CHAPTER 2

Identification, molecular cloning and characterization of B. malayi RNA Helicase
Introduction

Strategies to identify candidate vaccine antigens against filariasis have relied mainly on screening expression libraries with the sera of the putatively immune population (Freedman et al, 1989), differential screening of abundantly expressed mRNAs (Werner et al, 1989; Gregory et al, 2000), expressed sequence tags (EST) approach (Blaxter et al, 1996; Williams et al, 2000) and phage display method (Gnanasekar et al, 2004). Using these approaches several potential vaccine candidates have been identified and reported to have varying degrees of protection in animal models. Although there are effective drugs available for the control of filariasis, developing a vaccine remains a promising strategy for mass control of this mosquito-borne infection in areas of endemicity. Immunoscreening of cDNA library has been the most preferred choice to pick up desired gene from a pool of genome. Life stage libraries derived from various filarial parasite species have been screened with the sera from infected human or animals or even animals immunized with filarial antigens or live parasites. Other than vaccine candidates there is also an urgent need to identify and characterize novel drug targets and use them in discovery of better filaricidal drugs.

Irradiated infective larvae have been reported to offer strong resistance to larval challenge in various animal models (Lucius et al, 1991). Sera of putatively immune endemic normal patients differentially recognize antigen/s of L₃ stage of B. malayi (Freedman et al, 1989; Nutman et al, 1991). Therefore the immunoscreening of cDNA expression library of infective larval stage would be an ideal choice for identifying and characterizing immunodominant proteins and/or metabolic regulatory enzymes to further assess their role in development of protective immunity and host-parasite biology.

Since human filarial parasitic material is not available in sufficient amount, the construction of cDNA expression libraries and molecular cloning approaches are important methods for isolating and characterizing metabolic regulatory enzymes and protein antigens. High quality cDNA libraries covering the full diversity of the life-cycle of the parasite have been constructed to advance and facilitate molecular studies on human lymphatic filarial parasites. Several group have generated cDNA libraries from specific life-cycle stages of filarial nematodes by reverse transcription of RNA followed by polymerase chain reaction (RT-PCR) using the conserved nematode spliced leader (SL1) and oligo (dT) as primers (Seeber et al, 1993; Martin, 1995; Devaney et al, 1996; Blaxter et al, 1996). More than 20 new high quality stage-specific cDNA libraries of B. malayi are available from most of the transition
stages (Yenbutr and Scott, 1995; Blaxter et al, 1996) which have been distributed to over 100 research laboratories worldwide. So far more than 26000 expressed sequence tags (ESTs), derived from these cDNA libraries representative of the significant Brugia life-cycle stages (Williams et al, 2000; Parkinson et al, 2004; Williams, 2004) have been sequenced and submitted to dbEST, of which half are similar to genes already identified in other organisms and their functions can therefore be predicted, rest are unique to B. malayi and thus may be useful in the search for new targets for the development of drugs, vaccine and diagnostic tests. Some are sex-specific genes and some are active only at certain developmental stages of B. malayi. Stage-specific and transition stage libraries may be important in developing new ways to block the development of filarial parasites. The recombinant proteins identified by immunoscreening and tested for protective efficacy against filarial infections have not so far induced significant degree of immunity to challenge infection (Li et al, 1999; Peralta et al, 1999). There is still a need to identify more functional proteins/enzymes that can be used as vaccine candidate, drug-target or diagnostic proteins.

Material and Methods

1. Raising antiserum to irradiated L3

Aedes aegypti mosquitoes were fed on B. malayi infected microfilaraemic mastomys. Infecitve larvae were recovered from the gently crushed mosquitoes on day 9 ± 1 of infective feeding (described in detail in chapter 1). The larvae were cleaned in Ringer’s solution, exposed to Cobalt\(^{60}\) irradiation at a dose of 25 krad and inoculated subcutaneously to mastomys (\(~100\) L3 each) on three occasions at four week interval (Weil et al, 1992) and the sera of animals were checked for the development of high levels of anti-L3 antibodies.

