2

Parasite Biology

2.1 Human Malaria Parasites: Molecular Characterization

2.1.1 Genetic Structure of *Plasmodium vivax*

Plasmodium vivax genome sequence revealed abundant mini and microsatellites. Identification of polymorphic mini and microsatellite markers is crucial for studying the pattern of genetic diversity, population genetic structure and linkage disequilibrium of population for genotype-phenotype association mapping. To understand the population genetic structure of *P. vivax* among Indian sub continent, we used polymorphic mini and microsatellites.

Minisatellites for *P. vivax*

In this pioneer study, Indian *P. vivax* populations have been characterized using ten minisatellites which were identified in the laboratory. Single clone *P. vivax* isolates from five geographically separated regions, namely Delhi, Nadiad, Panna, Chennai and Sonapur were analyzed using multi-locus minisatellite markers. Amplified PCR fragments were run on high resolution metaphor agarose for allele sizing (Fig. 2.1.1). Extensive genetic polymorphism was observed among the five *P. vivax* populations (Fig. 2.1.2). Number of alleles observed per locus was 11–22 alleles, except one locus that showed six alleles. Analysis revealed average heterozygosity per locus was similar or slightly higher (*He* = 0.815–0.915) than the average heterozygosity revealed by the microsatellite marker. Magnitude of the genetic diversity and the diversity pattern was similar among the five geographical regions of India. Phylogenetic tree based on Nie genetic distance revealed that isolates from different populations cluster together suggesting *P. vivax* isolates circulating in five widely separated geographical regions possess same genetic structure (Fig. 2.1.3). High degree of allele sharing observed among the five geographical regions indicates high de-

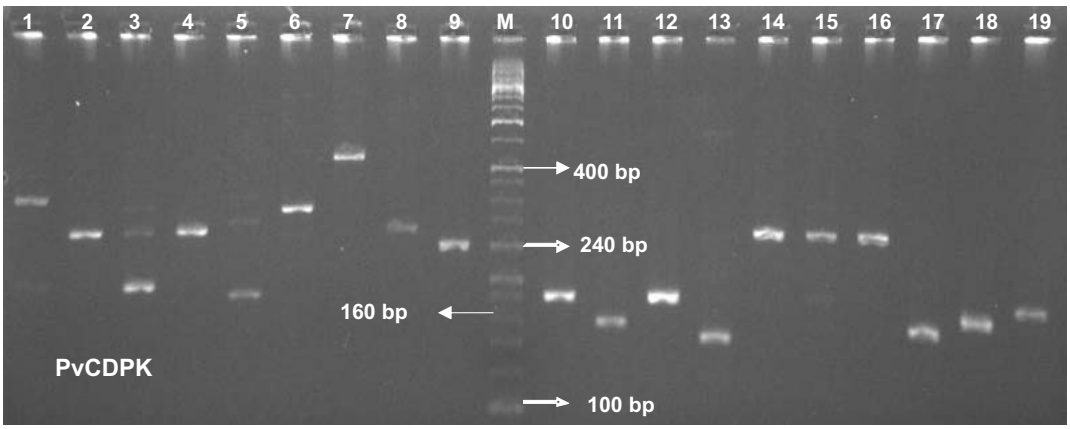

Fig. 2.1.1: Gel images of minisatellite variations in Indian *P. vivax* isolates. Amplicons were visualised in 3% high resolving Metophore agarose gel for allele sizing. M represents 20 bp DNA ladder

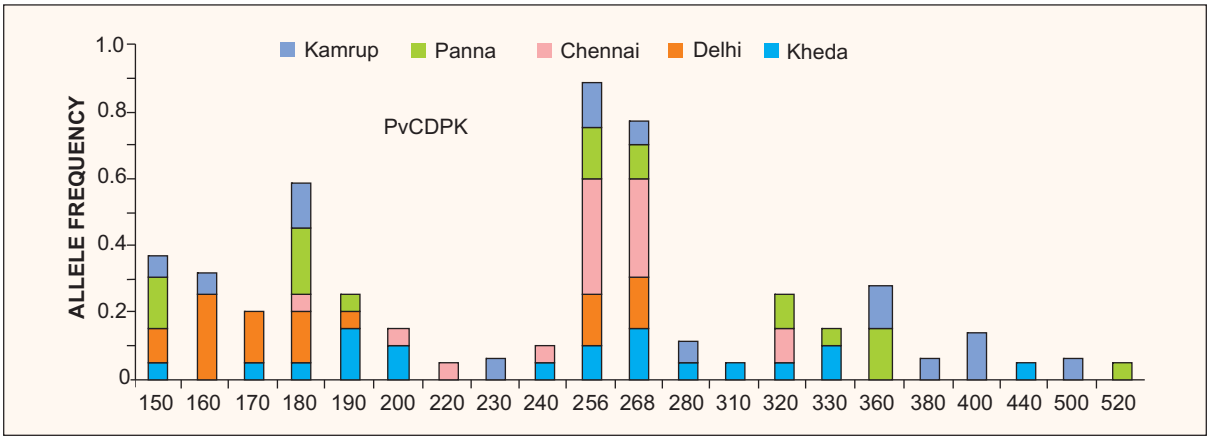

Fig. 2.1.2: Extent of genetic diversity and allele sharing in *P. vivax* isolates from five different populations at minisatellite locus

gree of gene flow among the widely separated geographical regions of India. The study suggests that minisatellites have potential resolving power for genetic diversity.

Microsatellite Markers

Microsatellite markers were identified by scanning *P. vivax* genome sequence. About 2–3 nucleotide repeat sequences with at least 12–15 copy number (repeat number) were selected for the study. Eight microsatellites out of ten studied were observed to be highly polymorphic. Number of alleles observed per locus was 6–13 and heterozygosity among the study populations varied between 0.65 and 0.90. High degree of allele sharing observed

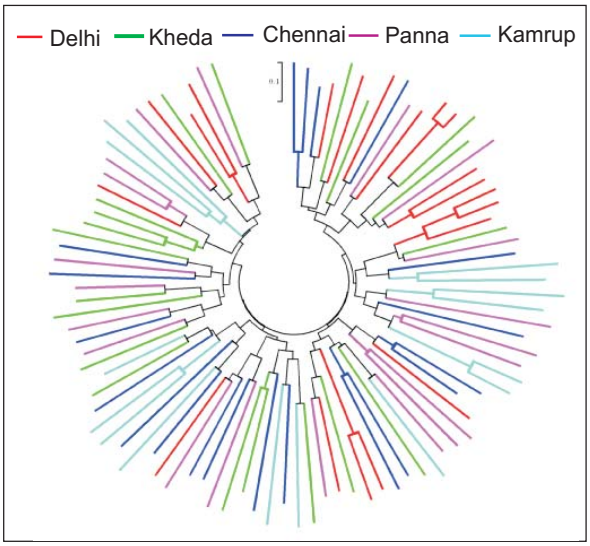

Fig. 2.1.3: Neighbour-Joining phylogenetic tree showing genetic relationship among Indian *P. vivax* isolates using ten minisatellites

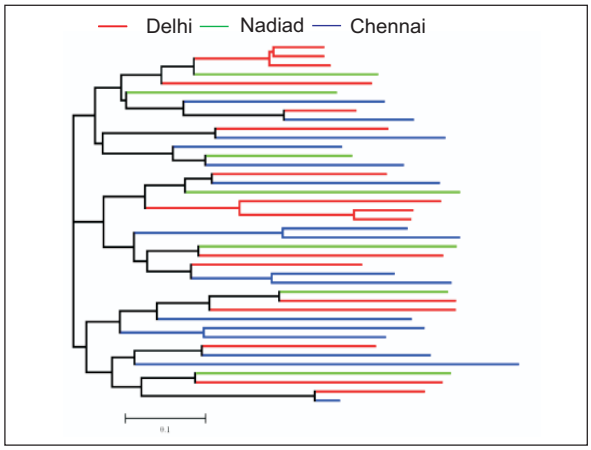

Fig. 2.1.4: Neighbour-Joining phylogenetic tree derived from genetic distance scored at eight microsatellites showing genetic relationship among Indian *P. vivax* isolates. Line colours in the phylogenetic tree represent geographical origins of isolates. Blue, red, and green coloured lines represent Chennai, Delhi and Nadiad population isolates respectively

among the isolates from three geographical regions namely, Delhi, Nadiad and Chennai indicates high degree of gene flow among the widely separated geographical regions of India. Phylogenetic analysis reflected that *P. vivax* isolates circulating in different geographical regions cluster together, suggestive of similar genetic structure among different geographical regions of India (Fig. 2.1.4).

2.1.2 Antigenic Repertoires of Vaccine Candidates

Antigenic diversity in the natural parasite populations is the major obstacle in the development and success of effective antimalarial control measures. Antigenic repertoires of

human malaria parasites have been widely studied. However, day-by-day new antigenic variants are being reported from different parts of the globe.

Antigenic repertoires of *P. vivax* vaccine candidates were investigated in five widely separated geographical regions (Delhi, Panna, Kheda, Chennai and Kamrup) of India to understand the local antigenic repertoires. Sequence analysis of five vaccine candidates from asexual [*Circumsporozoite Protein (CSP), Apical Membrane Antigen-1 (AMA-1)* and *Duffy Binding Protein-II (DBP-II)*] and sexual stages (*Pv25S* and *Pv28S*) revealed differential levels of antigenic repertoires. The asexual stage revealed high antigenic repertoires in comparison to the sexual stage. Extensive non-synonymous and synonymous nucleotide substitutions were found and the overall number of non-synonymous substitutions exceed over the synonymous substitutions suggesting signature of diversifying selection at vaccine candidates. Extensive non-synonymous substitutions found in the vaccine candidates in comparison to the housekeeping genes explained that high antigenic variation among antigenic genes is the adaptive mechanism of parasite to evade the host immune response. Substantial amount of local antigenic repertoires for each candidate in all the five regions have been uncovered. The antigenic repertoires of five vaccine candidates for all the regions were very high, however, each region showed a fraction of specific antigenic repertoires. The overall observed SNPs in five vaccine candidates revealed that a good number of antigenic repertoires are shared among different regions, however, signature of region-specific antigenic repertoire was also observed (Fig. 2.1.5).

"Local antigenic variations are very crucial in the understanding of total antigenic repertoires in a country like India and in turn, planning effective vaccine based control measures"

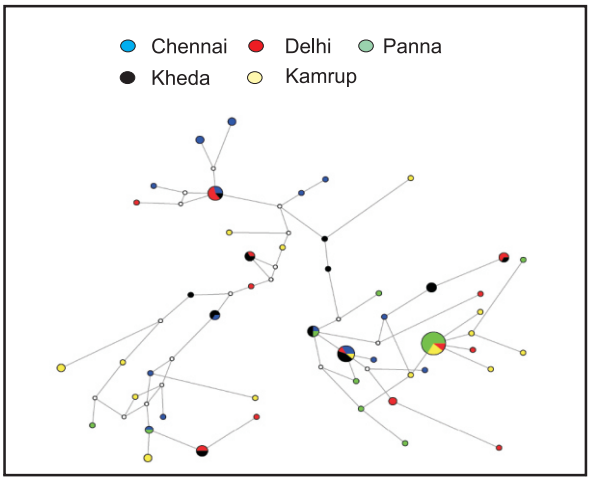

Fig. 2.1.5: Region-specific haplotypes and shared haplotypes of *P. vivax* as well as phylogenetic relationship between *AMA-1* haplotypes. Each circle represents different haplotype, colour of the circle represents geographical origin of isolates and different colours in a circle represent haplotypes shared between respective regions, while white circle represents missing haplotypes. Haplotypes are connected with the mutational events; length of connecting line is proportional to the number of mutational events and size of the circle is proportional to the frequency of haplotypes

2.1.3 Distribution and Genetic Relatedness of Two Sub-populations (Subtypes) in Indian *Plasmodium vivax*

P. vivax has been categorized into two distinct lineages, the 'New World' and 'Old World', distinguishable by gene conversion in the SSU rRNA S-type and mutations in an open reading frame (ORF 470) in the apicoplast genome. The distribution of the two subtypes of *P. vivax* (Old and New world) based on S-type 18S SSUrRNA was studied in field isolates from different locations in India. A total of 354 *P. vivax* field isolates collected from nine different geographical regions of India including coastal, mainland, forest and island regions were analyzed for length polymorphism in S-type SSUrRNA gene.

