Effect of residual antimalarials in malaria patients enrolled for therapeutic efficacy studies and its effect on spread of drug resistant parasites in high malaria endemic districts in India

A Thesis Submitted to Goa University

for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

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STATEMENT

As required under the University ordinance, I hereby state that the present thesis for Ph.D. degree entitled **"Effect of residual antimalarials in malaria patients enrolled for therapeutic efficacy studies and its effect on spread of drug resistant parasites in high malaria endemic districts in India"** is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area. The literature related to the problem investigated has been cited. Due acknowledgement has been made whenever facilities and suggestions have been availed of.

Place: Goa University

Date:

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CERTIFICATE

This is to certify that the thesis entitled **"Effect of residual antimalarials in malaria patients enrolled for therapeutic efficacy studies and its effect on spread of drug resistant parasites in high malaria endemic districts in India"** submitted by **Mr. Kamlesh Kaitholia** for the award of the degree of **Doctor of Philosophy** in **Biotechnology** is based on his original studies carried out by him under our supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institution.

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Dedicated to Father and Elder Father

For if you have been here today, I would have seen you the happiest!!!

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ABBREVIATIONS

ASHA	Accredited Social Heath Activist
ACPR	Adequate Clinical and Parasitological Response.
AIDS	Acquired Immune Deficiency Syndrome
AL	Artemether Lumifantrine
AS	Artesunate
ACT	Artemisinin base Combination Therapy
ANMs	Auxiliary Nurse Midwife
bp	Base Pair
BHC	Benzene Hexa Chloride
B/W	Body/Weight
CRF	Case Record Form
CG	Chhattisgarh
CSP	Circum Sporozoites Protein
CHC	Community Health Centre
CI	Confidence Interval
^{0}C	Degree Centrigreate
DNA	Deocyribo Nucleic Acid
dNTPs	deoxy Nucleotide Triphosphates
DDT	Di-chloro Di-phenyl Tri-chloro ethane
DHFR	Dihydrofolate Reductase
DHPS	Dihydropterote Synthase
ETF	Early Treatment Failure
FDC	Fixed Dose Combination
HPLC	High Performance Liquid Chromatography
HRP	Histidine Rich Protein
HIV	Human Immunodeficiency Virus
HCl	Hydrochloric Acid
IRS	Indoor Residual Spray
ITNs	Insecticide treated Nets

JH	Jharkhand
kg	Kilogram
kb	Kilo base
LCF	Late Clinical Failure
CPF	Late Parasitological failure
LDH	Lactase Dehydroganase
≤	Less Then
MP	Madhya Pradesh
MDA	Mass Drug Administration
MQ	Melfoquinine
mg	Milligram
mm	Millimetre
mM	Millimolar
M	Molar
≥	More Then
ng	Nanogram
NVBDCP	National Vector Borne Disease Control Program
NIMR	National Institute of Malarial Research
NS	Non-synonymous
NE	North-Eastern
OR	Odisha
PABA	Para Amino Benzoic Acid
PCT	Parasite Clearance Time
%	Percentage
pfdhfr	*Plasmodium falciparum dihydrofolate reductase*
pfdhps	*Plasmodium falciparum dihydropterote synthase*
pfcrt	*Plasmodium falciparum Chloroquine Resistant Transporter*
pfmdr-1	*Plasmodium falciparum Multi drug Resistant-I*
PCR	Polymarase Chain Reaction
PHC	Primary Health Centre
QN	Quinine

RDT	Rapid Diagnostic Kit
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribo Nucleic Acid
rpm	Rounds per Minute
SEA	South East Asia
SD	Standard Deviation
SP	Sulphadoxine-Pyrimethamine
$t_{1/2}$	Terminal half life
TBE	Trise Boric Acid EDTA
WBCs	White Blood Cells
WHS	World Health Statistics
WHO	World Health Organization

Chapter I

Introduction and Review of Literature

1.1 Introduction

Malaria, a life threatening disease, is widespread in the tropical and subtropical regions mainly around the equator. It is an infectious disease caused by protozoa of genus "*Plasmodium*" and transmitted to human by certain species of infected female Anopheles mosquitoes (WHO 2016). Mainly, there are five species of human malaria parasites namely *Plasmodium falciparum* (Welch 1897), *Plasmodium vivax* (Grassi & Feletti 1890), *Plasmodium* ovale (Stephens, 1922), *Plasmodium malariae* (Feletti & Grassi 1889) and *Plasmodium knowlesi* (Sinton and Mulligan 1933) which cause malaria to humans. However *Plasmodium malariae* is reported to infect both human and African apes (De Nys, Löhrich et al. 2017). Recently another species of parasite, *Plasmodium Knowlesi* has been also reported to cause malaria in apes and has been found to infect humans (De Nys, Löhrich et al. 2017). *Plasmodium falciparum* and *Plasmodium vivax* are the two major parasites that cause malaria in humans worldwide, but amongst them *P. falciparum* is more virulent and life threatening (WHO 2013). Worldwide, malaria is among the 5th position in causing mortality due to infectious diseases like HIV/AIDS, tuberculosis, measles and Hepatitis B. However, in African countries, it is the second most common cause of death after HIV/AIDS (WHS 2013).

1.2 Global epidemiology of malaria

According to WHO 2016 report, the world wide estimated cases of malaria were 265.3 million. Most of them are estimated from African region (216.7 million) followed by the South East Asia region (36.2 million), Eastern Mediterranean region (8.4 million),

Western Pacific region (2.2 million) and American region (1.6 million) (WHO, 2016). African region has reported highest confirmed malaria case (94.2 %) followed by Eastern Mediterranean region (3.9%) and SEA region (1.1%) (WHO 2016). In South East Asia region, India has reported the highest number of malaria cases (77.3 %) followed by Indonesia (14.3 %) and Myanmar (5.1%) (WHO, 2016) (**Fig. 1.1**).

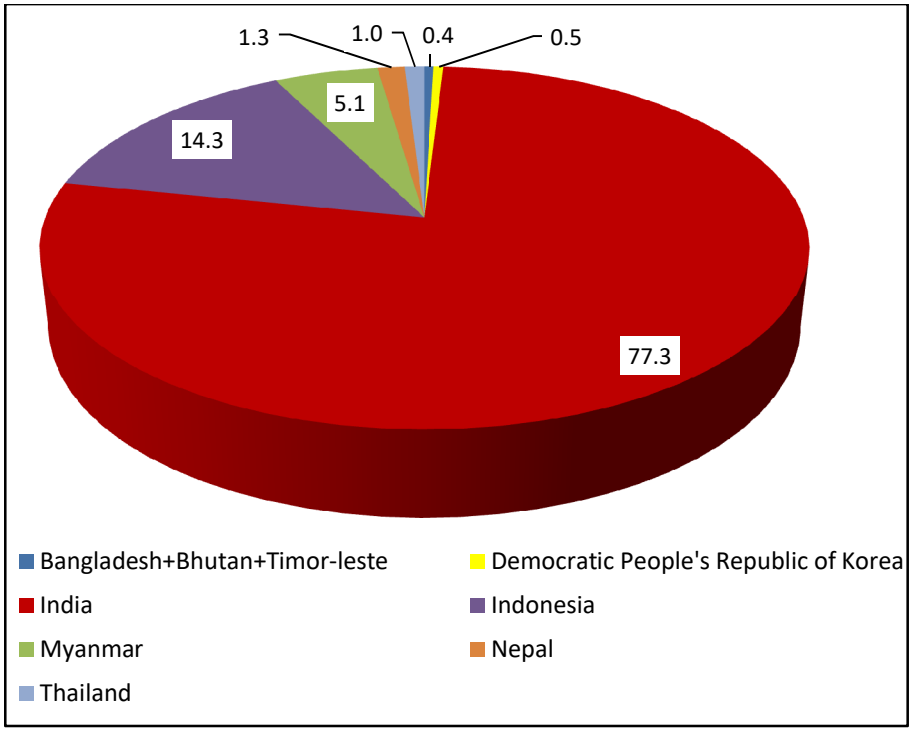

Fig. 1.1: Malaria cases reported from South East Asia Region

In 2016, highest deaths were reported from African region (98.4%) followed by Eastern Mediterranean (0.8%) and SEA region (0.5%) out of the total deaths occurring due to malaria (WHO 2016). In Africa region, 74% of deaths were reported in children less than 5 years of age (WHO 2015). In 2016, out of the total reported death cases in SEA region, India contributed 61.9% followed by Indonesia (25.8%), Myanmar (6%), Thailand (5.3%) and Bangladesh (1.5%).

Globally, between the years 2000-2015, malaria incidence and mortality rate have been reduced by 41% and 62%, respectively. There is a considerable decrease in malaria endemicity in the year 2000, with 108 countries and territories considered malaria endemic and this number has dropped down to 91 countries and territories in the beginning of the year 2016 (**Fig. 1.2**).

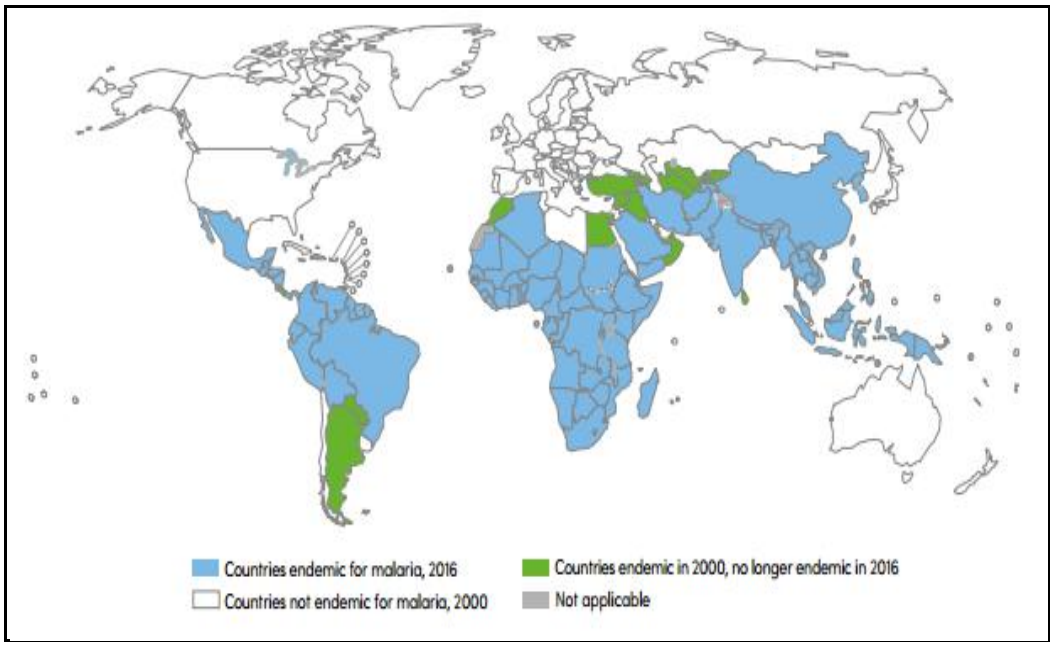

Fig. 1.2: Worldwide distribution of malaria in 2000 and 2016 (Source: WHO Malaria report 2016)

In India, a total of 1.05 million confirmed malaria cases were reported by the National Vector Borne Disease Control Programme in the year 2016 (NVBDCP 2016). Odisha, Chhattisgarh, Madhya Pradesh and Jharkhand states account for 71.3 % of reported malaria cases in the country (NVBDCP 2016). The highest number of malaria cases were reported from Odisha (42.4%) followed by Chhattisgarh (14.3%), 11.3% from Jharkhand, 3.3% from Madhya Pradesh and 28.7% cases from rest of the country (NVBDCP 2016) (**Fig. 1.3**).

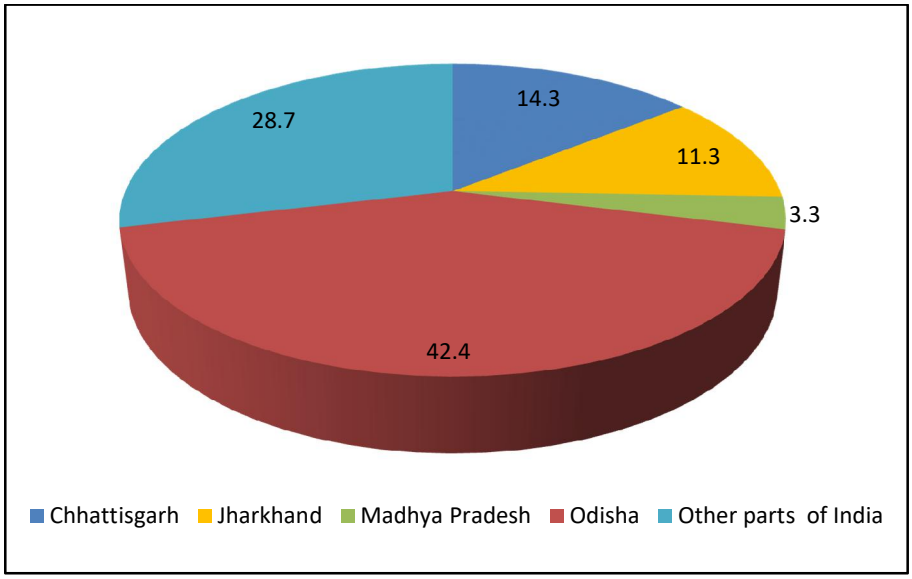

Fig. 1.3: Distribution of malaria cases in different endemic regions of India

Out of total *P. falciparum* cases reported in India, highest are from Odisha (55.2%), followed by Chhattisgarh (17.6%), Jharkhand (10.2%) and Madhya Pradesh (3.1 %) (**Fig. 1.4**). To accomplish the proposed study and to achieve maximum enrolment goal, study sites were selected on the basis of *P. falciparum* malaria endemicity.

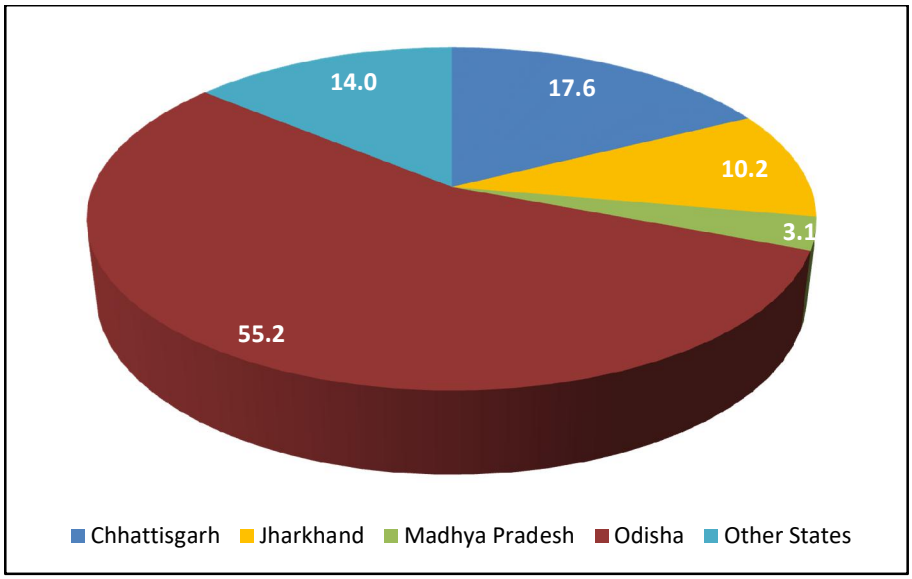

Fig. 1.4: Distribution of *P. falciparum* cases at a different endemic region of India

P. falciparum and *P. vivax* are the predominant species contributing to maximum malaria burden in the country. The trends of *P. falciparum* malaria cases show a gradual increase between 2012 to 2015, but a decline in percentage of *P. falciparum* cases was observed in 2016 (NVBDCP 2016) (**Fig. 1.5**).

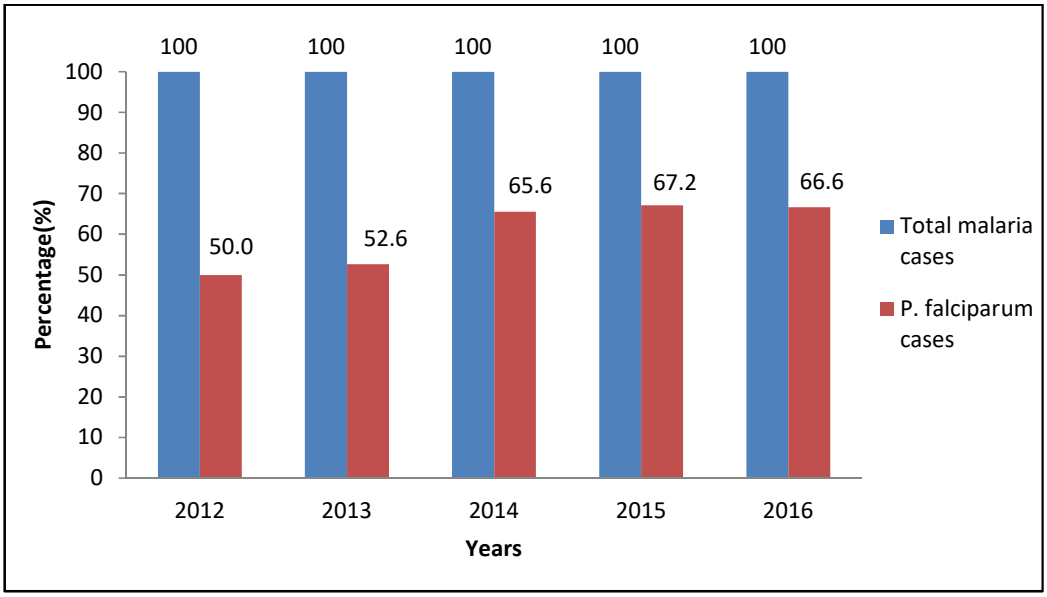

Fig. 1.5: Trends of *P. falciparum* cases in last five year in India

1.3 Malaria vectors and their control

Malaria is transmitted by female Anopheles mosquito; nearly 481species have been recognised throughout the world, out of which 58 species have been identified in India (Mosquito Taxonomic Inventory: http://www.mosquito-taxonomic-inventory.info). Mainly six anopheles species are primary malaria vectors namely An. *culicifacies*, An. *dirus*, An. *fluviatilis*, An. *minimus*, An. *sundaicus* and An. *Stephensi* (Christophers 1933; Roa1984; Kumar *et al*. 2007; Dash *et al*. 2007). Besides, some vectors of local importance are An. *philippinensis-nivipes*, An. *varuna*, *An*. *annularis* and An. *Jeyporiensis* (Dash *et al*. 2007).

Geographical distribution of malaria vectors vary from species to species; *An. fluviatilis* and *An.minimus* are found in the foot-hill regions, *An. stephensi* and *An. sundaicus* are found in the coastal regions (Rao 1984), while *An.culicifacies* and *An. philippinensis* are found in the plains (Park 1997). *An. dirus* is mainly prevalent in the forest and forest-fringe areas (Dutta *at el*. 1996). However, *An. stephensi* is highly adaptable and is found to be a potent vector of human malaria. *An. culicifacies* and *An. stephensi* are found to be abundant in rural and urban areas, respectively (Sharma 1998**)**. *An. culicifacies*is is widely distributed across the country and accounts for 60–65% malaria transmission in the country especially from rural and peri-urban areas (Subbarao *at el*. 1994). The main breeding sites for *An. culicifacies* are rice fields, borrow pits, irrigation channels etc.

Vector control plays important role in the global malaria control strategy. The aim is to reduce transmission of the disease which in turn reduces the levels of mortality and morbidity (WHO 2011). One of the most important strategies for vector control is to reduce breeding sites by filling ditches, pits, low lying areas, channelizing, streamlining, trimming of drains, water disposal and sanitation, emptying of water container once in a week, etc. In past years, indoor residual spray (IRS) with insecticides is an excellent tool for controlling malaria cases in many parts of the world. In 1966, use of DDT as IRS resulted in drop-down in malaria cases from 75 million to as low as 0.1 million cases in the country (Sharma 2003). The first reported resistance to DDT appeared in *An. culicifacies* in 1958 (Rahman *et al*. 1959) and benzene hexachloride (BHC, gamma isomer) was banned in year 1997. Resistance against BHC, dieldrin and malathion was reported quickly after its use (Sharma *et al*. 1962; Patel *et al*. 1958; Rajgopal 1977). Combination of malathion and pyrethroids

are being used in public health programme, specially synthetic pyrethroids (SPs) such as deltamethrin, cyfluthrin and lambdacyhalothrin have been introduced into public health programme (Dash *et al*. 2006).

Biological control of mosquito breeding can be achieved through biological agents like larvivorous fishes (*Gambusia* and Guppy) and by larvicides. Larvivorous fishes have also been found to be very effective in controlling malaria in certain situations in Karnataka (Ghosh *et al*. 2005). Personal protection measures are based on insecticide-impregnated materials such as insecticide-treated nets (ITNs), use of mosquito repellent creams, liquids, coils and mats etc. and installing wire mesh in doors and windows, wearing clothes that cover a maximum surface area of the body are some other useful protective measures.

1.4 Scientific classification of *Plasmodium*

- Kingdom: Protista
- Phylum: Protozoa
- Class: Sporozoa
- Order: Coccidiida
- Family: Plasmodiidae
- Genus: *Plasmodium*
- Species: *falciparum*, *vivax*, *malariae*, *ovale* and *knowlesi*

1.5 Life cycle of *Plasmodium*

Malaria parasite requires two hosts to complete its life cycle; definitive host where sexual cycle occurs i.e. *Anopheles* mosquito and intermediate host where asexual cycle take place i.e. human.

1.5.1 Life cycle of parasite in mosquito

When female *Anopheles* mosquito bites malaria infected human, mosquitoes take up erythrocytes infected with asexual schizogonic stage parasites as well as with gametocytes (first sexual stage of parasites). Inside the mosquito gut due to fall in temperature, gametocytes immediately initiate formation of gametes in the presence of mosquito factor-Xanthurenic acid (David & Herbert 2002). Within 10 minutes, female gametocytes escape from cytoplasmic "osmiophilic bodies" and the enveloped erythrocytes break down. The male gametocytes similarly escape from the enveloped erythrocytes and simultaneously it undergoes three mitotic divisions inside a single persistent nucleus. Then the male gamete fuses with the female gamete by a process of chemotaxis to form a zygote. After 18 – 24 hours, transformation of a zygote to ookinete takes place. This ookinete invades the mid gut wall which requires the expression of a secretary protein- Cirucm sporozoite eprotein(CSP) and CSP-Thrombospondin related protein (CTRP). A fraction of ookinete emerge through the mid gut cell to basal lamina, where it initiates its differentiation into an oocyst. All of these processes complete within 24–36 hours of blood feed time (David & Herbert 2002). Gradually, oocysts increase in size and eventually rupture the overlying basal lamina. Approximately 6^{th} day onwards, the oocyst tarts undergoing segmentation. The resulting increase in surface: volume ratio permits the emergence of about 8000 daughter cells/sporozoites. These sporozoites then become motile and are released into the oocyst. The oocyst wall becomes weakened and the sporozoites emerge through the cyst wall and basal lamina, which move towards salivary glands of the mosquito. When infected mosquito bite human and sporozoites injected into blood stream of human and sporozoites reach liver (**Fig. 1.6**).

1.5.2 Life cycle of malaria parasite in the human

In the human host, schizogony (asexual reproduction) takes place in two phases. The first is erythrocytic schizogony which is found in erythrocytes and the second is exo-erythrocytic schizogony which is found in hepatocytes. In humans, tissue phase of the parasite is confined to liver and it takes a single round of replicative development after which the parasite invades the erythrocytes. The life cycle of the parasite in human starts after an infected female *Anopheles* mosquito bites a person and injects its sporozoites into the blood stream of the host. Sporozoites infect liver cells and mature into schizonts, which rupture and release merozoites. Merozoites are then attached to the specific receptors on the erythrocytes and then penetrate its membrane and passes through the stages of trophozoite and schizont (**Fig. 1.6**).

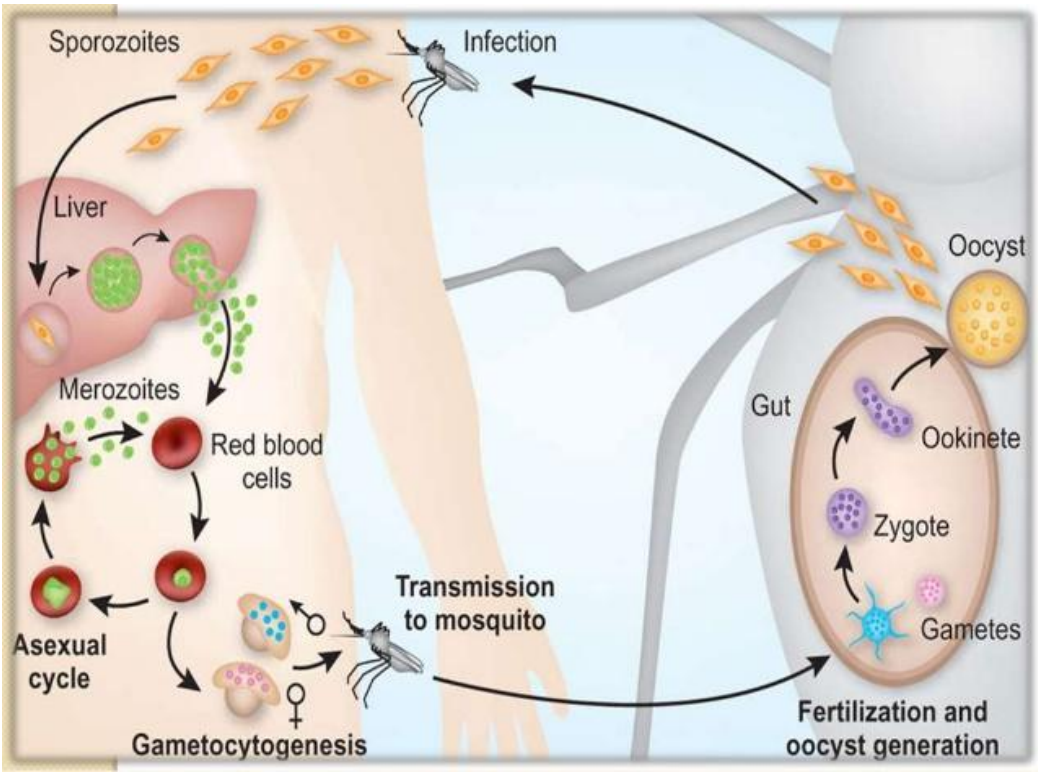

Fig. 1.6: Life cycle of malaria parasite (**source**: Nature 433, 113-114 (doi:10.1038/433113a)

Each cycle of erythrocytic phase lasts 48 to72 hours. Erythrocytic phase ends with the release of merozoites, which then infect new red blood cells. Some of the merozoites instead of developing into trophozoite or schizont give rise to the forms which are capable of sexual functions (gametocyte) after leaving the host. This sexual stage of parasite i.e. gametocytes are capable of infecting the female *Anopheles* mosquito when it bites an infected patient. After infecting the blood, the initial tissue phase of parasitaemia completely disappears in *P. falciparum* but in *P. vivax* and *P. ovale*, it persists in the liver and this is called the-erythrocytic cycle. This cycle is responsible for the relapses in *P. vivax* and *P. ovale*.

1.6 Diagnosis of malaria

Clinical diagnosis is based on the patient's signs and symptoms, and on the physical findings at examination. The early symptoms of malaria are very non-specific and variable, which include fever, headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhoea, nausea, vomiting and anorexia. The clinical diagnosis is still challenging because of the non-specific nature of the signs and symptoms, which overlap considerably with other common viral & bacterial infections (Mwangi *et al.* 2005). Prompt and accurate diagnosis is critical to the effective management of malaria. Malaria diagnosis involves identifying malaria parasites in patient blood. Various diagnostic techniques are available for malaria parasite identification such as microscopy, Rapid diagnostic test (RDT) kits, Quantitative Buffy Coat (QBC), and PCR based molecular methods.

1.6.1 Microscopy

For malaria diagnosis the direct microscopic examination of malaria parasites is widely accepted "Gold Standard" (Ndao *et al*. 2004; Mangold *et al*. 2005). Microscopic

examination of malaria parasite is most economic, preferred and reliable method for the identification of the prevalent parasite species, which can be diagnosed based on their distinguishing characteristics. Two types of blood films are traditionally made; thin film microscopic examination of the parasites can morphologically differentiate the various *Plasmodium* species. Thick film is used by microscopists using larger volume of blood and has been found to be twelve times more sensitive than thin film examination, thereby-picking up low levels of infection, which is easier on a thick film. In microscopy, staining and identification of malaria parasite are tedious, time-consuming, and require considerable expertise and trained manpower (Snounou *et al*. 1993; Milne *et al.* 1994; Kain *et al.* 1998). The major disadvantage of microscopic examination is its relatively low sensitivity for identification of malaria parasite at low parasitaemia levels (Guerin *et al.* 2002; Moody 2002).

1.6.2 Rapid diagnostic test

RDT is an alternate way for the rapid diagnosis of malaria infection by detecting specific malarial antigens in the patient's blood. The rapid diagnosis kits are available to detect antigens and it does not require any equipment. The majority of the RDTs target *P. falciparum* specific proteins, e.g. Histidine-rich protein-II(HRP-II) or lactate dehydrogenates (LDH) (Palmer *et al.* 1998 ; Moody 2002). RDTs have the benefit of parasite based diagnosis of malaria, particularly in remote malaria endemic areas. RDTs are the available option for the early diagnosis and treatment of malaria parasite in rural areas such as the sub-centres attached to primary health centres (PHCs) where electricity and health-facility resources are inadequate. However, the quality issue such as false positive results remains with RDTs (Moody 2002 ; Murray *et al*. 2008).

1.6.3 Quantitative Buffy Coat (QBC)

In this method, fluorescent dyes that stain nucleic acids have been used for the detection of blood parasites (Rieckmann *et al.*1989). In the QBC method, patient's samples collected in a tube containing acridine orange, an anticoagulant is centrifuged in a micro hematocrit centrifuge and then examined using a fluorescence microscope. Acridine orange is the preferred diagnostic method in the context of epidemiologic studies in asymptomatic populations in endemic areas, probably because of increased sensitivity at low parasitaemia (Ochola *et al*. 2006). QBC technique is simple, reliable, and user-friendly, but it requires specialized instrumentation, is more costly than conventional light microscopy, and is poor at determining species and numbers of parasites.

1.6.4 Polymerase Chain Reaction (PCR)

To overcome the drawbacks of previously mentioned diagnostic techniques for malaria detection, the modern molecular biological techniques were explored which showed that PCR technique is more sensitive than microscopy and RDT. The initial studies for the nucleic acid based malaria diagnosis exploited parasite's repetitive DNA sequences present throughout the *Plasmodium* genome as the diagnostic target. Therefore, after the sequencing of two small subunits (18s) rRNA genes from *P. falciparum* and *P. vivax* species specific regions of rRNA genes have been exploited in developing a sensitive and specific diagnostic procedure (Snounou *et al.* 1993 & 1993a; Myjak *et al.* 2002; Rougemont *et al.* 2004). PCR appears to have overcome two major problems of malaria diagnosis, sensitivity and specificity, but its use is limited by complex methodologies, higher costs and the need for trained manpower (Johnston *et al.* 2006). Recently, Loop mediated isothermal amplification (LAMP)

technique has been developed and now researchers are working to make it user-friendly and filed adaptability.

1.6.5 Other diagnostic methods

The first serological test to be used for malaria antibodies was immunofluorescence antibody testing (IFAT), which give quantitative results for both G and M specific immunoglobulin. Through its specificity and sensitivity largely rely on the laboratory technician's expertise. This technique is simple and sensitive but time-consuming and often useful in epidemiological surveys and for screening potential blood donors. Other limitations of IFAT are that it cannot be automated, and thus less number of sera can be studied; requires fluorescence microscope, specific reagents and trained manpower. This all limit the use of IFAT in remote settings. The indirect haem agglutination test (IHA) is simple and suitable for field studies, but its sensitivity and specificity are poor. The enzyme-linked immunosorbent assay (ELISA) has similar sensitivity and specificity characteristics as that of IFA test, but the interpretation of the results are yet to be standardized.

Advancement in immunological research has led to Flow cytometry techniques, which is based on detection of hemozoin pigment, and this method provides a sensitivity of 49-98%, and a specificity of 82-97% (Grobusch *et al.* 2003; Padial *et al.* 2005). However, this technique is time consuming, requires trained manpower, and costly equipment. Besides these disadvantages, false-positives are also reported with other bacterial or viral infections. Mass spectrometry is a novel method for *in-vitro* detection of malaria parasite with sensitivity of 10 parasites/µl of blood (Huh et al. 2008). However, rural areas in the country with intermittent electricity are not suitable

for existing high-tech mass spectrometers. Thus, for prompt and effective treatment, specific and sensitive diagnostic methods are needed to eliminate malaria parasites.

1.7 Malaria treatment

The primary objective of the treatment of uncomplicated malaria is to completely cure the infection and to prevent the progression of uncomplicated malaria to severe form. Another important consideration while treating malaria is to prevent the spread and emergence of resistance to antimalarials. Currently, Artemisinin Combination Therapy (ACT) is given as the first line of treatment to all the confirmed uncomplicated *P. falciparum* cases found positive by microscopy or RDT. The artesunate plus sulphadoxine pyrimethamine (AS+SP) is recommended in the National Programme throughout India except northeastern states, where the recommended dose of artesunate is 4 mg/kg body weight, daily for 3 days and sulphadoxine is administered as 25 mg/kg body weight with pyrimethamine (1.25 mg/kg body weight) on Day 0 followed by single dose of primaquine (PQ) (0.75 mg/kg body weight) on day 2. In the Northeastern states (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, and Tripura), due to the recent reports of late treatment failures to the current combination of AS+SP in *P. falciparum* malaria, the presently recommended ACT in National Drug Policy is- fixed dose combination (FDC) of Artemether-Lumefantrine (AL) (NVBDCP 2014).

Microscopically confirmed *P. vivax* malaria cases are treated with chloroquine in full therapeutic dose of 25 mg/kg divided over three days (First day 10mg, second 10mg and 5mg/kg B/W on third day). Patients infected with *P. vivax* may cause relapse due to the presence of hypnozoites (which remain dormant in the liver cells) and

gametocytes, which are responsible for transmission in the population. Reduce transmission of malaria parasites primaquine (PQ) has been given 0.25mg/kg body weight daily for 14 days (NVBDCP 2014).

Quinine is recommended in the first trimester and the ACT should be given for treatment of *P. falciparum* malaria in second and third trimesters of pregnancy, while *Plasmodium vivax* malaria can be treated with chloroquine. Mixed infections with *P. falciparum* should be treated as *falciparum* malaria. Since AS+SP is effective in *vivax* malaria, other ACT are also used. However, anti-relapse and gametocytocidal treatment by PQ has been recommended for 14 days.

1.8 Challenges in malaria treatment

Accurate diagnosis of malaria parasite species is needed for timely management of patients. Antimalarial drug resistance is a crucial obstacle to formulate new regimen for treatment of malaria patients. Treatment of malaria patients in India remains dynamic and is guided by evidence based data generated through surveillance studies on efficacy of antimalarials in the country. Therapeutic efficacy studies are *in-vivo* monitoring tools for confirmation of drug response against studied malaria parasites. Therapeutic efficacy studies are conducted to assess parasitological response through community-based trials, enrolling the age groups most affected by clinical disease (Shah *et al.* 2012).

In the past, chloroquine (CQ) was effective for treating nearly all malaria cases. However, CQ resistance in *P.falciparum* was first reported in Assam, India in 1973 (Sehgal *et al.*1973) and number of studies until 1977 indicated widespread presence of CQ resistant *P.falciparum* in Assam, Arunachal Pradesh, Mizoram and Nagaland.

Since then drug resistance has been reported from several other parts of the country (Dua *et al.* 2003; Baruah *et al*. 2005*;* Valecha *et at.* 2009).

To overcome the problem of CQ drug resistance, sulphadoxine-pyrimethamine (SP) combination was recommended as the second line of treatment by the National Programme in the country (National Antimalarial Programme 1982). SP was replaced by the ACT artesunate plus sulphadoxine-pyrimethamine (AS+SP) as the second line drug in 2005 for use in CQ treatment failure and as the first line antimalarial in the area where CQ resistance was documented. In 2007, AS+SP was recommended as the first-line treatment in districts where high numbers of cases for chloroquine resistance were documented. Due to high rate of chloroquine treatment failure, in 2010, AS+SP was used as the first-line treatment for uncomplicated *P. falciparum* malaria throughout India (NVBDCP, 2011). In North-Eastern (NE) states (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, and Tripura), due to the reports of high treatment failures to AS+SP in *P. falciparum* malaria, the fixed dose combination (FDC) of Artemether-Lumefantrine (AL) was recommended since 2013. Therefore, the present recommendation of National drug policy is that the ACT used in the national programme in NE states is AL and rest of India is AS+SP (NVBDCP 2014). India have lost many valuable antimalarials in different time points due to emergence and spread drug resistant parasites in different geographical areas (Valecha *et al.* 2009; Mishra *et al*. 2014).

1.9 Drug resistance

According to WHO, resistance to antimalarial drugs is defined as "the ability of a parasite strain to survive and /or to multiply despite the administration and absorption

of a drug given in doses equal to or higher than, those usually recommended, but within the limits of tolerance of the subject" (WHO 2011). Drugs act by hindering the cellular or biochemical pathways called 'targets'. The common drug targets are enzymes which are inhibited by the drug. The effective drug should have selective toxicity for the pathogen as compared to the host. Many factors are playing major role in drug resistance such as mutations in the drug target so that the drug does not bind or inhibit the target. Drug resistance can develop quickly in situations where a single point mutation can confer resistance (White 2004) or in the case of over expression of the target (Nair *et al.* 2008). This can be accomplished either through increased gene amplification, transcription and translation. It results in a requirement for higher levels of drugs to achieve the same level of inhibition. Decreasing drug accumulation or metabolizing the drug to nontoxic products result in lesser drug content reaching the target and can also contribute to drug resistance (Ecker *et al.* 2012).

1.10 Molecular mechanisms of drug resistance

1.10.1 Quinine

Quinine (QN) is a oldest remedy for the malaria cure. One of the postulated mechanism for the action of QN is the inhibition of heme detoxification in the parasite digestive vacuole (Hawley et al. 1998). However, the exact mode of mechanism of Quinine is yet to be explored fully (Achan *et al.* 2011). However. there are reports where decreased sensitivity of QN in the in vitro assays has been strongly linked with polymorphisms of *Plasmodium falciparum multidrug resistant transporter-I* (*pfmdr1*), *pfcrt* and the *Plasmodium falciparum Na+/H+ exchange* (*pfnhe1*) genes and also linked to its resistance (Bohórquez *et al*. 2012; Eyase *et al.* 2013). The first indication of QN resistance in Brazil was observed some 100 years ago, after which

Thai-Cambodian border became the epicentre of quinine resistance during the mid-1960s (Pickard and Wernsdorfer 2002). However, in India, the first report of QN resistance was reported from North Eastern states and also from Karnataka (Singh *et al.* 2009). Quinine is presently reserved as a second-line to treat cases of severe malaria. Reduced efficacy with quinine treatment has been observed in few case reports in India (David & Gilles, 2002) but treatment failure cases are little less common in India.

1.10.2 Chloroquine

CQ accumulates up to several folds in the food vacuole of the parasite (Bray *et al*. 2005). Mechanisms for this selective accumulation of CQ in the food vacuoles may have following pathways:

(1) Protonation and ion trapping of the CQ as the food vacuole has a low pH.

(2) Active uptake of CQ by a parasite transporter.

(3) Binding of CQ to a specific receptor in the food vacuole (Johnson et al. 2004, Martin et al. 2009).

Parasite food vacuole is a lysosome like organelle in which the breakdown of hemoglobin and the detoxification of heme occurs (heme to hemozoin) (Bray *et al.* 1998). Large quantities of heme are released as a result of hemoglobin digestion in the food vacuole; free heme can lyse membranes leading to the generation of reactive oxygen intermediates and inhibit many other processes (Sugioka *et al.*1988) (**Fig. 1.7**)**.** CQ exerts its toxic effect by interfering with the conversion of free heme to hemozoin. Up to 95% of free iron released during hemoglobin digestion is found in hemozoin (Egan 2008). X-ray crystallography and spectroscopic analysis indicates

that hemozoin has the same structure as β-hematin (Pagola *et al*. 2000). β-hematin is a heme dimer formed via reciprocal covalent bonds between carboxylic acid groups on the protoporphyrin-IX ring and the iron atoms of two heme molecules; these dimers interact through hydrogen bonds to form crystals of hemozoin. Therefore, pigment formation is best described as a biocrystallization, or biomineralization process (Hempelmann 2007; Egan 2008). Chloroquine is metabolized into desethychloroquine and bisethylchloroquine and these are detected in blood at concentrations ranging between 20 to 50% and 10 to 15% of CQ levels, respectively (Projean *et al.*2003).

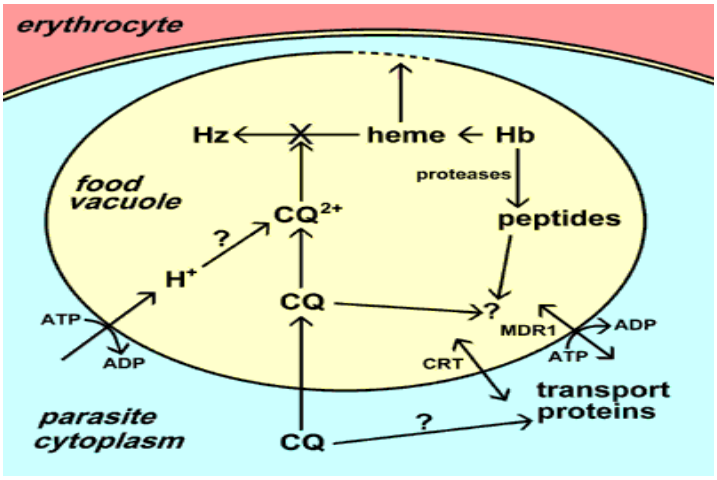

Fig. 1.7: Transport of chloroquine in food vacuole and mechanism of action of drug (Source: http://www.tulane.edu/~wiser/protozoology/notes/drugs)

CQ resistance is associated with a decreased accumulation of CQ in food vacuoles which is its main site of action. Some studies have shown that the decrease in drug accumulation is due to an increase in drug flux (Bray *et al.* 1998). A genetic cross and mapping studies between a CQ resistant clone and a CQ sensitive clone resulted in the identification of a 36 kb region on chromosome 7 found to be associated with CQ resistance. The gene has been designated as *pfcrt* and the protein is localized to the food vacuole membrane. Several mutations in *pfcrt* gene has been observed with CQ

resistance phenotype but the highest correlation has been observed with a substitution of lysine (K) to threonine (T) at codon 76 (K76T) leading to CQ resistance (Fidock *et al*. 2000). The substitution of lysine to threonine (K76T) at codon 76 of the *pfcrt* gene is associated with *in vivo* and *in vitro* CQ resistance in Africa, South America, and Southeast Asia (Warhurst 2003; Ojurongbe *et al*. 2007; Anvikar *et al.* 2012). Now, it has been proven with precision that the entire 72 to 76 amino acid residues (haplotypes) play a key role in CQ resistance (Mallick *et al.* 2012). Also, CQ resistance occurs due to polymorphisms in both the *pfcrt* and *pfmdr1* genes (Anderson *et al*. 2005).

1.10.3 Sulphadoxine-pyrimethamine

Malaria parasites have high replication rates during its multiplication phase, and therefore there is a continuous need of precursor (nucleotides) for synthesis of DNA. Reduced form of folate serves as a co-factor in biosynthesis of amino acids and nucleotides. The inability of the parasite to utilize exogenous folate and the inability of the human host to synthesize folate makes folate biosynthesis a good drug target as the parasite is dependent on *de novo* synthesis of folates. The basic building blocks of folate pathway are GTP, para aminobenzoic acid (PABA) and glutamate which involves the use of five enzymes (Muller *et al.* 2013) (**Fig. 1.8**).

Out of these enzymes, dihydrofolate reductase *(DHFR)* and dihydropterote synthase (*DHPS*) are the two enzymes involved in folate pathway which can serve as good drug targets. Sulpha based drugs such as Sulphadoxine and Dapsone are structural analogues of PABA which is converted to non-metabolizable additional product by *DHPS*, thereby inhibiting the folate biosynthesis and depleting folate pool. This

results in the reduction of thymidylate available for DNA synthesis. Dihydrofolate gets reduced to tetrahydrofolate by *DHFR* which is further involved in a biosynthetic pathway contributing to thymidylate pool. Inhibiting *DHFR* by drugs like pyrimethamine and proguanil further reduces the amount of thymidylate available for DNA synthesis and thus arrests parasitic growth. The involvement of these two enzymes in a single pathway makes these drugs act synergistically (Muller *et al.* 2013) (**Fig. 1.6**).

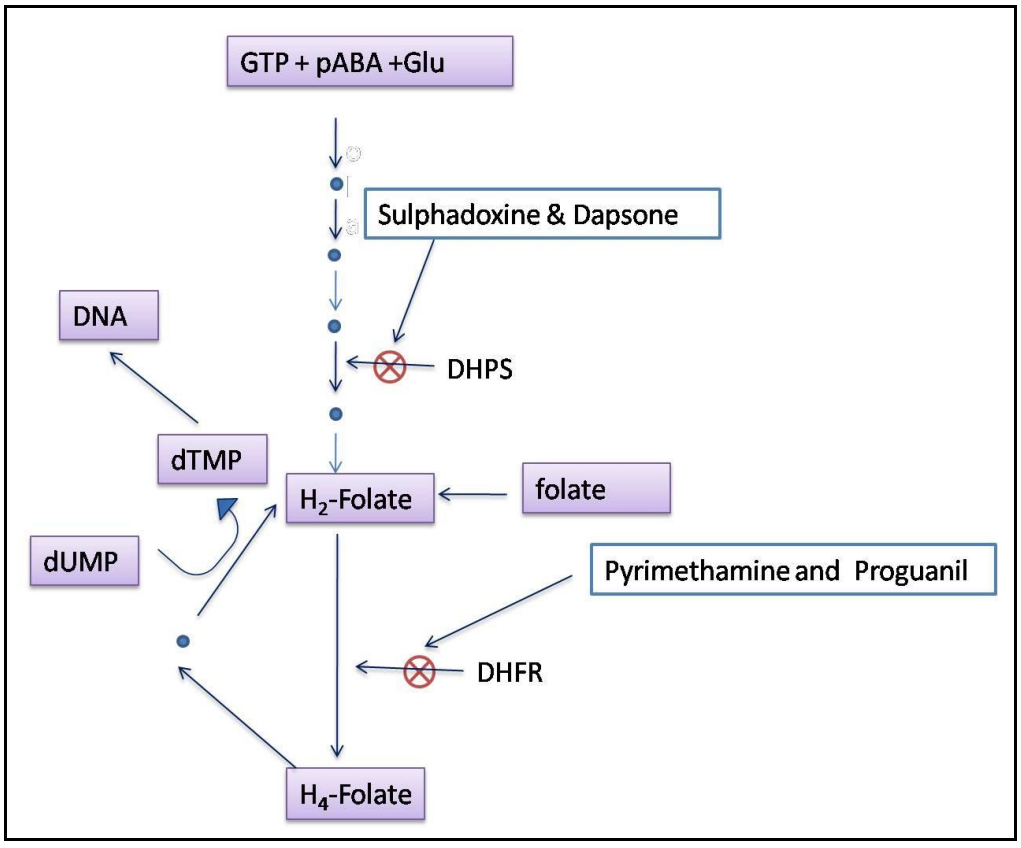

Fig. 1.8: Mode of action sulphadoxine-pyrimethamine to inhibition of folate pathway

Thus, Sulphadoxine and pyrimethamine drugs are inhibitors of DHPS and *DHFR* enzymes, respectively. Specific point mutations in *pfdhps and pfdhfr* genes encoding these enzymes lead to a lower binding affinity for sulphadoxine-pyrimethamine drugs.

Resistance tends to develop rapidly in the presence of drug pressure in situations where a single mutation can lead to drug resistance. The use of drug combinations will slow the development of resistance since two independent mutations must occur to develop resistance against both drugs. *P. falciparum* resistance to SP is conferred by mutations in the *pfdhps and pfdhfr* genes, respectively (Jelinek *et al*. 1998; Ngo *et al.* 2003). The mutations at codon *S*108N (S to N) in *pfdhfr* gene seems to be enough to confer resistance to pyrimethamine (Mockenhaupt *et al.* 2001). The presence of a mutation at position 51 (N to I) or 59 (C to R), together with S108N confer a considerable increase in resistance level to pyrimethamine when compared with S108N mutation alone (Tarnchompoo *et al.* 2002; Sirawaraporn *et al.* 1997). Mutations in codons 437 (A437G) and 540 (K540E) of *pfdhps* are also associated with resistance to sulphadoxine. The quintuple mutant (triple *pfdhfr*: 51I, 59R, 108N and double *pfdhps*: 437G, 540E) is considered as the molecular marker of SP treatment failure (Nzila *et al.* 2000: Kublin *et al.* 2002, Bwijo *et al.* 2003). Epidemiological studies have also demonstrated that the presence of mutations 59R in *pfdhfr* and 540E in *pfdhps* are significantly associated with resistance as well as with the presence of other aforesaid mutations (Kyabayinze *et al.* 2003). The study clearly highlights the increased level of resistance to SP in Thai isolates than Indian isolates as the number of mutations in all the studied codons of *pfdhfr* and *pfdhps* were more frequent in Thailand, most notably the frequency of mutation in *pfdhps* gene (Biswas *et al*. 2000). High frequency of mutations in *pfdhfr* gene at codon S108N followed by codon C59R and double mutant (S108N+C59R) genotypes are prevalent in India (Mishra *et al.* 2012). Apart from this, mutations at codon 51 and 164 are also responsible for increasing the tolerance of parasite towards the drug (Lumb *et al.*

2009). *P. falciparum* showed variable levels of resistance to sulphadoxine with sequence variation in *pfdhps* gene at codon S436 to A436, A437 to G437, K540 to E540, A581 to G581 and A613 to S/T613. This has been observed in a number of studies from India (Mishra *et al.* 2012; Ganguly *et al.* 2014). High frequency of mutations in *pfdhps* gene was observed in Car-Nicobar Island (Lumb *et al.* 2009a). Even, higher prevalence of mutations in *pfdhps* gene at codon 436, 437 and 540 (Mishra *et al.* 2014) has been also observed in Northeastern region of India, where high treatment failure of AS+SP regimen was observed due to partner drug resistance.

1.10.4 Mefloquine

Mefloquine was introduced in the 1970s (Trenholme *et al.* 1975) and is a long acting antimalarial with half-life of 14–18 days (White et al 2008). The copy number variation of *pfmdr1* gene is associated to mefloquine resistance (Preechapornkul *et al.* 2009). Although, the exact mechanism of action of MQ remains uncertain, yet *in vitro* experiments demonstrate that MQ can bind to heme and exert some antimalarial activity by inhibiting heme detoxification (Rohrbach, *et al. 2006;* Eastman & Fidock 2009). Mefloquine (MQ) resistance was first reported near the Thai-Cambodian border in the late 1980s (Wongsrichanalai *et al.* 2001). In India, MQ resistance was first reported from Gujarat (Sharma *et al*. 1996). The role of *pfmdr1* as the key modulator of mefloquine resistance has been strengthened following the transfection experiments in parasites which showed Ser1034, Asn1042, and Asp1246 mutations as the cause of resistance to MQ as well as the structurally related drugs quinine and halofantrine (Carrara *et al*. 2013).

1.10.5 Artemisinin

Artemsinins are rapidly acting antimalarials with short elimination half-life. Artemisinin derivatives are highly potent in clearing parasitaemia compared to other antimalarials. Artemisinin and its derivatives (e.g. artemether, artesunate, and dihydroartemisinin) are not used alone for clinical treatment as they are associated with high rates of recrudescence following monotherapy (Adjuik *et al.* 2004; Rehwagen 2006). Artemisinin and its derivatives have been used in combination with long-acting partner drugs. Following this evidence, the Drug Controller General of India imposed ban on the production, sale and marketing of oral artemisinin monotherapy in the year 2009 (Mishra *et al.* 2011). Transcriptomic studies, *in-vitro* experiments and mathematical modelling studies have suggested the role of reduced susceptibility of ring stage parasites causing slow parasite clearance time when treated with artemisinin or its derivatives (Ashley *et al.* 2014; Fairhurst *et al.* 2015). Recently artemisinin resistance has been strongly associated with point mutations in the kelch13 propeller region (pfk-13) of *P. falciparum* (Ashley *et al.* 2014). Non synonymous (NS) mutations (codons 533 (G-A), 549 (S-Y), 561 (R-H), and 578 (A-S) in the propeller region were observed in northeastern states of India (Mishra *et al.* 2015). Another study conducted by Mishra *et al*. in 2016, observed NS mutation at codon F446I, K189T and F446I in the north-eastern states (Mishra *et al.* 2016).

1.11 Factors affecting emergence and spread of resistant parasites

Many factors have cumulative effect on emergence and spread of resistant parasite in the community including parasites (biomass, genetic fitness, mutation rate and genetic basis of resistance), environment (transmission intensity & clonal multiplicity), vector (migration & parasite preference), host (pharmacokinetics and genetic factors, host

immunity and migration) and drug (drug pressure, elimination half-life, dosing, drug use and drug action) etc. (**Fig. 1.9**).

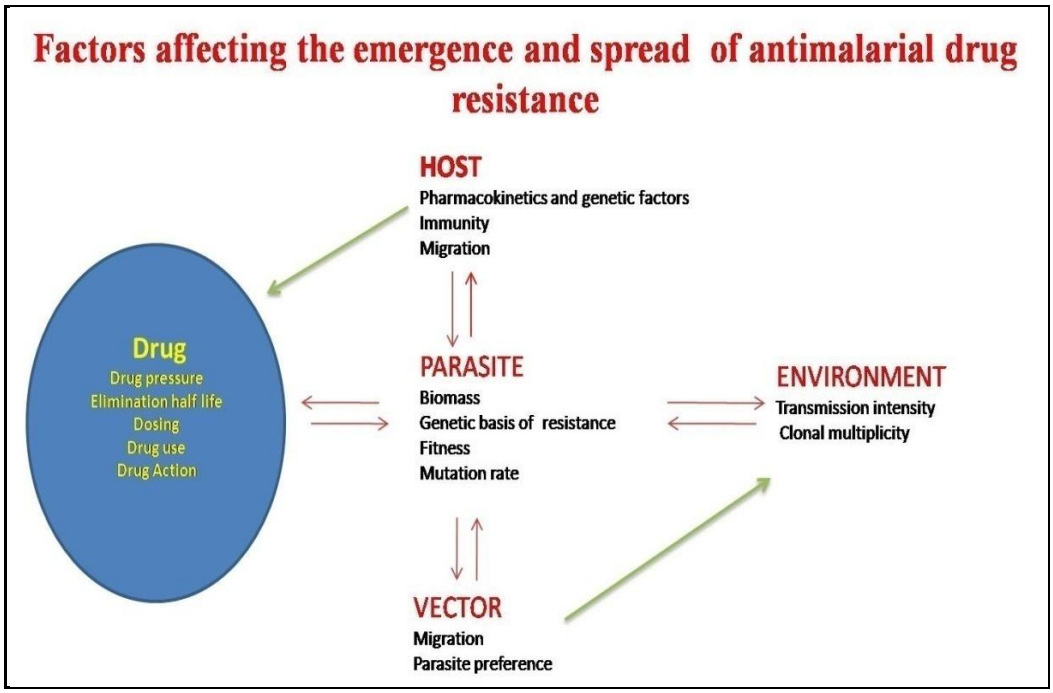

Fig. 1.9: Factors affecting the emergence and spread of antimalarial drug resistance and their interaction

1.11.1 Transmission Intensity

Drug resistance spread more rapidly in areas of high malaria transmission. In these settings, a decrease in transmission intensity of malaria is a great tool for delaying the spread of resistant parasite (Talisuna *et al.* 2007). However, low malaria transmission areas are also prone to emergence and spread of resistant parasites. To delay the spread of resistance, patients should be treated with rational use of antimalarial drugs after proper diagnosis. The unnecessary use of antimalarials increased the drug pressure in the community, consequently accelerating the spread of resistance (Talisuna *et al.* 2007).

1.11.2 Clonal multiplicity

Clonal multiplicity is defined by the average number of circulating clones per individual, determined by direct genetic analysis of parasites obtained from the blood (Anderson *et al.* 2005a). Clonal multiplicity increases the chances of sexual recombination of parasite clones. When more than one gene is involved in resistance then recombination occurs between two clones and there is a chance of evolution of resistant clones (Curtis *et al.* 1986; Hastings 1997; Hastings *et al.* 2000). If only a single gene is involved in resistance, in this situation, sexual recombination of clone has no effect.

