Drug Resistance

Monitoring Antimalarial Drug Resistance

In order to understand genetic composition of the isolates in respect to commonly used antimalarials, isolates from different geographical regions were tested for their drug response either by *in vitro* or *in vivo* assay. Status of drug sensitivity in malaria parasites is important to plan and identify appropriate chemotherapeutic regimens and drugs.

In vitro Studies

The drug sensitivity can be determined *in vitro* in *P. falciparum* culture by using standard 96 well microtitre plates, predosed or prepared with several dilutions of the test drug. The sensitivity of parasite to drug is assessed by schizont maturation inhibition of parasites over 24–48 h. At NIMR *P. falciparum* isolates from different parts of the country were tested to ascertain resistance to chloroquine (Fig. 15).

In vivo Studies

The standard WHO *in vivo* 28-day test system was originally developed for chloroquine and now is extended to other drugs with appropriate changes in the number of days for other drugs. In their performance, these tests follow set criteria for the administration of a standard treatment regimen of the appropriate drug, and daily parasitological blood examination for the stipulated period. As 28-day WHO *in vivo* method is time consuming and requires daily follow-up during the first week of treatment and also patients have to wait for at least seven days before starting the alternate treatment in resistant cases, a simplified *in vivo* 7-day test was validated by the Institute (Prasad *et al* 1990). Test requires blood examination on Day 0, 2 and 7 and infection can be declared resistant as early as on 2nd day of drug administration. Now this test is being used routinely in field studies. Both 28- and 7-day tests were conducted by Assam, Hardwar (Uttarakhand), Orissa, Car Nicobar, Madhya Pradesh field units of NIMR (Chaudhury *et al* 1987; Ghosh *et al* 1992; Dua *et al* 1993; Giri *et al* 1994; Singh *et al* 1989, 1995; Dua *et al* 2000). Variable response from RI to RIII was observed among isolates assayed from different areas. It may be mentioned that in Mathura (U.P.), during routine treatment, one case showed tolerance to chloroquine (Dua *et al* 1996).

Therapeutic Efficacy Studies

The standard *in vivo* tests consider mainly the parasitological responses for assessment. Thus, to take into consideration the clinical response as well, a simplified test system where the number of parasitological observations were reduced and complemented by standardized clinical observations has been introduced by the WHO. These studies

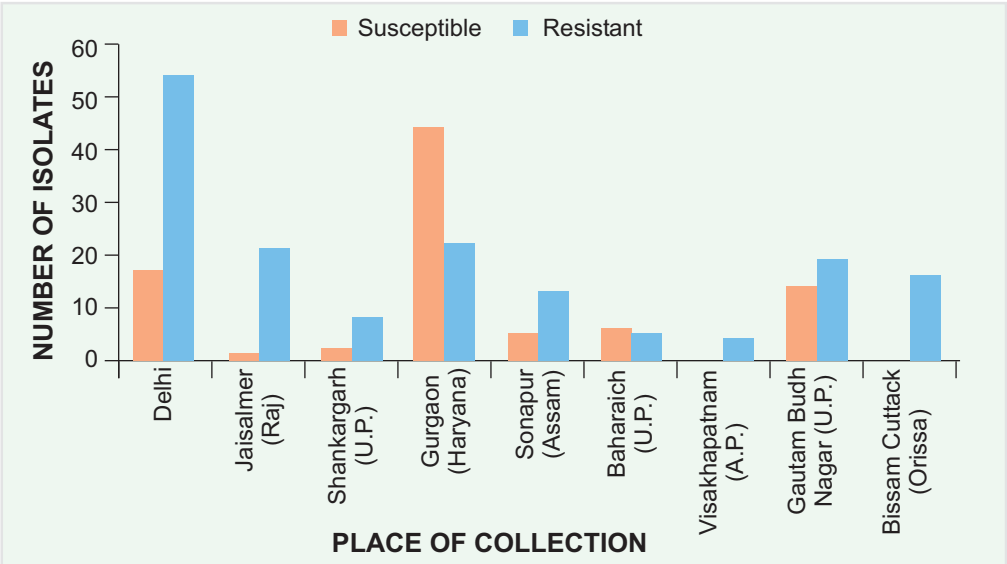

Fig. 15: Chloroquine sensitivity status of *P. falciparum* isolates *in vitro* (1992–2001)

