STUDYING ARTEMISININ RESISTANCE IN SELECTED MALARIA ENDEMIC SITES OF INDIA

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BIOTECHNOLOGY

By

RUCHI GUPTA

Under the Supervision of

Dr. ASHWANI KUMAR (Co-Guide)

Dr. NEELIMA MISHRA (Guide)

GOA UNIVERSITY
TALEIGAON, GOA – 403206

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STATEMENT

As required under the Goa University ordinance OB-9.9 (ii), I state that the present thesis entitled **“Studying artemisinin resistance in selected malaria endemic sites of India”** is my original contribution and the same has not been submitted to any other University/Institute on any previous occasion for any degree. To the best of my knowledge, the present work is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Place: Goa University **Ruchi Gupta**

Date:

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This is to certify that the thesis entitled **“Studying artemisinin resistance in selected malaria endemic sites of India”** submitted by **Mrs. Ruchi Gupta**, 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 other university or institution.

Place: Goa University

Date:

Dr. Ashwani Kumar (Co-guide)
Scientist ‘F’ & Officer-in-Charge
National Institute of Malaria Research
Directorate of Health Services Building
Campal, Panaji – 403001, Goa

Dr. Neelima Mishra (Guide)
Scientist ‘E’
National Institute of Malaria Research
Sector-8, Dwarka
New Delhi – 110077

CERTIFICATE FROM THE CANDIDATE

As suggested by the external examiner, appropriate corrections are incorporated into the thesis on relevant pages.

Ruchi Gupta
Ph. D student
Department of Biotechnology
Goa University, Goa

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ABBREVIATIONS

µl	Microliter
ACPR	Adequate clinical and parasitological response
ACT	Artemisinin based combination therapy
AL	Artemether-lumefantrine
ARDS	Acute respiratory distress syndrome
AS+SP	Artesunate+Sulphadoxine-pyrimethamine
bp	Base pair
CDC	Centres for Disease Control and Prevention
CHC	Community Health Centre
CQ	Choloroquine
Ct	Cycle threshold
DHA	Dihydroartemisinin
dhfr	Dihydrofolate reductase
dhps	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
EDPT	Early case Detection and Prompt Treatment
ETF	Early treatment failure
FDC	Fixed-dose combination
G6PD	Glucose-6-phosphate dehydrogenase
GPARC	Global Plan for Artemisinin Resistance Containment
HELI	The Health and Environment Linkages Initiatives
HIV	Human immunodeficiency virus
IEC	Institutional Ethics Committee
IVM	Integrated vector management
LCF	Late clinical failure
ldh	Lactate dehydrogenase
LPF	Late parasitological failure

MQ	Mefloquine
NE	North-east/eastern
NIMR	National Institute of Malaria Research
NTC	No template control
NVBDCP	National Vector Borne Disease Control Programme
PCR	Polymerase chain reaction
PCT	Parasite clearance time
Pf	*Plasmodium falciparum*
pfatpase6	*Plasmodium falciparum* sarco-endoplasmic reticulum Ca^{2+} ATPase
pfmdr1	*Plasmodium falciparum* multi drug resistant gene 1
PfTCTP	*Plasmodium falciparum* translationally controlled tumor protein
pgmet	unique methionine tRNA gene
PHC	Primary Health Centre
PQ	Primaquine
Pv	*Plasmodium vivax*
PyTCTP	*Plasmodium yoelli* translationally controlled tumor protein
QN	Quinine
RBM	Roll Back Malaria
RDT	Rapid Diagnostic Test
RFLP	Restriction fragment length polymorphism
SERCA	Sarco/endoplasmic reticulum Ca2+ ATPase
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
TFACT	Treatment failure of artemisinin combination therapy
UI	Uninfected human blood
WHO	World Health Organization
WTH	Withdrawal
β-tubulin	Beta tubulin

Chapter I

Introduction and Review of Literature

1.1 Malaria: A Global Problem, General Introduction and Epidemiology

Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected mosquitoes. The disease is caused by the parasites known as *Plasmodium vivax (P. vivax), Plasmodium falciparum (P. falciparum), Plasmodium malariae (P. malariae)* and *Plasmodium ovale (P. ovale)* with a recent addition to the list being that of *Plasmodium knowlesi (P. knowlesi).* Though the causative entities are these parasites, the disease is transmitted by bites of infected mosquitoes, Anopheles species, which are known as the vectors of malaria. Malaria due to *P. falciparum* is the most deadly form predominating in endemic countries. *P. vivax* malaria is less dangerous but more widespread (WHO 2012) while the other three species are reported less frequently.

Malaria is global problem as it is endemic in 97 countries and about 3.3 billion people are at risk including 1.2 billion at high risk i.e. >1 case/1000 population. In 2015, malaria caused an estimated 214 million cases (range 148-303 million) with 438,000 deaths (with an uncertainty range of 236,000 to 635,000), a decline of 48% from the year 2000 (WHO 2015). The good fact is that malaria is preventable and curable provided that the recommended interventions are properly implemented. Efforts made to prevent and control malaria are dramatically reducing the malaria burden in many places of the world. Taking into account the population growth, the estimated decrease in total malaria burden is about 37% between the year 2000 and 2015. Most global cases of malaria were accounted by African Region (88%) followed by the South-East Asia Region (10%) and the Eastern Mediterranean Region (2%) (WHO 2015, Malaria factsheet). Similarly, in the year 2015, most of the malaria deaths were accounted by WHO African Region (90%), followed by the WHO South-East Asia Region (7%) and the WHO Eastern Mediterranean Region (2%) (WHO 2015). In 2014, India reported a

total of 881,730 malaria cases among which 463,846 cases were due to *P. falciparum* causing 561 deaths (NVBDCP 2014).

1.2 Vectors of Malaria

Mosquitoes species are responsible for transmitting various mosquito-borne diseases including malaria which has undoubtedly detrimental impact on the population and economies of the affected countries. There are about 3500 species of mosquitoes and those which cause malaria belong to genus Anopheles. Out of the 430 known species of Anopheles, around 50 species are capable of transmitting malaria globally (CDC, 2012). In India, 58 species of Anopheles are reportedly found, out of which six are recognized to be primary malaria vectors. These species of Anopheles namely *An. culicifacies*, *An. dirus*, *An. fluviatilis*, *An. minimus*, *An. sundaicus* and *An. stephensi* with disparate geographical distribution are briefly described below.

1.2.1 *An. culicifacies* Giles 1901

An. culicifacies is a sibling species complex comprising of major malaria vectors in the country as they are responsible for transmitting 65% of the malaria cases especially in rural and peri-urban areas (Dev and Sharma 2013). It is distributed throughout the country residing indoors (endophilic), mainly in the cattle sheds being primarily zoophilic and zoophagic. Streams, rice fields, burrow pits, irrigation channels, river pools etc. are the main breeding sites preferred by the species.

1.2.2 *An. fluviatilis* James, 1902

It contributes to approximately 15% of the total malaria cases. It is found in the plains and foothills breeding in streams. Its presence is mainly found in human dwellings resting on walls (Sahu et al. 2011). Breeding has been reported from slow running

streams, their channels and water logged terraced paddy fields during monsoon and post-monsoon months (Rao 1984).

1.2.3 *An. minimus* Theobald, 1901

An. minimus is an efficient malaria vector species reportedly found only in north eastern regions and the adjoining areas. This species is also recognized to have three sibling species. Resting pattern is same as that of *An. fluviatilis* (Sahu et al. 2011). Breeding occurs throughout the year in slow-flowing streams with grassy banks and feeds mainly on human hosts (Dev 1996).

1.2.4 *An. stephensi* Liston, 1901

It is an urban malaria vector contributing to around 12% of total malaria in the country. Two forms of the species exist, the 'type form' and the 'var. mysoriensis'. The type form is a malaria vector found in urban areas while the var. mysoriensis is a non-vector found in rural settings. Breeds mainly in home containers and at construction sites and other industrial locations. Feeds on humans and domestic animals and rests mainly in poorly constructed structures rather than brick structures (Sinka et al. 2011).

1.2.5 *An. dirus* Peyton and Harrison, 1979

It is mainly prevalent in forest and forest fringe areas. It is a complex of seven sibling species, out of which two are found in the country. These two species are named as *A.baimaii* which is mainly found in north-eastern states while another one, *A.elegans* is found in Shimoga hills of Karnataka. Breeds primarily in temporary, standing or slowly moving water under shade. Since such sites are associated with forest areas, this species results in transmission of malaria mainly in forest workers (Obsomer et al. 2007).

1.2.6 *An. sundaicus* (Rodenwaldt 1925)

It is found in coastal areas and previously reported to have its presence in West Bengal, Odisha, Andhra Pradesh, Andaman & Nicobar Islands and Kutch of Gujarat. Currently it is only restricted to Andaman and Car Nicobar Islands. It prefers to breed in brackish water creeks. A fresh water form has also been reported. The preference to feed on humans also varies since this species was found primarily anthropophilic in coastal areas of Indonesia whereas in Car Nicobar island, India, it was reported predominantly zoophagic (Nanda et al. 2004). It is found resting indoors in human dwellings, cattle-sheds, Copra Machan (huts for ripening the endocarp of coconut), and outdoors in Keodi (Padanus larum) bushes and under dry coconut leaves and stumps (Nanda et al. 2004).

The wide distribution of malaria vectors and their complexity including the presence of multiple sibling species makes management of malaria and implications of controls measures even more difficult.

Besides the vectors, which plays role in transmission of the disease, the disease is actually caused by parasites.

1.3 Parasite Species causing Human Malaria

The important discovery of presence of malaria parasite inside the mosquito gut was made by Sir Ronald Ross in 1897. In May 1895, he observed the presence of early stages of malarial parasite inside a mosquito stomach. However, he faced failures due to interruption in his studies as he was deputed to Bangalore to manage and investigate an outbreak of cholera. Thereafter, he got a chance to visit malaria endemic region of Sigur Ghat near Ooty where he observed a mosquito on the wall in a peculiar posture and named it “dappled-winged” mosquito. In June, 1897, he was transferred to Secunderabad.

In July, he successfully cultured 20 adult brown mosquitoes from the larvae he collected and infected them using a patient's blood. He dissected the blood-fed mosquito and isolated a perfectly circular cell from the gut of dappled winged mosquito (which turned out to be the species of genus Anopheles) and confirmed the presence of malaria parasite inside the mosquito gut on 20th August 1897 (Wikipedia, Ronald Ross 2016).

Malaria causing parasites belong to genus Plasmodium. Although there are more than 100 species which are capable of causing malaria to birds, reptiles and various mammals but only five of them have been recognized to cause malaria to humans (CDC 2015). Out of these, one species *(P. knowlesi)* which was known to infect macaques has recently been recognized to be a cause of zoonotic malaria infections in humans. The five species of Plasmodium infecting humans are briefed herein:

1.3.1 *P. falciparum* (William H. Welch, 1897)

It is a cause of malignant tertian malaria. It is found worldwide in tropical and subtropical areas. It is responsible for majority of malaria deaths globally. It multiplies rapidly in blood causing severe blood loss (anaemia). When it occurs in brain, it is a cause of cerebral malaria as it infests and clog blood vessels making the condition fatal. Multiple organs may be involved causing organ failure. Thus, this species is life threatening and most of the malaria mortality is attributed to this species.

1.3.2 *P. vivax* (Grassi & Feletti, 1890)

It is the most frequent cause of benign tertian malaria. It is the second most significant malaria species after *P. falciparum*, found mostly in Asia, Latin America, and in some parts of Africa. *P. vivax* as well as *P. ovale* enters dormant liver stage termed 'hypnozoites' which can be reactivated leading to clinical symptoms even in absence of mosquito bite.

1.3.3 *P. ovale* Stephens, 1922

It is another less frequent cause of benign tertian malaria. It is found mostly in Africa and the islands of the western Pacific. It is both biologically and morphologically very similar to *P. vivax,* however, it can infect individuals who are negative for the Duffy blood group as against *P. vivax*, which is the case for many residents of sub-Saharan Africa. This explains the greater prevalence of *P. ovale* in Africa. As mentioned above it forms liver hypnozoites like that of *P. vivax* which can activate and invade the blood ("relapse") after several months or years of initial infection.

1.3.4 *P. malariae* (Feletti & Grassi, 1889)

It is the cause of benign quartan malaria (three days cycle) representing only a small percentage of infections like *P. ovale.* Though less frequent but its presence is reported worldwide. It can cause long-lasting, chronic infection that in some cases can last a lifetime, if left untreated. In case of chronic infections, it can also lead to serious complications such as the nephrotic syndrome.

1.3.5 *P. knowlesi* Sinton and Mulligan 1933

It is the cause of severe quotidian malaria in South East Asia since 1965. It is found throughout Southeast Asia. It is a natural pathogen of long tailed and pig tailed macaques. Though the mode of transmission from macaques to humans remains unclear, it has recently been shown to be a cause of zoonotic malaria in humans particularly in Malaysia. It has a 24 hour replication cycle, progressing rapidly from uncomplicated to severe malaria that can be fatal.

1.4 *P. falciparum* Malaria: A Major Concern

P. falciparum malaria is of major concern than *P. vivax* because of numerous reasons. Firstly, it is the cause for major deaths attributed to malaria as compared to *P. vivax*

which is less virulent than *P. falciparum* malaria. However, *P. vivax* can also lead to severe disease and death due to splenomegaly (a pathologically enlarged spleen) and acute respiratory distress syndrome (ARDS) like symptoms has been recently reported (Sarkar et al. 2010) but approximately 90% of the malaria deaths are due to *P. falciparum,* currently according to WHO (WHO 2015). Secondly, *P. falciparum* is prone to develop drug resistance which is the major cause of concern in fighting malaria. Thirdly and most significantly, *P. falciparum* affects the behaviour of red blood cells changing their shape and making them sticky for internal sequestration. These parasitized sticky cells then gets lodged into the blood vessels which leads to clogging of the fine blood vessels. The process of sequestration reduces the blood flow to the major organs leading to complications. The formation of knob-like protrusions and presence of EMP-1 (erythrocyte membrane protein-1) antigen on the infected RBCs helps in cytoadherence and sequestration (Chan et al. 2014). When sequestration occurs in the blood vessels of the brain, the condition is clinically recognized as cerebral malaria, complications can include impaired consciousness, coma and even death.

1.5 Life Cycle

The life cycle of Plasmodium is completed in two hosts; the vertebrate host and the insect host (Fig.1.1). The vertebrate host (human) is considered as the intermediate host where the exoerythrocytic and the erythrocytic stages are completed while the mosquito host is considered to be the definitive one where sexual reproduction takes place (Matteelli and Castelli 1997).

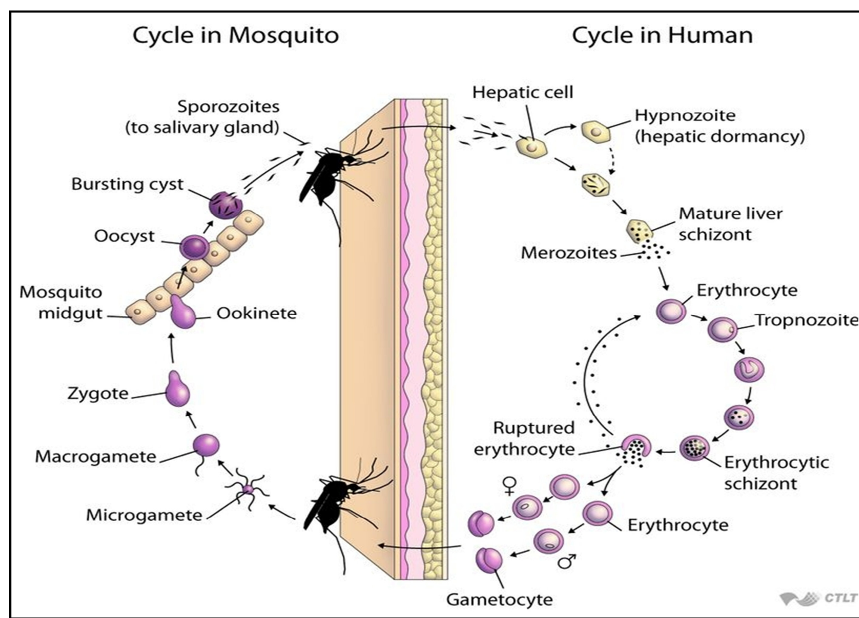
When an infected female Anopheles mosquito takes her blood meal from the human hosts, it injects dozens to thousands of sporozoites in the human blood. Although a single

sporozoite is capable of initiating the infection, the sporozoite load greatly affects the clinical picture, the greater the load, the less the incubation time and more serious the symptoms. The sporozoites circulate in the blood for a very short time (60 minutes maximum) before they enter the liver. Inside the liver, the Kuppfer cells do not allow the sporozoites to develop as they are phagocytosed whereas the hepatocytes allow the beginning of asexual exo-erythrocytic cycle. The liver strophozoite initially appears as a mononucleate cell inside the host cytoplasm subsequently converting to mature schizont finally releasing a large number of merozoites. The mature schizont is 30-70 μm in size and occupies the whole cell cytoplasm. The length of schizogonic liver cycle differs and constant in case of each species of Plasmodium and therefore, is considered to be a taxonomic character. The above character is termed as pre-patent period which is 5.5 days, 8, 9, and 15 days for *P. falciparum, P. vivax, P. ovale,* and *P. malariae*, respectively. The number of merozoites produced is also a species specific character, estimated as 2,000 for *P. malariae*, 10,000 for *P. vivax/P. ovale*, and up to 30,000 for *P. falciparum.* The liver cycle ends with the release of merozoites in the sinusoids of liver which then invades the red blood cells marking the beginning of erythrocytic cycle. Again, the time required to complete the erythrocytic cycle is the characteristic for each species of Plasmodium, *P. falciparum* and *P. vivax* take 48 hours, *P. ovale* takes 50 hours and *P. malariae* takes 72 hours to complete the erythrocytic schizogony. This duration determines the periodicity of clinical symptoms; however, since most parasitic populations are heterogenous, they result in continuous fever due to completion of asynchronized schizogonic cycles. The merozoites after getting released in the bloodstream invades the RBCs and gets converted to trophozoite, taking the shape of a ring due to formation of the vacuole by the parasite. As the cytoplasm expands, the ring is diminished while the parasitic pigment becomes visible within the cytoplasm. The stage

ends with the formation of schizont which produces numerous merozoites due to multiple nuclear divisions. The number of merozoites produced in case of *P. falciparum* is approximately 36 whereas in case of *P. vivax* and *P. ovale* approximately 24 merozoites are produced while *P. malariae* schizont produces approximately 12 merozoites. The schizogonic cycle is continued with the release of these merozoites in the circulation, until inhibited by specific immune response or chemotherapy, determining the typical malaria paroxysm. Some of the merozoites develop into sexual form of the parasite known as male and female gametocyte that appears in the blood approximately from third generation in case of *P. vivax, P. ovale* and *P. malariae* while atleast after ten generation in case of *P. falciparum.* This probably reflects the typical behaviour of slow maturation and sequestration leading to complications in case of this species.

When a female Anopheles mosquito seeks her blood meal from an infected human, it ingests the circulating gametocytes initiating the sporogonic cycle. The length of the 'sporogonic cycle' or 'extrinsic cycle' varies from 7 to 30 days depending upon the anopheline species, parasite species and temperature. When the gametocytes reach the midgut of the mosquito, the female and the male gametocyte transforms into the macrogamete and the microgamete, respectively. The nucleus of the microgamete produces eight flagellated sperm like structures and actively moves to fertilize the macrogamete. After fertilization, the resultant zygote develops into the sausage shaped motile ookinete. The ookinete penetrates the midgut of the mosquito and settles as non-motile oocyst. The nucleus of the oocyst divides repeatedly to produce as many as 10,000 nuclei leading to the formation of sporozoites which are motile in nature and travels to the salivary gland of mosquito settling into the salivary duct. When this mosquito feeds the vertebrate host it injects these sporozoites to begin another asexual replicative cycle (Fig. 1.1).

P. vivax and *P. ovale* might cause a relapsing infection even after treatment with drugs if they have no action on parasite liver stages. A relapse is a renewed clinical manifestation of the infection started by persistent liver hypnozoites which start an exo-erythocytic cycle months after the invasion of the hepatocyte. The length of the dormancy period depends on the subpopulations of the parasite and is an intrinsic property which is genetically determined (Matteelli and Castelli 1997). The host factors including the waning of immunity doesn't play a role in forming the biological basis of relapse.



Source: http://www.malariasite.com/life-cycle/

Fig. 1.1: Life cycle of Plasmodium

1.6 Management: Diagnosis, Treatment, Prevention and Control

Early diagnosis, prompt and complete treatment are essential components to effective prevention and control of malaria. Early diagnosis and prompt treatment (EDPT) of malaria aims at:

i. Complete cure

ii. Prevention of progression of uncomplicated malaria to severe disease

iii. Prevention of deaths

iv. Interruption of transmission

v. Minimizing risk of selection and spread of drug resistant parasites

vi. Reducing chances of relapse in case of *P. vivax* and *P. ovale*

1.6.1 Diagnosis

All cases which are clinically suspected of malaria should be diagnosed either by Microscopy and/or Rapid Diagnostic Test (RDT).

1.6.1.1 Microscopy

Thick and thin smears are prepared for diagnosis of malaria. Analysing these smears through microscopy remains the gold standard for diagnosis of malaria. It allows detection of the parasites at low densities, quantify parasite load, species differentiation and identifying different stages of the parasite.

1.6.1.2 RDT

Rapid Diagnostic Tests (RDTs) are immuno-chromatographic tests that detect parasite specific antigens in peripheral blood sample. Some RDTs detects only one species (monovalent) while others are capable of detecting more than one species (bivalent and multivalent). NVBDCP has recently introduced bivalent RDTs (which can detect both *P. falciparum* and *P. vivax*) for use in public health sector.

1.6.1.3 Molecular Methods

This involves the use of molecular techniques like PCR and Real time assay which targets the polymorphic and specific regions of parasite's nucleic acids by the use of short oligonucleotide sequences called 'primers'. It is mainly useful for confirming the species of malaria parasite. Though it is more sensitive than microscopy but it requires

specialized laboratory settings and moreover, it is expensive, and hence, it is of little use in field settings.

1.6.1.4 Others

Other methods include subjective diagnosis which is done in areas where laboratory diagnosis is not affordable/possible. This relies on history of subjective fever to treat for malaria. Another approach is to detect iron crystal by-product of haemoglobin that is found in malaria parasites feasting on red blood cells but absent in normal cells. This method can detect even a single infected cell amongst the million normal and it can be operated by nonmedical personal, produce zero false-positive readings, and it doesn't need a needle or any damage done (Lukianova-Hleb et al. 2014).

1.6.2 Treatment

Effective treatment is essential component in malaria management after confirmation of diagnosis of malaria. Treatment of *P. vivax* and *P. falciparum* malaria is briefed herein:

1.6.2.1 Treatment of *P. vivax*

According to National Drug Policy (NIMR and NVBDCP 2014), *P. vivax* malaria is treated with chloroquine in full therapeutic dose of 25mg/kg as per age wise dosage schedule given in Table 1.1 below.

Table 1.1: Dosage schedule of chloroquine for *P. vivax*

Age (years)	Number of tablets		
	Day 1 (10 mg/kg)	**Day 2 (10 mg/kg)**	**Day 3 (5 mg/kg)**
<1	½	½	½
1-4	1	1	½
5-8	2	2	1
9-14	3	3	1 ½
>15	4	4	2

Source: http://www.mrcindia.org/Diagnosis%20of%20Malaria%20pdf/Guidelines%202014.pdf

In approximately, 8% to 30% of cases, *P. vivax* patients may experience relapse which is prevented by administration of primaquine at a dose of 0.25mg/kg body weight daily for 14 days under supervision as per dosage described in Table 1.2.

Table 1.2: Dosage of primaquine for *P. vivax* (Daily dosage for 14 days)

Age (years)	Daily dosage (in mg base)
<1	Nil
1-4	2.5
5-8	5.0
9-14	10.0
>15	15.0

Source: http://www.mrcindia.org/Diagnosis%20of%20Malaria%20pdf/Guidelines%202014.pdf

Primaquine is contraindicated in pregnant women, infants and known G6PD deficient patients. While it has teratogenic effect, it may also lead to haemolysis in case of G6PD deficiency, therefore caution is taken while administration of primaquine and patients are made aware of the contraindications and preferably monitored when under primaquine therapy.

1.6.2.2 Treatment of *P. falciparum*

All the confirmed *P. falciparum* cases are administered artemisinin based combination therapy (ACT) as per the National Policy (NIMR and NVBDCP 2014). This is accompanied by single dose of primaquine on day 2 in a dose of 0.75mg/kg body weight. The recommended ACT in the National Programme in India except in north eastern states is artesunate (4mg/kg body weight) daily for three days and sulfadoxine (25 mg/kg body weight) – pyrimethamine (1.25 mg/kg body weight) [AS+SP] on day 0. The dosage schedule of AS+SP is given in Table 1.3.

Table 1.3: Dosage schedule of AS+SP and PQ for *P. falciparum* malaria

Age group (years)	1st day		2nd day		3rd day
	AS	SP	AS	PQ	AS
0-1	1 (25 mg)	1 (250+12.5 mg)	1 (25 mg)	Nil	1 (25 mg)
1-4	1 (50 mg)	1 (500+25 mg)	1 (50 mg)	1 (7.5 mg base each)	1 (50 mg)
5-8	1 (100 mg)	1 (750+37.5 mg)	1 (100 mg)	2 (7.5 mg base each)	1 (100 mg)
9-14	1 (150 mg)	2 (500+25 mg each)	1 (150 mg)	3 (7.5 mg base each)	1 (150 mg)
15 & above	1 (200 mg)	2 (750+37.5 mg each)	1 (200 mg)	4 (7.5 mg base each)	1 (200 mg)

Source: http://www.mrcindia.org/Diagnosis%20of%20Malaria%20pdf/Guidelines%202014.pdf

In the northeastern states (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, and Tripura), due to high failure rate of the combination AS+SP (Mishra et al. 2014), the policy has been modified to the combination artemether-lumefantrine (AL) (NIMR and NVBDCP 2014). The dosage schedule for the fixed dose combination (FDC), AL for different age groups is given in Table 1.4.

Table 1.4: Dosage schedule of AL

Wt. in kg (Age)	Total dose for co-formulated AL (twice daily for 3 days)	No. of tablets in the packing	Administration (twice daily for 3 days) tablets
5-14 (>5 months to <3 years)	20mg/120mg	6	1
14-24 (≥3 to <9 years)	40mg/240mg	12	2
25-34 (≥9 to <14 years)	60mg/360mg	18	3
>34 (≤ 14 years)	80mg/480mg	24	4

Source: http://www.mrcindia.org/Diagnosis%20of%20Malaria%20pdf/Guidelines%202014.pdf

1.6.3 Prevention

Malaria prevention can be done by preventing mosquito bites and chemoprophylaxis. Mosquitoes can be kept away from humans by spraying the home with insecticides that

prevent entry of mosquitoes inside. Other strategies include sleeping under a bed net, covering the skin, spraying the clothes and skin by use of permethrin and DEET respectively.

Chemoprophylaxis is recommended for travellers, migrant workers and military personnel in high malaria endemic areas. Use of insecticide treated bed nets is recommended for pregnant women, infants and other vulnerable population. Doxycycline and mefloquine are recommended as short term and long term chemoprophylaxis, respectively, for people travelling to malarious area (National Drug Policy, NIMR and NVBDCP 2014).

1.6.4 Control

Malaria control is pre-requisite to malaria elimination which is currently recommended and targeted.

Besides Early case Detection and Prompt Treatment (EDPT), as discussed above, malaria control strategies, according to NVBDCP, include:

1. Vector Control including Environmental Management & Source Reduction Methods
2. Community Participation
3. Monitoring and Evaluation of the National programme

According to WHO, prevention and control of malaria can be done by 'integrated vector management' (IVM) which reinforces linkages between health and environment while optimizing benefits to both (HELI, WHO).

Vector control relies mainly on chemical spraying however, IVM stresses on first understanding the vector ecology and pattern of disease transmission and then choosing the vector control tool from all the options available.

The other options include environmental management that can reduce or eliminate vector breeding grounds, biological control that kill the vector larvae by use of biological agents bacterial larvicides and larvivorous fish without generating the harmful impacts of chemical spray.

1.7 Antimalarials: Classification of different Antimalarials, Use, Mechanism of Action, Adverse Effects and Contraindications

Early diagnosis and prompt treatment are the principal components of the global strategy to treat malaria which depends on the efficacy, safety, availability, affordability and acceptability of antimalarial drugs. Antimalarial chemotherapy is the keystone of malaria control efforts. Amongst all the available antimalarials, cinchona alkaloids and qinghaosu was gifted to the world by Mother Nature. World War II led to the introduction of choloroquine, proguanil, amodiaquine and pyrimethamine. Vietnam War introduced mefloquine and halofantrine. These are the only drugs used to treat malaria. Not many new drugs have been developed to tackle malaria and therefore, the antimalarial drug pipeline is thin with limited potential candidates. For example, out of total, 1223 new drugs registered between the year 1975 and 1996, only three were antimalarials. Therefore, there is a need to preserve the present antimalarials for their indiscriminate and improper use which lead to development of drug resistance (Srinivas 2015).

1.7.1 Classification

The classification of antimalarials can be done based on the antimalarial activity and structure (Srinivas 2015).

1.7.1.1 According to Antimalarial Activity

1.7.1.1.1 Tissue Schizonticides for Prophylaxis

This mode of therapy is rather theoretical than practical since it acts on the liver stages of the parasite before clinical symptoms appear. Primaquine and pyrimethamine prevent the primary tissue phase of plasmodium during the pre-erythrocytic stage i.e. in liver hepatocytes and hence categorized under this category.

1.7.1.1.2 Tissue Schizonticides for Preventing Relapse

These drugs act on hypnozoites of *P. vivax* and *P. ovale* and hence prevents relapse. Eg. Primaquine is the prototype drug while pyrimethamine also has such activity.

1.7.1.1.3 Blood Schizonticides

These drugs supresses the clinical symptoms since they act on blood stages of the parasite, therefore, these are the most important class of antimalarials. The drugs include chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones, tetracyclines, doxycycline, etc.

1.7.1.1.4 Gametocytocides

These drugs destroy the sexual stages of the parasite and prevents transmission of malaria. The drugs chloroquine and quinine have gametocytocidal activity against *P. vivax* and *P. malariae* while primaquine acts against gametocytes of all plasmodia.

1.7.1.1.5 Sporontocides

These drugs prevent the formation of oocysts in the mosquito and ablate transmission. eg. Primaquine and choloroguanide (proguanil).

1.7.1.2 According to the Structure

1.7.1.2.1 Aryl Amino Alcohols

Eg. Cinchona alkaloids- quinine and quinidine; mefloquine and halofantrine are aryl amino alcohols.

1.7.1.2.2 4-aminoquinolines

The antimalarials chloroquine and amodiaquine are classified as 4-aminoquinolines.

1.7.1.2.3 Folate synthesis inhibitors

There are two categories i.e. Type-1 which are competitive inhibitors of dihydropteroate synthase, eg. Sulphones and sulphonamides; Type-2 which inhibits dihydrofolate reductase, eg. Biguanides like proguanil and chloroproguanil, diaminopyrimidine like pyrimethamine.

1.7.1.2.4 8-aminoquinolines

Primaquine and Tafenoquine are categorized under this category

1.7.1.2.5 Antimicrobials

The drugs tetracycline, doxycycline, clindamycin, azithromycin, fluroquinolones are antimicrobials which also have antimalarial activity.

1.7.1.2.6 Peroxides

Artemisinin and its derivatives fall under this category. Eg. Artemether, arteether, artesunate and artelinic acid.

1.7.1.2.7 Naphthoquinones

Atovaquone is the antimalarial which falls under this category.

1.7.1.2.8 Iron chelating agents

Eg. Desferrioxamine

1.7.2 Use, Mechanism of Action, Adverse Effects and Contraindications

1.7.2.1 Chloroquine

It is the prototype antimalarial drug used to treat all type of malaria infection. It is the cheapest, time tested and safe antimalarial agent. It is highly effective against erythrocytic stages of all three forms of malaria while only for sensitive strains of *P. falciparum* and gametocytes of *P. vivax*. It is more effective and safer than quinine.

Mechanism of action: It inhibits parasitic enzyme heme polymerase that converts toxic heme into non-toxic hemozoin, resulting in the accumulation of toxic heme in the parasite thereby causing its death.

Adverse effects: It is relatively safer drug. It can cause dizziness, headache, diplopia, disturbed visual accommodation, dysphagia, nausea and malaise. Even if it causes visual hallucinations, confusion and sometimes psychosis, these symptoms do not warrant stoppage of treatment. Intramuscular injections of the drug can cause hypertension and cardiac arrest particularly in children.