1.11.3 Vector migration

Migration of malaria vectors is an important source of spread insecticide drug resistance and as well as antimalarial drug resistance. Recent studies pointed out that oversea migration is important factor in explaining the present worldwide distribution of certain resistance genes (Raymond *et al.* 1991). Author analyzed combined effects of gene flow, genetic drift, and selection on the distribution of resistance genes in adjacent populations. There has a strong differentiation in the circulation of resistance genes is closely related with insecticide treatments (Chevillon *et al*. 1995).

1.11.4 Host immunity and pharmacokinetics

Immunity to malaria is a very complex process and poorly defined in malaria patients. In malaria high endemic areas, therapeutic responses vary with age, as young children have little or no immunity compared with older ones and adults. Immunity results in better therapeutic responses for any level of resistance (White 2004). In high transmission areas, people who have developed immunity against malaria parasite

remain asymptomatic carriers (White 1999). Terminal half-life of an antimalarial drug is a crucial factor in a propensity to develop resistance (Watkins *et al.* 1993). Pharmacokinetics and pharmacodynamic of the drug resistant parasites are influenced by the immune factors in human hosts (White 1999a).

1.11.5 Parasite biomass, mutation and genetic fitness

The emergence and spread of resistance in malaria parasites depend on several factors including; parasite biomass, rate of mutation in the parasite and; genetic fitness of parasite. Higher parasite biomass and low dose of drugs are the main source of antimalarial drug resistance (White 2009). A large parasite biomass is more likely to occur in areas of low transmission because of the lower host immunity (Hastings 2003; White 2004). This is the possible explanation for the origin and rapid rise of multi-drug resistance in SEA. However, besides this observation CQ and SP resistance seems to have originated at a few foci in areas of low transmission and spread through selective sweeps across the world (Wootton *et al.* 2002; Roper *et al.* 2003, Anderson *et al.* 2005).

Higher mutation rate facilitates the faster emergence of resistance and lead to lethal mutations. An increased mutation rate is beneficial for the adjustment to quickly changing environments (Sniegowski *et al.* 2000). This is exactly the situation where parasites are exposed to chang drug selection pressures. Some studies described an accelerated resistance to multiple drugs (ARMD) phenotype, present in isolates from SEA, which acquire drug resistance at a higher rate than other geographically distinct strains and *in vitro* experiments. Such ARMD parasites could explain the observation that resistance to new drugs often arises first in SEA (Rathod *et al.* 1997). The mutation associated with drug resistance often imposes a fitness cost, the selective advantage acquired by

becoming drug resistant is balanced by the biological cost arising from the altered function of the mutated protein. Such a fitness cost can be diminished by the acquisition of compensatory mutations during prolonged drug pressure (Petersen *et al.* 2011).

1.11.6 Half-life of antimalarials

Treatment with longer terminal half-life antimalarials, provide an elongated period after clearing the initial infection, during which consequent re-infection is suppressed by residual levels of the antimalarial drug (White 2008). However, possibly these drugs exert undesirable drug selection pressure during the period when their concentrations drop below a critical threshold level, that can still prevent re-infection by sensitive parasites but not by resistant ones (White 2004). Sub therapeutic level of long elimination half life antimalarials provide wide window period for selection of resistant parasite (White 2008). Thus, drugs with a very short terminal half-life, including artemisinin derivatives, should carry a lower risk of selecting resistant parasites than longer acting drugs (Seidlein & Greenwood 2003).

1.11 .7 Mass drug administration

Mass drug administration (MDA) was a component of many malaria elimination programme during the eradication era (Seidlein & Greenwood 2003). MDA has increased unnecessary drug pressure in the community. In this approach those patient who do not have malaria but treated with antimalarial drug were unknown and are accountable for the increased drug pressures on population and promote spread of drug resistance (Wernsdorfer 1992). Once resistant parasites emerged, exposure to sub-lethal drug concentrations associated with indirect MDA will have eliminated the highly and moderately sensitive parasites, providing a selective advantage for more-

resistant parasites (Wernsdorfer 1992). MDA have also been linked to the emergence of drug resistance (Wernsdorfer 1992; Payne 1987).

1.11.8 Irrational use of antimalarials and self-treatment

Irrational treatment practices like non-compliance to prescription and self-medication of antimalarials are a common phenomenon in malaria endemic areas. This problem mainly relates to the lack of training, absence of proper treatment guidelines and diagnostic facilities, especially in rural areas. Therefore, in areas where clinicians have no access to laboratory facilities, treatment is based mainly on clinical symptoms, which lead to over diagnosis of malaria and excessive use of antimalarial drugs (Ezenduka *et al.* 2014). Mannan *et al.* 2009, reported that 22.4% of patients who did not presented sign and symptoms of fever were treated with antimalarial drugs at health facilities in Khartoum, Sudan. Further, out of total treated patients, 35.4% of patients had malaria parasite negative blood films (Mannan *et al.* 2009). These populations do not have access to or do not use appropriate diagnostic and treatment services for various reasons, and participate in irrational drug use practices (Mannan *et al*. 2009). In Pakistan also, the irrational prescription of antimalarial drugs without laboratory confirmation has also been reported (Khan *et al.* 2012). Use of antimalarial drugs in the absence of malarial infection was common in the study area and continuous use of such drugs, not only in Pakistan but also in other parts of the world create a higher risk for emergence of drug resistance (Khan *et al.* 2012). The use of monotherapy, self-medication remains a significant risk undermining the treatment policy and suggesting that additional measures are required to directly target patients and healthcare providers for improved use of antimalarial drugs in Nigeria (Meremikwu *et al.* 2007). Unnecessary use of drugs increase s the drug pressure which ultimately promotes

development of drug resistance (White *et al.* 2004; Mishra *et al.* 2013). A number of challenges also pose threat to complete treatment of malaria in the patients. In high malaria endemic areas, use of antimalarial treatments for febrile episodes and self-treatment has been observed in the past (Nwanyanwu *et al.* 1996; Mahomva *et al.* 1996). The standard WHO protocol to monitor the efficacy of antimalarial medicines does not exclude patients with history of antimalarial intake (Souares *et al.* 2009; Hodel *et al.* 2009 & 2010). Out of 138 patients of febrile illness, 60% patients were treated at home with herbal remedies or medicines purchased from local medical shops, and only 18% patient's took treatment from health centre or hospital; no treatment was sought by the rest of the patients (Ruebush *et al*. 1995). The study findings confirmed that self-medication practice is common in rural communities in the studied area (Ruebush *et al*. 1995). Self-treatment with antimalarial drugs is a common practice and should be a concern for the policymakers (McCombie 2002).

1.11.9 Drug pressure

Several factors are involved in increasing the drug pressure in the community including the self-medication by patients (Jombo *et al.* 2011; Nsimba *et al*. 2005; Souares *et al.* 2009; Hodel et al. 2009 & 2010), irrational treatment practices by the physicians (Aborah *et al.* 2013; Khan *et al.* 2012), lack of awareness regarding the suitable antimalarial drug to be used for treating malaria, long acting antimalarials, post-treatment prophylaxis and mass drug administration (Stepniewska *et al.* 2008) etc. Globally, this information has been captured in number of studies, including quantification of residual antimalarials such as CQ or SP in urine/ blood of the patients (Hodel *et al*. 2009; Whichmann *et al.* 2007). Based on earlier studies, a significant trend in the higher frequencies of the resistance markers with increasing CQ concentrations

was observed in *P. falciparum* malaria patients i.e. prior use of CQ in enrolled patients may favour selection of resistant genes (Ehrhardt *et al.* 2005). However, pre-treatment blood CQ concentration has been observed to have an inverse relation with degree of *P. falciparum* resistance to CQ (Quashie *et al.* 2005). In addition, it has been observed that high pre-treatment blood CQ concentration assists in eliminating CQ resistant strains of the parasites during drug treatment (Quashie *et al.* 2005). However, the scope of examining the impact of pre-hospital CQ and SDX on the resolution of malaria following treatment with antimalarials such as ACT, which is the first-line drug for the treatment of *P. falciparum* malaria, still remains open. Keeping the above points in mind the following objectives were set for the proposed research work:

1. To monitor the residual antimalarial levels in malaria patients in high endemic districts in the country.
2. To correlate the residual antimalarial levels with molecular marker of drug resistance for chloroquine, sulphadoxine and pyrimethamine.
3. To establish links between presence of residual antimalarials and therapeutic outcome, if any.

Monitoring residual antimalarials levels in *P. falciparum* infected patients belonging to tribal dominated areas in India and correlating the levels of antimalarials with the molecular markers of antimalarials drug resistance for CQ, SP along with the correlation of the findings with the therapeutic outcome will provide evidence based data on the status of antimalarial drug resistance in the study population and will help in generating the awareness by increasing the information, education and counselling activities in the study areas.

Chapter II

Residual antimalarial levels in Plasmodium falciparum malaria patients from selected sites in India: An indication of drug pressure

2.1 Introduction

Malaria imposes a substantial socio-economic challenge and together with 4 other diseases (diarrhoea, HIV/AIDS, tuberculosis, measles, hepatitis B and pneumonia), it accounts for 85% of global infectious disease burden (Murray & Lopez 1997). Unfortunately, the control of malaria is hampered due to the emergence of antimalarial drug resistance in South East Asia region to commonly used antimalarial drugs particularly for *P. falciparum.* The resistance to anti-malarial drugs has also increased the global cost of controlling the disease, including the cost of new drug development. The north-eastern region of India has been the epicentre of antimalarial drug resistance in the past and the history of resistance against antimalarials in this region include chloroquine (CQ) resistance in *P. falciparum* in Karbi Anglong district in Assam in the 1973 followed by Sulphadoxine-Pyrimethamine (SP) resistance in the same region in the 1979 (Sehgal *et al*. 1997; Das *et al*. 1981). Ineffective treatment regimen like use of monotherapies with artemisinin, lack of compliance to the prescribed medications and intake of counterfeit medicines are associated contributing factors in promoting drug resistance (Petraland 1993; White 1999). Before 2005, CQ was the first line therapy for *P. falciparum* malaria in India. Despite the fact that ACT (AS+SP) became the first line treatment for uncomplicated *P. falciparum* malaria cases in India (NVBDCP, 2011); sulphadoxine-pyrimethamine (SP), quinine (QN), mefloquine (MQ) and chloroquine (CQ) were still found to be prescribed as well as circulating widely in the private health sectors (Mishra *et al*. 2013). A study conducted in six states of India revealed 14.8% prescription of artesunate (AS) monotherapy in Jharkhand, one of the malaria endemic sites in the country (Mishra *et al*. 2011).

In the high malaria-endemic region, use of antimalarial treatments for febrile episodes and self-treatment has been observed in the past (Nwanyanwu *et al.*1996; Mahomva *et al.*1996). Self-treatment with antimalarial drugs is a common practice and should be a concern for the policy makers (McCombie 2002). Mass drug administration (MDA) was a part of several malaria elimination programs during the eradication era (Seidlein & Greenwood 2003). These factors are increased drug pressure and could promote drug resistance. Keeping the above points in mind the following objective was set to fulfil my aim.

- To monitor the residual antimalarial levels in malaria patients in high endemic districts in the country.

The standard WHO protocol to monitor the efficacy of antimalarial medicines does not exclude patients with history of antimalarial intake (WHO 2009), however, the intake of antimalarial drugs prior to inclusion in an in-vivo study may interfere with the estimation of treatment outcome because of accumulation of residual or sub-therapeutic levels of antimalarials. Standard case record forms were filled to ascertain the use of antimalarials prior to in-vivo efficacy studies. However, with a recall period of a week or fortnight, this information remains unreliable and needs further confirmation. Thus, this information might not give an accurate and complete picture of drug use in that area. Therefore, it is important to measure the antimalarial drug levels using field adapted methods. Globally, this information has been captured by a number of studies, including quantification of residual antimalarials such as CQ or SP in urine or blood in the general population or patients (Whichmenn *et al.* 2007; Hodel *et al*. 2009 & 2010). But to the best of our knowledge this is the first study report from highly endemic sites of malaria in India where simultaneous quantification of five residual antimalarials

namely CQ, sulphadoxine (SDX), pyrimethamine (PYR), QN and MQ in human blood samples was carried out. In addition, association of residual antimalarial levels with parasite densities of P. falciparum was also analysed. This work will highlight the residual levels of antimalarials purchased over the counter by the patients participating in in-vivo therapeutic efficacy studies in highly endemic regions of malaria.

2.2 Materials and methods

2.2.1 Study sites

The study was carried out during the year 2011-2013 at Gaurella Community Health Centre (CHC) at Bilaspur district in Chhattisgarh, Ghodadongri Primary Health Centre (PHC) at Betul district in Madhya Pradesh, Kurdega CHC at Simdega district in Jharkhand and Bisra CHC at Sundergarh district in Odisha (**Fig. 2.1**). In these areas, malaria transmission takes place round the year due to favourable environmental conditions supporting vector survival. *Plasmodium falciparum* infections are predominantly recorded from October to December. Amongst the four study sites, Odisha contributes 55.2% cases, Chhattisgarh contributes 17.6 % cases, Jharkhand contribute 10.2% malaria cases and Madhya Pradesh contributes 3.1 % of the annually reported malaria cases in the country (NVBDCP 2016). At the time of the study, ACT (AS+SP) was the first line treatment for uncomplicated *P. falciparum* malaria in these areas and was available with Auxiliary Nurse Midwives (ANMs), Accredited Social Health Activists (ASHAs) and other community health volunteers.

All the study procedures were conducted in accordance with the Institutional Ethics Committee of the National Institute of Malaria Research (NIMR), Ministry of Health and Family Welfare, Govt. of India.

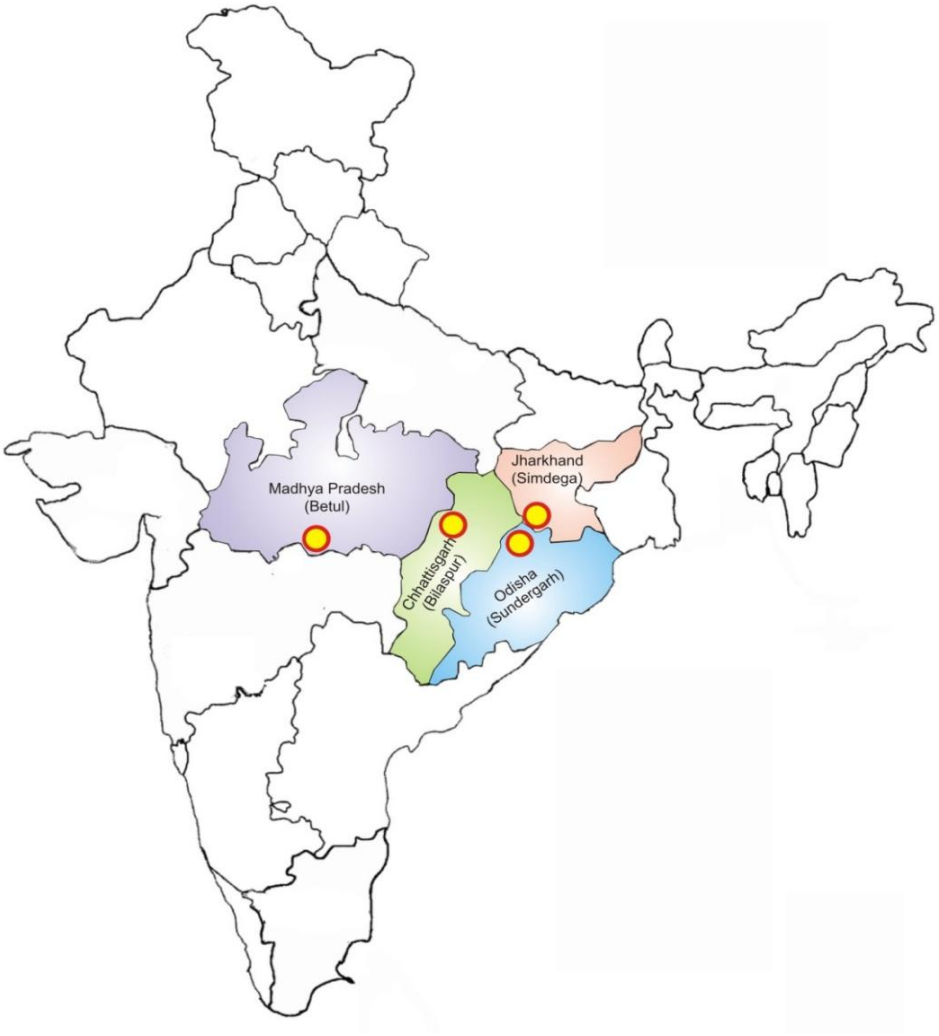

Fig. 2.1: Map showing study sites; Bilaspur (Chhattisgarh), Betul (Madhya Pradesh), Sundergarh (Odisha) and Simdega (Jharkhand)

2.2.2 Population screening

All patients reporting to local clinics with the complaint of fever or by active survey were examined for prevalence of parasites in blood smear.

2.2.2.1 Inclusion criteria

- Patients age above 6 months
- Mono-infection with *Plasmodium falciparum*

- Parasitaemia ranges from 500 to 100000/µl
- Auxiliary temperature ≥ 37.5°C or history of fever during the previous 24 hours
- Ability to come for stipulated follow-up visits; easy access to health facility
- Informed consent by patient/guardian for children.

2.2.2.2 Exclusion criteria

- Presence of one or more of the general danger signs or any sign of severe malaria
- Presence of mixed infection
- Presence of severe malnutrition
- Presence of febrile conditions caused by diseases other than malaria
- Contraindications related to the anti-malarial drugs used, especially history of allergy
- Pregnant women

2.2.3 Collection of samples

Patients were screened according to above inclusion and exclusion criteria, patients who fulfilled inclusion criteria were enrolled in our study. Informed consent was obtained by patient/legal guardian and Case Record Forms (CRF) were completed in case of each patient providing details on other demographic information such as body temperature, body weight, sex, age etc. Before drug administration, finger prick blood samples were used for counting parasite density on day 0 until 28 days of follow-up. Three hanging blood drops were collected on 3Chr Whatman filter (Whatman GE Health Care, UK) on day 0 for molecular studies and one hundred microliters (100 µl) of heparinised blood was taken on 31ET filter paper (Whatman GE Health Care, UK) for monitoring residual drug on day 0 and sulpahdoxine (SDX) drug level on day 7. The filter papers were then

allowed to dry at room temperature and stored in a zipper pouch with desiccant at 4°C in a refrigerator until analysed.

2.2.4 Treatment and follow-up

Following collection of samples from enrolled patients, directly observed treatments (DOTs) were given by qualified personnel. The patients were observed for 30 minutes after dosing and if they vomited during this period, full dose of antimalarial was repeated. The drugs supplied by the National Programme were used according to age and recommendation of National Drug Policy for Malaria. The follow-up examination of patients was done on 1, 2, 3, 7, 14, 21 & 28 day (**Table 2.1**).

Table 2.1: Summary of the follow up and samples collection

Day	**0**	**1**	**2**	**3**	**7**	**14**	**21**	**28**
Administration of the Drug	Yes	Yes	Yes	-				
	(AS+SP)	(AS)	(AS+PQ)					
Clinical assessment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Blood smear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Blood Sample on filter paper (molecular biology & drug concentration)	Yes				Yes	Yes	Yes	Yes
	2 samples				if smear positive 2 samples	if smear positive 1 sample	if smear positive 1 Sample	if smear positive 1 samples

2.2.5 Microscopy

Thick and thin smear of patients fingerpick blood were prepared for microscopy examination. Both blood smears (thick and thin) were stained using 10% Giemsa stain and observed under a light microscope on 100X oil immersion lens. Thin film was used

for parasites identification and quantification of parasite has been done using thick smears. Parasites were counted against white Blood Cells (WBCs), A blood smear was considered negative when no parasite was observed against 1000 WBCs counts/100field of thick smear. Parasite density per microliter of blood was obtained by computing parasites against 8000WBCs. The formula expressed for parasite count per microliter of blood is given below:

$$\text{Parasite density/µl blood} = \frac{\text{Number of parasites counted}}{\text{Number of WBCs counted}} \text{X } 8000$$

2.2.6 Residual antimalarial quantification

Monitoring the residual antimalarials in patients infected by *P. falciparum* malaria was done according to previously published work (Blessborn *et al.* 2010). Following this method, five different antimalarials (CQ, SDX, PYR, QN and MQ) could be quantified simultaneously in a single run.

2.2.6.1 Preparation of standard

CQ and QN were dissolved in 0.1 M hydrochloric Acid (HCl), PRY and MQ were dissolved in methanol: HCl (0.1 M) 50:50 (v/v) and SDX was dissolved in 0.1 M NaOH. Stock solutions of all the above drugs were prepared at 1 mg/ml concentration except SDX (5 mg/ml). Stock solutions were stored at 4^{o}C until further use.

2.2.6.2 Preparation of standard dried blood spots

Whole blood (drug free blood) was mixed with standards of antimalarials (CQ, QN, PYR & MQ) prepared in different concentration ranges (125, 250,500, 1000, 2000, 4000 and 8000 ng/ml), whereas the standard for SDX was prepared in the range of 625-4,00,000 ng/ml viz. 625, 1250, 2500, 5000, 10000, 20000 & 400000 ng/ml). Hundred

microliters blood was applied onto 31ET Whatman filter paper and dried at room temperature. Dried blood spots were stored at 4^{o}C in Zip lock pouch with desiccators for further analysis.

2.2.6.3 Extraction of antimalarials

For preparation of standard curves, antimalarials were extracted from pre-prepared dried blood spots of whole blood spots containing different concentrations of standard (for calibration) and 100 µl patient samples collected on day 0. The punches were cut and placed in a 2 ml micro centrifuge tube. Following this, 1.5ml of methanol: acetic acid (0.5 M) (20:80 v/v) was added. The mixture was vortexed on rotator mixer for approximately 1 hour. After mixing, the sample tubes were centrifuge at 7000g for 5 min. Thereafter, the samples were decanted into fresh 15 ml polypropylene tube. Before transferring to M-M column, 1ml acetic acid (0.5M) was added in the samples stored in 15ml tube (**Table 2.2**). Elute was collected in 3 ml glass tube and evaporated under a gentle stream of nitrogen gas at 700C. Dried samples were dissolved in 100 µl methanol: HCl (0.01M) 10:90 v/v. Fixed volume of 20 µl sample was injected into High Performance Liquid Chromatography (HPLC) system.

Table 2.2: Details of extraction of antimalarial using SPE column

SPE step	Sample from first extraction, solvents for Multimode column	Volume (ml)	Centrifugation duration (min)
Activation	Methanol	1.0	At 500 rpm for 2 min.
Conditioning	Acetic acid (0.5M)	1.0	At 500 rpm for 2 min
Loading	Sample	2.3	At 1500 rpm for 2 min
Washing	Acetic acid (0.5M)	1.0	At 1000 rpm for 2 min
Elution	Methanol:Triethylamine 97:3 v/v	1.0	At 1000 rpm for 2 min

2.2.6.3 Instrumentation and chromatographic conditions

Hitachi system consisting of binary pump (Model L-2100/2130 pump), multi wavelength UV detector (Model L-2420 UV-VIS detector) operated at 280nm wavelength and manual sample injector was used for HPLC analysis. The compounds were analyzed on a Tosoh ® 5 µm C18 (150 mm × 2 mm) column and protected by a pre-column Security Guard Tosoh C8 (4 mm × 2 mm). Mobile phase solution consisted of two type of solutions; solution (A)-acetonitrile: ammonium formiate (20mM with 1% volume of formic acid) (5:95v/v) and solution (B)-acetonitrile: ammonium formiate (10mM with 1% volume of formic acid) (80:20 v/v). Gradient were used with solution A and solution B for running the samples; Initial condition of solution-A: solution-B was 93:7 (v/v) ratio after that linear gradient was increased up to solution A: solution B ratio of 10:90 (v/v) over the 7 min. This condition was held for 3 min then back to initial condition within 1 min (solution A: solution B was 93:7). Following this, the flow is then equilibrated for 4 min before next injection. Data acquisition and quantification was performed using HystarTM and Data Analysis TM (Bruker, Bremen, Germany) (**Fig. 2.2**). Represent able calibration plots and drug detection on day 0 and 7 are shown in **Fig 2.3 and Fig 2.4**.

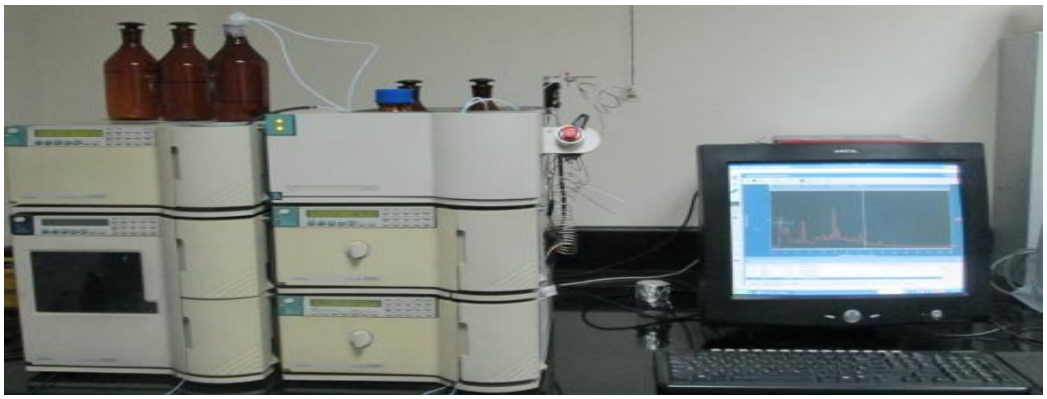

Fig. 2.2: High Performance Liquid Chromatography experimental setup in the laboratory

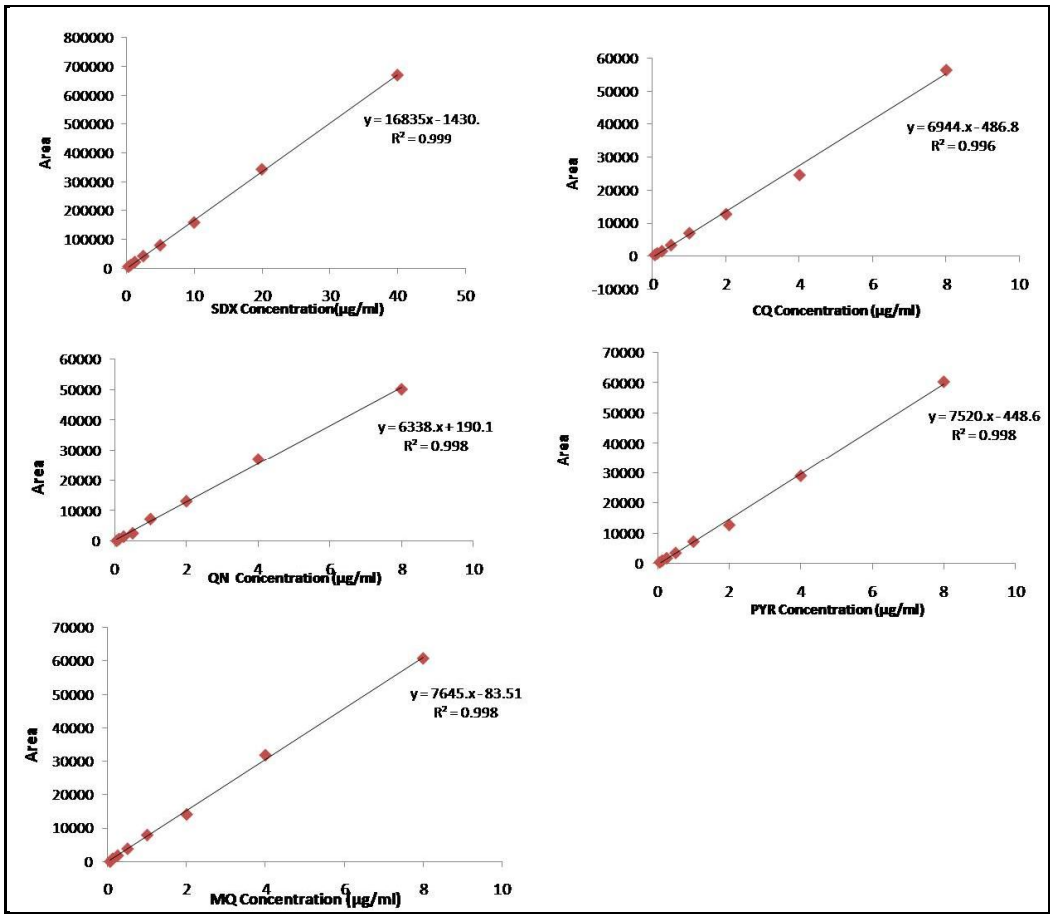

Fig. 2.3: Calibration curve of standard antimalarial drug namely CQ, SDX, QN, MQ and PYR

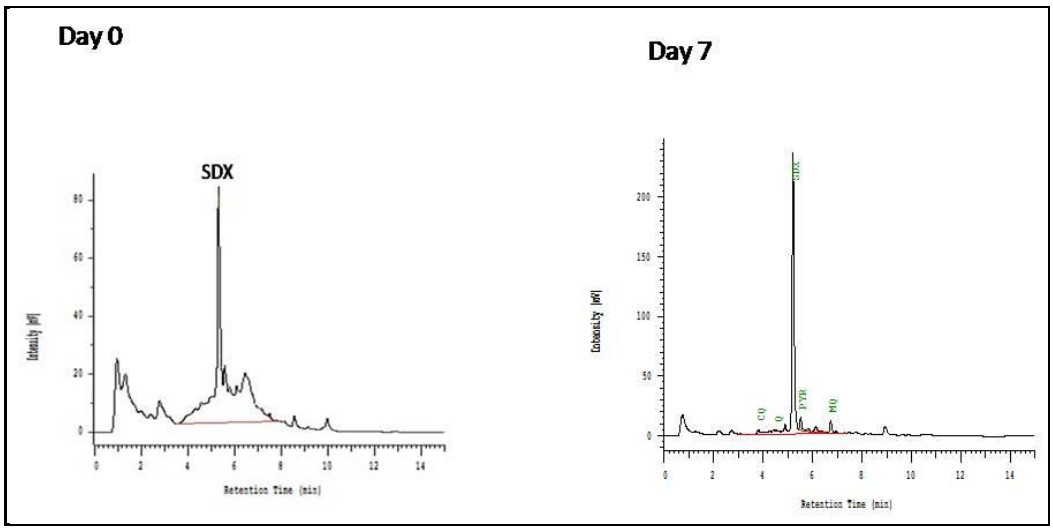

Fig. 2.4 : Chromatogram showed SDX concentration on Day 0 and Day 7, respectively

2.2.7 Estimation of dose intake time for sulphadoxine

To estimate the probable timing of drug intake, we compared the whole blood concentrations of SDX at baseline (C0) and on Day 7 (C7) after a complete treatment with AS+SP for the same patients. Assuming a terminal elimination half-life (t½) of 7.2 days for SDX, an inter-individual variability of 30% and a similar dosage on pre-study exposure and during the study, a back-calculation was done to estimate the intake time of the drug before baseline sampling:

$$\text{Intake time} = \ln(C_7/C_0).t_{1/2}/\ln(2)+7[\text{days}]$$

The variability on t½ was used to estimate a 90% confidence interval around this intake time, considering plausible inter-individual variations in elimination rate (White 1999b). Similar calculation could not be attempted for pyrimethamine because of its short half-life period (Hodel *et al.* 2009).

2.2.8 Statistical analysis

All statistical analyses were done using the SPSS software version 14. Geometric mean of parasite densities at 95% confidence interval (CI) was calculated. Frequencies were compared using the X^2 test. The differences were considered statistically significant at an error probability less than 0.05 ($p<0.05$).

2.3 Results

2.3.1 Baseline demographic data

A total of 295 eligible patients with microscopy-confirmed *P. falciparum* malaria (Chhattisgarh (n=70), Madhya Pradesh (n=80), Jharkhand (n=73) and Odisha (n=72), satisfying the inclusion/exclusion criteria were enrolled in the study. Common

symptoms of malaria such as fever, headache, chills/rigor and vomiting were reported by the patients at the time of recruitment. Baseline data including gender (male/female), age (geometric mean±SD), parasitaemia/µl **(**mean±SD & range) and previously drug intake information was recorded in CRF (**Table 2.3)**.

Table 2.3: Baseline demographics data of all study sites

Study Site	**Bilaspur (Chhattisgarh)**	**Betul (Madhya Pradesh)**	**Simdega (Jharkhand)**	**Sundergarh (Odisha)**
Characteristic	**(n=70)**	**(n=80)**	**(n=73)**	**(n=72)**
Sex [no. (%)]				
Male	44(62.9)	36(45.0)	38(52.1)	42(58.3)
Female	26(37.1)	44(55.0)	35(47.9)	30(41.7)
Age category [no. (%)]				
< 5 years	2(2.9)	9(11.3)	5(6.8)	3(4.2)
5-15 years	12(17.1)	45(56.2)	33(45.2)	23(31.9)
Adult	56(80.0)	26(32.5)	35(47.9)	46(63.9)
Temperature (˚C)				
Mean ± SD	37.8±1.1	37.7± 0.3	37.1± 0.7	37.7± 0.5
Range	36.1-39.8	36.9-39.3	35.9-39.0	37.0-39.5
Febrile (≥37.5)				
Yes [no. (%)]	36(51.4)	69(86.3)	19(26.0)	49(68.1)
No [no. (%)]	34(48.6)	11(13.8)	54(74.0)	23(31.9)
Parasite count (no/µl)				
Mean ± SD	16405.6 ± 17820.5	7641.0± 11561.9	10174.8± 28228.0	36766.7± 33320.6
Gametocytes on day 0				
[no. (%)]	3(4.3)	2(2.5)	6(8.2)	2(2.8)
Previous antimalarial intake				
Yes [no. (%)]	0(0.0)	0(0.0)	0(0.0)	0(0.0)
No [no. (%)]	62(88.6)	0(0.0)	0(0.0)	0(0.0)
Unknown [no. (%)]	8(11.4)	80(100.0)	73(100.0)	72(100.0)

2.3.2 Detection of residual antimalarials

Out of 295 patient samples, 289 samples were processed for monitoring residual antimalarials levels. Out of processed 289 samples, 70 (24.2%) patients had residue of antimalarials on day 0 (at enrolment time). Out of these 70 patients, 25(31.6%) patients from Madhya Pradesh had highest residual antimalarial followed by 18 (25.4%) patients from Jharkhand, 17(25%) patients from Chhattisgarh and 10 (14.1%) patients from Odisha. The details of 70 samples showed residual antimalarials namely; 52.9% of sulphadoxine (SDX), 25.7% of chloroquine (CQ), 1.4% of melfoquine (MQ), 1.4% of quinine(QN), 5.7% of SDX+CQ, 5.7% of SDX+PYR, 1.4% of SDX+QN, 1.4% of CQ+QN, 1.4% of SDX+PYR+CQ and 2.9% patients had SDX+CQ+QN in combination (**Fig. 2.5**).

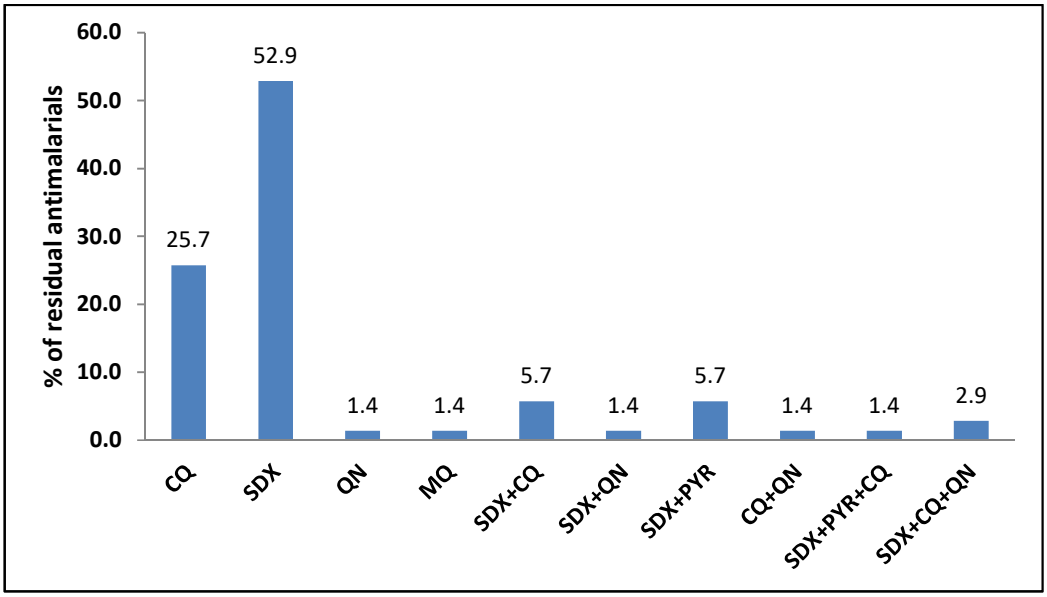

Fig. 2.5: Details of residual antimalarials detected on day 0 in patients.

The detail of residual antimalarial observed in patients enrolled from Chhattisgarh showed that 14.7% had SDX, 7.4% had CQ, 1.5% had SDX+ CQ+QN, 1.5% patient had

residual SDX+ CQ and no residual antimalarials were observed in 75% patients. Similarly residual antimalarial drug in patients from Madhya Pradesh showed that 15.2% had SDX, 10.1% had CQ, 5.1% had SDX+CQ+QN, 1.5% patient had residual MQ and remaining 68.4% patients did not have any residual antimalarial. Jharkhand site showed that 16.9% had SDX, 2.8% had CQ, 1.4% had QN, 1.4% had SDX+ CQ+QN, 1.4% had CQ+QN, 1.4% patient had residual SDX+QN and 74.6% patients did not show any previous antimalarial intake. In Odisha site, 9.9% patients had SDX, 4.2% patient had residual CQ and 85.9% patients had no residual antimalarial (Table 2.4).

Table 2.4: Details of residual antimalarials alone or in various combinations at all study sites

Antimalarial Drug	Chhattisgarh (n=68)	Madhya Pradesh (n=79)	Jharkhand (n=71)	Odisha (n=71)	Total (n=289)
CQ	7.4	10.1	2.8	4.2	6.2
SDX	14.7	10.1	16.9	9.9	12.8
QN	0.0	0.0	1.4	0.0	0.3
MQ	0.0	1.3	0.0	0.0	0.3
SDX+CQ	1.5	3.8	0.0	0.0	1.4
SDX+QN	0.0	0.0	1.4	0.0	0.3
SDX+PYR	0.0	5.1	0.0	0.0	1.4
CQ+QN	0.0	0.0	1.4	0.0	0.3
SDX+PYR+CQ	0.0	1.3	0.0	0.0	0.3
SDX+CQ+QN	1.5	0.0	1.4	0.0	0.7
NO residual	75.0	68.4	74.6	85.9	75.8

Abbreviation: CQ: Chloroquine, SDX: Sulphadoxine, QN: Quinine and MQ: Mefloquine, PYR: Pyrimethamine

2.3.3 Levels of residual antimalarials

Out of the 289 patients, the presence of antimalarial drug was detected in 70 patients (24.2%): 49 (17.0%) had significantly higher mean SDX concentration of 10765.3ng/ml

(100-54100ng/ml), 27(9.3%) of them showed a significantly higher CQ mean concentration of 147.0 ng/ml(51-263ng/ml), 5(1.7%) had significantly high mean PYR concentration of 980ng/ml (100-1600ng/ml), 4(1.4%) had significantly high mean QN concentration of 184.5ng/ml (100-279ng/ml), while MQ was present in only 2 (0.7%) patients at significantly higher mean concentration of 317ng/ml (267-367ng/ml (**Table 2.5**).

Table 2.5: Residual plasma concentration of antimalarials prior to treatment in malaria patients (ng/ml)

Antimalarial	No. of patients	ng/ml			
		Mean	Median	Minimum	Maximum
Sulphadoxine	49(17.0%)	10765.3	6190	100	54100
Chloroquine	27(9.3%)	147	135	51	263
Pyrimethamine	5(1.5%)	980	900	100	1600
Quinine	4(1.4%)	184.5	179.5	100	279
Mefloquine	2(0.7%)	317	-	267	367

2.3.4 Therapeutic level of SDX on day 7

The level of SDX was monitored on day 7 in patients (n= 219) treated with AS+SP. Quantification of SDX on day 7 samples was done in four study sites; Chhattisgarh (n=50), Madhya Pradesh (n=48), Jharkhand (n=58) and Odisha (n=63). Interestingly, the level of SDX on day 7 at all the sites ranged between mean ± SD value of 48.8±13.3 in Chhattisgarh, 45.8±18.2 in Madhya Pradesh, 43.7±18.8 in Jharkhand and 45.5±12.9 µg/ml in Odisha**.** The Mean ±SD of total analysed samples **(**n=219**)** of SDX on day 7 was 45.8±14.8µg/ml in blood.

2.3.5 Probable time of previous SDX intake

After comparing the whole blood concentration of SDX at baseline (day 0) and on day 7, the back estimation method indicated a mean of 29 days prior to study enrolment (range 5-69days; 90% CI), the most likely time for previous SDX intake. Majority of the patients i.e. 23 (46.9%) showed previous SDX intake estimated time of more than 28 days, and 22.4% patients before 3-4 weeks,12.2% patients before 2-3 weeks and 16.3% patients before 1-2 weeks took single dose of SP before the enrolment in study. Only one patient showed recent intake of SP dose.

2.3.6 Residual antimalarial in different age groups

The patient samples were divided in three groups on the basis of age viz. 6 month to <5 years, ≥5 years to <15 years and ≥15 years or above to see the effect of irrational practices in different age groups. Since, 70 patients had residual antimalarials on day 0, the age wise residual antimalarial drugs were 8.6%, 31.4% and 60.0%, respectively (**Fig. 2.6**).

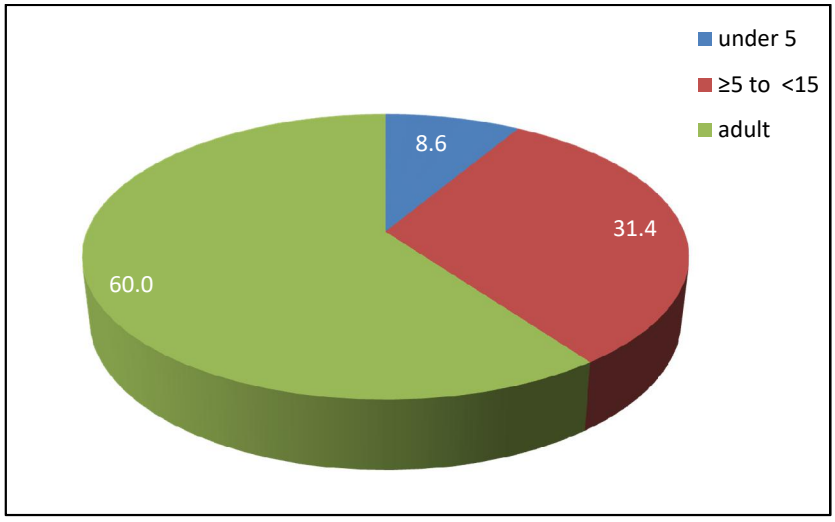

Fig. 2.6: Residual antimalarial drug (day 0) in different age groups

2.3.7 CRF information and residual antimalarial

A total of 295 patients were analysed for the previous drug intakes using case record form (CRF) information; 21.1% patients denied intake of antimalarial drug, 75.4% patients were not aware about previous intake and 3.5% of the patients were in category where information could not be recorded. However, HPLC analysis of blood samples on day 0 showed that 24.2 % patients had residual antimalarials (**Fig. 2.7**).

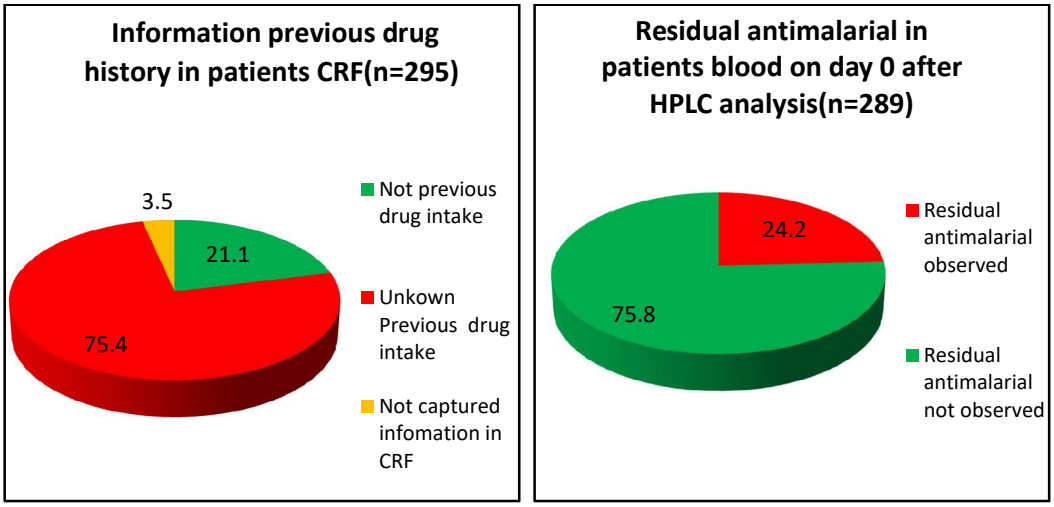

Fig. 2.7: Comparision of CRF information on previous drug history and HPLC analysis

2.3.8 Relationship between Parasite density and residual SDX

Parasite densities of *P. falciparum* patients ranged between 616-99200 asexual parasites/µl (mean±SD: 23627±2554 asexual parasites/µl). Parasitaemia was compared between patients having residual SDX in blood (n=49) on day 0; 1154 to 80238 asexual parasites/µl (mean ±SD: 16733±18077asexual parasites/µl, $P<0.05$) and those with no SDX on day 0 (n=240), that is, 616 to 99290 asexual parasites/µl (mean± SD: 25023±26665asexual parasites/µl, $P<0.05$) (**Fig. 2.8**). A significantly lower parasite density/µl was observed in patients having residual SDX.

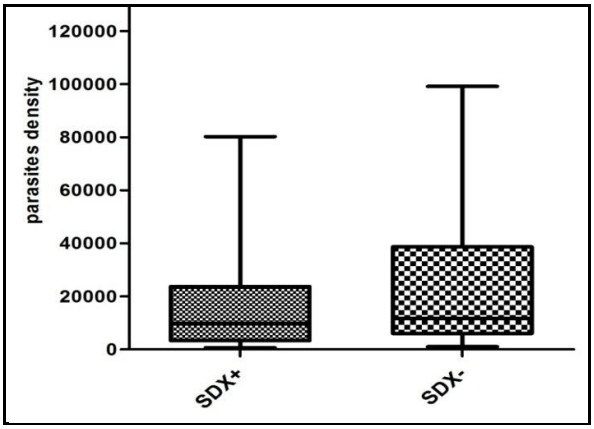

Fig. 2.8: Parasites density distribution with residual SDX(+) and without the residual SDX(-) on Day 0. Horizontal line in histogram shows mean value of the parasites densities. Bottom and upper value show the lowest and highest parasite density in the patients

2.3.9 Residual CQ and parasite density

Parasitaemia was compared among patients having residual antimalarials (n=27) on day 0; 1056 to 78240 asexual parasites/µl (mean ±SD: 20394±19735 asexual parasites/µl, p value = 0.27) and without residual CQ on day 0 (n=262), that is, 616 to 99290 asexual parasites/µl (mean± SD: 23687±25835 asexual parasites/µl) (**Fig. 2.9**). Lower parasite density/µl was observed in patients having residual CQ as compared to those patients with no previous CQ intakes.

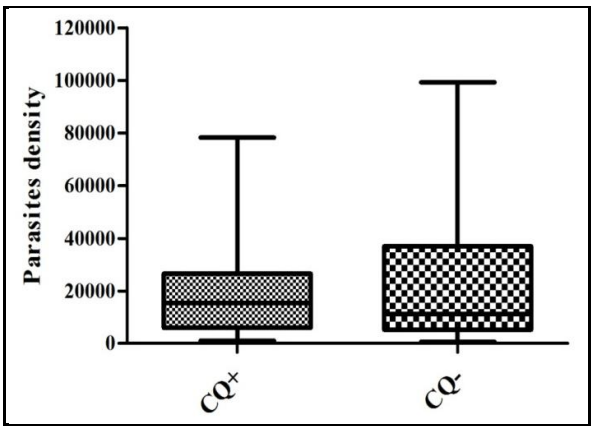

Fig. 2.9: **Parasite density with residual CQ** (+) **and without the residual CQ** (-) **on Day 0**. **Horizontal line in histogram shows the mean value of the parasites densities, bottom and upper value show the lowest and highest parasite density in the patients.**

2.4 Discussion

To our knowledge this is the first study to investigate the presence of five antimalarials (CQ, SDX, PYR, QN and MQ) in blood of malaria positive patients from Bilaspur (Chhattisgarh), Betul (Madhya Pradesh), Simdega (Jharkhand)and Sundergarh (Odisha) districts in India. These districts have high *P. falciparum* malaria burden. Out of these five antimalarial drugs, CQ is currently recommended as first line antimalarial for uncomplicated *P. vivax* malaria cases in the country, while SP is being recommended as partner drug in the recommended ACT for the treatment of uncomplicated *P. falciparum* malaria throughout the country except in the north-eastern India. In Northeast India region, artemether-lumefantrine is the first line of treatment for *P. falciparum*, as high treatment failure to SP was observed during 2012-13(Mishra *et al*., 2014). Quinine is recommended as rescue treatment in treatment failure cases to ACT while MQ is advised for long term chemoprophylaxis (NVBDCP 2005). The measurement of these five antimalarials allowed a comprehensive assessment of the circulating drugs in the community under study.

Malaria is a major public health problem in India and its transmission varies from place to place. The forest and tribal areas of Madhya Pradesh (MP) Chhattisgarh (CG) are situated in central part of India, while Jharkhand and Odisha are situated in the East, where in most high malaria burden areas, the disease is stable although malaria outbreaks are frequently recorded in unstable areas making its control difficult logistically; the probable reason being the inadequate surveillance, poor reporting, a time-lag in reporting to decision makers and inadequate geographic information system that could identify the trouble spots for a timely preventive action. Both *falciparum* and *vivax* species of *Plasmodium* are prevalent in these states with a preponderance of

P. falciparum. Drug resistance has remained a major problem for control of malaria in this region. Besides the genetic factors, drug pressure in the community also plays a major role in emergence of drug resistant parasites (Hodel *et al.* 2009). To assess the drug pressure, residual level of antimalarials were monitored by HPLC in the samples from patients enrolled at selected study sites under therapeutic efficacy studies.

We observed that out of 295 patients enrolled in the study, 289 samples could be used for monitoring residual level of antimalarials. Amongst them, 70 (24.2%) carried blood residual antimalarials above the lower limit of detection (50 ng/ml) at inclusion (Hodel *et al.* 2009). This could be a worrying factor and a possible indication of tolerance to residues which could act as a precursor for the development or precipitation of resistance. This is a matter of concern and the issue needs to be carefully monitored at state as well as national level.

Sulphadoxine-Pyrimethamine (SP)

SP is currently being used as a partner drug for the first line ACT (Artesunate+Sulphadoxine-Pyrimethamine) for the treatment of uncomplicated *P. falciparum* malaria in India since 2010, except in the north-eastern India as mentioned elsewhere. In this study, we observed residual level of SDX in blood of 49 (17%) patients on day 0 at a mean concentration of 10765.5 ng/ml. Frequency of residual SDX was different in low and high transmission areas. Samples from Betul district (Madhya Pradesh) had higher frequency of residual SDX as compared to Simdega district (Jharkhand), Bilaspur district (Chhattisgarh) and Sundergarh district (Odisha). Variation in frequency of residual antimalarial may be due to illiteracy, malaria transmission and accessibility to health facilities.

This finding corroborates with the results obtained from the study in which residual plasma concentration of antimalarials was detected prior to the treatment (Hodel *et al*. 2009). The SDX level in two hundred nineteen patients on day 7 was found to be 45.8±14.8 µg/ml. PYR is usually given as a combination therapy with SDX. A mean concentration of 0.98 µg/ml of PYR in the blood of five (3.5%) patients was observed in our study.PYR is usually given at a lower dose of 1.25 mg/kg in combination with SDX and its half-life is approximately 4 days; than that of SDX which has a half-life of 7.2 days (Hodel *et al*. 2009; Weidekamm *et al*. 2007). In present study, the mean concentration obtained for SDX and PYR was comparatively higher than the reported study; but there was no conspicuous difference in the concentration range of the residual antimalarials (Hodel *et al*. 2009). There are various factors which might be responsible for such differences in the mean concentration of residual antimalarials: (1) difference in the samples-dried blood spots & extraction procedure (2) instrumentation technique used i.e. the reported study have used liquid chromatography-tandem mass spectrometry which is highly sensitive as compared to HPLC used in the present study.

Based on the day 0 and day 7 SDX concentration (C0 and C7), and assuming that the patients with residual SDX in their blood had taken a single dose of SP according to their body weight, we can infer by using back estimation method that these patients must have taken SP approximately within one month (29 median days) prior to inclusion in our study (Hodel *et al*. 2009). Furthermore, it is also possible that these patients might have taken a sub-therapeutic dose or counterfeit form of SP more recently (Basco *et al*. 2004) which though is difficult to confirm.

Chloroquine (CQ)

Although, CQ has been withdrawn to be used as first-line of treatment for *P. falciparum* infection (Valecha *et al*. 2009), it was detected in the blood of 27 (9.3%) patients in our study at a mean concentration of 147ng/ml. Hodel *et al.* 2010 have also reported the residual plasma concentration of CQ, MQ and QN before initiating the antimalarials treatment in patients (Hodel *et al.* 2010). This might be due to easy availability of CQ in the private clinics and over the counter in pharmacy shops or due to its unregulated and unsupervised wide use in the treatment for a previous episode of *P.vivax* malaria. Previous study from our laboratory has reported that CQ is the most common antimalarial sold across the counter with high frequency of prescription in public as well as private health facilities as compared to other antimalarials (Mishra *et al.* 2011).

As we employed dried blood spot method for sample collection, it was not possible to detect any artemisinin compounds. Also, short half-life of artemisinin requires sophisticated techniques as well as plasma sample collection. However, the presence of residual artemisinin cannot be ruled out as high prescription rate of this antimalarial has been previously observed in the country (Mishra *et al.* 2011).

Both MQ and QN are not recommended for uncomplicated malaria currently in India, but their residual levels were still found to be present in the patients, although in very few samples and that too at low concentrations.

By detecting the presence of residual anti-malarial levels on day 0, it can be concluded that the probable reasons for this may be due to; (1) Self-medication due to illiteracy or non-availability of drug at the dispensing facility or by the treating practitioner leading to inadequate dosing and there by inability to control infection (Nsimba & Rimoy 2000;

Source *et al.* 2009; Hodel *et al.* 2009 & 2010; Jambom *et al.* 2011), (2) Irrational treatment practices by the physician at the study sites with high transmission intensity of malaria parasites, where all febrile patients were treated with a variety of available antimalarial drugs (Khan *et al.* 2012; Aborah *et al.* 2013), (3) Lack of awareness regarding the suitable antimalarial drug to be used for treating different species of malaria (4) Treatment of previous episode of infection with antimalarial drug and then re-infection with a new parasite resulting in accumulation of previously consumed drug as residue in blood (Quashile *et al.* 2005; Stepniawsha & White 2008). These factors contribute to increased drug pressure on the parasite thus encouraging resistance in *Plasmodium* species (Jamison *et al.* 2009). With these observations, it can be deduced that the entry criteria based on self-reporting of previous drug intake or information collected in case record forms are not reliable at least in these study populations.

The influence of age and sex on the probability of residual antimalarials at entry showed no significant relationship in our study (X^2=23.0; p=0.10) indicating uniform antimalarial prescription or intake behaviour in the population. These estimates are approximate, as indicated by the wide confidence interval explained by the fair degree of inter-individual variability and residual error. The correlation between the parasite density and levels of antimalarial drugs was also studied.

We observed that the lower mean parasite density of 16733 asexual parasites/µl (p<0.05) were present in blood samples with residual SDX, while 25023 asexual parasites/µl were present in those samples without residual SDX. Similarly, lower mean parasite density of 20394 asexual parasites/µl were present in blood samples with residual CQ, while 23687 asexual parasites/µl were present in those without residual

CQ. This confirms that the parasitaemia was comparatively lower in the patients who had residual antimalarial levels. This could be due to suppression of new-infection by the residual antimalarials levels in blood, however, the levels of residual antimalarial could not be enough to completely control parasite replication and ultimately relieving clinical symptoms. Here it is difficult to comment whether the residual drug levels were due to the result of full or incomplete treatment and the parasites causing the current episode of infection were from the same or a new infection.

