CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND PLASMIDS

*Escherichia coli* strains DH5α and TOPO-Zero blunt cloning vectors for sequencing were obtained from Invitrogen, CA, USA. Prokaryotic T7 expression vectors pRSET A and pRSET B were purchased from Invitrogen, CA, USA. Genotype of the *E. coli* strains employed in this study is given in Appendix I. Maps and restriction sites of the vectors pRSET A and pRSET B are shown in Appendix II.

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, Mumbai, India) and 5 g of sodium chloride in 1 L of distilled water and the pH adjusted to 7.2 with 1N NaOH. To prepare solid medium, 1.5% agar (HiMedia, Mumbai, India) was added to the liquid broth. Media was supplemented with 60 μg/mL of ampicillin whenever required. Luria Bertani broth without NaCl was used to propagate GJ1158. The chemicals were procured from Merck, Germany.

2.3 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 weight markers were obtained from Fermentas (Fermentas, MD, USA). High binding 2HB plates (Thermo Scientific, MA, USA) for peptides and Maxisorb (Nunc, Maxisorp, Nalge Nunc International, Denmark) 96 wells were purchased for ELISA. The other molecular grade chemicals were procured from HiMedia, India.

2.4 EXPRESSION SYSTEM USED IN THIS STUDY

All the three genes were expressed in pRSET 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 metal-binding domain (six-tagged histidine moieties) at the N-terminal end forms a fusion peptide and has a high affinity for the divalent ions (nickel, copper and cobalt) and facilitates purification of the protein using immobilized metal affinity columns (IMAC) (Crowe et al 1995).

2.5 RECOMBINANT PROTEINS USED IN THE PRESENT STUDY

WbSXP-1 (Rao et al 2000) and Wb14 clones identified from the genomic DNA amplification was re-cloned into T7 expression vector pRSET B, and WbTPx-1 was sub-cloned in T7 expression vector pRSET A and expressed in E. coli GJ1158. The recombinant proteins were further purified and were used for all the functional analysis.
2.6 BANCROFTIAN AND BRUGIAN SERUM GROUPS USED IN THE STUDY

The patients were grouped into the following clinical groups: endemic normal (EN; individuals residing in an endemic area who are free of infection), microfilaraemics (MF; individuals with *B. malayi* or *W. bancrofti* microfilaria (mf) in the peripheral circulation), chronic pathology patients (CP; individuals with filariasis-mediated chronic pathology) and non-endemic normal (NEN; populations outside the endemic area). Serum samples from blood banks (Surat Rakthadhan Kendra, Gujarat, India), known MF patients identified by Department of Public Health (DPH), Tamil Nadu, and Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sevagram, India, were used for optimization of assay and evaluation studies. The positives controls, mouse anti-SXP and mouse anti-BmTPx-1 used were raised previously in our laboratory.

2.7 PARASITE COLLECTION

*W. bancrofti* microfilariae (mf) were collected from 10 individuals residing in each of the four endemic region for filariasis in India (Sabesan et al 2000) representing different geographic regions of about 20–2000 km. In Pondicherry (PDY, Pondicherry State), samples were collected through Vector Control Research Center (VCRC), ICMR, Pondicherry; in Sevagram (SVG, Maharashtra state) through Mahatma Gandhi Institute of Medical Sciences (MGIMS), Wardha; in Bhubaneshwar (BHU, Orissa state) through Regional Medical Research Center (RMRC), ICMR, Bhubaneshwar; and in Vellore (VRE; Tamil Nadu state) through Department of Public Health (DPH), Govt. of Tamilnadu. About 5 mL of venous blood was collected from each mf carrier between 20 h and 22 h. Informed consent was obtained from all the patients before sample collection. Ethylenediamine tetraacetic acid
(EDTA; final concentration 1 mM) or heparin was added to the blood samples to prevent clotting, and the samples were stored at 4–8°C until use.

2.8 MICROFILARIA SEPARATION FROM BLOOD

The mf were separated from blood samples by membrane filtration technique (Dennis and Kean 1971), then pooled and used for genomic DNA extraction. About 5 mL blood of the MF patients was diluted with TBES (TBE diluted with saline) in the ratio 1:10 and passed onto a 0.5-µm filter (Millipore, MA, USA). The filter was then washed separately with TBES and incubated at 37°C and the mf gets released into the solution. The solution was then centrifuged at 4000 rpm for 15 min to pellet out the mf. The supernatant was discarded and the mf was resuspended in fresh 1X Phosphate-Buffered Saline (PBS) (pH 8). About 1 µL of mf suspended in 1X PBS was mounted on a micro-slide and counted by visualizing through microscope. The mf was stored at −20°C until use.

