Malaria is a major public health problem in tropical and subtropical countries including India. It is a widespread disease throughout the world. It causes ample morbidity, mortality and economic loss (Dye, 2013). The word malaria was originated from Italian word “mal aria” means “bed air”. Hippocrates (400 BC) was the first to know about malaria fever and spleen enlargement (Francis, 2010). The causative agent of malaria was firstly discovered in late 1800s by Laveran and Ross (Cox, 2010). Malaria is caused by the species of genus *Plasmodium* which is an obligate intracellular protozoan parasite and approximately, 172 species of *Plasmodium* infects birds, reptiles and mammals, which is transmitted by the bite of an infected female mosquito of genus *Anopheles* which bites mainly between dusk and dawn. There are four main species that causes malaria to human beings i.e. *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Out of these four species, *P. falciparum* and *P. vivax* are the most common species responsible for malaria in India. *P. falciparum* and *P. vivax* can also infect owl, squirrel, monkeys and chimpanzees (Fandeur *et al*., 1995; Contamin *et al*., 2000; Daubersies *et al*., 2000; Sullivan *et al*., 2001). Recently, *P. knowlesi* has also been reported from human being a species which causes malaria among monkeys and prevalent in forested areas of South-East Asia (WHO, 2011). Among all the species of *Plasmodium*, *P. falciparum* is accountable for the most severe form of disease along with severe complications which are potentially fatal.

The estimated prevalence of malaria is 198 million cases and 0.584 million deaths every year (WHO, 2014). Most deaths occur among the children under the age of five years living in Sub-Saharan Africa. Malaria mortality rates have fallen by more than 25% globally since 2000 and by 33% in the WHO African region (WHO, 2011). Most deaths occur among the children living in Africa where a child dies every minute from malaria (WHO, 2011). It is estimated that approximately 0.48 million children were died less than five years of age in 2012, i.e. 1300 children every day or one child almost every minute (WHO 2013). The scale-up of interventions helped to reduce malaria incidence rates by 29% globally and by 31% in the WHO African region in between 2000 and 2012. The global malaria mortality rate was reduced by 45% during the same period, while the decrease in the WHO African region was 49% (WHO, 2013).

Due to scale-up of malaria interventions program, an estimated 3.3 million lives were saved from 2000 to 2012 (WHO, 2013). Approximately 90% or 3 million, of these lives saved are in the under-five years of age group, in sub-Saharan Africa. There were an
estimated 207 million cases of malaria in 2012 and estimated 0.627 million deaths (WHO, 2013). It has been estimated that the malaria mortality rates decreased by 47% worldwide and by 54% in the WHO African region in 2013 (WHO, 2014).

In India, the reported incidence of malaria in last decade has been between 1.5 to 2.0 million cases with 700 to 1500 deaths annually (WHO 2014). WHO has reported that in India in the year 2009, the estimated number of malaria cases was 25 million with 30,000 deaths (WHO, 2009). The most alarming feature of malaria in India since resurgence in 1970s is the increase in the cases of *P. falciparum* from 9% in 1972 to around 50% in 2009 and 2010. The situation has been complicated even more, due to spread of the strains of this parasite which are resistant to chloroquine, the most effective, safe and cost effective drug for the treatment of malaria and other antimalarials like sulphadoxine and Pyrimethamine (SP). Resistance to newer antimalarial drug artemisinin based combinations was reported on the Thailand-Combodia Border in 2009 and has since been reported in Mayenmar and Viet Nam (WHO 2011).

2.1 Drug resistance in Malaria

WHO launched the national malaria control program (NMCP) in India in 1950s. Despite the impressive result of NMCP which declined the malaria infection in India from 75 million to 100,000, but program fail due to the social, technical, operational and financial difficulties. These problems lead to its resurgence in the late 1960s and early 1970s in many parts of the world including India. The control program has been hampered by the spread of drug resistance in parasite and insecticide resistance in mosquito vectors (Farooq, 2004). The most alarming feature of malaria in India is increase in the cases of *P. falciparum* which causes malignant form of disease along with fatal complications and the spread of the strains of this parasite which are resistant to chloroquine to most states of the country (Sharma, 1997). Resistance to Chloroquine was first appeared in Southeast Asia to Africa (Payne, 1987). In India, chloroquine resistance was first detected in Karbi Anglong district in Assam (Sehgal et al., 1973). Gradually it spread toward almost all part of the country.

Due to the chloroquine resistance, combination of sulfadoxine-pyrimethamine (SP) was used as a first line drug to cure malaria. However resistance in SP soon emerged and spread widely (Roper et al., 2003, Nair et al., 2003). In India resistance in *P. falciparum* to SP combination was first detected in Delhi in 1987 (Choudhury et al., 1987).