Residual levels of SDX and CQ above minimum inhibitory concentration (MIC) were observed in 18.4 % and 74.1 % patients respectively. This shows that the accessibility to CQ was more as compared to SDX and thus people could easily buy and consume CQ for the treatment of malaria episode. Also, in the past, presumptive treatment with CQ was practiced in the country to treat febrile episodes of malaria. This suggests that despite drug policy change at the national level and introduction of ACT for treatment of falciparum malaria, CQ is still being widely used for treating malaria.

2.5 Conclusion

The intense parasite transmission and the widespread presence of sub-curative drug levels in blood most likely constitute a predisposing environment for the selection and spread of resistant *P. falciparum* strains. As resistance gradually expands and intensifies, it is also likely that the drug is taken more frequently and at higher doses. As a consequence, more intense drug pressure will then select for more resistant parasites. The chances of drug resistant parasites to be selected depends on several factors, and is higher for patients with lower immunity (e.g., young children), drugs with long residence times and resistance being conferred through single point mutations, and for

infections with a large parasite biomass. In this scenario, the efficiency of treatment outcome also cannot be evaluated effectively as the clearance of parasites may be due to an additive effect of the current intake of treatment drug and the residual drug taken during previous episodes of malaria infection. To minimize the burden of drug resistance in the country, essentially requires awareness in the community as well as amongst the physicians and paramedical staff on national drug policy for treatment of malaria.

The findings of this study are revealing and must be confirmed in other settings as they have potential implications for both clinical research, surveillance (treatment efficacy and safety outcome) and malaria control policy.

Chapter III

Correlation of residual chloroquine levels with molecular marker of chloroquine resistance

3.1 Introduction

Chloroquine (CQ) has remained the choice of antimalarial for treatment of malaria in the India until recently despite documented resistance in some parts in early 1970s. Its use till early 2000s was rampant throughout in the country, however, in areas with known CQ resistance, sulphadoxine-pyrimethamine (SP) treatment was recommended as a second line treatment between 1982 to 2005. India introduced artemisinin based combination therapy (ACT) in 2005 and the available option with the national programme at that time was artesunate plus sulphadoxine-pyrimethamine as SP was already available as a second line treatment for uncomplicated *falciparum* malaria in chloroquine resistance areas in the country and artesunate was registered for treatment of severe malaria cases in the country (NVBDCP 2007). Due to increasing resistance of chloroquine in wide ranging areas in the country until early 2010, AS+SP was recommended as the first line treatment for all uncomplicated *falciparum* malaria cases in the country (NVBDCP 2011). Later in Northeast region, due to high treatment failure rate of AS+SP regimens, because of high prevalence of partner drug resistance due to mutations in *Plasmodium falciparum dihydrofolate reductase* (*pfdhfr*) and *Plasmodium falciparum dihydropteroate synthase (pfdhps)* gene, AS+SP regimens was replaced by AL in Northeast India in 2013 (Mishra *et al*. 2014; NVBDCP 2013).

The emergence and spread of antimalarial resistance could due to number of factors including lack of vector control measures, declining efficacy of recommended drugs, prevalent irrational treatment practices in the community, etc. CQ resistance has been well documented and its mechanisms have been elucidated in detail. The resistance to CQ in *P. falciparum* is facilitated primarily by mutant forms of the 'chloroquine

resistance transporter' (*pfcrt*) gene. Several mutations have been identified in the *pfcrt* gene (at codons 72-76), but the K76T mutation involving the amino acid change from positively-charged lysine to neutral threonine, which could facilitate drug efflux through a putative channel is the most important amongst all (Fidock *et al.* 2000). It has been strongly associated with chloroquine (CQ) susceptibility and clinical outcomes which has been found at higher frequencies throughout India (Mallick *et al.* 2012). Chloroquine resistance may have been worsened by misdiagnosis and consequently improper anti-malarial drug treatment, as CQ is the first-line drug *for P. vivax* malaria in India (NVBDCP 2016) and is readily available across counter.

Several studies have shown that self-medication, irrational treatment practices, mass drug administration, availability of counterfeit antimalarials and presumptive treatment with antimalarials in febrile patients are very common in high endemic areas (Nsimba & Rimoy 2000; Source *et al.* 2009; Hodel *et al.* 2009 & 2010; Jambo *et al.* 2011, Khan *et al.* 2012; Aborah *et al.* 2013). All these factors have predominantly contributed to CQ resistance in *P. falciparum* (Jamison *et al.* 2006). Residual CQ level in enrolled patients with prior use of CQ has been correlated with the significant trend for higher frequencies of the resistance markers of *pfcrt* (Ehrhardt *et al.* 2005). However, it has also been observed that pre-treatment of blood CQ concentration has an inverse relation with degree of *P. falciparum* resistance to CQ (Quashie *et al.* 2005). In this study, it has also been observed that high pre-treatment blood CQ concentration assists in eliminating CQ resistant strains of the parasites during drug treatment (Quashie *et al.* 2005).

To confirm this correlation between residual antimalarials and molecular markers of CQ resistance, mutation in *pfcrt* gene at codon K76T, were analysed in samples from

endemic areas in India and were correlated with residual CQ level. Till date, there is no published data on residual levels of CQ and its effect on mutation in *pfcrt gene* at codon K76T from India. To attempt this objective, a total of 295 patients from Chhattisgarh, Madhya Pradesh, Jharkhand and Odisha were enrolled in the study. Data on residual levels of CQ, parasite density and mutation in *pfcrt* gene at codon K76T was generated and attempts were made to correlate these parameters.

3.2 Materials and Methods

3.2.1 Study site, patients enrolment and sample collection

The study was carried out during the year 2011-2013 at Gaurella Community Health Centre (CHC) at Bilaspur district in Chhattisgarh, Ghodadongri Primary Health Centre (PHC) at Betul district in Madhya Pradesh, Kurdega CHC at Simdega district in Jharkhand and Bisra CHC at Sundergarh district in Odisha.

Patients were screened following the inclusion and exclusion criteria, and those patients, who fulfilled the inclusion criteria, were enrolled in the study. Informed consent was obtained by patient/guardian for children and Case Record Form (CRF) was completed for each patient. Other demographic information such as body temperature, body weight, sex, age etc. was recorded. Before drug administration, finger prick blood samples were collected on glass slide for blood smear preparation and also on filter paper for biochemical estimations including parasite counting on day 0 until 28 days of follow-up. Three hanging blood drops collected on 3Chr Whatman filter (Whatman GE Health Care, UK) on day 0 molecular studies and one hundred microliters (100 µl) of heparinised blood was taken on 31ET filter papers (Whatman GE Health Care, UK) for monitoring residual drug levels on day 0. The filter papers

were then allowed to dry at room temperature and stored in a zipper pouch with desiccant at 4°C in a refrigerator until analyzed. The patients were treated with ACT (Artesunate+Sulphadoxine-Pyrimethamine) according to national drug policy after blood collection.

3.2.2 Quantification of antimalarial by High Performed Liquid Chromatography

Baseline blood samples (day 0) collected from patients reporting no antimalarial intake prior to the study were screened for the presence of five antimalarial drugs such as CQ, sulphadoxine (SDX), Pyrimethamine (PYR), Quinine (QN) and Mefloquine (MQ) using a modified HPLC method **(**Blessborn *et al***.**, 2010**).** Extraction of the standard drugs (CQ, QN, SDX, PYR and MQ; Sigma Aldrich, USA) from whole blood spot (blank, control sample) and each of the collected samples were carried out according to the standard protocol with slight modifications **(**Blessborn *et al*., 2010**).** Extraction of antimalarials involved the use of multi-mode solid phase extraction column **(**M-M SPE, Biotage, USA**)** and elution of the samples by methanol**:**triethylamine (97:3 v/v) mixture**.** Elute was collected in glass tube and elutes were evaporated under a gentle stream of nitrogen gas at 70^0C**.** Twenty microliter (20µl) of extracted standards and patient samples were injected into the HPLC system. HPLC was performed on a Hitachi gradient system equipped with binary pump (Model L-2100/2130) and multi wavelength UV detector (Model L-2420 UV-VIS). Extracted drugs were analysed using two different mobile phases (A) acetonitrile:ammonium formate (20 mM in 1% formic acid) (5:95 v/v) and (B) acetonitrile:ammonium formate (10 mM in 1% formic acid) (80:20 v/v) and were run according to previously described gradient program (Blessborn *et al***.**, 2010). Antimalarial were analysed on a Tosoh ® 5 µm C_{18} (150 mm × 2 mm) column protected by a precolumn security guard C_8 (8mm x2 mm) (Tosoh

Bioscience, PA). The UV detector was monitored at 280nm. Data acquisition and quantification were performed using Hystar™ and Data Analysis™ (Bruker, Bremen, Germany).

3.2.3 Mutation analysis in *pfcrt* gene by PCR-RFLP

3.2.3.1 Parasite genomic DNA isolation

Genomic DNA was isolated from dried blood spots using QIAamp mini kit (QIAGEN, Germany), following the manufacturer's instruction with slight modification. The steps are as follows-

- Punched three circles (3mm) from a dried blood spot placed into 1.5 ml tube and added 180µl ATL buffer and kept at room temperature for overnight.
- Incubated at 85°C for 10 min and centrifuged to remove drops from inside the lid.
- Added 20 µl proteinase K stock solution (20mg/ml), mixed by vortex and incubated at 56°C for 1 hours.
- Briefly centrifuged to remove drops from inside the lid and added 200 µl AL Buffer to the sample and mixed by vortex.
- Incubated at 70°C for 10 min and briefly centrifuged to remove drops from inside the lid.
- Added 200µl absolute ethanol (96–100%) to the sample and mixed thoroughly by vortex.
- Briefly centrifuged to remove drops from inside the lid. Carefully applied the mixture into QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim and centrifuged at 8000 rpm for 1.5 min.

- Placed the QIAamp Mini spin column in 2 ml collection tube and filtrate was discarded.
- Add 500 µl wash Buffer AW1 in QIAamp Mini spin column without wetting the rim and centrifuged at 8000 rpm for 1.5 min.
- Placed the QIAamp Mini spin column in clean 2 ml collection tube and filtrate was discarded.
- Added 500 µl Buffer AW2 into QIAamp Mini spin column and without wetting the rim and centrifuged at 13,000 rpm for 3 min.
- Placed the QIAamp Mini spin column in a new 2 ml collection tube and centrifuged at 13,000 rpm for 1 min
- Placed the QIAamp Mini spin column in a 1.5 ml microcentrifuge tube and discarded the collection tube containing the filtrate.
- Added 150 µl distilled water in to QIAamp Mini spin column and incubated at room temperature for 1 min. Centrifuged at 8000 rpm for 1.5 min
- Eluted genomic DNA was stored at -20^{0}C before its use.

3.2.3.2 PCR amplification

PCR was used to amplify *pfcrt* gene from extracted DNA as described previously (Vasthala et al. 2004). In brief, 20 µl PCR reaction mixture contained 10 pmol of forward and reverse primers (Eurofins, Bangalore), 0.25 mM of each dNTPs (Genei, Banglore), 1X PCR buffer (Life technology Inc.), 2.5mM $MgCl_2$ (Life technology Inc.) 1unit of AmpliTaq Gold DNA polymerase (Life technology Inc.) and 2µl genomic DNA and 2µl genomic DNA (**Table 3.1**).

Table 3.1: Details of primers sequence and cycling conditions for amplification of *pfcrt* gene

PCR reaction	Primer sequence	Cycling conditions	Amplicon size (bp)	Fragment size after digestion
Primary PCR	F 5'-CCGTTAATAATAAATACAGGCAG-3' R 5'-CTTTTAAAAATGGAAGGGTGTATAC-3'	Initial denaturation: 95°C for 12 min Denaturation: 95°C for 30 sec Annealing: 60°C for 1 min Extension: 72°C for 1 min 30 sec Number of cycles: 40 cycles Final extension: 72°C for 5 min Hold at 12°C for ∞	1600pb	
Nested PCR	F 5'-GGCTCACGTTTAGGTGGA-3' R 5'-TGAATTTCCCTTTTTATTTCCAAA-3'	Initial denaturation: 95°C for 12 min Denaturation: 95°C for 30 sec Annealing: 52°C for 1 min Extension: 72°C for 1 min 45 sec Number of cycles: 40 cycles Final extension: 72°C for 5 min Hold at 12°C for ∞	264bp	W 136 +128bp M 264bp

Primary PCR product was diluted (1:50) with distilled water and 2µl of diluted PCR product was used as template DNA for nested PCR. In brief, 20 µl PCR reaction mixture contains 10 pmol of forward and reverse (Eurofins, Bangalore) 0.25 mM of each dNTPs (Genei, Banglore), 1X PCR buffer (Life technology Inc.), 2.5mM $MgCl_2$ (Life technology Inc.) 1units AmpliTaq Gold DNA polymerase (Life technology Inc.). The nested PCR product size was 264 bp (**Table 3.1, Fig. 3.1**).

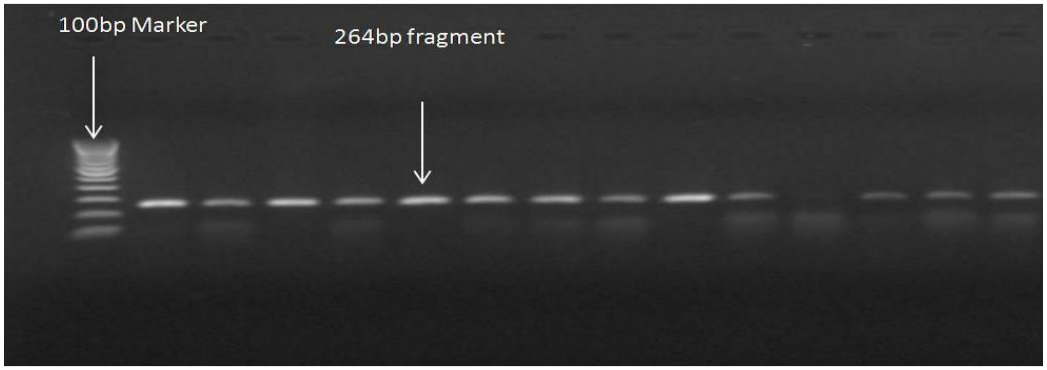

Fig. 3.1: Representative gel for nested PCR amplification of *pfcrt* gene

Five microlitres of nested PCR product was digested with *Apo*I restriction enzyme (NEB) and incubated at 37°C for 20 min, and then resolved on 1.5 % agarose gel containing ethidium bromide (0.5µg/ml). PCR products were visualized under UV transilluminator (280 nm) gel documentation system (**Fig. 3.2)**.

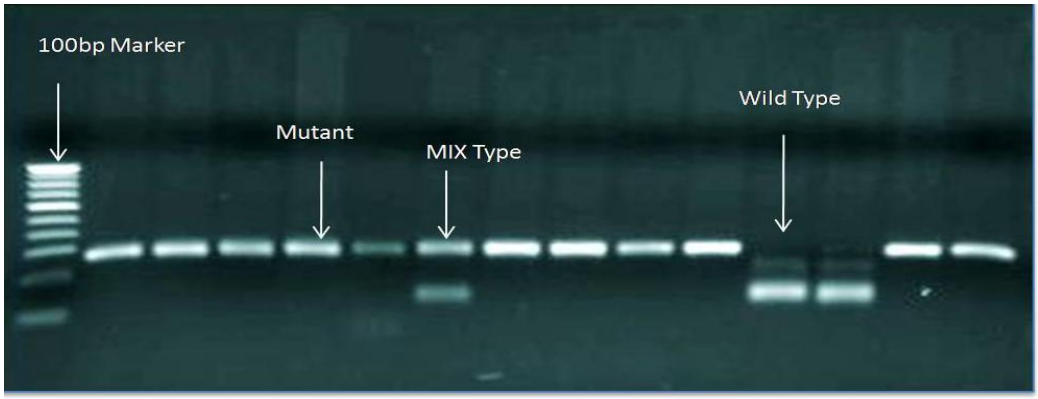

Fig. 3.2: Restriction digestion of nested PCR product with *Apo*I enzyme

3.2.4 Data management and statistical analysis

Statistical analysis of data was performed using the SPSS software version 14. Geometric mean of parasite densities at 95% confidence interval (CI) was calculated. Frequencies were compared using the X^2 test. The differences were considered statistically significant at an error probability less than 0.05 ($p<0.05$). Preparation of graphs, data entry and analysis was also done using Microsoft Office Excel 2007 and Graph Pad Prism version 5.03.

3.3 Results

3.3.1 Residual Chloroquine levels

Out of 295 enrolled patients, 289 patients samples were processed for monitoring the residual levels of CQ on day 0. Twenty seven (9.3%) patients had residual CQ levels above the detection limit (50ng/ml) in the blood. The incidence of residual CQ levels was observed in higher frequency in Madhya Pradesh (16.5%, P=0.0001) followed by Chhattisgarh (10.3%, P=0.0023), Jharkhand (5.6%, P=0.0048) and Odisha (4.2%, P=0.0157) (**Fig. 3.3**).

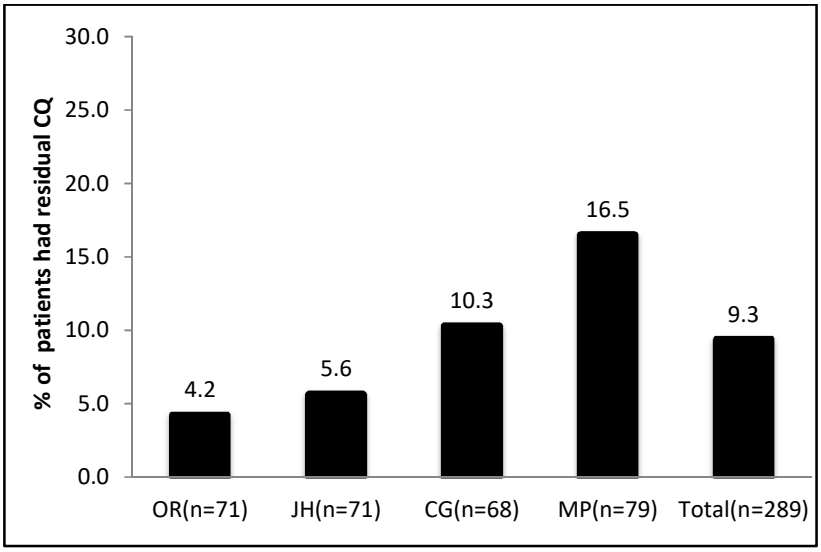

Fig. 3.3: Histogram shows the frequency of residual CQ in the patients on day 0 at different study sites; Odisha (OR), Jharkhand (JH), Chhattisgarh (CG) and Madhya Pradesh (MP).

The frequency of residual CQ levels was observed to be significant at all the study sites (p= <0.05). The median residual CQ levels observed in Chhattisgarh was 135ng/ml (51-263 ng/ml), 123 ng/ml (87-160 ng/ml) in Madhya Pradesh, 180ng/ml (70-230 ng/ml) in Jharkhand and 126 ng/ml (87-139 ng/ml) in Odisha (**Fig. 3.4**).

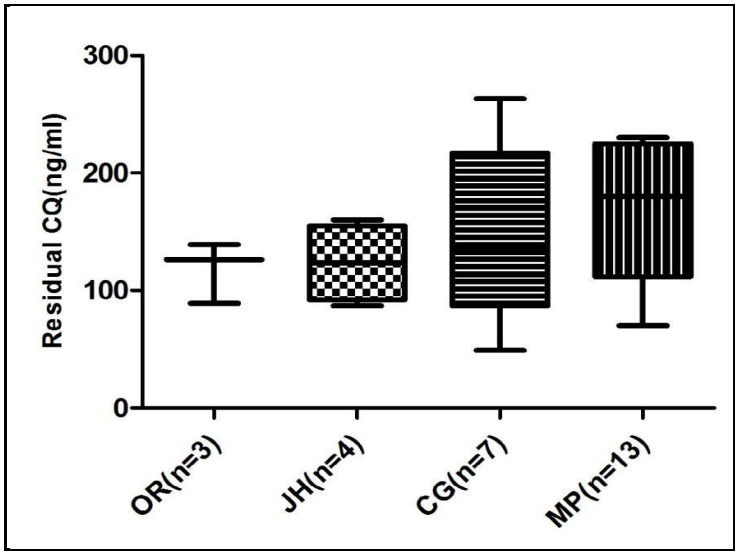

Fig. 3.4: Residual CQ blood concentration present, before the treatment (at day 0) in 27 patients with *P. falciparum* malaria. The number of patients, mean, 25th and 75th percentiles, lower and upper adjacent values, and outer value shows CQ concentration (ng/ml) on a logarithmic value for study sites; for Odisha (OR), Jharkhand (JH), Chhattisgarh (CG) and Madhya Pradesh (MP)

3.3.2 Correlation between residual CQ levels and parasite density

Parasite density in blood smears was compared amongst patients with and without residual CQ levels on day 0. Patients with CQ levels (n=27) on day 0 had asexual parasite density ranging between 1056 to 78240 /µl of blood (mean ±SD: 20394±19735 asexual parasites/µl, p =0.27), while patients who did not show residual CQ levels on day 0 (n=262), the asexual parasite density ranged between 616 to 99290/µl of blood (mean± SD: 23687±25835 asexual parasites/µl). Lower mean of parasite density/µl of blood was observed in patients having residual CQ as compared to those patients

without CQ intake. Radar plot shows the residual CQ levels with density/µl of blood (**Fig. 3.5**).

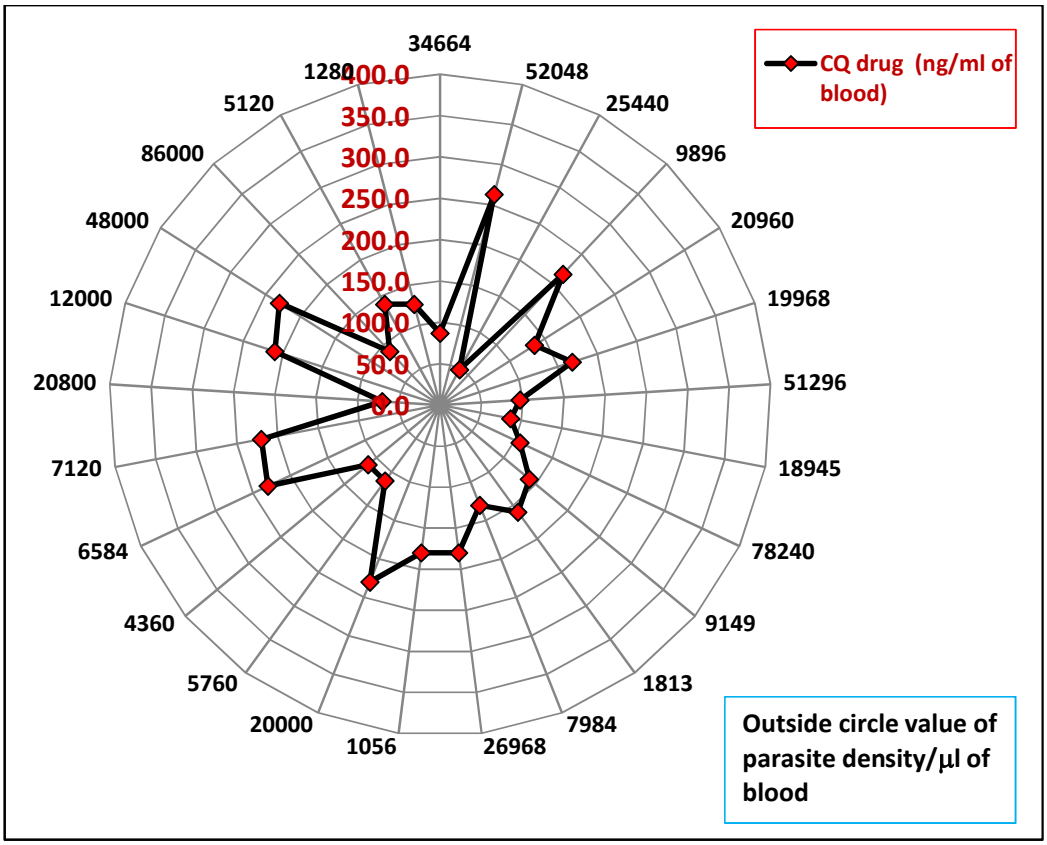

Fig. 3.5: Residual levels of CQ concentration (ng/ml) with corresponding parasite density per microliter of blood on day 0

3.3.3 Prevalence of *pfcrt* K76T mutation in different geographical region

To access the CQ drug resistance pattern, mutation in *pfcrt* gene at codon K76T was analyzed in the samples. A total of 295 samples were subjected for *pfcrt* gene mutation analysis. However, amplification was observed in 276 samples. Out of the total analyzed samples, 76.1% (210/276) were found to be mutant at codon 76T, 5.8% (16/276) samples showed mix type (K76 + 76T) pattern while 18.1% (50/276) samples had wild type pattern (**Fig. 3.6**).

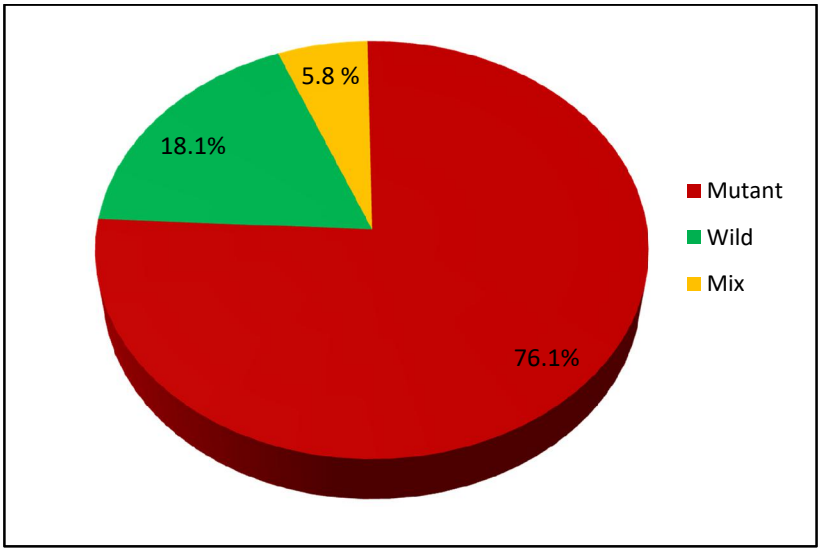

Fig. 3.6: Mutation pattern in *pfcrt* gene at codon K76T

Mutation at codon K76T of *pfcrt* gene was predominant in Madhya Pradesh (98.7%, 76/77) followed by Jharkhand (84.1%, 58/69), Chhattisgarh (81.3%, 52/64) and Odisha (36.4%, 24/66) in the studied sites. Nine samples (13.6%, 9/66) showed mixed type (K76 + 76T) pattern in Odisha, 9.4% (6/64) samples in Chhattisgarh and 1.3% (1/77) in Madhya Pradesh respectively (**Fig. 3.7**).

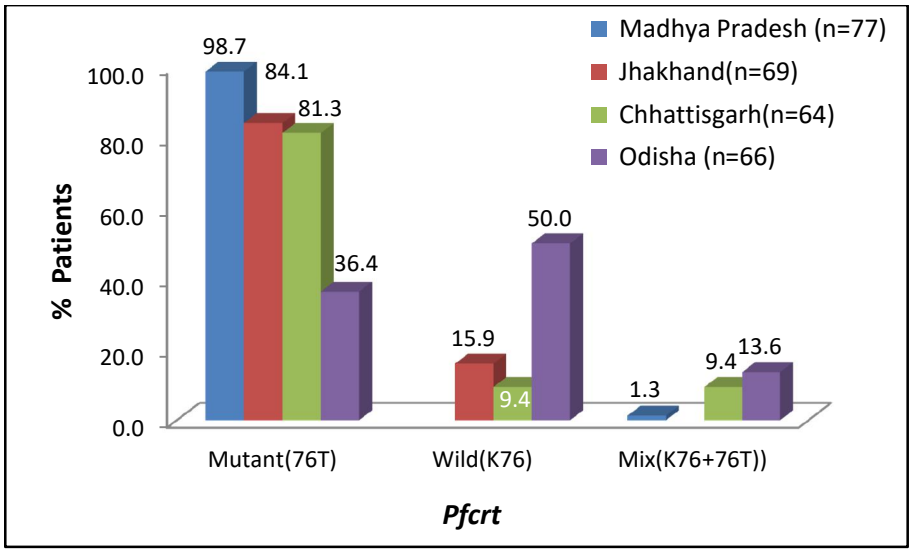

Fig. 3.7: Prevalence of *pfcrt* K76T mutation at study sites

According to NVBDCP 2016 data, the ratio of *P. falciparum: P. vivax cases* study areas in Odisha, Chhattisgarh, Jharkhand and Madhya Pradesh study sites were observed 6.4, 4.5, 1.6 and 1.5, respectively. Prevalence of *pfcrt* K76T mutation was significantly higher, where *P. falciparum* -*P. vivax* ratio is low ((95% CI, *df*=2, p<0.0001) (**Fig. 3.8**).

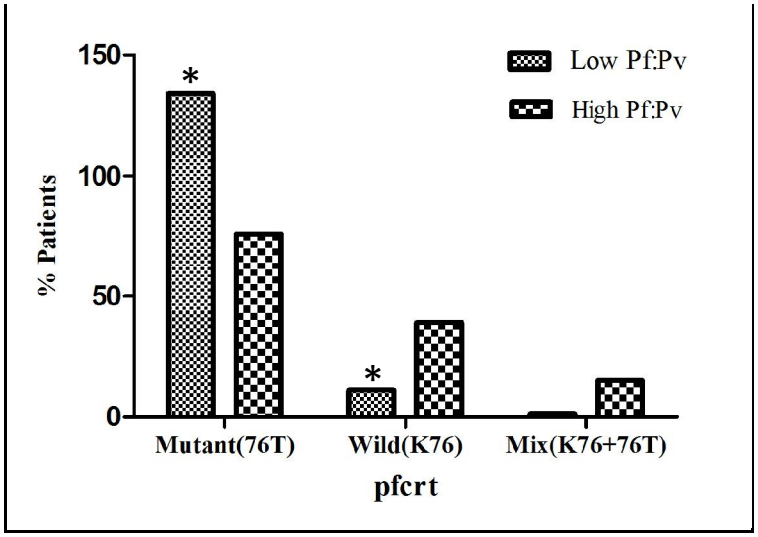

Fig. 3.8: Mutation in pattern in the *pfcrt* K76T mutation in high (Odisha,) and low (Madhya Pradesh) ration of *P. falciparum: P. vivax*. The number of patients' samples in each group is shown in parentheses. The chi-square test was used to compare values between two groups (*, significant, p< 0.05)

Correlation of residual CQ levels and *pfcrt* mutation

The mutation in the *pfcrt* gene at codon76 (Lysine to Tyrosine) was correlated with the residual CQ levels. It was found that patients who had residual CQ levels, showed higher frequency (96%) of mutation in *pfcrt* gene at codon K76T as compared to patients who did not have residual CQ levels (74.6%) (95% CI, *df*=1, p=0.0304) (**Fig. 3.9**). The patients who had residual CQ levels more than minimum inhibitory concentration (MIC) i.e.90ng/ml (n=20) showed 100% mutant genotype in *pfcrt* gene at codon K76T, while those who had residual CQ levels lower than MIC(n=5) showed 80% isolates showed mutant genotype (95% CI, *df*=1, p=0.042) and 20% wild genotype (**Fig. 3.10**).

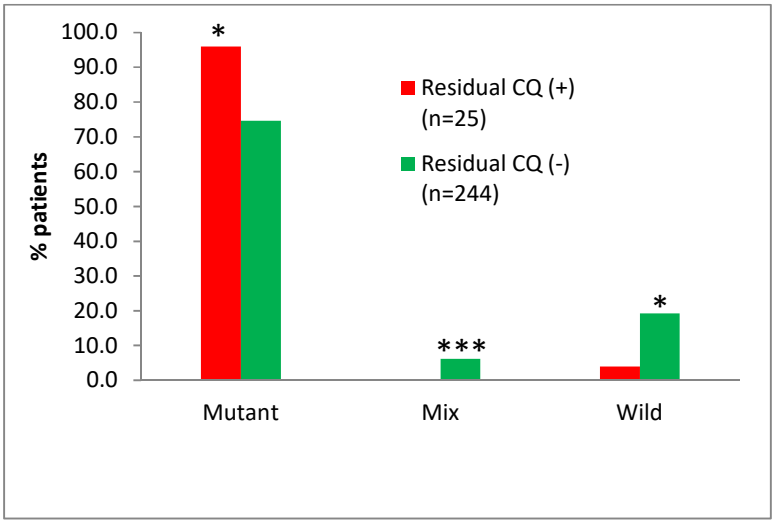

Fig. 3.9: Showing type mutation in *pfcrt* gene with and without residual CQ levels on day. The number of patients' samples in each group is shown in parentheses. The chi-square test was used to compare values between two groups (*, significant $p < 0.05$; *, not applicable).**

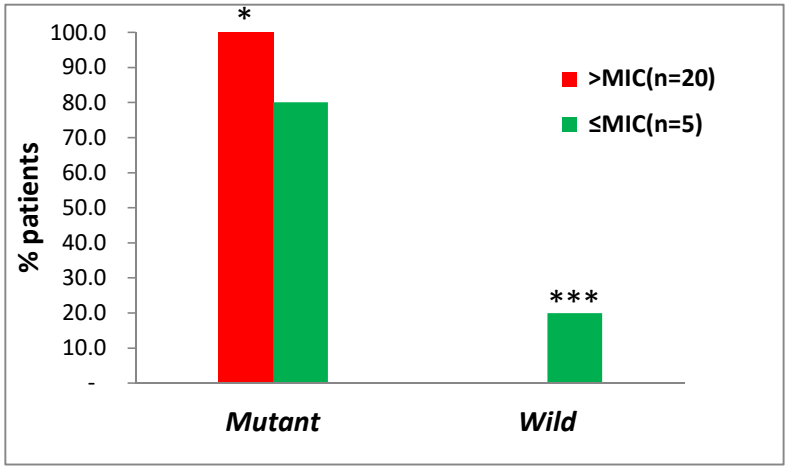

Fig. 3.10: Showing type of mutation in *pfcrt* gene at codon K76T in samples detected with residual CQ levels above and below MIC. The number of patients' samples in both grou is shown in parentheses. The chi-square test was used to compare values between two groups (*, Significant P < 0.05; *, not applicable)**

3.4 Discussion

Chloroquine, a 4-aminoquinoline, was introduced in the 1940s and remained highly efficacious, safe and tolerable drug for years, even in pregnant women. Due to these properties, it was used on a massive scale for treatment of malaria throughout the

endemic countries. Since Chloroquine has long half-life of approximately 60 days, it is exposed to the parasites for an extended time period before it falls to sub-therapeutic concentration, and thus may select for drug-resistant parasites. Chloroquine resistant parasites emerged first along the Thai–Cambodian border in the 1950s, and later this CQ resistant strains spread from Southeast Asia to Africa in the late 1970s.

To the best of our knowledge, this is the first study to investigate the presence of residual CQ levels in the blood of malaria patients from high *P. falciparum* malaria study districts before the onset of treatment in India. Currently, CQ is recommended as first-line antimalarial for uncomplicated *P. vivax* malaria cases in the country (NVBDCP 2016), although, case reports of CQ drug resistance has been documented from few sites in the country. Besides the genetic factors, drug pressure in the community also plays a major role in emergence of drug resistant parasites (Ehrhad *et al.* 2005; Hodel *et al*. 2009). Polymorphisms in the *pfcrt* gene has been associated with chloroquine resistance in the past. To assess the drug pressure, residual CQ levels were monitored by HPLC in the enrolled patients at selected study sites under therapeutic efficacy studies and mutation in *pfcrt gene* at codon K76T were analyzed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method.

Out of 295 enrolled patients, residual CQ levels were monitored in 289 patient's sample. Out of these, 27(9.3%) samples carried blood residual CQ levels above the lower limit of detection (50ng/ml). Residual CQ levels before initiating the antimalarials treatment in patients has also been observed by Hodel *et al* (Hodel *et al.* 2010). Although, CQ has been withdrawn to be used as a first-line drug for the

treatment of uncomplicated *P. falciparum* malaria in the country (Valecha *et al*. 2009), yet the present study showed the presence of residual CQ levels in blood samples of *P. falciparum* infected patients before the onset of treatment. A previous study has reported that CQ is the most common antimalarial sold across the country with high frequency of prescription in public as well as private health facilities as compared to any other antimalarial (Mishra *et al.* 2011). The frequency of residual CQ levels is high in those study sites, where *P. vivax* malaria cases are predominant. The frequency of residual CQ levels was highest (16.5%) in *P. falciparum* malaria patients at Betul study site and the epidemiological data supported the findings as during the study period, 68.6% *P. vivax* malaria cases were reported from Madhya Pradesh (NVBDCP 2012). The free availability of CQ in the public health system, unawareness of the physicians serving in the private clinics and pharmacists in the pharmacy or due to its non-judicious use in the treatment of malaria irrespective of diagnosis, and illiteracy in the population could be the probable reasons for the high intake/prescription in the population. As, the half-life of CQ is longer and bioavailability in patients' blood is more than a month (Hodel *et al.* 2009), there could be previous episode treatment effect, which has resulted in high residual effect in the population. Therefore, the presence of residual CQ levels on day 0, could be attributable to: (1) Self-medication (Hodel *et al.* 2009 & 2010), (2) Irrational treatment practices (Khan *et al.* 2012; Aborah *et al.* 2013), (3) Free access to the miracle drug CQ etc.

The mean parasite density of asexual parasites/µl in the blood was lower in those patients who showed residual CQ levels, while mean parasite density of asexual parasites/µl in the blood was higher in patients who did not had residual CQ levels. This

could be due to the presence of residual CQ levels in blood which subsequently suppressed the new-infections (Hodel *et al.*, 2009).

It has been reported that mutation in *pfcrt* gene at condon K76T is associated with CQ resistance (Fidock *et al.* 2000). To monitor the CQ drug resistance, mutation in *pfcrt gene* was analyzed in the samples obtained on day 0. Genomic DNA was isolated from 295 samples, out of these 295 samples, 276 samples could be amplified and analyzed for mutation in *pfcrt* gene at codon K76T. Out of the total analyzed samples, 210 (76.1%) samples had 76T mutation, 16 (5.8%) samples had mixed genotype (K76 + 76T) while 50 (18.1%) samples showed wild genotype. *pfcrt* K76T mutation was most prevalent in Madhya Pradesh with 98.7% followed by 84.1% in Jharkhand, 81.3% in Chhattisgarh and 36.4% in Odisha. The frequency of K76T mutation in *pfcrt gene* varied according to the pressure of CQ drug in the community and the prevalence of *P. vivax* cases. In India, CQ is being used as a first-line treatment for uncomplicated *P. vivax* malaria. It has been reported that CQ drug pressure could be high in the areas where *P. vivax* malaria is predominant since large number of patients are being treated with CQ. Chloroquine drug pressure could be one of the factors for *pfcrt* gene to get mutated. Chloroquine drug pressure affects the mutation frequency of *pfcrt* gene at codon K76T, it could be due to coexistence of *P. vivax* and *P. falciparum* infections. Exposure of CQ to *P. falciparum* can occur in these areas due to several reasons. Firstly, there could be a situation where the doctors continue to prescribe the antimalarial drug based on clinical symptom due to lack of the facility for immediate and accurate diagnosis of the parasite species in these areas. Secondly, economical issues which can also tempt patients to take CQ for the treatment of *P. facliparum* malaria since it is easily available and cheap. Thirdly, mixed infection of *P. vivax* and

P. falciparum could be treated with chloroquine in the first instance and later with ACT, which could add to the burden of CQ in the community**.**

Present findings are consistent with previous study done by Ehrhardt et al where higher frequency of K76T mutation was observed with previous drug intake of CQ concentrations (Ehrhardt *et al.* 2005).

Our data shows presence of higher frequency of mutation in *pfcrt* at codon K76T with presence of residual CQ levels as compared to those patients without residual CQ levels on day 0. The samples with residual CQ levels more than minimum inhibitory concentration (MIC) showed 100% mutant genotype in *pfcrt* gene at codon K76T. This could be due to presence of sub-therapeutic levels of CQ, which can kill the drug sensitive parasites. The role of mutation in *pfcrt* gene at codon K76T has been associated with decreased CQ accumulation in food vacuole of parasite (Anderson *et al.* 2005). In case of sensitive parasites, the accumulation of CQ occurs in food vacuole; recommended dose is sufficient for the interference of heme detoxification pathway resulting in the production of free radicals, which damage the membrane of parasites food vacuole (Fidock *et al.* 2000).

3.5 Conclusion

The present study highlights the presence of residual chloroquine levels in significant proportion of *P. falciparum* patients, before seeking treatment from public health facilities. The probable reason for the presence of residual CQ levels could be due to self-medication due to low cost and easy availability or due to irrational treatment provided by private clinicians, who do may not possibly be following the national treatment policy or the public health providers who have huge stocks of previous years available with them.

The emergence and spread of resistance are more prone in high transmission intensity areas, where self-medication and presumptive drug use is very common.

Our data also suggests significant association of the frequency of *pfcrt* gene mutation with the residual CQ levels. In our study, increased residual CQ levels more than MIC was highly associated with resistant genotype. Thus, it can be concluded that continuous increase in CQ drug pressure in patients can induce the emergence of drug resistant parasites in the population.

To prevent emergence and spread of drug resistance, there is a need to minimize the window of parasite exposure to sub-therapeutic doses which can be done by selecting appropriate drug combinations. In addition, it is the need of the hour that appropriate guidelines and policies are made available to the treating physicians for updated information and awareness along with programmes to make aware the community population which would help in reducing the problem of self-medication.

Chapter IV

Correlation of residual sulphadoxine and pyrimethamine levels with molecular markers of sulphadoxine-pyrimethamine resistance

4.1 Introduction

The emergence of resistance towards chloroquine, the recommended first-line drug in the country, led to policy makers to recommend antimalarials targeting parasite folate biosynthetic pathway, which were deployed as the second-line treatment for uncomplicated *Plasmodium falciparum* malaria in the country (Muller *et al*. 2013). The most commonly used antifolate drug is sulphadoxine-pyrimethamine (SP). Sulphadoxine is analogous to p-amino benzoic acid and competitively inhibits the dihydropteroate synthase (DHPS) enzyme. Pyrimethamine has been inhibit the dihydrofolate reductase (DHFR), a key enzyme involved in the reduction of dihydrofolate to tetrahydrofolate (Muller *et al.* 2013). The rapid development of resistant parasite against SP drug, this is mediated by the sequential acquisition of specific point mutations in the *Plasmodium falciparum dihydrofolate reductase* (*pfdhfr)* and *Plasmodium falciparum dihydropteroate synthase (pfdhps)* gene (Sibley *et al*. 2003). Specific point mutations in *pfdhps* & *pfdhfr* genes encoding these enzymes (DHFR & DHPS) lead to a lower binding affinity for the sulphadoxine-pyrimethamine drug (Costanzo *et al*. 2011). Resistance to sulphadoxine and pyrimethamine is confirmed by the presence of mutations in *pfdhps* and *pfdhfr* genes respectively (Ahmed *et al*. 2004). *In vitro* study has revealed that subsequent addition of single, double or triple mutations in *pfdhfr* and *pfdhps* gene progressively enhance resistance to many folds (Das *et al*. 2012). *Plasmodium falciparum*has developed resistance to SP globally as well as in India after its extensive use (Pinichpongse *et al*. 1982; Ejov *et al.* 1999; Biswas *et al.* 2000; Ahmed *et al.* 2004). In India, high frequency of double mutant (S108N+C59R) has been reported (Lumb *et al.* 2011, Das *et al.* 2013). It has been reported that point mutations in the *pfdhps* gene at codon

S436A, A437G and K540E was associated with sulphadoxine resistance (Ahmed *et al*. 2004; Mishra *et al*. 2014).

To combat the drug resistance; WHO has recommended the use of artemisinin based combination therapy (ACT) for the treatment of uncomplicated *falciparum* cases (WHO 2005). Following WHO guidelines, India has introduced artesunate plus sulphadoxine–pyrimethamine (AS+SP) combination therapy (NVBDCP 2007). Initially, it was implemented in selected parts of the country but in 2010, its use was extended across the country (NVBDCP 2011). High treatment failure rates of AS+SP regimen in Northeast region of India during 2012 was mainly due to a higher frequency of mutations in *pfdhp*s and *pfdhfr* genes (Mishra *et al*. 2014). In 2013, Artemether Lumefantrine (AL) has been introduced as a first-line drug for the treatment of uncomplicated *P. falciparum* malaria case in Northeast region of India (NVBDCP 2013).

Several factors are responsible for the rapid emergence of resistance against SP after its introduction. These include factors like long bioavailability in the blood stream, irrational treatment practices, self-medication, mass drug administration, use of sub-standard and counterfeit antimalarials, etc. (Source *et al.* 2009; Hodel *et al.* 2010; Khan *et al.* 2012). These factors increase the drug pressure in the community and promote the spread of drug resistance. Keeping the above points in mind, the following objective was chosen.

- Correlation of residual sulphadoxine and pyrimethamine levels with molecular markers of sulphadoxine-pyrimethamine resistance.

To confirm this correlation between residual antimalarials and molecular markers of sulphadoxine-pyrimethamine resistance, mutation pattern in *pfdhfr-pdhps* genes were analysed in the samples collected from patients residing in malaria endemic areas of India. The data generated on residual levels of sulphadoxine-pyrimethamine, parasite

density and mutation in *pfdhfr-pfdhps* genes at codon N51I, C59R, S108N, I164L, S436A, A437G, K540E, A581G and A613T was correlated with residual CQ level. In Indian scenario, till date, no data has been published on the correlation of residual levels of SP and its effect on mutation in *pfdhfr and pfdhps*.

4.2 Materials and Methods

4.2.1 Study site, patient enrollment and sample collection

The study was carried out during the year 2011-2013 at Gaurella Community Health Centre (CHC) Bilaspur district in Chhattisgarh, Ghodadongri Primary Health Centre (PHC) Betul district in Madhya Pradesh, Kurdeg CHC Simdega district in Jharkhand and Bisra CHC) Sundergarh district in Odisha.

Patients screening was done according to standard WHO protocol with due consideration to inclusion/exclusion criteria, and those patients who fulfilled inclusion criteria were enrolled in the study. Informed consent was obtained from each patient and assent was obtained in case of children with legal guardian. The Case Record Form (CRF) having details about each patient on demographic information such as body temperature, body weight, sex, age was recorded. Prior to drug administration, finger prick blood was used to prepare blood smear for counting parasite density on day 0 and each follow visits up to 28 days. Three hanging blood drops were collected on 3CHR Whatman filter (Whatman GE Health Care, UK) on day 0, for molecular markers and a hundred microliters (100 µl) of heparinised blood was taken on 31ET filter papers (Whatman GE Health Care, UK) for monitoring residual drug levels on day 0. The filter papers were then allowed to dry at room temperature and kept in a zipped pouch with desiccant and stored at 4°C in a refrigerator until analysis. The patients were treated according to the national drug policy (NVBDCP 2010).

4.2.2 Quantification of antimalarial by High Performance Liquid Chromatography

Hundred microliter blood was applied onto 31ET Whatman filter paper and dried at room temperature. Dried blood spots were stored at 4ºC in Zipped pouch with desiccators for further analysis. Baseline blood samples (day 0) collected from patients reporting no prior intake of antimalarial were screened for the presence of five antimalarial drugs viz CQ, sulphadoxine (SDX), pyrimethamine (PYR), quinine (QN) and mefloquine (MQ) using a modified HPLC method (Blessborn *et al*. 2010). CQ and QN were dissolved in 0.1 M hydrochloric Acid (HCl), PRY and MQ were dissolved in methanol: HCl (0.1 M) 50:50 (v/v) and SDX was dissolved in 0.1 M NaOH. Stock solutions of all the above drugs were prepared at 1 mg/ml concentration except SDX (5 mg/ ml). Stock solutions were stored at 4ºC until further use. Whole blood (drug free blood) was mixed with standards of antimalarials (CQ, QN, PYR & MQ) prepared in different concentration ranges (125, 250,500, 1000, 2000, 4000 and 8000 ng/ml), whereas the standard for SDX was prepared in the range of 625-4,00,000 ng/ml viz. 625, 1250, 2500, 5000, 10000, 20000 & 400000 ng/ml). The samples were further processed using the multi-mode solid phase extraction column (M-M SPE, Biotage, USA) and samples were eluted with buffer containing methanol:triethylamine (97:3 v/v) mixture. Elute was collected in glass tube and evaporated under a gentle stream of nitrogen gas at 70ºC. Twenty microliter (20µl) of extracted standards and patients sample was injected into the HPLC system. HPLC was performed on a Hitachi gradient system equipped with binary pump (Model L-2100/2130) and multi wavelength UV detector (Model L-2420 UV-VIS). Extracted drugs were analysed using two different mobile phases (A) acetonitrile:ammonium formate (20 mM in 1% formic acid) (5:95 v/v) and (B) acetonitrile:ammonium formate (10 mM in 1% formic acid) (80:20 v/v) and run according to previously described gradient program (Blessborn *et al*., 2010). Antimalarial were analysed on a Tosoh ® 5 µm C_{18} (150 mm × 2 mm) column

protected by a pre-column security guard C_8 (8mm x2 mm) (Tosoh Bioscience, PA). The UV detector was monitored at 280nm. Data acquisition and quantification was performed using Hystar™ and Data Analysis™ software (Bruker, Bremen, Germany).

4.2.3 Mutation analysis in *pfdhfr* and *pfdhps gene*

4.2.3.1 Genomic DNA isolation

Genomic DNA was extracted from clinical samples by using QIAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol with slight modification. The *pfdhfr* and *pfdhps* gene products were amplified using earlier reported method (Duaraising *et al*. 1998) and the PCR products were digested with restriction enzymes to detect the point mutations in *pfdhfr* (codon 51, 59, 108 and 164) and *pfdhps* (436, 437, 540, 581 and 613) genes.

4.2.3.2 Mutation in *pfdhfr* gene

Mutation analysis in *the pfdhfr* gene was done using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) approach. Primary PCR was done using previously reported primers (Duarai Singh *et al*. 1998). In brief, 20 µl PCR reaction mixture contains 10 pmol of forward and reverse (Eurofins, Bangalore) primers, 0.25 mM of each dNTPs (Genei, Banglore), 1X PCR buffer (Life Technology Inc.), 2.5mM $MgCl_2$ (Life Technology Inc.) 1unit of AmpliTaq Gold DNA polymerase (Life Technology Inc.) and 2µl genomic DNA **(Table 4.1)**.

Primary PCR product was diluted in a ration of 1:50 with distilled water and 2µl of diluted PCR product was used as template DNA for nested PCR. In brief, 20 µl PCR reaction mixture contains 10 pmol of forward and reverse primers (Eurofins, Bangalore), 0.25 mM of each dNTPs (Genei, Banglore), 1 X PCR buffer (Life Technology Inc.), 2.5mM $MgCl_2$ (Life Technology Inc.) 1 units AmpliTaq Gold DNA polymerase (Life Technology Inc).

Table 4.1: Details of primers sequence and cycling conditions for *pfdhfr* gene

PCR reaction	**Primer sequence**	**Product size (bp)**	**Thermal profile**	***Pfdhfr* gene**			
				N51I	**C59R**	**S108N**	**I164L**
pfdhfr primary	5'-TTTATGATGGAACAAGTCTGC-3' 5' -AATTGTGTGATTTGTCCACAA-3'	648	95^0C for 10 min; 94^0C for 30 s, 57^0C for 30s,72^0 C for 45sec × 40 cycles; 72^0 C for 5 min	***MluCI***	***XmnI***	***BsrI***	***DraI***
pfdhfr nested-I	5'-TTTATGATGGAACAAGaCTGgGACGTT-3' 5'-AAATTCTTGATAAACAACGGAACCTttTA-3'	522	95^0C for 10 min; 94^0C for 30 s, 55^0C for 30 s,72^0 C for 1 min × 40 cycles; 72^0 C for 5 min	Wild 154bp+120bp		Wild 522 bp	Wild 245bp+ 171 bp+107bp
				Mutant 218bp+120bp		Mutant 332bp+190 bp	Mutant 245bp+143bp+ 107bp
pfdhfr nested-II	5'-GAAATGTAATTCCCTAGATATGgAATATT-3' 5'-TTAATTTCCCAAGTAAAACTATTAGAgCTTC-3'	326	95^0C for 10 min; 95^0C for 30 s, 55^0C for 30 s,72^0 C for 45s × 40 cycles; 72^0 C for 5 min		Wild 37+189		
					Mutant 137+163		

Amplified nested I and nested II PCR products were observed in gel documentation system. Nested I PCR product was digested with *Tsp*509I, *Bsr*I and *Dra*I restriction enzymes for the analysis of mutation at codon N51I, S108N and I164L. Nested II PCR product was digested with *Xmn*I for analysis of mutation at codon C59R Digested PCR product (5-8 µl) was run on 1.5 % agarose gel containing ethidium bromide (0.5µg/ml) and 0.5X TBE running buffer (pH 8.0) and visualised under UV transilluminator and digitally captured the image with the help of gel documentation system (Alpha Imager EP, USA) (**Table 4.1**).

4.2.3.3 Mutation in *pfdhps* gene

The mutation analysis in *the pfdhps* gene was done using PCR-RFLP approach .In brief, 20 µl PCR reaction mixture contains 10 pmol of forward and reverse)Eurofins, Bangalore (0.25 mM of each dNTP)Genei, Banglore(, 1X PCR buffer)Life Technology Inc(., 2.5mM $MgCl_2$) Life Technology Inc (.1units AmpliTaq Gold DNA polymerase)Life Technology Inc (.and 2µl genomic DNA and 2µl genomic DNA (**Table 4.2**).

Primary PCR product was diluted 1:50 with distilled water and 2µl of diluted PCR product was used as template DNA for nested PCR. In brief, 20 µl PCR reaction mixture contains 10 pmol of forward and reverse (Eurofins, Bangalore) 0.25 mM of each dNTPs (Genei, Banglore), 1X PCR buffer (Life Technology Inc.), 2.5mM $MgCl_2$ (Life Technology Inc.) 1units AmpliTaq Gold DNA polymerase (Life Technology Inc.) (**Table 4.2**).

Table 4.2: Details of primers sequence and cycling conditions for *pfdhps* gene

PCR reaction	**Primer sequence**	**Product size (bp)**	**PCR cycling condition**	***Phdhps gene***				
pfdhps primary	5' -AACCTAAACGTGCTGTTCAA-3' 5' -AATTGTGTGATTTGTCCACAA-3'	710	95^0C for 10 min; 94^0C for 30 s, 56^0C for 30 s,72^0 C for 45 sec × 40 cycles; 72^0 C for 5 min	**S436A**	**A437G**	**K540E**	**A581G**	**A613T**
				MnlI	***MwoI***	***FokI***	***BstU1***	***BsaWI***
pfdhps nested -I	5'-TGCTAGTGTTATAGATATAGGatGAGcATC-3' 5'-CTATAACGAGGTATTgCATTTAATgCAAGAA-3'	438	95^0C for 10 min; 95^0C for 30 s, 45^0C for 30 s,72^0 C for 45s × 40 cycles; 72^0 C for 5 min	Wild 278bp+212bp+ 39bp	Wild 387bp+32bp+ 19bp	Wild 405bp+33bp		
				Mutant 317bp+121bp	Mutant 419bp+19bp	Mutant 320bp+85bp+ 33bp		
pfdhps nested -II	5'-ATAGGATACTATTTGATATTGGAccAGGATTcG-3 ' 5'-TATTACAACATTTTGATCATTCgcGCAAccGG-3'	161	95^0C for 10 min; 95^0C for 30 s, 45^0C for 30 s,72^0 C for 45s × 40 cycles; 72^0 C for 5 min				Wild 105bp+33bp+ 23bp	Wild 161bp
							Mutant- 138bp+23bp	Mutant 131bp+30bp

Amplified PCR product in nested I and nested II was observed in gel documentation system. Nested I PCR product was digested with *Mnl* I, *Mwo* I and *Fok* I restriction enzymes for mutation analysis at codon S436A, A437G and K540E respectively Nested II PCR product was digested with *BstU* I and *Basw* I restriction enzymes for mutation analysis at codon A581G and A613T, respectively. Digested PCR product (5-8 µl) was analysed on 1.5 % agarose gel containing ethidium bromide (0.5µg/ml) and 0.5X TBE running buffer (pH 8.0) and visualized under UV transilluminator (280 nm) gel documentation system **(Table 4.2)**.

4.2.4 Statistical analysis

Statistical analysis of data was performed using the Statistical Package for the Social Sciences (SPSS) software version 14 (Geometric mean of parasite densities at 95% confidence interval (CI) was calculated. Frequencies were compared using the X^2 test. The differences were considered statistically significant at an error probability less than 0.05 ($p<0.05$). Preparation of graphs, data entry and analysis was also done using Microsoft Office Excel 2007 and Graph Pad Prism version 5.03.