2. Antibody measurement by Enzyme-linked Immunosorbent Assay (ELISA)

The blood was collected just before the start of immunization and on day 7 of each immunizing dose. Serum antibody titre was assessed by ELISA using soluble somatic antigen of L3. ELISA strips (Nunc, Denmark) were coated overnight with the L3 antigen (1 \(\mu\)g/ml) in PBS (1x) at 4\(^\circ\)C. The strips were blocked with 1% gelatin in 1x PBS+Tween-20 (PBST) for 2 h at room temperature (RT). After washing the wells thrice with PBST, mastomys serum in the form of primary antibody raised against irradiated L3 was added at serial two-fold dilutions and kept for 90 min at RT. The pre-immunized serum was added in the control antigen coated strips in the same manner. Washing of wells was done with PBST thrice and goat anti mouse-IgG-HRP (Sigma, USA) at 1: 10000 dilution (in 1% gelatin in PBS) was
added to each well as secondary antibody and incubated for 90 min. at RT. Plates were washed again in the same manner and finally developed with buffer containing 12 mg of substrate Orthophenyalamine (OPD, Sigma) and 10 μl H₂O₂ in PBS. The reaction was stopped with 2.5 N H₂SO₄. Absorbance was read at 492 nm in multiplate reader (Infinite M200, Tecan, Switzerland) to determine the IgG antibody titre at different time points.

3. Screening of L₃ cDNA expression library

*B. malayi* L₃ cDNA library (SAW94WL-BmL3 library) constructed in the lambda UniZap XR vector (Stratagene) and was received as a kind gift from Prof. S.A. Williams, Smith College, Northampton, Massachusetts, USA. The protocol of Stratagene and Maniatis (Sambrook et al; 1998) was used with some modifications for screening of library. One loop of stock *E. coli* bacteria (XL-1 Blue MRF’ strain) was inoculated to 5 ml of 2.2% NZCYM medium (37°C, 224 rpm) overnight (O/N). Cultured bacteria was sub-cultured into 20 ml of fresh 2.2% NZCYM medium containing 15 μl of 20% maltose and incubated till OD₆₀₀=0.5. The bacterial culture was pelleted at 3,000 rpm for 20 min at RT. Pellet was suspended in 2 ml of 10 mM MgSO₄ and kept in ice till use. For screening, 2μl of stock cDNA library was diluted to 10⁻⁴ and kept in ice till use. 30 μl of this diluted library was mixed in 300 μl of grown XL1 Blue bacterial culture and incubated in a water bath at 37°C for 20 min for infection of bacteriophage. 8 ml of top agar heated at 50°C was mixed with bacteriophage infected bacterial cells. This mixture was overlayed slowly into the bactoagar plates uniformly and plates kept till solidification. The plates were then kept inverted in an incubator at 42°C for 3-4 hrs till clear plaques appear. Nitrocellulose filter discs (Schleicher and Schull) soaked in 10 mM IPTG and dried were laid down onto the bactoagar plates containing visible plaques. Plates were incubated at 37°C O/N in an inverted position. Plates were taken out and kept at 4°C for 2 hrs in order to avoid sticking of agar to filter discs. Plates were asymmetrically marked for identification by using waterproof ink after incubation. Filters were carefully peeled off from the plates and immediately kept in washing buffer (1XPBS) while plates were stored at 4°C till use. Blocking was done with 10% skimmed milk in PBS for 4 hrs on a moving platform. Each membrane was reacted with mastomys hyper immune serum (1:100) in 1% blocking buffer and incubated O/N at 4°C on a moving platform. Membrane was reacted with anti-mouse IgG-HRP labelled secondary antibody (1:1000) and agitated slowly on rotating platform for 2 h followed by washing with PBS. Filters were developed in DAB solution (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 7.5 mg DAB (Sigma), 1.5μl H₂O₂) till the signals appeared as dark brown spots on the membrane.
surface. The discs were matched with master plate in right orientation to pick up positive plaques from the plate and suspended individually in 1 ml of SM buffer. Few drops of chloroform added to the tubes and stored at 4°C for secondary screening. Secondary and tertiary screenings were carried out in the same manner to purify the positive plaques. In-vivo excision of purified recombinant pBluescript phagemid from λ UniZap vector was carried out with the help of ExAssist helper phage (Stratagen). XL-1 Blue MRF bacterial cells suspended in MgSO₄ (as described above) and O/N grown SOLR cells in LB broth (Stratagene) with OD₆₀₀= 1.0 were used for excision procedure. Briefly, 100 µl of XL1 Blue was mixed with 15 µl lysate, 5µl of ExAssist helper phage and incubated at 37°C for 20 min in a water bath. 2 ml of L.B. broth was added to this tube and culture allowed to grow for next 3.5 hrs at 37°C, 224 rpm. The grown culture was heated at 70°C for 20 min in a water bath and centrifuged at 10,000 rpm for 10 min at RT and supernatant containing phagemid was transferred to a fresh tube. For transformation in to SOLR cells, 200 µl of previously grown culture of SOLR cells was mixed with 100 µl of phagemid and incubated at 37°C for 20 min in a water bath and the mixture was spread on the LB ampicilin (LB amp+) plate uniformly. Plates were incubated overnight at 37°C to obtain positive colonies. Plasmid was isolated from the positive colonies by kit as per manufacturer’s protocol. The size of the B. malayi cDNA inserts was determined by double digestion with Eco RI and Xho I restriction enzymes. Partial nucleotide sequencing of the 5' and 3' ends of the inserts using T3 and T7 universal primers was performed at the UDSC Department of Biochemistry, University of Delhi, South campus, New Delhi, India using automatic sequencer ABI Prism (Version 3.0. Model 3100).

