CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Fine chemicals and chemicals were procured locally from Madras (India) or from Sigma Chemical Company, St. Louis, U.S.A. and were of analytical grade. Restriction enzymes, T-4 DNA ligase and calf intestinal alkaline phosphatase were obtained from New England Biolabs, Beverly, USA. Immunochemicals were from Jackson Immunoresearch Labs., USA. Radiochemicals were obtained from Bhabha Atomic Research Center, Trombay, India. Buffers, reagents and media were prepared from glass-distilled water. Solutions to be sterilized were autoclaved or constituted from sterile components and then filtered through sterile microfilters of 0.45 μm pore size.

2.2 SERA

Sera were collected during epidemiological surveys for bancroftian filariasis in endemic areas in and around Madras, India, and were categorized as having clinical filariasis (elephantiasis, hydroceles or chyluria) (age group 40 to 60), acute filariasis (tropical pulmonary eosinophilia) (age group 15 to 35) or having microfilaremia (age group 15 to 35). The endemic controls were from the residents of filarial-endemic areas negative in the above disease diagnosis (age group 5 to 60). The non-endemic controls were obtained from the uninfected volunteers living in non-endemic areas in USA and India (age group 25 to 40). *Brugia malayi* and *Onchocerca volvulus* sera were obtained from World Health Organization serum bank. Single infection sera from infections such as *Trichinella spiralis*, *Schistosoma mansoni*, *Ancylostoma duodenale*, *Litmosoides donovani*,
Trypanosoma gambiense and Plasmodium vivax were obtained from Germany, Africa, Mexico and Brazil and supplied by Span Research Center, Surat, India.

2.3 PARASITE ANTIGEN SOURCE

2.3.1 S.digitata parasites

Adult cattle filarial parasite worms of S.digitata were obtained from the peritoneal cavity of the animal immediately on slaughter. The parasites were transported in normal saline to the laboratory.

2.3.2 W.bancrofti genomic library

An expression library of W.bancrofti was constructed in λgt11 using a complete EcoRI digest of W.bancrofti genomic DNA in our laboratory by Ragaven et al (1991). Quantitation of the EcoRI-digested DNA indicated that of the total, approximately 70-75% was on the suitable size range (<7Kb) for packaging by λgt11. The DNA was then ligated with EcoRI-digested, dephosphorylated λgt11 DNA and packaged using Gigapack\textsuperscript{R} packaging extract (Strategene, La Jolla, CA). A single amplification of the library was performed to obtain a titre of 3 \times 10^{12} pfu ml\textsuperscript{-1}.

2.4 ANTIGEN EXTRACTION FROM THE CUTICLE OF S.DIGITATA

The cuticular antigens of adult S.digitata were isolated by EDTA extraction as described by Kent (1963) with modifications. The exsheathed cuticle from the parasites were homogenized in a glass homogenizer for 15 min at 4°C in a buffer containing 10 mM Tris and 10 mM EDTA, pH 8.0 (appendix 2). The homogenate was allowed to stand at 30°C for 2 hrs and then spun at 1500 x g for 15 min. The supernatant was collected and saved. The sedimented sheath fragments were again extracted with the same buffer but containing 100 mM
EDTA (appendix 2), for 2 hrs at 30°C. The mixture was spun as mentioned above and the supernatant labelled as *S. digitata* 100 mM EDTA extract. Extraction with 100 mM phosphate buffered saline (PBS) was performed similarly and was labelled as PBS extract. To all antigen supernatants a cocktail of protease inhibitors containing, 2mM phenyl methyl sulphonyl fluoride, 0.1 mM N-tosylamide L-phenylamine chloromethyl ketone, 1mM N-ethyl maleimide, 1 mM EDTA, 1 mM Ethylene Glycol-bis (2 Amino ethyl ether) N,N,N',N'-Tetra acetic acid and 0.2 mM N,1-P-tosyl L-lysyl chloromethyl ketone hydrochloride (all from Sigma) were added. Antigen extracts were aliquoted and stored at -20°C.

**2.5 AFFINITY PURIFICATION**

2.5.1 *S. digitata* cuticular antigen purification

2.5.1.1 Preparation of immunoadsorbent

2.5.1.1.1 Activation of C-L Sepharose 4 B beads

C-L Sepharose 4 B beads with 1% aqueous CNBr was activated by the following procedure (Cutrecasas and Afinsen, 1971) and the buffers used were listed in Appendix 3.

Ten ml of CL Sepharose 4 B beads were first washed with 3 M dipotassium hydrogen phosphate under vacuum in a 2 cm x 12 cm mini glass column. It is then washed with 10 ml of 5 M dipotassium hydrogen phosphate solution and the beads were transferred to a conical flask, placed in a ice bath. Ice cool suspension of 3.5% CNBr was added gradually to CL Sepharose 4 B beads, while in ice bath with continuous stirring for 15 min. The beads were transferred to the mini glass column and washed with 50 ml of chilled distilled water followed by 10 ml of 0.1 M Carbonate buffer (pH 8.5) containing 0.05 M NaCl.

Previous day of the activation of beads, the chronic filarial serum immunoglobulins were dialysed and the protein concentration adjusted to 10 mg/ml of the packed volume. The washed beads were transferred to a beaker containing
dialysed protein and 7 ml of 0.1 Carbonate buffer (pH 8.5) was added while in shaking. The entire mixture was allowed to stand undisturbed overnight at 4°C.

The following day the beads were packed on to a column, and the excess fluid removed by suction from a vacuum pump. The column was washed with 1 M ethanolamine (pH 8.0) for 1 hr at 37°C. It was given continuous washes with 50 ml Acetate buffer, 250 ml of Borate buffer, 50 ml of Tris acetate buffer containing 0.5 M NaCl, and 100 ml of Tris containing 0.1 M NaCl (appendix 3).

2.5.1.1.2 Adsorption

The adsorption was carried out by passing 3 ml of crude sheath antigen (4 mg protein/ml) for 30 min at 37°C. After the contact time of 30 min the column was washed with 50 ml of 0.05 M phosphate buffer containing 0.5 M NaCl.

2.5.1.1.3 Elution of the adsorbed antigens

The antigen was eluted with 3 M potassium thiocyanate at a flow rate of 10 ml/hr. Three ml fractions were collected and to detect protein antigen eluting out, the fractions were analyzed for absorbance at 280 nm.

Eluted fractions showing high optical density at 280 nm were pooled and dialysed against normal saline in Cellophane dialyzing tubing at 4°C for 48 hrs. Dialysate was concentrated by lyophilization.