2.9 W. BANCROFTI MICROFILARIA ISOLATED FROM ARCHIVED SLIDES

The mf +ve slides archived were also collected through National Vector Borne Disease Control Programme (NVBCP). W. bancrofti mf is sheathed and its body is gently curved. The tail is tapered to a point with the nuclear column (the cells that constitute the body of the microfilaria) is loosely packed; the nuclei can be visualized individually and do not extend to the tip of the tail. The mf was isolated by the method described Hoti et al (2003). The slides are marked for the presence of the mf and were treated with the lysis buffer. About 20 µL of lysis buffer was added to the regions marked for the presence of mf and incubated at room temperature for 20 min. The mf was released from the blood smear. Freely floating mf are picked with a fine brush under microscope and washed in TE buffer, pH 8, and were used for genomic DNA isolation.
2.10 GENOMIC DNA (gDNA) ISOLATION AND PURIFICATION

A pool of 100 mf from 10 microfilaraemic individuals (10 mf from each) of each region was used for the genomic DNA extraction by the method described by Hoti et al (2003). Individuals having more than 10 mf/60 cm$^3$ of the blood were included in this study. The mf were washed in phosphate-buffered saline (PBS) and digested in homogenization buffer (0.1 M Tris, pH 8.5; 0.05 M EDTA; 0.2 M NaCl; 1% SDS and 2 mg/mL Proteinase K) at 65°C for 30 min with occasional gentle inversion of the tube. The lysate was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform: isoamyl alcohol (24:1) mixtures. After the extraction, 1/10 volume of 8 M LiCl was added to the aqueous layer to remove RNA, and the DNA was precipitated at −20°C overnight after adding sodium acetate at 0.8 M concentration and 2.5 times volume of ethanol. The DNA precipitate was pelleted by centrifugation; the pellet was washed with 70% ethanol and dissolved in TE buffer (pH 8). The DNA was stored at −20°C, and 20 ng of the genomic DNA was used as a template for PCR amplification.

2.11 POLYMERASE CHAIN REACTION

2.11.1 SspI Polymerase Chain Reaction to Check gDNA Purity

A highly sensitive and specific polymerase chain reaction (PCR) assay, based on Ssp I repeat sequences, has been developed for detecting *W. bancrofti* in human blood and in vectors (Zhong et al 1996). The Ssp I repeat sequence primers are F 5′-CGT GAT GGC ATC AAA GTA GCG-3′ and R 5′-CCC TCA CTT ACC ATA AGA CAAC-3′. Each 50-µl PCR mixture contained 200 µM of each dNTP, 2.5 mM MgCl$_2$, 0.5 mM of each primer, 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3) and two units of high-fidelity vent polymerase (NEB, MA, USA). The PCR was carried out in MJ MiniCycler (Bio-Rad, CA, USA) with the following conditions: initial denaturation at 95°C for 5 min with cycling conditions at 95°C for 1 min; 53°C for 1 min and 72°C for 1 min. After 30 cycles, a final extension was
performed at 72°C for 5 min. The amplified products were resolved in 2% agarose gel, stained in ethidium bromide solution and observed under UV trans-illuminator.

2.11.2 Gene-Specific Polymerase Chain Reaction

The initial PCR optimization reaction was carried with *B. malayi* mf gDNA as the template. The PCR cycling conditions and the template concentration was optimized and standardized for all the genes. The same parameters were used to amplify the gene-specific products from *W. bancrofti*. The primers used for amplifying functional genes *WbSXP-1* (GenBank acc. no. AF098861), *WbTPx-1* (GenBank acc. no. AF045165) and *WbCol-4* (GenBank acc. no. DQ641263) are listed in Table 2.1, which also includes vector-specific M13 primers which were used in screening positive clones by lysate PCR. Each 50-µL PCR mixture contained 200 µM of each dNTP, 2.5 mM MgCl₂, 0.5 mM of each primer, 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3) and two units of high fidelity vent polymerase (NEB, MA, USA). The PCR was carried out in MJ MiniCycler (Bio-Rad, CA, USA) and the cycling parameters are given below.

