CHAPTER II

MATERIALS AND METHODS
PARASITES

*Setaria cervi*, a bovine filarial parasite, was collected from the peritoneal folds of freshly slaughtered Indian water buffaloes from the local abbatoir and brought to the laboratory in normal saline. The motile worms (both males and females) were washed thoroughly with normal saline to remove all the adhering materials and were used immediately for antigen preparation or stored at -20°C until used.

EXPERIMENT ANIMALS

Albino rabbits of either sex (weighing 1.5 to 2.0 Kg) and BALB/c mice (6-8 weeks old) from the inbred colony of Central Drug Research Institute's animal house, were used. All the animals were kept in separate cages and fed on balanced diet and housed in an air conditioned room.

CHEMICALS USED

Acrylamide, agarose, ammonium persulphate, anti-rabbit IgG (whole molecule) horse radish peroxidase conjugate, 2-beta
mercaptopethanol (βME), bisacrylamide, bovine serum albumin (BSA), bromophenol blue, coomassie brilliant blue (R~250), ethylene diamine tetra acetic acid (EDTA), hypoxanthine aminopterin thymidine (HAT), molecular weight markers, nitrocellulose paper (NCP), orthophenylene diamine (OPD), polyethylene glycol (PEG), sodium dodecyl sulphate (SDS), N N N'N' tetramethyl ethylene diamine (TEMED), Tris (Hydroxymethyl) aminomethane (TRIS Buffer) were procurred from Sigma Chemical Company, St. Louis, MO, USA, Freund's complete and incomplete adjuvants were obtained from Difco Detroit, MI, USA. The CNBr activated sepharose CL-4B was procurred from Pharmacia Biotech AB, Uppsala, Sweden. The powdered Dulbecco's modified eagle medium (DMEM) was obtained from Gibco Laboratories, NY, USA. All other chemicals used were of analytical grade. Triple distilled water was used in all the experiments.

**PREPARATION OF ANTIGEN**

The excretory-secretory (E-S) antigens were prepared by short term *in vitro* maintenance of bovine filarial parasites as described by Malhotra *et al.* (1987). The medium used for the culture was prepared by dissolving one packet of Dulbecco's modified eagle medium (DMEM), 2.38g of N-2 hydroxylethyl piperazine-N'2-ethane
sulphonic acid (HEPES), 3.8g sodium bicarbonate and 0.3g glutamine to make 1 ltr with triple distilled water. This solution is filtered through 0.22 μm millipore filter for sterilization and dispensed into sterile screw capped flask.

The adult motile worms (70-75) of *S. cervi* (both males and females) were maintained aseptically at 37°C for 32 hr in a protein free defined medium i.e. DMEM containing 1000 U/ml, penicillin and 100 g/ml streptomycin. The medium was changed at regular intervals. The spent medium was centrifuged at 1000 x g (3000 rpm) for 10 min to remove the microfilariae released in the medium. The supernatant was kept at -70°C until used. The E-S products were concentrated by lyophilization. The lyophilized E-S materials were reconstituted in minimal volume of normal saline and were dialysed against the normal saline. This was again centrifuged at 1000 x g (3000 rpm) for 10 min and the E-S products finally prepared were stored at -70°C until used.

**PROTEIN ESTIMATION**

The protein contents of the E-S products were determined by the microassay procedure of Bradford (1976) while the procedure of
Lowry's et al. (1951) was used to measure the protein contents of somatic antigen and antibodies.

**Bradford's method:** prepared several dilutions of 0.8 ml of protein standard (BSA 1 mg/ml) from 1 to 25 μg/ml and 0.8 ml of sample buffer for blank, then 0.2 ml of Biorad dye reagent (Biorad, USA, Coomassie brilliant blue in phosphoric acid). It was mixed gently and after 5 min optical density (O.D.) was measured at 595 nm in spectrophotometer.

