General Discussion

Molecular and genetic epidemiology is a hybrid discipline whose ultimate aim is to identify and to characterize population level factors that contribute to a disease. Genetic epidemiologists often pursue this aim through the design and implementation of studies that simultaneously invoke principles in population genetics, epidemiology, molecular biology and biostatistics. However, traditional research in genetic epidemiology has barely tapped the potential that these disciplines have to work together. It is our view that current and future genetic epidemiology inquiry will benefit greatly from stronger integration of these disciplines. The molecular and genetic epidemiology of malaria parasite is an excellent area of investigation which needs an integrated approach considering jointly the genetic polymorphism of the pathogen, the host and the vector, and their interactions for combating the disease concerning all the disciplines together.

One of the primary aims of our present research was to understand the epidemiology of malaria parasite in an endemic population and to identify and characterize genes that influence the pathogenicity of this particular disease. To start with, we undertook yearly surveys on mosquito vector and host populations of Calcutta metropolis, since the city was facing malaria epidemics in certain parts for last few years. We tried to identify the factors affecting susceptibility to malaria infections in both vertebrate as well as invertebrate hosts. Further, we attempted to comprehend the malaria parasite strain specificity and virulence in localized geographical niche. When certain facts responsible for the outcome of the disease were clarified, we approached for molecular characterization of a particular gene which is responsible for
pathogenecity of the disease. Assuming that the gene expression product may have a potential for developing anti-parasitic inhibitor, we finally explored the immune mechanism and innate factors of the host which are crucial for acquiring effective host resistance against invading pathogens by therapeutic approaches.

In the first chapter of the thesis we described the epidemiological survey on vector mosquitoes. Three year long (1995-1997) epidemiological survey revealed persistence of *Anopheles stephensi* as the major urban vector for malaria transmission in Calcutta metropolis. To recognize whether the density of the vectors in the specific biotopes is sufficient for malaria transmission, an in-depth survey of the distribution of mosquitoes in different ecogeographical zone was undertaken which revealed that for all these years, statistically significant positive correlation can be observed between larval and subsequent adult density. Determination of the density of anopheline mosquitoes by sampling the larva as well as adults revealed predominance of six major Anopheline in this metropolis. The picture of vector dispersal emphasized the necessity of understanding the susceptibility status of anophelines to malaria parasites. The comparative susceptibility study between *Anopheles stephensi* of two different geographical niche Calcutta and Delhi recognized the competence of vector mosquitoes with three different parasite load, viz. *Plasmodium vivax*, *Plasmodium falciparum* and *Plasmodium yoelii yoelii*. The observations suggested that in view of the differences in oocyst infection rate, Delhi anopheline species is much more susceptible in producing ample amount of viable oocyst than their Calcutta counterpart. Similar result was also found for sporozoite positivity and it was observed, in case of vivax and falciparum, the amount of sporozoite production was much higher compared to yoelii species of
Plasmodium. The above observations therefore indicated that the Plasmodium oocyst and sporozoite load is very much Anopheline strain specific and it varies as a function of oocyst and sporozoite production with place and/or population effect. To corroborate the above fact with malaria endemicity of Calcutta and Delhi in context of survival of anopheline hosts we conducted the infectivity assay by measuring the survival rate of the different strains of Anopheles stephensi with parasite load from both Delhi and Calcutta through immediate and delayed capture method. It has been observed from the result of the above experiment that there is a sharp decline in the survivality of A. stephensi from Delhi than that of Calcutta with malaria parasite load. The overall result of susceptibility and infectivity analysis summarized that the potential of malaria transmission is not only dependent on the capacity of vectors to bear parasite, it also depends on the longevity of malaria vectors with infected parasites in a particular eco-geographical niche. Therefore, it has been presumed that vectoral capacity of a particular strain of vector and its susceptibility is genetically determined and the competence of the vector to transmit infection is truly dependent on the malaria parasite retention capacity. To differentiate vector population according to their dispersal specificity in endemic areas, we tested some arbitrary oligo based molecular markers which were supposed to generate ample amount of polymorphisms for easy distinction of Anopheles stephensi strains. Molecular marker analysis revealed that some particular markers can be assigned specifically for distinct geographically isolated strains of Anopheles stephensi. Now, to identify the preferential adaptive genotypes of different strains in accordance with their endemically variable eco-geographical niche, we analyzed four different strains of Anopheles stephensi, with ascribed endemicity, by isozyme analysis. Thus, genotype preferences were identified for each strain of Anopheles stephensi. It has been understood from the present
study that in a defined malaria endemic area the possible presence of genetic lines within an insect vector species that may be more or less efficient in disease transmission would be important in terms of epidemiology and could influence appropriate vector control measures.

