSB-I

Medicinal Methylene Blue: 0.5 gm

Potassium dichromate {$K_2Cr_2O_7$}: 0.5 gm

Sulphuric acid (1% by volume) {H_2SO_4}: 3.0 ml

Disodium hydrogen phosphate dihydrate {$Na_2HPO_4.2H_2O$}: 3.5 gm

Distilled water {H_2O}: 500 ml

2.2.5.12 Method

Methylene blue was dissolved in water and sulphuric acid was added gradually while stirring to ensure through mixing. Potassium dichromate was then added which forms a purple precipitate, next disodium hydrogen phosphate was added and after stirring the solution for some time the precipitate appears to get dissolved. The solution was boiled in flask with a reflex condenser for one hour till blue colour of the solution deepens. This solution was stored in (brown colour) bottle and ready for use after maturation.

JSB II

Dissolved 1 gm of water-soluble eosin in 500 ml of distilled water and kept in a bottle (brown colour) for maturation.

Giemsa stain

Giemsa powder (Azure B type): 3.8 gm

Glycerol: 250 ml

Methyl alcohol: 250 ml

2.2.5.13 Method

- Place a small amount (e.g. 500mg) of dry stain in a mortar followed by small amount (e.g. 15ml) of glycerol.
- Grind the dye thoroughly, and then pour off into a clean dark bottle.
- Repeat the grinding in this manner until all the dye has been mixed with glycerol.
- Incubate the dye-glycerol mixture in a water bath at 55-60^{0}C for 6-8 hours with periodical shaking. Then cool the dye mixture to room temperature.
- Add 250 ml methanol to the dye mixture, mix well and keep at 37^{0}C for two or more weeks.
- Filter the dye mixture and store in a dark bottle until use.

2.2.5.14 Procedure for subculture (passaging)

- When the parasitaemia was above 2-3 % in the initial culture it was sub-cultured.
- Removed the old media and added fresh washed RBCs and complete media.
- Dispensed to more vials.

2.2.5.15 Procedure for synchronization of parasite culture

- Prepared 5% sorbitol in distilled water.
- To 1 ml of the cultured pellet added 9 ml of sorbitol.
- Kept it at room temperature for 5 minutes.
- This process killed all the stages except rings.
- Removed the supernatant by centrifugation.
- Washed the pellet twice in medium.
- Setup the culture by adding freshly washed erythrocytes

2.2.6 WHO Mark III micro test

The sensitivity of Indian isolates to the anti-malaria drugs was assessed by a modification of the standard WHO Mark III micro-test (WHO. 2001). The standard operating procedures for drug coating are in annexure 1.

- *In vitro* assays carried out on samples with parasitaemia > 0.3% by microscopy.
- Samples washed three times in basal medium (RPMI 1640).
- Supernatant and white blood cell interface removed after every wash.
- Erythrocytes re-suspended at 1:1 volume in RPMI (Complete media).
- The starting parasitaemia adjusted to 0.3-2.0%, by adding fresh uninfected erythrocytes.
- Microtitre plates coated with five antimalarials in concentrations recommended by WHO (Table 2.1).
- Each well of a 96-well filled with 90 µl drug plus 10 µl parasite suspension of about 5% hematocrit.
- Control wells with parasitized blood contained no drug.
- The plates were incubated at 37°C in a gas mixture of 90% N, 5% CO_2 and 5% O_2 for 25-30 hours.

Table 2.1: Table showing different concentration of drugs used for coating microtiter plates

Conc.(nmol/l)	**Well**	**CQ**	**AQ**	**MQ**	**QN**	**AS**
	A	**C**	**C**	**C**	**C**	**C**
1	B	25	5	2.5	50	0.25
2	C	50	10	5	100	0.5
4	D	100	20	10	200	1
8	E	200	40	20	400	2
16	F	400	80	40	800	4
32	G	800	160	80	1600	8
64	H	1600	320	160	3200	16
IC value cut off	**IC_{50}**	**160**	**80**	**640**	**800**	**10.5**

2.2.7 Growth inhibition assessment

- At the end of the incubation period, removed suspended medium.
- Thick smears on a microscope glass slide made.
- Smear air-dried, stained with Giemsa/JSB and examined at 100X under microscope.
- Number of schizonts with three or more nuclei against 200 asexual parasites counted for each sample.
- To confirm the results for reproducibility, all tests were carried out in duplicate.

2.2.8 HRP II ELISA

2.2.8.1 Predosing of plates

ELISA plates were dosed with appropriate concentrations of antimalarials Chloroquine (CQ), Amodiaquine (AQ), Quinine (QN), Artemisinin (ART) and Mefloquine (MQ).

2.2.8.2 Sample preparation and culture

- In a sterile disposable tube cell medium mixture (CMM) prepared by adding 0.94 ml of the parasitized blood sample to 24.06 ml of RPMI medium.
- Pre-dosed plates loaded with 200 µl of CMM to each well (starting with well A and proceeding to higher drug concentrations).
- The plates were incubated for 72 hours at 37ºC in CO_2 incubator.
- At an end of the incubation time the plates further processed immediately or stored below –20°C.
- After 72 hours, another slide was prepared to determine parasitaemia.
- Four to 10 fold increases in parasite density within 72 hours considered as adequate growth.

2.2.8.3 Hemolyzing

- The plates were transferred into a freezer for overnight.
- The plates were thawed at room temperature.
- Procedure repeated until complete haemolysis achieved (i.e. all wells look completely clear).

2.2.8.4 Coating ELISA plates

- Primary IgM antibody (MPFM-55A, Immunology Consultants Laboratories, Inc, Newberg, OR, USA) was dilute to 1.0 ug/ml in PBS. (if original solution as shipped from manufacturer is 11.5 mg/mL dilute 9 µl of original solution in 103.5 mL PBS – 100 mL is sufficient for coating 10 plates).
- 100 µl of diluted antibody per well were transferred to the ELISA plate using a multichannel pipette.
- Plates were sealed and incubated at 4ºC overnight. Contents were discarded of the ELISA plate and bang dry.

2.2.8.5 Blocking solution

- Bovine serum albumin (BSA) solution (2%) was prepare in PBS. For each plate, 0.4 g of BSA was dissolved (Sigma, CAS No. 9048-46-8) in 19.6 mL PBS).
- Blocking solution (200 µl) per well was added and was incubated at room temperature for 2 hours further it was discarded and banged to dry.
- PBS/Tween (0.05%) washing solution was prepare: by adding 0.5 ml Tween 20 to 999.5 mL PBS.
- Plate were washed 3 times in PBS/Tween (200 µl/well) it was banged and dried. Plates were sealed in airtight plastic bag and freeze at -20ºC.

2.2.8.6 ELISA Step 1

- Sample (100 µl) was transferred from the culture plate to the ELISA plate (starting parasite density before culture should be equivalent to around 0.05 %, 1.5% hematocrit – samples may be diluted before or after culture (Fig. 2.1).
- Samples were incubated for 1 hour at room temperature in humid chamber.
- Plates were washed 3 times in PBS/Tween (200 µl/well) and banged to dry.

2.2.8.7 ELISA Step 2

- Antibody conjugate was diluted and added to ELISA plate. Diluent for 2nd antibody was prepared which was a solution of 2% BSA+1% Tween 20 in 97 ml PBS (2 g BSA+1 ml Tween 20 + 97 ml PBS – prepare 10 ml per plate) and pH 7.4 was adjusted.
- Second antibody (MPFG-55P, Immunology Consultants Laboratories, Inc, Newberg, OR, USA) was diluted to 0.2 µg/ml (range 0.05 to 0.2 depending on the activity of the conjugate) in diluent.
- A 200x stock solution (40 µg/ml) of the second antibody was prepared (do not store this stock solution for extended periods of time)

2.2.8.8 Stock solution (200x)

- If original solution was shipped from manufacturer as 1 mg/ml dilute 40 µL of the original antibody solution in 1 ml diluent was used. This quantity was sufficient for around 20 plates.

2.2.8.9 Final working solution

- For each plate 50 µl of 200x antibody stock solution was added to 10 ml of diluent.
- 100 µl of diluted 2nd antibody per well was transferred to the ELISA plate using a multichannel pipette It was incubate for 1 hrs. at room temperature in humid chamber.
- Plates were washed 3 times in PBS/Tween (200 µl/well) and banged to dry.

2.2.8.10 ELISA Step 3

- 100 µl per well of TMB chromogen (TMB Single Solution Chromogen, Zymed Lab., Inc., San Frnacisco, CA, USA) was added in each well and plate was incubated for 5 to 10 minutes at room temperature in the dark (Fig. 2.2).
- The reaction was stopped with 50 µl of 1 M sulphuric acid (add 10 mL of sulphuric acid to 90 mL of water to prepare the stop solution) (Fig. 2.3).
- Absorbance was read using an ELISA plate reader at 450 nm

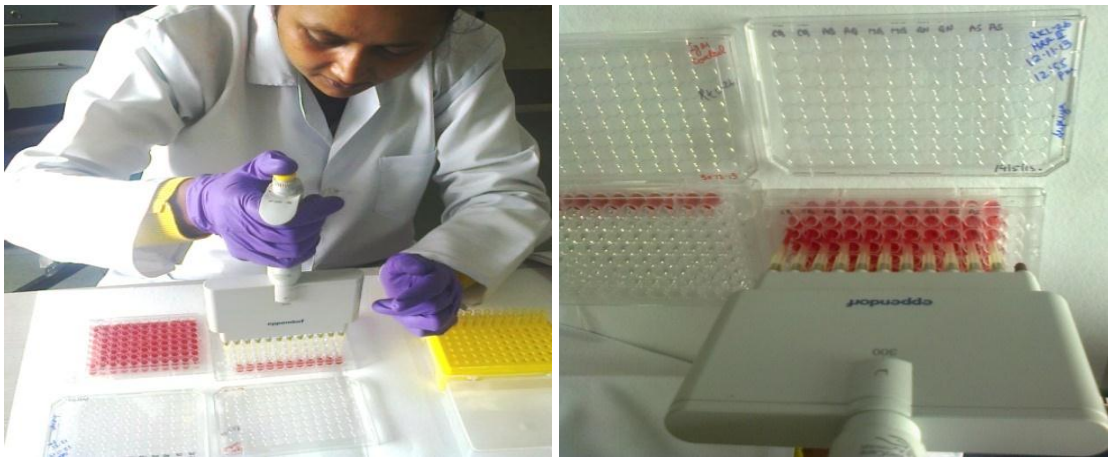


Fig. 2.1: Sample from the culture plate was transferred to the ELISA plate

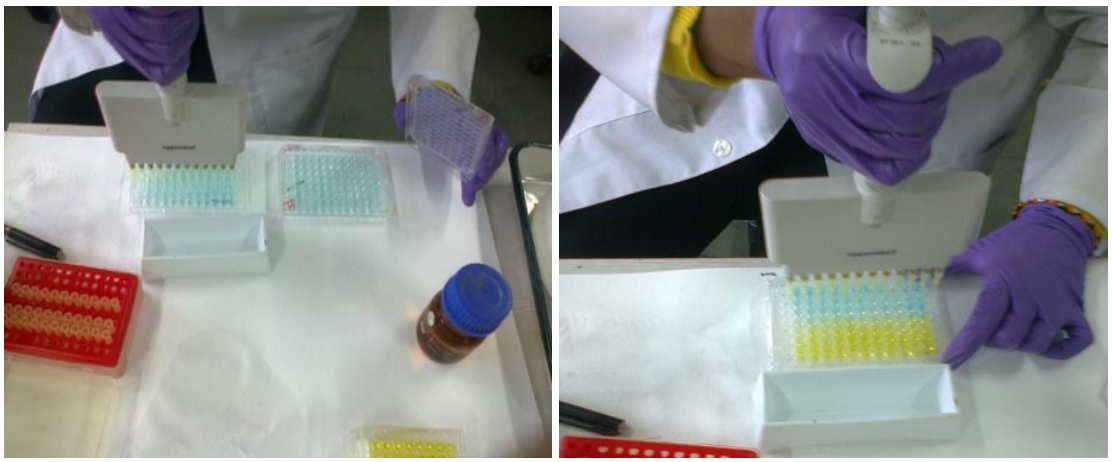


Fig. 2.2: TMB chromogen (100 µl) was added and plates were incubated for 5 to 10 minutes at room temperature in the dark

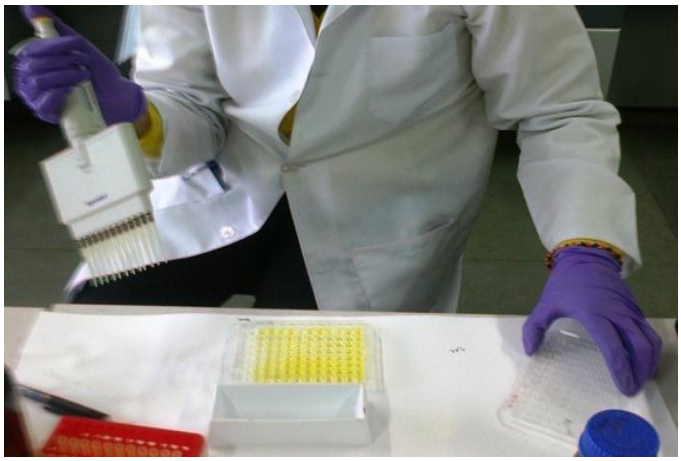


Fig. 2.3: Reaction was stopped by adding 1 M sulphuric acid

2.2.9 Data management and statistical analysis

The drug concentration that inhibits schizogony by 50% (IC_{50}) relative to the drug-free control samples of each *P. falciparum* isolate for both *in vitro* assay was estimated from dose-response curves analysis. This was done using non-linear regression analysis by HN-NonLinReg Analysis Malaria.farch.net; (Noedl et al. 2002).The cut-off values for determining sensitivity to antimalarials based on the WHO microtest protocol were; 160nM/l for CQ, 80nM/l for AQ, 640nM/l for MQ and 800nM/l for QN (World Health Organisation. 2001).The cut-off IC_{50} value of 10.5 nM/l for artesunate as described somewhere else (Pradines et al. 2002) was considered as WHO protocol for does not recommend cut-off for artemisinin. Confidence interval (CI) at 0.05 significance level was calculated by Graph pad prism software Ver 5.0. Individual inhibitory concentrations (IC_{50}, IC_{90}, and IC_{99}) for both assays were determined by nonlinear regression analysis. Bland-Altman plots were performed to assess agreement between two methods. Standard correlation analysis was used to establish linear associations between inhibitory concentrations obtained from different assays with various drugs. Those data that were not normally distributed, nonparametric procedures or log transformations were used.

2.3 Results

2.3.1 *In vitro* cultivation of samples

All samples collected from Odisha (Rourkela, n=10), Northeast (6 from Meghalaya, 4 Mizoram and 10 Tripura), Chhattisgarh (Raipur, n=10) and 10 routinely cultured parasites from MPB were successfully adapted using Trager and Jenson method in culture condition. An emphasis was made to use single lot of media and serum for a particular isolate for their successful adaption to culture conditions (Fig. 2.4 – 2.11).

Fig. 2.4: Labeling of slide for smear preparation

Fig. 2.5: Smear preparation

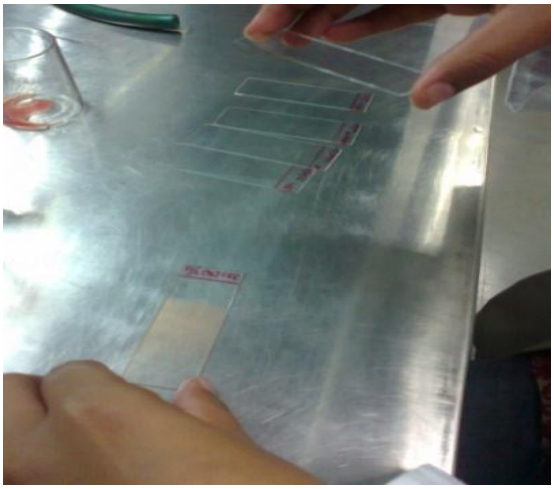


Fig. 2.6: Smear prepared

Fig. 2.7: Culture vial with parasite

Fig. 2.8: Changing media for routine culture

Fig. 2.9: Adding media for parasite revival

Fig. 2.10: Incubation at 37 ^{0}C at 5 % CO_2

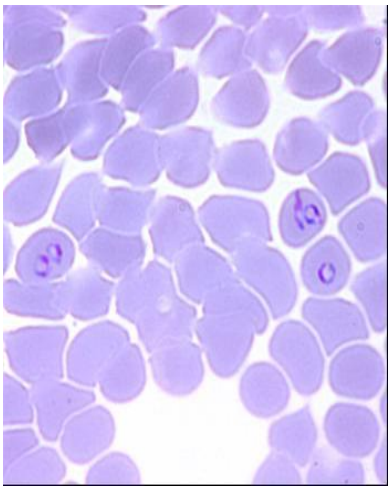


Fig. 2.11: Thin smear of *P. falciparum*

2.3.2 WHO Mark III result

Among the field and culture isolates, only 10% were found resistant to CQ with geometric mean IC_{50} value of 59.5 against the cut off value of 160 nM. All the isolates were found sensitive for AQ with the geometric mean IC_{50} ($GMIC_{50}$) value of 13.3 nM against 80 nM, MQ with $GMIC_{50}$ of 7.07 nM against 649nM, QN with 146.4 nM against 800 and ART with 0.43 nM against 10.5 nM (Table 2.2).

Table 2.2: Result of WHO Mark III test for five drugs against field isolates/culture isolates

Drug	No. of isolates	IC_{50}				% resistant isolates (n)
		Geometric Mean (nM)	Range (nM)	Threshold for resistance (nM)	Fold Change *	
CQ	50	59.5	49.35- 71.73	160	0.37	10(5)
AQ	50	13.31	11.06- 16.02	80	0.16	0(0)
MQ	50	7.07	5.93- 8.43	640	0.01	0(0)
QN	50	146.4	124.3- 172.6	800	0.18	0(0)
ART	50	0.43	0.37- 0.50	10.5	0.04	0(0)

2.3.3 HRP II ELISA result

Eighteen percent of field and culture isolates were resistant to CQ with $GMIC_{50}$ value of 78.34 against the cut off value of 160 nM. For AQ, 8% of the isolates were resistant with the $GMIC_{50}$ value of 22.02 nM against 80 nM cut off. Similarly 4% of the isolates were resistant to QN with $GMIC_{50}$ value 258.7 with respect to cutoff value of 800 nM. None of the isolates were found to be resistant to MQ and ART as all the IC_{50} values were below the cut off value of 640 nM and 10.5 nM respectively. (Table 2.3)

Table 2.3: Result of HRP II ELISA test for five drugs against field isolates/culture isolates

Drug	No. of isolates	IC_{50}				% resistant isolates (n)
		Geometric Mean (nM)	Range (nM)	Threshold for resistance (nM)	Fold Change *	
CQ	50	78.34	64.57-95.03	160	0.48	18(9)
AQ	50	22.02	18.03-26.9	80	0.27	8(4)
MQ	50	11.46	9.44-13.9	640	0.01	0(0)
QN	50	258.7	207.6-322.4	800	0.32	4(2)
ART	50	1.004	0.81-1.23	10.5	0.09	0(0)

2.3.4 State wise *in vitro* susceptibility

Table 2.4: Drug sensitivity pattern in Indian *Plasmodium falciparum* isolates

	Drug sensitivity pattern				
	Choloroquine resistant/ Total isolates (%) (95% CI)	**Amodiaquine resistance/ Total isolates (%) (95% CI)**	**Mefloquine resistance/ Total isolates (%) (95% CI)**	**Quinine resistance/ Total isolates (%) (95% CI)**	**Dihydro artemisinin resistance/ Total isolates (%) (95% CI)**
Chhattisgarh	1/10(10) (40.84-170.34)	0/10(0) (11.5946.64)	0/10(0) (4.85-39.95)	1/10(10) (122.8-1132.1)	0/10(0) (0.51-3.01)
MPB	3/10(30) (28.1-241.31)	1/10(10) (5.47-66.88)	0/10(0) (3.36-38.49)	0/10(0) (50-800)	0/10(0) (0.7-2.77)
Odisha	1/10(10) (20.1-398.54)	1/10(10) (5.66-95.16)	0/10(0) (3.87-55.61)	2/10(20) (61.2-1378.9)	0/10(0) (0.34-6.08)
Northeast	1/10 (10) (20-163.66)	1/10 (10) (6.97-118.2)	0/10 (0) (3.01-22.71)	1/10 (10) (78.57-993.93)	0/10(0) (0.42-6.28)
Tripura	2/10 (20) (39.09-249.56)	0/10 (0) (7.93-68.05)	0/10 (10) (6.42-22.71)	0/10 (10) (112.13-418.58)	0/10 (10) (0.42-1.34)

Across the five states, the incidence of chloroquine resistance was found to be 18% (9/50) in all the isolates. Moderately higher proportion of CQ resistance was observed in malaria parasite bank isolates (30%; 3/10) followed by 20% (2/10) in Tripura isolates and Odisha isolates. 10% (1/10) of CQ resistance was observed in isolates from North east and Chhattisgarh. Quinine resistance was observed in 8% (4/50) of all isolates, including the two isolates from Odisha 20% (2/10) and one isolate each 10% (1/10) from Chhattisgarh and North east. Resistance of amodiaquine was also recorded in 6% (3/50) of the studied isolates from Odisha, Northeast and culture isolate of parasite bank contributing to 10% (1/10) from each state. Among all isolates, a single isolate from Odisha showed to have cross resistance towards CQ, AQ and QN. None of the isolates were found resistant to mefloquine and artemisinin (Table 2.4).

2.3.5 Comparative results of two *in vitro* assays

The geometric mean IC_{50} for the 50 isolates in the modified WHO assay were 59.5 nM (95% confidence interval [CI] = 49.35-71.73 nM) for CQ whereas the corresponding result in HRP II was 78.34 nM (95% CI = 64.57-95.03 nM). Similarly, for AQ, the geometric mean by WHO assay was 13.31 nM (95% CI = 11.06–16.02 nM) which is slightly higher than the 22.02 nM (95% CI = 18.03–26.9 nM) by HRP II ELISA. The WHO assay result for MQ was 7.07 nM (95% CI = 5.93–8.43 nM) in comparison to 11.46 nM (95% CI = 9.44–13.9 nM) obtained by HRPII ELISA result. Also, the result of geometric mean (258.7 nM) for QN was slightly higher for HRP II ELISA (95% CI = 207.6–322.4 nM) compared to WHO assay result 146.4 nM (95% CI = 124.3–172.6 nM). A nominal difference (0.43 nM (95% CI = 0.37–0.50 nM) verses1.00 nM (95% CI = 0.81–1.23 nM) was observed in the geometric mean for ART among both assays respectively. Fig. 2.12 and Table 2.5.

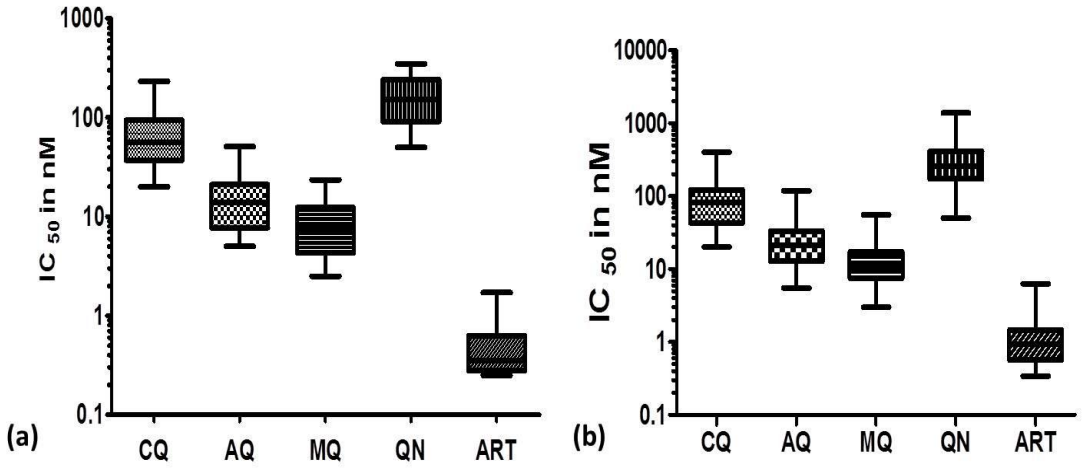


Fig. 2.12: Mean IC_{50} values of chloroquine, monodesethylamodiaquine, mefloquine, quinine and artemisinin for (a) WHO modified assay and (b) HRP II assay respectively.

