CHEPTER 2

MATERIALS AND METHODS

2.1 REAGENTS AND CHEMICALS

Chemicals of analytical grade were purchased from Sigma chemical company, St Louis, MO, USA and Merck Gmbh, Germany. Restriction enzymes were procured from New England Biolabs, UK, and IMAC chelating sepharose were procured from Amersham International, Birmingham, UK. DNA molecular weight markers and protein molecular eight markers were obtained from Fermentas (Fermentas, MD, USA). Maxisorb (Nunc, Maxisorp, Nalge Nunc International, Denmark) 96 wells microtiter plates were purchased for ELISA. The other molecular grade chemicals were procured from HiMedia, India.

2.2 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 (HiMedia, India) and 5 g of sodium chloride in 1 L of distilled water and the pH adjusted to 7.2 with 1 N NaOH. To prepare solid medium, 1.5 % agar (HiMedia, Mumbai, India) was added to the liquid broth. Luria Bertani broth without NaCl was used to propagate GJ1158. The chemicals were procured from Merck, Germany. Media was supplemented with 100µg/ml of ampicillin or 50 µg mL$^{-1}$ of kanamycin, 7 µg mL$^{-1}$ of gentamycin and 10 µg mL$^{-1}$ tetracycline. 100 µg mL$^{-1}$ X gal and 40 µg mL$^{-1}$ IPTG was used for blue-white selection of
DH10Bac colonies for recombinant bacmid. TC-100 insect cell medium (Hi Media, India) supplemented with 10% foetal bovine serum (GIBCO laboratories, New York, USA) and L-Glutamine (20.55 mL of 200 mM L-Glutamine in 1 L of TC-100 medium, Hi Media, India) used for growth of Sf21 insect cell line.

2.3 REAGENTS FOR CELL CULTURE AND HYBRIDOMA

Dulbecco’s modified Eagle’s medium (DMEM), Iscove’s modified Dulbecco’s medium (IMDM), medium 199 (M 199) and fetal bovine serum (FBS) were purchased from GIBCO laboratories, New York, USA. Sterile filtration units were obtained from Millipore Corporation, Bedford, USA. Tissue culture flasks and plates were obtained from Nunc, Denmark. Polyethylene glycol (PEG) 3500, hypoxanthine, aminopterin, thymidine, glutamine, nystatin and trypsin were purchased from Sigma Chemical Co, USA. Penicillin was obtained from Alembic chemical works, India. Streptomycin from Sarabhai chemicals, India, gentamycin was from the Pharmaceutical Company of India. Dimethyl sulphoxide (DMSO) was from E Merck. Micro titre plates were from Nunc, Denmark. Gelatin, bovine serum albumin (BSA), PEG 6000, Tween 20, Triton X-100 was obtained from Sigma Chemical Co, USA. Rabbit anti-mouse immunoglobulins conjugated to HRP was obtained from Dakopatts, Denmark. Tetra methyl benzidine (TMB) was obtained from Sigma Chemical Co, USA. CellFECTIN reagent for transfection of bacmid was obtained from Invitrogen.

2.4 CELL-LINE

The cell-line used for fusion was Sp2/0-Ag-14 myeloma cells. Sf21 cell lines (Invitrogen, USA) was used with baculovirus and originated at the USDA Insect Pathology Laboratory. The cell line was originated from the IPLBSF-21 cell line, derived from the pupal ovarian tissue of the fall army

### 2.5 BACTERIAL STRAINS AND PLASMID

The salt-inducible *E. coli* GJ1158 (Courtesy Dr. J. Gowrishankar, US Patent: 5830690 [19], Genotype: F- ompT hsdSB (rB- mB-) gal dcm endA1 lon- proUp::T7 RNAP::maIQ-lacZ (TetS) ) and IPTG inducible *E. coli* BL21 (Invitrogen, CA) were used as hosts. *E. coli* strains DH5α (Invitrogen, CA) were used for plasmid maintenance. MAX Efficiency DH10Bac competent cells and pFastBac donor plasmid with polyhedrin promoter for baculovirus expression system was obtained from Invitrogen, CA, USA.

WbSXP-1 clone: The recombinant clone pBSWbSXP-1 was identified from *W. bancrofti* L3 stage cDNA library by screening using BmSXP-1 as a DNA probe. The insert from pBSWbSXP-1 was subcloned in pRSETB (Invitrogen, CA) vector and transformed in *E. coli* BL21 for expression and named as rWbSXP-1; Accn. No. AFO98861 (Rao et al 2000).

### 2.6 EXPRESSION SYSTEM USED IN THIS STUDY

The gene encodes WbSXP-1 protein was expressed in pRSETB plasmid system based on T7 RNA polymerase (Studier and Moffat 1986). T7 promoter is highly specific for T7 RNA Polymerase and the transcription by T7 polymerase is selective and 5 times faster than *E. coli* RNA polymerase thus leading to higher expression of genes cloned under T7 promoter.

The SXP gene was cloned in baculovirus expression system and expressed in Sf21 insect cell line infected with recombinant baculovirus. Recombinant baculoviruses have become widely used as vectors to express heterologous genes in cultured insect cells and developed by researchers at
Monsanto (Luckow, et al). Heterologous genes placed under the transcriptional control of the strong polyhedrin promoter of the Autographa california nuclear polyhedrosis virus (AcNPV) are often abundantly expressed during the late stages of infection.

2.7 T7 EXPRESSION SYSTEM

A gene expression system based on T7 RNA polymerase (Studier and Moffat 1986), pRSETB, was employed in the present study to clone and express the SXP gene. This vector contains T7 promoter that is highly specific for T7 RNA Polymerase. Transcription by T7 polymerase is selective and 5 times faster than E. coli RNA polymerase thus leading to higher expression of genes cloned under T7 promoter. This expression vector (pRSETB) has the T7 promoter driven system originally developed by Studier et al (1990). So target gene cloned in plasmids is under the control of strong bacteriophage T7 transcription and translation signals and providing a source of T7 polymerase in the host cell. T7 RNA polymerase is selective and active that almost all of the cell’s resources are converted to target gene expression and the desired product can comprise more than the rest of the total cell protein after few hours of induction. The T7 RNA polymerase is able to make complete transcripts of almost all DNA that is placed under the control of a T7 promoter. Because of these properties along with the availability of the cloned gene makes it allows high level expression in E. coli. This vector (pRSETB) 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. This metal binding domain (six-tagged histidine moieties) on the fusion peptide has high affinity for the divalent ions (like nickel, copper and cobalt) and facilitates purification of the protein using (IMAC) immobilized metal affinity columns (Belew et al 1990, Crowe et al 1995).
Moreover histidine tag is found to be non-immunogenic due to its small size. Thus histidine tagged recombinant proteins can be used in immunological studies without employing tedious proteolytic cleavage procedures generally required in other recombinant fusion protein systems. However, if necessary "enterokinase" cleavage site facilitate the removal of histidine tag. The other advantages of the vector include its small size, presence of ribosomal binding site, multiple cloning site and ampicillin resistance marker (Studier et al 1986).

2.8 BACULOVIRUS EXPRESSION SYSTEM

The insert form pRSET B vector was subcloned in pFastBac1 donor vector (Invitrogen) and transformed into DH10Bac competent cells (Invitrogen) for site specific transposition of sxp into bacmid. Recombinant bacmid was transfected into Sf21 insect cells (Invitrogen) were cultured in TC100 medium supplemented with 10 % FBS and 2 mM glutamine. The cells were transfected with the recombinant bacmid containing the gene of interest. The Sf21 insect cells originated from the IPLBSF-21 cell lines, derived from the pupal ovarian tissue of the fall army worm, spodoptera frugiperda (O’Reilly et al 1992, Vaughn et al 1977) using CellFECTIN Reagent (Invitrogen).

Baculovirus Expression System is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in E. coli. This is a rapid and efficient method to generate recombinant baculoviruses was developed by researchers at Monsanto. The bacmid (bMON14272) contains low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the lacZα peptide from a pUC-based cloning vector. Inserted into the N-terminus of the lacZα gene is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-attTn7) that does not disrupt the reading frame
of the $\text{lacZ}_\alpha$ peptide. The bacmid propagates in Escherichia coli DH10Bac as a large plasmid that confers resistance to kanamycin and can complement a $\text{lacZ}$ deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate such as Blue-gal or X-gal and the inducer IPTG.