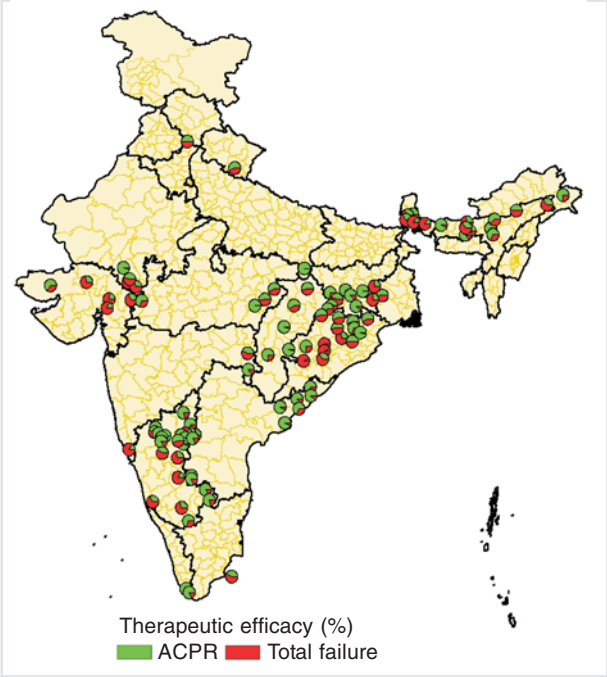

Fig. 16: Sites where antimalarial drug resistance monitoring conducted by NIMR

have been initiated by NIMR using the new WHO protocols (Fig. 16). Therapeutic efficacy of chloroquine in *P. vivax* and *P. falciparum* malaria was monitored at different sites in the country. Almost all the studies have shown high treatment failure to chloroquine and accordingly drug policy has been revised for *P. falciparum*. ACT has been recommended in about 200 districts. In addition, efficacy of ACT (AS + SP) will also be monitored.

Mechanism of Drug Resistance

Molecular Mechanism of Chloroquine (CQ) Resistance in *Plasmodium falciparum* Isolates

Polymerase chain reaction (PCR) polymorphism of 3' untranslated region of *Pfmdr 1* gene and mutational changes at nucleotide positions 754, 1049, 3598, 3622 and 4234 in the structural gene were attributed to CQ resistance. Our study revealed that PCR polymorphism of 3' untranslated region is not associated with CQ resistance. About 18 CQ sensitive and 22 CQ resistant isolates were studied to determine mutational pattern in Indian isolates. Analysis of mutations among CQ resistant isolates revealed mutations at 3 or more positions except for one isolate in which mutations at two positions were observed. In two of the isolates mutations were found at all five positions. In CQ sensitive isolates nucleotide changes were totally absent at four positions,1049, 3598, 3622 and 4234, while at 754 position mutation was present in five isolates only (Table 12). From this study, it can be said that among Indian *P. falciparum* isolates, CQ resistance is conferred by mutations at three or more nucleotide positions (mentioned above), indicating a strong association but incomplete correlation between mutational changes and chloroquine resistance. Analysis of more samples from different geographical regions may confirm the findings of the study (Bhattacharya *et al* 1999).

Biochemical Characterization of Chloroquine Resistance

Protein kinase C (PKC), a Ca++ and phospholipid dependent protein kinase which has a central role in the regulation of parasite growth, maturation and differentiation functions has been characterized from the trophozoite stage forms of the malarial parasite *P. falciparum*. PKC activity was found to be distributed in all the stages of the *P. falciparum* maturation. Activation of cytosolic PKC required Ca++, PSx and either diacylglycerol or phorbol esters (PMA). A nine fold increase in the activity was observed in schizonts as compared to the ring stage of the malaria parasite. Activation of the trophozoites with PMA resulted in the translocation of the PKC activity from cytosol to the membrane fractions. Our results showed that chloroquine (CQ) an antimalarial drug, directly inhibited the PKC activity in a dose dependent manner with an IC_{50} of 45 nM in trophozoites of chloroquine sensitive CQ(S) strains of the parasite whereas the activity was found to remain unaltered in the chloroquine resistant CQ(R) strain. Kinetic studies with Lineweaver-Burk double reciprocal plot showed that the inhibition of cytosolic PKC activity by CQ was noncompetitive with respect to ATP, histone and Phosphatidyl serine(PS). Above results indicated that PKC activity is developmentally