Table 2.5: Geometric mean IC_{50}, IC_{90}, and IC_{99} with 95% CIs, for chloroquine, monodesethyl amodiaquine, mefloquine, quinine and artemisinin determined by the HRP II drug susceptibility assay and a modified WHO schizont maturation assay

Drug and assay (n = 50)	IC nM (95% CI)		
	50	90	99
CQ			
WHO Mark III	59.5 (49.35–71.73)	370.3 (296.2–462.8)	910.9 (730.6–1136)
HRP II ELISA	78.34 (64.57–95.03)	598 (525.1–681)	945.9 (836.6–1069)
AQ			
WHO Mark III	13.31 (11.06–16.02)	81.3 (62.39–105.9)	171.3 (135.7–216.4)
HRP II ELISA	22.02 (18.03–26.9)	149.6 (131.9–169.6)	243.5 (220.8–268.6)
MQ			
WHO Mark III	7.076 (5.93–8.43)	43.72 (31.13–61.41)	102.8 (77.53–136.2)
HRP II ELISA	11.46 (9.44–13.9)	75.68 (65.41–87.56)	121.5 (108–136.7)
QN			
WHO Mark III	146.4 (124.3–172.6)	821.1 (632.9–1065)	1809 (1465–2233)
HRP II ELISA	258.7 (207.6–322.4)	1410 (1192–1668)	2256 (1930–2637)
ART			
WHO Mark III	0.43 (0.37–0.50)	1.604 (1.186–2.169)	2.655 (1.881–3.747)
HRP II ELISA	1.00 (0.81–1.23)	6.348 (5.193–7.76)	11.32 (10.05–12.75)

In a correlation analysis, the results obtained from the HRP II drug sensitivity tests with all four antimalarials showed a highly significant linear association with those obtained from the WHO assay at the IC_{50} level ($rIC_{50} = 0.94$, $r^2 = 0.92$, $p < 0.001$), as well as at IC_{90} level ($rIC_{90} = 0.96$, $r^2 = 0.94$, $p < 0.001$) (Fig. 2.13).

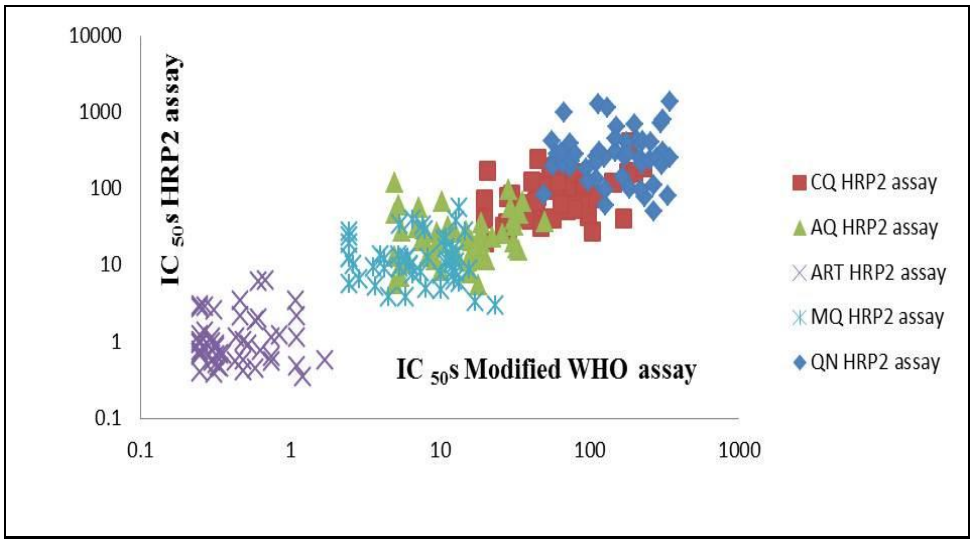


Fig. 2.13: Scatter plot (250 data points) for the individual field and culture isolates 50% inhibitory concentrations (IC_{50}s) for chloroquine, amodiaquine, mefloquine, quinine, and artemisinin determined by the HRP II field test and a modified WHO schizont maturation assay (n =50; r^2= 0.96, p < 0.0001)

When correlating the results obtained from both assays for individual drugs, the correlation coefficients at the IC_{50} level were r = 0.52 (r^2 = 0.34, p < 0.001) for CQ, r = 0.73 (r^2 = 0.53, p < 0.001) for AQ, r = 0.82 (r^2 = 0.76, p < 0.001) for QN, r = 0.72 (r^2 = 0.65, p < 0.001) for MQ and r = 0.98 (r^2 = 0.89, p < 0.001) for ART, respectively. The mean difference for chloroquine, amodiaquine, mefloquine, quinine and dihydroartemisinin, determined by the modified World Health Organization (WHO) schizont maturation assay and histidine-rich protein II (HRP II) assay was in limits of agreement 148.3 and 100.6 for CQ, 58.3 and 34.4 for AQ, 29.4 and 17.2 for MQ, 781.4 and 425.3 for QN and 3.4 and 1.7 for ART at the IC_{50} level and -0.037. The results obtained by the HRP II assay and the modified WHO assay were plotted as Bland-Altman plots (mean difference and limits of agreement) as shown in Fig. 2.14.

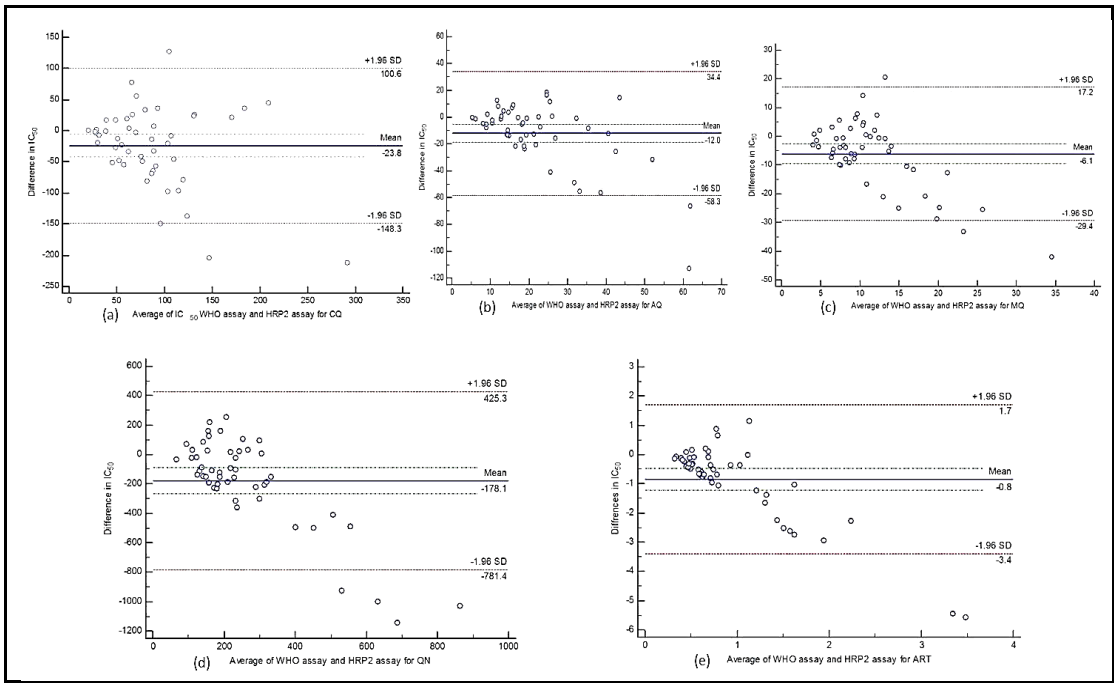


Fig. 2.14: Bland-Altman plot of the difference in log 50% inhibitory concentration (IC_{50}) values for (a) chloroquine (b) amodiaquine (c) mefloquine (d) quinine and (e) dihydroartemisinin, determined by the modified WHO schizont maturation assay and HRP II assay and a plotted against their mean values.

2.4 Discussion and Conclusion

In vitro assays are the technique for assessing the individual drug response on parasite growth irrespective of the host immune response and pharmacokinetic factors. It can detect even a minute change in the drug sensitivity pattern of the isolates of particular areas. Also it can produce an early sign of change in the parasite's drug susceptibility pattern even before it is detected clinically (Basco 2007). In this study, the drug sensitivity pattern of five antimalarials in *P. falciparum* field and culture isolates from diverse regions of India was assessed with respect to two *in vitro* methods namely modified WHO Mark III assay and HRP II ELISA assay. Isolates collected from malaria parasite bank showed higher percentage of resistance to CQ as compared to other states, followed by Tripura. Isolates from malaria parasite bank, Odisha and Northeast showed resistance towards amodiaquine whereas none of them were resistant

to mefloquine and dihydroartemisinin. Comparable results were seen in a study carried out in culture and field isolates of the country (Anvikar et al. 2012). None of the isolates were resistant to mefloquine and artemisinin *in vitro*. The IC_{50} value of mefloquine, 7.07 nM/l (95%C.I.5.93-8.43) in this study was less than as reported in year 2012 (Anvikar et al. 2012). This may be due to the fact that mefloquine has been used sporadically in the country that too only for short-term disease management. Monitoring of drug will be useful when its consumption increases in the country. Similarly, artemisinin showed lower IC_{50} value 0.43 nM/l (95% C.I. 0.37-0.50) than the previously published data from India (Anvikar et al. 2012). In an earlier study, the pre-dosed plates from WHO having the minimum drug concentration of 3 nM/l were used hence it could not detect the lower values whereas in this study plates were coated with the drug concentration of 0.25 nM/l as minimum drug concentration.

In South America, the first case of quinine resistance was observed nearly a century ago. In the mid-1960s, it was observed from the Thai-Cambodian border. Sporadic cases of quinine have been reported from South America and Africa (Wongsrichanalai et al. 2002a). Quinine has been used in combination with tetracyclin or doxycycline since last two decade to improve its efficiency. Reported cases of quinine resistance from India is from Northeastern states and Kolar district in Karnataka (Farooq et al. 2004).In this study, none of the isolates were found to be resistant to quinine with respect to WHO Mark III result whereas four isolates were found to be resistant to quinine via HRP II ELISA results with IC_{50} value 258.7 nM/l (95% C.I. 207.6-322.4). The variation in the result obtained may be attributed to numerous factors including the experimental methods standard deviations, host and parasite relationship, the environment factors, immunity of the population, the parasite polymorphism and

antimalarials recommended in the area. Additionally, transmission patterns are also varied in these regions. The *in vitro* assay data must be inferred with carefulness. This study data provide antimalarial drug susceptibility in a way equivalent to that of other alike sampled and performed *in vitro* assessments. Developments of *in vitro* susceptibility to drugs by *P. falciparum* may reproduce such trends in clinical treatment outcome. Though, an *in vitro* assay does not provide a direct measurement of risk of therapeutic failure (Noedl et al. 2003).

The reviewed and revised National Drug Policy in India recommends the use of artesunate + sulphadoxine and pyrimethamine for treating falciparum malaria cases all across the country. Recently in 2013, policy revised for the seven Northeastern states (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, and Tripura) with respect to the resistance developed to partner drug, artemether-lumefantrine has replaced the combination in these States (Anvikar et al. 2014). The *in vitro* result helps in observing the minimum inhibitory concentrations of artemisinin derivatives as well as its combination drug and they can guide the selection of the partner drug of ACT. Hence, this study put forwards mefloquine as a better combination drug than amodiaquine.

The monitoring and prevention of drug resistance have become highly significant than ever before as malaria parasites have advanced its approaches to escape almost any existing antimalarial treatment. A simple and effectual method for the surveillance of drug resistance is through *in vitro* drug sensitivity assays. The key factors for the success of drug resistance assays are high sensitivity, together with ease of implementation and execution. As reported by (Noedl et al. 2004) under the same

conditions when both assays were performed, the results of the HRP II field assay were close to those obtained with the WHO assay. It was true in our results also. Comparison of the HRP II drug sensitivity assay with modified WHO assay produced a method for assessing the quantification of antimalarial drug action. However, HRP II drug sensitivity assay combines the advantages of the both *in vitro* assays, although it overlooks most of their disadvantages. On comparing both *in vitro* assays, the HRP II assay was easier, less labor intensive and faster to perform than the WHO *in vitro* assay. Commercial ELISA kits availability for the quantification of HRP II protein of *P. falciparum,* excludes the necessity for standardization of the ELISA process, thereby making its application faster than that of any other assay.

The results of the HRP II assay are analogous to those obtained by the modified WHO schizont maturation assays, although both methods used different end points. The shared component of both the methods is that they are dealing with parasite growth and development. Due to the unique features of every assay, it is not possible to perform both assays in the same condition. A comparative of both assays is outlined in Table 2.6.

Table 2.6: Comparative features of WHO Mark III and HRP II ELISA assay

Descriptions	**WHO Mark III assay**	**HRP II ELISA assay**
Sample handling time (Incubation time)	~ 24-36 hrs	~ 72 hrs
Ease of use	Labor demanding	Comparatively less labor intensive
Cost effectiveness	Low cost	High cost
Interpretation of result	It is subjective (depends on efficiency of microscopist)	Colorimeter gives result
Availability of commercial Kit	Not available	Available in variant of HRP II protein
Accuracy of result	Not so accurate	HRP II protein is stable so result is more accurate

The modified WHO method is based on schizont maturation; it gives the estimate of a number of parasites which develops from ring stage (early trophozoite) to schizont. The assay is considered as more sensitive as it is based on the microscopic evaluation (Noedl et al. 2002). Also, it is more economical than the HRP II assay; however, it is more labour demanding with a requisition of skilled and trained technician to overcome the experimental inaccuracy caused by human errors. The rise in HRP II levels with respect to gradually increasing incubation time marked by the early schizont and trophozoite stages of the parasite is captured in the HRP II assay (Desakornl et al.1997). ELISA assays are sensitive but they need minimum incubation time as in the case of HRP II an increase in the protein level is directly gives measures of parasitaemia.

The sensitive and resistant status of the field and culture isolates to the antimalarials chloroquine, amodiaquine, mefloquine, quinine and artemisinin is defined reliably by both the assay. The equivalent result obtained by both assay reveals the consistency and reliabilities of *in vitro* assays for antimalarial susceptibilities testing. The benefits of using HRP II protein is its stability which results in the ability of this protein to be detected even when there is a small difference in parasite growth (Desakornl et al.1997). Although the cost of performing ELISA test is little higher than WHO *in vitro* assay but it also gives us options like it can be performed with any of the HRP II specific ELISA for *P. falciparum.* Nowadays, HRP II monoclonal antibodies are available in the market at very competitive price which can be used widely for the assay. The commercial kits have plus points like the easy to perform with standardized parameters, high sensitivity and reproducibility of assay.

Furthermore, to its prospective value both as an implement for drug resistance surveillance and as a screening tool for new antimalarials, numerous added practices for the assay are under concern. Like to evaluate the potential pharmacodynamics, collaborations of antimalarials may be tested in a dilution, or to measure the quantity of antimalarials in the blood, post intake of drug. Alternative potential use of these *in vitro* assays is the analysis of the inhibitory activities of specimens which are potential vaccine candidates (Wernsdorfer et al. 2002). Conclusively, the HRP II assay was found to be simple to operate, less labour intensive and results reading was comparatively faster.

Chapter 3

In vitro sensitivity pattern of chloroquine and artemisinin in Plasmodium falciparum

3.1 Introduction

Antimalarial drug resistance is a matter of great concern for the control and elimination of malaria worldwide. ACTs that is extensively used as first line therapy for falciparum malaria and thus monitoring resistance towards these antimalarials is needed urgently. These antimalarials have specific targets and mode of action and thus it is significant to elucidate the mode of action of antimalarials with similar targets and mode of action. To assess the possible correlation among two important antimalarials, artemisinin and chloroquine resistance, which can help in drug discovery (Ding et al. 2011).

3.1.1 Mode of action of artemisinin

Multiple-target models for the mechanism of action of artemisinin have been proposed but none has been proved (Krishna et al. 2006, Neill et al. 2010). There are many postulates which describe the mode of action of artemisinin, mainly four of them are considered as not mutually exclusive of each other. However, the conflict remains both in favour and against all these theories. The suggested mode of action reveals that intrinsic endoperoxide bridge located within the parasite's food vacuole, once cleaved it might act by:

a) Interfering the detoxification pathway of heme.in which free radicals of artemisinin alkylate these free heme molecules which leads to interference in their detoxification (Meunier et al. 2010)
b) Inducting alkylation in the tumour protein which is translationally controlled and also other proteins.
c) Inhibiting the sarco/endoplasmic reticulum membrane calcium ATPase 6 *(pfATPase6*) gene.
d) Interfering with mitochondrial function of Plasmodium.

The opinions for a given model are mostly based on the chemistry of artemisinin, or on the existence or absence of association between antimalarial activities of artemisinin derivatives and the strong point of mode of action with their respective interaction in the postulated targets.

3.1.2 Mode of action of chloroquine

Chloroquine is known as scizonticidal drug which acts on the blood stage of Plasmodium. The mode of action of chloroquine has not been fully explained. There are numerous theories which have been developed to explain the chloroquine mode of action, one of them states that CQ inhibits the various enzymes and/or transporters involved in the DNA binding (Slater et al.1993). The widely explored and acknowledged theory is chloroquine interferes with the hemoglobin digestion by the parasite (Bray et al. 1966). It is known that in the RBC when Plasmodium invades they eat the hemoglobin from the host cell cytosol and then accumulate it in the digestive vacuole (DV) which is an isolated lysosomal acidic compartment where hemeoglobin is absorbed by the parasite. The protein moiety is degraded during this process and heme along with related peptides are transformed into an inactive crystalline polymer known as hemozoin (HZ) (Slater et al.1993). The mode of action of chloroquine is also to prevent the polymerization of toxic heme released during proteolysis of hemoglobin in the Plasmodium digestive vacuole (Hawley et al. 1998). *P. falciparum* chloroquine resistant transporter (*pfcrt*) protein is localized in the digestive vacuole membrane of the parasite which is considered as the transporter for CQ into the food vacuole of parasites (Fidock et al. 2000, Johnson et al. 2004). Alteration in *pfcrt* protein as a result of amino acid change at codon 76 from lysine to tyrosine leads to reduce the accumulation of chloroquine inside the food vacuole. Thus chloroquine effluxes out of food vacuole as a result of which no CQ is left for the detoxification of heme (Wongsrichanalai et al. 2002b).

As artemisinin and chloroquine both have similarity in heme detoxification pathways here, we tried to understand the possible mechanism of action of ART by modified trophozoite maturation inhibition (TMI) assay (Chotivanich et al. 2014), WHO Mark III assay and molecular marker study for chloroquine resistance at K76T codon of *pfcrt* gene in cultured parasites (Vathsala et al. 2004).

3.2 Materials and Methods

3.2.1 *In vitro* assay

Two *P. falciparum* culture isolates from Malaria Parasite Bank, National Institute of Malaria Research (NIMR), MRC-2 and RKL-9 were revived and cultured for 4-6 days in RPMI 1640 complete medium (Trager et al. 1976).They were double synchronized at a gap of 4 hours, using 5% sorbitol in order to eliminate all the stages except the early trophozoites (ring stage) (Lambros et al.1979). After second synchronization, 96 well-plate was set for the TMI assay (Chotivanich et al. 2014). The sensitivity of cultured isolates to CQ and ART was assessed by standard WHO Mark III micro-test (WHO. 2001) with slight modification. The 96 well plates were labeled for MRC-2 and RKL-9 with drugs chloroquine and artemisinin. Each well filled with 100µl of RPMI-1640 incomplete medium. Drug concentrations were added in duplicates with well 'H' having highest concentration of both, CQ =12.8 µM and ART = 1.6 µM, other wells were filled by serial dilution method. First row of 96 well plates was used as control (without drug). Ten microliters of blood mixture containing 0.5-1% ring stage parasites with 2% haematocrit was added to each well starting from control well. After proper mixing the plates were incubated at 37°C in a gas mixture of 90% N, 5% CO_2 for 16-18 hours for modified TMI assay and for 25-30 hours for modified WHO Mark III assay. Thin and thick smear were prepared, fixed and stained with Jaswant Singh and Bhattacharya I and II (JSB) stains. The morphology was studied under light microscope

(Carl Zeiss, Germany) and results were tabulated for number of infected RBCs per 20 fields in each thin smear, where each field consists of 200 erythrocytes for modified TMI assay. At the end of the incubation period of 25-30 hours for modified WHO Mark III assay, suspended medium were removed while the blood within each well were used to make thick smears on a glass slide. These were air-dried, fixed and stained with JSB stain and examined under microscope at 100X magnification. The numbers of schizont with three or more nuclei against 200 asexual parasites were counted for each sample.

3.2.2 DNA sequencing for Kelch13 and *pfcrt* mutation analysis

Isolation of DNA was carried out using QIAamp mini kit (QIAGEN, Germany) according to manufacturer's protocol. This DNA was stored at -20^{0}C until processed further. The amplification of *pfcrt* gene was carried out according to previously published standard protocol (Vathsala et al. 2004). On a 1.5% agarose gel containing ethidium bromide the PCR product from the amplified reactions were seen by electrophoresis. Ten microliter of the nested PCR product was digested with *ApoI* cut smart restriction enzyme (NEB) for 20 min at 37°C as recommended by the manufacturer. Digested products were run on 1.5% agarose gel, and visualized by UV trans illumination. Further, to reaffirm SNP, primary PCR products of *pfcrt* gene were DNA sequencing at Xcelris Labs, Ahmedabad. The Kelch-13 amplification and mutation analysis was done as per previously published protocol (Ariey et al. 2014).

3.2.3 Data management and statistical analysis

The drug concentration that inhibits schizogony by 50% (IC_{50}) relative to the drug-free control samples of each *P. falciparum* isolate for both *in vitro* assay was estimated from dose-response curves by non-linear regression analysis using HN-NonLin Reg.Analysis (Noedl et al. 2002).The IC_{50} cut-off values were based on the WHO microtest protocol

for determining sensitivity to antimalarials which is 0.8µmol/l for CQ (WHO 2001).The cut-off IC_{50} value of 0.01 µmol/l for artesunate as described by Pradines (Pradines et al. 2002) was considered as WHO protocol does not recommend cut-off for artemisinin. Statistical analyses were done using SPSS software (Version 17, SPSS Inc., Chicago). The editing and alignments of DNA sequences were done using Mega 6 Software.

3.3 Results

Trophozoite and schizont maturation inhibition patterns were analyzed with different range of CQ (0-12.8 (µmol/l) and ART (0-1.6 (µmol/l) with respective CQ resistant and sensitive isolates collected from parasite bank. Comparison of TMI and WHO Mark III assay for CQ resistant and sensitive isolates (Fig. 3.1).

