PARASITE BIOLOGY

Studies on Drug Susceptibility

Twenty isolates characterized for their chloroquine sensitivity status were revived from cryopreserved condition and cultivated *in vitro* to retest their CQ sensitivity status and two third of 20 samples showed variation in their chloroquine sensitivity status.

P. falciparum isolates (sensitive and resistant to chloroquine) were cloned and the clones were tested for their CQ sensitivity. These clones would be characterized for genotypes of MSP-1 and MSP-2.

Molecular Characterization of *P. falciparum* Isolates

Parasite Bank

P. falciparum isolates available in the parasite bank of the Centre have been analyzed for polymorphism of MSP-1, MSP-2 and GLURP using nested PCR with family-specific primers. In MSP-1 system, all the three families namely K-1, MAD20 and R033 were observed with prevalence of first two (K1 and MAD20). In MSP-2 system, both the families namely FC27 and 3D7 were observed with approximately similar prevalence. In addition to MSP-1 and MSP-2, GLURP system was standardized and isolates were analyzed. GLURP exhibited polymorphism with 11 different size fragments. Most of the isolates were observed to be multiclonal. The results of family grouping analysis of MSP-1 and 2 of *P. falciparum* isolates are shown in Fig. 1.

HIGHLIGHTS

- Parasite bank has well characterized *P. falciparum* isolates
- Characterization of *P. falciparum* isolates in the Parasite Bank for MSP-1, MSP-2 and GLURP has revealed that each analyzed isolate has a different genotype.
- Genotyping of *P. falciparum* and *P. vivax* isolates from different regions of the country has shown higly polymorphic nature of the Indian isolates in respect of family grouping analysis of MSP-1 and MSP-2 in *P. falciparum* isolates and MSP-3? and GAM1 of *P. vivax*.
- Genotyping of recrudescence infection in five *P. falciparum* patients revealed different genotypes of MSP-1/2 in 3 patients on Day 14
- Inhibitory effect of Nitric Oxide on plasmepsin activity of *P. vivax* was observed suggesting to design strategies to selectively upregulate NO production.
- Analysis of blood samples from different endemic zones has shown high anti-GPL antibody in case of *P. falciparum* and *P. vivax* infections but not with normal or nonmalarial sera.
- HRP-II antigen studies has shown that antimalaria treatment neutralizes the antigen persistence by antibodies
- A multiplex PCR to differentiate *P. falciparum* and *P.vivax* in a single assay was standardized.

Field Isolates

Bloodspots of *P. falciparum* positive patients collected from the Jarawas tribe of Andaman and Car Nicobar islands were analyzed by nested PCR assay for MSP-1 and MSP-2 size variations in variable repeat regions. Results have shown presence of only one allele of each—450 bp for MSP-1 and 500 bp for MSP-2. Blood spots were further assayed using family specific primers. Results revealed presence of all the three families of MSP-1 with higher proportion of R033 family. In MSP-2, both the families were present with greater

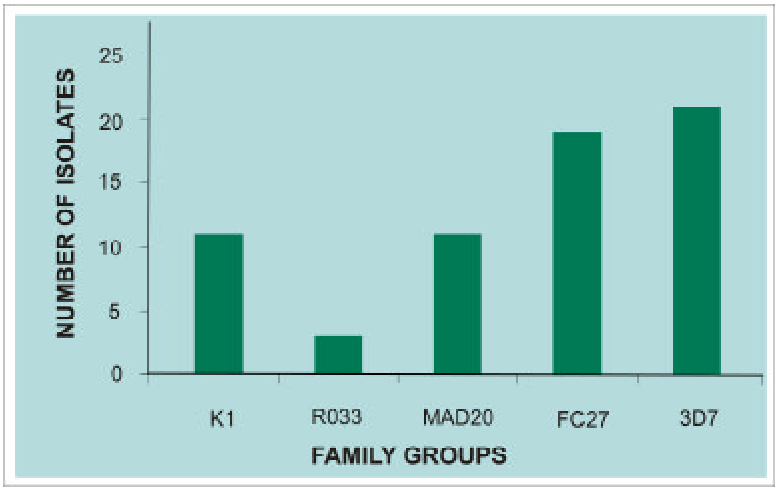

Fig.1: Distribution of family groups of MSP-1&2 of *P. falciparum* isolates of parasite bank