Recombinant bacmids are constructed by transposing a mini-Tn7 element from a pFastBac donor plasmid to the mini-attTn7 attachment site on the bacmid when the Tn7 transposition functions are provided in trans by a helper plasmid (pMON7124). The helper plasmid confers resistance to tetracycline and encodes the transposase. Each pFastBac donor plasmid vector has a baculovirus-specific promoter (i.e., the polyhedrin or p10 promoter from AcNPV) for expression of proteins in insect cells. The mini-Tn7 in a pFastBac donor plasmid contains an expression cassette consisting of a Gentamycin resistance gene, a baculovirus-specific promoter, a multiple cloning site, and an SV40 poly (A) signal inserted between the left and right arms of Tn7.

Recombinant baculoviruses have become widely used as vectors to express heterologous genes in cultured insect cells and insect larvae. Heterologous genes placed under the transcriptional control of the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcNPV) are often abundantly expressed during the late stages of infection. In most cases, the recombinant proteins are processed, modified, and targeted to their appropriate cellular locations, where they are functionally similar to their authentic counterparts.

2.9 HUMAN SERA SAMPLES

All serum samples used in this study were obtained from volunteers at Chennai, Vellore and Chengalpet, India. Informed consent was obtained
from all patients in accordance with U.S. Department of Health and Human Services Human Experimentation Guidelines and Department of Public Health, Chennai, Tamil Nadu, India. All the procedures followed were in accordance with the guidelines issued by Department of Public Health, Government of Tamil Nadu, India, for dealing with human subjects. The Institutional review board at the Center for Biotechnology, Anna University, India also approved the protocols. Sera were classified into MF, CP or EN based on the detection of circulating parasites, parasite antigens or by evaluating clinical symptoms of lymphatic filariasis. Circulating microfilariae were detected in the blood of subjects as described previously (Rao et al 2000). The presence of circulating antigen was detected using an Og4C3 kit (Lalitha et al 1998) and a WbSXP-based enzyme-linked immunosorbent assay (ELISA) (Rao et al 2000). Subjects with no circulating antigen or microfilariae were classified as EN, whereas subjects with circulating microfilariae and/or circulating antigen, as detected by ELISA, were considered as MF. Subjects showing lymphedema and other visible clinical symptoms of filariasis were grouped into CP. (Control non-endemic normal (NEN) sera were kindly provided by Professor Murray Selkirk, Imperial College London, London).

2.10 SUBCLONING OF SXP-1 GENE INTO pFastBac1 DONOR VECTOR

The SXP-1 gene from pRSETBSXP-1 was subcloned into pFastBac1 vector at the BamHI and EcoRI site.

2.10.1 Restriction Digestion

Restriction digestion of pRSETBSXP-1 was performed using BamHI and EcoRI (New England Bio lab, USA) in the manufacturer’s recommended buffer. The reaction was set up as follows:
DNA (3-4 µg) 2 µL
Buffer (10X) 2 µL
Enzymes (2-3 units µg⁻¹ of DNA) 1 µL
BSA 10 X 2 µL

The total reaction volume was made up to 20 µL with distilled water and the reaction mixture was incubated at 37 °C for 2 h. The completion of digestion of DNA was monitored by analyzing the samples on a 1 % agarose gel. The released insert was further purified by gel-extraction kit (Qiagen, Germany).

Similarly pFastBac1 vector was treated with BamH1and EcoR1. After complete digestion, the product was run on a 1 % agarose gel and the vector was gel-purified. Further to prevent recircularization of digested pFastBac1, it was treated with calf-intestinal alkaline phosphatase (CIP) (Amersham) for 1 h and purified.

2.10.2 Ligation

Ligation of digested vector (pFastBac1) and insert DNA (SXP-1) was performed as follows. The ligation mixture consisted of

10X ligation buffer 10 µL
Vector (50 ng) 2 µL
Insert (20-50 ng) 6 µL
T4 DNA Ligase (NEB, USA) 1 µL

The total reaction volume was made up to 20 µL with distilled water and ligation was performed for 16 h at 16 °C and after completion stored at -20 °C till use.
The ligation mixture was transformed into E.coli host *DH5α* cells. For screening transformants, a small portion of freshly grown transformed colony was picked using a sterile tooth pick and resuspended in 50 µl of 0.1X TE (1 mM Tris and 0.1 mM EDTA, pH 8.0) buffer. The cells were lysed by boiling for 10 min, snap-chilled on ice, centrifuged at 12000 X g for 10 min and 1 µL of the supernatant was used as template for PCR. Gene-specific primers were used to check for the presence of insert. The positive clones were further confirmed by digesting the plasmids extracted from those clones with BamH1 and EcoR1 and checked for the release of the insert.

2.10.3 Plasmid DNA Preparation and Agarose Gel Electrophoresis

All Plasmid DNA isolation from recombinant E. coli was done using commercial plasmid isolation kit (Eppendorf). For 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.

1. The electrophoresis was performed at 5-8 volts/cm at room temperature.

2. The gel-loading buffer contained 50 % glycerol with 0.01 % bromophenol blue and 0.01 % Orange-G in TE.

3. Agarose gels (1 %) were employed throughout the present study.

4. Gels were stained with approximately 0.5 µg mL⁻¹ of ethidium bromide and viewed under UV transilluminator (Fotodyne, Hartland, WI, USA).
5. Either 100 bp Ladder (Gibco BRL, MD USA) or λ Hind III digest DNA (New England Biolabs, MA, USA) were used as molecular weight markers.

6. Photographs were taken with Tracktel GDS-2 gel documentation system, Vision system, Germany.

7. The relative amounts of PCR products in the gel were determined by scanning the gel in Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA.

### 2.10.4 Restriction Digestion

The restriction digestions were performed using enzymes from New England Biolabs, USA in the manufacturer recommended buffers

1. Restriction enzyme digestions were performed by adding:
   - DNA (2-3 µg) 2 µL
   - 10 X Buffer 2 µL
   - Enzyme (2-3 units/µg of DNA) 1 µL
   - BSA 10 X 2 µL

2. Total volume was made upto 20 µL with triple distilled water and incubated at 37 °C for 3 to 4 h.

3. The completion of digestion was monitored by agarose gel (1 %) electrophoresis.

4. When double digestions were performed, the most appropriate buffer as recommended by the manufacturer was used. Simultaneously the efficiency of each enzyme was verified separately in the selected buffer using control DNA.
2.10.5  **Polymerase Chain Reaction**

PCR amplification was performed using the commercially available kit (Eppendorf) following conditions. PCR was performed either on MJ Thermal cycler. The optimal annealing temperature of 56 °C was used for all the primer sets. The PCR parameters used are:

1. Initial denaturation 95 °C, 5 min
2. Denaturation: 95 °C, 1 min
3. Primer annealing: 56 °C, 1 min
4. Primer extension: 72 °C, 1 min
5. Steps in 2 to 4 were cycled 30 times

PCR products were analyzed by 1% agarose (w/v) in TEB (0.5 x) buffer gel electrophoresis.

Table 2.1 Primers used in Polymerase chain reaction

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SXP forward</td>
<td>5’CGGGATCCATGGTCACTTCATCACTCAATC3’</td>
</tr>
<tr>
<td>SXP Reverse</td>
<td>5’CCGGAATTCCTAGTGGTGTGTTGTTGGTTGGTTGGTGGTGGTGGTTTTATTACTTTTTTGTTCG3’</td>
</tr>
<tr>
<td>M13 (-21) universal forward</td>
<td>5’-TGT-AAA-ACG-ACG-GCC-AGT-3’</td>
</tr>
<tr>
<td>M13/pUC reverse primer</td>
<td>5’-CAG-GAA-ACA-GCT-ATG-ACC-3’</td>
</tr>
</tbody>
</table>

2.10.6  **Transformation of E. coli with Plasmid DNA**

Transformation of E. coli with plasmid DNA was based on the method of Hanahan (1983) using CaCl$_2$, modified by Sambrook et al (1989)
for the preparation of competent cells. Briefly the following procedure was used.

1. A single colony of freshly revived E. coli culture was inoculated in 2 ml of LB and grown overnight at 37 °C. 100 µL of grown culture was inoculated into 50 mL LB medium in conical flask and allowed to grow at 37 °C till OD 600 reached 0.6.