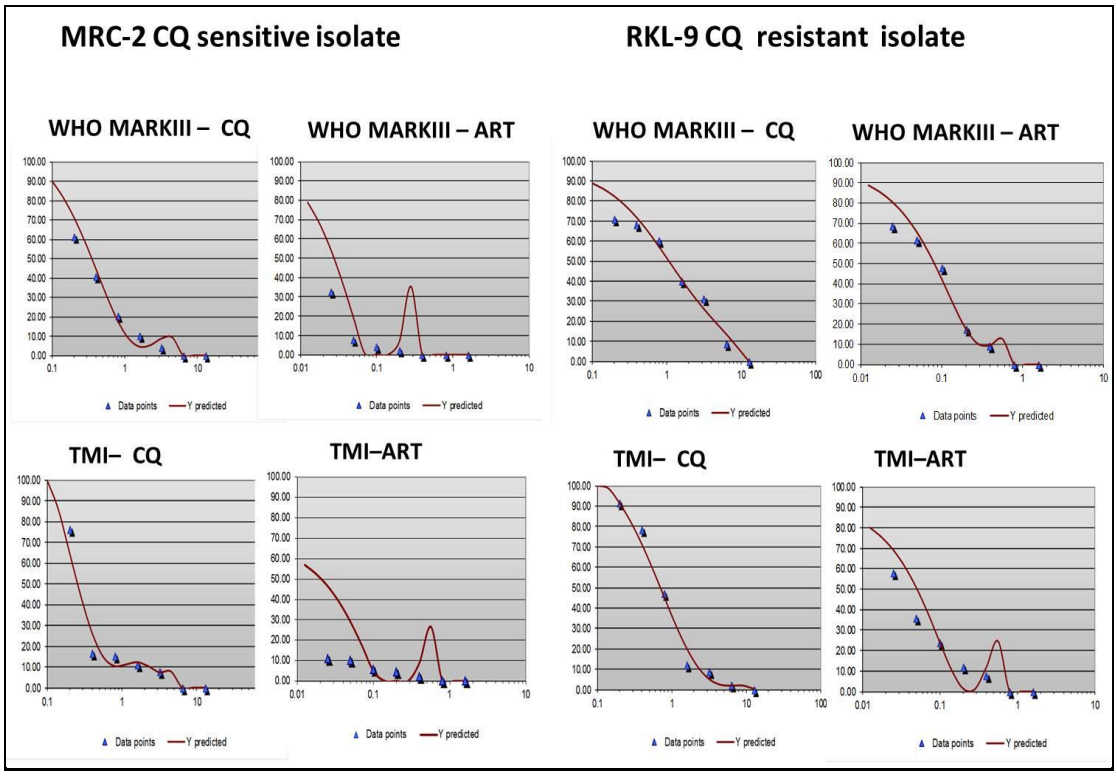


Fig. 3.1: Comparison of TMI and WHO Mark III assay for CQ resistant and sensitive isolates

The IC_{50} and IC_{99} values of CQ by TMI assay and WHO Mark III in CQ sensitive isolate were 0.25 µmol/l and 2.85 µmol/l and 0.35 µmol/l and 2.85 µmol/l respectively. The IC_{50} and IC_{99} values of CQ by TMI assay and WHO Mark III in CQ resistant isolate were 0.80 µmol/l and 11.80 µmol/l and 1.07 µmol/l and 11.72 µmol/l respectively. The IC_{50} and IC_{99} values of ART by TMI assay and WHO MARKIII in CQ sensitive isolate were 0.025 µmol/l and 0.23 µmol/l and 0.036 µmol/l and 0.36 µmol/l respectively. The IC_{50} and IC_{99} values of ART by TMI assay and WHO Mark III in CQ resistant isolate were 0.07 µmol/l and 0.24 µmol/l and 0.036 µmol/l and 0.36 µmol/l respectively (Table 3.1).

Table 3.1: *In vitro* susceptibility of CQ sensitive and resistant isolates of *P. falciparum*

Malaria parasite bank isolate	Drug	TMI assay		WHO Mark III assay	
		IC_{50} value (µmol/l)	**IC_{99} value (µmol/l)**	**IC_{50} value (µmol/l)**	**IC_{99} value (µmol/l)**
RKL-9	CQ	0.80	11.80	1.07	11.72
	ART	0.07	0.24	0.09	0.79
MRC-2	CQ	0.25	2.85	0.35	2.85
	ART	0.025	0.23	0.036	0.36

Chloroquine resistance in *P. falciparum* was determined by mutation in *pfcrt* gene at codon K76T. Mutation at codon K76T was found in RKL-9 while MRC-2 has shown wild genotype at this codon. DNA sequence evidence for haplotype analysis of the PCR products indicates that MRC-2 has CQ sensitive haplotype (CVMNK) and RKL-9 has CQ-resistant haplotype (SVMNT) (Fig. 3.2). We sequence RKL-9 and MRC-2 isolates for mutation analysis in Kelch13 propeller region, no SNP was observed in both isolates (Fig. 3.3).

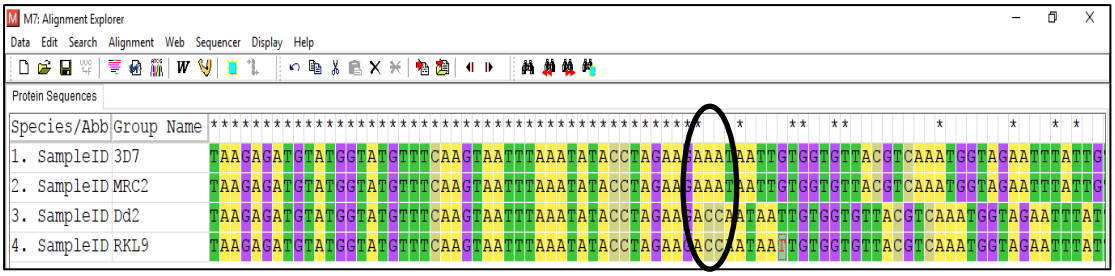


Fig. 3.2: DNA sequences alignment for the pfcrt gene for analyzing mutation at 76 position from lysine (K) encoded by either AAA or AAG to threonine (T) encoded by ACT, ACC, ACA or ACG. Here 3D7 (Chloroquine sensitive) and Dd2 (Chloroquine resistant) were reference strains.

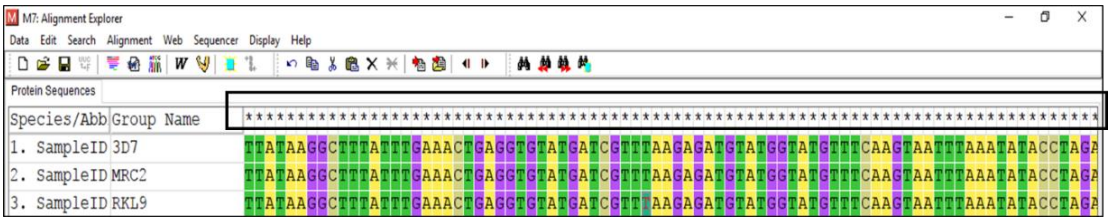


Fig. 3.3: DNA sequences alignment for the Kelch 13 propeller region gene for analyzing synonymous and non-synonymous mutation. * shows all have same sequence. No mutation recorded both samples were found to be sensitive with respect to mutation at Kelch 13 propeller region. 3D7 was reference strains.

3.4 Discussion

Artemisinin derivatives are effective antimalarial drugs and widely have been employed as a first line treatment globally. Different molecular modes of action have been postulated to explain the parasiticidal effect of these compounds; however, none has been evidently accepted and their physiological application is still questioned (Ding et al. 2011).

In the present study, we tried to understand how the parasite works against the antimalarials via *in vitro* assays. Also, we correlated chloroquine with action of artemisinin, through of TMI and WHO Mark III assay. A very rapid parasite clearance was observed in sensitive isolates for both drugs (CQ and ART) in TMI and WHO

Mark III assays. Inhibition pattern for trophozoite and schizont with CQ and ART were analogous in MRC-2 isolates. This could be due to accumulation of CQ in food vacuoles which interferes with heme detoxification pathway leading to inhibition of trophozoite and schizont maturation (Fidock et al. 2000). Interference of heme detoxification by alkylation is one of the postulated mechanism of action of artemisinin (Meunier & Robert 2010). Slow parasite clearance with both CQ and ART in TMI and WHO Mark III assay in CQ resistant isolate was observed. Further, parallel maturation inhibition in trophozoite and schizont with CQ and ART drugs was observed in CQ resistant isolate. The possible reason for this similarity could be mutation in *pfcrt* at codon K76T leading to decreased accumulation of CQ in food vacuoles; allowing parasite to detoxify heme and survive with given normal dose (Vathsala et al. 2004).This could be true with ART as well since the isolate showed mutation in *pfcrt* gene leading to lesser alkylation of heme by artemisinin. Hence, the parasite is able to survive for a longer duration.

With respect to the IC_{50} value CQ resistant isolate showed 3 fold increase for CQ whereas 2 fold increase for ART and IC_{99} value were increase 3 fold and 16 fold in resistant isolate as compared to sensitive isolate with CQ and ART drug, respectively. It suggests a probable analogue mechanism of action for both drugs. Also, SNP were analyzed for both the genes (*pfcrt* and *pfK13*) have reaffirmed the status of resistant and sensitive parasites. Our experiment upholds the heme detoxifixtion pathway of parasite is interfered by CQ and ART. It interferes with the heme detoxification via alkylation and may be correlated as one of the plausible mechanism of action of artemisinin.

Chapter 4

Status of drug resistance molecular markers in Indian Plasmodium falciparum field and culture isolates

4.1 Introduction

Antimalarial drug resistance has hampered the progress of malaria control and now elimination to a larger extent. Assessing the extent of problem using emerging tools and techniques is needed during all times. Molecular markers act as a promising public health tool with great potential value in antimalarial drug resistance studies. Currently, a number of genes have been proposed but the primary focus is on five genes that have been identified in detail that play a role in regulation of resistance to the antimalarials.

In this study, molecular markers of chloroquine resistance *P. falciparum* chloroquine resistance transporter (*pfcrt* codon K76T), *P. falciparum* multidrug resistance1 (*pfmdr1*codon N86Y**)**, genes conferring pyrimethamine resistance dihydropteroate reductase **(***pfdhfr* codon S108N, C59R, N51I, I164L and A16V**)**, sulphadoxine resistance gene (*pfdhps* codon S436F and A437G**)**, artemisinin resistance in Plasmodium ATPase6 (*pfATPase6* codon D639G and E431K**)** and Kelch 13 propeller region *pfK13* gene have been included.

4.2 Materials and Methods

4.2.1 Parasite genomic DNA isolation

Genomic DNA was isolated from both field isolates preserved on filter papers and from packed red cells of cultured isolates by QIAamp DNA mini kit (QIAGEN, California, USA) according to the manufacturer's instructions:

- Three circles of dried blood spot were punched out from filter paper into a 1.5 ml micro centrifuge tube and 180 µl of ATL buffer was added. It was kept overnight at room temperature.

- It was incubated at 85 ºC for 10 min and centrifuge briefly to remove drops from inside the lid.
- Proteinase K (20 µl) solution was added and mixed by vortexing and incubate at 56 ºC for 1 hrs and centrifuge briefly to remove drops from inside the lid.
- AL buffer (200 µl) was added, mixed thoroughly by vortexing and incubated at 70 ºC for 10 min
- Absolute ethanol (200 µl) was added and vortexed thoroughly.
- Carefully the mixture from step 5 was added to the QIAamp spin column (2 ml collection tube) without wetting the rim and centrifuge at 6000xg (8000 rpm) for 1 min. The QIAamp spin column was placed in a 2 ml collection tube and the tube containing the filtrate was discarded.
- The spin column was open carefully and 500 µl AW1 buffer was added without wetting the rim and centrifuged at 8000 rpm for 1 min. The QIA amp spin column was placed in a 2 ml collection tube and the tube containing the filtrate was discarded.
- Added 500 µl AW2 buffer without wetting the rim. The cap was closed and centrifuged at 14000 rpm for 3 min.
- The spin column was placed in a clean 1.5 ml micro centrifuge tube and the collection tube containing filtrate was discarded. The column was open carefully and 150 µl of AE buffer was added. It was incubated at room temperature for 1 min and then centrifuged at 8000 rpm for 1 min.
- Now eluted DNA was preserved in – 20 ºC till use.

4.2.2 Molecular markers

- Polymerase chain reaction was performed to indicate the presence of mutations encoding resistance to different antimalarial drugs through *pfcrt*, *pfmdr1*, *pfdhfr*, *pfdhps*, and *pfATPase6* genes.

4.2.3 *pfcrt* K76T mutation analysis

- The amplification of *pfcrt* gene was carried out by using nested PCR in a final reaction volume of 20 µl containing DNA template (2 µl), primers (10 pM for each primer), dNTPs (Bangalore GENEi, 200 µM for each dNTP), 1X reaction buffer, 2 mM $MgCl_2$ and 0.25 U Taq polymerase (Amplitaq Gold).
- Species specific primers were used as previously described by Vatsala et al. with slightly modification (Table 4.1)

Table 4.1: Primers and cycling conditions for *pfcrt* gene amplification

Target gene	Primer	Cycling condition	Amplicons size (bp)
pfcrt Primary PCR	F 5'CCGTTAATAATAAATACAGGCAG-3' R 5'CTTTTAAAAATGGAAGGGTGTATAC-3'	Initial denaturation: 95°C for 12 min Number of cycles: 40 cycles Denaturation: 95°C for 30 sec Annealing: 60°C for 1 min Extension: 72°C for 1 min 30 sec Final extension: 72°C for 5 min Hold at 12°C for ∞	1600 bp
pfcrt Nested PCR	F 5'GGCTCACGTTTAGGTGGA-3' R 5'TGAATTTCCCTTTTTATTTCCAAA-3'	Initial denaturation: 95°C for 12 min Number of cycles: 40 cycles Denaturation: 95°C for 30 sec Annealing: 52°C for 1 min Extension: 72°C for 1 min 45 sec Final extension: 72°C for 5 min Hold at 12°C for ∞	264 bp

- Mutation analysis was carried out by using PCR-RFLP using *ApoI* restriction enzyme.
- PCR products were digested using 10X NEB buffer, 10X BSA, *ApoI* (3U), and 5 µl PCR product as template DNA and incubated at 37 °C for 2 hrs
- Ten micro liters of PCR products were separated by electrophoresis on a 1.5% agarose gel.
- The fragment size of PCR product was determined by comparison with molecular marker (100 bp).

4.2.4 *pfmdr1* N86Y mutation analysis

- PCR amplification of *pfmdr1* gene was carried out in a final reaction volume of 25 µl containing DNA template, primers (0.5 µM for each primer), dNTPs (Bangalore GENEi, 200 µM for each dNTP), 1X reaction buffer,1.5 mM $MgCl_2$ and 0.12 U Taq polymerase (Amplitaq Gold). To amplify *pfmdr1* gene specific primers of Vatsala et al. were used (Table 4.2).

Table 4.2: Primers and modified cycling conditions used for *pfmdr1* gene amplification

Target gene	Primer	Cycling condition	Amplicons size (bp)
pfmdr1	F 5'ATGGGTAAAGAGCAGAAAGA 3' R 5'AACGCAAGTAATACATAAAGTCA 3'	Initial denaturation: 95°C for 12 min Number of cycles: 40 cycles Denaturation: 95°C for 30 sec Annealing: 52°C for 1 min Extension: 72°C for 1 min 45 sec Final extension: 72°C for 5 min Hold at 12°C for ∞	603 bp

- Mutation analysis carried out by PCR-RFLP using *Afl* III restriction enzyme.
- PCR products were digested by using 10X NEB buffer, 10X BSA, *Afl* III (3U), and 10 µl PCR product as template and incubated at DNA 37 °C for 2 hrs.
- Ten micro litres of PCR product was separated by electrophoresis on a 1.5% agarose gel.

- The fragment size of PCR products was determined by comparison with molecular weight marker (100 bp) using molecular weight analysis of gel documentation system.

4.2.5 *pfdhfr* mutation analysis

- The point mutation in five *pfdhfr* codons namely *S*108*N*, *C59R*, *N*51*I*, *I*164*L* and *A*16*V* were analyzed.
- The amplification of *pfdhfr* were carried out by using nested PCR in a final reaction volume of 25 µl containing DNA template, primers (10 pM for each primer), dNTPs (Bangalore GENEi, 200 µM for each dNTP), 1X reaction buffer, 2 mM $MgCl_2$ and 0.2 U Taq polymerase (Amplitaq Gold).
- The gene specific primers of Duraisingh et al. (1998) were used to amplify *pfdhfr* codons and details are provided in Table 4.3.

Table 4.3: Primers and cycling conditions for *pfdhfr* gene amplification

Target gene	Primer	Cycling condition	Amplicons size (bp)
pfdhfr	F 5'ATGGGTAAAGAGCAGAAAGA 3' R 5'AACGCAAGTAATACATAAAGTCA 3'	Initial denaturation: 95°C for 5 min Number of cycles: 40 cycles Denaturation: 94°C for 30 sec Annealing: 57°C for 30 sec Extension: 72°C for 45 sec Final extension:72°C for 5 min Hold at 12°C for ∞	648 bp
*pfdhfr for codon S*108*N*, *I*164*L*, *N*51*I*, and *A*16*V*	F 5'TTTATGATGGAACAAGTCTGCGA CGTT 3' R 5'AAATTCTTGATAAACAACGGAAC CTTTTA 3'	Initial denaturation: 95°C for 5 min Number of cycles: 40 cycles Denaturation: 94°C for 30 sec Annealing: 55°C for 45 sec Extension: 72°C for 45 sec Final extension:72°C for10 min Hold at 12°C for ∞	522 bp
*pfdhfr for codon S*108*N*, *C*59*R*	F 5'GAAATGTAATTCCCTAGATATG gAATATT 3' R 5'TTAATTTCCCAAGTAAAACTAT TAGAgCTTC 3'	Initial denaturation: 95°C for 5 min Number of cycles: 40 cycles Denaturation: 94°C for 30 sec Annealing: 55°C for 45 sec Extension: 72°C for 45 sec End cycle Final extension:72°C for10 min Hold at 12°C for ∞	326 bp

- Mutation analysis carried out by PCR-RFLP using various restriction enzymes detailed in Table 4.4.

Table 4.4: Restriction enzyme and digestion temperature for *pfdhfr* point mutation analysis

Restriction Enzyme	Codon	Product size	Digestion temperature
AluI	*pfdhfr* S108N	522 bp	37^0C
DraI	*pfdhfr* I164L	245 bp + 143 bp + 107 bp	37^0C
XmnI	*pfdhfr* C59R	189 bp + 137 bp	37^0C
Tsp509I(TasI)	*pfdhfr* N51I	154 bp	65^0C
NlaIII	*pfdhfr* A16V	429 bp + 93 bp	37^0C

- PCR products were digested by using 10X NEB buffer, 10X BSA, RE, and 10 µl PCR product as template DNA at specific temperature for 1-2 hrs.
- Ten micro litres of PCR product were separated by electrophoresis on a 1.5% agarose gel.
- The PCR products were analyzed by comparing it to molecular weight marker (100 bp).

4.2.5 *pfdhps* mutation analysis

- Analyze point mutation for two codons of *pfdhps* namely S436F and A437G.
- Amplification of *pfdhps* carried out by using nested PCR in a final reaction volume of 25 µl containing DNA template (2 µl), primers (10 pM for each primer), dNTPs (Bangalore GENEi, 200 µM for each dNTP), 1X reaction buffer,2 mM $MgCl_2$ and 0.2 U Taq polymerase (Amplitaq Gold).
- The gene specific primers as mention in Table 4.5 used to amplify *pfdhps* gene were taken from Duraisingh et al. (1998).

Table 4.5: Primers and modified cycling conditions for *pfdhps* gene amplification

Target gene	Primer	Cycling condition	Amplicons size (bp)
pfdhps codon S436F	F 5' AACCTAAACGTGCTGTTCAA 3' R 5' AATTGTGTGATTTGTCCACAA 3'	Initial denaturation: 95°C for 5 min Number of cycles: 40 cycles Denaturation: 94°C for 30 sec Annealing: 56°C for 30 sec Extension: 72°C for 45 sec Final extension:72°C for 5 min Hold at α	707 bp
pfdhps codon A437G	F 5' TGCTAGTGTTATAGATATAGGATGAGCATC 3' R 5' CTATAACGAGGTATTGCATTTAATGCAAGAA 3'	Initial denaturation: 95°C for 5 min Number of cycles: 40 cycles Denaturation: 94°C for 30 sec Annealing:45°C for 30 sec Extension: 72°C for 45 sec Final extension:72°C for10 min Hold at 12°C for ∞	438 bp

- Mutation analysis was carried out using PCR-RFLP with various restriction enzymes detailed in Table 4.6.

Table 4.6: Restriction enzyme and digestion temperature for *Pfdhps* point mutation analysis

Restriction Enzyme	Codon	Product size	Digestion temperature
MnlI	*pfdhps* S436F	317 bp + 121 bp	37^0C
AvaII	*pfdhps* 437G	404bp	37^0C

- PCR products were digested by using 10X NEB buffer, 10X BSA, RE, and 10 µl PCR product as template DNA at specific temperature for 1-2 hrs.
- Ten micro litres of PCR products were separated by electrophoresis on a 1.5% agarose gel.
- The fragment size of PCR products was determined by comparison with molecular weight marker (100 bp).

4.2.6 *pfATPase6* mutation analysis

- Four specific overlapping oligonucleotide primers were used to analyze at codon D639G, E431K, D443E and M813Q of *pfATPase6* gene.
- Amplification of *pfATPase6* was carried out in a final reaction volume of 20 µl containing DNA template, primers (10 pM for each primer), dNTPs (Bangalore GENEi, 200 µM for each dNTP), 1X reaction buffer,2 mM $MgCl_2$ and 0.2 U Taq polymerase (Amplitaq Gold).
- The gene specific primers of Kwansa et al. (2011) were used to amplify *pfATPase6* codon as mention in Table 4.7

Table 4.7: Primers and cycling conditions for *pfATPase6* gene amplification

Target gene	Primer	Cycling condition	Amplicons size (bp)
pfATPase6 for codon D639G	F 5' TCATCTACCGCTATTGTATGTGG 3' R 5' ATTCCTCTTAGCACCACTCCT 3'	Initial denaturation: 94°C for 5 min Number of cycle: 40 cycles Denaturation: 94°C for 15 sec Annealing: 55°C for 30 sec Extension: 72°C for 45 sec Final extension:72°C for 5 min Hold at 12°C for ∞	777bp
pfATPase6 for codon E431K	F 5' TCACCAAGGGGTATCAACAA 3' R 5' TGGCATAATCTAATTGCTCTTCC 3'	Initial denaturation: 94°C for 5 min Number of cycle: 40 cycles Denaturation: 94°C for 15 sec Annealing: 55°C for 30 sec Extension: 72°C for 45 sec Final extension:72°C for 5 min Hold at 12°C for ∞	692 bp
pfATPase6 for codon D443E	F 5' ATGTATAGCTGTTGTAATCAACCT AGA 3' R 5' TCACTATATGGATCAGCTTCATCA 3'	Initial denaturation: 94°C for 5 min Number of cycle: 40 cycles Denaturation: 94°C for 15 sec Annealing: 55°C for 30 sec Extension: 72°C for 45 sec Final extension:72°C for 5 min Hold at α	822 bp
pfATPase6 for codon M813Q	F 5' CCAGTACATTGAATGAAAATG 3' R 5' ACGTGGTGGATCAATAATACCT 3'	Initial denaturation: 94°C for 5 min Number of cycle: 40 cycles Denaturation: 94°C for 15 sec Annealing: 55°C for 30 sec Extension: 72°C for 45 sec Final extension:72°C for 5 min Hold at 12°C for ∞	605 bp

The Kelch13 propeller region was amplified by using previously described method (Ariey et al. 2014). The PCR products were purified using a gel extraction kit (Qiagen, Hilden, Germany) and sequenced using Sanger sequencing (Xcelris Labs,Ahmedabad, India). Sequencing data was aligned using the online BioEdit software version 7.0 for single nucleotide polymorphisms.