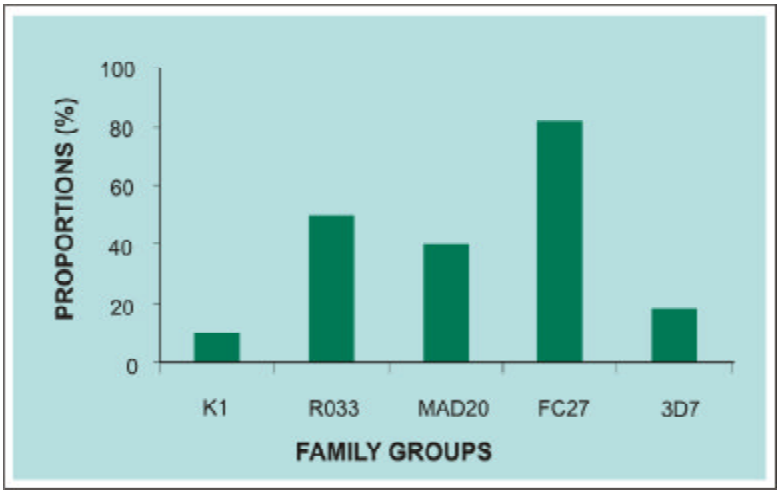

Fig. 2: Distribution of family groups of MSP-1&2 in *P. falciparum* isolates (Jarawas tribes of Andaman & Nicobar Islands)

proportion being that of FC27. The proportion of isolates with different families of MSP-1 and MSP-2 is shown in Fig. 2.

Genetic Diversity Studies of *P. falciparum* and *P. vivax* in India using Molecular Markers (ICMR Funded Project under Genomics)

P. falciparum

Field isolates collected from Assam and Orissa have been analyzed for polymorphism of MSP-1 & MSP-2. Results revealed polymorphism of both the systems among the isolates of the areas.

Twenty isolates of *P. falciparum* from Assam area have been analyzed for family grouping and observations revealed presence of all the three families of MSP-1 (K1, MAD20 and R033) and both of MSP-2 (FC27 and 3D7) (Table 1). Proportion prevalence of family-specific markers is 51% for MAD20, 31% for K1 and 17% for R033 of MSP-1 and 50% for FC27 and 3D7 of MSP-2. Out of twenty samples analyzed 50% (out of these 50% of isolates with all the three families of MSP-1) were multiclonal. Only two isolates were categorized as single clonal based on the genotyping of MSP-1 and MSP-2.

Twenty-two samples from Assam were also analyzed for allelic polymorphism using nested protocol. Among the isolates analyzed size variants of MSP-1 ranging from 400 to 600 bp were observed and in MSP-2 variants ranging between 400 and 750 bp were observed. Twenty-eight isolates of *P. falciparum* from District Sundargarh, Orissa, have been analyzed for MSP-1 and MSP-2 family grouping. All the three families of MSP-1 and two of MSP-2 were observed with a prevalence of 40% for K1, 38% for MAD20, 22% for R033 of MSP-1 and 58% for FC27 and 42% for 3D7 of MSP-2 (Table 1). About 57% of the isolates were multiclonal based on genotypes of both MSP-1 and MSP-2.

Table 1. Family grouping analysis of MSP-1 and MSP-2 among Assam and Orissa samples

Area	MSP-1		MSP-2			% of multiclonal isolates
	K1	MAD20	R033	FC27	3D7	
Assam	11 (31.4)	18 (51.4)	6 (17.1)	10 (50.0)	10 (50.0)	50
Orissa	15 (40.5)	14 (37.8)	8 (21.6)	36 (58.1)	26 (41.9)	57

Figures in parentheses are per cent proportions.

Differentiation of Recrudescence from Fresh Infection

Isolates collected from three patients on Day 0 and Day 14 (day of recrudescence) showed different genotypes of MSP-1/MSP-2 suggesting new infection. However, in another two patients, isolates collected on Day 0 and Day 14 showed same genotype of both MSP-1 and MSP-2. To get more conclusive results, analysis of these samples using GLURP is in progress. Fig. 3 shows the genotypes of paired samples.

P. vivax

Twenty *P. vivax* isolates collected from Delhi have been analyzed for GAM1 and MSP-3?. GAM1 system is observed to be polymorphic with alleles in the range of 400 bp to1.2 kb. In MSP-3? PCR product after restriction digestion has shown different RFLP patterns with Hha I and Alu I enzymes. Study is being continued to analyze more isolates from Delhi and Chennai.