**Lowry's method:** several dilutions of 0.4 ml of protein standard (BSA, 500 μg/ml) from 10 to 50 μg/ml or above were made and 0.4 ml of sample buffer for blank was taken. To all the samples 2.0 ml copper reagent was added (0.5 ml of Na, K, tartarate, 0.5 ml of 1% copper sulphate, 5H₂O and 50 ml of 2% sodium carbonate dissolved in 0.1 N sodium hydroxide), incubated for 10 mins 0.2 ml of Folin's reagent (1N) was added and the mixture was mixed thoroughly and optical density was measured at 625 nm after 30 min.

**SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS**

The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of *S. cervi* E-S antigen was done according to Laemmli (1970) using
Pharmacia slab gel electrophoresis apparatus. A separating gel of 10% acrylamide was used. The composition of the mixture was 10 ml of stock acrylamide (30%) and bisacrylamide (0.8%), 7.5ml of 1.5M Tris-HCl (pH 8.8), 300 μl of 10% sodium dodecyl sulphate (SDS), 100 μl of 10% ammonium persulphate (APS), and 10 μl of N,N,N',N'-tetra methyl ethylene diamine (TEMED) in a total volume of 30 ml made up with triple distilled water. The stacking gel (4.5%) contained 1.49 ml of acrylamide stock solution (30% acrylamide and 1% bisacrylamide), 2.63 ml of 0.5M Tris-HCl (pH 6.8), 0.1 ml of 10% SDS, 75 μl of 10% APS and 10 μl of TEMED in a total volume of 10 ml made up with distilled water. The samples were prepared by mixing equal volumes of the E-S antigen with SDS-PAGE sample buffer (containing 12.5 ml of 1M Tris-HCl buffer, pH 6.8; 20 ml of glycerol; 10 gm of SDS; 2 ml of 0.1% bromophenol blue; 10 ml of 2β-mercaptoethanol (βME) in a total volume of 100 ml) in 1:1 ratio and keeping for 5 min in boiling water bath.

Electrophoresis was carried out at a constant current of 25 mA per gel for about 4-5 hr till the tracking dye has reached 1 cm above the bottom of the gel. After electrophoresis, the gel was stained with 0.25% coomassie brilliant blue R-250 (dissolved in 30% methanol and 10% acetic acid) and destained with 30% methanol and 7% acetic acid. The gel was also stained by silver staining method (Merril
et al., 1981). Briefly, the gel was fixed with 50% methanol and 12% acetic acid, excess SDS was removed by washing three times with 200 ml of 10% ethanol and 5% acetic acid. The gel was soaked for 10 min in 200 ml of 0.0034 M potassium dichromate and 0.0032N nitric acid. The gel was then washed 4 times with 200 ml of distilled water and placed in 0.2% silver nitrate solution for 30 min. The gel was again washed twice with distilled water, followed by rapid staining with two 300 ml of the image developing solution, containing 0.28 M sodium carbonate and 0.5 ml formalin per ltr. Gel was gently agitated in a third portion of the developing solution until the image has reached the desired intensity. The reaction was stopped by putting the gel in 100 ml of 1% acetic acid.

**IMMUNIZATIONS**

**Rabbits**

The polyclonal hyperimmune serum against the S. cervi E-S antigens were raised in albino rabbits of either sex. The rabbits were immunized intramuscularly with an emulsion of 0.5 mg of S. cervi E-S product in Freund's complete adjuvant. One month after the first injection, three subsequent injections were given at seven days
interval with E-S products emulsified in Freund's incomplete adjuvant. The rabbits were bled one week after each injection starting from third injection. The serum was obtained by centrifugation of blood at 900 x g (2500 rpm) for 10 min. The immunization and bleeding of rabbits were continued and done alternatively at 15 days interval in order to get the hyperimmune sera. The serum obtained was stored at -20°C until used. Similarly rabbits were immunized with bovine serum albumin (BSA, 2mg/rabbit) in order to raise the hyperimmune sera against BSA.