In the second chapter of the thesis, we emphasized on understanding the human epidemiology in malaria prone areas, factors that influence malaria transmission and immune selection and identifying genotypes which are preferred by natural selection against pathogenesis of malaria. As all diseases and all phenotypes generally are, under the simultaneous influence of genes and environmental stimuli, we identified rain and temperature as important climatic factors and, age and gender specificity as crucial parameters affecting malaria pathogenesis. To understand the dynamics of the host parasite balance, its relationship to transmission, and to identify those factors that contribute to the development of the disease in human, epidemiological studies were carried out in well defined cohorts in which malaria parameters are recorded longitudinally. Clinically, the most striking observation was the very short duration of a high proportion of fever episodes during malaria attacks regardless of the age of the patients and the lack of any marked diminution of the severity of most presenting symptoms according to age. Moreover, we found that seasonality in malaria incidences was different for *Plasmodium vivax* and *Plasmodium falciparum* infections. The seasonal indices for vivax malaria is found highest in the month of June and lowest in the month of December which is just reverse in case of falciparum infection. From the overall distribution of vivax and falciparum malaria over four years (1995-1998), it has become clear that vivax infections remain in the niche always in a penetrable limit though in dry season falciparum cases go quiescent. Relationships between climate and disease
occurred at varying levels. In Calcutta metropolis, maximum precipitation is expected in July and August, while the dry season starts from November and persists till May. In case of Temperature, almost above ambient temperature persists throughout the year in day time and it reaches the highest peak in May and June. Our result indicates that there is a statistically significant correlation between mean rainfall and arithmetic mean of Plasmodium vivax and Plasmodium falciparum cases. Statistically significant correlation was found between mean temperature and arithmetic mean of malaria cases for Plasmodium vivax but not for Plasmodium falciparum cases. Since changes in the precipitation (rainfall) and temperature can have marked effect on the intensity of the transmission of the malaria parasite, our study on vector dispersal (in the first chapter) and malaria transmission in human population correlates to the varying degree of malaria endemicity and seasonality of malaria incidences in Calcutta metropolis. While investigating the physiological variables concerned to malaria transmission, it has been found that gender bias has a direct association with host innate factors and age distribution has a correlation with immune selection in the pathogenicity of malaria. In the present survey, it has been found that irrespective of the type of infection (either Plasmodium falciparum or Plasmodium vivax) males are more prone to malaria infection than females. During exploration of age distribution in malaria cases it has been found that there are certain peak shifts in the infection levels, significantly associated with different age groups. This fact may be attributed to persistence of high amount of polymorphism among malaria parasite strains which probably helps the parasite to evade immune response of the hosts in selective age groups. Finally, to identify the preference of genotypes of innate resistant factor in human host in malaria prone individuals for clinically defining the disease status, heterogeneity in gene expression of G6PD was
investigated. This results indicate that against vivax infection, there is protective preference to particular homozygotes and in falciaprum cases, protective preference owes to heterozygotes. Moreover, our observations clearly indicates that G6PD deficient individuals does not necessarily provides protection against malaria.

In the third chapter of the thesis we explored the genetic diversity of malaria parasites (both *Plasmodium vivax* and *Plasmodium falciparum*) in Indian subcontinent, including Calcutta metropolis, in context of epidemiological tracking of malaria incidences, as discussed in chapter two. Some abrupt peaks in annual malaria incidences are presumed to be associated with emergence of new strains of malaria parasites in a localized endemic area. In recent years, reports have described *Plamodium* alleles associated with differences in disease outcome. The diversity of such population structures associated with these alleles is, in part, influenced by intensity of transmission. Human genes associated with susceptibility to malaria are likely to influence the parasite population structure. HLA (Human Leucocyte Antigen) type has been shown to affect the the parasite strains associated with clinical malaria. We therefore intended to know how much clonality prevails in native *Plasmodium* population structure. Random markers are chosen in the hope that they will be linked to a quantitative trait loci (QTL) influencing the susceptible trait of pathogenecity. Among these random markers, while RAPD can generate enormous amount of genetic polymorphisms from malaria parasites, SSR can powerfully detect the genetic identity of malaria parasites from a single infected sample and subsequently overcomes the sampling bias. In our study, the extent of genetic differentiation among the isolates of *Plasmodium falciparum* analyzed by RAPD and microsatellite markers revealed that there was a significant sign of
clonal propagation among different strains. In case of *Plasmodium vivax*, the structure of parasite population is prominently more diverse. It can be argued from our findings that modifications in the density of a specific genotype, distributed throughout different geographical locations, could be due to strain competition and strain specific immune response. Our study emphasizes that, it is essential to consider host factors while studying the parasite population structure with respect to clinical manifestations susceptibility genes and immunologic responses.

