

7. Molecular Biology of Malaria Parasites & Vectors

7.01 The *Plasmodium vivax* Genome Sequence and comparative analysis with *Plasmodium falciparum*

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The project to sequence the complete genome of *Plasmodium vivax* Salvador I isolate began in 2002 at The Institute for Genomic Research (TIGR), with the aim of producing a sequence as good as if not better than that of the first human malaria parasite to be sequenced, *Plasmodium falciparum*. The project is now close to completion three years after its initial start date. The majority (23 Mb) of the ~27 Mb genome is contained within ~50 contigs, with a number of smaller, most likely subtelomeric contigs of high [A+T] content composing the remainder. Whole genome synteny analysis with the *P. falciparum* genome and a composite rodent malaria genome show conservation of several synteny break points with the rodents, but less conservation with the *P. falciparum* genome. Approximately 5,400 genes have been predicted and characterized in the nuclear genome, similar to the number found in *P. falciparum*. Significant differences exist in the gene repertoire found in subtelomeric regions of the genome, a characteristic in common with other *Plasmodium* species, and several novel gene families have been identified in these regions. Studies are ongoing into other characteristics of the genome, such as the comparison of low-complexity regions of genes between *P. falciparum* and *P. vivax*, and differences in [G+C] content between genes.

7. Molecular Biology of Malaria Parasites & Vectors

7.02 Role of falcipain-2, a principal cysteine protease of *P. falciparum* in merozoite egression

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In malaria the process of merozoite release involves rupture of the parasitophorous vacuole membrane and erythrocyte plasma membrane. Although use of different protease inhibitor(s) have demonstrated the involvement of proteases in the merozoite release, actual parasite molecule (s)/protease(s) involved in the merozoite release has not been identified. To identify and elucidate the role of cysteine proteases in the merozoite release, we treated *P. falciparum* cultures with siRNAs corresponding to *falcipains-1, -2* and *-3*. Treatment of malaria parasites with either of the falcipain siRNAs considerably reduced parasite growth. Morphological examination of siRNA treated parasite cultures revealed that most of the parasites in the *falcipain-2* siRNA treated cultures were arrested at the schizont stage. Treatment of a transgenic line expressing chimeric-GFP with *falcipain-2* siRNA revealed that *falcipain-2* plays an important role in the rupture of erythrocyte membrane at the time of merozoite release.

To understand the mechanism of RNA interference (RNAi) in malaria parasite, we have recently identified, cloned and characterized one of the proteins involved in RNAi pathway, Tudor-SN in *P. falciparum*. Tudor staphylococcal nuclease (Tudor-SN) protein is a conserved ~100kDa protein that has four intact staphylococcal/micrococcal nuclease domains and a Tudor domain. The recombinant Pf Tudor-SN protein exhibits RNA binding as well as nuclease activity. Interestingly, treatment of *P. falciparum* with pdTp, a specific inhibitor of Tudor-SN protein, inhibits in vitro growth of the parasite. PdTp is equally effective in retarding the growth of both chloroquine (CQ) sensitive and chloroquine (CQ) resistant strains of *P. falciparum*. These results suggest that Pf Tudor-SN is an essential enzyme in the parasite life cycle and can be an important target for malaria drug development given its low homology with human Tudor-SN proteins.

7. Molecular Biology of Malaria Parasites & Vectors

7.03 Molecular epidemiology of drug resistant malaria

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Malaria remains uncontrolled and requires newer drugs and vaccines. Till the malaria vaccine and newer class of antimalarial drugs become available, the existing drugs need to be used cautiously. This is because the irrational use of antimalarial drugs can cause the drug resistant malaria. Effective usage of existing antimalarial drugs for malaria control strategies require continuous input of the drug resistance pattern in the field. While *in vitro* and *in vivo* methods are available for this purpose, they may not be feasible for the larger scale studies. Molecular studies using various markers however, can provide the advance information on the emergence of drug resistance pattern in the field. The data can then be used to design malaria control strategies. We have used such markers to study the point mutations in the DHFR and DHPS genes of *P.falciparum* to monitor sulfadoxine-pyrimethamine resistance, and *Pfcr1* and *Pfmdr1* mutations for chloroquine resistance. Results indicate that mutations in these genes are increasing with time. This is an indication of the continued drug pressure in the field and should be taken in to consideration by the malaria control program managers of the country.

7. Molecular Biology of Malaria Parasites & Vectors

7.04 Molecular studies on antifolate resistance to malaria parasites in Africa

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Molecular genetic markers have become useful tools to track emerging drug resistance in malaria. Single nucleotide polymorphisms (SNP) in dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes have been associated with *Plasmodium falciparum* sulphadoxine-pyrimethamine resistance. Mutations at *dhfr* codons N51I, C59R, and S108N/T in *dhfr* (triple mutant allele) have been linked to high levels of resistance to pyrimethamine. A fourth mutation at codon I164L yields the highest level of parasite resistance to this drug. This mutation also increases the parasite resistance to chlorproguanil, a drug that is being evaluated for use in combination with dapsone and artesunate in Africa. So far the presence of I164L mutation has not been well established in Africa and there is a concern that appearance of 164L mutation in Africa may become a challenge for the use of new combination therapies that contain chlorproguanil. Mutations at *dhps* codons S436A/F, A437G, K540E, A581G, and A613S/T are associated with resistance to sulphadoxine. The prevalence of these mutations at *dhfr* and *dhps* differs in various geographical areas. Microsatellite markers are powerful tools that can be used to track the spread of drug resistance genes in populations. Recent findings using microsatellite markers spanning *dhfr* suggest that triple mutant parasites in Africa and Southeast Asia may have a common origin and could have arisen from a single event. These findings need to be validated in other studies to confirm whether these observations can be extrapolated to other parts of the world, and to determine if these markers could be used as reliable markers for tracing drug resistance in surveillance studies. This presentation will detail the latest findings about the occurrence of 164L mutation and the prevalence of various microsatellite haplotypes associated with triple mutant parasites in Africa. These findings will be discussed in the light how different sets of molecular markers can be used for tracking drug resistant *P. falciparum* in different parts of the world.

