

Chapter I

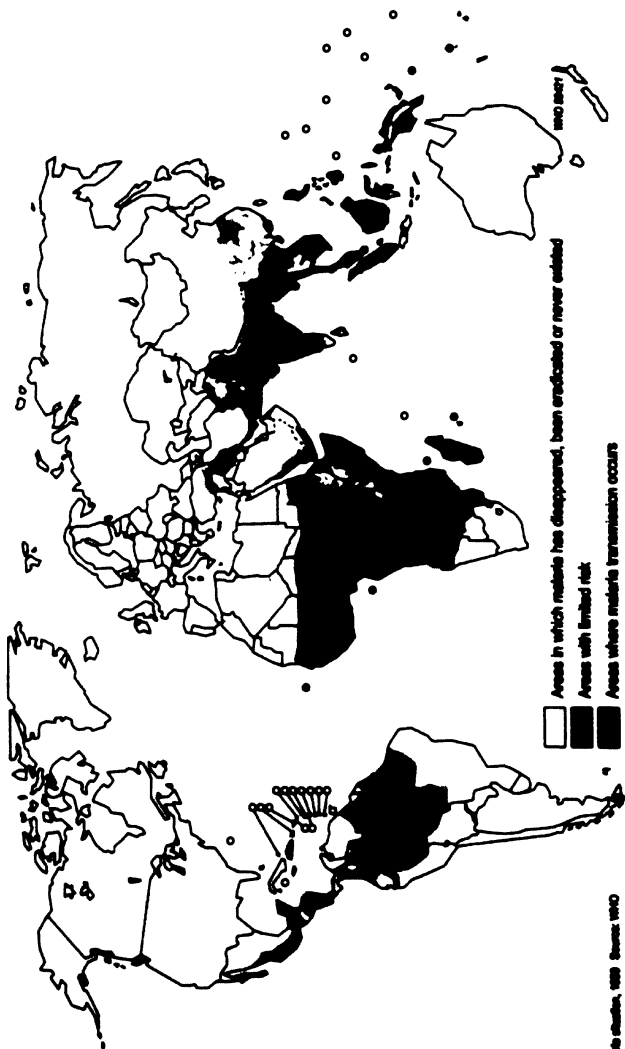
General introduction on the disease, rationale and objectives
of the study.

General Introduction:

Of all human afflictions, the greatest death toll has been extracted by malaria and even today, it disables and kills more people than any other infectious disease (Sherman, 1998). Malaria is a mosquito-borne parasitic disease of human, which is endemic in 80 countries (Fig 1.1) and responsible for 300 million clinical cases resulting in at least 1 million deaths annually (WHO, 2002). Every 40 seconds a child dies of malaria, causing a daily loss of more than 2,000 young lives worldwide (Sach and Malaney, 2002). Malaria in humans is caused by four species of protozoan parasites of the genus *Plasmodium*; *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Among these four species, *P. falciparum* is responsible for majority of deaths. The dream of completely eliminating malaria pursued during 1950s and 1960s slowly faded away and the disease is re-emerging. At present time, its containment has become more difficult with the currently existing tools of diagnosis of drug resistant parasite, control measures and other infrastructures. The most alarming situation is the spread of drug resistance strain of *P. falciparum*, which is one of the important factors for increasing disease morbidity and mortality. If new intervention measures other than current measures like vector control and chemotherapeutic treatment are not introduced the predicted clinical cases and death will double by year 2020 (Bermen, 2001). At the present situation, few believe that global eradication of malaria will be possible in the foreseeable future, but the recent goals and belief of malaria control are the reduction of clinical cases and mortality.