Due to the resistance in SP from different part of the world, artemisinin class drugs have been widely adopted as first-line drugs. This class of drugs are highly efficacious in eliminating *P. falciparum*-infected erythrocytes and they are well tolerated by almost all
patients (Schlitzer, 2008). However, evidence of growing *P. falciparum* resistance to artemisinin and its derivatives has been reported along the Thai-Cambodian border (Dondorp *et al*., 2009). Artemisinin-based combination therapy (ACT) is the most rapid, reliable and effective treatments to cure patients infected with *P. falciparum* malaria worldwide. In order to maximize the effectiveness of artemisinin and its derivatives and to protect them from the development of resistance, WHO has repeatedly recommended that they be combined with other drugs that have different mechanisms of action and longer half-lives (WHO, 2001; 2006; 2010). Five combinations are currently recommended: artemether–lumefantrine, artesunate–amodiaquine, artesunate–mefloquine, artesunate–sulfadoxine–pyrimethamine and dihydroartemisinin–piperaquine (WHO, 2010). However all the artemisinin combination drug show treatment failure rates in different part of the world except Artemether–lumefantrine combination drug (WHO, 2000-2010). Artemether–lumefantrine remains highly effective in most parts of the world, with the exception of Cambodia (Dondorp *et al*., 2009). The low efficacy of combination drugs might be due to pre-existing high resistance in combination drug with artemisinin. In India, due to the reports of resistance in artesunate -sulfadoxine–pyrimethamine combination drug from North Eastern states, artemether – lumefantrine has been recommended (National Drug Policy on Malaria, 2013). Therefore, due to the resistance in most of the combination drugs, there is a need to develop vaccine which would form a powerful additional component in control strategies for malaria and would be a cost effective adjunct. A number of *Plasmodium* antigens expressed at different stages of the parasite’s life cycle have been characterized for the development of vaccine (Targett and Greenwood, 2008).

### 2.2 Vaccine candidate antigens of *Plasmodium falciparum*

There is a need to develop an effective malaria vaccine, which may prove to be cost effective adjunct to the existing malaria control measures considering the magnitude of the public health problem posed by *P. falciparum* in the tropical areas. Extreme efforts have been made in the development of a human malaria vaccine. Many lines of evidence indicates that human beings may be vaccinated against malaria. Individuals born in endemic areas who survive the first years of exposure continue to develop parasitaemia on natural exposure, but become resistant first to severe, life-threatening malaria and then to clinical disease. Frequent re-exposure is required to maintain this condition of immunity with infection in man, acquired immunity against the blood stages of *Plasmodium* is mediated at least partly by antibody (Cohen *et al*., 1961), demonstrating that clinical protection from malaria is possible, and that immunoglobulin targeting malaria antigens can play a critical role. There are currently no licensed vaccines against malaria or any other human parasite. One research
vaccine against *P. falciparum*, known as RTS,S/AS01, is the most advanced and the first to undergo large-scale phase 3 clinical trial in Africa (Agnandji *et al.*, 2012;).

In search for a vaccine against *P. falciparum*, the agent of the often fatal malaria, many antigens have been characterized, their genes identified and cloned and their sequences are now known. Potential vaccine candidates from the asexual stages of the parasite’s life cycle, include antigens found in Rhopty organelles and on the surface of invasive merozoites. Of particular importance is a family of merozoites surface antigens (MSA-1, 2, 3 & 4), apical membrane antigen-1, Glutamate rich protein (GLURP), Soluble antigen (S-antigen), Erythrocyte Binding Antigen-175 (EBA-175), and a sporozoite antigen, the circumsporozoite proteins (CSP). The design of antimalarial vaccine is hampered by extensive polymorphism in *Plasmodium* proteins, particularly those expressed on the parasites surface, which are obvious targets for the development of highly specific vaccines (Kaslow *et al.*, 1994; Conway *et al.*, 1997; Takala and Plowe, 2009). Highly polymorphic regions have been observed in the genes encoding surface antigenic proteins such as circumsporozoite protein (CSP) merozoite surface proteins 1 & 2 (MSP-1, MSP2) (Anders and Saul, 1994) of *P. falciparum*.