P. vivax S-type SSUrRNA genotyping for Old world and New world isolates was carried out by one step touch down PCR assay. Two 18S SSUrRNA S-type were confirmed by sequenc-

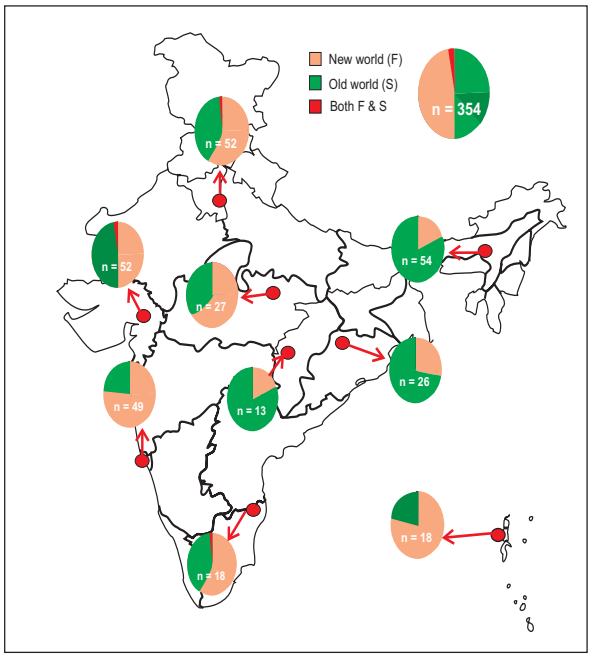

Fig. 2.1.6: Distribution of two *P. vivax* SSUrRNA subtypes in India

ing. Dimorphic nature of SSUrRNA S type gene was observed among the isolates. Based on the fragment size, a slow moving (S) 480 bp fragment (Old world or type I) or fast moving (F) 454 bp (New world or type II) were designated. Distribution of both types of S-type 18S SSUrRNA was nearly equal among the study isolates, however, their proportions varied among isolates of different regions (Fig. 2.1.6). In Delhi, both 'Old' and 'New world' isolates were in equal proportions, while Nadiad and Chennai isolates showed 55 and 58% 'New world' isolates respectively. Similarly, Panna and Goa isolates were predominantly of 'New world' type. Isolates of Car Nicobar also showed higher proportion of 'New world' (80%) isolates. On the other hand, isolates of Sonapur, Rourkela and Raipur were dominated by 'Old world' type and their proportions were about 80%.

"Both Old and New world P. vivax subtypes are equally prevalent all over India"

To understand the genetic structure and relatedness of two sub-types (Old and New world), multi-locus genotyping was initiated. We used highly polymorphism marker, MSP-3α to identify single clonal isolates using PCR-RFLP method. Analyses revealed about 20% of the total isolates are multi-clonal and the rest are single clones. One hundred single-clone isolates, comprised of 50 'Old world' and 50 'New world' types were selected from the pooled 354 *P. vivax* isolates for further molecular characterization of point mutations in *dhfr* and *dhps* genes, known to be responsible for the pyrimethamine and sulphadoxine resistance respectively. In all, 100 isolates of successful amplification for *Pvdhfr* and *Pvdhps* genes were obtained. Amplified PCR products were purified with the commercially available gel extraction kit and sequenced. DNA sequences were edited and aligned with reference sequence (wild type) for both the genes to identify mutant isolates.

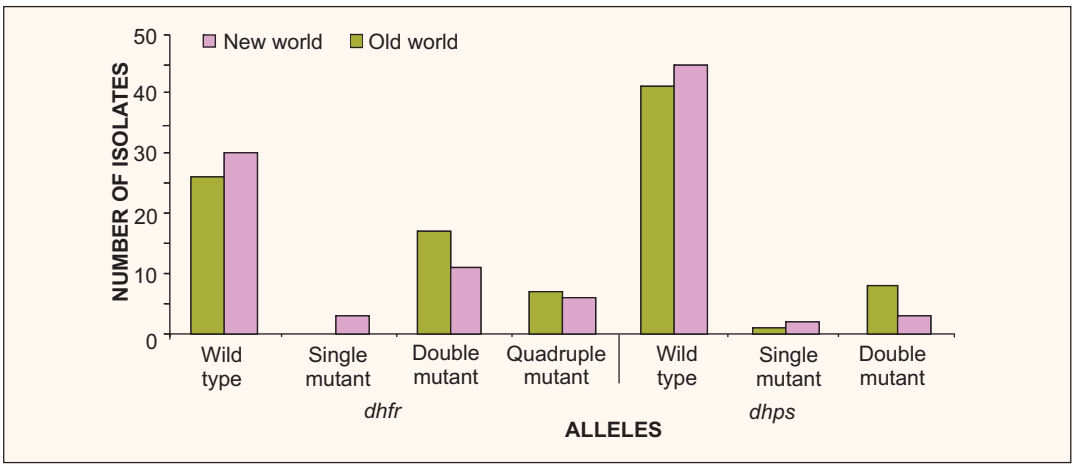

Fig. 2.1.7: Genotype wise distribution of dhfr/dhps alleles among 'Old' and 'New world' sub-populations of *P. vivax*

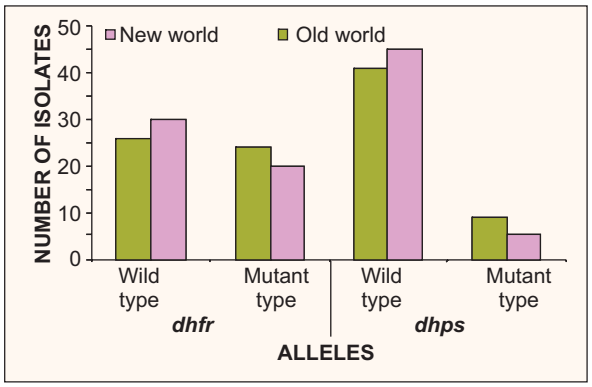

Fig. 2.1.8: Distribution of dhfr/dhps mutant genotypes among 'Old' and 'New world' sub-populations of *P. vivax*

Sequences obtained were submitted to GenBank vide accession numbers EU145878-EU145947 for *dhfr* and EU149665-EU149764 for *dhps*.

In the *Pvdhfr* gene, single, double and quadruple mutants were observed alongwith wild type genotype. In *Pvdhps* gene, limited point mutations (single and double mutants) were observed. Proportion of mutant *dhfr* alleles were more in Old world types (48%) compared to New world types (40%). Similar scenario was observed for mutant *dhps* alleles, where mutant alleles were more in 'Old world' isolates (18%) compared to 'New world' isolates (10%). Proportions of wild and mutant alleles at both *dhfr* and *dhps* loci in 'Old world' and 'New world' isolates are given in Figs. 2.1.7 and 2.1.8. Similarly, proportions of mutant alleles conferring drug resistance (double mutant) and higher level of resistance (quadruple mutant) were more in 'Old world' isolates compared to 'New world' isolates, at both *dhfr* and *dhps* loci but these differences were not statistically significant ($\chi^2 = 1.98$, df = 3, p = 0.577).

"Proportion of mutant alleles (dhfr and dhps) conferring SP resistance was higher in Old world isolates than New world isolates, though differences were not statistically significant"

2.1.4 Molecular Determination of Chloroquine Resistance in Indian *P. falciparum* Isolates

Widespread use of chloroquine (CQ) for the past few decades has led to chloroquine resistant parasites and emergence of clinical failure of chloroquine treatment. Previous studies on the *Pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter) gene, that is responsible for the chloroquine resistance, revealed a heterogeneous situation of chloroquine resistance in Indian *P. falciparum* isolates. These studies analyzed the randomly collected samples and were not supported with clinical assessment of chloroquine efficacy, however, their results indicate *Pfcrt* gene as an attractive target for studying the epidemiological and molecular aspects of drug resistance. About 200 malaria patients enrolled for the chloroquine (CQ) therapeutic efficacy studies and 68 uncomplicated malaria patients from different geographical regions were assessed for genetic basis of chloroquine resistance using molecular markers. The status of point mutation responsible for CQ resistance was assessed by PCR amplification of *Pfcrt* gene. Three types of amino acid haplotypes, encoding 72aa–76aa (amino acid) of Pfcrt protein, namely SVMNT, CVIET and CVMNK were observed. We observed the prevalence of SVMNT haplotype in both clinically sensitive (70%) and resistant (74%) isolates. The wild type CVMNK was found only in

"SVMNT (mutant) haplotype was observed in both clinically sensitive (70%) and resistant (74%) isolates, however, the wild type CVMNK was found only in clinically sensitive cases (20%). Prevalence of SVMNT haplotype is observed in all geographical regions irrespective of endemicity of malaria"

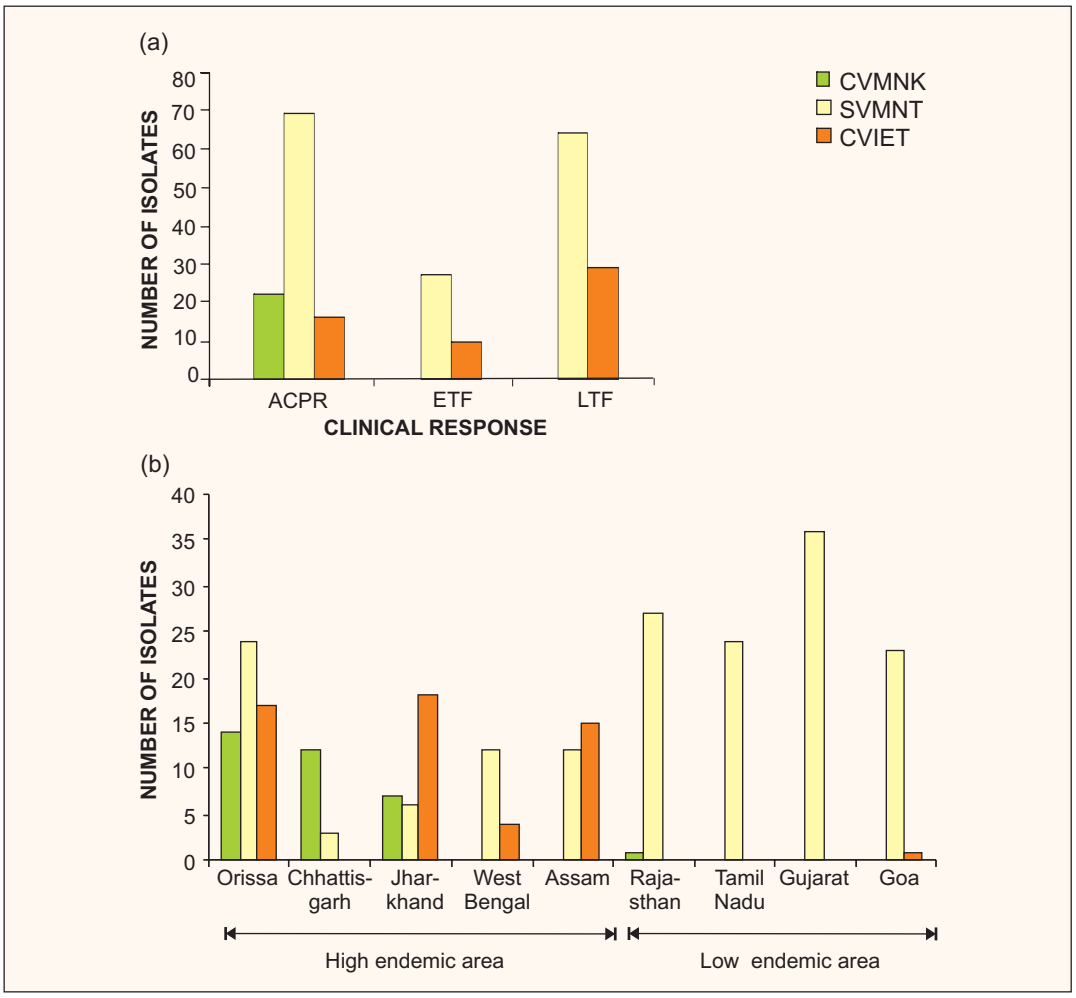

Fig. 2.1.9: Distribution of Pfcrt haplotypes among field isolates. (a) Prevalence of haplotypes in chloroquine-treated malaria cases and (b) Prevalence of haplotypes in high and low malaria endemic areas. ACPR— adequate clinical and parasitological response; ETF— early treatment failure; and LTF—late treatment failure

clinically sensitive cases (20%) (Fig. 2.1.9a). Prevalence of SVMNT haplotype was observed in all geographical regions irrespective of endemicity of malaria. Proportion of SVMNT was nearly 100% in low endemic areas, while in high endemic areas all the three haplotypes were observed (Fig. 2.1.9b).

2.1.5 Characterisation of the *P. falciparum* Strains Prevalent in Northeastern States

Objectives of this study were: (i) to identify drug resistant cases using therapeutic efficacy protocol in *P. falciparum* and to validate using molecular markers; (ii) to generate data on parasitic diversity using microsatellite markers, anchored primer amplification of DNA (APAD) and resistant markers for chloroquine (CQ) and sulfadoxine-pyrimethamine (SP); and (iii) to correlate clinical and parasitological data with genotypic data.

Molecular Characteristics of Drug Resistance Associated Mutations in *Plasmodium falciparum* from Northeastern India

The therapeutic efficacies of commonly used antimalarials were ascertained for the treatment of uncomplicated *P. falciparum* malaria patients who were enrolled for the follow-up of *in vivo* antimalarial response according to WHO protocol with regular clinical and parasitological assessment.