The history of malaria is as old as human origin. Malaria antigens were detected in skin and lung samples of mummies dating back to 3200 and 1304 B.C (Miller *et al.*,

Figure 1.1. Map shows the global distribution of malaria Source Roll Back Malaria. World Health Organization (www.who.rbm.net)



Malaria situation, 1989. Source: WHO

1994). The writings of Vedic period (1500-800 B.C) refer to autumnal fevers as the “King of diseases” with symptoms of enlarged spleen establishing epidemic forms in India. Eventually the “Roman fever” gave rise to the Italian word *mal'aria*, meaning “bad air” to describe the cause of sickness. On 20th October 1880, Charles Louis Alphonse Laveran, an army doctor, while examining microscopically the blood of a soldier suffering from intermittent fever noticed crescent shaped bodies and later found several mobile filamentous forms emerging from these crescent bodies. He named it as *Oscillaria malariae* and his announcement was received with scepticism. Six years after Laveran’s discovery, Camillo Golgi using a thin smear of fresh blood discovered the asexual development and showed that fever coincides with rupture of red blood cells. Laveran received Nobel Prize in 1907 for his discovery of the causative agent of malaria, the one-celled protozoan *Plasmodium*.

Plasmodium is classified phylogenetically as follows

Phylum	Apicomplexa
Class	Sporozoa
Subclass	Coccidia
Order	Haemosporida
Suborder	Aconoidina
Family	Haemospoidae
Genus	<i>Plasmodium</i>

On 20th August 1897, Sir Ronald Ross called the day as “Mosquito day” – the day he demonstrated mosquito transmission of malaria (Ross, 1923) and wrote a letter to his wife:

This day designing God
Hath put into my hand
A wondrous thing
And God be praised. At this command
I found thy secret deed
Oh million murdering Death. I know that this
Little things
A million men will save
Oh death where is thy sting? The victory oh
Grave?

This great discovery provided very important information for the control and knowledge of the transmission of the dreaded disease, malaria. Sir Ronald Ross received the Nobel Prize in 1902 for this achievement.

Life Cycle of human malarial parasite:

The life cycle of malaria parasite is complex (Fig 2.1). Malarial infection is not restricted to humans alone but is also found in other vertebrate hosts like simian, rodent, birds, reptiles and amphibians. There are 29 simian malarial parasites, which infect higher apes, monkey and lemurs. The widely used primate malarial parasites for model are *P. knowlesi* and *P. cynomolgi* for experimental purposes. The rodent malarial parasites are *P. berghei*, *P. yoelii*, *P. vinckei*, *P. chabaudi* and birds are infected with *P. lophurae* and *P. gallinaceum*. The parasites are highly host specific but the human malarial parasites are used and adapted in some higher apes by experimental infection. There are rare reports of natural transmission of simian malarial parasites to human host also. The life cycles of malarial parasites invariably are completed in two different hosts: a vertebrate and an invertebrate mosquito host.

Figure. 1.2 Diagrammatic sketch of the life cycle of human malarial parasite, *Plasmodium falciparum* showing various stages of developments in mosquito and human host

TRYPANOSOMA FEEDING ON INFECTED MAN



SECTION OF HUMAN SKIN



Oocysts

DEVELOPMENT IN MOSQUITO TO STOMACH SPOOROZOYTES DEVELOPMENT IN MAN SPOOROZOYTES



Sporozoites

Salivary Glands

Infective Mosquito Introducing Sporozoites

Section of Human Skin

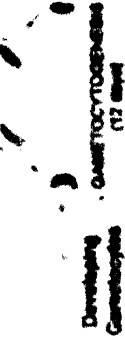
Sporozoites

Macrogametocyte

Macrogametocyte

Trophozoite

Mauzer's Dot



Developing Gametocytes

DEVELOPING GAMETOCYTOGENESIS (12 days)

DEVELOPING SYPHONITE

ASEXUAL CYCLE (48 HOURS)

