CHAPTER 2

MATERIALS AND METHODS

2.1 REAGENTS AND CHEMICALS

Chemicals of analytical grade were purchased from Sigma Chemical Company, St. Louis, USA and HiMedia, Mumbai, India. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, MA, USA and Taq DNA polymerase was from Gibco BRL, USA. Oligonucleotide primers for cloning the genes were synthesized from Integrated DNA technologies (IDT), Lexington, USA. QIAquick PCR product purification columns were obtained from Qiagen GmbH, Hilden, Germany. DNA molecular weight markers were purchased from New England Biolabs, MA, USA and protein molecular weight markers were obtained from BioRad, USA. Recombinant 6X Histidine tagged fusion protein purification gel matrix, enhanced chemiluminescence substrates and Hibond N+ Nitrocellulose membranes were procured from Amersham International, Birmingham, UK. Radioactive $^{45}$CaCl$_2$ was purchased from ICN biomedical Inc, USA.

Enzyme Immuno assay detection kit for histamine was purchased from Immunotech, Marseille, France. Antibodies to mouse and human Immunoglobulins were either obtained from Sigma Chemical Company, St. Louis, USA or Genei, Bangalore, India. Mouse anti-phosphotyrosine monoclonal antibody was obtained from BD Pharmingen, USA. The endotoxin assay kit and urea detection kit were purchased from Sigma Chemical
Company, St. Louis, USA. ELISA (96 well) plates were purchased from Nunc, USA and Pierce, Rockford, IL, USA. Frozen *Brugia malayi* L3, adult male, adult female and live mf were obtained from filarial parasite repository, University of Georgia, GA, USA.

### 2.2 BACTERIAL STRAINS, PLASMIDS AND cDNA LIBRARIES

*E. coli* strains DH5α, BL21 (DE3) and BL21 (DE3) pLys S were obtained from Invitrogen, CA, USA. BLT5403 and Novablue singles cells was from Novagen, Madison, WI, USA and XL-10 Gold cells were from Stratagene, CA, USA. T7 expression vector pRSET B was purchased from Invitrogen, CA, USA and pPCR-Script AMP SK (+) cloning kit was obtained from Stratagene, CA, USA. T7 bacteriophage display cloning kit and pSTBlue-1 perfectly blunt cloning kit were obtained from Novagen, Madison, USA.

Genotypes of the *E. coli* strains that are employed in this study are given in Appendix 1. *B. malayi* L3, L4 and *W. bancrofti* mf cDNA libraries (Appendix 2) were obtained from Dr. Steven Williams through the Filarial Genome Project Resource Center at Smith College, MA, USA. Maps and restriction sites of the vectors pRSET B and T7 bacteriophage are shown in Appendix 3.

### 2.3 CULTURE MEDIA

Luria Bertani (LB) broth was used for propagation of *E. coli* strains. The LB broth was prepared by dissolving 10 g of Tryptone, 5 g of sodium chloride in 1 litre of distilled water and the pH adjusted to 7.2 with 1 N NaOH.
To prepare solid medium, 1.5% agar was added to the liquid broth. M9LB medium was prepared by mixing 100 ml of LB broth with 5 ml of 20X M9 medium (2g NH₄Cl, 6g KH₂PO₄, 12g Na₂HPO₄ dissolved in 100 ml deionized water), 2ml of 20% glucose and 0.1ml of 1M MgSO₄. This medium was used to propagate T7 bacteriophage. Media was supplemented with 100 μg/ml of ampicillin or 35 μg/ml of chloramphenicol wherever required.

2.4 SERA AND BLOOD SAMPLES

Areas endemic for *W. bancrofti* infections were identified in and around Chennai through Department of Public Health and Preventive Medicine, Tamil Nadu, India. Sera and blood samples were collected after getting prior consent of the individuals residing in the endemic areas. Clinical status of the individuals were classified according to the parasitological manifestations by night blood smear, membrane filtration of 1 ml of heparinized blood (Nucleopore, Pleasanton, CA, USA) and by Og4C3 assay kit.

Individuals positive for circulating mf in the peripheral blood and circulating filarial antigen by Og4C3 were classified as microfilaremics (MF) and those who were negative for mf in the peripheral blood and for Og4C3 antigen with clinical symptoms of lymphoedema and lymphatic obstruction were classified as Chronic pathology (CP). Endemic Normals (EN) were classified based on the absence of mf in the night blood smear with no clinical presentations. Nonendemic normal (NEN) sera was a gift from Dr. Thomas Nutman, Helminthology Division, Laboratory of Parasitic Diseases, NIAID, NIH, MD, USA.
2.5 RECOMBINANT CLONES USED IN THE PRESENT STUDY

Bm-TCTP was identified from *B. malayi* L4 cDNA library by EST analysis as part of the filarial genome project at our Centre (Rao 1998). The gene in pBluescript vector was not in proper reading frame and was cloned in T7 expression vector, pRSET B. A homologue of this gene was cloned in pRSET B from *W. bancrofti* mf cDNA library using Bm-TCTP forward primer and T7 promoter primer and the clone was named as Wb-TCTP.

*B. malayi* L3 λ ZAP cDNA library was cloned in T7 bacteriophage display system and biopanning of the library with endemic normal (EN) sera led to the identification of three antigens ‘BmALT-2, BmVAH and BmTPX-2’ displayed on the surface of the phage. These clones were of partial length as the genes had an internal *Hind* III site.