2.5.2 Purification of 15 KDa specific filarial antibodies from filarial sera

The *S.digitata* surface antigen was run on a preparative SDS polyacrylamide gel (7.5%) (Lammeli 1970) and Western blotted as described by Towbin *et al* (1979). The region of nitrocellulose membrane with 10 to 20 KDa peptides was excised and blocked with 3% bovine serum albumin and 0.05% Tween 20 in 0.1 M Phosphate buffered saline, pH 7.2. The strips were incubated
for overnight at 4°C with diluted (1:200), pooled human sera of *W. bancrofti* infection (microfilariaemia stage). The blots were washed 6 times in phosphate buffered saline containing 0.05% Tween 20. The bound monospecific antibodies were eluted by a 2 min rinse with 0.2 M Glycine-Hydrochloride buffer, pH 2.2. The eluate was quickly neutralized with 3 M Tris- Hydrochloride (pH 7.4) containing 0.5% bovine serum albumin (Smith and Fisher, 1984). These affinity purified antibodies were used for immunoscreening of the *W. bancrofti* genomic library.

2.6 *W. bancrofti* genomic library of Agt11-immunoscreening and isolation (Sambrook et al, 1989).

2.6.1 Preparation of plating bacteria

A single bacterial (*E. coli*, Y1090) (appendix 1) colony was inoculated into 50 ml of LB medium, supplemented with 0.2% maltose in a 250 ml flask for overnight. Following day the growth medium was centrifuged at 4000 x g for 10 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in sterile (0.01 M) MgSO₄, to an appropriate density of approximately 1.5x10⁹ cells/ml (OD₆₀₀ = 2). The cells were stored at 4°C and was used within two days.

2.6.2 Recombinant phage infection and plating

Phage infection was performed by incubating 0.1 ml of the plating bacteria (Y1090) and 0.1 ml of the recombinant phage stock *W. bancrofti* genomic library in Agt11 for 20 minutes at 37°C. Three ml of LB agarose (0.7%) medium placed at a 47°C waterbath was immediately transferred to the first tube containing phage bacteria mixture. At once the contents of the tube was mixed thoroughly and was poured over a preformed agar (hard LB agar) plate. The plate swirled gently to ensure uniform distribution of the top agar. The plates were inverted after
solidification. Recombinant plaques appeared within 8 to 16 hours of incubation at 42°C.

2.6.3 Immobilization of *W. bancrofti* recombinant phage proteins on to nitrocellulose filters

The recombinant plaques appeared in the plates were screened (Young and Davis, 1983) using enzyme visualization method. In order to achieved this, the phage clones were induced and the recombinant proteins were blotted onto a filter. The phage infected plates were overlaid with the nitrocellulose filters (Schleicher and Schuell, BA 85, 0.45 μm 1 per plate) pre soaked and dried in 10 mM IPTG. The orientation of the filters were marked and the plates were incubated for two hours at 37°C. At the end of incubation the filters were removed gently and were washed in PBST buffer for 5 min and then incubated with 3% skimmed milk for 2 hrs at 37°C. At the end of incubation the blots were washed and further processed as shown below. The marked plates containing the plaques were preserved at 4°C.

2.6.4 Preliminary screening of the plaques using the affinity purified monospecific antibodies

The blotted filters, were incubated (2 hrs) with the diluted human filarial antibodies affinity purified from the 15 KDa *S. digitata* antigen as described earlier. The filters were washed with PBST thrice and was incubated with antihuman alkaline phosphatase conjugate (1:2000 dil) for 1 hour at 37°C. On incubation, the filters were washed again with PBST and was subjected to the substrate solution containing Nitro blue tetrazolium (NBT) (0.033%) and Bromochloro indolyl phosphate (BCIP) (0.017%). Positive plaques appeared as purple signals at the respective regions on the blot.
2.6.5 Isolation of specific plaques and plaque purification

Using the purple signals on the filters the respective positive plaque on the plate was identified. Agar plugs of 5 mm was removed from the positive plaque using a pasteur pipette and was resuspended in phage dilution buffer (SM) containing a drop of chloroform. A plaque yielded of $10^7$ infective phage and was stored at 4°C (Sambrook et al, 1989).

Replating and rescreening of the above phage stock was performed in the similar manner and was checked for plaque purity.

2.6.6 Secondary screening of plaques for filarial IgG4, subclass specific clones

The identified positive clones were replated and rescreened using affinity purified human antibodies (15 kDa specific) as before however this time a monoclonal antihuman sub class specific antibodies were employed (1:1000) to choose IgG4 specific clones. The filter were incubated for 3 hrs at 37°C and was then washed thrice with PBST. The washed filters incubated using antimouse alkaline phosphatase conjugate (1:5000) for an hour at 37°C. The filter were washed again with PBST and the substrate solution containing NBT (0.033%) and BCIP (0.017%) were added to develop the color. The positive clones were plaque purified three times further before they were stored as high titer lysate at 4°C (Sambrook et al, 1989).

2.6.7 Lysogen preparation

Recombinant proteins from positive clones were extracted from small-scale transient infections of hfl, lysis defective E.coli Y1089 cells (Young and Davis, 1983). Approximately $10^6$ phage from high titre lysate were incubated with $10^5$ log phase E.coli Y1089 cells in 1 ml Luria broth containing 50 ug/ml ampicillin, at 30°C for 3 hrs. At an absorbance (600 nm) of approximately 0.6, the cultures were induced for 20 min at 42°C and IPTG was added (2 mM final concentration) and
was incubated for a period of 3 hrs at 37°C. The recombinant antigen thus obtained was used for dot blot and western blots to study the antigen.

2.6.8 Dot immunoanalysis

The protein obtained from the expressed recombinant clones were applied to the nitrocellulose paper, under suction in a dot-blot apparatus (S and S, Germany), at the required protein concentrations. Soon after application the blots were dried at room temperature for 10 min and was blocked using 3% skim milk in phosphate buffered saline (PBS/T) containing 0.05% Tween 20 for 2 hrs (37°C). The blot washed and was incubated with the ideally diluted patients serum for 1 hr at 37°C. On completion of the incubation, the blot was washed again with PBS/T and incubated with the anti human antibody conjugated to alkaline phosphatase for 30 min at 37°C. At the end of incubation the blots were washed and transferred to the substrate solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2) containing Nitroblue tetrazolium (NBT) (0.033%) and Bromo chloro indolyl phosphate (BCIP) (0.017%) for color development.