Multiple infection

Multiple infection

Macrogametocyte

Macrogametocyte

Macrogametocyte

Macrogametocyte

Macrogametocyte

Macrogametocyte

Developing Siphonite

Developing Siphonite

Developing Siphonite

Developing Siphonite

Developing Siphonite

Developing Siphonite

Macrogametocyte

Macrogametocyte

Macrogametocyte

Macrogametocyte

Macrogametocyte

Macrogametocyte

Mature Siphonite Liberating Microgametes

Mature Siphonite

Mature Siphonite

Mature Siphonite

Microgametes

Microgametes

Microgametes

Pre-erythrocytic Siphonite

Pre-erythrocytic Siphonite

Pre-erythrocytic Siphonite

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Pre-erythrocytic Siphonite

Pre-erythrocytic Siphonite

Pre-erythrocytic Siphonite

In vertebrate host or human, the life cycle starts with the infectious bite of female Anopheline mosquito, which releases sporozoite stages of the parasite in to the capillaries while sucking the blood. These extra cellular sporozoites with in few minutes invade the liver cells and multiply asexually to mature liver schizonts of 10,000- 30,000 nucleated cells. The liver cells rupture and the multinucleated parasite schizont releases uninucleated stage called merozoites in to the blood stream. This liver cycle takes about 8-10 days, but in *P. falciparum* it completes in a shorter period of 5-6 days. This phase of development of malarial parasite inside the liver is called exo-erythrocytic cycle. In case of *P. vivax*, *P. malariae* and *P. ovale* the liver stages remain dormant and rupture at different periods and lead to relapse of malaria symptoms.

The released merozoites invade the red blood cells, with this begins the erythrocytic cycle of the parasite. The merozoite grows inside the safe cabin of human's most precious tissue, and develops to ring stage, trophozoite stage and then divides to become multinucleated schizont stage. Then, the schizont ruptures and from each schizont 8-32 merozoites are released in to the blood stream by breaking the red blood cell membrane. The released merozoites reinfect fresh blood cell and cycle continues. The complete erythrocytic cycle takes 48 to 72 hours depending on the species. The rupture of the infected blood cells releases toxic materials into the blood stream and the onset of symptoms like fever, headache, chill and rigor begin. Some merozoites differentiate in to sexual stages (male and female gametocytes) of the parasite, which enters another host system to complete the life cycle.

When the female Anopheline feeds on the infected human host, the gametocytes are ingested in to the mosquito gut along with blood meal. In the gut of the mosquito host the gametocytes are released from the RBC and are now called gametes. The male gamete transform into active motile form by producing 8 flagellar structures called as microgamete. Then the microgamete fuses with the macrogamete, the female gamete, and diploid zygote is formed. The zygote matures into motile ookinete stage, which penetrate the lumen of the mid gut and forms oocysts on the external surfaces of the gut. The oocyst undergoes meiotic division followed by rapid mitotic division to form millions of spindle shaped sporozoites. The oocysts burst after maturation, releasing sporozoites into the hemocoel of the mosquito, which migrate to the salivary gland of mosquito. The mosquito is now infective and ready to spread malaria to a new individual. The mosquito cycle takes place about 8-12 days depending on parasite species and climatic conditions. The infective mosquito sucks the blood and injects million of sporozoites in to the blood stream of a new host, thus transmitting the most notorious killer disease of human being known as "Malaria".

Pathogenesis of the disease in human:

The clinical features of malaria infection cover a spectrum of clinical manifestations from asymptomatic infection to fulminate disease leading to death. Important determinants of the clinical pattern are species of parasite, age and immune status of the host and the degree of malaria endemicity (Miller *et al.*, 1994; Marsh, 1998). The pathogenesis of malarial infection is associated with erythrocytic cycle of the parasite and host parasite interaction. The well-known three stages of malarial infection

shaking chill, intense fever and drenching sweats is characteristic but not universal (Bruce-Chawatt, 1988). There have been many attempts to devise algorithms, which help in making diagnosis and in distinguishing malaria from other common childhood conditions (Rougment *et al.*, 1991; Redd *et al.*, 1996). However malaria is so ubiquitous, both as an infection and as a disease, it cannot be diagnosed by clinical features with any degree of certainty for practical purposes. The symptoms of malaria develop within 8-30 days of infection. The rupture of blood cells release huge amount of toxic substances like hemozoin in to the blood stream, which initiate the symptoms of malaria fever. In endemic areas the symptoms vary with different age groups. The adults living in endemic regions show mild symptoms like headache, low fever but children are the worst sufferers. The clinical symptoms of the disease are also intense among international travelers or individuals from non-endemic regions.