Table 2.1 List of primers used in PCR amplification of functional genes and vectors

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Primer</th>
<th>Sequence of the Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WbSXP-1 F</td>
<td>5'-CGCGGATCCGATGAAATATTITATTITTC-3'</td>
</tr>
<tr>
<td>2</td>
<td>WbSXP-1 R</td>
<td>5'-CCGGAATTCCCTA TTTATTCTTTTTTGT-3'</td>
</tr>
<tr>
<td>3</td>
<td>WbCol F</td>
<td>5'-ATCGGGATCCAAATTTTAGGTGACAAAGGAAAGT-3'</td>
</tr>
<tr>
<td>4</td>
<td>WbCol R</td>
<td>5'-CGCGGAATTCTTATTATGATCGAGAAGCATA-3'</td>
</tr>
<tr>
<td>5</td>
<td>TPX F</td>
<td>5'-ATCGGGATCCAAATTTTAGGTGACAAAGGAAAGT-3'</td>
</tr>
<tr>
<td>6</td>
<td>TPX R</td>
<td>5'-CGCGGAATTCTTATTATGATCGAGAAGCATA-3'</td>
</tr>
<tr>
<td>7</td>
<td>M13 F</td>
<td>5'-GTAAAACGACGGCCAG-3'</td>
</tr>
<tr>
<td>8</td>
<td>M13 R</td>
<td>5'-CAGGAAACAGCTATGAC-3'</td>
</tr>
<tr>
<td>9</td>
<td>T7 F</td>
<td>5'-ATTAACCCTCACTAAAGGGA-3'</td>
</tr>
<tr>
<td>10</td>
<td>T7 R</td>
<td>5'-TAATACGACTCACTATAGGG-3'</td>
</tr>
</tbody>
</table>
PCR conditions used for amplification

i. Sequencing vector-specific PCR cycling parameters

Step 1. Initial denaturation : 95°C, 5 min  
Step 2. Denaturation : 95°C, 1 min  
Step 3. Annealing : 53°C, 1 min  
Step 4. Extension : 72°C, 1 min  
Step 5. Cycling from step 2 to 4 for 30 more times.  
Step 6. Final extension : 72°C, 5 min  
Step 7. End

ii. *WbSXP-1* gene PCR cycling parameters

Step 1. Initial denaturation : 95°C, 5 min  
Step 2. Denaturation : 95°C, 1 min  
Step 3. Annealing : 56°C, 1 min  
Step 4. Extension : 72°C, 1 min  
Step 5. Cycling from step 2 to 4 for 34 more times.  
Step 6. Final extension : 72°C, 10 min  
Step 7. End

iii. *WbCol-4* gene PCR cycling parameters

Step 1. Initial denaturation : 95°C, 5 min  
Step 2. Denaturation : 95°C, 1 min  
Step 3. Annealing : 62°C, 1 min
Step 4. Extension : 72°C, 1 min

Step 5. Cycling from step 2 to 4 for 30 more times.

Step 6. Final extension : 72°C, 10 min

Step 7. End

iv. \textit{WbTPx-1} gene PCR cycling parameters

Step 1. Initial denaturation : 95°C, 8 min

Step 2. Denaturation : 95°C, 1 min

Step 3. Annealing : 60°C, 1 min

Step 4. Extension : 72°C, 1 min

Step 5. Cycling from step 2 to 4 for 34 more times.

Step 6. Final extension : 72°C, 8 min

Step 7. End

v. Lysate PCR with T7-specific primers cycling parameters

Step 1. Initial denaturation : 95°C, 5 min

Step 2. Denaturation : 95°C, 1 min

Step 3. Annealing : 56°C, 1 min

Step 4. Extension : 72°C, 1 min

Step 5. Cycling from step 2 to 4 for 30 more times.

Step 6. Final extension : 72°C, 5 min

Step 7. End
2.12 AGAROSE GEL ELECTROPHORESIS

All the PCR amplicons were separated on horizontal submerged gels at 1 or 1.2% agarose (GE Healthcare, CA, USA) and resolved using TBE buffer of pH 8.3 (98 mM Tris, 89 mM boric acid and 2 mM EDTA) at 5–8 V/cm of the gel (Sambrook et al 1989). The gel loading buffer (Fermentas, MD, USA) was used for loading the amplicons and they were stained with 5 µg/mL of ethidium bromide and viewed under UV trans-illuminator (Bio-Rad, CA, USA). Either 100 bp ladder or 1000 bp ladder (Fermentas, MD, USA) was used as molecular weight markers. The relative amounts of PCR products in the gel were determined by scanning the gel in personal densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

2.13 PCR PRODUCT PURIFICATION FROM AGAROSE GEL

Amplified gene products from various geographical locations were gel purified individually using Qiaquick gel extraction kit (Qiagen, Hilden, Germany) as described below:

**Protocol**

1. The expected amplified gene product was excised using a sterile scalpel blade from the agarose gel.
2. Binding buffer, thrice the weight of the excised gel piece, was added and incubated at 50°C until the gel gets melted completely.
3. Equal volume of isopropanol of the gel weight was added and mixed well.
4. The contents were then transferred to the column and centrifuged at 13,000 rpm for 1 min and the filtrate was discarded.