2.7 POSITIVE W.BANCROFTI RECOMBINANT \PHAGE CLONES- RECLONING FOR OVER EXPRESSION (Sambrrok et al, 1989).

2.7.1 Large scale preparation of positive recombinant phage \lambda clones (infection of high multiplicity)

Ten ml of LB in 50 ml flask was inoculated with a single colony of E.coli (Y 1089) host. The flask was incubated overnight with vigorous agitation (300 rpm) at 37°C. A prewarmed (37°C) 500 ml LB in 2 L flask was inoculated using 1 ml of the overnight culture, and was agitated (300 rpm) at 37°C until the OD600 of the culture reached 0.5 (3-4 hrs). At this stage the flask was inoculated with 10^9 pfu of the positive recombinant bacteriophage \lambda and was incubated (37°C) continuously with vigorous shaking for 3-5 hrs until lysis occurred. A fully lysed culture contained a considerable amount of bacterial debris. Chloroform (10 ml)
was added to the flask and incubated for a further period of 10 min at 37°C with agitation to obtain a high titre phage lysate.

2.7.2 Purification of recombinant phage λ clones

The lysed cultures were cooled to room temperature and to a final concentration of 1 μg/ml, DNAse I and RNAse were added and incubated for 30 min at room temperature. Solid 29.2 g of NaCl was added to 500 ml (1M final) of the lysed culture and was dissolved by swirling and was incubated for 1 hr on ice. The lysate was centrifuged at 11000 x g for 10 min at 4°C to remove the debris. To the supernatant, solid polyethyl glycol (PEG 8000) was added to a final concentration of 10% w/v (ie 50 g per 500 ml of supernatant) and it was dissolved by slow stirring. To allow formation of precipitate the lysate was cooled for at least 1 hr on ice. The precipitates were centrifuged at 11000 x g for 10 min at 4°C and the pellet was resuspended gently using 8 ml of SM for the 500 ml lysate. The PEG and cell debris were removed from bacteriophage by extraction with chloroform for 30 sec by vigorous mixing in an vortex. The aqueous phase was separated from the organic phase by centrifugation at 3000 x g for 5 min. The aqueous phase contained the recombinant bacteriophage particles.

The aqueous phase was centrifuged at 15000 x g for 2 hrs at 4°C to pellet the bacteriophage particles. The phage particles appeared as a glassy pellet. Two ml of the SM buffer was added to the pellet and was allowed to resuspend overnight at 4°C. The following morning the solution was pipetted gently up and down to ensure complete resuspension.

2.7.3 Extraction of DNA from recombinant phage/clones

To the above bacteriophage suspension, proteinase K (50 μg/ml final concentration) (appendix 4) and SDS solution (0.5% final concentration) was added to the tube and was incubated at 56°C for 1 hr. The digestion mixture was cooled to room temperature. Equal volume of equilibrated phenol was used to
form an emulsion. The two phases were separated by centrifugation at 3000 g for 5 min and the aqueous phase was collected. The aqueous phase was extracted once with a 50:50 mixture of phenol and chloroform. The aqueous phase was collected again and was extracted once with chloroform. The aqueous phase is dialysed in dialysis membrane overnight at 4°C against 2 to 3 changes of 1000 fold volume of TE (pH 8.0).

2.7.4 Restriction and elution of the *W. bancrofti* DNA inserts from phage λ clones

About 50 μg of recombinant bacteriophage λ DNA was suspended in 1xTE (pH 8.0) to give a final volume of 170 μl. Twenty μl of (10X) the restriction buffer (EcoRI unique buffer, Biolabs) was added and a three fold excess of the EcoRI enzyme was used and incubated for 1 hr at 37°C. When the digestion was complete, EDTA was added to a final concentration of 5 mM and was extracted once with phenol Chloroform and once with chloroform. The DNA from the aqueous phase was precipitated using ethanol and the DNA pellet redissolved in 100 μl of TE (pH 8.0).

The restricted DNA was run on a 0.8% preparative agarose gel and was stained with ethidium bromide to visualize the EcoRI cut *W. bancrofti* insert DNA. The EcoRI cut *W. bancrofti* DNA was intercepted on the gel with a strip of DEAE (NA-45, S and S) membrane which was already activated by 10 mM EDTA (for 10 min) and 0.5 M NaOH (for 5 min) and was rinsed with distilled water, before use). On interception with the strip, the DNA band to be eluted was run onto the strip and over it. The electrophoresis was stopped and the DNA bound membrane was washed in NET buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris pH 8.0). The strip was placed in a 500 μl microfuge tube and was incubated in 200 μl of high salt NBT buffer (1.0 M NaCl. 0.1 mM EDTA 20 mM Tris pH 8.0) 45 min at 65°C with occasioned swirling. The buffer was collected and extracted with 3 volumes of water saturated n-butanol to remove ethidium bromide the DNA was initially precipitated with 2.5 volumes of ethanol (5 hrs at -
20°C) and was reprecipitated using 0.3 M sodium acetate to remove any NaCl residues (Winberg and Hammers jold, 1988).

2.7.5 Dephosphorylation of the pMAL vector after ECORI digestion

When necessary, vector DNA was dephosphorylated to remove the 5’phosphates at the termini of the molecules. Calf intestinal phosphatase was used for the purpose. The buffer composition for the reaction was: 0.5 M Tris HCl (pH 9.0), 10 mM Magnesium chloride, 1 mM Zinc chloride, 10 mM spermidine (Sambrook et al, 1989).

Procedure

a) DNA which was precipitated by ethanol after restriction digestion was dissolved in 10 μl of TE in a microtube; 5 μl of buffer and 35 μl of distilled water were added to the DNA.

b) Phosphatase (0.01 units) was added to the mixture which was incubated for 30 min at 37°C.

c) The same amount of enzyme was added again and the incubation was continued for 30 min.

d) TE (50 μl) and 100 μl of buffered phenol were added to the tube containing the dephosphorylated DNA and the tube contents were mixed thoroughly by vortexing and the phases were separated by centrifugation. The aqueous phase was reextracted with phenol. DNA present in the aqueous phase was precipitated by ethanol and subsequently dissolved in 20 μl of TE.

2.7.6 Ligation of vector and passenger DNA

The 10X buffer used for ligation contained; 0.5 M Tris HCl (pH 7.4), 0.1 M Magnesium chloride, 0.1 m DTT, 10 mM spermidine, 10 mM ATP, 1 mg/ml nuclease-free bovine serum albumin [Sambrook et al, 1989].
Procedure

a) appropriate amounts of passenger and vector DNA, 2 µl of 10X ligase buffer and distilled water to 19 µl were mixed in a microtube.

b) T-4 DNA ligase (1 unit) was added and the contents were gently mixed. The tubes were incubated for 18 h at 12°C for EcoRI termini.

c) At the end of the incubation period the ligated DNA was used directly for transforming competent *E.coli* cells.

