PARASITE BIOLOGY

Parasite Bank

The parasite bank is involved in the collection and characterization of more field isolates of malaria parasite species from different areas. From Bissamcuttack, Orissa, a total of 22 *Plasmodium falciparum*, five mix (*P. falciparum* and *P. vivax*), four *P. vivax* and two *P. malariae* isolates were collected. These samples were collected from Christian Hospital, Bissamcuttack and tribal villages—Tado and Kakaromaska. Three *P. falciparum* and 17 *P. vivax* isolates collected from Delhi were also cryopreserved in liquid nitrogen (Table 5). Chloroquine sensitivity status of 19 of these isolates was carried out using WHO tests kits. Out of these, three samples collected from Delhi were found to be sensitive to chloroquine and all the 16 samples from Orissa were resistant to chloroquine. Among the 16 samples tested from Orissa, four were resistant up to 64 pmol and three were resistant up to 32 pmol (Table 6).

Table 5. Human malaria parasites collected during 2001

Place of collection	*P. falciparum*	*P. vivax*	*P. malariae*	Mix (*Pf*+*Pv*)	Total
Delhi	3	17	–	–	20
Christian Hospital, Bissamcuttack (Orissa)	9	3	1	1	14
Village–Tado, Bissamcuttack (Orissa)	9	1	–	3	13
Village–Kakaromaska, Bissamcuttack (Orissa)	4	–	1	1	6
Total	25	21	2	5	53

Table 6. Chloroquine sensitivity status of *P. falciparum* isolates

Place of collection	No. of isolates tested	Sensitivity status				
		Sensitive (1– 4 pmol)	Resistant (pmol)			
			8	16	32	64
Delhi	3	3	–	–	–	–
Christian Hospital, Bissamcuttack (Orissa)	6	–	3	–	2	1
Village–Tado, Bissamcuttack (Orissa)	8	–	–	5	1	2
Village–Kakaromaska, Bissamcuttack (Orissa)	2	–	–	1	–	1
Total	19	3	3	6	3	4

Twenty-five *P. falciparum* samples were added this year and at present the total number of *P. falciparum* isolates preserved at the parasite bank is 580. In addition to characterization of *P. falciparum* isolates for sensitivity to antimalarials, 20 isolates were characterized for size variation in MSP 1 and MSP 2 antigens by nested polymerase chain reaction (PCR) assay. A total of twenty isolates collected from Rajasthan, Uttar Pradesh, Delhi, Orissa, Tamil Nadu were characterized. Maximum number of alleles observed were five in an isolate. Multiplicity of infection in the isolates was about 3.15 by MSP 1 and about 1.95 by MSP 2. A total of nine alleles in MSP 1 ranging between 400 and 650 bp and eight alleles in MSP 2 ranging between 450 and 750 bp were observed. The allelic polymorphism observed in the study samples is shown in Fig. 11. Using these two systems 20 isolates were shown to have 19 different types.