Blood samples were collected from two sites; (i) PHC Kumarikata, District Nalbari (Indo-

Bhutan border area), Assam (KNA); (ii) CHC Dalu, District Tura (Indo-Bangladesh border area), Meghalaya (DTM). Patients reported with fever were clinically examined. Both thick and thin blood smears were checked by microscopy for the presence of *P. falciparum*. Four to five drops of blood were spotted onto sterile filter paper (Whatman No. 3) strips in triplicate for molecular studies. Finger-prick blood samples (59 of KNA and 55 of DTM) were taken from each patient before treatment. Post-treatment sample was also taken as and when the patients reported with parasitaemia during the 28-day follow-up period. DNA from blood spots was extracted with Qiagen DNA mini kit according to manufacturer's protocol.

Parasitized blood before and after treatment was analyzed by PCR assay for variants in the target genes for *pfcrt*, *dhfr* and *dhps*. The nested mutation-specific PCR methods were used to determine the prevalence of *pfcrt* allele Lys-76 and Thr-76 (K76T), *dhfr* polymorphism at 16, 51, 59, 108 and 164 and *dhps* polymorphism at 436, 437, 540, 581 and 613 codon sites, respectively.

The unequal distribution of genotypes was observed in two areas. We found some of the isolates had mixed genotypes of both wild and mutant (Fig. 2.1.10). Majority of the isolates had Thr_{76} mutation in *pfcrt*. The mutant genotypes Ile_{51}, Arg_{59} and Asn_{108} of *dhfr* and Gly_{437} and Glu_{540} of *dhps* were significantly associated with the recrudescent parasites.

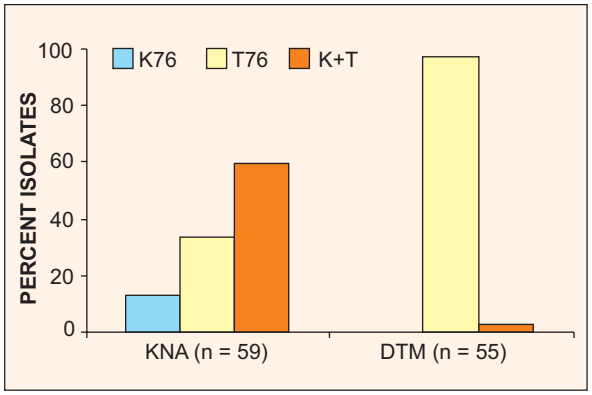

Fig. 2.1.10: Prevalence of wild, mutant and mixed genotypes of *Pfcrt* (KNA—Kumarikata, Nalbari district, Assam; DTM—Duke, Tura district, Meghalaya)

Analysis of Recrudescent Infection

Analysis of recrudescence in *P. falciparum* infection was done by genotyping of paired *P. falciparum* samples. Three paired *P. falciparum* samples collected on Day 0 and on the day of reappearance of parasitaemia were analyzed to distinguish between recrudescence and new infection. Two genetic markers, MSP-1 and MSP-2 were used for the genotyping by studying length variations in repeat nucleotide sequence regions. The genotyping of recrudescence infections using three surface protein markers, MSP-1, MSP-2 and GLURP revealed that of five chloroquine failure cases, only three had same genotype suggestive of true drug failure (Fig. 2.1.11). Similarly, for ACT, of six failure cases, four had different genotypes suggestive of new infections. In 2007, studies based in Dalu CHC of West Garo Hill district of Meghalaya along Indo-Bangla border population groups revealed that ACT was seemingly effective for the treatment of *P. falciparum* cases. Of 54 cases, 51 (94%) were ACPR, and only 3 (6%) were late clinical failures (LCF).

"Majority of the isolates from north-eastern states had Thr_{76} mutation in pfcrt. The mutation of Ile_{51}, Arg_{59} and Asn_{108} of dhfr and Gly_{437} and Glu_{540} of dhps were significantly associated with the recrudescent parasites"

Genetic Diversity Study

Genetic diversity study in *P. falciparum* isolates was done with MSP-1, MSP-2, GLURP and microsatellite markers. Ninety-six *P. falciparum* isolates collected from Kumari Kata District Nalbari, were analyzed with GLURP and the results revealed polymorphic nature of the field isolates and a total of 14 alleles were found. Proportion of multi-clone isolates were less compared to Dalu isolates. Microsatellite analysis using two loci (ARA2 and TAA60)

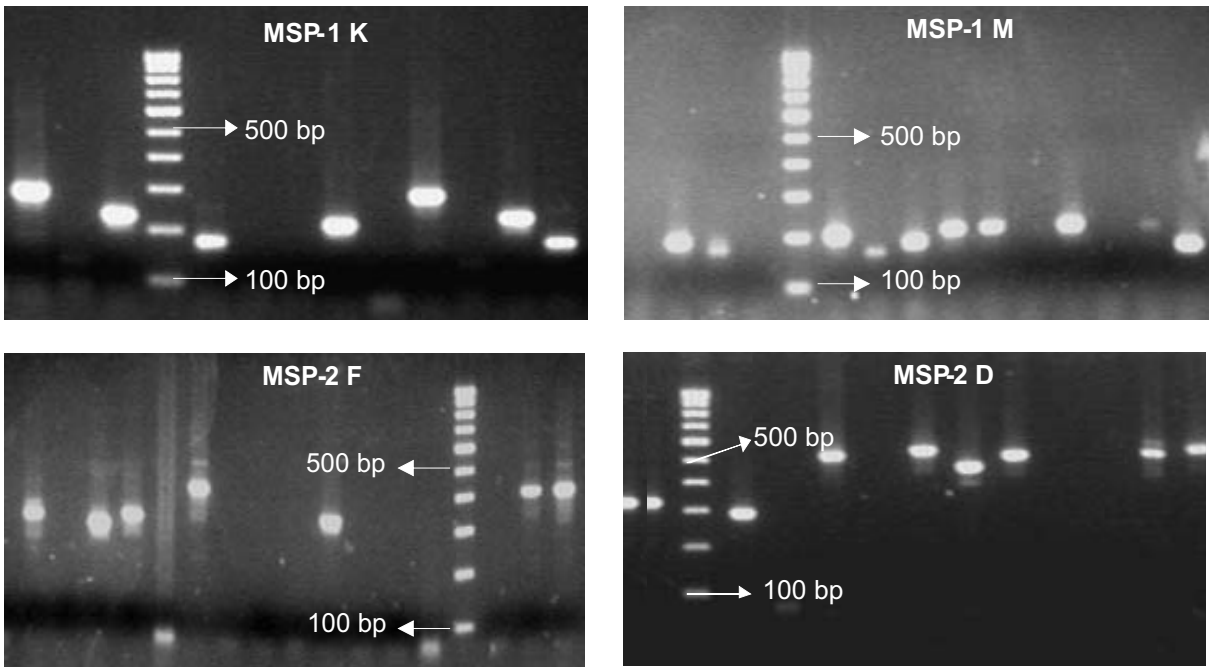

Fig. 2.1.11: Gel image showing variations in *MSP-1* and *MSP-2* gene allelic families in the *Plasmodium falciparum* field isolates

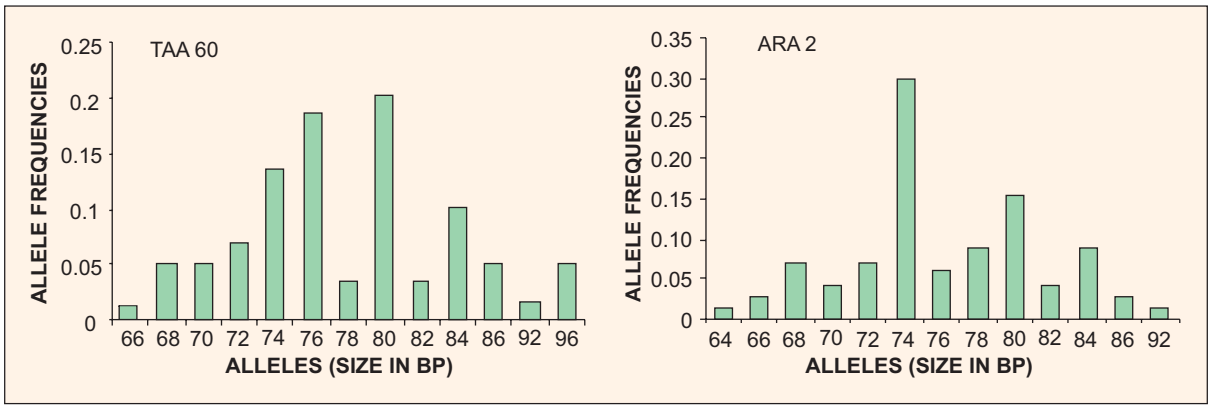

Fig. 2.1.12: Allelic frequencies of two microsatellite loci TAA60 and ARA 2 alleles in the population

revealed both loci were highly polymorphic in the field isolates. Allele per locus was 13 and heterozygosity at both loci were 0.86 (ARA2) and 0.89 (TAA60). Allele frequencies per locus per allele were variable in the population (Fig. 2.1.12). This high degree of genetic diversity at both loci in the field isolates of *P. falciparum* suggests that microsatellite could be a potential genetic marker for the recrudescence infection study.

Forty-eight *P. falciparum* isolates from Dalu collected during 2007 were analyzed for *MSP-1* and *MSP-2* genes to assess the genetic diversity. The results revealed that the *P. falciparum* isolates from Dulu were highly polymorphic and diverse. Proportion of multi-clone infections (48.83%) was very high. *MSP-1* gene has three allelic families, namely K, RO33 and MAD, of them only RO33 was monomorphic, whereas K and MAD were having wide allele range in the population. Similarly, *MSP-2* gene has two allelic families 3D7 and FC27 and both were polymorphic in the population. The distribution of allelic families in the MSP-1 and MSP-2 varied in the population (Fig. 2.1.13). Studies on microsatellite markers are in progress for more number of loci to resolve the genetic diversity and their pattern in the populations.

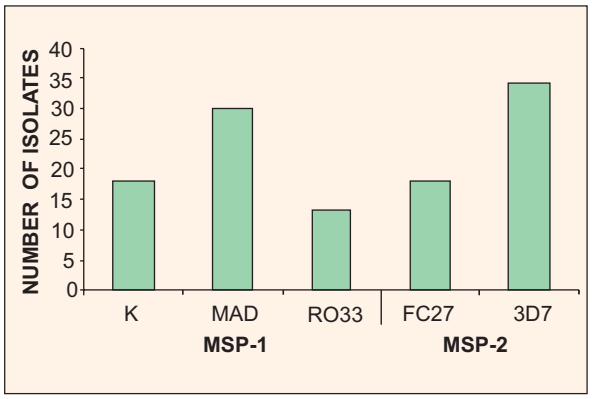

Fig. 2.1.13: Distribution of MSP-1 and MSP-2 allelic families in the population

Data generated on clinical efficacy of drugs will be validated by molecular markers as well as prevalence of drug resistance related markers among the field isolates, thus, generating accurate data on problem of drug resistance in the area.

2.1.6 Genotyping of *Plasmodium falciparum* parasites using Anchored Poly-A and Poly-T oligonucleotides from Northeastern India

Malaria parasites provide an excellent system to study the genomic effects of strong selection in a recombining eukaryote. The genetic structure of *P. falciparum* shows deletion and rearrangement of chromosome segments, expansion or shortening in the number of repetitive elements, point mutations and size polymorphism. These changes seem to be one of the important mechanisms to help the parasites in evasion of host immunity for their survival. A couple of markers have been developed for genotyping of parasites; anchored primer amplification of DNA (APAD) is one of them. This is a recently developed method, which readily displays genetic difference in A-T rich DNA sequences.

Blood samples of *P. falciparum* patients collected from two sites: (i) PHC Kumarikata, District Nalbari (Indo-Bhutan border area), Assam (KNA); and (ii) CHC Dalu, District Tura (Indo-Bangladesh border area), Meghalaya (DTM) were analysed by APAD. DNA extraction was performed with Qiagen DNA mini kit according to manufacturer's protocol. APAD was performed by using different sets of Poly-A and

"The APAD method could be a useful tool for parasite genotyping"

Poly-T primers with di- to hexa-nucleotide anchored at 3'end. Out of these primers, some were giving smear or no bands, so were excluded from the study and some selected primers giving discrete bands were used for further study. Mostly di- and tri-nucleotide anchored primers produced smears or no bands. Most of the penta– and hexa-nucleotide anchored primers produced discrete bands.

