CHAPTER I:

Introduction
1. **INTRODUCTION**

Malaria remains a major global health problem with 300 to 500 million clinical infections and more than a million deaths reported each year. In 2009, World Health Organization (WHO) reported 225 million malaria cases and 781000 deaths from 106 endemic countries. Majority of the cases were from African countries (78%) and the contribution from South East Asian and Mediterranean regions were 15% and 5% respectively. About 85% of the deaths caused by malaria were in children under 5 years of age\(^1\). It is commonly associated with poverty and is also an important cause of poverty slowing down economic growth by 1.3% per year in endemic areas. A 10% reduction in disease was associated with 0.3% higher growth\(^2\). In India, retrospective analysis of burden of malaria showed that disability adjusted life years lost due to it was 1.86 million years\(^3\).

In the South East Asia Region (SEAR), approximately 70% of the total population is at risk of malaria, of which three countries, India (65%), Myanmar (20%) and Indonesia (12%) accounted for 94% of the reported cases\(^1\). 60.5% of all infection were caused by *Plasmodium falciparum*. Malaria is endemic in the Indian state of Assam contributing more than 5% of the total cases recorded in the country annually. Malarial outbreaks characterized by enhanced morbidity and mortality are common across the state\(^4\). The endemicity of malaria is not uniform across the state with many pockets along forest fringes, forest and foothill villages particularly along the inter-country/inter-state border vulnerable to malaria outbreaks\(^5\). These high risk zones are predominantly inhabited by indigenous tribal populations of distinct ethnic backgrounds. Most of these affected areas report widespread resistance to first line drugs like chloroquine, sulphadoxine-pyrimethamine and even quinine resistance is being reported. In fact, resistance to chloroquine was first reported from Karbi Anglong district of Assam in India\(^6\).

Human malaria is caused by four distinct species of *Plasmodium* viz *P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax*. The malarial parasite has a
complex life cycle (Fig 1.1) involving both female anopheline mosquito (sexual phase) and human (asexual phase) as host.

Fig 1.1: Life cycle of *Plasmodium falciparum*. (Adapted from Crompton *et al.* 2010).

Malaria causes a wide variety of symptoms ranging from asymptomatic infection to very mild symptoms to severe (complicated) disease and even death. Based on the clinical presentation, the disease may be divided into uncomplicated and complicated malaria as per WHO guidelines.

Strategies to control malaria have been redefined with effective diagnosis and treatment measures along with vector control interventions like Insecticide
Residual spray and use of insecticide treated mosquito nets. However, with emergence of insecticide resistant mosquitoes and the parasites developing resistance against conventional antimalarial drugs, it poses challenge to malaria control. Recently, artemisinin resistance has been reported from the Greater Mekong subregion and the threat of its spread to other areas is a serious issue\(^1\). Notably, chloroquine resistance in \(P.\) \(falciparum\) was first detected from South East Asia region\(^8\).

Vaccination is considered an important control measure which has long been pursued but with little progress. Studies of various vaccine candidates including those of the liver stage and asexual blood stage candidate reported limited success in earlier attempts\(^9\). Combination B vaccine consisting of merozoite surface proteins 1 and 2 (MSP-1, MSP-2) and ring stage infected erythrocyte surface antigen (RESA) showed a 62% reduction in parasite density in vaccinees\(^10\). Recently a malaria vaccine candidate RTS, S, has entered phase III trial in Kenya\(^11\). The difficulties in development of a malaria vaccine may be attributed to the complex life cycles and antigenic polymorphism exhibited by the parasite and compounded by a poor understanding of protective immune mechanism\(^12\). Besides, the disease shows variation in epidemiology across different endemic regions\(^13\).

The genetic structure of \(P.\) \(falciparum\) has been demonstrated to be highly diverse world over\(^14\). Major vaccine candidate genes exhibit antigenic diversity in the form of allelic polymorphism where the alternate forms of antigen coding genes exist. Mention can be made of the merozoite surface proteins (MSPs), the serine repeat antigen (SERA) genes families. Additionally diversity may be generated by clone multiplicity where a clonal lineage of parasite expresses alternate forms of an antigen without changes in genotype. Variations in genes encoding antigens are also generated by non meiotic recombination such as strand slippage events, gene conversion as well as homologous recombination during meiosis\(^16\). This extensive diversity in \(Plasmodium falciparum\) antigens is seen as immune evasion mechanism of the parasite. Repetitive sequences constitute immunodominant epitopes in parasite
proteins, therefore sequence variation in allelic forms of the molecules may prevent the population from developing immunity to different strains of the parasite\textsuperscript{17}. Elucidating the extent of genetic variation in the malaria parasite will therefore be central to decreasing the malaria disease burden\textsuperscript{18}. The non uniform population structure of \textit{P. falciparum} worldwide depends upon local factors related to parasite, vector and host factors\textsuperscript{19}. Factors like transmission intensity and drug treatment too influence the genetic diversity of the parasite\textsuperscript{14}.