4.2.7 Data analysis

The data was analyzed using Software Mega6. Sequence Alignment was carried out using BLAST (Basic Local Alignment Search Tool) from NCBI (National Center for Biotechnology Information).

4.3 Results

4.3.1 Molecular analysis of *pfcrt* K76T and *pfmdr1* N86Y gene

To determine the genetic polymorphism for drug resistance nested PCR for *pfcrt* and *pfmdr1*genes was performed. The position of the amplicon was confirmed by digesting the PCR products with *Apo1* and *Afl III* restriction enzymes respectively for *pfcrt* and *pfmdr1*genes. The nested product of PCR for *pfcrt* gene showed an amplicon of 264 base pair (bp). CQ sensitive isolates showed digested product of size 136 bp with *ApoI* enzyme, suggesting the presence of wild type K76 (Fig. 4.1). The product of nested PCR for *pfmdr1*, all the culture and field isolates showed the *pfmdr1* codon 86 region amplicon with the product size of 603 bp. The sensitive isolates were not digested by *Afl III* enzyme (Fig. 4.2). However, *AflIII* generated fragments of 350 bp from the amplicon of resistant isolates indicating a mutant allele at codon 86 (86T). Those isolates which showed both bands (digested and undigested) together were considered as mixed isolates, considered as resistant type in final analysis.

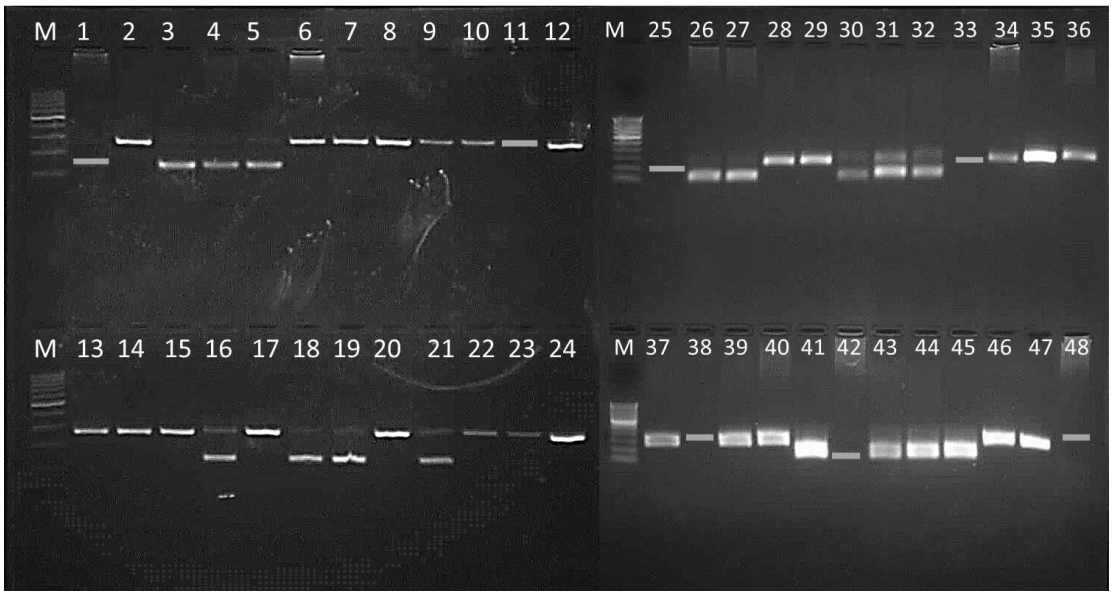


Fig. 4.1: Restriction fragment length polymorphism pattern with restriction enzyme *Apo*I for *Pfcrt* K76T mutation analysis (a) Upper lane:1-10- Odisha 11-12-Northeast isolates, Lower lane-13-20 Northeast isolates, 21-24 – Malaria Parasite Bank isolates, 31-40 – Chhattisgarh isolates, 41-48 Tripura isolates. The undigested DNA fragments (264 bp) are the resistance genes while the digested DNA fragments with 136 bp are the sensitive strains. M represent the molecular size marker

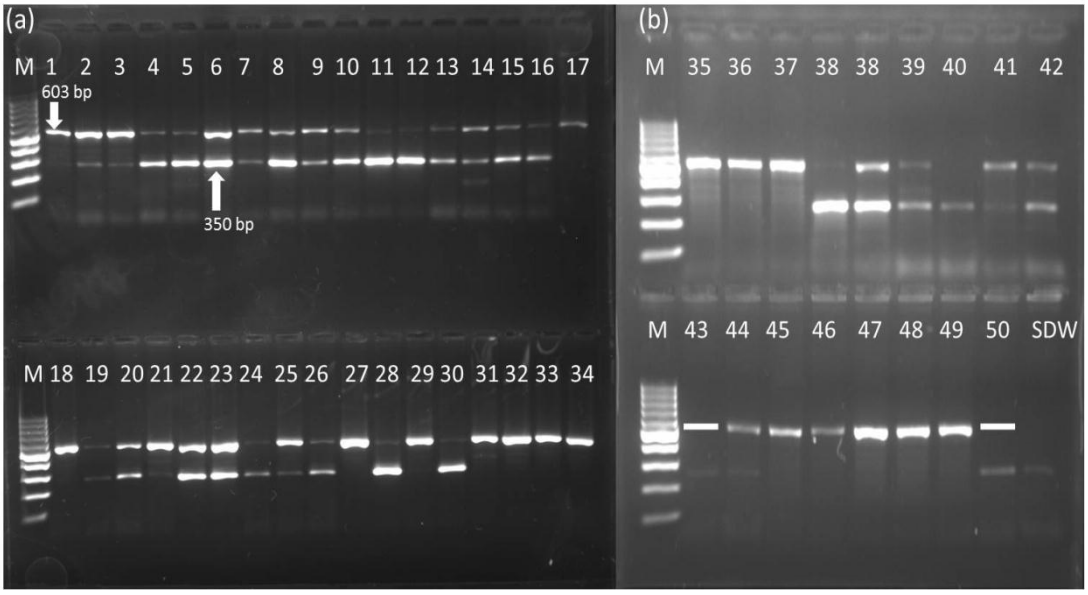


Fig. 4.2: Restriction fragment length polymorphism pattern with restriction enzyme *Afl*III for *pf*mdr N86Y mutation analysis (a) Upper lane:1-10- Odisha 11-17-Northeast isolates, Lower lane-18-20 Northeast isolates, 21-30– Malaria Parasite Bank isolates, 31-34–Chhattisgarh isolates, (b) 35-40- Chhattisgarh isolates, 41-42- Tripura isolates, Lower lane 43-50- Tripura isolates, SDW-Sterile distilled water. The undigested DNA fragments (603 bp) are the sensitive/wild type while the digested DNA fragments with 350 bp are the resistance types. M - Molecular size marker

Out of 50 isolates studied for *pfcrt* K76T and *pfmdr1* N86Y mutation, 22% and 40% were sensitive, 64% and 4% were resistant and 14% and 56% were mixed for both genes respectively. All of the isolates from Chhattisgarh were resistant to *pfcrt*K76T and 90% were resistant to *pfmdr1* N86Y. The culture isolates of MPB showed 80% and 100% mutation for both genes respectively which is comparable to the field isolates of North east, having 100% mutation for *pfcrt* whereas 80% for *pfmdr1*. Similarly, fifty percent of isolates of Tripura were resistant to *pfcrt* and 90% for *pfmdr1*. Odisha field isolates showed 60% resistance for *pfcrt* and 90% for *pfmdr1* (Table 4.8).

Table 4.8: State wise prevalence of mutation at *pfcrt* K76T and *pfmdr1* N86Y

Field isolates/Cultured line	**N**	***pfcrt* K76T (%)**			***pfmdr1* N86Y (%)**		
		K	**T**	**K+T**	**N**	**Y**	**N+Y**
Chhattisgarh	10	0	8	2	7	1	2
MPB	10	2	6	2	3	0	7
Northeast	10	0	7	3	2	0	8
Odisha	10	4	6	0	1	0	9
Tripura	10	5	5	0	7	1	2
Total	**50**	**22%**	**64%**	**14%**	**40%**	**4%**	**56%**

4.3.2 Prevalence of *pfATPase6* mutation

Out of 50 samples, 30 isolates were successfully amplified for *pfATPase6* gene. We got bands of expected size from the amplified PCR products (Fig. 4.3 and 4.4). Standard chromatogram peaks showing clear signal and no background noise was obtained for 26 samples. Sequencing analysis of *pfATPase6* gene revealed mutation in only one sample of Tripura with E431K mutation (1/26; 3.8%). No other relevant SNPs were recorded.

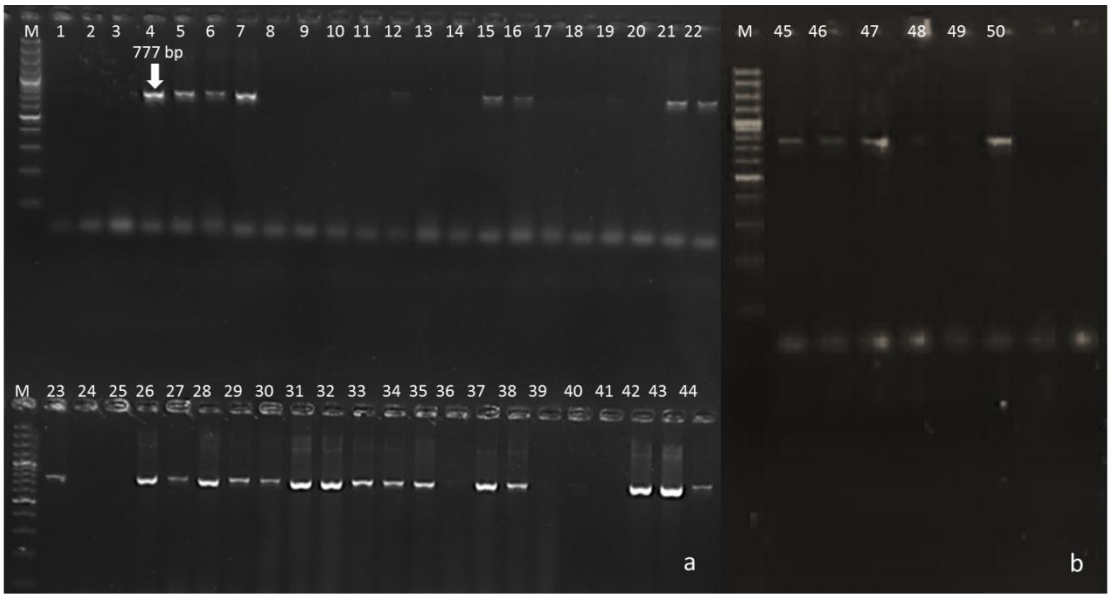


Fig. 4.3: DNA bands of *P.falciparum* sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) *pfATPase*6 genes codon D639G.The isolates with band at position 777bp may contain mutation which needs to be correlated with sequence analysis. (a) Upper Lane:1-10-Odisha isolates,11-20-Northeast isolates,21-22-Malaria Parasite Bank isolates, Lower Lane: 23-30-Malaria Parasite Bank isolates,31-40-Chhattisgarh isolates, 41-44-Tripura isolates. (b) 45-50-Tripura isolates. M - Molecular size marker of 100bp

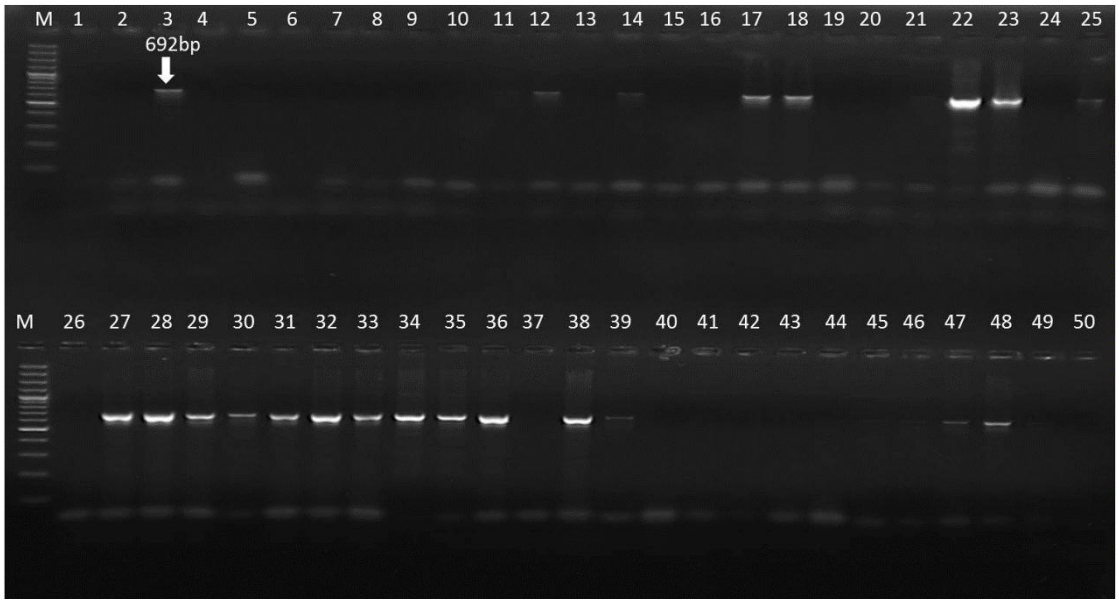


Fig. 4.4: DNA bands of *P.falciparum* sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) *pfATPase*6 genes codon E431K.The isolates with band at position 692bp may contain mutation which needs to be correlated with sequence analysis. Upper Lane:1-10-Odisha isolates,11-20-Northeast isolates,21-25-Malaria Parasite Bank isolates, Lower Lane: 26-30-Malaria Parasite Bank isolates,31-40-Chhattisgarh isolates, 41-50-Tripura isolates M- Molecular size marker of 100bp

Prevalence of *pfdhps* and *pfdhfr* mutations

The presence of two *pfdhps* mutations (S436F/A and A437G) and five *pfdhfr* mutations (A16V, N51I, C59R, S108N and I164L) known to be involved in resistance to SP were evaluated (Fig. 4.7– 4.13). Mutation analysis was successful in all 50 isolates for *pfdhps* and *pfdhfr* codons. Mutant codons were common at both the loci among the field and culture isolates. Among all mutations studied for *pfdhfr*; 164 codon showed 2% (Fig. 4.5), 59 codon showed 42% and 108 codon showed 56% mutation. The mutant *pfdhfr* allele 16 and 51 were completely absent for both field and culture isolates. The mutant allele of *pfdhfr* codon 59 and 108 were more common in Chhattisgarh with a prevalence of 80% for both, followed by 70% in Odisha and 60% and 70% in North-east isolates. Among *pfdhps* gene, 437 codon showed 32% and 436 codon showed 20% mutation (Fig. 4.6).

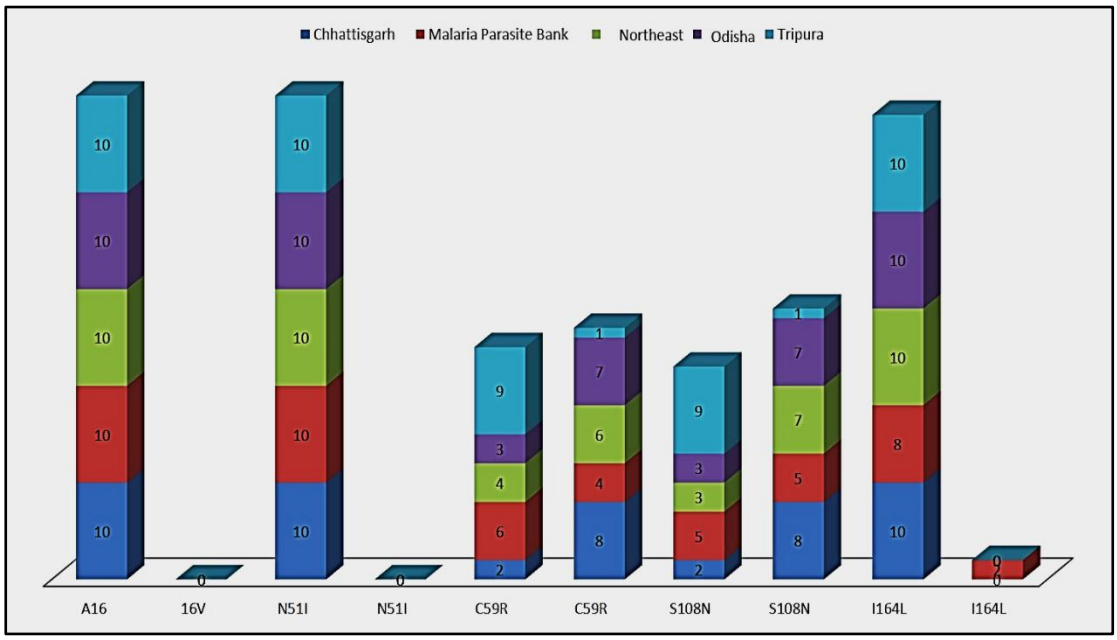


Fig. 4.5: Site wise prevalence of mutation in *pfdhfr* gene

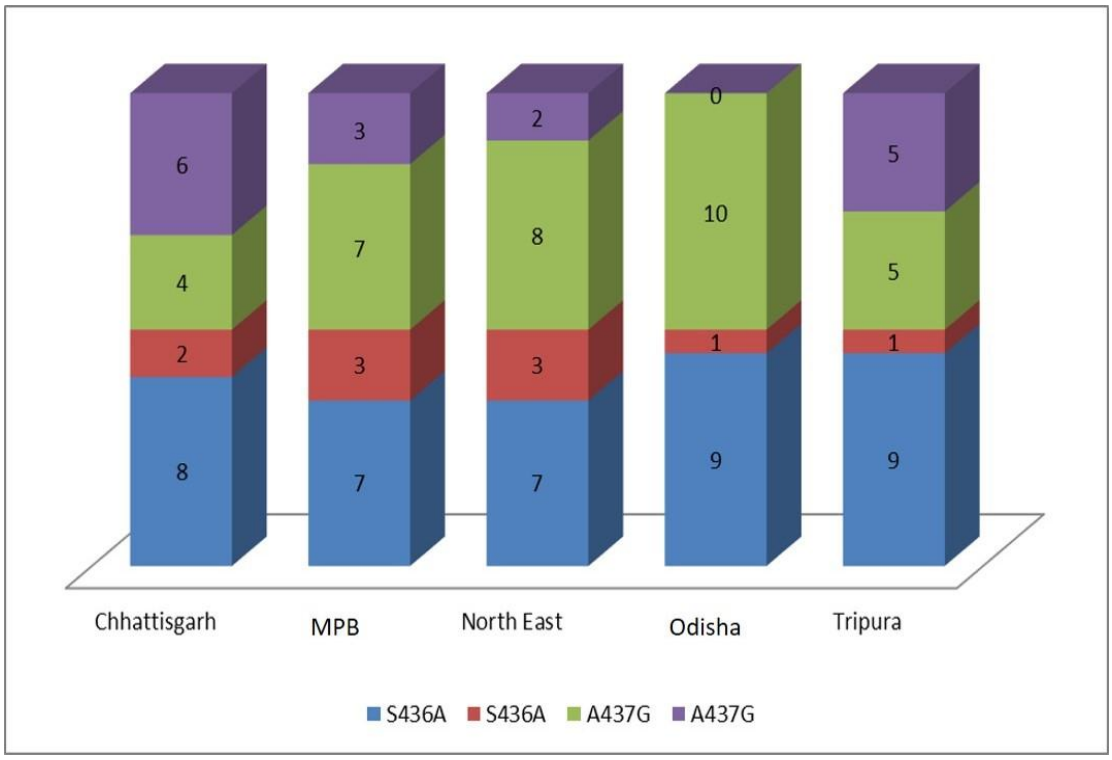


Fig. 4.6: Site wise prevalence of mutation in *pfdhps* gene

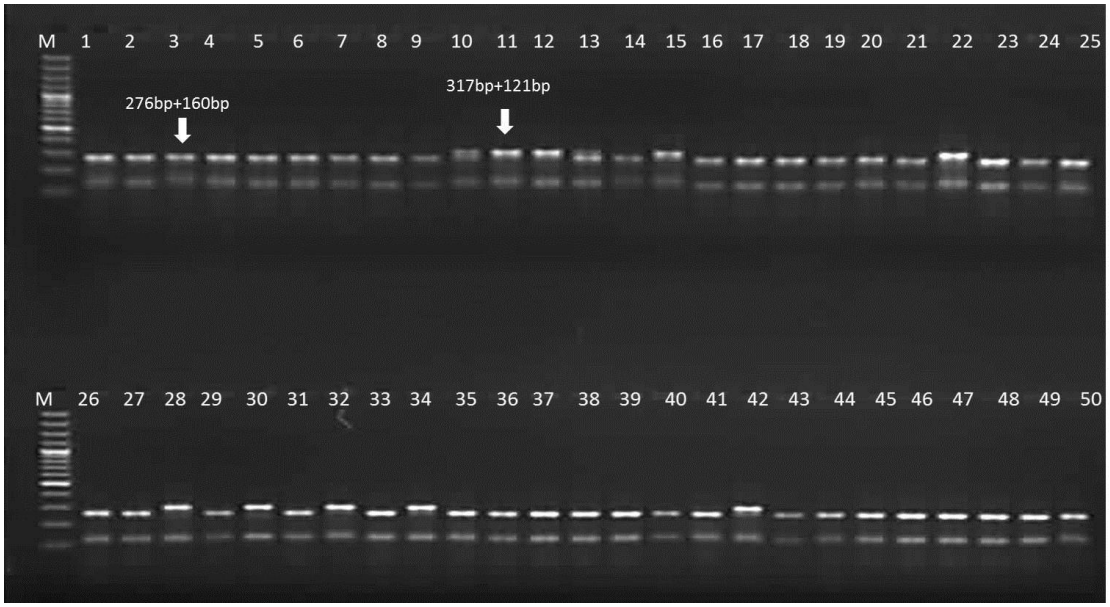


Fig. 4.7: Restriction fragment length polymorphism pattern with restriction enzyme MnII for *pf*dhps S436F -Upper Lane: 1-10-Odisha isolates, 11-20-Northeast isolates, 21-25-Malaria Parasite Bank isolates, Lower Lane: 26-30-Malaria Parasite Bank isolates, 31-40-Chhattisgarh isolates, 41-50-Tripura isolates. The DNA fragments of size 317+121 bp are the resistant while the digested DNA fragments with 276+160 bp are the sensitive strains. M- Molecular size marker of 100bp