**ENZYME LINKED IMMUNOSORBENT ASSAY**

Enzyme linked immunosorbent assay (ELISA) was performed according to Voller *et al.* (1974), with few modifications, in a 96 well polystyrene plates. Briefly, the microtitre ELISA plate was coated with different concentrations of *S. cervi* E-S antigens (7.5 ng-1000 ng/well) diluted in phosphate buffered saline (PBS, 0.05M, containing 0.04M disodium hydrogen phosphate, 9.5mM sodium dihydrogen phosphate, and 0.15M sodium chloride, adjusted to pH 7.4) and incubating the plate for 18-20 hr at 37°C. The plate was washed thrice with PBS to remove the unbound antigen. The uncoated sites of the plate were blocked with 5% non-fat dry milk in
PBS for 2 hr at 37°C. The plate was washed thrice with PBS containing 0.05% Tween-20 (PBS-Tween). Then, the plate was incubated with 100 ml of fixed dilutions of antibody to each well and kept for 2 hr at 37°C. Again the plate was washed with PBS-Tween and then incubated with peroxidase conjugated secondary antibody at 1:1000 dilution in 1% milk for 90 min at 37°C. Finally, the plate was washed with PBS-Tween and was developed with substrate i.e. orthophenylenediamine (OPD 1mg/ml) in 0.05M citrate buffer (0.02M citric acid and 0.05M disodium hydrogen phosphate), pH 5.2, containing hydrogen peroxide (H₂O₂, 1 μl/ml). The reaction was stopped after 10 min with 5N H₂SO₄ and the colour intensity was read at 490 nm in an ELISA reader.

CROSSED IMMUNOELECTROPHORESIS

Crossed immunoelectrophoresis (CIE) was performed according to Axelsen et al. (1973). The S. cervi E-S antigens were separated in 1.5 mm thick layer of 1% agarose in 0.02 M barbital buffer, pH 8.6 (20 mM diethylbarbituric acid, 80 mM Tris, 20 mM sodium azide, 0.8 mM calcium lactate) for 1 hr at a constant voltage of 150 V. After separation in the first dimension, 1x6 cm agarose strip containing the separated antigens was transferred to another plate (6x9 cm). Anodic
(5x6x0.12 cm) and cathodic (2x6x0.12 cm) gel containing 5-10% immune rabbit serum was poured onto the plate. An intermediate gel (1x6x0.13 cm) containing no antibody, was poured between the anodic gel and the first dimension gel containing the separated antigen strip. The plate was electrophoresed at right angle to the first dimension for 18-20 hr at 2-3 V/cm. The CIE plate was washed three times with isotonic saline for 45 min each and finally with distilled water for 15 min and alternate pressing of the plate was done under several sheets of filter paper. The plate was air dried, stained with 0.25% w/v of commassie brilliant blue (R~250) made in 45% ethanol and 10% acetic acid and destained with the same.

CROSSED LINE IMMUNOELECTROPHORESIS

Crossed line immunoelectrophoresis (CLIE) is the modification of CIE and was carried out in the similar manner except that in CLIE the test antigen (50-100 µg) was introduced into the intermediate gel. After electrophoresis the plate was washed and stained as described earlier for CIE. On staining, the common antigen gave a line at the base of the antigenic peaks.
TANDEM CROSSED IMMUNOELECTROPHORESIS

Tandem crossed immunoelectrophoresis (tCIE) is almost similar to that of CIE and is carried out in the same manner except that in tCIE the test antigen was mixed with the antigen (1:1 ratio) and electrophoresed together in the first dimension. The second dimension electrophoresis is similar to that of CIE. After electrophoresis the plate was washed, dried and stained as described earlier in CIE. On staining the common antigen showed the increase in height of the precipitin peaks as compared with the control CIE.