### 2.2.1 Merozoite Surface Protein – 1 (MSP-1)

Merozoite surface protein–1 (MSP-1) is a high molecular weight (180 – 230 kDa) immunodominant protein also known as P-190 (Mackay *et al.*, 1985), GP-195 (Weber *et al.*, 1986) and precursor to major merozoite surface antigen (PMMSA) (Peterson *et al.*, 1988). MSP-1 synthesized in the schizonts and is expressed on the surface of the merozoites (Holder, 1988). According to Tanabe *et al.*, 1987, MSP-1 of *P. falciparum* is divided into 17 blocks (at the amino acid level) which constituted by conserved, semi conserved and polymorphic region. There are seven blocks (blocks 2, 4, 6, 8, 10, 14, and 16) are highly variable; five blocks (blocks 7, 9, 11, 13, and 15) are semi-conserved, and five blocks (blocks 1, 3, 5, 12, and 17 are conserved (Figure-2.1). The major parts of the variable blocks are dimorphic and represent basically two allelic forms. The block 2 region is the exception which contains set of repetitive tripeptides (Certa *et al.*, 1987, Peterson *et al.*, 1988). On the basis of tripeptide repeats in Block -2 region, three different allelic forms are found *i.e* K-1, MAD-20 and RO33 (Viriyakosol *et al.*, 1995, Farooq *et al.*, 2006). Thus there is large variability in the structure of MSP-1 of *P. falciparum*. Extensive genetic polymorphism in MSP-1 gene of *P. falciparum* has been reported from different part of world (Snewin *et al.*, 1991, Konate *et al.*, 1999, Zakeri *et al.*, 2005). Similarly, MSP-1 gene polymorphism has also been reported from north-western and central part of India (Ranjit *et al.*, 1999, Joshi *et al.*, 2007, Farooq *et al.*, 2006).
It has been reported that the polymorphic blocks of MSP-1 are immunogenic and capable of producing antibodies (Cavanaugh et al., 1998). The nature of diversity in 19 kDa region (C-terminal) of MSP-1 can be used to design vaccine constructs that induce immunity against all known variant forms of parasite (Qari et al., 1998). Vaccination with MSP142 formulated in water-in-oil adjuvants in Aotus monkey induced protection in Aotus nancymai monkeys experimentally infected with P. falciparum, and protection from high parasitemia was correlated with antibody levels and in vitro growth inhibition (Singh et al., 2006). Further in Aotus monkey adjuvant dependent antibody responses was observed, higher levels of protection seen in the groups receiving antigen with complete/incomplete Freund’s Adjuvant or ISA-720 compared to that receiving antigen with AS02A (Lyon et al., 2008). In another study on Aotus, a vaccine FMP1/AS02A, which was based on the 3D7 allele of MSP-1 had shown good immunogenicity but inadequate clinical efficacy in a Phase 2b trial (Ogutu et al., 2009). A phase Ia clinical trial was reported in healthy Adults of a vaccine ChAd63-MVA MSP1, which induced strong mixed CD4/CD8 T cell induction and in addition some IgG responses was also observed (Sheehy et al., 2011). This viral vectored vaccine was safe and generally well tolerable but incapable of impacting on initial parasite growth rates in the blood of malaria-naive adults following mosquito bite challenge (Sheehy et al., 2012). Thus more alternative strategy are required that could achieve protective efficacy in humans.

2.2.2 Merozoite Surface Protein – 2 (MSP –2)

Merozoite Surface Protein –2 (MSP-2) glycoprotein antigen is expressed on the surface of merozoites. MSP-2 is a small polymorphic glycoprotein, with molecular weight ranged from 35-56 kDa (Clark et al., 1989). The parasite has polymorphic central variable region of the protein, which is flanked by a short N-terminal, and a longer C-terminal conserved region. From the conserved N and C terminal regions T–cell epitopes have been
identified (Theander et al., 1997). The central variable region contain extensive domain of repeats. The repeats are flanked by non-repetitive variable regions which define two allelic families called the FC27 and IC-1 family. Most of the MSP-2 gene belongs to these two allelic family and therefore MSP-2 like MSP-1 is considered as dimorphic in character (Kemp, 1992). The MSP-2, like MSP-1, is antigenically diverse (Clark et al., 1989) and exhibit extensive antigenic variation (with specific monoclonal antibodies) and genetic diversity (Fenton, 1991; Snewin et al., 1991; Miller et al., 1993; Viriyakosol et al., 1995; Haddad et al., 2000). In India genetic diversity was reported from central, northwest state (Ranjit and Sharma, 1999), eastern and North eastern India (Ranjit et al., 2005, Joshi et al., 2007), north and north western India (Farroq et al., 2009).

Polymorphism in surface antigens can be a selective advantage to the parasite, as MSP-2 is strongly recognized by naturally acquired antibodies (IgG) from individuals exposed to malaria. These antibodies were found to be directed against the central sero-group (A&B) specific variable region of the molecule rather than against the conserved N’ and C’ terminal amino acids (Al-yaman et al., 1994; Taylor et al., 1995; Engelbrecht et al., 1995, 2000). Considering the antibody response and cellular immune response, large number of vaccine trials has been reported on MSP-2 from different part of the world. A phase 1-2b trial was reported on combination B vaccine from Papua New Guinea. Combination B vaccine comprises full-length 3D7 MSP2, small N-terminal fragment of MSP1 and recombinant Plasmodium falciparum ring-infected erythrocyte surface antigen. A significant reduction in parasitemia was observed among the 30 vaccinated children not pretreated with sulfadoxine-pyrimethamine, with mean parasitemia reduced by 62% (Genton et al., 2002). A Phase 1 trial was reported in healthy, malaria-native adults of a new MSP2 vaccine (MSP2-C1) containing the 3D7 and FC27 forms of MSP2, formulated with equal amounts with adjuvant Montanide 720. It has been observed that majority of subjects vaccinated with MSP2-C1 developed an antibody responses to both forms of MSP2, and that these antibodies mediated cellular inhibition provide further support for MSP2 as a malaria vaccine candidate (McCarthy et al., 2011).