Contraindications: Chloroquine should be used with caution in patients with hepatic disease, gastro intestinal, neurological and blood disorders.

1.7.2.2 Quinine

It is the chief alkaloid which comes from cinchona bark, a tree found in South America. The source of quinine still remains the same due to the difficulty in synthesizing the complex molecule.

Mechanism of action: It is a blood schizonticide and mechanism of its action is same as that of chloroquine, death of the parasite occurs by deposition of toxin heme in the food vacuole. It also has gametocytocidal action against *P. vivax* and *P. malariae*. However, as a schizonticidal drug, it is less effective and more toxic than chloroquine but it plays a major role in management of *P. falciparum* malaria especially in resistant and failure cases where it is administered as rescue treatment.

Adverse effects: It causes cinchonism which is characterized by ringing in the ears, headache, nausea and disturbed vision. Gastrointestinal symptoms like vomiting, abdominal pain and diarrhoea may also be seen. Other symptoms include rashes, sweating, angioedema, excitement, confusion, delirium, etc. It can also cause coma, respiratory arrest, hypotension and even death when overused. It may also result in renal failure, massive haemolysis and haemoglobinuria especially in pregnancy or with overdose. Hypoprothrombinemia and agranulocytosis is also reported in some cases. Since quinine also stimulates insulin secretion, it can result in hypoglycaemia which should be monitored regularly while administering quinine. Therefore, maintenance of 10% dextrose infusion is advisable with the administration of this drug.

Contraindications: Treatment with quinine should be stopped in case hypersensitivity in the form of rashes, angioedema, visual and auditory symptoms or haemolysis is reported. Patients with tinnitus (functional impairment of eighth nerve), optic neuritis and myasthenia gravis should not be administered with this drug. Caution is recommended in patients with atrial fibrillation.

1.7.2.3 Chloroguanide (Proguanil)

It is popularly called as proguanil with the active metabolite called as cycloguanil pamoate. It controls acute infection of *P. falciparum* and clinical attacks of vivax malaria. It is also used as a prophylactic drug along with chloroquine.

Mechanism of action: It acts by inhibiting the parasitic enzyme *dihydrofolate reductase (dhfr)*.

Contraindications: Occasional nausea and diarrhoea is reported though otherwise it is a safe drug which can even be used in pregnancy.

1.7.2.4 Sulfadoxine+Pyrimethamine

It is a very useful adjunct in the treatment of uncomplicated falciparum malaria while it occupies place in National drug policy in combination with artesunate.

Mechanism of action: Sulfadoxine inhibits the utilization of para-aminobenzoic acid in the synthesis of dihydropteroic acid which is an intermediate in the synthesis of folic acid by inhibiting the enzyme *dihydropteroate synthetase (dhps)*. Folic acid is vital to the synthesis, repair, and methylation of DNA which is important for cell growth of the parasite. With this nutrient lacking, the parasite has difficulty in reproducing and this is how sulfadoxine plays its role. Pyrimethamine inhibits *dhfr* enzyme of plasmodia thereby blocking the synthesis of purines and pyrimidines which is essential for DNA synthesis and cell multiplication. Since both these drugs inhibits the enzyme of the same pathway, the combination of these offers a two-step synergistic blockade of plasmodial division.

Adverse effects: Pyrimethamine occasionally causes skin rash and depression of hematopoiesis while sulfadoxine has numerous side effects like agranulocytosis, aplastic

anemia, hypersensitivity reactions, fixed drug eruptions, erythema, multiforme of the Steven Johnson type, exfoliative dermatitis, serum sickness, liver dysfunctions, etc.

Contraindications: Sulpha drugs are contraindicated in patients with known hypersensitivity, infants below two months of age, patients having advanced renal disease and first and last trimester of pregnancy.

1.7.2.5 Halofantrine

It is a phenanthrene methanol structurally related to quinine. It is no longer used for treatment of drug resistant uncomplicated *P. falciparum* malaria.

Mechanism of action: It forms toxic complex with ferritoporphyrin IX damaging the cell membrane of the parasite.

Adverse effects: Abdominal pain, diarrhoea, prolongation of QT interval (electric depolarization and repolarization of ventricles) and arrhythmias.

Contraindications: Patients having prolonged QT interval, pregnant and lactating women, infants and the cases who have received mefloquine in preceeding three weeks.

1.7.2.6 Mefloquine

Born during Vietnam War, it was discovered in search for newer drug to treat multidrug resistant falciparum malaria. It is administered as oral preparations since otherwise it causes severe local reactions.

Mechanism of action: Similar to that of chloroquine and halofantrine, it forms toxic complexes with free heme damaging the parasite membrane.

Adverse effects: Well tolerated in therapeutic doses while when dose exceeds 1g, it causes nausea, vomiting, abdominal pain and dizziness. It may also cause nightmares,

sleep disturbances, ataxia, sinus bradycardia, sinus arrhythmia, postural hypotension and acute brain syndrome, though these are reported less frequently.

Contraindications: Should be used with caution in patients with heart block and those taking beta blockers, having history of epilepsy and psychiatric disease. It is also avoided during first and last trimester of pregnancy, preceding treatment within three months, and concomitant use with other antimalarials due to increased risk of cardiotoxicity and risk of convulsions. It also increases risk of seizures in patients taking valproate. Patients taking this drug should refrain from driving or operating machinery. It may also compromise immunization by typhoid vaccine.

1.7.2.7 Atovaquone

It is a synthetic hydroxynapthoquinone found useful against Plasmodia as well as Toxoplasma and *Pneumocystis carinii*. A fixed dose of atovaquone along with proguanil is administered for both treatment and prophylaxis of malaria.

Mechanism of action: Interferes with the mitochondrial electron transport and thereby ATP and pyrimidine biosynthesis. In Plasmodium, it targets cytochrome bc1 complex disrupting the membrane potential.

1.7.2.8 Pyronaridine

It is structurally similar to amodiaquine and has been found to be very effective against chloroquine resistant strains in China. Administered as combination therapy with artesunate (Pyramax).

1.7.2.9 Piperaquine

Similar to chloroquine, it has been registered as combination therapy with dihydroartemisinin.

1.7.2.10 Lumefantrine

It is an aryl alcohol related to quinine, mefloquine and halofantrine. It is administered as a popular combination with artemether (AL).

1.7.2.11 Other Drugs with Antimalarial Activity

Clindamycin, ciprofloxacin, norfloxacin and azithromycin, etc have also been found to be effective against malaria parasites. Tetracyclines are amongst the first antibiotics to come into human use to treat malaria.

1.8 Artemisinin based Combination Therapy (ACT)

Treatment of *Plasmodium falciparum* malaria depends on administration of artemisinin based combination therapy abbreviated as 'ACT'. ACT is a combination of drugs with different modes of action belonging to different class of antimalarials. In order to provide adequate cure and delay drug resistance artemisinin class of drugs having short half-life (White 1999) is administered with another class of drug with different mode of action having long half-life. The artemisinin partner being highly potent takes care of the initial high burden of the parasites reducing the parasite biomass to 10^4 fold per 48 hour of life cycle whereas the weaker partner drug then takes care of the remaining parasitaemia so as to delay drug resistance.

Companion drugs include lumefantrine, mefloquine, amodiaquine, sulfadoxine/ pyrimethamine, piperaquine and chlorproguanil/dapsone.

The available artemisinin combinations are as follows:

1. AS+SP (artesunate+sulfadoxine-pyrimethamine)
2. Artemether-Lumefantrine (AL) also called as Coartem®

3. Dihydroartemisinin+Piperaquine (DHA+PQ) registered as Eurartesim®
4. Artesunate+Mefloquine (AS+MQ): Fixed-dose combination (FDC) called as artequin granules.
5. Artesunate+Amodiaquine (AS+AQ) registered as Coarsucam®
6. Artesunate+Pyronaridine also called as Pyramax®
7. Artemisinin+Naphthoquine: Registered and promoted in more than 10 countries as ARCO®

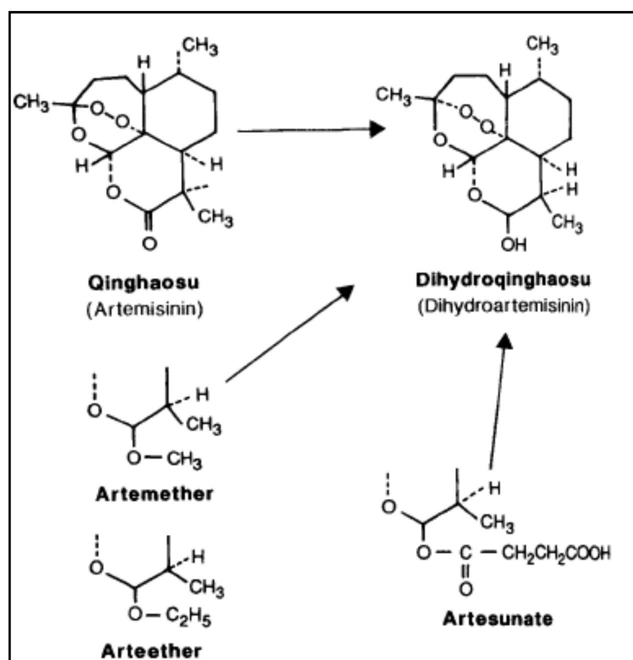
Apart from this, artesunate monotherapies are administered as injectable only in case of severe malaria. However, WHO urged to withdraw artemisinin monotherapy from market as it was considered to be a major contributing factor towards developing drug resistance to artemisinin. In view of this, Drugs Controller General of India banned the use and marketing of artemisinin monotherapy in the country in the year 2009 (Anvikar et al. 2014).

1.9 Artemisinin: Chemistry, Structure, Mechanism of Action

Discovery and evaluation of a group of antimalarial drugs from Chinese medicinal herb qing hao (*Artemisia annua L.*) was described in December 1979. The drug was developed by a group of Chinese scientists working on a secret “Project 523” (Dondorp et al. 2011). The Chinese herb from which artemisinin was derived is also known as annual or sweet wormwood. Out of the seven sesquiterpene compounds that were isolated from qing hao in 1972, one was found to have principal anitimalarial activity. This compound was named Qinghaosu (“ching-how-soo”), literally the active principal of qing hao. The western name for the compound is artemisinin.

It was also noticed that the antimalarial properties of Qinghaosu were different from that of other antimalarials as this agent was rapidly parasiticidal and produced characteristic ultrastructural changes in growing trophozoite parasites. Moreover, this compound and its derivative were considerably less toxic than chloroquine (Hien and White 1993).

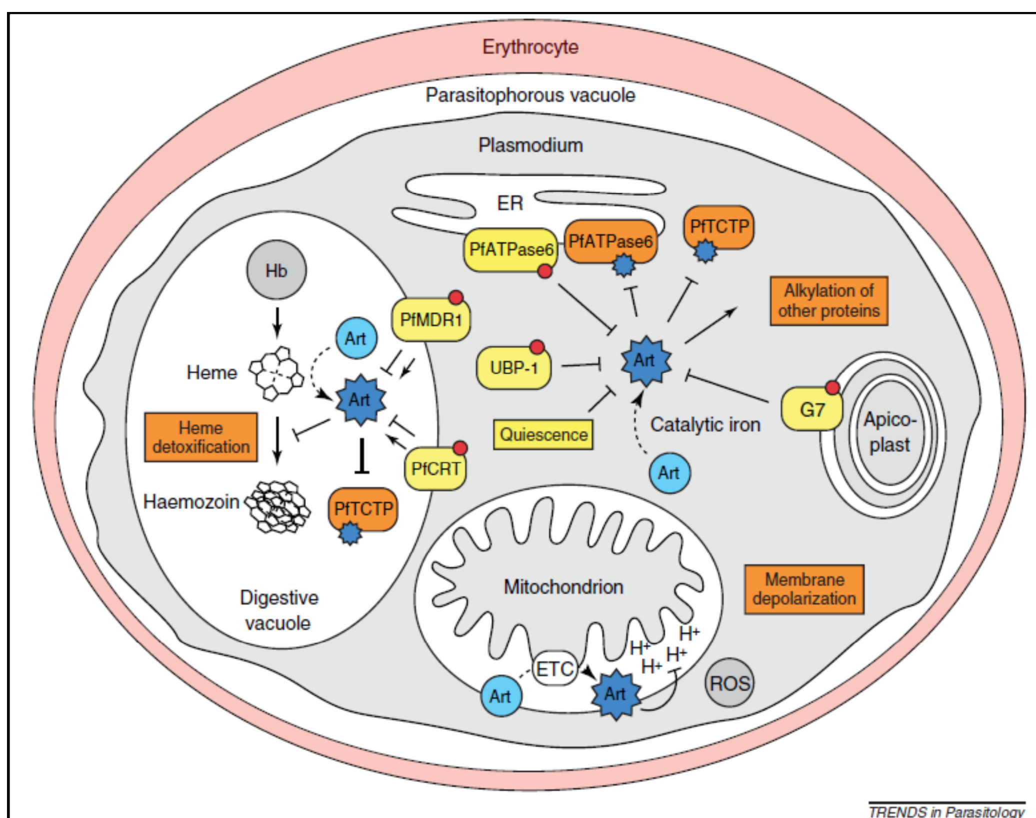
Qinghaosu (artemisinin) is sesquiterpene lactone peroxide. The empirical formula is $C_{15}H_{22}O_5$. It is essentially insoluble in water but decomposes in other protic solvents via opening of the lactone ring. The endoperoxide moiety, which is rarely found in natural products, is essential for the antimalarial activity. Clinical studies conducted in the year 1972 revealed it to be active against both vivax and falciparum malaria. Four pharmaceutical formulations were produced thereafter, artemisinin and dihydroartemisinin while artemether and artesunate were manufactured commercially (Hien and White 1993). The chemical structure has been depicted in Fig. 1.2.



Source: http://www.ncbi.nlm.nih.gov/pubmed/8094838

Fig. 1.2: The chemical structure of artemisinin and its derivatives

The mechanism of action of artemisinin is not defined clearly but four different models have been proposed to explain artemisinins mode of action against Plasmodia. There are evidences both in favour of and against each of these models and so these models have been widely discussed (Ding et al. 2011). Briefly, once activated through cleavage of endoperoxide bond, artemisinins might (i) interfere with the heme detoxification pathway; (ii) induce the alkylation of the translationally controlled tumor protein (PfTCTP) and other proteins; (iii) inhibit the sarco/endoplasmic reticulum membrane calcium ATPase6 (*PfATPase6*); or (iv) interfere with Plasmodium mitochondrial functions (Ding et al. 2011) (Fig. 1.3). The arguments in favour and against each of these postulates are discussed hereby.



Source: doi:10.1016/j.pt.2010.11.006 Trends in Parasitology, Feb 2011, Vol. 27, No.2

Fig. 1.3: Postulated artemisinins' modes of actions and resistance factors in *P. falciparum*

(i) Artemisinins interfere with heme detoxification pathway is favoured by the fact that artemisinin alkylate heme which is a Plasmodium specific property of artemisinin and heme iron activates artemisinin. The counter argument says that non-heme iron might activate artemisinin and that artemisinins alkylates other proteins as well. Also, artemisinin when prevented to interact with heme yet showed antimalarial activities and an upstream block of haemoglobin degradation does not affect artemisinins' activity.

(ii) Immunoprecipitation studies showed interaction of PfTCTP with dihydroartemisinin and an unstable artemisinin resistant strain of murine malaria parasite, *P. yoelli*, showed slight increase in PyTCTP protein levels suggesting that PyTCTP might be a target for artemisinin. However, the postulates which are against the above arguments are that PfTCTP is poorly characterized lacking functional data and specificity of upregulated PyTCTP was not demonstrated in the above experiment.

(iii) It is believed that artemisinins inhibits *Pfatpase6* which seems to be logical as thapsigargin, which is similar to artemisinin, inhibits sarco/endoplasmic reticulum Ca2+ ATPases (SERCAs), mammalian orthologue of *Pfatpase6*. Experiments in Xenopus oocytes revealed that active artemisinin specifically inhibited *Pfatpase6* activity supposedly mediating parasite death. Mutation in a single residue L263E directly modulated this activity, however, the counter argument is that L263E is never seen in any of the field isolates as yet (Kwansa-Bentum et al. 2011; Tanabe et al. 2011). Moreover, different enatiomers of artemisinins, which is a chiral

molecule, shows similar binding ability to *Pfatpase6*, which probably requires a certain steric formation.

(iv) Artemisinins interfere with Plasmodium mitochondrial function was supported by the observation that it inhibited growth of *Saccharomyces cerevisiae* only in the absence of fermentable carbon source. Active artemisinin induced membrane depolarization of mitochondria in *P. berghei*, which does not happen in mammals and also does not happen in presence of inactive deoxyartemisinin. Membrane depolarization is reversible and correlates with ROS production in response to active artemisinin. Also, interfering with mitochondrial electron transport chain of *P. berghei* through inhibition of electron donor NADH dehydrogenase antagonized action of artemisinin but not chloroquine. Co-localization studies showed fraction of artesunate localizing on parasites' mitochondria in infected erythrocytes, however, this experiment lacked significance as it used artesunate and not artemisinin and that too at an unspecified concentration (Ding et al. 2011).

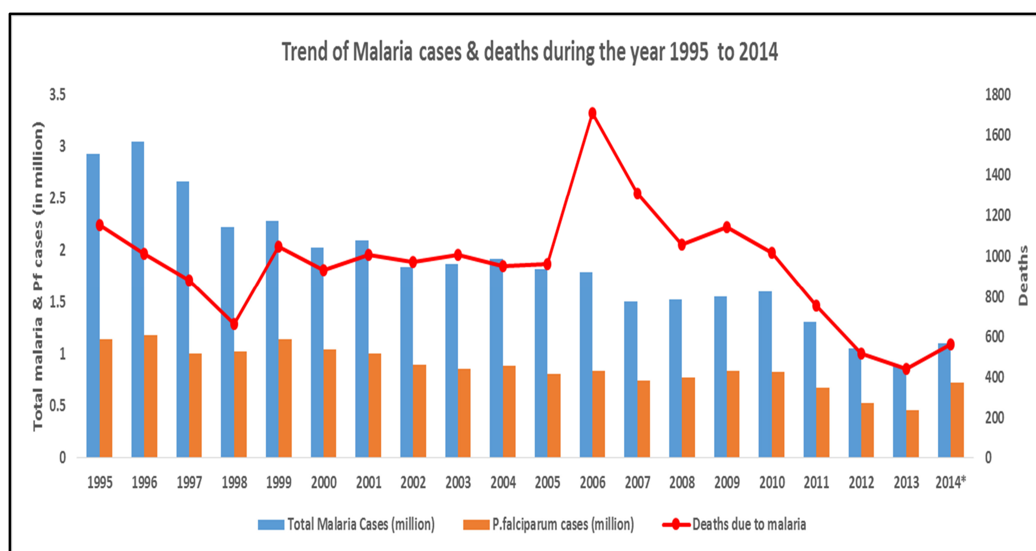
The above studies clearly marks that mode of action of artemisinin and the molecular factors involved in it needs to be studied further.

1.10 Drug Resistance: History and Severity of the Problem, Factors Affecting Drug Resistance, Mechanism of Development of Drug Resistance

Drug resistance is defined as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject" (Bloland, WHO, 2001).

1.10.1 History and Severity of the Problem

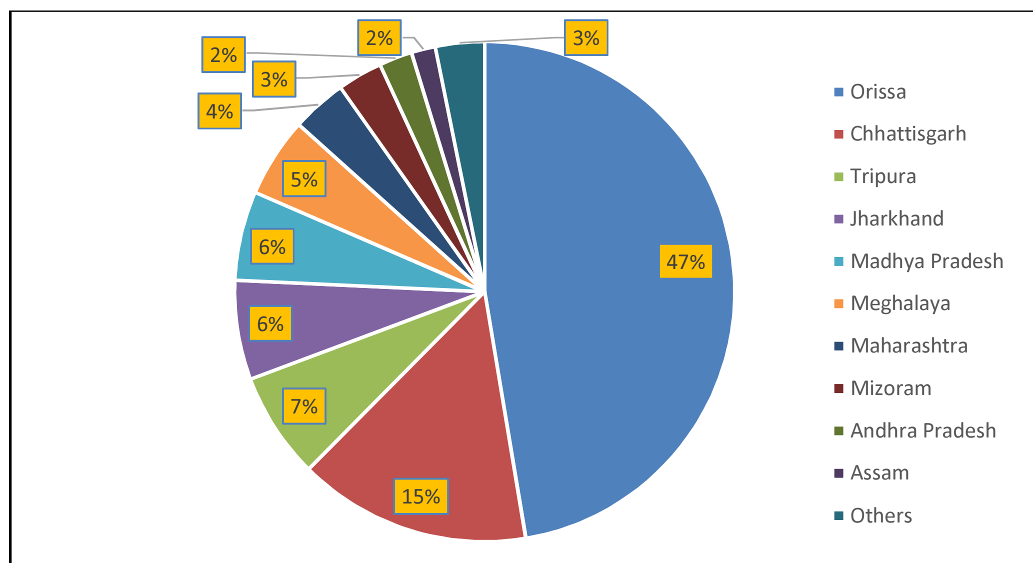
India has a diverse epidemiological malaria background with almost 85% of the population living in malarious zone (Sharma 1999). *P. falciparum* malaria is of major concern in India because it is responsible for high mortality. The percentage of *P. falciparum* arose to nearly about 65.6% in the year 2014 as compared to 39% of the total malaria cases in the year 1995 although the annual incidence of *P. falciparum* declined from 1.14 million cases to 0.72 million cases during the same period (NVBDCP 2014) (Fig. 1.4). Generally, apart from *P. falciparum*, *P. vivax* accounts for 25 to 30% while mixed infection accounts for 4 to 8% of total malaria cases annually (Rana 2007).



**Note: Data for the year 2014 is provisional*
Source: www.nvbdcp.org

Fig. 1.4: Trend of declining malaria cases over the years from 1995 to 2014 though the relative percentage of Pf increased over all these years

In India, Odisha contributes to the maximum burden of *P. falciparum* malaria (47%) followed by Chhattisgarh (15%), Tripura (7%) and Jharkhand (6%). The graph below (Fig. 1.5) depicts the burden of *P. falciparum* malaria in India (NVBDCP 2014).



Source: www.nvbdcp.org

Fig. 1.5: Contribution of individual states in total *P. falciparum* cases in India in the year 2014

The plausible reason behind relative increase in *P. falciparum* burden in India is that *P. falciparum* malaria is more prone to drug resistance (Shah et al. 2011).

Plasmodium vivax is relatively less prone to develop drug resistance. CQ resistance in *P. vivax* has only been reported in two cases from Mumbai (Maharashtra) and a case report each from Mathura and Aligarh (Uttar Pradesh) while three case reports from Kanpur (U.P.) (Valecha et al. 2006). Futhermore, a study carried out at Daltonganj (Jharkhand) reported few cases to be chloroquine resistant (Singh 2000). Contrary to this, during the same period, Nandy et al. (2003) reported 100% cure rates of CQ in *P. vivax* by day 7 in a study carried out in 480 patients. Therefore, CQ resistance in *P. vivax* is not of much concern in the country.

Emergence of Antimalarial Resistance

History of emergence of drug resistant malaria originates from North-eastern states of India. Chloroquine resistant *P. falciparum* malaria was first reported in the year 1973

from Karbi Anglong district of Assam (Sehgal et al. 1973) followed by sulfadoxine-pyrimethamine (SP) resistance in the year 1979 again in Karbi-Anglong (Das et al. 1981). SP was introduced as second line treatment in chloroquine resistant areas in the year 1982 when National drug policy was introduced to improve malaria case management. Later, artesunate+sulfadoxine-pyrimethamine (AS+SP) was introduced as a second-line treatment in chloroquine resistant areas in the year 2005. Owing to the larger spread of chloroquine resistance, AS+SP was introduced as a first line drug to treat uncomplicated *P. falciparum* malaria throughout the country (NVBDCP 2010).

Dissemination of Drug Resistance and its Later Emergence in other parts of the Country

Gradual spread of CQ resistant malaria occurred from Karbi Anglong to 55 districts (311 PHCs in 14 states) and then to 110 districts (1410 PHCs in 18 states) in the year 1982 when SP was introduced as second line treatment for CQ resistant cases (Sharma 2007). Shah et al. (2011) extensively compiled and summarised the studies conducted on CQ resistant *P. falciparum* between the years 1978 to 2007. The report revealed that out of the 337 studies conducted for investigating the efficacy of CQ in 17189 patients, the number of studies and proportion of failures varied between regions and states within a region. It was also observed that the proportion of failures detected was higher in 28-day follow-up than 7-day follow-up suggesting prevalence of late treatment failures after CQ treatment. The interquartile range (IQR) of failures detected after 28 day follow-up between these years was 13.0-58.2, median being 35.1%. The increasing failure trend of CQ was clear when 2/17 studies revealed >10% failure (threshold to

switch to second line treatment in India) during the year 1978 to 1979 while 35/40 studies revealed >10% of CQ failure during the year 2006-07. To further dig up the details, the regions where CQ efficacy studies were conducted and reported >10% failures, were looked into. These regions comprised a total of 115 districts representing 20/28 states and 2/5 Union Territories. Others either had low incidence of *P. falciparum* or antimalarial drug trials were not conducted in these regions (Shah et al. 2011). Travelling from northeast to eastern regions to the west and south of India, finally to the North-central, CQ resistance gradually spread covering almost the entire country (Shah et al. 2011, Farooq and Mahajan 2004). However, with the altered therapy and reduced CQ usage, the drug pressure is reduced and a reversal trend is expected in this scenario of CQ resistance as has been reported in some parts of Africa following withdrawal of CQ (Palleau et al. 2015).

According to National Drug Policy (1982), Sulfadoxine-Pyrimethamine was recommended as second line of treatment prescribed to CQ resistant cases. However, the widespread use of this drug precipitated resistance soon. The molecular markers of SP resistance i.e. *dhfr* and *dhps* have also been identified and showed increasing trends towards developing mutation. During the year 1978 to 2007, 26 studies covering 1431 patients were conducted to determine the efficacy of SP. Different derivatives of SP were tested in different studies e.g. the studies conducted in Assam (n=3) made use of sulfalene whereas, others used sulfadoxine. After 28-day follow-up the IQR for failure was 0·7–33·1 median being 15%. During 1984 to 1996, 19/246 (7.7%) patients failed SP treatment compared to 307/1185 (25.9%) during 1997-2007. Most of the studies were done in NE regions, the highest rate of treatment failure was seen in Arunachal

Pradesh which is close to Chinese and Myanmar border (Shah et al. 2011). Due to high drug pressure of SP, increased resistance led to the introduction of artemisinin based combination therapy (ACT-AS+SP) in India in the year 2005 in CQ resistant areas which became the first line treatment in the year 2010 throughout the country. However, due to increased treatment failure of AS+SP in North-eastern (NE) states recently (Mishra et al. 2014), the drug policy has been changed to artemether-lumefantrine (AL) in NE regions of the country (NIMR and NVBDCP 2014). The other regions of the country are still confined to AS+SP as first line treatment for uncomplicated falciparum malaria (NIMR and NVBDCP 2014).

Quinine (QN) is used to treat complicated malaria cases and also given to patients who do not respond to CQ, SP and ACT. QN is administered as a rescue treatment. Though reduced efficacy with quinine treatment has been seen but treatment failure cases are not very common in India. QN resistance in India was first reported from NE states and also from Kolar district in Karnataka (Farooq and Mahajan 2004).

Mefloquine resistance in India was first reported from Surat district of Gujarat state (Sharma 1996).

1.10.2 Factors Affecting Drug Resistance

Numerous factors contribute to the spread of drug resistance. The figure below (Fig.1.6) explains the factors affecting spread of drug resistance and their interdependence on each other. Apart from human behaviour, vector and parasite biology, pharmacokinetics and economics play a definitive role in development and intensification of drug resistance.

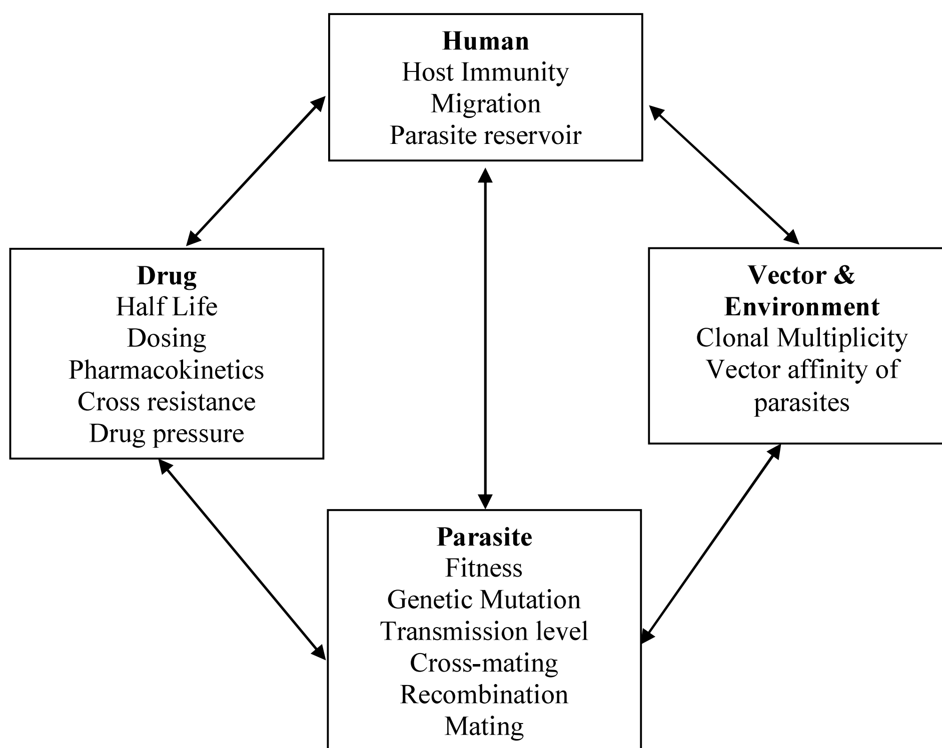


Fig. 1.6: Factors and characteristics affecting spread of malaria drug resistance

1.10.2.1 Biological Influences on Resistance

The residuum parasites that are able to survive treatment facilitate development of drug resistance. Factors that affect the ability of immune system in clearing the residuum parasite also contribute to the development of drug resistance. When parasites are cycled in non-immune individuals the non-specific immune response in these individuals is less effective than specific immune response in semi-immune individuals which contribute to the intensification of drug resistance. This also explains poorer treatment response among young children and pregnant women. Moreover, infection with human immunodeficiency virus (HIV) and malnutrition facilitate the development and intensification of drug resistance. Some combinations of resistant parasites and vector species enhance transmission of drug resistance while others inhibit it. Also,

some antimalarials facilitate development of resistance to others. The use of drugs having long life increases the likelihood of developing resistance due to prolonged elimination periods. Recrudescent or reinfecting parasites that are exposed to drug levels that are high enough to exert a selective pressure but cannot provide prophylactic or suppressive protection also intensifies development of drug resistant parasites. When two drugs which are used in combination have different half-lives, it plays a significant role in development of drug resistance due to mismatched pharmacokinetics (Bloland, WHO, 2001).

1.10.2.2 Programmatic Influences on Resistance

Programmatic influences on resistance include overall drug pressure, inadequate drug intake (poor compliance or inappropriate dosing regimens), pharmacokinetic and pharmacodynamic properties of the drug or drug combination, and drug interactions. Reliance on presumptive treatment and mass drug administration has the greatest impact on drug resistance. Ease of access and greater use of drugs also play a significant role in development of drug resistance. Confusion over proper dosing regimen and concurrent treatment with other drugs increases the likelihood of treatment failure and development of drug resistance. Use of counterfeit drugs or deterioration of drugs due to inadequate handling and storage, drugs may not contain sufficient quantities of the active ingredients which also facilitates development of drug resistance (Bloland, WHO, 2001).

1.10.3 Mechanism of Development of Drug Resistance

Generally, spontaneous mutations is the main cause of development of drug resistance to a given drug or a class of drugs. Sometimes, single point mutations whereas at other

times multiple mutations are required to confer resistance. If these mutations are not deleterious for the survival or reproduction of the parasite, drug pressure will remove sensitive parasites while resistant parasites multiply establishing its population. This scenario will reverse only when long persisting drug pressure is removed (Bloland, WHO, 2001).