Following primers produced discrete bands:

(1) A_{14}GCATCG
(2) A_{14}GGTTCC
(3) A_{14}CGACGA
(4) A_{14}CATGCC
(5) A_{15}GTGTA
(6) T_{14}CGACGA
(7) T_{14} GCAGCA
(8) T_{15} GTCTA

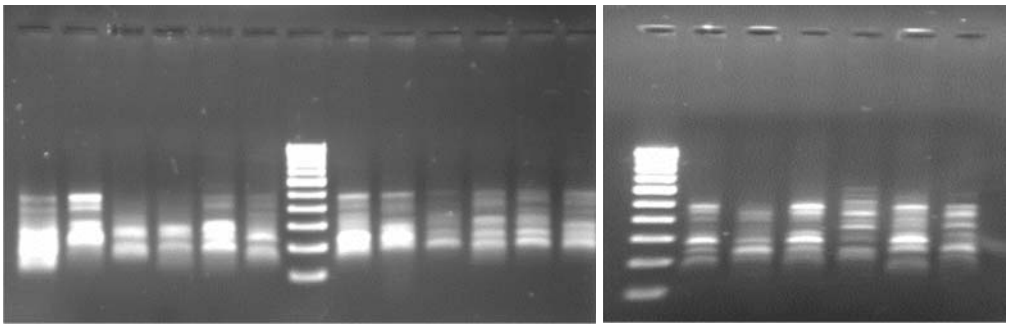

Fig. 2.1.14: Different isolates of *P. falciparum* showing variations by the primer A_{14}GCATCG by APAD; M=100 bp

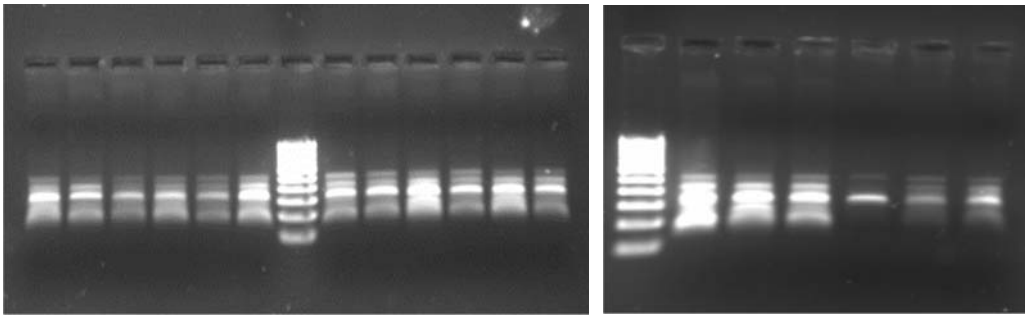

Fig. 2.1.15: Different isolates of *P. falciparum* showing variations by the primer A_{14}GGTTCC by APAD; M=100 bp

Twenty isolates have been studied for genetic diversity study. Some primers gave multiple and distinct bands with all the isolates and some show slight differences in the pattern of bands. Differences in the patterns of bands with the same primer in *P. falciparum* parasites revealed diversity among the isolates obtained from different patients (Figs. 2.1.14 and 2.1.15).

This method can be very useful for identifying polymorphism that can distinguish closely related parasite isolates. This method is very useful for rapid parasite typing because large number of loci in the genome can be checked quickly. This method also provides a potentially powerful tool for developing genetic markers from different parasite species.

2.2 Parasite Immunology

2.2.1 Naturally Acquired Immune Responses to Stage-specific *P. falciparum* and *P. vivax* Antigens in a Population of Central India

The study has been initiated with objectives: (i) to characterize immune responses to stage-specific *P. falciparum* and *P. vivax* antigens in children and adults naturally exposed to malaria; (ii) to study the development and maintenance of immune responses in different age groups with emphasis of infants, their older siblings and mothers, including identification of epitopes that correlate with protection; (iii) to determine the role of stage-specific antigens in the development and maintenance of natural immunity to malaria; and (iv) to evaluate the immune mechanisms, those are involved in pathogenesis of malaria, especially anaemia, cerebral malaria and placental malaria. The study was conducted in three populations. They are: (i) infants, children and adults from the community; (ii) pregnant women from the community; and (iii) Hospitalised patients with severe malaria. Peripheral blood, placental and cord blood at delivery were taken for determining the antibodies against species and stage-specific antigens by enzyme immunoassay. The antibody levels were quantified using known antimalarial antibody (positive controls) and this allowed us to estimate antibody levels in O.D. values. Sera from non-endemic healthy subjects were taken as negative control (Figs. 2.2.1 and 2.2.2). Cellular response was determined in peripheral blood mononuclear cells by lymphocyte transformation test in the presence of *P. falciparum* and *P. vivax* antigens and cytokines (IL-4 and IFN-γ) level was estimated in activated T-lymphocytes culture supernatant by sandwich ELISA. The peripheral blood mononuclear cells (PBMC) were isolated from venous blood of subjects who have past experience of malaria and the proliferative responses against *P. falciparum* and *P. vivax* antigens (5 each) were determined in individual set of PBMCs. Only a subset of adults and older children above 14 years were participated in this study. In general, the study subjects responded to T-cell epitopes. The mean stimulation index (SI) was not significantly different among positive responders. The SI of >2 was taken as cut-off to determine the positive responders. The *in vitro* stimulation of T-cells

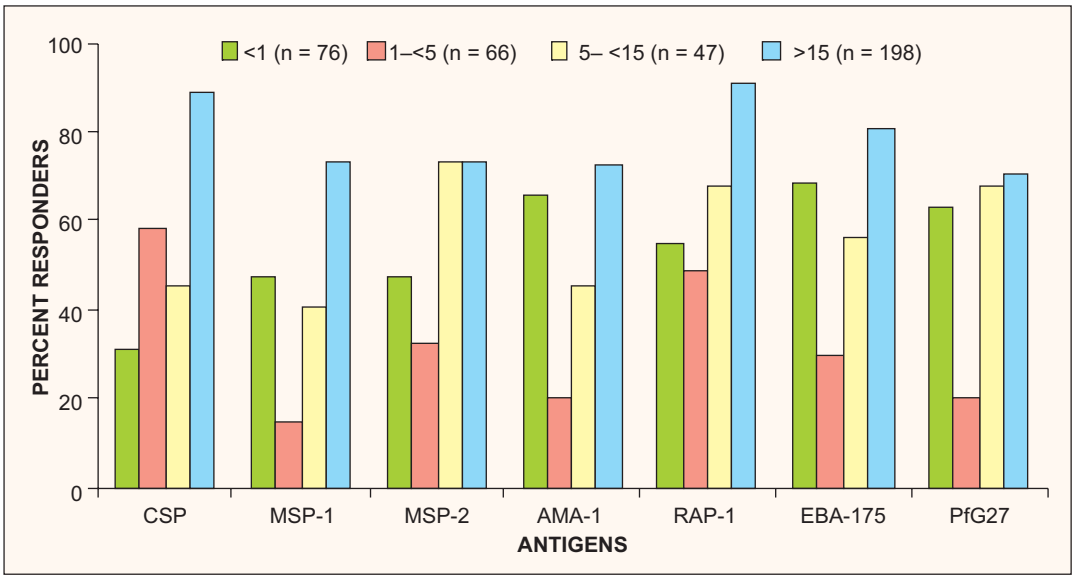

Fig. 2.2.1: Responder frequency to *Plasmodium falciparum* antigens

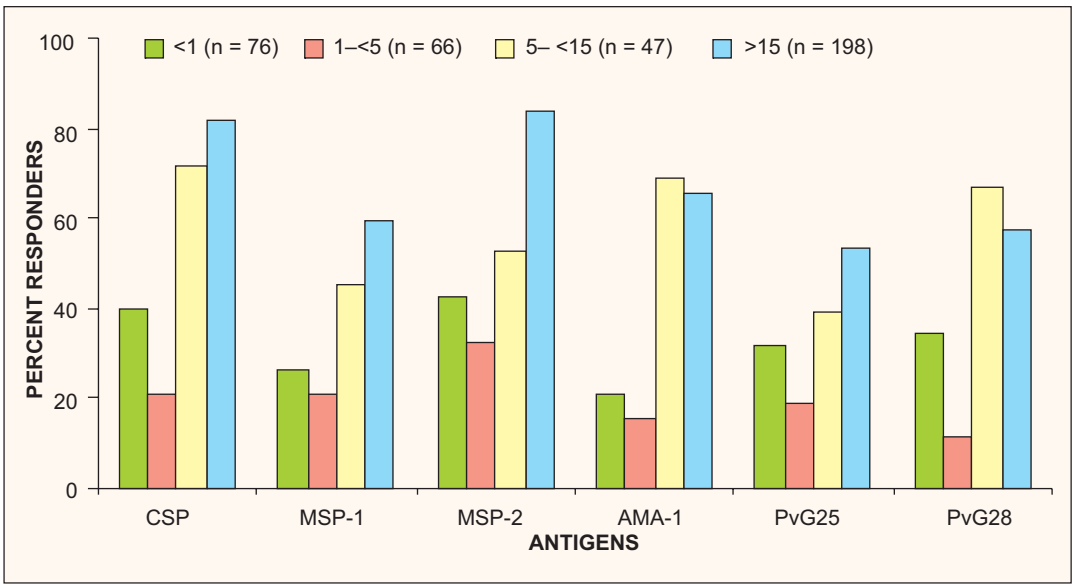

Fig. 2.2.2: Responder frequency to *Plasmodium vivax* antigens

from malaria-exposed donors results in the production of IL-4 and IFN-γ in concordance with the serum concentrations of antibodies specific for the antigens used for lymphocyte stimulation (Figs. 2.2.3 and 2.2.4). Peripheral blood from healthy cohort participants and *P. falciparum* malaria patients were taken for the estimation of cytokines (IL-4, IL-10, IP-10, IFN-γ and TNF-α) in plasma using commercially developed two-site ELISA assay kits.

Study needs to identify the antigen-specific antibody responses in the serum of pregnant women who do not suffer from the placental malaria, which may suggest that these antibodies are important for protecting the mother against infection. Antibodies produced by adults (mothers) were correlated with protection. Therefore, it is important to determine if infants mount antibody responses to species and stage-specific antigens and the association between infant's antibodies with protection. An important component of this study is to understand the role of immunologic factors in the pathogenesis of severe malaria. The current understanding of

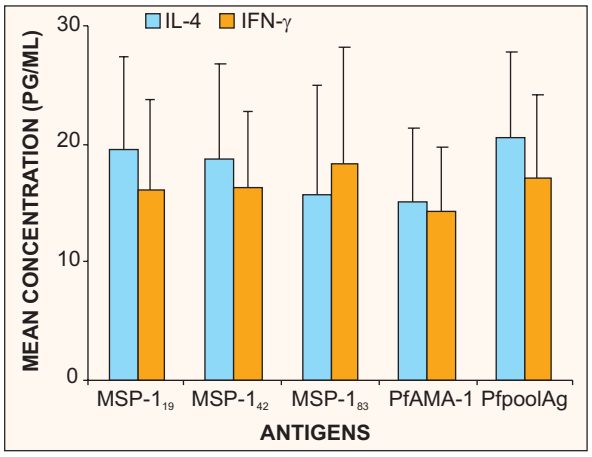

Fig. 2.2.3: Cytokine level in lymphocyte culture supernatant with *Plasmodium falciparum* antigens

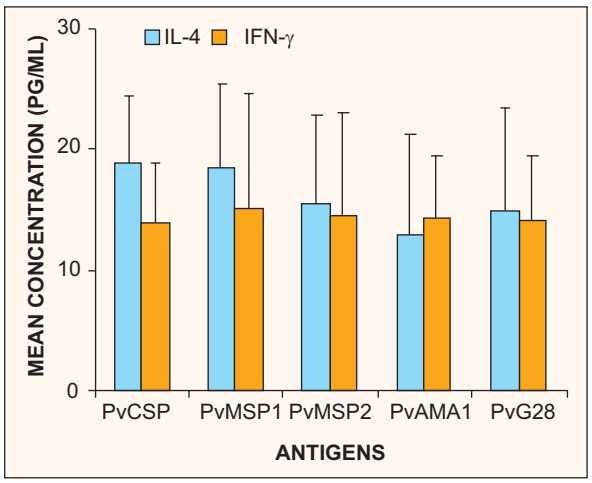

Fig. 2.2.4: Cytokine level in lymphocyte culture supernatant with *Plasmodium vivax* antigens

the immunology of severe malaria is mostly based on African studies. However, Indian populations face a different epidemiologic setting due to differences in the malaria transmission and occurence of both *P. falciparum* and *P. vivax*.

As proposed in the project, data on all above parameters would be generated to fulfill the objectives in more number of samples for determining the role of stage-specific antigens in the development and maintenance of immune responses in different age groups with emphasis on infants and mothers. Cellular immune responses and cytokine profiles in both *P. falciparum* and *P. vivax* patients would also be determined by a hospital-based survey.