5. Column was washed with the wash buffer in the ratio 1:4 (wash buffer: alcohol) and centrifuged at 13,000 rpm for 1 min and the filtrate was discarded.

6. The empty column was centrifuged again at 13,000 rpm for 1 min to remove the excess alcohol.

7. The column was then placed in a new collecting tube and 30 µL of sterile water was added and incubated for 1 min and centrifuged at 13,000 rpm for 1 min.

8. The filtrate containing the purified PCR gene product was analyzed in 1.2% agarose gel and quantified.

2.14 CLONING THE PCR PRODUCTS IN SEQUENCING VECTOR

The amplified PCR products have to be confirmed for the presence of the specific genes. The PCR products were not sequenced directly to avoid the PCR-induced errors and to avoid artifact deletions and insertions that normally occur in direct sequencing. The three genes amplified from different geographical locations were purified individually and were cloned separately in the Zero-blunt cloning vector (Appendix 1) for sequencing (Invitrogen, CA, USA). The gene products were ligated with the sequencing vector as follows:

**Ligation mixture used in cloning PCR product**

- Fresh PCR product 0.5–4 µL (~100 ng)
- Salt solution 1 µL
TOPO vector 1 µL
Sterile water add to a final volume
Final volume 6 µL

1. Mix the reaction mixture gently and incubate for 5–15 min at room temp.
2. Add 2 µL of the ligated mixture into the vial of competent *E. coli* cells.
3. Incubate in ice for 5–30 min.
4. Heat shock the cells for 90 s in 42°C.
5. Immediately transfer the tubes into ice.
6. Add 250 µL of room-temperature SOC medium (supplied with the kit).
7. Cap the tubes and incubate at 37°C for 1 h.
8. Spread 10–20 µL of each transformants on prewarmed LB agar plate with ampicillin at final concentration of 60 µg/mL (HiMedia, Mumbai, India) as selective marker and incubate overnight at 37°C.

### 2.15 SCREENING RECOMBINANT CLONES BY LYSATE PCR

For screening the recombinant clones, a small portion of freshly grown transformant-positive 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 min, snap chilling and centrifugation for 5 min, and 1 µL of the supernatant was used as template in PCR (Sambrook et al 1989). Vector-specific M13 forward and M13 reverse primer sites were used
in PCR. A direct analysis of the lysate PCR will reveal the possible presence of the gene insert. The clones were selected based upon the insert site and archived for further analysis.

2.16 GENE SEQUENCE ANALYSIS

Full-length sequencing was done with M13 forward and reverse primers using an Automated DNA sequencer (ABI Prism Applied Biosystems, Foster City, CA, USA). For each geographical region, at least 20 clones were sequenced to exclude the PCR-derived errors and to confirm the variations. The sequences were confirmed by performing a BLAST search (Altschul et al 1990) using online NCBI tools. The sequences having similarity with the submitted reference sequences were further analyzed individually to understand the polymorphism of the appropriate genes. The sequences of each region, viz., Sevagram (SVG), Vellore (VRE), Bhubaneshwar (BHU) and Pondicherry (PDY), were analyzed and compared with reference sequences separately. Sequences were compared by multiple sequence alignment within each region and compared with the known reference sequence, and the percentage similarity or homology is determined. The nucleotide variations observed were translated into the amino acid sequence using online tool Expasy translate (http://www.expasy.ch/tools/dna.html), and the nucleotide variations were studied for the synonymous and non-synonymous substitutions. The amino acid sequences were compared with the reference sequences submitted in the GenBank: *WbSXP-1* (GenBank acc. no. AAC70783), *BmTPx-1* (GenBank acc. no P48822) and *WbCol-4* (GenBank acc. no ABG22161).

2.17 COMPARATIVE GENOMICS

The secondary structure predictions were performed on Lasergene software (DNASTAR Inc, WI, USA) and the probable immunogenic regions
were predicted. The variations observed and the impact of the substitutions were assessed by their influence in the antigenicity and surface probability using DNASTAR prediction. The immunogenic epitopes were predicted and the conservation among the epitopes were studied.

2.18 PLASMID DNA ISOLATION

Freshly grown recombinant clones of WbSXP-1, Wb14 and WbTPx-1 were maintained in E. coli DH5α maintaining host, deficient for T7 RNA polymerase and hence does not express the foreign protein were used for plasmid DNA isolation. All the plasmids were isolated using plasmid mini isolation kit as per the manufacturer’s instructions (Qiagen, Hilden, Germany). The plasmids isolated were estimated and used for further studies.

2.19 RESTRICTION DIGESTION

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

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

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

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.

