SUMMARY

Malaria is a parasitic infection caused by a protozoan parasite of the genus *Plasmodium*, which undergoes a complex multistage developmental cycle that alternates between a vertebrate and an invertebrate vector host, the female anopheles mosquito. Despite the continuous efforts and major eradication programmes of WHO, malaria still remains one of the major world health problems and the greatest killer of mankind particularly in the tropical and subtropical regions of the world. For a brief period in early 1960s it appeared that malaria might soon be brought under control. Extensive spraying of dichlorodiphenyl trichloroethane (DDT) greatly helped in reducing anopheline mosquito population while effective chemotherapeutic measures were equally successful in controlling the disease. A few novel drugs such as chloroquine and mefloquine were successfully used for treating the patients. But ten years later by the mid 1970s with the resurgence of malaria, the situation was entirely different, the chlorinated insecticides, such as DDT, were proving to be ineffective due to a newly acquired phenomenon, the insecticidal resistance in mosquitoes and a more alarming situation has arisen due to acquired resistance in *Plasmodium falciparum* to various antimalarial drugs. Currently this constitutes the most important threat towards our efforts for effective control of the disease. Chloroquine and multiple drug resistant strains of *P. falciparum* are spreading in various regions of the world including India. So in view of this grim situation it seems that the repertoire of tools and methods available for malaria control are not keeping pace with the increasing complexity of the disease over the last three decades.

The failure of the conventional measures to control and eradicate malaria has led the scientists and research organisations to the revival of their interest in malaria research not only for finding the better chemotherapeutic measures but also towards finding an immunological solution to the disease. The development of malaria vaccine and the better immunodiagnostic methods still remain the two main objectives of the scientific working groups of the WHO on immunology of malaria since it was set up in 1976. Although, significant advances have been made in developing the vaccine against different developmental stages of the malarial parasites viz. sporozoite stage, asexual blood stages as well as gametocyte stages, the slow progress of the malaria vaccine development may be attributed to the complex multistage developmental cycle of the parasite and the nature of its interaction with its host as well as lack of suitable animal model for human malaria parasites.
As the morbidity, its clinical symptoms and mortality due to malaria is mainly caused by the asexual blood stages, therefore, the blood stages of malaria parasite remains the main focus of malaria research not only for developing the new antimalarial drugs but also for developing the antimalarial vaccine, as well as for immunodiagnostic test. The biological and morphological similarities between human and nonhuman primate malarias make the later good animal model for human malaria providing suitable laboratory host available. Therefore the proposed study reported in this thesis deals with the immunochemical studies on asexual blood stages of primate malaria parasites, *P. knowlesi* (a simian malaria parasite) and *P. falciparum* (the most dreaded of human malaria), in order to identify the common or cross reactive antigens between these two parasites. So the information obtained from the proposed study may be useful and may further be explored for developing better immunodiagnostic test and immunoprophylactic measures of human malaria.

The studies were initiated with the purification of *P. knowlesi* schizont infected RBCs (SI-RBCs), which were purified more than 95% (free from platelets and leucocytes) using CF-11 cellulose and glass beads column followed by one step percoll gradient (56%) centrifugation. The purified SI-RBCs were found to be intact and completely free from broken SI-RBCs, free parasites, membranes and the early infected erythrocytes. These purified *P. knowlesi* SI-RBCs were also found biologically active and viable as they were able to produce infection when inoculated in monkeys and they were also found invading the RBCs in *in vitro* culture system.

The purified *P. knowlesi* SI-RBCs were used to immunize the rabbits for raising the polyvalent hyperimmune serum. Two rabbits were immunized over a period of 3-4 months. The immune rabbit sera collected after each injection (from I to VI injections) as well as their γG fractions (prepared 3 times concentrated from pooled immune rabbit sera by ammonium sulphate precipitation) were tested for the parasite specific antibodies by employing the techniques of enzyme linked immunosorbent assay (ELISA), indirect haemagglutination assay (IHA). In both these assays, an increase in titre values with increasing number of immunization were observed in case of both the rabbits, however, the titre values became constant after IV and V injections. The ELISA was found more sensitive than IHA. The maximum values of reciprocal antibody titre were found 128000 as determined by ELISA and 12800 by IHA in the immune rabbit sera collected after IV and subsequent injections in
case of both the rabbits, however, the γG fractions of the pooled immune sera gave the increased values of reciprocal antibody titre of 256000 (by ELISA) and 25600 (by IHA) in case of both rabbits.

The presence of protective antibodies in immune rabbit sera was demonstrated by examining their effect on multiplication of *P. knowlesi* parasites in *in vitro* culture system. The short term *in vitro* culture of *P. knowlesi* was done by candle jar method of Trager and Jensen (1976), separately in the presence of normal control and immune (anti-*P. knowlesi* SI-RBC) rabbit sera. Though significant rise of parasitaemia in normal rabbit sera was observed while no parasites were observed in presence of immune rabbit sera. Therefore, these results indicated the presence of protective antibodies in immune rabbit sera.