4. DNA sequence analysis

The open reading frame (ORF) of cDNA clones was deduced using ORF Finder at National Centre for Biotechnology Information (NCBI; Bethesda, Md). Sequences were compared with nucleotide and protein sequences available in non-redundant databases and B. malayi draft genome using NCBI basic local alignment search tool BLAST 2. Comparison with expressed sequence tag (EST) sequences was performed using tBLASTn (NCBI) and NemaBLAST (Washington University BLAST, version 2) and multiple sequence alignment by CLUSTAL-W algorithm.
Of the 5 cDNA clones picked up, one was found to code for an interesting novel protein (~1.16 Kb) of *Brugia malayi* i.e. RNA Helicase, designated as BmL3-Helicase and therefore taken up for detailed characterization.

5. Sub-cloning of BmL3-Helicase cDNA clone into pET28a expression vector

5.1 Preparation of competent cells of DH5α and BL-21(DE3)

5.0 ml Luria Bertani (LB) broth (pH- 7.5; Hi Media, India) was inoculated with one loop of DH5α strain (stored at -70°C) and grown overnight (O/N) at 200 rpm in a shaker incubator (HT Infors, Bottmingem, UK) at 37°C. 150 μl from this grown culture was inoculated in to 25 ml of fresh LB broth and grown further till OD_{600} reached 0.4. Cells were centrifuged at 5,000 rpm for 7 min at 4°C, pellet suspended in 10 ml of solution I (0.1M CaCl2), incubated for 1 h in ice and further centrifuged at 4,000 rpm for 5 min at 4°C. The pellet was resuspended in 2 ml of solution II (0.1M CaCl2; 15% glycerol) and 0.2 ml aliquots were made and stored at -70°C for later use. Competent cells of BL-21(DE3) were also prepared in the same manner.

5.2 Plasmid isolation from transformed SOLR cells

A single colony from the transformed plate was inoculated in 3 ml of LB broth containing 3 μl of ampicillin (100 μg/ml). The culture was grown at 200 rpm at 37°C, O/N in a shaker incubator and the plasmid was isolated using QIAprep Spin Miniprep kit (Qiagen, Germany) as per manufacturer’s protocol. Briefly, bacterial culture was pelleted in a micro-centrifuge tube at 6000 x g for 15 min at 4°C and vortexed in 250 μl resuspension buffer (P1). Homogenization buffer (P2; 250μl) was added and mixed gently followed by addition of 250 μl lysis buffer (N3) for cell lysis. Tube was left for 2 min followed by centrifugation at 17,000 x g for 10 min. Supernatant was transferred to a column containing collection tube and centrifuged at 17,000 x g for 1 min. to discard the supernatant and 0.5 ml buffer PB (Plasmid Binding) was added to column followed by re-centrifugation at 17000 x g for 1 min and supernatant again discarded. Column was then washed with 0.750 ml buffer PE (Plasmid Elution) and centrifuged to discard supernatant and tube was re-centrifuged for removal of residual wash buffer. Elution was done by adding 50 μl buffer EB (Elution Buffer) or deionized water to the center of the spin column to collect supernatant containing plasmid in a fresh centrifuge tube. Plasmid (1.0 μl) was electrophoresed on 1% agarose gel containing ethidium bromide (Sigma, USA; 0.5 μg/ml), visualized under UV light (Trans illuminator
Bioview, Germany) and photographed. The remaining plasmid was stored at 4°C for further use.