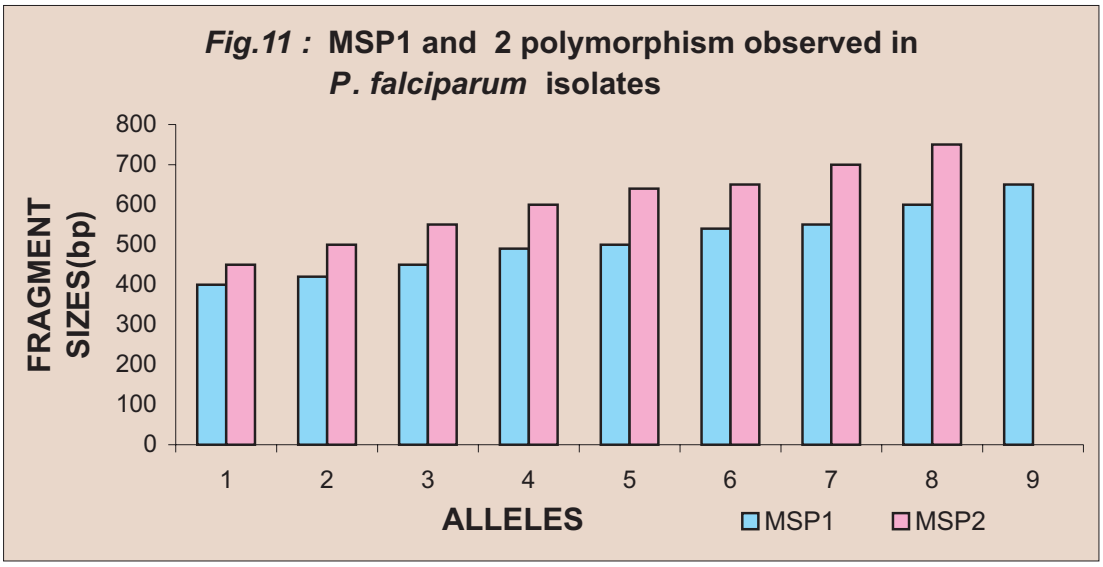


Fig.11 : **MSP1 and 2 polymorphism observed in *P. falciparum* isolates**

Biological materials including non-human and human plasmodia preserved/maintained at the parasite bank were supplied to various research organizations. The non-human parasites, especially *P. berghei* (both chloroquine resistant and sensitive) are being used for the *in vivo* screening of extracts/fractions from medicinal plants. The parasite bank is actively involved in the collection of medicinal plants from various places and in preparing the extracts and fractions for testing their antimalarial activities.

Characterization of Human Malaria Parasites

Plasmodium falciparum

Molecular Analysis of Cytoadherent Phenotype and Invasion Pathways of Indian Isolates of *P. falciparum*

These studies aim to define the cytoadherent phenotypes and the invasion profile of Indian field isolates of *P. falciparum*, collected and cryopreserved at the parasite

bank. The experiments on erythrocyte invasion inhibition using anti-EBA-175 showed encouraging result. Purified anti-EBA-175 RII (F_2) antibody was used to test its ability to inhibit invasion of erythrocytes by the parasites. One laboratory isolate 3D7 and two field isolates, RKL-9 and JDP-8, collected and preserved/maintained at the parasite bank, with known invasion properties were selected for these studies. This antibody raised against EBA-175 showed about 80% inhibition compared to that in controls indicating that this antibody is highly effective in blocking erythrocyte invasion by these parasites. Protein matched serum/pre-immune serum was used as control and the experiments were repeated and the same results were obtained. Four more isolates were characterized for their cytoadherent properties, of which one from Assam and another from Delhi showed high binding to ICAM-1.

Prevalence of Point Mutations in Dihydropteroate Synthetase Gene and *in vitro* Pyrimethamine Sensitivity of *P. falciparum* Isolates Collected from India

The sulphonamide/sulphone, the type-1 antifolate drug is used extensively in the treatment of bacterial diseases as well as in many parasitic infections involving *Cryptosporidium*, *Pneumocystis*, *Toxoplasma* and *Plasmodium*. Members of this group of compounds, such as sulphadoxine and dapsone, inhibit malarial dihydropteroate synthetase (DHPS), a component of the folate biosynthetic pathway. In case of *P. falciparum*, sulphadoxine has been used for prophylaxis. Usually, potentiating mixtures of type-2 (pyrimethamine) along with type-1 antifolates inhibit all growing stages of the parasite. In chloroquine resistant areas the synergistic combination of two antifolates is the alternative treatment for uncomplicated falciparum malaria. *P. falciparum* strains with variable level of sensitivities to sulphadoxine demonstrated sequence variation in DHPS. Sulphadoxine resistant strains showed mutations at 436 Ser to Phe; 437 Ala to Gly; and 613 Ala to Ser or Thr; as well as a single point mutation at 581 Ala to Gly. A further mutation at 540 Lys to Glu was also reported in isolates from Thailand, Bolivia, Kenya and Tanzania.