In the fourth chapter of the thesis we dealt with a novel gene of malaria parasite which directly influences pathogenicity of the disease. Malaria parasites are observed to be very sensitive when exposed to oxidative stress and therefore role of this particular gene, G6PD, remains crucial in this organism. Studies on the metabolic pathways of Plasmodium have led to the identification of many characters unique to the parasite. The first step to the rational development of parasite specific inhibitor involves identification of enzyme targets in the parasite. Since G6PD is involved in the nucleic acid synthesis of malaria parasite and also responsible for the outcome of the disease, characterization of this enzyme may open up the possibility of design of a new anti-parasitic inhibitor against malaria. The advantage of G6PD is that, since it is a house-keeping gene, intraspecific is more or less limited. We first characterized the G6PD protein in *Plasmodium vivax* and found that it has an unusually large size which is in near homology to already characterized *Plasmodium falciparum* G6PD. We therefore studied the gene structure of G6PD in the same parasite species and compared with that of *Plasmodium falciparum*. Upon sequencing, a 120 base homologous region was identified between both the parasite species which was presumed to code for a 40 residue peptide. This homologous region
did not show any similarity with human G6PD. The predicted peptide sequence was analyzed for its biochemical nature and it has been found that there is high probability of the peptide to act as an antigen and this has been supported by Antigenic index analysis. Our result demonstrated that the G6PD protein of Plasmodium vivax and Plasmodium falciparum has an accurate sequence homology at the DNA level and the predicted protein structure indeed supports the hypothesis of utilising G6PD as a cytoplasmic antigen for drug or vaccine development.

Finally, we tried to understand the immunological status and related interaction among host and pathogen in the fifth chapter of the thesis. Current models in immunity to blood stages of plasmodium invoke a primary role for T cell mediated immune response in malaria. Such interaction involves different human innate factors like HLA antigens and T cell receptor repertoire. Functional contribution of T cells in malaria encompasses HLA antigen heterogeneity and selective alteration in the molecular structure of T cell receptor which ultimately persuades protective immunity against human malaria. To understand the structural as well as functional influence of the innate immune factors in malaria pathogenesis, we investigated the expression of primary T cell subtypes, their interaction with HLA antigens and structural modifications in T cell receptor subsets which actually can beseech therapeutic approaches in malaria infection. Our study on the activation of CD4 and CD8 T cells in acute malaria infection provided strong evidence for upregulation of CD8+ cytotoxic T cells. An overall increase in the percent distribution of CD8+ T cell subset over CD4+ cells has been observed both for Plasmodium falciparum and Plasmodium vivax cases. Specific HLA class I and Class II antigen restrictions are observed in infection with malaria parasite. it has been
found that the allelic frequency of HLA A26 in malaria patient is predominantly higher than those in the control group. Similar association with HLA A11 allele was also observed, although in low frequency, among malaria patients. Further study implicated a protective advantage to individuals expressing HLA B35 over malaria patients and an association between malaria and HLA DR3 was also established. Finally we look forwarded to see the alteration in the structure of γδ T cell receptors since this T cell population expands in the peripheral blood in an oligoclonal or polyclonal fashion and remains elevated for weeks after successful chemotherapy. Our intention was to know which particular allelic aggregation in the V-D-J joining region of δ chain of γδ T cell receptors actually interplays in Plasmodium infection. Two different types of transcripts were recognized from the sequencing data: germline Jδ1 spliced to Cδ and germline Jδ2 spliced to Cδ. Though limited germline diversity has been identified, there was unprecedented variability observed in the V-J junctional region. It has been observed that most of the transcripts arose from Vδ2. Two different V region transcripts are observed: Vδ1 and Vδ2. Nine V-D-J rearrangements were sequence analysed and each was unique indicating polyclonality of the population of cells studied. Extensive N region insertions were also observed in the 5' and 3' regions of the δD sequences. Though there was a specificity in Dδ2 and Dδ3 regions, frequent mutations were identified in their usage as well as in the arrangements of recombination signals. It has been further identified that most of the Vδ2 was recombined either with either Jδ1 or Jδ3 among which usage of Jδ1 is predominant. Expression of the diverse set of TCR δ chains in malaria patients supports the hypothesis that these cells contribute to the immunopathology of malaria and is consistent with the T cell activation reported in the early part of the chapter. This findings indicate that different combinatorial arrangements of the gamma-delta heterodimer structures
could be anticipated in adoptive immunotherapy in malaria infections for an highly endemic area in due course.

The complex human and parasite determinants that influence the disease severity in *Plasmodium* malaria reflect thousands of years of selective pressure. We hope, our small contribution in the field of research in the molecular and genetic epidemiology of malaria will provide an insight in understanding the disease malaria at large.