2.2.3 Circumporozoite Proteins (CSP)

Circumsporozoite protein (CSP) is an acidic polypeptide expressed on the surface of sporozoites, and early liver stages of parasitic infection. CSP is involved in the adhesion of the sporozoite to the hepatocyte and invasion of the hepatocyte. The molecular weight of CSP is 40-60 kDa. The structure of CSP is characterized by polymorphic central tandemly repeated peptide units known to contain immunodominant B cell epitopes (Zavala et al.,
1983) and flanking less polymorphic 5’ and highly polymorphic 3’ end, which have T cell epitopes (Doolan et al., 2000). These two regions (Central and terminal) interact with heparin like oligosaccharide and are important for uptake of sporozoite by hepatocytes (Ying et al., 1997). Although the CSP gene is largely invariant yet sequence variation has been noted mainly in the T-cell epitope regions of P. falciparum (de la-Cruz et al., 1987; Doolan et al., 1991). The central repeat portion of P. falciparum contains a four amino acid repeat peptide NANP/NVDP (Dame et al., 1984). Two T- helper cell epitopes (TH2R and Th3R) have been identified at the C-terminal end of the CSP flanking the highly conserved R-II region, which are spanning from amino acid residues 326 to 343 (Th2R) and 361 to 380 (Th3R) respectively (Figure-2.2) (Good et al., 1987; Jalloh et al., 2006). Extensive polymorphism found in this T helper cell epitopes region in P. falciparum isolates collected from different part of Africa (Escalante et al., 2002). However, relatively less polymorphism was observed from other part of world including Brazil, Papua New Guinea, Vanuatu, Thialand and Myanmar (Escalante et al., 2002; jalloh et al., 2006). Similarly, genetic diversity of T-helper cell epitopic region was reported in CSP of P. falciparum isolates from India (Bhattacharya et al., 2006; Singh et al., 2009).

![CSP Diagram](image)

**Figure-2.2: Structure of CSP with conserved RI, RII and repeat region.**

The fact that CS is the most predominant surface antigen of sporozoites (Nussenzweig and Nussenzweig, 1989) and therefore considered as the most popular antigen for use in pre-erythrocytic vaccine candidates. The initial vaccine construct of the repeat region of the circumsporozoite protein mainly copies of four amino acids sequence ‘NANP’ (Ballou et al., 1987). The NANP vaccine failed in this first trial and the failure was attributed due to lack of T-cell epitope. The failure of NANP vaccine lead to the most effective malaria vaccine RTS,S which is combination of the central repeat (‘R’) fused to the C-terminal region known to contain T cell epitopes (hence ‘T’) fused in turn to the hepatitis B surface antigen (‘S’) (Stoute et al., 1997). RTS,S, a hybrid protein particle, formulated in a multi-component
adjuvant named AS01. RTS,S/AS01E has been reviewed extensively and is by far the most advanced candidate malaria vaccine (Cohen et al., 2009; Ballou, 2009). Recently a phase 3 trial was conducted of RTS,S/AS01 vaccine in children and young infant at eleven African site. The vaccine efficacy was found higher in case of children than in infants (The RTS,S Clinical Trials Partnership, 2014). Another vaccine spf66, developed in colombia in the late 1980s. This vaccine was combination of synthetic peptide consisting of CS and merozoite surface protein 1 (MSP1) epitopes adjuvanted with alum. Several studies in different phases and countries were assessed and vaccine efficacy has been found relatively low (Kashala et al., 2002; Graves and Gelband, 2006). Several other vaccine were analysed based on CSP gene (Schwartz et al., 2012) and still efforts are going on to formulate a vaccine based on this immunogenic gene indicating the importance of this gene as a vaccine candidate against malaria.

2.2.4 Erythrocyte Binding Antigen-175 (EBA-175)

The 175 kDa Erythorocyte binding antigen (EBA-175) is a *P. falciparum* protein located in the apical micronemes of merozoites. The merozoite of *P. falciparum* uses EBA-175 for the invasion of erythrocytes and start multiplication inside erythrocyte. EBA-175 binds to the RBC glycophorin A (GpA) and the interaction constitutes a major invasion pathway. The erythrocyte binding domain of EBA-175 is situated in region-II (Figure-2.3). The region II, a highly conserved 616 amino acid fragment, with the use of truncated portions of EBA-175 expressed on COS cells (Sim et al., 1994). Therefore the receptor binding domain (region II) assessed as an important candidate for receptor blocking therapy and vaccines.