In the present study the prevalence of point mutations in DHPS gene have been determined at three codon sites, namely 436, 581 and 613 in *P. falciparum* clinical isolates and their *in vitro* sensitivity to sulphadoxine was also tested to draw comparison with molecular assay. Forty finger prick blood samples were collected aseptically from *P. falciparum* patients of all age groups from Assam (n = 15), Ghaziabad (n = 17) and Delhi (n = 8). Primary PCR amplified DHPS gene using two flanking primers yielding a product of 1.15 kb. Amplified DHPS domain from the first round of PCR was used in mutation-specific second round PCR using two flanking and seven mutation-specific primers to detect point mutations at codon sites 436, 581 and 613. Known sulphadoxine sensitive (3D7) and resistant (V1/S) clones were taken as control. *In vitro* assay for sulphadoxine sensitivity was done for all 40 isolates. Parasites were cultured in conditioned media, RPMI-1640 LPLF containing 10%

AB+ serum at a haematocrit of 2.5% and an initial parasitaemia of 0.1–1% in the presence of various concentrations of sulphadoxine (0.4–5000 µM). Parasite growth was monitored microscopically. Rate of schizont maturation as an indicator of parasite growth was calculated for each case and minimum inhibitory concentration (MIC) was determined.

The results of the study confirmed the presence of a few sulphonamide resistant isolates among symptomatic patients and are in agreement with *in vitro* drug sensitivity results. Of the 40 clinical isolates studied, 10% (4/40) presented double mutated forms of S436F and A613T; single mutant type allele A581G was detected in 5% (2/40) isolates. Parasites carrying double or single mutant types showed elevated MIC values above 100 µM (200–515 µM), whereas wild-type parasites sensitive to sulphadoxine were having MIC less than 100 µM (35–100 µM). None of these isolates showed single point-mutation at positions 436 and 613. It is not known to what extent the correlation between molecular techniques and *in vitro* drug sensitivity assay is relevant to the clinical efficacy of sulpha-pyrimethamine; a systematic study on therapeutic efficacy of these compounds at epidemiological level may strengthen the utility of this assay.

P. falciparum Dihydrofolate Reductase Mutation at Thr-108 and Val-16 and Resistance to Antifolate Drug — A Case Study

A patient suffering from *P. falciparum*, who was a traveller to the NE-state from Thailand-Myanmar border, came to Delhi, did not respond to the recommended doses of chloroquine and also to sulpha-pyrimethamine (SP). Although parasitaemia fell rapidly after treatment with SP regimen, the infection had not resolved six days later because of the poor response to treatment selected resistant sub-population. The blood samples collected from this patient prior to treatment with SP regimen and also on Day 2 and Day 6 were tested *in vitro* for antimalarial drug sensitivity and for dihydrofolate reductase gene mutation. *In vitro* drug sensitivity assays demonstrated that the blood samples collected on different days had higher MIC for CQ, pyrimethamine, sulphadoxine and cycloguanil when compared with reference sensitive and resistant strains. Polymerase chain reaction and restriction digest-based methods revealed that the digestion with restriction enzymes Alu I and Bsr I produced a single fragment of 708 bp, whereas with Scr FI produced two fragments of 386 and 322 bp. The Nla III digestion produced two fragments of 568 and 140 bp. The banding pattern with Scr FI and Nla III resembled the mutant FCR3 clone. Overall results on drug sensitivity and RFLP indicated that at the known drug resistant loci, the isolate had a genotype of DHFR Val-16 and Thr-108, previously found associated with cycloguanil resistance. As per the published reports, this type of paired mutations in natural isolates are rare. It is of considerable interest to carry out studies on alleles of this gene in relation to resistance at epidemiological level.

Antimalarial Activity of Amineperoxides *in vitro* in Chloroquine Sensitive and Resistant *P. falciparum* Isolates

Naturally occurring peroxides, such as artemisinin, dihydroascaridole show potent antimalarial activity by oxidative free radical damage to parasite membrane system. The endoperoxide moiety in artemisinin and its analogues is essential for antiparasitic activity which is mediated by activated oxygen or carbon free radicals. The amines containing t-butoxyperoxides show antimalarial activity *in vitro* against *P. falciparum*, but are inactive *in vivo*. A panel of 12 reactive peroxyamines have been synthesized and their chemical structures were determined by a group of researchers under the supervision of Prof. S.V. Bhat of IIT, Bombay. Biological evaluation of these compounds was done by assessing their antimalarial activity *in vitro* in two well adapted *P. falciparum* strains, one chloroquine sensitive and the other resistant. Assay was done at 10% haematocrit containing 1% ring stage parasites in a 96 well flat-bottom tissue culture plate. Compounds were dosed in wells in duplicate at concentrations of 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01 µmol per well. Artemisinin was taken as reference drug and also dosed with similar concentrations. To determine the activity of various compounds, assay was done for 24 and 72 hours. The growth of the parasites was monitored microscopically. Percentage schizont maturation and total growth inhibition were calculated to determine the inhibitory concentrations. Nine out of 12 compounds showed antimalarial activity *in vitro*, one compound was found to exhibit maximum antiparasitic activity.