ABSORPTION OF *S. CERVI* E-S PRODUCTS ON ANTI-BSA \( \gamma \)-GLOBULIN SEPHAROSE

The anti-BSA sera raised in rabbits were used to prepare \( \gamma \)-globulin fraction by ammonium sulphate fractionation method. The anti-BSA \( \gamma \)-globulin fraction was coupled to cyanogen bromide activated sepharose CL-4B (Pharmacia) as follows: 100 mg anti-BSA \( \gamma \)-globulin fraction was mixed with 10 ml of the preswellon sepharose CL-4B in 45 ml coupling buffer (0.1M NaHCO\(_3\) and 0.5M NaCl, pH 8.3) by shaking end over end overnight at 4°C. The unbound
protein was removed by washing the beads with Tris-HCl buffer (pH 8.0) and the residual active groups were blocked with 1M diethanolamine (pH 8.0), overnight at 4°C. The sorbent was washed alternatively with 0.1 M acetate buffer (1M NaCl, 0.012 M sodium acetate.3H₂O and 0.096M glacial acetic acid), pH 4.0, and 0.1 M borate buffer (0.1 M boric acid, 0.025 M sodium tetraborate and 0.075 M NaCl), pH 7.9, with 10 min. minimum exposure to each buffer. Finally, the sorbent was stored in 0.005M borate buffer containing 0.15 M NaCl, pH 8.0, having 0.01% sodium azide.

For the absorption, one ml of *S. cervi* E-S products were mixed with 2 ml of the anti-BSA sepharose beads and was rocked gently for 24 hr at 4°C, centrifuged and taken out. Again the preabsorbed E-S products were mixed with 1 ml anti-BSA sepharose beads and rocked gently for 8 hrs at 4°C, centrifuged and taken out. This preabsorbed E-S products were stored at -70°C until used.

**ABSORPTION OF RABBIT ANTI-*S. CERVI* E-S SERUM WITH BOVINE SERUM ALBUMIN-SEPHAROSE**

The bovine serum albumin (BSA) was coupled to cyanogen bromide activated Sepharose CL-4B (Pharmacia) as follows: 5 mg
BSA was conjugated to 1 ml moist gel in 45 ml coupling buffer (0.1 M NaHCO₃ containing 0.5M NaCl, pH 8.3) by shaking end over end at 25±2°C for 2 hr. Unbound protein was removed by washing the sorbent with Tris-HCl buffer (pH 8.0) and residual active groups were blocked by incubation with 1M diethanolamine (pH 8.0) for 2 hr at 25±2°C. Excess diethanolamine was removed by alternate washing of sorbent with 1% ammonium bicarbonate (0.1 M, pH 8.0) and 0.1 M acetate buffer, pH 4, containing NaCl (0.5 M) with 10 min minimum exposure to each buffer prior washing. One and a half ml of anti-S. cervi E-S serum was mixed with 1 ml of BSA-Sepharose and 0.5 ml serum was taken out after centrifugation (unabsorbed). The beads were mixed, rocked gently for 1 hr at 25±2°C, centrifuged and serum was again taken out (absorbed). The beads were washed three times with borate buffered saline pH, 8.4, and bound antibodies were eluted with 0.2 M glycine, pH 2.8, and immediately neutralized with 2.0 M Tris-HCl, pH 8.0. The eluted antibodies were dialysed and concentrated to 1.0 ml (eluted). Both the absorbed and eluted antibodies were stored at -20°C until used.

COUNTER CURRENT IMMUNOELECTROPHORESIS

The counter current immunoelectrophoresis (CIEP) was performed according to Siber and Skapriwsky (1978) by pouring 1%
agarose, w/v (1.5 mm thick) on a glass plate. Parallel rows of wells 2 mm in diameter and 5 mm apart were punched out on the glass plate with the aid of a template. Each well was filled with five microlitre of patient sera and 10 µl of rabbit anti-E-S serum. Wells containing antisera were placed on the anodic side of the electrophoretic chamber, those containing the antigen on the cathodic side. Electrophoresis was performed at a constant voltage of 100 volts for 30 min. The plate was placed in a moist chamber at 37°C for 1 hr. The CIEP plate was read unstained immediately after incubation with the use of oblique lighting against a dark background. The plate was then placed in normal saline overnight at room temperature. The plate was washed, dried and stained similarly as described previously in CIE. Positive reactions were defined by precipitation lines between antigen and the sera. Positive and negative controls were included with each test.

