CHAPTER IV

SUMMARY AND CONCLUSIONS
Filariasis is a major health problem in tropical and subtropical countries. It is caused by the parasites belonging to class filaroidea, which have complex life cycle that alternates between an arthropod and vertebrate host. Though, the disease is not fatal, but these parasites cause lymphatic damage that leads to elephantiasis and hydrocoele which is responsible for considerable disability and social stigma. Important pathogenic species infecting humans are *Wuchereria bancrofti*, *Brugia malayi* and *B. trimori*. In India over 90% of the disease is caused by *Wuchereria bancrofti*.

The unequivocal diagnosis of filarial infection still largely depends on the detection of the microfilariae in the blood. Nocturnal periodicity of microfilariae imposes a practical limitation on the effective use of microfilaria detection of lymphatic filariasis because of the need for night blood sampling. The parasitological method is inconvenient for both the patient as well as the investigator and fails to detect the amicrofilaraemic stage of the disease or when mf are present in very small numbers. This has put forward an emphasis for improved immunodiagnostic methods for species and accurate diagnosis of active filarial infection.

Earlier studies on immunodiagnosis of filariasis was focussed mainly on the demonstration of antibodies to filarial antigens. The major disadvantage of the immunodiagnostic test based on antibody
detection is their inability to distinguish between the past exposure and current infections as well as their cross-reactivity with other helminth parasites. The detection of filarial circulating antigen in blood and other body fluids of infected individuals has been shown to be a better approach for diagnosing active filarial infections. Though a number of antigen detection assays have been developed but the problem of sensitivity and specificity of these assays still remains. There is still a need to identify specific filarial antigen for developing better immunodiagnostic reagents.

Due to the non-availability of human filarial parasites in sufficient quantities, heterologous antigens from animal filarial parasite including *S. cervi* (bovine filarial parasite) have been used for developing immunodiagnostic tests. However, the use of antibodies prepared against crude antigenic preparation results in false positive reactions. This may be due to complex nature of these antigens and their cross-reactivity with other helminths parasites. Polyclonal antibodies against purified or simple antigenic preparations or monoclonal antibodies against filaria-specific epitopes are required for developing specific immunodiagnostic methods. The excretory-secretory antigens released by the living parasites in the host are known to be less complex in nature and may contain antigen(s) equivalent to filarial circulating antigen. The present study was undertaken on the characterization of excretory-secretory products of *S. cervi* (bovine
filarial parasite) to identify the antigen equivalent to filarial circulating antigen, production of antibodies against these antigens.

The studies were initiated with the preparation of *S. cervi* E-S products, by maintaining the adult parasites aseptically in protein free medium (DMEM) for 32 hrs at 37°C. Substantial amount (12-15 mg protein/100 worms) of E-S products were obtained from *S. cervi* adult by maintaining in DMEM and the worms remained motile for the maximum period of time without change of medium. The release of large amount of protein facilitated the fractionation and characterization of *S. cervi* E-S products. The SDS-polyacrylamide gel electrophoretic analysis of *S. cervi* E-S products showed the presence of 14-15 protein bands in molecular weight range of 10-200 kD. These results revealed less complex nature of ScE-S products as compared to *S. cervi* somatic antigenic preparation which showed 30-35 protein bands using hyperimmune rabbit serum. The ScE-S products were used to immunized the rabbits for raising hyperimmune polyvalent serum. Three rabbits (R1, R2 & R3) were immunized over a period of 5-6 months. The immune rabbit sera collected after each immunization starting from the 3rd injection and were tested for the parasite specific antibodies by employing enzyme linked-immunosorbent assay (ELISA). An increase in the antibody titre, with increasing number of immunization, was observed and the titre value became constant after VIIIth injection in all the three rabbits (IRS 1,
IRS 2 and IRS 3). The immune serum pool from IRS 1 showed the reciprocal antibody titre of 128,000, while the reciprocal antibody titre of 256,000 were observed for IRS 2 and IRS 3 pools. The antigenic components of S. cervi E-S antigens were analysed by employing the techniques of CIE using rabbit anti-S. cervi E-S serum. The best resolution in CIE was obtained by using 5-10% concentration of anti-S. cervi E-S serum and it revealed the presence of 10-12 precipitin peaks in E-S products.

Parasite worms are known to adsorb host serum proteins (mainly albumin) on their surfaces in order to evade the immunologically hostile environment of the host. The S. cervi adult worms resides on the peritoneal folds of buffaloes and absorption of normal host proteins and their subsequent release during in vitro maintenance is likely to occur. In the present study, the S. cervi E-S products were also analysed for the presence of host serum protein contaminants by using rabbit anti-normal buffalo serum and it showed the presence of 3-4 host proteins. Further, efforts were made to characterize these host protein contaminants by using the techniques such as crossed line immunoelectrophoresis (CLIE) and tandem crossed immunoelectrophoresis (TCIE). The CLIE was done by adopting two ways, firstly the BSA was introduced in the intermediate gel and secondly by introducing the rabbit anti-BSA serum in the intermediate gel, and the rabbit anti-E-S serum was used in both the cases. In the
former case, when BSA was used in the intermediate gel, two precipitin peaks of the E-S products showed lines at their bases. The reduction in the heights of the same precipitin peaks was observed in the latter case. In case of TCIE, BSA was run along with the E-S products in the first dimension, using the rabbit anti-E-S serum, and it showed considerable increase in the heights of the same two corresponding precipitin peaks as observed in case of CLIE. The above results with CLIE and TCIE indicates that the major components of host serum proteins is albumin.