2.2.2 Parasite Growth Inhibition and ELISA IgG Responses by Antibody Dependent Cellular Inhibition (ADCI) Assay

Batches of high antibody titer sera from clinically immune subjects were tested for their effect in growth of *P. falciparum in vitro* by antibody dependent cellular inhibition (ADCI) assay in the presence of peripheral blood monocytes. The isolation of monocytes was done from peripheral whole blood of healthy adults, who had no past malaria history. A well-adapted, stable culture line of *P. falciparum* was used for determining the effect of various sera in the parasite growth. The average rate of multiplication of this strain was 8–10 fold after 96 h. Sera of clinically immune individuals (n = 20) with high antibody titer were taken for the assay. They were tested at 1 : 10 dilution for their effect on the parasite growth in the absence of monocytes by growth inhibition assay or in the presence of monocytes by ADCI assay in an established *P. falciparum* culture line. Culture synchronized at ring stage was added in 96-well flat bottom tissue culture plates at 5% hematocrit with 1% parasitaemia. Assay was done for 48 h at 37 °C by Candle-jar technique. Growth of the parasite (development and multiplication) was monitored microscopically in thin smears counting number of ring and early trophozoite stages. The sera showed growth inhibition at a range of 33–67%, but same sera showed enhancement of 49–82% in the presence of monocytes. The control serum pool also

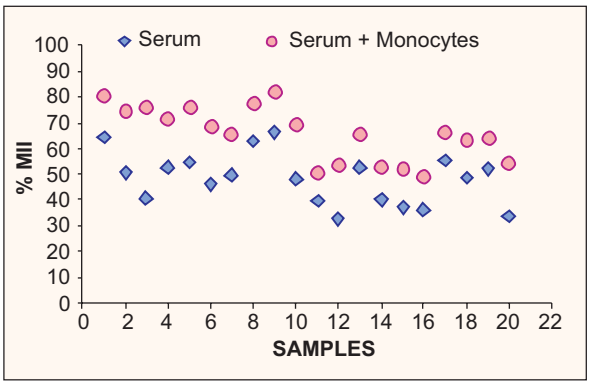

Fig. 2.2.5: Merozoite invasion inhibition (MII) in the absence or presence of monocytes

showed an average of 12.3% inhibition in the presence of monocytes. Therefore, test serum showing monocyte-mediated inhibition above 15% (Mean ± 2 SD; 12.3 ± 2.7) was considered significant. All 20 sera, which showed ability to promote monocyte-mediated cytotoxicity to *P. falciparum* had substantial level of antigen-specific IgG (Fig. 2.2.5). However, there was no significant correlation between the presence of antibodies and merozoite invasion inhibition.

The biological function of antibodies detected by ELISA was assayed in *P. falciparum* culture for their ability to inhibit the parasite growth. All 20 clinically immune donors had antibodies to merozoite stage antigens substantially. However, all of them could not cause significant inhibition of parasite multiplication. The addition of monocytes to cultures containing these antibodies affected parasite multiplication to some extent with all the sera. It is to be expected that sera containing cytophilic or opsonic antibodies mediate in phagocytosis of free merozoites causing inhibition in merozoite invasion of new RBC. The lack of significance of correlations between serum-dependent monocytes-mediated cytotoxicity and antibodies may indicate the failure of the sera to recognize appropriate antigens on the surface of infected erythrocytes. It was observed that most of the sera, despite the presence of high antibodies to blood stage antigens, could not substantially control parasite growth alone. Antibody-dependent monocytes-mediated antimerozoite activity has been suggested as one of the important *in vivo* antiparasite mechanisms. To monitor such mechanisms, appropriate assays would be essential to provide feed back in the vaccine development.

2.2.3 Purification and Characterization of Monoclonal Antibodies against Erythrocytic Stages of *Plasmodium vivax*

This is in continuation of the earlier work related to characterization of monoclonal antibodies. Of the 27 hybridomas, 15 lines were revived. They were grown *in vitro*. These clones were taken for antibody production and characterization. Supernatant of each culture line was tested by ELISA for antibody against *P. vivax*, *P. falciparum* and human-NRBC lysate. Fifteen *P. vivax* antibody positive clones were characterized by immunoglobulin isotyping by ELISA. Of the 15 hybridomas, 13 produced IgG type of antibody, one showed both IgG and IgM responses and one produced IgM type antibody. Of the 13 IgG producing hybridomas, 11 showed stable growth and by subclass analysis these were found to be IgG1 type (Fig. 2.2.6). Culture supernatant from 11 growing hybridomas was tested for

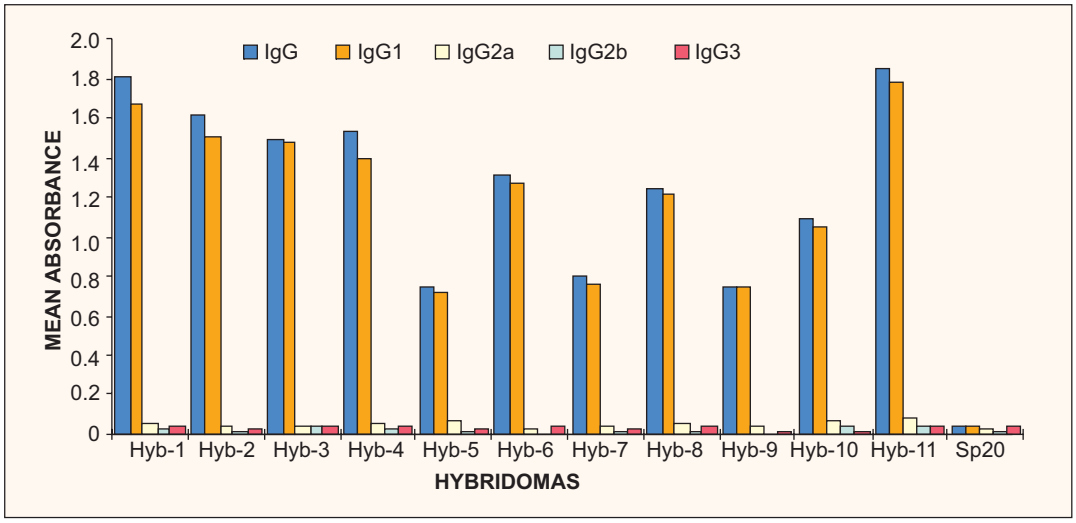

Fig. 2.2.6: Immunoglobulin subclass analysis of hybridomas

their reactivity with *P. vivax* erythrocytic stages by IFA test. All 11 MAbs reacted with blood stages of *P. vivax* with varying intensity. Of the 11 monoclonals, 8 were tested on dot-blots with patients' blood samples. The *P. vivax* crude antigen (PvC) was subjected to SDS-PAGE to check the band patterns on the gel. Proteins from the unstained SDS-PAGE slab gel were transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane. They were used to develop Western blot by reacting with six MAbs. On Western blot, each MAb reacted with proteins of *P. vivax* lysate. Seroreactivity of 6 MAbs was also determined against PvC antigen by competitive binding assay in an indirect ELISA. The addition of second MAb showed a rise in seroreactivity as observed in percent increase in antibody binding. Dot ELISA showed specific reaction of monoclonal antibodies with *P. vivax* infected patients' blood.

"Humoral responses to the erythrocytic stage antigens were acquired in an age-dependent manner during natural course of infection, and these sera containing antigen-specific antibodies mediated in parasite phagocytosis"

2.2.4 Circulatory Cytokines in Clinically Active *Plasmodium vivax* Infection

Malaria parasites have evolved to acquire diverse immune evasion mechanisms that evoke poor immune responses and allow infection to individuals previously exposed. The malarial parasite, *P. vivax* is sensitive to inflammatory response, and thus, the circulating cytokine evaluation has attracted interest in the field of diagnosis, therapeutics and monitoring in view of malarial and clinical complications. Our knowledge of the cytokine profile and imbalance in cytokine network in clinical severity, pathogenesis, protection and susceptibility of vivax malaria is very limited. Thus, to establish its relevance to active clinical vivax malaria as well as the clinical response of pro- and anti-inflammatory cytokine in severity, pathogenesis and diagnosis of disease, we investigated the impact of the content of TNF-α, IL-2, IL-6, IL-8, IFN-γ, IL-10 and IL-12 in serum of adults with an emphasis of clinically defined heterogeneous groups. These cytokines were evaluated because of their biological relevance in inflammatory and immunomodulatory responses in order to obtain a global measure of the patients' actual reactivity towards *Plasmodium* challenge. We also studied the association and specific involvement in terms of clinically heteroge-

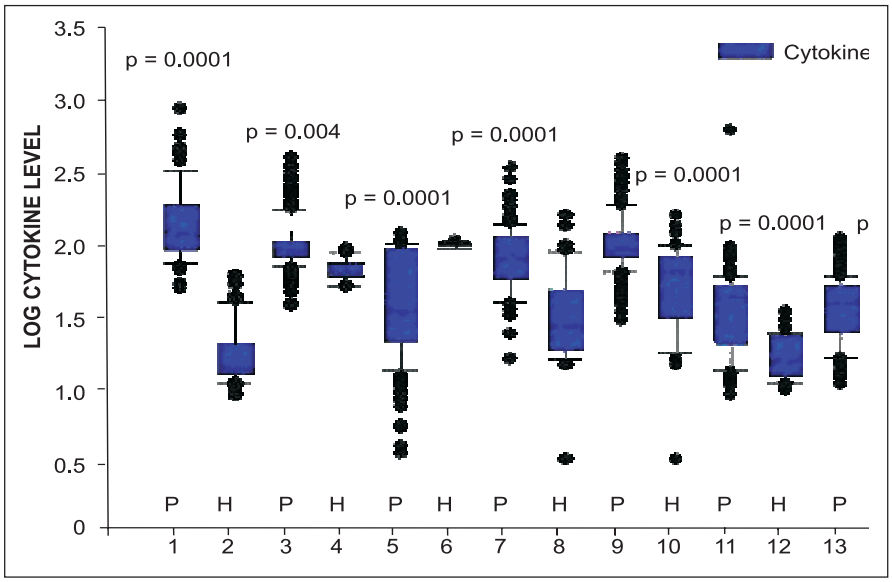

Fig. 2.2.7: Differences in serum cytokine levels of TNF-α (1, 2), IL-6 (3,4), IL-10 (5,6), IFN-γ (7,8), IL-8 (9,10), IL-12 (11,12) and IL-2 (13,14) (in pg/ml) in clinical vivax malaria as compared to healthy subjects. Horizontal bars indicate cytokine wise medians and SE for each cytokine is denoted by error bars; P— Patients; H— Healthy subjects

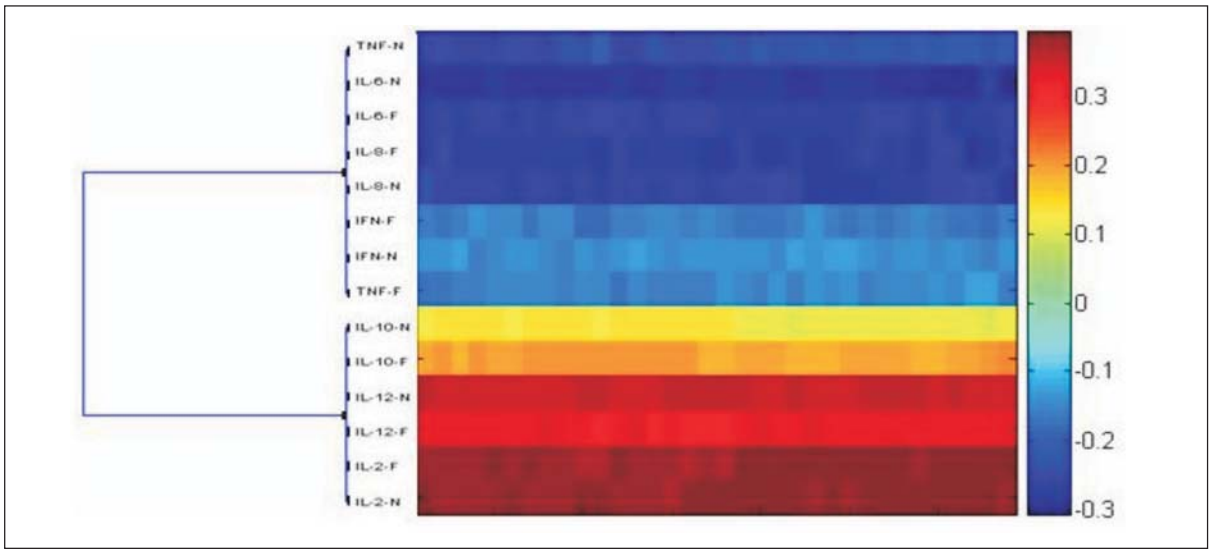

Fig. 2.2.8: Two-way coupled cluster analysis. Each cell in the 2-dimensional graph indicates the measure of a single cytokine in one sample with standardized levels indicated by colour according to the scale on the right. Sample clustering resulting from the algorithm applied is shown at the left side of the graph as a vertical dendrogram, with an indication of the group to which each individual sample belongs. Major clusters discriminated the clinical groups exactly. Cytokine clustering is depicted analogously in the vertical order to the left of the graph. TNF—Tumor necrosis factor; IL—Interleukin; IFN—Interferon

neous groups/clusters between serum cytokine network/profile and clinical parameters like temperature, weight and age.