5.3 Transformation of recombinant pBluescript plasmid into DH5α cells
200 µl of DH5α competent cells were mixed with 5 µl of recombinant plasmid (BmL3-Helicase+pBluescript) and kept on ice for 15 min. Heat shock was given at 42°C for 90 seconds in water bath. 800 µl of fresh LB broth was added and cells were further grown for 1 h at 37°C at 200 rpm. 100 µl of this culture was spread on LB ampicillin (LB Amp+) plate with sterile glass spreader and the plate was incubated at 37°C, O/N in an incubator to look for the positive colonies appearing on the plate the next day.

5.4 Isolation of the pBluescript plasmid (BmL3-Helicase) and pET28a plasmid from DH5α
A loop of transformed DH5α carrying the pBluescript plasmid (stored at -70°C) was inoculated in to 5 ml of LB containing 5 µl ampicillin (100 µg/ml) and grown O/N at 200 rpm at 37°C. Similarly, a loop from the glycerol stock of DH5α carrying pET 28a was inoculated in 5 ml of LB containing 50 µg/ml kanamycin. Both the tubes were incubated O/N at 37°C at 200 rpm. Plasmid was isolated as described above, double digested with Eco RI and Xho I and run on 1% agarose gel.

5.5 Ligation of BmL3-Helicase cDNA with pET28a expression vector
For the purpose of directional cloning of B. malayi cDNA insert (BmL3-Helicase) in E. coli expression vector, pET 28a and pBluescript (containing insert BmL3-Helicase) were digested with Eco RI and Xho I. Digested DNA fragments (vector and insert) were resolved on 1% agarose gel. The DNA bands of vector and insert were cut from the agarose gel and purified by Gel Elution kit (Qiagen) following manufacturer’s instructions. In brief, the individual gel pieces were heat treated and loaded on tube columns to collect DNA which was washed and eluted. The purified vector and DNA insert, in an optimized molar ratio of 1:3, were subjected to ligation in a total volume of 10 µl containing 1 unit of T4 DNA ligase in 1x T4 DNA ligase buffer (Invitrogen) at 16°C for 16 h. The ligated plasmid was freshly transformed into competent DH5α cells (stored at -70°C) and the recombinant colonies were selected on LB kanamycin (LB Kan+) plates.
5.6 Selection of the positive colonies

Colonies appearing on the LB agar plate were screened to identify recombinant colonies. Briefly, single colony from LB agar plate was inoculated in 5 ml LB broth and grown at 37°C, with O/N shaking at 200 rpm. Plasmids were isolated from the grown culture by QIAprep Spin Miniprep kit and cloning of ~1.16 Kb cDNA insert (BmL3-Helicase) was confirmed by restriction digestion of the plasmids with Eco RI and Xho I.

5.7 Transformation of BmL3-Helicase+pET 28a construct into BL21 (DE3) and DH5α E. coli cells

Expression vector pET28a was procured from Novagen, USA, and the plasmid was transformed into DH5α and BL21 (DE3) cells to collect the plasmid in bulk for cloning purpose. For transformation, 5.0 μl of pET28a plasmid was added to 200 μl of competent DH5α cells and the mixture was kept in ice for 15 min. A heat shock was given at 42°C for 90 seconds to these cells in a water bath. 800 μl fresh LB broth was added to the vial and incubated at 37°C in a shaker incubator, at 225 rpm for one hour. 100 μl of transformed culture was spread on LB Kan+ plates which were then kept for O/N incubation at 37°C in incubator. Two to three O/N grown colonies were picked up and looked for the presence of pET 28a plasmid after isolating the plasmid by QIAprep Spin Miniprep kit. 25% glycerol stock was prepared from a single colony carrying transformed plasmid and stored at -70°C for further use.