Table 12. Analysis of point mutations observed in *Pfmdr* gene of *P. falciparum* isolates

Nucleotide positions	CQ sensitive isolates		CQ resistant isolates											
	(13)	(5)	(1)	(3)	(1)	(1)	(3)	(1)	(3)	(2)	(2)	(2)	(1)	(2)
754	–	+	–	+	+	+	+	+	–	+	+	+	–	+
1049	–	–	+	–	+	+	–	+	+	+	–	+	+	+
3598	–	–	–	+	–	+	+	–	+	–	+	+	+	+
3622	–	–	+	–	+	–	+	–	–	+	+	+	+	+
4234	–	–	–	+	–	–	–	+	+	+	+	–	+	+

Figures in parentheses are number of isolates; with mutation (+) or absence (–).

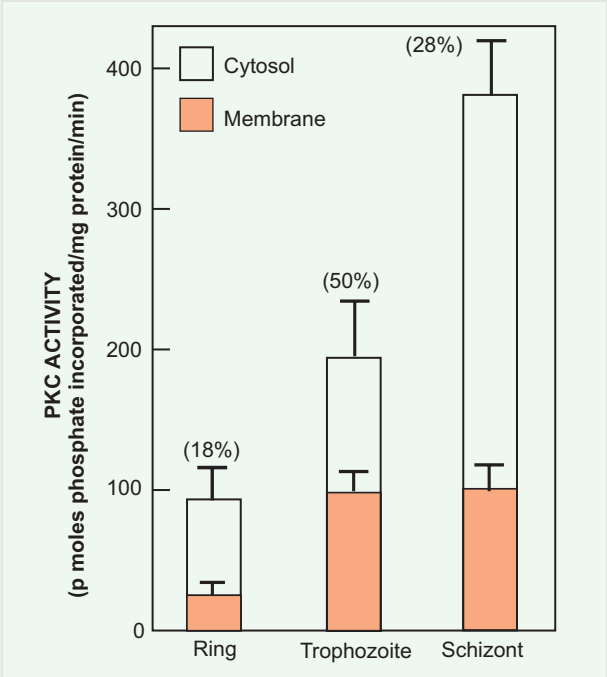

Fig. 17: PKC activity in the developmental stages of *P. falciparum* — (Solid bar–pellet; and Open bar–cytosol). Values in parentheses indicate percent of PKC activity in the membrane fraction of each parasite stage

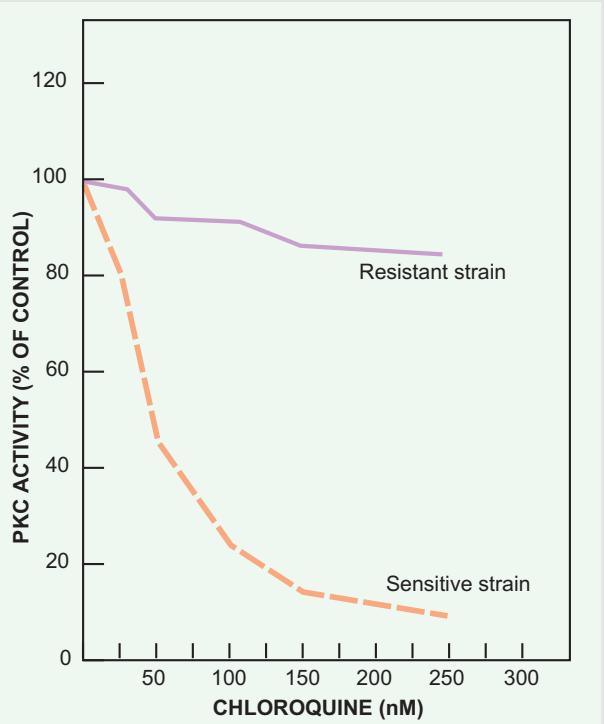

Fig. 18: Inhibition of PKC by CQ in cytosol of CQS and CQR strains of *P. falciparum* trophozoites. Each point is the average of triplicate determinations

expressed during parasite development and its inhibition by antimalarial drug chloroquine in the CQ(S) strain and no inhibition in CQ(R) strains (Figs. 17 and 18) may provide new insights into the possible explanation for the mechanism of action of CQ and development of resistance.