In case of *P. falciparum* infection, it often leads to severe and complicated malaria called as cerebral malaria. Mostly young children below the age of five are major sufferers of the severe disease and it accounts for 90% *P. falciparum* related of death. The prediction of infected cases, which will lead to cerebral malaria is difficult to obtain as in some cases the onset is extremely rapid, with a child apparently well and playing immediately before becoming comatose. At the other extreme, children may have been symptomatic for up to a week before leading to severe form of the disease. The defining feature of clinical syndrome of cerebral malaria is deep coma. The other symptoms are convulsion, abnormality in respiratory patterns, retinal abnormalities and raised intracranial pressure, Metabolic acidosis, hypoglycemia, sever anemia are also associated

features of severe malaria (Crawley *et al.*, 1998; Lawallen *et al.*, 1993; Newton *et al.*, 1991). The recovery from coma in survivors has heterogeneous clinical syndrome. The most persisted neurological sequelae after the recovery from severe disease were hemiplegia, behavioral disorders and epilepsy. The less frequently found permanent disorder was blindness and generalized spasticity (Marsh, 1998). Pregnant women are also more susceptible to severe form of malaria. In addition to increased risk, the disease is associated with abortion, still birth, premature delivery, low birth weight and maternal mortality (Nosten *et al.*, 1991; Marsh, 1998)

Host genetics and malaria:

Infectious diseases have been a major selective force and might have greatly modified the genetic make up of human evolution (Haldane, 1948). Malaria is one among the infectious diseases, which played important role for genetic selection in the evolution of human population (Hill, 1992; Miller, 1994). Haldane (1948) was first to suggest that the high frequency of thalassaemia in Mediterranean populations might have resulted from heterozygote protection against malaria. The studies showed that hemoglobin S provides 80-95% protection against *P. falciparum* infection, notably, severe anemia and cerebral involvement (Hill *et al.*, 1991) and where there is (or has been) malaria there is thalassaemia, often at very high frequencies (Weatherall, 1996). Glucose-6-phosphatase dehydrogenase deficiency is a very common X-linked trait affecting millions of individuals also protect from severe malaria (Ruwende *et al.*, 1995). Duffy negative blood group is resistant to invasion of *P. vivax* and very high prevalence of Duffy negative populations in Africa reflects the natural selection might have taken place in the

past to protect against *P. vivax* infection (Miller, 1994). The other host genetic factor like human leukocyte antigen (HLA) and major histocompatibility complex (MHC) genes have also come under similar selection to give protection against the malarial infection (Hill *et al.*, 1991; Jepson *et al.*, 1997).

Host immunity and malaria:

Natural immunity to malaria develops after repeated exposure and builds up slowly in individuals living in high endemic regions (Sergent and Parrot, 1956). In endemic areas, most individuals develop an immune response to control the parasite replication, which lower the parasite burdens and decrease the clinical impact of malaria and are termed as "immune" (Bruce-Chawatt, 1952). Numerous epidemiological studies conducted in areas of stable malaria transmission report an age- dependent increase in *Plasmodium*-specific responses, as well as an age related decrease in malaria-dependent morbidity (Al-Yaman *et al.*, 1997; Rajgopalan *et al.*, 1990; Braid, 1992; Warsame *et al.*, 1997). During first few months of a child's life passive immunity from mother confers some protection. The mortality is observed highest in first few years' life and later gradually lower parasite density and fewer clinical symptoms manifest acquired immunity. This immunity is short-lived, however, and in the absence of repeated infections e.g., among those who have spent less than a year away from malaria-endemic areas was once again susceptible to clinical disease (Marsh and Greenwood, 1992). Similarly subjects living in low endemic areas and travelers from non-endemic areas develop severe symptomatic disease to almost each and every infective bite of vector (Luxemburger *et al.*, 1996). Earlier studies show that passive transfer of antibodies

purified from individuals residing in endemic areas protects recipients from on-going infection (Cohen *et al.*, 1961; McGregor *et al.*, 1963). Collectively, these experiments and studies established that immune response develops slowly and mounts an anti-parasite response.

