

Molecular Characterisation

Assessment of Molecular Markers for the Identification of Recrudescence Infection in *P. falciparum* from Fresh Infection

Blood spots collected from *P. falciparum* positive patients after microscopic examination during therapeutic efficacy studies of various antimalarials (chloroquine and sulphadoxine-pyrimethamine) were analysed for molecular markers namely MSP-1, MSP-2 and GLURP. During the study, paired samples of Day 0 and the day of recrudescence were analysed from Orissa, Goa, Tamil Nadu and West Bengal. Analysis revealed same genotype of all the three marker systems in about 56.4% of isolates in Orissa (Sundargarh district), 86.7% in Goa, 87.0% in Rameswaram, Tamil Nadu and 63.6% in Darjeeling district of West Bengal (Fig. 15). The study revealed highest efficiency of MSP-2 marker for identification of different genotypes in paired samples of Day 0 and the day of recrudescence (Fig. 16).

Genetic Diversity Studies of Human Malaria Parasites

P. falciparum isolates collected from Nadiad taluka (Kheda), Gujarat; Sundergarh district, Orissa; and Kamrup district (Sonapur), Assam were analysed for polymorphism in MSP-1 and MSP-2 to get information about extent of diversity existing among the field isolates.

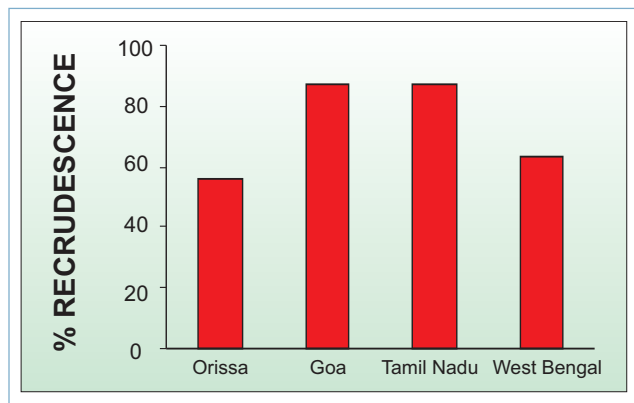


Fig. 15. Proportion of isolates with identical genotypes of MSP-1, MSP-2 and GLURP

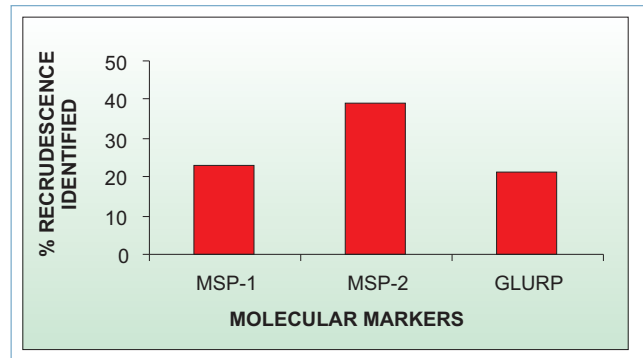


Fig. 16. Efficiency of markers for the differentiation of recrudescence

In all the three areas, both the markers MSP-1 and MSP-2 were polymorphic with three families of MSP-1 namely (K1, MAD20 and RO 33) and two of MSP-2 (FC27 and 3D7). Proportion of multiclonal isolates was 26.3% in Kheda, 35% in Sonapur and 52% in Orissa (Fig. 17).

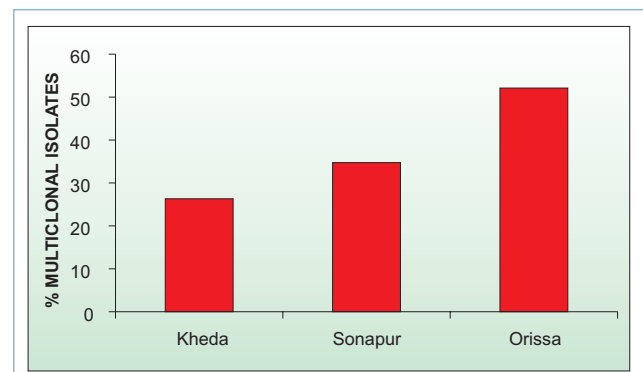


Fig. 17. Proportion of isolates with multiclones among the study samples

Molecular Monitoring of Sulphadoxine-Pyrimethamine Resistance among *P. vivax* Field Isolates