Allelic diversity and size polymorphism of merozoite surface protein 1(MSP-1) gene have been employed as a molecular marker in studies of malaria transmission dynamics and host immunity in \textit{P. falciparum} malaria. MSP-1 gene has also been extensively studied as it has a role in invasion, disease and immune evasion\textsuperscript{14}. MSP-1 gene is divided into 17 blocks, based on analysis of sequence diversity. Block 2 region near the N terminus and block 17 at the C terminus are major targets of host immunity. The block 2 of MSP-1 is described by 3 distinct allelic families MAD20, K1 and RO33 and contains antigenically unique tripeptide repeats with extensive diversity in the number of repeats\textsuperscript{20}. Significantly, block 2 repeats are targeted by \textit{P. falciparum}-inhibitory monoclonal antibodies and naturally acquired antibodies associated with clinical immunity in humans\textsuperscript{15}. A comprehensive understanding of the immune responses lies in dissecting the antigenic diversity of \textit{P. falciparum}.

Immune response to malaria is complex and is species and stage specific. Immunity to malaria can be either anti disease immunity which confers protection against clinical disease or anti infection immunity conferring protection against parasitaemia\textsuperscript{21}. Naturally acquired immunity to malaria develops slowly and is rarely sterile. In holoendemic or hyperendemic areas older population generally develops immunity showing asymptomatic parasitaemia while in areas of low malaria transmission, individuals do not develop immunity in an age dependent manner. The infection and disease can therefore occur in both children and adults\textsuperscript{22}. The picture that emerges from human studies is that immunity to malaria infection is relatively slow to develop.
and incomplete, although immunity to disease is acquired more quickly and may be important after a single episode. Acquired immunity involves both the antibody mediated and cell mediated immune response.

Malaria infection induces strong humoral immune responses, involving production of predominantly IgM and IgG but also other immunoglobulin isotypes. Species as well as stage specific antibodies against wide variety of parasite antigens have been reported while a large proportion of the antibodies are non-malaria specific. The importance of antibodies as mediators of protective immunity to malaria is well established in both animal and human infections. Mice lacking B cells were unable to clear parasites from *P. chabaudi* infection, rather such mice developed chronic parasitaemia. Passive transfer of monoclonal antibodies against parasite antigens conferred protection in naive mice by reducing parasitaemia and clinical disease. In humans, treatment of *P. falciparum*-infected patients from Thailand with IgG extracted from African immune adults, resulted in reduction of parasitemia and clinical symptoms. Studies in humans and mice show that memory B cells are either poorly induced or short lived as a result of infection. Recent studies showed that memory B cells and antibodies increased gradually over many years and were dependent on cumulative exposure to malaria. Despite the importance of antibody responses for protection against malaria, not all antibodies are reported to be protective. Polyclonal antibody specific to MSP-2 but not monoclonal specific to the same antigen enhanced invasion of multiple merozoites into RBC.

Protective antibodies primarily target the asexual stage antigens of which MSPs are leading blood stage vaccine candidate molecules of *Plasmodium falciparum*. MSP-1 is the major surface protein of the parasite, which is synthesized as a 190 kDa precursor protein. It undergoes proteolytical cleavage into four fragments and remains attached to merozoite surface by glycosylphosphatidylinositol anchor prior to invasion. During erythrocyte invasion, majority of the MSP-1 complex is shed of which only the 19 kDa C-terminal fragment remains anchored on the merozoite surface. Antibodies
targeted to this fragment are shown to inhibit erythrocyte invasion and associated with protection from clinical malaria\textsuperscript{30}. MSP-1\textsubscript{19} fragment has a highly conserved sequence however at least six single nucleotide polymorphisms (SNPs) have been identified in its two epidermal growth factor like (EGF-like) domains\textsuperscript{31}. Allele specific as well as cross-reactive antibody responses to variants of MSP-1\textsubscript{19} fragment have been reported\textsuperscript{32}. Further, certain MSP-1\textsubscript{19} polymorphisms have been implicated as particularly important to immunity\textsuperscript{31}.

Cell mediated immunity (CMI) has crucial roles in protective immunity to malaria but also has the potential to cause tissue pathology and contribute to the development of severe malaria\textsuperscript{33}. Vaccine formulations with T cell epitopes induce a strong memory response\textsuperscript{9}. The CD4 T-cell subset is of major importance for the induction of blood-stage immunity in both murine and human malaria, while the CD8 subset has been shown to be cytolytic against liver stages of the parasite\textsuperscript{28}. In experimental mice CD4 T cells could act independent of B cells in resolution of parasitaemia\textsuperscript{34}. Humans lacking previous exposure to \textit{P. falciparum} as well as malaria exposed individuals have CD4 T cells that proliferate and secrete IFN\gamma in response to parasite antigen and inhibit parasite growth in vitro\textsuperscript{35}. Another subset of CD4+ T lymphocytes, T-regulatory (T-reg) cells is postulated to be involved in immunosuppression where effectors responses were enhanced in the absence of Tregs\textsuperscript{33}.