2.6 CLONING OF FILARIAL GENES

T7 expression vector pRSET B was used for cloning and expression of Bm-TCTP and Wb-TCTP. Insert specific primers with appropriate restriction enzyme sites were designed for PCR amplification of *Brugia malayi* TCTP. Bm-TCTP forward and T7 promoter primer was used for amplification of Wb-TCTP from mf cDNA library. T7 select 1-1b vector was used for cloning and display of BmL3 cDNA gene products. T3 and T7 primers were used for amplification of *B. malayi* L3 λ ZAP cDNA. Map of the cloning vectors pRSET B and T7 Select 1-1b are given in Appendix 3. The sequence of the primers with the restriction sites and the corresponding annealing temperatures are given in the Table 2.1.
Table 2.1 Primers used for cloning the filarial genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ – 3′)</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bm-TCTP (Forward)</td>
<td>CGGGATCCTATGTTGATCTTCAAGGATG</td>
<td>55°C</td>
</tr>
<tr>
<td>Bm-TCTP (Reverse)</td>
<td>CCGAATTCTCAATTGTTTTTCTTCAATGAG</td>
<td>55°C</td>
</tr>
<tr>
<td>Wb-TCTP (Forward)</td>
<td>CGGGATCCTATGTTGATCTTCAAGGATG</td>
<td>55°C</td>
</tr>
<tr>
<td>Wb-TCTP (Reverse)</td>
<td>TAATACGACTCATATAGGG</td>
<td>55°C</td>
</tr>
<tr>
<td>T3 universal promoter</td>
<td>AATRAACCCTCACTAAAGGG</td>
<td>50°C</td>
</tr>
<tr>
<td>T7 universal promoter</td>
<td>TAATACGACTCATATAGGG</td>
<td>50°C</td>
</tr>
</tbody>
</table>

2.7 SEQUENCE ANALYSIS

DNA sequencing was performed on both the strands using vector specific and insert specific primers on ABI prism 377 automated cycle sequencer using ABI prism dye terminator cycle sequencing kit (Perkin Elmer, CT, USA). The sequences obtained were analyzed with other reported sequences in the database using Basic Local Alignment Search Tool (BLAST) family of programs (Altschul et al 1990) available on the Worldwide Web Site of NCBI, MD, USA. Multiple sequence alignment and secondary structure
prediction analysis were performed using Lasergene software available from DNASTAR Inc., WI, USA.

2.8 POLYMERASE CHAIN REACTION

The amplification of the genes was performed in MJ Research minicycler and in Perkin-Elmer 9600 PCR machines. The PCR was performed as follows:

The following reaction mixture prepared in 20 or 100μl reaction volume

a) 200 μM of dNTPs
b) 1X PCR buffer (50 mM Kcl, 10 mM Tris-Cl, pH 8.3)
c) 2.5 mM MgCl₂
d) 5 pM of each primer
e) 1 unit of Taq DNA polymerase
f) 10 ng of DNA template

were mixed thoroughly and an optimal annealing temperature of either 50°C or 55°C were set for all the reactions as given in Table 2.2.

Amplification of the PCR products was analyzed by agarose gel electrophoresis. PCR products were either purified on QIAquick PCR columns as per the instructions of manufacturers (Qiagen, Hilden, Germany) or ethanol precipitated and were digested with appropriate restriction enzymes and ligated to pRSET B vector. Ligation mixture transformed into DH5α, the resultant transformants were selected on LB agar plates supplemented with ampicillin. Transformants were screened by PCR using insert specific primers.
Table 2.2 The optimized PCR parameters

<table>
<thead>
<tr>
<th>PCR parameter</th>
<th>Temperature (°C)</th>
<th>Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) Initial denaturation</td>
<td>96</td>
<td>5</td>
</tr>
<tr>
<td>ii) Denaturation</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>55/50</td>
<td>1</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>Steps in (ii) were cycled for 30 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iii) Final extension</td>
<td>72</td>
<td>5</td>
</tr>
</tbody>
</table>

For screening the transformants, a small portion of freshly grown transformant colony was picked using a sterile toothpick and resuspended in 100 μl of 0.1X TE (1 mM Tris and 1 mM EDTA). The cells were lysed by boiling for 10 minutes, snap chilled, centrifuged for 5 minutes and 1 μl of the supernatant was used as template in PCR.

2.9 RESTRICTION DIGESTION AND LIGATION OF DNA

Restriction enzyme digestions were performed using Bam HI, Eco RI and Hind III enzymes in the buffers recommended by the manufacturer (New England-Biolabs, MA, USA). Restriction enzyme digestions were performed as follows:

- DNA (1-3μg) 5 μl
- Buffer (10X) 2 μl
- Restriction Enzyme 1 μl
- (2-3 units/μg of DNA) 12 μl
The above reaction mixture was incubated at 37°C for 2-3 hours. The completion of digestion of DNA was monitored by agarose gel electrophoresis. The digested DNA was purified on QIAquick PCR columns and used for ligation with the appropriate digested vector. Ligation of digested vector and insert DNA was performed as follows:

- Vector DNA (~50 ng) 1µl
- Insert DNA (~20-50 ng) 1µl
- Ligase buffer (10X) 1µl
- 10mM ATP 1µl
- T4 DNA ligase (10 Weiss units) 1µl
- Deionized water 5µl

Ligations were performed at 16°C for 15 hours. Molar ratios of 4:1, 6:1 of insert to vector were used in the ligation reactions.

2.10 CONFIRMATION OF RECOMBINANT CONSTRUCTS BY SEQUENCING

The nucleotide sequences of the cloned inserts of the filarial genes were deduced using either vector specific primers or insert specific primers. The nucleotide sequence and the cloning sites were verified.