![EBA-175](image)

**Figure-2.3: Structure of EBA-175 with receptor binding domain region-II.**

The RII domain is currently considered as the vaccine candidate antigen as the vaccine of this domain was shown to be safe and immunogenic, producing antibodies that inhibit invasion, with protection of 1 of 3 vaccinated *Aotus* monkeys from disease (Sim et al.,
It has been observed that antibodies raised against EBA-175 RII in rabbits inhibit invasion regardless of the invasion pathway utilized (Jiang et al., 2011). Prior experiments show that total IgG acquired by malaria-exposed individuals has the ability to inhibit erythrocyte invasion (Persson et al., 2008). In a Phase 1 study (based on RII domain) the safety and immunogenicity of a recombinant EBA-175 vaccine with aluminium phosphate adjuvant was evaluated in healthy young adults living in the United States. The vaccine resulted in a safe and immunogenic response at varying doses (Sahly et al., 2010). Currently a Phase 1 study on EBA-175 RII-NG vaccine in semi-immune adult volunteers of Ghana is underway (NIAID, 2013). Recently EBA-175 RIII-V domain was identified as a conserved antigen that induces potent cross strain neutralizing antibodies (Healer et al., 2013). Thus EBA-175 is considered as prime vaccine candidate antigen and need to be explore more for developing future vaccine against malaria.

2.2.5 Soluble Antigen (S-ANTIGEN)

S-antigen of *P. falciparum* is a heat stable protein, secreted into the parasitophorous vacuole and released upon schizont rupture. It exhibits a remarkable degree of size and serological diversity. The molecular weight of S-antigen is ranging from 50 kDa to 250 kDa (Anders and Smythe, 1989). The s-antigen contains a single exon with a large central block of tandem repeats. The sequence of the central block of tandem repeats provides a useful means to distinguish the S antigen alleles and also define the serotype of an S antigen whereas the amino acid carboxy terminal sequences defined the S antigen allelic family. Four allelic families of s-antigen have been described i.e.VI/K1/NF7 (Nichols et al., 1980), FC-27 (Saint et al., 1987), Welcome (Brown et al., 1987) and 3D7 (Bickle et al., 1992). The comparison of the four S antigen families reveals that they differ considerably from each other with variation being pronounced in the carboxy-terminal region (Bickle et al., 1992). The sequence of V1, K1 and NF7 alleles are nearly identical but they differ significantly from FC27 and Welcome alleles (Saint et al., 1987; Brown et al., 1987). These differences are the cause of the remarkable size polymorphism that has been observed in the S-antigen system with relative molecular masses ranging from 50,000 to 250,000 (Nicholls et al., 1988; Mattei, 1988). However the non-repetitive flanking sequences in S-antigens are more conserved than the repeats region (Anders and Smyth, 1989).

S-antigen of *plasmodium falciparum* could be another vaccine candidate antigen, since monoclonal antibodies to s-antigen inhibit the invasion of erythrocyte by *P. falciparum* merozoites *in-vitro* (Saul et al., 1985). A specific s-antigen of *P. falciparum* has been shown in some primary isolates from Papua New Guinea, Brazil, Thialand (Schofield et al., 1985).
2.2.6 Glutamate-rich Protein (GLURP)

GLURP is expressed in all stages of the parasite life cycle in humans, including on the surface of newly released merozoites (Borre et al., 1991). The molecular mass of glutamate-rich protein is 220-kDa. The gene encoding GLURP in *P. falciparum* consists of three regions namely, N-terminal nonrepetitive region (R0), central repetitive region (R1) and an immunodominant C-terminal repetitive region (R2) (Figure-2.4) (Borre et al., 1991). It is highly antigenic and the gene encoding GLURP shows little polymorphism in geographically different *P. falciparum* isolates (Theisen et al., 1995; Stricker et al., 2000). Recently genetic polymorphism was also reported from India in R2 regions of GLURP (Kumar et al., 2014).

![GLURP Diagram](image)

**Figure-2.4: Structure of GLURP; R0 indicates the non-repeat region, R1 indicates the central repeat region, and R2 indicates the carboxy-terminal repeat region.**

The *Plasmodium falciparum* glutamate-rich protein (GLURP) is an antigen considered to be one of the leading malaria vaccine candidates. Previous immune epidemiologic studies performed in high transmission areas have shown a high prevalence of antibodies against GLURP in adults (Dziegieł et al., 1991; Boudin et al., 1993; Dziegieł et al., 1993), as well as a significant association of high levels of GLURP specific antibodies with low parasite densities (Hogh et al., 1992; Hogh et al., 1993) and protection against clinical malaria (Dodoo et al., 2000; Oeuvray et al., 2000; Soe et al., 2004). Studies performed in areas highly endemic for malaria have demonstrated a high prevalence of antibodies against two well-defined regions within *P. falciparum* GLURP, the relatively conserved N-terminal nonrepeat region (R0) and the immunodominant repeat region (R2) (Theisen et al., 1998; Oeuvray et al., 2000). A Phase 1a study of the GMZ2 vaccine in malaria naïve German volunteers showed acceptable safety and reactivity along with induced anti-GLURP and anti- MSP3 antibodies and memory B-cells (Esen et al., 2009). A Phase 1b clinical trial in semi-immune adults from Gabon also showed acceptable safety, a boosted anti-GMZ2 cytophilic IgG response compared with elevated baseline levels, and the
induction of memory B-cells (Mordmuller et al., 2010). A phase 1b study in Gabonese children was reported (Belard et al., 2011) with both IgG and memory B cell induction confirmed for GMZ2.