In low transmission areas, drug resistance emerges *de novo*. The emergence of resistance can be considered in two parts: first the initial genetic event which produces the resistant mutant, and consequently the selection process wherein the survival advantage in the presence of the drug leads to preferential transmission and the spread of resistance. The first event for the development of resistance is termed as *'de novo'* (White and Pongtavornpinyo 2003)

1.11 Artemisinin Resistance: Definition and Debates

Artemisinin resistance is an important topic to be addressed in a fight against malaria. To properly assess and address the danger posed by artemisinin resistance and enable appropriate measures, definitions of 'artemisinin resistance' and 'ACT resistance' are needed at both, clinical and parasitological levels (Ferreira et al. 2013). On the one hand there are arguments that artemisinin resistance should be correlated with treatment failure (TFACT) for it to be called as 'artemisinin resistance' and that calling 'delayed PCT' as sole marker for artemisinin resistance is not appropriate. The report also claims that artemisinin treatment failure is best assessed later and it is not associated with prolonged PCT (Krishna and Kremsner 2013). This argument was supported by valid and logical facts. Artemisinin in monotherapy should be given for 7 days while in combination it is only given for 3 days which is supposedly considered as incomplete

treatment increasing the risk of treatment failure and this should be not be called as resistance (Krishna and Kremsner 2013). This report also cited example of the study where >50% patients had delayed PCT but >94% of the patients were cured at the end of 28-day follow-up of the study, dissociating delayed PCT with treatment failure while stressing on assessing markers for partner drug (Krishna and Kremsner 2013). The other questionable arguments which emphasizes which dissociates delayed PCT and artemisinin resistance are like patients who have undergone splenectomy have prolonged PCT, the state of the parasites which circulate for >72 hours without increased risk of treatment failure is not known, the viability of the parasites where delayed PCT is correlated with treatment failure, lack of conventional *in vitro* correlates, declining efficacy of partner drug while insufficient dosing with artemisinin, etc. According to Krishna and Kremsner (2013), in order to get a complete picture of artemisinin resistance, the markers of drug resistance for partner drug should be assessed essentially to predict outcome. For the same, *in vitro* assay should be correlated with markers of partner drug and treatment response in individual patients should be assessed (Krishna and Kremsner 2013). However, on the other hand, there is an opinion that waiting till the time when artemisinin resistance would turn into treatment failures would be irresponsible (Dondorp and Ringwald 2013). According to this latter group of researchers, development of artemisinin resistance is clearly a danger at present because of the fact that efficacy of artemisinin has declined in reducing the parasite burden to 10^2 fold instead of initial 10^4 fold (Dondorp and Ringwald 2013). Slow clearance of parasitaemia renders large parasite burden of around 10^8 parasites exposed to weaker partner drug thereby increasing the probability of development of resistance to the weaker partner drug and if that happens ACTs

would start to fail (Meshnick 2012). For example, in Cambodia, mefloquine resistance reduced the 42 day PCR corrected cure rate after treatment with AS+MQ. Also, at Thai-Myanmar border prolonged clearance coincided with declining efficacy of ACT. Moreover, in Western Cambodia, high failure rates with DHA+PQ are seen when >50% patients showed persistence of parasitaemia after 3 days of ACT treatment (Dondorp and Ringwald 2013). Still after much of this debate, delayed PCT finds room in WHO's definition of artemisinin resistance where suspected resistance is defined as an increase in parasite clearance time as evidenced by ≥10% of cases with parasites detectable on day 3 after treatment with an ACT, an increase in parasite clearance time, as evidenced by greater than 10% of cases with parasites detectable on day 3 following treatment with an ACT and confirmed resistance is defined as a treatment failure as evidenced by presence of parasites at day 3 and either persistence of parasites on day 7 or recrudescence after day 7 of parasites within 28/42 days, after treatment with an oral artemisinin-based monotherapy, with adequate blood concentration (WHO 2014). Another report argues that falciparum malaria became resistant to every other drug, then artemisinin is no different and artemisinin resistance seems to be developing in the same geographical areas at Thai-Cambodia border where CQ, SP and MQ resistance had developed (Meshnick 2012). Same ACTs clears parasite slowly than earlier as when artemisinin was introduced. Though the same report describe messy inconsistencies related to artemisinin resistance that there is little evidence of ACT clinical failure caused by artemisinin resistance (Meshnick 2012) and every definition of drug resistance includes clinical failure (WHO 2002). There are evidences where all the patients with delayed PCT were clinically cured at the end of the study and *in vitro* studies revealed these isolates to be susceptible to artemisinin (Dondorp et al. 2009,

Phyo et al. 2012, Noedl et al. 2008). Cheeseman et al. (2012) described that delayed PCT is associated with specific parasite genotype which is heritable. The counter perspective marks the increased failure rate of AS+MQ of nearly 30% at North West border of Thailand. It also highlights the reason why clinical efficacy does not translate to marked *in vitro* susceptibility changes because mostly *in vitro* tests are done on mature parasite stages when ring stage susceptibility is lost. This also results in delayed parasite clearance and reduced overall parasiticidal effect (White 2012). Hoping but not relying on fact that artemisinin resistance may not occur is good (White 2012). ACT failure from partner drug may occur but this does not explain the reduced efficacy of the combination AS+MQ which reduced to six times as compared to when ACTs were introduced in the year 1994. The failure rate of MQ in the year 1994 was 50% yet addition of artesunate reduced it to 5% which is not the same as now. This is clearly due to artemisinin and not just MQ. This could be marked as declining susceptibility of artemisinin partner. Delayed PCT could be a harbinger of disaster or may be a false alarm (White 2012) but due to very limited safe antimalarials possessing qualities like that of artemisinin derivatives but raising the alarm is appropriate to adopt cautious approaches to delay resistance to this highly valuable antimalarial drug.

1.12 Molecular Markers: Delayed PCT, Parasite Clearance Curve, Factors Affecting PCT, *Pfatpase6*, *Pfmdr1* Copy Number, K13

Confirmed molecular marker to monitor artemisinin resistance is not known as yet (Pantaleo et al. 2015). In the past, attempts have been made and are still being made to describe artemisinin's mode of action and the molecular markers associated with it. The only success till now is the evidence for the association of mutations in Kelch 13 propeller domain with artemisinin resistance (Ariey et al. 2014, Miotto et al. 2015). The

putative molecular candidates associated with artemisinin resistance are being described here:

1.12.1 Delayed PCT

Artemisinin is a fast acting drug which is known to rapidly reduce the parasite biomass within 48 hours of administration. Slow clearance results in the presence of parasites after 72 hours of drug administration. This phenomenon is termed as 'delayed parasite clearance time' (delayed PCT) and it has been associated with artemisinin resistant malaria. Delayed PCT has remained a debatable topic amongst the peers and is subject to a number of caveats at present. In Thailand and Cambodia too, the basis for categorizing Plasmodium strains as artemisinin resistant was based on parasite clearance time where the median parasite clearance time was 84 hours (interquartile range, 60 to 96) and 48 hours (interquartile range, 36 to 66) in Pailin, western Cambodia and Wang Pha, northwestern Thailand, respectively (Dondorp et al. 2009). This being an alarming situation, containment efforts were initiated (WHO 2011) since it appeared in the same region which was the epicentre of development of drug resistant malaria. Therefore, delayed PCT is considered as a marker trait to monitor artemisinin resistant malaria.

1.12.2 Parasite Clearance Curve

The graphic plot of the parasite densities following the start of antimalarial treatment is termed as parasite clearance curve. Parasite clearance rates are important measures of antimalarial drug efficacy particularly in assessment of artemisinin resistance which accelerate the ring stage clearance. The focus of therapeutic assessment is the slope of

the log linear segment in the middle of parasite clearance curve that has the least inter-individual variance (White 2011).

1.12.2.1 Factors Affecting Parasite Clearance Curve

There are various factors which affect parasite clearance curve which are being discussed here (White 2011)

1.12.2.1.1 Frequency of Sampling

To define individual parasite clearance profiles, sampling needs to be done at least twice daily and to define lag phase, sampling needs to be done at 6 hourly interval.

1.12.2.1.2 Parasitaemia

Peripheral blood sample in case of *P. falciparum* does not give a clear picture of total parasite burden. In *P. falciparum* infection, only one third of the parasitized red blood cells circulate freely in the 48-hour asexual cycle and rest are sequestered. Counts may vary over four orders of magnitude from approximately 100 to 1,000,000 parasitized erythrocytes/µl of blood. As parasite clearance is a first order process, the higher the initial parasitaemia, the longer the parasites will take to become undetectable i.e. the longer the PCT.

1.12.2.1.3 Stage of Development

Since *Plasmodium falciparum* has the tendency to sequester, mature parasites are seldom seen in the peripheral blood. In synchronous infection, large rings are sequestered resulting in abrupt fall of parasitaemia and onset of fever. Similarly, when schizonts rupture, it releases tiny rings which increases fever resulting in apparent slowing of initial parasite clearance.

1.12.2.1.4 Drug Effects

Drug effects are characterized by the stage of parasite it clears, like most antimalarials clear the more mature trophozoite stages of parasite. Furthermore, till the time the antimalarial reaches the therapeutic levels, there is a variable delay in drug absorption and hence, it might affect the parasite clearance rates.

1.12.2.1.5 Host Defences

Host defences are related with non-specific and specific immune responses to malaria. Haemoglobinopathies and G6PD deficiency contributes significantly against malaria. Immunity enhances therapeutic responses. Inter-individual differences in anti-malarial pharmacokinetics, splenic function, other non-specific host defences and any acquired immunity may alter therapeutic response.

1.12.3 ***Pfatpase6***

Pfatpase6 is a 139.4 kDa protein which comprises of 1228 amino acids. It is believed that *Pfatpase6* which is a mammalian orthologue of Sarco endoplasmic reticulum Ca^{2+} -ATPase (SERCA), which regulates calcium homeostasis in *P. falciparum* (Kimura et al. 1993, Varotti et al. 2003). Calcium is an important intracellular messenger controlling range of cell functions like host cell invasion and motility in apicomplexan parasites. The cytosolic concentration of calcium in Plasmodium and also in the uninfected RBC is low (Nagamune et al. 2008). To overcome this, Plasmodium has compartment similar to mammalian endoplasmic reticulum to store calcium (Garcia et al. 2008).

Though the overall homology between *Pfatpase6* and mammalian SERCA is relatively low, the key residues related with calcium transport is conserved in both of them (Kimura et al. 1993). Further, it was demonstrated that SERCA inhibitor, thapsigargin,

inhibits *Pfatpase6* too, suggesting that *Pfatpase6* is functionally related to higher mammal homologues and that it is involved in calcium homeostasis in *P. falciparum* (Varotti et al. 2003). Moreover, thapsigargin is also a sesquiterpene lactone like artemisinin. Hence, it was thought that artemisinin acts by inhibiting *Pfatpase6.* This hypothesis was supported by the experiments carried out in *Xenopus laevis* where artemisinin specifically inhibited *Pfatpase6*, as thapsigargin. Therefore, *Pfatpase6* was suggested to be a target of artemisinin (Eckstein-Ludwig et al. 2003). The key residues in the transmembrane domains M3, M5 and M7 determines SERCA and thapsigargin binding in humans. Mutations within these domains affects this binding (Xu et al. 2004). Homology modeling and docking studies suggests that these residues are also important for artemisinin binding to *Pfatpase6* (Jung et al. 2005). Residue 263 was shown to be important for inhibition of artemisinin by *Pfatpase6* (Ulhemann et al. 2005), however, natural variant of this mutation could not be found as yet (Kwansa-Bentum et al. 2011; Tanabe et al. 2011) which indicates that this is a very well conserved and functionally important region of the protein. Natural variant of *Pfatpase6* was reported when in French Guiana, S769N SNP was associated with decreased *in vitro* susceptibility to artemether (Legrand et al. 2008) but this could not be confirmed in subsequent studies (Jambou et al. 2005; Pillai et al. 2012; Zakeri et al. 2012). The combination of additional two SNPs, E431K and A623E from Senegal was associated with increased IC_{50} with artemether in fresh isolates (Jambou et al. 2010). This has been reported from several other studies conducted in context with artemisinin resistance.

1.12.4 *Pfmdr1* Copy Number

Variations in *Pfmdr1* gene copy number has been shown to affect susceptibility of mefloquine and artemisinin in *Plasmodium falciparum* (Carrara et al. 2009, Lim et al.

2009, Price et al. 2004). At Thai-Cambodia border, amplifications in *Pfmdr1* gene copy number was reported as a molecular marker for artesunate-mefloquine treatment failure. The trait has also been reported by Pickard et al. (2003) in Southeast Asia and was regarded as an important surveillance tool for monitoring mefloquine and artemisinin resistance.

1.12.5 Kelch 13 Propeller Domain

In the year 2014, PF3D7_1343700 kelch 13 propeller domain ('K13-propeller') was discovered as an affirmative molecular marker for artemisinin resistance (Ariey et al. 2014). Whole genome sequencing of artemisinin resistant cell lines from Africa and clinical isolates from Cambodia reveals that mutant K13-propeller allele correlates with *in vitro* parasite survival rates and *in vivo* parasite clearance rates (Miotto et al. 2015). Further analysis on some parasite populations revealed that polymorphism in certain molecular markers like fd (ferredoxin), arps10 (apicoplast ribosomal protein S10), mdr2 (multidrug resistance protein 2) and crt (chloroquine resistance transporter) forms the genetic background on which K13 mutations are likely to arise indicating the involvement of specific predisposing genetic factors in parasite population (Miotto et al. 2015). Emergence of mutations in K13 seems to be a recent phenomenon as there was difference in two studies carried out in Dakar, Senegal where the one study which was carried out in the year 2012 and 2013 showed wild type K13 while the other conducted in the isolates from year 2013 and 2014 showed mutation in propeller domain of K13 (Boussaroque et al. 2015). In Southern China, a single mutation in F446I was associated with delayed PCT (Huang et al. 2015). Very recently high frequency of K13 mutations and amplification in *pfmdr1* copy number was reported across Myanmar in context with artemisinin resistance (Win et al. 2016). Various other studies have tried and found out

mutations in kelch13 gene with reference to artemisinin resistance. In India, Mishra et al. (2015) first reported mutations in kelch13 in four isolates from northeastern states of India but it did not correlate with ACT treatment failure. Since the frequency of kelch13 mutations were significantly low and it seems to be a recent phenomenon, hence it opens doors for further studies in the country.

1.13 Contributions made by this Study in Increasing the State of Knowledge

Artemisinin resistance studies are needed to be conducted as a regular practice in view of *Plasmodium falciparum* history of developing drug resistance to every other drug. As briefed before, NE states of India have remained as an epicentre to report initial cases of development of drug resistance. Furthermore, since confirmed molecular marker to study artemisinin resistance is not known as yet and owing to the fact that artemisinin stays as most important antimalarial for the treatment of *P. falciparum* infections, regular monitoring and containment of spread of drug resistance towards artemisinin is the only way left to battle the deadly malaria. In urge of this, WHO, too, in collaboration with its stakeholders has launched the Global Plan for Artemisinin Resistance Containment (GPARC) under the Roll Back Malaria (RBM) Programme in the year 2011 (WHO, 2011). The threat of emerging artemisinin resistance and the need to contain it was well addressed by Dondorp et al. (2011). It was also said that elimination of artemisinin resistant malaria using ACT can only be achieved by eliminating all malaria since "the last man standing is most resistant" which, if not contained, will increase its population (Maude et al. 2009), making the situation even more difficult. It would be prudent to assume the worst and be cautious before the clock ticks (White 2010). In view of all this, number of parameters needs to be studied to monitor artemisinin resistance whereby deployment of sensitive and sophisticated tools

like Real time PCR, studying putative molecular candidates, and correlation of clinical and molecular data seemed fruitful to us.

With this background we designed this study and found our results really interesting and more so revealing. The study has put light on important aspects of artemisinin resistance whereby it revealed that delayed PCT is indeed associated with artemisinin resistance and it needs to be monitored continuously with the help of sensitive tools like herein. It also gave insights that important SNPs of *Pfatpase6* gene need to be monitored on a regular basis in malaria endemic sites to find its conclusive association with artemisinin resistance or its mode of action.

Chapter II

Monitoring of Parasite Clearance Time in *Plasmodium falciparum* Infected Malaria Patients Treated with ACT in Selected Endemic Sites of the Country

2.1 Origin of the study

Artemisinin resistance is currently a burning issue being addressed by researchers all across the globe. Since artemisinin is an important and currently widely used drug to treat life threatening *P. falciparum* infections, losing it to resistance may pose a bigger challenge to combat the deadly malaria caused by *P. falciparum*. Moreover, mechanism of action of artemisinin is still a debatable topic. Dondorp et al. (2009) have already reported presence of *P. falciparum* strains at Thai-Cambodia border which were shown to have delayed PCT after treatment with artesunate and these strains were thereby concluded to be artemisinin resistant. Besides, India shares border with malaria endemic neighbouring country, Myanmar towards the East which is close to this epicentre of drug resistant malaria and where lowering of sensitivity to artesunate and development of resistance has been confirmed. Owing to the fact that India is a malaria endemic country, the threat of development and/or introduction of artemisinin resistant *P. falciparum* clones is more pronounced here, and hence, this study finds its importance. Studying delayed parasite clearance time (PCT) is the best way to capture emerging artemisinin resistance in the north east and other highly *P. falciparum* endemic regions of the countries which are at risk of developing resistance to this important drug. With this aim, the present study was undertaken.

2.2 Objective

With this objective under consideration, parasite clearance time was assessed in *Plasmodium falciparum* infected patients treated with ACT (AS+SP) with the help of

two techniques, i.e. microscopy, the gold standard for diagnosis, and a more sensitive, Real time PCR. The two study sites were chosen based on the endemicity and the history of origin of drug resistant malaria. These include Bisra Community Health Centre (CHC), district Sundergarh, Odisha and Miao Public Health Centre (PHC), district Changlang, Arunachal Pradesh.

2.3 Materials and Methods

2.3.1 Ethics Statement

The study was approved by the Institutional Ethics Committee (IEC) of National Institute of Malaria Research, New Delhi. The present cross-sectional study is a part of larger study on monitoring therapeutic efficacy of anti-malarial drugs in India (Mishra et al. 2012). Before enrolment, informed consent was obtained in all the cases. In case of subjects who were minor, informed consent was obtained from parent/guardian (Annexure I).

2.3.2 Study Sites

One of the study site was Bisra Community Health Centre (CHC), Sundergarh district, Odisha. The sample collection and follow-up was done during Jun-Sep, 2012. Another study site was selected as Miao Primary Health Centre (PHC), Changlang district, Arunachal Pradesh. The sample collection and follow-up at this site was done during Sep.-Oct., 2012. Fig. 2.1 represents the sites in the proposed states where the study was conducted.

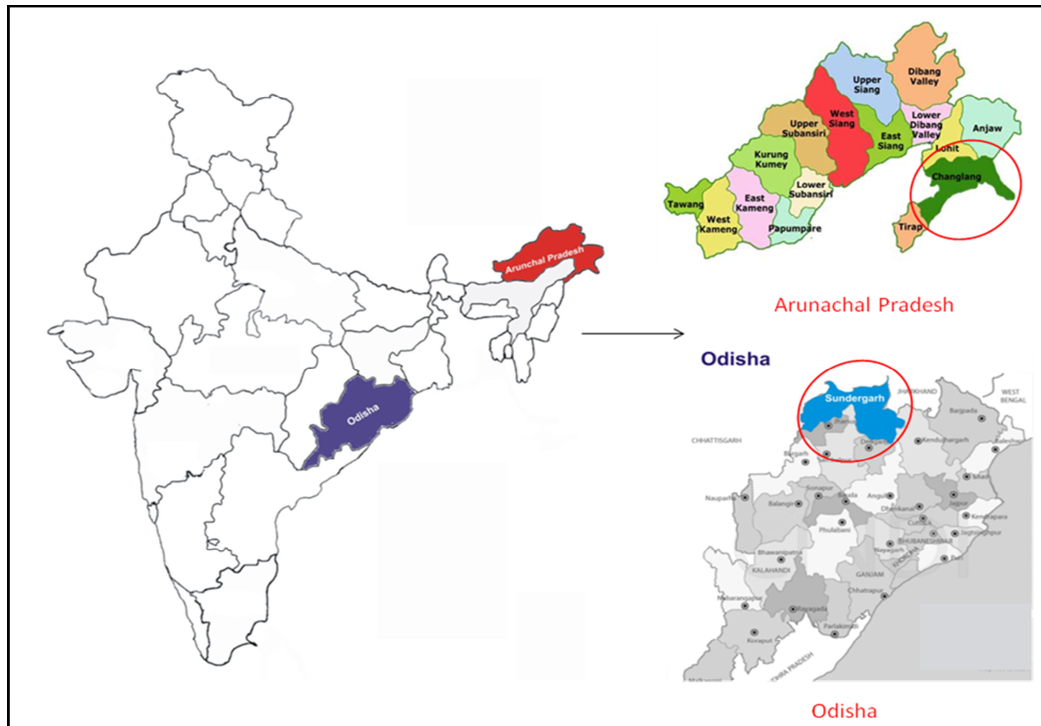


Fig. 2.1: Study sites at Odisha and Arunachal Pradesh where the study was conducted

2.3.3 Sample Collection

All the suspected malaria patients were screened for *Plasmodium falciparum* infection by microscopy and, in some cases with both RDT and microscopy. Those found positive were enrolled in the study after following all the inclusion and exclusion criteria (listed below).

Inclusion Criteria

- Patients aged above 6 months
- Mono-infection with *Plasmodium falciparum*
- Parasitaemia in the range of 1000 to 100000/µl
- Axillary temperature ≥37.5˚C or history of fever during the previous 24 hrs
- Able to come for the stipulated follow-up visits; easy access to the health facility
- Informed consent by the patient or by parent/guardian for children

Exclusion Criteria

- Presence of one or more of the general danger signs or any signs of severe malaria
- Presence of mixed infection
- Presence of severe malnutrition
- Presence of febrile conditions caused by diseases other than malaria
- Contradictions related to the antimalarial drugs used, especially history of allergy
- Pregnancy

After instituting informed consent and screening, blood samples were collected which included a slide for microscopic examination in the form of a blood smear and a filter paper blood spot which was dried for storage and subsequent isolation of genomic DNA for molecular analysis, on day 0 of infection/enrolment. ACT (AS+SP) was administered as per the dosing regimen and further sampling was done in the similar manner on day 1, 2, 3 and then weekly on day 7, 14, 21, 28, 35 and day 42 of follow-up as per the WHO schedule (WHO 2009). Though WHO prescribes follow-up of patients for 28 days after administration of AS+SP, for academic purposes, herein the patients were followed upto day 42 to detect late treatment failure.

2.3.4 Microscopy

Thick and thin smears of patient's peripheral blood were prepared for microscopic analysis. Both thick and thin smears were stained using Giemsa stain and observed under 100X oil immersion lens. Parasites were counted against 200 WBCs in thick smear, if the presence of the parasite is detected. A blood slide was considered negative when examination of 1000 white blood cells or 100 fields of thick smear reveals no asexual parasites. A count of parasite per µl of blood was obtained by calculating

parasitaemia against 8000 WBCs. The formula used for calculating parasitaemia is given below:

$$\text{Parasite/}\mu\text{l of blood} = \frac{\text{No. of parasites counted}}{200} \times 8000$$

2.3.5 Data Entry and Quality Check

Clinical and demographic details of the samples were double entered in WHO therapeutic efficacy database (WHO 2009) to confirm the entry. The WHO sheet compares Entry1 and Entry 2 automatically after entry. The word 'TRUE appeared in the check cell if data in both the sheet is same, in case of discrepancy, the word 'FALSE' appeared. In this case, the data was cross checked to verify the entry. Quality check of the blood smears was done for 10% of the randomly selected slides from the study site at Odisha with the help of another expert microscopist. Those samples which were found positive on day 3 by Real time were again cross checked with microscopy to confirm the findings. 100% of the slides were cross checked for the study site at Arunachal Pradesh due to high failure rate of the drug, AS+SP.

2.3.5.1 Kaplan-Meier Survival Estimates

Kaplan-Meier survival estimates was done using WHO therapeutic efficacy database (WHO 2009). This data sheet has been developed by WHO Global Malaria Programme to facilitate entry and interpretation of the data collected during therapeutic efficacy tests. In this, the Entry1 is the main data entry page where site information (like name of health facility, locality, district, province, date of the study) treatment information (name of drug/drug combination, manufacturer, batch number, expiry date, dosage) and patient data (ID, place of residence, age, sex, weight, height, drug, total dose, fever, etc.) are entered (Fig. 2.2).

World Health Organization 2008

A — Name of health facility / Locality / District / Province / Date of study from ... to

B — Name of drug or drug combination / Manufacturer / Batch number / Expiry date / Dosage per tablet

C

No	ID	Place of residence/ study sites	Age (months)	Age (years)	Age Category	Sex	Weight (kg)	Height (cm)	Drug	Total dose (tablets or mg)	Previous antimalarial intake	Date D0	History of fever D0	Temp D0	Para D0	Gametocy D0
1																
2																
3																
4																
5																

Source: http://apps.who.int/iris/bitstream/10665/44048/1/9789241597531_eng.pdf

Fig. 2.2: Data entry sheet developed by WHO for conducting Therapeutic Efficacy Tests

The variables like history of fever, temperature, parasitaemia, gametocytes and danger sign/severe malaria, are collected repeatedly throughout the 42-day schedule as summarized in the chart below (Fig. 2.3).

	Day										
	0	1	2	3	7	14	21	28	35	42	Unsched*
Danger sign/severe malaria**		•	•	•	•	•	•	•	•	•	•
History of Fever	•				•	•	•	•	•	•	•
Temp	•	•	•	•	•	•	•	•	•	•	•
Para	•	•	•	•	•	•	•	•	•	•	•
Gametocytes	•				•	•	•	•	•	•	•

**Unsched=unscheduled day, day/date of unscheduled day must be recorded*
***complete information on danger signs and corresponding parasitaemia should be recorded so as to decide on treatment failures.*
Source: http://apps.who.int/iris/bitstream/10665/44048/1/9789241597531_eng.pdf

Fig. 2.3: Schedule described by WHO to collect the data over the 42-day period

The data was double entered in this sheet to confirm the entry and thereby PCR corrected and uncorrected cure rate was found out.

2.3.6 Genomic DNA Isolation

Genomic DNA was isolated from three punched out filter paper blood spots using QIAamp® DNA Blood Mini Kit (Qiagen) as per the manufacturer's instructions. DNA

was isolated for all the study samples from both the study sites for molecular analysis. Isolation of DNA was done on day 0, 1, 2 and day 3 of infection for analysing PCT by Real time PCR. DNA was also isolated from 3d7 and Dd2 strain of *Plasmodium falciparum* which were procured from the malaria parasite bank of the National Institute of Malaria Research, New Delhi which served as controls.

2.3.7 PCR for Checking Preparation of gDNA

To check the preparation of genomic DNA, end point PCR was set up with primers designed for Real Time PCR. In addition to 3D7 and Dd2, MRC2 and RKL9 positive control strains from Parasite bank of NIMR were also included. The cross reactivity of the primers was checked by setting up PCR on the parasite DNA (MRC2, RKL9, Dd2 & 3d7) and the human DNA (uninfected human blood) with both set of primers i.e. those specific to parasite and to human. It was confirmed, thereby, that the primers were specific since human DNA could only be amplified using Human *β tubulin* primers while parasite DNA could be amplified using parasite specific primers (Fig. 2.4).

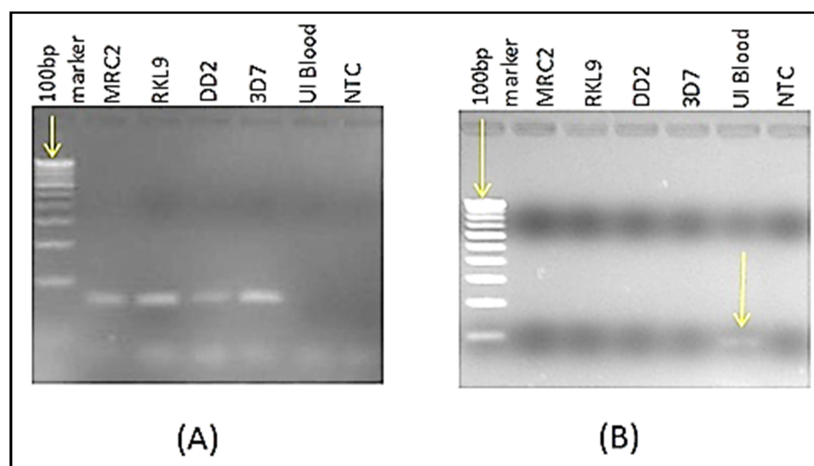


Fig. 2.4: (A) Gel picture showing amplification of parasite DNA by Plasmodium specific primers where uninfected human blood DNA served as NTC; (B) Amplification of Human DNA by Human tubulin primers, here Plasmodium DNA served as NTC; NTC denotes no-template control, UI denotes uninfected human blood

2.3.8 Real time PCR

Standardization of Real Time PCR was done on Roche's LightCycler® 480 using SYBR green dye. The primers, probes (listed in Table 2.1) and the cycling conditions were used as described elsewhere (Beshir et al. 2010). Standard curve was plotted for checking the efficiency of primer for parasite target gene, *pgmet* and human endogenous control, *β- tubulin* by preparing 10 fold dilutions (a total of five dilutions) of DNA isolated from 3D7 culture and uninfected human blood (UI), respectively. Melt curve analysis was done at 95°C for 10 sec, 65°C for 1 min and 95°C continuous, to check if the primers are specific i.e. amplifying specific products and not having dimers and thus, producing single peaks. Analyses of the data and plotting of these curves was done with the help of software LightCycler® 480 SW 1.5.

Table 2.1: Sequence of Primers and probes used for calculation of PCT by Real time

Gene name	Primer/Probe	5' Fluorophore	Sequence 5'→3'	Quencher	Reference
pgmet	PgMET_Forward		TGAAAGCAGCGTAGCTCAGA		Beshir et al. 2010
	PgMET_Reverse		CGCGTGGTTTCGATCCACG		
	PgMET_probe	FAM	GGGGCTCATAACCCCCAGGA	BHQ2	
β_tubulin	HumTuBB_Forward		AAGGAGGTCGATGAGCAGAT		
	HumTuBB_Reverse		GCTGTCTTGACATTGTTGGG		
	HumTuBB_Joe	JOE	TTAACGTGCAGAACAAGAACA GCAGCT	BHQ2	

Real Time PCR on patient's samples was conducted using SYBR green dye on Roche's LightCycler® 480. Primers were designed on *pgmet* gene of Plasmodium species as described elsewhere (Beshir et al. 2010) and *human tubulin* gene was used as

endogenous control. For every sample, two sets of reactions were set up, one for each gene. Each reaction comprised of 2x LightCycler® 480 SYBR Green Master (Roche), 0.3μM of each forward and reverse primer, 5μl of template DNA, adjusting the total reaction volume to 20μl with molecular grade water. The thermal profile included pre-incubation at 95°C for 6 mins; amplification at 95°C for 15 sec, 60°C for 1 min and 72°C for 10 sec (acquisition mode set as single at this temperature) for a total of 50 cycles. A cut off of 44 cycles was set to define positive samples. Every set up was accompanied by a No Template Control (NTC) for both sets of primers, to check on contamination and non-specific amplification.

Each reaction was set up in triplicate and the mean of the triplicate was taken for calculation of relative reduction in parasitaemia. Difference of more than 0.4 Ct between the two replicates was discarded.

2.3.9 End Point Diagnostic PCR

To confirm the findings of Real time PCR, single step diagnostic PCR was set up using two different methodologies as described by Patsoula et al. (2003) and Demas et al. (2011), respectively. The primers used for end point PCR have been listed in Table. 2.2. This PCR was done on every sample from Odisha study site which was positive on day 3 according to the results of Real time PCR. The PCR was set up on day 0, 1, 2 and 3 of infection to determine if they are positive up till day 3 by end point PCR also. The product of the amplification reaction which yielded a 346bp and 220bp Pf specific band, respectively, in case of positive samples was checked on 1.5% 2D agarose gel.

Table 2.2: Primers used for analyzing Pf specific band by end point diagnostic PCR

Primer	Primer sequence (5'→3')	Reference
Primary	Forward: CCATTTTACTCGCAATAACGCTGCAT	Demas et. al 2011
	Reverse: CTGAGTCGAATGAACTAGTCGCTAC	
Nested	Forward: CCGGAAATTCGGGTTTTAGAC	
	Reverse: GCTTTGAAGTGCATGTGAATTGTGCAC	
PL3	Forward: ATGGCCGTTTTTAGTTCGTG	Patsoula et al. 2003
PL5	Reverse: ACGCGTGCAGCCTAGTTTAT	

2.3.10 Data Analysis

For the Real time data analysis, assuming day 0 parasitaemia as 100%, relative decrease in parasitaemia was calculated using $2^{(-\Delta\Delta Ct)}$ with the help of LightCycler® 480 SW 1.5 and Microsoft Office Excel 2007. Preparation of graphs was also done using Microsoft Office Excel 2007.

