CHAPTER IV

SUMMARY AND CONCLUSION
Ancylostomiasis is one of the major public health problems in tropical countries. The world incidence of hookworm infections are 1000 million with the death rate of 55 thousand per year. The currently available anthelmintics are not very effective. This clearly emphasises the need to intensify the control measures in hookworm endemic areas. An alternate approach for the permanent control of the disease would be to develop immunoprophylactic measures against hookworm parasites. However, the existing knowledge about the molecular immunology of hookworm infection is still in its infancy. Hookworm parasites evoke functional protective immunity and some reports are available regarding the protective potential of live parasitic stages as well as of somatic extracts and excretory-secretory products from adult and larval stages, however, not much is known about the molecules/antigens which are involved in evoking these protective responses. A basic knowledge about the total antigenic composition of the parasites is essential not only for understanding the host parasite relationships but also for the identification of antigenic targets of protective immunity. We have, therefore, directed our studies towards the characterisation of antigens from adult and larval stages of human hookworm parasite; *Ancylostoma ceylanicum*, which has earlier been used for the screening of anthelmintic compounds by several workers. Total protein and antigenic composition of adult and larval stages of *A. ceylanicum* were studied employing SDS-polyacrylamide gel
electrophoresis (SDS-PAGE) and immunoelectrophoretic techniques using hyperimmune rabbit sera. The levels of parasite specific antibodies in immune sera were determined by enzyme linked immunosorbent assay (ELISA). Similar techniques were used for antigenic analysis of the excretory-secretory products from adult *A. ceylanicum*. The protective potential of somatic extracts from adult and larvae as well as the excretory-secretory products from adult *A. ceylanicum* was also studied by immunising the naive hamsters with these antigens (somatic extracts from adult and larvae and excretory-secretory products from adults) followed by challenge infection.

The somatic extracts from adult (AcA) and larval stages (AcL) of *A. ceylanicum* were separated on SDS-polyacrylamide gradient gel electrophoresis (SDS-PAGE), which showed the presence of 47 protein bands in adults and 43 in larvae in the molecular weight range of 10-170 kD. Most of the protein bands were found to be common between these two stages (adult and larvae), however, 8 (130, 125, 42, 30, 20, 17, 16.5 and 10 kD) and 4 (170, 160, 153 and 85 kD) protein bands specific to adult and larval stages respectively were observed. The quantitation of parasite specific antibodies in immune rabbit sera was done by enzyme linked immunosorbent assay and reciprocal antibody titers of 256,000 and 128,000 were obtained for rabbit anti-adult and rabbit anti-larval sera respectively using AcA antigen at optimum concentration of 0.25 ug/well. Both these sera showed the reciprocal anti-
body titers of 256,000 with AcL at optimum concentration of 0.50 ug/well.

The antigenic analysis of somatic extracts from adult and larval stages of *A. ceylanicum* was initially done by immunoelectrophoresis (IEP), which revealed 10-12 precipitin lines in AcA with rabbit anti-AcA serum and 6-7 precipitin bands in AcL with rabbit anti-AcL serum. Further characterisation of these somatic antigens was carried out employing crossed immunoelectrophoresis (CIE) which showed the presence of 34 antigenic components in AcA and 19 antigens in AcL. Out of 34 AcA antigens, 25 were anodic and 9 cathodic while 17 anodic and 2 cathodic antigens were observed in AcL. In order to study the cross-reactive or common as well as stage/parasite specific antigens of *A. ceylanicum*, crossed immunoelectrophoresis employing heterologous sera and crossed line immunoelectrophoresis (CLIE) were used. When AcA and AcL were reacted with heterologous immune rabbit sera in CIE, most of the antigens were found common or cross-reactive between the two stages, however, 12 antigens specific to adult and 5 antigens specific to larval stage were observed. The subsequent analysis of CLIE also revealed the same number of common or cross-reactive as well as stage specific antigens in AcA and AcL. In order to analyse *A. ceylanicum* antigens having cross-reactivity with rat hookworm - *Nipponstrongylus brasiliensis*, CIE using immune rabbit sera raised against adult (NbA) and infective larval (NbL) somatic extracts of *N. brasiliensis* and
CLIE using NbA and NbL in the intermediate gel, were done. Most of the antigens (22-26) were found to be AcA specific, only 12 and 8 antigens of AcA were found to be common/cross reactive with NbA and NbL respectively. On analysing the cross-reactive or common antigens of AcL with NbL and NbA, 13 common or cross reactive and 6 AcL specific antigens were observed.