The recombinant clone \textit{WbSXP-1} was restricted with \textit{BamHI} and \textit{EcoRI}, and \textit{WbTPx-1} was restricted with \textit{BamHI} and \textit{HindIII}.

\subsection*{2.20 CLONING IN T7 EXPRESSION VECTOR}

The positive clones identified from the above were recloned in the T7 expression system pREST A and pRSET B (Studier and Moffatt 1986). \textit{WbSXP-1} and \textit{Wb14} were recloned in pRSET B while, \textit{WbTPx-1} was expressed in pRSET A. The restricted gene product and the appropriate T7 vector were gel purified as described above and ligated using T4 DNA ligase (NEB, USA) in a ratio of 3:1 as per the manufacturer’s recommendation.

\subsection*{2.21 TRANSFORMATION OF \textit{E. coli} WITH PLASMID DNA}

Recombinant plasmids were chemically transformed using CaCl\textsubscript{2} (Sambrook et al 1989) in \textit{E. coli} GJ1158. Competent cells were prepared as stated below:

1. Pre-inoculum from a single colony of freshly revived \textit{E. coli} culture was made in a 3-mL LB broth without NaCl and grown o/n at 37°C and the control was grown with ampicillin (50 \(\mu\)g/mL).
2. 100 \(\mu\)l of overnight culture was inoculated into 50 mL LB medium without NaCl in conical flask and allowed to grow at 37°C till OD\textsubscript{600} reached 0.6.
3. Culture was chilled on ice for 5 min by gentle swirling and centrifuged at 3500 rpm for 10 min at 4°C.
4. The cell pellet was resuspended in 10 mL of 100 mM ice-cold MgCl₂ and incubated on ice for 30 min.

5. Cells were pelleted as in step 3 and the pellet was resuspended in 2 mL of 100 mM ice-cold CaCl₂ and incubated on ice for 1 h.

6. Approximately 10–20 ng of DNA was added to 100 μL of above cells and further incubated for 30 min on ice.

7. A heat shock at 42°C was given for 90 s and chilled again on ice for 10 min.

8. About 400 μL of LB medium without NaCl was added to the tubes and allowed to grow in rotary shaker at 37°C for 45 min.

9. Around 100 μL was plated onto LB agar without NaCl and the plates were supplemented with appropriate antibiotics.

10. A positive control plasmid (10 ng of pRSET B) was used in all the experiments to verify the transformation efficiency. Cells with no DNA added served as negative controls.

2.22 EXPRESSION OF THE RECOMBINANT PROTEINS IN E. COLI

E. coli strains were transformed with WbSXP-1:pRSET B, Wb14:pRSET B and WbTPx-1:pRSET A. Recombinant protein expression was studied after inducing with specific inducers.

1. A single colony of fresh transformant was inoculated into 1.5 mL LB/ON and grown overnight (o/n) at 37°C.

2. About 50 μL of the o/n culture was inoculated into 50 mL LB/ON (LB without NaCl) and grown at 37°C with 150 rpm shaking, until OD₆₀₀ of the culture reached 0.6.
3. The culture was induced with NaCl at a final concentration of 300 mM and grown for 3 h at 37°C with 150 rpm shaking.

4. The culture was centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and *E. coli* pellet containing the recombinant protein was suspended in 1X PBS and analyzed by SDS–PAGE for protein expression.

### 2.23 PROTEIN EXPRESSION ANALYSIS

#### 2.23.1 SDS–polyacrylamide gel electrophoresis

Recombinant proteins extracted from the above clones were analyzed by SDS–PAGE (Laemmli 1970). The gel compositions of the SDS–PAGE are described below:

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) Electrophoresis buffer: 0.025M Tris–Cl, 0.192M glycine, 0.1% SDS, pH 8.3.

v) Ammonium persulphate (APS): 120 mg/mL (12%).

vi) SDS: 10% solution.

vii) TEMED

viii) Sample solubilizing buffer (SSB) (5X): 10% SDS, 10% (v/v) β-mercaptoethanol, 50% sucrose, 0.025% bromophenol blue in stacking gel buffer. 1X SSB was added to the cell pellet and resuspended with appropriate volume of 1X PBS and kept in boiling water bath for 10 min.
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 (Lowry et al 1951) and equal amounts of total protein were loaded in each well. Electrophoresis was performed at room temperature with constant current of 20 mA 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) overnight and destained with 45% methanol, 10% acetic acid solution until a clear background was obtained.