Remarkable antigenic variations in several antigens of *P. falciparum* have been reported from different parts of the world. This is based on the studies of genes coding for these antigens. This polymorphism is particularly important in those antigens which are known to elicit the immune response against the *Plasmodial* infection and are the targets for the development of an effective malaria vaccine. In addition to polymorphism in antigen, another major obstacle in vaccine development is polymorphism in MHC gene. Human MHC molecules are known as human leukocyte antigens (HLA). Each human individual expresses up to six HLA-I molecules and up to a dozen HLA-II molecules. HLA genes show extensive polymorphism. The diversity of HLA molecules increases the probability that any foreign antigen will contain HLA-binding peptides suitable as vaccine targets. The amino acids within the binding groove determine the specificity of peptide binding to a given HLA molecule. Across multiple HLA molecules, the polymorphic residues that form the binding groove determine the repertoire of binding peptides to a particular HLA molecule. Tens of thousands of allele-specific and promiscuous HLA binders and T-cell epitopes have been identified in humans and mice while smaller numbers have been identified in other model animals, such as monkeys and rats (Rammensee et al., 1999; Peter et al., 2005). The majority of peptides bind to one or two HLA alleles but not to many others (Donnes and Kohlbacher, 2006). This restricts the utility of peptides as a vaccine in global perspective. The solution is promiscuous Peptide to develop an effective vaccine based on polymorphic genes which are immunogenic in nature (Gowthaman and Agrewala, 2008). The promiscuous peptides can bind to more than one HLA allelic variants and able to elicit immune response. The promiscuous peptide recognized by the T cells mediated immunity may have the potential in developing peptide based vaccine. Bioinformatics tools have the potential to accelerate research into the vaccines and diagnostic tests by exploiting genome sequences.

### 2.3 Computational Methods for Prediction of HLA Class-II Peptides

*In-silico* Approaches have been far more developed considering their low computational cost and independency from available crystallographic structures. The *in-silico* method started from simple statistical sequence analysis to new algorithms. Such as binding matrix-based strategy that takes into account residue frequencies at each epitope position; scoring matrices are built on the sequences of experimentally known binders and comprise information about position-specific frequencies and binding affinity. An improvement of
binding matrices algorithms is represented by the stabilized matrix method (SMM); The combination of this SMM with a pair coefficient that calculate a score for peptide residue pairs is included in the IEDB database and, together with artificial neural network (ANN) algorithms. Another algorithm evolved and adopted machine-learning approaches such as ANNs, Hidden Markov model (HMM), and support vector machine (SVM); these algorithms have the advantage to perform predictions handling nonlinear data. ANN algorithms are some of the best predictors; they represent epitopes features as amino acid descriptors and perform complex pattern recognition after being trained with a dataset of epitopic and non-epitopic peptides.

2.3.1 NetMHCIIpan2.1

One of the best performing pan-specific HLA class II prediction method is the NetMHCIIpan method (Nielsen et al., 2008). This method is a pan-specific version of the earlier published allele-specific NN-align algorithm and does not require any pre-alignment of the input data. This allows the method to benefit also from information from alleles covered by limited binding data. The method is evaluated on a large and diverse set of benchmark data, and is shown to significantly out-perform state-of-the-art MHC-II prediction methods.

2.3.2 IEDB SMM align

Immune Epitope Database (IEDB), and further performed in-silico analysis to estimate the promiscuity at the population level. IEDB SMM-align is a freely available server utilizing a large set of quantitative HLA class II peptide binding data which has been made publicly available on the IEDB databases (Toseland et al., 2005). The data set contains peptide data with IC50 binding affinities with more than 14 HLA (human MHC) and also with several mouse MHC class II alleles. SMM-align is a novel method for quantitative HLA class II binding predictions. The method is an extension of the stabilization matrix method proposed by Peters et al. (Swets, 1988; Peters and Sette, 2005). The SMM-align method seeks to identify a weight matrix that optimally reproduces the measured IC50 values for each peptide in the training set. The relative binding ability of different peptides to a specific MHC molecule can be directly assessed by competition experiments. Typically, it is expressed as the ratio between the half-maximal inhibitory concentrations (IC50) of the standard peptide to that of the test peptide. In the context of MHC-peptide binding, IC50 is the concentration of the test peptide required to inhibit binding of the standard peptide to MHC by 50%. The method allows for identification the HLA class II binding motif in terms of a position specific
weight matrix. The output of the SMM-align method is IC$_{50}$ binding affinity values, enabling direct readout of the peptide:HLA binding affinity (Nielsen et al., 2007).

2.3.3 IEDB NN-align

IEDB NN-align is a novel artificial neural network-based method, that allows for simultaneous identification of the HLA class II binding core and binding affinity. NN-align is trained using a novel training algorithm that allows for correction of unfairness in the training data due to redundant binding core representation. Incorporation of information about the residues flanking the peptide-binding core is shown to significantly improve the prediction accuracy. The method is evaluated on a large-scale benchmark consisting of six independent data sets covering 14 human HLA class II alleles, and is demonstrated to outperform other state-of-the-art HLA class II prediction methods (Nielsen et al., 2009).