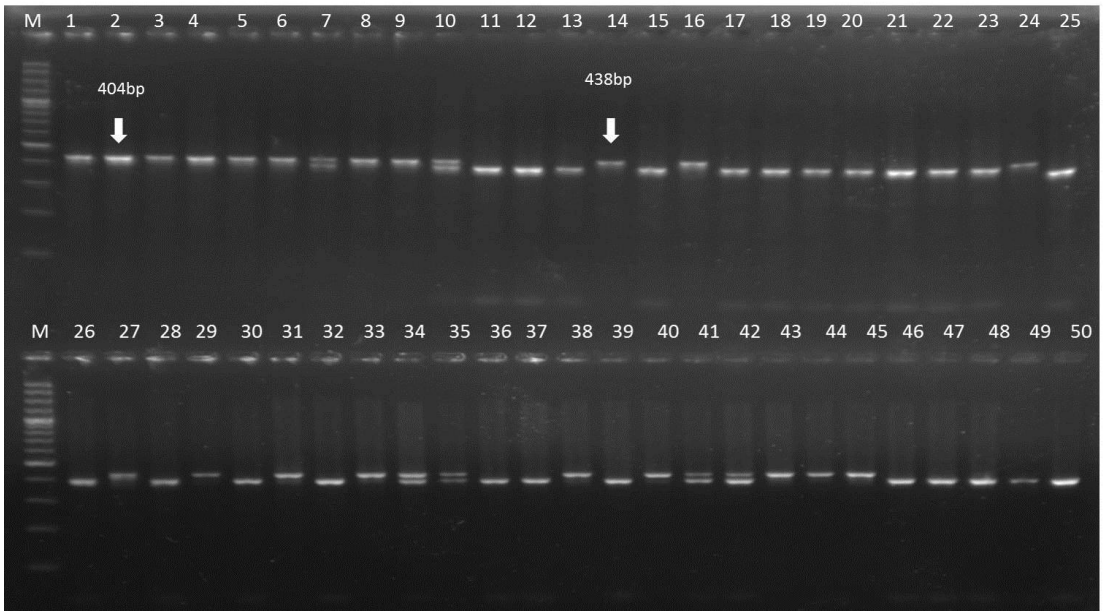


Fig. 4.8: Restriction fragment length polymorphism pattern with restriction enzyme AvaII for *pf*dhps A437G -Upper Lane:1-10-Odisha isolates,11-20-Northeast isolates,21-25-Malaria Parasite Bank isolates, Lower Lane: 26-30-Malaria Parasite Bank isolates,31-40-Chhattisgarh isolates, 41-50-Tripura isolates. The undigested DNA fragments of 438 bp are the resistant while the digested DNA fragments with 404 bp are the sensitive strains. M- Molecular size marker of 100bp

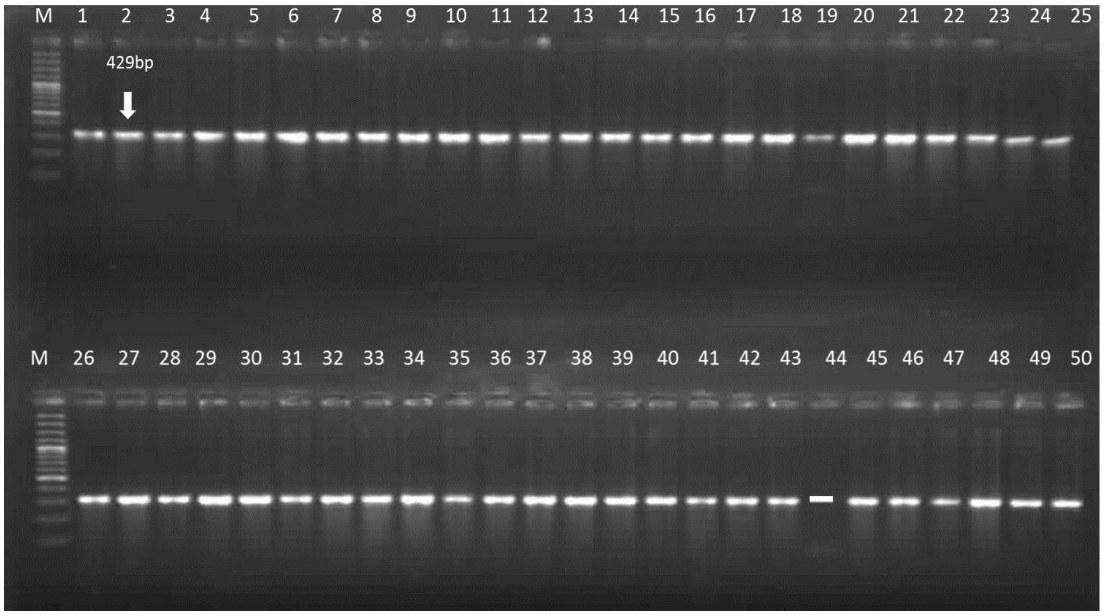


Fig. 4.9: Restriction fragment length polymorphism pattern with restriction enzyme NlallI for *pfdhfr* A16V -Upper Lane:1-10-Odisha isolates,11-20-Northeast isolates,21-25-Malaria Parasite Bank isolates, Lower Lane: 26-30-Malaria Parasite Bank isolates,31-40-Chhattisgarh isolates, 41-50-Tripura isolates. The DNA fragments of 376+76 bp are resistant the while the digested DNA fragments with 429bp are the sensitive strains. M-Molecular size marker of 100bp

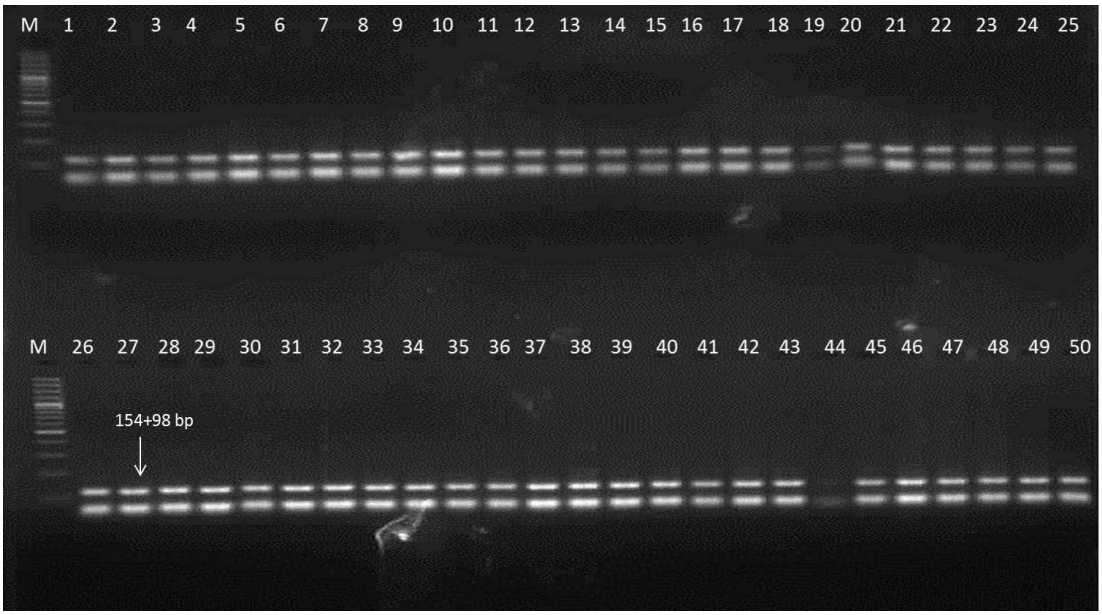


Fig. 4.10: Restriction fragment length polymorphism pattern with restriction enzyme Tsp509I for *pfdhfr* N51I-Upper Lane:1-10-Odisha isolates,11-20-Northeast isolates,21-25-Malaria Parasite Bank isolates, Lower Lane: 26-30-Malaria Parasite Bank isolates,31-40-Chhattisgarh isolates, 41-50-Tripura isolates. The DNA fragments of 154+98 bp are wild/sensitive while the digested DNA fragments with 218+120bp are resistant/mutant strains. M- Molecular size marker of 100bp

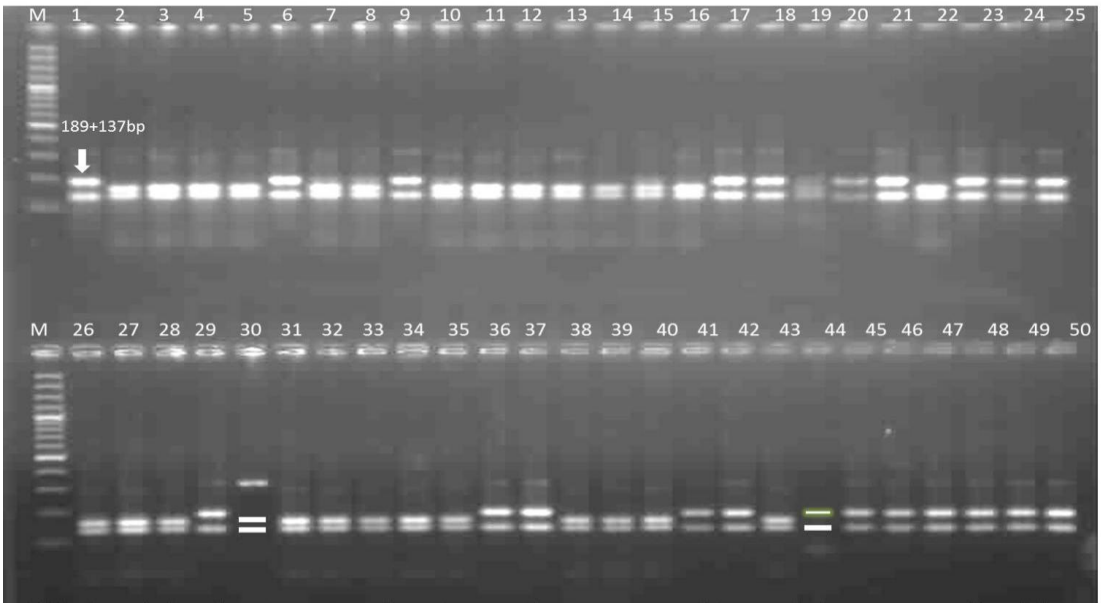


Fig. 4.11: Restriction fragment length polymorphism pattern with restriction enzyme XmnI for *pfdhfr* C59R -Upper Lane:1-10-Odisha isolates,11-20-Northeast isolates,21-25-Malaria Parasite Bank isolates, Lower Lane: 26-30-Malaria Parasite Bank isolates,31-40-Chhattisgarh isolates, 41-50-Tripura isolates. The DNA fragments of 189+137 bp are wild/sensitive while the digested DNA fragments with 163bp are resistant/mutant strains. M- Molecular size marker of 100bp

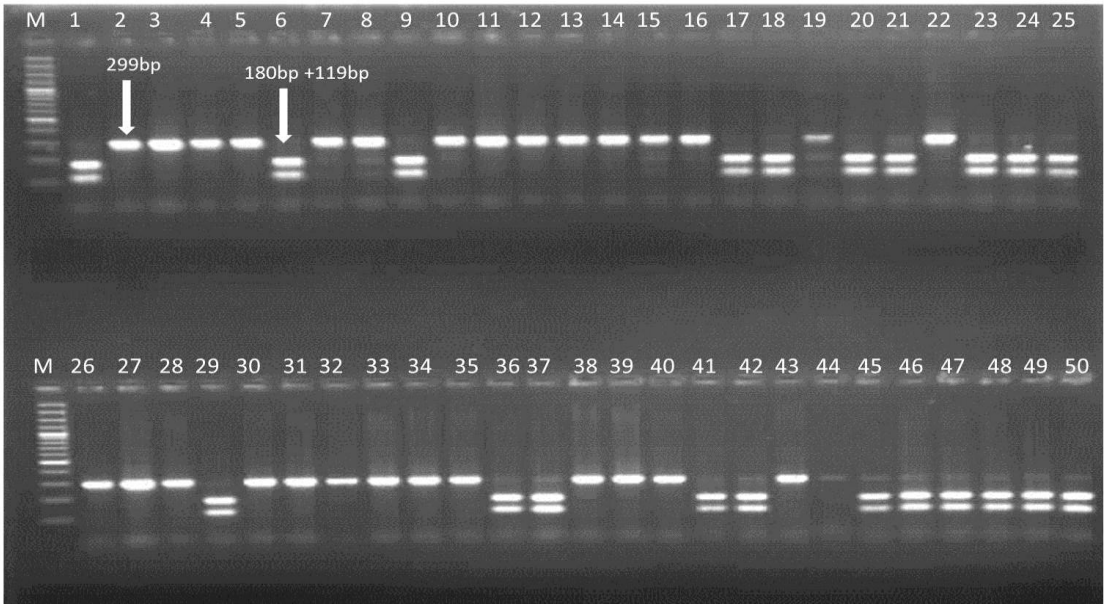


Fig. 4.12: Restriction fragment length polymorphism pattern with restriction enzyme AluI for *pfdhfr* S108N -Upper Lane:1-10-Odisha isolates,11-20-Northeast isolates,21-25-Malaria Parasite Bank isolates, Lower Lane: 26-30-Malaria Parasite Bank isolates,31-40-Chhattisgarh isolates, 41-50-Tripura isolates. The undigested DNA fragments of 299 bp are the resistant while the digested DNA fragments with 180bp + 119 bp are the sensitive strains. M- Molecular size marker of 100bp

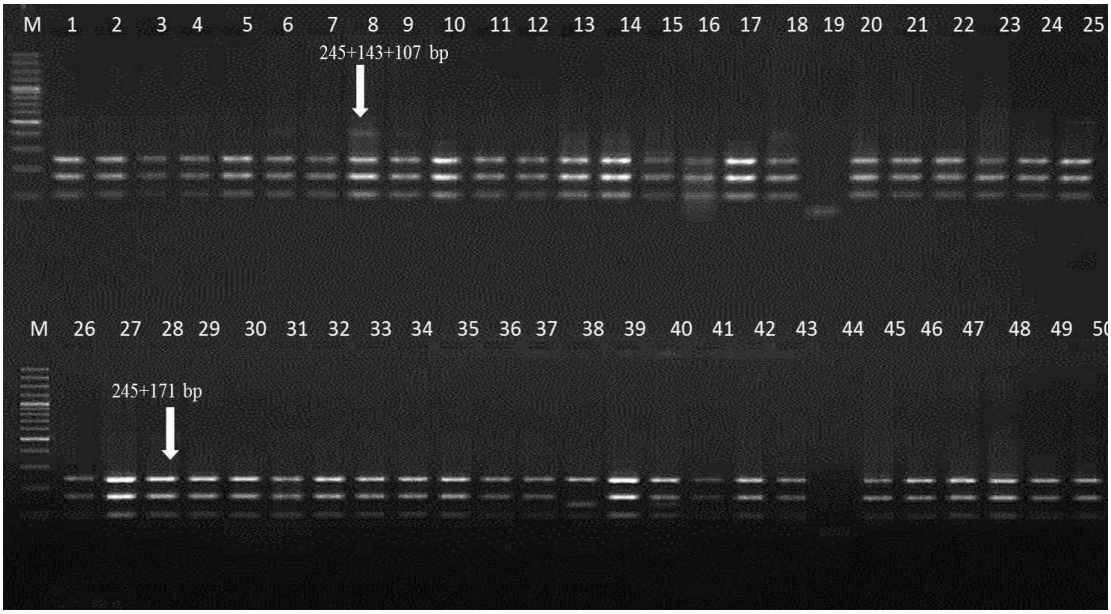


Fig. 4.13: Restriction fragment length polymorphism pattern with restriction enzyme DraI for *pfdhfr* I164L-Upper Lane:1-10-Odisha isolates,11-20-Northeast isolates,21-25-Malaria Parasite Bank isolates, Lower Lane: 26-30-Malaria Parasite Bank isolates,31-40-Chhattisgarh isolates, 41-50-Tripura isolates. The DNA fragments of 245+171 bp are wild/sensitive while the digested DNA fragments with 245+143+107bp are resistant/mutant strains. M- Molecular size marker of 100bp

4.4 Discussion

A major hurdle in the control of malaria is the fast rate of emergence of antimalarial drug resistance. The worth and durability of antimalarial drugs remains imperative and will analytically depend on the systematic research headed for the search of *in vitro* markers of resistance and the accomplishment of *in vitro* and *in vivo* monitoring programs, such as those supported by the Worldwide Antimalarial Resistance Network (Sibley et al. 2008, Plowe et al. 2007). Precisely, molecular markers are needed to identify antimalarial drug resistance which helps in active surveillance of time-based drifts in parasite susceptibility (Plowe et al. 2007). The advances in the molecular techniques is the key for the rapid detection of drug resistant parasites in the epidemiological studies and details on the selection of antimalarial management programs. The polymorphism in the parasite genes which are linked to the alteration in drug response have been marked as molecular markers of antimalarials. It acts as an adjunct to the measures of clinical and *in vitro* drug resistance (Barnes et al. 2008). In this chapter, the point mutations in the *pfcrt, pfmdr1, pfATPase6, pfdhfr* and *pfdhps* genes were studied for the assessment of partner drug efficacy.

The first report of CQ resistance was reported in 1950's, since then it has spread worldwide. In the year 1973, the first case of CQ resistance was reported from Karbi-Anglong district in Assam, India. That time onwards, it has slowly spread across West and South regions. The development of resistance in the *pfcrt* gene is a result of cumulative process where many genes with one or more variation(s)/mutation(s) leads to resistance for the drug (Shrivastava et al. 2014).

Detection of point mutation in *pfcrt*K76T which involves the substitution from lysine (K) to threonine (T) at position 76 (K76T) was observed as CQ resistance. These findings uphold well with the previous findings. In *pfcrt* gene the analysis of K76T mutation has revealed its role in CQ resistance (Vathsala et al. 2004). In this study, 78% of isolates were found to be resistant to CQ with respect to the *pfcrt* K76T analysis. The highest proportion of resistance was found in Chhattisgarh isolates followed by MPB isolates. It is significant to note that MPB isolates are in continuous culture and hence they can show a higher proportion of resistance genes, owing to the rate of change of their genes due to immense culture condition, putting pressure over the times (Desakornl et al.1997). These are useful for tracking of CQ resistance as it diagnose quickly and may have prospective use in the surveillance in malaria endemic areas for *in vivo* therapeutic efficacy of CQ (Cooper et al. 2005).

The role of *pfmdr1* polymorphisms is still disputed. Recently, single nucleotide polymorphism has been linked to lower *in vitro* and *in vivo* efficacies in *P. falciparum* towards antimalarials such as mefloquine, amodiaquine and quinine resistance (Chaijaroenkul et al. 2010). In this study, overall 60% isolates were found to be resistant to the *pfmdr1* gene with no sensitivity in culture isolates from MPB, followed by decreased sensitivity in the North-east isolates which corresponds to the recent report of declined sensitivity in North-east (Shrivastava et al. 2014) . Our result also indicate that the *pfmdr1* N86 can foretell decreased susceptibility to amodiaquine, the metabolite of monodesethyl amodiaquine, and the findings are in parallel to previous studies (Wurtz et al. 2014).

The results reported by Chaijaroenkul et al.2010 also supports the role of *pfmdr1* in controlling the degree of MQ, QN and AS susceptibilities. Amplification of *pfmdr1*

seems to be the candidate molecular marker of decreased susceptibility of *P. falciparum* isolates to MQ, AS, and QN in a multidrug resistance area. The clinical studies can rely on the characteristics of this candidate gene in producing resistance to ACTs. *In vivo* resistance to MQ and artesunate have been linked to point mutations at codons 86, 1034, 1042, and 1246 for *pfmdr1* with in genetically adapted parasite lines. Studies of *P. falciparum* isolates showed a link between mutation at codon 86 and a rise in susceptibility to MQ (Price et al. 1999, Duraisingh et al. 2000). Regardless of the information on the mechanism of action of artemisinin, till date it is a matter for debate and one of the many proposed mechanism is the study of the interaction of parasite with sarcoplasmic reticulum Ca2+ATPase 6 transporter (*pfATPase6*). Till date, E431K mutation at lower prevalence has been reported from few study sites in India (Saha et al. 2012 , Gupta et al. 2015). Saha et al reported E431K in 19% of the studied isolates whereas Gupta et al reported E431K mutation in 16.7 % of the sample. This study also finds single sample (1/26, 3.8 %) with this point mutation, with increased IC_{50} values. Hence, it has become important to study this point mutation in the country. It can be used as putative gene markers for the assessment of *pfATPase6* mutation which has been known for its likely role in artemisinin resistance.

Artemisinin-based combination therapy with artesunate plus pyrimethamine (AS+SP) has been recommended as the first-line drug for the treatment of uncomplicated malaria throughout the country by the Government of India in 2010, which was later revised with artemether lumefantrine (AL) for Northeast India (NVBDCP 2013). Artemisinin and its derivatives decreases most of the parasite biomass due to their early, fast action and an active long-acting companion drug can destroy the residual parasite load. The two essential enzymes of folate biosynthesis pathways namely dihydrofolate reductase

and dihydropteroate synthase are target of pyrimethamine and sulfadoxine respectively. Through *in vivo* and *in vitro* studies, the point mutations in these genes have been linked to the SP treatment failure (Kublin et al. 2002) . In the present study, molecular analysis of the five codons of *pfdhfr* and two codons of *pfdhps* was carried out to assess the status of SP resistance in the study region. Triple mutation in the codons C59R, S108N and C59R for *pfdhfr* was found along with the double mutant for *pfdhps* S436F and A437G codons. These results are parallels to the reports of (Mishra et al. 2014 , Sharma et al. 2015). Antifolate resistance related to point mutations in *pfdhfr* and *pfdhps* genes were observed among the field samples of Tripura, Chhattisgarh and MPB culture isolates. Continued molecular surveillance is not only needed for better execution of the drug policy, but also to limit the expansion of complex level of resistance. Nevertheless, further studies from different parts of the country will gives us a better perceptive and knowledge of the resistance to ACTs.

Chapter 5

Correlation of chloroquine sensitivity and other antimalarials with the partner drug resistance to Plasmodium falciparum malaria in selected sites of India

5.1 Introduction

Antimalarial drug resistance is a major hurdle in the successful treatment of malaria. *Plasmodium falciparum (P. falciparum)* has developed resistance to almost all antimalarials currently in use (World Health Organization. 2015). Resistance to all known antimalarial drugs, including artemisinin based combination therapy (ACTs) varies worldwide. *P. falciparum* resistance to artemisinin has now been detected in five countries; in the Greater Mekong sub region, Cambodia, Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam (World Health Organisation. 2015). The antimalarial drug resistance is monitor by various methods such as therapeutic efficacy studies, *in vitro* tests, molecular markers, and measurement of drug concentrations etc. (Otsuji et al. 2010). As compared to *in-vivo* tests, *in vitro* tests provide meticulous experimental environment and fast analysis. It has relatively low cost and is simple to conduct as compared to *in-vivo* assessments.

A naturally occurring histidine and alanine rich protein named Histidine rich protein II (HRP II) plays an important role as an indicator for the growth and resistance towards antimalarials. In a recent study, HRP II *in vitro* assay has been found to be better than WHO Mark III assay for *in vitro* drug sensitivity test (Sharma et al. 2016).

Molecular markers are considered as a promising public heath tools for identifying drug resistance in malaria, which has a great potential value. Molecular marker studies are comparatively faster and less expensive than clinical studies (Fidock et al. 2008).The non-synonymous mutations confer parasite resistance to drugs and are targets for molecular studies. However, the genetic mechanisms of *P. falciparum* drug resistance have not been completely elucidated till date.

The mutations in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene have been linked to chloroquine (CQ) resistance and are important indicator of *in vitro* resistance as well as therapeutic failure (Johnson et al. 2004, Sidhu et al. 2005).The amplification and/or polymorphisms of *pfmdr 1*(*P. falciparum multidrug resistance1*) gene have also been shown to affect the susceptibility to antimalarial drugs such as mefloquine (MQ), artesunate (AS), lumefantrine (LF) and quinine (QN) (Price et al. 1999). Resistance against sulfadoxine and pyrimethamine (SP) is conferred by single or multiple mutations in the dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) genes respectively (Wang et al. 1997, Watkins et al. 1988). Mutations in Plasmodium ATPase6 gene (Jambou et al. 2005) and *pfk13* propeller region are important markers for artemisinin resistance. Recently, Kelch 13 propeller polymorphism has been established as a useful molecular marker for large-scale surveillance efforts to frame artemisinin resistance (Ariey et al. 2014). To provide a better understanding of resistance pattern at the study sites, the present study was planned to correlate *in vitro* HRP II ELISA assay with molecular markers. *In vitro* susceptibilities for five antimalarial drugs and molecular markers; *pfcrt* (codon K76T), *pfmdr 1* (codon N86Y), *pfdhfr* (codon S108N, C59R, N51I, I164L and A16V), *pfdhps* (codon S436F and A437G), *pfATPase6* (codon D639G and E431K) genes and *pfK13* gene have been studied.