2.23.2 Immunoreactivity of the expressed recombinant proteins

After electrophoresis, the SDS–PAGE gel was transferred for Western blotting as described by Towbin et al (1979). The separating SDS–PAGE gel and nitrocellulose membrane (NC) (HyBond, Amersham Pharmacia, U.K) cut to the exact size of separating gel was incubated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS) for 10 min. The nylon mask was laid in the apparatus to block the extra area of transfer. Without trapping air bubbles, the NC was overlaid on the gel and sandwiched between filter papers and scotch brite pads. Electrophoretic transfer was carried out at 120 mA for 90 min using Hoefer TE 70 semi-dry electroblotting apparatus (Amersham Pharmacia Biotech, U.K). After transfer, the molecular weight marker lane was cut and stained with amido black (100 mg amido black in 45% methanol, 10% acetic acid). The rest of the NC was stained with Ponceau S (0.2% Ponceau S [Sigma, St Louis, USA] in 0.3% trichloroacetic acid and 0.3% sulfosalicylic acid) 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 NC was washed in wash buffer (PBS with 0.05% Tween-20) thrice for 5 min, followed by washing in 1X PBS thrice and then incubated with appropriately diluted primary antibody at room temperature for 1 h. The membrane was washed again as described above and was incubated in recommended dilution of secondary antibody conjugated with alkaline phosphatase for 1 h. After extensive washing, the blot was incubated in detection buffer (100 mM Tris–Cl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) for 10 min. The colour development was achieved using 33 μL of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/mL in dimethyl formamide; USB, Amersham Pharmacia) and 66 μL of nitroblue tetrazolium (50 mg/mL in 70% dimethyl formamide; USB, Amersham Pharmacia) in 10 mL of detection buffer. The reaction was stopped after 15 min by adding 10 mM EDTA.

Primary antibodies, mouse monoclonal anti-His (Sigma, St Louis, USA), 1:3000 diluted in 1X PBS was used in detecting recombinant fusion protein expressed. Various human clinical sera such as microfilareamics (MF), chronic pathology (CP), endemic normals (EN) and non-endemic normals (NEN) in dilutions 1:200 was used in human sera immunoblot analysis. Mouse anti-WbSXP-1 (1:2000), mouse anti-Wb14 (1:2000) and mouse anti-BmTPx-1 (1:5000) were used. Secondary antibodies used were anti-human (Sigma, St. Louis, USA) 1:1000 and anti-mouse (Sigma, St. Louis, USA) 1:1000.

2.24 PROTEIN PURIFICATION OF RECOMBINANT PROTEIN USING IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC)

Immobilized metal affinity chromatography (IMAC) is an excellent method for purifying recombinant protein having poly-histidine fusions. The His-tag have affinity to metal ions such as copper and zinc or transition metal
ions such as cobalt or nickel which binds to protein selectively by reacting with imidazole group of histidine residues (Porath et al 1975). The recombinant proteins expressed were purified as described below.

2.2.4.1 Sample Preparation

The recombinant protein cultures WbSXP-1 and Wb14 were centrifuged and the cell pellet was resuspended at 2.5 g wet weight per mL of lysis buffer comprising 50 mM Na₂HPO₄–NaH₂PO₄, 50 mM Tris, 400 mM NaCl, 10 mM imidazole, pH 8, and 0.3 mg/mL lysozyme. The suspension was incubated at 4°C for 1 h and subjected to five cycles of French pressure (1000 psi) (Janardhan et al 2007). To the cell lysates, 1 mM PMSF and 1 mM β-mercaptoethanol were added and centrifuged at 12000 rpm for 10 min prior to purification. The clear supernatant was loaded in the column for purification. WbTPx-1 cells were disrupted by sonication and centrifuged at 12000 rpm at 4°C for 10 min. The clear supernatant was loaded in the IMAC column for purification.

2.2.4.2 Immobilization of the Nickel to the Matrix

The chelating Sepharose fast-flow matrix (Amersham Pharmacia, U.K) was used in the present study. It has iminodiacetic acid as the chelating ligand, which is coupled to the agarose (Sepharose fast flow) by epoxy coupling method. The column was prepared as per the manufacturer's instructions.

2.2.4.3 Charging the Column

Column was charged by passing the solution of nickel chloride (6%) prepared in distilled water at neutral pH 7. The progress of column charging was monitored by detecting the presence of nickel using sodium carbonate in the fractions collected. After charging, the column was washed
thoroughly with distilled water. This is followed by equilibration of the matrix with the binding buffer (0.1M phosphate buffer pH 8, 0.01M Tris pH 8).