6. Over-expression and purification of recombinant BmL3-Helicase

6.1 Expression and localization studies on BmL3-Helicase in BL21 (DE3) cells

The E. coli strain BL21 (DE3) was used for expression of recombinant BmL3-Helicase. The BL21 (DE3) competent cells were transformed with BmL3-Helicase-pET28a+ plasmid and selected on LB kan+ plate. The transformed single colonies were inoculated into 5 ml culture and grown at 37°C at 220 rpm. Cultures in logarithmic phase (OD600=0.5–0.6) were induced for 3 h with 1.0, 0.5 and 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG), harvested by centrifugation at 7000 rpm for 5 min and lysed in 5 ml sample buffer (0.313 M Tris–HCl, pH 6.8, 50% glycerol, 10% SDS, and 0.05% bromophenol blue) for further analysis on 10% SDS–PAGE (Laemmli et al, 1970) along with uninduced vector control culture. To observe the solubility of recombinant protein, the cell pellet was resuspended in
1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5; 200 mM NaCl; 100 mM DTT), sonicated at 10 dB/10s in a Soniprep150 sonicator in cold. The cell lysate was centrifuged at 14,000 rpm for 30 min. to collect the supernatant containing soluble fraction while the remaining pellet containing insoluble fraction was resuspended in 1 ml of lysis buffer. Soluble and insoluble fractions were then analyzed in parallel on 10% SDS-PAGE and visualized by Coomassie blue staining to observe the solubility of recombinant protein.

6.2 Western blotting of over-expressed BmL3-Helicase with anti-His antibody

To detect the hexa-His tag in recombinant BmL3-Helicase protein, E. coli lysates were resolved on 10% SDS-PAGE. The resolved proteins were electro-blotted onto nitrocellulose membrane (Schleicher & Schuell, Pore size 0.45 μm) at 100 volts for 2 h in transfer buffer using wet transfer method of Towbin (1979) as described earlier (Verma et al, 2008). Membrane was first blocked at 4°C O/N with 5% skimmed milk in PBS and incubated with mouse anti-His IgG antibody (Invitrogen) at a dilution of 1:1000 in blocking solution (5% skimmed milk in PBS) for 2 h at 37°C. Primary antibody was removed and 4 washings were given in PBS-T to re-incubate the membrane with secondary antibody i.e. goat anti-mouse IgG (Sigma, USA) coupled to horseradish peroxidase (HRP) at a dilution of 1:1000 in blocking buffer for 1 h at 37°C. Membrane was washed thrice with PBS-T followed by development in 20 ml PBS containing 8.8 mM H2O2 and 3 mg DAB (Sigma, USA) till bands of desired intensity appeared (approximately 2-3 min). The membrane was washed with tap water, dried and photographed.

6.3 Purification of recombinant BmL3-Helicase

The soluble fraction containing adequate amount of recombinant protein tagged to hexa-His was purified under non-denaturing conditions by affinity chromatography using Ni-nitrilotriacetic acid resin (Ni-NTA; Qiagen, Germany). Column was equilibrated with 60 mL of binding buffer containing 10 mM imidazole. The cell lysate was applied to the column and allowed to bind. The bound protein was eluted by applying various concentrations (10-250 mM) of imidazole containing 10% glycerol. Fractions were collected and presence of recombinant protein in the eluted fraction was confirmed by loading on 10% SDS-PAGE. The fractions containing single band of recombinant protein were pooled, dialyzed (50 mM Tris-HCl, pH 7.5; 50 mM NaCl) for 12 h and concentrated to 1 ml volume using Amicon ultra filters (Millipore, USA) with 33 kDa cut off.
protein concentration was determined by Bradford’s method using bovine serum albumin as standard (Bradford, 1976).