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

Haemoglobin is an important nutrient source for intraerythrocytic malaria organisms. Aspartic proteases play a key role in the degradatve process. Most of the work on aspartic proteases has been carried out in *P. falciparum*, however, no work has till date been reported on the isolation and characterization, and role of aspartic proteases in *P. vivax*. Inhibition of hemoglobin catabolism or other essential functions catalyzed by aspartic proteases in *P. vivax* offers attractive targets for therapeutic interventions.

In continuation of earlier studies, samples from Chennai have been processed for the aspartic protease activity in *P. vivax* parasites. Aspartic protease activity was isolated from a pooled extract of *P. vivax*

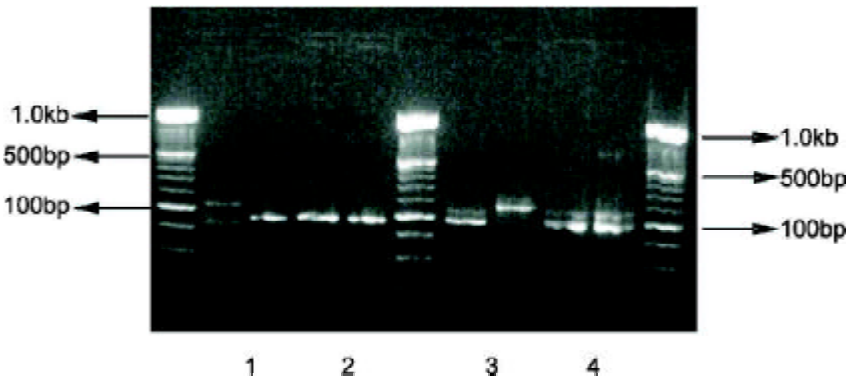

Fig. 3: Gel electrophoretogram showing genotypes of paired samples. Pairs 1 and 3 are different size variants and pairs 2 and 4 are with same size

Table 2. Purification of *P. vivax* aspartic protease

	Protein (mg)	Activity (nmoles/min)	Sp. activity (nmoles/min/mg)	Purity	Yield %
Triton extract	0.758	5.56	0.142	1	100
DEAE extract	0.139	6.92	3.250	23	124
pH 4.5 cut	0.034	2.61	6.960	49	47
Hydroxylapatite	0.013	0.97	23.660	167	17
HPLC	0.006	0.44	40.150	282	8

samples by conventional chromatography on DEAE, hydroxylapatite and gel filtration columns. The resultant peak activity was 282 fold purified over the starting material. The pH optima of the purified enzyme was found to be 4.5–5.0. The IC_{50} for the inhibitor pepstatin was 5nM. PMSF, leupeptin inhibitors of serine and cysteine proteases had no effect on the reaction. For NH_2 terminal sequencing partially purified enzyme was subjected to electrophoresis blotted on PVDF membrane and the 40kD band excised and sequenced. Tentative sequence had revealed 9 of 22 residues identical to most specific mammalian aspartic protease—renin (Table 2). Now a study is being 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*; to clone and express it in *E. coli*; and to purify and refold the recombinant protein for further studies on its role in parasite functions.

Nitric Oxide Inhibits Plasmepsin, a *P. vivax* Aspartic Protease involved in Haemoglobin Degradation

Nitric oxide (NO) has been known to possess antiparasitic activity in *Plasmodium* species. Parasite proteases are promising targets for antimalarial chemotherapy. In the present study, we have studied the inhibitory effect of NO on the plasmepsin activity, the pepsin like aspartic protease involved in the cleavage of haemoglobin degradation in *P. vivax*. NO donors (±) (E)-4-ethyl-2-[(E) hydroxyimino]-5-nitro-3-hexenamide (NOR-3), S-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP) and activators of nitric oxide production IL-6, IFN-? and TNF-?, were found to inhibit the plasmepsin activity in a dose dependent manner in *P. vivax* extracts, an effect attributable to the nitrosylation of the cysteine residue at the catalytic site. However, the inhibitor of aspartic protease activity namely, pepstatin A was also found to suppress the enzyme activity. These results, therefore, represent new insights into the pathophysiological mechanisms and help in designing strategies to selectively upregulate NO production in *P. vivax* infections for antimalarial chemotherapy.

Glycophospholipid Antigen from *P. falciparum* Culture Supernatant: Isolation, Chemical Analysis and Detection of *Pf* Infection

A *P. falciparum* malaria blood stage antigen was isolated from *in vitro* parasite culture supernatant. The antigen was identified as a mixture of glycophospholipids (GPL). The serological activities of the GPL from *P. falciparum* culture were examined by ELISA against *P. vivax* and *P. falciparum* infected patient's serum. Cross-reactivity of GPL was tested against serum from