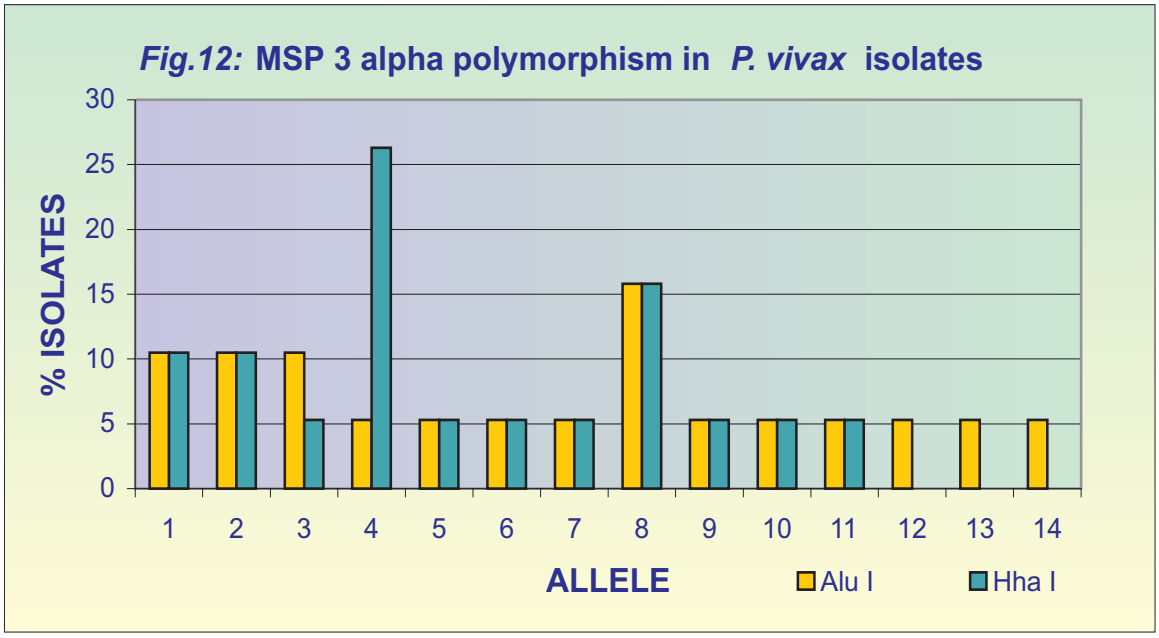
Studies on Genetic Diversity of T-Helper Cell Epitopic Regions of Circumsporozoite Protein of *P. falciparum* Isolates from India

In a previous study on genetic polymorphism of T-helper cell epitopic regions (Th-2R and Th-3R) of CSP of 41 *P. falciparum* isolates from different geographical regions of India, it was found that the T-helper cell epitopes could be categorized into four groups. Both Th-2R and Th-3R variants could be categorized into four groups. Some of the groups categorized based on the sequence variation in Th-2R and Th-3R showed homology with the sequence reported previously. The variations were regionally unbiased as different islolates collected from different regions showed identical sequence variations and belonged to the same group. However, few isolates showed random polymorphism and could not be categorized into groups. In continuation of this study 12 more isolates collected from different geographical regions (Rajasthan, Madhya Pradesh, Delhi and Assam) were studied. Sequence variations in Th-2R and Th-3R showed that 11 isolates exhibited sequence homology with the four groups categorized in the previous study and one isolate showed random variation. Variations in Th-2R and Th-3R in the present study were also found to be regionally unbiased.

Plasmodium vivax

With an aim to select suitable and highly polymorphic markers, isolates collected at malaria clinic, Nanak Enclave, Delhi and samples collected during epidemic investigation in Gautam Budh Nagar, U.P. were analyzed for polymorphism of MSP 3α and DBP (Duffy binding protein).