A total of 69 *P. vivax* infected blood spots were analysed for mutations of the *Pvdhfr* gene, associated with sulphadoxine/pyrimethamine resistance. Mutations are good markers for the molecular epidemiological studies of drug resistance. Maximum number of isolates (69.6%) showed mutation at 117

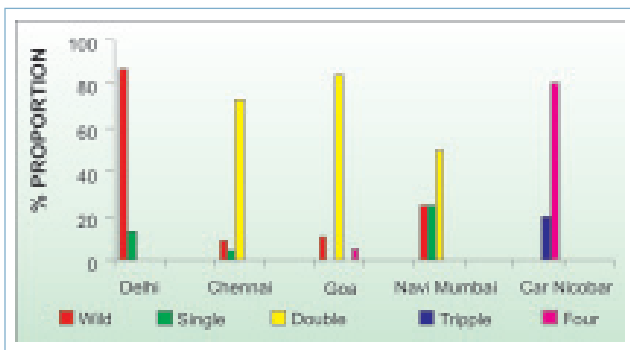


Fig. 18. Distribution of Pvdhfr genotypes among Indian field isolates

residue followed by at 58 residue (62.3%). Limited number of isolates showed mutations at residue 57 (10.1%) and 61 (7.2%). Among the study samples, proportion of double mutants was highest (56.5%) followed by wild type (27.5%). Wild type genotype was maximum in Delhi region (86.6%), while Chennai and Goa isolates had double mutants in 86.4 and 84.2% of isolates respectively. Navi-Mumbai isolates had shown in between picture with 50% isolates being double mutants.

Car Nicobar isolates had totally different picture with tripple and quadruple mutants. Fig. 18 shows the areawise distribution of Pvdhfr mutations in *P. vivax* field isolates.



◆ Genotyping of treatment failure cases has revealed that MSP-1, MSP-2 and GLURP could be used to identify fresh infection from recrudescence infection in case of *P. falciparum*

Immunological Characterisation

P. vivax Monoclonal Antibodies: Purification and Characterisation

Ten hybridomas rose against *P. vivax* erythrocytic stages showed reactivities in immunofluorescence and enzyme immunoassays. These lines were expanded *in vitro* for producing large volume of culture. Culture supernatant from 10 hybridoma lines was tested for immunoglobulin isotyping. These 10 antibodies were found to be IgG₁ type. After testing by IFA, individual batch of supernatant was subjected to ammonium sulphate precipitation and affinity adsorption with Protein-A sepharose for isolation of IgG fraction. Purified IgG fractions were labelled with Fluorescine isothiocyanate and Rhodamine. Labelled conjugates were tested in *P. vivax* smears by IFA to check their differential reactivities.

One earlier batch of monoclonal antibodies reactive to *P. vivax* erythrocytic stages has been tested in clinical isolates to establish its potential as a diagnostic reagent. Antibody secreted by the clone, demonstrated reactivity with *P. vivax* isolates by Inhibition ELISA. This antibody detected patients' blood samples, positive with *P. vivax* at or above 1000 parasites per microlitre. Work is continued on isolation of *P. vivax* proteins reacting with monoclonal antibodies and isolation of high affinity parasite antibodies from existing panel of hybridomas to develop diagnostic reagent.