7. Molecular Biology of Malaria Parasites & Vectors

7.05 Translation within the apicoplast of *Plasmodium falciparum* is targeted by thiostrepton

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The apicoplast of *Toxoplasma* and *Plasmodium* has generated immense interest as a putative drug target. Although transcripts for gene encoded by the 35 kb circular plastid DNA (pDNA) had been detected, the actual presence of their protein products was only postulated. We have analyzed the translation of elongation factor Tu (EF-Tu), the product of the apicoplast *tufA* gene of *P.falciparum*, which is one of the best-conserved proteins encoded by pDNA.

Using antisera generated against *P.falciparum* apicoplast EF-Tu, expressed as a fusion protein in *E.coli*, we have demonstrated the presence of this protein in *P.falciparum*. Analysis of parasitized RBCs by immunofluorescence assay localized EF-Tu within the apicoplast. Co-immunolocalization experiments confirmed that the protein was confined to the apicoplast. EF-Tu was found to be synthesized throughout the erythrocytic cycle with maximal expression seen in the late trophozoite stage. Our results provide the first conclusive evidence for translation within the apicomplexan plastid.

We assessed the activity of thiostrepton, a potent inhibitor of prokaryotic translation, on plastid EF-Tu levels. Thiostrepton has a binding site in apicoplast LSU rRNA and exhibits antimalarial activity in culture. Our results indicated that translation within the apicoplast is the primary site of action of drug. Additionally, differential effects of thiostrepton were seen on levels of parasite cytoplasmic proteins.

7. Molecular Biology of Malaria Parasites & Vectors

7.06 Molecular assessment of *Plasmodium falciparum* resistance to antifolate drugs in endemic malarious areas in Iran

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Malaria has been endemic in Iran for centuries. Now, it is limited to three southeastern provinces as endemic areas with some sporadic cases-almost as imported malaria-in all over the country.

There are 10000 to 30000 cases annually with three species: *Plasmodium vivax*, *P. falciparum* and *P. malariae* with 70, 26, 3 percent respectively and 1 percent mixed.

Plasmodium falciparum resistance to chloroquine-first line drug for malaria therapy-was reported in 1988 using currency *in vivo* method. Due to some preferences in using molecular biology methods, we establish and using mutation specific PCR assay in malaria researches for first time in Iran. We have studied the most important reported mutations in dihydrofolate reductase (DHFR) gene of *P. falciparum* isolates from malarial patients from endemic areas in Iran.

We found four genotypes in position 108 as: Ser-108, Thr-108, Asn-108 and mixed Ser-108+Thr-108 with frequency of 82.8,2.8,11.6 and 2.8 percent respectively.

Molecular data were compared with clinical condition and confirmed by microscopic observations.

7. Molecular Biology of Malaria Parasites & Vectors

7.07 Single-nucleotide polymorphisms and genome diversity in *Plasmodium vivax*

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In India *Plasmodium vivax* malaria constitutes more than 50% of the total malarial cases. Although rarely fatal the parasite causes debilitating disease that severely affects the quality of life and economic productivity of its victims. The *P. vivax* genome is distributed among 14 haploid chromosomes. Only a few genome markers mostly orthologs of previously identified *P. falciparum* antigen genes, have been used for population studies of *P. vivax*, in contrast to 1000 polymorphic microsatellites and hundreds of restriction fragment length polymorphism and single nucleotide polymorphism (SNP) markers described for *P. falciparum*. The lack of genetic markers for the *P. vivax* genome has severely hampered an indepth analysis of the population structure and evolutionary history of the parasite. SNP's have received considerable attention recently because of their potential as markers for genetic mapping for studying molecular evolution and population dynamics. SNP's are relatively easy to assay and are often present at high frequency, making them ideal genetic markers. Isolate for different geographical parts of India i.e. Delhi, Chennai, Navi Mumbai, Goa, Car Nicobar, Bihar and Rourkela are being used to study SNPs in five house keeping genes namely, ribosomal protein L35e gene, serine/threonine protein kinase gene, adenylate cyclase protein gene, acyl carrier protein gene and exonuclease domain protein gene. Blood spots collected on sterile Whatmann filter paper were used for PCR. Sequencing was carried out using Big Dye terminator chemistry on ABI 3100 automatic DNA sequencer. L35e ribosomal protein revealed SNP in about 450bp fragment. Size variation was noticed in Acyl Carrier protein (ACP). All SNP's were validated by sequencing independent PCR products.