2.3.4 IEDB-ARB

A methodology commonly used to predict HLA binding affinity is the matrix or linear coefficients method. Average Relative Binding (ARB) matrix methods directly predict IC-50 values allowing combination of search involving different peptide sizes and alleles into a single global prediction. This is a computer based program that was developed to automate the generation and evaluation of ARB predictive tools. Using an in-house HLA binding database, generated a total of 85 and 13 HLA class I and class II matrices, respectively. Results from the automated evaluation of tool efficiency are presented. This automation framework will be generally applicable to the generation and evaluation of large numbers of HLA predictive methods and tools, and will be of value to centralize and rationalize the process of evaluation of HLA predictions (Bui et al., 2005).

2.3.5 SVMHC

Prediction of HLA class II binding peptides is based on the matrices published by Sturniolo et al., 1999. By sequence similarity studies, they defined modular pockets in the HLA molecule involved in peptide interaction. These pockets are independent of the rest of the binding cleft and a limited number of pockets can be combined into virtual binding matrices for a wide range of HLA class II alleles. These matrices are also a part of the TEPITOPE prediction software and they have been used to identify candidate binding peptides for both HIV and Tuberculosis vaccines. Prediction is available for 51 different HLA class II alleles (Donnes and Kohlbacher, 2006).

2.3.6 Propred

Propred is specifically aimed to predict HLA class-II binding regions in an antigen sequence, using quantitative matrices derived from publish literature by Sturniolo et al.,
This server is a useful tool in locating the promiscuous binding regions that can bind to a total of 51 alleles belonging to nine serologically defined HLA-DR molecules encoded by the DRB1 and DRB5 genes (Singh and Raghava, 2001).

2.4 Structure based in-silico method

Although the HLA binding prediction algorithm reached high performances but there are reports that suggest these tools may not be very efficient (Gowthaman and Agrewala, 2009). The efficiency of the predicted promiscuous peptides by sequence based in-silico methods can be verified by structure based HLA binding in-silico methods.

One of the main categories in structure-based HLA binding in-silico methods is docking. Computational docking is used to predict the binding modes of two or more molecules. Many successful methods for docking of ligands to receptor targets have been developed (Sousa, 2006). Autodock is a program which has shown continued success in docking analysis. Autodock predicts the optimal bound confirmation of ligand to receptor. The in-silico methods can be further combined with experimental techniques for T-cell epitope mapping (Martin et al., 2003; Somvanshi and Seth, 2009).

2.5 Immunology

T-cell epitopes mapping can be done by cell based technique which mainly involve the screening of synthetic peptides on T-cell population to evaluate binding specificity. A broadly used cell-based approach is the enzyme linked immunospot assay (ELISPOT) (Czerkinsky et al., 1983). ELISPOT evaluate T-cell cytokine secretion level after antigen recognition. The main advantage from this technique mainly consist in its high resolution and high throughput results that can be further improved by the use of dedicated scanners which allow the scaling-up of the technique.

Other cell-based assay uses highly efficient multi parameter flow cytometry to quantitate and, both phenotypically and functionally, characterize Ag-specific CD4 memory/effector T cells in the human (Waldrop, 1997). This technique has important advantages over both traditional measures of Ag-specific T cell responses such as Ag-induced proliferation, and other single cell quantitative techniques such as limiting dilution (LD) and enzyme-linked immunospot (ELISPOT) assays.

Another cell based assay, lymphoproliferation assays are most frequently used for T cell epitope mapping and also rely on cytometric analysis. Earlier the cell proliferation was commonly assessed by measuring the tritiated thymidine incorporation (\(^3\)H-TdR) (Maghni et al., 1999). However it is not compatible with recovery of viable daughter cells for further
characterization. Also the handling and disposing of radioisotopes determined the need of other less hazardous alternative.

Cell-tracking reagents such as the green-fluorescent protein labeling dye CFSE is the best reagent currently available to monitor cell proliferation by flow cytometry in heterogeneous cell populations responding to immune stimuli (Wallace et al., 2008). The cell tracking dyes have been previously proven to be useful for qualitative and quantitative monitoring of cell division (Hawkins et al., 2007).

CFSE spontaneously and irreversibly couples to both intracellular and cell surface proteins by reaction with lysine side chains and other available amine groups. When cells divide, CFSE labeling is distributed equally between the daughter cells, which are therefore half as fluorescent as the parents. A major advantage of using flow cytometry and cell-tracking dyes to monitor extent of cell division is that the cells can also be stained for expression of other cell surface or intracellular markers to define lineage, functionality, activation state, cytokine expression.

The nature of the immune responses can be obtained through an analysis of the cytokine profiles. T-cells producing type 1 cytokines such as interferon-γ (IFN-γ) which have been associated with delayed type hypersensitivity responses. On the other hand T-cells which produce type 2 cytokines such as IL-4 have been associated with promoting B-cell function.