Antimalarial Antibody Profile against Defined *P. falciparum* Antigens in Chloroquine Responder and Non-responder Group of Patients

The study subjects included the inhabitants of Kathiatali, Nowgaon and Sonapur, Kamrup districts of Assam. Finger-prick blood samples were collected from 90 patients found positive with *P. falciparum*

◆ Monitoring of S/P resistance revealed point mutation in Pvdhfr gene, a predominance of wild type gene among Delhi isolates, while isolates of South (Chennai), Coastal (Goa) and Central (Navi-Mumbai) regions had shown predominance of double mutants

infection during July to October 2002. Of the 90 patients, 61 responded to CQ as no asexual parasite was observed during 28 days follow-up. This group was denoted as responder who showed ACR. The other group of 29 patients showed treatment failure (TF) since their blood smears detected positive with falciparum ring during 28 days follow-up. Sero-reactivity of these patients were compared with known falciparum patients from Delhi and Ghaziabad (n=24), who showed adequate clinical responses (ACR) to CQ; also with known malaria negative healthy individuals (n=16). Sera were tested for antimalarial IgG antibody against five *Pf* stage-specific synthetic peptides (CSP, MSP-1/19, EBA175, AMA1 and PfG27; procured from Molecular vaccine Section, CDC, Atlanta) and *P. falciparum* infected erythrocyte lysate (*Pf* crude).

The sero-reactivity of TF and ACR groups was compared. Sera of TF patients showed lower antibody profile against all six antigens. The differential reactivity profiles of these two groups found to be significant ($p < 0.01$). Antibodies detected against CSP, MSP-1/19, AMA1 and PfG27 were lower in patients from study group than known *Pf* positive group. However, average level of anti-EBA175 antibody was almost alike in two groups. The healthy normal subjects showed very low sero-reactivity.

Immunocytochemical Peroxidase Test (ICPT) and Dot Immunobinding Assay (DIBA) for the Detection of Antimalarial Antibody in Patients' Sera

Finger-prick blood samples were collected from 46 subjects of age group 5–25 yr belonging to Nanoo village of PHC Loni, Distt. Ghaziabad during October–November 2003, after obtaining informed consent. They reported with fever and came to mobile clinic for malaria diagnosis. Among them, three were positive for *P. falciparum*. Almost all of them had previous history of malaria. Indirect ELISA was done to estimate antimalarial IgG antibody. Of the 46 samples, 19 had high, 23 had

moderate and 4 had low level of *Pf*-antigen specific IgG. The ELISA results of 46 individuals were compared with that of healthy individuals (negative sera) and malaria immune subjects (positive sera). In this group of 46, none showed very high titre of antibodies as compared to immune sera.

In ICPT, sera were allowed to react with the whole parasite on a microslide, whereas in DIBA sera were allowed to react on antigen blotted nitrocellulose membrane after inactivation of endogenous peroxidase. Parasite antigen-antibody complexes were trapped with antihuman IgG-HRPO conjugate. The assay was read after addition of enzyme-specific substrate, amino ethyl carbazol/hydrogen peroxide. The results of both the assays were comparable. However, DIBA found to be more sensitive than ICPT.

Partial Characterisation and Growth Inhibition Reaction of a Glycophospholipid Antigen from *P. falciparum* Culture Supernatant

Glycophospholipids (GPL), a distinct class of antigens are particularly abundant in parasites where they are found as free lipids and attached to proteins. It has been found that *P. falciparum* synthesise GPL and its biosynthesis is crucial for the development and survival of the parasites. Determination of a detailed structure requires isolation of pure GPL which is released in the spent media during schizogony is difficult to obtain in adequate amounts from host cell free components. We are able to purify *P. falciparum* GPL to homogeneity and partially characterised the structural components. Parasite culture supernatant was collected, concentrated, dialysed, gradient centrifuged and lyophilised. Parasite antigen was purified by differential chloroform extraction to remove most nonglycosylated lipids, and the extract containing free GPL was subjected to Folsch's wash. Finally the GPL was purified by successive fractionation using HPLC and silica gel column chromatography. One

◆ One IgG₁ type monoclonal antibody demonstrated reactivity in *P. vivax* infected patients' blood samples by Inhibition ELISA

◆ The sero-reactivity of chloroquine responder (ACR) and non-responder (TF) groups were compared. Sera of TF group showed lower antibody profile against all six antigens than ACR group

gram of lyophilised culture supernatant yielded 60 mg of GPL by differential extraction method.