**FRACTIONATION OF S. cervi E-S PRODUCTS USING DEAE-SEPHACEL COLUMN**

A DEAE-Sephacel (Pharmacia) anion exchange column was prepared having a bed dimension of 2x25 cm and was equilibrated with 20mM Tris-HCl, pH 7.5. The *S. cervi* E-S products (5 mg proteins) was applied to the DEAE-Sephacel column. The unabsorbed
E-S protein was removed by passing 20 ml of 20mM of Tris-HCl, pH 7.5. The absorbed E-S products were eluted by a linear chloride gradient formed from 20 ml of starting buffer (20mM Tris-HCl, pH 7.5) and 20 ml of 100mM sodium chloride, 250mM NaCl and 500mM NaCl gradient. Fractions were collected of 3 ml volume at a flow rate of 1 ml/5 min and the absorbancy of the fractions was measured at 280 nm in a double beam spectrophotometer (Shimadzu). The fractions were tested in ELISA against the anti-E-S rabbit antibodies and the monoclonal antibodies.

HYBRIDOMA PRODUCTION

Immunization of mice

The BALB/c mice from the animal facility of Central Drug Research Institute, Lucknow (India) were immunized by subcutaneous (s.c.) injection of 20 μg S. cervi E-S purified antigens emulsified in Freund’s complete adjuvant. Second injection was given at 15 days interval after the first antigen. The mice were bled on 7th day from the second injection and was tested for antibody to S. cervi E-S product by enzyme linked immunosorbent assay (ELISA). The mice chosen for
fusion were boosted with 20 μg of *S. cervi* E-S products and were immunized intervenously 4 days before fusion.

**Fusion**

The fusion was performed according to Kohler and Milestein (1975). Briefly, the mice were sacrificed and the spleen cells were taken out. The spleen cells and the myeloma cells were washed twice with DMEM before fusion and was centrifuged at 200xg for 5 min. The erythrocytes present in the spleen cells were lysed with 0.9% ammonium chloride. The viability of the cells was checked by trypan blue exclusion and the cells were counted in haemocytometer. The spleen cells from the immune mice were fused with sp2/O myeloma cells (in a ratio of 1:10) with the addition of 1 ml polyethylene glycol (PEG, mol. wt. 1500) in 1 min and were cultured in a hypoxanthine aminopterin thymidine (HAT) selection medium in 24 well culture plates.

**GROWTH IN HAT SELECTION MEDIUM**

The pellat was suspende in hypoxanthine-aminopterin-thymidine (HAT, 13.6 mg/ml hypoxanthine, 0.019 mg/ml aminopterin and 0.388 mg/ml thymidine) medium with 10% FCS-supplemented DMEM. The suspension was diluted to 2x10^6 cells/ml and immediately 1.0 ml of
this was aliquoted into the wells of costar, 24 well culture plates. The plates were kept in humified atmosphere, 5% CO₂ incubator at 37°C. Next day half of the medium was replaced with fresh HAT selection medium and the growing hybrids were identified by visible colonies in an inverted phase contrast microscope.

HYBRIDOMA SCREENING PROCEDURE

The cell culture supernatants were initially screened for anti-filarial antibodies to S. cervi E-S products in ELISA. After few days of regular screening, once the positivity of the wells was established, they are subjected to growth in HAT selection medium. The positive wells were cloned twice, by limiting dilution techniques in a 96 well microtitre plate to ensure the monoclonality. The positive wells in each cloning were screened by ELISA. Subsequently, the clones, secretory anti-filarial antibodies were grown in DMEM containing 10% FCS, in a 25 cm² tissue culture flasks.