2.4 Results

2.4.1 Sample Collection from Odisha and Arunachal Pradesh

A total of 72 and 43 samples were enrolled in the study from Bisra CHC, Sundergarh district, Odisha and Miao PHC, Changlang district, Arunachal Pradesh, respectively. The clinical and demographic details of the enrolled patients from the two study sites have been tabulated in Table 2.3.

Table 2.3: Baseline details of samples collected from Bisra CHC, Sundergarh district, Odisha and Miao PHC, Changlang district, Arunachal Pradesh

Characteristic		**No. of Patients**	
		Odisha	**Arunachal Pradesh**
Age	≤ 5 yrs	6	12
	5-20 yrs	33	22
	20-60 yrs	33	8
Sex	Male	42	24
	Female	30	18
Parasites density/µl (Day0 count)	≤ 10,000	26	16
	10,000-30,000	12	10
	30,001-80,000	24	14
	80,001-99,999	10	2
Outcome	Total enrolled	72	42
	ACPR	64	22
	LCF	-	2
	LPF	-	4
	WTH	8 (Due to loss to follow-up)	5 (increased to 14 after PCR correction)

2.4.2 Standardization of Real Time PCR

Standardization of Real time PCR was done for checking the primer efficiency by preparing 10 fold dilution curve of DNA isolated from 3D7 culture and uninfected human blood. The primer efficiency for the parasite gene, *pgmet*, came out to be 93% while that for the human endogenous control, *β-tubulin*, was found out to be 97%. Melt curve analysis has been done to check if the primers are not having dimers and thus, producing single peaks.

The amplification curve, standard curve and melt curve for parasite target gene *(pgmet)* (Fig. 2.5, 2.6, 2.7) and those for the human endogenous control *(β-tubulin)* (Fig. 2.8, 2.9, 2.10), respectively, are depicted as below:

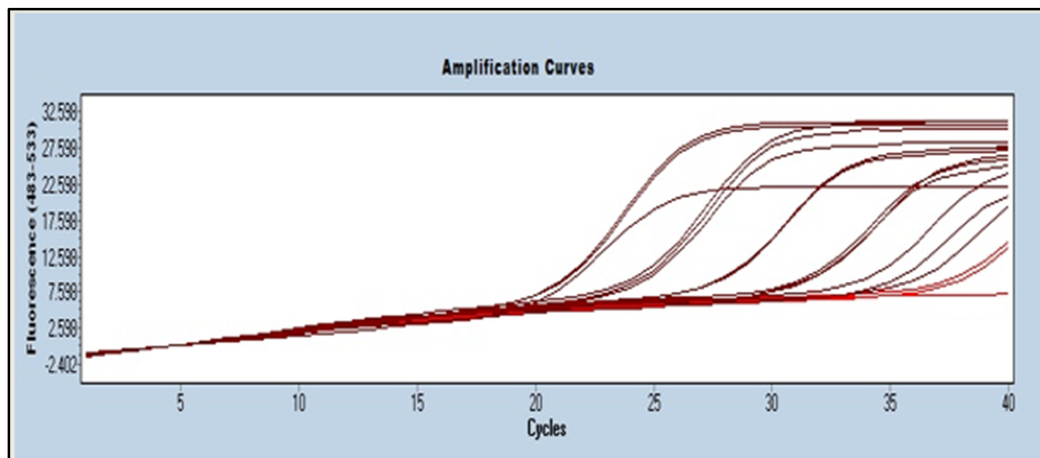


Fig. 2.5: Amplification curve for the parasite gene; *pgmet*

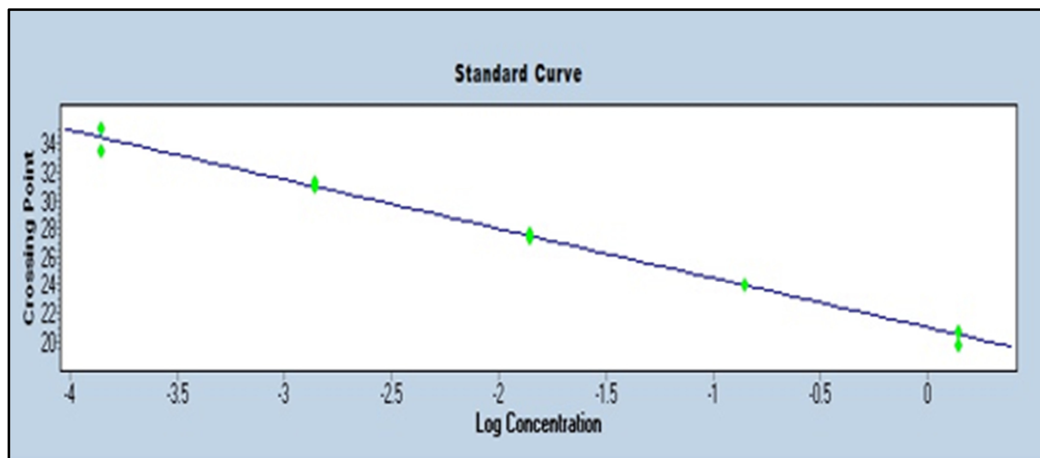


Fig. 2.6: Standard curve for the parasite gene; *pgmet*

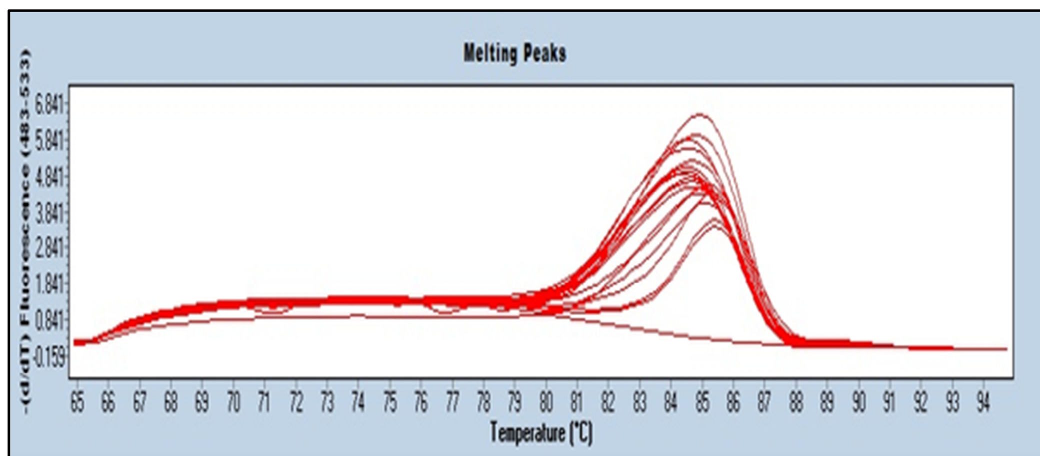


Fig. 2.7: Melting curve for the parasite gene; *pgmet*

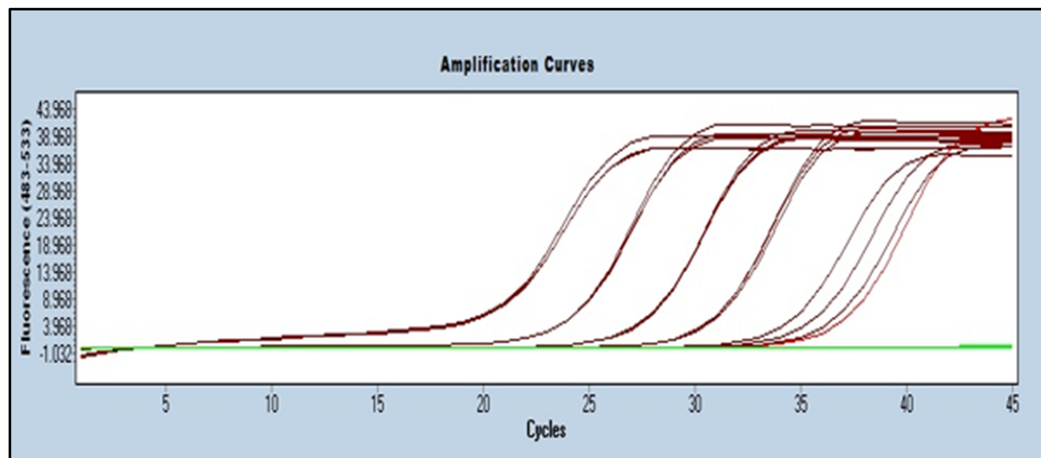


Fig. 2.8: Amplification curve for the human tubulin gene; *β-tubulin*

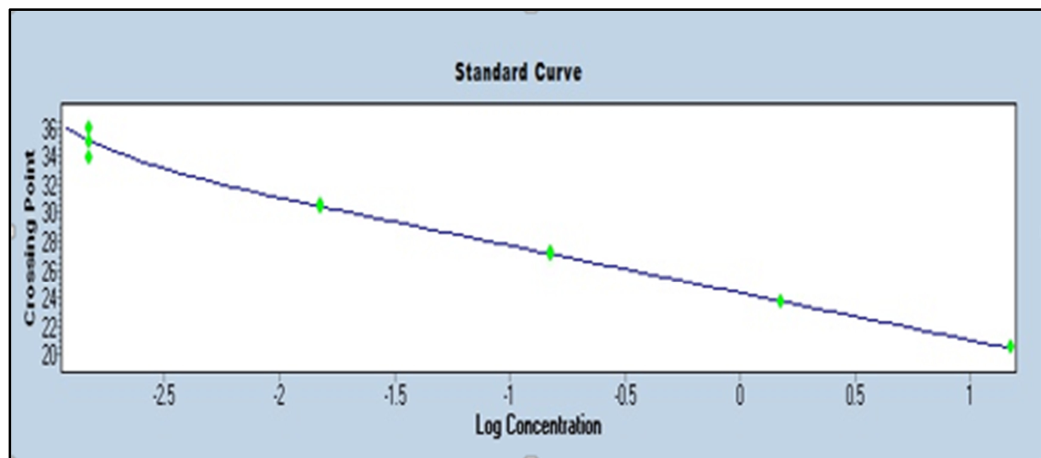


Fig. 2.9 Standard curve for the human tubulin gene; *β-tubulin*

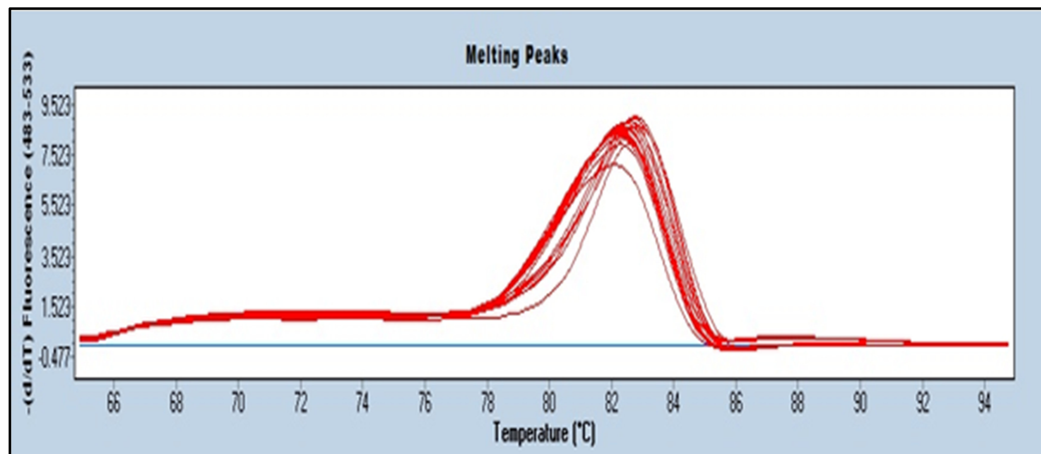


Fig. 2.10: Melting curve for the human tubulin gene; *β-tubulin*

2.4.2 Microscopic Analysis: Treatment Failures and ACPR

2.4.2.1 Bisra CHC, Odisha and Miao PHC, Arunachal Pradesh

The Kaplan Meier survival estimates from Odisha study site reveals the high efficacy of AS+SP in *P. falciparum* attaining 100% adequate clinical and parasitological response (ACPR) (defined in Annexure II). The PCR corrected and uncorrected cure rate therefore remains the same as 100%. However, high failure rate of 17.7% have been observed in Arunachal Pradesh. The PCR uncorrected and corrected cure rate was determined as 59.5% and 82.3%, respectively. After PCR correction, nine samples were found out to be Pf reinfection, four were late parasitological failure (LPF) while two were late clinical failure (LCF) (defined in Annexure II)

2.4.3 PCT Real Time

2.4.3.1 Odisha

Parasite clearance time was analysed and compared by microscopy and Real time PCR. Microscopic analysis of the PCT revealed none of the samples to be day 3 positive. Real time PCR was conducted on 43 of the total 64 samples as these samples had parasitaemia ≥10,000/µl of infected blood which was chosen as the baseline. The results of these 43 samples were compared and interestingly, it was found that 12 out of these 43 samples are day 3 positive according to Real time. The comparison of relative decrease in percentage log parasitaemia in these 12 samples as determined by these two techniques is depicted in Fig. 2.11. Surprisingly, all these 12 samples had high initial parasitaemia ranging from 52,800 to 98,000 parasites/µl of blood, mean parasitaemia being 72,750 parasites.

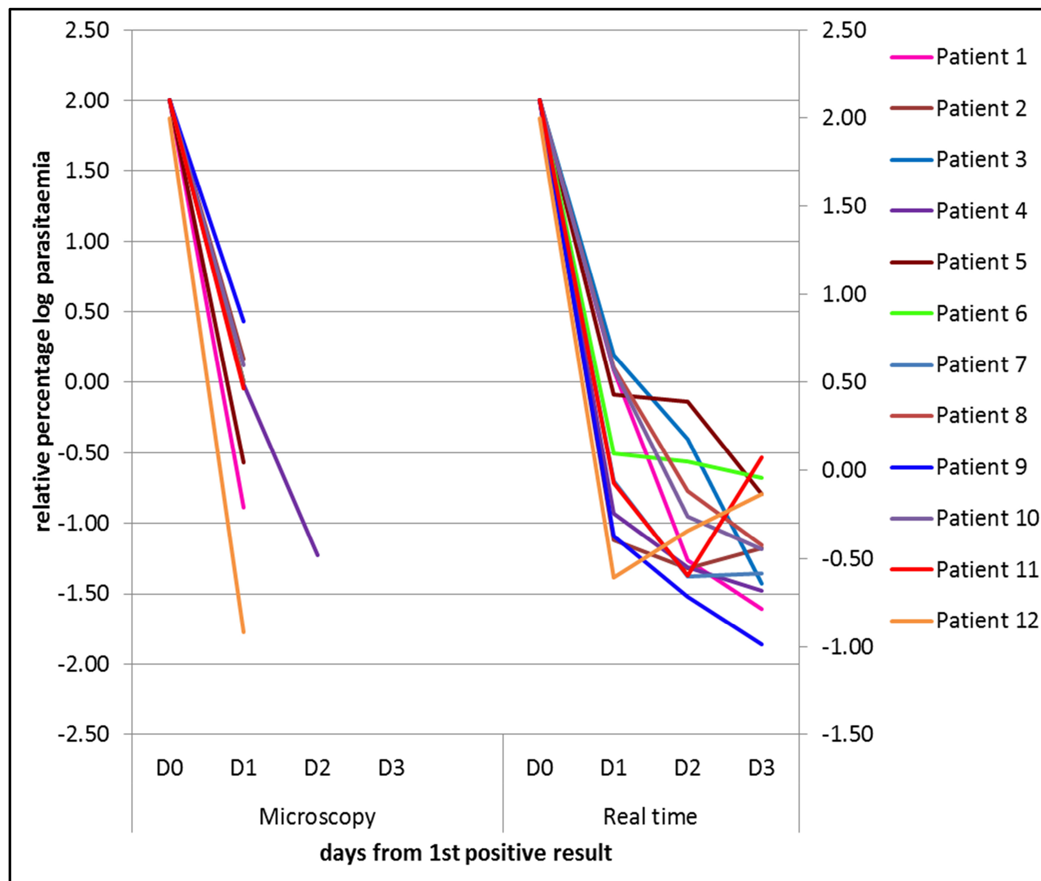


Fig. 2.11: Relative decrease in percentage log parasitaemia as determined by Microscopy and Real time in the representative samples from Odisha study site

2.4.4 Comparison: PCT Real Time and PCT Microscopy

Microscopic measures of PCT for the samples from Odisha revealed most of the patients to be parasite negative by the end of day 1, except three samples (7%) which were parasite positive upto day 2 (Fig. 2.12), while none of the samples had detectable parasitaemia on day 3. The graph below depicts the number of cases which cleared parasitaemia in less than 24 hours (negative on day 1 itself), within 24 to 48 hours (negative on day 2), within 48 to 72 hours (negative on day 3) and greater than 72 hours (positive on day 3).

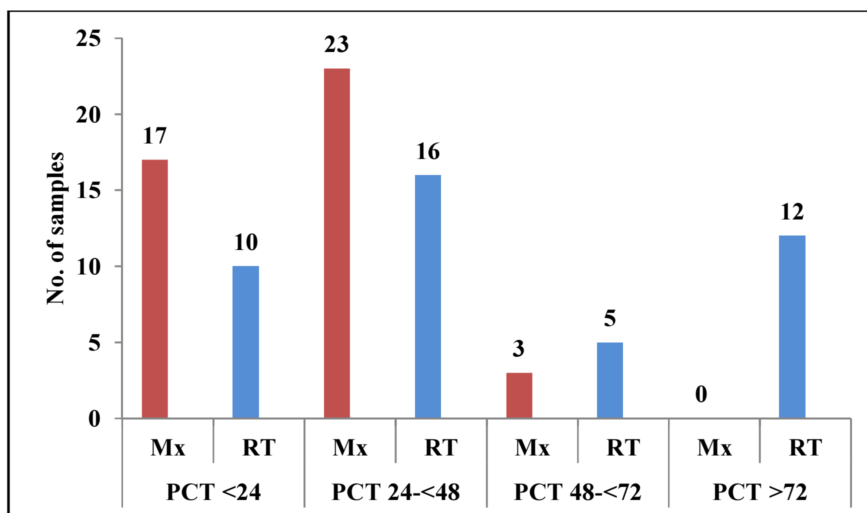


Fig. 2.12: Difference in PCT determined by Microscopy (Mx) and Real time PCR (RT) in samples from Odisha

Real time PCR revealed 17 cases (39.5%) to be parasite positive by the end of day 2 while 33 (76.7%) were positive on day 1 in comparison to 26 cases (60.5%) according to microscopic data. The total difference in the analysis done for 43 samples for sensitivity of detection of parasitaemic patients as determined by the two methods is highlighted in Fig. 2.13.

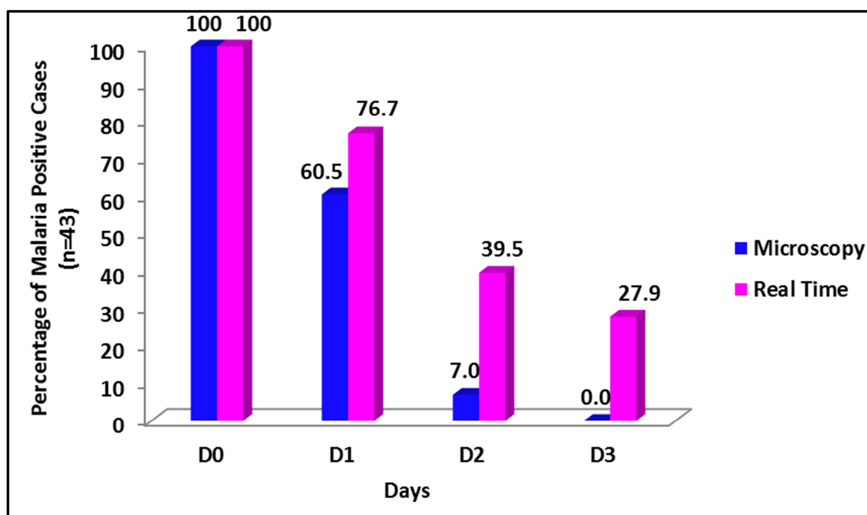


Fig. 2.13: Comparison of percentage decrease in malaria positive cases after treatment with AS+SP as determined by microscopy and Real time PCR at Sundergarh district, Odisha

The results clearly indicate higher sensitivity of Real time PCR over microscopy in detecting the threshold parasitaemia. Table 2.4 shows the relative percentage decrease in parasitaemia, for the 12 samples which were found to be parasite positive on day 3 by Real time PCR, from day 0 to day 3 after the administration of the drug as determined by microscopy and Real time PCR.

Table 2.4: Relative decrease in percentage parasitaemia over three days of ACT administration in the representative samples from Odisha as per Microscopy and Real time PCR

Sample ID	MXD0	MXD1	MXD2	MXD3	RTD0	RTD1	RTD2	RTD3
Patient 1	100.00	0.13	0.00	0.00	100.00	1.22	0.05	0.02
Patient 2	100.00	1.46	0.00	0.00	100.00	0.08	0.05	0.07
Patient 3	100.00	0.00	0.00	0.00	100.00	1.56	0.40	0.04
Patient 4	100.00	0.96	0.06	0.00	100.00	0.12	0.05	0.03
Patient 5	100.00	0.27	0.00	0.00	100.00	0.83	0.73	0.16
Patient 6	100.00	0.00	0.00	0.00	100.00	0.32	0.27	0.21
Patient 7	100.00	0.00	0.00	0.00	100.00	0.20	0.04	0.04
Patient 8	100.00	0.00	0.00	0.00	100.00	1.28	0.17	0.07
Patient 9	100.00	2.72	0.00	0.00	100.00	0.08	0.03	0.01
Patient 10	100.00	1.33	0.00	0.00	100.00	1.23	0.11	0.07
Patient 11	100.00	0.90	0.00	0.00	100.00	0.19	0.04	0.29
Patient 12	100.00	0.12	0.00	0.00	100.00	0.25	0.45	0.73

MX=Microscopy, RT=Real time; D0= day 0, D1=day 1, D2=day 2, D3=day 3

2.4.5 End Point Diagnostic PCR

To further confirm the findings of Real time PCR, diagnostic PCR was conducted using two different set of Pf specific primers (listed in Table 2.2) (Patsoula et al. 2003, Demas et al. 2011, respectively) on day 0, 1, 2 and day 3 samples of the 12 cases from Odisha study site which were found to be day 3 positive by Real time. Significantly, 10 out of these 12 samples were positive upto day 3 by either set of primers (Fig. 2.14 & 2.15, respectively). However, one of the remaining two samples was found positive only upto day 2 of infection by both set of primers while yet another sample could not be amplified except on day 0 of infection by either set of the primers.

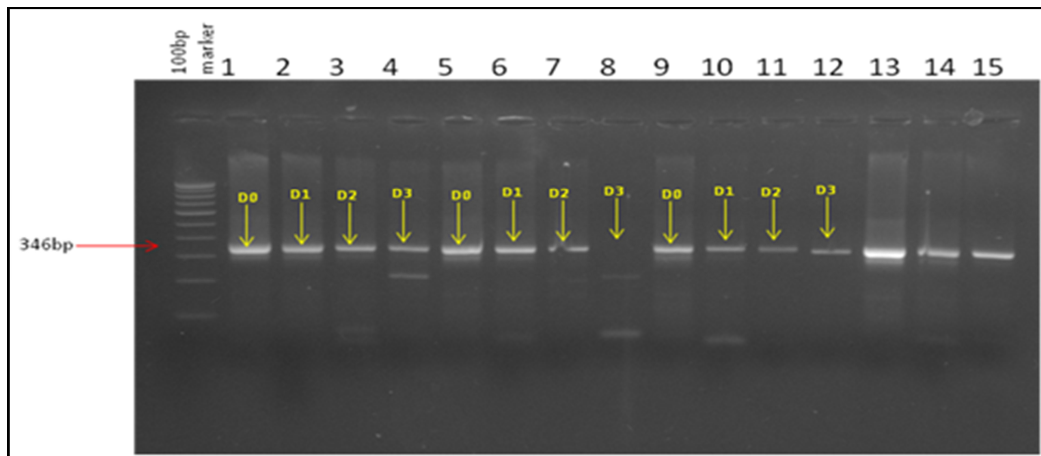


Fig. 2.14: Single step diagnostic PCR for detection of parasites in day 3 positive samples by Real time

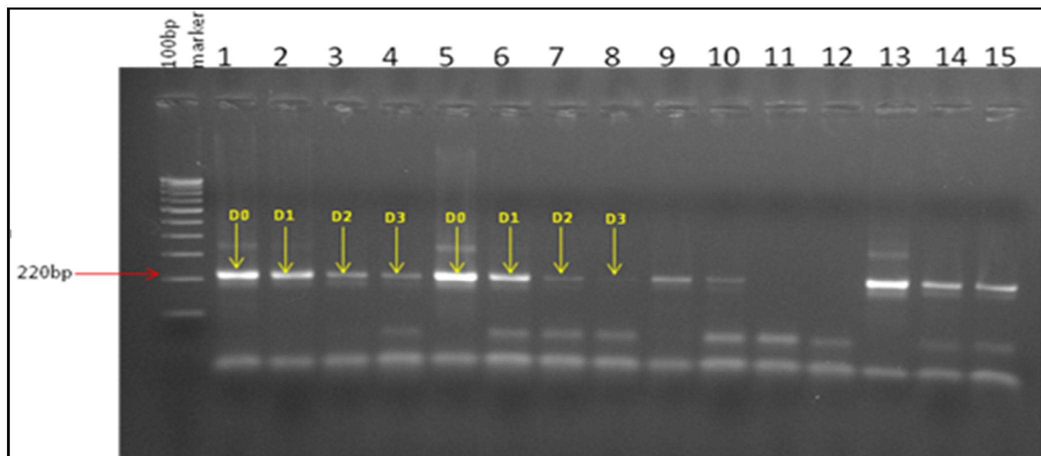


Fig. 2.15: Single step diagnostic PCR for detection of parasites in day 3 positive samples by Real time

2.4.3.2 Arunachal Pradesh

Real time PCR was done on 27 of the total 42 samples which included all the failure samples (n=6) and those which had parasitaemia ≥10,000/µl of blood. There were 10 cases which did not have consensus between microscopic and real time analyses of PCT within three days of ACT administration. Fig. 2.16 depicts the difference in PCT of the representative 10 samples as determined by microscopy and real time PCR.

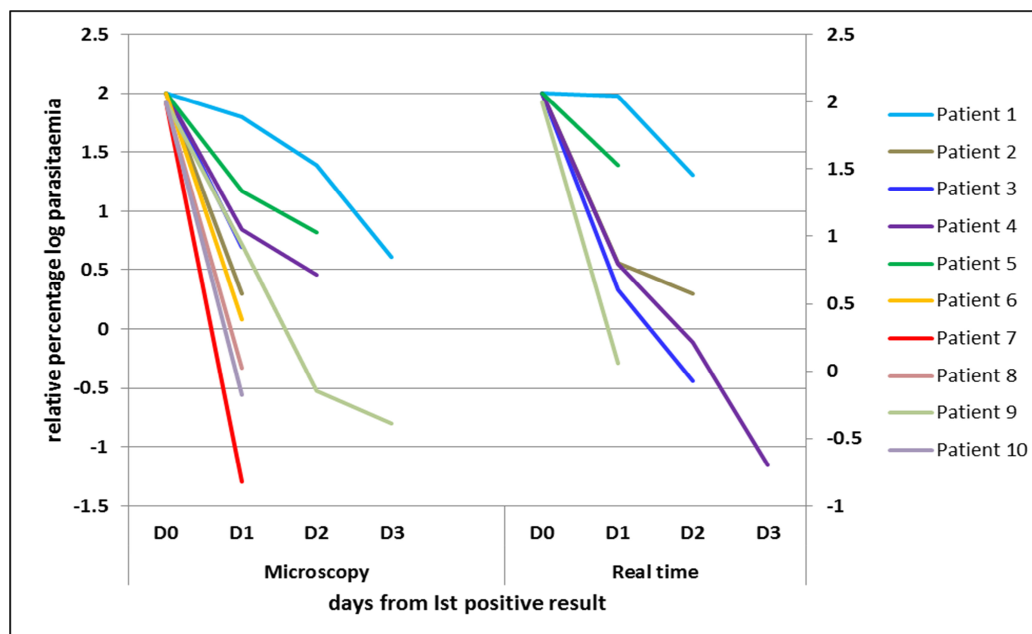


Fig. 2.16: Relative decrease in percentage log parasitaemia as determined by Microscopy and Real time PCR in the representative samples from Arunachal Pradesh study site

The relative decrease in percentage parasitaemia in 10 samples as determined by the two techniques is listed in Table 2.5.

Table 2.5: Relative decrease in percentage parasitaemia over three days of ACT administration in the representative samples from Arunachal Pradesh as per Microscopy and Real time PCR

Sample ID	MXD0	MXD1	MXD2	MXD3	RTD0	RTD1	RTD2	RTD3
Patient 1	100.00	63.03	24.3	4.05	100.00	94.82	20	0.00
Patient 2	100.00	1.99	0.00	0.00	100.00	3.62	1.99	0.00
Patient 3	100.00	4.9	0.00	0.00	100.00	2.16	0.36	0.00
Patient 4	100.00	7.04	2.87	0.00	100.00	3.58	0.77	0.07
Patient 5	100.00	14.72	6.6	0.00	100.00	24.34	0.00	0.00
Patient 6	100.00	1.2	0.00	0.00	100.00	0.00	0.00	0.00
Patient 7	100.00	0.15	0.00	0.00	100.00	0.00	0.00	0.00
Patient 8	100.00	1.06	0.00	0.00	100.00	0.00	0.00	0.00
Patient 9	100.00	8.79	0.72	0.41	100.00	1.14	0.00	0.00
Patient 10	100.00	0.67	0.00	0.00	100.00	0.00	0.00	0.00

MX=Microscopy, RT=Real time; D0= day 0, D1=day 1, D2=day 2, D3=day 3

2.4.5 Comparison: PCT Real Time and PCT Microscopy

PCT analysis revealed 11 cases to clear the parasite within 24 hours of drug administration as per Real time in contrast to only six cases as per microscopy. While nine cases cleared the parasites within 24 to 48 hours of drug administration according to Real time PCR, there were 14 cases in this category. The PCT analysis done for all the samples (n=24) is depicted in Fig. 2.17

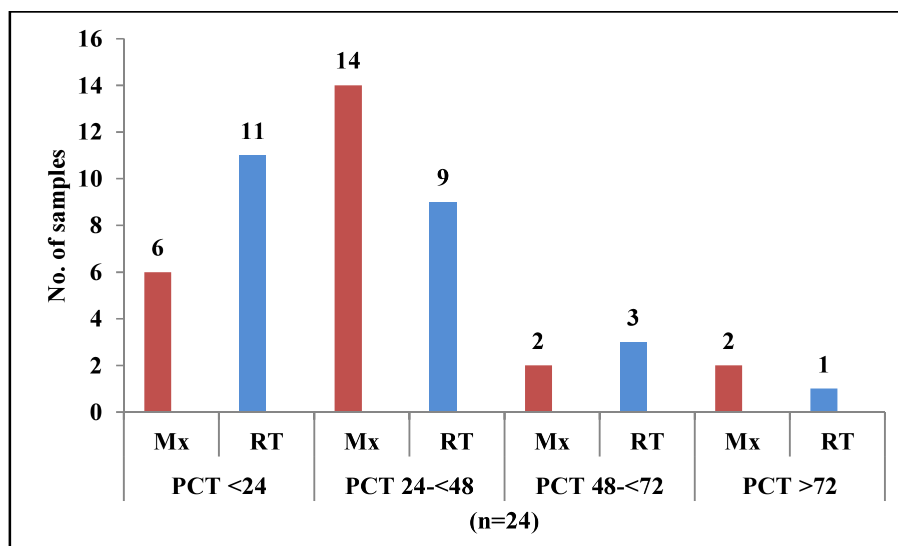


Fig. 2.17: Difference in PCT determined by Microscopy (Mx) and Real time PCR (RT) in samples from Arunachal Pradesh

Overall, two cases were parasite positive upto day 3 according to microscopy as compared to a single case as per Real Time PCR. This case was different than those (n=2) which were day 3 positive by microscopy making a total of three cases (12.5%) positive on day 3 as determined by both the techniques. Microscopy revealed two cases to be parasite positive upto day 2 (8.3%) as compared to real time PCR where three cases had parasitaemia on day 2 making a total of five cases to be parasite positive on day 2 when analyzed using the two techniques. Three samples could not be amplified by Real time PCR and hence was not included in the comparative analysis. Fig. 2.18

depicts the overall comparison of microscopy and Real time PCR of the total analyzed samples.