The mass spectrometry of the purified three GPL fractions isolated from silica gel column chromatography showed the presence of glycerophosphorylcholine, nonadecanoyl, tetradecanoyl and docasanoyl moieties. Each fraction contains glycerophosphocholine, nonadecanoyl and docasanoyl as core components. Identification of GPL fractions by HPLC revealed the presence of rhamnose, galactosamine, fucose, mannose, galactose and glucose. Partial structural analysis indicates that GPL is a new kind of antigen so far has been published.

Among three GPL purified fractions, 50 and 70% fractions were used for parasite growth inhibition assay. This test suggests GPL fractions have toxic effect on parasite growth. A small amount of GPL can inhibit 50% growth in comparison to control GPL fractions.

The variation in parasite count between replicate wells was estimated. In treatment groups, count was lower when the concentration of GPL antigen was increased. We also determined that the schizonts started surviving when antigen concentrations were lowered from 25 $\mu\text{g}/\text{ml}$ to 0.62 $\mu\text{g}/\text{ml}$. A 50% growth inhibition with the parasitised 1 $\mu\text{g}/\text{ml}$ GPL was noticed while in control GPL 50% growth inhibition was observed when the concentration was increased to 26 $\mu\text{g}/\text{ml}$. Our experiment suggests GPL from parasitised culture supernatant is more lethal than the control GPL.

Growth Inhibition of *P. falciparum* in the Presence of 50 and 70% GPL Fractions

Silica gel column chromatography of 50 and 70% methanol eluted fractions isolated from glycerophospholipid were tested for schizont growth inhibition. In *in vitro* parasite culture, dose dependent GPL antigen fractions were added in different concentration ($\mu\text{g}/\text{ml}$). GPL isolated from parasitaemic and nonparasitaemic (control GPL) culture supernatant were used. About 26 $\mu\text{g}/\text{ml}$

◆ **Dot immunobinding assay found to be more sensitive than immunocytochemical test for detection of antimalarial antibody in patients' sera**



control GPL was needed for inhibiting 50% schizont growth while 1.0 $\mu\text{g}/\text{ml}$ GPL was good enough to reach to the above condition. The test indicates GPL has toxic effect on parasite growth.

Biochemical Characterisation

Purification and Characterisation of a Haemoglobin Degrading Aspartic Protease from *P. vivax*

Elucidation of structure/activity and biochemical strategies of the enzyme is necessary to facilitate the development of potent specific inhibitor for potential application as antimalarial drugs. In order to validate whether recombinant forms of plasmepsins are appropriate for use in systematic investigation into inhibitor drug design and development it was considered vital to isolate, characterise and establish the properties of the naturally occurring enzymes in terms of activity and specificity to reflect those in their recombinant forms. Thus isolation, characterisation and inhibition of haemoglobin catabolism catalysed by aspartic proteases in *P. vivax* offers attractive target for chemotherapeutic intervention studies if these classes of enzymes are to be exploited as drug targets. We have now defined the purification and characterisation of *P. vivax* aspartic proteases and have shown that they also share properties similar to that of *P. falciparum* and may play a pivotal role in the development of/as new drugs/drug targets.

◆ **Glycerophospholipid from parasitised culture supernatant is more lethal than the nonparasitised one and the GPL has toxic effect on parasite growth**