2.11 EXPRESSION OF THE RECOMBINANT PROTEINS IN \textit{E. coli}

The recombinant gene constructs pRBm-TCTP and pRWb-TCTP, was maintained in \textit{E. coli} host DH5α that is deficient for T7 RNA polymerase
and hence does not express the foreign protein. A T7 gene expression vector system, pRSfT B based on T7 RNA polymerase (Studier and Moffatt 1986) was employed in the present study to clone and express the recombinant constructs in T7 expression host BL21(DE3) pLysS. Transcription by T7 RNA polymerase is selective and 5 times faster than *E. coli* RNA polymerase thus leading to higher expression of genes cloned under T7 promoter. The vector also contains a nucleotide sequence that encodes a metal binding domain, a series of six consecutive histidine amino acids expressed as N-terminal fusion to the protein of interest. The histidine tag facilitates the easy purification of the recombinant protein by metal affinity chromatography (Crowe et al 1995). Moreover the histidine tag is found to be non-immunogenic due to its small size. Such histidine tagged recombinant proteins can be used in immunological studies. However, if necessary enterokinase cleavage site facilitates removal of the histidine tag. The other advantages of the vector include its small size, presence of multiple cloning site and ampicillin resistance marker. The availability of these vectors in three possible reading frames A, B, C gives the researcher the choice of selecting vector that maintains the right reading frame of their insert.

BL21(DE3) *E. coli* strain contains a chromosomal copy of T7 RNA polymerase gene under the control of lac UV5 promoter and hence expression of genes under the control of T7 promoter was induced with the gratuitous inducer IPTG at a mM concentration. Further, BL21 (DE3) being a lon protease deficient strain, protects the expressed heterologous proteins from proteolytic cleavage.

Briefly the following protocol was used for expression of the recombinant proteins (LB was supplemented with 100 μg/ml of ampicillin and 35 μg/ml of chloramphenicol for all the experiments).
a) *E. coli* BL21 (DE3) pLysS was transformed with pRBm-TCTP and pRWb-TCTP gene constructs.

b) A single colony of fresh transformant was inoculated into 1.5 ml LB and grown overnight at 37°C, at static condition.

c) 50 μl of the overnight culture was inoculated into 10 ml LB in 100 ml conical flask and grown at 37°C with 150 rpm shaking, till O.D₆₀₀ of the culture reached 0.6.

d) IPTG was added to a final concentration of 1 mM and the culture was grown for 3 hours at 37°C with 150 rpm shaking.

e) The culture was centrifuged at 10,000g for 5 minutes. The supernatant was discarded and the bacterial pellet containing the recombinant protein was stored at -20°C until further use.

2.12 PURIFICATION OF Bm-TCTP and Wb-TCTP PROTEINS USING IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC)

The DNA inserts were expressed with 6 histidine residues as a N-terminal fusion peptide. The metal binding domain in the fusion peptide allows simple one step purification of recombinant protein by IMAC. The recombinant proteins was expressed as inclusion bodies, hence the proteins were purified under denaturing conditions (8M Urea).

Briefly the following protocol was adopted for purification:

a) Cells were harvested by centrifugation at 12,000g after induction with IPTG for 3 hrs.
b) The cell pellet was solubilized with binding buffer (0.1M Phosphate buffer pH 8.0, 0.01 M Tris pH 8.0 and 8M Urea) overnight at 4°C on a rocker for denaturing purification.

c) The column was equilibrated with binding buffer (pH8.0).

d) Samples were applied to the column, allowed to bind.

e) Column was washed with solubilization buffer (pH8.0), followed by elution with increasing concentrations of Imidazole (10 - 100 mM) to remove all contaminating proteins.

f) The protein was eluted at 200 mM imidazole concentration. The protein was checked on SDS-PAGE and dialyzed against PBS overnight at 4°C. The protein concentration was determined (Bradford, 1976) and stored in aliquots at -20°C until further use.

2.13 PRODUCTION OF POLYCLONAL ANTIBODIES AGAINST Bm-TCTP RECOMBINANT ANTIGEN

Four to six weeks old Swiss albino outbred mice were immunized with the purified recombinant protein Bm-TCTP at a concentration of 2 µg/100 µl per mice. The protein was emulsified with equal volume of Complete Freund's Adjuvant and immunized in mice. First booster dose of similar antigen concentration was given in incomplete Freund's adjuvant followed by three booster doses, at two-week intervals. Mice were bled through retro-orbital plexus and ear vein. Serum separated by centrifugation at 2,000g for 10 minutes. The serum was stored at -20°C until further use.
2.13.1 Quantitation of antibody titre by ELISA

a) The 96 wells plate were coated with purified Bm-TCTP recombinant antigen at 100 ng/well in coating buffer (NaHCO₃/Na₂CO₃ 0.067 M pH 9.6) and incubated at 4°C for overnight.

b) The plates were washed in PBS containing 0.05% Tween 20 and blocked with 5% BSA, incubated at 37°C for 2 hrs.

c) After further washes, 100 μl of different dilutions of pre-immune sera and sera from mice immunized with Bm-TCTP (1:100, 1:500, 1:1000, 1:2000, 1:5000 & 1:10000) were added to the wells and incubated at 37°C for 2 hours.

d) The plates were washed with PBST and incubated with goat anti-mouse IgG alkaline phosphatase or anti-mouse IgG alkaline phosphatase at 37°C for 2 hrs.

e) The plates were washed with PBST, pNPP substrate was added and color developed was measured at 405 nm using a microplate ELISA reader (BIO-TEK EL311sx).