4.3 Results

4.3.1 Residual SDX levels

Out of 295 collected samples, 289 samples could be analysed for the monitoring of the residual levels of SDX on day 0. A total of 49 (17%) patients had residual SDX levels above the detection limit (50 ng/ml) in the blood. The incidence of residual SDX levels was observed in higher frequency in Madhya Pradesh (20.3%), followed by Jharkhand (19.7%), Chhattisgarh (17.6%), and Odisha (9.3%) (**Fig. 4.1**). The median of residual SDX levels observed was 16302 ng/ml (265-44600 ng/ml) in Chhattisgarh, 4440 ng/ml

(100- 54100 ng/ml) in Madhya Pradesh, 655 ng/ml (200-2250 ng/ml) in Jharkhand and 3670 ng/ml (360-10310 ng/ml) in Odisha (**Fig. 4.2**). Pyrimethamine was observed only in five malaria patients with median of 980 ng/ml (100-1600 ng/ml) concentration in blood.

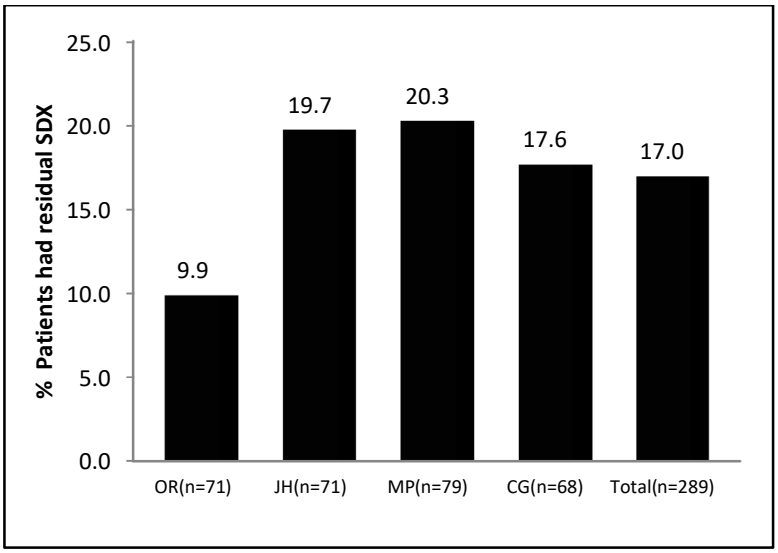

Fig. 4.1: Histogram showing frequency of residual SDX in patients blood on day 0 at study sites; Odisha (OR), Jharkhand (JH), Chhattisgarh (CG) and Madhya Pradesh (MP).

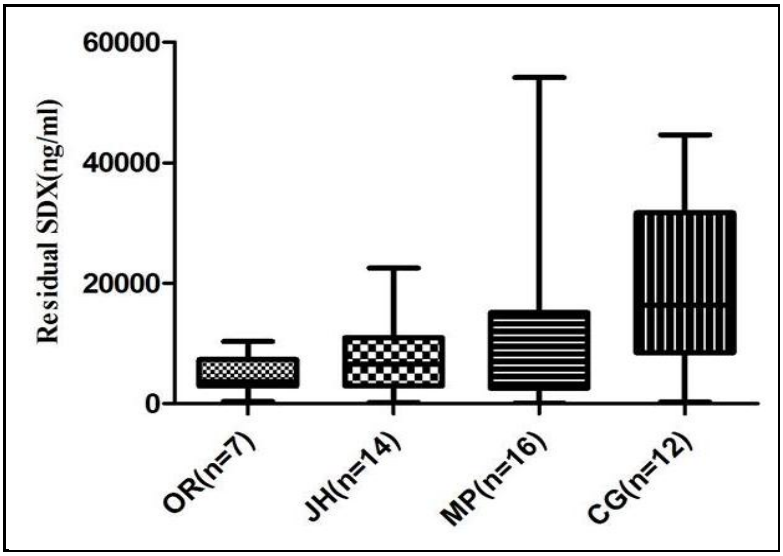

Fig. 4.2: Residual SDX blood concentration present, before the treatment (at day 0) in 49 patients with *P. falciparum* malaria. The number of patients, mean, 25th and 75th percentiles, lower and upper adjacent values, and outer value show SDX concentration (ng/ml) on a logarithmic value at all the for study sites.

4.3.2 Correlation between residual SDX levels and parasite density

Parasite densities in *P. falciparum* infected patients (n=289) ranged between 616-99200 asexual parasites/µl (mean-23627 asexual parasites/µl ± SD: 25584). The mean parasite densities were significantly different between the patients with (n=49) and without (n=240) residual SDX concentrations on day 0 (mean ± SD: mean ±SD: 16733±18077 Vs 25023±26665 asexual parasites/µl, p=0.042). The patients who had residual SDX levels showed lower mean parasite density when compared to those who were not detected with residual SDX levels. There was weak negative correlation observed between parasite densities and blood residual SDX levels on day 0 which was non-significant (95%CI, -0.525 to 0.246); r=-0.084,p=0.573) (**Fig. 4.3**).

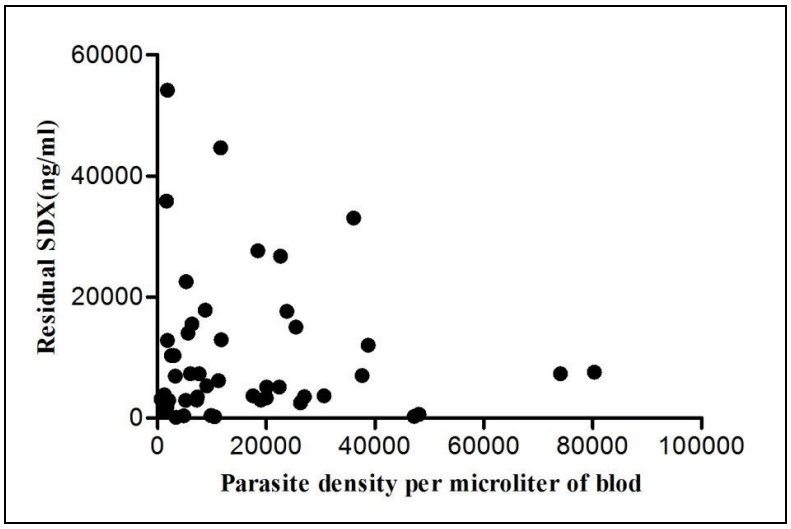

Fig. 4.3: Association between residual levels of blood SDX concentration (ng/ml) and parasites density in blood on day 0

4.3.3 Molecular markers of drug resistance

4.3.3.3.1 Mutation in *pfdhfr* gene

To access pyrimethamine drug resistance pattern, mutation pattern in *pfdhfr* gene was analysed in the samples collected from four study sites. A total of 295 samples were

subjected for mutation analysis. Out of which, 288 samples were successfully amplified and mutation were analysed after restriction digestion (**Fig. 4.4 to 4.9**). The results showed that 2.4% (7/288) samples had triple mutation (51+59+108 or 59+108+164), 71.9% (207/288) samples had double mutation (59+108), 9%(26/288) samples had single (59 or 108) and 16.7% (48/288) samples showed wild type pattern (**Fig. 4.10**).

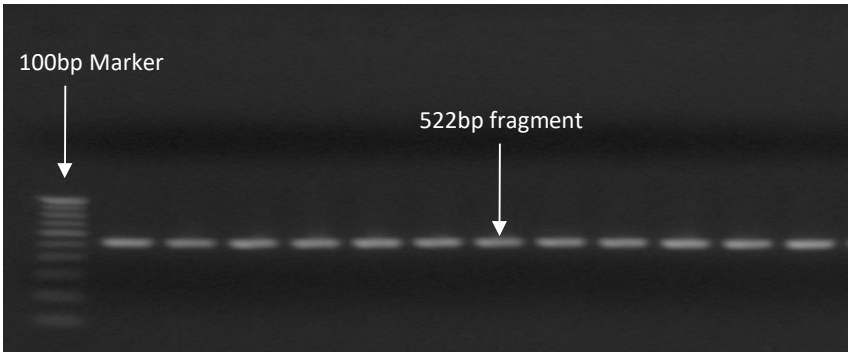

Fig. 4 .4: Confirmatory gel image for nested I PCR of *pfdhfr*

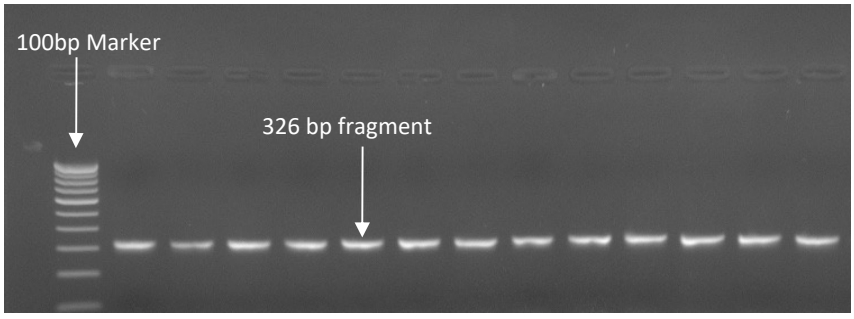

Fig. 4.5: Confirmatory gel image for nested II PCR of *pfdhfr*

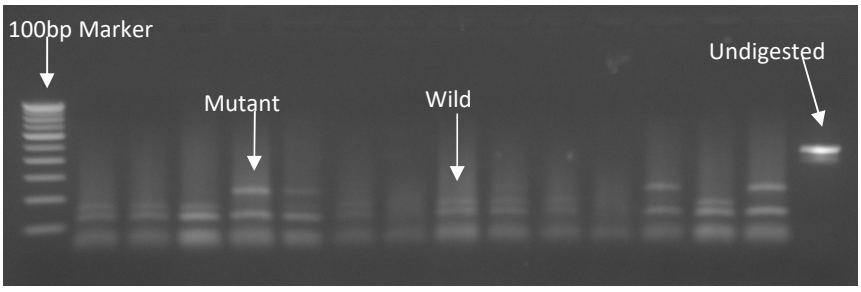

Fig. 4.6: Gel image after restriction digestion of nested I PCR product with *Tsp509*I restriction enzyme for codon N51I

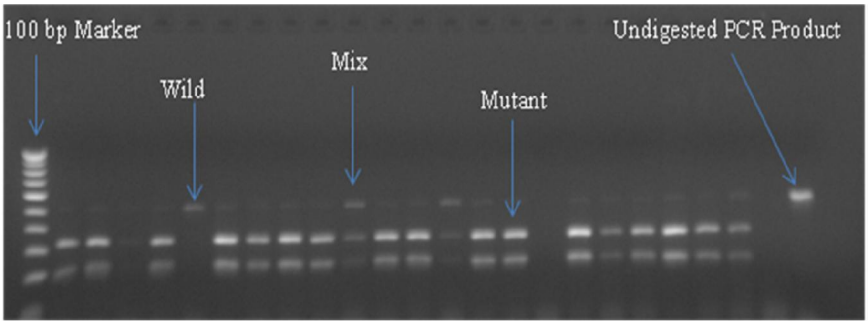

Fig. 4.7: Gel image after restriction digestion of nested I PCR product with *Bsr* I restriction enzyme for codon S108N

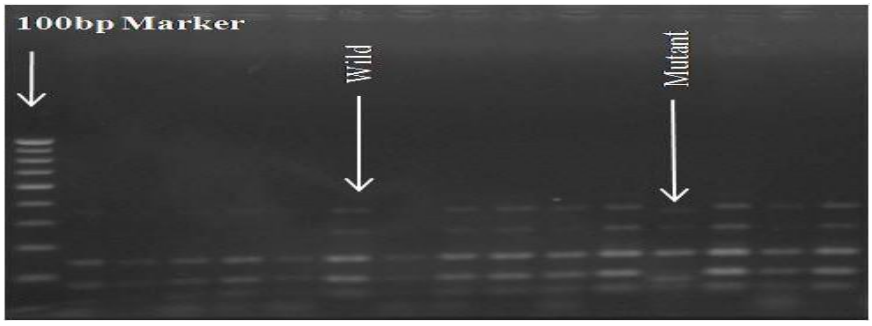

Fig. 4.8: Gel image after restriction digestion of nested I PCR product with *Dra* I restriction enzyme for codon I164L

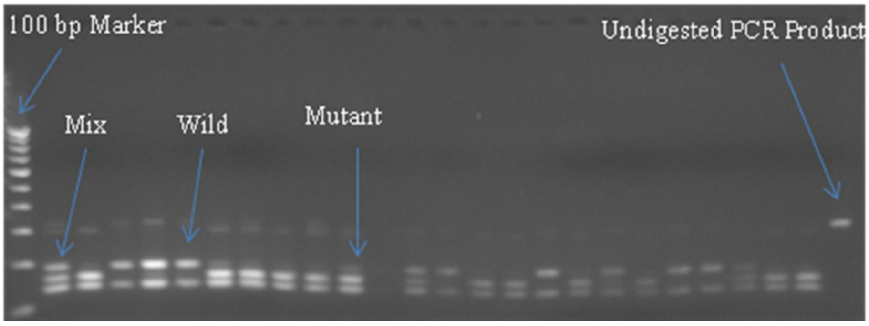

Fig. 4.9: Gel image after restriction digestion of nested II PCR product with *Xmn*I restriction enzyme for codon C59R.

Frequency of double mutation (59+108) was highest in Madhya Pradesh (97.5%) followed by Chhattisgarh (75.4%), Jharkhand (64.8%) and Odisha (46.5%). A single mutation was observed in varying frequencies of 18.3%, 12.7%, 3% and 2.5% in Odisha, Jharkhand, Chhattisgarh and Madhya Pradesh samples, respectively. The triple

mutation was observed in 9.1% of samples collected from Chhattisgarh and in 1.4% of Jharkhand samples (**Fig. 4.10**).

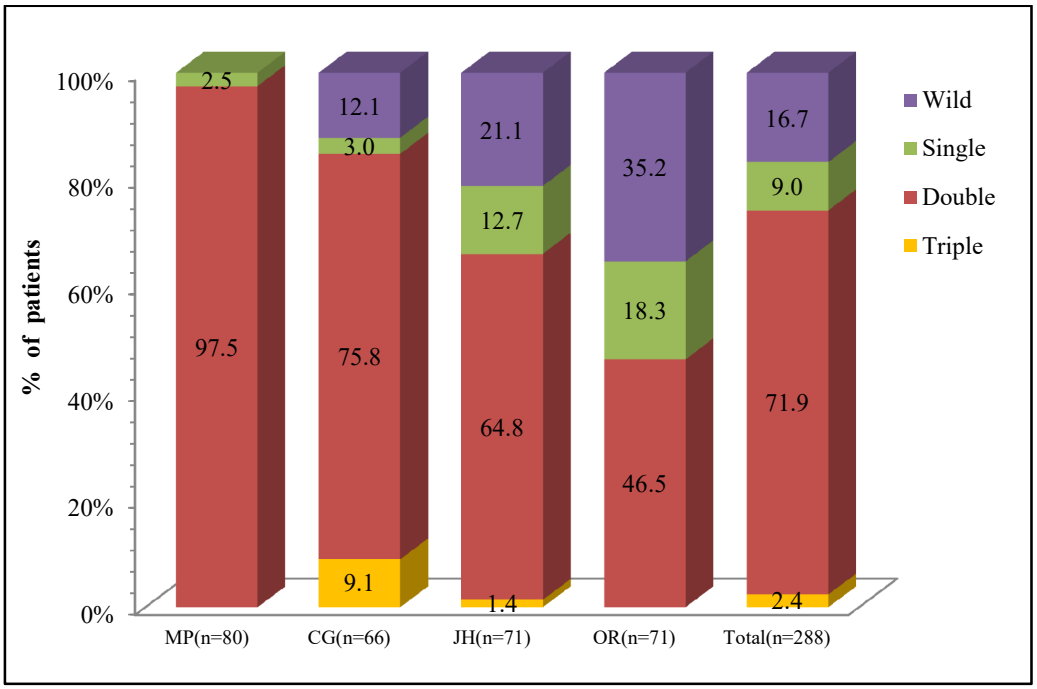

Fig. 4.10: Histogram showing types of mutation in *pfdhfr* gene in samples collected from Madhya Pradesh, Chhattisgarh, Jharkhand and Odisha study sites

4.3.3.2 Mutation in *pfdhps* gene

To monitor the sulphadoxine drug resistance pattern, mutations in *pfdhps* gene were analysed in the samples collected from four study sites. A total the 295 samples were subjected to mutation analysis. Out of these, 288 samples were successfully amplified for pfdhps and mutation were analysed after restriction digestion (**Fig. 4.11 to 4.17**). The results revealed that 26% (75/288) samples had triple mutation (436+437+540), 11.8% (34/288) samples had double mutation (436+437 or 436+540 or 437+540), 11.1% (32/288) samples had single (436 or 437 or 540) and 51% (147/288) samples showed wild type pattern (**Fig. 4.18**). Triple (436+437+540) mutation in the *pfdhps* gene was observed in the frequency of 52.1%, 31.3%, 16.9% and 1.5% in the isolates collected

from Jharkhand, Madhya Pradesh, Odisha and Chhattisgarh, respectively. However, the frequency of double mutation was highest in Madhya Pradesh (30.0%) as compared to Chhattisgarh (7.6%), Odisha (4.2%) and Jharkhand (2.8%) isolates, respectively. A single mutation in *pfdhps* was found in 18.3%, 16.7%, 7.5% and 2.8% of the samples from Jharkhand, Chhattisgarh, Madhya Pradesh and Odisha, respectively (**Fig. 4.18**).

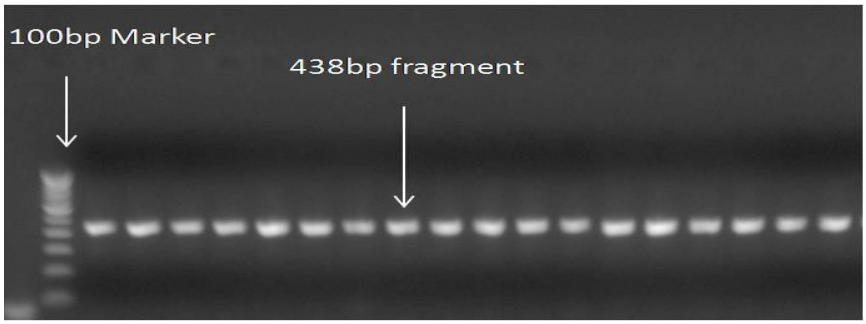

Fig. 4.11: Confirmatory gel image for nested I PCR of *pfdhps*

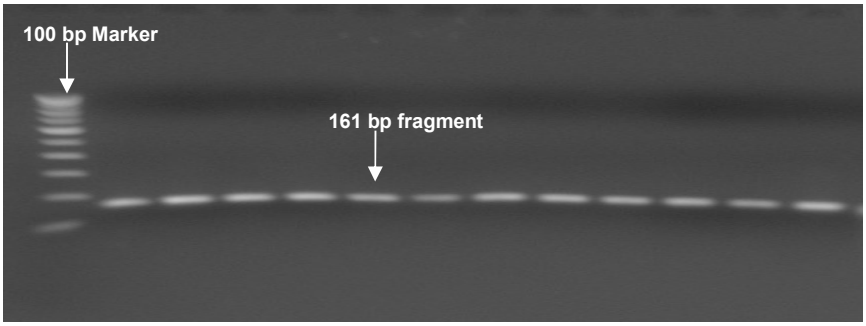

Fig. 4.12: Confirmatory gel image for nested II PCR of *pfdhps*

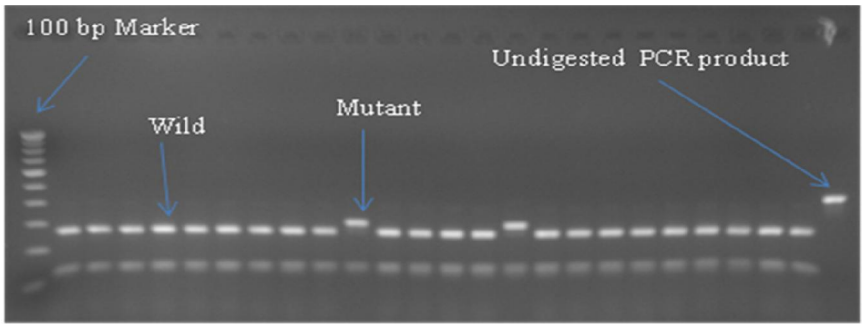

Fig. 4.13: Gel image after restriction digestion of nested I PCR product with *Mnl* I restriction enzyme for codon S436A

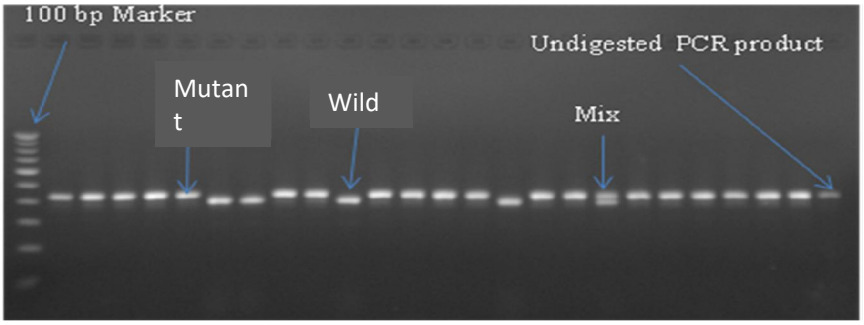

Fig. 4.14: Gel image after restriction digestion of nested I PCR product with *Mwo*I restriction enzyme for codon A437G

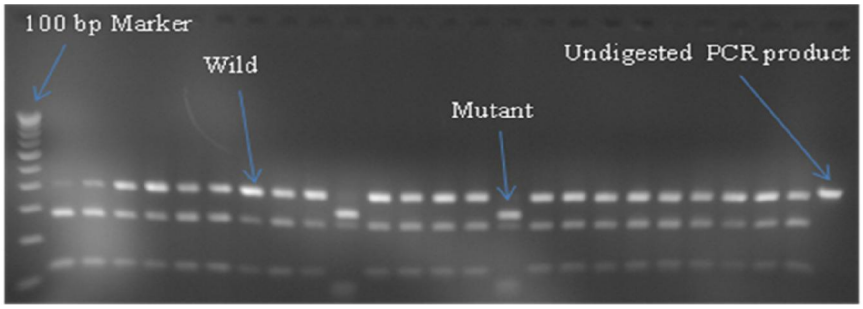

Fig. 4.15: Gel image after restriction digestion of nested I PCR product with *Fok* I restriction enzyme for codon K540E

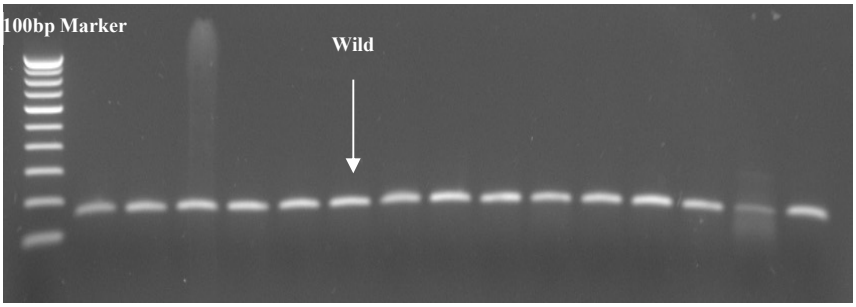

Fig. 4.16: Gel image after restriction digestion of nested I PCR product with *BstU* I restriction enzyme for codon A581G

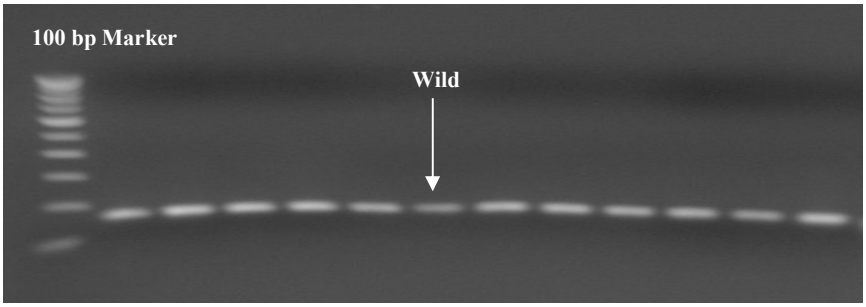

Fig. 4.17: Gel image after restriction digestion of nested I PCR product with *Basw*I restriction enzyme for codon A613T

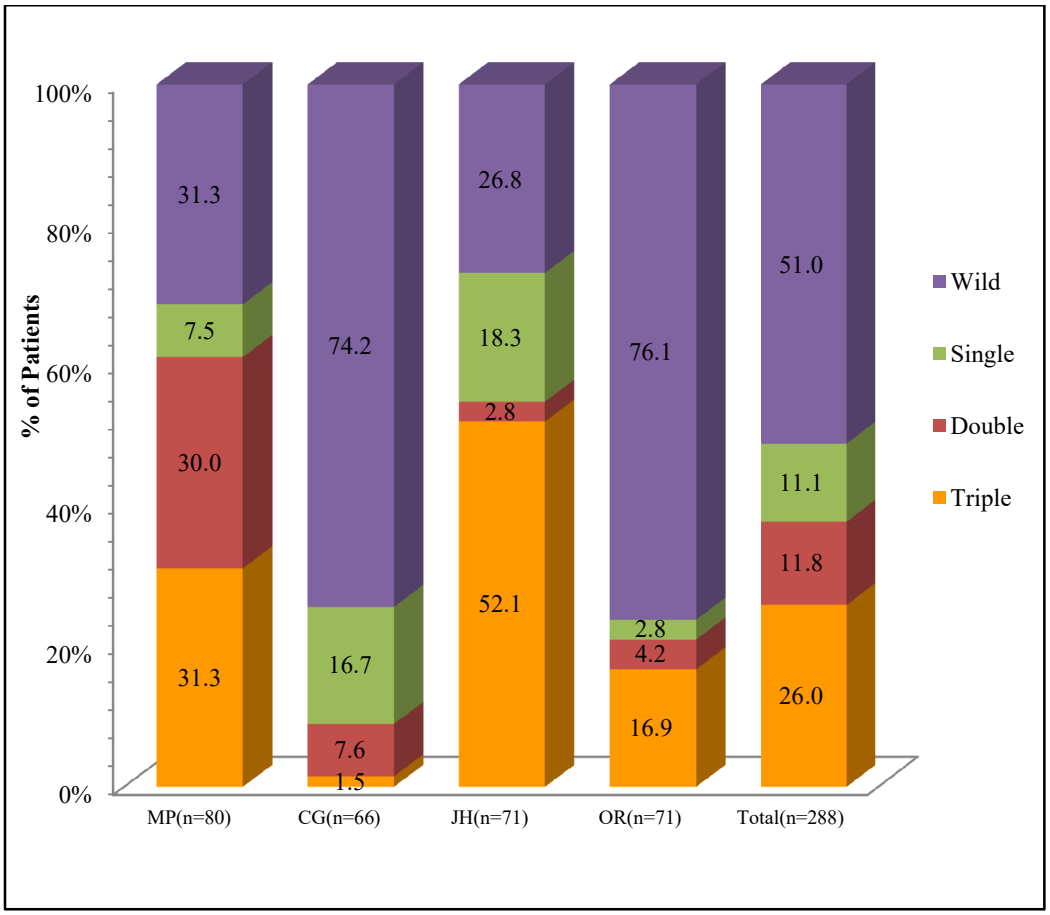

Fig. 4.18: Histogram showing mutation pattern in the *pfdhps* gene in samples collected from Madhya Pradesh, Chhattisgarh, Jharkhand and Odisha study sites

4.3.3.3 Codon wise mutation in *pfdhfr-pfdhps* gene in different geographical regions

4.3.3.3.1 Chhattisgarh

Mutation in *the pfdhfr* gene at codon 108 and 59 was predominant (86.4%). For codon 164, 9.1% samples showed mutant genotype. No mutation was observed at codon 51. In the *pfdhps* gene, namely at codon 436, 437 and 540, mutations were observed in the frequency of 3%, 13.6% and 19.7% of samples, respectively. However, no mutation was observed at codon 581 and 613 of *pfdhps* gene (**Fig. 4.19**).

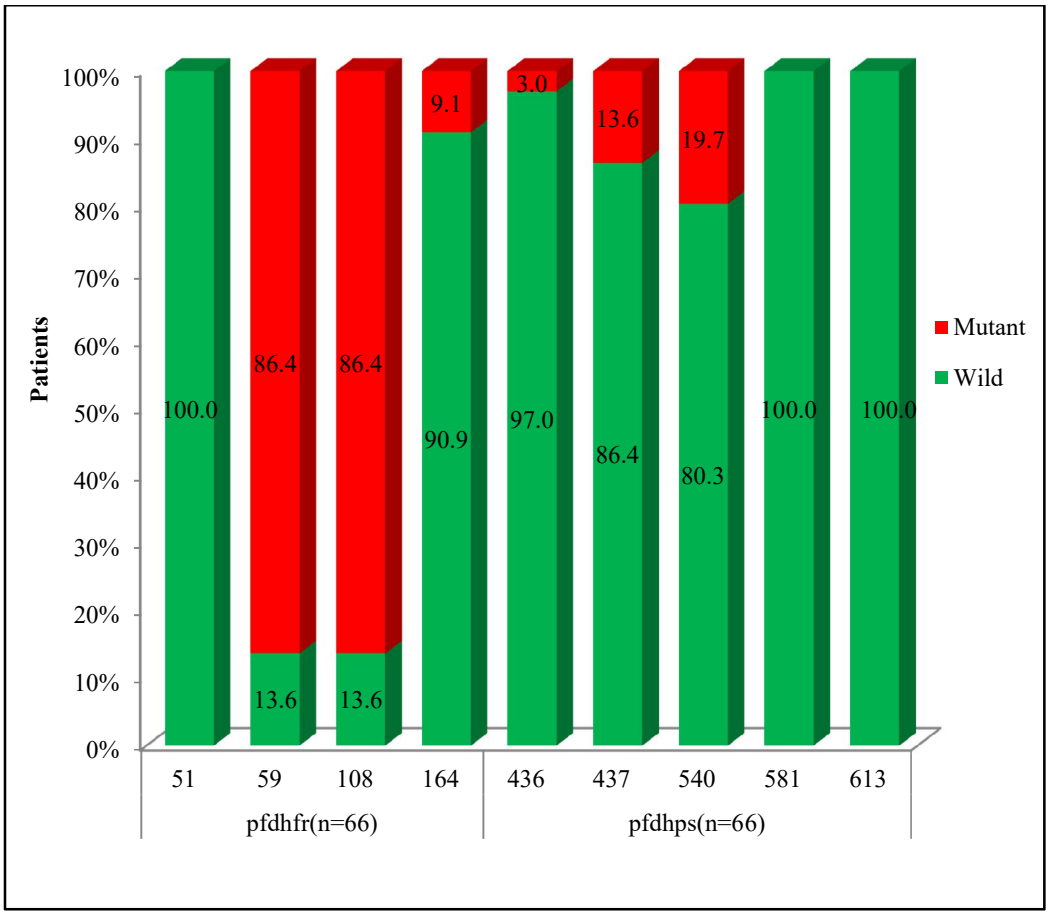

Fig. 4.19: Histogram showing codon wise mutation in *pfdhfr* and *pfdhps* genes

4.3.3.3.2 Madhya Pradesh

Mutation in *the pfdhfr* gene at codon 108N was observed in all samples (100%) followed by codon 59 (97.5%) mutation. All the samples were wild type for codon 51 and 164 (100%). In *the pfdhps* gene, namely at codon 436, 437 and 540, mutations were observed in the frequency of 43.3%, 50% and 57.5% of samples, respectively. However, no mutation was observed at codon 581 and 613 (**Fig. 4.20**).

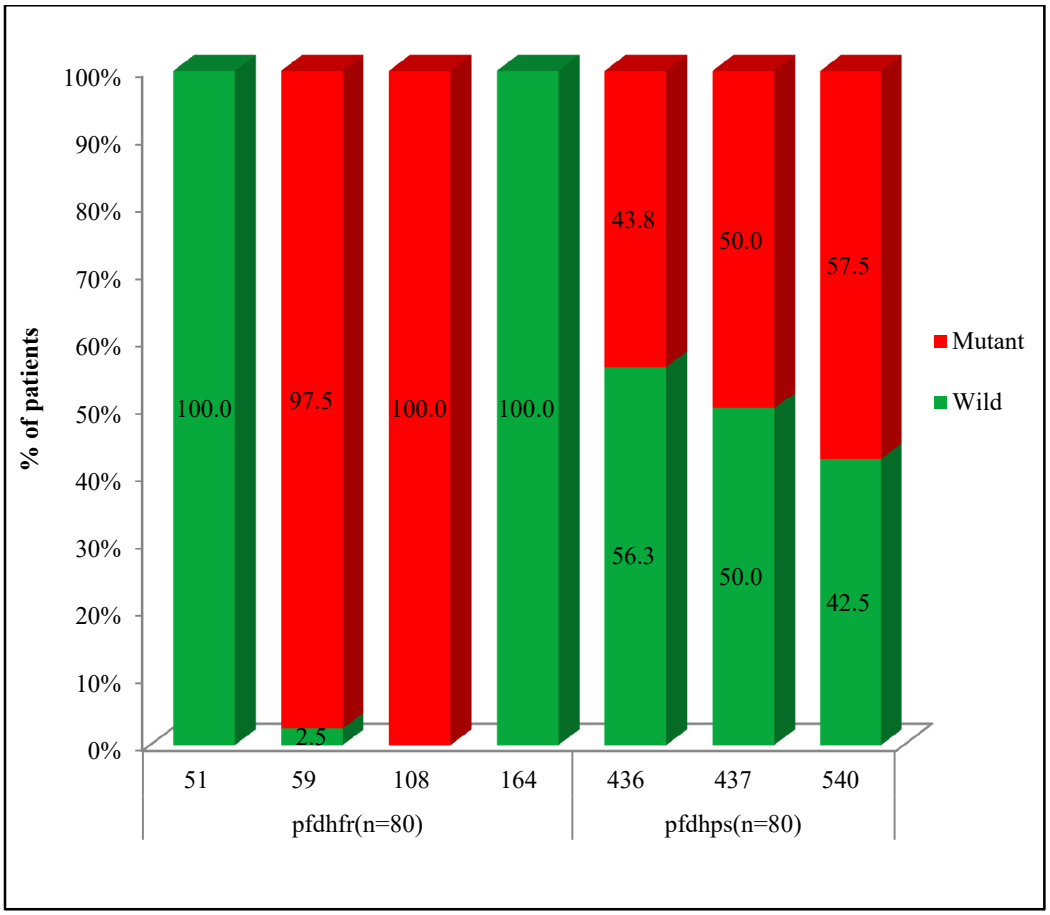

Fig. 4.20: Histogram showing the codon wise mutation in *pfdhfr* and *pfdhps* genes

4.3.3.3.3 Jharkhand

In the *pfdhfr* gene, a mutation at codon 108 was most prevalent (77.5 %) followed by codon 59 (67.6%) while 1.4% had mutant genotype for codon 51. The mutation was not observed at codon 164 in the samples collected from this study site (100% wild type). In the *pfdhps* gene, mutant genotype was observed at codon 436, 437 and 540 with frequency 72.1%, 58.8% and 57.4% respectively. However, no mutation was observed at codon 581 and 613 (**Fig. 4.21**).

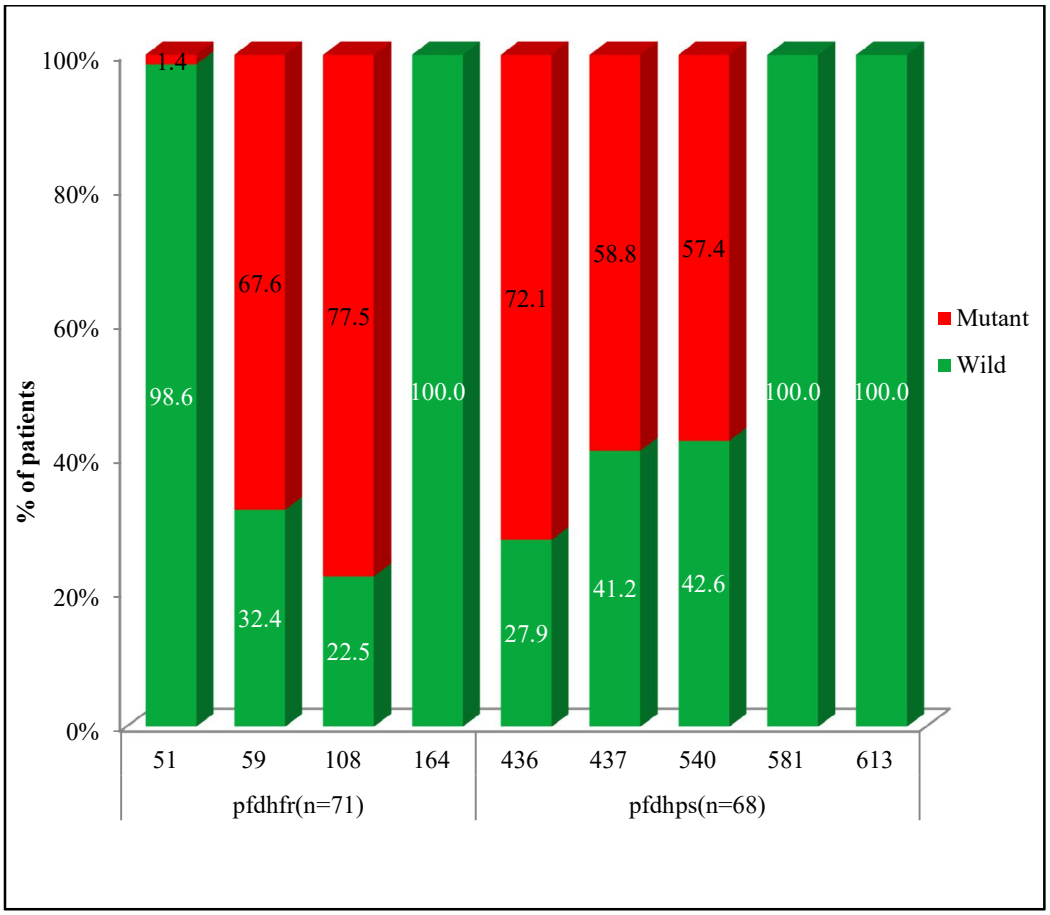

Fig. 4.21: Histogram showing codon wise mutation in *pfdhfr* and *pfdhps* gene

4.3.3.3.4 Odisha

Mutation in *the pfdhfr* gene at codon 108 was observed in highest frequency (64.8%) followed by codon 59 (46.8%). All the samples had the wild genotype for codon 51 and 164. In *pfdhps* gene at codon 436, 437 and 540, the wild type pattern was observed in 76.1%, 78.9% and 83.1% of the samples, respectively. However, codon 581 and 613 showed wild genotype in all the samples (**Fig. 4.22**).

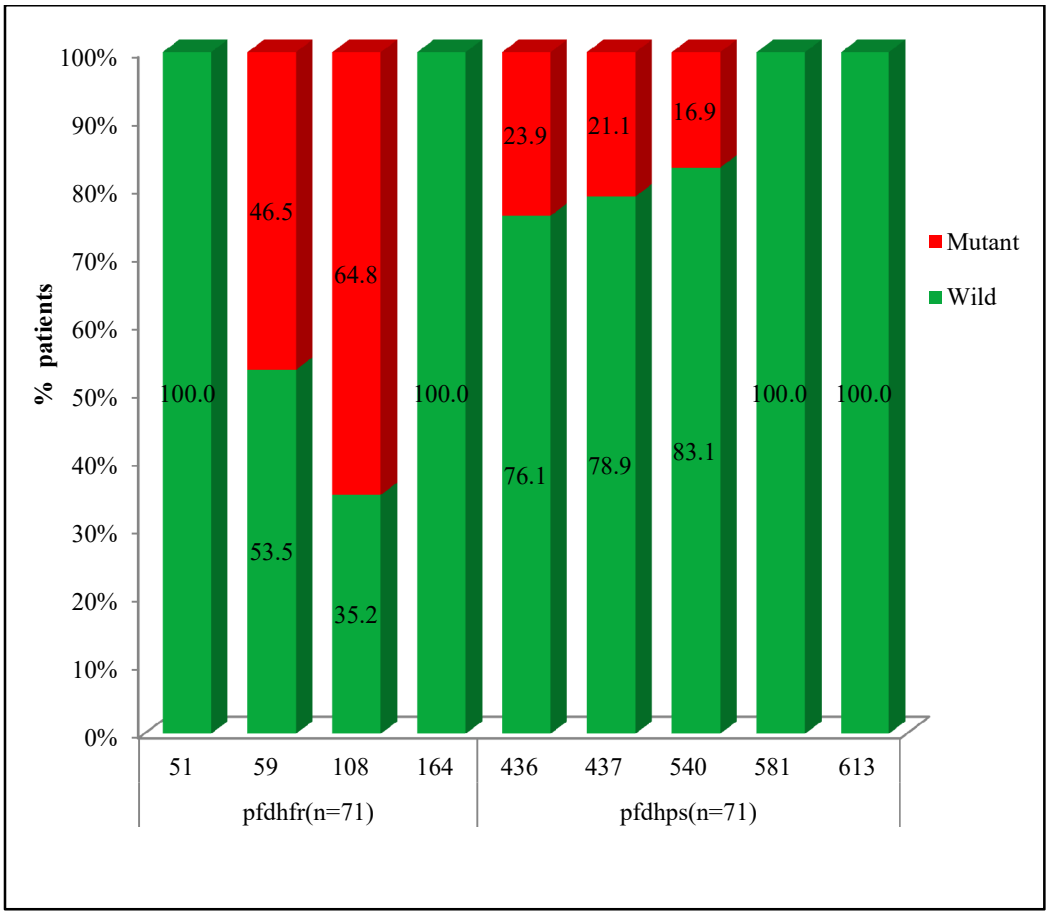

Fig. 4.22: Histogram showing codon wise mutation in *pfdhfr* and *pfdhps* gene

4.3.3.4 Combined haplotype in *pfdhfr-pfdhps locus*

In 288 samples, the double locus *pfdhfr-pfdhps* haplotype analysis was carried. Four codons of *pfdhfr* and five codons of *pfdhps* gene were taken into consideration for this analysis. Twenty different *pfdhfr-pfdhps* combined two locus haplotypes were observed. Out of 20 haplotypes, five haplotypes were predominant; which includes $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$ haplotype (33.5%) followed by $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$ (22.9%), $N_{51}C_{59}S_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$(10.2%), $N_{51}C_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$ (4.9%), $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$ (4.9%).

Table 4.3: Combined haplotypes of *pfdhfr-pfdhps* in the studied sites

S.No.	dhfr -dhps haplotypes	CG (n=66)	MP (n=80)	JH (n=71)	OR (n=71)	Total (n=288)	No. of mutations
1	$N_{51}C_{59}S_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	13.6	0.0	1.5	26.8	10.5	0
2	$N_{51}C_{59}S_{108}I_{164}$-$\mathbf{A}_{436}A_{437}K_{540}A_{581}A_{613}$	0.0	0.0	6	1.4	1.8	1
3	$N_{51}C_{59}S_{108}I_{164}$-$\mathbf{A}_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$	0.0	0.0	1.5	0.0	0.4	2
4	$N_{51}C_{59}S_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	0.0	0.0	0.0	2.8	0.7	2
5	$N_{51}C_{59}S_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$	0.0	0.0	7.5	4.2	2.8	3
6	$N_{51}\mathbf{R}_{59}S_{108}I_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	0.0	0.0	1.5	0.0	0.4	2
7	$N_{51}C_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	1.5	1.3	4.4	12.7	4.9	1
8	$N_{51}C_{59}\mathbf{N}_{108}I_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	0.0	1.3	0.0	0.0	0.4	2
9	$N_{51}C_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}A_{437}K_{540}A_{581}A_{613}$	0.0	0.0	3.0	1.4	1.0	2
10	$N_{51}C_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$	0.0	0.0	1.5	4.2	1.4	4
11	$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	50.0	30.0	17.9	36.6	33.5	2
12	$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}A_{437}K_{540}A_{581}A_{613}$	0.0	0.0	6.0	0.0	1.4	3
13	$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	4.5	7.5	1.5	0.0	3.5	3
14	$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$	12.1	6.3	1.5	0.0	4.9	3
15	$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	1.5	1.3	1.5	1.4	1.4	4
16	$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$	6.1	8.8	0.0	0.0	3.9	4
17	$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$	0.0	7.5	0.0	0.0	2.1	4
18	$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$	1.5	36.3	43.3	8.5	22.6	5
19	$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	9.1	0.0	0.0	0.0	2.1	3
20	$\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$	0.0	0.0	1.5	0.0	0.4	6

Out of 20 haplotypes, four haplotypes $N_{51}C_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$, $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$,$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$, and $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$ were present at all the study sites. Haplotype $N_{51}C_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$ showed higher frequency in Odisha (12.7%)

followed by Jharkhand (4.5%), Chhattisgarh (1.5%) and Madhya Pradesh (1.3%). Haplotype $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$ was observed with the highest frequency (50%) in Chhattisgarh followed by Odisha (36.6%), Madhya Pradesh (30%) and Jharkhand (17.9%) samples. Haplotype, $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$ was predominant in Jharkhand isolates (43.3%) followed by Madhya Pradesh (36.3%), Odisha (8.5%) and Chhattisgarh (1.5%) samples (**Table 4.3**).

4.3.3.5 Correlation between *pfdhfr* gene mutation and residual PYR & SDX

PYR resistance is associated with the mutations in *pfdhfr* gene, especially at codon 108 and consequently increased with cumulative mutation at codon 51, 59, 164. PYR is usually given as a combination therapy with SDX. It was observed that 5(3.5%) patients in this study were found to have a mean concentration of 0.98 µg/ml of PYR in their blood. PYR is usually given at a lower dose of 1.25 mg/kg in combination with SDX and its half-life is approximately 4 days; than that of SDX which has a half-life of 7.2 days (Hodel *et al*., 2009). Comparative analysis to detect type/s of mutation in *pfdhfr* gene was made to ascertain the extent of resistance and also the types of mutation in *pfdhfr* gene were correlated with the patients who had (n=48) or who did not have residual SDX levels (n=235) on day 0. Significant difference was not observed in triple mutation (95%, *df*=1, =0.84), double mutation (95%, *df*=1, *p*=0.78), single mutation (95%, *df*=1, *p*=0.488) and wild type genotype (95%, *df*=1, *p*=0.34) in those patients who had and who did not have residual SDX levels on day 0 (**Fig. 23**). Codon wise mutation result was compared in among the two groups i.e in-patients who had and who did not have residual antimalarial level. Statistically non-significant difference was observed in mutation at codon N51I (95%, *df*=1, *p*=0.650), C59R (95%, *df*=1, *p*=0.488), S108N (95%, *df*=1, *p*=0.488) and I164L (95%, *df*=1, *p*=0.488) (**Fig. 24**).

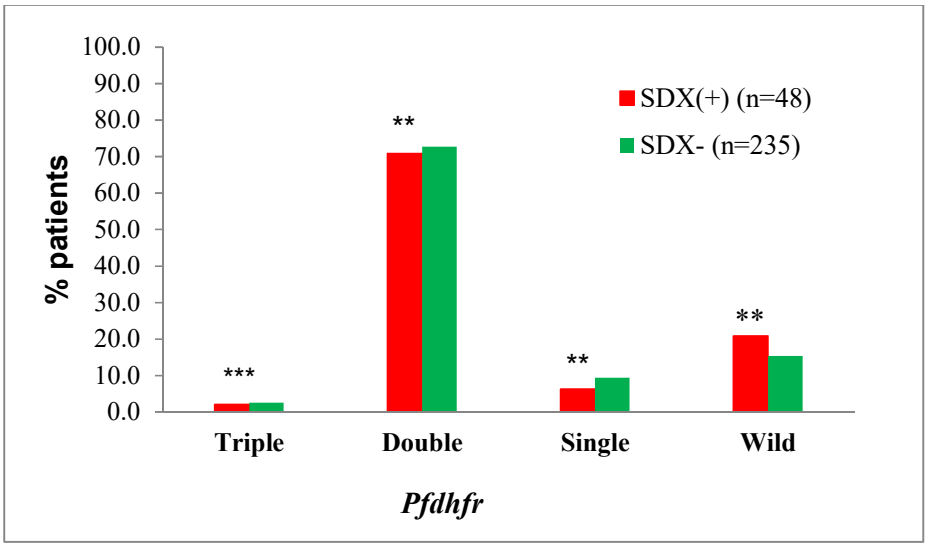

Fig. 23: Comparison of types mutation in of pfdhfr gene with or without residual SDX on day0. The number of patients' samples in each group is shown in parentheses. The chi-square test was used to compare values between two groups (, not significant, P >0.05)**

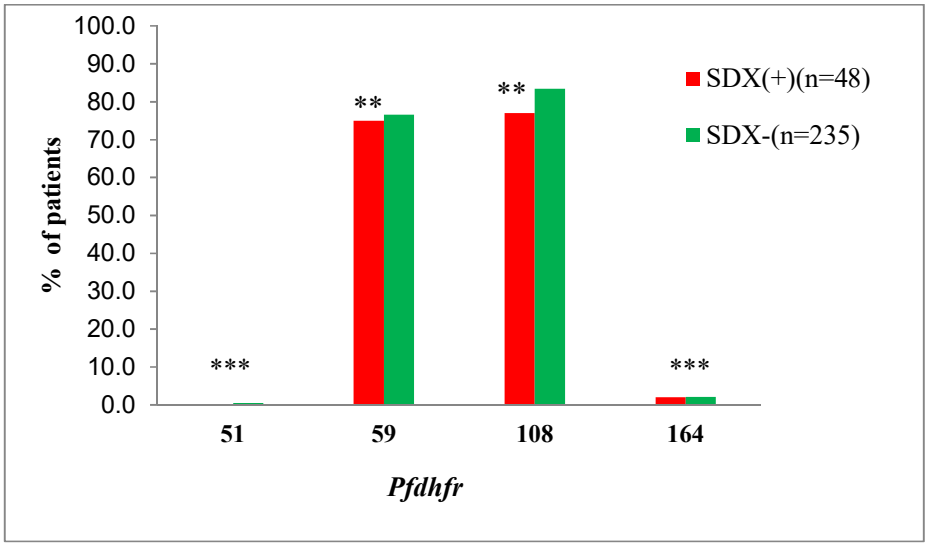

Fig. 4.24: Comparison of codon wise mutation in *pfdhfr* gene with or without residual SDX on day 0. The number of patients' samples in each group is shown in parentheses. The chi-square test was used to compare values between two groups (, not significant, P >0.05, ***, not applicable).**

4.3.3.6 Correlation between *pfdhps* gene mutation and residual sulphadoxine levels

The type of mutation in *pfdhps* gene was correlated with the patients who had (n=45) or did not have residual SDX levels (n=235) on day 0. Significant difference was not observed, values varied from 33.3 to 25.5% in triple mutation (95%, *df*=1, =0.278), 13.1 to 11.5% in double mutation (95%, *df*=1, *p*=0.725), 11.1 to 11.5% in single mutation (95%, *df*=1, *p*=0.941) and 42.2 to 51.5% had wild type genotype (95%, *df*=1, *p*=0.254) in those patients who had residual SDX and who did not have residual SDX levels day 0 (**Fig. 4.25**).

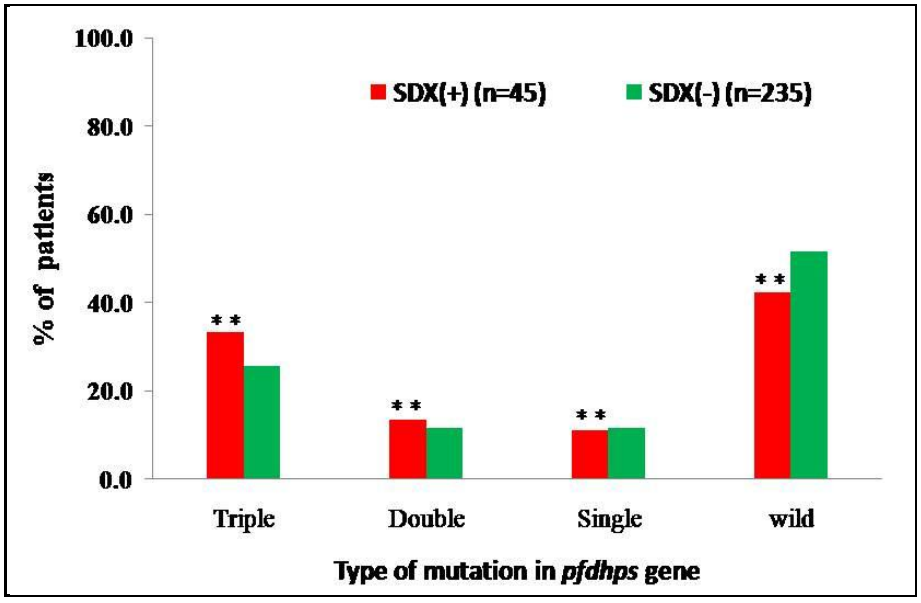

Fig. 4.25: Comparison of types of mutation *pfdhps* present with or without residual SDX on day 0. The number of patients' samples in each group is shown in parentheses. The chi-square test was used to compare values between two groups (, not significant, P >0.05)**

The mutation in the *pfdhps* gene at codon 436, 437 and 540 were correlated with the patients who had or did not have residual SDX levels. It was found that patients who had residual SDX levels showed higher frequency (57.8%) of mutation in codon K540E as compared to those patients who did not had residual levels of SDX (35.7%) on day 0 (95%, *df*=1, *p*=0.0001).

Mutation at codon S436A was also higher (44.4%) in those patients who had SDX as compared to those who did not have residual levels of SDX (35.3%) on day 0, but difference was not statistically significant (95%, *df*=1, *p*=0.244) (**Fig. 4.26**).

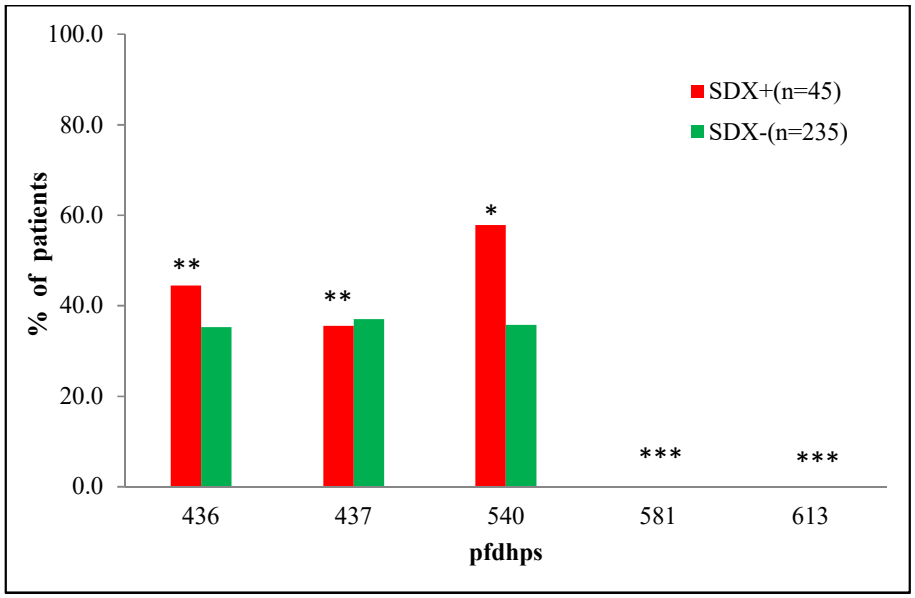

Fig. 4.26: Comparisonof codon wise mutation in *pfdhps* gene with or without SDX on day 0. The number of patients' samples in each group is shown in parentheses. The chi-square test was used to compare values between two groups (*, P < 0.05; **, not significant, P >0.05, *, not applicable)**

4.4 Discussion

In this study, out of 295 enrolled malaria patients, residual SDX levels were monitored in 289 malaria infected samples. Of which 49 (17%) patients carried residual SDX levels in their blood above the detection limits (50ng/ml). These residual SDX levels were detected in the patient's samples before initiating the antimalarials treatment. SDX is a long acting partner drug of currently recommended ACT for treatment of uncomplicated *P. falciparum* malaria cases across the country, except NE region. The similar observations have also been reported earlier, where residual SDX levels prior to initiating of antimalarial

drug treatment were studied (Hodel *et al*. 2009). The findings of the study indicate that some patients had taken SDX for a previous febrile episode. This could be due to easy availability of SDX in the private clinics and pharmacy or due to its wide use in the current market due to previous recommendations as second line for control of resistant *P. falciparum* or as per the low cost of treatment. Since, the half-life of SDX is longer, therefore its bioavailability in patient's blood remains for more than a month (Hodel *et al.* 2009). In the present study, as none of the patients declared about the details of any previous antimalarial drug intake episodes, the presence of residual SDX levels on day 0, which was detected through HPLC could be due to self-medication and irrational treatment practices, being carried out in the studied community which they may not be admitted out of fear or lack of knowledge about type of drug consumed if prescribed by a quacks or a practitioner. The significantly decrease in mean parasite density in patients' blood having residual SDX levels was due to the effect of residual SDX which might have suppressed any new-infection (Hodel *et al.* 2009). The possible reason could be that the drug could screen out sensitive parasites and only resistant parasite population could survive with sub-therapeutic levels of SDX. The residual (SDX) levels of drug in blood were not enough to completely block parasites replication and to overcome clinical symptoms in the patients (Hodel *et al*. 2009).