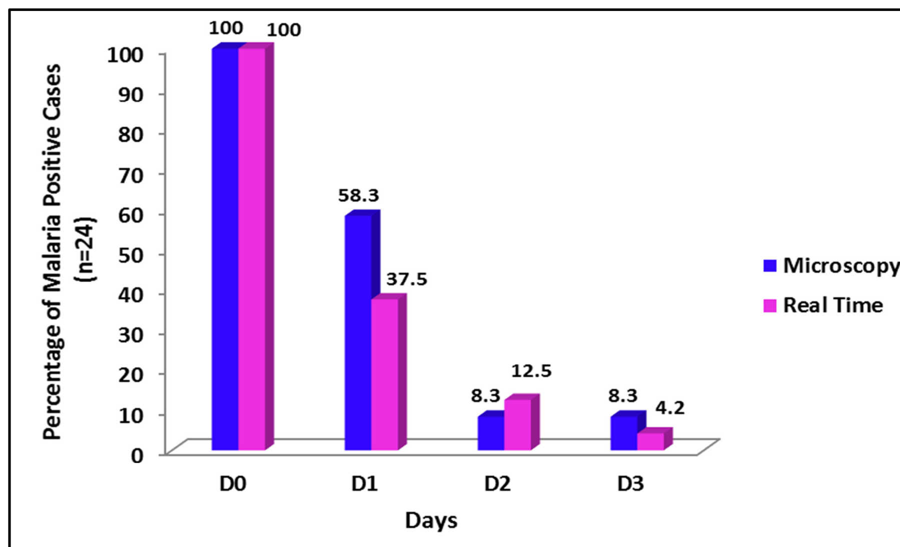


Fig. 2.18: Comparison of percentage decrease in malaria positive cases after treatment with AS+SP as determined by microscopy and Real time PCR at Changlang district, Arunachal Pradesh

Though Real time PCR is more sensitive in detecting threshold parasitaemia as also evident from the results of study site at Odisha, the differences in the results of microscopy and real time PCR of study site at Arunachal Pradesh is a limiting factor. Therefore, it becomes difficult to explain the difference of samples being day 3 positive by microscopy and not by Real time PCR. The most plausible reason for this difference is the presence of PCR inhibitory factors in the DNA sample. These factors bind directly to the DNA making it unavailable for interaction with the polymerase enzyme or affect by interfering with the DNA polymerase itself. Alternatively, they interfere with the co-factors such as Mg2+ which are required by the polymerase for its activity, thus inhibiting PCR (Bessetti 2007). The same reason explains the fact that three out of the 27 analysed samples could not be amplified at all by Real time assay even after they

were repeated twice. However, more attempts on repeating the analysis could not be made as the amount of DNA sample was also limiting due to the fact that these samples were a part of another important study (Mishra et al. 2014).

2.5 Discussion

Delayed PCT is indicative of artemisinin resistant malaria. Artemisinin resistance has been reported in the year 2009 on the Thai-Cambodian border where resistance was characterized by slow clearance rate of *Plasmodium falciparum* in patients treated with artesunate (Dondorp et al. 2009) though the evidence of first two cases with prolonged clearance time after treatment with artesunate in the same region was reported in the year 2008 (Noedl et al. 2008). Most of the efforts made to understand mechanism of action of artemisinin and to find its molecular marker have gone in vain except a recent study which revealed Kelch-13 propeller domain as an affirmative molecular marker associated with artemisinin resistance in *Plasmodium falciparum* (Ariey et al. 2014, Miotto et al. 2015). Due to this fact delayed PCT finds room in WHO's definition of artemisinin resistance (WHO 2014) and hence, acts as a reliable marker to study artemisinin resistance as of now. With this background, this study was designed to capture signs of emerging artemisinin resistance in selected sites of India, being prone to the threat of emergence of artemisinin resistant malaria due to endemicity and location. To address the issue promptly and for even closer monitoring of the trait, Real time PCR was deployed in the study apart from microscopy and the results for parasite clearance determined by both the techniques were compared. The results were further authenticated by end point PCR and efforts were also made to associate it with clinical outcome. Since PCT is a first order process, it depends largely on initial parasite load

i.e. the higher the initial parasitaemia, the longer the PCT (White 2011), which was confirmed by our findings too.

In the Odisha study site, microscopy revealed all the samples (n=64) to be parasite negative by the end of day 3 in contrast to Real time PCR which found 12 samples to be parasite positive on day 3 of administration of ACT. Ten of these were also positive by end point diagnostic PCR which is even lesser sensitive than Real time PCR. Moreover, all the samples (n=12) which were day 3 positive had initial parasitaemia (ranging from 52,800 to 98,000 parasites/µl of blood), correlating with the fact that initial parasitaemia does affect PCT (White 2011). This suggests that though day 3 positivity or delayed PCT is not yet confirmed by microscopy in the studied samples, nor it is translated to clinical outcome, still the trait needs to be continuously monitored as advocated (Jambou et al. 2005) for early attention and prompt action. It was also commented that waiting till the time when artemisinin resistance would turn into treatment failure would be irresponsible.

There were three samples in the Arunachal Pradesh study site which were day 3 positive according to combined results of microscopy and Real time PCR along with high failure rate of the ACT combination which was mainly because of the SP partner as resistance to SP has emerged long back in the region (Shah et al. 2011). Therapeutic efficacy study conducted by NIMR, therefore, became the evidence for the National programme to change the ACT combination from AS+SP to artemether-lumefantrine (AL) in North-east (NE) states of the country (Mishra et al. 2014). In an ACT, the partner drug plays an equally important role which is also related to slow clearance because slow clearance renders higher parasite load on the partner drug ultimately

calling ACTs to fail (Meshnick 2012) thus, becoming a determining factor in defining artemisinin resistance (WHO 2014). In contrast to this there are examples when all the samples with delayed PCT were clinically cured at the end of the study and were also susceptible to artemisinin *in vitro* except in two cases with modest elevation in IC50 which leads to messy inconsistencies and controversies (Meshnick 2012). To expand the understanding further, the argument about the role of declining efficacy of the partner can be explained with the fact that when ACT was introduced in the year 1994 in North West Thailand where mefloquine (MQ) alone was used, failures increased to about 50%, ACTs restored it to as low as nearly 5%. The susceptibility of MQ is still the same as then in 1994 but the efficacy of ACTs is not the same and the failure rates with AS+MQ combination has increased to about 30% in the region (White 2012). This definitely is suggestive of reduced susceptibility to artemisinin partner. Although there are several other reasons like host immunity, pharmacokinetics factors like absorption and metabolism of the drug, etc which play important roles in waning of therapeutic efficacy of any drug (Dondorp and Ringwald 2013), still defining artemisinin resistance and factors determining artemisinin resistance have remained controversial and matter of protracted debate and concern.

Chapter III

Assessment of Putative SNPs Namely L263E, E431K, A623E and S769N of Pfatpase6 Gene and Copy Number Changes in Pfmdr1 Gene

3.1 Origin of the Study

In order to understand artemisinin resistance more closely, it was necessary to study its putative associated markers to derive a valid correlation between delayed PCT and polymorphism in the molecular markers, if any.

3.2 Objective

The objective was to find polymorphisms in molecular markers associated with artemisinin resistance. The most related and putative candidate genes being *Pfatpase6* and *Pfmdr1*, specifically the most reported SNPs of *Pfatpase6* namely L263E, E431K, A623E and S769N and the changes in *Pfmdr1* copy number were assessed.

3.3 Materials and Methods

3.3.1 *Pfatpase6* Mutation Analysis by Sequencing

For sequencing of *Pfatpase6* gene, the selected region of interest from 6 to 3216 bp out of the total 4032 bp was opted for sequencing. The selected region was divided into six overlapping fragments as described elsewhere (Imwong et al. 2010) and then amplification was done initially on eight of the randomly selected *P. falciparum* malaria patient samples from endemic regions. After this, 14 other patient samples were selected for sequencing based on their clinical outcome as delayed PCT or ETF (early treatment failure) (defined in Annexure II) for full length gene sequencing. The successfully amplified amplicons were purified as template for cycle sequencing reaction which was set with both forward and reverse primers (listed in Table 3.1) and run on Applied Biosystem's 3730XLGA for sequencing. After successful completion of sequencing reaction, these samples were analysed with the help of softwares, Finch TV

from Geospiza for reading the electropherogram and MEGA 5.10 for aligning the sequences. The reference sequence used as wild type *Pfatpase6* was retrieved from NCBI's GenBank (Accession no. AB121053.1).

Table 3.1: Primers used for analyzing mutations in *Pfatpase6* by sequencing

Fragment	Primer sequence (5'→3')	Reference
Fragment 1	Forward: CTTATTATATCTTTGTCATTCGTG	Imwong et al. 2010
	Reverse: CCACATACAATAGCGGTAGATG	
Fragment 2	Forward: AATAAAACTCCCGCTGATGC	
	Reverse: TTCTCCATCATCCGTTAAAGC	
Fragment 3	Forward: TTGCTTTAGCTGTTGCTGCT	
	Reverse: TTGTTGATACCCCTTGGTGA	
Fragment 4	Forward: AAGATGAAGGAAATGTTGAAGC	
	Reverse: CCCAATTTTGAGTGGAAACAA	
Fragment 5	Forward: GGCAACAACAAATGGATATGA	
	Reverse: TCCTTTTCATCATCTCCTTCA	
Fragment 6	Forward: GAGCATTAAGAACACTTAGCTTTGC	
	Reverse: CTGTTGCTGGTAATCCGTCA	

3.3.2 *Pfatpase6* Mutation Analysis by PCR-RFLP

PCR-RFLP was deployed for analysis of mutations in codon 263, 431, 623 and 769 of *Pfatpase6* gene. Single step PCR was conducted using primers (listed in Table 3.2) described elsewhere (Zakeri et al. 2012) followed by ApoI and MboII digestion for codon 263 and 431, respectively. PCR was done using 2X DreamTaq DNA Polymerase (Thermo Scientific), thermal profile included initial denaturation at 94°C for 4 min, followed by 35 cycles of amplification at 94°C for 1 min, 58°C for 2 min, 72°C for 1

min and final extension at 72°C for 5 min. Confirmation of PCR amplification was done on 1.5% agarose gel.

ApoI and MboII (New England Biolabs, Inc.) digestion was done for 15 mins at 50°C and 37°C respectively, using 10X CutSmart™ buffer supplied with the enzyme in a total of 20µl reaction. Digested products were checked on 2.5% agarose gel.

In silico analysis was done using Gene Runner to differentiate between patterns of wild and mutant type isolates after digestion with the restriction enzyme. The expected digested products upon digestion of 948bp PCR product in case of wild type codon 431 were 128+107+49+241+299+124bp while that of mutant type were 128+107+49+241+423bp (Fig. 3.1). However, in case of wild type codon 263, the 948bp product remains undigested while the mutant get digested into 312+633bp fragments.

PCR-RFLP analysis for detecting mutation in codon 623 and 769 was done as described elsewhere (Zakeri et al. 2012) followed by Cac8I and DdeI restriction digestion, respectively.

Table 3.2: Primers used for analyzing mutations in *Pfatpase6* by PCR-RFLP

Reaction	Primer sequence (5'→3')	Reference
Primary 1	Forward: TTGGTAATAAAACTCCCGC	Zakeri et. al 2012
	Reverse: TATTCCTCTTAGCACCACTCC	
Primary 2	Forward: AAGAAGGATAAATCACCAAG	
	Reverse: AAATACACGTATACCAGCC	
Nested 2a	Forward: TAACCATTCTAATTATACTACAGCgCAGG	
	Reverse: TGTGTTGATGTGGTATTTATTTTATTACCC	
Nested 2b	Forward: AGAACATTTAGCTTTGCTTATAAAAAAcTAA	
	Reverse: ATATGGCATAATCTAATTGCTCTTCCTAC	

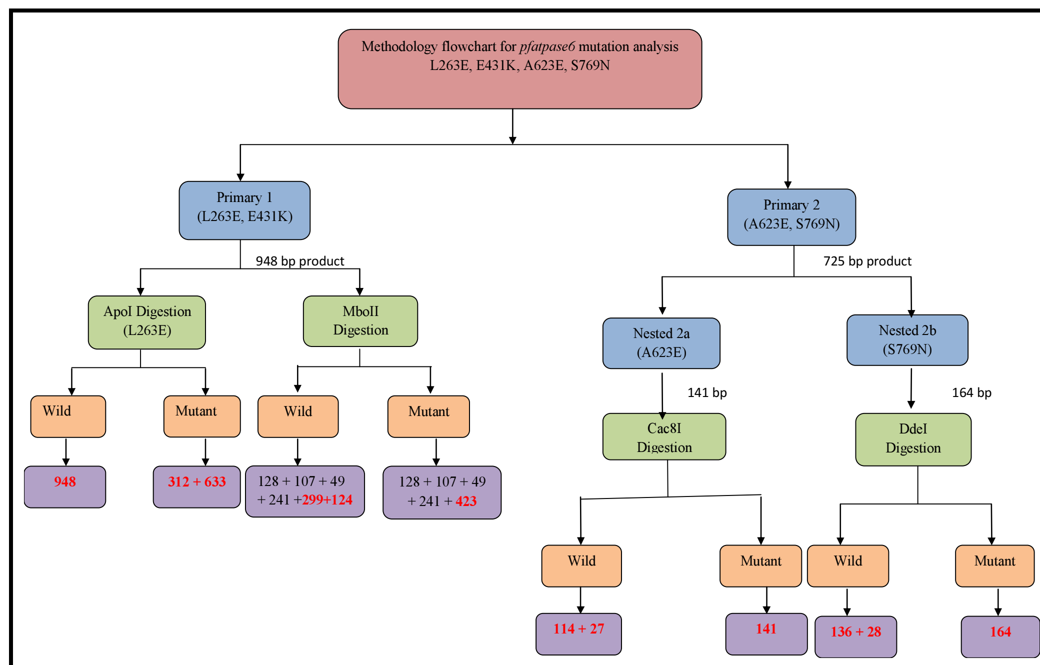


Fig. 3.1: Methodology flowchart for analysis of mutations in *Pfatpase6* gene

3.3.3 *Pfmdr1* Copy Number Polymorphism Analysis by Real Time PCR

Real time PCR was deployed for analysis of *pfmdr1* copy number on Roche's LightCycler® 480 using hydrolysis probe. The ratio of *pfmdr1* gene w.r.t. endogenous control *ldh* and *β-tubulin* was determined using ΔΔCt method. The reaction efficiency of each gene was checked by preparing standard curve using 10 fold serial dilutions of 3d7 template. Primers and probes for *Pfmdr1* and *ldh* gene were picked from Pickard et al. (2003) while that of *β-tubulin* gene (listed in Table. 3.3) were derived from Price et al. (2004). 3d7 was used as calibrator having one copy of *pfmdr1* while Dd2 was used as a multicopy strain. Each reaction was set up in triplicate and difference of more than 0.4 Ct between the replicates was discarded and was not considered for calculation of the mean Ct (Defined in Annexure II). 3d7 being the calibrator was fixed to have one copy number and Dd2 was masked. Copy number calculation was done using formula $2^{-\Delta\Delta Ct}$ and the data was accepted if the copy number of Dd2 ranged between 2-3 copies.

The data of the unknown sample was discarded if it was less than 0.6 and the run was repeated for that particular sample.

Table 3.3: Sequence of primers and probes used for analyzing *pfmdr1* copy number by Real time

Gene name	Primer/ Probe	5' Fluorophore	Sequence 5'→3'	Quencher	Reference
Pfmdr1	Pfmdr1_Forward		TTAAGTTTTACTCTAAAAGAAGGGAAA ACATAT		Pickard et al. 2003
	Pfmdr1_Reverse		TCTCCTTCGGTTGGATCATAAAG		
	Pfmdr1_probe	FAM	CATTTGTGGGAGAATCAGGTTGTGGGA AAT	TAMRA	
β_tubulin	b-tubulin_Forward		TGATGTGCGCAAGTGATCC		Price et. al 2004
	b-tubulin_Reverse		TCCTTTGTGGACATTCTTCCTC		
	b-tubulin_probe	VIC	TAGCACATGCCGTTAAATATCTTCCAT GTCT	TAMRA	
ldh	Ldh_Forward		ACGATTTGGCTGGAGCAGAT		Pickard et al. 2003
	Ldh_Reverse		TCTCTATTCCATTCTTTGTCACTCTTTC		
	Ldh_probe	FAM	AGTAATAGTAACAGCTGGATTTACCAA GGCCCCA	TAMRA	

3.3.4 Data Analysis

Data analysis, preparation of graphs and statistics was done using Microsoft Office Excel 2007 and Statistical Package for the Social Sciences 14.0 (SPSS 14.0). Data analysis for Real time experiments for calculation of PCT and analysis of *Pfmdr1* gene copy number was done using Roche's software LightCycler® 480 SW 1.5.

3.4 Results

3.4.1 Sequencing Results of *Pfatpase6* Gene

Standardization of DNA sequencing was done on eight of the randomly chosen patient samples. The results revealed that four (sample no. 2, 3, 6 & 8) of the eight samples could be amplified successfully for all the six fragments after making adjustments in

thermal profile and $MgCl_2$ concentration (Fig. 3.2). Confirmation gel (Fig. 3.3) and nano measurements for final concentration/µl of DNA were also recorded for purified gel fragments.

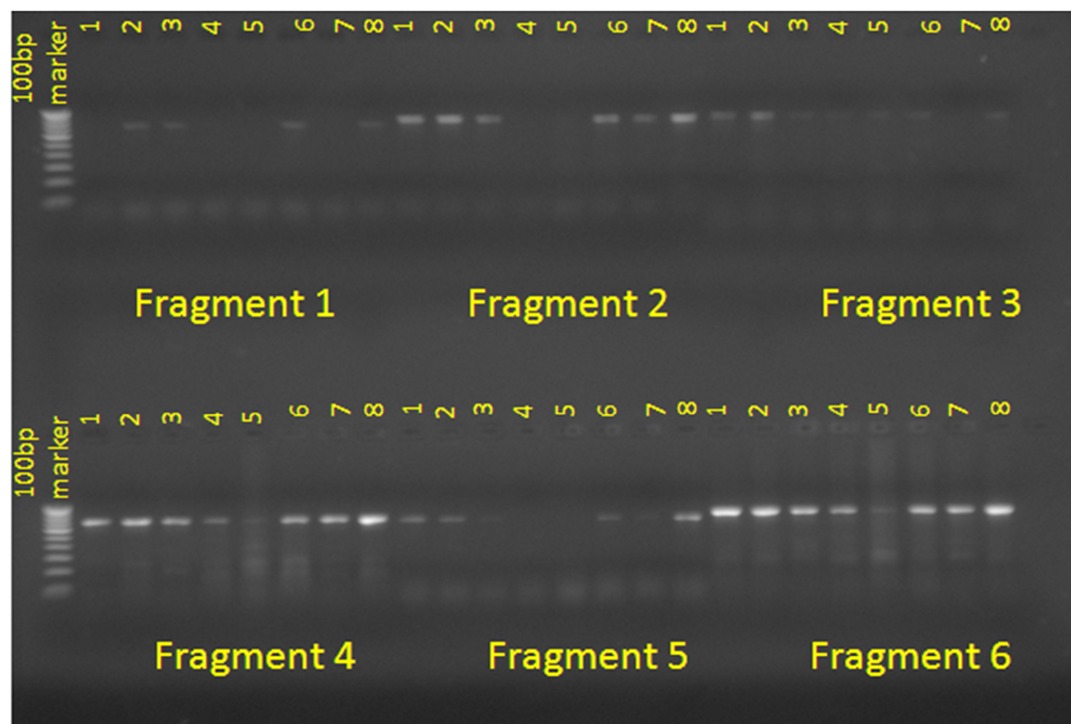


Fig. 3.2: Amplification of *Pfatpase6* gene fragments from *P. falciparum* infected patient samples for sequencing

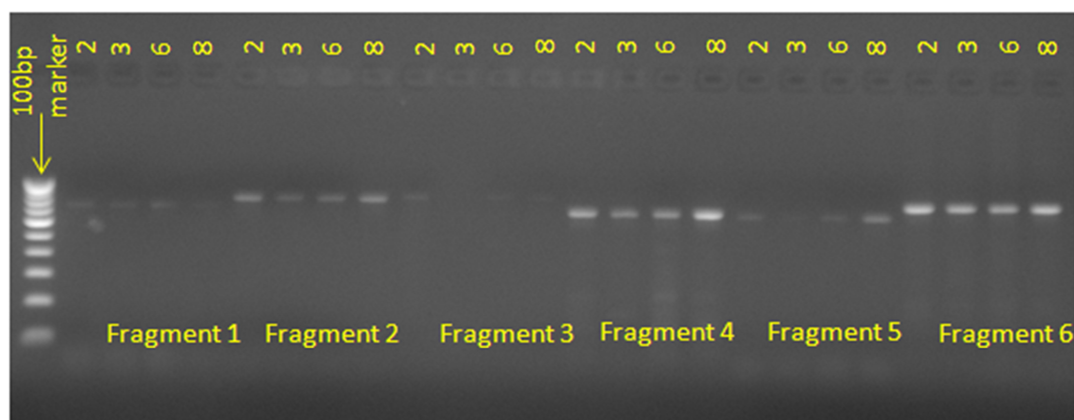


Fig. 3.3: Successfully amplified and purified samples for sequencing of *Pfatpase6* gene of *P. falciparum*

After analyzing the results for all the four samples which were successfully sequenced, none of the reported SNP could be seen, however, one synonymous change in fragment 2 at position 1116bp (V372V) was observed in one of the samples (sample no. 8) as could be seen in the figures below (Fig. 3.4, 3.5 & 3.6). This observation could be confirmed in the sequences obtained from both forward and reverse primer of the same sample. After reading the electropherogram, this change was observed in heterozygous form which could be due to mix type of population infecting a single patient i.e. isolates with different genotype infecting a single patient. All the above four samples which were sequenced for *Pfatpase6* gene were clinically cured as ACPR.

DNA Sequences | Translated Protein Sequences

Species/Abbrv	
1. gi|56342160|	CGTTAGGATGTACAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCGTGTTTCATTTGTTTAGAGAAT
2. 9	CGTTAGGATGTACAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCGTGTTTCATTTGTTTAGAGAAT
3. 13	CGTTAGGATGTACAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCGTGTTTCATTTGTTTAGAGAAT
4. 10	CGTTAGGATGTACAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCGTGTTTCATTTGTTTAGAGAAT
5. 14	CGTTAGGATGTACAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCGTGTTTCATTTGTTTAGAGAAT
6. 11	CGTTAGGATGTACAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCGTGTTTCATTTGTTTAGAGAAT
7. 15	CGTTAGGATGTACAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCGTGTTTCATTTGTTTAGAGAAT
8. 12	CGTTAGGATGTACAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCGTATTTCATTTGTTTAGAGAAT
9. 16	CGTTAGGATGTACAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCGTATTTCATTTGTTTAGAGAAT

Fig. 3.4: MEGA 5 alignment results for fragment 2 highlighting the change from GTG to GTA

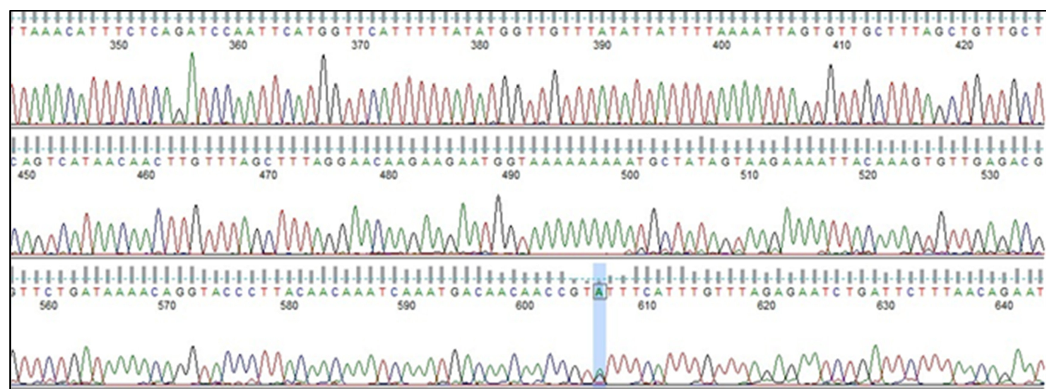


Fig. 3.5: Electropherogram for sequences of fragment 2 obtained from forward primer of the representative sample highlighting the mutated base

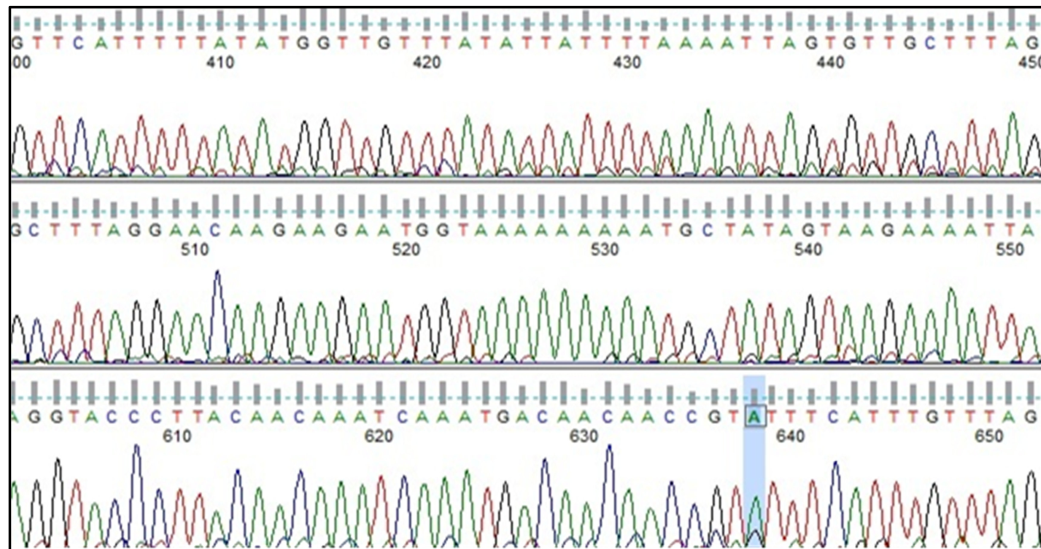


Fig. 3.6: Electropherogram for sequences of fragment 2 obtained from reverse primer of the representative sample highlighting the mutated base

After standardization, the same approach was used for sequencing *Pfatpase6* gene in samples (n=14) showing delayed PCT (day 3 positive by microscopy) or early treatment failure (ETF). The results were used to study selected and important SNPs through PCR-RFLP.

The MEGA 5 alignment results at the nucleotide and amino acid level for the SNPs E431K, A438D and N569K are depicted in the figures below (Fig.3.7, 3.8, 3.9 & 3.10).

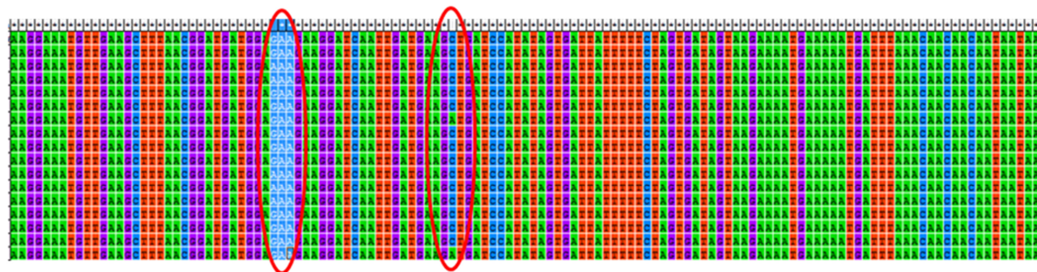


Fig. 3.7: MEGA 5 alignment results for E431K and A438D highlighting the change from GAA to AAA and GCT to GAT respectively

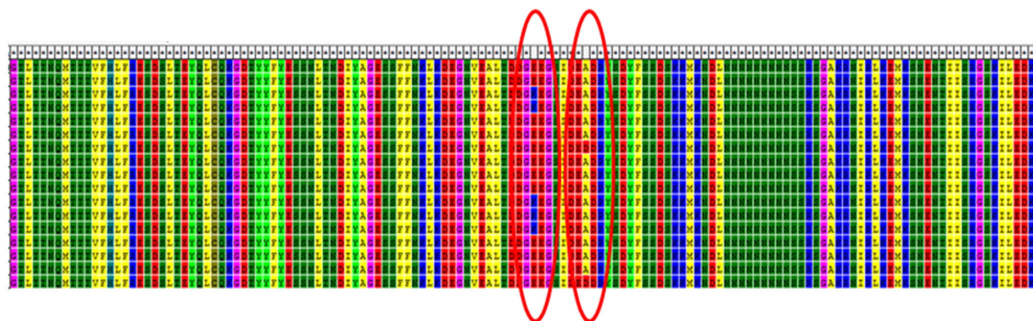


Fig. 3.8: MEGA 5 alignment results for translated protein sequence for SNP E431K and A438D

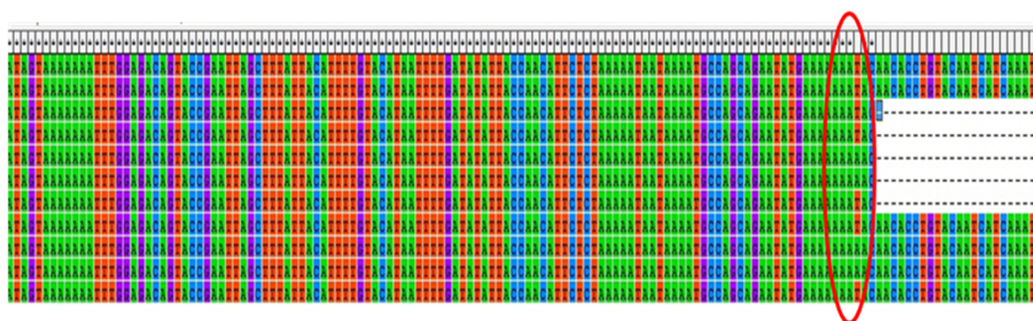


Fig. 3.9: MEGA 5 alignment results for N569K highlighting the change from AAT to AAA

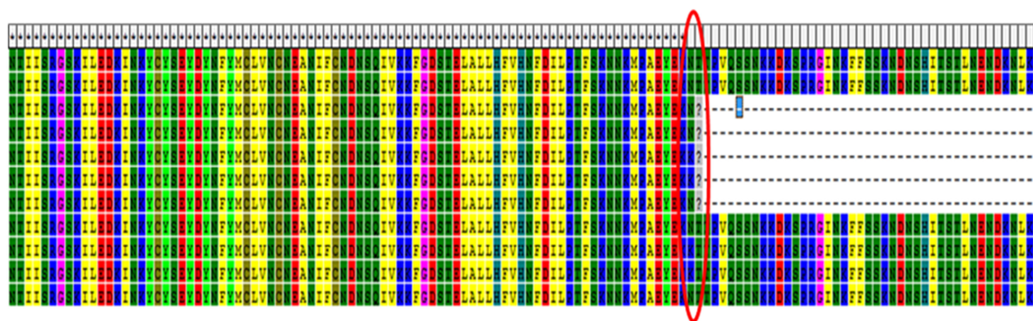


Fig. 3.10: MEGA 5 alignment results for translated protein sequence for SNP N569K

The sequencing results of the above samples revealed prevalence of E431K in highest frequency with other mutations making lesser impacts. Also, N569K has been observed in few isolates, but neither its functional relevance could be sought nor does it correlate with increased IC50 with artemisinin or any of its derivatives in the studies conducted so far (Krishna et al. 2010).

The above results made us to focus on four important and widely studied SNPs of *Pfatpase6* gene namely L263E, E431K, A623E and S769N (Tahar et al. 2009; Jambou et al. 2010; Krishna et al. 2010; Zakeri et al. 2012). These four mutations were then studied through more economic, less cumbersome and less time consuming technique of PCR followed by RFLP.

3.4.2 PCR-RFLP Mutation Analysis of *Pfatpase6* Gene

To standardize less cumbersome, economic and high throughput technique which may reveal information about few important SNPs in *Pfatpase6* gene, PCR-RFLP approach was deployed. Amplicon for primary 1 PCR for detection of L263E and E431K has been shown in Fig. 3.11. ApoI digestion (for L263E) has been shown in Fig. 3.12 and MboII (for E431K) in Fig. 3.13. The expected digested fragments after digestion of the primary product was determined by *in silico* analysis using GeneRunner.