2. Culture was chilled on ice for 5 min by gentle swirling and centrifuged at 3500 X g for 5 min at 4 °C.

3. The cell pellet was resuspended in 10 mL of 100 mM ice-cold MgCl₂ and incubated on ice for 20 min.

4. 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 h.

5. Approximately 10-20 ng of DNA was added to 100 µL of above cells and further incubated for 1 h on ice.

6. A heat shock at 42 °C was given for 90 sec and chilled again on ice. To this tube 800 µL of LB medium was added, allowed to grow for 1 h at 37 °C.

7. Around 100 µL was plated onto LB agar plates supplemented with appropriate antibiotics.

8. A positive control plasmid (10 ng of pRSETB) was used in all the experiments to verify the transformation efficiency. Cells with no DNA added served as negative controls.
2.10.7 Transposition of Insert into Bacmid

DH10Bac E. coli cells were transformed with pFastBac-SXP-1 for the transposition of inset into bacmid. Briefly the following procedure was used.

1. A single colony of freshly revived DH10Bac culture was inoculated in 2 mL of LB and grown overnight at 37 °C. 100 µL of overnight culture was inoculated into 50 mL LB medium in conical flask and allowed to grow at 37 °C till OD 600 reached 0.6.

2. Culture was chilled on ice for 5 min by gentle swirling and centrifuged at 3500 X g for 5 min at 4 °C.

3. The cell pellet was resuspended in 10 mL of 100 mM ice-cold MgCl$_2$ and incubated on ice for 20 min.

4. Approximately 20 ng of DNA was added to 100 µL of above cells and further incubated for 1 h on ice.

5. A heat shock at 42 °C was given for 90 sec and chilled again on ice. To this tube 800 µl of LB medium was added, allowed to grow for 1 h at 37 °C.

6. Around 100 µL was plated onto LB agar plates supplemented with 50 µg mL$^{-1}$ kanamycin, 7 µg mL$^{-1}$ gentamicin, 10 µg mL$^{-1}$ tetracycline and 100 µg mL$^{-1}$ X-gal, 40 µg mL$^{-1}$ IPTG.

7. Plates were incubated for 24 to 48 h at 37 °C.
2.10.8 Isolation of Recombinant Bacmid DNA

White colonies of DH10Bac transformed with pFastBac-SXP1 contain recombinant bacmid and selected for bacmid isolation. DH10Bac white colonies were streaked on fresh agar plates and incubated at 37 °C for 16 h. The following procedure used for isolating bacmid DNA (> 100 kb).

1. A single isolated colony was inoculated in 2 mL LB medium supplemented with 50 µg mL⁻¹ kanamycin, 7 µg mL⁻¹ gentamicin, 10 µg mL⁻¹ tetracycline and grown at 37 °C up to 24 h.

2. Culture was transferred to 1.5 mL microcentrifuge tube and centrifuge at 14000 X g for 1 min.

3. Supernatant was removed and each pellet was resuspended in 0.3 mL of Solution I [15 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg mL⁻¹ RNase A].

4. 0.3 mL of solution II (0.2 N NaOH, 1 % SDS) was added, gently mixed and incubated at room temperature for 5 min.

5. 0.3 mL of 3 M potassium acetate (pH 5.5) was added slowly and gently mixed during addition. Sample was placed on for 5 to 10 min and centrifuged for 10 min at 14,000 X g.

6. Supernatant was transferred to microcentrifuged tube containing 0.8 mL of isopropanol. Sample was mixed gently by inverting tube and placed on ice for 10 min.

7. Sample was centrifuged for 15 min at 14,000 X g at RT. Supernatant was removed and added with 0.5 mL 70 % ethanol and centrifuged at 14,000 X g for 5 min at room temperature.
8. Supernatant was removed and pellet was air dried at room temperature and suspended into 40 µL of TE buffer.

2.10.9 PCR Analysis of Recombinant Bacmid

The confirmation of transposition of insert into bacmid was done with PCR using pUC/M13 amplification primers. The pUC/M13 amplification primers are directed at sequences on either side of the miniattTn7 site within the lacZα (complementation region of the bacmid). If transposition has occurred, the PCR product produced by these primers is 2,300 bp plus the size of the insert. Alternatively, recombinant bacmid was amplified using gene specific forward primer and pUC/M13 reverse and vice versa and gene-specific primers.

PCR amplification was performed using the commercially available kit (Eppendorf) following conditions. PCR was performed either on MJ Thermal cycler. The optimal annealing temperature of 55 ºC was used for all the primer sets. The PCR parameters used are:

1. Initial denaturation 95 ºC, 3 min
2. Denaturation: 95 ºC, 45 sec
3. Primer annealing: 55 ºC, 45 sec
4. Primer extension: 72 ºC, 5 min
5. Steps in 2 to 4 were cycled 30 times
6. Final extension 72 ºC, 5 min. End.

PCR products were analyzed by 1 % agarose (w/v) in TEB (0.5 x) buffer gel electrophoresis.
2.10.10 Transfection of Sf21 Cells with Recombinant Bacmid DNA

1. 9x10⁵ Sf21 cells in mid-log phase with a viability of > 97% seeded in per 35-mm well (of a 6-well plate) in 2 mL of TC-100 medium with 10% FBS.

2. Cells were allowed to attach at 28 °C for 1 h.

3. Two solutions were prepared in 12 x 75-mm sterile tubes.

4. **Solution A**: 10 µL of mini-prep bacmid DNA was diluted into 100 µL of TC-100 serum free medium.

5. **Solution B**: 6 µL of CellFECTIN Reagent was diluted into 100 µL of TC-100 serum free medium.

6. Two solutions were combined, mix gently, and incubated for 15 to 45 min at room temperature.

7. After incubation 0.8 mL of TC-100 medium was added to tube containing lipid-DNA complexes.

8. Cells were washed with serum free medium once and overlaid with diluted lipid-DNA complexes. Cells were incubated for 5 h at 28 °C.

9. Transfection mixture was removed after 5 h of incubation and 2 mL of TC-100 with 10% FBS were added to cells. Cells were incubated for 5 days.

10. Virus was harvested from cell culture medium after five days post-transfection, and stored at 4 °C.
2.10.11 Titration of Virus by TCID\textsubscript{50} (End Point Dilution)

The TCID\textsubscript{50} was used to determine the titre of recombinant virus. The infectious units were expressed as TCID\textsubscript{50} unit mL\textsuperscript{-1} and were converted to the titre in pfu mL\textsuperscript{-1}. (Summers and Smith 1987)

**Method**

1. Serial log (1 in 10) dilution was made of the recombinant virus of range of 10\textsuperscript{-1} to 10\textsuperscript{-7} to be titred in TC100 growth medium.

2. 100 \(\mu\)L of diluted viruses were (from 10\textsuperscript{-1} to 10\textsuperscript{-7}) added to 900 \(\mu\)L of Sf21 cell suspension (2x 10\textsuperscript{5} cells mL\textsuperscript{-1}) and mixed.

3. 100 \(\mu\)L of cell-virus suspension (10\textsuperscript{-1} dilution) was added to the first row of wells in a 96-well microtitre plate. The last two rows, non-infected cells were added as control. Same were repeated with 10\textsuperscript{-2} dilution in the next row and so on, until all the samples have been plated.

4. Plate was incubated at 28 °C for 5 days, in a box lined with moist tissue paper to prevent drying out. Cells were monitored daily for signs of infection in the lower dilutions.