The other arm of protection mechanism from malaria is displayed by the cellular immunity. Experiments in mice and field studies in Gambia have provided evidence that cytotoxic T lymphocytes (CTL) play a protective role against *P. falciparum* infection (Aidoo *et al.*, 1995). T-cells are critical for immunity to malaria, not only because they function as helper cells for an antibody response, but also they serve as effector cells. The role of cytokines involved in both protection as well as pathogenesis of malaria was reported. One potential role for IFN- γ immunity to blood stage infection is its macrophage activating capacity (Gyan *et al.*, 1994) and other cytokines like GM-CSF, IL-8 and IL-10 implicated to play an immunoregulatory role in malaria immunity (Peyron *et al.*, 1994, Kumaratilake *et al.*, 1996, Brugmann *et al.*, 1995). A dual role of cytokines like TNF was reported. It exhibited antiparasitic effect by inducing nitric oxide (Anstey *et al.*, 1996). Several studies have also reported a strong positive correlation between plasma TNF levels and cerebral malaria (Grau *et al.*, 1989; Kwiatkowski, 1990).

Malaria control and its pitfalls:

Malaria is endemic in tropical and sub-tropical developing countries. Even after hundred years of its discovery of mode of transmission, the efforts to control the disease

have failed and the disease has reemerged with more dangerous form. The malaria control at presently is mainly based on chemotherapeutic treatment with antimalarial drugs and vector control. Antimalarial drugs are used to prevent the onset of the disease, to treat the clinical cases, to prevent the disease transmission in the population. The drugs like chloroquine, primaquine, quinine, sulphadoxine-pyremithamine and mefloquine are being used widely for treatment and prevention of the disease. The emergence of drug resistant strains of most virulent parasite *P. falciparum* has reemphasized concerns about the use and efficacy of present line drug. The first report of drug resistant strain of *P. falciparum* to chloroquine was reported in Thailand and Cambodia border in 1960s, and has since spread to all parts of the world. There is also widespread increasing resistance to other antimalarial drugs like pyrimethamine, fansidar, mefloquine and halofantrine (Krogstad, 1996, Valero *et al.*, 1997) and is major concern for future treatment of the clinical and sever cases. At present the artemisin and its derivatives have been proved to be an effective chemotherapy for multi-drug resistance severe falciparum malaria in adults and children (Klayman, 1985; Frederich, 2002; Warrel *et al.*, 2002). The malaria parasites mutate the drug targets very fast to make the drug no more effective for their survival in the human host system. It is necessary to identify novel potential drug targets for effective and durable control measures.

The other important arm of malaria control depends heavily on vector control measures like residual insecticide spraying, larviciding, reducing the man mosquito contact and bio-enviromental measures (Das *et al.*, 1993; Curtis and Mnzava, 2000; Philips, 2001; Sharma, 1999). Residual spraying using DDT, malathion, carbamates,

pyrethrin and pyrethroids compounds are used in all the endemic areas as a front line malaria control measures. Unfortunately, mosquitoes in many parts of the world have become resistant to the most of the pesticides and have adapted to avoid the sprayed surfaces (Beals and Giles, 2002). In recent days, use of protective clothing, insect repellents, mosquito coils, and impregnated bed nets have shown to be reducing morbidity and mortality and are being adapted in most of the countries for controlling the disease (Rozendall, 1989; WHO, 2002). This method is currently used to control the disease but has also some limitations regarding its cost and proper use in the poorest section of the human population living in endemic areas of the tropical countries. Another novel strategy to control the vector is development of transgenic mosquitoes refractory to the *Plasmodium* infection and spread of refractory genes in the mosquito populations (Kidwell and Ribeiro, 1992; O'Brochta *et al.*, 1997), which has to go a long way to reach field trials.