5.2 Materials and Methods

5.2.1 Study sites and parasites collection

Total 50 *P. falciparum* isolates were collected from five states (Chhattisgarh, Meghalaya, Mizoram, Odisha and Tripura) of India from December 2011 to September

2014. These samples also included culture adapted samples from the Malaria Parasite Bank (MPB) of National Institute of Malaria Research (NIMR) New Delhi.

5.2.2 *In vitro* HRP II ELISA assay

The collected samples were revived for fortnight using *in vitro* Trager and Jensen method (Trager et al. 1976) in RPMI-1640 medium. These revived samples were used for *in vitro* test and molecular analysis. The *in vitro* test was done as per the protocol described earlier(Wernsdorfer et al. 2002). Briefly, the cell medium mixture (CMM) was prepared by adding 0.94 ml of the parasitized blood sample to 24.06 ml of RPMI medium in a sterile disposable tube. 200 µl of the resulting CMM was added to drug coated plates. The plates were incubated for 72 hours at 37ºC in a CO_2 incubator. Primary IgM antibody was coated and kept at 4^0C overnight. It was washed with PBS tween solution and stored at 4^0C until further use. One hundred microliter of sample from the culture plate was transferred to the ELISA plate. Secondary antibody (IgG) was diluted to 0.2 µg/ml in diluent and 100 µl of the same transferred to the ELISA plate. The reaction was stopped by adding 50 µl of 1 M sulphuric acid and absorbance read using an ELISA plate reader (Spectrostar Nano, BMG LABTECH, Germany) at 450 nm (Noedl et al. 2005).

5.2.3 Molecular markers

DNA was isolated from the blood samples spotted on filter paper using Qiagen Blood DNA isolation Kit (Qiagen, Germany) as per manufactures instructions. Nested polymerase chain reaction was performed to specify the presence of mutations showing resistance to antimalarial drugs. *Pfcrt, Pfmdr1* and Kelch13 propeller region genes were

the genes of interest. The results of the molecular markers study were correlated with *in vitro* sensitivity of the same isolates. The PCR products of Kelch13 propeller region genes were purified using a gel extraction kit (Qiagen, Germany) and sequenced using Sanger sequencing at Xcelris Labs (Ahmedabad, India) using below described primers sets (Table 5.1).

Table 5.1: Primers and its sequence for amplification for propeller region of Kelch 13

Primer	Sequence
K13-Pri F	CGGAGTGACCAAATCTGGGA
K13-Pri R	GGGAATCTGGTGGTAACAGC
K13-Nes F	GCCAAGCTGCCATTCATTTG
K13-Nes R	GCCTTGTTGAAAGAAGCAGA

Kelch13 propeller region sequences obtained were aligned using the online BioEdit software version 7.0.9.0, and analyzed at codons 427-727 bp for single nucleotide positions mutations. The editing and alignments of DNA sequences were done using Mega 6 Software.

5.2.4 Statistical analysis

The IC_{50} of each *P. falciparum* isolate for *in vitro* assay were estimated by dose-response curves of non-linear regression analysis using HN-NonLinReg. Analysis (Malaria.farch.net) (Noedl et al. 2002). A chi square test was performed for categorical data and IC_{50} geometric mean was calculated for continuous data. Further, parametric Pearson correlation test was performed for continuous data using SPSS version 17 (statistical package for social science). Assessment of standard cross-resistance of antimalarial drugs with each other was estimated by the Pearson correlation coefficient

(r). Univariate linear regression analysis was performed to identify CQ as independent predictor of other antimalarial drugs (AQ, MQ, QN, and ART). Model 1 linear regression analysis was performed for predicting other antimalarial with respect to CQ as predictor. A p value of less than 0.05 was considered significant.

5.3 Results

5.3.1 Correlation between antimalarial drugs

In vitro HRPII assay showed significant positive correlation between the responses of CQ vs. AQ (r=0.527, p=<0.0001), CQ vs. QN (r=0.320, p=0.023), AQ vs. QN (r=0.394, p=0.005), AQ vs. ART (r=0.409, p=0.003), QN vs. ART (r=0.501, p=<0.0001). Interestingly, MQ showed strong significant positive correlation with QN (r=0.582, p=<0.0001) and ART (r=0.505, p=<0.0001) (Table 5.2).

Table 5.2: Pearson correlation between antimalarial drugs

Drugs	**Correlation coefficient**	**p-value***
CQ vs AQ	0.527	<0.0001
CQ vs MQ	0.086	0.554
CQ vs QN	0.320	0.023
CQ vs ART	0.188	0.192
AQ vs MQ	0.147	0.309
AQ vs QN	0.394	0.005
AQ vs ART	0.409	0.003
MQ vs QN	0.582	<0.0001
MQ vs ART	0.505	<0.0001
QN vs ART	0.501	<0.0001

* p-values < 0.05 is significant

Table 5.3: Chloroquine as predictor for other antimalarial drugs

Drugs	Standardized coefficient (Beta)	r^2	p-value *
AQ	0.527	0.278	<0.0001
MQ	0.086	0.007	0.554
QN	0.320	0.103	0.023
ART	0.188	0.035	0.192

Predictor: CQ; Dependent variable: AQ, MQ, QN, ART
*p-values were calculated using linear regression. * p-values < 0.05 is significant

In univariate linear regression analysis as shown in table 5.3 for HRP II assay CQ showed a strong significant positive correlation as independent predictor of other antimalarial drugs i.e. AQ, and QN with r^2 score 0.278, and 0.103 respectively.

Table 5.4: Site wise CQ prediction pattern for other antimalarial drugs

Site	AQ		MQ		QN		ART	
	r^2	p-value	r^2	p-value	r^2	p-value	r^2	p-value*
Odisha	0.114	0.340	0.023	0.674	0.009	0.792	0.041	0.576
Northeast	0.561	0.013	0.775	0.001	0.193	0.204	0.010	0.784
MPB	0.023	0.675	0.225	0.166	0.021	0.689	0.473	0.028
Chhattisgarh	0.226	0.165	0.002	0.896	0.084	0.418	0.003	0.879
Tripura	0.564	0.012	0.127	0.312	0.487	0.025	0.049	0.541

Predictor: CQ; Dependent variable: AQ, MQ, QN, ART
*p-values were calculated using linear regression. * p-values < 0.05 is significant

The result of CQ as predictor for other antimalarial drugs hold true for state wise analysis as well as shown in Table 5.4. Here, CQ showed a strong significant positive

correlation as independent predictor of other antimalarial drugs. In north east (Meghalaya and Mizoram) including Tripura isolates CQ showed significant positive correlation as independent predictor of AQ with r^2 score 0.561 and 0.564 respectively. In north east isolates CQ showed significant positive correlation as independent predictor of MQ with r^2 score 0.775. Only in Tripura, CQ showed significant positive correlation as independent predictor of QN with r^2 score 0.487. Similarly, in malaria parasite bank culture isolates CQ showed significant positive correlation as independent predictor of ART with r^2 score 0.473.

5.3.2 Association between antimalarial drugs and *pfcrt K76T* and *pfmdr1* N86Y gene

We observed, both wild and mutant types of isolates in the polymorphism distribution of *pfcrt* K76T with antimalarial drugs (Table 4). Among all drugs CQ showed both wild type and mutant with the *pfcrt* K76T genotypes. In resistant (mutant) group, *pfcrt* 76**T** showed 80% (n=4) resistant and 20% sensitive, Moreover, this difference was not significant (p= 0.559). Furthermore AQ, MQ, QN and ART drug did not show any resistant isolates, so p-value was not applicable.

The polymorphism distribution of *pfmdr1* with CQ, MQ, AQ, QN and ART drug are represented in table 5. As previously discussed, CQ was the only drug which showed both wild and mutant type isolates with the *pfmdr1* N86Y in this analysis also. In mutant type isolates for CQ (n=5), *pfmdr1* gene showed 80% (n=4) mixed and only 20% sensitive with p-value 0.353. Likewise AQ, MQ, QN and ART drug did not show any resistant type isolates, so p-value was not applicable.

Table 5.5: Association between *Pfcrt* K76T mutation and antimalarial drugs

Drug	**Profiles**	**n**	**n (%), of strains with polymorphism: *pfcrt* K76T**			
			CVIEK	**CVIET**	**Mix**	**p-value***
CQ	Resistant	5	1(20)	4(80)	0(0)	0.559
	Sensitive	45	13(28.9)	26(57.8)	6(13.3)	
MQ	Resistant	0	0	0	0	NA
	Sensitive	50	14(28)	30(60)	6(12)	
AQ	Resistant	0	0	0	0	NA
	Sensitive	50	14(28)	30(60)	6(12)	
QN	Resistant	0	0	0	0	NA
	Sensitive	50	14(28)	30(60)	6(12)	
ART	Resistant	0	0	0	0	NA
	Sensitive	50	14(28)	30(60)	6(12)	

* p-values < 0.05 is significant

Table 5.6: Association between *pfmdr-1*N86Y mutation and antimalarial drugs

Drug	**Profiles**	**n**	**n (%), of strains with polymorphism : *pfmdr1*N86Y**			
			N86	**86Y**	**Mix**	**p-value***
CQ	Resistant	5	1(20)	0(0)	4(80)	0.353
	Sensitive	45	20(44.4)	4(8.9)	21(46.7)	
MQ	Resistant	0	0	0	0	NA
	Sensitive	50	21(42)	4(8)	25(50)	
AQ	Resistant	0	0	0	0	NA
	Sensitive	50	21(42)	4(8)	25(50)	
QN	Resistant	0	0	0	0	NA
	Sensitive	50	21(42)	4(8)	25(50)	
ART	Resistant	0	0	0	0	NA
	Sensitive	50	21(42)	4(8)	25(50)	

* p-values < 0.05 is significant

5.4 Discussion

This study reveals increasing trend of *in vitro* resistance in *P. falciparum* to CQ, AQ and QN and the correlation between reduced *in vitro* sensitivity of parasites to CQ, AQ and QN and the polymorphisms in the *pfcrt* K76T and *pfmdr1* N86Y genes in the study sites. In India, for the first time CQ resistance was reported from Karbi-Anglong district, Assam in 1973. Interestingly, CQ showed significant positive correlation as independent predictor of drug resistance for AQ, QN and MQ. As the mode of action of both drugs CQ and QN is to constrain the formation of hemozoin in the parasite digestive vacuole and till date, to the best of our knowledge no such prediction analysis has been reported so far for these antimalarials, our result gives a good correlation. Although, reports of cross reactivity between CQ and AQ are documented in malaria endemic areas (Folarin et al. 2011). In addition, four samples were found to be resistant to QN through *in vitro* assay with increased IC_{50} value. A few case reports of QN resistance have been reported in the past, particularly from the Northeastern states and Kolar district in Karnataka (Farooq et al. 2004). In this study, AQ resistance was also recorded in four samples which is similar to the finding of decreased sensitivity to AQ and polymorphisms in *pfcrt* and *pfmdr1* genes in Southwest Nigeria (Folarin et al. 2011)

The analysis *pfcrt* K76T showed that majority of the samples were found to be resistant to CQ. These findings uphold well with the previous finding (Vathsala et al. 2004).In a recent study conducted in Thai-Myanmar border, CQ resistant isolates were found with increased IC_{50} value and all resistant isolates were detected with mutation in *pfcrt* at positions 76 (Muhamad et al. 2016). Our findings also suggest that *pfcrt* K76T could be a useful marker for decreased susceptibility of CQ *in vitro* assay.

The role of *pfmdr1* gene polymorphism is still unclear. However, there is lack of sufficient information to describe the SNPs association with decreased *in vitro* susceptibilities to antimalarials (Wurtz et al. 2014). *pfmdr1* N86Y mutant samples (60%) were observed to be associated with decreased sensitivity in Northeast; which is quite similar to recent reports of declined sensitivity in Northeast (Shrivastava et al. 2014) . Recent study has revealed the role of *pfmdr1* in controlling the degree of MQ, QN and ART susceptibilities (Chaijaroenkul et al. 2010). Interestingly, an increased IC_{50} value for quinine was observed in this study.

The *pfmdr1* 86Y allele can predict increased *in vitro* susceptibility to MDAQ (monodesethyl-amodiaquine), a metabolite of AQ (Wurtz et al. 2014). In contrast, the 86Y mutation observed in this study was not significantly related with increased susceptibility to AQ (p=0.98), a finding similar to study conducted in Nigeria (Folarin et al. 2011). In Benin, no difference in AQ IC_{50} between N86 and 86Y haplotypes has been observed (Dahlstrom et al. 2014). The connection of N86Y in QN resistance is quiet disputed; in a few studies, *pfmdr1* N86Y mutation has been associated with increased susceptibility (Phompradit et al. 2011, Cheruiyot et al. 2014). The N86Y mutation was not linked with increased susceptibility to QN (p=0.65), which is quite similar to previous study (Folarin et al. 2008).

Regardless of ample information on mode of action of artemisinin, till date it is a matter of debate and research. One of the proposed mechanisms is the study of parasite interaction with sarcoplasmic reticulum Ca2+ATPase 6 (*pfATPase6*). However, no such association was observed between *pfATPase6 and in vitro* increased susceptibility in the present study, which is also reaffirmed by previous studies (Mugittu et al.

2006)'(Zhang et al. 2008). This study also observed single sample of E431K point mutation. Few reports on *pfATPase6* gene mutation at codon E431K are available from India(Saha et al. 2012,Gupta et al. 2015). Studies with larger samples for E431K mutation analysis can confirm its role as recognized markers for the assessment of artemisinin resistance in the country. Interestingly, no mutation was observed in Kelch13 propeller region, which is similar to reports from Kolkatta (Chatterjee et al. 2015), although there are reports, which have documented non-synonymous mutations from India (Mishra et al. 2015, Bharti et al. 2016).

Artemisinin-based combination therapy has been recommended by the Government of India since 2010 as the first-line therapy for the treatment of uncomplicated malaria (NVBDCP 2013b). The point mutations in *pfdhfr* and *pfdhps* genes have been linked to the SP treatment failure (Kublin et al. 2002). However, no *in vitro* study for SP could be done; attempts have been made to undertake molecular analysis of the five codons of *pfdhfr* and two codons of *pfdhps* to assess the AS+SP-ACT efficacy. A triple mutant for *pfdhfr* gene at codons C59R, S108N and C59R and double mutant for *pfdhps* at codon S436F and A437G were observed. These results are parallels to the recent publication (Sharma et al. 2015). Antifolate resistance related to point mutations in *pfdhfr* and *pfdhps* genes were observed among the field samples of Tripura, Chhattisgarh and MPB.

5.4.1 Conclusion

This is the first study to document the correlation between IC_{50} and molecular markers with respect antimalarial drug resistance in the study sites in India. Overall, the data from the *in vitro* susceptibility and molecular analysis may specify the tendency of

declining sensitivity of *P. falciparum* isolates towards CQ, AQ and QN in India, still, all the samples were found to be sensitive to ART and MQ during the study period. Surveillance study involving large sample size is required to confirm the association of *in vitro* susceptibility of anti-malarial drugs with the molecular markers in the country. In conclusion, results of this study suggest that *in vitro* susceptibility of *P. falci*parum isolates to antimalarials along with molecular marker is a valuable and dependable tool with increased prediction efficiency for drug resistance.

Summary

Malaria, a deadly protozoan parasitic disease is known worldwide for high morbidity and mortality. South East Asia (SEA) region is the second highest contributor of the total malaria cases after Africa. India alone accounts for > 80 % of malaria cases in SEA region. India has collaboration with WHO (World Health Organization) and set a target for malaria elimination by the year 2030 in the country. Antimalarial drug resistance is the biggest challenge for malaria elimination. To ascertain the status of antimalarial drug resistance at the study sites, this study was designed with the aim of assessing the *in vitro* sensitivity of *P. falciparum* isolates to antimalarials by standard tests including WHO Mark III (Micro III) and Histidine Rich Protein II enzyme linked immuno sorbent assay (HRP II ELISA) and correlating the findings with molecular markers of antimalarial drug resistance.

The *in vitro* results have revealed that HRP II ELISA assay was more consistent and reliable in assessing the resistance to antimalarials than the WHO Mark III test. As clearly, the finding of this study indicates that with respect to HRP II ELISA, 18% of study samples were resistant to chloroquine (CQ), 8% to quinine (QN) and 6% to amodiaquine (AQ) and none were found to be resistant to mefloquine (MQ) and artemisinin (ART). However, WHO Mark III assay could detect only 10% resistance of CQ and no resistance was recorded for other antimalarials.

On analyzing the results of geographically distinct isolates using ELISA assay, a higher proportion of CQ resistance was observed in culture isolates of Malaria Parasite Bank (MPB) (30%) followed by field isolates from Tripura (20%) and Odisha (20%). Only 10% of CQ resistance was observed in North east and Chhattisgarh isolates. Quinine

resistance was observed in two isolates of Odisha (20%) followed by Chhattisgarh (10%) and North-east (10%). Field isolates from Odisha, Northeast and culture isolate of MPB were found resistant to amodiaquine (10%). Among all isolates, a single field isolate of Odisha was found to have cross resistance of CQ, AQ and QN. Neither culture isolates nor field isolates were found to be resistant to mefloquine and artemisinin from any studied areas.

Notably, WHO Mark III assay could define resistance only for CQ WHO Mark III assay results showed similar finding for CQ resistance in culture isolates of MPB (20%), followed by Odisha and North-east isolates (10%). Evidently, these findings reveal that HRP II ELISA is more sensitive in analyzing the *in vitro* drug resistance. In addition, the assay is user friendly and convenient to operate.

The benefit of using HRP II protein is its stability which results in the ability of this protein to be detected even when there is a small difference in parasite number. Although, the cost of performing ELISA test is little higher than WHO *in vitro* assay but it has an advantage that any HRP II based ELISA kit for *P. falciparum* can be used for this assay. Nowadays, HRP II monoclonal antibodies are available in the market at very competitive price which can be used widely for the assay. These commercial kits have additional features like the ease to perform with standardized parameters, high sensitivity and reproducibility of the assay.

The correlation result of all five antimalarials for HRP II ELISA assay showed a high significant linear association with those obtained using WHO MARKIII assay at the IC_{50} level ($r_{IC50} = 0.94$, $r^2 = 0.92$, $p < 0.001$), as well as at IC_{90} level ($r_{IC90} = 0.96$, $r^2 = 0.94$,

$p < 0.001$). Correlation coefficients obtained from both assays for individual drugs at IC_{50} level were $r = 0.52$ ($r^2 = 0.34$, $p < 0.001$) for CQ, $r = 0.73$ ($r^2 = 0.53$, $p < 0.001$) for AQ, $r = 0.82$ ($r^2 = 0.76$, $p < 0.001$) for QN, $r = 0.72$ ($r^2 = 0.65$, $p < 0.001$) for MQ and $r = 0.98$ ($r^2 = 0.89$, $p < 0.001$) for ART, respectively. The univariate linear regression analysis for HRP II assay revealed CQ as independent predictor of drug resistance for other antimalarial drugs i.e. AQ and QN with r^2 score 0.278, and 0.103 respectively. The mean difference in IC_{50} level determined by the modified WHO and HRPII assay for antimalarial drugs were in limits of agreement, 148.3 and 100.6 for CQ, 58.3 and 34.4 for AQ, 29.4 and 17.2 for MQ, 781.4 and 425.3 for QN and 3.4 and 1.7 for ART by Bland Altman plot.

These outcomes give us evidence that both assays are statistically correlated. However, CQ was found to be an independent predictor of resistance towards other antimalarial drugs i.e. AQ, and QN by univariate analysis of HRP II ELISA results.

The genetic diversity study of the drug resistant genes was studied using *pfcrt* codon 76, *pfmdr 1* codon 86, *pfdhfr* (codon 16,51,59,108 and 164) and *pfdhps* (codon 436 and 437). The results showed that *pfcrt* K76T and *pfmdr1* N86Y gene had more of mixed species which were also concluded as resistant. The mutation analysis of *pfcrt* K76T gene among geographically distinct isolates showed resistance for antimalarials in all field isolates from Chhattisgarh, North east followed by culture isolates of MPB (80%), field isolates of Odisha (60%) and Tripura (50%). Contrastingly, all culture isolates of MPB showed mutation for *pfmdr1* N86Y followed by field isolates from Chhattisgarh (90%), Odisha (90%), Tripura (90%), and Northeast (80%). Overall, *pfdhfr* gene showed mutation at codon 108 (56%), 59 (42%) followed by 164 (2%). The mutant allele for

codon 59 and 108 in *pfdhfr* gene were more common in field isolates of Chhattisgarh with a prevalence of 80% for both, followed by 70% in Odisha and 60% and 70% in North-east isolates. The culture isolates of MPB showed 50%, 40 % and 10% mutation for codons 108, 59 and 164 in *pfdhfr* gene respectively. However, mutation at codon 164 *of pfdhfr* gene was observed only in culture isolates of MPB while at codons 16 and 51 were completely absent from all field and culture isolates. Among mutations studied in *pfdhps* gene, at codon 437 and 436 showed 32% and 20% mutation respectively. The culture isolates of MPB showed 30% mutation for codon 436 which is similar to the field isolates from north east (30%) followed by Chhattisgarh (20%) and Odisha and Tripura (10% each). Similarly, mutation at codon 437 in *pfdhps* gene was 30% in culture isolates of MPB as compared to Chhattisgarh (60%), Tripura (50%) and other North-east states (20%). Analysis of *pfATPase6* gene revealed E431K mutation in single sample from field isolate of Tripura (1/26; 3.8%) with an increased IC_{50} value of 6.28 nM. No other relevant SNPs were recorded. The analysis of propeller region (427-727bp) of Kelch13 gene did not showed any mutation**.**

The *in vitro* assays along with molecular studies provide valuable information on emerging drug resistance and thus maximize the efforts for effectiveness and prolonged existence of antimalarial drug monitoring for malaria control.

The correlation between the molecular and *in vitro* results for *pfcrt* K76T mutation was not associated with susceptibility responses to the five antimalarial drugs (p=0.84 for CQ, 0.49 for AQ, 0.18 for MQ, 0.88 for QN, and 0.65 for ART), all by Wilcoxon rank sum test. The association between *pfmdr1* N86 and 86Y and *in vitro* results did not

significantly influence the IC_{50} for CQ (p=0.12), AQ (p=0.98), QN (p=0.65), MQ (p=0.39), and ART (p=0.82). Pearson correlation was applied to study the correlation among the *in vitro* data and molecular markers namely *pfcrt* K76T and *pfmdr1* N86Y for field (n=40) and culture isolates (n=10). The results showed no significant correlation (p-value 0.559) among them.

The polymorphism study has revealed that *pfcrt* K76T and *pfmdr1* N86Y mutation were correlated for the mixed, mutant and wild type genotype for few isolates however the same was not true statistically. Similarly, there were isolates with increased IC_{50} value for artemisinin (5.193-7.76 nM) which showed amplification for *pfATPase6* gene but no significant point mutation was recorded except in one sample.

This is the first study to document the correlation between IC_{50} and molecular markers with respect to antimalarial drug resistance in the study sites in India. The outcome of this study significantly suggests the importance of studying single nucleotide point mutations as molecular markers which can be specifically involved as surveillance tools and help in detection of drug resistant parasites even before they escalate as public health problem. Also, these findings are helpful in providing early signs of emerging drug resistance pattern in the country.