5. Total numbers of positive and negative wells were counted. TCID\textsubscript{50} or end point dilution\textsubscript{50} was determined as the dilution which would give rise to 50 % positive wells and 50 % negative wells, as extrapolated from the data obtained. For the test to be accurate the dilution used should range from those that give 100 % positive and negative wells.
Calculation

a) First calculate the proportionate distance [- PD] (which has the negative value): 
   \[-PD = - \left( \frac{\text{rate of dilution next above 50 \%}}{\text{rate of dilution next below 50 \%}} \right) \]

b) Calculate log TCID\textsubscript{50}: 
   \[\text{log TCID}_{50} = \text{log dilution (next to and above 50 \% positive)} + - [PD] \]

c) Convert log TCID\textsubscript{50} into TCID\textsubscript{50}.
   \[\text{TCID}_{50} \text{ value} = 10^{\text{log TCID}_{50}} \]

d) The titre of the virus in infection doses per unit of inoculums is obtained from calculating the reciprocal of the TCID\textsubscript{50} value. The unit dose is 1ml.

e) To convert TCID\textsubscript{50} to pfu the following approximate conversion were used: 
   \[\text{TCID}_{50} \text{ mL}^{-1} \times 0.69 \text{pfu mL}^{-1} \]

2.10.12 Amplification of Viral Stocks

For amplification of viral stocks, a monolayer culture was infected at a MOI (multiplicity of infection) of 0.1 according to the following formula:

\[
\text{Inoculum required (mL)} = \frac{\text{desired MOI (pfu cell}^{-1}) \times (\text{Total number of cells})}{\text{Titer of viral inoculum (pfu mL}^{-1})}
\]

2.10.13 Storage of Recombinant Baculovirus

Recombinant baculovirus was harvested from post-infection supernatant and stored in sterile, capped tube at 4 °C with 2 % FBS.
2.10.14 Analyzing Expression by Sf21 Insect Cells Infected with Recombinant Viruses

Analysis of expression was carried out in a 6-well plate with the amplified viral stock.

1. Two million of Sf21 cells were seeded per well and allowed to attach for 1 h.
2. Cells were rinsed with fresh serum free medium and replaced with 2 mL of fresh serum free medium.
3. 100 µL of P5 viral stock was added to each well with one uninfected control well with Sf21 cells.
4. Cells were incubated at 28 °C and supernatant was collected from day 1 to 5.
5. Expression profile was checked with 12 % SDS-PAGE and confirmed with western blotting.

2.11 PROTEIN PROFILE ANALYSIS

2.11.1 SDS-Polyacrylamide Gel Electrophoresis

Proteins present in cell extracts were analyzed by SDS-PAGE according to the method of Laemmli (1970) with some modifications. The gels were stained with Coomassie Blue R-250 or silver staining (Harlow et al 1988). Protein estimation was done by Bradford method (Bradford 1979) using commercial reagent (BIO-RAD CA).

i) Monomer solution: 29.2 % acrylamide and 0.8 % N, N-methylene bis acrylamide in distilled water. The solution was filtered through Whatmann filter paper no.1 and stored in brown bottles at 4 °C.
ii) Separating gel buffer: 1.5M Tris pH 8.8

iii) Stacking gel buffer: 1.5M Tris pH 6.8

iv) Ammonium per sulphate (APS) – 140 mg mL\(^{-1}\) (14 %)

v) SDS-10 % solution.

vi) TEMED

Electrophoresis running gel buffer: 0.025 M Tris-HCl, 0.192 M glycine, 0.1 % SDS, pH 8.3. Sample solubilising buffer (5X): 4.0 mL of 10 % SDS, 10 % (v/v), (0.5μL mL\(^{-1}\)) β-mercaptoethanol, 50 % sucrose or 4.0 mL glycerol, 0.025 % Bromophenol blue in 1.25 mL 0.5M Tris pH 6.8/ stacking gel buffer made upto 10 mL with water.

The samples were mixed with sample buffer was boiled for 5 min and loaded before start. For a 12 % w/v separating gel, 6.0 mL of monomer solution, 3.8 mL separating gel buffer, 5.0 mL water, 150 μL 10 % SDS, 75 μL APS and 5 μL TEMED were used. For a 4 % w v\(^{-1}\) stacking gel, 1.0 mL of acrylamide solution, 1.5 mL stacking gel buffer, 60 μL 10 % SDS, 3.4 mL water, 60 μL APS and 3 μL TEMED were used.

Stacking gel was approximately 1/5 of the separating gel. Equal amounts of total proteins were loaded in each well depending on size of gel. In the case of time course experiments, equal volumes of samples were preferred. Electrophoresis was performed at room temperature with constant current of 20 mA for stacking gel and 30 mA for separating gel. When the dye front reached 1 cm from the bottom of the gel, electrophoresis was stopped. The gel was removed and soaked in staining solution.
2.11.2 Coomassie Blue Staining

1. After electrophoresis, the gel was transferred to a clean glass or plastic container, 5 gel volumes of 0.25 % coomassie brilliant blue R-250, 50 % methanol, and 10 % acetic acid were added.

2. Incubated 4 h to overnight at room temperature with shaking.

3. Stainer was removed and saved (the staining solution can be used many times 20-40) before replacing.

4. The gel was destained by successive incubations in 10 % methanol, 7.5 % acetic acid.

2.11.3 Silver Staining

1. After running a standard gel, it was placed in 5 gel volumes of 30 % ethanol, 10 % acetic acid for 3 h with gentle shaking.

2. Ethanol/acetic acid solution was removed and 5 gel volumes of 30 % ethanol were added.

3. Ethanol solution was removed and 10 gel volumes of deionized water was added. The gel was placed on a shaker and incubated for 10 min at room temperature.

4. The water wash was repeated twice. 5 gel volume of 0.1 % AgNO₃ solution was added and incubated for 30 min at room temperature with gentle shaking.

5. To remove AgNO₃ solution, gel was washed with deionized water for 10 min.

6. 5 gel volumes of 2.5 % sodium carbonate, 0.02 % formaldehyde were added and incubated at room temperature with shaking.
7. The stained bands appear after several minutes (Incubated until
the background begins to darken).

8. The reaction was stopped by washing in 1 % acetic acid. Then
washed with several changes of deionized water (10 min each).

2.11.4 Total Protein Analysis

Total protein in culture supernatants were quantified by using the
colormetric Bio-Rad protein assay based on the Bradford method. The Bio-
Rad assay is based on the colour change of Coomassie Brilliant Blue G - 250
dye due to binding with primarily basic (especially arginine) and aromatic
amino acid residues of the protein. The protein concentrations were quantified
in the microplate format, as recommended by the assay kit and the absorbance
readings were measured at 595 nm using an ELISA reader (Labsystems
Multiskan MS).

1. Prepare dye reagent by diluting 1 part Dye Reagent
Concentrate with 4 parts distilled, deionized (DDI) water. Filter
through 0.2 μ filter (or equivalent) to remove particulates.

2. Prepare three to five dilutions of a protein standard which is
representative of the protein solution to be tested. The linear
range of the assay for BSA is 1.2 to 10.0 μg mL⁻¹.

3. Pipette 800 μL of each standard and sample solution into a
clean, dry test tube. Protein solutions are normally assayed in
duplicate or triplicate.

4. Add 200 μL of diluted dye reagent to each tube and vortex.

5. Incubate at room temperature for at least 5 min. Absorbance
will increase over time; samples should incubate at room
temperature for no more than 1 h.
6. Measure absorbance at 595 nm and estimate with standard protein concentration.

2.11.5 Confirmation of the Immune-Reactivity of Expressed rBAC-WbSXP-1 Protein (Western Blotting)

Western blotting of rWbSXP-1 was performed for hourly samples during batch course to confirm its immuno-reactivity, using slightly modified sample preparation method of Rao et al (2000) to suit highly cell density fermentation samples. Microfilaraemic positive human serum (Courtesy, Department of Public Health, Govt. of Tamilnadu, India), monoclonal and polyclonal antibodies against rWbSXP, anti-human, anti-mouse, anti-rabbit IgG-ALP (Sigma-Aldrich) was used for primary and secondary detection. NBT-BCIP (Sigma-Aldrich) was used as substrate (Harlow et.al.1988). Anti-His monoclonal antibodies (Sigma) were used as secondary at working dilution as described by manufacturer.

1. Electrophoresis was carried out and the SDS-PAGE gel was incubated for 10 min in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol). Nitrocellulose membrane (NCP) cut to the exact size of separating gel was also incubated for 10 min in transfer buffer. Without trapping air-bubbles the NCP was overlaid on the gel and sandwiched between filter papers and scotch brite pads.

2. Electrophoretic transfer was carried out in the cold room at 200 mA for 3 h using LKB transphor 2005 electroblotting apparatus. After transfer, the molecular weight marker lane was cut and stained with amido black (100 mg Amido block in 45 % methanol, 10 % acetic acid).
3. In case of pre-stained protein molecular weight markers, NCP was not cut out. 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.