2.5.1 Interleukin-4 (IL-4):

IL-4 is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells and it promotes the growth of differentiated Th2 cells resulting in the production of an antibody response. The human Interleukin 4 (IL-4) gene located on the chromosome 5 (5q31-33) encodes for an anti-inflammatory cytokine produced by CD4+ Th2 cells, basophils and mast cells. IL-4 regulates variety of cell types playing an essential role in differentiation of Th2 effector cells and suppression of Th1 signaling (Gyan et al., 2004).

It has been demonstrated that the basophils stimulated by IL-3 produce IL-4 in large amount during severe anaemia caused by malaria and reduced immunity against blood stage parasites (Kumaratilake et al., 1992). Epidemiological studies indicated that IL4 play a key role in the induction of specific antibodies during blood stage P. falciparum infection (Elghazali et al., 1999). However, several mechanisms are involved in reducing blood-stage parasite immunity and one of them is that IL-4 which interferes with Th1 cell development and reduces the production of IFN-γ (Zhu et al., 2006). Another study has revealed that INF-γ...
levels were significantly elevated during early stages of malaria, whereas the IL-4 levels were elevated during intermediate and late stages (Tangteerawatana et al., 2007). Concomitantly, Jha et al., (2012) reported that IL-4 Haplotype -590T, -34T and Intron-3 VNTR R2 reduce the malaria risk by downregulating the overexpression of proinflammatory cytokines TNF-α and INF-γ among Indian tribal populations.

In severe anaemia (SA) and cerebral malaria (CM) the concentration of IL-4 is found to be higher. This higher concentration of IL-4 plays an important role in developing CM by inhibiting the destruction of blood-stage parasites and thereby favoring sequestration in microvasculature. IL-4 participates in the aggravation of cerebral malaria in 3 ways: by increasing parasite mass; by promoting infiltration by monocytes, basophils, and eosinophils; and by increasing parasite sequestration (Cabantous et al., 2009).

Studies in mice infected with Plasmodium yoelii have shown that IL-4 is required for the development of CD8+ T lymphocytes and for the development of a memory response against liver-stage parasites (Morrot et al., 2005). This raises the possibility that IL-4 may also exert opposite and indeed positive effects on the control of P. falciparum infection. Once an infection is established, IL-4 is expected to facilitate parasite multiplication via its multiple effects on immunity.

2.5.2 Interferon-γ (IFN-γ)

IFN-γ is an important cytokine produced primarily by Th1 cells, although it can also be produced by Tc and NK cells, which in turn activates macrophages to destroy parasitized red blood cells, promotes the production of opsonizing antibody and contributes to destroying Plasmodium in hepatocytes (Bashyam et al., 2007). McCall et al., 2010, reported that immunity to infection with blood-stage Plasmodium parasites is critically dependent on IFN-γ and requires coordinate and timely innate and adaptive immune responses involving dendritic cells (DC), NK cells, CD4+ T helper cells, and B cells. Consistent with this, another study showed the Natural Killer cells are the first lymphocytes which respond to P. falciparum infected red cells by producing IFN-γ to control the malaria parasite burden (Agudelo et al., 2012).

Studies in malaria endemic areas of Africa have documented that IFN-γ and IL-10 responses to Liver Stage Antigen-1 (LSA-1) correlate with protection from infection (John et al., 2000). In addition, a study in children from a malaria holoendemic area of western Kenya reported that IFN-γ response to a pooled group of pre-erythrocytic antigens including LSA-1 was associated with protection from anaemia (Ongecha et al., 2003). These findings suggest that IFN-γ and IL-10 responses to LSA-1 will be important markers of immunogenicity in
LSA-1 containing vaccines. IFN-γ responses to LSA-1 are thought to mediate protection from infection by eliminating infected hepatocytes through the induction of nitric oxide pathway (Hoffman et al., 2000).

Evidently it was shown that IFN-γ contributes to protection against cerebral malaria at least in those who recover from the disease (Cabantous et al., 2005), and also have significant protective effects against Severe Malaria (Marquet et al., 2008).

However, a recent finding revealed that IFN-γ also has pro-inflammatory effects that could contribute to disease severity. The effect of IFN-γ was shown to be harmful for the survival of *Plasmodium falciparum*-specific CD4 T cells by regulating its contraction (Sun et al., 2012). In addition, the study conducted in Mozambique population showed the higher levels of IL-10 and IFN-γ in children with severe *P. falciparum* malaria, thereby suggesting their role in increasing the pathogenesis of malaria (Quelhas et al., 2012). The contradictory role of IFN-γ indicated that the role of this cytokine is not clear in malaria.

Keeping these points in view, the present study was planned for computational prediction of promiscuous peptides from conserved region of MSP-1, MSP-2, CSP, GLURP, S-antigen and EBA-175 antigens of *P. falciparum*. The predicted promiscuous peptides were further planned to experimentally confirm by immunological assay (lymphoproliferative assay) and cytokine level was determined by Th1 and Th2 markers i.e. IFN-γ and IL-4.