With the inadequacies of vector control as well as chemotherapeutic measures, malariologists focused their attention to develop malaria vaccine together with identification of drug targets, drugs and improved control measures. Vaccination is one of the important tools of eradication or control of any disease. Epidemiological studies in endemic areas revealed that the severity of the disease slowly decreases with increasing age of the individuals (Cohen *et al.*, 1961; Beadle *et al.*, 1995) and short lived in the absence of exposure (Hoffman and Miller, 1996). The individual continually exposed to infection by the parasite protozoan responsible eventually develops immunity to the disease, and the passive transfer of antibodies has a dramatic effect on clearing blood

stages of the parasite (Cohen *et al.*, 1961). Immunization of mice, non-human primates and human volunteers with radiation-attenuated sporozoites induces complete sterile immunity (Nussenzweig *et al.*, 1967; Clyde *et al.*, 1973; Rieckmann *et al.*, 1974; Gwadz *et al.*, 1979). These observations give a hope that a vaccine is possible in near future.

Malaria vaccine can be divided in to three different categories. The anti-infection vaccine would direct against the invading sporozoites and liver stages of the parasite and may prevent the development of disease. The anti-morbidity/mortality vaccine was directed against erythrocytic stages designed to prevent disease and mortality. It is essential to save million of children from death. The third kind of vaccine is to block infection in the mosquito is called as "Transmission blocking vaccine", where human host antibodies are directed against gametes, zygotes, ookinete stages, proved to be effective to reduce the development of parasite in mosquito host (WHO, 2000). Attempts to develop a malaria vaccine began early in the twentieth century (Desowitz, 2000), and in spite of enormous developments of biomedical research, no effective vaccine is available for wide spread use against malaria. The main obstacles in vaccine development are the multiple mechanisms of immune evasion evolved by *Plasmodium* species, which also include stage specific antigen expression and antigenic polymorphism. Therefore, a vaccine directed against single stage in the life cycle of malaria parasite may not be effective because parasites that progress to the next stage, may express a new set of antigens. The other very important drawback is most of the vaccine candidates identified are polymorphic in field populations (Roger and Hoffman, 1998). Hence, identification of new potential antigens from different stages of the parasite life cycles is important for

effective future vaccine development. To check the spread of drug resistant parasites and human mortality, identification new drug targets and development of new drugs are highly essential in present time and in future. Malaria parasites quickly mutate the drug targets and make the drug ineffective. Based on the two major problems like antigenic polymorphism, and mutation of drug targets, exploitation of conserved antigen genes might be an ideal choice for vaccine as well as drug targets in malaria. It is well known that some of the conserved genes are known to be having very vital functions for parasite survival and are also having very low mutational tendency. Hence, identification and characterization of these molecules may pay dividends in future control of this dreaded disease. The thesis work is carried out in this direction with following rationale and objectives.

Rationale and objectives of the present study:

Malaria vaccine development is compared to “A Game of Chess” (Mendis, 1991). The high antigenic polymorphism in malarial parasite is one of the major hurdles for vaccine development (Conway, 1997; Reeder & Brown, 1996; Phillips, 2001). Secondly, the mutation in the drug target sites of the parasites has resulted in the development of quick resistance to available anti-malarial drugs like chloroquine and antifolate drugs (Brook *et al.*, 1994; Trigalia *et al.*, 1997). It is one of the important causes for high mortality, re-emergence and spread of the malarial diseases. To overcome these twin problems of high antigenic polymorphism and introduction of early mutations in the drug target sites, identification of conserved genes having important function for the parasite survival may be a reasonably good strategy for moving towards drug and