7. Reactivity of BmL3-Helicase with human bancroftian sera
Reactivity of antibodies present in the sera of *B. malayi* infected animals and human subjects with the parasite recombinant Helicase was observed in blots. For bancroftian serum samples, blood was collected from *W. bancrofti* endemic area in the outskirts of Lucknow, India and was categorized as endemic normal, asymptomatic microfilaria carriers, microfilaraemic symptomatic and amicrofilaraemic symptomatic. Sera from humans living in filaria free zone like Jammu and Kashmir, India served as non-endemic control. Microfilarial presence or absence was determined in 2 ml night blood by membrane filtration technique (Singh et al, 1997). Endemic normals remained microfilaria negative and free from any clinical filarial symptom when followed for five consecutive years. Purified recombinant protein along with prestained molecular weight marker was run on a preparative 10% SDS-PAGE, transferred to nitrocellulose membrane and processed for immune-recognition with human (1:200) and rodent (1:400) sera pools from 10 subjects of each category. Rodent sera (10 pooled) were obtained from *B. malayi* infected and normal uninfected mastomys. HRP-conjugated goat anti-human and anti-mouse IgG (1:10,000 dilutions each) were used as secondary antibodies and reaction was developed by DAB as mentioned earlier.

8. Immunization of BALB/c mice with recombinant BmL3-Helicase for production of polyclonal antibodies
Male BALB/c mice weighing 10-15 g maintained in CDRI animal house facility were used for immunization with BmL3-Helicase. A group of ten BALB/c mice were vaccinated subcutaneously with the recombinant protein (25 µg/animal) in three doses, first dose with FCA and two booster doses with FIA. Control group received equivalent volume of adjuvant alone. The animals were euthanized by terminal anaesthesia 1 week after the last injection. Blood for isolation of pre-immunized serum was collected from the retro-orbital plexus before immunization and also on day 7 of last immune booster (the day of autopsy). Serum antibody titration was done by ELISA as described above using L3 soluble somatic antigen prepared as mentioned in chapter 1.
9. Stage-specific expression of BmL3-Helicase in *B. malayi*

Presence of BmL3-Helicase was assayed in the various life cycle stages (mf, L₃, adult male, adult female) of *B. malayi* which were isolated from vertebrate or vector hosts (described in detail in chapter 1). A 10 µg protein of each life-stage antigen in sample buffer was resolved on 10% SDS-PAGE, transferred onto nitrocellulose membranes and probed with mouse anti-BmL3-Helicase serum (1:200 dilutions) for 90 min at R/T. After washing the membrane three times with Tris buffer saline (TBS) containing 0.05% Tween-20, HRP-labelled goat anti-mouse IgG (Sigma, USA) was added at 1:1000 dilutions and membrane incubated for further 90 min. The membrane was developed using 3'-3' diaminobenzidine (DAB) and H₂O₂. Purified BmL3-Helicase was used as a positive control.

10. ATPase Assay

10.1 RNA and DNA substrates for ATPase assay
Randomly synthesized dsRNA containing 26 random nucleotides (without any overhangs) (Qiagen) and 1 µg of Yeast total RNA as ssRNA were used as substrates. pBluescript vector linearized with *Eco RI* was used as a dsDNA substrate. 22 nucleotide sequence obtained from Sigma was used as a substrate for ssDNA.

10.2 ATP Hydrolysis assay
To determine the enzymatic activities of the bacterially expressed recombinant BmL3-Helicase, we examined the ATPase activity of the recombinant enzyme by measuring the released inorganic phosphate during ATP hydrolysis using a direct colorimetric assay (Chan et al, 1986; Pugh et al, 1999; Youliang and Zhi-Ren, 2002) with some modifications. The method is based on the quantification of blue-green complex formed between malachite green (MG) molybdate and free phosphate at 630 nm. A typical ATPase assay was carried out in 50 µl reaction volumes, containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM MgCl₂, 5 mM DTT, 1-2 µg of appropriate RNA, 40 units of RNase A, 4 mM NTP, and 10 µl of recombinant helicase. The ATPase reactions were incubated at 37°C for 30 min. and 20 µl of malachite green-molybdenum reagent (prepared freshly) was added and reactions were further incubated at R/T for 5 min. The absorption (A) at 630 nm was then measured on a microplate reader (Infinite M200, Tecan, Switzerland) and the concentrations of inorganic phosphate were determined by matching the A₆₃₀ nm in a standard curve of A₆₃₀ nm *versus* known standard phosphate concentrations.
Results