Protein tyrosine kinases (PTKs) are the principal signal enzymes enabling cell to cell communication, growth regulation and differentiation. PTKs are therefore potentially important drug targets due to their role as positive regulators of cell proliferation. Growing resistance of *P. falciparum* to chloroquine and other antimalarials has led to the search of other antimalarials including natural plant products. In this context, a search for naturally occurring plant products with inhibitory activity towards PTK could yield new antimalarials for the study of protein phosphorylation leading to new drug designs and more potent inhibitors. We have reported that one such compound piceatannol, an antileukemic principle in the seeds of *Euphoria lagascae*, has been found to be inhibitory to *P. falciparum* protein tyrosine kinase during the asexual maturation of the parasites in both CQ (S) and CQ (R) *in vitro* (Table 13). The results suggest that the PTK activity may be of use as chemotherapeutic drug target and new drug development in *P. falciparum* resistant malaria (Mishra *et al* 1999; Sharma *et al* 1999).

Detection of Mutations in Dihydrofolate Reductase (DHFR) and Dihydropteroate Synthetase (DHPS) Genes Associated with Resistance to Sulfadoxine-pyrimethamine in *Plasmodium falciparum*

The antimalarial compound sulfadoxine-pyrimethamine in combination is the drug of choice

Table 13. Distribution and inhibition of cytosolic protein tyrosine kinase activity in developmental stages of *P. falciparum*

Stages	Protein tyrosine kinase activity (p moles phosphate incorporated/mg protein/min)					
	Chloroquine sensitive strain (FJB-D9)			Chloroquine resistant strain (FJB-D4)		
	Status	Chloroquine (0.1 mM)	Piceatannol (0.25 mM)	Status	Chloroquine (0.1 mM)	Piceatannol (0.25 mM)
Ring	4.4 ± 0.4	3.6 ± 0.3	3.9 ± 0.3	4.3 ± 0.4	3.9 ± 0.4	3.4 ± 0.2
Trophozoite	6.1 ± 0.5*	3.7 ± 0.4	3.8 ± 0.2	5.6 ± 0.4*	5.6 ± 0.5	3.8 ± 0.2
Schizont	6.6 ± 0.5*	4.0 ± 0.3	4.1 ± 0.3	7.2 ± 0.5*	5.9 ± 0.4	4.0 ± 0.3
Merozoite	4.3 ± 0.3	2.7 ± 0.2	2.7 ± 0.2	5.0 ± 0.4	4.7 ± 0.5	2.5 ± 0.1

*$p < 0.05$; Values are for triplicate assays for five independent culture experiments and are expressed as mean ± SEM.

in patients suffering from *P. falciparum* malaria and fail to respond to chloroquine. They target the activities of folate biosynthetic pathways inhibiting DHFR and DHPS enzymes. A remarkable feature of *Pf*-DHFR and DHPS mutations is that the specific codon sequences involve changes at a number of sites. Pyrimethamine resistant parasites show mutations at codon sequences involving the changes—Ser 108–Asn 108/Thr 108; Ala 16 –Val 16; Ile 164 –Leu 164 and Cys 59 –Arg 59. In case of sulfadoxine resistant strains, mutations are observed in the DHPS domain altering Ser 436 –Phe 436; Ala 613 –Ser or Thr 613 and Ala 581 –Gly 581. Based on the mutations observed in the resistant strains PCR based studies have been designed to examine mutations in DHFR and DHPS genes in natural *P. falciparum* isolates collected from various parts of India. The results showed presence of 81% DHFR mutant type Asn 108 and only 6% DHPS mutant type Phe 436 and Ser 613. Though, the treatment failure against this combination drug has been reported from highly endemic areas of India, conditions are not yet so acute like in Thailand (Biswas *et al* 2000, 2001). ❑