vaccine development. Such an approach of identification and characterization of conserved gene(s) or antigen(s) and its uses in cocktail vaccine or drug target might be more successful by providing a means to reduce the risk of selection of antigenic polymorphism and delay in development of resistant forms of the malaria parasite. The existence of conserved antigen analogs throughout *Plasmodium* genus demonstrated by cross-hybridization and by comparison of the amino acid sequences of cloned proteins of different species were reported (De Portillo *et al.*, 1991; Kaslow *et al.*, 1989). The earlier studies (Ray *et al.*, 1994) showed that convalescent-phase mouse anti-*P. yoelii* antibodies inhibit the intra-erythrocytic stage growth of *P. falciparum*, invasion of red blood cells *in-vitro* and cross react with at least 15 polypeptides of *P. falciparum*. These results prove that the rodent malarial parasites (*P. yoelii*) share some of the common epitopes of human malarial parasite (*P. falciparum*) and which might be essential for parasite survival. These conserved genes of *P. falciparum* can be exploited as a target for a widely effective vaccine or drug targets in future endeavors of malarial controls. Therefore, the aim of present study was to identify, clone, analyze sequence, express and carry out molecular characterization of a conserved novel antigen of *P. falciparum* and the objectives are as follows:

- i To identify novel cross-reactive antigens of *P. falciparum* through the construction and differential immunoscreening of the *P. falciparum* genomic expression library using anti- *P. yoelii* sera and pooled sera from malaria endemic area and

- ii. To carry out molecular characterization of the identified conserved antigen (s).

To achieve these objectives, we constructed a genomic expression library of *P. falciparum* in λ gt-11 expression vector and immunoscreened the library with mice sera convalescent from *P. yoelii* infection. Out of 100,000 recombinant plaques 20 plaques were found immuno-reactive with anti- *P. yoelii* sera. These positive clones were further probed with healthy individual sera living in highly *P. falciparum* endemic areas. One of the clones (KP1) found strongly reactive to both *anti-P. yoelii* as well as immune sera collected from endemic areas. The KP1 clone had an insert size of 1132 bp and hybridized to *P. falciparum* DNA and blood stage RNA by Southern and Northern blot hybridization, respectively. Dot blot hybridization showed that the gene was conserved at the DNA level among different *Plasmodium* species. The insert was 70% dA+dT rich and having a complete open reading (Accession no AF256227). Blast analysis revealed the insert encodes a novel partial protein homologue to karyopherin beta, a receptor protein involved in transport and trafficking mechanism. The recombinant protein was expressed in *E. coli* as a GST fusion protein, purified and used to raise antibodies against the protein. Antibodies against recombinant protein identified a 120 kDa native protein band in *P. falciparum* lysate, as determined by SDS- PAGE/Western immunoblot. Reverse transcriptase PCR and immunofluorescence microscopy confirmed that this protein is highly expressed in blood stages of the parasite. Polyclonal murine antibodies against the recombinant fusion protein partially inhibited *in-vitro P. falciparum* growth. Using RACE and newly extant *Plasmodium* genome data bases the full length karyopherin beta gene was characterized in *P. falciparum* and *P. yoelii*. The DNA /Protein homology

between two different geographically isolated strains of *P. falciparum* (India and Africa) showed absolute homology, suggesting that it is highly conserved among different geographically isolated strains. The comparative protein sequence analysis of karyopherin beta between *P. falciparum* (human malaria parasite) and *P. yoelii* (rodent malaria parasite) showed 92 % similarities, which also proves that karyopherin beta is highly conserved across different *Plasmodium* species. However, karyopherin of *P. falciparum* showed only 26-28% homology with karyopherin of human, yeast, drosophila and nematode. As per published report in other organisms, karyopherin beta is a receptor protein, involved in nucleo-cytoplasmic transport and trafficking and proved to be vital molecule for survival of the organisms. These results suggest that this strategy is useful to identify molecules for vaccine and drug targets against human pathogens for which animal models and multiple related species exists.