1. **Immunoscreening of larval stage cDNA Library**

About $2 \times 10^5$ recombinant phages were screened with the *B. malayi* L$_3$ resistant mouse polyclonal serum. Five strongly positive clones were picked up and purified by secondary and tertiary screening (Fig 1). In-vivo excision of each purified clone in pBluescript phagemid from λ UniZap vector was performed with the help of ExAssist helper phage (Stratagen). The excised clones were transformed into SOLR strain of *E. coli* cells. Positive colonies on LB amp$^+$ plates were grown in LB broth for plasmid isolation. The insert size of the clones was determined by double digestion of plasmid with EcoRI and XhoI. Sequencing and homology matching (using BLASTx at NCBI) of the five immunodominant clones led to selection of only one cDNA clone having an insert of 1.16 kb (Fig 2) as it was a novel and unreported gene. This cDNA sequence indicated similarity with DEAD/DEAH box Helicase family protein of *B. malayi* (GenBank accession no. EDP36872), *Caenorhabditis briggsae* (GenBank accession no. CAE62876) and *C. elegans* (GenBank accession no. AAB71200) giving a very significant e-value of $4 \times 10^{-97}$. The homology is schematically represented in Fig. 3. The amino acid sequence of this cDNA clone contained DEAD box conserved domain which are present in almost all Helicase super-family proteins.

2. **Deduced Nucleotide sequence of BmL3-Helicase clone (1.16 kb)**

S'GGCACGAGATTTCGGTGTGATTCCATCATGGTAACAAAGAAAAAAGAAATGATAAAACGAAATAAATTGGATGAAACGACAAATTTGGGACGTGAAGATGAAAGTGATGAACGTGGTTTAATAGAGAAAGCTGCTTCGAATAAGTGCGAAGAAGTGACCAAATCTTCTTTGCAAAAACATAAACGGAAAAAATCTAAACAATACGTTGCTCAGCCATCAGCATGTTTTGAAAATGTAGAGTTGGACACAGATGTAGAATCAGTAATGACTTTCAAAGATTTTGGTCTGGATGAACAATTGTTAAAAGTTATTGGTGAGTTTGGATGGGAGCGTCCGACATTGATTCAGAGCCGAATGATTCCAACTGCTTTTGAAAATAAGAATATTCTTGCACGAGCACGAACGGGCTAGGGCAAAAACACGCCGCACTTATGCTTCTCCTGCTGACAAAAAAGTTTTTCGCAACGTGAATATTCTTCAAGTAACGGAGATGCTCCGATTTTTTCCCGCTCTTCCTAAGAGAGCTGCCAACACAGACTTTATTCATGTGCTATGCAAATTAACAGAGAATATCCTGGCTTTAATTGCGGTGGGTGGTACCTTCTGATGAGAAATGGCCAGCTGATCAGAGATGAGGCTGATAAAAAATTTTTTCCCAGCACACCAGCAAAATTTACTCAATTCCACCAAAATACGACAGCTACTTGTGCACGAGCAGAACAGCATATGAAATCAGAGAAGGACAGCTACCGAGTACAACAGCTTCTTCACAGTACATATTTTGTCCACACGAGACCGTTTTGCAACGTGGAATGGAATTTGATATGATATCAGGCGAATCGCAAGCAGTGATGAAAGATGAAGATGATGAAGATATATCTAAAAAGAAAAAAAAAA

AAAAAAAAA3'
3. Expression and Localization of recombinant BmL3-Helicase

The cDNA of BmL3-Helicase was found to be in frame with pET28a (Novagen) bacterial expression vector producing hexa-Histidine tag fusion proteins. The BmL3-Helicase consisted of an open reading frame of 1160 nucleotides predicted to encode 386 amino acids encoding about 42-kDa protein. This construct was transformed into E. coli BL-21(DE3) host cells, which provide phage T7 RNA polymerase for expression of heterologous genes producing a single fusion protein with a molecular mass of ~50 kDa. Various conditions such as temperature, IPTG concentration and different E. coli host cells were tried so as to obtain maximal expression of recombinant protein. BmL3-Helicase was found to be optimally expressed at 37°C for 3 h after induction with 0.5 mM IPTG (Fig 4 [A]). It was also ascertained that the majority of recombinant protein expressed was present in the soluble fraction by using various host cells (Fig 4 [B]). The expressed protein was purified using Ni-NTA affinity column and eluted to obtain single band native protein as shown in Fig 4 [C]. The expression of recombinant protein was also confirmed in western blot after reaction with anti-His antibody (Fig 5). A single band of ~50 kDa was recognized in blot with anti-His antibody thus confirming the expression of a single protein by the insert-vector construct.