We have described *pfdhfr* mutations among *P. falciparum* samples from four different geographical regions of India. These regions are far apart from each other and show a diverse level of drug resistance and malaria transmission intensity. In the *pfdhfr* gene, single or double mutation alone cannot cause treatment failure to SP but a single mutation in a *pfdhps* gene besides a double mutation in *pfdhfr* or triple mutation alone in *dhps* can cause higher levels of resistance to SP (Mombo-Ngoma *et al.* 2011; Abdullah *et al*. 2013*;*

Mishra *et al*. 2014). Triple mutation in the combination of N51I+C59R+S108N or C59R+S108N+I164L is an indicator of high levels of treatment failure of pyrimethamine. Our data showed a prevalence of 2.4% triple mutant population in the studied samples (Mombo-Ngoma *et al.* 2011*;* Mishra *et al*. 2014). Prevalence of double mutation in *pfdhfr* (59+108) is highest (71.9%) in the study areas, higher frequency of double mutation has been also reported of earlier (Mishra et al. 2012).

The mutation rate or SP resistance vary from place to place and associated with drug pressure, transmission intensity of malaria, etc. (White 1999). The high transmission intensity of malaria parasites provides multiple chances of high recombination frequency which will lead to higher genetic variation. This could also lead to the emergence of more and more drug resistance genotypes and faster spread of PYR resistance genotypes in these states. Mutations in the *pfdhps* gene at codons; S436A, A437G, K540E, A581G, and A613T has been associated with SDX resistance (Muller et al. 2013). Mutation at codon S436A in *dhps* gene reduces the binding affinity of SDX drug to DHPS enzyme followed by cumulative mutations at Codon G437, E540, G581, and T/S613, subsequently, reducing the binding affinity (Sibley *et al*. 2001).

Our data shows that wild genotype was present in higher frequency as compared to the triple, double and single mutation in *a pfdhps* gene. Previous studies have also reported sequence variation in single, double and triple mutation in *pfdhps* gene from different part of the India (Ahmed *et al.* 2006, Lumb *et al.* 2009 & 2011, Mishra *et al.* 2012). A Recent study from Jalpaiguri, India has also shown 9.5% failure rate in AS+SP arm, pointing to SP treatment failure, due to mutations in *pfdhfr* (I51+R59+N108) with *pfdhps* (G437+E540) genes (Saha *et al.* 2012). Subsequently, NIMR also observed

higher treatment failures rates for AS+SP regimen in North-eastern states of India with higher frequency mutations in *pfdhfr* and *pfdhps* genes. In 2013, AS+SP was replaced by AL as first line treatment for uncomplicated *P. falciparum* cases in this region (Mishra *et al*. 2014). Quintuple mutations, combination of two or three mutations in combination of *pfdhfr-pfdhps* gene including *pfdhfr* three codons (N108 + I51 + R59) and two *pfdhps* codons (G437+ 540E) is a strong indicator of SP treatment failure (Abdullah *et al.* 2013). Such combination of quintuple mutation was not observed in our studies, assuring higher efficacy of partner drug-SP, as evident from higher efficacy observed through in-vivo studies, confirming and complementing both the findings in the study areas. As the PCT also remains much below the threshold of <72 hours, there does not remain any pre-indication of treatment failure with a long acting partner drug in the study areas. Residual levels of SDX did not show any effect on the mutation frequency in *pfdhfr* gene in the studies population. With the influence of SDX drug pressure in our study, higher mutation in *pfdhps* gene, specially mutation at codon K540E was observed, which is strongly associated with the presence of residual levels of SDX on day 0, indicating that SDX drug pressure might have selected K540E mutation. SDX drug pressure also influences the S436A mutation but the findings did not show statistical significance.

4.5 Conclusion

The present study shows that falciparum malaria patients had residual SDX levels in their blood before the initiation of treatment regimen, pointing to the prevailing practices in the community residing in high transmission intensity areas, where self-medication, liberal prescription by practitioners and presumptive drug use may be very common.

Mutation pattern in *pfdhps* has suggested that SDX resistant parasites might have evolved in the study region due to SDX drug pressure in the community. While studies on drug resistance markers are not enough for expecting clinical treatment failure, these findings provide early warning signs and warrants the policy makers on the life span of AS+SP drugs combination therapy particularly in malaria endemic regions and thus the probable consequence should be to reassess the existing chemotherapeutic strategies for control of malaria. Our data also suggests that the patients with residual SDX levels showed significant association with mutation in *pfdhps* gene at codon K540E. The continuous increase in drug pressure of SDX on parasite may promote the emergence of drug resistant parasite population.

To prevent the emergence and spread of drug resistant parasites, we need to minimize the chance of sub-therapeutic exposure of parasite to residual drug levels that will reduce the selective window by introducing appropriate combinations of antimalarials. In addition, the presumptive use of long acting antimalarial drugs should also be minimized or stopped altogether in public health settings.

Chapter V

Effect of residual antimalarials on therapeutic outcome

5.1 Introduction

World Health Organization (WHO) recommends use of artemisinin-based combination therapy (ACT) to offset the development of resistance in *Plasmodium falciparum* (*P. falciparum*) to antimalarials and to achieve rapid reduction of parasitaemia and morbidity (WHO 2016). The National drug policy has been revised in India in favor of using ACT. Although at present artesunate plus sulphadoxine-pyrimethamine (AS+SP) is recommended for the treatment of uncomplicated *falciparum* malaria in the country except in Northeast region of India where Artemether-Lumefantrine (AL) is the first line of treatment for falciparum malaria (NVBDCP 2014). Higher prevalence of mutations in *Plasmodium falciparum dihydropterote synthase (pfdhps)* gene at codon S436A, A437G and K540E has been observed in the past in North east India, with high treatment failure of AS+SP regimen due to partner drug resistance (Mishra *et al.* 2014).

The duplication of antimalarial treatment for pyretic fever and self-treatment are common in malaria endemic areas. Prior to participating in an *in-vivo* study, the intake of antimalarials has an effect on the interpretation of both efficacy and safety outcome, and it may also be a source of subtherapeutic drug treatment which may lead to drug resistance. In fact, residual drug may affect the current treatment in several ways. Higher drug exposure resulting from cumulative levels may lead to enhanced efficacy or more toxicity (Hodel *et al.* 2000), and disease causing parasites at the time of enrollment may be less sensitive, due to selection by the residual antimalarial levels and patients would present with drug exposure at the time of inclusion.

In addition, the therapeutic efficacy study informs the *in-vivo* effects of antimalarials in uncomplicated *P. falciparum* malaria patients and is defined as the capacity of therapeutic effect of a given intervention (WHO 2009). As per WHO definition, the

therapeutic outcome is classified as early treatment failure (ETF), late treatment failure (LTF), and adequate clinical and parasitological response (ACPR). The late treatment failure (LTF) is further classified in to two groups; late clinical failure (LCF) and late parasitological failure (LPF). ACPR is indicating the absence of parasitaemia upto 28days, irrespective of auxiliary temperature of patients who did not meet any of the criteria of early treatment failure, late clinical failure or late parasitological failure.

In this chapter, attempt has been made to observe the effect of residual antimalarials on therapeutic outcomes in malaria enrolled patients in therapeutic efficacy study.

5.2 Materials and Methods

5.2.1 Patient screening, sample collection and follow-up

Screening of patients was done according to the inclusion and exclusion criteria as defined by WHO, and eligible patients fulfilling the inclusion criteria were enrolled in the present study. Hundred microliters (100 µl) of heparinised blood was taken on 31ET filter paper (Whatman GE Health Care, UK) for monitoring residual drug level on day 0. For *in vivo* therapeutic efficacy studies, the drugs were supplied by the National Programme and were given to the patients under direct observation, according to age and recommendation of National Drug Policy for Malaria. Patients were followed-up on 1, 2, 3, 7, 14, 21 & 28 day at all study sites.

5.2.2 Data entry and Kaplan-Meier survival estimate

Demographic and clinical details were double entered in WHO therapeutic efficacy database (WHO 2009) to confirm the entry. Kaplan-Meier survival estimates was done using WHO therapeutic efficacy database (WHO 2009). The data sheet has been prepared by WHO to facilitate entry and interpretation of the data collected during therapeutic efficacy study.

5.2.3 Statistical analysis

Patient's information was entered in WHO excel sheet and Kaplan-Meier Survival plot was generated. Preparation of graphs, data entry and analysis was also done using Microsoft Office Excel 2007.

5.3 Results

5.3.1 PCR uncorrected and corrected therapeutic outcome

5.3.1.1 Gaurella CHC, Bilaspur district, Chhattisgarh

Seventy patients were enrolled from Gaurella CHC, Bilaspur district, Chhattisgarh for monitoring of therapeutic efficacy study and to analyse residual antimalarial level on day 0. The patients were followed-up to 28 days. Sixty seven patients could complete the follow–up till 28 days, and achieved Adequate Clinical and Parasitological Response (ACPR). The therapeutic outcome, i.e the PCR uncorrected and corrected cure rates was observed 100% **(Fig. 5.1)**. During the study, there was one consent withdrawal and two lost to follow-up.

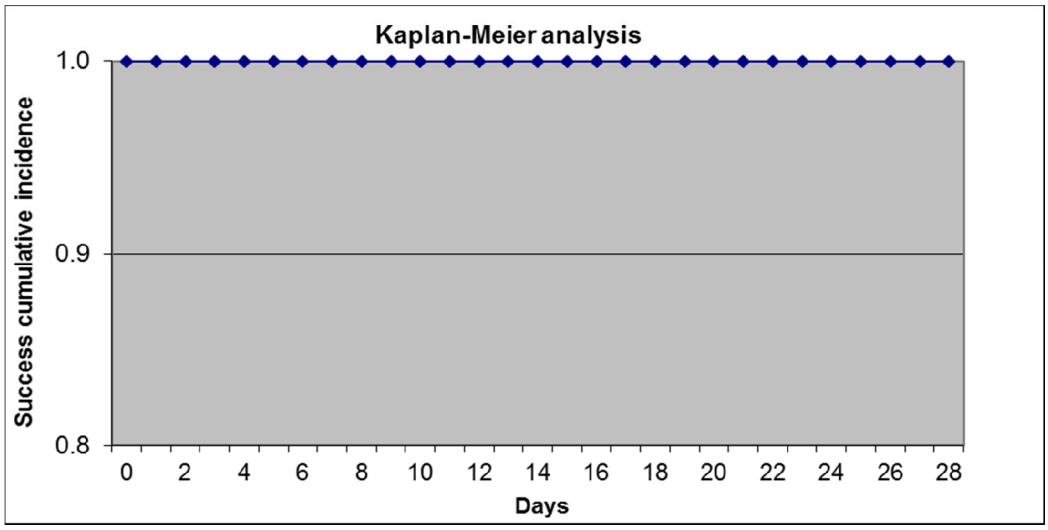

Fig. 5.1: Kaplan-Meier Survival analysis for patients from Gaurella CHC Bilaspur district, Chhattisgarh

5.3.1.2 Ghodadongri PHC, Betul district, Madhya Pradesh

Eighty patients were enrolled from Ghodadongri PHC, Betul district, Madhya Pradesh for monitoring the therapeutic efficacy study and to analyse residual antimalarial levels on day 0 and were followed-up to 28 days. Sixty nine patients could complete the follow-up till 28 days and achieved 100% ACPR. The therapeutic outcome in terms of the PCR uncorrected and corrected cure rate was 100% **(Fig. 5.2)**. During this study, 11 patients had consent withdrawal from the study.

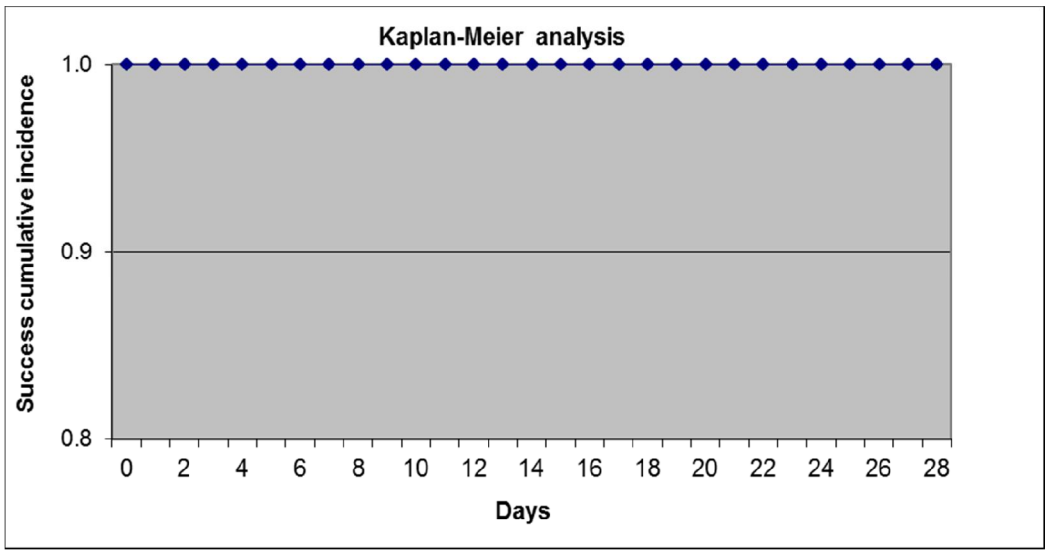

Fig. 5.2: Kaplan-Meier Survival analysis for patients from Ghodadongri PHC, Betul district, Madhya Pradesh

5.3.1.3 Kurdeg CHC, Simdega district, Jharkhand

Seventy-three patients were enrolled at Kurdeg CHC, Simdega district, Jharkhand for enrolment in the study and were monitored for residual antimalarial drug levels on day 0 and later followed-up for therapeutic outcome. Seventy patients could complete the follow-up till 28 days, and achieved ACPR and thus, the PCR uncorrected and corrected cure rate was 100% **(Fig. 5.3)**. Only one patient withdrawal was documented (consent) while two were lost during the follow-up.

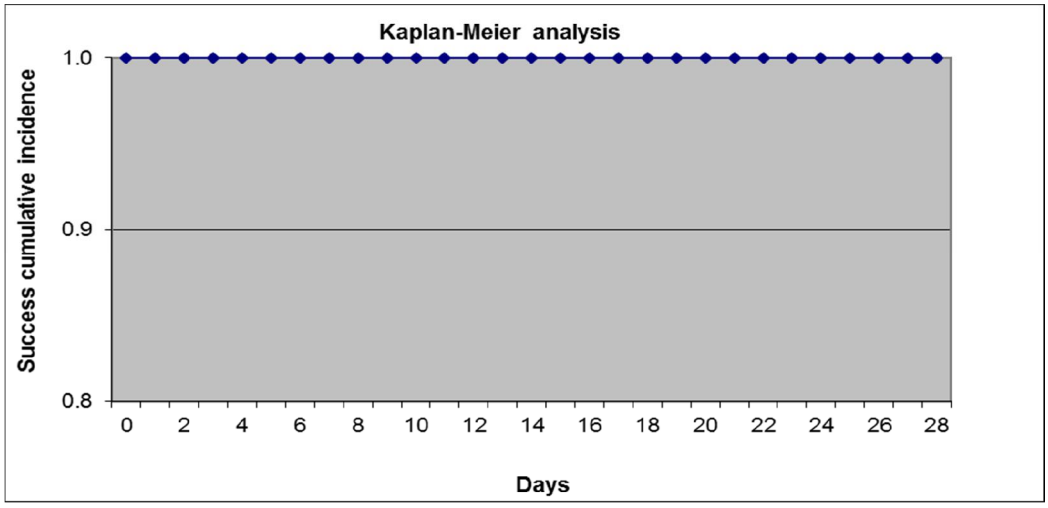

Fig. 5.3: Kaplan-Meier Survival analysis for patients from Kurdeg CHC, Simdega district, Jharkhand

5.3.1.4 Bisra CHC, Sundergarh district, Odisha

Seventy two patients were enrolled from Bisra CHC, Sundergarh district, Odisha for monitoring residual antimalarial on day 0 and followed-up for therapeutic outcome. Sixty seven patients could complete the follow-up to 28 days. All the patients achieved ACPR and thus, the PCR uncorrected and corrected cure rate remained 100% **(Fig. 5.4)**. During this study, five patients had consent withdrawal.

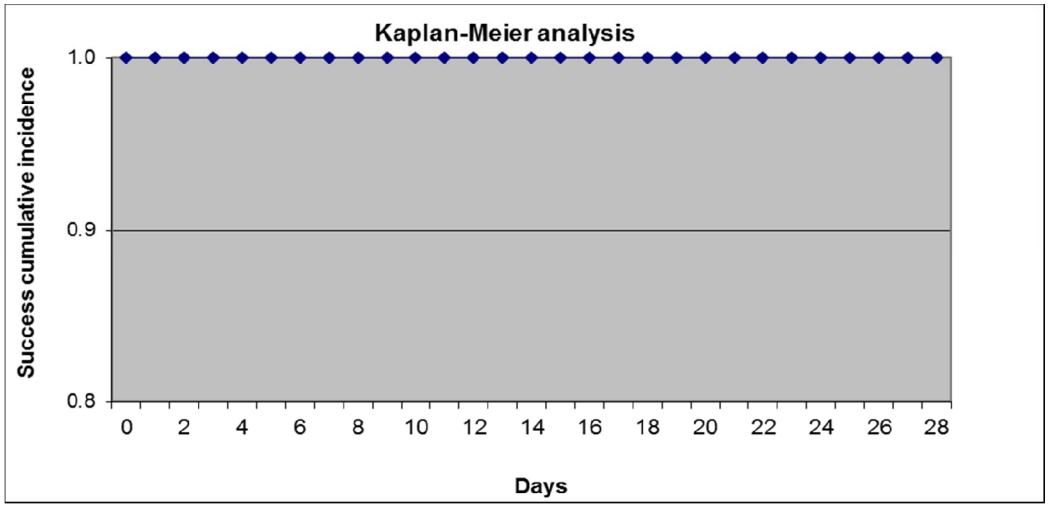

Fig. 5.4: Kaplan-Meier Survival analysis for patients from Bisra CHC, Sundergarh district, Odisha

5.3.2 Parasite clearance with residual CQ

Parasite clearance time (PCT) is an indicator of the potency of the antimalarial prescribed, and is being monitored for evaluating the efficacy of antimalarial through in-vivo studies. PCT depends on the half-life of referred antimalarial and the duration of follow up depends on the half life. For short acting antimalarials, six hourly PCT is being monitored as per WHO recommendation and for long acting antimalarials, 24 hourly PCT is monitored to evaluate the parasite clearance following ACT administration. The effect of residual chloroquine level on the parasite clearance was studied by comparing the parasite clearance in patients who had residual chloroquine on day 0 compared to the patients who did not have residual chloroquine on day 0. Parasite clearance time (PCT) with residual CQ on day 0 showed that 83.3% (20/24) patients had parasite clearance of <24 hours, and 100% patients (24/24) had PCT between 24 hours to<48 hours (100% PCT in 48 hours to <72 hours as well). While malaria patients who did not have residual CQ levels on day 0 showed that 78.8% (193/241) patients had parasite clearance of less than 24 hours, and 98.4% (241/245) cleared parasites between 24 hours to <48 hours and 100% (245/245) had PCT between 48 to <72 hours **(Fig. 5.5)**.

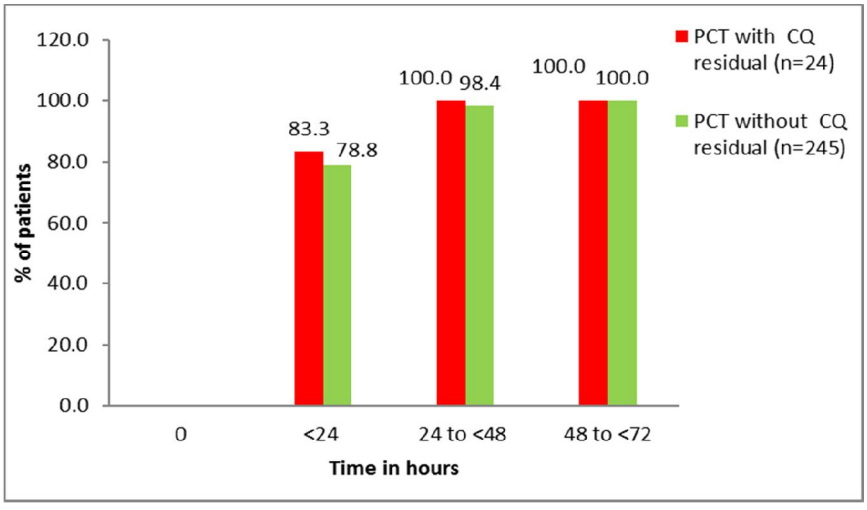

Fig. 5.5: Effect of residual CQ on parasites clearance in enrolled malaria patients

5.3.3 Parasite clearance with residual SDX

PCT was monitored to evaluate the effect of residual Sulphadoxine (SDX) on parasite clearance following ACT administration and was studied by comparing the parasite clearance time in patients who had residual sulphadoxine on day 0 compared to the patients who did not have residual sulphadoxine levels on day 0**.** Parasite clearance time in patients with residual sulphadoxine on day 0 were; 81.8 % (36/44) patients had parasite clearance of < 24 hours, 100% (44/44) had PCT between 24 hours to <48 hours (100% PCT in 48 hours to <72 hours as well). While parasite clearance in malaria patients who did not have SDX residual levels on day 0; 78.7% (193/245) patients had parasite clearance of < 24 hours, 98.2% (241/245) had PCT between 24 hours to <48 hours, 100% (245/245) had PCT between 48 hours to <72 hours **(Fig. 5.6)**.

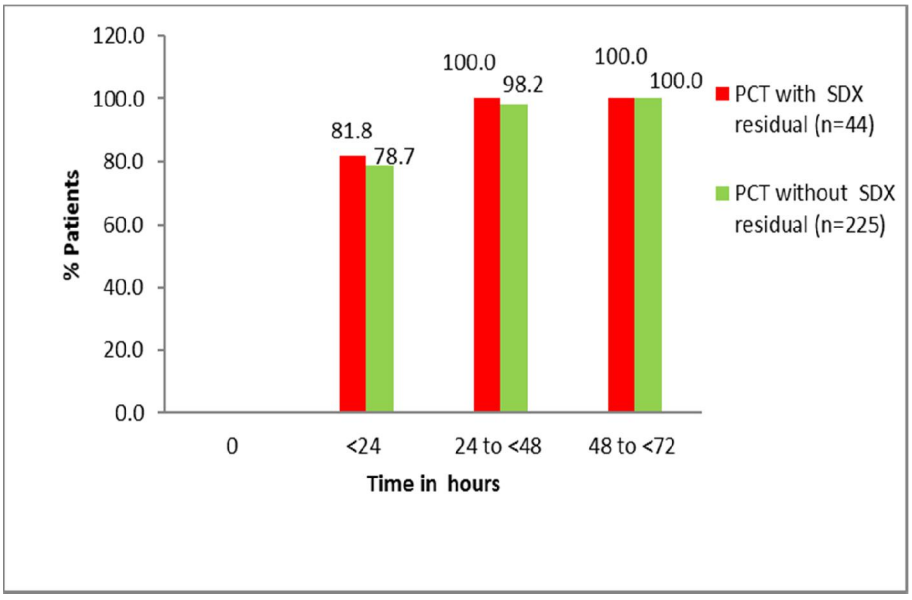

Fig. 5.6: Effect of residual SDX on parasites clearance in in enrolled malaria patients

5.3.4 Parasite clearance with residual CQ & SDX

Parasite clearance rate is important to measure the efficacy of the antimalarial drug. More rapid decline in parasitaemia, patients were treated with the artemisinin derivatives. The rapid decline in parasitaemia is a consistent finding in all studies of the artemisinin derivatives (White 2011). Parasite clearance rate was monitored to evaluate the effect of residual CQ and SDX following AS+SP administration and compared the parasite clearance rate in patients who had residual CQ and SDX on day 0 compared to the patients who did not have residual CQ and SDX levels on day 0**.** Rapid decline in parasitaemia was observed with residual CQ and SDX drug levels (<48 hours) as compared to patients who did not have residual CQ and SDX levels (48 to < 72 hours) (**Fig. 5.7**)

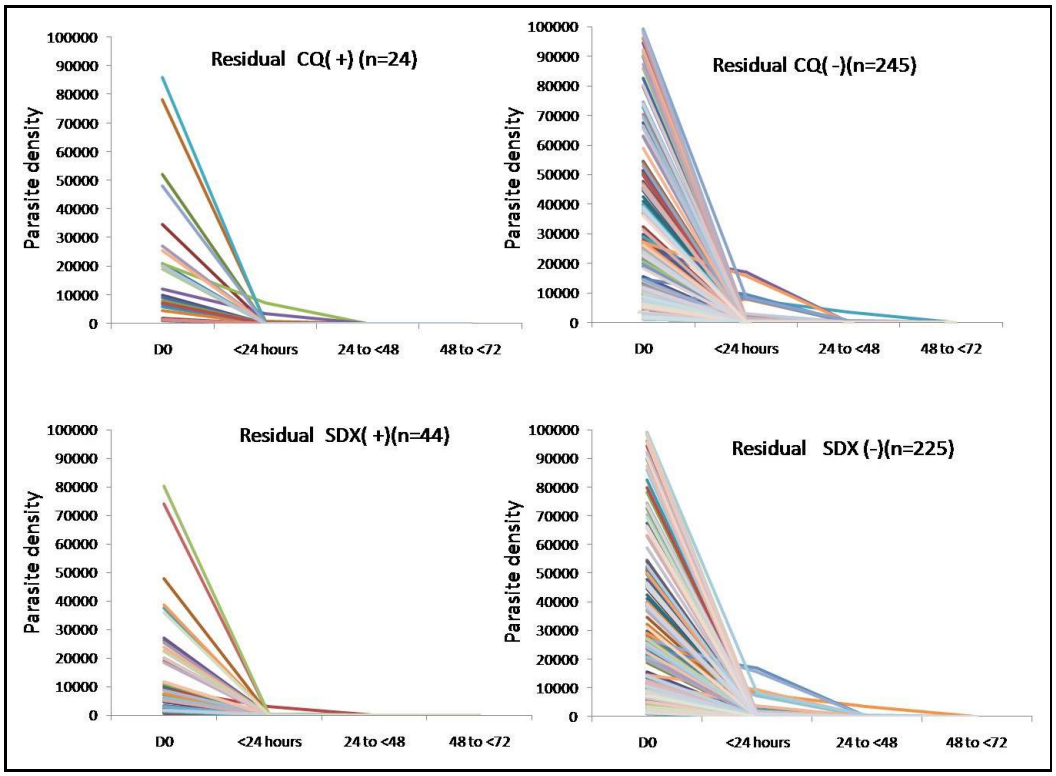

Fig. 5.7: Parasite clearance rate with presence of residual CQ and SDX on day 0.

5.4 Discussion

A total of 295 patients were enrolled in the study. Of these 295 patients, 273 patients completed the follow-up at all the four sites, namely Jharkhand, Madhya Pradesh, Chhattisgarh and Odisha and were followed up to 28 days. Clinical examination was done as per schedule. Previous drug intake leading to residual antimalarial could affect the treatment outcome in several ways; higher drug exposure resulting from cumulative drug levels could lead to better response to malaria infection. In the present study, all the patients (100%) had adequate clinical and parasitological response, suggesting that the efficacy of recommended antimalarial medicine was high and in addition, the presence of residual antimalarial might have helped to resolve the current malaria infection leading to effective cure. In addition, toxic effects due to higher drug exposure resulting from cumulative drug levels, was not observed in the studied patients. Parasites causing the disease to enroll patients at the time of enrolment could belong to a less sensitive population, selected by the previous treatment, and or the patients might have experienced treatment failure before inclusion in the study due to incomplete treatment or non-compliance towards prescribed antimalarials. We observed that with the presence of residual CQ and SDX levels in patients on day 0, the parasite clearance was within 48 hours. However in patients without residual antimalarial on day 0, parasites clearance time was increased to 48 to <72 hours (1.6% patients had parasites in the CQ group and 1.8% in the SDX group). The probable reason could be due to higher drug exposure resulting from cumulative drug levels (Hodel *et al.* 2009), a small fraction of population might have started resisting and thus could remain in the system for a longer duration. The increased PCT is a measure of increasing tolerance in the exposed malaria parasite population and will later result in treatment failure of the

recommended antimalarials. Since here, the population showing PCT between 48 to <72 hours is less, it could be due to increasing partner drug resistance in the exposed population of *P. falciparum* malaria parasites

5.5 Conclusion

In summary, the presence of residual levels of antimalarials, not only aid in increasing the cure rate of prescribed antimalarials for the malaria infection but also may assist in increasing the tolerance towards antimalarials and thereby might assist in spreading drug resistance.

Summary & Conclusion

In this study, 24.2% patients had residual antimalarial levels, above the detection level i.e LOD (Limit of detection) of 50 ng/ml of blood. The residual antimalarials were presentin all the study sites and it varied in range 14.1% - 31.6 % among all the study samples. Among 295 patients, 17% and 9.3% patients had residual SDX and CQ levels respectively across various study sites which was also above LOD. Therapeutic levels of SDX on day 7 were found ranging between 43.7 to 48.8µg/ml of the blood. Although, the CRF showed no previous drug intake of antimalarial but HPLC analysis showed presence in 24.2% samples. Lower parasite density/µl was observed in patients having residual CQ as compared to patients who had no residual CQ on day0 but not statistically significant. Lower parasite density/µl was also observed in patients having residual SDX levels as compared to patients without residual SDX on day0. This finding was statistically significant. Higher frequency (71.9%) of double mutation (C59R & S108N) in *pfdhfr* gene was observed in studied samples. Triple mutation (2.4%) in *pfdhfr* gene was observed in lower frequency in studied samples. Wild type genotype was more prevalent (51%) followed by triple mutant (26%), double mutant (11.8%) and single mutant (11.1%).76.1% samples had mutant genotype for codon K76T in *pfcrt* gene. Mutation rate in *dhps* gene was higher in those patients who had residual SDX (57.8%) as compared to without residual SDX (48.5%) concentration on day on day 0. Mutation in codon 540E was higher (57.8%) with presence of residual levels of SDX as compared to those patients who did not having residual SDX on day 0 (p value<0.05). The frequency of 76T mutation was significantly higher in those having residual CQ (96%) as compared to those not having residual CQ (74.6%). Early clearance of parasitemia was observed with residual antimalarial (<48), but without residual antimalarial persistence>48 hours.

The presence of residual antimalarials indicates the circulating drug pressure in the study areas which may be due to previous drug episodes/self-intake/irrational treatment/mass drug treatment. A residual level in CQ and SDX on day 0 indicates high mutation in *pfcrt* and *pfdhps* at both genes. It also, suggests that residual levels of antimalarials may encourage the emergence and spread of drug resistant parasites. The residual levels of antimalarials drug may also be helpful in overcoming the current infection. The need of the hour is to educate all medical staff as well as community peoples to reduce the irrational, presumptive treatment practices and self-intakes in malaria.

Way forward

Our data on presence of residual antimalarials from four studies site namely Chhattisgarh, Madhya Pradesh, Jharkhand and Odisha have significance as this provides evidence based data on the circulating antimalarial levels in the community. As irrational treatment practices, mass drug administration and self medication are responsible for increased drug pressure in the parasites population in community it may promote the emergence and spread of resistance parasite population in the study area.

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Annexures

ANNEXURE I: INFORMED CONSENT FORM

Title of the project: "Effect of residual antimalarials in malaria patients enrolled for therapeutic efficacy studies and its effect on spread of drug resistant parasites in high malaria endemic districts in India"

Submitted to ICMR for Junior Research Fellowship Project.

Name of the guide: Dr. Neelima Mishra Scientist 'D'

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 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 confirm the effect of residual antimalarial on therapeutic efficacy study of the medicine CQ or ACT recommended by the Ministry of health.

You/your child will receive 3 doses of medicine 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 the effect of residual antimalarial on the efficacy of the medicine on sick patients who have malaria and live in this area.

Your participation/your decision to have your child participate in this study are 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 CQ/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.

Hundred microliter bloods will be taken from you/your child's before drug administration on day 0 on a piece of filter paper. This will be used for measuring residual antimalarial levels. Also two or three drops of blood will also be taken from you/your child's finger onto a piece of filter paper. This helps to analyze the characteristic of the parasites. Your child will feel pain when the needle sticks but this will go away very quickly. Your child will receive the first dose of treatment.

During the follow-up, blood will be taken 7 times from you/your child's in a finger onto a slide or 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 determine residual antimalarial levels on day 0. The analysis of the parasites will be done after the study but this has no implication on the outcome of the illness. Blood will also be used to study of molecular markers for drug resistance.

At all the visits you/your child will be examined by a physician. At the second visit, your child will be given the second dose of treatment. On the third visit, your child will be given the third dose of treatment and two or three drops blood will be taken from your child's finger onto a slide for the parasite count. On the fourth visit two or three drops blood will be taken from your child's finger onto a slide for the parasite count. On the fourth, fifth, sixth and seventh visits, two or three drops blood will be taken from your child's finger onto a slide for the parasite count and onto a piece of paper to analyze the characteristic of the parasites.

The surveillance study takes place over 28 days. During that time, it will be necessary for you to come to the health facility 7 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 aware of currently. 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 or reactions. Or we may stop the use of one or more medicines. If this is 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 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 and your child will still have all the benefits that you would otherwise have at this centre. You may stop participating/your child from participating in the

surveillance study at any time that you wish without you/your child losing any of your rights as a patient here. Your treatment/your child's treatment at this centre will be affected in any way.

If you do not wish to take part/your child 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 any of the following.

Dr. Neelima Mishra, NIMR, New Delhi — Phone (Office): +911125307333
Mr. Kamlesh kaitholia, NIMR,New Delhi — Phone (Mobile): +919015212807

…….
…….
……..

This proposal has been reviewed and approved by 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:
Mr. Kamlesh Kaitholia
ECR Division,
National Institute of Malaria Research
Sector – 8, Dwarka,
New Delhi – 110077

Guided by:
Dr. Neelima Mishra, Scientist 'D'
National Institute of Malaria Research
Sector – 8, Dwarka,
New Delhi – 110077

Certificate of Consent >18 years old

I have been invited to participate/have my child participate in surveillance on the efficacy of antimalarial medicine and the effect of residual antimalarial on the efficacy of antimalarial medicines . I understand that it will involve me/my child receiving 1-3 doses and 7 follow-up visits. 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 to either myself/my child personally except for getting treated for malaria and that I will not be compensated beyond travel expenses. 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 in any way affecting my medical care.

Name of Participant_____

thumb print Participant

Signature of Participant _____

Date_____ day/month/year Name of

witness_____

Signature of witness _____

Date _____ day/month/year

Name of investigator/ MO_____

Signature of investigator / MO_____

Date _____ day/month/year

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

Informed Consent for Patient of 6 months to < 18 years old

Name of Patient: _____ Age of patient _____years _____ month(s)

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 about the research answered to my satisfaction. I am aware the participation is entirely voluntary. I understand that the patient may 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 Parent / Legal Acceptable Representative (LAR)

_____ _____

Signature of Parent / LAR Date

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 Witness

_____ _____

Signature of Witness Date

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 Person Who Obtained Consent

_____ _____

Signature of Person Who Obtained Consent Date

ANNEXURE II: CASE RECORD FORM

STUDY SITE	Health Facility's Name:				Town:		District/province:		
PATIENT	Identity Number:	Full Name:			Age (years):	Sex (M/F):	Weight (kg):	Height (cm):	
	Name of guardian:				Contact home address:				
ANTIMALARIAL DRUG	Drug name:		Manufacturer:		Batch number:		Expiry date:	Total dose (mg base):	
	Previous intake (Y/N/Unknown):		Age Category:		Manufacturing date:		Name of Urinary test Result:		
DAY	DAY 0	DAY 1	DAY 2	DAY 3	DAY 7	DAY 14	DAY 21	DAY 28	Unscheduled day
Date									
Danger signs (Y/N)									
History of fever Last 24 hrs (Y/N)									
Axillary Temperature (ºC)									
Urine Test for Pregnancy (Women, child bearing capacity)									
Parasite count Asexual count (per µl)									
Sexual count (per µl)									
Treatment No of tablets									
Time of Dosing									
Concomitant Treatment									
PQ treatment and Dose									
Rescue treatment as indicated									
Adverse effects if any (Y/N)									
If Yes, Name AE & Fill PV form									
Observations									

Overall assessment: Early treatment failure (ETF) | Late clinical failure (LCF) | Late parasitological failure (LPF) | Adequate clinical and parasitological response (ACPR) | Withdrawn (WTH) | Loss to follow-up (LOSS)

Day of assessment: Name of Investigator _____ Signature _____

Publications

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Residual Antimalarial Levels in *Plasmodium Falciparum* Malaria Patients from Selected Sites in India: An indication of Drug Pressure

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Abstract*: Irrational treatment practices and self-medication of antimalarials are common in malaria-endemic areas. Such types of practices may affect the treatment outcome as well as could promote the spread of drug resistance in the community. The aim of the study was to monitor residual antimalarials level in Plasmodium falciparum infected patients belonging to Bilaspur and Betul districts in India. A total of 139 patients were enrolled for the treatment of antimalarials as per national treatment policy, and their clinical follow-up was done as per WHO guidelines (2009). Heparinised blood was taken on 31ET filter paper for monitoring residual antimalarials on day 0 and post-treatment sulphadoxine levels on day 7 using High Performance Liquid Chromatography, 27.3% of the patients showed residual antimalarials on day 0. Surprisingly, the residual levels did not correlated well with the information collected on case record forms. A significantly lower parasite density/µl of blood was observed in samples having residual antimalarials than the samples without residual antimalarials (P<0.05). The residual antimalarials detected in the blood might be due to previous drug episodes, self drug intake, and irrational treatment practices. Awareness towards rational treatment practices are the need of hour.*

Introduction

Malaria imposes a substantial socio-economic challenge and together with six other diseases, it accounts for 85% of global infectious disease burden [1]. Annually, approximately 3.4 billion people worldwide expose to malaria, 1.2 billion are at high risk amongst them[2].India alone contributes 50% of the 2 million reported cases in the South-East Asia region; majority of which are reported from Odisha, Chhattisgarh, Jharkhand, Madhya Pradesh, Rajasthan and North-Eastern states in India[2, 3].

Unfortunately, the control of malaria is hampered due to development of antimalarial drugresistance emergence of antimalarial drug resistance in South East Asia region to commonly used antimalarial drugs particularly for *Plasmodium falciparum (Pf).* The resistance to anti-malarial drugs has also increased the global cost of controlling the disease, including the cost of new drug development. The north-eastern region of India has been the epicentre of antimalarial drug resistance in the past and the history of resistance against antimalarials in this region include chloroquine (CQ) resistance in *Pf* in Karbi Anglong district in Assam in the 1973 followed by sulphadoxine-pyrimethamine (SP) resistance in the same region in the1979[4, 5].Ineffective treatment regimen including the use of monotherapies with artemisinin, lack of compliance to the prescribed medications and intake of counterfeit medicines are associated contributing factors in promoting drug resistance[6,7]. Before 2005, CQ was first line therapy for *Pf* malaria in India. Despite the fact that ACT (AS+SP) became the first line treatment for uncomplicated *Pf* malaria cases in India[8]; SP, quinine (QN), mefloquine (MQ) and CQ were still found to be prescribed as well as circulating widely in the private health sectors [9]. A study conducted in six states of India revealed 14.8% prescription of artesunate (AS) monotherapy in Jharkhand, one of the malaria endemic site in the country [10].

The standard WHO protocol to monitor the efficacy of antimalarial medicines does not exclude patients with history of antimalaria intake [11], however, the intake of antimalarial drugs prior to inclusion in an *in-vivo* study may interfere with the estimation of treatment outcome because of accumulation of residual or sub-therapeutic levels of antimalarials. Standard case record forms were filled to ascertain the use of antimalarials prior to *in-vivo* efficacy

studies. However, with a recall period of a week or fortnight, this information was unreliable and needs further confirmation. Thus, this information might not give an accurate and complete picture of drug use in that area. Therefore, it is important to measure the antimalarial drugs level using field adapted methods. Globally, this information has been captured by a number of studies, including quantification of residual antimalarials such as CQ or SP in urine or blood in the general population or patients [12-14]. But to the best of our knowledge this is the first study report from highly endemic sites of malaria where simultaneous quantification of five residual antimalarials namely CQ, sulphadoxine (SDX), pyrimethamine (PYR), QN and MQ in human blood samples was done. In addition, association of residual antimalarial levels with parasite densities of *Pf* was also analysed. This work will highlight the use of over the counter residual antimalarial levels in the patients participating in *in-vivo* therapeutic efficacy studies in highly endemic regions of malaria

Materials and methods

Study sites

The study was carried out during the year 2011-2012 at Gaurella Community Health Centre at Bilaspur district, Chhattisgarh and Ghodadongri Primary Health Centre (PHC) at Betul district, Madhya Pradesh (Figure 1). In these areas, malaria transmission takes place round the year due to favourable environmental conditions supporting vector survival. *Pf* infections are predominantly recorded from October to December. Amongst the two study states, Chhattisgarh contributes 13.3% and Madhya Pradesh contributes 8.7% of annually reported malaria cases in the country with 2.2 % cases from Bilaspur district and 2.6% of cases from Betul district respectively [3]. At the time of the study, ACT (AS+SP) was the first line treatment for uncomplicated *Pf* malaria in these areas[8] and was available with Auxiliary Nurse Midwives (ANMs), Accredited Social Health Activists (ASHAs) and other community health volunteers. All the study procedures were conducted in accordance with the Institutional Ethics Committee of the National Institute of Malaria Research (NIMR), Ministry of Health and Family Welfare, Govt of India.

Figure 1. Map showing regions of Bilaspur (Chhattisgarh) and Betul (Madhya Pradesh)

Population screening and sample collection

Finger prick blood samples of 139 patients (70 from Chhattisgarh and 69 from Madhya Pradesh) in the age group of >6 months to ≤65yrs and infected with uncomplicated *Pf* malaria were collected on day 0 (D0). The inclusion/exclusion criteria for selection of patients was according to WHO protocol (2009) [11]. A written informed consent was obtained from all participating patients. From all eligible patients, a medical history was taken and clinical examination was made. Finger prick blood samples were used for counting parasite density on day 0 until 28 days of follow-up. Also, hundred microliters (100 µl) of heparinised blood was taken on 31ET filter papers (Whatman GE Health Care, UK) for monitoring residual drug on day 0 and SDX drug level on day 7. The filter papers were then allowed to dry at room temperature and stored in a zipper pouch with desiccant at 4°C in a refrigerator until analyzed. Treatment with standard first line therapy of AS+SP (ACT) was initiated in appropriate dosages according to age.

Treatment and follow-up

In this study, all eligible patients with non-severe malaria presenting at the outpatients clinic were enrolled for a 28-day follow up according to the WHO protocol [11]. All of them received oral doses of artesunate (AS) + sulphadoxine-pyrimethamine (SP) (age wise combi-blister pack) on day 0 and primaquine (PQ) (0.75 mg/kg bwt.) on day 2, as per the national guidelines[8]. Subjects were observed for 30 minutes after ingesting the drugs to ascertain

its retention. Those who vomited the first dose were retreated with an identical dose. Subjects who vomited twice were dropped from the study. Patients were asked to return for follow-up visits on days 1, 2, 3, 7, 14, 21 and 28 days. Parasitological assessment and temperature measurement was done during each follow-up visits.

Parasites identification and quantification
Thick and thin blood films were prepared and stained with 10% Giemsa solution .While thin film were studied for species identification, the parasite density was determined by counting the number of parasites in thick film per 200 white blood cells (WBCs), based on a mean WBC count of 8,000/µL. A slide was considered negative when no parasite was observed against count of 1000 white blood cell. Each of these slides were examined under a light microscope on days 0, 1, 2, 3, 7, 14, 21, 28 by experienced microscopist (s) at the site and were cross-checked for quality control at National Institute of Malaria Research, New Delhi by third experienced microscopist.

HPLC analysis
Baseline blood samples (day 0) collected from patients reporting no antimalarial intake prior to the study were screened for the presence of five antimalarial drugs such as CQ, SDX, PYR, QN and MQ using a modified HPLC method [15]. The level of partner drug (SDX) of AS+SP was also determined on day 7. Extraction of the standard drugs (CQ, QN, SDX, PYR and MQ; Sigma Aldrich, USA), blank whole blood spot (control sample) and each of the collected samples were carried out according to the protocol of Blessborn *et al*., 2010, with slight modification[15]. This involved the use of multi-mode solid phase extraction column (M-M SPE, Biotage, USA) and elution of the samples by methanol:triethylamine (97:3 v/v) mixture. Eluates were dried under a gentle stream of air at 70°C and were then dissolved in 100 µl of methanol:HCl (0.01 M) 10:90 v/v. Twenty microliter (20 µl) of each of these standards and samples were injected into the HPLC system. HPLC was performed on a Hitachi gradient system equipped with binary pump (Model L-2100/2130) and multi wavelength UV detector (Model L-2420 UV-VIS). Analytes extracted from the M-M SPE column were analyzed using two different mobile phases (A) acetonitrile:ammonium formate (20 mM in 1% formic acid) (5:95 v/v) and (B) acetonitrile:ammonium formate (10 mM in 1% formic acid) (80:20 v/v) and were run according to previously described gradient program[15]. The compounds were analyzed on a Tosoh ® 5 µm C_{18} (150 mm × 2 mm) column protected by a precolumn security guard C_8 (8mm x2 mm) (Tosoh Bioscience, PA). The UV detector was monitored at 280nm. Data acquisition and quantification were performed using Hystar™ and Data Analysis™ (Bruker, Bremen, Germany).

Estimation of dose intake time for sulphadoxine
To estimate the probable timing of drug intake, we compared the whole blood concentrations of SDX at baseline (C_0) and on Day 7 (C_7) after a complete treatment with AS+SP for the same patients. Assuming a terminal elimination half-life (t½) of 7.2 days for SDX, an inter-individual variability of 30% [12] and a similar dosage on pre-study exposure and during the study, a back-calculation was done to estimate the intake time of the drug before baseline sampling:

$$\text{Intake time} = \ln(C_7/C_0).t_{1/2}/\ln(2)+7[\text{days}]$$

The variability on t½ was used to estimate a 90% confidence interval around this intake time, considering plausible inter-individual variations in elimination rate [16]. Similar calculation was not attempted for pyrimethamine because of its short half-life period [12].

Statistical analysis
All statistical analyses were done using the SPSS software version 14. Geometric mean of parasite densities at 95% confidence interval (CI) was calculated. Frequencies were compared using the X^2 test. The differences were considered statistically significant at an error probability less than 0.05 ($p<0.05$).

Results

Baseline demographic data
A total of 139 (70 patients from Bilaspur and 69 from Betul) eligible patients with microscopy-confirmed *Pf* malaria and who satisfied the inclusion/exclusion criteria were enrolled in the study. Common symptoms of malaria such as fever, headache, chills/rigor and vomiting were reported by the patients at the time of recruitment. The total number of males and females included in the population study were 53.2% and 46.8% respectively. The patients were categorised on the basis of age as: Infants; < 5yrs (3.6%), 5-15 yrs (38.8%) and adults (57.6%). 62(88.6%) patients from Bilaspur reported to have not taken any prior course of antimalarial drug before the initiation of our study while 8(11.4%) patients from Bilaspur and 69 (100%) from Betul did not aware about the intake of any antimalarials. The clinical and demographic characteristics of these patients have been tabulated in **table 1**.

Detected levels of antimalarial drugs in population

The presence of antimalarial drugs above the detection limit (50 ng/ml) was observed in the blood of 38(27.34%) patients: 25(18.0%) had SDX, 17(12.2%) had CQ, 5(3.5%) PYR while 1(0.7%) each had QN and MQ (**Table 2**). On day 0, the median (range) blood concentration was 5300 ng/ml (100-54100ng/ml) for SDX, 180 ng/ml (51-263 ng/ml) for CQ, 900 ng/ml (100-1600 ng/ml) for PYR. QN and MQ residual level was observed to be 279 ng/ml and 367 ng/ml in one patient each (**Fig. 2 & Table 2**). Residual levels of SDX above the minimum inhibitory concentration (MIC) i.e. 20µg/ml were detected in six patients[17]. In addition, fourteen patients had residual levels of CQ more than the MIC (MIC; 90ng/ml)[18].

Among the 38 patients with residual drug concentrations, 9(23.7%) had residual level for more than one drug: 1(2.6%) patient had residual levels of SDX+PYR+CQ, 4(10.5%) patients had SDX+PYR, 1(2.6%) patient with SDX+QN+CQ while 3 (7.9%) patients had residual level of SDX+CQ concentration.

The SDX levels were also analysed on day 7 in blood samples of eighty six patients (38 with residual antimalarials and 48 without). The mean ±SD of SDX concentration for these patients was found to be 48.4±16.2 µg/ml (range: 15.3-99.1 µg/ml).

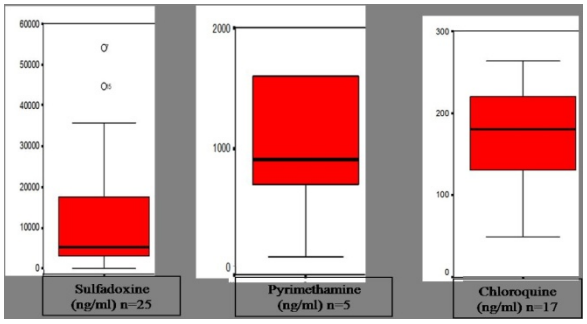

Figure 2: Residual blood concentrations of antimalarials present, before treatment (at day 0) in 38 patients with *P. falciparum* malaria from Bilaspur (Chhattisgarh) and Betul (Madhya Pradesh). The number of patients, median, 25th and 75th percentiles, lower and upper adjacent values, and outlier values are shown for sulphadoxine, pyrimethamine, and chloroquine. The data for mefloquine and quinine is not shown here because of the small sample size (n=1) for both

Probable time of previous SDX intake

After comparing the whole blood concentration of SDX at baseline (day 0) and on day 7, the back estimation method indicated a median of 30 days prior to study enrolment (range 6-71 days; 90% CI), the most likely time for previous SDX intake. Majority of the patients i.e. 13 (52%) showed previous SDX intake estimated time of more than 28 days.

Table 1: Clinical and demographic characteristics of patients (n=139) enrolled in studies

Study Site	**Bilaspur (Chhattisgarh)**	**Betul (Madhya Pradesh)**
Characteristic	(N=70)	(N=69)
Sex [no. (%)]		
Male	44 (62.9)	30 (43.5)
Female	26 (37.1)	39 (56.5)
Age category [no. (%)]		
< 5 yr	2 (2.9)	3 (4.3)
5-15 yr	12 (17.1)	42 (60.9)
Adult	56 (80.0)	24 (34.8)
Temperature (˚C)		
Mean ± SD	37.8 ± 1.1	37.8 ± 0.1
Range	36.1-40.6	37.5 - 38.1
Febrile (≥37.5)		
Yes [no. (%)]	36 (51.4)	69 (100.0)
No [no. (%)]	34 (48.6)	-
Parasite count (no/µl)		
Mean ± SD	233 59.8 ± 17820.4	12168. 6 ± 11561.9
Gametocytes on day 0		
[no. (%)]	3 (4.3)	2 (2.9)
Previous antimalarial intake		
Yes [no. (%)]	0 (0.0)	0 (0.0)
No [no. (%)]	62 (88.6)	0 (0.0)
Unknown [no. (%)]	8 (11.4)	69 (100.0)

CRF information & its correlation with residual antimalarial drugs

History of previous drug intake was collected using case record form (CRF) for each patient. This information was correlated with detected residual antimalarial levels in the patient's samples. The CRF information showed that 62(44.6%) patients reported no antimalarial intake, while 77 (55.4%) were not sure about the previous intake. However, HPLC analysis revealed that 38 (27.3%) patients had residual antimalarials in their blood while 101 (72.7%) patients did not have residual antimalarials levels on day 0 before the onset of treatment course **(Fig.3).** A weak association was observed between the information provided in CRF about theantimalarials intake and baseline residual antimalarials detected before initiation of the treatment ($\chi^2 = 1.27$; P=0.17).

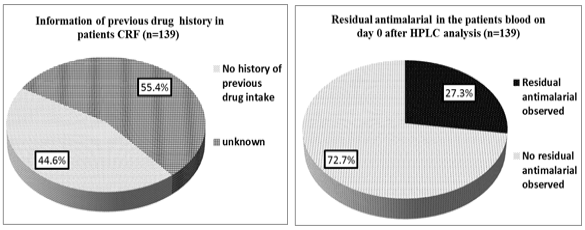

Figure 3: Comparison of CRF information of previous drug history and after HPLC analysis

Residual antimalarials: correlation with parasite densities, age and sex

Parasite density (Mean ± SD) in *Pf* infected patients was found to be 17804.4 ± 16005.9 asexual parasites/µl (range: 616-74040). Parasite densities were comparable between the patients with residual antimalarial concentrations (n=38) and those with no antimalarials (n=101) {15522.0±13020.1 asexual parasites/µl Vs 18203.2± 16733.2 asexual parasites/µl, $p<0.05$} on day 0. Residual antimalarial levels was found to be negatively correlated with parasite density and statistically non-significant (r=-0.47, p=0.778). The influence of age and sex on the probability of detecting residual antimalarials at baseline showed no significant relationship (X^2=23.0; *P*=0.10).

Table 2: Residual concentration of antimalarial drugs in blood samples of 38 patients from Bilaspur and Betul regions at Day 0, before the onset of treatment

Antimalarials	No. of patients	ng/ml			
		Mean	Median	Minimum	Maximum
Sulphadoxine	25(18%)	1341 4.8	5300 .0	100.0	54100. 0
Chloroquine	17(12.2%)	172. 3	180. 0	51.0	263.0
Pyrimethamine	5(3.5%)	980. 0	900. 0	100.0	1600.0
Quinine	1(0.7%)			-	-
Mefloquine	1(0.7%)			-	-

Table 3: Parasites clearance time with presence of residual antimalarials (alone/ combinations) and without residual of antimalarial.

Previous drug intake(Y/N)		Percentage of patients showing Parasites clearance time in days			
		Do (0 hr)	D1 (<24 hr)	D2 (≥24 to<48)	D3 (≥48 to <72hr)
Single drug(n=29)	SDX(n=16)	0	81.2	100	100
	CQ(n=12)	0	83.3	100	100
	MQ(1)	0	100	100	100
Combination of drug (n=9)	SDX+ PYR(n=4)	0	100	100	100
	SDX+ CQ(n=3)	0	100	100	100
	SDX+CQ+ QN(n=1)	0	100	100	100
	SDX+PYR +CQ(n=1)	0	100	100	100
No residual antimalarial present(n=101)		0	87.1	99.0	100

Treatment outcome and follow-up

Among the 139 patients enrolled in this study, 1 patient voluntarily withdrew on day 2, and 2 patients were lost to follow-up on day 7; while, remaining 136 study participants completed the 28-day follow-up. Among them, 120(86.3%) patients cleared their parasitemia in less than 24 hrs, 18(12.9%) cleared between 24 hrs and 48 hrs, while remaining 1(0.7%) cleared between 48 hrs and 72 hrs. 100% parasite clearance was observed in all the patients by day 3. There was no reappearance of parasitemia in these patients during follow-up till day 28, indicating adequate clinical and parasitological response (ACPR). Interestingly, On day 2 parasite clearance was observed in the patients detected with residual level of SDX and CQ; complete parasite clearance was observed on day 1 in the patients who had combinations of residual antimalarials; whereas, parasitemia was seen up to day 2 in case of the patients with no residual levels of antimalarials. The percentage of patients showing parasite clearance on day 1 was 81.2 and 83.3 for SDX and CQ respectively (**Table 3**).