2.14 PREPARATION OF SOLUBLE ANTIGENS AND EXCRETORY-SECRETORY PRODUCTS OF B. MALAYI

Different stages of B. malayi (adult male, adult female, L3 and mf) were obtained from Jung Jun, University of Georgia Research Foundation, Athens, GA. To prepare soluble extracts, parasites were homogenized in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl pH 7.4) in the presence of
protease inhibitor cocktail (Sigma, St.Louis, MO) and insoluble material was pelleted by centrifugation at 10,000 g for 15 minutes at 4°C. The soluble antigenic fraction in the supernatant was collected and protein concentrations were estimated using a BCA kit (Pierce, Rockford, IL). Excretory-secretory products (ES) of *B. malayi* mf were prepared by incubating 2×10^6* *mf in RPMI medium for 6 hours at 37°C. Following incubation the culture supernatant was collected, sterile filtered and protein concentration estimated.

### 2.15 STAGE SPECIFIC EXPRESSION AND TYROSINE PHOSPHORYLATION OF TCTP

Soluble antigenic extracts from different life cycle stages of *B. malayi* were resolved on 12% SDS-PAGE and transferred onto nitrocellulose membranes (BioRad Laboratories, Hercules, CA) and probed with 1:1000 dilution of mouse anti-Bm-TCTP serum or 1:1000 mouse monoclonal anti-phospho tyrosine antibody for 1 hour at room temperature. After washing the membrane three times with phosphate buffered saline containing 0.05% Tween 20, HRP conjugated anti-mouse antibody (Pierce Chemicals, Rockford, IL) was added at 1:5000 dilution and signal developed using a chemiluminescence substrate (Amersham Pharmacia Biotech, Piscataway, NJ). Purified Bm-TCTP was used as a positive control.

### 2.16 ^45^CaCl₂ OVERLAY ASSAY

The proteins were resolved on 12 % SDS-PAGE, transferred to nitrocellulose membranes and washed briefly with distilled water. The blots were incubated with 20 μCi/ml of ^45^CaCl₂ (ICN biomedical Inc., USA) for
10 minutes followed by washing in distilled water for 5 minutes, airdried and exposed to X-ray films for 10 minutes.

2.17 HISTAMINE RELEASE ASSAY

2.17.1 Mouse peritoneal mast cells

C57BL/6 mouse Peritoneal cells were collected by washing the peritoneal cavity with 2ml of protein free RPMI twice. 100 μl of peritoneal cells (6 \times 10^5) suspended in histamine release buffer was used for the histamine assay. The peritoneal cells were incubated with 20 μg/ml of Bm-TCTP, Wb-TCTP and 100 μg/ml of substance 48/80 (Sigma) for 30 minutes at 37°C. The cells were centrifuged at 900 g for 5 minutes at 4°C to recover the histamine present in the sample. The concentration of histamine was determined by Enzyme Immunoassay kit (Immunotech, France).

2.17.2 Rat peritoneal mast cells

Highly enriched peritoneal mast cells (PMC) (3 \times 10^4) (>95%) were incubated in triplicates at 37°C for 10 minutes with different concentrations of purified Bm- and Wb-TCTP and calcium ionophore A23187 at 2.5 μM as positive control. Histamine levels were measured by fluorometric assay (Shore et al 1959). Spontaneous histamine release was obtained from PMC in medium alone and was subtracted from the stimulated release to yield specific secretion. Viability of the PMC was examined using tryphan blue exclusion test after treatment with Bm-TCTP, Wb-TCTP and A23187.
2.17.3 Whole blood histamine release

Blood samples (1-2ml) were collected from individuals (MF, CP and EN) residing in an endemic area for bancroftian infections. 50 μl of whole blood was diluted to 1/7 in histamine release buffer and 100 μl of cells were used for cell challenge and spontaneous histamine release assays. Cells were incubated with 5 μg/ml of Wb-TCTP, 20 μg/ml of Bm-TCTP and 100 μg/ml of substance 48/80 for 30 minutes at 37°C. Untreated cells were used for spontaneous histamine release. Histamine in the samples were recovered by centrifuging the cells for 5 minutes at 900g at 4°C. Total histamine was obtained by lysing the cells by repeated freeze thawing. 100 μl of supernatant was used for the histamine assay. The histamine in the samples was quantitated by Enzyme Immuno assay kit method (Immunotech, France).

2.17.3.1 Principle of the assay

The enzyme immunoassay for histamine is based on the competition between the histamine to be assayed and its enzyme conjugate, histamine-alkaline phosphatase, used as tracer for binding to antibody, coated onto microwells. The monoamine histamine is too small to occupy completely the binding site on the antibody. High affinity monoclonal antibodies directed against modified histamine have therefore been obtained. The histamine in the sample must be derivatized in the same manner as the histamine of the conjugate. This is achieved readily and reproducibly with an acylating reagent at slightly alkaline pH.

The acylated histamine of the sample, and the histamine-alkaline phosphatase conjugate, when added to the microtiter wells, compete for binding
to a limiting number of antibody sites. After incubation, wells are rinsed in order to remove non-bound components. The bound enzymatic activity is then measured by the addition of a chromogenic substrate (pNPP). The intensity of the color depends inversely on the concentration of histamine in the sample. The concentration is calculated on the basis of a standard curve obtained with standards supplied with the kit.

Briefly the following protocol was adopted to determine the histamine present in the sample

a) 100 μl of standard or sample was acylated by mixing with 25 μl of acylation reagent and acylation buffer.

b) To antibody coated wells, 50 μl of acylated standard or sample and 200 μl of enzyme conjugate (Histamine-alkaline phosphatase) was added and incubated for 18 hours at 2-8°C.

c) Wells were rinsed for three times with wash solution provided in the kit followed by the addition of pNPP substrate. The plates were incubated for 30 minutes in the dark.

d) 50 μl of stop solution was added to arrest the reaction and the color developed was read at 405 nm.