4. The NCP was washed in wash buffer (PBS with 0.05 % Tween-20) thrice with 5 min duration each, and then incubated with appropriately diluted primary antibody for 3 h at room temperature.

5. After washing in the wash buffer the membrane was incubated for 1 h with recommended dilution of secondary antibody conjugated with alkaline phosphatase.

6. After extensive washing the blot was incubated in pre detection buffer (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) for 10 min.

7. The color development was carried out with 33 μL of 5-bromo 4-chloro-indolyl phosphate (50 mg mL⁻¹ in diethyl formamide) and 66 μL of Nitroblue Tetrazolium (50 mg mL⁻¹ in 70 % diethyl formamide) in 10 mL of detection buffer. The reaction was stopped after 15 min by addition of 10 mM EDTA.

2.11.6 PAS Staining

PAS staining of glycosylated protein has been described by (Fairbanks et al 1971).
1. After electrophoresis, the gel was transferred to a clean glass or plastic container incubated in a fixing solution having 25% isopropyl alcohol, 10% acetic acid, overnight.

2. Isopropyl alcohol / acetic acid solution was removed by washing with distilled water.

3. The glycoproteins were oxidized by immersing the gel in 1.0% periodic acid containing 3% acetic acid for 1h at room temperature.

4. The gel was washed with distilled water for 8-10 times to remove the periodic acid.

5. The gel was immersed in Schiff reagent in dark for 20-30 min.

6. Glycoproteins on the gel appeared reddish purple color in about 20 min.

7. The reaction was stopped by washing in 0.1% sodium metabisulfite in 3% acetic acid.

2.11.7 Purification of Expressed rBAC-WbSXP-1 from Recombinant Baculovirus Infected Sf-21 Cells Supernatant Using Immobilized Metal Affinity Chromatography (IMAC)

The recombinant protein was expressed with 6 histidine residues as a C-terminal fusion peptide. The metal binding domain in the fusion peptide allows simple one step purification of recombinant protein by IMAC.

Medium with infected cells was centrifuged at 1500 rpm for 10 min and the supernatant was collected and microfiltration using 0.2µ filter was carried out to remove any suspended particles. This was concentrated with 10 kDa ultrafiltration membrane. The concentrated medium was dialysed against
Tris-phosphate buffer with detergent (50 mM Tris buffer, 50 mM Phosphate, 150 mM NaCl and 0.5% L-lauroyl sarcosine, pH 8.0).

The column was equilibrated with 5 column volumes of binding buffer (50 mM Tris buffer, 50mM Phosphate, 150 mM NaCl and 0.5% L-lauroyl sarcosine, pH 8.0). Dialyzed samples were applied to the NiCl₂ charged Ni-NTA column (Amersham Pharmacia Biotech, Hong kong) at the rate of 5 mg of recombinant protein per ml of column matrix, and allowed to bind. Column was washed with wash buffer (50 mM Tris-phosphate buffer pH 8.0), to remove all contaminating proteins, followed by elution with increasing concentrations of imidazole (50 mM - 250 mM in elution buffer). The protein was eluted at 250 mM imidazole concentration. The purity of the protein fractions were analyzed on SDS-PAGE and the presence of recombinant proteins were confirmed by immunoblotting with anti-SXP MAbs and polyclonal antibodies.

The protein fractions were dialyzed against 0.1X PBS overnight at 4 °C, in snake-skin dialysis bags (Pierce, Rockford, USA) and then concentrated by vacuum concentrator. The concentration of the protein was determined by Lowry method and stored in aliquots at – 80 °C in aliquots till further use.

2.11.8 Enzyme-Linked Immunosorbent Assay

A solution of rWbSXP-1 at 10 µg mL⁻¹ 50 mM of carbonate buffer, pH 9.6 was coated in the wells of ELISA plates (Maxisorp, Nunc, USA) and incubated at 37 °C for 2 h. Blocking was done with 0.5 % gelatin in PBS for (2 h), followed by incubation (1 h) with Primary antibody (monoclonal or polyclonal or human clinical sera) as the case may be. After thorough washing with PBS containing 0.05 % Tween-20 (PBS-T) thrice, rabbit antibodies to mouse Ig-conjugated to horse radish peroxidase (r a-mIg-HRP) (DAKO, Glostrup, Denmark) or mouse antibodies to rabbit IgG, conjugate
with r a-mIg-HRP was added as the case may be, and incubated for 1 h. The plates were washed again with PBS-T and the bound peroxidase activity was detected using tetramethyl benzidine (TMB) at a concentration of 60 µg mL\(^{-1}\) in citrate phosphate buffer pH 5.5 containing 0.03 % H\(_2\)O\(_2\). The reaction was stopped with 1M H\(_2\)SO\(_4\), and absorption was read at 450 nm using multiscan reader (Labsystems).

### 2.11.9 Enzyme-Linked Immunosorbent Assay (ELISA) for Antibody Isotype Determination in Human Sera

The pattern of antibody subclass in human clinical samples elicited by rBAC-WbSXP-1 antigen was measured. The plates were coated with the antigen, and the human clinical samples were used as primary antibody. After washing, mouse anti-human IgG isotypes (IgG1 [1:500], IgG2 [1:2000], IgG3 [1:5000] and IgG4 [1:5000]), IgM (1:5000), IgE (1:5000), IgA (1:1000) were added as secondary antibodies (Sigma, St Louis, USA) and incubated at 37 °C for 1 h. After washing the plates with PBS-T followed by PBS, rabbit antibodies to mouse Ig-conjugated to horse radish peroxidase (r a-mIg-HRP) (DAKO, Glostrup, Denmark) was added and incubated for 1hr. The plates were washed again with PBS-T and the bound peroxidase activity was detected using tetramethyl benzidine (TMB) at a concentration of 60 µg mL\(^{-1}\) in citrate phosphate buffer pH 5.5 containing 0.03 % H\(_2\)O\(_2\). The reaction was stopped with 1M H\(_2\)SO\(_4\), and absorption was read at 450 nm using multiscan reader (Labsystems).

### 2.12 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

#### 2.12.1 Extraction of RNA

RT-PCR was carried out as described previously (Hall et al 1998). Isolation of RNA was carried out with adequate precautions to eliminate
RNase activity. Insect cells (Sf21) infected with recombinant virus and harvested at 48 h, were used for RNA extraction. Cells were lysed in 1 mL total RNA isolation reagent TRIZOL (Invitrogen, USA) in 1.5 ml centrifuge tubes and incubated for required time points. 200 µL of chloroform was added and spun at 12000 rpm for 15 min at 4 °C. The aqueous phase was removed and transferred to a new 1.5 mL centrifuge tube. 500 µL of isopropanol was added and incubated on ice for 15 min. Later it was spun at 12000 rpm at 4 °C for 15 min. The supernatant was discarded and the RNA pellet was washed with 1 mL of 75 % ethanol. The pellet was air dried and dissolved in 4.5 µL of DEPC-treated autoclaved water.

2.12.2 Reverse Transcription Reaction

Reverse transcription reaction was carried out as follows: 200 ng of random hexamer was added to 4.5 µL of total RNA and was made up to 10 µL using DEPC-treated water. The mix was incubated in a water bath at 72 °C for 3 min and snaps chilled on ice. 200 units of Melony Murine Leukimia Virus (MMLV) reverse transcriptase (2uL), 2uL of 10x reverse transcriptase buffer, 2.5 mM of dNTPs were added, and the reaction mix was made up to 20 µL. The reaction mix was incubated at 42 °C in a water bath for about one hour. The reaction mix was then heated at 90 °C for a minute to inactivate MMLV reverse transcriptase. The cDNA synthesized was further used for PCR.

2.12.3 Polymerase Chain Reaction of cDNA

For PCR reaction, 1 µL of the cDNA mixture prepared as described above, was added to a PCR reaction mixture consisting of 1 X PCR buffer (50 mM KCl, 10 mM Tris.Cl,pH 8.3), 200 µM dNTP mix, 5 picomoles of
each primer, 1 unit of Taq polymerase (NEB, MA, USA), and sterile water in a total volume of 20 μL.