T cells also play a central role in the elimination of blood stage malaria parasites through the release of cytokines that activate other effectors cells\textsuperscript{28}. Specific cytokine profiles are associated with different clinical manifestations. Th1 cells activated macrophages and other cells to produce mediators through release of inflammatory cytokine\textsuperscript{36}. Th1 were seen to be responsible in the initial resolution of acute parasitemia through production of IFN\gamma while Th2 were required for eventual clearance of the parasites via T-B cell cooperation\textsuperscript{37}. The balance between the Th1 and Th2 immune response may determine the level of parasitaemia and disease outcome. An early and sustained Th1 response was critically linked to IL-12 through IFN-\gamma production\textsuperscript{38}. Recent evidence suggest that rapid cell mediated immune responses are contributed by effector cells of
NK and T cells lineages which contain the initial stages of malaria through IFN-γ production\(^39\).

Natural killer (NK) cells are lymphocytes of the innate immune system that are involved in the early defense against foreign cells and autologous cells undergoing various forms of stress, such as microbial infection or tumor transformation. NK cells also influence adaptive immunity by modulating dendritic cell function and by inducing Th1 polarization via IFN-γ production\(^40\). NK cells and NK Receptor (NKR) positive cells are suggested to significantly control susceptibility and resistance to both malaria infection and severe disease syndromes. This was seen to depend on the receptors encoded within the Natural Killer Cell Receptor Complex (NKC)\(^41\). NK cells were seen to have direct contact with \(P. falciparum\) infected red blood cell via NK Cell receptors\(^42\). Further, NKR positive γδT cells were seen as the major source of IFN γ in response to \(P. falciparum\) infection in humans as well as in murine models which were in part controlled by NKR loci\(^41\).

NKRs are largely encoded by two genetic loci in humans: the Natural Killer Complex (NKC) on chromosome 12 and the Killer cell Immunoglobulin-like receptors (KIRs) region on chromosome 19. Diverse set of inhibitory and activating NKRs controlled NK cell activation and the outcome of NK cell activity is the balance of signals from activating and inhibitory receptors\(^43\).

The KIR gene complex is characterized by variation in gene content. Studies suggest that they are rapidly evolving genes which lack conservation among species and exhibit remarkable diversity as haplotypic variation in gene number and content and allelic polymorphism of individual genes\(^43, 44\). Two broad haplotype groups termed A and B are used to define variation in KIR genetic profile\(^44\). Frequencies of specific \(KIR\) haplotypes vary across different ethnic populations. The important role of KIR in the immune response and its genomic diversity coupled with its specificity for HLA ligands, affects resistance and susceptibility to pathogenesis of a number of infectious and autoimmune diseases\(^43\). NK cells response to parasitized RBCs was noted to vary significantly.
between individuals and that the variation was associated with KIR genotype of an individual\textsuperscript{12}.

North East India is inhabited by people of various ethnic origins with a history of malaria endemicity. Malaria remains a major public health problem in the state of Assam. Nearly 65\% of the total population of the state (26.6 million) is estimated to be living in high-risk areas\textsuperscript{45}. Malaria transmission is perennial and continues to be uninterrupted supported by major vectors namely \textit{An. minimus, An. fluviatilis} and \textit{An. dirus}. The region is highly receptive to malaria transmission due to excessive and prolonged rainfall (2–3 m) promoting vector breeding and longevity due to high humidity (60–90\%) and warmer climates (22–33°C) for most of the year\textsuperscript{46}. \textit{P. falciparum} is the major malarial infection and accounts for 58–68\% of the cases and the remainder are due to \textit{P. vivax}. Despite efforts to contain the disease mortality and morbidity are significantly high due to emergence of drug resistant Pf malaria, delayed diagnosis due to non-availability of facilities in interior villages, hilly areas and tribal belts\textsuperscript{4}. Malaria in Assam is poorly investigated with respect to diversity of the circulating local \textit{Plasmodium falciparum} and immune responses of the population. Besides it is populated by individuals of different ethnic origin. The present study was thus undertaken to understand the diversity of the \textit{Plasmodium falciparum} of the region and the host parasite interactions with an aim to evaluate the protective immune mechanism and markers of protection if any.
Objectives:

1) To study the clone multiplicity of the parasite genotypes existing in the study area

2) To elucidate the protective humoral immune response.

3) To elucidate the protective cell mediated immune response