2.17.4 Human basophils

The histamine release assay was performed in a final volume of 100 μl in the presence of Ca\textsuperscript{2+}. An approximate number of $2 \times 10^4$ basophils were used per reaction tube. Cells were primed for 15 minutes at 37°C. At the end of the incubation, 900 μl of cold (4°C) PIPES/albunin/glucose (PAG) was
added to stop histamine release, and tubes were centrifuged for 2 minutes at 1000g. Cell-free supernatants were collected and assessed for histamine content by the automated fluorometric assay (Siraganian 1974). Results were based on the mean of duplicate determinations and were expressed as a percentage of histamine release by dividing the total histamine after subtracting the spontaneous release of unstimulated cells. Total histamine was obtained by lysing the cells with 2.0 % perchloric acid.

2.18 MEASURING CELLULAR RESPONSES TO FILARIAL TCTP IN THE MOUSE PERITONEUM

C57BL/6 mice were initially sensitised by injecting 200 µg of ovalbumin (Sigma, St.Louis, MO) intraperitoneally. One week later sensitization, 5 µg of Bm-TCTP or 100 µl of sterile saline was injected into the peritoneum. At 24 hours after injection, peritoneal cells were collected as described above and a differential count was made on a cytospin smear preparation of the cells stained with Giemsa.

2.19 T7 BACTERIOPHAGE DISPLAY SYSTEM

T7 bacteriophage cloning kit containing pre-digested vector T7 select 1-1b (Eco RI and Hind III digested), packaging extract, propagation host E. coli BLT 5403, T7 up and T7 down primers were obtained from Novagen, Madison, WI, USA.

2.19.1 Cloning of B. malayi L3 λ ZAP cDNA in T7 phage display vector

A µl of the BmL3 λ ZAP cDNA in 100 µl reaction was PCR amplified using T3 and T7 primers. The PCR product was purified by
QIAquick PCR columns and then digested with Eco RI and Hind III. The digested PCR product was ligated with pre-digested (Eco RI and Hind III) T7 select 1-lb vector by assembling the following components at 16°C for 16 hours.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>BmL3 cDNA (1μg)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>T7 select vector arms (0.5μg)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>10X Ligase buffer</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>10mM ATP</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>100mM DTT</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>T4 DNA Ligase (0.4-0.6 Weiss units)</td>
<td>1.0 μl</td>
</tr>
</tbody>
</table>

2.19.2 *In vitro* Packaging

Ligation reaction mixture was directly added to T7 packaging extracts for *in vitro* packaging. Briefly the following protocol was used.

a) The T7 select packaging extract was allowed to thaw on ice. 5 μl of the ligation mixture was added to 25 μl packaging extract provided with the kit and mixed gently by stirring with a pipette tip. To test the packaging efficiency, 0.5μg T7 select packaging DNA was used as a positive control.

b) The reaction mixture was incubated at 22°C for 2 hours, and stopped by adding 9 volumes of sterile LB media.

c) A plaque assay was performed to determine the titer of the primary library.
2.19.3 Plaque assay

a) BLT5403 E. coli strain was inoculated in LB medium, supplemented with 50μg/ml of ampicillin and incubated overnight in a shaker at 37°C to an OD_{600nm}=1.0. The host cells obtained as above can be stored at 4°C for a period of 48 hours.

b) A sufficient volume of top agarose providing 5 ml for each dilution to be plated was melted and maintained at 45-50°C in a water bath till plated.

c) A serial dilutions (10^{-3} to 10^{-6}) of the library was prepared in LB medium.

d) To 4 ml sterile tubes, 250 μl of host cells (BLT5403) and 100 μl of the diluted phage were added followed by addition of 3 ml of top agarose. The contents of the tubes were mixed evenly and poured on the prewarmed (37°C) LB ampicillin agar plates and left undisturbed till the top agarose hardens, then the plates were inverted and incubated for 3-4 hours at 37°C.

e) The titer of the plaques was calculated as follows

\[ \text{Phage titer} = \text{No. of plaques on the plate} \times \text{Dilution factor} \times 10 \]

2.19.4 Amplification of the phage library

A single round of amplification of the phage library is necessary prior to biopanning. Plate lysate amplification was performed to amplify the primary library. Briefly the following protocol was used for amplifying the primary phage display library:
a) 1 ml of the overnight culture of BLT5403 was inoculated in 50 ml TB and incubated in a shaker at 37°C till the OD reaches 0.6 –1.0.

b) $10^6$ phage were mixed with 10 ml of cells and 1 ml of the phage mixture was aliquoted in 15 ml sterile tubes.

c) To the tubes added 10 ml molten top agarose and the contents were poured evenly on LB ampicillin plates and allowed to sit undisturbed till the agar solidifies. The plates were incubated at 37°C for 3-4 hours.

d) The plaques formed were eluted with 10 ml of phage extraction buffer (100mM NaCl, 20mM Tris-HCl, pH 8.0 and 6mM MgSO$_4$) by placing the plates on a level surface at 4°C for overnight.

e) The phages were harvested by tapping the plate slightly and pipetting the liquid into a sterile container. The extracts from all the plates were combined in a single tube and centrifuged at 3000g for 5 minutes to clarify the lysate and the supernatants were transferred to a sterile tube. The titer of the amplified library was determined by plaque assay. The amplified library was either stored at 4°C or as glycerol stocks at -70°C.

2.19.5 Biopanning of the library with Endemic Normal (EN) sera

The T7BmL3 library was incubated with 1: 100 Non Endemic Normal (NEN) sera, followed by Microfilaremic (MF) and Chronic Pathology (CP) sera to remove non-specific clones. The absorbed library containing $10^{11}$ plaques
added to the wells coated with 1:100 diluted EN sera and incubated for 30 minutes. The wells were washed with PBST to remove unbound phages and then eluted by incubation with 200 μl of TBS containing 1% SDS for 15 minutes. The eluted phages were amplified on BLT5403 E. coli host cells and biopanning with EN sera was repeated till saturation in enrichment of the phage was attained. Titer of the phages obtained after each rounds of elution and amplification was determined by plaque assay. The phages from final round of biopanning contain a large percentage of EN specific phages. The schematic representation of biopanning is given in Figure 2.1. High specific recombinant phages were further selected by plaque lift protocol.