2.7.7 Transformation of bacteria by plasmid DNA

Transformation of *E.coli* PR722: The procedure for rendering cells of *E.coli* competent for transformation is based on that described by Sambrook *et al* [1989] with some modifications as described below:

a) *E.coli* PR722 was inoculated from a glycerol stock into 10 ml LB broth and was incubated static for 12 h at 37°C.

b) One ml of this preculture was used to inoculate 50 ml prewarmed LB broth contained in a 500 ml Erlenmeyer flask. The flask was immediately placed on a rotary shaker operated at 350 rpm and maintained at 37°C.

c) At an optical density of 0.2 at 600 nm, the flask was removed from the shaker and transferred to an ice-water bath in which it was agitated so as to cool its contents rapidly.

d) After 10 min, 40 ml of the chilled culture was transferred to a sterilized and chilled screw-cap centrifuge tube and the cells pelleted at 3500 rpm in the RPR 20-2 rotor of a Hitachi HIMAC SCR20BA high speed centrifuge for 10 min and at 10°C.

e) The supernatant was discarded and the pellet was gently resuspended in 20 ml ice-cold sterile 50 mM calcium chloride. The tube was kept in an ice-water bath for 15 min and centrifuged as
before. This time the pellet was resuspended in 2.5 ml ice-cold sterile calcium chloride and the tube was stored in ice. The cells were now competent for transformation.

f) Glass test tubes containing the DNA samples were placed in ice. After the tubes cooled, 200 μl of the competent cell suspension was added rapidly to each tube and also to a tube not containing DNA; this tube served as a negative control. The tubes were incubated at 0°C.

g) After 45 min, the tubes were transferred for 2 min to a 42°C water bath.

h) Then, 0.8 ml prewarmed LB broth was added to each tube and the tubes incubated for 90 min at 37°C.

i) The tube contents, in 50 μl volumes (for samples of ligation mixtures) were plated onto LB agar plates containing suitable antibiotics.

j) The plates were incubated at 37°C for 18 hrs by which time colonies arising from transformed cells appeared on the plates whereas plates receiving control cells remained sterile.

2.7.8 Preparation of plasmid DNA

2.7.8.1 Small scale preparation of plasmid DNA

Procedure

Plasmid DNA was isolated from *E. coli* by the method of Birnboim and Doly (1979). All centrifugation steps in this procedure were performed at 8000xg at room temperature. Comparison of the buffers used were shown in the Appendix 4.

a) A 1.5 ml culture of a plasmid bearing strain of *E. coli* was grown in LB broth containing antibiotics at 37°C with aeration till the
stationary phase. The culture was transferred to a 1.5 ml microtube and centrifuged for 45 sec. The medium was decanted and centrifuged again for 10 sec. The residual medium was removed by aspiration.

b) The cell pellet was resuspended in 100 µl of TEG buffer and vortexed thoroughly.

c) Alkaline SDS (200 µl) was added to the cells which was inverted gently ten times and then placed in an ice-water bath for 5 min.

d) Sodium acetate (150 µl) was added and the contents were mixed gently. The samples were incubated at -70°C for 30 min, thawed, mixed thoroughly and spun for 10 min.

e) The supernatant liquid was transferred to a fresh tube and 1 ml ethanol was added, mixed by inversion and incubated at room temperature for 10 min.

f) The sample was spun for 5 min and the supernatant removed as in step a. The pellet which contained plasmid DNA, contaminating chromosomal DNA and RNA was dissolved by vortexing in 50 µl TE. Ethanol (150 µl) and sodium acetate (10 µl) (step d) were added to the tube and incubated at room temperature for 10 min.

g) The sample was centrifuged for 5 min and the supernatant was discarded. The precipitation by ethanol was repeated twice and the final pellet was dissolved by adding 100 µl sterile TE buffer. Chloroform (25 µl) was added as preservative and the DNA stored at 4°C.

**Note**

Incubating the solution (of step f) with a 1/10 volume of sodium acetate and two volumes ethanol at room temperature for 5 min was sufficient to precipitate all the nucleic acid present but at other times, especially when the nucleic acid concentration was low or if the volumes being handled were large, the incubation time was increased to one or more hours and the temperature of incubation was lowered to -70°C to precipitate all the DNA. The subsequent centrifugation time
was also increased to 15 min. In the rest of this thesis the term ethanol precipitation is used to describe this procedure relevant to the context.

2.7.8.2 Large scale preparation of plasmid DNA

The cells were harvested by centrifugation at 5000 rpm in the RPR12-2 rotor of a Hitachi HIMAC SCR20BA centrifuge at 4°C. The pellet obtained from one liter culture was resuspended in 10 ml of TEG buffer. The volumes of other reagents were appropriately scaled up and the centrifugation parameters modified. Each spin was performed at 4°C and 12000 rpm for 10 min. The final pellet was dissolved in 1 ml TE buffer.

2.7.9 Further purification of plasmid DNA by ultracentrifugation

Recombinant plasmids extracted by the procedures described above were sufficiently pure for most purposes including restriction enzyme mapping and subcloning. However, the pPR683 used for the initial cloning experiments were purified by centrifugation in gradients of cesium chloride containing ethidium bromide [Sambrook et al, 1989].

Procedure

a) The plasmid preparation obtained from a one litre culture was diluted to 2.5 ml with TE buffer in a 5 ml ultracentrifuge tube and 2.5 g cesium chloride was added to the solution. The tube was inverted repeatedly till the salt dissolved. Ethidium bromide (0.4 ml of a 10 mg/ml solution in distilled water) was added and the tube was filled nearly to capacity with a solution of Caesium chloride in TE prepared by dissolving 10 g of cesium chloride in 10 ml TE.

b) The samples were centrifuged for 24 h in the RPV65T rotor of a Hitachi HIMAC SCP70H ultracentrifuge at 40000 rpm and 20°C.
c) The tube was illuminated by long wave ultraviolet light and the lower of the two fluorescent bands was recovered by puncturing the centrifuge tube just below the band and withdrawing most of the fluorescing material in that band.

d) Ethidium bromide was removed from this solution by three extractions with water-saturated n-butanol. The solution was then diluted three fold, dialysed extensively with TE and the plasmid DNA was precipitated with ethanol. After two precipitations, the plasmid DNA was stored as a solution in TE over chloroform.

e) The concentration of DNA was then estimated by measuring the absorbance at 260 nm of an appropriately diluted sample. (Double-stranded DNA at a concentration of 50 ug/ml gives an absorbance of 1). The ratio of absorbances at 260 and 280 nm should be close to 1.8 for a DNA preparation free of significant contamination by protein (Sambrook et al, 1989).