An overall significant elevation of serum TNF-α, IL-6, IFN-γ, IL-2, IL-8 and IL-12 content ($p < 0.05$), whereas highly significant depletion of IL-10 content ($p = 0.0001$) was observed in vivax patients (Fig. 2.2.7). Two-way coupled cluster analysis revealed two clusters of cytokines relevant to clinical subgroups stratified according to patient's cytokine level, with (P-F) and without fever (P-NF). The first cluster, composed of TNF-α, IL-6, IL-8 and IFN-γ, revealed predominance of pro-inflammatory cytokines, with elevated levels of all, except TNF-α. Whereas the second cluster, composed of IL-10, IL-12 and IL-2, displayed anti-inflammatory response and immunomodulatory cytokines, with elevated levels of all cytokines, except IL-10. Moreover, in both the cluster groups the levels were found to be statistically significant ($p < 0.05$) in all cytokine groups during infection (Fig. 2.2.8). Furthermore, a considerable qualitative and quantitative inter-individual variability in the Th1 and Th2 type cytokines was found and a statistically significant difference ($p < 0.05$) with an initial dominance of Th1 response (Figs. 2.2.9 and 2.2.10a).

"The sequential and prospective characterization of cytokine levels during infection with vivax malaria could provide additional insight into their pathogenic and protective role in the disease and might ultimately suggest therapeutic interventions"

A significant upsurged ratio of pro-inflammatory response compared to anti-inflammatory ($p = 0.001$) response was observed with the onset of vivax infection and this eventual imbalance is a vital determinant of pathological conditions, host defense, hemopoiesis and inflammation (Fig. 2.2.10b). In addition, our findings suggest that variability in the circulating level of immunomodulatory cytokine ratio is of biological significance and may play important roles in host defense mechanisms against vivax infection by enhancing cell-mediated immunity and stimulating the protective immunological cascade (Fig. 2.2.10c).

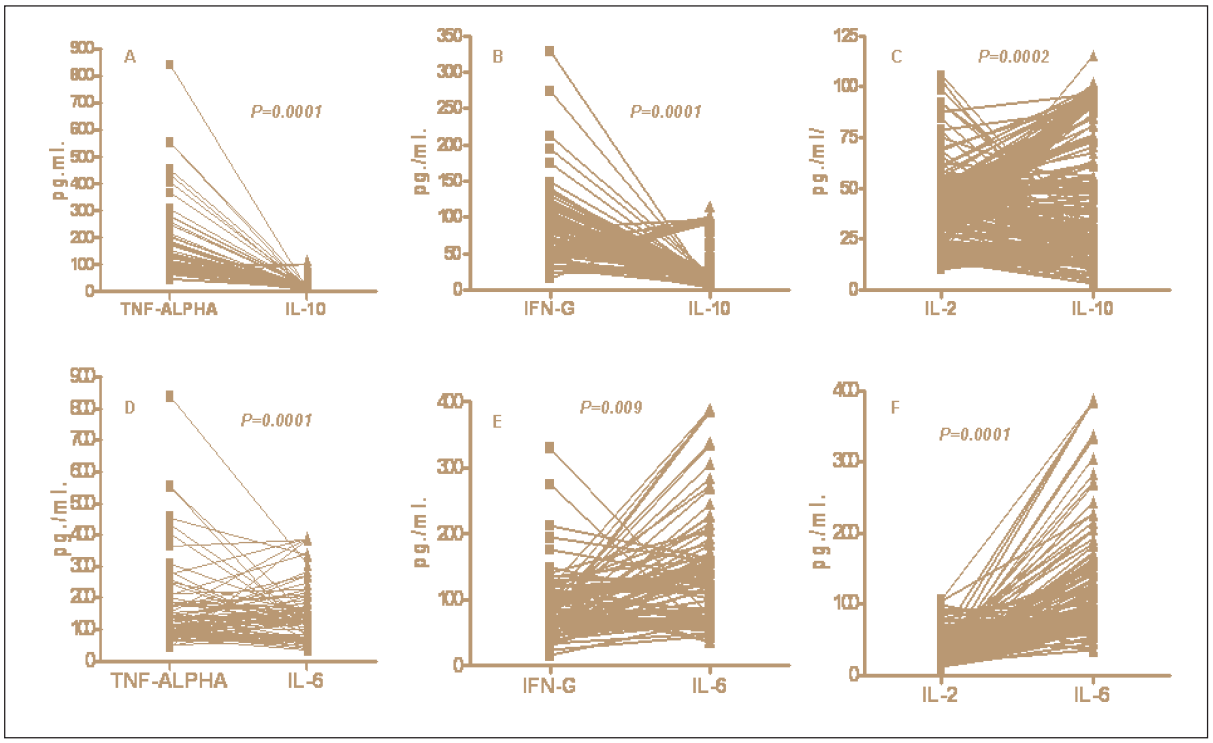

Fig. 2.2.9: Relationship between the individual values of Th1-type cytokine (TNF-α, IFN-γ and IL-2) *vs* Th2-type cytokine (IL-10 and IL-6) of *P. vivax* exposed individuals. The differences between the Th1-type cytokine *vs*. Th2-type cytokine were statistically significant (p <0.05)

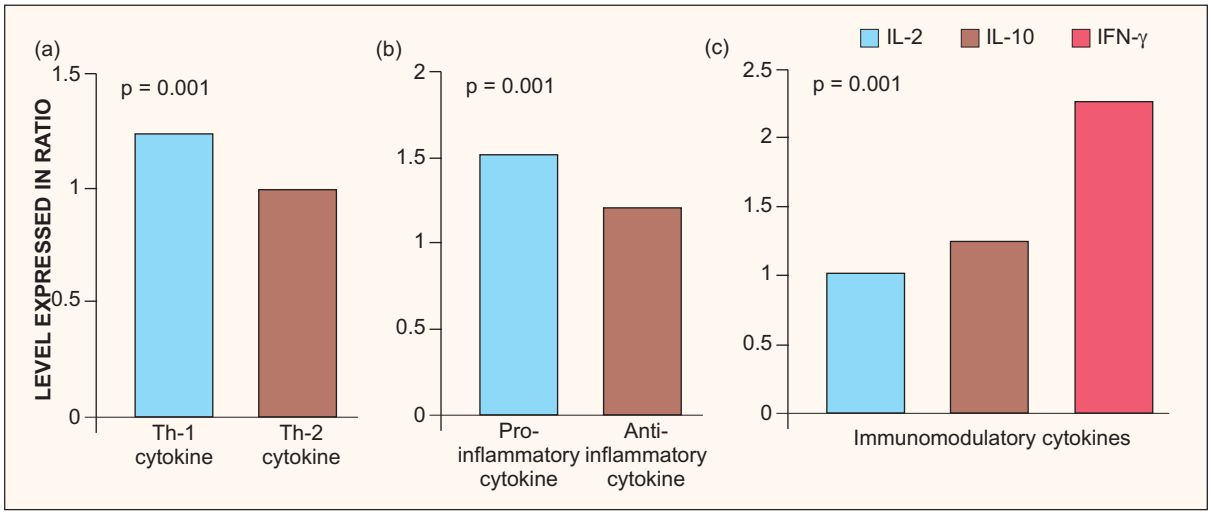

Fig. 2.2.10: (a) Level of Th1 cytokines (TNF-α, IL-2 and IFN-γ) and Th2 cytokines (IL-10 and IL-6) cytokines in vivax infected patients. The differences between the ratios of two groups were statistically significant; (b) Level of pro-inflammatory (TNF-α and IL-6) and anti-inflammatory (IL-10 and IL-12) cytokines in vivax infected patients. The differences between the ratios of two groups were statistically significant; and (c) Level of immunomodulatory cytokines (IL-2, IL-10 and IFN-γ) in vivax infected patients

In view of the present findings of cytokine profile and other clinical parameters of vivax infection, we suggest that pro-inflammatory responses are associated with rapid control of parasite growth at the cost of developing clinical symptoms, suggesting a profound consequence of initial cellular response on disease outcome and may be considered a reliable immunological marker, a promising rational for diagnostic potential and immuno-therapeutic interventions in clinical vivax malaria.

2.3 Bioinformatics

2.3.1 Comparative Evolutionary Genomics of *Plasmodium falciparum* and *P. vivax*

Complex and rapidly evolving behaviours of the two human malaria parasites, *P. falciparum* and *P. vivax* have always been mysterious to the evolutionary biologists as the former is the most virulent and the latter is most prevalent malaria parasite species across the globe. With the availability of whole genome sequence data, it is now feasible to pinpoint genomic similarities and differences between the parasites with comparative evolutionary genetic approaches and thus, define new measures for malaria control. We herewith utilized available genome information of these two species and compared functional genes of *P. falciparum* with partially-assembled whole genome sequences of *P. vivax*. Four different kinds of genes are present in the entire genome of *P. falciparum*. The distribution of these genes are shown in Fig. 2.3.1. About 82% of total functional genes of *P. falciparum* were found to be conserved in *P. vivax* and rest 18% to be unique to *P. falciparum*. Although both types of genes were distributed across all 14 chromosomes of *P. falciparum*, the distribution was slightly biased towards two separate chromosomes for each category (Fig. 2.3.2). About a half of the conserved genes was intron-less, whereas almost all unique genes have introns. However, number of introns was comparatively higher (usually >2) in the intron-possessing conserved genes than in the unique genes (mostly <2) (Fig. 2.3.3). Statistically significant positive correlations between total intron length and gene lengths were detected in 11 chromosomes for unique genes, whereas only in three chromosomes for conserved genes. Three most conserved genes (Actin, Elongation factor alpha 1 and Ribosomal protein L 10 putative) between *P. falciparum* and *P. vivax* were found to be highly conserved in four other species of *Plasmodium* (except Actin gene in *P. chabaudi*) and were

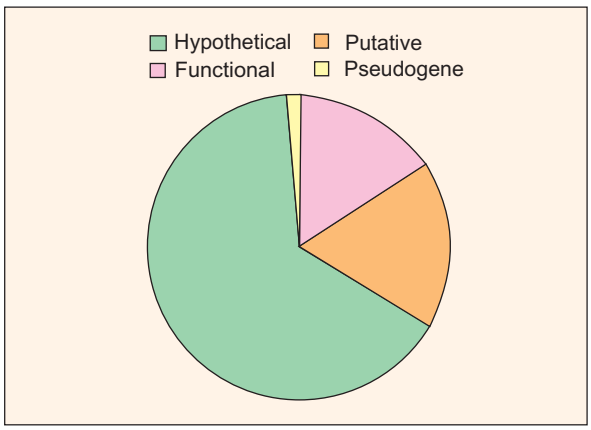

Fig. 2.3.1: Total genes in *P. falciparum* genome. Note that the putative and functional categories (together considered as functional) of genes have been utilized in the present study

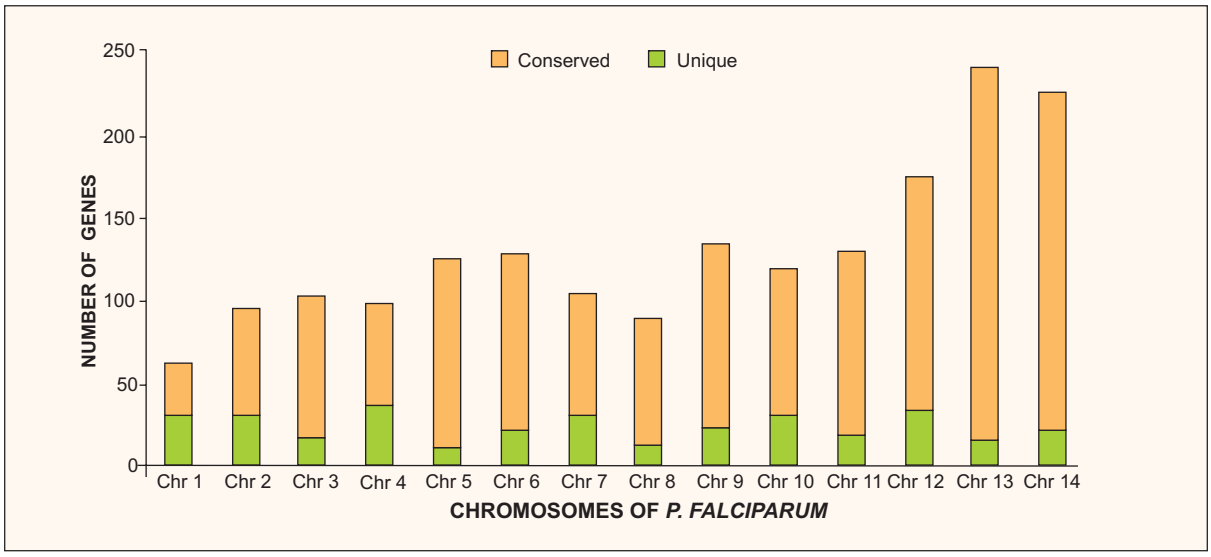

Fig. 2.3.2: Different proportions of unique and conserved genes in chromosomes of *P. falciparum*