### 2.19.6 Plaque lift protocol

a) The plaques on the plates were chilled at 4°C to minimize the tendency of the top agar to stick to the membrane. The plates were carefully overlaid with the membranes and the alignment of the membrane was marked using a pin. After one minute of contact, the membranes were carefully peeled off the plate and allowed to air dry for 10 – 20 minutes.

b) The membranes were blocked with 5% BSA in 1X TBST for 30 minutes with gentle rocking followed by washing 5 times with 1X TBST.

c) The membranes were incubated with 1:100 endemic normal (EN) sera for 2 hours at 37°C followed by washing 5 times with 1X TBST.

d) To the membranes, 1:5000 Goat antihuman-IgG-Horse raddish peroxidase (Pierce, Rockford, IL, USA) conjugate was added
Figure 2.1 Schematic representation of biopanning technique
and incubated for 1 hour at 37°C. Then the blots were washed with 1X TBST.

e) The membranes were developed by incubating in solution A and Solution B (Amersham International, Birmingham, USA) for 1 minute, rinsed briefly with distilled water and exposed to X-ray films for 3 minutes.

f) The strongly reacted plaques were isolated by aligning the X-ray film on the master plate and subjected to sequence analysis.

2.19.7 Sequence analysis of selected phage recombinants

The isolated plaques were amplified in 50 ml LB and the lysates were centrifuged at 10000g for 10 minutes and an aliquot of the lysate was serially diluted, plated on LB ampicillin plates and incubated 2-3 hours till the plaques were formed.

2.19.7.1 PCR amplification of Plaques

a) A portion of the top agar of an individual plaque was scraped using a sterile pipette tip and dispersed in a tube containing 100 μl of 0.1X TE.

b) The tubes were boiled for 10 minutes; snap chilled on ice, followed by centrifuging at 12000 rpm for 5 minutes to clarify the lysate.
The following components were mixed for the PCR reaction

a) 200 µM of dNTPs
b) 1X PCR buffer (50 mM Kcl, 10 mM Tris-Cl, pH 8.3)
c) 2.5 mM MgCl$_2$
d) 5 pM of T7 select up and down primer
e) 1 unit of Taq DNA polymerase
f) 1 µl of phage lysate

The following conditions were set for the PCR amplification

i) Initial denaturation 95°C 5 minutes
ii) Denaturation 95°C 1 minute
   Primer annealing 50°C 1 minute
   Primer extension 72°C 1 minute
   Steps in (ii) were cycled for 30 times
iii) Final extension 72°C 5 minutes

The amplified PCR products were purified on QIAquick PCR columns (Qiagen, Hilden, Germany) and cloned in either pPCR Script Amp SK (+) or pSTBlue-1 vector for sequence analysis.

2.19.7.2 Cloning of PCR product in pPCR-Script Amp SK (+)

The PCR product was cloned in pPCR-Script Amp SK(+) obtained from Stratagene, CA, USA. The reaction was set as follows:
Vector (10ng/μl) 1.0 μl
10X reaction buffer 1.0 μl
10mM ATP 0.5 μl
PCR product 4.0 μl
T4 DNA ligase (4U/μl) 1.0 μl
Sterile water 2.5 μl

The ligation reaction was gently mixed and incubated for 1 hour at room temperature followed by heat inactivation at 65°C for 10 minutes. 2 μl of the ligation reaction mix was used to transform into Epicurian coli XL-10 gold ultracompetent cells. The resultant transformants were screened by PCR using vector specific primers provided with the kit to select the recombinants.

2.19.7.3 Cloning of PCR product in pSTBlue-1 vector

The PCR products were cloned in pSTBlue-1 vector obtained from Novagen, Madison, WI, USA. The PCR products were treated with end conversion mix provided with the kit for blunt end ligation.

End conversion of the PCR product was performed as follows:

PCR product 2.0 μl
End Conversion mix 5.0 μl
Nuclease free water 3.0 μl
Total volume 10 μl

The reaction mix was incubated at 22°C for 15 minutes followed by inactivating the reaction at 75°C for 5 minutes and cooled on ice. The ligation reaction was performed as follows:
End conversion reaction mix 10μl
Vector (50ng) 1 μl
T4 DNA ligase 1 μl

The reaction mix was incubated at 22°C for 15 minutes and 2μl of ligation mix was used to transform Novablu singles competent cells obtained from Novagen. The resultant transformants were screened by PCR using vector specific primers provided with the kit to select the recombinants.

2.19.7.4 Sequence analysis

Plasmids were prepared from the recombinant transformants and subjected to sequencing on ABI prism 377 automated cycle sequencer using ABI prism dye terminator cycle sequencing kit (Perkin Elmer, CT, USA). DNA sequencing was performed on both the strands using vector specific primers and the sequences obtained were analyzed for the presence of open reading frames using Vector NTI software of Informax Inc, MD, USA and compared with other reported sequences in the database using Basic Local Alignment Search Tool (BLAST) family of programs (Altschul et al 1990) available on the Worldwide Web Site of NCBI, MD, USA.