2.8 OVER EXPRESSION AND EXTRACTION OF FUSION PROTEIN

The recombinant pGT7 clones were grown on LB till a $O.D_{600}$ reaches 0.6, added to a final concentration of 1 mM IPTG and the incubation continued at 37°C for 4 hrs. At the end of 4 hrs the broth was centrifuged and the pellet was resuspended in 3 ml of lysis buffer (appendix 2) per gm of pellet weight. This step was carried out on ice. For every gram of pellet 8 $\mu$l of 50 mM PMSF (Phenyl methyl sulphonyl fluoride) and 80 $\mu$l of lysozyme (10 mg/ml) were added and kept in a ice bath for 45 min, with occasional stirring. The resulting solution was passed through French Pressure cell at 1000 psi for 4 cycles. The resulting solution was centrifuged at 8000 x g for 15 min, at 4°C and the supernatant (Sup I) was stored in ice. The remaining pellet was resuspended in 9 times its volume in lysis buffer and kept at room temperature for 15 min and the solution was centrifuged at 8000 x g for 15 min and the supernatant designated as sup II. To the remaining pellet 3 ml of lysis buffer was added. In addition PMSF to a final concentration of 0.1 M per gram of pellet and 8 M Urea were added to the lysis buffer. This was kept...
at room temperature for 1 hr. To the above solution 9 volumes of 50 mM Potassium di hydrogen phosphate, pH 10.7, 1 mM EDTA and 50 mM NaCl were added drop by drop and incubated at room temperature for 30 min. The pH of the solution was increased to 10.7 with KOH and then the pH was adjusted to 8.0 with HCl and kept at room temperature for 30 min. Further, the solution was centrifuged at room temperature for 15 min and the supernatant (Sup III) was stored on ice. The pellet was resuspended in 3 ml of lysis buffer and was also analy along with other fractions.

2.9 AMYLOSE AFFINITY PURIFICATION

2.9.1 Cross linking of amylose resin

Ten grams of amylose (Sigma cat No.A-7043) was dissolved in 40 ml of water and was continuously stirred in a 1000 ml beaker. The contents were warmed to 50°C. In a fume hood, 60 ml of 5 N NaOH and 30 ml of epichlorohydrin (Sigma cat No.E-4255) was added with rapid stirring. The suspension was continuously stirred until it forms into a solid gel and was allowed to cool to room temperature. The gel was cut into small pieces and was washed three times with 1000 ml of water. Using a Warring blender the gel was chopped to required fine size. The gel was washed twice with 1000 ml of 50 mM glycine-HCl containing 0.5M NaCl, pH 2.0 (appendix 3). It was again washed with 10 mM Tris-Cl pH 7.2 three times. The gel was suspended in 10 mM Tris-Cl pH 7.2 until use at 4°C.

2.9.2 Purification

The gel (0.5 g) was swollen using column buffer (appendix 3) containing 0.25% Tween 20 and 10 mM EDTA. Degassing was done with an aspirator. The gel was poured into a 1 x 4 cm column. The column was washed with three column volume of column buffer. The crude bacterial cell lysate was diluted 1:5 times with column buffer containing Tween, so that the protein concentration of
the solution to remain at 2.5 mg/ml. The diluted protein solution was passed through the column three times in cycle and the column was then washed with three column volumes of column buffer containing Tween. The column was again washed with 5 column volumes of column buffer without Tween. Elution of the bound fusion protein was performed using 10 mM Maltose in column buffer. Three ml fractions were collected and was dialysed in 10 mM Tris-Cl, pH 8.0 containing 100 mM NaCl and used in assays.

2.10 DNA HOMOLOGY STUDIES

2.10.1 Restriction mapping

Partial restriction maps of recombinant plasmids were constructed from the sizes of DNA restriction fragments separated by agarose gel, electrophoresis. Restriction enzymes of hexa nucleotide specificity which had unique sites or no sites in the vector portion of the plasmid were used. Single and double enzyme digestions were performed.

Sizes were derived by a graphical method in which the fragment mobilities were interpolated on a curve obtained by plotting the migration of standard restriction fragments against their sizes on semilogarithmic paper. The standards, lambda (cI857) DNA digested by HindIII [Sigma, USA] were electrophoresed in parallel with fragments whose sizes were to be determined (Sambrook et al, 1989).

2.10.2 DNA dot blot

DNA of different filarial parasites at 3 ug, 2 ug, and 1 ug were denatured by using 3 N NaOH, kept at room temperature for 1 min and was immediately cooled and 2 M ammonium acetate was added and spotted on gene screen plus membranes. After spotting by mild suction the membrane was allowed to dry at room temperature and was used for further hybridization studies.
2.10.3 Nick translation

Nick translation of the DNA fragment was carried out by using BRL, Nick translation system (Bethesda Research Lab), kits and the protocol as described by the manufacturer. 500-700 ng. Test DNA of 500 to 700 ng was nick translated by using alpha -32 P dATP (sp. activity 3000 curie/mmol) (appendix 5).

2.10.4 Hybridization of DNA

The membrane with DNA was pre-hybridized with 6x SSC (20x SSC: 175.3 g of NaCl and 88.2 g of Sodium citrate in 800 ml of water, the pH was adjusted to 7.0 with a few drops of 10 N NaOH and the volume made up to one liter), 0.5% SDS, 5x Denhardts solution (Ficoll 5 g, polyvinylpyrrolidone 5 g, BSA 5 g and water to 500 ml) and 100 ug/ml denatured Salmon sperm DNA and incubated for 4 hrs submerged in a water bath at 68°C. After pre-hybridization, the nick translated DNA probe (denatured) and EDTA to a final concentration of 0.01 M were added and incubated at 68°C for 12 hrs.

After hybridization the filter was removed and immediately submerged in a tray containing 2x SSC (appendix 5) and 0.5% SDS at room temperature. After 5 min, the filter was incubated in a solution containing 2x SSC and 0.1% SDS and incubated for 15 min at room temperature with gentle agitation. The filter was further incubated at 68°C for 2 hrs with 0.1x SSC and 0.5% SDS. The filter was dried at room temperature wrapped in between saran wrap and exposed for autoradiography.