The reaction mixture was placed in a PCR thermal cycler for cyclic reactions. The PCR reaction was set up as per the nature of primer and size of amplified product. The cycling protocol for amplification was an initial activation and denaturation step of 5 min at 95 °C, followed by 35 cycles each consisting of 1 min at 94 °C, 1 min at 56 °C annealing temperature, 1 min at 72 °C, and a final extension was given at 72 °C for 10 min. All amplification were carried out using MJ Minicycler (Perkin Elmer, USA).

The PCR products were run on 1.2 % agarose gels stained with 0.5 μg mL⁻¹ ethidium bromide and photographed by gel documentation system.

2.13 MAINTENANCE OF INSECT CELLS

2.13.1 Insect Cell Culture Media

Invertebrate cell cultures are very sensitive to environmental factors. In addition to chemical and nutritional culture factors, physical factors can also affect invertebrate cell growth and require optimization to maximize in vitro cell growth. Some important general areas include:

**Temperature.** The optimal range for growth and infection of cultured insect cells is 27 °C to 30 °C.

**pH.** A range of 6.1 to 6.4 appears to work well for most culture systems.

**Shear Forces.** Suspension culture techniques generate mechanical shear forces. Serum concentration ranges of 5 % to 20 % in medium provide
adequate protection from cellular shear forces. In the absence of serum, the medium may require supplementation with a shear force protectant such as Pluronic F-68.

2.13.2 Serum-Supplemented Culture

Spodoptera frugiperda (Sf21) cells were cultured in TC-100 Insect Medium (Hi Media, India) containing 5 % to 10 % FBS and have a doubling time of 20 to 30 h. Alternatively, cells may be cultured in Grace’s Insect Cell Culture Medium containing 3.3 g L\(^{-1}\) of yeastolate, 3.3 g L\(^{-1}\) lactalbumin hydrolysate, and 5 % to 10 % HI-FBS.

2.13.3 Monolayer Culture Procedure

1. Medium and floating cells from a confluent monolayer was aspirated and discarded.

2. 4 ml of complete growth medium was added to 25-cm\(^2\) flask (12 mL to a 75-cm\(^2\) flask) and cells were resuspended by pipetting the medium across the monolayer with a Pasteur pipet.

3. Cell monolayer was observed using an inverted microscope to ensure adequate cell detachment and viable cell count was performed on harvested cells using trypan blue dye exclusion.

4. \(2 \times 10^5\) viable cells mL\(^{-1}\) was inoculated into respective culture vessels and incubated at 28 °C. Cells were subcultured when the monolayer reaches 80 – 100 % confluency.
2.13.4 Shaker Culture Procedure

A shaker method which works well uses 250 mL shaker flasks containing 50 to 125 mL total volume. Oxygen tension is not rate limiting under these conditions. 0.1 % Pluronic F-68 was added to media formulations.

1. The standard flask employed for the growth of Sf21 was 250 mL disposable sterile Erlenmeyer for a 100 mL medium volume. Orbital shaker was set at 100 rpm for cultures adapted to and maintained in suspension culture.

2. The orbital shaker flask assembly was maintained in a 28 °C, non humidified, ambient air regulated incubator. 250 mL Erlenmeyer flask with 100 mL of medium inoculated with $1 \times 10^5$ viable cells mL$^{-1}$.

3. Culture was incubated until it reaches $2 \times 10^6$ to $3 \times 10^6$ viable cells mL$^{-1}$. The shaker flask culture was subcultured to approximately $3 \times 10^5$ viable cells mL$^{-1}$. For consistent optimal cell growth, the culture should be in mid-log-phase of growth when subcultured and did not allow the cultures to reach stationary phase. For consistent growth, the cells must be subcultured when they are in mid-log (exponential) growth.

2.14 TESTING AND EVALUATION OF RAPID FLOW THROUGH FILARIAL DIAGNOSTIC KITS AND REAGENTS

Kits were manufactured and assayed as described (Basker et al 2004) at the research facility, SPAN Diagnostics Ltd. Briefly, the kit contains a test spot with rWbSXP-1 and a control spot with goat-anti human IgG on nitro cellulose paper. The anti-SXP-1 antibodies if present in test serum bind
to rWbSXP-1, whereas, any IgG binds to control spot. Following a wash, the protein-A-colloidal gold signal reagent was used to detect bound IgG.

### 2.14.1 Preparation of colloidal gold and Conjugation of protein-A

Gold chloride (HauCl4) was procured from Amresco, OH, USA. Colloidal gold with an average diameter of 25-30 nm (validated by electron microscopy, Amresco) was prepared by controlled reduction of a boiling solution of 0.02% chloroauric acid with 1% sodium citrate according to the method of Frens (1973) and standardized by SPAN diagnostics Ltd., (Basker et al 2004).

The solution was stored in refrigeration 4°C away from light until use. Criteria for the colloidal gold solution batch having maxima between 525–530 nm and A1cm 527 nm = 2.0 + 0.05 was used for preparing the conjugate with antibody (Basker et al 2004).

To colloidal gold sol, 0.01% of freshly prepared 1% K2CO3 solution was added to adjust the pH of sol to 6.5. To this 15-20 mg of protein-A were added and centrifuged for 5 minutes at 5000 rpm. 950 μL of supernatant was discarded and the pellet was suspended in the remaining 50 μL of supernatant.

### 2.15 MONOCLONAL ANTIBODY PRODUCTION

#### 2.15.1 Immunization of Mice with rWbSXP-1 for Hybridoma

Six-eight week old female BALB/c mice were immunized subcutaneously with 100 μL of emulsion containing 50 μg of purified rWbSXP-1 protein in PBS emulsified with equal volume of Freund’s complete adjuvant. The first booster was given 3 weeks later, by subcutaneous injection of 50 μg of antigen in incomplete Freund’s adjuvant. Second booster was
given 3 weeks later from first, and the blood sample was collected 10 days later. Antibody titre was determined by ELISA. After resting for 1 month, 4 days prior to fusion, the mice were injected intraperitoneally with 200 µg of the antigen in saline.

2.15.2 Preparation of Myeloma Cells and Splenocytes

The cell-line used for fusion was Sp2/0-Ag-14, originally derived from a fusion between spleen cells from BALB/c mice with X63-Ag8. Sp2/0 myeloma cells were maintained in IMDM supplemented with 36 mM sodium bicarbonate, penicillin (100 U mL\(^{-1}\)), streptomycin (100 µg mL\(^{-1}\)), gentamycin (50 µg mL\(^{-1}\)), nystatin (5 U mL\(^{-1}\)), 10 % (v v\(^{-1}\)) FBS and β-mercaptoethanol (5 x 10\(^{-5}\) M). Prior to fusion with splenocytes, Sp2/0 cells in the log phase were harvested, pelleted down by centrifugation and washed twice with IMDM to remove serum. After excision of spleen from the immunised mice under aseptic conditions, the splenocytes were recovered using needle and piston assembly, washed twice and resuspended in 10 mL of IMDM. An aliquot of the cells suspension was counted. About 80- 100 x 10\(^{6}\) splenocytes could be recovered from one mouse.

2.15.3 Preparation of Macrophage Feeder Layer

Mice were sacrificed and macrophages collected by flushing the peritoneal cavity with 10 mL of ice cold IMDM. About 5-7 x 10\(^{6}\) cells could be obtained from a normal mouse.

2.15.4 Fusion of Cells

A suspension of the SP2/O cells and splenocytes in a 1:5 ratio was centrifuged to obtain a tight pellet. To the dry pellet, 0.5 mL of the PEG-4000 solution (1 g in 0.8 mL of IMDM and 0.2 mL of dms Merck, Rahway, NJ) was added drop wise over 1 min, with gentle tapping of the tube throughout
the course of addition and exposed to PEG for another 1 min. This was followed by slow addition of 5 mL of IMDM over 5 min, first 1 mL being added drop wise over one minute. The cells were incubated at 37 °C for 20-60 min. After centrifugation, the cell pellet was resuspended gently in HAT supplemented IMDM.