7. Molecular Biology of Malaria Parasites & Vectors

7.08 An efficient detection of chloroquine resistance marker in pfCRT gene of *Plasmodium falciparum* from complicated malaria samples by PCR-SSCP analysis

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Malaria has plagued humans throughout recorded history and results in the deaths of approximately 1.5 - 2.7 million people per year, which is still worsened by the spread of chloroquine resistance throughout the world posing a major problem to combat this disease. Despite earlier successes in reducing human malaria, the disease is now rampant in many tropical and sub-tropical countries taking a heavy toll of human life. Among the several drugs of choice, chloroquine is the most chosen one because it is less toxic. In the present study, an efficient Polymerase Chain Reaction - Single Strand Conformational Polymorphism (PCR-SSCP) based assay has been demonstrated to detect pfCRT K76T point mutation, which is a marker for chloroquine resistance. For the first time, we have used PCR-SSCP based technique here to identify the mutation in a single step labeling reaction during PCR and SSCP gel electrophoresis. This assay is 100% efficient giving no false positive or negative results and can be carried out within short bench time. We have successfully analyzed 120 blood samples with multi organ failure collected from SCB Medical College using PCR-SSCP method for the detection of chloroquine resistance marker and found 91 out of the 120 samples showing pfCRT T76 mutation while 72% (65 out of 91 samples) showing correlation of chloroquine resistance from the clinical data's of the patients. The advantage of our method lies in the fact that a single step PCR reaction is enough for the detection of K76T mutation. The SSCP method requires less consumable and the results are highly reproducible. The PCR-SSCP analysis is a very sensitive technique in terms of detection of K76T mutation in pfCRT gene. This PCR-SSCP technique can be applied for surveillance of chloroquine resistance malaria in malaria endemic localities around the world and administration of appropriate drug can be predicted. 76% out of 120 samples showed K76T mutation, which denotes a higher percentage of drug resistance occurs in complicated malaria.

7. Molecular Biology of Malaria Parasites & Vectors

7.09 Molecular study of *Anopheles fluviatilis* species complex in different geographical regions of Orissa

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Orissa is an endemic zone for malaria with a very high mortality rate in India. *An. fluviatilis* is a major vector in the foothill regions of Orissa. The state of Orissa is divided into four distinct geo-physiographical regions i.e. eastern ghat, coastal track, central table land & northern plateau. Mosquitoes were collected from Angul district (central table land), Keonjhar district (northern plateau), Kalahandi & Phulbani (eastern ghat). The species were identified using the available identification key. *An. fluviatilis* collected were processed both for chromosomal and molecular identification of sibling species. In the molecular analysis, the D3 region of the ribosomal DNA were analyzed using specific primers developed by MRC. The result of the molecular study were compared with that of chromosomal analysis of sibling species determination. In the Angul district, the individuals were 90% of S type & 10% of T type. In the Keonjhar district 95% of S type & 5% of T type. In the Kalahandi district 76% of S type & 20% of T type & 4% U type. In the Phulbani district, the individuals were found to be 85% of S type & 15% of T type. This study concludes the presence of three types of sibling species of *An. fluviatilis* in Orissa.

7. Molecular Biology of Malaria Parasites & Vectors

7.10 The *Pf*CRT (K76T) point mutation favours clone multiplicity and disease severity in *Plasmodium falciparum* infection

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Malaria has been a common disease and it continues to be one of the most widely spread health hazards. It is the major parasitic infection in many tropical and subtropical regions leading to more than one millions deaths out of 400 millions cases every year. More than half of the world's population lives in the areas where they remain at the risk of malarial infection. Moreover, in recent years the situation has worsened in many ways mainly due to malaria parasite becoming resistant to several antimalarial drugs. This resistance concerns numerous drugs, but is thought to be most serious with chloroquine, the cheapest and most widely used drug to treat malaria. The ethnopharmacological approach for the search of new antimalarial compounds from the plants sources has proved to be more predictive. Hence several research groups are now working to develop new antimalarial compounds as an alternative to chloroquine and artemisinin (a plant based antimalarial drug isolated from Chinese plant *Artemisia annua*). A new antimalarial compound namely (1) was isolated from the leaves of herbs collected from north eastern region of Himalayas, together with compound (2). The structure of (1) was determined by chromatographic and spectroscopic method. Absolute conformation of (1) was finally determined by single crystal XRD. Compound (1) and (2) have been found to be potent against *P.falciparum* *in vitro* IC₅₀ 38.6µg/ml and 3.8µg/ml. In order to increase the potency, ten novel derivatives of compound (1) have also been synthesized and evaluated *in vitro*. All the compounds displayed antimalarial properties *in vitro* against chloroquine sensitive and chloroquine resistant strains of *P.falciparum*. MN-5 displayed the highest potency with IC₅₀ 1.72 µg/ml, which is well within the acceptable range of WHO (1-5 µg/ml).