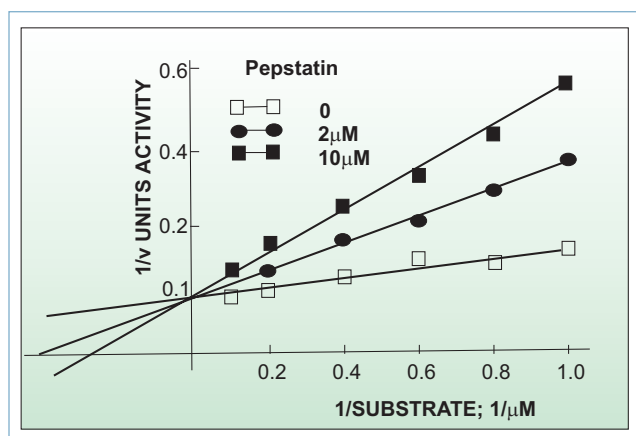


Fig. 19. Kinetic analysis of inhibition of the aspartic protease activity Lineweaver Burk double reciprocal plot of various concentrations of pepstatin (0,2 and 10 μM)

Kinetic Analysis of the Aspartic Protease Activity

To understand the kinetics of inhibition of the aspartic protease activity by pepstatin we have conducted experiments with increasing concentrations of pepstatin (2, 5, 10 μM) and plotted the inhibition kinetics by Lineweaver Burk double reciprocal plot (Fig. 19). Our results demonstrate that the inhibition is competitive with respect to substrate.

Haemoglobin Degradation

To compare the fragments generated by the haemoglobin degradation by purified aspartic protease with those of the fragments produced by the parasite *in vivo* we have carried out experiments with

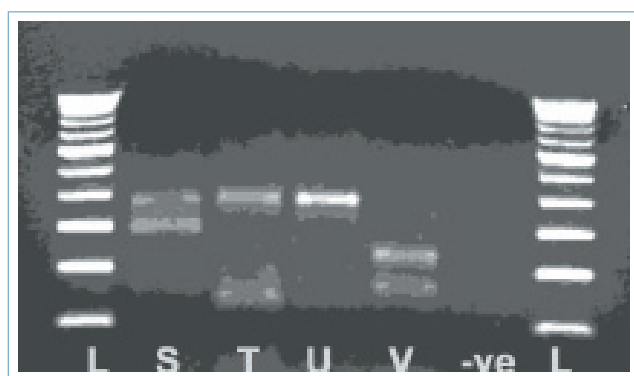


Fig. 20. SDS-PAGE of the proteolysis products: Haemoglobin (4 μM) was incubated with *P. vivax* malaria aspartic protease (0.14 p moles/min) for 30 min at pH 4 in the presence and absence of aspartic protease inhibitor, pepstatin. An 8–25 % gradient gel was run under denaturing reducing conditions. Haemoglobin control (Lane A); Haemoglobin + aspartic protease + 10 μM pepstatin (Lane B); and Haemoglobin + aspartic protease (Lane C). Arrows 1 and 2 mark the two peptide fragments

purified haemoglobin. Haemoglobin was incubated for 30 min with our isolated aspartic protease and also in the presence and absence of pepstatin and then analysed on SDS-PAGE (Fig. 20). Arrow 1,2 in the figure indicates the two peptide fragments with the increasing time the two primary cleavage products accumulated and can be seen in absence of pepstatin. The hydrolysis was found to be abolished by the inclusion of pepstatin.

Effects of the Antimalarials

The effect of antimalarial drugs on the enzyme activity was also investigated. The enzyme was incubated with each drug at different concentrations for 15 min at 30° C followed by the measurement of the residual activity under standard assay conditions

Drug	Concentration (mM)	% Activity
Quinine	1.0	80
Chloroquine	1.0	100
Primaquine	1.0	85
Mefloquine	1.0	65

(Table 5). The protease activity was found to be insensitive to known classes of antimalarials. Mefloquine ((1.0 mM) inhibited the enzyme activity to some extent.

Parasite Killing in *P. vivax* Malaria by Nitric Oxide: Implication of Aspartic Protease Inhibition

The generation of NO as a direct result of circulating cytokines may mediate the host pathology seen in malaria infections and in the production of reaction nitrogen intermediates (RNI). The antiparasitic effects of the cytokines observed in different murine malaria infections may be mediated via the production of RNI and may be due to the inhibition of protease activity. Thus, RNI production may be important for understanding the pathophysiology of *P. vivax* infections.