2.24.4 Binding and Elution of Protein from Nickel Immobilized Column

The supernatant containing the solubilized recombinant protein was passed through the column twice and allowed to bind for 2 h. The unbound protein was removed by washing the column with 5–10 column volumes of elution buffer (0.1M phosphate buffer pH 8). The bound recombinant protein was eluted by increasing the imidazole concentration (10–300 mM) in elution buffer. Initially 5–10 column washes was given with each imidazole concentration and the fractions were collected and analyzed for the presence of the protein by SDS–PAGE. The column washes were optimized based upon the purity of the protein eluted. The concentration of the protein was determined (Lowry et al 1951) and stored in aliquots at –20°C, until further use.

2.25 IMMUNOREACTIVITY OF THE ANTIGENS WITH HUMAN CLINICAL SAMPLES

The optimum dilutions for assay reagents were determined by titration, and the blocking/assay conditions were determined by a series of comparative trials. WbSXP-1, Wb14 and WbTPx-1 antigens (100 ng/well) diluted in coating buffer (0.1M carbonate/bicarbonate, pH 9.6) were used to coat the 96-well plates (Nunc Maxisorp, Nalge Nunc International, Denmark) and they were incubated o/n at 4°C. After washing three times with PBS-T, the plates were blocked with 5% BSA (Sigma, St Louis, USA) at 37°C for 1 h. Human clinical sera of various stages, viz., MF, CP, EN and NEN, were diluted in PBS-T (1:200) and added to the wells (100 µL/well), then incubated at 37°C for 1 h. After washing with PBS-T, human anti-IgG alkaline
phosphatase conjugate (Sigma, St Louis, USA), diluted at a final concentration of 1:1000 in PBS-T, was added (100 µL/well) and incubated for 1 h at 37°C. Plates were washed three times with PBS-T and bound conjugate was reacted with pNPP (p-nitrophenyl phosphate, disodium salt) substrate system (Sigma, St Louis, USA). The optical density of the reaction product was read at 405 nm after 30 min.

2.25.1 Estimation of IgG Subclasses

Human antibody isotypes were determined to measure the efficacy of the antigens in eliciting the immune responses. The pattern of IgG subclass elicited by different antigens was measured. The plates were coated with the antigens, and the human clinical samples were used as primary antibody as described above. After washing, mouse anti-human IgG isotypes (IgG1 [1:500], IgG2 [1:2000], IgG3 [1:5000] and IgG4 [1:5000]) 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, mouse anti-human IgG-ALP (Sigma, St Louis, USA) conjugate was added (1:1000 dilution) as secondary antibody and incubated for 1 h at 37°C. Plates were washed three times with PBS-T and bound conjugate was reacted with pNPP (p-nitrophenyl phosphate, disodium salt) substrate system (Sigma, St Louis, USA). The optical density of the reaction product was read at 405 nm after 30 min. The ALP conjugate was used since they are more sensitive than the HRP conjugates.

For isotype ELISA of peptides, wells of polystyrene microtitre plates (Immulon IIHB, Dynatech, ThermoFisher) were coated with 500 ng of peptides in carbonate buffer and incubated at 4°C o/n. Plates were washed with three changes in PBS-T:PBS and blocked with PBS containing 5% BSA in PBS/T for 2 h at 37°C. About 100 µL of 1:100 dilution of sample human
sera in PBS was added in duplicate, and incubated at 37°C for 2 h. After washing respective monoclonal antibodies IgG₁ (1:500), IgG₂ (1:2000), IgG₃ (1:5000), and IgG₄ (1:5000), IgM (1:5000) and IgE (1:5000) (Sigma, St. Louis, USA) were added and incubated at 37°C for 2 h. The plates were washed and incubated with alkaline phosphatase-conjugated anti-mouse IgG for 1 h at 37°C. After washing the plates, 100 µL of substrate pNPP (1 mg/mL) in sodium carbonate substrate buffer (pH 8.6) was added and colour was developed. The reaction was arrested after 30 min by adding 100 µL of 3M NaOH per well and the absorbance was read at 405 nm.

2.26 IMMUNE RESPONSE OF Wb14

Six-week-old inbred BALB/c mice were used for immunization studies. Mice were separated into two groups with five mice in each group. About 100 µg of Wb14 suspended in alum as adjuvant was immunized intraperitonially for one group. The other group was immunized with alum and used as control. All the immunizations were given at 15-day interval. Hyperimmune serum was collected on the day previous to subsequent immunization through tail pricks. Pre-bled collected were check for the immune response.