7. Molecular Biology of Malaria Parasites & Vectors

7.11 Characterization of plastid DNA Elongation factor *Tuf A* from *Plasmodium vivax*

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Apicomplexan parasites such as *Plasmodium* and *Toxoplasma* are characterized by a three - four membrane organelle called Apicoplast. The organelle is considered as the putative drug target and is the site for Type II fatty acid synthesis pathway and non - mevalonate pathway for isoprenoid biosynthesis. It also contains a 35 kb circular DNA (plastid DNA) having its own origin of replication and found in copies of 3-8. The complete 35kb genome has been reported from *Plasmodium falciparum*. The genome contains many conserved regions and ORFs. These show high conservation with the chloroplast genome and appear to have left out the photosynthetic genes. The major regions present in this 35kb plastid DNA include large and small subunit rRNA genes, *suf B* (ORF470) gene, *Clp C* (caseinolytic protease) gene, *Tuf A* (elongation factor Tu) gene and various other ribosomal protein genes. Besides this, 7 ORFs of varying sizes and unknown functions are also present in the genome. Our study aims at the characterization of the *Tuf A* region from this genome of *Plasmodium vivax* from India. Primers were designed from the *P. falciparum* plastid DNA sequence obtained from NCBI database. After repeated trials, the *Tuf A* gene was amplified from the *P. vivax* field samples. The amplified fragments were cloned in pRSET A vector and sequenced. A comparative analysis of *Tuf A* gene from *P. vivax* and *P. falciparum* sequence was done. Structural analysis was carried out based upon the crystallographic structure of *E.coli* and hypothetical protein structure of *P. falciparum*. The differences between the *Tuf A* structure of *P. vivax* and *P. falciparum* have been elucidated with the emphasis on the active sites of the molecule. Phylogenetic analysis using this *Tuf A* sequence suggests a major divergence of the Indian *P. vivax* from the reported *P. vivax* cluster using Clustal W and Phylip tools.

7. Molecular Biology of Malaria Parasites & Vectors

7.12 Expression of T-helper cell epitopic regions of circumsporozoite protein of *Plasmodium falciparum* in *Escherichia coli*

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Importance of T-cells in malaria immunity has been appreciated for long. Two T-helper cell epitopes has been located at the C-terminal end flanking the highly conserved region RII of CSP. Th-epitopes shows variation. Even then if the variations are restricted and can be grouped, then the prototype variants from the group could be included into subunit polyvalent vaccine against sporozoites. We have studied the genetic variation in Th-epitopes of 148 *P. falciparum* isolates collected from different epidemic and endemic regions of India. Variations have been found to be regionally unbiased in the sense that similar type of variation have been found in different regions. The allelic variants could be categorized into 4 groups. Since the number of variants are small, then the prototype variants could be included into a subunit polyvalent vaccine against sporozoites.

We have cloned the prototype variants into Pqe-40 expression vector and expressed it as a fusion protein fused to DHFR (Dihydrofolate reductase) gene tagged to 6 HIS residue and expressed the T-cell epitopes as a fusion protein. We have purified the fusion protein and the study of the immunogenicity of fusion protein is in progress.

7. Molecular Biology of Malaria Parasites & Vectors

7.13 Molecular cloning and functional characterization of a putative choline kinase from the human malaria parasite *Plasmodium falciparum*

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Generation of phosphocholine by choline kinase is important for phosphatidylcholine biosynthesis via Kennedy pathway and phosphatidylcholine biosynthesis is essential for intra-erythrocytic growth of malaria parasite. No choline kinase has been purified and characterized from *Plasmodium falciparum* till to date. A putative gene (Gene ID PF14_0020) in chromosome 14, having highest sequence homology with choline kinase has been identified by BLAST searches from *P. falciparum* genome sequence database. This gene has been PCR amplified, cloned and over-expressed. Recombinant protein (PfCK) catalyzes the formation of phosphocholine by phosphorylating choline in presence of ATP. RT-PCR studies indicate that PfCK is expressed stage specifically in trophozoite-schizont stage and it is a cytosolic protein as revealed by westernimmunoblotting. A model structure of PfCK was constructed based on the crystal structure of choline kinase of *C. elegans* to identify inhibitors based on affinity to PfCK by molecular docking. H-89, an isoquinolinesulfonamide derivative showed highest affinity to PfCK as compared to others in this series of compounds. To validate molecular docking experimentally, effect of H-89 on the activity of PfCK as well as parasite growth was studied. H-89 inhibited both purified PfCK as well as choline kinase activity in *P. falciparum*. H-89 also inhibited membrane phosphatidylcholine biosynthesis and parasite growth in culture. Thus, PfCK may be a potential target for antimalarial drug development.

7. Molecular Biology of Malaria Parasites & Vectors

7.14 Evolutionary puzzle of *Plasmodium falciparum*: New hopes with advances in genomics and bioinformatics

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Inference on the evolutionary history (the origin, recent time of common ancestry, migration pattern, past demographic events and the historical role of natural selection) of a species primarily depends on the distribution of genetic variation among the populations across its whole distribution. Understanding genetic variations in the malarial parasite, *Plasmodium falciparum* populations is of major importance to fight against this devastating disease, especially as we contemplate widespread programmes of vaccination. This is because if a vaccine controls only a part of the parasite population, then it might alter the genetic composition of the rest of the surviving populations and 'select' for variants that are even more virulent. Interestingly however, strong disagreement persists regarding the level of genetic variability of existing *P. falciparum* populations across the globe, and by inference, their evolutionary history. For example, based on the available molecular genetic data, some have argued a recent time of common ancestry to around 3,000 - 10,000 years ago and others to about 50,000 years ago or more. Such wide variations of these estimates are often attributed to the types of genes examined and/or the methods of data analyses. Likewise, the origin, migration pattern, historical events of demographic changes and the role of natural selection have been very poorly understood in this species. We have initiated a study that follows combined genomic and bioinformatic approaches to determine the distribution of genetic variations (both at functionally neutral genes and at genes conferring resistance to anti-malarial drugs) in Indian *P. falciparum* and to construct a database on genome-wide genetic variation. This would help in finding answers not only to above-mentioned evolutionary questions in Indian context, but also to design population-specific anti-malarial drugs/vaccines.