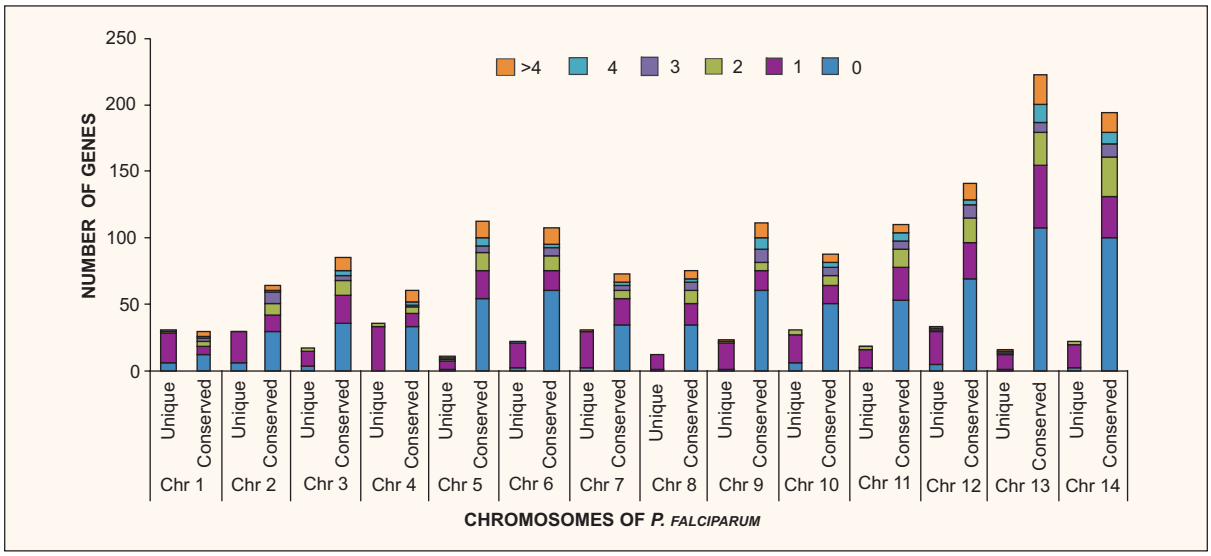

Fig. 2.3.3: Distribution of genes with different intron numbers in conserved and unique genes in chromosomes of *P. falciparum*

mostly intron-less. Phylogenetic trees were constructed separately for each of the three genes (Fig. 2.3.4); in two genes (Actin and Elongation factor alpha 1) (Fig. 2.3.4 a, b) different *Plasmodium* species were placed in almost similar positions, whereas Ribosomal protein L 10 putative show different relationships between *Plasmodium* species (Fig. 2.3.4c). Three unique gene families in three *Plasmodium* species (*P. falciparum*, *P. vivax* and *P. knowlesi*) were studied in detail for total intron length and correlations between intron lengths and gene lengths, which corroborate findings on the overall patterns of whole unique genes of *P. falciparum*. The results are discussed in terms of chromosome and intron evolution in *Plasmodium* in general, relevance of introns in differential functions of *P. falciparum* genes and genetic similarities and differences between *P. falciparum* and *P. vivax* and its implications in malaria, in particular.

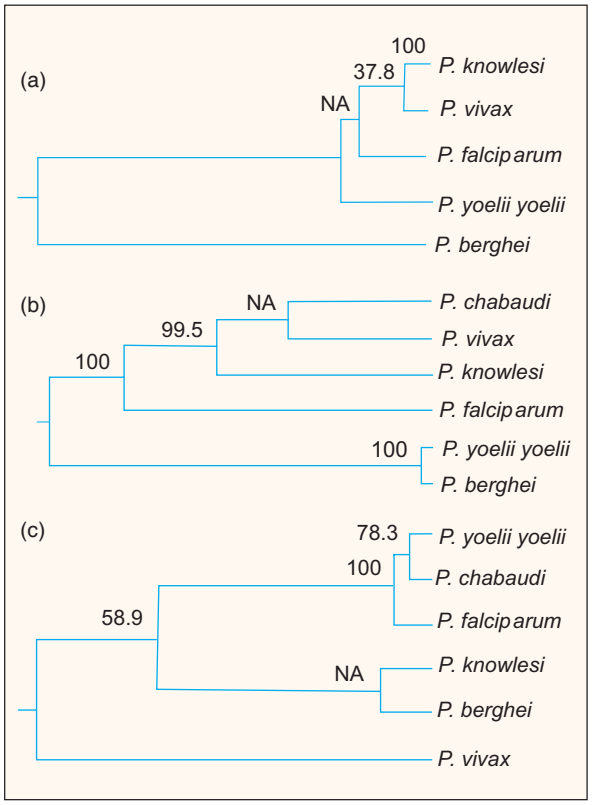

Fig. 2.3.4: (a) Phylogenetic status based on actin; (b) Phylogenetic status based on elongation factor alpha 1; and (c) Phylogenetic relationship based on ribosomal protein L10 putative

2.3.2 Genetic Characterization and Evolutionary Analysis of Human CD36 Gene

Understanding evolutionary genetic details of immune system genes responsible for infectious diseases is of prime importance concerning disease pathogenecity. Considering malaria as a devastating disease in the world including India, detail evolutionary understanding on human immune system gene is essential. The primary aim of this study is to initiate work on such genes. To start with, we have considered the human CD36 gene that is responsible in malaria pathogenesis. DNA sequences of the human CD36 gene present in chromo-

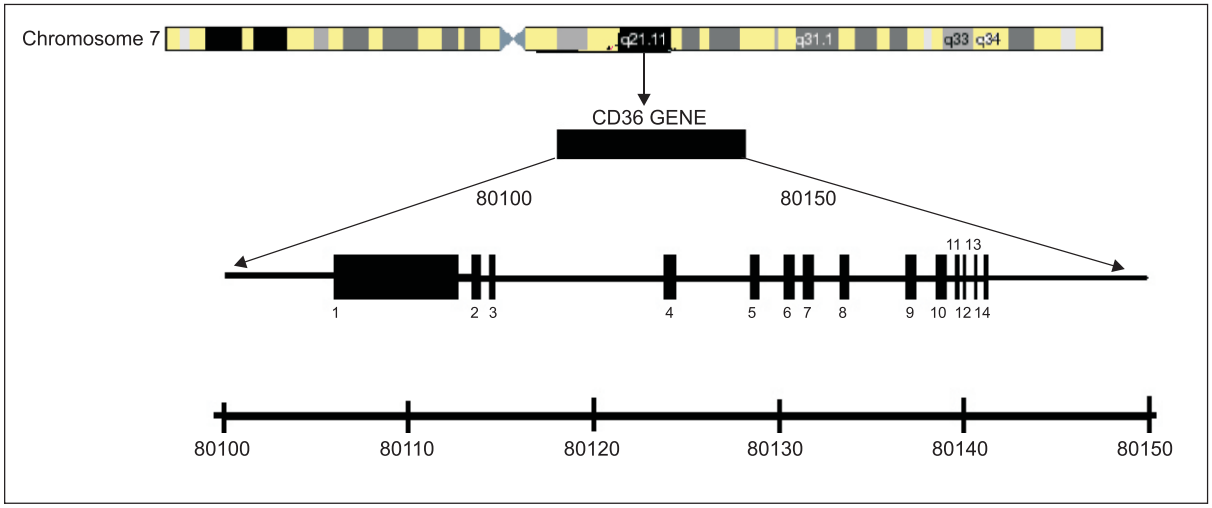

Fig. 2.3.5: Location of human CD36 gene at the locus 7q21.11 on the chromosome 7 showing 14 exons (excluding the untranslated region) (Figure not in scale)

some 7 (Fig. 2.3.5) was retrieved from public domain and fine-scale details were characterized (Fig. 2.3.6). Both comparative and evolutionary analyses were performed with sequences from six other taxa, namely *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Macaca mulatta*, *Canis familiaris* and *Gallus gallus*, where CD36 homologs are present. Different statistical analyses were also performed. We detected differential distribution in the lengths of exons and introns in CD36 gene across seven taxa (Fig. 2.3.7). The cpG islands were also found to be distributed unevenly across the gene and taxa. We constructed neighbour-joining tree and observed that the chimpanzee and human are diverged at the CD36 gene relatively recently. The chicken, *Gallus gallus* was found to be diverged from rest of the taxa sig-

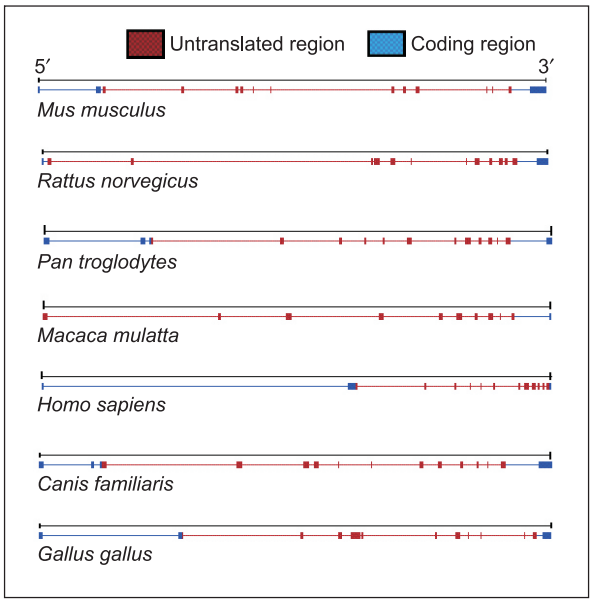

Fig. 2.3.6: Detailed characterization of CD36 gene in different taxa showing the position of coding (exons) and non-coding (introns) (Figure not in scale)

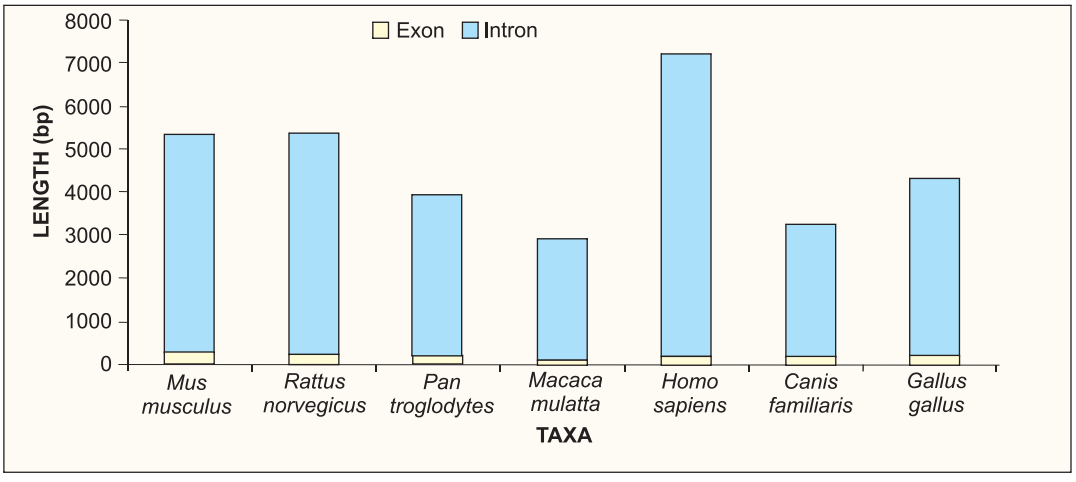

Fig. 2.3.7: Relative composition of exon and intron in CD36 gene among different taxa

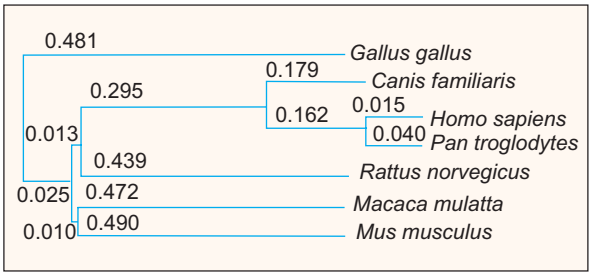

Fig. 2.3.8: Phylogenetic status of different taxa at CD36 gene. Values depict length of each branch leading to a single taxon

nificantly (Fig. 2.3.8). Gene copy number variation was observed across different taxa (Fig. 2.3.9). Comparative genomic study of a human immune-system gene was conducted for the first time in CD36 gene, which revealed relationships among different taxa at the evolutionary level. The information can be used for future study in this gene at the molecular level, especially the level of genetic diversity in malaria endemic zones and correlate it further with malaria pathogenecity.