Discussion

To our knowledge this is the first study to investigate the presence of five antimalarials (CQ, SDX, PYR, QN and MQ) in blood of patients from Bilaspur (Chhattisgarh) and Betul (Madhya Pradesh) districts with high *Pf* malaria before the onset of treatment. Out of these five antimalarial drugs, CQ is currently recommended as first line antimalarial for uncomplicated *P. vivax* (*Pv*) malaria cases in the country, while SP is being recommended as partner drug in the recommended ACT for the treatment of uncomplicated *Pf* malaria throughout the country except in the north-eastern India. In this region, artemether-lumefantrine is the first line of treatment for *Pf* in the north-east India, as high treatment failure to SP was observed during 2012-13[19]. Quinine is recommended as rescue for case where treatment failure cases to ACT is observed while MQ is advised for long term chemoprophylaxis. The measurement of these five antimalarials allowed a comprehensive assessment of the circulating drugs in the community under study.

Malaria is a major public health problem in India and its dynamics vary from place to place. The forest and tribal areas of Madhya Pradesh and Chhattisgarh situated in central part of India where malaria outbreaks are frequently recorded; its control is logistically difficult reason being the inadequate surveillance, poor reporting, a time-lag in reporting to decision makers and a poor geographic information system that could identify the trouble spots for a timely preventive action. Both *Pf* and *Pv* are prevalent species in these states with a preponderance of *Pf*. Drug resistance is a major problem for control and eradication of malaria. Besides the genetic factors, drug pressure in the community also plays a major role in emergence of drug resistant parasites [12]. To assess the drug pressure, residual level of antimalarials was monitored by HPLC in the patients enrolled at selected study sites under therapeutic efficacy studies.

We found that out of 139 patients enrolled in our study, 38(27.34%) carried blood residual antimalarials above the lower limit of detection (50 ng/ml) at inclusion[13]. This could be a worrying factor and a possible indication of tolerance to residues which could act as a precursor for the development precipitation of resistance. This is a matter of concern and the issue needs to be carefully monitored at state as well as national level.

Sulphadoxine-Pyrimethamine (SP)

SP is currently being used as a partner drug in first line ACT (Artesunate+Sulphadoxine-Pyrimethamine) for uncomplicated *Pf* malaria in India since 2010, except in the north-eastern India as mentioned elsewhere. In our study, we found residual level of SDX in blood of 25 (18%) patients on day 0 at a mean concentration of 13.4µg/ml. This finding corroborates with the results obtained from the study in which residual plasma concentration of antimalarials was detected prior to the treatmen[12]. The SDX level in ninety six patients on day 7 was found to be 48.4±16.2 µg/ml. PYR is usually given as a combination therapy with SDX. 5(3.5%) patients in our study were found to have a mean concentration of 0.98 µg/ml of PYR in their blood. PYR is usually given at a lower dose of 1.25 mg/kg in combination with SDX and its half-life is approximately 4 days; than that of SDX which has a half-life of 7.2 days[12, 20]. In present study, the mean concentration obtained for SDX and PYR was comparatively higher than the reported study; but there was no conspicuous difference in the concentration range of the residual antimalarials[12]. There are various factors which might be responsible for such differences the in mean concentration of residual antimalarials: (1) Differences in the Samples (dried blood spots) extraction procedure (2) Instrumentation technique used i.e. they have used liquid chromatography-tandem mass spectrometry which is highly sensitive as compared to HPLC used in the present study.

Based on the day 0 and day 7 SDX concentration (C0 and C7), and assuming that the patients with residual SDX in their blood had taken a single dose of SP according to their body weight, we can infer by using back estimation method that these patients must have taken SP approximately within one month (30 median days) prior to inclusion in our study [12]. Furthermore, it is also possible that patients might have taken a sub-therapeutic dose or counterfeit form of SP more recently[21].

Chloroquine (CQ)

Although, CQ has been withdrawn to be used as first-line of treatment for *Pf* infection [22] ; it was detected in the blood of 17 (12.2%) patients in our study at a mean concentration of 0.172 µg/ml. Hodel *et al.,* 2010 also reported the residual plasma concentration of CQ, MQ and QN before initiating the antimalarials treatment in patients[13]. This might be due to easy availability of CQ in the private clinics and pharmacy shops or due to its wide use in the treatment for a previous episode of *Pv* malaria. Previous study from our laboratory has reported that CQ is the most common antimalarial sold across the counter with high frequency of prescription in public as well as private health facilitiesas compared to other antimalarials[10].

As we employed dried blood spot method for sample collection, it was not possible to detect any artemisinin compounds. Also, short half-life of artemsinin requires sophisticated techniques as well as plasma sample collection. However, the presence of residual artemisinin cannot be ruled out as high prescription rate of this antimalarial has been previously observed in the country [10].

Both MQ and QN are not recommended for uncomplicated malaria, but their residual levels were still found to be present in the patients, although in very few samples and that too at low concentrations.

By detecting the presence of residual anti-malarial levels on day 0,it can concluded that this be due to; (1) Self-medication due to non-availability of reachable drug to the dispensing facility or the practitioner leading to inadequate dosing and there by inability to control infection[12, 13, 23-25] (2) Irrational treatment practices by the physician at the study sites with high transmission intensity of malaria parasites, where all febrile patients were treated with a variety of available antimalarial drugs[26-27], (3) Unawareness regarding the suitable antimalarial drug to be used for treating malaria (4) Treatment of previous episode of infection with antimalarial drug after then re-infection with a new parasite resulting of previously consumed episode of drug as residue in

blood [28-29]. These factors contribute to increased drug pressure on the parasite thus encouraging resistance in Plasmodium species [30]. With these observations, it can be deduced that the entry criteria based on self reporting of previous drug intake or information collected in case record forms are not reliable at least in this population.

The influence of age and sex on the probability of residual antimalarials at entry showed no significant relationship in our study (X^2=23.0; *P*=0.10) indicating uniform antimalarial prescription or intake behaviour in the population. These estimates are approximate, as indicated by the wide confidence interval explained by the fair degree of inter-individual variability and residual error. The correlation between the parasite density and levels of antimalarial drugs was also studied. We found that 15522 asexual parasites/µl (P<0.05) were present in blood samples with residual antimalarials, while 18203.2 asexual parasites/µl (P<0.05) were present in those without residual antimalarials. This clearly showed that the residual levels of drug in blood were not enough to control parasite replication and to overcome clinical symptoms in the patients, although the parasitemia levels were little lower in the patients with residual antimalarials than without. Here it is difficult to comment whether the residual drug levels were due to the result of full or incomplete treatment and the parasites causing the current episode of infection were from the same or a new infection.

Residual levels of SDX and CQ above MIC were observed in 24% and 82.3 % patients respectively. This shows that the accessibility to CQ was more as compared to SDX and thus people could easily buy CQ for the treatment of malaria episode. Also, earlier presumptive treatment with CQ was practiced in the country to treat febrile episodes of malaria. This suggests that despite drug policy change at the national level and introduction of ACT for treatment of falciparum malaria, CQ is still being widely used for tackling this species.

One hundred and thirty six patients (136) out of a total of 139 recruited in the present study completed the 28-days follow-up after treatment with AS+SP. No microscopically visible parasitemia was observed in any patient on day 3. There was no reappearance of parasites up to 28 days of follow-up. This suggests that AS+SP was 100% efficient in treating uncomplicated *Pf* malaria in patients and it showed adequate clinical and parasite response (ACPR).

Prior drug intake may have an effect on the current treatment in ways that; it may lead to increased drug exposure from cumulative levels resulting either in better efficacy or more toxicity. The prevalent parasitaemia in the presence of residual antimalarials at the time of patient enrollment suggested that these clones causing the disease were already thriving in the patients and constituted less sensitive population selected by the previous treatment, which may also impact, in addition to precipitation of resistance, on the outcome of the treatment under investigation.

Conclusion

The intense parasite transmission and the widespread presence of sub-curative drug levels in blood most likely constitute a predisposing environment for the selection and spread of resistant *Pf* strains. As resistance gradually expands and intensifies, it is also likely that the drug is taken more frequently and at higher doses. As a consequence, more intense drug pressure will then select for more resistant parasites. The chances of drug resistant parasites to be selected depends on several factors, and is higher for patients with lower immunity (e.g., young children), drugs with long residence times and resistance being conferred through single point mutations, and for infections with a large parasite biomass. In this scenario, the efficiency of treatment outcome also cannot be evaluated effectively as the clearance of parasites may be due to an additive effect of the current intake of treatment drug and the residual drug taken during previous episodes of malaria infection. To minimize the burden of drug resistance in the country, it essentially requires awareness in the community as well as amongst the physicians and paramedical staff on national drug policy for treatment of malaria.

The findings of this study are revealing and must be confirmed in other settings as they have potential implications for both clinical research, surveillance (treatment efficacy and safety outcome) and malaria control policy.

Conflict of interest

The authors declare that they have no conflict of interest.

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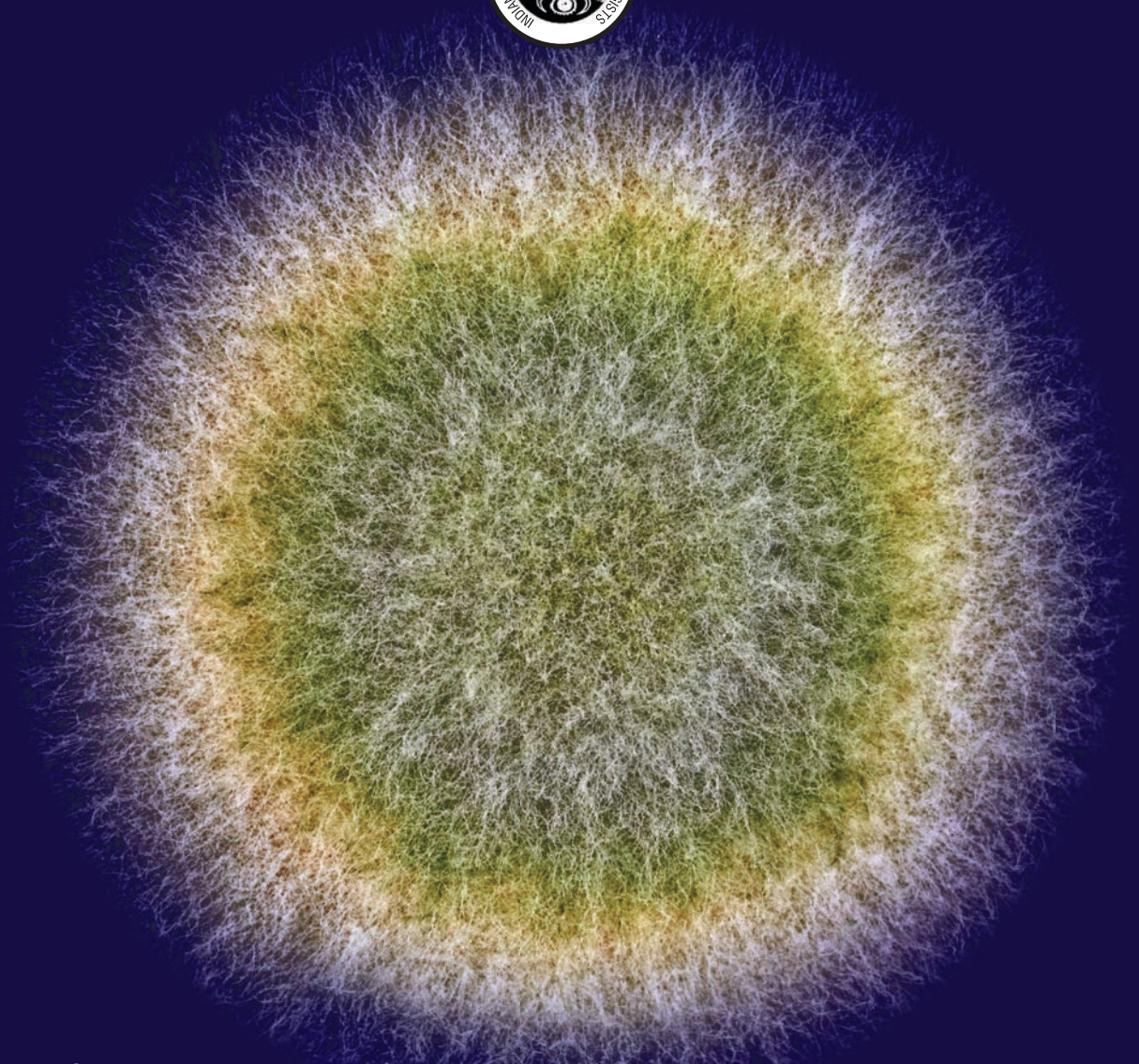

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Brief Communication

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

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

Abstract

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

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

Introduction

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

Materials and Methods

In vitro assay

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

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

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

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

Data management and statistical analysis

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

Results

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

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

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

Discussion

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

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

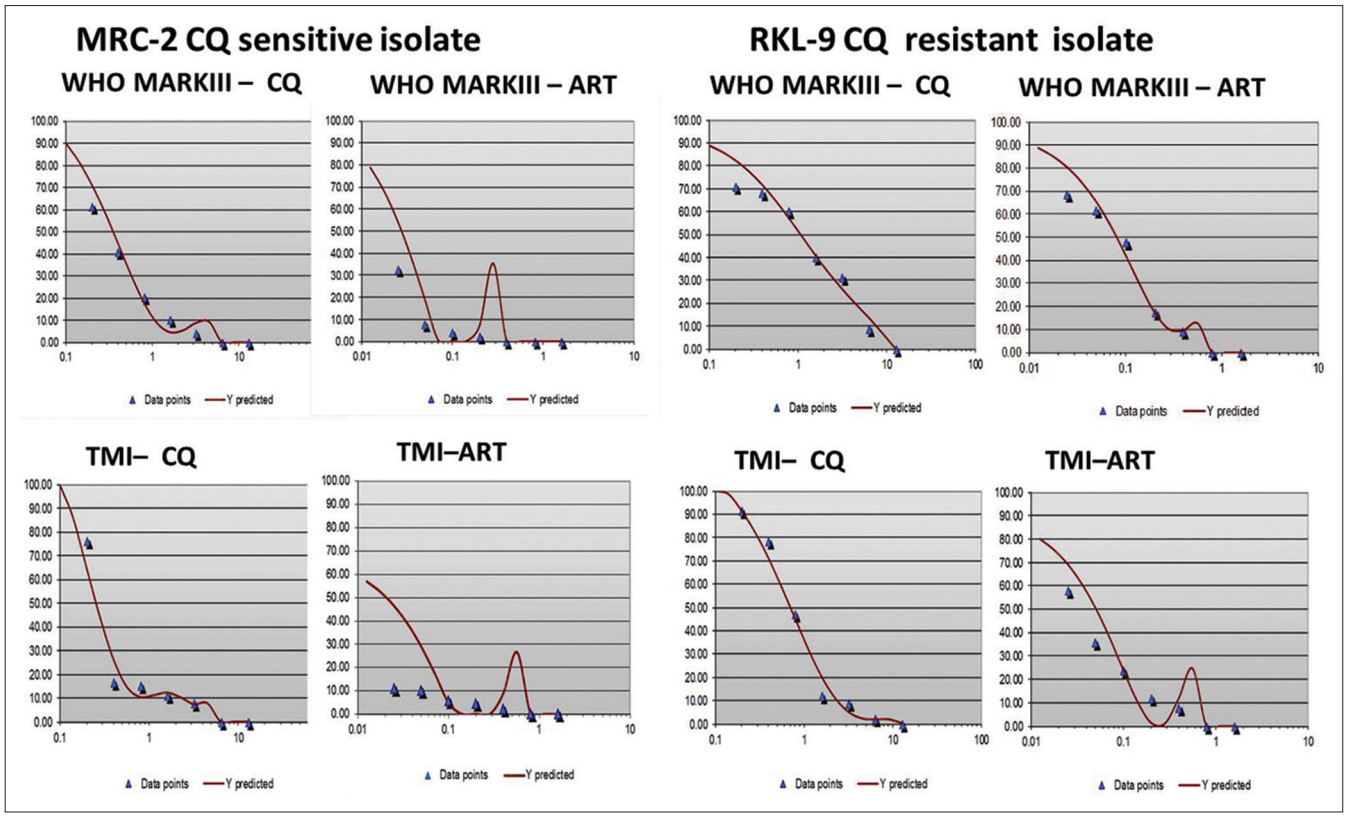

Figure 1: Comparison of TMI and WHO MARK III assay for CQ resistant and sensitive isolates

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

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

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

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Conflicts of interest

There are no conflicts of interest.

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Monitoring the efficacy of antimalarial medicines in India *via* sentinel sites: Outcomes and risk factors for treatment failure

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ABSTRACT

Background & objectives: To combat the problem of antimalarial drug resistance, monitoring the changes in drug efficacy over time through periodic surveillance is essential. Since 2009, systematic and continuous monitoring is being done through nationwide sentinel site system. Potential early warning signs like partner drug resistance markers were also monitored in the clinical samples from the study areas.

Methods: A total of 1864 patients with acute uncomplicated malaria were enrolled in therapeutic efficacy studies of artesunate plus sulphadoxine-pyrimethamine (AS+SP) for *Plasmodium falciparum*; those infected with *P. vivax* were given chloroquine (CQ). Polymerase chain reaction (PCR) was used to distinguish post-treatment reinfection from treatment failures. Isolates of *P. falciparum* were also analysed for dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) gene mutations.

Results: Overall, 1687 (91.7%) patients completed the follow-up. In most of the falciparum patients the parasitaemia was cleared within 24 h of treatment, except 12 patients who remained parasite positive after 72 h. Presence of *dhfr* and *dhps* quintuple mutation was observed predominantly in treatment failure samples. A daily dose of artesunate of < 3 mg/kg of body weight, age of <5 yr, and fever at enrolment were associated with an increased risk of treatment failure. The AS+SP in *P. falciparum* was effective in > 95% cases in all the sentinel sites except in Northeastern region (NE). Chloroquine remained 100% efficacious in case of *P. vivax* infections.

Interpretation & conclusion: Till 2012, India's national antimalarial drug resistance monitoring system proved highly efficacious and safe towards first-line antimalarials used in the country, except in Northeastern region where a decline in efficacy of AS+SP has been observed. This led to change in first-line treatment for *P. falciparum* to artemether-lumefantrine in Northeastern region.

Key words Artemisinin-based combination therapy; artesunate+sulphadoxine-pyrimethamine; chloroquine; dihydrofolate reductase (*dhfr*); dihydropteroate synthase (*dhps*); *Plasmodium falciparum*; *P. vivax;* primaquine

INTRODUCTION

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Antimalarial drug resistance in *Plasmodium falciparum* has been a major contributor to resurgence of malaria in the last two decades[1]. Southeast Asia (SEA) has remained the epicentre of drug resistance for all antimalarials in the past and valuable antimalarial drugs have become ineffective due to development of resistance in parasites over a period of time. Chloroquine (CQ) resistance in *P. falciparum* malaria was first reported in 1957 in SEA region, later followed by sulphadoxine-pyrimethamine (SP) resistance after 10 yr[2–4]. India shares international boundaries with countries known to be the epicentre for antimalarial drug resistance. India too experienced increase in falciparum malaria cases with resistance to first-line antimalarial CQ in 1973 in Northeast India which later spread to other areas in the country[5].

Increasing resistance to CQ forced the national programme to abandon CQ for treatment of *P. falciparum*[6–8]; however CQ remains the first-line antimalarial treatment for *Plasmodium vivax.* In 1995, a single dose of sulphadoxine-pyrimethamine (adult single dose 1500/75 mg) was recommended for the treatment of falciparum malaria in chloroquine-resistant areas[9], however, resistance to SP was observed in early 2000[10].

Since 2005, treatment of uncomplicated *P. falciparum*

malaria in the country is based on artemisinin-based combination therapy (ACT), following the recommendation of World Health Organization (WHO)[11]. The ACT recommended in the country consists of artesunate plus SP (AS+SP).

Since 2009, systematic and continuous monitoring of recommended antimalarials has been carried out through nationwide sentinel site system[12]. In 2009–10, the efficacy of AS+SP against falciparum malaria was high (94–100%) and the efficacy of chloroquine for treatment of vivax malaria was 100%[13]. However, focal resistance to SP has been reported in the country from time-to time[10], putting the use of AS+SP in danger. The mechanism of SP resistance has been well documented compared to other antimalarials[14]. Point mutations in the genes coding essential enzymes in the folate biosynthesis pathway[14] serve as potential early warning signs of drug resistance. These include point mutations in the dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) genes which were also monitored in selected study areas.

This study was a part of nationwide sentinel site system initiated in 2009 with the aim to address the continued problem of antimalarial drug resistance in the country. Continuous monitoring of ACT efficacy has been carried out at the 29 identified sentinel sites[12]. This study reports the findings of the clinical as well as molecular studies at these study sites in the endemic regions of the country.

MATERIAL & METHODS

Study sites

The study sites (Fig. 1) were from five eco-epidemiological regions which included Northeastern (NE) region with sites from Mizoram, Tripura, Assam and Arunachal Pradesh states. These sites contribute 71.4% of malaria cases with 0.2% deaths in the region. The eastern region included sites from Jharkhand, Odisha and West Bengal states having annual parasite incidence (API) rate higher in Odisha (6.2) followed by Jharkhand (3.9) and West Bengal (0.6). In the central region, study sites were included from Chhattisgarh and Madhya Pradesh states with API of 4.8 and 1 respectively. The southern region included sites from Andhra Pradesh and Karnataka states with low API (< 0.5) among the study sites. In the western region, studies were conducted in six districts namely Silvassa, Surat Ahmedabad, Baran, Gadchiroli and Mumbai from the four states, *i.e.* Dadra & Nagar Haveli, Gujarat, Rajasthan and Maharashtra

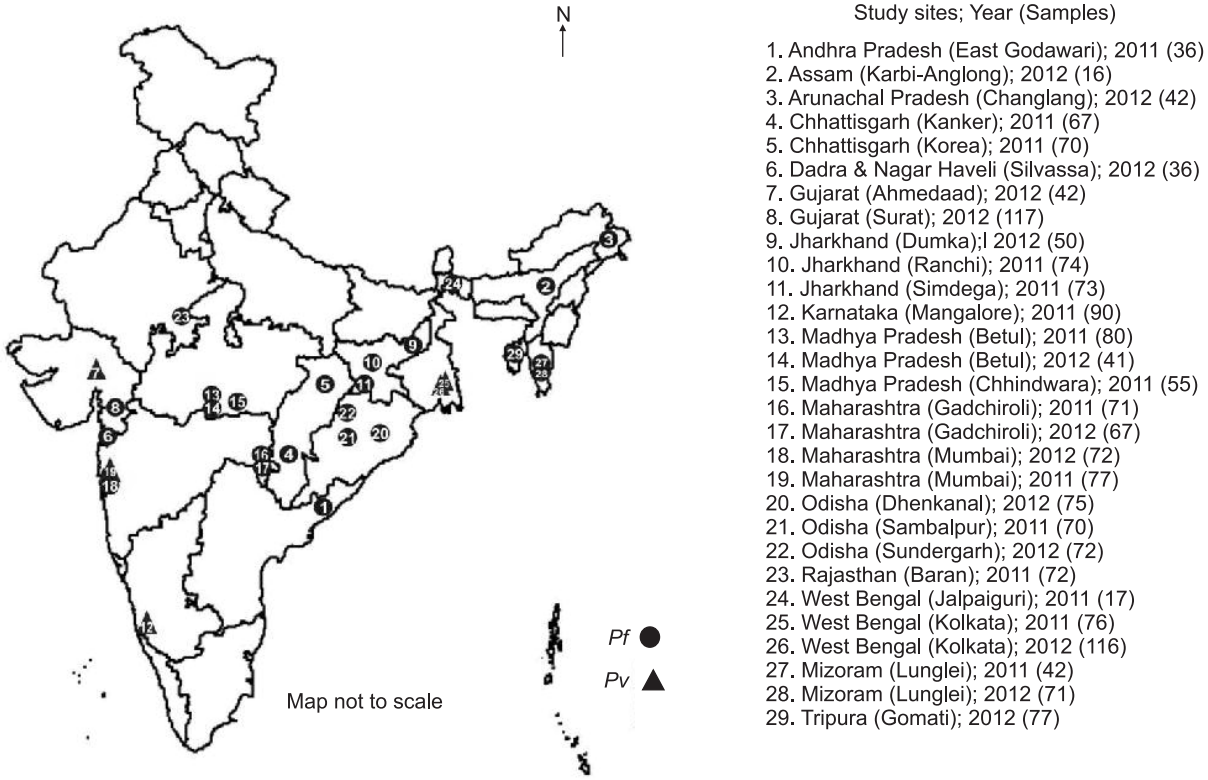

Fig. 1: Details of study site from the national antimalarial drug resistance monitoring system, India, 2011–12.

respectively. These include sites, with the API ranging from 0.5 to 13.6. The highest API was observed in Dadra & Nagar Haveli[15].

Study design

It was an open label one arm prospective study of clinical and parasitological responses after administration of ACT treatment; and WHO protocol for *in vivo* monitoring was followed[16].

Ethical considerations

The Ethics Committee of the National Institute of Malaria Research (NIMR), New Delhi approved the study protocol. Written informed consent or assent was obtained from the patients or guardians of the children before inclusion in the study.

Study population and sample size

The patients included were adults and children ≥6 months of age presenting with fever (axillary temperature ≥37.5°C) at the time of visit or a history of fever for the preceding 24 h. Patients with *P. falciparum* monoinfection (500–100,000 asexual parasites per µl of blood) or *P. vivax* monoinfection (>250 asexual parasites per µl) were enrolled in these studies. Pregnant or lactating women, children with body weight <5 kg, patients with sign of severe malaria, adults who declined to give informed consent and children with legal guardians who declined to give consent were excluded.

As these studies have been conducted in accordance with the WHO 2009 protocol, the target sample size of at least 50 patients per site, based on an expected proportion of treatment failure rate < 15% with desired confidence level (95%) and precision (5 or 10%) were enrolled from each site[16]. At few sites (n = 8), the recruitments of patients was low due to non-availability of patients fulfilling inclusion criteria.

Treatment and follow-up

The study followed the standard WHO protocol for assessment of therapeutic efficacy of antimalarial drugs for uncomplicated falciparum malaria for moderate transmission[16]. At enrolment (*i.e.* on Day 0), a complete medical history, *i.e.* presenting symptoms, current medications and previous antimalarials drug use was recorded for each patient. Female patients of child bearing age were tested for pregnancy. A pre-treatment blood sample was collected from each patient enrolled in the study and used to make thick and thin smears and dried blood spots on filter paper. Patients were then given doses of antimalarial drug according to their age, as recommended in the national drug policy[17]. Patients infected with *P. falciparum* were given AS+SP (the recommended dose is 4 mg artesunate per kg of body weight, for three days, plus single dose of SP that is equivalent to 25 mg sulphadoxine per kg and 1.25 pyrimethamine per kg) with primaquine on Day 2 (0.75 mg per kg body weight). Patients infected with *P. vivax* received chloroquine for three days (the recommended dose is 25 mg per kg for three days and then after completing the 28 days of follow-up, primaquine for 14 days (at the recommended dose of 0.25 mg per kg body weight). The study team ensured the compliance to treatment on all three days and quality assured drugs were used through state government supply.

Patients were observed for few minutes after administering the study drug to ensure that they did not vomit. Repeat dose was given to the subjects who vomited within 30 min. At 15 sites follow-ups were scheduled for Days 1, 2, 3, 7, 14, 21, 28, 35 and 42 (28 days follow-up at 15 sites including five *P. vivax* study sites and 42 days of follow-up at 14 sites). On each of these days, clinical and parasitological assessments were made. For genotyping and molecular marker studies, 2–3 hanging drops of blood samples were obtained on 3 mm Whatman filter papers on Day 0 and every other day of follow-up. Polymerase chain reaction (PCR) based genotyping was done for distinguishing recrudescence from new infections by comparing merozoite surface proteins (*msp1*, *msp2*) and glutamate rich protein (*glurp*) gene loci of pre- and post-treatment sample pairs. The outcomes of treatment with PCR correction were based on the number of true recrudescence excluding cases of new infections. In addition, *pfdhfr* and *pfdhps* mutations for partner drug (SP) resistance were used as molecular markers to analyze different samples.

Laboratory based analyses

Thick blood films were collected and stained with Giemsa. All slides were examined on the same day by experienced microscopist(s). Slides were also prepared on Days 1, 2, 3, 7, 14, 21, 28, 35 and 42 to determine parasite density by counting the number of parasites against 200 WBCs, and were expressed assuming WBC count to be 8000/µl. A slide was considered negative when counting 1000 WBC in thick smear did not show asexual parasites. Quality check of blood smears from study sites was done as per standard WHO guidelines, which included 10% random selection and cross check of all the treatment failure samples. Cent percent cross check of slides was done where unusual high treatment failures were observed during the study period.

Paired blood samples of patients collected on Day 0

and the day of recurrent parasitaemia (14–42 days) were analyzed sequentially starting with the highest discriminatory marker for genotyping *msp2* or *glurp*. The third marker analyzed was *msp1* to differentiate recrudescence from new infections[18]. A new infection is a subsequent occurring parasitaemia in which all the alleles in parasites from the post-treatment sample are different from those in the admission sample, for one or more loci tested. In a 'recrudescence' at least one allele at each locus is common to both paired samples[18]. In addition, SP resistance associated markers, namely mutations at codon 51, 59, 108 and 164 of *pfdhfr* gene and at codon 436, 437, 540, 581 an 613 of *pfdhps* gene were also analyzed on the Day 0 samples.

Genomic DNA was isolated from blood spots using QIAamp DNA mini kit, Germany. Genotyping PCR assays were carried out following the protocols reported earlier[19]. Separate nested PCR reactions were performed for the three allelic families of *msp1* (MAD20, K1, and RO33), two allelic families of *msp2* (Fc 27 and Ic) and *glurp* marker gene. The *pfdhfr* and *pfdhps* gene products were PCR amplified using earlier reported methods[20] and then digested using restriction enzymes for *dhfr* and *dhps* genes. Applied Biosystem thermocycler was used for all PCR amplification reactions. Digested PCR product (5–8 µl) was analyzed on 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) and 0.5x TBE running buffer (pH 8.0). PCR products were visualized under UV transilluminator (280 nm) and digitally captured with the help of gel documentation system (Alpha Imager EP). Molecular sizes of PCR fragments were calculated using gene tool (Alpha Inotech EP, version 3.0; 302 and 365 nm wavelengths).

Rescue medication

Due to treatment failure or drug resistance, some *P. falciparum* infected patients may not respond to treatment. In such instances, alternative ACT or quinine with doxycycline is recommended by the national drug policy[17]. However, doxycycline is contraindicated in pregnant and lactating women and children aged up to 8 yr. Thus, all the patients with early treatment failure (ETF) or late treatment failure (LTF) were treated with standard recommended antimalarial medicines in the national programme.

Data management

Data entry: The case record forms (CRF) were completed for each patient and all the clinical and parasitological data from Days 0 to 42 were recorded. Close monitoring of parasite clearance time (PCT) up to Day 3, *i.e* ≥72 h was done and patients who were found positive at ≥72 h and showed treatment response as ETF, were administered rescue treatment as per national policy. The data were entered in WHO software (WHO, 2009 version 7.2) and both per protocol and Kaplan-Meier analysis were performed to classify response as ETF, LTF and adequate clinical and parasitological response (ACPR). In the secondary analysis, patients were withdrawn or censored, if classified as new infection by PCR or if PCR was missing. The primary study end points were classified according to the standard criteria defined in recent WHO's guidelines[16] and parasite clearance intervals (< 24 h, 24 to < 48 h, 48 to < 72 h and ≥ 72 h) were used as secondary end points.

Data analysis: Patient's demographic variables that could be associated with treatment failure were investigated. Body weights recorded on Day 0 were used for calculating the doses of artesunate given to patients with *P. falciparum* and were categorized as being ≥ 87.5, < 87.5 to 75% and < 75% of the recommended dose per kg. The PCT was recorded in CRF till Day 2 of the treatment, although slides were made on Day 3 as well. However, the delayed PCT (PCT ≥72 h) was calculated on Day 3 based on the mean PCT up to Day 2 ± SD.

Kaplan-Meier survival analyses of treatment failure and parasite clearance were conducted with and without including the results of the PCR-based identification of the parasitaemia that resulted from post-treatment reinfection. Log-risk models were used to evaluate the univariate and multivariate associations observed between risk factors and end-points.

Multivariate analysis was based on full models that included age, sex, fever at enrolment, level of parasitaemia at enrolment, infection with a parasite that harboured any of the investigated mutations in *dhfr* or *dhps,* and artesunate dose, as well as the interactions between age and artesunate dose, age and level of parasitaemia at enrolment, and between presence of mutations and level of parasitaemia at enrolment. Geometric mean of parasite densities at 95% confidence interval (CI) was calculated and correlation was used to find the association between the dose of artesunate and treatment failure. A strategy of backward elimination was followed in which *p*-value of <0.10 and <0.15, respectively, were used as the cut-offs in eliminating individual factors and interaction terms as statistically significant risk factors for treatment failures. All data analysis was performed using a software package developed by WHO's Global Malaria Programme, version 7.2 for evaluating therapeutic efficacy (WHO, Geneva, Switzerland) and SPSS version 17.

RESULTS

Patient enrolment and characteristics

Overall, 1864 patients were enrolled, of which 970 patients were from 15 sentinel sites between August 2011 and January 2012; and 894 patients from other 14 sites between June 2012 and December 2012 (Fig. 1). Twenty five patients subsequently withdrew their consent or could not satisfy the inclusion criteria. Out of 1839 eligible patients, 1687 (91.7%) completed the follow-up (Fig. 2). Most of the patients investigated were males who were febrile at the time of enrolment (Table 1). A history of fever was nearly universal but the intake of antimalarial drugs in the previous episode was observed only in one patient. The geometric mean level of asexual parasitaemia on Day 0 was 10,142 parasites per µl (range: 520–100,000) for *P. falciparum* and 4229 parasites per µl (range: 368–98,452) for *P. vivax*.

Plasmodium falciparum

Primary and secondary end points: Of the 1438 patients infected with *P. falciparum* who completed follow-up (Table 2), 73 were initially categorized as treatment failures. However, when parasites in paired dried blood spots from these 73 patients were genotyped by PCR, only 39 (55.7%) patients were confirmed as treatment failure, 27 were found to have *P. falciparum* reinfection and seven had *P. vivax* infections on Day 21, 28 and 35 during follow-up or after quality check. Out of total 39 treatment failures, majority were observed up to Day 28; 9 on Day 21 and 4 on Day 28. Subsequently, other treatment failures were observed on Day 35 (n = 3) and Day 42 (n = 6).

Three ETFs were observed in Surat (Gujarat); all three patients had parasitaemia and fever on Day 3. Also four ETF cases were observed in Gomati (Tripura); two had parasitaemia and fever on Day 3 while two had parasitaemia on Day 2 where parasite count on Day 2 was >25% of Day 0.

The site wise crude and PCR-corrected Kaplan-Meier survival estimates are shown in Table 2. The 28-days follow-up showed 100% efficacy of AS+SP in *P. falciparum* malaria patients at majority of the sites which include East Godavari, Karbi-Anglong, Kanker, Korea, Dumka, Ranchi, Simdega, Betul, Chhindwara, Gadchiroli, Mumbai, Sundergarh, Sambalpur, Baran and Jalpaiguri districts. However, the efficacy at Surat, Silvassa, Betul

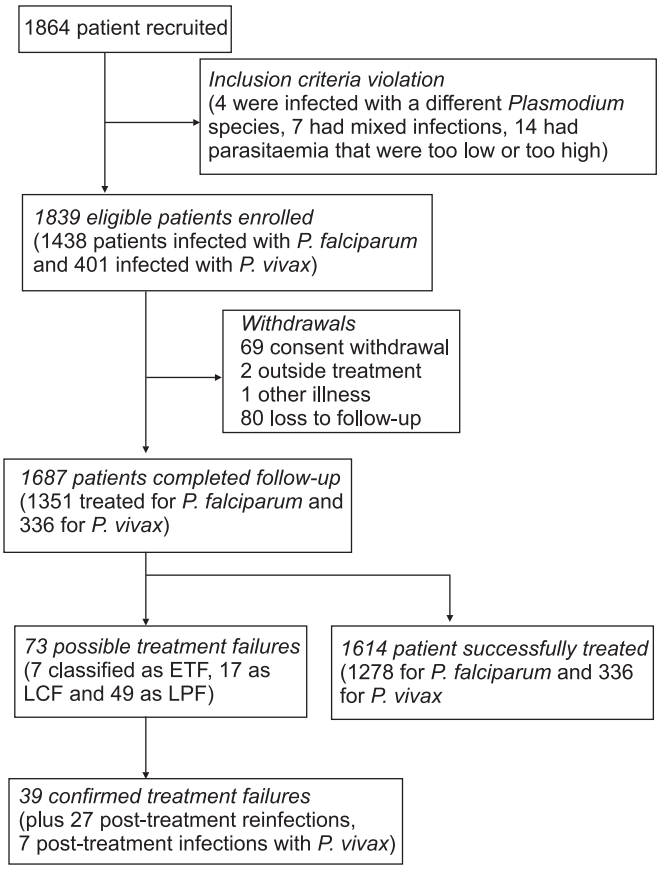

Fig. 2: Flowchart for patient recruitment and follow-up in the efficacy trials within India's national antimalarial drug resistance monitoring system, 2011–12.

Table 1. Characteristics of malaria patients enrolled in studies conducted under India's national antimalarial drug resistance monitoring system, 2011–12

Characteristics	Number of patients infected with	
	P. falciparum (n = 1438)	*P. vivax* (n = 401)
Sex		
Male	801 (55.7)	331 (82.5)
Female	637 (44.3)	70 (17.5)
Age category		
< 5 yr	140 (9.7)	1 (0.2)
5–15 yr	633 (44)	44 (11)
Adult	665 (46.2)	356 (88.8)
Febrile (≥37.5)[b]		
Yes	1019 (71.9)	345 (86)
No	399 (28.1)	56 (14)
History of fever[b]		
Yes	1412 (99.6)	401 (100)
No	6 (0.4)	0 (0)
Previous antimalarial drug intake[ab]		
Yes	1 (0.1)	0 (0)
No	366 (25.8)	130 (32.4)
Unknown	1051 (74.1)	271 (67.6)
Parasite count (No/µl)[b]		
<5000	449 (31.7)	232 (57.9)
5000–50,000	771 (54.4)	166 (41.4)
≥50,000	198 (14)	3 (0.7)

[a]At enrolment, pre- treatment; [b]Data were not available for 20 patients; Figures in parentheses indicate percentages.

Table 2. Site-wise results of therapeutic efficacy and parasite clearance in studies of the national antimalarial drug resistance monitoring system, India, 2011–12

Year	State/UT	District/City	Drug	Follow-up days	Therapeutic efficacy											PCT (h)			
					n	ACPR[a]	ETF[b]	LCF[c]	LPF[d]	WTH[#]	PF[†]	28 days follow-up		42 days follow-up		<24	24 to <48	48 to <72	≥72
												Survival	95% CI	Survival	95% CI				
2011	Andhra Pradesh	East Godavari	AS+SP	28	36	27	0	0	0	9	0	100	—	—	—	28	0	0	0
2012	Assam	Karbi-Anglong	AS+SP	42	16	16	0	0	0	0	0	100	—	100	—	14	2	0	0
2012	Arunachal Pradesh	Changlang*	AS+SP	42	42	22	0	2	4	5	9	80	61.4, 92.3	78.6	59, 91.7	15	18	2	2
2011	Chhattisgarh	Kanker	AS+SP	28	67	65	0	0	0	2	0	100	—	—	—	59	8	0	0
2011		Korea	AS+SP	42	70	70	0	0	0	0	0	100	—	100	—	50	19	0	0
2012	Dadra & Nagar Haveli	Silvassa	AS+SP	42	36	32	0	0	1	3	0	97.1	85.1, 99.9	97	84.2, 99.9	10	16	9	0
2012	Gujarat	Ahmedabad	CQ	28	42	30	0	0	0	12	0	100	—	—	—	14	2	14	5
2012		Surat	AS+SP	42	117	85	3	0	1	28	0	95.9	89.8, 98.9	95.5	88.9, 98.8	56	35	22	4
2012	Jharkhand	Dumka	AS+SP	42	50	44	0	0	0	5	1	100	—	100	—	1	29	26	0
2011		Ranchi	AS+SP	28	74	70	0	0	0	3	1	100	—	—	—	60	14	0	0
2011		Simdega	AS+SP	28	73	70	0	0	0	3	0	100	—	—	—	66	7	0	0
2011	Karnataka	Mangalore	CQ	28	90	76	0	0	0	14	0	100	—	—	—	1	49	34	4
2011	Madhya Pradesh	Betul	AS+SP	28	80	69	0	0	0	11	0	100	—	—	—	51	17	1	0
2012		Betul	AS+SP	42	41	37	0	1	0	3	0	97.4	86.2, 99.9	97.4	86.2, 99.9	31	10	0	0
2011		Chhindwara	AS+SP	28	55	55	0	0	0	0	0	100	—	—	—	0	30	25	0
2011	Maharashtra	Gadchiroli	AS+SP	28	71	70	0	0	0	1	0	100	—	—	—	71	0	0	0
2012		Gadchiroli	AS+SP	42	67	65	0	0	0	2	0	100	—	100	—	65	2	0	0
2012		Mumbai	AS+SP	42	72	72	0	0	0	0	0	100	—	100	—	29	35	8	0
2011		Mumbai	CQ	28	77	77	0	0	0	0	0	100	—	—	—	56	12	8	1
2012	Odisha	Dhenkanal	AS+SP	42	75	65	0	1	0	8	1	98.5	92, 100	98.5	91.8, 100	56	14	1	0
2011		Sambalpur	AS+SP	28	70	66	0	0	0	4	0	100	—	—	—	41	29	0	0
2012		Sundergarh	AS+SP	42	72	67	0	0	0	5	0	100	—	100	—	37	29	3	0
2011	Rajasthan	Baran	AS+SP	28	72	67	0	0	0	5	0	100	—	—	—	55	16	0	0
2011	West Bengal	Jalpaiguri	AS+SP	42	17	14	0	0	0	3	0	100	—	100	—	1	7	9	0
2011		Kolkata	CQ	28	76	75	0	0	0	1	0	100	—	—	—	39	33	4	0
2012		Kolkata	CQ	28	116	78	0	0	0	38	0	100	—	—	—	44	40	1	1
2011	Mizoram	Lunglei	AS+SP	28	42	40	0	0	0	2	0	100	—	—	—	4	27	10	0
2012		Lunglei*	AS+SP	42	71	47	0	3	8	8	5	87.7	76.3, 94.9	81	68.6, 90.1	47	15	1	0
2012	Tripura	Gomati*	AS+SP	42	77	43	4	3	8	9	10	84.1	72.7, 92.1	74.1	61, 84.7	25	26	12	6

n = No. of patients; ACPR—Adequate clinical and parasitological response; ETF—Early treatment failure; LCF—Late clinical failure; LPF—Late parasitological failure; WTH—Withdrawal; PCT—Parasite clearance time; [†]PF—*P. falciparum* reinfection; AS+SP—Artesunate plus sulphadoxine-pyrimethamine; [a]Absence of parasitaemia on Day 42 without previous criteria for failure; [b]Marked by the following, alone or in combination: Danger signs for severe malaria on Day 1, 2 or 3 in the presence of parasitaemia; Parasitaemia level on Day 2 higher than on Day 0; Parasitaemia level on Day 3 more than 25% higher than on Day 0; Parasitaemia on Day 3 plus fever; [c]Danger signs for sever malaria and/or fever plus parasitaemia from Day 4 to 42; [d]Parasitaemia from Day 7 to Day 42 even if patient afebrile; *Published data[21]; [#]Loss to follow-up/Consent/Protocol violation.

and Dhenkanal ranged from 95–100% except the three sites in NE region where the observed efficacy was below the WHO cut-off for policy change (range: 84.1–87.7%).

Generally, in the *P. falciparum* patients treated with AS+SP, the parasite clearance was rapid; Out of 1463, 872 (59.6%) in <24 h, 405 (27.7%) in intervals of 24 to <48 h, 129 (8.8%) in 48 to <72 h and 12 (0.8%) of the patients in ≥72 h (Tables 2 and 3). In most of the patients parasitaemia was cleared within <24 h.

Even in the LTF patients (39) the parasitaemia cleared rapidly; 14 (35.9%), 12 (30.8%), 7 (17.9%) and 6 (15.4%) of these patients, showed parasite clearance intervals of <24 h, 24 to <48 h, 48 to <72 h and ≥72 h, respectively. Out of total, in 23 patients (1.3 %) the parasitaemia was not cleared within 72 h. Out of these, 0.8% patients were from *P. falciparum* sites which included Gomati, Tripura (n = 6), Surat, Gujarat (n = 4) and Changlang, Arunachal Pradesh (n = 2). Out of these, six were identified as treatment failures (five ETF and one LPF), while two were re-infection cases and four achieved complete cure. However, the proportion of patients remaining parasite positive at Day 3 in these study sites was much below the cut-off prescribed by WHO. No adverse event was noted at any site during the 42-days' follow-up.

Plasmodium vivax

A total of 336 patients infected with *P. vivax* had 100% chloroquine efficacy, in contrast to the patients treated with AS+SP for *P. falciparum*. The *P. vivax* patients treated with CQ (401) cleared their parasitaemia rapidly, 154 (38.4%) in <24 h, 136 (33.9%) in the intervals of 24 to <48 h, 61 (15.2%) in 48 to <72 h, and 11 (92.7%) of the patients in ≥72 h (Tables 2 and 3). In most of the patients infected with *P. vivax* the parasitaemia was cleared within 48 h. The parasitaemia in 11 patients persisted for ≥72 h. Out of these 11 patients one was withdrawn on Day 3 and one was loss to follow-up on Day 7. Chloroquine treatment was well tolerated.

Table 3. Treatment outcomes among eligible malaria patients in studies conducted

Outcome	Number of patients infected with	
	P. falciparum	*P. vivax*
Primary classification		
ACPR	1278 (88.9)	336 (83.8)
Early treatment failure	7 (0.5)	0 (0)
Late clinical failure	20 (1.4)	0 (0)
Late parasitological failure	46 (3.2)	0 (0)
Lost to follow-up	38 (2.6)	42 (10.5)
Withdrawal after Day 0	49 (3.4)	23 (5.7)
PCR-corrected results		
Recrudescence	39 (2.7)	0 (0)
Reinfection	27 (1.9)	0 (0)
Infection with other species	7 (0.5)	0 (0)
Parasite clearance interval (h)		
<24	872 (61.5)	153 (42.4)
24 to <48	405 (28.6)	136 (37.7)
48 to <72	129 (9.1)	61 (16.9)
≥ 72	12 (0.8)	11 (3)

ACPR—Adequate clinical and parasitological response; PCR—Polymerase chain reaction; Figures in parentheses indicate percentages.

Molecular markers of drug resistance

Out of the total 333 isolates, 292 were successfully genotyped for *dhfr* and 327 for *dhps* mutation analysis. In *dhfr*, double 57.2% (n = 167), single 14.7% (n = 43), wild 11.6% (n = 34) and triple mutations 15.8% (n = 46) were observed. The most frequent haplotype was double mutant 108/59 followed by single mutant at codon 59. Only two isolates were found to have quadruple mutations (0.7%).

In *dhps*, double mutations in 9.8% samples (n = 32) at codon 436+437, 436+540 and 437+540 and triple mutations in 20.5% samples (n = 67) at codon 436+437+540 were common. Amongst the *dhps* codons, higher frequency of mutations in 437 and 540 codon was observed. However, the most frequent haplotype was wild type (60.9%) followed by triple mutant (20.5%) (Table 4).

Out of the 39 confirmed treatment failures samples, *dhfr* quadruple mutations were observed only in one sample (2.6%), while triple mutations in 19 samples (48.7%), double mutations in 10 samples (25.6%) and single mutation in one sample (2.6%). However, eight samples could not be amplified (20.5%). In addition, 19 (48.7%) samples had *dhps* triple mutations, four (10.3%) had double mutations, seven (17.9%) were single mutants and one (2.6%) was found to be wild type for *dhps* gene. Quintuple mutations were observed in 35.9% of the treatment failure samples (14/39), majority from the NE region.

Predictors of treatment failure and parasite clearance interval

In both univariate and multivariate analysis, an age of <5 yr (relative to an age of ≥ 15 yr) and a daily dose of artesunate of either <3 or <3.5 mg (relative to higher doses) were significantly associated with treatment failure. Fever at the time of enrolment was also positively and significantly associated with treatment failure (Table 5). Out of the total 39 treatment failure cases, 31 were found to be febrile at enrolment. The risk of failure increased (4.4 to 22.4%) as the dose of artesunate decreased. Similarly, the risk of failure increased (2.2 to 10.7%) in

Table 4. Molecular markers of antifolate resistance in *P. falciparum* isolates collected through the national antimalarial drug resistance monitoring system, India, 2011–12

Result	*dhfr* (n = 292)		*dhps* (n = 327)	
	n	Haplotype	n	Haplotype
Quadruple	2	**I**51/**R**59/**N**108/**L**164		
Triple	43	**I**51/**R**59/**N**108/I164	67	**A**436/**G**437/**E**540
Triple	3	N51/**R**59/**N**108/**L**164		
Double	166	N51/**R**59/**N**108/I164	10	**A**436/A437/**E**540
Double	1	**I**51/C59/**N**108/I164	9	**A**436/**G**437/K540
Double			13	S436/**G**437/**E**540
Single	37	N51/C59/**N**108/I164	4	**A**436/A437/K540
Single	6	N51/**R**59/S108/I164	6	S436/A437/**E**540
Single			19	S436/**G**437/K540
Wild	34	N51/C59/S108/I164	199	S436/A437/K540

Mutations in bold letters.

Table 5. Predictors of treatment failure among patients in studies conducted under India's national antimalarial drug resistance monitoring system, 2011–12

Predictor	Failure	ACPR	Risk	RR[a]	95% CI	*p*-value
Artesunate dose (mg/kg)[b]						
≥ 3.5	31	1159	0.026	—		
3 to < 3.5	5	108	0.044	0.24	0.06, 0.89	0.059
< 3	3	11	0.214	8.55	2.96, 24.86	<0.001
Age (yr)						
< 5	13	109	0.107	5.91	3.15, 11.09	<0.001
≥ 15	26	1169	0.022	—		
Parasitaemia (asexual parasites/μl)						
<5000	30	404	0.069	—		
≥ 5000	9	874	0.010	0.15	0.07, 0.31	<0.001
Fever at enrolment (≥37.5°C)						
Yes	31	914	0.033	1.53	0.71, 3.29	0.098
No	8	364	0.022	—		

ACPR—Adequate clinical and parasitological responses, RR—Relative risk; CI—Confidence interval; [a]Relative risk for the failure of treatment of *P. falciparum* infection with a combination of AS and SP; [b]The dose given per day.

the patients under age < 5 yr, as compared to ≥ 15 yr age category. Day 0 parasitaemia of ≥ 5000 asexual parasites per μl was associated with reduced risk of treatment failure. Presence of *dhfr* + *dhps* quintuple mutations was observed only in treatment failure samples, however in lower frequency. There were insufficient observations to determine the risk factors for a parasite clearance interval of ≥72 h.

DISCUSSION

The national antimalarial drug resistance monitoring system has been functional since 2009 and has recruited 3597 number of patients till 2012, out of which 1864 patients were recruited during 2011–12[12]. These 1864 patients completed therapeutic efficacy trials in 29 sites across India during 2011–12. The results indicate that the first-line therapies for *P. falciparum* and *P. vivax* malaria recommended by the national antimalarial drug policy (*i.e.* AS+SP and chloroquine, respectively) remain efficacious at 26 sites in 15 states, while declining efficacy of AS+SP was observed at three sites in Northeastern region. The 28-day efficacy of AS+SP in *P. falciparum* malaria patients remained above the WHO recommended threshold (>90% efficacy) at majority of the study sites (n = 3/29) as compared to study sites (n = 26/29) during 2009–10[12]. Also, AS+SP treatment failures have been observed in 2.7% samples (n = 39) and the increased parasite clearance intervals has doubled after AS+SP treatment over the years (0.8% compared to 0.4%). However, these were clustered in just a few sentinel sites. This emphasises the clustering design of the national monitoring system, where wide geographical coverage increases the chances of detecting hotspots for resistance (as well

as longitudinal studies help in tracking the emerging trends). Higher frequencies of SP treatment failure have been reported in the past[21–22], with increasing trend in *dhfr–dhps* point mutations over years prompting to focus more in the NE region sites where declining efficacy of AS+SP has been observed at all the three sentinel sites. This increase in treatment failure showed increasing trend from 2009 (1.2%) to 2012 (2.7%). The observation that four of the six patients who showed parasite clearance intervals of ≥ 72 hr were confirmed to be treatment failures indicates the potential usefulness of measuring clearance intervals as a predictor of AS+SP treatment failure. Post-treatment reinfection with *P. falciparum* was observed in 36.9% patients in the present study.

A strong indicator for SP treatment failure is the quintuple mutations in three *pfdhfr* codons (108asn+51ile+59arg) and two *pfdhps* codons (437gly+540glu)[23]. No quintuple mutations have been observed in the studies conducted during 2009–10.

Majority of the isolates (87.6%) of *P. falciparum* that were amplified showed double mutations (S108N/C59R) in the relevant *dhfr* codons, showing partial resistance to pyrimethamine. Such mutations have been found to increase the median inhibitory concentration (IC50) of pyrimethamine upto 10-fold[13]. While five isolates possessed genotypic evidence of the I164L mutation that has been associated with high-level resistance[20], the prevalence of triple mutations was 15.8% with low prevalence of quadruple mutants among the genotyped isolates (0.7%). As seen in a previous study based on the same methods[21], the typing of *dhps* appeared to be relatively difficult as it was unsuccessful for 20.5% of the isolates for which typing was attempted. In contrast to 2009–10, the prevalence of single or double *dhps* mutations among the isolates that were successfully genotyped was low (18.6%) as compared to triple mutants (20.5%) and wild type (60.9%).

Till 2010, *dhps* remained wild in majority of the samples, however, increasing trend in *dhps* point mutations was observed in 2011, particularly in sites of Northeastern region. This prompted the nationwide sentinel site monitoring system to focus more in Northeastern region with selection of three sites at different ecological conditions for efficacy studies of AS+SP in *P. falciparum.* During 2012, quintuple mutations ranging from 0.6 to 4.2% have been observed at all the three sites of NE region and at Surat (Gujarat) being highest in Tripura[21]. In addition, quadruple mutations have increased from 2.8 to 8.6% over the period from 2009–10 to 2011–12. Similarly, high prevalence of quintuple mutations (35%) have been observed in samples collected from Kolkata[24]. These increasing trends in the prevalence of resistance-related mutations in *dhfr* and *dhps* genes, points to the threat of treatment failure with the AS+SP combination posed by resistance to SP in *P. falciparum,* and can be evaluated as early markers, independent of any observations for the clinical response.

Negative correlation was observed between the dose of artesunate (in mg per kg body weight) and probability of treatment failure.

Out of the observed treatment failure samples, 46.5% reinfection has been recorded during these studies as compared to 33.3% observed during 2009–10. The rate of reinfection may be correlated with the length of the follow-up period, but is also dependent on the intensity of transmission in the study sites as reinfection was highest in Silachari district (13.7%), Tripura followed by Lunglei district (5.5%), Mizoram and Changlang district (12.3%), Arunachal Pradesh. The intensity of malaria transmission in Northeastern India is generally higher than in many other parts of the country.

In the present study, younger age, fever at the time of enrolment and a low level of parasitaemia at enrolment – all potential markers of relatively low immunity to parasite antigens were associated with recrudescence following AS+SP treatment. Fever at the time of enrolment was positively and significantly associated with treatment failures. Another association observed, the negative correlation between the dose of artesunate (in milligram per kg body weight) and the probability of treatment failure, was not surprising. Although, the recommended daily dose of artesunate is 4 mg per kg, 7.9% of the subjects of the present study who were given AS+SP received 3 to < 3.5 mg of artesunate per kg, and 1.1% received < 3 mg per kg. Earlier, routine use of age, rather than body weight, as a guide for determining the dose of antimalarial drug needed by a patient was probably a cause of suboptimal dosing, however, with the introduction of blister packs and dispensing of drugs according to body weight, the problem of underdosing has reduced as described previously[21]. The relationship between the administered dose and pharmacodynamics response is however complex and the adequate clinical and parasitological response may still be achieved when the dose is lower than recommended in standard guidelines[23].