MSP 3 alpha : A total of 19 isolates were analyzed by PCR-RFLP assay for size and sequence variations. Size variations were observed in the range of 1.2 to 1.8 kb and RFLP assay with Alu I and Hha I has shown changes in the restriction site positions in the alleles. A total of 14 alleles by Alu I digestion and 11 alleles with Hha I digestion were observed. Fig. 12 shows distribution of alleles among the isolates. Among 19 isolates, a total of 16 different genotypes were observed.



Sequence diversity in DBP RII region : With an aim to study sequence diversity in *P. vivax* DBP RII in the Indian field isolates (*P. vivax* duffy binding protein region II has been used in vaccine development), primers have been designed to amplify RII region of DBP from Indian isolates. With these primers, PCR assay has been standardized using genomic DNA and a fragment of about 1 kb has been amplified. Further, amplification, purification and cloning was done for four DNA samples isolated from blood spots collected from *P. vivax* positive patients. Sequencing of the clones is under progress.

Studies on Monoclonal Antibodies against *P. vivax* Erythrocytic Stages

A panel of fifteen hybridomas was raised, eight of them were cultured for harvesting a large volume of supernatant. All of them showed reactivity by IFA test in *P. vivax*

smears with varying degree of fluorescence intensity. By ELISA test, out of 8 lines, 7 were IgG type; one showed positivity for both IgG and IgM. Clones secreting IgG type antibody gave strong signal in IFA and higher optical density in ELISA. Immunoglobulin G isolated from these supernatants were coupled with peroxidase and tested by dot-blot assay on *P. vivax* blots to check the reactivity in clinical specimens. Antibodies showed detection limit in patient's blood having parasitaemia above 0.05%.

In continuation of earlier work, one IgG1 type antibody producing clone was taken for production of large quantities of antibody. On western blot, this monoclonal antibody reacted with a protein above 30 kDa of *P. vivax* crude erythrocytic stage antigen. N-terminal sequencing of the protein was done at IMTECH, Chandigarh. The sequences produced with significant alignments were homologous to one *P. falciparum* cDNA library, *P. berghei* 34 kDa phosphoprotein mRNA and to mouse blastocyst cDNA clone. Since the protein preparation was not completely pure, overlapping of amino acids in the sequences has been observed. This antibody was coupled with horse-radish peroxidase enzyme. The reactivity of the antibody-enzyme conjugate was tested in 20 clinical isolates by dot-blot ELISA. The colour signal was strong with *P. vivax* positive blots.

Biochemical Characterization and Expression of *P. vivax* Aspartic Proteases

The key role of an aspartic protease in initiating haemoglobin digestion in food vacuoles has been defined and two aspartic haemoglobinase I and II (plasmepsins) have been purified in *P. falciparum*. No work has till date been reported on the isolation and characterization and role of aspartic proteases in *P. vivax*. Inhibition of haemoglobin catabolism or other essential functions catalyzed by aspartic proteases in the parasite offers attractive targets for therapeutic intervention studies.

In continuation of our earlier studies, samples from Chennai were processed for the aspartic protease activities in *P. vivax* parasites. Gelatin gel PAGE and ELISA have been used to characterize and assay the protease activities. Two aspartic protease activities have been identified in *P. vivax* having a molecular weight of 37 kDa and 40 kDa which were found to be similar to the activities of plasmepsins in *P. falciparum*. The aspartic protease activities in parasite samples were identified using specific inhibitor namely pepstatin A which shows IC_{50} at 3 µM. The pH dependence of the aspartic protease activity has shown a pH maxima of 4. Kinetic analysis of the inhibition of the enzyme aspartic proteases by Line weaver double reciprocal plot has shown that pepstatin A is a competitive inhibitor of the enzyme with respect to the substrate concentration.