2.11 MONOCLONAL ANTIBODY PRODUCTION AND SCREENING

2.11.1 Immunization of mice

An antigen concentration of 50 ug in 100 µl of PBS, was taken and emulsified using equal volume of complete Freund’s adjuvant. The mice were
immunized using a 22 G needle, intraperitoneally. A total of 5 mice were immunized. First booster was given 3 weeks later, by intraperitoneal injection of 30 μg of antigen in incomplete Freund's adjuvant (total 200 μl emulsion/mouse). Second booster was given 2 weeks later from first, and the serum titre determined by ELISA. When antibody titre reached approximately 1/1000 a final booster similar to the first boost was given 3 days before fusion but at least 2 weeks after the previous immunization. Three days after the immunization spleen was removed for cell fusion.

2.11.2 Cell viability test by Trypan blue and viable cell counting by exclusion

To determine the number of viable cells in the cell culture, trypan blue staining was performed just before observing under microscope. One part of trypan blue solution (0.4% trypan blue in phosphate buffered saline (PBS)) and one part cell suspension was mixed together and applied to a Hemocytometer chamber. The viable cells have clear cytoplasm whereas the dead cells have blue cytoplasm. The viable cells present in all four corner squares were counted (including those that lie on the bottom and left-hand perimeters but not those that lie on the top and right-hand perimeters). Any clump present was counted as one cell. The mean number of cells per 0.1 mm³ volume was calculated and multiplied by 10⁴ to obtain the number of cells/ml (ie. cells/cm³/ml). The dilution factor used for trypan blue (2x) was applied to obtain the number of cells per ml of culture.

Viable cells (%) = \( \frac{\text{Number of viable cells}}{\text{Total number of cells (dead and viable)}} \times 100 \)

2.11.3 Preparation of mouse feeder cells

Sacrifice a unimmunized, normal mouse to obtain sufficient feeder cells (macrophages and other cells) by cervical dislocation. Immerse mouse in 70% ethanol and was placed on a dissection board. The skin a diaphragm level snipped
and the skin pulled back exposing the lower part of the rib cage and abdomen. The needle (18G) was inserted into the peritoneal cavity at the base of the sternum and the chilled sterile sucrose (0.34 M) was injected. The abdomen was squeezed gentle three times before withdrawing peritoneal cells. Transfer solution to a siliconized glass centrifuge tubes containing equal volume of chilled HAT medium centrifuged at 100 xg for 5 min at room temperature. The cell pellets were suspended in chilled HAT medium at 1 x 10^5 cells/ml concentration. The above feeder cell suspension was distributed to 96 well plates (100 µl/well/1x10^4 cells) and incubated overnight at 37°C in a CO2 incubator in 8% CO2 in air with 98% relative humidity.

2.11.4 Preparation of myeloma cells

The liquid nitrogen frozen SP2/0 murine myeloma cell line (American type culture collection No; CRL 1581) was recovered by warming the cells in cryotubes at 37°C water bath for less than one minute. Warmed (37°C) 5 ml complete culture medium was added and the tubes centrifuged at 100 xg for 5 min and the supernatant was aspirated. The cell pellet was resuspended in warm (37°C) complete culture medium and transferred to 25 cm² tissue culture flask. The flask was incubated overnight at 37°C in a CO2 incubator in 8% CO2 in-air with 98% humidity. Flasks were placed upright. The following day 5 ml of the warm complete culture medium was added and the flasks laid flat. The cells were harvested by flushing the medium on to flask surfaces and the suspension centrifuged to obtain the cells. A seeding cell density of 5x10^4 cells/ml worked well with SP2/0 cells. A total of 1x10^7 SP2/0 cells (ie. 1:10 ratio to immune spleen cells) was used for fusion.

2.11.5 Preparation of immune spleen cells

The immunized mouse was sacrificed by anesthetizing with diethyl ether in a closed beaker. The mouse was immersed in 70% ethanol and was laid on a dissection board. Using sterile forceps, the skin over the thorax was gently lifted
over thorax and snipped with sterile scissors. The skin peeled to expose the left side of the rib cage. Using another set of sterile forceps and scissors the spleen from the left abdomen was removed. In the sterile hood, the surface fat and other adhering tissues were carefully removed. Using a sterile syringe and needle pump the culture medium into the spleen and press it against a stainless steel strainer. The dispersed cell suspension was layered over the histoplaque solution in a centrifuge tube and was centrifuged. The red blood cells tend to sediment, where the spleen cells remained suspension in a narrow layered in between the histoplaque and the medium which was then gentle collected using a pasteur pipette. By counting in a hemacytometer using trypan blue, a viable count of $1 \times 10^8$ spleen cells were taken for fusion.

### 2.11.6 Fusion of myeloma cells with immune spleen cells

$1 \times 10^7$ viable myeloma cells were added to $1 \times 10^8$ viable immune spleen cells (1:10 ratio) in a 50 ml tube. The tube was filled with DMEM (appendix 7) fully and centrifuged at 200xg for 5 min at room temperature. The pellet was resuspended in supplemented DMEM and was centrifuged as above. The pellet was warmed by placing in a waterbath at 37°C for 2 min and was loosened by flicking the tip of the tube gently. The sterile polyethylene glycol (PEG 4000) solution (appendix 7) (50% v/v) was added drop by drop onto the cell pellet as shown below:-

- **Over the first 1 min**: 1 ml of PEG solution is added at 37°C and was gently mixed.
- **Over the next 2 min**: Centrifuged at 100xg (2 min total time)
- **Over the next 3 min**: 4.5 ml of supplemented DMEM was added
- **Over the next 2 min**: 5 ml of supplemented DMEM was added.

In the end of the above period, the tubes were filled with supplemented DMEM and centrifuged for 5 min at 100xg. The supernatant aspirated and the cell pellet was resuspended in HAT medium (approximately 35 ml) (appendix 7).
above cell suspension was incubated in CO$_2$ incubator for 45 min (8% CO$_2$ in air with 98% relative humidity). At the end of this period, the above cell suspension was distributed (100 $\mu$l/well) to a preconditioned 96 well tissue culture plate with peritoneal macrophages and was incubated in the CO$_2$ incubator (day 1). On day 5, 100 $\mu$l of HAT medium was added to each well. On day 7, 100 $\mu$l from each well was aspirated and 100 $\mu$l of fresh HAT medium was added to each well. Every alternate days, removal and addition of the medium was performed as shown above until the 50% confluence of hybrid growth observed. The supernatant from the wells were screened for antibodies. At the end of two weeks (from day 1) the hybrids were grown in HT medium.