7. Molecular Biology of Malaria Parasites & Vectors

7.15 Genetic polymorphism among human *Plasmodia* species in India

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Malaria is one of the most serious public health problems in India and is prevalent in whole of the country except in areas 1800 meters above sea level. Two most prevalent human *Plasmodium* species prevalent in India are *P. falciparum* and *P. vivax*. Malaria control is becoming difficult due to development of resistance by parasites against commonly used antimalarials. Therefore, it is important to understand the genetic diversity present among the field isolates of the species to plan appropriate control programmes and to develop and test novel control strategies. In this paper, we will present our observations in the genetic diversity existing among field isolates of India in respect of surface proteins genes, neutral markers e.g. micro satellites and house keeping genes as well as drug resistant related markers. Indian isolates have exhibited high degree of polymorphism in respect of above markers and a complex nature with co-existence of more than one genetically different parasite types in an isolates.

7. Molecular Biology of Malaria Parasites & Vectors

7.16 Study of the size and sequence specific polymorphism of Rhoptry Associated Membrane Antigen

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The spread of drug resistance in parasite and insecticide resistance in mosquito vector has hampered the malaria control programme and thus gave way to a new avenue of thought i.e. vaccine for malaria. Rhoptry-associated proteins are among several proteins that are investigated as candidate vaccine.

Rhoptries are intracellular organelles present in the merozoite stage of the *Plasmodium sp.* and necessary for invasion into the erythrocyte. The contents of the rhoptries are transiently accessible to antibodies but seroepidemiological studies have demonstrated the development of antibody responses to rhoptry proteins RhopH3, RAP1 and RAP2 following infections.

RAMA (Rhoptry associated membrane antigen) has been characterized recently. It is a 170kD protein which is proteolytically processed into a 60kD protein in the rhoptries. The C-terminal end of the protein is believed to bind to the RBC membrane. Confocal microscopy shows the release of 60kD RAMA protein from the rhoptries and its presence in the parasitophorous vacuole. RBC binding assays also shows that RAMA is released from the rhoptries and it binds to the RBC membrane components. The receptor of RAMA protein on the erythrocyte membrane is not known as yet. RAMA protein has been expressed in fragments A, B, C, D, E. The antigenic responses to RAMA protein has been tested by Elisa in the Vietnamese population. Atleast two distinct epitopes in the p60 region and additional epitopes are predicted to be present in the full length protein.

Our study will stress on looking at the polymorphism of RAMA gene in the Eastern Indian population to know the conserved and variable region. Blood spots have been collected from *P. falciparum* positive patients diagnosed positive by microscopy. DNA has been isolated from blood spots using Quigen DNA mini kits. Specific primers have been designed based on reported sequences and PCR protocols were standardized. Results of PCR, SSCP and sequencing will be discussed.

7. Molecular Biology of Malaria Parasites & Vectors

7.17 DNA-protein interactions at replication *ori* of the *P.falciparum* apicoplast genome

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Apicomplexan parasites contain a plastid-like organelle, the apicoplast, that is the site for several biochemical pathways within the parasite. The *P. falciparum* apicoplast contains a 35 kb circular DNA (pIDNA) that replicates at the late trophozoite/early schizont stage of the erythrocytic cycle. Critically, inhibition of pIDNA replication causes parasite death.

Previous studies from our laboratory have shown that multiple origins of replication of *P. falciparum* pIDNA are located within the inverted repeat region (IR) and are activated differentially during pIDNA replication. In order to investigate the nature of DNA-protein interactions at *ori* sites, we carried out EMSAs using the identified *ori* regions as probes. Two specific shifts were obtained with the 194 bp DNA fragment that is located within the replication initiation zone. These shifts were specific to the organelle-enriched protein fraction and were not seen when parasite nuclear protein extract was used for binding. Initial characterization of the complexes revealed that the upper DNA-protein complex was more stable at higher binding temperatures. Both the upper and lower DNA-protein complexes did not require divalent cations for interaction and ionic interactions seemed to play a very minor role. Specific inhibition of binding indicated that the protein(s) approached the DNA through the minor groove of the DNA double helix. Southwestern analysis revealed that molecular weight of these proteins is between ~66 kDa and ~75 kDa. Moreover, the proteins interact at the 3'-end of the 194 bp DNA fragment as revealed by DNaseI footprinting analysis. The identity of these proteins is currently being investigated.

7. Molecular Biology of Malaria Parasites & Vectors

7.18 Correlation analysis of *Pf*CRT K76T mutation and *in vivo* response to chloroquine

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The problem of drug resistance is a key obstacle in tackling the malaria problem. In India, resistance to chloroquine was reported for the first time in 1973 from Karbi Anglong in Assam and subsequently spread to other parts of the country. Increased *Plasmodium falciparum* chloroquine resistance in country emphasized the regular monitoring in the field for effective malaria control strategies. Methods available for monitoring of CQR in the field are *in vitro* and *in vivo* test. However, both the tests are lengthy and need skilled laboratory persons for microscopy and we always requires a simple, rapid and effective system to monitor CQR in the field. The *Pf*CRT (*Plasmodium falciparum* chloroquine resistance transporter) gene, which encodes a transmembrane protein located in the *P.falciparum* digestion vacuole, was recently characterised. A correlation was found between *in vitro* response to chloroquine and sequence polymorphism at the *Pf*CRT locus. *In vitro* chloroquine resistance was associated with the substitution of lysine for threonine at position 76 in the field and laboratory strains and it has been proposed as a molecular marker for the faster detection of chloroquine resistant *falciparum* malaria in field. Here, we have investigated the evaluation of this marker in Indian *P.falciparum* field isolates, which were clinically CQR or CQS and collected during therapeutic efficacy test of chloroquine in *P.falciparum* infected patients. Isolates were from three different area, namely Orissa, highly malarious area with malaria related deaths, Tamilnadu (Rameshwaram), area with malaria endemicity, but isolates were collected during an epidemic outbreak of *P. falciparum* and Rajasthan (Udaipur), area with malaria epidemic. Therapeutic efficacy revealed about 56% resistance among the isolates of Orissa, 59% resistance in Rameshwaram isolates and 96% susceptibility among Udaipur isolates. However, PCR RFLP analysis for *Pf*CRT K76T mutation revealed 100% (46 of 46) chloroquine resistance among Orissa and Rameshwaram isolates and 95%(20 of 21) chloroquine resistance among Udaipur isolates. Results obtained *in vivo* and PCR-RFLP will be discussed.