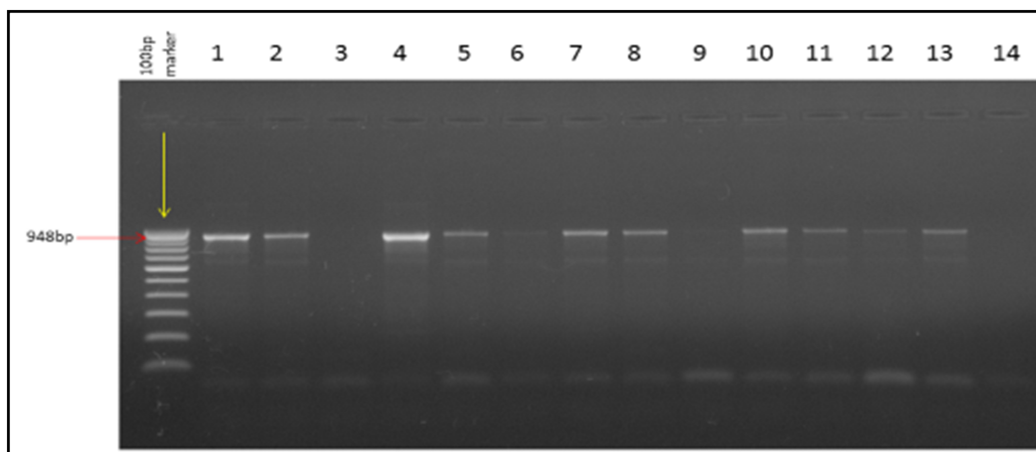


Fig. 3.11: 2D gel for Primary1 PCR product before digestion

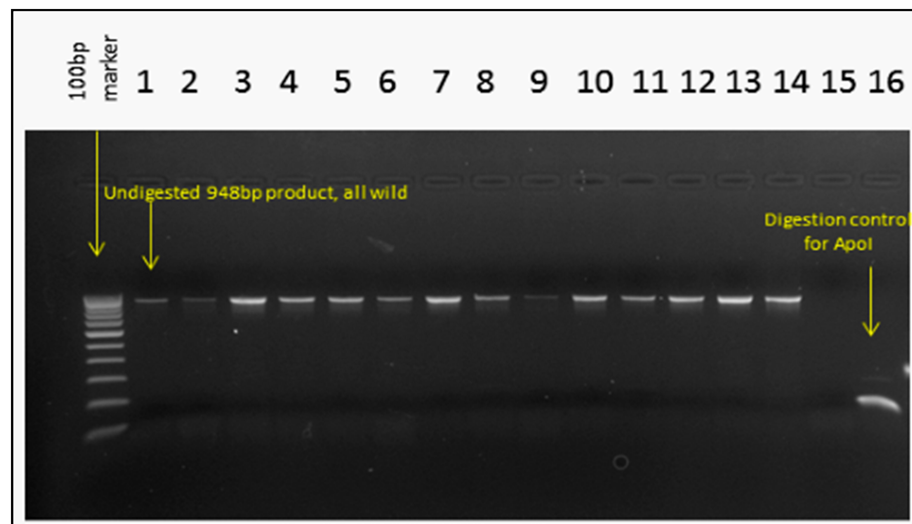


Fig. 3.12: Restriction digestion pattern with ApoI on 948bp primary1 product for detection of L263E mutation

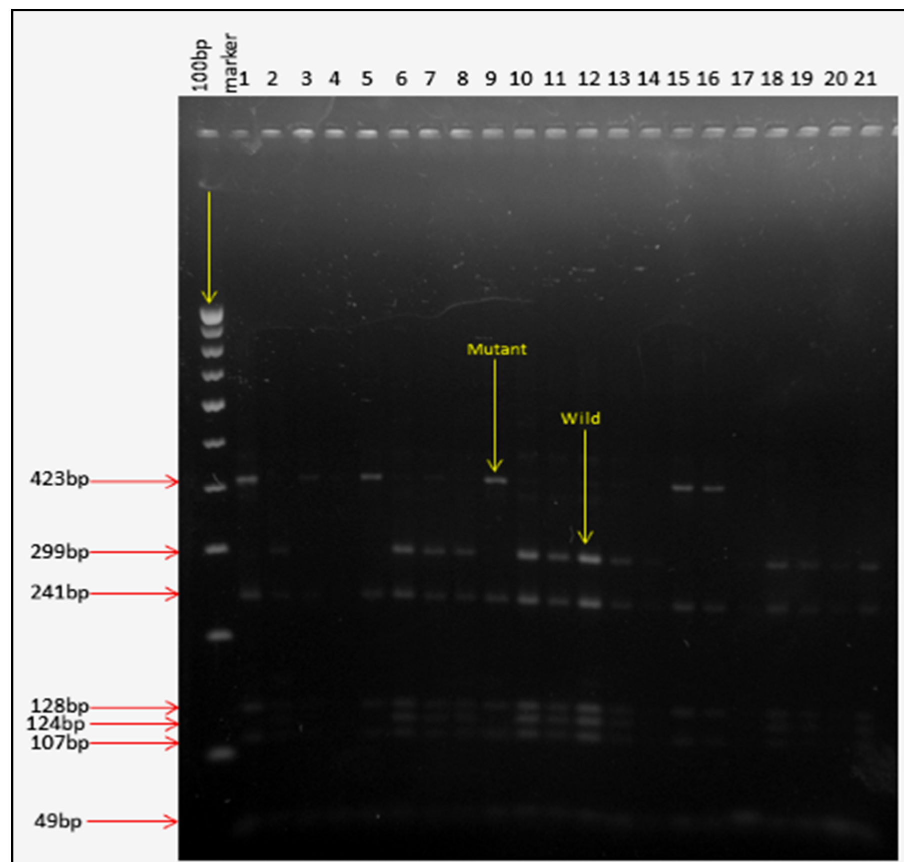


Fig. 3.13: Restriction digestion pattern with MboII on 948bp primary1 product for detection of E431K mutation

Primary 2 PCR for detection of A623E and S769N is depicted in Fig. 3.14 which was followed by nested 2a (Fig. 3.15) and nested 2b (Fig. 3.17) PCR which was finally digested by Cac8I (for A623E) (Fig. 3.16) and DdeI (for S769N) (Fig. 3.18), respectively.

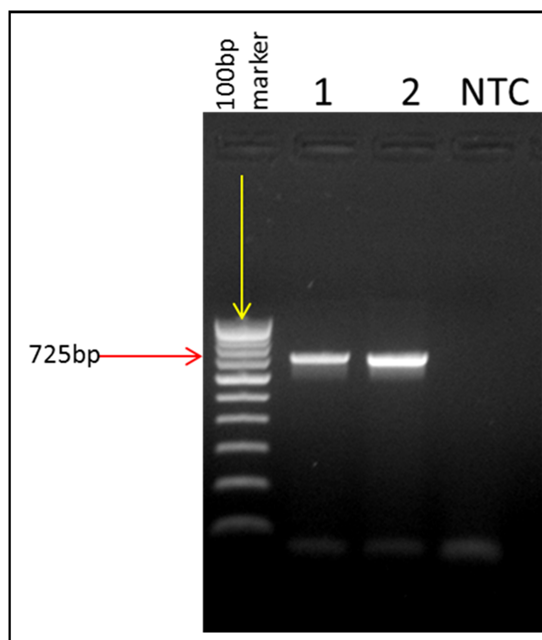


Fig. 3.14: 2D gel for Primary 2 PCR product before nested PCR

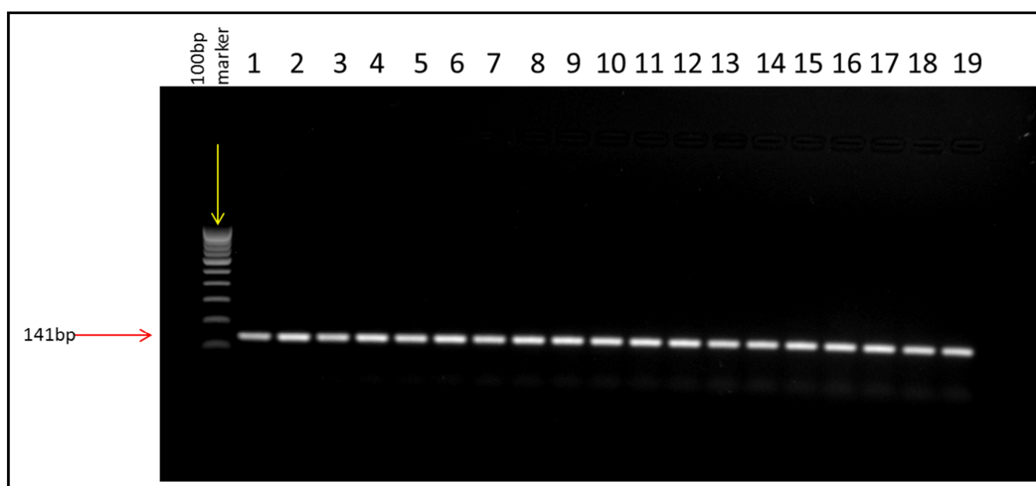


Fig. 3.15: 2D gel for nested2a PCR product

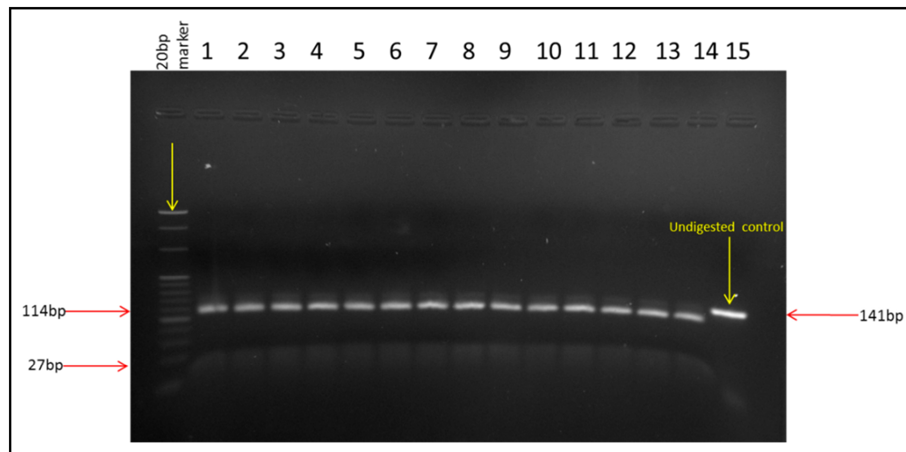


Fig. 3.16: Restriction digestion pattern with Cac8I on 141bp nested2a PCR product for detection of A623E mutation

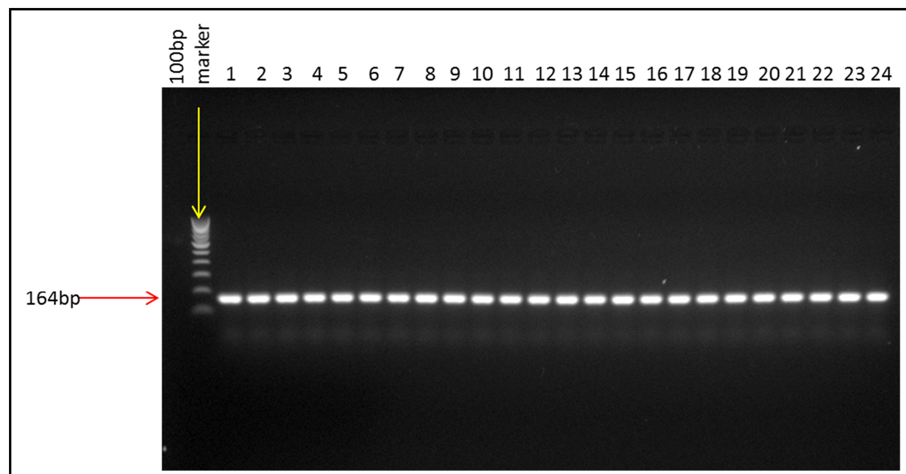


Fig. 3.17: 2D gel for nested2b PCR product

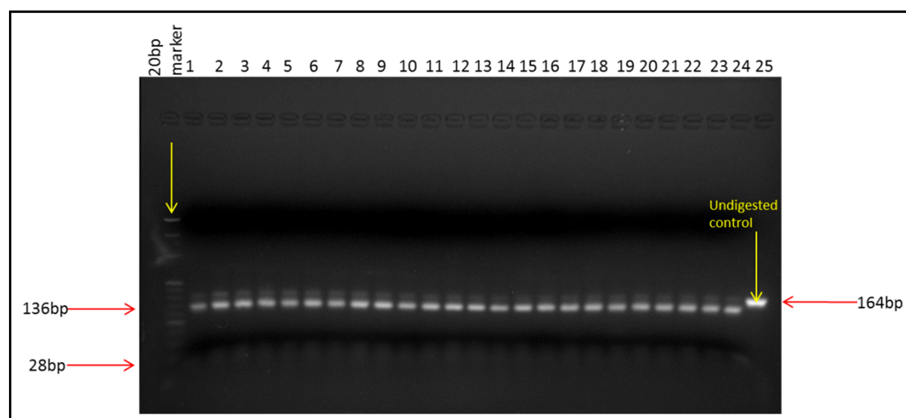


Fig. 3.18: Restriction digestion pattern with DdeI on 164bp nested2b PCR product for detection of S769N mutation

3.4.2.1 Odisha

Mutation analysis in codon 263, 431, 623 and 769 of *Pfatpase6* gene was done in 30 samples including the 12 which were found to day 3 positive by Real time while the other 18 served as controls from the study site of Odisha. Interestingly, presence of E431K was observed in two samples out of the 12 samples while one mix type pattern was observed in the control group (n=18). Other three codons showed wild type genotype in all the analysed samples. The results of prevalence of E431K have been summarized in Fig. 3.19.

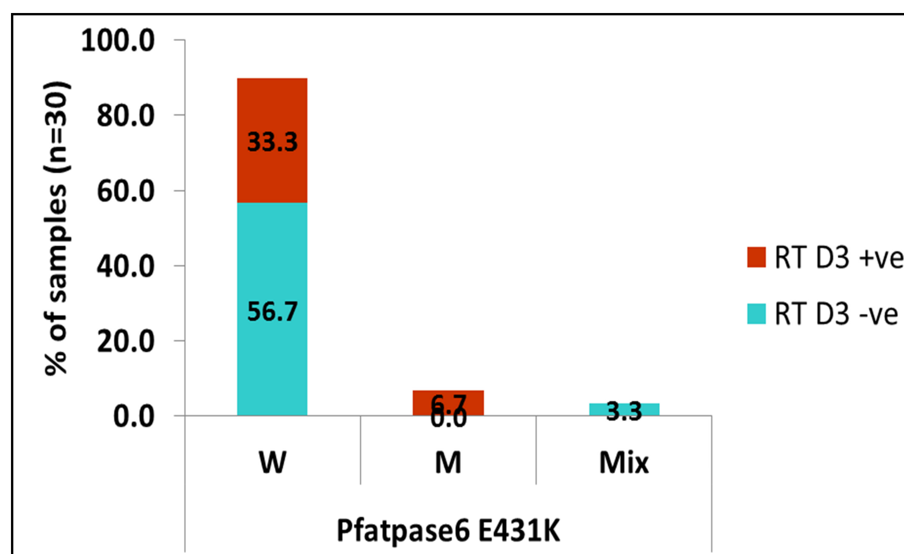


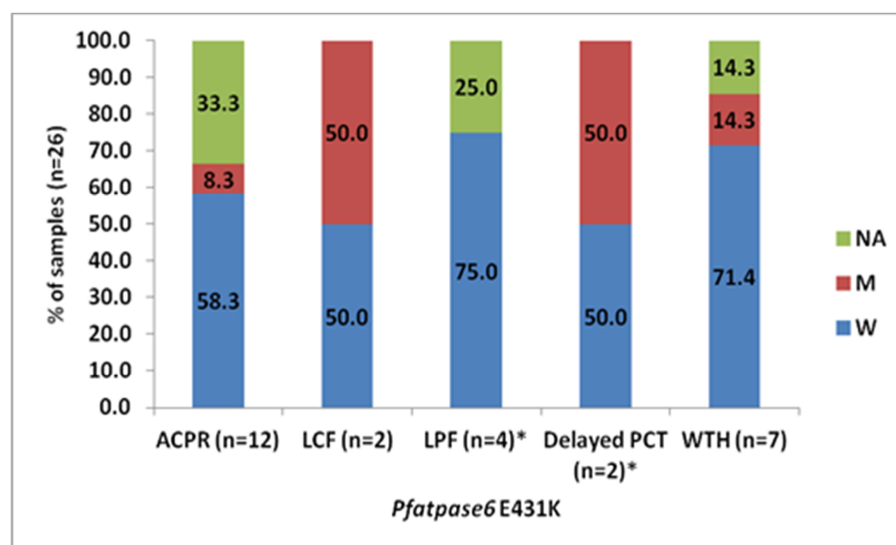
Fig. 3.19: Prevalence of E431K mutation in Real time day 3 positive samples from Odisha

3.4.2.2 Arunachal Pradesh

Pfatpase6 mutation analysis was done in a total of 26 samples from the study site at Arunachal Pradesh which included six failure samples. Two samples were found to have day 3 positivity which were categorized as delayed PCT. Out of the total analysed samples four were found to be mutant for E431K (15.4%) while others were all wild (n=16). Six samples could not be amplified in the primary 1 PCR for *Pfatpase6* which

covers codon 263 and 431. However, all the 26 samples were found to be wild type for codon 623 and codon 769. Similarly, all the samples which could be amplified for L263E analysis (n=20) were found to be wild type for the said codon.

Fig. 3.20 depicts the summary of E431K SNP analysis of the studied samples with its clinical outcome. There was a single case which was found to be LPF along with day 3 positivity and hence categorized under delayed PCT too. However, this was found to have wild type genotype for codon 431.



**Note: Includes a common case, hence total number of cases exceeding to 27 instead of 26*

Fig. 3.20: Prevalence of E431K mutation in *Pfatpase6* gene in samples from Arunachal Pradesh

3.4.3 *Pfmdr1* Copy Number

3.4.3.1 Odisha

Pfmdr1 gene copy number analysis was done in representative samples (n=12) and also in the control group (n=18). None of the sample was found to have amplification in *pfmdr1* gene copy number. The copy number of Dd2 ranged between 2-3 copies, mean

copy number from all the run was 2.3 while those of unknown samples ranged between 0.67 to 1.18 copies of *Pfmdr1*, mean copy number being 0.92

3.4.3.2 Arunachal Pradesh

Pfmdr1 gene copy number was studied in 11 samples of the studied site which included six failure samples while others were taken as controls. No sample was found to have amplification in *Pfmdr1* gene copy number. The mean copy number of the 11 samples was found out be 1.16 copies of *Pfmdr1* (range 0.83 – 1.24).

3.5 Discussion

There are evidences that *Pfatpase6* which is homologue of mammalian thapsigargin is indeed target of activated artemisinins. *In vitro* experiments revealed that artemisinins act by inhibiting *Pfatpase6* in *Plasmodium falciparum* after activation by iron (Eckstein-Ludwig et al. 2003). However, in the presence of these evidences too, mode of action of artemisinin still remains an enigma. Krishna et al. (2010) in their review article revisited and summarised the biological basis of the controversial issue that artemisinins acts by inhibiting *Pfatpase6*. This review concluded that sequence changes in *Pfatpase6* is indeed associated with artemisinin resistance and that further studies are needed to be carried out in this direction in light of artemisinin's use and its importance.

Jambou et al. (2010) conducted and compiled the studies in isolates collected from multiple sites of Asia, Africa and South America and concluded that though the coding sequence diversity of *Pfatpase6* was large but the functional domains were very well conserved except S769N mutation which was reported in the isolates from French Guiana (Legrand et al. 2008). This was located close to the headpiece linking calcium

homeostasis with drug resistance. Nevertheless, S769N has made only occasional impacts from few studies (Jambou et al. 2005; Pillai et al. 2012; Zakeri et al. 2012) while there are many reports which could not detect this mutation (Mugittu et al. 2006; Sisowath et al. 2007; Zhang et al. 2008; Ferreira et al. 2008; Menegon et al. 2009; Wangai et al. 2011; Kwansa-Bentum et al. 2011; Tanabe et al. 2011; Brasil et al. 2012; Saha et al. 2013) or even denied its association with artemisinin resistance (Phompradit et al. 2011; Cui et al. 2012). L263E was shown to alter the susceptibility of artemisinin *in vitro* but this mutation could not be found in any of the field isolates in the studies conducted so far (Kwansa-Bentum et al. 2011; Tanabe et al. 2011). Apart from this, the other SNP frequently reported is E431K which has also been reported in association with A623E. Altogether these four SNPs were the most studied and widely discussed SNPs of *Pfatpase6* which have been studied in context with artemisinin resistance (Tahar et al. 2009; Jambou et al. 2010; Krishna et al. 2010; Zakeri et al. 2012). The result of this too coincides with the above findings as we could not find mutation in any of the three codons in any of the studied isolates from both the study sites except E431K. Presence of E431K was detected in significant number of isolates from both the study sites like other studies suggesting the possible involvement of this mutation in artemisinin resistance. However, more studies are needed in this direction in much larger number of isolates to be conclusive. The reports which support the above findings are the ones which detected presence of E431K (Eshetu et al. 2010), and A623E, while L263E and S769N was found absent (Menegon et al. 2008, Dahlstrom et al. 2008) in these studies. Not many studies have been conducted in India except the two studies conducted by Saha et al. (2012, 2013) in Jalpaiguri and Kolkata districts, respectively, which also support the above findings where in one study, the presence of

E431K was detected while in another absence of S769N was confirmed in the studied isolates. Since our report became the second report from India to confirm the presence of E431K in different study sites than Jalpaiguri, it suggests us to be vigilant, if at all E431K is associated with artemisinin resistance and to ascertain whether it is also present in other regions of the country.

Chapter IV

Correlation of Clinical and Molecular Data

4.1 Origin of the Study

In clinical studies, it becomes necessary to validate the data once the findings of the study are correlated with the *in vivo* data. It is with this aim this research objective was designed so that the molecular findings could be correlated with the clinical observations after 42 day follow-up.

4.2 Objective

This objective made it possible to correlate the molecular data obtained from this study with the clinical data of the ongoing studies. Since the samples were a part of larger study on monitoring therapeutic efficacy of antimalarial medicines in India, the larger clinical data was available in order to derive this correlation. It may be noted that the samples were followed upto day 42 as per schedule of follow-up prescribed by WHO (WHO 2009) and also to fulfil this objective.

4.3 Materials and Methods

4.3.1 Statistical Analysis

Statistical analysis was done using Microsoft Office Excel 2007 and Statistical Package for the Social Sciences 14.0 (SPSS 14.0), GraphPad Prism 5. Preparation of graphs, data entry and analysis was also done using Microsoft Office Excel 2007. Correlation findings and scatter plots were prepared using GraphPad Prism 5.

4.4 Results

4.4.1 PCR Uncorrected and Corrected Cure Rate

4.4.1.1 Odisha

All the samples from Bisra CHC which could complete the 42 day follow-up attained ACPR and hence, the PCR uncorrected and corrected cure rate remains the same as 100% (Fig. 4.1).

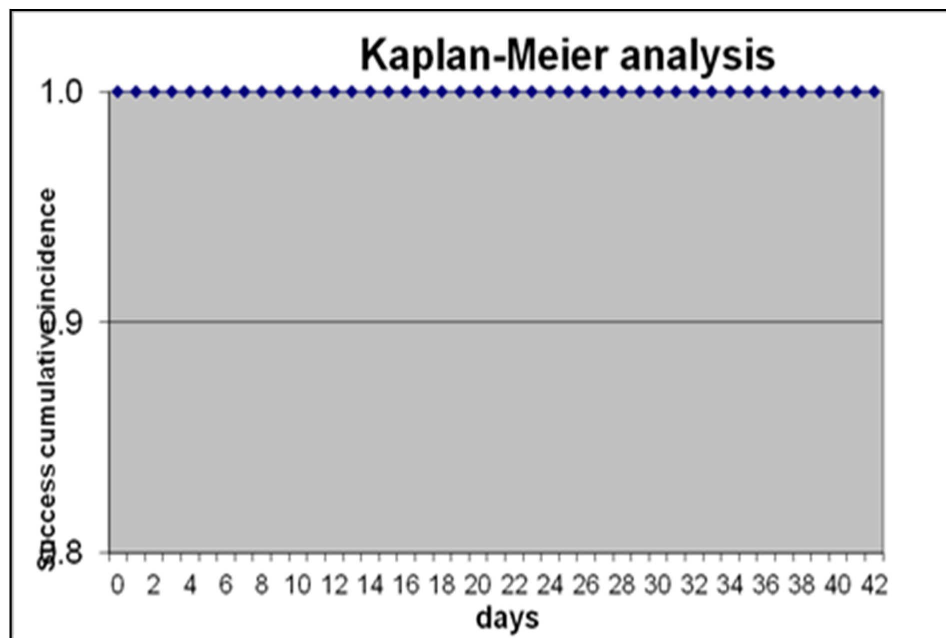


Fig. 4.1: Kaplan-Meier survival analysis for samples collected from Bisra CHC, Sundergarh district, Odisha

4.4.1.2 Arunachal Pradesh

The samples from this study site showed high failure rate after 42 day follow-up. The PCR uncorrected and corrected cure rate as determined by Kaplan-Meier survival analysis was 59.5% (Fig. 4.2) and 82.3% (Fig. 4.3), respectively. After PCR correction, nine samples were found out to be *Pf* reinfections, four were cases of late parasitological failure (LPF) (defined in Annexure II) while two were cases of late clinical failure (LCF) (defined in Annexure II). Therefore, after PCR correction the failure rate reduced to 17.7% as against 40.5% found without PCR correction. However, it was an important piece of evidence for the National Programme to change the drug policy in north eastern states of India (Mishra et al. 2014).

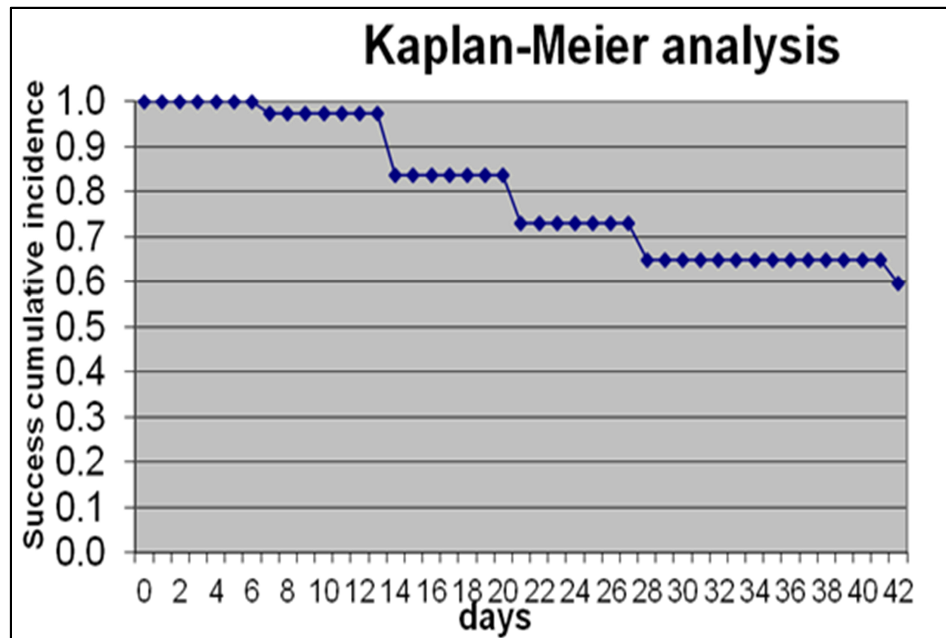


Fig. 4.2: Kaplan-Meier survival analysis for PCR uncorrected data for samples collected from Miao PHC, Changlang district, Arunachal Pradesh

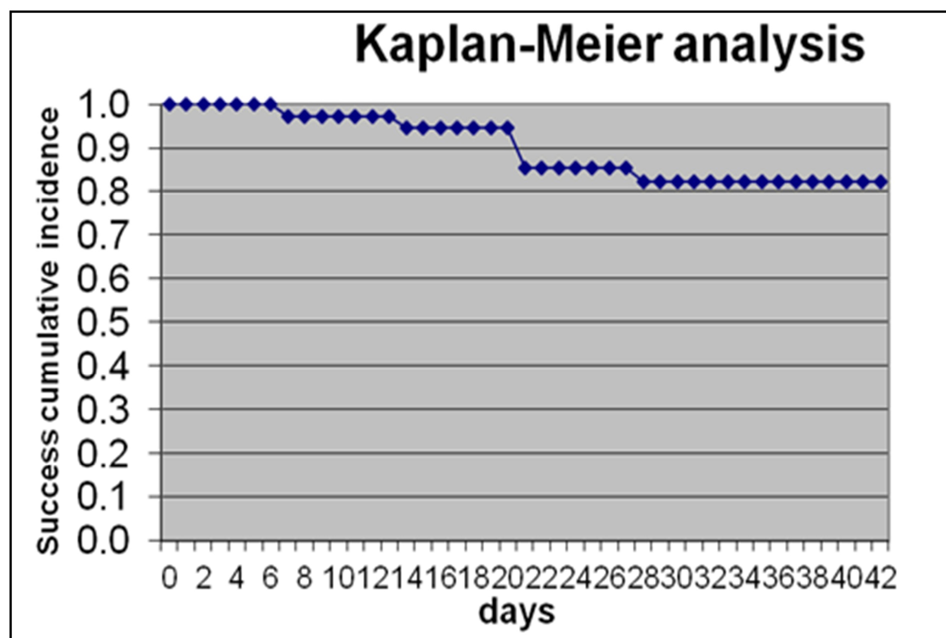


Fig. 4.3: Kaplan-Meier survival analysis for PCR corrected data for samples collected from Miao PHC, Changlang district, Arunachal Pradesh

4.4.2 PCT Correlation with Initial Parasitaemia

4.4.2.1 Odisha

Microscopically all the samples were negative on day 3 but real time PCR revealed 12 samples to be positive on day 3. Surprisingly, all these 12 samples had high initial parasitaemia ranging from 52,800 to 98,000 parasites/µl of blood, mean parasitaemia being 72,750 parasites.

4.4.2.2 Arunachal Pradesh

At this site, three samples were day 3 positive according to combined results of microscopy and real time PCR, one as per real time while two as per microscopy, respectively. Interestingly, here also two of these samples were those which had the highest parasitaemia amongst all the enrolled samples at the said site. Specifically, the initial parasitaemia of the samples which were day 3 positive according to microscopy was 22,720 and 77,800/µl of blood while for the samples which was day 3 positive according to real time had highest initial parasitaemia as 79,560/µl of blood.

4.4.3 Mutation in *Pfatpase6* Gene with Treatment Outcome

4.4.3.1 Odisha

Mutation in E431K codon of *Pfatpase6* gene was observed in two of the samples from this site. Both these samples belonged to the group which were day 3 positive according to real time PCR (n=12), however, the treatment outcome for all the samples from this site was ACPR.

4.4.3.2 Arunachal Pradesh

Herein, four isolates showed mutation in E431K codon of *Pfatpase6*. Detailed analysis of E431K mutation revealed that the four mutant isolate had varied clinical outcome.

One sample was reported to be LCF while another one was categorized as delayed PCT. Out of the remaining two mutants, one was found to be ACPR while the other one belonged to the withdrawal category (WTH) as it was found to be a case of Pf reinfection on day 14 of follow-up.

4.4.4 Correlation of Age, Fever and Initial Parasitaemia

4.4.4.1 Odisha

Pearson correlation was analysed between age and initial parasitaemia and also between fever and initial parasitaemia (n=72). It was thereby found out that at Odisha study site age and initial parasitaemia are negatively correlated that the lesser the age of the patient, the higher the parasitaemia and it was significantly correlated (Pearson r = -0.3218, p value = 0.0058, 95% CI = -0.5151 to -0.09737). The scatter plot has been depicted in Fig. 4.4.