Conclusion

The continuous monitoring of *in vivo* response together with the *in vitro* sensitivity, and molecular markers of resistance, in combination with treatment outcome is crucial to distinguish true resistance from treatment failure.

The comparative analysis of both *in vitro* assay used in this study conclude that the HRP II ELISA assay showed a reliable sensitivity complemented with distinguishing features such as ease to perform, and was notably consistent than WHO Mark III assay. Univariate analysis of HRP II results in addition, suggested CQ as an independent predictive marker for drug resistance for other antimalarial drugs i.e. AQ, and QN. Our correlation study finding on action of CQ and ART revealed that CQ interferes with heme detoxification pathway in food vacuoles of parasite and this may be correlated as one of the plausible mechanisms of artemisinin resistance.

Besides being the prominent marker for CQ resistance, our study suggested that the amplification of *pfmdr1* gene seems to be the candidate molecular marker of decreased susceptibility of *P. falciparum* isolates to QN and AQ. Also, our finding of triple mutation in *pfdhfr* along with double mutant for *pfdhps* gene raise an alarm for considering them as potential molecular marker for all ongoing surveillance studies of AS+SP in the country. Although, low frequency of non-synonymous mutations in the propeller region of Kelch13 gene has been reported from India, we did not find any such mutations in our samples. Molecular surveillance is not only needed for better informed drug policy, but also to limit the expansion of complex level of resistance.

Overall, the data from the *in vitro* susceptibility and molecular analysis has specified the tendency of declining sensitivity of CQ for *P. falciparum* isolates in India.

Nevertheless, further detail studies from different parts of the country with larger sample size will give us a better perceptive and knowledge of the antimalarials drug resistance in the country.

Way forward

Continuous monitoring using *in vitro* tests and molecular marker studies will provide information which can be useful in predicting emerging antimalarial resistance predicting self-life of drug which can be used by national programme. HRP II ELISA assay together with *pfmdr1* molecular marker study can contribute as better monitoring tool for the estimation of the antimalarial drug resistance in the country. Further, other *in vitro* tools such as developed by GlaxoSmithKline (GSK) in Spain for assessing the potential fast-acting antimalarial compounds which acts as throughput screening measures are needed for the control and probably the eradication of malaria. Moreover, this study finding for CQ as independent predictor of drug resistance for other antimalarial drugs i.e. AQ, and QN may provide boost for focusing on CQ resistance study.

In depth studies with more sophisticated and sensitive techniques can provide information for potential antimalarial drug resistance markers. Long term *in vivo* surveillance of ACT and recurrent monitoring of artemisinin drug resistance by using new techniques, such as the *in vitro* ring stage assay (RSA) and whole gene analysis of 13 chromosome including Kelch13 polymorphisms could be an ideal tool for studying emerging resistance.

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Annexure

Coating of ELISA plates with antimalarials

Standard Operating Procedure for Chloroquine (Formula weight=515.9)

Stock Solution

- 10 ml glass tubes
- Balance-Clean and Calibrate
- Measure 6.6 mg of drug into the tube
- Add 10 ml of distilled water to achieve a concentration 0.66 gm/l (1280 µmol/l)

Solution A

Dilute stock solution with distilled water (1:100) to achieve drug concentration of 12.8 µmol/l

Coating

Put 25 µl distilled water to well A to G. Add 50 µl of solution A to H well and remove 25 µl from well H and add to well G. Repeat the procedure till well B and discard 25 µl mix from well B (serial dilution).Keep in laminar flow overnight. Store at 4^0 C.

Standard Operating Procedure for Amodiaquine (Formula weight=464.8)

Stock Solution

- 10 ml glass tubes
- Balance-Clean and Calibrate
- Measure 1.19 mg of drug into the tube
- Add 10 ml of distilled water to achieve a concentration 0.119 gm/l (320 µmol/l)

Solution A

Dilute stock solution with distilled water (1:100) to achieve drug concentration of 3.2 µmol/l.

Coating

Put 25 µl distilled water to well A to G. Add 50 µl of solution A to H well and remove 25 µl from well H and add to well G. Repeat the procedure till well B and discard 25 µl mix from well B (serial dilution).Keep in laminar flow overnight. Store at 4^0 c.

Standard Operating Procedure for Mefloquine (Formula weight=414.77)

Stock solution

- 10 ml glass tubes
- Balance-Clean and Calibrate
- Measure 2.65 mg of drug into the tube
- Add 10 ml of methanol to achieve a concentration 0.265 gm/l (160 µmol/l)

Solution A

Dilute stock solution with methanol (1:100) to achieve drug concentration of 1.6 µmol/l.

Coating

Put 25 µl distilled water to well A to G. Add 50 µl of solution A to H well and remove 25 µl from well H and add to well G. Repeat the procedure till well B and discard 25 µl mix from well B (serial dilution).Keep in laminar flow overnight. Store at 4^0 C.

Standard Operating Procedure for Quinine (Formula weight=324.4)

Stock Solution

- 10 ml glass tubes
- Balance-Clean and Calibrate
- Measure 4.62 mg of drug into the tube
- Add 10 ml of methanol to achieve a concentration 0.462 gm/l (3200 µmol/l)

Solution A

Dilute stock solution with methanol (1:100) to achieve drug concentration of 32 µmol/l.

Coating

Put 25 µl distilled water to well A to G. Add 50 µl of solution A to H well and remove 25 µl from well H and add to well G. Repeat the procedure till well B and discard 25 µl mix from well B (serial dilution).Keep in laminar flow overnight. Store at 4^0 C.

Standard Operating Procedure for Artemisinin (Formula weight=282.3)

Stock Solution

- 10 ml glass tubes
- Balance-Clean and Calibrate
- Measure 3.6 mg of drug into the tube
- Add 6.4 ml of methanol and 3.6 ml of bicarbonate to achieve a concentration 0.36 gm/l (16 µmol/l)

Solution A

Dilute stock solution with methanol (1:100) to achieve drug concentration of 0.16 µmol/l.

Coating

Put 25 µl distilled water to well A to G. Add 50 µl of solution A to H well and remove 25 µl from well H and add to well G. Repeat the procedure till well B and discard 25 µl mix from well B (serial dilution).Keep in laminar flow overnight. Store at 4^0 C.

Publications

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Comparison of WHO Mark III and HRP II ELISA for *in vitro* sensitivity of *Plasmodium falciparum*

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ABSTRACT

Background & objectives: Antimalarial drug resistance is a serious challenge to malaria control worldwide. *In vitro* sensitivity assays provide an early indication of emerging drug resistance. *In vitro* susceptibility of field and culture adapted *Plasmodium falciparum* isolates to different antimalarials was compared using two methods: World Health Organization (WHO) micro-test (MARK III) and histidine rich protein II (HRP II) based enzyme-linked immunosorbent assay (ELISA).

Methods: In total, 50 *P. falciparum* isolates were collected from five states, *viz*. Chhattisgarh, Meghalaya, Mizoram, Tripura and Odisha of India during December 2011-September 2014. The isolates were revived and evaluated for their susceptibility to chloroquine (CQ), monodesethylamodiaquine (AQ), mefloquine (MQ), quinine (QN) and artemisinin (ART) using the WHO micro-test (Mark III) and HRP II ELISA. The data were analyzed using non-linear regression analysis.

Results: The geometric mean (GM) IC_{50} values of different antimalarials for WHO Mark III assay were comparatively lower than HRP II ELISA assay. The GM IC_{50} value for CQ was 59.5 nM (95% confidence interval [CI]: 49.35–71.73 nM) and 78.34 nM (95% CI: 64.57–95.03 nM) for Mark III and HRP II ELISA, respectively. Similarly, the values of GM IC_{50} for AQ, MQ, QN and ART by Mark III and HRP II ELISA were 13.31, 7.07, 146.4, 0.43 nM and 22.02, 11.46, 258.7, 1.00 nM, respectively. On analyzing statistically, the results of both assays were comparable ($R^2 = 0.96$, $p < 0.001$; mean log difference at $IC_{50}= 0.037$).

Interpretation & conclusion: The HRP II ELISA assay showed a reliable sensitivity in comparison to WHO Mark III micro-test complemented with distinguishing features such as high specificity, ease of performance, and notable consistency.

Key words Drug sensitivity; HRP II ELISA assay; *in vitro;* malaria; *Plasmodium falciparum;* WHO Mark III assay

INTRODUCTION

The drug resistance against antimalarials has become a serious challenge in the control of falciparum malaria worldwide. Hence specific treatment programmes as well as malaria control policies are in need based on thorough understanding of drug sensitivity[1]. Basically, drug susceptibility of antimalarials is assessed *via* two methods namely, *in vivo* and *in vitro* methods. *In vitro* sensitivity assays provide early indication of emerging drug resistance, are easy to perform and can be carried out for multiple antimalarials simultaneously. However, their results do not relate to host immunity and other host associated factors such as drug failure. They provide better understanding of the intrinsic drug sensitivity than *in vivo* tests. Except pricking for blood collection, *in vitro* tests do not involve any intervention and risk to patient[2]. Principally, all *in vitro* tests are based on the measure of drug effect on the parasite maturation and growth. The widely used *in vitro* methods/assays are World Health Organization (WHO) Mark III micro-test assay, methods based on lactate dehydrogenase, [3H]-hypoxanthine incorporation assay[3], SYBR green I fluorescence assay[4] and histidine rich protein II (HRP II) assay. All these methods have advantages as well as disadvantages[4–5].

Modified WHO Mark III and HRP II ELISA tests are based on the schizont maturation as indicator for the parasite growth[5–6]. The Mark III micro-test involving the schizont maturation assay needs little technical knowledge, is useful for samples with low parasite densities, and generally requires only 24 h of incubation. The total numbers of parasites are counted on thick films prepared from the cultured samples and schizonts are counted against total parasite. Schizont maturation assay is not commonly used by microscopists as this is labour-intensive technique and requires highly skilled persons to identify and limit the single inconsistency in counting and determining the different stages of the parasites.

The HRP II protein is a histidine and alanine rich, water-soluble protein, which is localized in several cell compartments including the cytoplasm of *P. falciparum*.

The antigen is expressed only by *P. falciparum* trophozoites. The stability of HRP II protein is major advantage for *in vitro* drug susceptibility assays[6]. The amount of HRP II secreted varies between parasite strains. The amount of HRP II found in culture samples is directly related parasite growth and development[7]. The HRP II assay practices a longer culture incubation time (72 h instead 48 h) than most other assays which give advantage of testing the slow metabolizing drugs with no change in the procedure. A simple, commercially available double-site sandwich, ELISA test kit (or basically any ELISA that is specific to HRP II protein) can be used for the assessment of parasite growth and development simply by measuring the amount of HRP II produced[1]. The simplicity of use and low resource settings has made ELISA-based *in vitro* tests a successful tool for antimalarial susceptibility testing[8].

The aim of this study was to assess the *in vitro* susceptibilities of field and culture adapted *P. falciparum* isolates to different antimalarials through two widely used *in vitro* assays, *viz*. modified WHO Mark III micro-test and HRP II ELISA test. These *in vitro* studies may be very helpful in evaluating the national guideline for malaria treatment.

MATERIAL & METHODS

Study sites & parasites collection

In total 50 *P. falciparum* isolates were collected from five states of India [Chhattisgarh (n = 10), Meghalaya (n = 6), Mizoram (n = 4), Odisha (n = 10), and Tripura (n = 10)] from December 2011 to September 2014. These samples also included culture adapted samples (n = 10) from the Malaria Parasite Bank of National Institute of Malaria Research (NIMR), New Delhi.

Ethical consideration

This work is a part of the project "Characterization of Indian *P. falciparum* isolates with special reference to *in vitro* susceptibilities and molecular markers for antimalarials resistance", which has been approved by the ethical committee of NIMR, New Delhi.

Processing of the samples

The collected and cultured samples were revived *in vitro* using Trager and Jensen[9] method in RPMI (Roswell Park Memorial Institute)-1640 medium. The parasite isolates were revived and maintained in culture for 7–8 cycles.

WHO Mark III micro-test

The sensitivity of Indian *P. falciparum* isolates to different antimalarials was assessed by using a modified version of the standard WHO Mark III micro-test[5]. *In vitro* assays were carried out on samples with parasitaemia >0.3% by microscopy. The initial parasitaemia was adjusted to 0.3–1% when required, by the addition of fresh uninfected erythrocytes. Microtitre plates were coated with five different antimalarials in concentrations recommended by WHO, *viz.* chloroquine (CQ) = 20–1280 ng/ml, amodiaquine (AQ) = 5–320 ng/mL, mefloquine (MQ) = 2.5–160 ng/ml, quinine (QN) = 50–3200 ng/ml, and artemisinin (ART) = 0.3–16 ng/ml. Each well of a 96-well tissue culture plate was filled with 90 µl drug solution of appropriate concentration plus 10 µl parasite suspension of about 5% haematocrit[5]. Control wells with parasitized blood contained no drug. The plates were incubated at 37°C in CO_2 incubator having a gas mixture of 90% N, 5% CO_2 and 5% O_2 for 25–30 h. At the end of the incubation period, suspended medium was removed while the blood within each well was used to make thick smears on a microscope glass slide. These were air-dried, stained with JSB (Jaswant Singh-Bhattacharji) stain[10] and examined under microscope at 100 × magnification. The number of schizonts with three or more nuclei against 200 asexual parasites was counted for each sample. All the tests were carried out in duplicate.

HRP II ELISA

ELISA plates were dosed with appropriate concentrations of antimalarials same as done for WHO Mark III test. The cell medium mixture (CMM) was prepared by adding 0.94 ml of the parasitized blood sample to 24.06 ml of RPMI-1640 medium in a sterile disposable tube. The pre-dosed plates were loaded with 200 µl of the resulting CMM to each well (starting with well A and proceeding to higher drug concentrations). The plates were incubated for 72 h at 37°C in CO_2 incubator. After incubation the plates were further processed immediately or stored below –20°C. After 72 h, another slide was prepared to determine parasitaemia. Four to 10 fold increase in parasite density within 72 h was considered as adequate growth[6]. The plates were removed from the incubator and separately transferred into a freezer, and kept there until all wells were completely frozen (preferably overnight). The plates were thawed. Primary IgM antibody (MPFM-55A, Immunology Consultants Laboratories, Inc, Newberg, OR, USA) was diluted to 1 µl/ml in phosphate buffer saline (PBS); 100 µl of the diluted antibody was transferred to each well of the ELISA plate using a multichannel pipette. The plates were sealed and incubated at 4°C overnight. Contents of the ELISA plate were

discarded and the plate was dried by gentle tapping. About 200 µl of blocking solution (2% bovine serum albumin in PBS) was added to each well followed by incubation of the plates at room temperature for 2 h. The content was discarded and the plate was dried. The plates were washed three times with PBS-Tween 20 (0.05%) washing solution, sealed air-tight in plastic bag and freezed at –20°C.

In total, 100 µl of sample from the culture plate was transferred to the ELISA plate. Plate was incubated for 1 h at room temperature in humid chamber, washed three times in PBS/Tween (200 µl/well) and banged dry. Secondary antibody (IgG) (MPFG-55P, Immunology Consultants Laboratories, Inc. Newberg, OR, USA) was diluted to 0.2 µg/ml in 2% BSA and 1% Tween 20 and 100 µl of the same was transferred to the ELISA plate. It was incubated for 1 h at room temperature in humid chamber, washed three times in PBS/Tween and banged to dry. Then 3,3',5,5'-Tetramethylbenzidine (TMB) chromogen (Ameresco, US) (100 µl) was added and plates were incubated for 5–10 min at room temperature in dark. The reaction was stopped by adding 50 µl of 1 M sulphuric acid and absorbance was read using an ELISA plate reader (Spectrostar Nano, BMG LABTECH, Germany) at 450 nM[8].

Data management and statistical analysis

The drug concentration that inhibits schizogony by 50% (IC_{50}) relative to the drug-free control samples of each *P. falciparum* isolate for both *in vitro* assays was estimated from dose-response curves by non-linear regression analysis using HN-NonLin software v1.1[11]. The IC_{50} cut-off values for determining sensitivity to antimalarials based on the WHO micro-test protocol were: 160 nM/l for CQ, 80 nM/l for AQ, 640 nM/l for MQ, and 800 nM/l for QN[4]. The cut-off IC_{50} value of 10.5 nM/l for artesunate was based on a previous study[12], as WHO protocol does not recommend cut-off for artemisinin. The confidence interval (CI) at 0.05 significance level was calculated by GraphPad Prism software v 5.0 package (GraphPad Software, San Diego, CA, USA).

Individual inhibitory concentrations (IC_{50}, IC_{90} and IC_{99}) for both the assays were determined by non-linear regression analysis. Bland-Altman plots[13] were performed to assess agreement between two methods. Standard correlation analysis was used to establish linear association between inhibitory concentrations obtained by two assays with selected drugs. Non-parametric procedures or log transformations were used for data that were not normally distributed.

RESULTS

WHO Mark III result

Among the field and culture isolates, only 10% were found resistant to CQ with geometric mean (GM) IC_{50} value of 59.5 against the cut-off value of 160 nM. All the isolates were found sensitive for— AQ with the $GMIC_{50}$ value of 13.3 nM against 80 nM, MQ with $GMIC_{50}$ of 7.07 nM against 649 nM, QN with 146.4 nM against 800 nM and ART with 0.43 nM against 10.5 nM.

HRP II ELISA result

The HRP II assay revealed that 18% of field and culture isolates were resistant to CQ with $GMIC_{50}$ value of 78.34 against the cut-off value of 160 nM. For AQ, 8% of the isolates were resistant with the $GMIC_{50}$ value of 22.02 nM against 80 nM cut-off. Similarly 4% of the isolates were resistant to QN with $GMIC_{50}$ value 258.7 with respect to the cut-off value of 800 nM. None of the isolates were found to be resistant to MQ and ART as all the IC_{50} values were below the cut-off value of 640 nM and 10.5 nM, respectively.

Comparative results of two in vitro assays

The $GMIC_{50}$ for 50 isolates in the modified WHO Mark III assay was 59.5 nM (95% confidence interval [CI] : 49.35–71.73 nM) for CQ whereas the corresponding result in HRP II ELISA assay was 78.34 nM (95% CI = 64.57–95.03 nM). Similarly, for AQ, the $GMIC_{50}$ by WHO Mark III assay was 13.31 nM (95% CI: 11.06–16.02 nM) which is slightly higher than the 22.02 nM (95% CI: 18.03–26.9 nM) by HRP II ELISA assay. The WHO Mark III assay result of $GMIC_{50}$ for MQ was 7.07 nM (95% CI = 5.93–8.43 nM) in comparison to 11.46 nM (95% CI: 9.44–13.9 nM) obtained by HRP II ELISA result. Also, the result of $GMIC_{50}$ was higher for QN, *i.e.* 258.7 nM (95% CI: 207.6–322.4 nM) by HRP II ELISA assay than the WHO Mark III assay result, *i.e.* 146.4 nM (95% CI: 124.3–172.6 nM). A nominal difference was observed in the GM for ART in both the assays, *i.e*. 0.43 nM (95% CI: 0.37–0.50 nM) versus 1.00 nM (95% CI: 0.81–1.23 nM) for WHO Mark III assay and HRP II ELISA assay, respectively (Table 1 and Fig. 1).

The correlation analysis results obtained by the HRP II ELISA drug sensitivity tests with all five antimalarials showed a highly significant linear association with those obtained by the WHO Mark III assay[14] at the IC_{50} level (RIC_{50} = 0.94, R^2 = 0.92, p <0.001), as well as at IC_{90} level (RIC_{90} = 0.96, R^2 = 0.94, p < 0.001) (Fig. 2). When correlating the results obtained with both the assays for

Table 1. Geometric mean IC_{50}, IC_{90} and IC_{99} with 95% confidence intervals for chloroquine (CQ), monodesethylamodiaquine (AQ), mefloquine (MQ), quinine (QN) and artemisinin (ART) determined by the HRP II ELISA assay and a modified WHO Mark III micro-test

Drug and assay (n = 50)	IC nM (95% CI)		
	50	90	99
CQ			
WHO Mark III	59.5 (49.35–71.73)	370.3 (296.2–462.8)	910.9 (730.6–1136)
HRP II ELISA	78.34 (64.57–95.03)	598 (525.1–681)	945.9 (836.6–1069)
AQ			
WHO Mark III	13.31 (11.06–16.02)	81.3 (62.39–105.9)	171.3 (135.7–216.4)
HRP II ELISA	22.02 (18.03–26.9)	149.6 (131.9–169.6)	243.5 (220.8–268.6)
MQ			
WHO Mark III	7.076 (5.93–8.43)	43.72 (31.13–61.41)	102.8 (77.53–136.2)
HRP II ELISA	11.46 (9.44–13.9)	75.68 (65.41–87.56)	121.5 (108–136.7)
QN			
WHO Mark III	146.4 (124.3–172.6)	821.1 (632.9–1065)	1809 (1465–2233)
HRP II ELISA	258.7 (207.6–322.4)	1410 (1192–1668)	2256 (1930–2637)
ART			
WHO Modified	0.43 (0.37–0.50)	1.604 (1.186–2.169)	2.655 (1.881–3.747)
HRP2 ELISA	1.00 (0.81–1.23)	6.348 (5.193–7.76)	11.32 (10.05–12.75)

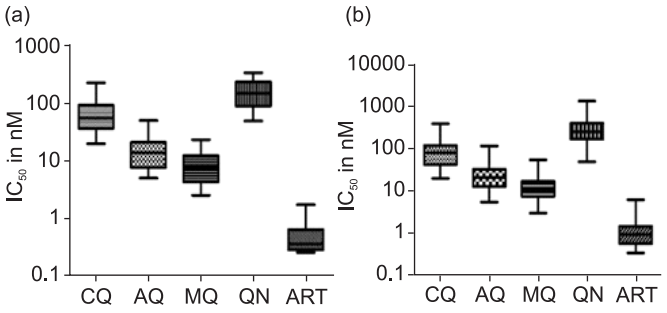

Fig. 1: Mean 50% inhibitory concentrations (IC_{50}) values of chloroquine (CQ), monodesethylamodiaquine (AQ), mefloquine (MQ), quinine (QN) and artemisinin (ART) for: (a) modified World Health Organization (WHO) Mark III micro-test assay; and (b) histidine-rich protein II (HRP II) ELISA assay, respectively.