Another set of antigens analysed in the present study is the excretory-secretory (E-S) products from adult _A. ceylanicum_. The conditions for the preparation of E-S products were optimised with respect to medium as well as the incubation time. Out of three different media: RPMI-1640, DMEM and Ringer's solution tried, Ringer's solution proved to be the best as the motility of the worms was maximum in this medium and the worms could be maintained upto 6h without changing the medium. The amount of protein released was found to be 95±15 _ug/100 adults_ in RPMI-1640 and DMEM as compared to 125±25 _ug/100 adult_ worms in Ringer's solution. Immunoelectrophoretic analysis using rabbit anti-AcA serum showed the presence of 5-7 precipitin bands in E-S products prepared in these media. As there was not any significant difference in the amount of protein released as well as the antigenic patterns of E-S products prepared in three media, Ringer's solution (containing salts and glucose) was chosen to obtain the E-S products from _A. ceylanicum_ adults because this is much simpler and more economical in comparison to other two media (RPMI-1640 and DMEM).
A polyvalent hyperimmune rabbit serum was raised against *A. ceylanicum* adult E-S products and the titer of parasite specific antibodies in immune rabbit sera was determined by enzyme linked immunosorbent assay using optimum concentration of 0.50 ug E-S antigen/well. A reciprocal antibody titer of 256,000 was obtained with rabbit anti-E-S serum. The E-S antigens were also tested with the immune rabbit sera raised against somatic extracts of *A. ceylanicum* in ELISA and a titer value of 128,000 was obtained for both rabbit anti-adult and rabbit anti-larval sera. Crossed immunoelectrophoresis (CIE) using rabbit anti-E-S serum revealed 16 antigens in *A. ceylanicum* E-S products, out of which 13 were anodic and 3 moved towards the cathode. E-S products have also been found to share antigenic determinants with the somatic extracts from adult and larval stages. IEP analysis of E-S products showed the presence of 6 and 4 precipitin bands with rabbit anti-AcA and rabbit anti-AcL sera respectively. On further analysing the E-S products by CIE using rabbit sera raised against somatic extracts 12 and 7 antigens of E-S products were found to share antigenic determinants with AcA and AcL respectively. Tandem crossed immunoelectrophoresis (TCIE) of E-S products with AcA and AcL also revealed almost the same number of common/cross-reactive antigens between the E-S products and the somatic extracts. The presence of host serum proteins in the E-S products was also analysed by CIE using rabbit serum raised against normal
A polyvalent hyperimmune rabbit serum was raised against *A. ceylanicum* adult E-S products and the titer of parasite specific antibodies in immune rabbit sera was determined by enzyme linked immunosorbent assay using optimum concentration of 0.50 μg E-S antigen/well. A reciprocal antibody titer of 256,000 was obtained with rabbit anti-E-S serum. The E-S antigens were also tested with the immune rabbit sera raised against somatic extracts of *A. ceylanicum* in ELISA and a titer value of 128,000 was obtained for both rabbit anti-adult and rabbit anti-larval sera. Crossed immunoelectrophoresis (CIE) using rabbit anti-E-S serum revealed 16 antigens in *A. ceylanicum* E-S products, out of which 13 were anodic and 3 moved towards the cathode. E-S products have also been found to share antigenic determinants with the somatic extracts from adult and larval stages. IEP analysis of E-S products showed the presence of 6 and 4 precipitin bands with rabbit anti-AcA and rabbit anti-AcL sera respectively. On further analysing the E-S products by CIE using rabbit sera raised against somatic extracts 12 and 7 antigens of E-S products were found to share antigenic determinants with AcA and AcL respectively. Tandem crossed immunoelectrophoresis (TCIE) of E-S products with AcA and AcL also revealed almost the same number of common/cross-reactive antigens between the E-S products and the somatic extracts. The presence of host serum proteins in the E-S products was also analysed by CIE using rabbit serum raised against normal
hamster serum, and 6 antigens in the E-S products appeared to be of host origin.

The protective potential of somatic extracts from adult and larval stages as well as excretory-secretory products from adult *A. ceylanicum* was tested by immunizing the hamsters with these antigens and evaluating the immunized hamsters for protective immunity. The sera obtained from immunized hamsters, before challenge, showed significant levels of parasite specific antibodies. The immunodiffusion and immunoelectrophoretic analysis revealed the presence of precipitin antibodies in immune hamster sera and 7 precipitin bands in adult somatic extracts and 4 in both larval somatic extracts and E-S products. The hamsters immunized with larval somatic extracts showed maximum protection (93% reduction in worm burden) while 75% and 63% protection was observed in hamsters immunized with excretory-secretory products and adult somatic extracts respectively.

Therefore, in the present study we have been able to characterise the protein and antigenic components of adult and larval stages of *A. ceylanicum* using hyperimmune rabbit sera having high titers of parasite specific antibodies and certain biochemical and immunochemical techniques. The common or cross-reactive antigens between the two life stages of *A. ceylanicum* and also between the two hookworm parasites (*A. ceylanicum* and *N. brasiliensis*) as well as stage and parasite
specific antigens have also been identified. We have been able to prepare the excretory-secretory products of *A. ceylanicum* adults using Ringer's solution which is much simpler and a more economical medium. Antigenic characterisation of E-S products revealed less complex nature of these products as compared to the adult somatic extracts. The protective potentials of the somatic extracts from adult and larval stages and also of E-S products from adult *A. ceylanicum* was also studied. The larval somatic extracts, though contain less number of antigens, showed maximum protection followed by excretory-secretory products and adult somatic extracts. These studies provide basic information about the antigenic compositions of somatic extracts and excretory-secretory products of *A. ceylanicum* as well as the comparative efficacy of these antigens in inducing protective immunity against this parasite. However, further studies on molecular characterisation of these antigens are certainly required for identifying the antigenic targets of protective immunity.