Experiments were initiated to characterize the aspartic protease gene in *P. vivax* using the known primers of *P. falciparum*. However, it was not possible to amplify the *P.*

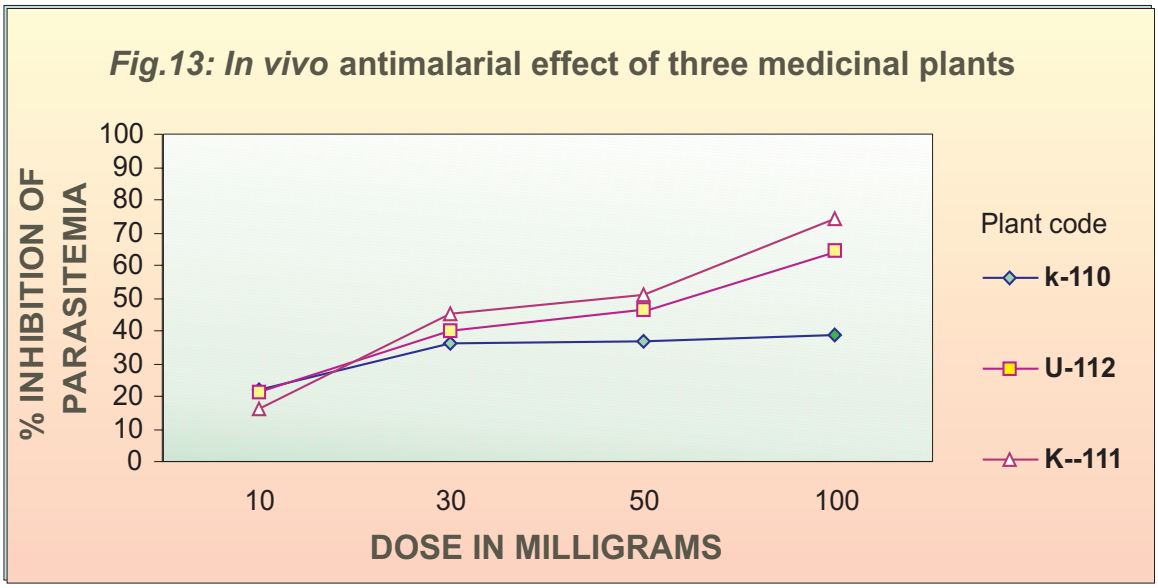
vivax gene using known primers of *P. falciparum*. It is now planned to identify and characterize aspartic protease gene in *P. vivax* using degenerate primers synthesized from the N-terminal amino acid sequences of the plasmepsins from *P. vivax* clone and express it in *E. coli*, purify and refold the recombinant protein for further studies on its role in parasite functions.

Primary Screening of Herbal Products for their Antimalarial Activity

Our earlier studies on the *in vivo* effect of the crude extracts of *Azadirachta indica*, *Phyllanthus niruri*, *Ocimum sanctum* against *P. berghei* showed encouraging results. Studies on this aspect was continued and the antimalarial effect of ethanol extracts of more medicinal plants was studied.

The medicinal plants or parts of the plants used for the treatment of fever in rural/ tribal areas were collected from different geographical regions of India and tested for their antimalarial properties. Fifty per cent ethanol extracts of nine medicinal plants were tested *in vitro* for their antimalarial activity. Some of these extracts, which were showing encouraging results *in vitro*, have been tested *in vivo* this year. The plants were collected, washed and dried in shade and powdered before the preparation of their extracts. The standard methods were followed for the preparation of 50% ethanol extracts using a soxhlet apparatus. The extracts were concentrated and lyophilized for testing their antimalarial properties. The *in vitro* test was done using both chloroquine sensitive and resistant isolates of *P. falciparum*. The assay used was schizont maturation inhibition assay. The *in vivo* test with three medicinal plants was carried out following Peter's 4-day test in which different concentrations of the plant extracts were given orally to the batches of mice after inoculating *P. berghei* intraperitoneally to mice on Day 0. Three more doses were given to mice and on Day 4 slides were prepared from all the mice to quantitate the percentage parasitaemia.

***Fig.13: In vivo* antimalarial effect of three medicinal plants**



The control animal received only parasites. The percentage inhibition of parasitaemia was calculated against the parasitaemia of the control to see the antimalarial effect of the plant products (Fig. 13). All the plants tested showed varying degree of antiplasmodial effect.

These extracts were further fractioned with chloroform, butanol, hexane and ethyl acetate to get the most effective fractions. These studies are in progress. Besides these, four more new plants collected from Kerala, Uttaranchal and Assam have been tested *in vitro* for their antimalarial activity. The antimalarial effect of these plants was not as good as that of the plants tested in our laboratory earlier.