2.11.7 Screening of hybridoma supernatants

The hybridoma supernatants were screened for the presence of W. bancrofti recombinant antigen (fusion protein) specific antibodies. The ELISA polystyrene plates were coated with the above fusion protein and a non-filarial component (MBP) of the above fusion protein alone was also coated separately as a control. Thereby the antibodies developed against the maltose binding protein (MBP) were eliminated.

The antigen (100 $\mu$l/well) was coated at 10 ug/ml concentration in coating buffer (appendix 8) and was incubated overnight at 4°C. The antigen solution was aspirated and the wells now washed thrice this with wash buffer (Appendix 8). 100 $\mu$l of hybridoma supernatant was applied to each well and incubated for 1 hr at 37°C. Plates were washed with thrice with wash buffer. 100 $\mu$l of horse radish peroxidase anti mouse IgG was diluted to 1:1000 times in diluting buffer and was applied to each well. Incubating for 30 min at 37°C the plates were washed and the substrate solution (100 $\mu$l/well) was added. After 30 min at room temperature. The plates were read using 405 nm further in ELISA reader.
2.11.8 Cloning of hybridoma cell lines by limiting dilution

The hybridoma to be cloned were diluted to 0.8 cells/well. The dilution provided 36% of wells with 1 cell/well by Poisson statistics. When cultures were 50% confluent, the antibody was assayed by ELISA. Two cloning procedures were carried out to obtain, above 90% wells counting, single clones showing positive for antibody production.

The antigen-specific antibody producing hybridomas were transformed to 24 well plates that has been preconditioned with feeder cells. From the overnight cultures of the 24 well plate, the dilutions were made. 1:100 dilution in a total of 3 ml was made in the first well. 80 cells/ml in 5 ml and 8 cells/ml in 10 ml of medium was made in second and third wells respectively.

Using a multichannel pipette, 100 μl from 8 cells/ml dilution (ie. 0.8 cells/well) and 100 μl from 80 cells/ml (ie 8 cells/well) dilution were distributed in a 96 well culture plate and was incubated in CO2 incubator.

On day 6, the cultures were fed with 100 μl/well fresh medium (HAT). Every alternate days the cultures were refed with 100 μl of the fresh medium. once 50% confluent growth seen under inverted microscope the supernatant were assayed by ELISA. The cloning procedure was repeated until a stable, and single hybridoma cell line was established. The hybrids were transferred to 24 well plates culture medium and a part of the cell population was frozen in liquid nitrogen for preservation.

2.11.9 Freezing hybridoma cell lines

The hybridoma cells were centrifuged at 100xg for 5 min at room temperature and the pellet resuspended in freezing medium (10% v/v DMSO and 90% (v/v) fetal calf serum). Suspension as to have a final cell density of $1 \times 10^7$ viable cells/ml, 0.5 ml of the above cell suspension was disturbed per cryotube (ie
5 x 10⁶ cells/tube). The tubes were frozen in ice/ethanol and glycerol bath for 60 min. Tubes were then transformed to liquid nitrogen freezer for preservation.

2.12 ANTIBODY ELISA

2.12.1 Qualitative assessment of total filarial IgG

The total filarial IgG antibodies were detected from filarial and normal individuals from endemic regions. The Immulon (Dynatech, USA) plates were coated with 1 µg/ml of the purified recombinant antigen in coating buffer (appendix 8) and was blocked with 3% BSA in PBST. The test sera was incubated for 1hr and the anti-human (IgG) alkaline phosphatase conjugate (1:1000 dilution) was employed to visualize using p-nitrophenyl phosphate substrate at 405 nm. All steps inbetween requires washings four times, with PBST.

2.12.2 Qualitative assessment of filarial IgG₄ and IgE

The filarial specific IgG₄ or IgE antibodies were determined using the purified *W. bancrofti* recombinant antigen in ELISA. Immulon 2 plates were coated with 10 µg/ml of the purified protein antigen in the coating buffer and kept overnight at 4°C. The plates were blocked with 3% BSA for two hours at 37°C. Serial dilutions of each pooled sera was incubated in duplicates for 2 hours at 37°C and then mouse monoclonal antibody to the IgG₄ or IgE were added at the optimal dilution of 1:1000 for overnight at 4°C. The plates were incubated with goat anti-mouse IgG conjugated to alkaline phosphatase. Similarly *E.coli* extracts were also coated and identical reactions were carried out. Between each step the plates were washed six times with PBST (appendix 8) (Tween 20, 0.05%). The color reaction was then read in a microplate reader (Bioteck Instruments). The MBP and *E.coli* readings were subtracted from the recombinants and then plotted.
2.13 ANTIGEN ELISA

2.13.1 Quantitative assessment of the circulating filarial antigens by sandwich ELISA

The rabbit anti *S. digitata* polyclonal antibodies diluted in NaHCO₃ buffer (0.1 M) pH 9.6 (appendix 8) (10 µg/ml) was coated onto polyvinylchloride microtiter plates (100 µl/well) to bind overnight at 4°C. The wells were washed in phosphate buffered saline containing 0.05% Tween 20 (PBST) and was saturated with 0.5% bovine serum albumin in PBST. The test sera was incubated for 2 hrs at 37°C and was washed with PBST. The mouse monoclonal or polyclonal antibody developed against the *W. bancrofti* recombinant (pGT7) antigen was employed to reveal the already captured antigen on the plates. The above polyclonal antibody was incubated in the wells for an hour while the monoclonal antibody needed 3 hrs at 37°C for efficient signalling. The plates washed again with PBST and the alkaline phosphatase-conjugated goat antimouse immunoglobulin (Jackson Immunoresearch, USA) was employed at a dilution of 1:500. The wells washed again and p-nitrophenyl phosphate (Sigma) (1mg/ml) was added as substrate and the color was read after 30 min at 405 nm with an ELISA reader. Optimum dilutions of the reagents were determined by checkerboard titrations. Test results were considered positive if the observed mean antigen level (ng/ml) exceeded the mean antigen + 4 SD of the known negative sera (non-endemic normals).

2.13.2 Standard curve determination

Known concentration of the recombinant pGT7 antigen was added to the pooled control (non-endemic normal) sera and was detected using polyclonal or monoclonal antibodies developed for the above antigen. Concentrations of filarial antigen such as 5000 ng, 2500 ng, 1250 ng, 625 ng, 312.5 ng, 156.25 ng, 78.13 ng, 39.1 and 19.5 ng were added to the control sera for detection. Since the filarial antigen is a 62 KDa peptide of the 105 KDa total fusion protein, the concentration was calculated on molar ratio. Accordingly 8470 ng, 4235 ng, 2117.5 ng, 1058.75...
ng, 529.4 ng, 264.7 ng, 132.3 ng, 66.17 ng and 33.09 ng of fusion protein was employed for standard graph determination. Serial two fold dilutions were performed to obtain the required concentration.