7. Molecular Biology of Malaria Parasites & Vectors

7.19 PCR based detection of *Plasmodium falciparum* sporozoites in *Anopheles annularis*

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Keonjhar district of Orissa is highly endemic for malaria and show continued transmission of malaria (SPR 17.6% and Pf 96.1%). Ghatgaon PHC of the above district reported more malaria deaths during last five years. Impregnated bed nets were distributed in Ghatgaon PHC by the state Government to interrupt transmission during January 2000. After two years evaluation were made to see the impact of bednet on transmission and an attempt was made to reassess the vectorial capacity of Anopheline mosquitoes by detecting the sporozoite using PCR technique. Indoor resting mosquitoes were collected from human dwellings of endemic villages between 6AM to 9AM using sucking tube and mechanical aspirator during November, 2002 to January, 2003. They were brought to the laboratory and identified with the help of keys of Christopher's, 1933 and Puri, 1937. Some of the females were then dissected in normal saline and examined for oocyst and Sporozoite infections in gut and salivary gland. Rest of the mosquitoes were brought to the laboratory and kept in individual vials for processing them by PCR technique for detection of Sporozoite. Dissection and microscopic examination of the salivary glands of *An. annularis*(132), *An. culicifacies*(14), *An. subpictus*(45), *An. vagus*(52) and *An. hyrcanus*(10) revealed no sporozoite. Rest of the mosquitoes from all species were processed by PCR, among which only seven *An. annularis* out of 204 were found positive for sporozoite, thus giving a sporozoite infectivity rate of 3.4%.

7. Molecular Biology of Malaria Parasites & Vectors

7.20 Functional characterization of origin recognition complex (ORC) subunits 1, 2 and 5 in *Plasmodium falciparum*

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In eukaryotes, the origin recognition complex (ORC) is essential for the initiation of DNA replication. In all cases studied, ORC binding to the replicator is the first step in the establishment of pre-replicative complex (pre-RC) that acts as a "landing pad" to localize other factors to the site of initiation. ORC, a six-protein complex contains ORC1 that has a regulatory role in origin activation while other ORC subunits play a crucial role in complex formation and stability. Here we report the cloning and functional characterization of *Plasmodium falciparum* ORC1, 2 and 5 homologs. All these genes contain unique amino terminal extensions and conserved carboxy terminal regions. By RT-PCR we show the expression of all three genes in *Plasmodium falciparum*. Using immunofluorescence and immunoelectron microscopy, we further show that these ORC subunits are expressed in the nucleus during the late trophozoite and schizont stages where maximum amount of DNA replication takes place. Homology modelling of the carboxy terminal region of PfORC1 (781 to 1033) using *Saccharomyces pombe* Cdc6/Cdc18 homologue as a template reveals the presence of a similar AAA+ type nucleotide-binding fold. This region shows ATPase activity *in vitro* that is important for the origin activity. To our knowledge, this is the first evidence of an individual ORC subunit that shows ATPase activity. These observations strongly suggest that the PfORCs might be involved in DNA replication initiation during the blood stages of the parasitic life cycle.

7. Molecular Biology of Malaria Parasites & Vectors

7.21 AFLP Polymorphism of Vaccine Candidate Var Genes in *Plasmodium falciparum* Malaria Parasites

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Var genes of *Plasmodium falciparum* encode the variant surface antigen PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein) which has an important role in pathogenesis (cytoadherence) and immune evasion. Var genes are members of a highly polymorphic multi-copy gene family. Within the 3D7 genome there are 59 var genes. DBLa (Duffy binding like domain) which is presented by the malaria parasite on the surface of infected RBCs has a conserved flanking region and a variable middle region. To understand the nature of genetic diversity with respect to var gene repertoire of these parasites, we have examined the DBLa domain in the genomic DNA in the isolates of India, Thailand and in the 3D7 parasites. AFLP-amplified fragment length polymorphism analysis of the various strains of *Plasmodium falciparum* reveals a polymorphism with respect to the sizes and intensity of amplified fragments. The pattern obtained with isolates from India and Thailand differed significantly from that obtained with 3D7. The genomic DNA of all these parasite lines when analyzed for RESA, MSP-1 and MSP-2 genes showed distinct pattern confirming the purity of parasites used. Thus, our study clearly shows variations in the var gene pattern in the isolates belonging to different geographical regions. The extreme polymorphism and heterogeneity in the var genes as observed in the present analysis with respect to geographical location emphasizes the complexity of the problem. The understanding of the mechanisms underlying the diversity in the repertoire of var genes will give insight into pathophysiology of the disease and help in designing vaccine candidate for malaria.