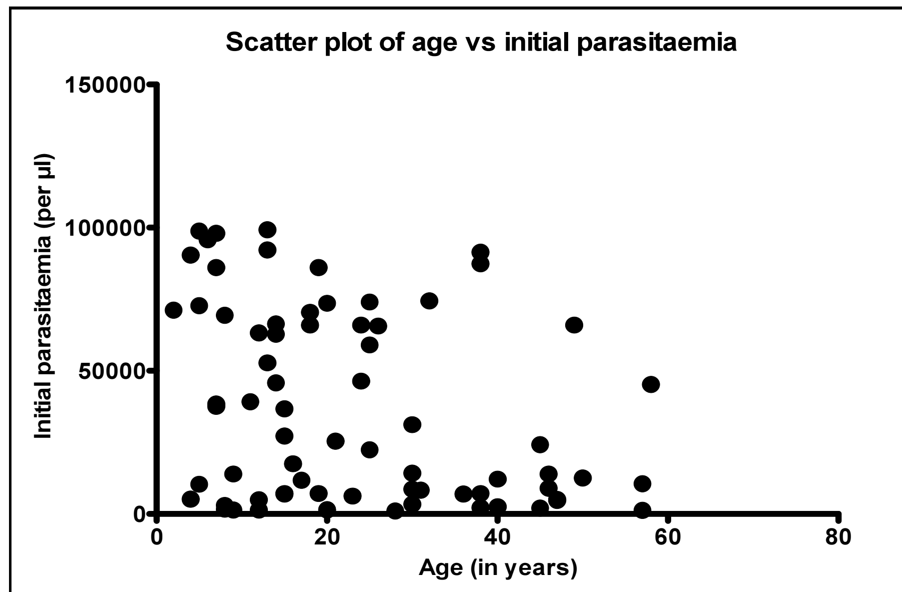


Fig. 4.4: Scatter plot for depicting correlation between age and initial parasitaemia of patients from Odisha study site

The correlation between fever and initial parasitaemia was done in all the 72 samples and it was found out to be positive i.e. the more the parasitaemia, the more the fever (Pearson r = 0.2904, p value = 0.0134, 95% CI = 0.06287 to 0.4892). The scatter plot is depicted in Fig. 4.5.

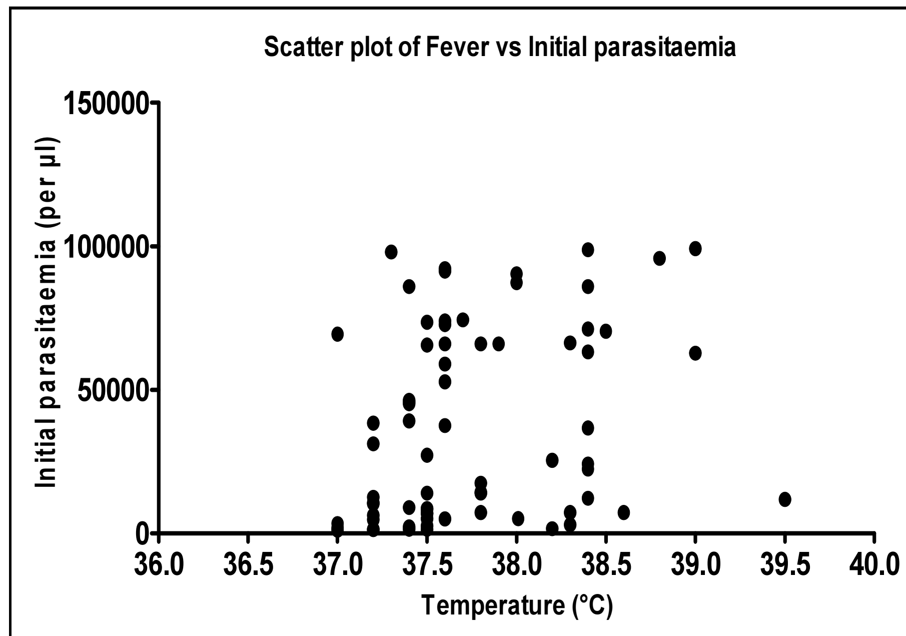


Fig. 4.5: Scatter plot for depicting correlation between fever and initial parasitaemia of patients from Odisha study site

4.4.4.2 Arunachal Pradesh

At Arunachal Pradesh study site, age and initial parasitaemia does not correlate, however, like Odisha study site, herein too, fever and initial parasitaemia correlates positively when analysed in all the 42 samples (Pearson r = 0.5903, p value < 0.0001, 95% CI = 0.3489 to 0.7582). The scatter plot is depicted in Fig. 4.6.

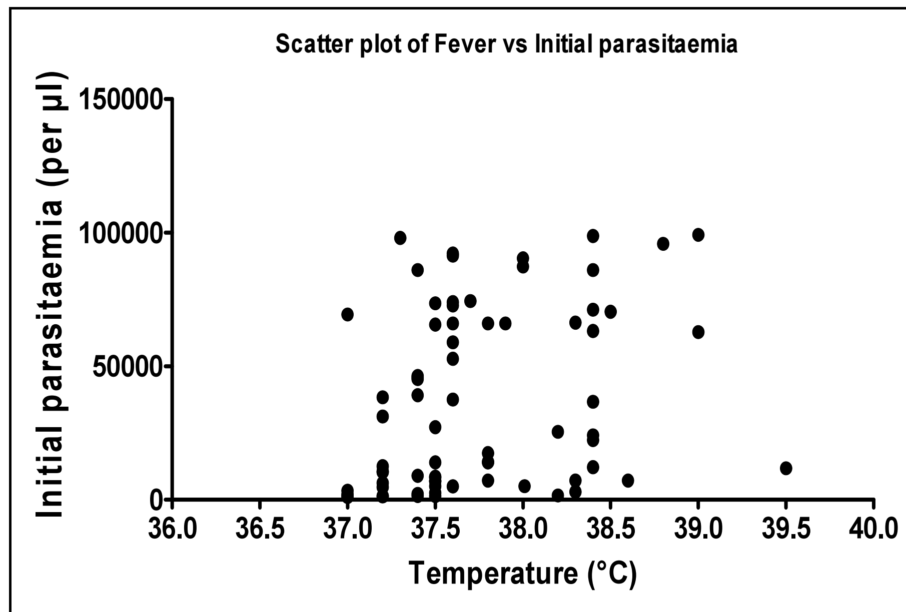


Fig. 4.6: Scatter plot for depicting correlation between fever and initial parasitaemia of patients from Arunachal Pradesh study site

4.4.5 Correlation of Day 3 Positivity with Age, Fever and Initial Parasitaemia

4.4.5.1 Odisha

Day 3 positivity was observed in 12 of the 43 samples analysed. Pearson correlation coefficient was found out for percentage parasitaemia on day 3 vs age, fever and initial parasitaemia, separately (n=43). None of above trait correlated with day 3 positivity (p value > 0.05).

4.4.5.2 Arunachal Pradesh

Day 3 positive samples (n=3) was correlated with age, fever and initial parasitaemia separately in the total number of samples analysed (n=26). However, herein, too, the trait could not be correlated significantly (p value > 0.05), which could be due to low frequency of samples being day 3 positive.

4.4.6 Correlation of E431K Mutation with Day 3 Positivity, Age, Fever and Initial Parasitaemia

4.4.6.1 Odisha

Correlation between E431K and day 3 positivity, age, fever and initial parasitaemia has been shown in Table 4.1. There were three samples at Odisha study site (Mutant=2, Mix=1) which were shown to have mutant genotype for E431K. All these three patients had high initial parasitaemia as evident from the table below while the fever ranged from 37.4 °C to 38.3 °C.

4.4.6.2 Arunachal Pradesh

The findings for Arunachal Pradesh study site was even more interesting where all the four mutant for E431K belonged to the lower age group (3 – 6 years), had high fever (37.8 – 39.1°C) with high initial parasitaemia (34120 – 77800/µl of blood). Table 4.1 details the summary of correlation of E431K mutation with day 3 positivity, age, initial parasitaemia, fever and clinical outcome in patients from both the study sites.

Table 4.1: Correlation of codon 431 mutant genotype with day 3 positivity, age, initial parasitaemia, fever and clinical outcome in samples from Odisha and Arunachal Pradesh

Study Site	Sample ID	E431K	Day 3 positivity (Mx)	Day 3 positivity (RT)	Age (in years)	Initial Parasitaemia (per µl)	Fever (≥37.5°C)	Clinical Outcome
Bisra, Odisha	Patient A	Mutant	No	Yes	14	66400	Yes	ACPR
Bisra, Odisha	Patient B	Mutant	No	Yes	49	66000	Yes	ACPR
Bisra, Odisha	Patient C	Mix	No	No	11	39200	No	ACPR
Miao, Arunachal Pradesh	Patient A	Mutant	No	No	5	72920	Yes	LCF
Miao, Arunachal Pradesh	Patient B	Mutant	No	No	6	58000	Yes	WTH
Miao, Arunachal Pradesh	Patient C	Mutant	No	No	5	34120	Yes	ACPR
Miao, Arunachal Pradesh	Patient D	Mutant	Yes	No	3	77800	Yes	Delayed PCT/WTH

Mx=Microscopy, RT=Real time

4.4.7 Summary

4.4.7.1 Odisha

The summary of the overall result obtained after analysis from Odisha study site is depicted in Fig. 4.7.

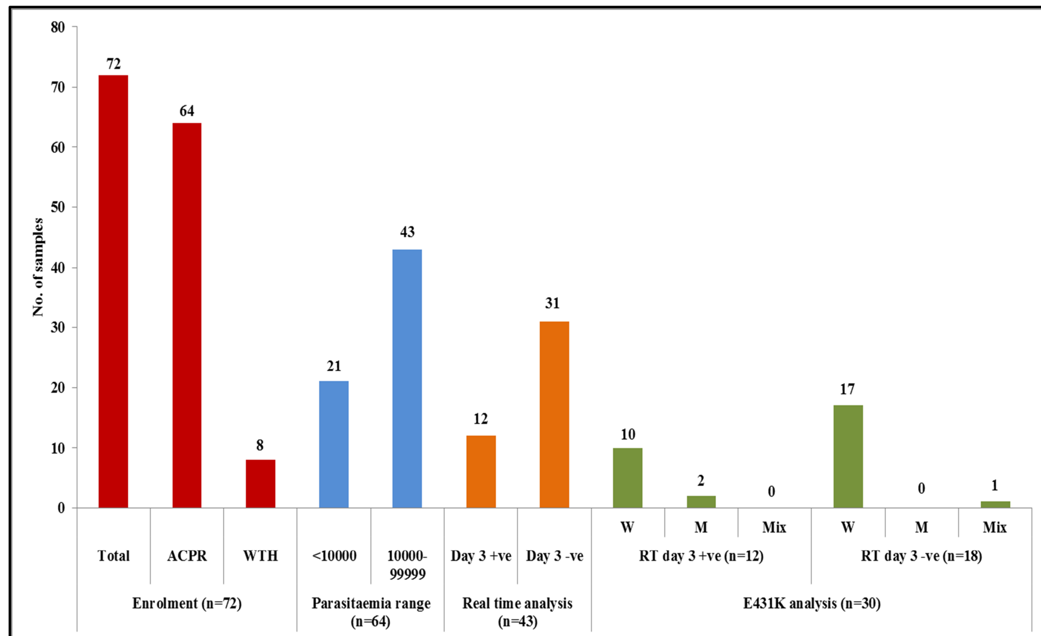


Fig. 4.7: Graphical representation of the total work done on samples from Bisra CHC, Sundergarh district, Odisha

4.4.6.2 Arunachal Pradesh

The summary of the overall result obtained after analysis from Arunachal Pradesh study site is depicted in Fig. 4.8.

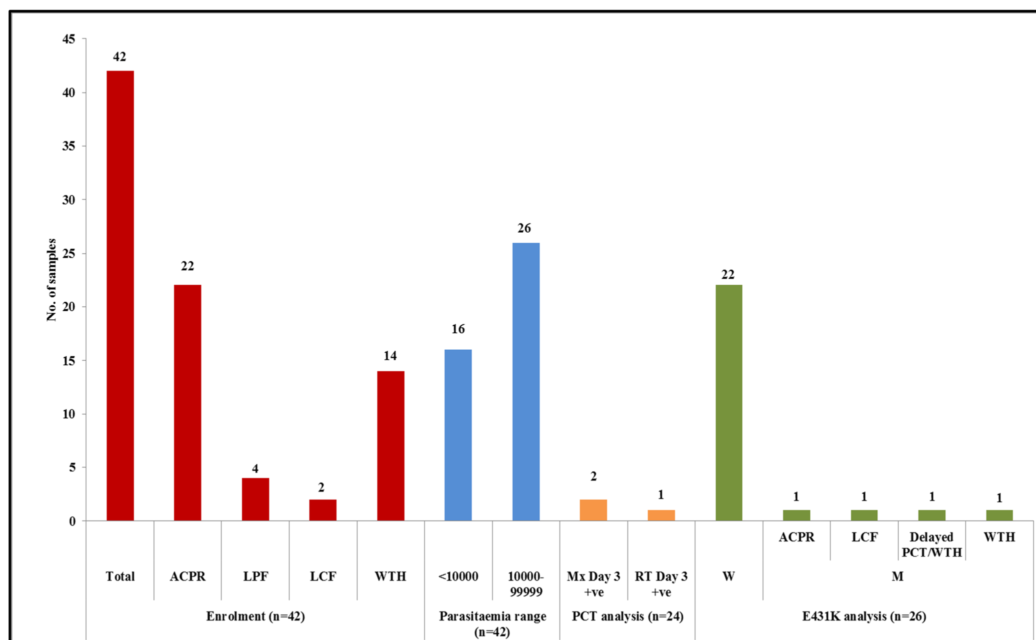


Fig. 4.8: Graphical representation of the total work done on samples from Miao PHC, Changlang district, Arunachal Pradesh

4.5 Discussion

In vitro data needs to be correlated with *in vivo* data for better understanding of clinical efficacy of any drug. Herein, artemisinin resistance was monitored at the genomics level and thereby correlated with the clinical data of the study available after 42 follow-up. It indeed gave deeper insights in to the risk of development of artemisinin resistance in two of the endemic sites of the country whereby it revealed that all the strains of *Plasmodium falciparum* which were day 3 positive either by Real time PCR or microscopy had high initial parasitaemia. Reports suggests that hyperparasitaemia does affect parasite clearance time and is associated with artemisinin resistance (Stepniewska et al. 2010). Findings of this study correlates the day 3 positive isolates with high parasite density on admission from both the study sites. Infact, from Arunachal Pradesh study site the two samples with highest initial parasitaemia were day 3 positive by

microscopy and Real time PCR, respectively. Also, all the 12 isolates from Odisha study site too revealed high initial parasite densities amongst the enrolled. The positive correlation of fever and initial parasitaemia also emphasizes the outcome as initial parasite load greatly affects the clinical picture (Matteelli and Castelli 1997). It is also evident that children have low background immunity as compared to adults, this was validated by findings of the present study wherein age correlated inversely with initial parasite load at one of the studied site (Odisha). However, more efforts on correlating age of the patients, temperature, initial parasitaemia with day 3 positivity at both the study sites could not fetch valid conclusions. This might be due to low frequency of samples being day 3 positive.

Presence of E431K mutation in isolates which were day 3 positive (Odisha) or which turned out be clinical failure (Arunachal Pradesh) is noteworthy since E431K is an important SNP in context with artemisinin resistance as discussed in Chapter III. Moreover, its correlation with high fever and lower age group especially in samples from Arunachal Pradesh is even more revealing, suggesting a possible involvement of this trait in imparting resistance. However, to be conclusive, the trait needs to be monitored in greater number of isolates from different sites of the country.

Chapter V

Summary and Conclusion

5.1 Summary

Owing to malaria's high mortality rate and the disease burden exposing 3.3 billion at risk every year, the concern becomes huge. The disease is transmitted by bites of infected mosquitoes, Anopheles species, though the causative entities are the species of parasite Plasmodium. Out of all the five known species of Plasmodium which cause the disease in human beings, *Plasmodium falciparum* is the most threatening and deadly form causing concern throughout the globe owing to the highest mortality rate and problem of drug resistance posed by this species. This species has developed resistance to every other antimalarial used in the past except artemisinin and its derivatives which is used at present for its treatment. The signs of emerging artemisinin resistance have been reported at Thai-Cambodia border raising the concern since at present there is no other drug which can replace artemisinin. The region where artemisinin resistance has been reported has always remained the epicentre of development of drug resistance, even in the past. Moreover, it shares close border with India's epicentre of drug resistance, the north eastern states. History of development of antimalarial drug resistance comes from north eastern states of the country. Initial reports of development of chloroquine resistance and SP resistance comes from Karbi Anglong district of Assam. It is with all this background that artemisinin needs to be contained from developing resistance. In view of this, WHO has initiated the Global Plan for Artemisinin Resistance Containment (GPARC) to preserve this precious antimalarial succumb to resistance.

Currently, artemisinin based combination therapy (ACT) is administered for the treatment of uncomplicated falciparum malaria based on WHO's recommendation. Artemisinin and its derivatives are the sesquiterpenes class of drugs which are known to

rapidly reduce the parasite biomass to 10^4 fold per 48 hour of life cycle. In this regard, it has emerged as one of the safest and fast acting life-saving drug and ever since its discovery, artemisinin and its derivatives have overshadowed quinine, which has been considered a standard life saving rescue drug since about one century. If artemisinin succumbs to resistance problem, it may pose even bigger challenge in combating malaria. Therefore, through this study, delayed parasite clearance time (PCT), which is a marker trait for studying artemisinin resistant malaria, was studied with the help of two techniques in two highly malaria endemic sites of the country. The selection of site was such that at one hand we did study in the highest disease burden state and on the other hand, the study state had been the epicentre of antimalarial drug resistance in the past. The results of PCT determined by microscopy and Real time PCR were compared and correlated with treatment outcome after 42 days of follow-up of AS+SP administration. There were 12/43 samples at Odisha study site while 3/24 samples at Arunachal Pradesh study site which were found to have increased PCT according to the combined results of microscopy and Real time PCR. The prevalence of E431K mutation in *Pfatpase6* gene in 6.7% and 15.4% of the samples, its correlation with day 3 positivity, lower age of the patients, high fever and clinical failure at study sites in Odisha and Arunachal Pradesh, respectively, was another major finding which further emphasizes recurrent surveillance of artemisinin resistance in disparate malaria endemic sites of the country. The results indicate that Real time PCR can be a very useful tool in artemisinin resistance monitoring studies which can guide the National Programme on the status of development of resistance from time to time. Since the sensitivity of Real time PCR is high, it can detect fewer parasites, which may be present on day 3 and thus could serve as an early warning signal for the emerging artemisinin

resistance. For this, resistance monitoring at key sentinel sites could be a significant approach. This exercise will help to preserve artemisinin drug and its derivatives effectively and assist the experts in devising suitable strategy to thwart the onset of resistance to one of the most valuable antimalarial drugs known to mankind.

5.2 Conclusion

The present study was conducted to determine Parasite Clearance Time (PCT) post administration of AS+SP drug by enrolling *P. falciparum* patients at sites from two malaria endemic states of India, Odisha and Arunanchal Pradesh. The PCT monitoring in the study patients was done with the help of microscopy which is still considered a gold standard technique. The samples were also assayed with more sensitive technique Real time PCR at the two proposed sites. In Odisha, the efficacy of AS+SP combination was found out to be 100% upon 42-day follow-up which was also correlated with the fact that no patient was parasitaemic on day 3 as per microsopy. From the combined results of Real time PCR, end point diagnostic PCR and prevalence of E431K mutation in *Pfatpase6,* it can be concluded that the signals of artemisinin resistance has started emerging in the country and therefore, there is a need to be vigilant about development of artemisinin resistant malaria, as well as its entry in to the country from neighbouring eastern countries well known for antimalarial resistance to multiple drugs.

The high failure rate of AS+SP at the Arunachal Pradesh study along with presence of strains having increased PCT are thus a cause of concern. The prevalence of E431K mutation in *Pftatpase6* gene is another trait which needs to be studied for its role in imparting artemisinin resistance. Correlation studies revealing significant correlation between age, fever and initial parasitaemia and those suggesting prevalence of E431K

mutation in treatment failure, day 3 positive samples with low age, high fever and high initial parasitaemia emphasizes the outcome. The above results conclude that artemisinin resistance monitoring should be a routine practice in endemic areas of the country. It also indicate that the deployment of more sensitive tools like Real time PCR could prove useful for conducting such studies in addition to gold standard microscopy.

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Annexures

Annexure I: Informed Consent Form

Title of the project: "Studying artemisinin resistance in selected malaria endemic sites of India"

Name of the Guide: Dr. Neelima Mishra, Scientist 'E'
Name of the Organization: National Institute of Malaria Research

Information Sheet Introduction

We are doing a surveillance study on the treatment of malaria, which is very common in this country. I am going to give you information and invite you/your child to participate in this surveillance study. Before you decide, you can talk to anyone you feel comfortable with. There may be some words that you may not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask them to me, the study doctor or the staff.

Malaria is one of the most common and dangerous diseases in this region which needs treatment with medicines. The purpose of this study is to monitor artemisinin resistance in *Plasmodium falciparum* infected malaria patients aged above 6 months in this area.

You/your child will receive 3 doses of medicine ACT (AS+SP) over a period of 3 days.

We are inviting all adults and children to take part in this surveillance study because it is important that we confirm parasite clearance time in patients treated with ACT and thus, monitor emerging artemisinin resistance in sick patients who have malaria and live in this area.

Your participation/your decision to have your child participate in this study is entirely voluntary. It is your choice whether to participate or not/to have your child participate or not. If you choose not to consent, all the services you/your child receive at this clinic will continue and nothing will change. You may also choose to change your mind later and stop participating, even if you agreed earlier, and the services you/your child receives at the clinic will continue.

The medicine ACT is recommended by the Ministry of Health. It is called an antimalarial drug because it helps to kill the parasites in your blood. This antimalarial drug is known to be very effective. You should know that this medicine has some minor side effects.

If we find that the medicine that is being used does not kill all the parasites in your body, we will use what is called a "rescue medicine". The name of the medicine is quinine or mefloquine.

Upon first visit you/your child will be examined by a physician and two or three drops of blood will be taken from your/your child's finger onto a blood slide. This helps to characterize and count the parasite. Three drops of blood will also be taken from

you/your child's finger before drug administration on Day 0 onto a piece of filter paper. This will be used for monitoring parasite clearance time. You/Your child will feel pain when the needle sticks but this will go away very quickly. You/Your child will receive the first dose of treatment.

During the follow-up, blood will be taken 7-8 times from you/your child's finger onto a slide and a small piece of filter paper. The blood will be used to count the parasite in the blood, to study the characteristics of the parasites and to monitor parasite clearance time. This analysis will be done after the treatment but has no implication on the outcome of the illness. Blood will also be used to study molecular markers and if feasible, drug levels too.

At the second visit (Day 1) and third visit (Day 2), you/your child will be given the second dose and third dose of treatment respectively and three drops of blood will be taken onto a glass slide and filter paper. On the fourth visit (Day 3), no drug will be given but only three drops of blood will be taken from your/your child's finger onto a slide and a piece of filter paper. If presence of parasite is detected on Day 3, blood will also be taken in similar manner on the glass slide and a piece of filter paper on Day 4. Further follow up will be done in similar manner on Day 7, Day 14, Day 21 and Day 28 respectively.

The surveillance study takes place over 28 days. During that time, it will be necessary for you to come to the health facility 8-9 times, for 1 hour each day. At the end of one month, the surveillance will be finished.

As already mentioned, the medicine can have some unwanted effects or some effects that we are not currently aware of. However, we will follow you/your child closely and keep track of these unwanted effects or any problems. We will give you a telephone number to call if you notice anything out of the ordinary, or if you have concerns or questions. You can also come/bring your child to this health facility at anytime and ask to see local investigators ………………………………………We may use some other medicines free of charge for you to decrease the symptoms of the side effects/reactions or we may stop the use of one or more medicines. If necessary we will discuss it together with you and you will always be consulted before we move to the next step.

By participating in this surveillance study it is possible that you/your child may experience a bit of pain or fear as the finger is pricked. The pain should disappear in one day.

If you/your child participate(s) in this surveillance study, you/he/she will have the following benefits: any illnesses related to malaria or to the malaria treatment will be treated at no charge to you. There may not be any other benefit for you/your child but your/his/her participation is likely to help us find the answer to the surveillance study and this will benefit to the society and to the future generations.

You will not be provided any incentive to take part in this surveillance study. With this surveillance study, something out of the ordinary is being done in your community. It is possible that if others in the community are aware that you are

participating, they may ask you questions. We will not be sharing the identity of those participating in the surveillance study. The information that we collect from this surveillance study will be kept confidential. Information about you/your child that will be collected from the surveillance study will be put away and no-one but the team involved in the study will be able to see it. Any information about you/your child will have a number on it instead of your/his/her name. Only the study team members will know what your/his/her number is and we will lock that information up with a lock and key. It will not be shared with or given to anyone.

The knowledge that we get from this study will be shared with you before it is made widely available to the public. Confidential information will not be shared. There will be small meetings in the community and these will be announced. Afterwards, we will publish the results in order that other interested people may learn from our surveillance study

You do not have to take part/to agree to your child taking part in this surveillance study if you do not wish to do so and refusing to participate/to allow your child to participate will not affect your treatment/your child's treatment at this centre in any way. You/your child will still have all the benefits that you would otherwise have at this centre. You/your child may stop participating in the surveillance study at any time you wish without losing any of your rights as a patient here. Your/your child's treatment at this centre will not be affected in any way.

If you/your child do not wish to take part in the surveillance study, you/your child will be provided with the established standard treatment available at the centre/institute/hospital. People who have malaria are treated according to National Policy.

If you have any questions you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact the following.

Ms. Ruchi Gupta, NIMR, New Delhi Phone (Mobile): +919311199588

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This proposal has been reviewed and approved by Institutional Ethics Committee of National Institute of Malaria Research, which is a committee whose task it is to make sure that study participants are protected from harm.

By

Ms. Ruchi Gupta
ECR Division
National Institute of Malaria Research
Sector – 8, Dwarka, New Delhi – 110077

Guided by:

Dr. Neelima Mishra, Scientist 'E'
National Institute of Malaria Research
Sector – 8, Dwarka, New Delhi – 110077

Title of the project: "Studying artemisinin resistance in selected malaria endemic sites of India"

Certificate of Consent for patient >18 years old

I have been invited to participate in surveillance on "Studying artemisinin resistance in selected malaria endemic sites of India". I understand that it will involve me receiving 3 doses of medicine and 8-9 visits to the health facility. I have been informed that the risks are minimal and may include pain at the finger. I am aware that there may be no benefit personally except for getting treated for malaria and that I will not be compensated. I have been provided with the name of an investigator who can be easily contacted using the number I was given for that person.

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this surveillance study and understand that I have the right to withdraw from the surveillance study at any time without having effects on my medical care in any way.

Name of the participant__________________ Thumbprint of participant

Signature of the participant ___________________

Date ___________________________ (day/month/year)

Name of the witness _____________________ Thumbprint of witness

Signature of the witness ______________________

Date ________________________ (day/month/year)

Name of the investigator/ MO _______________________

Signature of the investigator / MO_______________________

Date ___________________________ (day/month/year)

A copy of this Informed Consent Form has been provided to participant

Title of the project: "Studying artemisinin resistance in selected malaria endemic sites of India"

Assent form for Patient of 6 months to < 18 years old

Name of Patient: __________________ Age of patient ______years ______ month(s)

I have been invited to have my child participate in a study entitled **"**Studying artemisinin resistance in selected malaria endemic sites of India". I have read the attached written information and / or received verbal information on the above study. I have been given the opportunity and time to have any questions answered to my satisfaction. I am aware the participation is entirely voluntary. I understand that my child can withdraw at any time without giving a reason and without this affecting his/her future care. I understand I will receive a copy of this information and consent form.

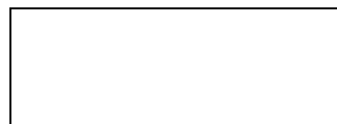
By signing this information and consent form I agree that the personal data of the patient may be used as described in this consent form and may be consulted by qualified representatives from the sponsor, the Ethics Committee or the health authorities.
I allow ______________________________________ to participate in the study.

Printed Name of the Parent / Legal Acceptable Representative (LAR)

____

Thumbprint of the parent/guardian:

Signature of the Parent / LAR __________________



Date __________________ (day/month/year)

If Parent /LAR cannot read the form themselves, a witness must sign here:

I was present throughout the entire informed consent process with the volunteer's Parent/Legal representative. All questions were answered and the patient's parent/ legal representative has agreed to have the patient take part in the research.

Printed Name of the witness __________________

Signature of the witness __________________

Date __________________ (day/month/year)

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

Printed Name of the person who obtained consent ____________________

Signature of person who obtained consent ____________________

Date ____________________ (day/month/year)

Name of the investigator/ MO _________________________

Signature of the investigator / MO__________________________

Date _____________________ (day/month/year)

A copy of this Informed Consent Form has been provided to the participant

Annexure II: Important Definitions

Cycle threshold (Ct): In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold i.e. exceeds background level (Source: https://www.google.co.in/search?q=Ct+definition&oq=Ct+definition&aqs=chrome..69i57.7885j0j7&{google:bookmarkBarPinned}sourceid=chrome&{google:omniboxStartMarginParameter}ie=UTF-8#q=cycle+threshold+definition).

Early treatment failure (ETF): Development of danger signs or severe malaria on Day 1, 2 or 3, in the presence of parasitaemia; parasitaemia on Day 2 higher than on Day 0, irrespective of axillary temperature; parasitaemia on Day 3 with axillary temperature >37.5°C; and parasitaemia on Day 3, >25% of count on Day 0 (NIMR and NVBDCP 2014).

Late clinical failure (LCF): Development of danger signs or severe malaria in the presence of parasitaemia on any day between Day 4 and Day 28 (Day 42) in patients who did not previously meet any of the criteria of early treatment failure; and presence of parasitaemia on any day between Day 4 and Day 28 (Day 42) with axillary temperature >37.5°C in patients who did not previously meet any of the criteria of early treatment failure (NIMR and NVBDCP 2014).

Late parasitological failure (LPF): Presence of parasitaemia on any day between Day 7 and Day 28 with axillary temperature <37.5°C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure (NIMR and NVBDCP 2014).

Adequate clinical and parasitological response (ACPR): Absence of parasitaemia at day 28 irrespective of axillary temperature and without previously meeting any of the WHO criteria for early or late treatment failure, or late parasitological failure (Source: http://bestpractice.bmj.com/best-practice/evidence/glossary/0919/sr-0919-g4.html).

Publication

Monitoring artemisinin resistance in Plasmodium falciparum: *comparison of parasite clearance time by microscopy and real-time PCR and evaluation of mutations in* Pfatpase6 *gene in Odisha state of India*

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ORIGINAL PAPER

Monitoring artemisinin resistance in *Plasmodium falciparum*: comparison of parasite clearance time by microscopy and real-time PCR and evaluation of mutations in *Pfatpase6* gene in Odisha state of India

Ruchi Gupta[1] · Neelima Mishra[1] · Ashwani Kumar[2] · Roma Rana[1] · Bina Srivastava[1] · P. K. Tyagi[3] · Anupkumar R. Anvikar[1] · Neena Valecha[1]

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Abstract Antimalarial drug resistance including artemisinin resistance in *Plasmodium falciparum* malaria is a major concern in combating malaria throughout the world. Delayed parasite clearance time (PCT) is indicative of emergence of artemisinin resistance. Herein, PCT has been monitored with the help of gold standard technique microscopy accompanied by a more sensitive real-time assay for academic purpose. After the administration of artemisinin based combination therapy, artesunate+sulfadoxine pyrimethamine (AS+SP), all the subjects were followed up to day 42 for monitoring the therapeutic efficacy of AS+SP in Bisra Community Health Centre (CHC), Sundergarh district in the state of Odisha in India. Further, representative samples were analyzed for L263E, E431K, A623E and S769N SNPs in *Pfatpase6* gene and copy number polymorphisms in *Pfmdr1* gene. Though all the samples were found parasite negative according to microscopy by the end of day 3 and attained adequate clinical and parasitological response (ACPR) at the end of day 42, real-time PCR showed day 3 positivity in 12 of the total analyzed samples (n=43). This was further validated by end-point diagnostic PCR and correlated with high initial parasite load. E431K mutation was observed in 2 of the 12 samples (16.7 %) while the controls (n=18) were all wild. L263E, A623E and S769N were wild in all the analyzed samples (n=30). *Pfmdr1* copy number analysis showed no change in the said trait. Conclusively, real-time PCR could support microscopy for better monitoring of PCT and may provide as an additional but useful research tool for artemisinin resistance studies.