MONOCLONAL ANTIBODY PRODUCTION AND PURIFICATION

The hybridomas were maintained in cultures and the supernatant collected was stored at −20°C. The Ig globulin fractions were isolated
from supernatant by 45% ammonium sulphate precipitatoin method. In between the cells were detracted from the culture and were injected i.p. to pristaned (Sigma) primed BALB/c mice for ascites production after 10 days the ascitic fluid was formed and this was collected by bleeding the mice. The ascitic fluid was centrifuged at 800xg for 30 min to remove the cellular materials and the supernatant collected was aliquoted and stored at -70°C until used.

The Ig globulins were isolated from ascitic fluid by 45% ammonium sulphate precipitation and was left overnight at 4°C. The precipitate was collected after centrifugation at 10 000 g for 20 min the pellat was solubilised in 0.15M NaCl and was dialysed against it and kept at -70°C until used. Further purification of IgG monoclonal antibodies were achieved by batch wise treatment with protien-A Sepharose (Pharmacia) column in 0.05M Tris-HCl with 0.05M NaCl, pH 8.2. The bound IgGs were eluted at pH values between 6-3, which is characteristic of their subclass (Ey et al., 1978). Briefly, to 100 ml culture supernatant add 5ml of 1M Tris-HCl, pH 8.2. At room temperature pass the supernatant slowly over a column containing 1ml protein-A Sepharose was swollen and washed in 0.05M Tris-HCL pH 8.2 containing 0.15M NaCl. The column was washed with 10 ml of the previous buffer and elution was done with 4ml of 0.05 M sodium citrate ,pH 5 containing 0.05 M NaCl into 2 ml of 1M Tris-HCl, pH
8.2 to raise the pH. The eluate was dialysed against 0.05M Tris-HCl, pH 8.2 and was stored in aliquotes at -70°C. The protein-A Sepharose column was recovered by washing with 10 ml of 0.05M Tris-HCl containing 0.15M NaCl, pH 8.3 and can be reused.

INHIBITION ELISA

The inhibition ELISA was done according to Stahli et al. (1983). The microtitre polyvinyl plates were coated with 0.25 mg/well S. cervi E-S purified antigen overnight at 37°C. The plate was washed with PBS. The uncoated sites were blocked by 5% non-fat dry milk in PBS (w/v) for 2 hr at 37°C. The plates were washed with PBS-Tween. Wells were then exposed with two fold dilutions of purified antigen and increasing concentration of monoclonal antibodies at 37°C for 2 hr. After washing the plate was incubated with anti-mouse horse radish peroxidase for 1 ½ hr at 37°C and finally developed with OPD as done in ELISA above and the reaction was stopped after 10 min with 5N H₂SO₄ and OD was read at 490 nm in an ELISA reader.

SANDWICH ELISA

The sandwich ELISA was done as described by Zheng et al. (1987) with few modifications. Briefly, the wells of microtitre plates
were coated with 100 ml of rabbit anti-\textit{S. cervi} E-S IgG (20 mg/ml) in PBS, pH 7.4 by keeping at 37°C for 3 hr. The plate were blocked by incubation with 5% non-fat dry milk in PBS (PBS-milk) for 2 hr at 37°C. After washing the plates with PBS-Tween (PBS containing 0.05% Tween 20). 100 ml of 1:1 diluted filarial patient sera or serum pool (pool of 10 filarial patient sera positive for circulating antigen in counter immunoelectrophoresis with rabbit anti-E-S serum) or normal human sera or serum pool were added to the wells and incubated at 37°C for 3 hr. The culture supernatants (100 ml) from different hybridoma clones were added to the wells and incubation was done overnight at 4°C. The plates were washed with PBS-Tween, incubated at 37°C for 90 min with peroxidase conjugated rabbit anti-mouse Ig (1:2500 in PBS-milk Tween) and developed as described for ELISA.