2.27 COMPARATIVE GENOMIC ANALYSIS OF ANTIGENS

2.27.1 Comparative Analysis of WbSXP-1, Wb14 and WbTPx-1 Antigens

Sequences corresponding to the SXP/Wb14 and WbTPx-1 proteins were analyzed. The most probable immunogenic peptides of the WbSXP-1, WbTPx-1 and Wb14 proteins were analyzed using Lasergene software (DNASTAR Inc, WI, USA). Highly immunogenic epitopes were predicted and compared using the online epitope predictions tools.
http://tools.immuneepitope.org and the most conserved amino acids with high immunogenicity were used for further studies. Synthetic peptides corresponding to the immunodominant epitopes were synthesized and used in further polymorphic analysis. The indel variations were compared and the effect of phenotypic variations were analyzed.

2.28 SYNTHESIS OF PEPTIDES

All the peptides corresponding to the B-cell epitope were derived from the amino acid sequence of WbSXP-1 and were synthesized using Fmoc chemistry and assembled on Wang resin. After cleavage and purification, all the peptides were authenticated by physico-chemical methods and further purity was checked by high-performance liquid chromatography and amino acid analysis. The linear epitopic peptides N: RADESKTDPQTEA, N1: GGVYQARFEQFKQEMKKQFAQYDKV, N2: IAESKQLTVKQKTEQIK, and N3: FSKWRKNHMRQKSNK, the conjugate or chimeric peptides N:N1–RADESKTDPQTEAGGYQARFEQFKQEMKKQFAQYDKVH, N1:N2–GGVYQARFEQFKQEMKKQFAQYDKVHIAESKQLTVKQKTEQIK, and C-terminal 29 amino acid extension peptide Ext: FCVISVLLNVTIKIFSKWRKNHMRQKSNK were synthesized. The other peptides corresponding to the B-cell epitopes of Wb14, which had substitutions with reference to SXP, are named by their position as P1:GGVYQARFEQFKQEMKKQKAQYDKVH and P2: IAESKQLTGKQKTEQIK. Since Wb14 lacks the 29 amino acid extension in C-terminal region, only these two peptides were synthesized.

2.29 DIRECT BINDING ASSAY

Direct binding assay was done to check the reactivity of WbSXP-1 and Wb14 antisera with different peptides [N, N1, N2 and N3] corresponding to the WbSXP-1 epitopes and [P1, P2] representing Wb14 peptides. The
plates were coated with peptide (500 ng/well) in high-binding 96-well titre plates (Thermo Scientific, MA, USA) and incubated o/n at 4°C. The plates were washed with PBS-T followed by PBS and blocked in 3% skimmed milk powder as described above. The anti-WbSXP-1 and anti-Wb14 sera raised in mice were diluted in 1% skimmed milk powder (1:1000) and incubated at 37°C for 2 h. After washing as described above, anti-mouse IgG-ALP (1:1000) (Sigma, St Louis, USA) was added and kept at 37°C for 1 h, washed and reacted with pNPP (p-nitrophenyl phosphate, disodium salt) substrate system (Sigma, St Louis, USA). The optical density of the reaction product was read at 405 nm after 30 min.

2.30 IMMUNODIAGNOSTIC EFFICACY OF THE PEPTIDES

The immunodiagnostic efficacy of the peptides synthesized was evaluated by ELISA. In preliminary experiments, the amount of antigen was optimized by running a checkerboard ELISA on a 96-microtiter plate, using 2-fold dilutions of antigen in each row and 2-fold dilutions of a positive control serum in each column. About 100–1000 ng of the peptides diluted in coating buffer (0.1M carbonate/bicarbonate, pH 9.6) were coated in 96-well microtitre plates (Thermo Scientific, MA, USA) and incubated o/n at 4°C. Non-specific binding sites were blocked with 5% milk powder at 37°C for 1 h and washed thrice with PBS-T followed by PBS. The primary sera were diluted in 1% skimmed milk powder and incubated at 37°C for 2 h. About 100 μL of the primary sera of either mouse anti-SXP-1 (1:1000), mouse anti-Wb14 (1:1000) or human clinical samples MF (1:500), EN (1:500), CP (1:500) and NEN (1:500) were used. The plates were briefly washed as described above and incubated with 100 μL anti-mouse or anti-human secondary antibody (1:1000) ALP conjugated (Sigma, St Louis, USA) at 37°C for 1 h. Plates were washed thrice with PBS-T and bound conjugate was reacted with pNPP (p-nitrophenyl phosphate, disodium salt) substrate system
(Sigma, St Louis, USA). The optical density of the reaction product was read at 405 nm after 30 min.

2.31 STATISTICAL ANALYSIS

All statistical analyses were done using Graphpad prism software version 5.0 and SPSS software version 13. The difference in two means was compared using non-parametrical analysis of Student’s t-test using Graphpad prism software. The comparison of more than two means was done using post-hoc one-way ANOVA tests. The probability (P) value ≤0.05 was considered as significant.