2.20 SPECIFICITY OF PHAGE DISPLAYED ANTIGENS

To test the specificity of the identified antigens, patients sera at 1: 100 dilution from EN, CP, MF and NEN groups were coated in 96 well plates. 100 μl of 10^{11} pfu/ml of phage antigens were added in each well and allowed to incubate for 10 minutes, followed by washing with PBST for 5 times. The
bound phages were eluted with 200 μl of TBS containing 1% SDS and the titer was determined as described earlier.

2.21 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Total IgG antibodies to purified recombinant antigens Bm-TCTP, Wb-TCTP and phage-displayed antigens (T7BmALT-2, T7BmVAH and T7BmTPX-2) were measured in various clinical groups of filariasis by ELISA (Hussain et al 1987). Purified recombinant antigens and phage antigens at various concentrations 500 pg, 1 ng, 10 ng, 100 ng and 1 μg/ well and sera at 1:50, 1:100 and 1:200 dilutions were tested in a pilot experiment. Optimal antigen concentration of 100 ng/well of Bm- and Wb-TCTP; 1 μg/well of phage-displayed antigens and optimal sera dilution of 1:100 were selected and used in all further experiments. Goat anti-human IgG alkaline phosphatase (ALP) conjugate, Sheep anti-mouse biotin conjugate, extravidin ALP conjugate dilutions were used as recommended by the manufacturer (Sigma, USA).

Briefly the following protocol was adopted for the quantitation of total IgG by ELISA.

a) 100 μl containing 100ng of purified recombinant antigens, Bm-TCTP and Wb-TCTP and 100 μl comprising 1 μg of phage antigens in coating buffer (NaHCO₃/Na₂CO₃ 0.067 M pH 9.6) were added to wells of 96 well microtitre plate and incubated overnight at 4°C.

b) The plates were washed 4 times with PBS containing 0.05% tween-20 (PBST).
c) The unbound or non-specific sites were blocked with 100 μl of 3% bovine serum albumin (BSA) at 37°C for 2 hours.

d) The plates were washed 4 times with PBST.

e) 100 μl of 1:100 primary antibody was added in duplicates and incubated at 37°C for 2 hours. The plates were washed 4 times with PBST.

f) 100 μl of 1:1000 alkaline phosphatase (ALP) labeled antihuman IgG conjugate was added and incubated at 37°C for 1 hour. The plates were washed 4 times with PBST.

g) 100μl of substrate (1 mg/ml) pNPP (p-nitrophenyl phosphate) in substrate buffer (100 mM Tris-Cl pH 9.5, 100 mM NaCl, 5 mM MgCl₂) was added to develop color. The reaction was arrested after 20 minutes of incubation by addition of 100 μl of 3 N NaOH solution per well. The absorbance was measured at 405 nm in a microplate reader.

For the measurement of IgE, sera were depleted of IgG using gamma-bound sepharose beads (Pharmacia, Uppsala, Sweden). For depletion of IgG, to 50 μl of serum, 25 μl of gamma bound sepharose beads were added and the total volume was made up to 200 μl with PBS. The mixture was kept at 4°C for overnight on a rocking platform. The suspension was centrifuged at 12000g for 5 minutes and the supernatant was taken for measurement of IgE. A final serum dilution of 1:10 was employed for IgE ELISA (Hussain et al 1981).

The sensitivity of IgE assay was enhanced using avidin-biotin system. After adding respective MAb for 2 hours and washing, the plates were incubated with anti-mouse biotin conjugate (Sigma, USA) for 2 hours. After
washing thrice with PBST, extravidin conjugated ALP was added, incubated for 1 hour and the color was developed as described before.

The data was expressed as optical density (OD) at 405 nm. All the assays were performed in duplicates and the mean values of the OD were taken. A positive control serum and a negative control serum were included on all the plates.

2.22 GENERAL METHODS

General techniques in molecular biology such as plasmid DNA preparation, agarose and SDS-PAGE electrophoresis, transformation and western blotting used in this study were adopted (Sambrook et al, 1989) with minor modifications as described below:

2.22.1 Plasmid DNA preparation

a) Plasmid DNA isolation from recombinant E. coli was based on the method of Birnboim and Doly (1979) (All centrifugation steps in this procedure were performed in a microfuge at 12000g).

b) A 1.5 ml of overnight grown culture of plasmid bearing E. coli was centrifuged for 1 minute and the supernatant was discarded. The residual medium was removed by brief centrifugation followed by aspiration.

c) The cell pellet was resuspended in 100 μl of ice-cold TEG buffer (25 mM Tris HCl, 10 mM EDTA, 50 mM glucose) by
vigorous mixing and incubated at room temperature for 5 minutes.

d) Freshly prepared 200\mu l of alkaline-SDS (1% SDS in 0.2 N NaOH) was added to the cell pellet, the tubes gently inverted 3-4 times and placed on ice.

e) After 5 minutes, 150 \mu l of potassium acetate solution (3 M pH 5.2) was added, mixed by gentle inversion, placed on ice for 15 minutes and centrifuged for 15 minutes at 4°C.

f) The supernatant was carefully transferred into a fresh tube. To this RNase was added to a final concentration of 10\mu g/ml and incubated at 37°C for 1 hour.

g) The sample was extracted once with equal volume of Tris buffered phenol:chloroform:iso-amyl alcohol (25:24:1) and once with equal volume of chloroform:iso-amyl alcohol (24:1).

h) The DNA in the aqueous phase was precipitated by adding 2.5 volumes of ethanol on ice for 15 minutes and pelleted by centrifugation for 15 minutes at 4°C.

i) The supernatant was discarded, and the DNA pellet was washed using 70% ethanol. The traces of ethanol were dried by speed-vac concentrator and the DNA was dissolved in appropriate volume of either double distilled water or TE (10 mM Tris-Cl, pH 8.0, 1mM EDTA) and stored at -20°C.