The rabbit anti-E-S serum was evaluated for its potential to detect circulating antigen in filarial patient serum. Initially, the rabbit anti-ScE-S serum showed non-specific reaction with the normal human serum. This may be due to the presence of host serum albumin in the \textit{S. cervi} E-S products. Therefore, efforts were made to absorb the host serum proteins from the \textit{S. cervi} E-S products using anti-BSA sepharose beads. The preabsorbed \textit{S. cervi} E-S products were analysed using SDS-PAGE, CIE and ELISA. The SDS-PAGE analysis of the preabsorbed E-S products showed the removal of major 66 kD protein band, which was present in the unabsorbed \textit{S. cervi} E-S products. The antigenic analysis by CIE, using rabbit anti-E-S serum, revealed the presence of 10 precipitin peaks in the preabsorbed E-S products. On testing the preabsorbed \textit{S. cervi} E-S products in ELISA against rabbit anti-ScE-S serum, the antibody titre was of 1:120 000.
Similarly, the absorption of rabbit anti-\textit{S. cervi} E-S serum on BSA-sepharose beads, resulted in the removal of the antibodies against the host protein components. The preabsorbed rabbit anti-ScE-S serum, showed the removal of the antibodies against the two major host protein contaminants of E-S products. This preabsorbed rabbit anti-ScE-S serum, when tested in ELISA against \textit{S. cervi} E-S products, gave an antibody titre of 1:120 000 as compared to the reciprocal antibody titre of 250 000 obtained for the unabsorbed anti-E-S serum. The preabsorbed anti-E-S serum was evaluated for the detection of circulating filarial antigen in patient sera by counter current immunoelectrophoresis. This absorbed rabbit anti-E-S serum showed no cross-reactivity with the normal human serum, and could successfully detect the circulating antigen in the filarial patient sera.

The fractionation of \textit{S. cervi} E-S products on DEAE-Sephacel column yielded 3 major protein peaks. Six antigen fraction pools were made according to their reactivity with rabbit anti-E-S serum and rabbit anti-BSA serum. The reactivities of the antigen fraction pools were tested against the monoclonal antibody (detecting the filarial circulating antigen) in ELISA. The ScESPFI showed high reactivity with the monoclonal antibody, whereas, all other antigen fraction pools did not show any significant reactivity. Therefore, the ScESPFI which may be having the antigen equivalent to the filarial circulating antigen, was used to immunize the mice for hybridoma production.
The production of monoclonal antibodies against *S. cervi* E-S products was done by the fusion of spleen cells from mice (immunized against ScESPFI) with myeloma cells. A fusion efficiency of 96% was obtained. The 69 hybridoma clones showed reactivity with *S. cervi* antigens, 27 with ScESFPI, 36 with *B. malayi* adult antigen and 21 with *W. bancrofti* microfilarial antigens. A total of 12 monoclonal antibodies having consistent reactivity with both bovine and human parasites were selected and out of these four monoclonal antibodies (FES 2, FES 7, FES 9 and FES 11) were found to have high affinity for the filarial circulating antigen. All the 4 monoclonals showed high reactivity with human (*B. malayi* and *W. bancrofti*) and bovine filarial (*S. cervi* E-S, ScESFPI, ScA and ScMf) antigen but the highest reactivity in all the cases was observed with FES 9 monoclonal antibody. None of these monoclonal antibodies showed reactivity with any significant the non-filarial antigens (ascaris, hookworm, malaria, amoebae, bacteria) indicate the specificity of the monoclonal antibodies to filarial antigen. Isotyping analysis revealed that one (FES 2) out of four monoclonals, was found IgG2b one (FES 11) was IgM and two (FES 7 and FES 9) were IgG1 isotype. The target epitope of the three monoclonal antibodies (FES 7, FES 9, FES 11) was found to be same as that of Sc1 monoclonal antibody, while FES 2 monoclonal was directed against a different epitope as shown by inhibition ELISA studies.
Therefore, in the present study we have been able to analyse and characterize the excretory-secretory products of *S. cervi* adult worms. The hyperimmune sera raised against the *S. cervi* E-S product showed high antibody titres in *S. cervi* E-S products and showed the presence of 10-12 antigenic components. Out of these 3-4 antigens were identified as host serum protein components and one of them being albumin. The host serum proteins present in *S. cervi* E-S products could be removed by absorbing with anti-BSA antibodies which resulted in the removal of host serum protein bands. The absorption of rabbit anti-*S. cervi* E-S serum with BSA removed the antibodies the against host serum proteins. The preabsorbed rabbit anti-ScE-S serum successfully detected the circulating antigen in filarial patients' sera. Fractionation on DEAE-Sephacel column resulted in isolating a purified antigen fraction common with human filarial parasite and showing high reactivity with the monoclonal, (detecting filarial circulating antigen), the four monoclonal antibodies (FES 2, FES 7, FES 9 and FES 11) produced against this purified antigen fraction (ScESPFI) showed high reactivity with filarial antigen and did not cross react with non-filarial antigens thereby, suggesting their filaria specificity. Out of these four monoclonals, three (FES 7, FES 9 and FES 11) were found to be directed against the same epitope as that of Sc1 monoclonal antibody, while the fourth (FES 2) was against a different epitope. The FES 9 monoclonal antibody in combination with rabbit ScE-S polyclonal, showed higher sensitivity for the detection of
circulating antigen in filarial patient sera. These findings suggest the potential of anti-ScE-S antibodies for developing specific immunodiagnostic test based on antigen detection for human filariasis.