2.15.5 Selection of Hybridoma

The HAT selection medium consists of IMDM supplemented with 20 % FBS, hypoxanthine \((1 \times 10^{-4} \text{ M})\), aminopterin \((4 \times 10^{-7} \text{ M})\) and thymidine \((1.6 \times 10^{-5} \text{ M})\). After resuspending in HAT medium, 0.2 mL aliquots containing 0.2 \(\times 10^6\) splenocytes and 3-5 \(\times 10^3\) macrophages were distributed in the wells of a 96 well micro titer plate. The plates were kept in a humidified incubator containing 5 % CO\(_2\) in the air at 37 °C. Medium from individual wells was replaced with fresh medium as above but without aminopterin after 7 days. The unfused Sp\(_2/0\) cells are killed within 72-96 h during selection in HAT medium. After 10-12 days following fusion, supernatants from wells containing hybrids that were 50 % confluent were tested for their ability to secrete specific antibody by ELISA using rWbSXP-1 as antigen.

Single cluster clones secreting antibodies specific to rWbSXP-1 was selected, expanded and subsequently subcloned to monoclonality by the method of limiting dilution on feeder cells. Monoclonality was confirmed by subclass isotyping using MAb isotyping kit II (ImmunoPure, PIERCE).

2.15.6 Analysis of Serum Samples and Monoclones by rWbSXP-1 Antigen Based ELISA

The ELISA method for the detection of antibodies to rWbSXP-1 was standardized in our lab.
1. The rWbSXP-1 antigen (1 µg well\(^{-1}\)) in PBS, pH 7.2, was placed in the wells of a polystyrene plate for overnight incubation followed by blocking of the unoccupied sites with a 1 % solution of gelatin in PBS. Followed by incubation with monoclonal or polyclonal antibodies for 2 h.

2. The unbound antibodies were removed by three washes (3 min each) with PBS containing 0.1 % Tween (PBST) followed by three washes with PBS.

3. This was followed by incubation for 1 h with rabbit anti-mouse Ig conjugated to horse radish peroxidase (HRP) at the dilution of 1:2000 in PBS containing 0.2 % of BSA (RIA buffer).

4. After washing with PBST and PBS, the immunoreactivity of the MAbs was visualized by addition of 60 µg mL\(^{-1}\) of the substrate 3,3',5,5'-tetramethyl benzidine (TMB) in citrate phosphate buffer, pH 5.5, containing 0.03 % H\(_2\)O\(_2\). The reaction was stopped with 1 M H\(_2\)SO\(_4\), and measuring A\(_{450\text{nm}}\) in ELISA reader (Biotek).

5. For the serum antibodies in positive and control group was based on the titer criteria, Mean ± 3 SD. The cutoff for a positive response was considered when the ELISA OD value was at least 3 times higher than the mean control value.

### 2.15.7 Expansion of Secretor Clones

Antibody secreting clones were expanded by transferring them from 0.2 mL culture wells to 1 mL culture wells of 24 well culture plates in the presence of 3-5x10\(^3\) macrophages. During subsequent subcloning or expansion, the cells were weaned off HT medium, by replacement with
serially diluted concentrations of HT (hypoxanthine and thymidine). Before transferring to plastic culture flasks (25 cm$^2$), cells with more than 75 % confluency in the 1 mL wells were confirmed for stable antibody secretion, by ELISA.

2.15.8 Cloning under Limited Dilution (Subcloning)

Subcloning was carried out after cell-lines were well established. The cells in the log phase of growth were diluted ten-fold so as to obtain one cell in 0.2 mL of IMDM containing 20 % FBS and 3-5 x 10$^3$ macrophages. Three to four days after plating in the 96 culture plate, wells were examined microscopically to determine the number of clones in the well. Wells containing single hybridoma were replenished with 0.2 mL of fresh medium and when the clones reached 50 % confluency, the supernatants were assayed for the presence of antibody. Antibody producing clones (monoclonsals) were expanded as described above.

2.15.9 Subclass Isotyping of Monoclonal Antibodies

Subclass isotyping was done with Rapidot Kit {(mouse Immunoglobulin isotyping) Department of Aquaculture, college of fisheries, Mangalore} to check the isotypes of all five monoclonal antibodies.

2.15.10 Maintenance of Cell-Lines

Hybridoma cell lines were maintained in IMDM, supplemented with 36 mM sodium bicarbonate, penicillin (100 U mL$^{-1}$), streptomycin (100 µg mL$^{-1}$), gentamycin (50 µg mL$^{-1}$), nystatin (5 U mL$^{-1}$), 8-10% (v v$^{-1}$) FBS and β-mercaptoethanol (5 x 10$^{-5}$ M). All the cultures were grown at 37 °C in a humidified incubator containing 5 % CO$_2$ incubator.
2.15.11 Freezing and Thawing of Cells

Myeloma, hybridoma cells were stored frozen (in liquid nitrogen) at various stages during the course of the experiment, so as to be able to revive them when required. The composition of freezing mixture includes 50 % IMDM/DMEM, 40 % FBS and 10 % DMSO. For freezing, cells in the log phase of the growth were centrifuged and the cell pellet was resuspended in the chilled freezing mixture by drop wise addition and transferred to – 70 °C in the freezing vials and subsequently to liquid nitrogen. For reviving the cells, the vials were removed from liquid nitrogen and warmed rapidly to 37 °C. Freezing mixture was removed by centrifugation and the cells were transferred to culture flasks containing 5 mL culture medium.

2.15.12 Affinity Measurement of Monoclonal Antibodies [Calculation of Dissociation Constants (Kd)]

The affinity of antibodies raised against rWbSXP-1 was measured by estimating the disassociation constant (Kd). For the measurement of the Kd in solution, the method of Friguet et al. (1985) was used.

1. The rWbSXP-1 antigen (1 µg well⁻¹) in PBS, pH 7.2, was placed in the wells of a polystyrene plate for overnight incubation followed by blocking of the unoccupied sites with a 5 % solution of non-fat milk in PBS.

2. The monoclonal antibodies were incubated with gradient of rWbSXP-1 antigen concentration for 16 h at 25 °C so as to attain antigen–antibody equilibrium. The starting concentration of inhibiting antigen was 50 µg mL⁻¹ and was carried out twofold serial dilutions.
3. These complexes were transferred onto the wells of the microtitre plates previously coated with the respective antigen and blocked and were incubated for 2 h at 37 °C.

4. After three washes with PBS containing 0.1 % Tween (PBST) followed by three washes with PBS, rabbit anti-mouse Ig conjugated to horse radish peroxidase (HRP) at the dilution of 1:2000 in PBS containing 0.2% of BSA was added and incubated for 1h at 37 °C.

5. After washing with PBST and PBS, the immunoreactivity of the MAbs was visualized by addition of 60 µg mL⁻¹ of the substrate 3, 3’, 5, 5’-tetramethyl benzidine (TMB) in citrate phosphate buffer, pH 5.5, contains 0.03 % H₂O₂. The reaction was stopped with 1 M H₂SO₄, and measuring A₄50nm in ELISA reader (Biotek).

6. Dissociation constant (Kd) was calculated using the following equation derived from Scatchard and Klotz (Friguet et al 1985):

\[
\frac{A₀}{A₀ - A} = \frac{1 + Kd}{a₀}
\]

Wherein A₀ and A: absorbance measured for antibody in absence and presence of antigen respectively; Kd: disassociation constant; a₀: total antigen concentration.

2.15.13 Effect of Urea Treatment on Monoclonal Antibodies Reactivity with rWbSXP-1 and W. bancrofti mf Crude Antigen

The ELISA for monoclonal antibodies reactivity with rWbSXP-1 and Wb mf crude antigen were performed as previously. The protocol
followed in present assay was previously described by Binley et al (1997) and used with modification.

1. The 1 µg well⁻¹ of rWbSXP-1 antigen and 2 µg well⁻¹ of Wb mf crude antigen in PBS, pH 7.2, was placed in the wells of a polystyrene plate for overnight incubation followed by blocking of the unoccupied sites with a 5 % non-fat milk solution in PBS. Followed by incubation with two fold dilution of monoclonal or polyclonal antibodies in PBS containing 0.5 % non-fat milk for 1 h.

2. For avidity measurement, plate was divided in such a way that rows A, B, and C are coated with antigen (PBS wash) and rows F, G, and H are coated with antigen for avidity (8M Urea treatment) rows D and E are blank.

3. For avidity, each plate was washed three times, 5 min each, with 200 mL well⁻¹ as follows:
   a. rows A, B, and C with PBS
   b. rows D, E, and F with 8 M urea in PBS

4. This was followed by incubation for 1 h with rabbit anti-mouse Ig conjugated to horse radish peroxidase (HRP) at the dilution of 1:2000 in PBS containing 0.5 % of non-fat milk solution in PBS.