The *P. vivax*-infected patients enrolled in the present study were cured by chloroquine treatment despite of the sporadic case reports of chloroquine-resistant *P. vivax* in India in the past[25]. Out of the 29 studies, five *P. vivax* studies were confined to southern and western parts where significant proportion of migrant workers from other parts of the country resides. However, efforts are ongoing to

undertake more trials to investigate the therapeutic efficacy of chloroquine against *P. vivax* infections in the north and east of India. As per national drug policy, confirmed *P.* v*ivax* cases are treated with chloroquine along with primaquine for 14 days to prevent relapses. Unfortunately, no standard protocol exists for evaluating the therapeutic efficacy of primaquine, alone or in combination with chloroquine[26]. Also, treatment of mixed infections where patients are found to be coinfected with *P. falciparum* and *P. vivax* remains a challenge although guidelines have been issued for treatment of mixed infections, similar to *P. falciparum*[21]. No data on the efficacy of AS+SP against *P. vivax* malaria were collected during the present studies. High incidence of *P. vivax* malaria following the treatment of *P. falciparum* infection has been reported from Southeast Asia, primarily as a result of the reactivation of the liver stages of *P. vivax*[27]. In the present study, *P. vivax* infections were detected during the follow-up of four cases of *P. falciparum* malaria who had been treated with AS+SP. Three of these four cases were observed at Changlang (Arunachal Pradesh) and one at Silachari (Tripura), and represented about one in every 20 patients treated with AS+SP at these two sentinel sites.

According to WHO recommendation, four to eight sites per country are sufficient for national representation though, due consideration to the geographical size, population distribution and density, malaria epidemiology or ecology should be given; however, for country as large as India with higher disease burden, a larger number of sentinel sites in the current system are required to provide adequate information. Coordinated use of resources in different malaria ecotypes were the main driving factors which helped in generating high quality data under India's national antimalarial drug resistance monitoring system. Under this system, national representation, and pooled data analysis were accomplished. Alternation of sentinel sites will be useful in tracking the longitudinal trends.

Thus, while in 2010, the drug policy was revised to incorporate the use of AS+SP for treating falciparum malaria cases across the country, in 2013, another policy change has been recommended in the seven Northeastern states (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland and Tripura) in view of the resistance to partner drug SP. Thereafter AS+SP was replaced by artemether lumefantrine[21]. Effective treatments implemented on large-scales can reduce malaria transmission and might even reduce existing drug resistance in other settings. Thus, minimisation of uneven drug pressure through use of an effective drug throughout a large geographical unit, such as a district or even cluster of districts, is an effective strategy to prevent the spread of drug resistance[28]. Such a change in treatment policy will also need fewer drug-efficacy studies and reduce operational challenges related to drug supply.

CONCLUSION

In India, chloroquine remains safe and effective for the treatment of uncomplicated *P. vivax* malaria while the efficacy of ACT (AS+SP) in *P. falciparum* remains effective in the majority of the study sites up to 2012, except in Northeastern region, for the treatment of uncomplicated malaria.

Conflict of interest

The authors declare that they have no any conflict of interest.

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Surveillance of Artemisinin Resistance in *Plasmodium falciparum* in India Using the kelch13 Molecular Marker

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Malaria treatment in Southeast Asia is threatened with the emergence of artemisinin-resistant *Plasmodium falciparum*. Genome association studies have strongly linked a locus on *P. falciparum* chromosome 13 to artemisinin resistance, and recently, mutations in the kelch13 propeller region (*Pfk-13*) were strongly linked to resistance. To date, this information has not been shown in Indian samples. *Pfk-13* mutations were assessed in samples from efficacy studies of artemisinin combination treatments in India. Samples were PCR amplified and sequenced from codon 427 to 727. Out of 384 samples, nonsynonymous mutations in the propeller region were found in four patients from the northeastern states, but their presence did not correlate with ACT treatment failures. This is the first report of *Pfk-13* point mutations from India. Further phenotyping and genotyping studies are required to assess the status of artemisinin resistance in this region.

The emergence and spread of drug resistance is a major obstacle to combating malaria. Resistance in *Plasmodium falciparum* to chloroquine and antifolates (1), which arose in Southeast Asia, spread across India and Africa and resulted in substantial increases in global malaria morbidity and mortality. This eventually led to the introduction of artemisinin (ART)-based combination therapy (ACT) (2) as first-line treatment for uncomplicated falciparum malaria. Until 2005, chloroquine (CQ) was the drug of choice for treatment of malaria in India, except in CQ-resistant cases, where sulfadoxine-pyrimethamine (SP) was recommended. However, with the increasing cases of resistance to SP during 2003 to 2004 (3) and the WHO recommendations, artemisinin-based combination therapy (ACT) of artesunate with sulfadoxine-pyrimethamine (AS+SP) was introduced in 2007 as the first-line antimalarial for the treatment of confirmed falciparum malaria cases in chloroquine-resistant areas in the country. In 2010, this ACT was used universally across the country for treating falciparum malaria cases (4).

ACTs have been well tolerated, safe, and highly effective. Unfortunately, artemisinin resistance in *P. falciparum* has emerged in Southeast Asia (Myanmar, Thailand, Cambodia, Vietnam, and Laos), threatening the recent gains in malaria control and elimination. Westward spread to India and Africa is a major concern (5–10). The mechanism of action of the artemisinin drugs remains unclear. Although artemisinin resistance is characterized by slow parasite clearance, several molecular markers have been proposed (11–13). Genome association studies strongly linked a locus on *P. falciparum* chromosome 13 to artemisinin resistance, and this was recently explained by the discovery that mutations in the kelch13 propeller region (*Pfk-13*) are strongly linked to resistance (14). *k-13* is a 1-exon gene that codes for a putative kelch protein and has three domains: a plasmodium-specific domain, a BTB/POZ, and a C-terminal six-blade propeller (15). Mutations in the propeller region are linked to resistance. *Pfk-13* is well conserved across *Plasmodium* species and is thought to mediate protein-protein interactions (14).

Three point mutations (G533A, R539T, and C580Y) in the kelch motif of *Pfk-13* were correlated with the artemisinin resistance phenotype in the original study (14). A protein structure-modeling study showed that these mutations can alter the biological function of this putative protein (14). More than 30 single nucleotide polymorphisms in the propeller region of *Pfk-13* have since been associated with artemisinin resistance, although each resistant isolate has only one of these mutations.

Given that CQ-resistant *P. falciparum* strains spread to India from Southeast Asian countries through the northeastern states, a similar scenario may be expected for the spread of artemisinin-resistant *P. falciparum* from the epicenter in Southeast Asia (5–10). If artemisinin resistance does spread to or emerge in India, the public health consequences will be immense. India is also experiencing declining efficacy of its currently recommended first-line ACT (artesunate plus sulfadoxine-pyrimethamine [AS+SP]) in the northeastern states of the country (16, 17). In the present study, point mutations in *Pfk-13* were studied in *P. falciparum* isolates collected from patients enrolled in ACT efficacy studies from different sites in India. Although artemisinin resistance is defined by slow parasite clearance, these studies did not include detailed assessments of parasite dynamics.

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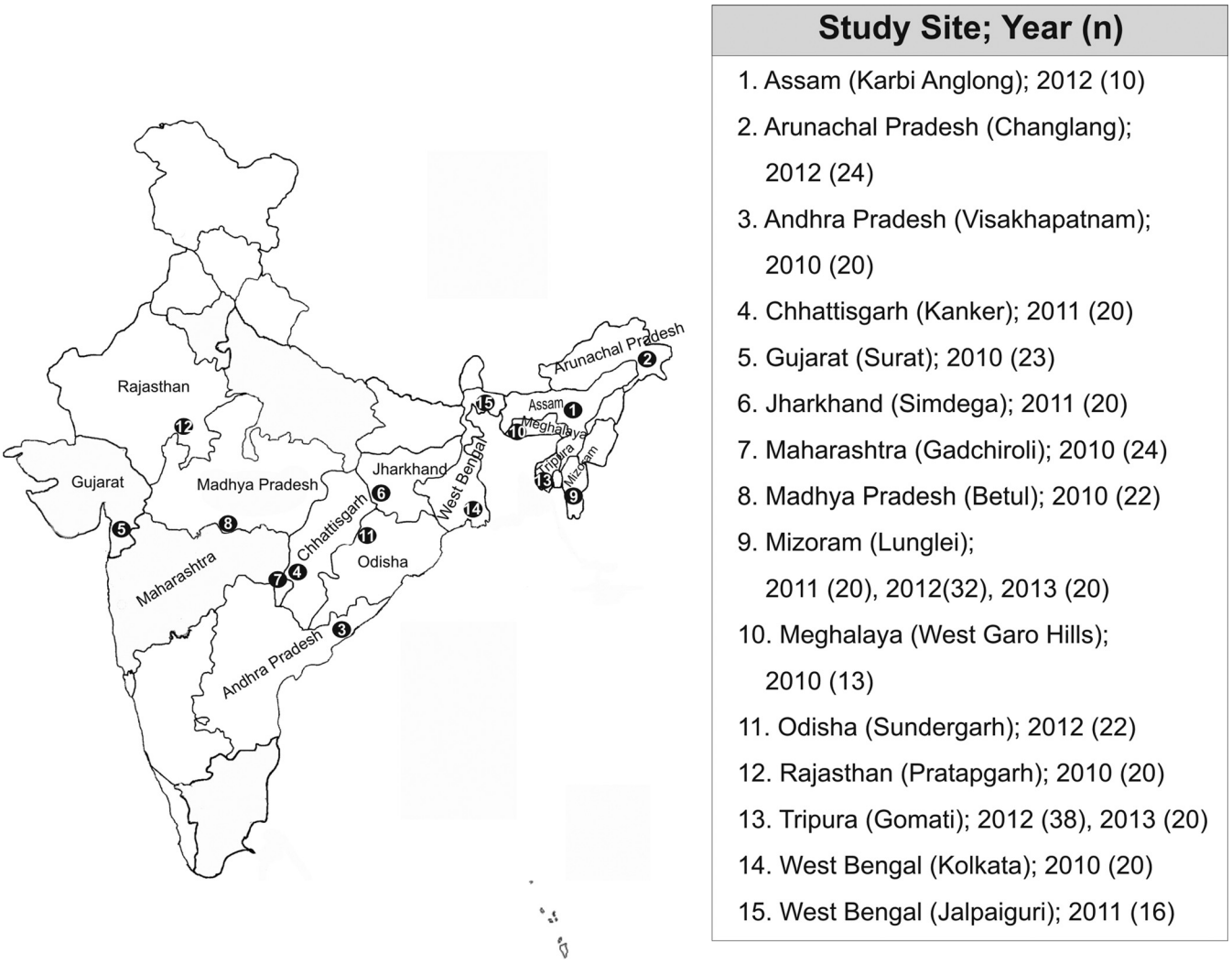

FIG 1 Details of *Plasmodium falciparum* ACT efficacy study sites in India.

MATERIALS AND METHODS

A total of 389 *P. falciparum* samples were collected from patients enrolled in prospective ACT (AS+SP) therapeutic efficacy studies conducted between 2009 and 2013 as part of the nationwide sentinel site antimalarial drug therapeutic assessment system (16, 17). Directly observed treatments with quality-assured drugs from standard Indian manufacturers were given to the enrolled patients. These drugs were supplied by the state health departments and were used within their expiry period. We obtained informed, written consent from each enrolled adult and from a legal guardian of each enrolled child. The study protocol was approved by the Government of India, Ministry of Health and Family Welfare, and the scientific advisory committee of the National Institute of Malaria Research (NIMR). The institutional ethics committee (IEC) approved the secondary study.

During 2008, NIMR and the National Vector Borne Disease Control Programme (NVBDCP) selected 25 sentinel sites for routine monitoring of antimalarial drug resistance. These sites were purposely selected to provide a representative cross-section of malaria ecotypes, transmission intensities, and geographic regions. The blood samples used for the study were from 15 sentinel sites spread across different periods and geographic regions. Open-label, single-arm prospective studies were conducted (16) as per WHO protocol and patients were followed up to day 28 during 2011 and 2013 and up to day 42 during 2012. The studies included five districts from the northeastern region (Gomati in Tripura, Lunglei in Mizoram, West Garo Hill in Meghalaya, Karbi Anglong in Assam, and Changlang in Arunachal Pradesh); two from West Bengal (Jalpaiguri and Kolkata); and one each in Odisha (Sundergarh), Jharkhand (Simdega), Chhattisgarh (Kanker), Maharashtra (Gadchiroli), Rajasthan (Paratapgarh), Madhya Pradesh (Betul), Gujarat (Surat), and Andhra Pradesh (Vishakhapatnam) (Fig. 1). Details about each study site, such as the prevalence of malaria, introduction of ACT as first-line treatment, and relevant geographic information, as well as the sample details, are provided in the supplemental material.

Genomic DNA was extracted using the QIAamp mini DNA kit (Qiagen, Germany) from microscopy-diagnosed *P. falciparum*-positive blood spotted on Whatman filter paper (3-mm) strips.

Paired blood samples, i.e., from day 0 and the day of reappearance of parasitemia, were analyzed using three well-established molecular markers (*msp-1*, *msp-2*, and *glurp*) for differentiating recrudescence from reinfection. *Pfk-13* genes from all samples were amplified by PCR and DNA sequenced (Macrogen, South Korea) per protocols reported previously (14). This primer set covered all mutations reported to be associated with artemisinin resistance, which were from codon 427 to 727 of *Pfk-13* (see Fig. S1 in the supplemental material) (10). High-fidelity Taq DNA polymerase (Kapa Hi-Fidelity Hot Start master mix) was used to minimize PCR-incorporated nucleotide errors. All mutations observed in the study

were validated with another independent PCR and by resequencing *Pfk-13*.

Nucleotide sequence accession numbers. DNA sequences of representative samples showing wild and mutant types were submitted to GenBank and assigned accession numbers KP780808, KP790255, KP790256, KP790257, KP790258, KP790259, KP790260, and KP790261.

RESULTS

Samples from patients who responded (adequate clinical and parasitological response [ACPR]) to ACTs were obtained from all study sites, while PCR-corrected confirmed recrudescences (n = 42) were obtained from seven sites: Arunachal Pradesh (n = 6), Tripura (n = 15), Mizoram (n = 11), Gujarat (n = 3), Maharashtra (n = 4), Madhya Pradesh (n = 2), and West Bengal (n = 1) (see Fig. S2 in the supplemental material) (16, 17). *Pfk-13* was PCR amplified successfully from all responders, but five samples from nonresponders could not be amplified. DNA sequence analysis of *Pfk-13* from the 384 clinical isolates of *P. falciparum* showed six point mutations and one deletion. The mutations were synonymous in two and nonsynonymous (NS) in four, and there was one deletion causing frameshift. Synonymous substitutions were found at nucleotide positions 1377 (G-A) and 1752 (T-C), and NS substitutions were observed at codons 533 (G-A), 549 (S-Y), 561 (R-H), and 578 (A-S) (Table 1). The frameshift mutation was observed at nucleotide position 1991 (deletion of A nucleotide). Only NS mutations were used for further analysis. The 561 (R-H) and 578 (A-S) mutations were reported previously in association with slow parasite clearance, but the 533 (G-A) and 549 (S-Y) mutations have not been reported to date (10, 14). The mutations were observed at very low frequencies (0.26%, 1/384).

Among the four NS substitutions, three were observed in northeastern states and one was observed in Jalpaiguri, which is the gateway to northeastern states (Assam) (Fig. 1). Thus, no NS mutations were observed in isolates collected from other parts of India (Table 2).

In addition, a higher prevalence of point mutations in the *P. falciparum* dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) genes was also observed in the northeastern states compared with isolates from other parts of India (Table 3) (16, 17).

DISCUSSION

This is the first report of *Pfk-13* point mutations from India. Does this mean that artemisinin resistance has already spread to India? Without corresponding phenotyping, it is premature to conclude that artemisinin resistance has arrived. The recent large multinational Tracking Resistance to Artemisinin Collaboration (TRAC) study (10), which conducted detailed parasite clearance assessments and genotyping, clearly associated *Pfk-13* propeller region mutations with slow parasite clearance in Southeast Asia, but such mutations were also found in the Democratic Republic of the Congo, where they were not associated with resistance. Other genotyping studies suggested a low background frequency of such mutations in parasite populations across the world (18, 19). However, a recent study in sub-Saharan Africa identified novel coding substitutions of unclear phenotypes (20). Clearly, more information is needed, but it seems at present that other genetic changes in *P. falciparum* may be required in order to confer a stable artemisinin-resistant phenotype. There is an urgent need to assess with clinical and laboratory phenotyping in addition to genotyping whether artemisinin resistance is present in this region.

TABLE 1 Point mutations in *Plasmodium falciparum k-13* gene from Indian clinical isolates

Pfk-13 point mutation description (nonsynonymous)[a]															No. of isolates (n = 380)
G449A	N458Y	T474I	A481V	Y493H[b]	T508N	G533S	**G533A**	N537I	R539T[b]	I543T	**S549Y**	P553L	R561H	V568G	P574L	A578S	C580Y[b]	D584V	S623C	
							1													1
											1									1
													1							1
																1				1

[a] Bold type indicates novel point mutation. An empty cell indicates no point mutation.
[b] Mutation in *Pfk-13* that confers ART resistance in Cambodian *P. falciparum* isolates (14).

TABLE 2 Point mutations in *Plasmodium falciparum k-13* gene from India

Study site	Yr of sample collection	Sample size (no.)	*k-13* mutation description[a]			
			G533A	**S549Y**	R561H	A578S
Gomati, Tripura	2012[b]	38	**1**			
	2013[c]	20				
Lunglei, Mizoram	2011[c]	20				
	2012[b]	32				
	2013[c]	20				1
Karbi Anglong, Assam	2012[c]	10				
West Garohills, Meghalaya	2010[b]	13				
Changlang, Arunachal Pradesh	2012[b]	24			1	
Jalpaiguri, West Bengal	2011[c]	16		**1**		
Kolkata, West Bengal	2010[b]	20				
Sundergarh, Odisha	2012[c]	22				
Simdega, Jharkhand	2011[c]	20				
Kanker, Chhattisgarh	2011[c]	20				
Betul, Madhya Pradesh	2011[c]	22				
Surat, Gujarat	2010[b]	23				
Vishakhaptnam, Andhra Pradesh	2010[b]	20				
Pratapgarh, Rajasthan	2010[b]	20				
Gadchiroli, Maharashtra	2010[b]	24				
Total		384	1	1	1	1

[a] Bold type indicates novel point mutation. An empty cell indicates no point mutation.
[b] See references 16 and 17.
[c] Our unpublished data.

TABLE 3 Point mutations in *Plasmodium falciparum k-13* gene from ACT resistance cases

Study site	Clinical outcome[a]	Sample size (no.)	*k-13* mutation (no.)				Prevalence of *Pfdhfr/Pfdhps* mutant genotype (%)		Reference or source for *Pfdhfr/Pfdhps* mutation
			G-533-A	S-549-Y	R-561-H	A-578-S	*Pfdhfr*	*Pfdhps*	
Gomati, Tripura	ACPR	25					100	97.3	17
	TF	15[b]	1						
Lunglei, Mizoram	ACPR	21					100	97.0	17
	TF	11							
Changlang, Arunachal Pradesh	ACPR	21			1		100	97.0	17
	TF	6[c]							
Kolkata, West Bengal	ACPR	19					100	95.2	16
	TF	1							
Betul, Madhya Pradesh	ACPR	20					80	3.30	21,22
	TF	2							
Surat, Gujarat	ACPR	20					100	24.60	Our unpublished data
	TF	3							
Gadchiroli, Maharashtra	ACPR	20					100	ND[e]	Our unpublished data
	TF	4							
Total	ACPR	146			1				
	TF	37[d]	1						

[a] ACPR, adequate clinical and parasitological response; TF, treatment failure.
[b] Two samples were not PCR amplified.
[c] Three samples were not PCR amplified.
[d] Five samples were omitted from total; shaded areas represent northeastern states.
[e] ND, not done.

In the northeastern states, the higher prevalence of point mutations in the *Pfdhps* and *Pfdhfr* genes, both coding for essential enzymes in the folate biosynthesis pathway, is likely to be responsible for ACT treatment failures in this region (17).

All the NS substitutions were observed in northeastern states, with one in Jalpaiguri, which is the gateway to northeastern states (Assam). Treatment responses were good in each of the four patients harboring NS *Pfk* mutations, despite the declining efficacy of the partner drug sulfadoxine-pyrimethamine in northeast India.

The history of the introduction of chloroquine resistance to India suggests that the northeastern region is the gateway and therefore a likely physical route for possible introduction of artemisinin-resistant *P. falciparum* strains in the near future. Artemisinin-resistant parasites are prevalent in adjacent Myanmar. Continued monitoring of ART resistance in clinical studies measuring parasite clearance half-lives supported by molecular genotyping is required, particularly in the northeastern states of India. This study had a few limitations. Most of the treatment failure samples from patients were late treatment failures that were due to the partner drug component of ACT rather than to artemisinin. To confirm the presence of artemisinin resistance, a detailed assessment of parasite clearance dynamics by 6-hourly parasite measurements and whole kelch13 gene mutation needs to be made. However, in the light of emerging reports, there was an urgent need to study the mutations in the reported propeller region associated with *in vivo* and *in vitro* artemisinin resistance. Thus, the study was confined to the reported propeller region, and samples from surveillance studies were included. Since these samples were from surveillance studies, the data on the detailed assessment of parasite clearance dynamics were a limitation. Also, due to the limited number of observed mutations in the kelch propeller region, the correlation between the observed mutations in the samples from patients who responded to ACT therapy and those in patients who failed the treatment remains inconclusive.

The present observations serve as a necessary baseline. Further phenotyping and genotyping studies are needed to determine whether artemisinin resistance has spread to or emerged in northeast India.

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RESEARCH Open Access

Declining efficacy of artesunate plus sulphadoxine-pyrimethamine in northeastern India

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Abstract

Background: Anti-malarial drug resistance in *Plasmodium falciparum* in India has historically travelled from northeast India along the Myanmar border. The treatment policy for *P. falciparum* in the region was, therefore, changed from chloroquine to artesunate (AS) plus sulphadoxine-pyrimethamine (SP) in selected areas in 2005 and in 2008 it became the first-line treatment. Recognizing that resistance to the partner drug can limit the useful life of this combination therapy, routine *in vivo* and molecular monitoring of anti-malarial drug efficacy through sentinel sites was initiated in 2009.

Methods: Between May and October 2012, 190 subjects with acute uncomplicated falciparum malaria were enrolled in therapeutic efficacy studies in the states of Arunachal Pradesh, Tripura, and Mizoram. Clinical and parasitological assessments were conducted over 42 days of follow-up. Multivariate analysis was used to determine risk factors associated with treatment failure. Genotyping was done to distinguish re-infection from recrudescence as well as to determine the prevalence of molecular markers of antifolate resistance among isolates.

Results: A total of 169 patients completed 42 days of follow-up at three sites. The crude and PCR-corrected Kaplan-Meier survival estimates of AS + SP were 60.8% (95% CI: 48.0-71.4) and 76.6% (95% CI: 64.1-85.2) in Gomati, Tripura; 74.6% (95% CI: 62.0-83.6) and 81.7% (95% CI: 69.4-89.5) in Lunglei, Mizoram; and, 59.5% (95% CI: 42.0-73.2) and 82.3% (95% CI: 64.6-91.6) in Changlang, Arunachal Pradesh. Most patients with *P. falciparum* cleared parasitaemia within 24 hours of treatment, but eight, including three patients who failed treatment, remained parasitaemic on day 3. Risk factors associated with treatment failure included age < five years, fever at the time of enrolment and AS under dosing. No adverse events were reported. Presence of *dhfr* plus *dhps* quintuple mutation was observed predominantly in treatment failure samples.

Conclusion: AS + SP treatment failure was widespread in northeast India and exceeded the threshold for changing drug policy. Based on these results, in January 2013 the expert committee of the National Vector Borne Disease Control Programme formulated the first subnational drug policy for India and selected artemether plus lumefantrine as the new first-line treatment in the northeast. Continued monitoring of anti-malarial drug efficacy is essential for effective malaria control.

Keywords: Plasmodium falciparum, Artesunate + sulphadoxine-pyrimethamine (AS + SP), Artemisinin combination therapy (ACT), *Dihydrofolate reductase* (*dhfr*), *Dihyropteroate synthase* (*dhps*)

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Background

Anti-malarial drug resistance, particularly in *Plasmodium falciparum* was a major contributor to global resurgence of malaria in the 20th Century. Southeast Asia (SEA) has been the focus of drug resistance for all anti-malarials with loss of valuable drugs from time to time. Chloroquine resistance in *P. falciparum* malaria was first reported in 1957 in SEA region, later followed by sulphadoxine-pyrimethamine resistance after ten years [1-3]. India shares international boundaries with countries known to be the epicentre for drug resistance. India too experienced increase in falciparum malaria cases with resistance to first-line anti-malarial chloroquine (CQ) in 1973 in northeast India which later spread to other areas in the country [4]. However, resistance to second-line anti-malarial sulphadoxine-pyrimethamine (SP) was also detected in the same area in the country, spreading even more rapidly to other parts of the country. Increasing resistance to CQ and SP forced the national programme to abandon CQ for treatment of *P. falciparum* [5-7], however CQ remains the first-line anti-malarial treatment for *Plasmodium vivax,* another parasite that contributes equally to human malaria in the country.

Since 2005, treatment of uncomplicated *P. falciparum* malaria in the country is based on artemisinin combination therapy (ACT) as per the recommendation of World Health Organization (WHO) [8]. The ACT recommended in the country is artesunate plus SP. Presently, all the five formulations of ACT recommended by WHO except dihydroartemisinin [DHA] + piperaquine, are registered with the Drugs Controller General of India. Prior to introduction of ACT in the country, SP has been used for the treatment of uncomplicated falciparum malaria in India since 1980s as second line [9]. However, resistance to partner drug SP has already been reported in the country [10], which threatens the useful life of this ACT. The mechanism of SP resistance has been well documented compared to other anti-malarials. Point mutations in the *dihydropteroate synthas*e (*dhps*) and *dihydrofolate reductase* (*dhfr*) genes, both coding for essential enzymes in the folate biosynthesis pathway, led to resistance to antifolate drugs [11].

Recent reports on artemisinin resistance in the SEA region [12] also required continuous monitoring of ACT in the country. Artemisinin resistance, defined by delayed parasite clearance following complete treatment, was first reported in SEA along the Thai-Cambodia border in 2006 [12,13]. Recently, parasites with much slower clearance profiles have also been identified in Western Thailand on the border with Myanmar, along the Myanmar-China border and in Vietnam [14-17].

The Indian northeastern region has varied ecological diversity, inaccessible areas where vector control measures as well early treatment and diagnosis facilities are difficult to assess. Malaria control remains a challenge in these areas. ACT was introduced in the study areas in 2007 and since 2009 continuous monitoring of recommended anti-malarials has been done through nationwide sentinel site system. Also, partner drug resistance markers (*dhfr* plus *dhps*) are potential early warning signs; the same were also monitored for the study sites which prompted more focus in these study areas.

This study was part of nationwide sentinel site system initiated in 2009 with the aim to address the continued problem of anti-malarial drug resistance in the country. Continuous monitoring of ACT efficacy was done at the developed sentinel sites. This study reports the findings of the clinical as well as molecular studies at three study sites in the northeastern region.

Methods

Study sites

The study was conducted from May to August 2012 in Mizoram (district Lunglei, Tlabung Sub divisional hospital); from August to September 2012 in Tripura (district Gomati, Silachari Primary Health Centre (PHC)); from September to October 2012 in Arunachal Pradesh (district Changlang, PHC Miao). The study sites (Figure 1), which include three different regions of northeastern India representing different epidemiological situation of malaria due to international borders, are described below.

Mizoram

Lunglei district in the state of Mizoram presents ideal ecological conditions for malaria transmission with undulating uplands intersected by forested hills, rocky streams and jhum cultivation land. The area is characterized by a tropical, humid climate with cool summer and cold winter and heavy rainfall during May to late September with an average annual rainfall of 3,006 mm under the influence of southwest monsoon, and the mean annual temperature ranges between 20 and 26°C. The state contributes 0.8% to the country's malaria burden [18]. *Plasmodium falciparum* accounts for 80 to 90% of the total malaria cases and the proportion of *P. falciparum* has increased from 83 to 96% over the last five years in the study district. The study area of Tlabung subdivisional hospital in Lunglei district is highly endemic for malaria, characterized by year-round malaria transmission.

Tripura

The state contributes 1.3% to the country's malaria burden [18]. The peak malaria season is from August to November. The study districts are highly endemic, with a high proportion of *P. falciparum* (87-91%) cases.

Tripura is one of the states in the northeastern region which shares a long international border with Bangladesh. The hilly and undulating terrain and the movement of

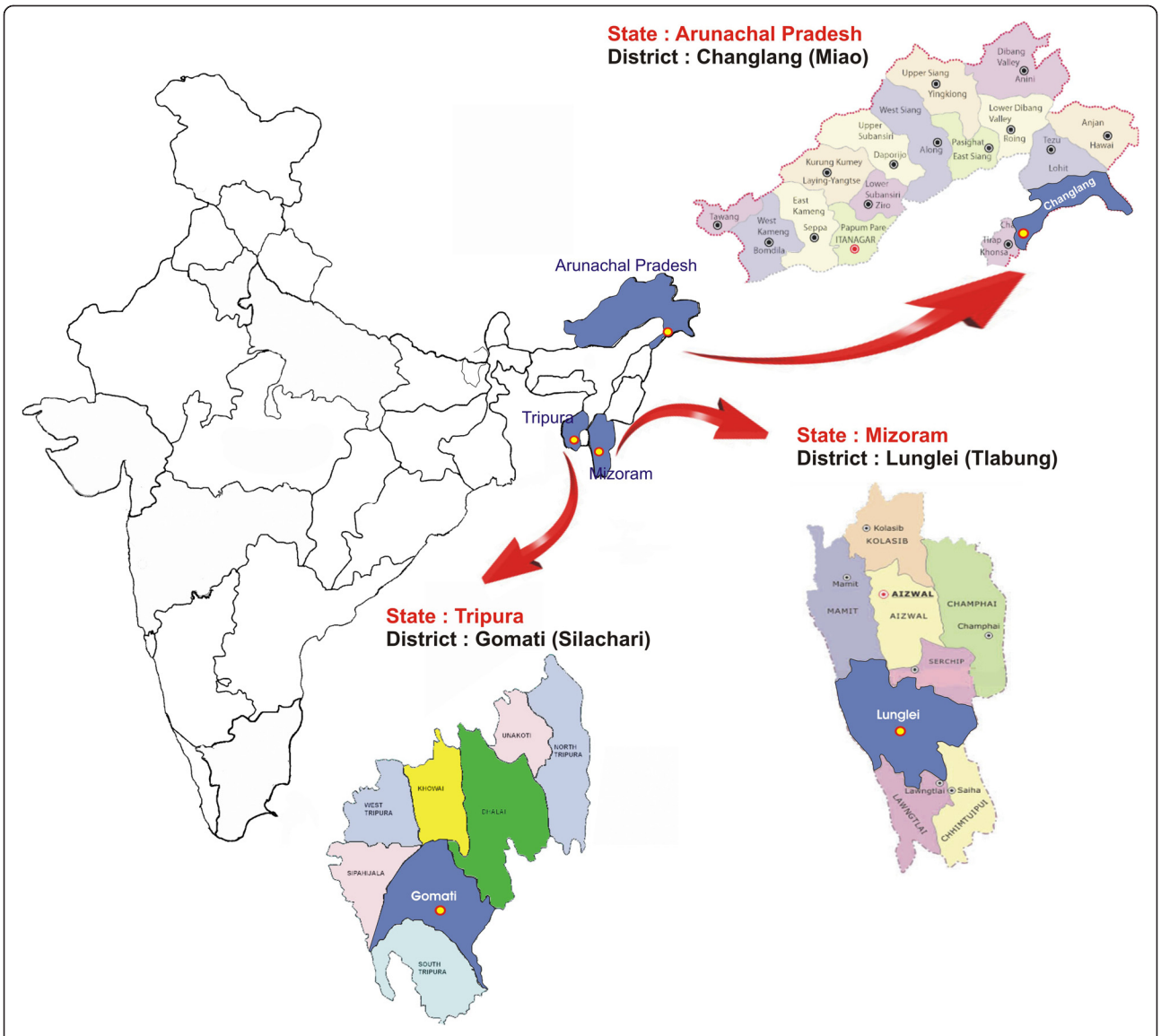

Figure 1 Details of study site from the National Antimalarial Drug Resistance Monitoring System, northeast India, 2012–2013.

people across the border have led to persistence of malaria in villages near the border. The regions adjacent to the Indo-Bangladesh border are mostly covered with thick forests and have poor communication and health infrastructure. *Plasmodium falciparum* is a major malaria parasite in this region, causing 70 to 90% of malaria infections. The hot and humid climate in the region is ideal for survival and multiplication of malaria vectors [19].

Arunachal Pradesh

Changlang district in the state of Arunachal Pradesh is bounded by Assam and Arunachal Pradesh in the north and Myanmar in the southeast. The state contributes 1.1% of the country's malaria burden [18]. The capital town of Miao, in Changlang district, was selected as the study site where the percentage of *P. falciparum* has increased from 56 to 64% in the last five years.

Study design

This was an open-label, one-arm prospective study of clinical and parasitological responses after administration of ACT treatment and WHO protocol for *in vivo* monitoring was followed [20].

Ethical considerations

Informed, written consent was obtained from enrolled adults and from a legal guardian of each child enrolled. The study protocol was approved by the Institutional Ethics Committee of the National Institute of Malaria Research (NIMR) in New Delhi. These studies were part of

monitoring and surveillance studies jointly carried out by NIMR and National Vector Borne Disease Control Programme (NVBDCP) with added quality assurance, monitoring and molecular markers.

Study population and sample size

Adults and children over 6 months presenting with fever (axillary temperature ≥ 37.5°C) at visit or a history of fever for the preceding 24 hours were included in the study. Other criteria for inclusion were mono-infection with *P. falciparum* with parasitaemia between 500 and 100,000 asexual parasites/μl blood, absence of other febrile conditions and informed consent. Patients having severe malnutrition as per WHO guidelines, severe malaria or danger signs and inability to come for follow-up visits were excluded from the study.

Treatment and follow-up

Patients with uncomplicated *P. falciparum* received artesunate (AS) + SP (AS 4 mg/kg for three days plus SP 25/1.25 mg/kg single dose on day 0) and primaquine (PQ) (0.75 mg/kg) on last day of treatment. The treatment was directly observed on all three days and used quality assured drugs through state government supply which consisted of manufacturers (site name in brackets): Medicamen Biotech Ltd, India (Lunglei), Medico Remedies Pvt Ltd, India (Gomati), ZEST Pharma, India (Changlang). These drugs are supplied in the trade name of 'Antimalarial combi blister pack'. All drugs were used within their expiry period and batch number and expiry dates were recorded in each case record form (CRF). The dosing was based on age with five categories of combi blister packs with age category < one year, one to four years, five to eight years, nine to 14 years and >15 years (adult pack). The study followed the standard WHO protocol for assessment of therapeutic efficacy of anti-malarial drugs for uncomplicated falciparum malaria for moderate transmission [20]. On enrolment day (day 0), upon fulfilment of all inclusion criteria, consent was obtained from the patients to participate in the study. A brief history was recorded and clinical was done. A pretreatment blood sample was collected from each eligible patient and used to make thick and thin smears and dried blood spots on filter paper. All medicines were given under direct supervision. Patients were observed for a few minutes after administering the study drug to ensure that they did not vomit. Patients who vomited the dose within 30 minutes were retreated with the same drug and dose. Follow-ups were scheduled for days 1, 2, 3, 7, 14, 21, 28, 35, and 42. On each of these days, clinical and parasitological assessments were performed. Blood samples were obtained on filter papers for genotyping and molecular studies on day 0 and every other day of follow-up. Polymerase chain reaction (PCR) genotyping was done for distinguishing recrudescence from new infections by comparing *msp1*, *msp2* (merozoite surface protein) and *glurp* (glutamate rich protein) gene loci of pre- and post-treatment sample pairs. The outcome of treatment with PCR correction was based on the number of true recrudescence excluding cases of novel infections. In addition, *pfdhfr* and *pfdhps* mutations for partner drug resistance were used as molecular markers to analyse different samples.

Laboratory methods

Thick and thin blood films were collected and stained with Giemsa. Slides were examined on day 0 by experienced microscopist(s) for species identification and quantification of parasites. Slides were also prepared on days 1, 2, 3, 7, 14, 21, 28, 35, and 42 to determine asexual and sexual parasite density by counting the number of parasites against 200 white blood cells (WBCs), and were expressed assuming WBC count to be 8,000/microlitre. A slide was considered negative when counting 1,000 WBC in thick smear did not show asexual parasites. All the slides were cross-checked at NIMR, Delhi.

Paired blood samples of patients collected on day 0 and the day of recurrent parasitaemia (14–42 days) were analysed sequentially starting with the highest discriminatory marker, *msp2* or *glurp*. The third marker analysed was *msp1* to differentiate recrudescence from new infections [21]. A new infection is a subsequent occurring parasitaemia in which all the alleles in parasites from the post-treatment sample are different from those in the admission sample, for one or more loci tested. In a 'recrudescence' at least one allele at each locus is common to both paired samples [21]. In addition, SP resistance-associated markers, namely mutations at codon 51, 59, 108, 164 of *pfdhfr* gene and *pfdhps* gene at codon 436, 437, 540, 581, 613 were also analysed in the day 0 samples.

Genomic DNA was isolated from blood spots using QIAamp DNA minikit, Germany. Genotyping PCR assays were carried out following the protocols reported earlier [22]. Separate nested PCR reactions were performed for the three allelic families of *msp1* (MAD20, K1 and RO33), two allelic families of *msp2* (Fc 27 and Ic) and *glurp. Pfdhfr* and *pfdhps* gene products were PCR amplified using earlier reported methods [23] and then digested using restriction enzymes for *dhfr* and *dhps* gene. Applied Biosystem thermocycler was used for all PCR amplification reactions. Digested PCR product (5–8 microlitre) was analysed on 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and 0.5X TBE running buffer (pH 8.0). PCR products were visualized under UV transilluminator (280 nm) and digitally captured with the help of gel documentation system (Alpha Imager EP). Molecular sizes of PCR fragments were calculated using gene tool (Alpha Inotech, version 3.0.3.0).

The case record forms were completed for each patient and all the clinical and parasitological data from

days 0 to 42 were recorded. The data were entered in WHO software [20] and both per protocol and Kaplan Meier analysis were performed to classify response as early treatment failure (ETF), late treatment failure (LTF) and adequate clinical and parasitological response (ACPR). In the secondary analysis, patients were withdrawn or censored, if classified as new infection by PCR or if PCR was missing.

Rescue medication

All the patients with ETF or LTF were treated with oral quinine in standard dose of 10 mg/kg body weight, three times for seven days. CRFs were used to capture adverse events, if any.

Data analysis

Patient and demographic variables that could be associated with treatment failure were investigated. Using the body weights recorded on day 0, the doses of AS given to patients with *P. falciparum* were categorized as being ≥ 87.5%, < 87.5 to 75% and < 75% of the recommended dose per kg. The parasite clearance time (PCT) was recorded in CRF till day 2 of treatment, although slides were made on day 3 as well. However the delayed PCT (PCT ≥ 72 hours) was calculated on day 3 based on the mean PCT up to day 2 ± SD. Kaplan-Meier survival analyses of treatment failure and parasite clearance were conducted with and without the results of the PCR-based identification of the parasitaemia that resulted from post-treatment re-infection. Log-risk models were used to evaluate the multivariate associations observed between risk factors and endpoints. Multivariate analysis was based on full models that included age, sex, fever at enrolment, level of parasitaemia at enrolment, infection with a parasite that harboured any of the investigated mutations in *dhfr* or *dhps,* and AS dose, as well as the interactions between age and AS dose, age and level of parasitaemia on enrolment, and between presence of mutations and level of parasitaemia on enrolment. A complete case (per protocol) analysis was performed. A strategy of backward elimination was followed in which p-value of < 0.10 and < 0.15, respectively, were used as the cut-offs in eliminating individual factors and interaction terms as statistically significant risk factors for treatment failures. All data analysis was performed using version 7.2 of a software package developed by WHO's Global Malaria Programme for evaluating therapeutic efficacy or SPSS version 14.

Results

A total of 190 patients with uncomplicated *P. falciparum* receiving AS + SP treatment on first three days and PQ on last day of treatment were enrolled from Lunglei district in Mizoram (71), Gomati district in Tripura (77) and Changlang district in Arunachal Pradesh (42), the three far- flung regions of northeast India. Fifteen patients were withdrawn after cross-checking due to mix infection, presence of other species, or out of range parasitaemia at the time of enrolment. Thus, 175 patients were found to be eligible, 169 (96.6%) of whom completed the 42 days of follow-up (Figure 2).

Almost half of the patients were of either sex and were febrile at the time of enrolment (Table 1) at two sites except Lunglei. The intake of anti-malarial drug in the previous week was rare. High gametocyte carriage was observed in Changlang followed by Gomati and Lunglei districts. The combined point mutation in *dhfr-dhps* gene, indicating partner drug resistance was observed at all the three sites with majority of isolates showing multiple mutations.

For calculating the primary and secondary endpoints of the 169 patients infected with *P. falciparum* who completed 42 days of follow-up, 57 patients were initially categorized as treatment failures. However, when parasites in paired dried blood spots from these 57 patients were genotyped by PCR, only 32 (56%) of the patients were confirmed as treatment failure, 24 were found to have *P. falciparum* re-infection and one had *P. vivax* infection on day 21. These 32 patients who were confirmed to have failed AS + SP treatment came from Lunglei (11), Gomati (15) and Changlang (six) (Table 2). Four ETF occurred in Gomati (Tripura); two patients had parasitaemia and fever on day 3 while two patients had parasitaemia on day 2 (parasitaemia on day 2 was > 25% of day 0). The crude and PCR-corrected Kaplan-Meier survival estimates were 60.8% (95% CI: 48.0-71.4) and 76.6% (95% CI: 64.1-85.2) for Gomati (Tripura); 74.6% (95% CI: 62.0-83.6) and 81.7% (95% CI: 69.4-89.5) for Lunglei, Mizoram; and 59.5% (95% CI: 42.0-73.2) and 82.3% (95% CI: 64.6-91.6) for Changlang, Arunachal Pradesh, respectively. In general, the *P. falciparum* patients treated with AS + SP cleared their parasitaemia rapidly, 52.1% (88 of 169) in < 24 hours, 35.5% (60 of 169) in intervals of 24 to <48 hours, 8.8% (15 of 169) in 48 to < 72 hours and 4.7% (eight of 169) of the patients in ≥ 72 hours, respectively (Table 2). The highest number of patients clearing parasitaemia within <24 hours was in Lunglei, Mizoram. Even the LTF patients cleared their parasitaemia rapidly; 14.8% (13 of 88), 16.7% (ten of 60), 40% (six of 15) and 37.5% (three of eight) of the patients who showed parasite clearance intervals of < 24 hours, 24 to < 48 hours, 48 to < 72 hours and ≥ 72 hours, respectively. Six of the patients from Gomati, Tripura and two from Changlang, Arunachal Pradesh did not clear their parasitaemia within 72 hours (Table 2). Three of them were identified as treatment failures (including two ETF and one late parasitological failure), two were re-infection whereas the three achieved complete cure. However, the proportion of patients remaining parasite positive at day 3 in these

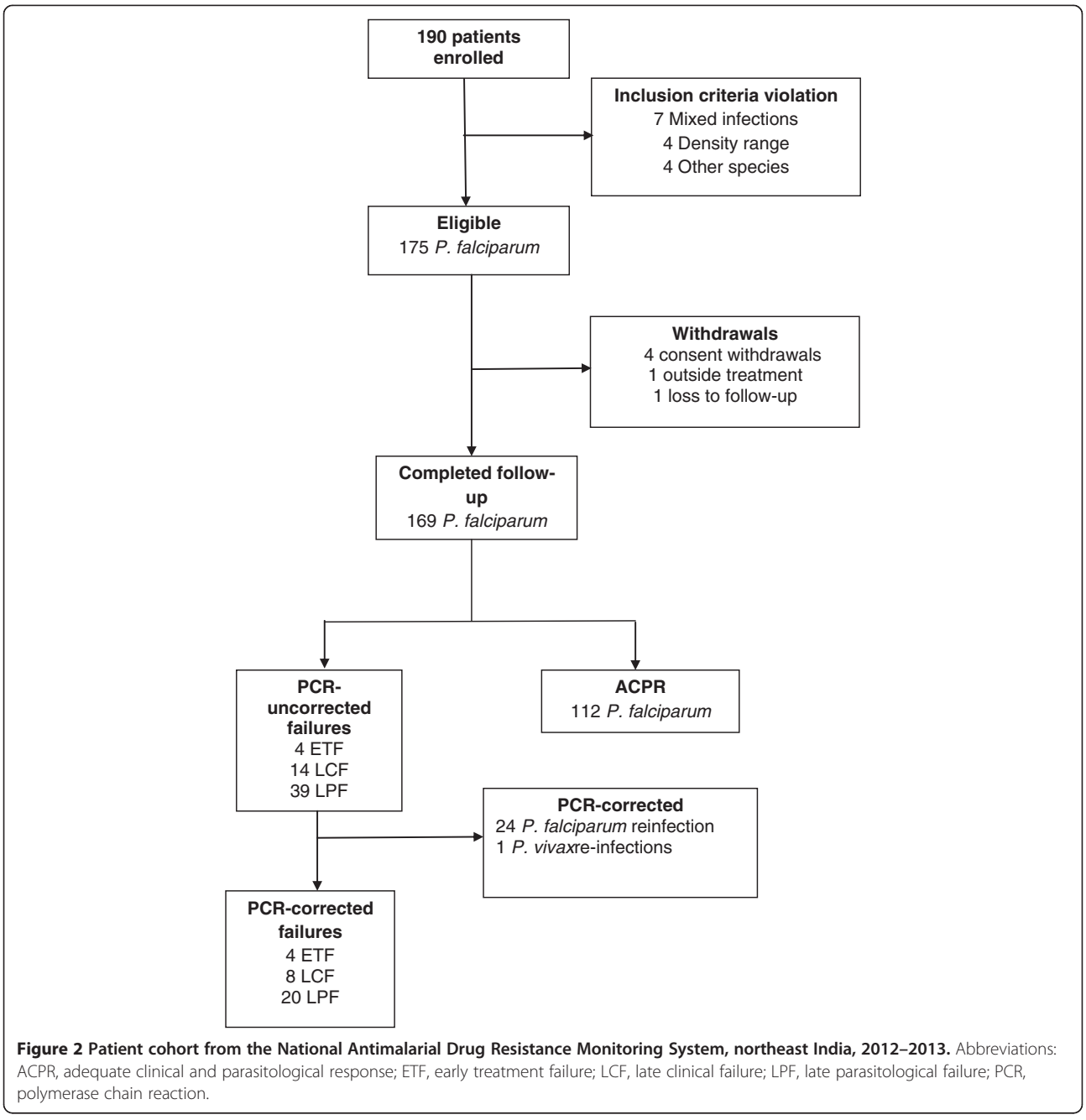

Figure 2 Patient cohort from the National Antimalarial Drug Resistance Monitoring System, northeast India, 2012–2013. Abbreviations: ACPR, adequate clinical and parasitological response; ETF, early treatment failure; LCF, late clinical failure; LPF, late parasitological failure; PCR, polymerase chain reaction.

studies was much below the cut-off prescribed by WHO. No adverse effect was noted at any site during the 42 days' follow-up.

The study compared the mean parasitaemia at enrolment, dose of AS or any adverse event reported between patients who had parasitaemia ≥72 hours with those who cleared parasite within 48 hours. No significant difference was observed between the two groups. However, host immunity had a definite role as six out of eight patients were < five years of age.

In multivariate analysis, younger age category (< five years) relative to adults 15 years or older and low administered dose of AS (< 3 mg/kg, 3–3.5 mg/kg) relative to 3.5 mg/kg or more were associated with higher risk. The risk of failure increased as the dose of AS decreased. However, optimal or higher dose of SP was observed in 90.6% patients. Fever at enrolment was positively and significantly associated with treatment failures. Also, relative risk was highest with the presence of triple or quadruples mutation (Table 3). There were insufficient observations

Table 1 Clinical and demographic characteristics of eligible patients in studies of the National Antimalarial Drug Resistance Monitoring System, northeast India, 2012-13

Study site	Lunglei (Mizoram)	Gomati (Tripura)	Changlang (Arunachal Pradesh)
Characteristic	(N = 66)	(N = 72)	(N = 37)
Sex (no (%))			
Male	39 (59.1)	39 (54.2)	22 (59.5)
Female	27 (40.9)	33 (45.8)	15 (40.5)
Age category (no (%))			
< 5 yr	6 (9.1)	24 (33.3)	7 (18.9)
5-15 yr	26 (39.4)	30 (41.7)	18 (48.6)
Adult	34 (51.5)	18 (25.0)	12 (32.4)
Temperature (°C)			
Mean ± SD	36.3 ± 1.3	38.1 ± 0.4	38.2 ± 0.5
Range	36.2-39.3	37.5 - 39.2	(37.5 - 39.1)
Febrile (≥ 37.5)			
Yes (no (%))	13 (19.7)	72 (100.0)	37 (100.0)
No (no (%))	53 (80.3)	-	-
Parasite count (no/μl)[a]			
Mean ± SD	18899.3 ± 23979.5	18507.6 ± 20937.3	31186.5 ± 27451.6
< 5,000	22 (33.3)	26 (36.1)	7 (18.9)
5,000-50,000	36 (54.5)	37 (51.4)	20 (54.1)
≥ 50,000	8 (12.1)	9 (12.5)	10 (27.0)
Gametocytes on day 0			
(no (%))	3 (4.2)	17 (23.9)	12 (32.4)
Number of mutations			
0-2	6 (9.1)	3 (4.2)	1 (2.7)
2-4	4 (6.1)	5 (6.9)	2 (5.4)
≥ 5	56 (84.8)	64 (88.9)	34 (91.9)

[a]At enrolment, before treatment.

to determine the risk factors for parasite clearance interval of ≥ 72 hours.

Gametocytes on day 0 at the enrolment were reported in 20.1% patients with highest number in Changlang followed by Gomati. There was increase in the number of patients carrying gametocytes on day 7 at Gomati, Tripura (Table 4). The presence of gametocyte was observed in 2.3% patients as late as day 42 although radical treatment with single dose of PQ (0.75 mg/kg) was given on last day of treatment. PQ use is however contra-indicated in pregnant women and children aged < one year.

Table 2 Site-wise results of therapeutic efficacy and parasite clearance in studies of the National Antimalarial Drug Resistance Monitoring System, northeast India, 2012-2013

				Therapeutic efficacy										PCT (hours)			
State/UT	District/city	Drug	n	ACPR[a]	ETF[b]	LCF[c]	LPF[d]	LFU	WTH	PV	PF*	Surv	95% CI	< 24	24- < 48	48- < 72	≥ 72
Tripura	Gomati	ASP	77	43	4	3	8	0	8	1	10	74.1	61.0, 84.7	25	26	12	6
Mizoram	Lunglei	ASP	71	47	0	3	8	1	7	0	5	81.0	68.6, 90.1	47	15	1	0
Arunachal Pradesh	Changlang	ASP	42	22	0	2	4	0	5	0	9	78.6	59.0, 91.7	15	18	2	2

Abbreviations: *ACPR* adequate clinical and parasitological response, *ETF* early treatment failure, *LCF*, late clinical failure, *LPF* late parasitological failure, *LFU* loss to follow-up, *WTH* withdrawal, *PCT* parasite clearance time, **PF P. falciparum* re-infection, *PV* protocol violation.
[a]Absence of parasitaemia on day 28 without previous criteria for failure.
[b]Marked by the following, alone or in combination: danger signs for severe malaria on day 1, 2 or 3 in the presence of parasitaemia; parasitaemia level on day 2 higher than on day 0; parasitaemia level on day 3 more than 25% higher than on day 0; parasitaemia on day 3 plus fever.
[c]Danger signs for sever malaria and/or fever plus parasitaemia from day 4 to day 28.
[d]Parasitaemia from day 7 to day 28 even if patient afebrile.

Table 3 Risk of *Plasmodium falciparum* treatment failure among patients in studies of the National Antimalarial Drug Resistance Monitoring System, northeast India, 2012-2013

Predictor	Value	Fail[a]	ACPR	Risk	RR	95% CI
Artesunate dose (mg/kg)	≥ 3.5	24	84	0.222		
	3.0-3.5	5	21	0.192	0.64	0.19, 2.19
	< 3.0	3	7	0.300	1.35	0.49, 3.71
Age	< 5	13	18	0.419	2.17	1.11, 2.46
(years)	5-15	11	46	0.193	1.35	0.58, 3.11
	≥ 15	8	48	0.143	–	
Parasite count	< 5000	6	38	0.136	0.65	0.28, 1.53
(/μL)	5000 - 50000	17	64	0.210	0.44	0.23, 0.84
	≥ 50000	9	10	0.474	–	
[#]Antifolate	Wild	0	0	0.000	–	
mutations	1-2	0	6	0.000	–	
	3-4	5	11	0.313	1.48	0.65, 3.34
	5-6	22	82	0.212	0.68	0.29, 1.53
	7-8	5	11	0.313	–	
Fever	Yes	25	73	0.255	1.68	0.78, 3.59
(≥ 37.5°C)	No	7	39	0.152	–	
Previous	Yes/unknown	17	69	0.198	0.76	0.42, 1.41
Drug intake	No	15	43	0.259	–	

Abbreviations: *ACPR* adequate clinical and parasitological response, *RR* relative Risk, *CI* confidence interval, [#]*dhfr* + *dhps*.
[a]25 patients were excluded from the analysis as confirmed by PCR.

Out of the 190 isolates, 155 could be successfully genotyped for *dhfr* and *dhps* mutation analysis. In *dhfr*, double (n = 71) and triple mutations (n = 69) were common. The most frequent haplotype was double mutant 108/59 followed by triple mutant 108/59/51. Only five isolates were single mutant while three were found to have quadruple mutation (5.2%).

In *dhps*, triple (n = 75) and quadruple mutations (n = 35) were common. Mutations in codon 437 and 540 were most frequent and the most frequent haplotype was triple mutant 540/437/436 followed by quadruple mutant 581/540/437/436 (Table 5).

Among the 32 PCR-corrected treatment failures, two samples were not amplified, one was *dhfr* quadruple mutant, 21 were triple mutants, seven were double mutants and one was single mutant. However, seven samples were *dhps* quadruple mutant, 14 triple mutants, eight were double, and one was single mutant.

Interestingly, *pfdhfr* L164 mutation has been observed in three out of 11 treatment failures in Lunglei, two out of 15 treatment failures in Gomati and in Changlang district. Earlier, *pfdhfr* L164 mutation has been found to be associated with higher level of SP resistance in addition to decreased efficacy of chloroguanil/dapsone. Also *dhps* $\mathbf{T}_{613}$, $\mathbf{G}_{581}$ and $\mathbf{A}_{436}$ have also been observed at all the three sites, majority in ACPR samples except one $\mathbf{G}_{581}$*dhps* mutation in treatment failure sample out of the total four failures in Changlang district.

Discussion

The sentinel site monitoring studies at three sites in northeastern region indicate that the efficacy of the ACT (i.e., AS + SP) recommended for *P. falciparum* malaria was declining. The treatment failure rates above the 10% threshold warranted a change of drug policy in the region. These treatment failures were mostly LTF with

Table 4 Gametocyte carriage detected by microscopy in patients

S No	State	District	Gametocytes/μl (%)						
			D0	D7	D14	D21	D28	D35	D42
1	Tripura	Gomati	17/69 (24.6)	22/69 (31.9)	20/69 (29.0)	14/69 (20.3)	6/69 (8.7)	-	3/69 (4.3)
2	Mizoram	Lunglei	1/63 (1.6)	2/63 (3.2)	0/63 (0)	0/63 (0)	1/63 (1.6)	0/63 (0)	0/63 (0)
3	Arunachal Pradesh	Changlang	12/37 (32.4)	0/37 (0)	1/37 (2.7)	0/37 (0)	0/37 (0)	0/37 (0)	1/37 (2.7)

Table 5 Molecular markers of anti-folate resistance in *Plasmodium falciparum* isolates collected through the National Antimalarial Drug Resistance Monitoring System, northeast India, 2012-2013

	dhfr (N = 155)		*dhps* (N = 155)	
Mutations	**n**	**Haplotype**	**n**	**Haplotype**
Quadruple	5	**I**51/**R**59/**N**108/**L**164	1	**A**436/**G**437/**E**540/A581/**T**613
			35	**A**436/**G**437/**E**540/**G**581/A613
Triple	69	**I**51/**R**59/**N**108/I164	75	**A**436/**G**437/**E**540/A581/A613
	7	N51/**R**59/**N**108/**L**164	6	**A**436/**G**437/K540/**G**581/A613
			5	S436/**G**437/**E**540/**G**581/A613
Double	71	N51/**R**59/**N**108/I164	11	S436/**G**437/**E**540/A581/A613
			1	**A**436/A437/**E**540/A581/A613
			2	S436/A437/**E**540/**G**581/A613
			9	S436/**G**437/K540/**G**581/A613
Single	3	N51/C59/**N**108/I164	1	S436/A437/**E**540/A581/A613
			2	S436/**G**437/K540/A581/A613
			1	S436/A437/K540/**G**581/A613
Wild			6	S436/A437/K540/A581/A613

*Mutations in bold.

molecular markers for partner drug resistance showing triple or quadruple mutation in *dhfr* and *dhps* genes. Four ETF were observed at Tripura. These patients received optimal dose of AS and SP. As per WHO definition of suspected artemisinin resistance, increased parasite clearance as evidenced by > 10% of cases with parasites detectable on day 3 following treatment with an ACT warrants studies to confirm presence of artemisinin resistance. However, the proportion of patients remaining parasite positive at day 3 in these studies was much below the cut-off prescribed by WHO. In addition, artemisinin efficacy studies are being undertaken at two of these sites to confirm the efficacy of artemisinin in the region. It is worth mentioning that three out of four patients were below the age of five years and had higher frequency of quintuple mutation as compared to patients with ACPR, confirming the role of host immunity.