2.14 GEL ELECTROPHORESIS

2.14.1 Agarose gel electrophoresis of DNA

Horizontal submerged gels were employed in this study. The buffer for electrophoresis was TEB (89 mM Tris, 89 mM Boric acid and 2 mM EDTA, pH 8.3). The gel loading buffer was a solution of 20% sucrose and 0.01% Bromophenol blue in TE. Samples containing an appropriate quantity of DNA in TE, and previously treated with pancreatic RNase A, were mixed with a 1/4 volume of a gel-loading buffer, heated for 5 min at 65°C and then chilled on ice (RNase treatment of samples was routinely done to prevent RNA from obscuring DNA fragments). The heating step helped to dissociate proteins such as restriction enzymes from DNA and in the case of samples containing bacteriophage lambda, the heating and chilled denatured the 12 base cohesive ends which would otherwise cause fragments containing them to anneal [Sambrook et al., 1989].

Depending on the size of fragments to be separated, 0.7-1.2% agarose gels were used. The gels were 10 cm long and 3 mm thick. Electrophoresis was performed at 10 V/cm in a cold-room and stopped when the dye reached 1 cm from the bottom of the gel. Gels were stained in a 1 ug/ml solution of ethidium bromide in water for 5 min and viewed under illumination of 300 nm. Photographs were taken with a Polaroid MP-4 camera and Polaroid type 665 film; a red filter (No.22A) was used to reduce background.

2.14.2 SDS polyacrylamide gel electrophoresis

Proteins present in cell extracts were analysed by SDS-PAGE according to the method of Laemmli [1970] with some modifications. The compositions of the
various components of the systems are described below. Buffers and reagents are given in Appendix 6.

For a 10% (w/v) separating gel, 15 ml monomer solution, 4.5 ml separating gel buffer, 10.5 ml distilled water, 300 μl SDS, 150 μl ammonium persulfate, and 15 μl TEMED (n,N,N',N'-tetramethylene diamine) were used. For 7.5% gels 11 ml of the monomer solution and 4.5 ml of the separating gel buffer was used and the rest of them were the same as that for the 10% gels.

For a 4% (w/v) stacking gel, 2 ml monomer solution, 2.4 ml stacking gel buffer, 150 μl SDS, 5.6ml distilled water, 5 μl TEMED were used.

The protein estimations were carried out as per the protocol of Lowry et al (1951).

Electrophoresis was performed at room temperature at a constant current of 30 milliamperes. When the bromophenol blue dye reached 1 cm from the bottom of the gel electrophoresis was stopped. The gel was removed from between the glass plates and soaked in staining solution (0.25 g Coomassie brilliant blue R250 in 45 ml methanol, 10 ml glacial acetic acid and 45 ml distilled water). After 3 hrs the gel was rinsed briefly to remove excess stain and was then immersed in destaining solution. Destaining was stopped when the gel background became colorless.

2.15 TRANSFER OF DNA/PROTEIN

2.15.1 Southern transfer

After staining the gel, the DNA was nicked by exposing the gel to short wave UV light for 15 min. The gel was placed in 500 ml of denaturation buffer and was shaken gently for 30 min. The buffer removed and the neutralization buffer was used for incubation for 30 min. (Wicks were cut from Whatman 3mm
filter paper : Nitrocellulose sheet of the same size as the gel). The nitrocellulose sheet (NCP) was initially dipped in distilled water and then in 10 x SSC (appendix 5). (Paper towels were cut having the same size as the gel). The wicks were wetted using 10x SSC and was centered over the gel support placed in the blotting tray. The denatured and the renatured gel was gently placed on top of the wicks and was centered without air bubbles. Paper towels were placed on top making a stack over which a glass plate was placed with 500 ml bottle of water. The transfer occurred well when done overnight. When the transfer was complete, the NCP was washed with 3x SSC for 15 min. The NCP was placed on a aluminum foil, with the transferred side facing up. The NCP was blotted to remove the moisture. The NCP was baked in a vacuum oven at 80°C for 2 hours. The NCP was now hybridized.

2.15.2 Western transfer

After the electrophoresis was complete, the gel was incubated for 10-15 minutes in the transfer buffer (appendix 6) to eliminate swelling. In the mean time, the NCP, cut to the desired size was incubated for 5-10 minutes in transfer buffer (Tris 25 mM, glycine 192 mM, methanol, 20% and SDS 0.1%). The nitrocellulose was overlaid on the gel (by avoiding air bubble) and sandwiched between the filter paper and scotch Brite pads. The gel was placed 'cathodic' to the NCP. The transfer was carried out at 300 mA for 4 hrs in the cold room by using LKB transphor 2005 electroblotting apparatus. After the transfer was complete, the molecular weight marker lane was stained with amido black (100 mg amido black in 45 ml of methanol and 10 ml of acetic acid, made up to 100 ml of distilled water) for 2-3 minutes and then destained with several changes with destainer until the background stain was eliminated. The rest of the NCP was blocked for 1 hr at room temperature with 3% skim milk in PBST (PBS with 0.3% Tween-20).

The NCP was washed in wash buffer for three times of five minutes duration. The NCP was incubated for one hr with the desired primary antibody diluted in PBST. After intensive washing in the wash buffer, the NCP was incubated for another 1 hr with secondary antibody (1/1000 dilution) conjugated
with horse radish peroxidase or alkaline phosphatase. In the case of monoclonal antibodies after primary sera, the incubation was carried out with the monoclonal antibody at 37°C for 2 hrs followed by 4°C overnight.

In case of dot-blot ELISA individual samples were spotted using S and S Mini-dot blot apparatus and the NCP sheets were dried at room temperature and processed as in western blot.

In the case of horse radish peroxidase, the color development was carried out by using diaminobenzidine 15 mg/10 ml of PBS and 0.1% of 30% hydrogen peroxidase.

For alkaline phosphatase staining, the blots were incubated in predetection buffer (100 Tris HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl) for 10 minutes and the color development was carried out by using 30 μl of bromo-chloro-indoly phosphate (50 mg/ml in 100% diethylformamide) and 16.5 μl of nitroblue tetrazolium (50 mg/ml in 70% diethylformamide).