7. Molecular Biology of Malaria Parasites & Vectors

7.22 Polymorphism of the *Plasmodium falciparum* chloroquine resistance transporter genes and *in vivo* susceptibility to chloroquine resistance in a highly endemic area of Sundargarh district, Orissa, India

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A mutation in the *Plasmodium falciparum* chloroquine resistance transporter (Pfcrt) gene was examined to assess the associations with *in vivo* chloroquine resistance in an endemic region of Sundargarh district, Orissa, India. Out of the 70 cases which could be followed up completely, 23 cases were found to be early treatment failure, 9 cases as late treatment failure & 38 cases had adequate clinical response. 31% patients has Pfcrt T76, 56% had both Pfcrt T76 & Pfcrt K76, 12% patient showed only Pfcrt K76. Though a strong correlation between the Pfcrt K76T mutation & *in vitro* CQ resistance has been seen earlier, in endemic area, other factors like host resistance may play a crucial role to predict *in vivo* sensitivity to CQ.

7. Molecular Biology of Malaria Parasites & Vectors

7.23 Impact of S/P (Sulfadoxine-Pyrimethamine) over *Plasmodium vivax* populations in Indian subcontinent

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The world wide spread of chloroquine (CQ) resistant strains of *Plasmodium falciparum* has led to the use of S/P (Sulfadoxine/ Pyrimethamine) as first line antimalarial drug in South East Asian countries such as Thailand, Indonesia and Papua New Guinea. Resistant to S/P in *P. falciparum* has developed in short time and reports on high level of S/P resistant in *P. falciparum* have been reported from different regions. Analysis of associated point mutations in *Pfdhfr/dhps* genes in Thailand and Indonesia has shown prevalence of triple/quadruple mutations. Though S/P is not drug of choice for vivax malaria but in areas with co-infection of *P. falciparum* and *P. vivax*, S/P use for chloroquine resistant has shown an impact on *P. vivax* also as indicated by mutations in *Pvdhfr/dhps* gene. High level of S/P resistance as well as prevalence of triple and quadruple mutations have already been reported in *P. vivax* field isolates from S.E. Asia and Oriental region. India, is another region with prevalence of both the species of *Plasmodia*, *P. falciparum* as well as *P. vivax* in equal proportions. Therefore, with an aim to understand the status of *Pvdhfr* gene among Indian field isolates, a study on the monitoring of *Pvdhfr* gene from different region was undertaken. A differential picture in relation to *Pvdhfr* was observed in northern region in comparison to coastal regions of central and southern states. Results will be discussed.

7. Molecular Biology of Malaria Parasites & Vectors

7.24 Biochemical and Molecular Characterization of Nitric Oxide Synthase in *Anopheles culicifacies*: Relevance for refractory mechanism

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The drive to identify novel control strategies has, in part, focussed on identifying mosquito gene products that impart refractory phenotypes. Our goal is to develop tools for altering the vector competence of *Anopheles culicifacies* which requires the understanding of the mechanism of vectorial resistance to the malaria parasite including biochemical and molecular studies of vector parasite interactions. We have planned to use AcNOS (*Anopheles culicifacies* Nitric oxide synthase) responses in mosquito vectors to Plasmodium as a tool to explore critical components of parasite development in mosquitoes and correlate it to mechanism of refractoriness.

Haemolymph nitrate/nitrite ($\text{NO}_2^-/\text{NO}_3^-$) concentrations were measured using a modified cadmium reduction/ Griess reagent microassay at 7, 9-10 and 14-15 days pBM. Elevated levels of $\text{NO}_2^-/\text{NO}_3^-$ at this time may be the result of sustained production of AsNOS induction at 9 days. Specific activities of AsNOS in lysates of non blood fed, blood fed uninfected or *P. falciparum* infected mosquitoes at 6 and 9 days pBM. Activity was measured with or without NOS inhibitor L-NAME (1mM). Difference in AsNOS specific activities was much higher in infected and uninfected mosquitoes at day 9 than at day 6 days in control reactions. The effect of L-NAME on AsNOS activity in infected mosquitoes was relatively unchanged by L-NAME at 6 days, whereas activity in infected mosquitoes was significantly inhibited by the inhibitor three days later at 9 days. Four Primers were designed complementary to the *An. stephensi* exonic regions 1,7,15 and 17 encoding for the co-factors heme, FAD PPI, FAD NADPH respectively. All the four primers have shown the amplification of the NOS gene by PCR. Amplification of 200 base pairs against Exon 1 and 100 base pairs against exon 17 were observed in *An culicifacies*. Exon 7 and 15 have also shown the amplification of 163 base pairs. This understanding is critical to evaluating the potential for manipulating *Anopheles* NO synthase (NOS) gene expression to generate a refractory phenotype and this work will also reveal novel aspects of *Plasmodium* physiology for transmission blocking strategies.