The catalytic activity of proteolytic enzymes is modulated by NO through binding to metal centres and also by chemical modification of the reactive residues. In our efforts to establish the role of NO compounds in malaria we have conducted

Table 6. Killing of *P. falciparum* in vitro by reactive nitrogen intermediates

Reactive nitrogen intermediates*	IC ₅₀ μ M (Mean \pm SEM)	n#
NaNO ₃	36270 + 7,240	5
NaNO ₂	10,450 + 3,490	5
Sodium nitroprusside	>25000	3
ON-S-Glutathione	41.8 + 18.45	4
ON-S-Cysteine	38.20 + 10.75	4

*Nitric oxide-releasing Compounds: ON-S-, nitrosothiol group; #No. of experiments.

experiments *in vitro* with *P. falciparum* in culture (Table 6). We have observed that the most potent compounds tested were S-nitrosoglutathione and S-nitroso compounds, the reaction mixture which requires 1000 times less material on a molar basis than for either nitrate or nitrite. The parasitocidal effect of these compounds is primarily because of their nitrosothiol contents, and they were found to be a thousand times more active (50% growth inhibitory concentration, approx. 40 μ M) than nitrite. Once the nitrogen oxides have diffused into erythrocytes, nitrosothiol groups are formed on proteins, or more toxic chemical species such as peroxy-nitrites or hydroxyl radicals, which could lead to inactivation of enzymes and thus changes in protein functions are produced. The constant generation of nitric oxide may be required for it to be parasitocidal because it reacts to cross-linked sulfhydryl groups, therefore, we are now conducting experiments to purify and express the nitric oxide synthase in order to test this hypothesis.

The present data thus indicate that the plasmepsins of *P. vivax* are inactivated by NO donors and also NO, probably through S-nitrosylation, thus representing a novel approach for the inhibition of *P. vivax* infections. GNSO and nitroso-L-cysteine are known to kill *P. falciparum* *in vitro*, probably through the inhibition of a cysteine protease (falcipain). The concentrations of GNSO, NOR-3, SIN-1 and SNP used in the present study are consistent with the concentrations of GNSO and nitroso-L-cysteine used to kill malaria parasites (4.0×10^{-5} μ M).

NO has been shown to exhibit direct microbiocidal activity by interacting with enzymes, sulphhydryl groups or superoxides. Moreover, it has also been shown that NO regulates IL2 and IFN- γ production in Th-1 cells, and might be important in

the regulation of switching between Th-1 and Th-2 type immune responses. This can facilitate the design of strategies for upregulating NO-mediated S-nitrosylation of proteases in *P. vivax* infections, and for *P. falciparum* antimalarial chemotherapy through blocking of essential metabolic pathways for haemoglobin degradation by the parasite. The demonstration of the involvement of proteases and NO inhibitors of this activity could constitute a new approach for the treatment of malaria.

Screening of Medicinal Plants for their Antimalarial Property

Due to the development of resistance in parasites to almost all antimalarials available, efforts are being made to explore the possibility of having new antimalarials from indigenously available medicinal plants. About 25 medicinal plant extracts were tested and some of them showed good anti plasmodial activity *in vitro* against *P. falciparum*. Three extracts



were tested *in vivo* also. These extracts were giving up to 75% inhibition with 50 mg/kg body weight. Further work is in progress for the purification of compounds from these extracts.

The collaborative project entitled, "Primary screening of the medicinal plants from Northeastern states of India for their anti plasmodial activity" is an ongoing project. Under this project 25 crude extracts and eight fractions were tested *in vitro* till now. Out of this one crude extract tested *in vivo* gave about 50%

◆ Plant extracts received from different parts of India are being tested *in vitro* and *in vivo* for their antimalarial properties

inhibition with 50 mg/kg body weight. Further studies are in progress.

Three compounds (endoperoxidase) were received from the Department of Pharmaceutical Sciences,

Guru Nanak Dev University, Amritsar, Punjab for testing their antiplasmodial activity *in vitro*. These compounds showed very high anti plasmodial activity. The work is in progress.