In subsequent studies, the protein and antigenic components of *P. knowlesi* SI-RBC were analysed by employing the techniques of SDS-polyacrylamide gradient gel electrophoresis (SDS-PAGE) and immunoelectrophoretic techniques. The normal RBC ghost antigenic extract was also analysed along with for comparative purpose. The SDS-PAGE resolved the *P. knowlesi* SI-RBC into 30-35 major protein bands in the mol. wt. range of 15-230 KD on 5-12% gradient of polyacrylamide. The number of protein bands present in *P. knowlesi* SI-RBC were found much greater in number as well as having several additional and heavily stained protein bands than that in normal rhesus RBC ghost, thereby indicating certain qualitative and quantitative differences in the protein patterns of *P. knowlesi* SI-RBC and normal RBC ghost.

The antigenic components of *P. knowlesi* SI-RBC were analysed initially by employing the technique of immunoelectrophoresis (IEP) using rabbit anti-*P. knowlesi* SI-RBC sera. The IEP analysis showed an increase in the number of precipitin arcs with increasing number of immunization. The number of precipitin arcs become constant at 12 with sera obtained after IV and V injections. However, only 3-4 precipitin arcs were formed with normal rhesus RBC ghost antigen against rabbit anti-*P. knowlesi* SI-RBC sera. So these results indicated the presence of 8-9 parasite specific antigens in *P. knowlesi* SI-RBC.

The antigenic components of *P. knowlesi* SI-RBC and normal rhesus RBC ghost were further analysed more clearly by employing the more sensitive technique of crossed immunoelectrophoresis (CIE) using rabbit anti-*P. knowlesi* SI-RBC gamma globulin.
fraction (γG, prepared from the pooled hyper immune rabbit sera). Out of the different concentrations (5%, 7.5%, 10% and 15%) of gamma globulin fraction tried, better resolution of antigenic peaks was observed with 7.5% gamma globulin concentration with *P. knowlesi* SI-RBC and 10% gamma globulin concentration with normal rhesus RBC ghost antigens. The CIE analysis revealed 40 antigenic peaks with *P. knowlesi* SI-RBC while only 6 antigenic peaks were observed with normal rhesus RBC ghost antigens, thereby suggesting the presence of 34 parasite specific antigens in *P. knowlesi* SI-RBC.

In order to focus attention on the common or cross reactive antigens between *P. knowlesi* and *P. falciparum* SI-RBCs, the triton extracts of the $^{35}$S-methionine labelled SI-RBC of *P. falciparum* were immunoprecipitated with immune sera from patients having malaria (*P. falciparum*) infection and rabbit anti-*P. knowlesi* SI-RBC hyperimmune sera. The immune complexes formed were further analysed on SDS-PAGE followed by autoradiography. Instead of individual patient sera, immune serum pools (high, medium and low titred) were used for immunoprecipitation so as to include the widest spectrum of antibodies. The antibody titres of individual patient sera as well as immune serum pools were quantitated by IFA using *P. falciparum* SI-RBC as well as *P. knowlesi* SI-RBC antigens. The antibody titres were obtained more with *P. falciparum* SI-RBC as compared to *P. knowlesi* SI-RBC.

The $^{35}$S-methionine labelled proteins of *P. falciparum* after immunoprecipitation were analysed on SDS-PAGE, which revealed that 10-12 antigens were recognized by patient sera (*P. falciparum*) while 15-16 antigens were recognized by hyperimmune rabbit sera (anti-*P. knowlesi* SI-RBC). On comparing, 5 major antigens of 195, 120, 97, 94 and 74 KD were found to be common or cross reactive between these two parasites, one common antigen of 74 KD was found most strongly recognised by all the three patients serum pools as well as hyperimmune rabbit serum.

In conclusion, we have been able to purify *P. knowlesi* SI-RBC more than 95% (free from platelets and leucocytes) using glass beads and CF-11 cellulose column and subsequently by applying one step percoll gradient centrifugation. Immune rabbit sera raised against *P. knowlesi* SI-RBC showed high titres of parasite specific antibodies, as detected by (ELISA) and (IHA), thus showing the immunogenic nature of *P. knowlesi* SI-RBC. These hyper immune rabbit anti-*P. knowlesi* SI-RBC sera were found to contain protective antibodies, as they inhibited the multiplication of *P. knowlesi* parasites in *in-vitro* culture.
These SDS PAGE analysis of *P. knowlesi* SI-RBC revealed the presence of 30-35 major protein bands in the molecular weight range of 15-230 KD. The immunoelectrophoretic analysis revealed the presence of 34 parasite specific antigens in *P. knowlesi* SI-RBC. The immunoprecipitation and SDS-PAGE analysis of ³⁵S- methionine labelled antigens of *P. falciparum* SI-RBC with malaria (*P. falciparum*) patient sera and hyper immune rabbit sera (anti-*P. knowlesi* SI-RBC) pools revealed the presence of 5 common or cross reactive antigens of mol.wts. 195, 120, 97, 94 and 74 KD between these two parasites, of these, the 74KD antigen was found to be a major common antigen.