7. Molecular Biology of Malaria Parasites & Vectors

7.25 Characterization of Dihydrofolate Reductase gene of *Plasmodium vivax* severe malaria cases

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Plasmodium vivax infects an estimated 80 million people each year, causing severe and debilitating febrile illness. The risk of infection by this parasite is increasing in areas of endemicity, and the geographic range of risk has expanded rapidly during the past 10 years. Drug resistance in *Plasmodium* species has become a major obstacle in management and control of malaria. Molecular probes that reliably predict susceptibility of *P.vivax* to various drugs like sulfadoxine or pyrimethamine provide information supporting rational policy decisions and practice with regard to malaria treatment. Dihydrofolate reductase (DHFR) is used by the parasite for its de novo DNA synthesis. It is encoded by a bifunctional gene (*dhfr-ts*), which also encodes Thymidylate synthetase (TS). The drug Pyrimethamine acting as analogue of p-aminobenzoic acid (PABA), a folate precursor, binds to the active sites of the enzyme and inhibits its actions. Specific point mutations in *P. vivax* DHFR due to single nucleotide polymorphisms in the encoding gene have been associated with *in vitro* resistance to these drugs. These mutations are thought to be producing changes in the active sites of the enzyme where the drug binds but is unable to perform its action. Two major mutations that have been marked out are S117N, S58R. In our quest to identify and understand the use of DHFR as a molecular probe, we have analysed DHFR gene sequences from some cases of severe malaria including cerebral malaria caused by *P. vivax* in Bikaner, India. We have come across variations in the sequences that represent both drug resistant and drug sensitive parasite strains. Some of these variations are unique to these strains and have not been reported in *P. vivax* DHFR from other countries. Homology modeling of the corresponding protein was also carried out to identify whether these variations are producing any effect on the structure and the active sites of the protein.

7. Molecular Biology of Malaria Parasites & Vectors

7.26 A novel signalling pathway in *Plasmodium falciparum*

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Sequencing of the *Plasmodium* genome revealed the existence of several genes that may have a role in cell signalling in this parasite. It has close to 65 protein kinases, which are major players in eukaryotic signalling. However, there is little information about how these enzymes regulate signalling in the parasite. We have identified a novel signalling pathway in the malaria parasite, which involves PfPKB, a protein kinase B (PKB) like enzyme. Although PfPKB shares high sequence homology with the catalytic domain of mammalian PKB, it does not contain a pleckstrin homology (PH) domain¹. PKB interacts with 3'phosphorylated-phosphoinositides via this domain. We have evidence which suggests that PfPKB may be regulated in a phosphoinositide-independent manner. Our results suggest that PfPKB is regulated by calcium/calmodulin *in vitro* and in the parasite. Intracellular levels of calcium needed for this pathway to function are controlled by Phospholipase C, which acts as an upstream regulator of this pathway. Molecular mechanism of PfPKB regulation by this pathway will be discussed in detail. This is one of the first multi-component pathways to have been characterized in the malaria parasite. In addition, we have evidence which suggests that signalling via this cascade may be important for late stages of malarial infection.

7. Molecular Biology of Malaria Parasites & Vectors

7.27 Molecular markers associated with chloroquine resistance in *Plasmodium falciparum* isolates from India

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The development of resistance against Chloroquine in *Plasmodium falciparum* is complicating effective treatment and control of malaria. Mutational changes in three genes, *pfmdr1*, *pfcr1* and *cg2* has been attributed to chloroquine resistance. However alleles of a single gene could not be accounted for CQ-resistance. An association of alleles of these genes is believed to be responsible for CQ-resistance. We have studied thirty CQ-resistance and thirty CQ-sensitive *Plasmodium falciparum* isolates from different geographical regions of India to determine the association of CQ-resistant molecular markers. Genomic DNA was prepared from 30 CQ-resistance and 30 CQ-sensitive isolates. The portion of the gene, harbouring mutational site were PCR amplified using primers flanking the mutational sites and sequenced after purification.

Since it is not possible to sequence all samples we also opted for restriction enzyme digestion. It has been found that some of the resistant strains contain the *pfmdr1* mutation N86Y, while others not. Same is the situation with *pfcr1* K76T. All the *pfcr1II* amplified products could not be digested with Apol, so *pfcr1II* is not solely responsible for CQ-resistance. Thus it appears that mutation in any single gene is not responsible for CQ-resistance, but together they confer CQ-resistance. However the role of *cg2* gene is ambiguous. Therefore it can be concluded that, association of mutation in *pfmdr1* and *pfcr1* are responsible for CQ-resistance.

7. Molecular Biology of Malaria Parasites & Vectors

7.28 Studies on the genotype of *Plasmodium falciparum* isolates from India using anchored primer amplification of DNA, revealed much simpler genotype compared to isolates from other regions of the world

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The study of Genome organisation and Genotyping of *Plasmodium falciparum* is important for therapeutic efficacy and other purposes. Several methods have been employed to study the genotypes of malaria parasite especially for *P. falciparum*, but they have their limitations. A new method, Anchored Primer Amplification of DNA was developed by Su et al. In this APAD method anchored poly A and poly T oligonucleotide primers were used. Using these oligonucleotide primers anchored at the 3'end, the genetic differences in the AT rich DNA sequences can be displayed. So far we have used single nucleotide anchored primers to pentanucleotide anchored primer. Genomic DNA of sixty *P.falciparum* isolates was prepared from blood samples collected from different geographical regions of India, both endemic and epidemic areas. The genomic DNA was PCR amplified using single anchored poly A or poly T primers. With different primers, different genotypes were obtained. With dinucleotide anchored primer in most of the cases, smear was obtained, even at increased concentrations of MgCl₂. Whereas with tri, tetra and penta nucleotide anchored primers, though different pattern of bands were observed but they were less complex compared to the bands seen in the study by Su et al. Thus it can be concluded that the genotype of Indian *P.falciparum* is much simpler than the isolates from other geographical regions of the world.