2.22.2 Agarose gel electrophoresis

Horizontal submerged gels were used to separate the DNA fragments. TBE buffer of pH 8.3 (98 mM Tris, 89 mM Boric acid and 2 mM EDTA) was
used. The electrophoresis was performed at 5-8 v/cm at room temperature. The gel loading buffer contained 20% glycerol with 0.01% Bromophenol blue/0.01% Orange-G in TE.

1% agarose gels were employed throughout the present study. Gels were stained with approximately 0.5 μg/ml of ethidium bromide, viewed under UV transilluminator (Fotodyne, Hartland, WI, USA). Either 100 bp, 1 Kb ladder or lambda Hind III marker (Gibco BRL, MD, USA) (New England Biolabs, MA, USA) were used as molecular weight markers.

2.22.3 Transformation of E. coli with plasmid DNA

Briefly the following procedure was used for transformation:

a) A single colony of freshly revived E. coli culture was inoculated in 2 ml of LB and grown at 37°C for overnight.

b) 100 μl of overnight culture was inoculated into 50 ml LB medium in conical flask and allowed to grow at 37°C till OD_{600nm}=0.6.

c) Culture was chilled on ice for 5 minutes by gentle swirling and centrifuged at 3500g for 5 minutes at 4°C.

d) The cell pellet was resuspended in 10 ml of ice-cold 100 mM MgCl₂ and incubated on ice for 20 minutes.

e) Cells were pelleted as in step c and the pellet was resuspended in 2 ml of 100 mM ice-cold CaCl₂ and incubated on ice for 1 hour.
f) Approximately 10-20 ng of DNA was added to 100 µl of above cells and further incubated for 1 hour on ice.

g) A heat shock at 42°C was given for 90 seconds and chilled on ice for 5 minutes.

h) To this tube 800 µl of LB medium was added, allowed to grow for 1 hour at 37°C and 100 µl of the cells was plated onto LB agar plates supplemented with appropriate antibiotics.

i) A positive control plasmid (10 ng of pRSET B/pPCRscript AMP SK (+)/pSTBlue-1) was used in all the experiments to verify the transformation efficiency. Cells with no DNA added served as negative control.

2.22.4 SDS-Polyacrylamide Gel Electrophoresis

Proteins extracted from recombinant *E. coli* were analyzed by the method of Laemmli (1970) with minor modifications. The various buffers used are as follows:

a) Monomer solution: 20% acrylamide and 0.8% N, N-methylene bis acrylamide in distilled water. The solution was filtered through whatman filter paper and stored in brown bottles at 4°C.

b) Separating gel buffer: 1.5 M Tris-Cl, pH 8.3

c) Stacking gel buffer: 0.5 M Tris Cl, pH 6.8
d) Electrophoresis buffer: 0.025 M Tris-Cl, 0.192 M glycine, 0.1% SDS, pH 8.3

e) Sample solubilizing buffer (5X): 10% SDS, 10% (v/v) β-mercaptoethanol, 50% sucrose, 0.025% bromophenol blue in stacking gel buffer.

Depending on the proteins to be separated 10-15% separating gel and 5% stacking gels were used. Stacking gel was approximately 1/5 of the separating gel. Protein estimations were performed (Bradford, 1979) and equal amounts of total protein were loaded in each well. Electrophoresis was performed at room temperature with constant current of 20mA for stacking gel and 30 mA for separating gel. Gels were stained with staining solution (0.25 g of Coomassie Brilliant Blue R-250 in 45% methanol, 10% acetic acid) for 3 hours and destained with 45% methanol, 10% acetic acid solution until a clear background was obtained. Photographs were taken with Tracktel GDS-2 gel documentation system (Vision systems, Germany). The relative amounts of recombinant protein in whole cell lysate were determined by scanning the gels in Personal Densitometer, Molecular Dynamics, CA, USA.

2.22.5 Western blotting

After electrophoresis, the SDS-PAGE gel was incubated for 10 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS) as described by Towbin et al (1979) with little modifications in the protocol. Nitrocellulose membrane (NCP) cut to the exact size of separating gel was also incubated for 10 minutes in transfer buffer. Without trapping air-bubbles the NCP was overlaid on the gel and sandwiched between filter papers. Electrophoretic transfer was carried out using Trans-Blot SD-semi dry
electrophoretic cell blotting apparatus (Bio-Rad, Hercules, CA, USA). After transfer, the molecular weight marker lane was cut and stained with amido black (100 mg amido block in 45% methanol, 10% acetic acid). The rest of the NCP was stained with Ponceau S (0.2% Ponceau S, Sigma, USA in 0.3% trichloro acetic acid and 0.3% sulfosalicylic acid) staining to ensure the transfer of the proteins. Membrane was washed in PBS and blocked overnight at 4°C with 5% non-fat milk powder in PBS.

The NCP was washed in wash buffer (PBS with 0.05% Tween-20) thrice for 5 minutes duration each, and then incubated with appropriately diluted primary antibody for 3 hours at room temperature. After washing in the wash buffer, the membrane was incubated for 1 hour with recommended dilution of secondary antibody conjugated with horse raddish peroxidase. After extensive washing, the blot was developed with DAB (3,3’ Diaminobenzidine) substrate kit obtained from Pierce, IL, Rockford, USA. The reaction was stopped after 10 minutes by addition of 10 mM EDTA.

2.23 STATISTICAL ANALYSIS

Mann-Whitney U test was used to analyze the humoral immune response data and eosinophil infiltration studies. Histamine release data were analyzed by Mann-Whitney U test and Wilcoxon signed Rank test. A probability value of P<0.05 was considered statistically significant. All the above statistical analysis were performed through SYSTAT version-8 software.