"Cluster analysis of cytokines revealed that balance of inflammation mediates host defence against vivax infection and differential cytokine responses correlate with clinical outcomes and triggers clinical immunity during active clinical malaria"

2.3.3 *In silico* Genetic Characterization and Evolutionary Inference of TNF-α

TNF-α is an important human cytokine that imparts dualism in malaria pathogenecity. At high dosages TNF-α is believed to exhibit pathogenecity against cerebral malaria and at lower dosages TNF-α is protective against severe human malaria. In order to understand the human TNF-α gene closely and to ascertain evolutionary aspect of its dualistic nature on malaria pathogenecity, we first characterized this gene in detail in six different mammalian taxa. The avian taxa, *Gallus gallus* were

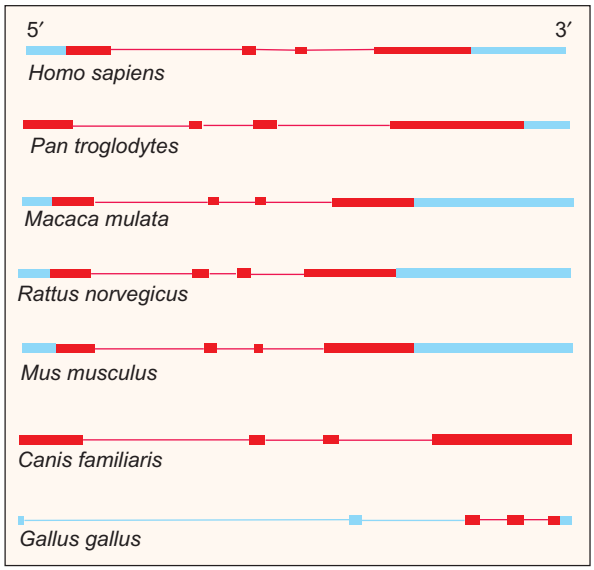

Fig. 2.3.10: Fine-scale characterization of TNF-α gene among six mammalian taxa with coding exons (red) and untranslated region (UTR) or non-coding exons (blue). For *G. gallus* information on the TNF-β has been provided. The length of non-coding exons, coding exons and introns are not in scale.

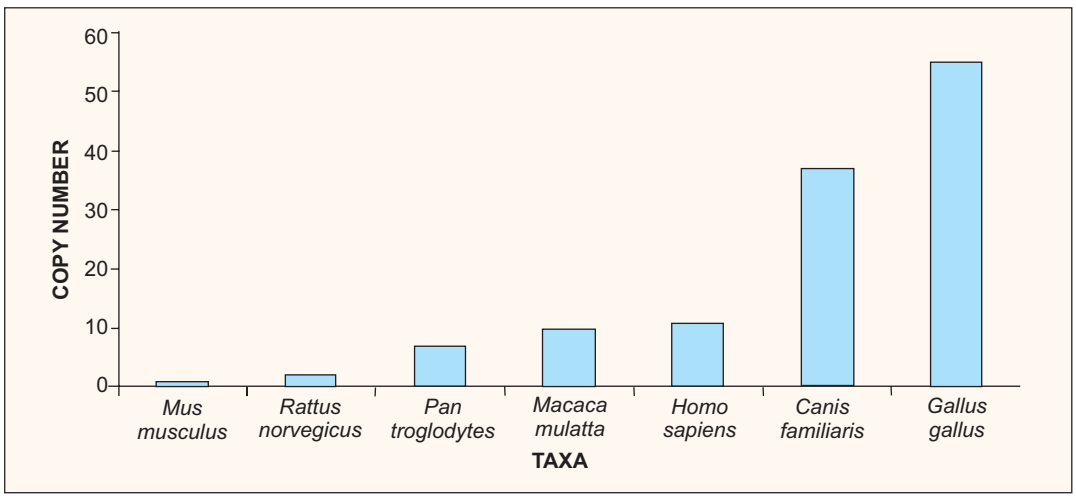

Fig.2.3.9: Copy numbers of CD36 gene in different taxa

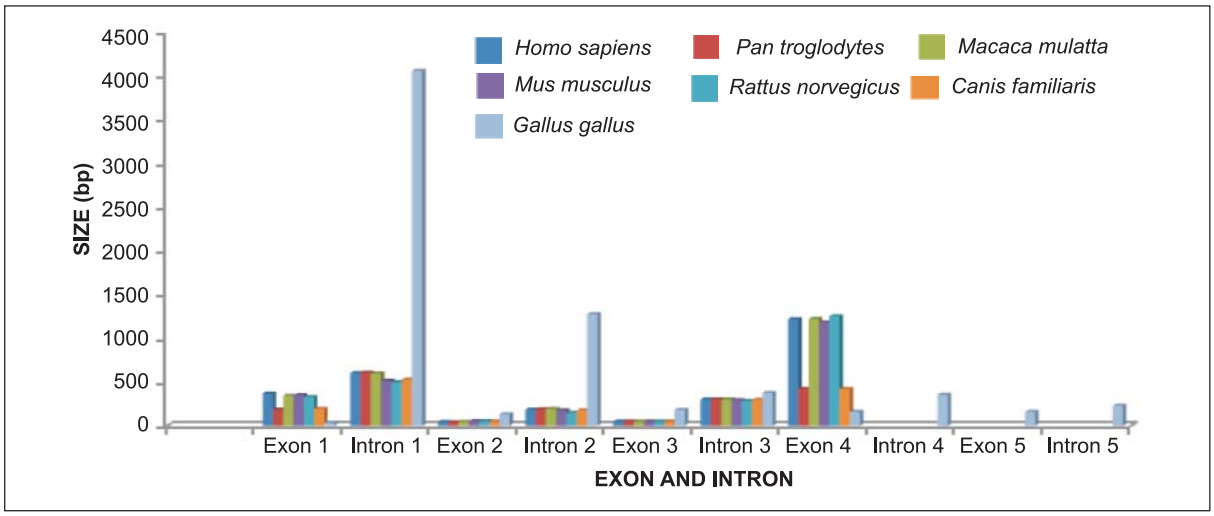

Fig. 2.3.11: Size distribution of exons and introns across all the seven taxa

included in the present study, as TNF-α is not present in birds, therefore, a tandemly placed duplicate of TNF-α (LT-α or TNF-α) was included in this study (Fig. 2.3.10). Comparative study was performed on nucleotide length variation, intron and exon size and number variation, differential compositions of coding to the non-coding bases etc. to look for similarities/dissimilarities at the TNF-α gene across all seven taxa (Fig. 2.3.11 & 2.3.12). The

"Genetic characterisation of human TNF-α gene and comparison among six mammalian taxa ascertain evolutionary aspects and dualistic nature in malaria pathogenecity"

"Comparative study of P. falciparum functional genes with other Plasmodium species signifies chromosome and intron evolution"

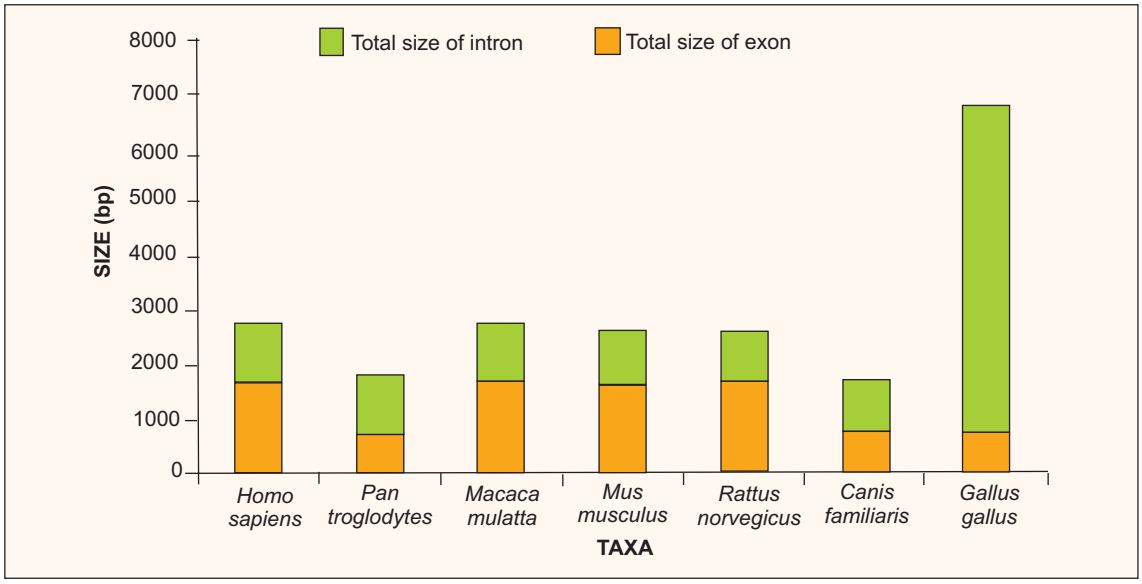

Fig. 2.3.12: Proportion of total coding and non-coding nucleotide in TNF-α gene across different taxa

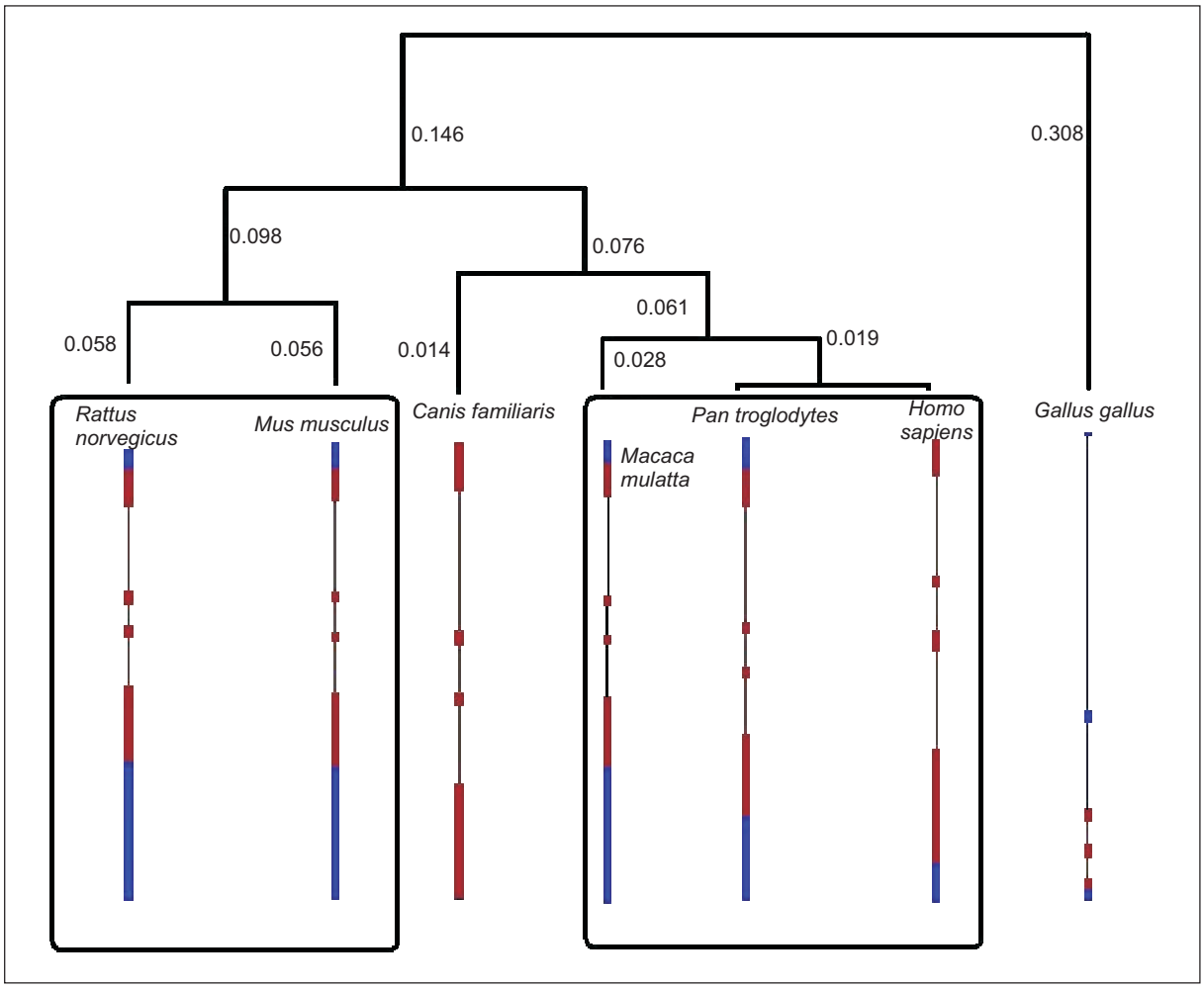

Fig. 2.3.13: Taxonomic position of different taxa in an unrooted Neighbour-Joining tree at the TNF-α gene. The branch length values are shown at each branch leading to taxa. In case of *G. gallus*, the TNF- β gene has been considered, as the TNF-α gene is not present in the birds

phylogenetic study revealed the pattern found in other genes, as human, chimpanzee and rhesus monkey were placed in a single clade and rat and mouse in another, with the *G. gallus* in a clearly separate branch (Fig. 2.3.13). We further focused on these three taxa and aligned the amino acid sequences and found fewer differences between human and chimpanjee but great differences were observed in rhesus monkey from the other two taxa. Further, comparison of coding and non-coding nucleotide length variations and coding to non-coding nucleotide ratio between TNF-α and TNF- β among these three mammalian taxa provided a first-hand indication on the role of TNF-α gene, not its duplicate TNF-β in dualistic nature of TNF-α in malaria pathogenecity. ❑