Keywords *Plasmodium falciparum* · Delayed PCT · Artemisinin resistance · *Pfatpase6* · E431K · Real-time PCR

✉ Neelima Mishra
neelima.nimr@gmail.com

[1] National Institute of Malaria Research, Sector 8, Dwarka, New Delhi 110077, India

[2] National Institute of Malaria Research Field Unit, Panaji, Goa 403001, India

[3] National Institute of Malaria Research Field Unit, Rourkela, Odisha 769002, India

Introduction

Artemisinin-resistant malaria is at present a major topic of research and cause of concern for scientists across the world. This is because of the reason that the drug is at the peril of losing its efficacy as evidenced at the Thai–Cambodia border where delayed parasite clearance time was observed and is believed to be associated with artemisinin resistance (Dondorp et al. 2009). Losing artemisinin to resistance may pose a bigger challenge in combating malaria (White 2004). Lately, definition of artemisinin resistance has remained under controversy when some scientists claim that delayed PCT urges alarm for declining efficacy of artemisinins (Meshnick 2012; Dondorp and Ringwald 2013), while others claim that delayed PCT should be correlated with treatment failure after administration of ACT (TFACT) and in vitro susceptibility assays for it to be called as 'artemisinin resistance' (Krishna and Kremsner 2013). However, WHO's definition of artemisinin resistance still remains unchanged and it gives room to day 3 positivity in defining artemisinin resistance (WHO 2014). It is also said that delayed PCT will not necessarily lead

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to treatment failure (WHO 2014). This is well explained by the fact that in the Greater Mekong Subregion, failure following treatment with ACT was only observed where resistance to partner drug exists regardless of the status of artemisinin resistance (WHO 2014).

Early monitoring tools for monitoring artemisinin resistance includes standard therapeutic efficacy tests with ACT, which allows monitoring trends of prevalence and detecting rate of patients positive at day 3. If >10 % of the patients are parasite positive at day 3, these samples need further confirmation and investigation (Ringwald, WHO Global Malaria Programme; WHO 2014). Apart from this, novel phenotypic assays have also been developed recently to detect artemisinin resistance (Witkowski et al. 2013). Genome study of *Plasmodium falciparum* is yet another method to catch artemisinin resistance (Winzeler and Manary 2014). Monitoring of the trait can also be done by in vitro method called WHO microtest (Mark III), in vitro and ex vivo Ring-stage Survival Assay (RSA^{0-3h}) performed on 0- to 3-h post-invasion rings obtained from culture-adapted and freshly collected parasites from patients' blood, respectively (WWARN 2014).

Monitoring artemisinin resistance is the need of the time as artemisinin is currently the drug of choice being administered as combination therapy with weaker partner drug for the treatment of uncomplicated falciparum malaria as recommended by World Health Organization (WHO 2010a, b). Artemisinin being more potent and having shorter half life (White 1999), reduces the initial parasite burden to such an extent that the weaker partner drug having longer half life is then able to take care of the remaining parasitaemia, so as to delay resistance. This way both the partners of ACT work hand in hand. According to WHO, resistance in either of the partner drug of ACT can hamper the efficacy of the other partner, which is also evident from the above explanation (WHO 2010a, b).

In India, AS+SP is the ACT recommended by the National Programme, for the treatment of uncomplicated *P. falciparum* infections except in North-east (NE) states where the policy has been modified to artemether–lumefantrine (AL) recently (NIMR and NVBDCP 2014). Resistance to SP has already been reported from northeastern parts of the country and, that is why the efficacy of artemisinin partner is at stake in these regions (Mishra et al. 2014). Therefore, continuous and diligent surveillance of artemisinin resistance is essential in endemic areas of the country.

In the year 2013, Odisha contributed to 44 % of the total *P. falciparum* malaria cases in the country. Also, it shares border with four other malaria endemic states namely Jharkhand, Chhattisgarh, West Bengal, and Andhra Pradesh which contribute to significant numbers of total malaria cases every year (NVBDCP 2014). Transmission in Odisha is perennial with a seasonal peak from July to October. Moreover, drug-resistant Plasmodium strains have already been reported in high frequency in many studies conducted in the state (Mishra et al. 2012; Srivastava et al. 2013; Das Sutar et al. 2013). Keeping these facts in mind, this study was designed to monitor PCT and to correlate it with treatment outcome so as to capture a better picture of efficacy of the recommended ACT in Odisha. Although microscopy still remains the gold standard, for closer monitoring of PCT, the use of more sensitive tool, real-time PCR has been deployed and the results were compared with microscopy. Parasite clearance being the first order process depends largely on initial parasite load, i.e., higher the initial parasitaemia, longer the PCT (White 2011). Thus, 10,000 parasites/μl of infected blood was chosen as the baseline parasitaemia for analysis of PCT by real-time PCR.

Furthermore, based on preliminary results of this comparison, promising strains were analyzed for L263E, E431K, A623E, and S769N SNPs in *Pfatpase6* gene, which is thought to be a candidate gene involved in playing a pivotal role in artemisinin resistance (Eckstein-Ludwig et al. 2003; Jambou et al. 2005; Li and Zhou 2010; Ding et al. 2011) in studies conducted across the globe. These studies are focussed on analyzing mutations in this gene, the major reports being those of the above-stated SNPs (Tahar et al. 2009; Jambou et al. 2010; Krishna et al. 2010; Zakeri et al. 2012). Another putative candidate related to artemisinin resistance is amplification in *Pfmdr1* gene copy number. The trait has been reported from Southeast Asia region (SEA) and associated with increased IC_{50} after treatment with artesunate, artemisinin along with mefloquine (Pickard et al. 2003) consistent with the observation that these regions are always the epicenter for emergence of antimalarial drug resistance including artemisinin. However, no such report is published on Indian isolates which further increased our quest to look for prevalence of *pfmdr1* copy number polymorphisms in Indian strains of *P. falciparum* from the state of Odisha.

Methods

Ethics statement

The study was approved by the Ethical Committee of National Institute of Malaria Research, New Delhi, India. The present cross-sectional study is a part of larger study on monitoring therapeutic efficacy of anti-malarial drugs in India.

Study site

The study was conducted at Bisra CHC, Sundergarh district of Odisha state in India during June to September 2012. The purpose of the study was explained to the participants and informed consent was obtained in each case. In case of minors, informed consent was obtained from parent/guardian.

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Sample collection

All the suspected malaria patients, reporting to the local CHC and those found during field survey, were screened for malaria by microscopy and, in some cases with both RDT and microscopy. Positive *P. falciparum* infections were enrolled in the study following inclusion and exclusion criteria as per WHO protocol (WHO 2009). Malnutrition, age (<6 months to >60 years), pregnancy, lower Hb content (than normal), and mixed infection were some of the major exclusion criteria including others. Others fulfilling the inclusion criteria were enrolled in the study. Thick and thin smears and filter paper blood spots were prepared on days 0, 1, 2, and 3 as counted from the day of administration of drug (AS+SP). Further, follow-up was done as per WHO schedule on days 7, 14, 21, 28, 35, and 42, respectively, to monitor the clinical outcome (WHO 2009).

Microscopic examination of blood films

Giemsa-stained blood smears were examined under oil immersion (100x) lens. In order to count the parasites, 200 white blood cells (WBCs) were counted in case of positive slide or else 100 thick films were screened to declare a slide negative. A count of 8,000 WBCs/μl of blood was assumed to obtain the final density of parasite per microliter of blood.

Genomic DNA isolation

Isolation of genomic DNA was done from three punched out blood spots of filter paper, using QIAamp® DNA Blood Mini Kit (Qiagen) according to manufacturer's instructions. DNA was isolated from the samples collected on the day before administration of drug (day 0) and on each subsequent day of ACT administration (days 1, 2 and day 3) for monitoring PCT by real-time PCR.

Real-time PCR

Real-time PCR was conducted using SYBR green dye on Roche's LightCycler® 480. Primers were designed on *pgmet* gene of Plasmodium species as described elsewhere (Beshir et al. 2010) and *human tubulin* gene was used as endogenous control. For every sample, two sets of reactions were set up, one for each gene. Each reaction comprised of 2X LightCycler® 480 SYBR Green Master (Roche), 0.3 μM of each forward and reverse primer, 5 μl of template DNA, adjusting the total reaction volume to 20 μl with molecular grade water. The thermal profile included pre-incubation at 95 °C for 6 mins; amplification at 95 °C for 15 s, 60 °C for 1 min and 72 °C for 10 s (acquisition mode set as single at this temperature) for a total of 50 cycles. A cutoff of 44 cycles was set to define positive samples. Every set up was accompanied by a No Template Control (NTC) for both sets of primers, to check on contamination and non-specific amplification. Melt curve analysis was done at 95 °C for 10 s, 65 °C for 1 min and 95 °C continuous, for checking if the primers are specific and thus, producing single peaks. Standard curve was prepared using 10-fold dilutions of 3D7 culture and uninfected human blood at five different concentrations, for calculating the reaction efficiency of *pgmet* and *human tubulin* genes, respectively.

Assuming day 0 parasitaemia as 100 %, relative decrease in parasitaemia was calculated using $2^{(-\Delta\Delta Ct)}$ with the help of LightCycler® 480 SW 1.5 and Microsoft Office Excel 2007. Each reaction was set up in triplicate and the mean of the triplicate was taken for calculation of relative reduction in parasitaemia. Difference of more than 0.4 Ct between the two replicates was discarded.

Single-step diagnostic PCR

To further confirm the findings of real-time PCR, single-step diagnostic PCRs were conducted using two different methodologies in the representative samples (n=12) which were found to be parasite positive on day 3 as per real-time. The primers PL3, PL4, and PL5 were used (Patsoula et al. 2003) to amplify DNA sample isolated for Odisha site on days 0, 1, 2, and 3 of enrolment using Dream Taq (Thermo scientific) master mix. *Pf* specific bands were expected to be seen at 346 bp which was analyzed in 2 % agarose gel. Diagnostic PCR was also set up using primers Pfr364 Alt-Forward and Pfr364 Alt-Reverse as described by Demas et al. (2011) which yields 220 bp band specific for *Pf*.

PCR-RFLP for *Pfatpase6* mutation analyses

PCR-RFLP was deployed for analysis of mutations in codon 263, 431, 623, and 769 of *Pfatpase6* gene. Single step PCR was conducted using primers described elsewhere (Zakeri et al. 2012) followed by ApoI and MboII digestion for codon 263 and 431, respectively. PCR was done using 2X DreamTaq DNA Polymerase (Thermo Scientific), thermal profile included initial denaturation at 94 °C for 4 min, followed by 35 cycles of amplification at 94 °C for 1 min, 58 °C for 2 min, 72 °C for 1 min, and final extension at 72 °C for 5 min. Confirmation of PCR amplification was done on 1.5 % agarose gel.

ApoI and MboII (New England Biolabs, Inc.) digestion was done for 15 min at 50 and 37 °C, respectively, using 10X CutSmart™ buffer supplied with the enzyme in a total of 20 μl reaction. Digested products were loaded on 2.5 % agarose gel.

In silico analysis was done using Gene Runner to differentiate between patterns of wild and mutant-type isolates after digestion with the restriction enzyme. The expected digested products upon digestion of 948 bp PCR product in case of

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wild-type codon 431 were 128+107+49+241+299+124 bp while that of mutant type were 128+107+49+241+423 bp. However, in case of wild-type codon 263, the 948-bp product remains undigested while the mutant get digested into 312+633-bp fragments.

The analysis of mutation in codon 623 and 769 was followed same as described by Zakeri et al. (2012).

Pfmdr1 copy number analysis

Real-time PCR was deployed for analysis of *pfmdr1* copy number on Roche's LightCycler® 480 using hydrolysis probe. The ratio of *pfmdr1* gene w.r.t. endogenous control *ldh* and *β-tubulin* was determined using ΔΔCt method. The reaction efficiency of each gene was checked by preparing standard curve using 10-fold serial dilutions of 3d7 template. Primers and probes for *Pfmdr1* and *ldh* gene were picked from Pickard et al. (2003) while that of *β-tubulin* gene were derived from Price et al. (2004). 3d7 was used as calibrator having one copy of *pfmdr1* while Dd2 was used as multicopy strain. Each reaction was set up in triplicate and difference of more than 0.4 Ct between the replicates was discarded and was not considered for calculation of the mean Ct. 3d7 being the calibrator was fixed to have one copy number and Dd2 was masked. Copy number calculation was done using formula $2^{-\Delta\Delta Ct}$ and the data was accepted if the copy number of Dd2 ranged between two and three copies. The data of the patient sample was discarded if it was less than 0.6 and the run was repeated for that particular sample.

Data entry and quality check

Clinical and demographic detail of each patient was double entered in WHO therapeutic efficacy database to confirm the entry. Kaplan–Meier survival analysis for treatment failure was done using this data. Statistical analysis of data was done using Microsoft Office Excel 2007 and Statistical Package of Social Sciences 14.0 (SPSS 14.0). For quality check of microscopic count of parasitaemia, 10 % of the randomly selected slides were cross checked with the help of another expert microscopist.

Results

Sample collection and demographic details

A total of 72 samples were collected from *P. falciparum* infected malaria patients reporting to the CHC. These samples were a part of a larger study where therapeutic efficacy of AS+SP was studied. The demographic details of these samples have been detailed in Table 1. Sixty four samples completed the study whereas eight cases were withdrawn from the study, due to loss to follow-up (LFU). Forty-three samples had parasitaemia ≥10,000 parasites/μl and were processed for monitoring PCT by real-time.

Table 1 Baseline details of the patients enrolled in the study at Bisra CHC, Sundergarh, Odisha, India

Study site	Sundergarh (Odisha)
Characteristic	*N*=72
Sex [*n* (%)]	
Male	42 (58.3)
Female	30 (41.7)
Age category [*n* (%)]	
Adults	3 (4.2)
5 to 15	23 (31.9)
Under 5	46 (63.9)
Age (years)	
Mean (sd)	23.2 (15.1)
Range (min–max)	2–58
Weight (kg), day 0	
Mean weight (sd)	39.5 (14.3)
Range (min–max)	10–66
Temperature (°C), day 0	
Mean temperature (sd)	37.7 (0.5)
Range (min–max)	37–39.5
Parasitemia (μl), day 0	
Mean parasitemia (sd)	36,766.7 (33,320.6)
Range (min–max)	1,080–99,200

Therapeutic efficacy

All of the 64 samples which could complete the 42-day follow-up were cured by the end of the study (ACPR). The Kaplan–Meier survival analysis done for these samples showed an efficacy of 100 % in both crude and PCR corrected data (95 % CI). Therefore, the therapeutic efficacy of the combination AS+SP was observed as 100 % (Fig. 1).

Microscopic examination

Out of the 64 patients, 50 % (32) of the patients were found to be parasite negative by the end of day 1. However, day 2 positivity was seen in only three cases while all the cases were found parasite negative by day 3, which correlates with the clinical efficacy of the ACT combination in use.

Real-time PCR

Out of 64 patients, 67 % (*n*=43) patient samples which had baseline parasitaemia ≥10,000 were analyzed for real-time PCR. According to real-time analyses, day 3 positivity was observed in 12 cases (27.9 %) where initial parasite count

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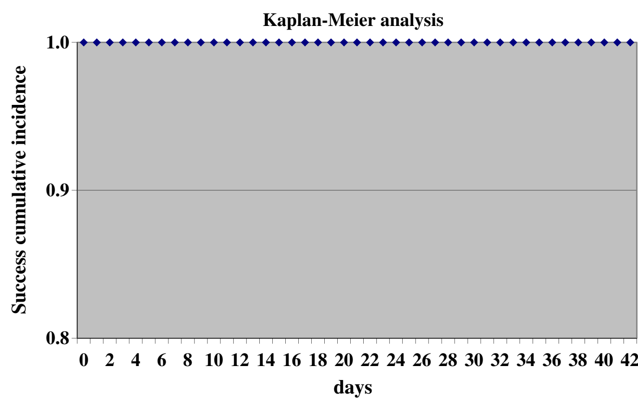

Fig. 1 Kaplan–Meier survival analysis showing an efficacy of 100 % for the studied drug AS+SP after 42-days clinical follow-up at Bisra CHC, Sundergarh, Odisha

ranged from 52,800 to 98,000 parasites/µl of blood. Seventeen cases (39.5 %) were parasite positive by day 2 while 33 were positive on day 1 (76.7 %) (Fig. 2).

Microscopy vs Real-time

Comparison between microscopy and real-time measure of PCT was made in 43 cases. The comparison of malaria positive cases as determined by real-time and microscopy after administration of ACT (AS+SP) has been depicted in Fig. 2. As far as day-3 positivity is concered, results of real-time PCR were revealing which showed 27.9 % cases to be day-3 positivity as compared to microscopy where none of the patient was found to have parasitaemia on day 3. The slides of these patient samples were repeated for microscopic examination with the help of another microscopist which revealed no siginificant difference between the initial count and the final one. The difference in parasite clearance curve in these 12 samples as evident by microscopy and real-time is depicted in Fig. 3. Relative reduction in parasite biomass was measured w.r.t. day 0 parasitaemia by both the techniques. The sensitivity of

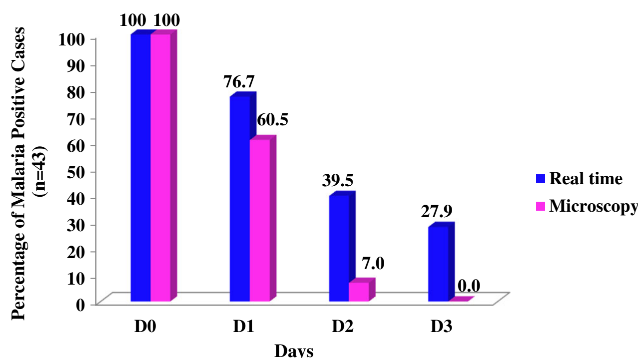

Fig. 2 Comparison of percentage decrease in malaria positive cases after treatment with AS+SP as determined by microscopy and real-time PCR at Bisra, Odisha

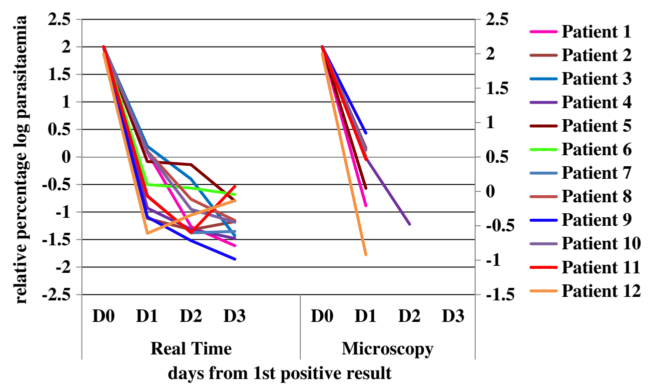

Fig. 3 Relative decrease in percentage log parasitaemia as determined by real-time PCR and microscopy in the representative samples from Odisha study site

real-time was found out to be more as compared to microscopy on each day of measurement.

End-point diagnostic PCR

Real-time PCR revealed 12 samples to be positive till day 3. This was further confirmed by end-point diagnostic PCRs. Ten out of 12 samples were found positive till day 3 by either of the two techniques. However, one of these samples was found positive up to day 2 by both the diagnostic techniques and another one could not be amplified except on day 0 of infection by both the techniques corresponding to the limitations of end-point PCR over real-time PCR. The agreement between the two diagnostic techniques further confirmed the findings. Figures 4 and 5 depicts the gel pictures with *Pf* specific bands on days 0, 1, 2 and 3 (D0, D1, D2, and D3) obtained after diagnostic PCR.

PCR-RFLP mutation analysis for E431K

Pfatpase6 mutation analysis was done on 30 of 43 samples which had parasitaemia ≥10,000/µl of blood. Twelve samples were the ones which were day-3 positive by real-time while the other 18 were included in the analysis to serve as normal/control samples. Interestingly, E431K mutation was observed only in two samples (16.7 %) out of the 12 positive on day 3 while the other controls (n=18) were all wild. However, one mixed-type isolate (5.6 %) was found in the control group. Figure 6 depicts the prevalence of E431K mutation in the total analyzed samples from Odisha. The other three codons, 263, 623, and 769, were 100 % wild in all the analyzed samples (n=30).

Pfmdr1 copy number analysis

Pfmdr1 gene copy number analysis was done in representative samples (n=12) and also in the control group (n=18). None of the sample was found to have amplification in *pfmdr1* gene

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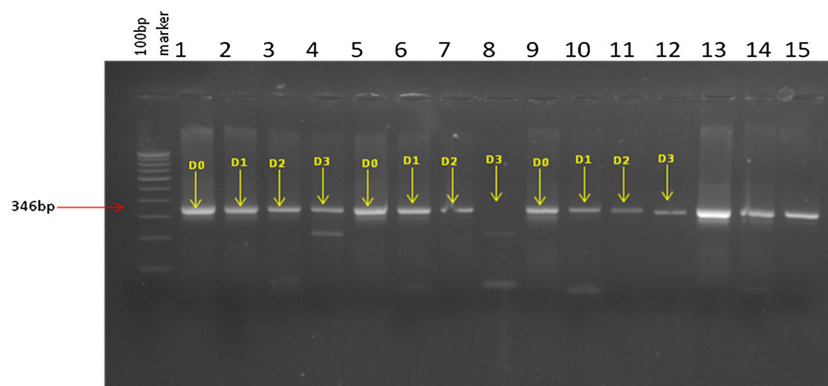

Fig. 4 Single-step diagnostic PCR as described by Patsoula et al. for detection of positivity in samples which are real-time day-3 positive

copy number. The copy number of Dd2 ranged between two and three copies, mean copy number from all the run was 2.3 while those of the patient samples ranged between 0.67 to 1.18 copies of *Pfmdr1*, mean copy number being 0.92.

Discussion

The study was conducted in Bisra CHC, Sundergarh district of Odisha, a state which is known to be highly endemic for malaria. The results revealed real-time PCR as a supporting tool for monitoring artemisinin resistance as it was shown to have greater sensitivity than microscopy. This further emphasized that for close monitoring of artemisinin-resistant malaria, use of molecular tools like real-time assay can be advantageous for timely update and better information. Herein, the findings revealed day-3 positivity (delayed PCT) in significant number of cases according to real-time PCR unlike microscopy. Moreover, it was further validated by diagnostic PCR and correlated with high initial parasite load and E431K mutation in putative candidate, *Pfatpase6*.

Delayed PCT though prone to caveats is still indicative of artemisinin resistance (Dondorp and Ringwald 2013), a debatable but important study objective across the globe. After much controversies, increased parasite clearance time still finds room in WHO's standard definition of suspected or confirmed artemisinin resistance (WHO 2014). However, host immunity, pharmacokinetics factors including absorption and metabolism of drug and others play a definitive role in waning of therapeutic efficacy of any drug (Dondorp and Ringwald 2013). Herein, partner drug also plays an equally important role. Slow clearance renders higher parasite load on weaker partner drug ultimately calling for ACTs to fail (Meshnick 2012) which is one of the determinant of artemisinin resistance. There have been incidences when all the samples with delayed PCT were clinically cured at the end of the study and were also susceptible to artemisinin in vitro except in two cases with modest elevation in IC_{50} which leads to messy inconsistencies and controversies (Meshnick 2012). Although the role of declining efficacy of partner drug is again arguable, as when ACT was introduced in the year 1994 in North West Thailand when mefloquine (MQ) alone failures increased to about 50 %, ACTs restored it to as low as nearly 5 %. The susceptibility of MQ is still the same as then in 1994 but the efficacy of ACTs is not the same and the failure rates with AS+MQ combination has increased to about 30 % in the region. This is definitely suggestive of reduced susceptibility to artemisinin partner (White 2012). Nevertheless, risks and threats involved with too much dependence on ACT have also been discussed in the past (Giha 2010). Finding the way out of

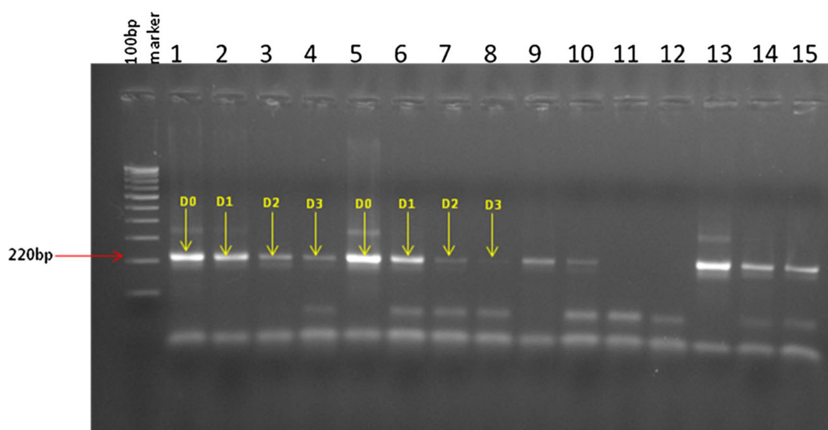

Fig. 5 Single-step diagnostic PCR as described by Demas et al. for detection of positivity in samples which are real-time day-3 positive

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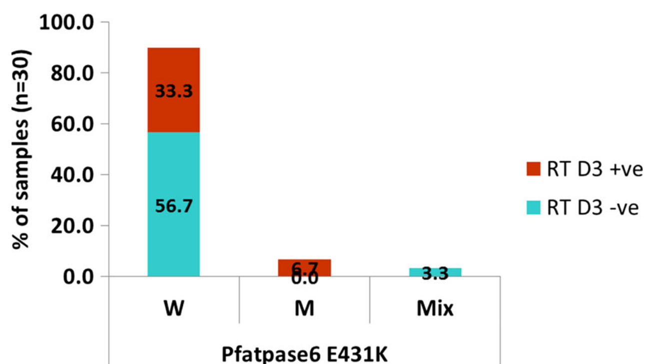

Fig. 6 Prevalence of E431K mutation in real-time day-3 positive samples from Odisha

these controversies, in the present study we aimed to monitor artemisinin resistance by monitoring day 3 positivity and correlating it with the clinical efficacy of AS+SP after 42-day follow-up. The putative suspects were tested for important SNPs in *Pfatpase6* gene reported to be involved in artemisinin resistance.

Real-time PCR was deployed for monitoring the PCT and the results were compared with microscopy. Advantages of deploying quantitative PCR include minimizing errors associated with microscopy which could be both systematic and random. Human error, excessive or insufficient blood on a slide, poor slide preparation or staining, uneven distribution of parasites within the thin smear or difficulty to identify parasite in thick films are the systematic errors. Individual expertise of microscopist which could be fixed or random further encouraged deployment of automated counting or real-time PCR methods. At low parasite densities, especially close to the limit of detection, the errors are maximized (White 2011). Real-time PCR being more sensitive has limitations too as it does not take care of the account of dead parasitic DNA present in the patient's blood, thus, overestimating the actual positivity. However, herein, the results of the 12 samples which were positive on day 3 correlated positively with higher initial parasitaemia which forms the basis of acceptance of these results as high initial parasitaemia is associated with day 3 positivity (White 2011). Clearance of parasite by 72 h depends on initial parasite density as three doses of artemisinin in an ACT is not sufficient to clear high densities of parasite as artemisinin reduces 10^4-fold of parasite density during each developmental cycle. Though end-point PCR is less sensitive than real-time PCR, results of end-point diagnostic PCR added to the authenticity and acceptability of the real-time data.

Furthermore, to derive a valid correlation and role of putative candidate, *Pfatpase6* gene mutation analysis was added in the study. The four important SNPs in *Pfatpase6* gene reported to be associated with artemisinin resistance in different studies are L263E, E431K, A623E, and S769N (Tahar et al. 2009; Jambou et al. 2010; Krishna et al. 2010; Zakeri et al. 2012). However, L263E was not observed in any of the field isolates in all the studies conducted so far (Kwansa-Bentum et al. 2011; Tanabe et al. 2011) but only detected in vitro (Uhlemann et al. 2005). The studies on *P. falciparum* isolates from Suriname too revealed L263, A623, and S769 codons in *Pfatpase6* gene to be wild type (Adhin et al. 2012). Also, in Southern-Iran, L263 and A623 were reported to be wild type (Zakeri et al. 2012). The presence of S769N also made occasional impacts from few studies (Jambou et al. 2005; Pillai et al. 2012; Zakeri et al. 2012) like that of the French Guiana (Legrand et al. 2008) while there are reports which observed its absence in the *P. falciparum* isolates that were analyzed (Mugittu et al. 2006; Sisowath et al. 2007; Zhang et al. 2008; Ferreira et al. 2008; Menegon et al. 2009; Wangai et al. 2011; Kwansa-Bentum et al. 2011; Tanabe et al. 2011; Brasil et al. 2012; Saha et al. 2013) or even denied the association of this SNP with artemisinin resistance (Phompradit et al. 2011; Cui et al. 2012). Another study from Yaounde, Cameroon, where all these four mutations were tested, high prevalence of E431K was warranted and alarmed (Tahar et al. 2009). Herein, we also found the prevalence of E431K mutation in *Pfatpase6* gene while the other three codons were wild corroborating with the above studies.

So far, the presence of E431K mutation has been reported in a single study from India. Indeed, two such studies were carried out by Saha et al. in Jalpaiguri (2012) and Kolkata (2013) districts respectively where in one they compared the efficacy of AS+SP, AM+LF, and AS+MQ and also studied polymorphisms in *Pfatpase6* gene thoroughly and found the presence of E431K in 19 % of the studied isolates (Saha et al. 2012). In another study, the efficacy of AS+SP and the prevalence of S769N was studied which could not find any polymorphism in the said SNP (Saha et al. 2013). Therefore, this then becomes another important report from India to detect E431K mutation in *Pfatpase6* gene and to derive a correlation of this trait with delayed PCT and thus, providing evidence for possible involvement of this trait in artemisinin resistance. Nevertheless, the emergence of this mutation in India seems to be a recent phenomenon as suggested by the above study conducted in Jalpaiguri. Analysis of the *Pfmdr1* gene copy number polymorphism revealed no variation in the copy number of the analyzed samples (n=30).

Very recently, mutations in the *PF3D7_1343700* kelch propeller domain ('K13-propeller') were associated with artemisinin resistance (Ariey et al. 2014). A large multicentre genome wide study conducted across 15 locations in SEA regions, identified and associated around 20 mutations in K-13 propeller and BTB/POZ domains with slow parasite clearance after treatment with artemisinin derivatives. It was thereby concluded that polymorphisms in *fd* (ferredoxin), *arps10* (apicoplast ribosomal protein S10), *mdr2* (multidrug resistance protein 2) and *crt* (chloroquine resistance transporter) genes forms the genetic background on which K-13

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mutations are likely to arise (Miotto et al. 2015). However, in our study, we could not conduct analysis on K-13 mutations due to logistic constraints of time and funds.

To sum up, the study provides evidence that though the study site from Odisha attained 100 % ACPR, there is a need for continuous monitoring of artemisinin-resistant malaria as urged otherwise also (Jambou et al. 2005). The evidence of relative increase in PCT as determined by real-time PCR in significant number of samples along with the prevalence of E431K mutation in *Pfatpase6* gene further emphasizes the outcome.

Conclusion

The study has helped to monitor PCT and to correlate it with artemisinin resistance. Herein, PCT was monitored with the help of gold standard technique microscopy which was further aided and compared by a more sensitive technique real-time PCR. The efficacy of AS+SP combination was found out to be 100 % after 42-day follow-up which was also correlated by microscopic measures of PCT which revealed no patient to be parasite positive on day 3. This concludes that there is no evidence of presence of artemisinin-resistant malaria at the studied site and that the partner drug is also working efficiently. However, combined results of real-time PCR, end-point diagnostic PCR and prevalence of E431K mutation in *Pfatpase6* gene led us to conclude that continuous surveillance to monitor the sensitivity of *P. falciparum* to artemisinins is required to detect emerging resistance, if any in the country. This way India will be vigilant and ready to counteract 'artemisinin resistance', as and when it knocks our door. Hereby, this way we could point out that real-time PCR could definitely be supporting microscopy for closer and better monitoring of artemisinin-resistant malaria in future studies.

Limitations

Though the study emphasizes a strong outcome, there are limitations too. One of the important limitations is the time of sampling. Closely spaced blood sampling of minimum six hourly differences is required to define the log of parasite clearance curve and also to define actual PCT. To determine PRR_{48} and PCT_{95}, i.e., the parasite reduction ratio between the time of admission and 48 h later and the time required to clear 95 % of the parasites, respectively also needs closely spaced blood samples as artemisinin is fast acting drug. Herein, the blood sampling was done on the day of admission and thereafter on days 1, 2, and 3 of infection due to logistic constraint of constant follow-up in field settings and also because the sampling otherwise involves admission of patient to the hospital thereby leading to concerned ethical issues. Another limitation of the study is that the detection limit of real-time PCR is very low and that it does not differentiate between live and dead parasites present in the patient's blood but only quantify the amount of parasitic DNA which could overestimate day 3 positivity. Nevertheless, combined with microscopy it could definitely be deployed as an important surveillance tool in artemisinin resistance studies. Recent studies on artemisinin resistance reported strong association of mutations in K-13 propeller domain with slow parasite clearance which could not be studied under the defined objectives of the present study limiting the outcome.

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