This study provides the first report on the emerging treatment failure of AS + SP in remote areas of the country, including areas directly across the border from Bangladesh and Myanmar where artemisinin resistance has been confirmed recently. Treatment failure to this ACT is now evident at all the three study sites of northeast India. The failure of AS + SP was likely as the molecular marker of partner drug resistance showed increasing trend since 2009 in the nationwide sentinel site monitoring where 20% random samples were assayed to monitor drug resistance at molecular level. The trend showed increase from single to double mutation in majority of the samples. These studies were part of nationwide sentinel site monitoring system, where 15 sentinel sites are being monitored across the country to ascertain the efficacy of recommended anti-malarials against the predominant species of Plasmodium.

Based on the results of this study, the expert committee of the NVBDCP created the first subnational drug policy for India and selected artemether plus lumefantrine as the new first-line treatment in the northeast [24].

ACT treatment failure > 10% is the cost-effective threshold and requires change of treatment policy for malaria along with continuous monitoring in adjacent areas [20]. In the present study high treatment failure of AS + SP was observed at all the three sites ranging between 17 and 23%. A recent study conducted in the neighbouring area of northeast also showed AS + SP treatment failure of 9.5% [25]. This indicates that the reported treatment failure of AS + SP is frequent and widespread as the sites were present in four distant corners of the region and thus is likely to be present in between areas as well. The treatment failures reported in the study could be due to resistance to partner drug SP as there has been long history of use and documented SP failure in the region [5]. Also, irrational treatment practices as well as non-availability of age-based combi blister packs for children might have caused drug pressure in the population leading to failure of partner drug [26,27]. However, combination therapy with ineffective partner drug is equivalent to artemisinin monotherapy on a large scale.

Until recently, the efficacy and safety of recommended ACT (AS + SP) was high at multiple sites in the country [28]. The nationwide sentinel site monitoring system conducted 40 studies up to 2011 and the efficacy of AS + SP during 28 days' follow-up was above WHO recommended cut-off for treatment failure at all the sites. The nationwide sentinel site system also monitored partner drug resistance through molecular studies given the long half life of SP to detect emerging resistance. Despite low prevalence of treatment failures to AS + SP, molecular markers of partner drug resistance were monitored to capture early signs of treatment failure. Emerging increasing trend of partner drug resistance markers and to capture LTF, if any, follow-up in the sentinel site monitoring system was extended to 42 days since 2012. Until 2010, *dhps* remained wild in majority of the samples, however, increasing trend in *dhps* point mutations were observed later, particularly in northeastern region. These findings prompted more focus in northeastern region. Thus, three sites at different ecological conditions were selected and efficacy of AS + SP in *P. falciparum* was studied.

Data on molecular studies for SP resistance are conclusive with increased frequency of quintuple mutation over time. Increased frequency of quadruple mutation in *dhfr* and *dhps* gene was observed from all the three study sites with higher frequency in *dhps* gene. Sulphadoxine

resistance in *P. falciparum* is associated with mutations at five *pfdhps* codons; 436ala/phe, 437gly, 540glu, 581gly, and 613ser [10]. The *pfdhps* mutation at codon A436 causes alteration in binding of sulphadoxine followed by sequential mutations at G437, E540, G581, and T/S613 which may cause increase in sulphadoxine resistance [29]. Mutation at codon G581 along with 437 and 540 may be associated with earlier treatment failure [30].

A strong indicator for SP treatment failure is the quintuple mutations in three *pfdhfr* codons (108asn + 51ile + 59arg) and two *pfdhps* codons (437gly + 540glu) [31]. Quintuple mutation has been observed in 39% of treatment failure samples at all the three sites with highest in Tripura, confirming the role of SP failure. In the present study, quintuple as well as more combination of mutations have been observed at all the three sites with highest in Tripura. Similarly, high prevalence of quintuple mutation (35%) has been observed in samples collected from Kolkata [25].

Following these findings, further new studies on tracking resistance to artemisinin are being undertaken at two sites in this region. Recently association of K13-propeller polymorphism with artemisinin resistance *in vitro* and *in vivo* has been proposed [32]. Further studies to map K13 gene polymorphism, in addition to the clinical phenotype of slow clearance, are being undertaken.

Various factors including host immunity and pharmacokinetics of the drug play an important role in achieving complete cure or treatment failure in patients. In the present study, younger age category (<five years) relative to adults 15 years or older, fever at enrolment and low administered dose of AS (< 3 mg/kg, 3–3.5 mg/kg) relative to 3.5 mg/kg or more were associated with higher risk of treatment failure. Negative correlation was observed between the risk of failure with the dose of AS (in mg/kg body weight). Although, the recommended daily dose of AS is 4 mg/kg, 15.6% of treatment failure patients received 3.0 to < 3.5 mg of AS per kg, and 9.4% received < 3.0 mg/kg. Due to variation in body weight within age categories, dosing of drugs based on age category presents the risk of under-dosing or over-dosing. Thus, the criteria to use age rather than body weight for calculating the dose of antimalarial needed for a patient is the probable cause of suboptimal dosing. However, optimal or higher dose of SP was observed in majority of the patients with only 9.4% patients receiving under-dose of SP. Fever at enrolment was positively and significantly associated with treatment failures. Earlier results of nation-wide sentinel system also observed younger age, fever and low AS dose as the potential risk factors for treatment failure [28]. Partner drug resistance markers are well defined and can ascertain the falling efficacy of the administered drug. The relative risk was highest with the presence of triple or quadruple mutation, which signifies the role of SP in treatment failure. Parasite clearance time is multifactorial and is affected by initial parasite load, host immunity, pharmacokinetics of the drug. No significant difference in mean parasitaemia, dose of AS at enrolment or any adverse event reported was observed between the patients who had parasitaemia ≥ 72 hours with those who cleared parasite within 48 hours. Host immunity had a definite role as six out of eight patients were less than five years of age. Also, poor absorption of drug in individuals may play a role in delayed PCT besides immunity. However, there were insufficient observations to determine the risk factors for parasite clearance interval of ≥ 72 hours.

Recently increasing failure rates of artesunate mefloquine in Thailand and dihydroartemisinin piperaquine in Cambodia have been reported [16,33]. With increasing cases of ACT failure, more studies to optimize the dose and duration of recommended ACT or new combination is required. The newly recommended ACT: artemether-lumefantrine (AL) has been used by neighbouring countries in the past; hence cross-border monitoring of AL with countries that use this regimen is urgently required. In addition, the newly introduced ACT in the region has certain challenges as the compliance to twice daily regimen and the fat dependant absorption in diet need to be considered besides the molecular markers for lumefantrine, as well as other partner drugs. Expanded *in vitro* monitoring in the northeast for early warning of this ACT is required.

Conclusions

The efficacy of AS + SP was declining in northeast India and exceeded the threshold for changing drug policy. Based on the results of the study, the expert committee of the National Vector Borne Disease Control Programme formulated the first subnational drug policy for India and selected artemether plus lumefantrine as the new first-line treatment in the northeast. Continued monitoring of antimalarial drug efficacy in northeastern region is essential for effective malaria control.

Limitations

The nation-wide sentinel site monitoring system created in 2009 accelerated the assessment of emerging ACT treatment failure in areas with international borders and is vigilant to detect delayed parasite clearance, if any. Few of the earlier reported limitations of nationwide sentinel system were overcome during 2012. Firstly, follow-up was extended up to 42 days, delayed parasitaemia was monitored by looking at parasite clearance interval of ≥ 72 hours; partner drug resistance markers were analysed in all the isolates. The other limitations include 24 hours sampling for parasite monitoring, as the patients were from distant places. The sample size was relatively low in Changlang district, Arunachal Pradesh. To ascertain the role of

partner drug resistance, drug levels of SP could not be done as the samples for drug concentration could not be collected.

Competing interests
The authors declare that they have no competing interests.

Authors' contribution
NM, NV and GSS were responsible for the design of the project proposal and monitored progress. NM, KK, BS, JPN and RSB were involved in quality check of data and molecular studies. NM, VD and SP monitored the studies at the site. NM, RR and NKS compiled and performed statistical analysis. NM and NKS wrote the first draft and ACD, GSS, ARA and NV corrected the draft and all authors read and approved the final manuscript.

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Monitoring antimalarial drug resistance in India via sentinel sites: outcomes and risk factors for treatment failure, 2009–2010

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Objective To describe India's National Antimalarial Drug Resistance Monitoring System, measure the efficacy of first-line malaria treatments, and determine risk factors for treatment failure.

Methods In 2009–2010, prospective studies with 28 days of follow-up were conducted at 25 sentinel sites. Patients infected with *Plasmodium falciparum* were given artesunate plus sulfadoxine-pyrimethamine (AS+SP); those infected with *P. vivax* were given chloroquine. Polymerase chain reaction was used to distinguish post-treatment reinfection from treatment failure. Isolates of *P. falciparum* were checked for *dhfr* and *dhps* mutations.

Findings Overall, 1664 patients were enrolled. Kaplan–Meier survival analysis showed an efficacy of 98.8% for AS+SP. Most patients with *P. falciparum* parasitaemia cleared their parasitaemias within 24 hours of treatment initiation, but six, including four with treatment failure, remained parasitaemic after 72 hours. Double mutants in *dhfr* were found in 68.4% of the genotyped isolates. Triple or quadruple mutants in *dhfr* and mutations in *dhps* were rare. A daily dose of artesunate of < 3 mg per kg of body weight, age of less than 5 years, and fever at enrolment were associated with an increased risk of treatment failure. Chloroquine remained 100% efficacious and generally cleared *P. vivax* parasitaemias within 48 hours. Vomiting (seen in 47 patients) was the most common adverse event.

Conclusion India's National Antimalarial Drug Resistance Monitoring System provides wide coverage. The first-line antimalarials used in the country remain safe and efficacious. The treatment of malaria in young children and the relative benefits of age- and weight-based dosing need further exploration.

Abstracts in عربي, 中文, Français, Русский and Español at the end of each article.

Introduction

Resistance to antimalarial drugs in the parasites that cause malaria is hampering the effective global control of the disease in humans. *Plasmodium falciparum* rapidly develops resistance to new therapies, and the presence in south-eastern Asia of strains of this parasite that are resistant to many drugs, including artemisinin, is alarming.[1] *P. vivax* can develop resistance to chloroquine and is innately less susceptible to antifolate compounds than *P. falciparum*, and the sensitivity of the parasite to primaquine during its dormant stages is difficult to assess.[2] The long-term public health response to the problem of antimalarial drug resistance must include a reduction in drug pressure through a rational approach to treatment and the creation of a pipeline for the identification and production of alternative drugs. The routine management of parasite resistance is based on the monitoring of the efficacies of current therapies and the subsequent selection of appropriate treatments based on the data collected. Several monitoring techniques are used to inform policy-makers, including in vitro tests of drug sensitivity, pharmacokinetic studies, investigation of the molecular markers of drug resistance, and, as the "gold standard", in vivo trials of therapeutic efficacy.[3]

In India the national government has monitored antimalarial drug resistance over many decades. Although chloroquine-resistant *P. falciparum* was first reported near the India–Myanmar border in 1973,[4] chloroquine-resistant *P. vivax* was unknown in India until 1995, when two cases of infection with resistant *P. vivax* were detected in Mumbai.[5] In 1978, the National Malaria Eradication Programme (now the National Vector Borne Disease Control Programme, or NVBDCP) created six regional monitoring teams.[6] For many years, the Malaria Research Centre (now the National Institute of Malaria Research, or NIMR) and other organizations supported a wide range of monitoring efforts in addition to the routine work of these regional teams.[7] Between 1978 and 2007, at least 380 in vivo trials of chloroquine and/or sulfadoxine-pyrimethamine for the treatment of *P. falciparum* malaria were conducted in India, with involvement of almost 19 000 patients.[8] Worryingly, the median percentage of cases failing to show an adequate response to sulfadoxine-pyrimethamine within 28 days of treatment increased from 7.7% in 1984–1996 to 25.9% in 1997–2007.[8] Indian isolates of *P. falciparum* were also frequently found to carry mutations in the genes that code for the targets of sulfadoxine and pyrimethamine: dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*), respectively.[9] In 2005,

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the combination of artesunate with sulfadoxine-pyrimethamine (AS+SP) replaced sulfadoxine-pyrimethamine as the nationally recommended first-line treatment for *P. falciparum* malaria in India.[10] While the efficacy of AS+SP, again measured after 28 days of follow-up, was found to be high (96–100%) in nine studies conducted in India between 2005 and 2007, the numbers of cases investigated were quite small given the large size of the country.[3] A major concern is that, since the efficacy and lifespan of artemisinin combination therapies depend largely on the partner drug, any pre-existing resistance to sulfadoxine-pyrimethamine could endanger the new combination.

To address the continued problem of antimalarial drug resistance in India, a joint NVBDCP–NIMR surveillance system – the National Antimalarial Drug Resistance Monitoring System – was set up in 2008. This system has several innovations: (i) only about 50% of the sites are monitored each year (so that each site is monitored every 2 years, there is widespread coverage and information on long-term trends can be collected); (ii) *P. vivax* trials are routinely conducted to track the emergence of chloroquine resistance in this species; (iii) blood smear examinations and data analysis undergo central quality control; (iv) routine genotyping is performed to separate post-treatment reinfections from any recrudescent infections resulting from treatment failures; (v) molecular markers of drug resistance are genotyped simultaneously; and (vi) in-vivo trials of drug efficacy are integrated with supplementary studies, such as the evaluation of plasma drug concentrations and other pharmacokinetic parameters. The focus of the present study was on the data collected, nationwide, during the first 2 years of the new surveillance system's operation. These data were used to evaluate the efficacies of AS+SP against *P. falciparum* and of chloroquine against *P. vivax* , to determine the prevalences of several molecular markers of sulfadoxine-pyrimethamine resistance in *P. falciparum* (and so assess, independently, the probable efficacy of the "partner drug" in the artemisinin combination therapy) and to determine the clinical, demographic and/or parasite-related risk factors for treatment failure.

Methods

Study sites and subjects

Through its extensive surveillance system, which screens more than 100 million people in India for malaria each year, the NVBDCP identified 1.6 million malaria cases in 2009 and 2010.[11] In 2008, during an annual review of data on antimalarial drug resistance, the NIMR and NVBDCP selected 25 sentinel sites for the routine monitoring of such resistance: 24 districts (each with a population of 1 to 4 million) and one city (Appendix A, available at: http://mrcindia.org/appendix_a.pdf). These sites were purposively selected to provide a representative "cross-section" of transmission intensities, malaria ecotypes and geographical regions. Open-label, single-arm prospective studies have since been conducted in each of the sites in accordance with the World Health Organization (WHO) 2009 protocol for a therapeutic efficacy trial with a target sample size of at least 50 patients per site.[12] Patients with *P. falciparum* monoinfection (500–100 000 asexual parasites per µl of blood) or *P. vivax* monoinfection (> 250 asexual parasites per µl) and either current fever (i.e. an axillary temperature of ≥ 37.5 °C) or a history of fever over the preceding 24 hours were enrolled in these studies. Pregnant or lactating women, children weighing < 5 kg, patients with signs of severe malaria, adults who declined to give informed consent and children with legal guardians who declined to give consent were all excluded.

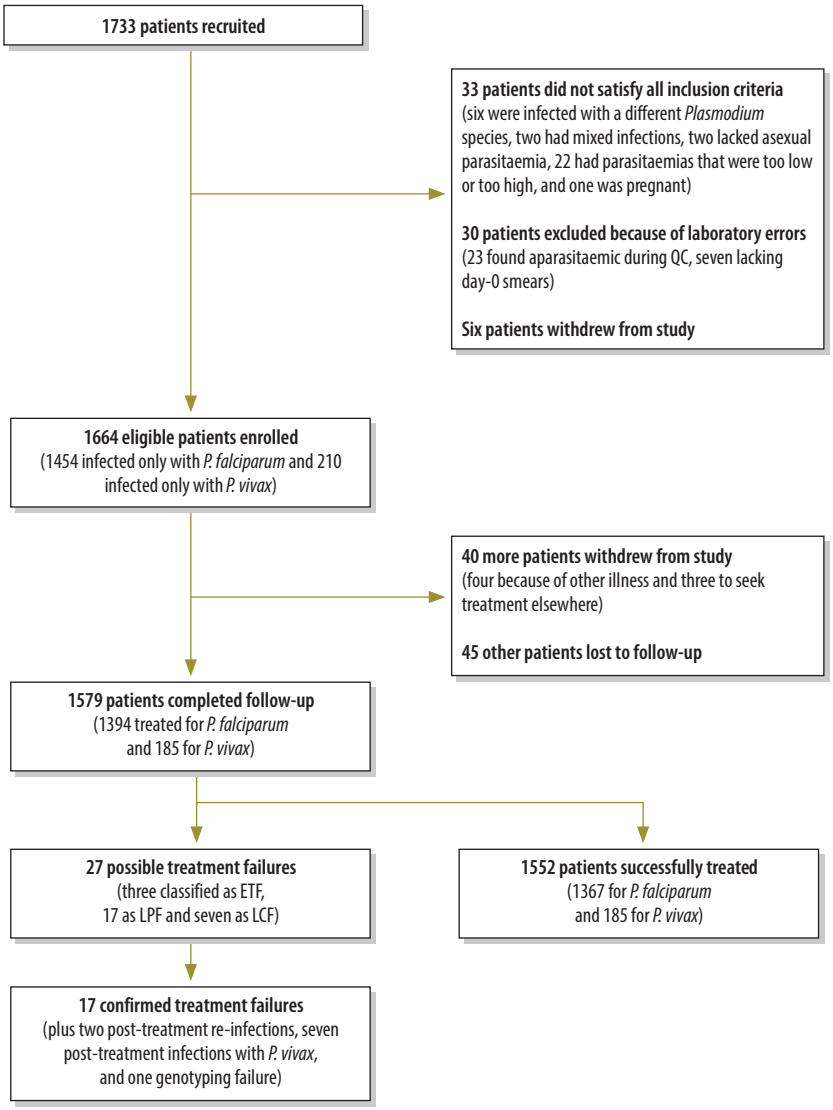
Data collection, treatment and follow-up

At enrolment (i.e. on "day 0"), a complete medical history (i.e. presenting symptoms, current medications and previous antimalarial drug use) was recorded for each patient. Female patients of child-bearing age were tested for pregnancy. A pre-treatment blood sample was collected from each eligible patient and used to make thick and thin smears and dried blood spots on filter paper. Patients were then given doses of an antimalarial drug according to their age, as recommended in the national drug policy.[10] Patients infected with *P. falciparum* were given AS+SP (the recommended dose is 4 mg artesunate per kg of body weight, given daily for 3 days, plus a single dose of sulfadoxine-pyrimethamine that is equivalent to 25 mg sulfadoxine per kg and 1.25 mg pyrimethamine per kg). Patients infected with *P. vivax* received chloroquine for 3 days (the total recommended dose is 25 mg mg per kg, given over 3 days) and then, after completing the 28 days of follow-up, primaquine for 14 days (the recommended daily dose is 0.25 mg per kg). The drug doses administered, which were based on age rather than body weight, may not have matched the recommended doses. The drug tablets used were quality-assured and obtained via the relevant state government. The intake of each treatment dose was observed, and patients who vomited within 30 minutes of a dose were retreated with the same drug and dose. Patients who did not complete treatment were withdrawn from the study. Patients were followed up on days 1, 2, 3, 7, 14, 21 and 28 in the clinics where they were treated, their residences or places of work. Patients were recommended to return to a clinic at any time if they experienced fever or other symptoms. At each follow-up visit, each patient was given a physical examination and a fresh sample of blood was collected and used to make both dried blood spots on filter paper and smears on a microscope slide.

Laboratory procedures

Each blood sample was used to make a thick and a thin smear on a single slide. The smears were Giemsa-stained. The level of parasitaemia (i.e. the number of asexual parasites per µl of blood) was determined from the thick smears by counting the asexual parasites against 200 leukocytes and assuming that each patient had 8000 leukocytes per µl of blood. Slides were declared negative if no parasites were detected in 100 high-power fields. Technicians with more than 10 years' experience rechecked the smears from all patients with treatment failure, plus a 10% random sample of all other smears from each sentinel site, at the NIMR's central quality control unit in New Delhi. Whenever parasitaemia was detected in a patient who had previously been found to be aparasitaemic, the malaria parasites in that patient's dried blood samples from day 0 and from the day of apparent recrudescence were genotyped by polymerase chain reaction (PCR) to enable post-treatment reinfection to be distinguished from treatment failure.[13] The dried day-0 blood samples from

Fig. 1. **Flowchart for patient recruitment and follow-up in the efficacy trials within India's National Antimalarial Drug Resistance Monitoring System, 2009–2010**



ETF, early treatment failure; LCF, late clinical failure; LPF, late parasitological failure; QC, quality control.

patients who failed treatment and from 25% of other patients selected at random were also checked for mutations in *dhfr* (at codons 51, 59, 108 and 164) and *dhps* (at codons 436, 437 and 540), using the primers described earlier.[14]

Trial end-points

Primary study end-points were classified according to the standard criteria defined in WHO's current guidelines.[12] Parasite clearance intervals (categorized as ≤ 24, > 24 to 48, > 48 to 72 or > 72 hours) were used as a secondary end-point, with an interval of > 72 hours used as the indicator of risk in the analysis of risk factors.

Data analysis

Patient and demographic factors that could be associated with treatment failure were investigated. Using the body weights recorded on day 0, the doses of artesunate given to patients with *P. falciparum* were categorized as being ≥ 87.5%, < 87.5% to 75% and < 75% of the recommended dose per kg. Kaplan–Meier survival analyses of treatment failure and parasite clearance were conducted with and without including the results of the PCR-based identification of the parasitaemias that resulted from post-treatment reinfection. Log-risk models were used to evaluate the univariate and multivariate associations observed between risk factors and end-points. Multivariate analysis was based on full models that included age, geographical area, sex, fever at enrolment, level of parasitaemia at enrolment, infection with a parasite that harboured any of the investigated mutations in *dhfr* or *dhps*, and artesunate dose, as well as the interactions between age and artesunate dose, age and level of parasitaemia on enrolment, and between presence of mutations and level of parasitaemia on enrolment. A strategy of backward elimination was followed in which *P*-values of < 0.10 and < 0.15 were used, respectively, as the cut-offs in eliminating individual factors and interaction terms as statistically significant risk factors for treatment failure. To account for the clustering of data by study site, clustered robust standard errors were used in the multivariate models. All data analysis was performed using version 7.2 of a software package developed by WHO's Global Malaria Programme for evaluating therapeutic efficacy (WHO, Geneva, Switzerland) or Stata version 10 (StataCorp. LP, College Station, United States of America).

Ethics

Informed, written consent was obtained from each adult enrolled and from a legal guardian of each child enrolled. The study protocol was approved by the Government of India's Ministry of Health and Family Welfare and by the Ethics Committee of the National Institute of Malaria Research in New Delhi.

Results

Patient enrolment and characteristics

Overall, 1733 patients were enrolled: 895 patients were from 13 sentinel sites between June 2009 and January 2010; 838 were from the 12 other sites, between June 2010 and December 2010 (Fig. 1). Some of these patients subsequently withdrew their consent or were found not to satisfy the inclusion criteria. This left 1664 eligible patients, 1579 (94.8%) of whom completed the 28 days of follow-up (Fig. 1). Most of the patients investigated were men who were febrile at the time of enrolment (Table 1). A history of fever was nearly universal but the intake of antimalarial

Table 1. **Characteristics of malaria patients enrolled in studies conducted under India's National Antimalarial Drug Resistance Monitoring System, 2009–2010**

Characteristic	No. (%) of patients infected	
	Plasmodium falciparum	*Plasmodium vivax*
Area		
Central	829 (57.0)	0
Western	391 (26.9)	55 (26.2)
North-eastern	234 (16.1)	0
Southern	0	155 (73.8)
Season		
Monsoon	415 (28.5)	115 (54.8)
Post-monsoon	645 (44.4)	95 (45.2)
Winter	394 (27.1)	0
Sex		
Male	835 (57.4)	172 (81.9)
Female	619 (42.6)	38 (18.1)
Age (years)		
< 1	2 (0.1)	0
1–4	120 (8.3)	2 (1.0)
5–9	290 (19.9)	14 (6.7)
10–14	252 (17.3)	18 (8.6)
15–49	689 (47.4)	154 (73.3)
≥ 50	101 (7.0)	22 (10.4)
Parasitaemia (asexual parasites/µl)[a]		
< 1 000	14 (1.0)	10 (4.8)
1000–4999	548 (37.7)	69 (32.9)
5000–9999	251 (17.3)	42 (20.0)
10 000–49 999	526 (36.2)	83 (39.5)
≥ 50 000	115 (7.8)	6 (2.8)
Febrile (≥ 37.5 °C)		
No	451 (31.0)	63 (30.1)
Yes	1003 (69.0)	146 (69.9)
History of fever		
No	31 (2.1)	4 (1.9)
Yes	1423 (97.9)	206 (98.1)
Previous antimalarial drug intake[b]		
No	1428 (98.2)	207 (98.6)
Yes	9 (0.6)	0 (0.0)
Unknown	17 (1.2)	3 (1.4)

[a] At enrolment, before treatment.
[b] No data were available for 25 patients enrolled at one sentinel site.

drugs in the previous week was rare. The geometric mean of the level of asexual parasitaemia on day 0 was 7975 parasites per µl (range: 560–99 707) for *P. falciparum* and 7133 parasites per µl (range: 378–123 317) for *P. vivax* .

Plasmodium falciparum

Primary and secondary end-points

Of the 1394 patients infected with *P. falciparum* who completed follow-up (Table 2), 27 were initially categorized as treatment failures. However, when parasites in paired dried blood spots from these 27 patients were genotyped by PCR, only 17 (63%) of the patients were confirmed as treatment failures. Of the other 10 patients, 7 were found to have developed *P. vivax* parasitaemia during follow-up, 2 were found to have *P. falciparum* reinfection on day 28, and one had an untypeable *P. falciparum* parasitaemia on day 28. The 17 patients who were confirmed to have failed AS+SP treatment came from 10 different sentinel sites. Two early treatment failures occurred in Betul district (Madhya Pradesh); both patients had parasitaemia and fever on day 3. Another occurred in Angul (Odisha) in a patient whose level of parasitaemia on day 3 was more than 25% higher than on day 0. In Gadchiroli district (Maharashtra), five patients failed treatment on day 7. The crude and PCR-corrected Kaplan–Meier survival estimates were 98.6% (95% confidence interval, CI: 97.8–99.1) and 98.8% (95% CI: 98.1–99.3), respectively (Fig. 2). Among the 25 sentinel sites, the PCR-corrected survival estimates varied from 93.8% to 100% (Appendix A). In general, the *P. falciparum* patients treated with AS+SP cleared their parasitaemias rapidly; 62% were aparasitaemic within 24 hours of the first dose (Table 3). Overall, 0.7% (6 of 873), 1.3% (5 of 384) and 1.3% (2 of 154) of the patients who showed parasite clearance intervals of ≤ 24 hours, > 24 to 48 hours and > 48 to 72 hours, respectively, were identified as treatment failures. Six patients infected with *P. falciparum* (including four from Betul) did not clear their parasitaemias within 72 hours. Four (67%) of them were identified as treatment failures (including three early treatment failures), whereas the other two achieved cure. In general, the AS+SP treatment was well tolerated. The most common adverse events reported during follow-up were vomiting, fever and jaundice, which were seen in 46 patients, three patients and one patient, respectively

Molecular markers of drug resistance

All but 31 of the 373 *P. falciparum* isolates genotyped for *dhfr* were successfully amplified. Single ($n = 65$) and double mutations ($n = 234$) in this gene were common. The most frequent haplotype was the double mutant 108/59, followed by single mutant 108 (Appendix A). In terms of the *dhfr* codons investigated, only 10 isolates were identified as triple mutants and only one was found to be a quadruple mutant. The typing of *dhps* proved more difficult and was only successful for 261 of the 373 isolates investigated. Mutations in *dhps* were only detected in six (2.3%) of the isolates that were successfully investigated; five isolates carried single mutations and one carried a double mutation, with a predominance of mutations in codon 437. When the day-0 samples from the 17 confirmed treatment failures were investigated, one sample did not amplify, 12 samples were found to be *dhfr* double mutants, four were found to be *dhfr*

Table 2. **Treatment outcomes among eligible malaria patients in studies conducted under India's National Antimalarial Drug Resistance Monitoring System, 2009–2010**

Outcome after 28 days	No. (%) of patients infected	
	Plasmodium falciparum	*Plasmodium vivax*
Primary classification		
Adequate clinical and parasitological response[a]	1367 (94.0)	185 (88.1)
Early treatment failure[b]	3 (0.2)	0 (0.0)
Late clinical failure[c]	7 (0.5)	0 (0.0)
Late parasitological failure[d]	17 (1.2)	0 (0.0)
Lost to follow-up	33 (2.3)	12 (5.7)
Withdrawal after day 0	27 (1.8)	13 (6.2)
PCR-corrected results		
Recrudescence	17 (63.0)	–
Reinfection	2 (7.4)	–
Infection with other species	7 (25.9)	–
Unknown	1 (3.7)	–
Parasite clearance interval (h)		
≤ 24	873 (61.6)	20 (10.5)
> 24 to 48	384 (27.1)	88 (45.8)
> 48 to 72	154 (10.9)	78 (40.6)
> 72	6 (0.4)	6 (3.1)

PCR, polymerase chain reaction.
[a] Absence of parasitaemia on day 28 without previous criteria for failure.
[b] Marked by the following, alone or in combination: danger signs for severe malaria on days 1, 2 or 3 in the presence of parasitaemia; parasitaemia level on day 2 higher than on day 0; parasitaemia level on day 3 more than 25% higher than on day 0; parasitaemia on day 3 plus fever.
[c] Danger signs for severe malaria and/or fever plus parasitaemia from day 4 to day 28.
[d] Parasitaemia from day 7 to day 28 even if patient afebrile.

single mutants and 16 were found to be wild-type for *dhps*.

Predictors of treatment failure and parasite clearance interval

In both the univariate (Appendix A) and multivariate analyses (Table 3), an age of < 5 years (relative to an age of ≥ 15 years) and a daily dose of artesunate of either < 3 or 3 to < 3.5 mg per kg (relative to higher doses) were each significantly associated with treatment failure. In the multivariate analysis, fever at enrolment was also positively and significantly associated with treatment failure (Table 3). The risk of failure increased as the dose of artesunate decreased. A day-0 level of parasitaemia ≥ 5000 asexual parasites per µl was associated with a reduced risk of treatment failure. No association was detected between infection with parasites harbouring three or more mutations in the genes investigated (i.e. *dhfr* and *dhps*) and failure of AS+SP treatment. There were insufficient observations to determine the risk factors for a parasite clearance interval of > 72 hours.

Plasmodium vivax

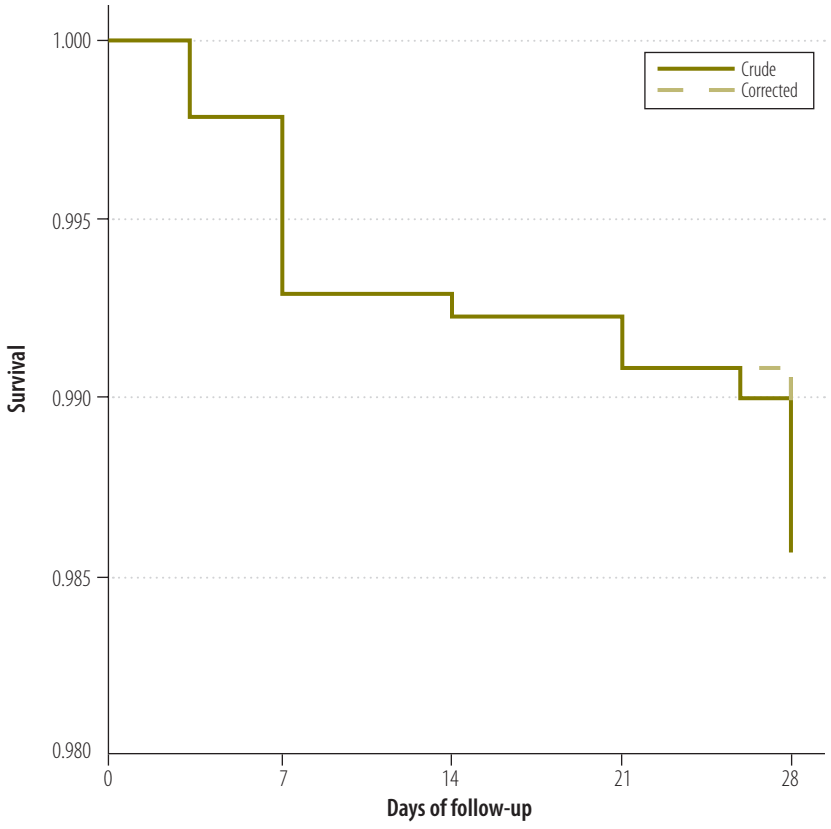
In three trials involving a total of 185 patients infected with *P. vivax* , chloroquine had 100% efficacy, although, compared with the patients infected with *P. falciparum*, larger percentages of the patients infected with *P. vivax* withdrew from the study or were lost to follow-up (Table 1). Most (56.2%) of the patients infected with *P. vivax* cleared their parasitaemias within 48 hours of the first dose of chloroquine (Fig. 3). The parasitaemias in six patients persisted for > 72 hours, without any concurrent symptoms, but cleared by day 7 of follow-up. Chloroquine treatment was well tolerated, with just one case each of gastritis, stomatitis and vomiting.

Discussion

India's National Antimalarial Drug Resistance Monitoring System recruited 1733 patients and completed therapeutic efficacy trials in 25 sites across India during its first 2 years. The results indicate that the first-line therapies for *P. falciparum* malaria and *P. vivax* malaria recommended by the national antimalarial drug policy (i.e. AS+SP and chloroquine, respectively) remain efficacious. The 28-day efficacy of AS+SP for the treatment of *P. falciparum* infection, for example, was found to be > 98%. Although AS+SP treatment failures and parasitaemias showing prolonged clearance intervals after AS+SP treatment were rare, those identified were clustered in just a few sentinel sites. This clustering validates the design of the new monitoring system, which uses wide geographical coverage to increase the chances of detecting hotspots for resistance (as well as longitudinal studies to track emerging trends). No evidence of resistance to AS+SP was collected in the sentinel sites in north-eastern India, even though this is the region of the country where the highest frequencies of sulfadoxine-pyrimethamine treatment failure have been reported.[15] The observation that four of the six patients who showed parasite clearance intervals of > 72 hours were confirmed to be treatment failures indicates the potential usefulness of measuring clearance intervals as a predictor of AS+SP treatment failure. Post-treatment reinfection with *P. falciparum* was rarely detected in the present study. While the frequency of reinfection recorded is likely to be correlated with the length of the follow-up period, it also depends on the intensity of transmission in the study sites. The intensity of malarial transmission in India is generally lower than in many other parts of the world.[16]

Most (87.1%) of the isolates of *P. falciparum* that were successfully typed showed genotypic evidence of partial resistance to pyrimethamine, with either single (S108N) or double mutations (S108N/C59R) in the relevant *dhfr* codons. Such mutations have been found to increase the median inhibitory concentration (IC_{50}) of pyrimethamine 10-fold.[14] While seven isolates possessed the I164L mutation that has been associated with high-level resistance,[17] the prevalence of triple or quadruple mutants among the genotyped isolates was low (3.2%). As seen in a previous study based on the same methodos,[9] the typing of *dhps* appeared to be relatively difficult; it was unsuccessful for 27.6% of the isolates for which typing was attempted. The prevalence of single or double *dhps* mutations among the isolates that were successfully

Fig. 2. **Crude and corrected Kaplan–Meier survival curves, India, 2009–2010**



Note: The data used to create these curves came from patients with *Plasmodium falciparum* infection who had been treated with a combination of artesunate and sulfadoxine-pyrimethamine. The corrected data reflect the results of using a polymerase chain reaction to separate recrudescences resulting from treatment failure from post-treatment reinfections with *P. falciparum*.

Table 3. **Predictors of treatment failure among patients in studies conducted under India's National Antimalarial Drug Resistance Monitoring System, India, 2009–2010**

Predictor	RR[a] (95% CI)
Artesunate dose (mg/kg)[b]	
≥ 3.5	–
3.0 to < 3.5	2.45 (0.58–10.3)
< 3.0	6.10 (1.04–35.8)
Age (years)	
< 5	4.46 (1.35–14.7)
≥ 5	–
Parasitaemia (asexual parasites/µl)	
≥ 5 000	0.47 (0.22–1.00)
< 5 000	–
Fever at enrolment (≥ 37.5 °C)	
Yes	3.94 (1.40–11.1)
No	–

RR, relative risk; CI, confidence interval.
[a] Relative risk for the failure of treatment of *Plasmodium falciparum* infection with a combination of artesunate and sulfadoxine-pyrimethamine, as estimated in a multivariate analysis.
[b] The dose given per day.

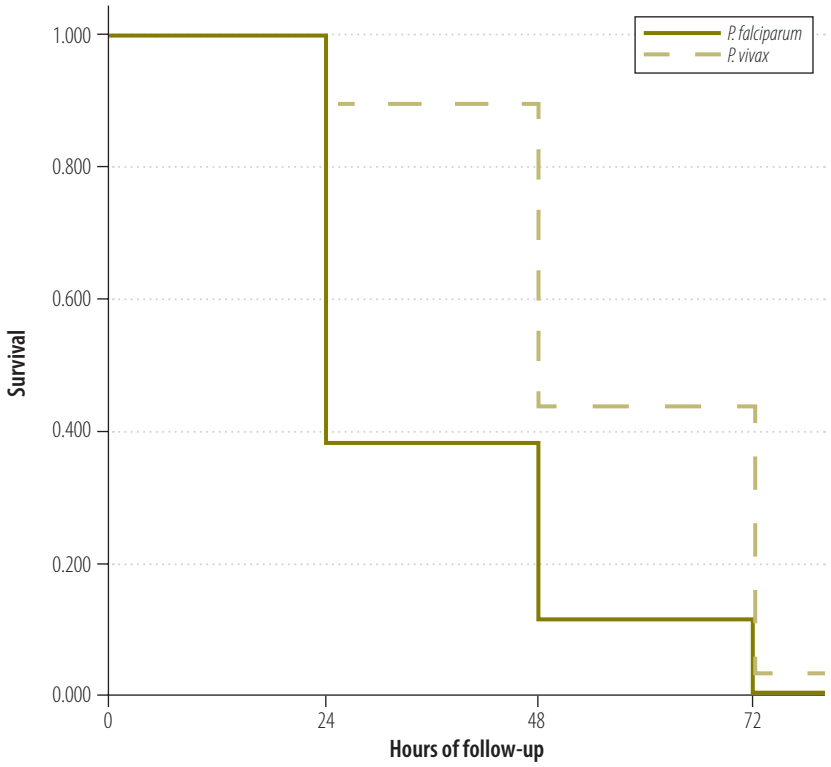
genotyped was low (2.3%), although the possibility that *dhps* mutations caused non-amplification cannot be excluded. By monitoring trends in the prevalences of resistance-related mutations in *dhfr* and *dhps*, the threat to treatment with the AS+SP combination posed by resistance to sulfadoxine-pyrimethamine in *P. falciparum* could be evaluated, independent of any observations of the clinical response.

Treatment failure reflects a combination of drug resistance, host immunity and pharmacokinetics.[18] In the present study, younger age, fever at enrolment and a low level of parasitaemia at enrolment – all potential markers of relatively low immunity to parasite antigens – were associated with recrudescence following AS+SP treatment. Another association observed, the negative correlation between the dose of artesunate (in mg per kg body weight) and the probability of treatment failure, was not surprising. Although the recommended daily dose of artesunate is 4 mg per kg, 8.8% of the subjects of the present study who were given AS+SP received 3.0 to < 3.5 mg of artesunate per kg, and 1.9% received < 3.0 mg per kg. The routine use of age, rather than body weight, as a guide for determining the dose of antimalarial drug needed by a patient is probably a cause of suboptimal dosing worldwide. The relationship between the administered dose and pharmacodynamic response is complex, however, and therapeutic levels may still be achieved when the dose is lower than recommended in standard guidelines.[19]

In spite of sporadic case reports of chloroquine-resistant *P. vivax* in India,[20] all of the *P. vivax* -infected patients investigated in the present study appeared to be cured by chloroquine treatment. Although many of the patients in sentinel sites in southern and western India who were given chloroquine in the present study were migrant workers from elsewhere in India, more trials to investigate the therapeutic efficacy of chloroquine against *P. vivax* infections are needed in the north and east of India.

Primaquine treatment to prevent relapses forms a critical component in the effective treatment of *P. vivax* infections. Unfortunately, no standard protocols for evaluating the therapeutic efficacy of primaquine, alone or in combination with chloroquine, exist.[2]

Fig. 3. **Kaplan–Meier survival curves for *Plasmodium falciparum* and *P. vivax* parasitaemias, India, 2009–2010**



Note: The data used to create these curves came either from patients with *Plasmodium falciparum* infection who had been treated with a combination of artesunate with sulfadoxine-pyrimethamine or from patients with *P. vivax* infection who had been treated with chloroquine.

Another remaining challenge is the treatment of mixed infections. In the present study, no data on the efficacy of AS+SP against *P. vivax* malaria were collected, although, according to India's national drug policy, AS+SP is the recommended treatment for a patient found to be co-infected with *P. falciparum* and *P. vivax* . Recent reports across south-eastern Asia have described a high incidence of *P. vivax* malaria following the treatment of *P. falciparum* infection, presumably the result of the reactivation of the liver stages of *P. vivax* .[21] In the present study, *P. vivax* infections were detected during the follow-up of seven cases of *P. falciparum* malaria who had been treated with AS+SP. Six of these seven cases were observed at Angul and Dhenkenal (both in the state of Odisha) and represented about one in every 20 patients treated with AS+SP at these two sentinel sites.

India's National Antimalarial Drug Resistance Monitoring System was designed to give high data quality, national representation of different malaria ecotypes, coordinated use of resources and pooled data analysis. While the number of sentinel sites in the current system exceeds the minimum recommended by WHO, more may be required in a country as large as India. The alternation of sentinel sites will help to provide information on long-term trends. Those employed to design and implement the national system faced several challenges. While overall coordination in the joint NVBDCP-NIMR system was satisfactory, a few administrative issues delayed the recruitment of patients at some sentinel sites. Other difficulties encountered involved the central procurement of reagents, sample transport to New Delhi (for quality control and molecular studies) and the local recording of data in a digital format. Each of the problems was reviewed and addressed. Furthermore, NIMR investigators are currently using data and biological samples collected through the system to investigate the blood levels of residual antimalarial drugs at enrolment and their association with treatment outcome, the role of the pharmacokinetics of sulfadoxine-pyrimethamine in treatment failure, and pharmacovigilance for the antimalarial drugs in use.

The present study had several limitations. First, follow-up was confined to 28 days (the minimum period recommended by WHO). If drug efficacy is high, treatment failures are likely to occur relatively late, especially if the drugs used have long half-lives (as is the case with sulfadoxine-pyrimethamine). Follow-up in more recent studies within the drug resistance monitoring system has therefore been extended to 42 days. Second, the rarity of treatment failure limited our ability to identify the risk factors for, or predictors of, such failure. Also, once-a-day monitoring of the level of parasitaemia is not enough to accurately estimate mean clearance times or stage-specific clearance.[22] However, by determining the proportion of aparasitaemic patients once each day, useful data on trends can still be collected. Third, the resources and time needed to check *P. falciparum* isolates for the molecular markers of resistance limited the number of isolates that could be typed in this manner. Finally, no attempt was made to determine the costs or cost-effectiveness of the national system. This would be a major challenge as the system uses resources from two different institutions and is supported by funding at both state and central levels.

Based on the data collected in the first two years of the existence of the National Antimalarial Drug Resistance Monitoring System, we recommend that: (i) the sentinel-site-based monitoring of antimalarial drug efficacy is maintained; (ii) the use of a parasite clearance interval of > 72 hours as a predictor of subsequent treatment failure be explored further, particularly in Betul; (iii) the nested PCR currently used for genotyping be replaced by a newer technology, such as real-time PCR, to enable the genotyping of more isolates; (iv) supplementary studies be undertaken on young children (because of their relatively high risk of AS+SP treatment failure); and (v) the relative benefits and disadvantages of age- and weight-based dosing be assessed. As part of the latter assessment, an increase in the doses of AS+SP in blister packs,

to compensate for higher-than-average body weights, should be considered.

Conclusion

In India, AS+SP remains safe and effective for the treatment of uncomplicated *P. falciparum* malaria and chloroquine remains safe and effective for the treatment of uncomplicated *P. vivax* malaria. ■

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Competing interests: None declared.

ملخص

رصد مقاومة الأدوية المضادة للملاريا في الهند عن طريق المواقع الخافرة: الحصائل وعوامل الاختطار الخاصة بفشل العلاج، في الفترة من 2009 إلى 2010

الغرض وصف النظام الوطني لرصد مقاومة الأدوية المضادة للملاريا في الهند وقياس نجاعة علاجات الخط الأول للملاريا وتحديد عوامل الاختطار الخاصة بفشل العلاج.

الطريقة في الفترة من 2009 إلى 2010، تم إجراء دراسات استطلاعية بلغت مدة متابعتها 28 يوماً في 25 موقعاً خافراً. وتم إعطاء المرضى المصابين بعدوى المتصورة المنجلية الأرتيسونات بالإضافة إلى السلفاديوكسين – بيريميثامين (AS+SP)؛ وتم إعطاء المصابين بالمتصورة النشيطة الكلوروكين. وتم استخدام تفاعل البوليميراز المتسلسل لتمييز معاودة الإصابة عقب العلاج عن الفشل في العلاج. وتم فحص مستفردات المتصورة المنجلية من أجل طفرات هيدروفوليت ريدكتيز وسينثاز دايهيدوربتيرويت.

النتائج بشكل عام، تم تسجيل 1664 مريضاً. وتبين من تحليل كابلان – ماير للبقاء على قيد الحياة نجاعتها بنسبة 98.8 ٪ بالنسبة للأرتيسونات بالإضافة إلى مادة السلفاديوكسين – بيريميثامين. وتم التخلص من تطفلن الدم لدى معظم المرضى المصابين بتطفلن دم المتصورة المنجلية في غضون 24 ساعة من بدء العلاج ولكن ظل تطفلن الدم لدى ستة أشخاص، من بينهم أربعة فشلوا في العلاج بعد 72 ساعة. ووجدت طافرات مضاعفة في إنزيم هيدروفوليت ريدكتيز لدى 68.4 ٪ من المستفردات ذات الأنماط الجينية. وكانت الطافرات الثلاثية أو الرباعية في إنزيم هيدروفوليت ريدكتيز والطافرات في إنزيم سينثاز دايهيدوربتيرويت نادرة. وكانت جرعة الأرتيسونات اليومية الأقل من 3 ملجم لكل كيلوجرام من وزن الجسم، والسن الأقل من 5 سنوات، والحمى لدى التسجيل، مرتبطة بزيادة خطورة الفشل في العلاج. وظل الكلوروكين ناجعاً بنسبة 100 ٪، وعموماً تم التخلص من تطفلن دم المتصورة النشيطة في غضون 48 ساعة. وكان القيء (الذي شوهد لدى 47 مريضاً) أكثر الأحداث الضائرة شيوعاً.

الاستنتاج يوفر النظام الوطني لرصد مقاومة الأدوية المضادة للملاريا في الهند تغطية واسعة. وتظل أدوية الخط الأول المضادة للملاريا المستخدمة في البلد آمنة وناجعة. ويحتاج علاج الملاريا لدى صغار الأطفال والفوائد النسبية للجرعة المستندة على العمر والوزن إلى مزيد من البحث..

摘要

通过监测哨点监测印度抗疟药物的耐药性：2009-2010 年效果和治疗失败的风险因素

目的 描述印度国家抗疟药物耐药性的监测系统，测量一线疟疾治疗的疗效，并确定治疗失败的风险因素。

方法 2009-2010 年，在25 个监测哨点开展前瞻性研究，并进行28 天的随访。对感染恶性疟原虫的患者给予青蒿琥酯联合乙胺嘧啶（AS+SP）药物治疗；对感染间日疟原虫的患者提供氯喹药物治疗。使用聚合酶链反应区分治疗后的再感染和治疗失败。检查恶性疟原虫菌株，了解是否有dhfr和dhps突变。

结果 总计有1664 例患者参加。Kaplan-Meier生存分析显示，AS+SP治疗具有98.8%的疗效。多数感染恶性疟原虫的患者在开始治疗的24 小时内消除血症，但有6 例（其中包括4 例治疗失败的患者）在74 小时后血症依然不消。在68.4%的基因型菌株中发现dhfr双突变。三重或四重dhfr突变和dhps突变则罕见。每千克体重<3 毫克的青蒿琥酯每日剂量、年龄小于5 岁以及在参加时发热等因素与更高的治疗失败风险有关。氯喹保持100%疗效，一般在48 小时内清除间日疟原虫血症。呕吐（47 例）是最常见的副作用。

结论 在印度，监测抗疟药物耐药性的国家系统覆盖面广。国内一线抗疟药物依然保持安全和有效。幼儿疟疾治疗和基于年龄及体重计算剂量的相对好处需要进一步探索。

Résumé

Surveillance de la résistance aux antipaludiques en Inde par l'intermédiaire de sites sentinelles: résultats et facteurs de risque de l'échec du traitement, 2009-2010

Objectif Décrire le système national indien de surveillance de la résistance aux médicaments antipaludiques, mesurer l'efficacité des traitements antipaludiques de première ligne et déterminer les facteurs de risque de l'échec du traitement.

Méthodes En 2009-2010, des études prospectives, avec 28 jours de suivi, ont été menées dans 25 sites sentinelles. On a administré aux patients infectés par le *Plasmodium falciparum* de l'artésunate et de la sulfadoxine-pyriméthamine (AS+SP). Ceux qui étaient infectés par le

P. vivax ont reçu de la chloroquine. La réaction en chaîne par polymérase a été utilisée pour distinguer la réinfection après traitement de l'échec du traitement. Des isolats de *P. falciparum* ont été vérifiés pour déceler les mutations de *dhfr* et de *dhps*.

Résultats Un total de 1664 patients ont participé à l'étude. L'analyse de survie de Kaplan-Meier a montré une efficacité de 98,8% de l'AS+SP. La plupart des patients infectés par le *P. falciparum* ont vu disparaître leurs parasitémies dans les 24 heures suivant le début du traitement, mais 6, dont 4 avec échec du traitement, sont restés parasitémiques après 72 heures. Des doubles mutations de *dhfr* ont été trouvées dans 68,4% des isolats génotypés. Les triples ou quadruples mutations de *dhfr* et les mutations de *dhps* étaient rares. Une dose quotidienne d'artésunate inférieure à 3 mg par kg de poids corporel, un âge inférieur à 5 ans, et de la fièvre au moment de l'enregistrement ont été associés à un risque accru d'échec thérapeutique. La chloroquine restait efficace à 100% et faisait généralement disparaître les parasitémies de *P. vivax* dans les 48 heures. Les vomissements (observés chez 47 patients) étaient l'effet indésirable le plus fréquent.

Conclusion En Inde, le système national de surveillance de la résistance aux médicaments antipaludiques offre une large couverture. Les antipaludéens de première ligne utilisés dans le pays restent sûrs et efficaces. Le traitement du paludisme chez les jeunes enfants et les avantages relatifs de la posologie en fonction de l'âge et du poids nécessitent une étude plus poussée.

Резюме

Мониторинг лекарственной устойчивости к противомалярийным препаратам в Индии на постах эпиднадзора: результаты и факторы риска для неудачного лечения, 2009–2010 гг.

Цель Описание национальной системы мониторинга устойчивости к противомалярийным препаратам в Индии, оценка эффективности первой линии лечения малярии, определение факторов риска неэффективности лечения.

Методы В 2009-2010 годах на 25 участках эпиднадзора были проведены перспективные исследования, включающие принятие последующих мер в течение 28 дней. Пациентам, инфицированным *Plasmodiumfalciparum,* назначался артезунат в сочетании с сульфадоксином-пириметамином (AS+SP); пациентам, инфицированным *P. vivax* , назначался хлорохин. Для различения между повторным заражением после проведенного лечения и неэффективным лечением был использован метод полимеразной цепной реакции. Изоляты вируса *P. falciparum* были проверены на мутации *dhfr* и *dhps*.

Результаты Всего в обследование было включено 1664 пациентов. Анализ выживаемости Каплана-Мейера для лечения препаратами AS+SP показал эффективность 98,8%. У большинства пациентов паразитемия типа *P. falciparum* была устранена в течение 24 часов после начала лечения, а 6 пациентов, в том числе 4 с неудачным лечением, остались зараженными после 72 часов. Двойные мутации в *dhfr* были обнаружены в 68.4% генотипированных изолятов. Тройные и четвертные мутации в *dhfr* и мутации в *dhps* встречались редко. Суточная доза артезуната <3 мг на кг массы тела, возраст менее 5 лет и лихорадка при начале лечения были связаны с повышенным риском неудачи лечения. Хлорохин продолжал показывать эффективность 100% и обычно позволял устранить паразитамию *P. vivax* в течение 48 часов. Рвота (наблюдалась у 47 пациентов) была самым частым побочным эффектом.

Вывод Национальная система мониторинга лекарственной устойчивости к противомалярийным препаратам в Индии обеспечивает широкий охват. Противомалярийные препараты первой линии, используемые в стране, показали себя как безопасные и эффективные. Методы лечения малярии у детей раннего возраста и относительные преимущества дозировок, основанных на возрасте и весе, нуждаются в дальнейшем исследовании.

Resumen

El seguimiento de la resistencia a los fármacos contra el paludismo en India por medio de sitios centinela: resultados y factores de riesgo en el fracaso del tratamiento, 2009-2010

Objetivo Describir el sistema nacional de seguimiento de la resistencia a los fármacos contra el paludismo en India, medir la eficacia de los tratamientos de primera línea contra el paludismo y determinar los factores de riesgo en el fracaso del tratamiento.

Métodos En los años 2009-2010, se llevaron a cabo estudios prospectivos con un seguimiento de 28 días en 25 sitios centinela. Se proporcionó artesunato con sulfadoxina-pirimetamina (AS+SP) a los pacientes infectados con *Plasmodium falciparum***,** y cloroquina a los pacientes infectados con *P. vivax* . Se empleó la reacción en cadena de la polimerasa para distinguir la reinfección postratamiento del fracaso del tratamiento y se examinaron aislados de *P. falciparum* en busca de mutaciones de DHFR y DHPS.

Resultados Se inscribieron un total de 1664 pacientes. Los análisis de supervivencia Kaplan-Meier mostraron una eficacia del 98,8% para el AS+SP. En la mayoría de pacientes con parasitemia por *P. falciparum,* ésta despareció en las 24 horas posteriores al inicio del tratamiento. Solo seis, incluidos los cuatro en los que el tratamiento fracasó, siguieron teniendo parasitemia pasadas 72 horas. Se encontraron mutantes dobles en DHFR en el 68,4% de los aislados genotipados. No fue frecuente encontrar mutantes en DHPS ni mutantes triples o cuádruples en DHFR. Una dosis diaria de artesunato de < 3 mg por kg de peso corporal, una edad inferior a 5 años y fiebre en el momento de la inscripción se asociaron con un riesgo superior de fracaso del tratamiento. La cloroquina fue eficaz en el 100% de los casos y eliminó, por lo general, las parasitemias por *P. vivax* en un plazo de 48 horas. El acontecimiento adverso más frecuente fueron los vómitos (presentes en 47 pacientes).

Conclusión El sistema nacional de seguimiento de la resistencia a los fármacos contra el paludismo en India proporciona una cobertura amplia. Los fármacos antipalúdicos empleados en primera línea en el país continúan siendo seguros y eficaces. Se debe seguir examinando el tratamiento del paludismo en los niños pequeños y las ventajas relativas de la edad y las dosis basadas en el peso corporal.

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