5. The unbound antibodies were removed by three washes (3 min each) with PBS containing 0.1 % Tween (PBST) followed by three washes with PBS.
6. The immunoreactivity of the MAbs was visualised by addition of 60 µg mL\(^{-1}\) of the substrate 3, 3’, 5, 5’-tetramethyl benzidine (TMB) in citrate phosphate buffer, pH 5.5, containing 0.03 % \(\text{H}_2\text{O}_2\). The reaction was stopped with 1 M \(\text{H}_2\text{SO}_4\), and measuring \(A_{450\text{nm}}\) in ELISA reader (Biotek).

**Data presentation:** Midpoint titers are defined as the antibody dilutions giving half-maximal binding (after background subtraction). The avidity index is defined here as \((A/B \times 100 \%)\), where \(A\) is the absorbance value with urea treatment and \(B\) is the absorbance value without urea treatment at a given dilution/concentration of antibody. The value of \(B\) in every avidity index calculation was derived from titration curves, where the absorbance value \(A\) was then read at the same antibody dilution, correcting for background for both values. Avidity indices calculated are the average of two replicates. Antibodies with avidity indices of < 30 % are designated low-avidity antibodies, those with values of 30-50 % are designated intermediate-avidity antibodies, and those with values > 50 % are designated high-avidity antibodies.

- When a urea wash was used in ELISAs, we define the binding property of monoclonal or polyclonal antibodies as its avidity, although it should be noted that monoclonal antibodies, owing to their clonal nature, cannot have avidity per se. We use this term here to conveniently distinguish binding observed after a urea wash step from that without the urea wash.

2.15.14 Production of Polyclonal Antibody Against rWbSXP-1

Laboratory bred rabbits were immunized with the purified recombinant protein WbSXP-1 to produce polyclonal antibodies, as per protocol described (Harlow et al 1988).
Briefly, the rabbit was immunized subcutaneously with 250 µg of purified rWbSXP-1 protein emulsified in Freund’s complete adjuvant, followed by administration of 125 µg of the antigen in Freund’s incomplete adjuvant. Animals were pre-bleed before immunization to be used as control. Serum samples were collected 2 weeks after the final immunization and tested for immunoreactivity against the rWbSXP-1 antigen by Western blotting and the antibody titers by ELISA. The antibody titre in immunized animals was estimated by serial dilution and compared with control or pre immune serum. The criteria for serum titre were Mean + 3 SD against the control. The cutoff for a positive response was fixed at least 3 times higher than the mean control value.

2.15.15 Purification of Monoclonal Antibody

Culture supernatant of IgM MAb (2E12E3, 3E4F1 and 3E3D7) supernatant was purified using Hi Trap IgM Purification HP, 1mL column. The matrix was equilibrated with binding buffer (20 mM NaH$_2$PO$_4$, 0.8M (NH)$_2$SO$_4$, pH 7.5). The MAb supernatant was loaded and eluted with 12 column volume of elution buffer (20 mM NaH$_2$PO$_4$, pH 7.5).

Culture supernatants of murine MAb IgG2a (1F6H3 and 3G12F7) were equilibrated against 50mM glycine-NaOH buffer, pH 8.5 containing 2M NaCl and loaded onto a protein A-Sepharose column (Amersham, USA). After washing, the bound MAb were eluted with 0.1M glycine-HCl, pH 3.0, and neutralized with 1 M Tris-HCl, pH 8.0.

2.15.16 Enrichment of MAbs and Polyclonal Antibodies with Ammonium Sulphate Precipitation Method

Monoclonal and polyclonal antibodies raised against rWbSXP-1 were precipitated with 50 % and rinsed twice with 40 % ammonium sulphate
to remove albumin fraction. Concentrated antibodies were dissolved and
dialized against 50 mM PBS and estimated.

2.16 STANDARDIZATION OF SXP ANTIGEN CAPTURE ELISA

Sandwich ELISA was standardized for antigen detection with monoclonal and polyclonal antibodies in combination as capture antibody and
detection antibody to detect antigens. The methods previously described by

1. Flat bottom 96-well microtitre plates (Immunolon 4,
   Dynatech Laboratories, Inc., Alexandria, VA) were coated
   with 1µg/well of anti-SXP MAb (500 ng of 1F6H3 and
   2E12E3) diluted in 50 mM PBS pH 7.2 and kept overnight at
   4 °C.

2. The plates were washed in phosphate buffered saline (PBS)
   containing 0.05 % Tween 20 (Sigma) and blocked with
   blocking buffer, PBS containing 5 % skimmed milk for two h
   at 37 °C.

3. After six washes, patient's serum sample (1:50) was mixed
   with equal volumes of glycine (0.15 M; pH 2.0/Tris (0.1 M;
   pH9.0) and added to the wells in duplicates and the plates
   were incubated at 37 °C for 2 h.

4. The plates were washed as before and incubated with rabbit
   anti-SXP antibody (dilution of 1:1000) and incubated at 37 °C
   for an h. After washing the plate, goat anti rabbit IgG HRP
   conjugate was added and incubated at 37 °C for an h.
5. The bound peroxidase activity was detected using tetramethyl benzidine (TMB 60 µg mL\(^{-1}\)) in citrate phosphate buffer pH 5.5 containing 0.03 % H\(_2\)O\(_2\). 1M H\(_2\)SO\(_4\) was used as stopper. The optical absorbance (450 nm) was measured by multiskan reader (Labsystems, Finland).

2.16.1 Statistical Analysis

All statistical analyses were done using GraphPad prism software version 5.0 (GraphPad Software, San Diego, CA). The difference in 2 means was compared using nonparametric Man Whitney analysis. The comparison of more than 2 means was done using 1-way analysis of variance (ANOVA) post hoc tests.

Sample sera were considered positive for rBAC-WbSXP-1 IgG4 antibody assays when the samples had exhibited optical density (OD) higher than mean non-endemic normal control sera plus 3 standard deviations (mean OD NEN +3SD).

The WbSXP-1 antigen assay was considered positive when the OD\(_{450}\) nm was higher than mean + 3 standard deviations of non-endemic normal control sample (mean OD NEN +3SD). The Mann-Whitney U test was used to compare the WbSXP-1 antigen levels among the MF, CP, EN and NEN.

A value of p < 0.05 was considered statistically significant for all the experiments. The sensitivity and specificity of the assays were calculated as follows:

\[
\text{Sensitivity} = \frac{\text{Number of microfilaremic patient positive by the assay}}{\text{Total number of microfilaremic patient}} \times 100
\]
Specificity = \( \frac{\text{Number of microfilaremia free patient negative by the assay}}{\text{Total number of microfilaremia free patient}} \times 100 \)

2.17 DEVELOPMENT OF RAPID DIPSTICK DIAGNOSTIC ASSAY FOR DETECTION

Prototype of dipstick device was developed and assayed as described at the research facility, SPAN Diagnostics Ltd.

1. Briefly, the prototype contains a test line of capture anti-SXP monoclonal 2E12E3 and a control line with goat-anti mouse IgG on nitro cellulose membrane.

2. The sample adsorbent pad contains detection reagent with colloidal gold conjugated monoclonal anti-SXP antibody (1F6H3).

3. The patient blood sample will be drawn in the adsorbent pad and any native antigen present will bind with the colloidal gold conjugated monoclonal and will be carried further across the test and control line.

4. The indication of positive reaction will be seen as two magenta coloured lines in test and control regions respectively.

5. The negative reaction will be represented as a single magenta coloured line in the control region.

2.17.1 Preparation of Colloidal Gold

Gold chloride (HauCl4) was procured from Amresco, OH, USA. Colloidal gold with an average diameter of 25-30 nm (validated by electron
microscopy, Amresco) was prepared by controlled reduction of a boiling solution of 0.02 % chloroauric acid with 1 % sodium citrate according to the method of Frens (1973) and standardized by SPAN diagnostics Ltd., (Basker et al 2004).

The solution was stored in refrigeration 4 degree C away from light until use. Criteria for the colloidal gold solution batch having maxima between 525–530 nm and $A_{1\text{cm}}$ 527 nm = 2.0 + 0.05 was used for preparing the conjugate with antibody (Basker et al 2004).