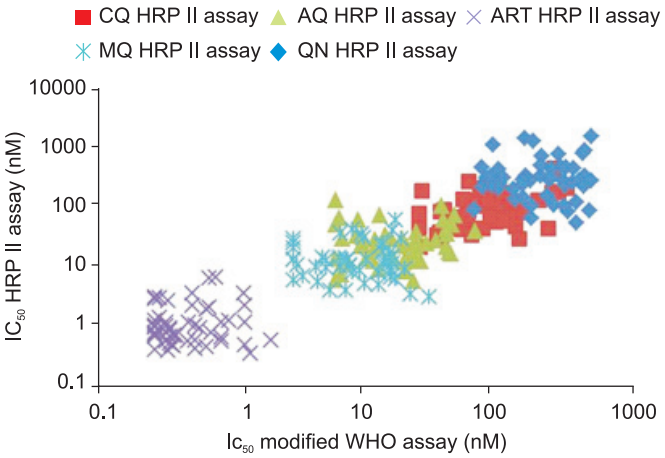

Fig. 2: Scatter plot (250 data points) for the association of individual IC_{50} of field and culture isolates for chloroquine (CQ), amodiaquine (AQ), mefloquine (MQ), quinine (QN), and artemisinin (ART) determined by the HRP II ELISA and a modified WHO Mark III micro-test assay (n = 50; $R^2 = 0.96$, $p < 0.0001$).

individual drugs, the correlation coefficients at the IC_{50} level were R = 0.52 ($R^2 = 0.34$, $p < 0.001$) for CQ, R = 0.73 ($R^2 = 0.53$, $p < 0.001$) for AQ, R = 0.82 ($R^2 = 0.76$, $p < 0.001$) for QN, R = 0.72 ($R^2 = 0.65$, $p < 0.001$) for MQ, and R = 0.98 ($R^2 = 0.89$, $p < 0.001$) for ART, respectively. The mean difference for CQ, AQ, MQ, QN and ART, determined by the modified WHO Mark III assay and HRP II ELISA assay was in limits of agreement, *i.e.* 148.3 and 100.6 for CQ, 58.3 and 34.4 for AQ, 29.4 and 17.2 for MQ, 781.4 and 425.3 for QN, and 3.4 and 1.7 for ART at the IC_{50} level. The agreement of the results (mean difference and limits of agreement) obtained by both the assays are plotted as Bland-Altman plots shown in Fig. 3

DISCUSSION

The monitoring and prevention of drug resistance have become highly significant than ever before, as malaria parasites have advanced their approaches to escape almost any existing antimalarial treatment. *In vitro* sensitivity assays are simple and effective methods for surveillance of antimalarial drug resistance. The key factors for the success of drug resistance assays are high sensitivity, together with ease of implementation and execution. These can detect even a minute change in the drug

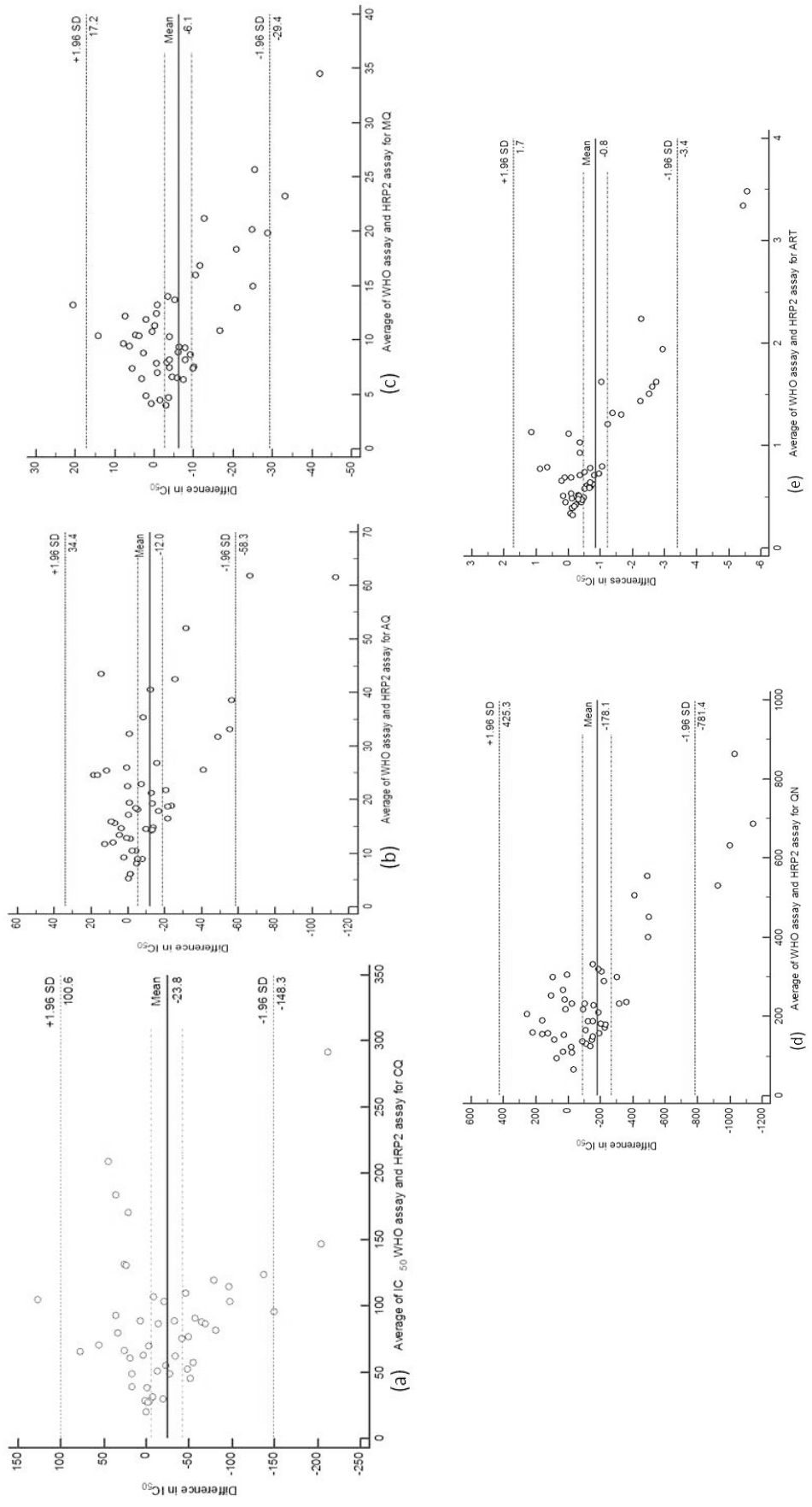

Fig. 3: Bland-Altman plot of the difference in log IC_{50} values for: (a) chloroquine (CQ); (b) amodiaquine (AQ); (c) mefloquine (MQ); (d) quinine (QN); and (e) dihydroartemisinin (ART), determined by the modified WHO Mark III micro-test and HRP II assay plotted against their mean values.

sensitivity pattern of the isolates of particular areas and can produce an early sign of change in the parasite's susceptibility pattern even before it is detected clinically. In this study, the drug sensitivity pattern of five antimalarials in field and culture isolates of *P. falciparum* from diverse regions of India was evaluated by two *in vitro* methods namely, modified WHO Mark III assay and HRP II ELISA assay.

When both assays were performed under the same conditions, results of HRP II field assay were close to those obtained with the WHO assay, similar to the results of another study[14]. Additionally, HRP II drug sensitivity assay combines the advantages of the both *in vitro* assays, although it overlooks most of their disadvantages (Table 2). Further, the HRP II assay was easier, less labour intensive and faster to perform than the WHO Mark III assay. Commercial ELISA kits availability for the quantification of HRP II protein of *P. falciparum*, eliminates the necessity for standardization of the ELISA process, thereby making its execution faster than that of any other assay.

Table 2. Comparison of WHO Mark III (micro-test) assay and HRP II ELISA assay

Factors/ Characteristics	WHO Mark III assay	HRP II ELISA assay
Sample handling time (Incubation time)	~ 24–36 h	~ 72 h
Ease of use	Labor intensive[5]	Comparatively less labour intensive[5]
Cost effectiveness	Low cost	High cost
Interpretation of result	It is subjective (depends on efficiency of microscopist)[6]	Colorimeter gives result
Availability of commercial kit	Not available	Available in variant of HRP II protein[6]
Accuracy of result	Not so accurate	HRP II protein is stable so result is more accurate
Equipment required	Microscope	Microscope and ELISA reader (Colorimeter)
Utility of assay for slow metabolizing drugs	Not helpful	Useful as incubation time is longer
Sensitivity	Comparatively lower than HRP II ELISA assay	High sensitivity[9]

The results of the HRP II assay are analogous to those obtained by the modified WHO schizont maturation assays, although both methods used different end points. The shared component of both the methods is that they deal with parasite growth and development. Due to the unique features of every assay, it is not possible to perform all assays in the same condition. The modified WHO method is based on schizont maturation; it gives the estimate of a number of parasites which develops from ring stage (early trophozoite) to schizont. The assay is considered as more sensitive as it is based on the microscopic evaluation[5]. Also, it is more economical than the HRP II assay; however, it is more labour demanding and requires highly trained personnel to overcome the experimental inaccuracy caused by human errors.

On the other hand, HRP II assay measures the rise in HRP II levels with respect to gradually increasing incubation time, marked by the early schizont and trophozoite stages of the parasite[8]. ELISA assays are sensitive but they need minimum incubation time as in the case of HRP II, and an increase in the protein level directly gives measure of parasitaemia.

The comparable results obtained by both the assays reveal the consistency and reliabilities of *in vitro* assays for testing antimalarial susceptibilities. The benefits of using HRP II protein is its stability which results in the ability of this protein to be detected even when there is a small difference in parasite growth[6]. Although, the cost of performing ELISA test is little higher than WHO *in vitro* assay, it provides options for its execution with any of the HRP II specific ELISA for *P. falciparum*. Now-a-days, HRP II monoclonal antibodies are available in the market at very competitive price which can be used widely for the assay. The commercial kits have plus points like the ease of performance with standardized parameters, high sensitivity and reproducibility of assay.

Furthermore, to its prospective value, both as an implement for drug resistance surveillance and as a screening tool for new antimalarials, numerous added practices for the assay are under concern, such as, to evaluate the potential pharmacodynamics of antimalarial drugs, or to measure the quantity of antimalarials in the blood post-intake of drug. Alternative potential use of these *in vitro* assays is the analysis of the inhibitory activities of specimens which are potential vaccine candidates[6].

CONCLUSION

Conclusively, the HRP II assay was found simple to operate, less labour intensive and result reading was comparatively faster. Besides being user friendly the sensitivity of this method was found to be better than WHO Mark III.

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Conflict of interest

The authors declare no conflict of interest.

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Brief Communication

In vitro sensitivity pattern of chloroquine and artemisinin in *Plasmodium falciparum*

Supriya Sharma, Kamlesh Kaitholia, Neelima Mishra, Bina Srivastava, CR Pillai, Neena Valecha, *Anupkumar R Anvikar

Abstract

Artemisinin (ART) and its derivatives form the mainstay of antimalarial therapy. Emergence of resistance to them poses a potential threat to future malaria control and elimination on a global level. It is important to know the mechanism of action of drug and development of drug resistance. We put forwards probable correlation between the mode of action of chloroquine (CQ) and ART. Modified trophozoite maturation inhibition assay, WHO Mark III assay and molecular marker study for CQ resistance at K76T codon in *Plasmodium falciparum* CQ-resistant transporter gene were carried out on cultured *P. falciparum*. On comparing trophozoite and schizont growth for both CQ-sensitive (MRC-2) and CQ-resistant (RKL-9) culture isolates, it was observed that the clearance of trophozoites and schizonts was similar with both drugs. The experiment supports that CQ interferes with heme detoxification pathway in food vacuoles of parasite, and this may be correlated as one of the plausible mechanisms of ART.

Key words: *Antimalarial resistance, artemisinin, chloroquine, malaria*

Introduction

Malaria is a major health problem worldwide, and drug resistance is one of the various challenges for its control. Artemisinin (ART) and its derivatives are effective antimalarials that are extensively used worldwide as first-line therapy for falciparum malaria and thus resistance to these is a matter of great concern. It is important to know the mode of action of ART resistance, which can help in drug discovery.[1] Multiple target models for the mechanism of action of ART have been proposed, but none has been proved.[2,3] ART supposedly acts by heme-dependent activation of an endoperoxide bridge located within the parasite's food vacuole. It has been anticipated that free radicals of ART alkylate these free heme molecules which lead to interference in their detoxification.[4] The mode of action of chloroquine (CQ) is also to prevent the polymerisation of toxic heme released during proteolysis of haemoglobin in the plasmodium digestive vacuole.[5] *Plasmodium falciparum* CQ-resistant transporter (*pfcrt*) protein is localised in the digestive vacuole membrane of the parasite which is considered as the transporter for CQ into the food vacuole of parasites.[6,7] Alteration in *pfcrt* protein as a result of amino acid change at codon 76 from lysine to tyrosine leads to reduce the accumulation of CQ inside the food vacuole. Thus, CQ effluxes out of food vacuole as a result of which no CQ is left for the detoxification of heme.[8] As both drugs have a similarity in heme detoxification pathways here, we made an attempt to understand the reasonable mechanism of action of ART.[10]

Materials and Methods

In vitro assay

Two *P. falciparum* culture isolates from Malaria Parasite Bank, National Institute of Malaria Research (NIMR), MRC-2 and RKL-9 were revived and cultured for 4–6 days in RPMI-1640 complete medium.[11] They were then double synchronised in a gap of 4 h, using 5% sorbitol to eliminate all the stages except the early (ring stage) trophozoites.[12] After the second synchronisation, 96 well plates was set for the TMI assay.[9] The sensitivity of cultured isolates to CQ and ART was assessed by a

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modification of the standard WHO Mark III micro-test.[13] The 96 well plates were labelled for MRC-2 and RKL-9 with drugs CQ and ART. Each well was filled with 100 μl of RPMI-1640 incomplete medium. Drug concentrations were added in duplicates with well 'H' having the highest concentration of both, CQ – 12.8 μM and ART – 1.6 μM, other wells were filled in by serial dilution method. The first row of 96 well plates was used as control (without drug). Ten microlitres of blood mixture containing 0.5%–1% ring-stage parasites with 2% haematocrit were added to each well starting from control well. After proper mixing, the plates were incubated at 37°C in a gas mixture of 90% N, 5% CO_2 for 16–18 h for modified TMI assay and for 25–30 h for modified WHO Mark III assay. Thin and thick films were prepared, fixed and stained with Jaswant Singh Bhattacharya (JSB) I and II stains. The morphology was studied under light microscope, and results were tabulated for number of infected RBCs per twenty fields in each thin film, where each field consists of 200 erythrocytes for modified TMI assay. At the end of the incubation period of 25–30 h for modified WHO Mark III assay, suspended medium was removed while the blood within each well was used to make thick smears on a glass slide. These were air-dried, fixed and stained with JSB stain and examined under microscope at ×100 magnification. The numbers of schizont with three or more nuclei against 200 asexual parasites were counted for each sample.

DNA sequencing for kelch13 and Plasmodium falciparum chloroquine-resistant transporter mutation analysis

Isolation of DNA was carried out using QIAamp mini kit (QIAGEN, Germany) according to manufacturer's protocol. This DNA was stored at −20°C until processed further. The amplification of *pfcrt* gene was carried out according to previously published standard protocol.[10] The polymerase chain reaction (PCR) product from the amplification reactions was seen by electrophoresis on 1.5% agarose gel containing ethidium bromide. Ten microlitres of the nested PCR product were digested with *ApoI* cutsmart restriction enzyme (NEB) for 20 min at 37°C as recommended by the manufacturer. Digested product was run on 1.5% agarose gel and visualised by UV transillumination. Further, to reaffirm SNP, primary PCR products of *pfcrt* gene were DNA sequencing at Xcelris Labs, Ahmedabad. The Kelch13 amplification and mutation analysis were done as per previously published protocol.[14]

Data management and statistical analysis

The drug concentration that inhibits schizogony by 50% (IC_{50}) relative to the drug-free control samples of each *P. falciparum* isolate for both *in vitro* assay was estimated from dose-response curves by nonlinear regression analysis using HN-NonLin Reg. Analysis.[15] The IC_{50} cut-off values for determining sensitivity to antimalarials were based on the WHO micro-test protocol which is 0.8 μmol/l for CQ.[13] The cut-off IC_{50} value of 0.01 μmol/l for artesunate as described by Pradines *et al*.[16] was considered as WHO protocol does not recommend cut-off for ART. Statistical analyses were done using SPSS software (Version II, SPSS Inc., Chicago, IL, USA). The editing and alignments of DNA sequences were done using Mega 6 software (Tamura, Stecher, Peterson, Filipski, and Kumar 2013).

Results

Trophozoite and schizont maturation inhibition patterns were analysed with different range of CQ (0–12.8 [μmol/l] and ART (0–1.6 [μmol/l]) with respective CQ-resistant and sensitive isolates collected from parasite bank. Comparison of TMI and WHO Mark III assay for CQ-resistant and sensitive isolates is shown in Figure 1.

The IC_{50} and IC_{99} values of CQ by TMI assay and WHO Mark III in CQ-sensitive isolate were 0.25 μmol/l and 2.85 μmol/l and 0.35 μmol/l and 2.85 μmol/l, respectively. The IC_{50} and IC_{99} values of CQ by TMI assay and WHO Mark III in CQ-resistant isolate were 0.80 μmol/l and 11.80 μmol/l and 1.07 μmol/l and 11.72 μmol/l, respectively. The IC_{50} and IC_{99} values of ART by TMI assay and WHO Mark III in CQ-sensitive isolate were 0.025 μmol/l and 0.23 μmol/l and 0.036 μmol/l and 0.36 μmol/l, respectively. The IC_{50} and IC_{99} values of ART by TMI assay and WHO Mark III in CQ-resistant isolate were 0.07 μmol/l and 0.24 μmol/l and 0.036 μmol/l and 0.36 μmol/l, respectively [Table 1].

CQ resistance in *P. falciparum* was determined by mutation in *pfcrt* gene at codon K76T. Mutation at codon K76T was found in RKL-9 while MRC-2 has shown wild genotype at this codon. DNA sequence evidence for haplotype analysis of the PCR products indicates that MRC-2 has CQ-sensitive haplotype (CVMNK) and RKL-9 has CQ-resistant haplotype (SVMNT). We sequence RKL-9 and MRC-2 isolates for mutation analysis in *Kelch13* gene, no SNP seen in both isolates, which are correlate to delayed parasite clearance time.[14]

Discussion

ART derivatives are effective antimalarial drugs and widely employed as first-line treatment globally. Different molecular modes of action have been postulated to explain the parasiticidal effect of these compounds; however, none has been evidently accepted, and their physiological application is still questioned.[1]

In the present study, we tried to understand how the parasite works against the drugs *in vitro* assays, to correlate it for action of ART, put it abreast with CQ mechanism of action by virtue of TMI and WHO Mark III assay. A very rapid parasite clearance was observed in sensitive isolates for both drugs (CQ and ART) in TMI and WHO Mark III assays. Inhibition pattern for trophozoite and schizont with CQ and ART was analogous in

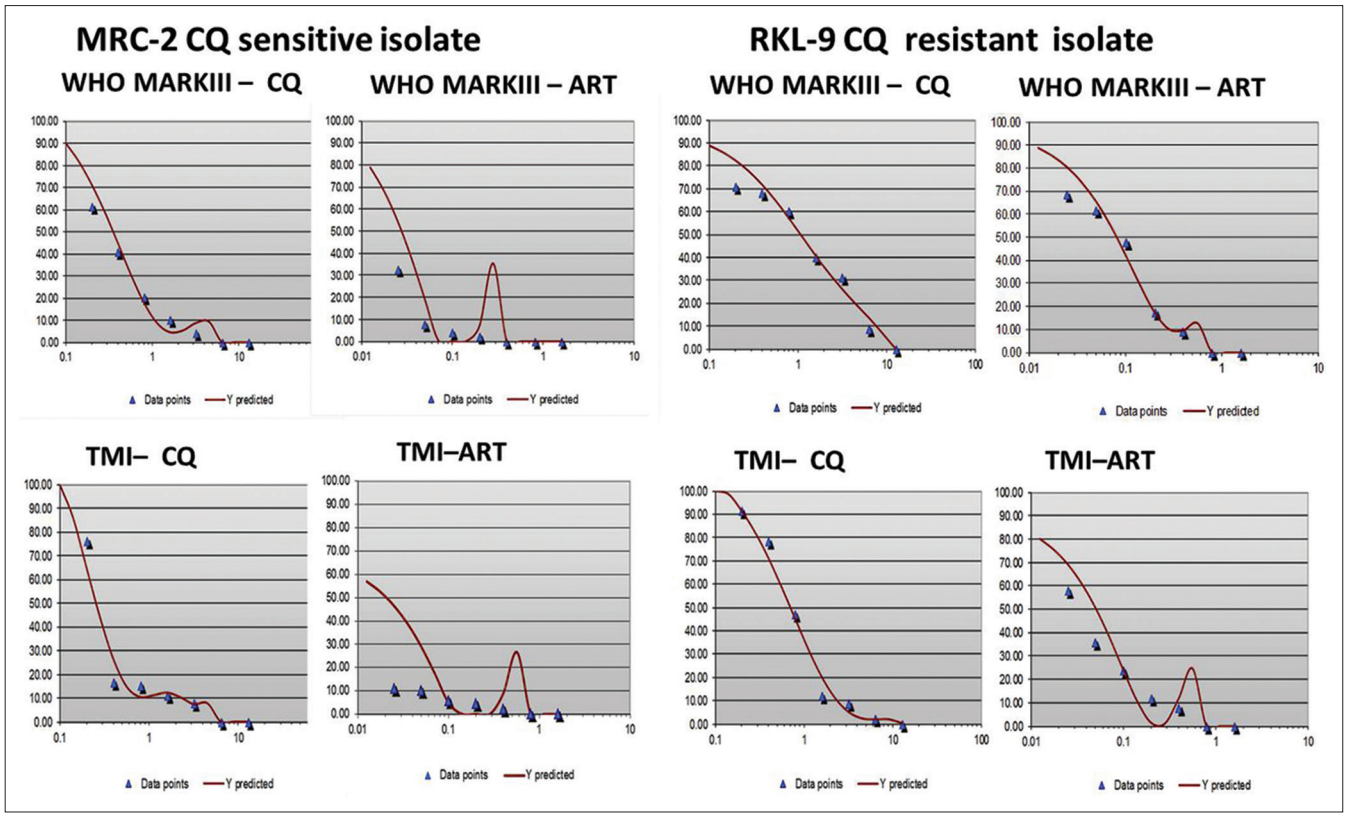

Figure 1: Comparison of TMI and WHO MARK III assay for CQ resistant and sensitive isolates

Table 1: *In vitro* susceptibility of CQ sensitive and resistant isolates of *P. falciparum*

Malaria parasite bank isolate	Drug	TMI Assay		WHO MARK III Assay	
		IC_{50} value (µmol/l)	IC_{99} value (µmol/l)	IC_{50} value (µmol/l)	IC_{99} value (µmol/l)
RKL-9	CQ	0.80	11.80	1.07	11.72
	ART	0.07	0.24	0.09	0.79
MRC-2	CQ	0.25	2.85	0.35	2.85
	ART	0.025	0.23	0.036	0.36

MRC-2 isolates. This could be due to accumulation of CQ in food vacuoles which interferes with heme detoxification pathway leading to inhibition of trophozoite and schizont maturation.[6] Interference of heme detoxification by alkylation is one of the postulated mechanisms of action of ART.[4] Slow parasite clearance with both CQ and ART in TMI and WHO Mark III assay in CQ-resistant isolate was observed. Further parallel maturation inhibition in trophozoite and schizont with CQ and ART drugs was observed in CQ-resistant isolate. The possible reason for this similarity could be the because of mutation in *pfcrt* at codon K76T leading to decreased accumulation of CQ in food vacuoles, allowing parasite to detoxify heme and survive with given normal dose.[10] This could be true with ART as well since the isolate showed the mutation of *pfcrt* gene leading to lesser alkylation of heme by ART. Hence, the parasite is able to survive for a longer duration. With respect to the IC_{50} value, CQ-resistant isolate showed 3-fold increase for CQ whereas 2-fold increase for ART, and IC_{99} value were increase 3-fold and 16-fold in resistant isolate as compared to sensitive isolate with CQ and ART drug, respectively. It suggests a probable analogues mechanism of action of both drugs. Furthermore, SNP analyses for both the genes (*pfcrt* and K-13) have reaffirm the status of resistant and sensitive parasites. Our experiment upholds, the heme detoxifixtion pathway of parasite is interfered by CQ and ART interferes the heme detoxification through alkylation and herby it may be correlated as one of the plausible mechanisms of action of ART.

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Conflicts of interest

There are no conflicts of interest.

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