4. Cross-reactivity with human bancroftian sera

Western blot analysis was done with the lysate of transformed BL21 (DE3) induced with 0.5 mM IPTG at 37°C for 3 h. The strong reactivity of purified BmL3-Helicase could be observed with the antibodies present in the pooled human bancroftian sera of various disease categories from endemic area along with sera from B. malayi infected mastomys. Sera of humans from filaria-free zone or from uninfected mastomys did not react with the protein as shown in Fig 6.

5. Stage-specific expression of BmL3-Helicase in different life-cycle stages of B. malayi

Immunoblot analysis of the extracts of L3, microfilariae and adult male and female parasite stages revealed that the Helicase content was high in microfilariae, L3 and both the sexes of adult B. malayi (Fig. 7). These findings thus suggest that BmL3-Helicase is highly expressed by all the major life-stages of B. malayi used in the study.

6. ATPase assay of BmL3-Helicase

The ATPase assays used in the present study demonstrated that the recombinant protein hydrolyzed ATP in a polynucleotide-dependent manner (Fig. 8 [A]). As a control, no ATP
hydrolysis was observed in the same assay using BSA. Our experiments also showed that the BmL3-Helicase enzyme also hydrolyzed dNTPs apart from ATP. The enzyme led to increased hydrolysis of dATP and dTTP as compared to dCTP and dGTP. To examine the nucleic acid-dependent ATPase activity of the bacterially expressed recombinant BmL3-Helicase, we carried out ATPase assays in the presence of ssRNA, dsRNA (Qiagen), dsDNA (pBluescript, linearized with EcoRI) or ssDNA (Oligonucleotide of 22bp). ATPase activity of the enzyme was markedly triggered by both the ssRNA and the dsRNA, however, to much less extent by the ssDNA and dsDNA (Fig. 8 [B]). The ATPase activity of the protein was almost doubled in the presence of the dsRNA as compared to that in the presence of the same molar amounts of the ssRNA. Thus these findings indicate that ATPase activity of the recombinant BmL3-Helicase is strongly stimulated by dsRNA.

Discussion
Immunoscreening of cDNA expression libraries and phage display libraries using patients' sera especially from endemic normal or monoclonal antibodies (Gnanasekar, 2004) has remained one of the most sought after approaches to search for filarial vaccine candidates/drug targets (Rao, 2000). In the present study, a cDNA clone of 1.16 kb was isolated by immunoscreening of L3 stage cDNA library of B. malayi. The cDNA clone showed about 58% similarity with C. elegans RNA Helicase, 78% with B. malayi RNA helicase sequence generated from the recently published draft genome. It contained a DEAD box conserved domain which is the signature sequence present in almost all RNA Helicases. Interestingly, the 2 day old irradiated L3 library of B. malayi also contained the ESTs of ATP dependent RNA Helicase. The serum used for immunoscreening of L3 library in the present study was also raised against irradiated infective larvae of B. malayi. RNA Helicases have been identified as potential drug target candidates in case of herpes simplex virus (HSV), the hepatitis C virus (HCV) and malaria parasite (Tuteja, 2007; David, 2003); however, it has still not been explored in case of filarial nematodes. B. malayi has the genome size of ~90 megabase (Mb) and about 11,500 protein coding genes are predicted to be present. The DEAD Box RNA Helicases are the sixteenth most abundant domains in B. malayi, C. elegans, and C. briggsae amongst the twenty most abundantly expressed domains as revealed by the recently released B. malayi draft genome data (Elodie et al, 2007). Also the analysis of a subset of randomly selected-clones sequenced from the
porcine nodule nematode *Oesophagostomum dentatum* male-specific cDNA library demonstrated that ESTs of DEAD box RNA Helicase were present as Major sperm proteins (MSPs) from a male-specific cDNA library suggesting its role in development, sexual differentiation and reproduction of parasitic nematodes (Alasdair et al, 2004). This also gives an idea that filarial Helicase may possibly be exploited for interfering with worm fertility and reproduction to block filarial transmission. The RNA Binding Motif in RNA helicase A having a DExH Helicase domain of *Xenopus laevis* is found to be involved in the unwinding of duplex of nucleic acids (Cheng et al, 2005) explaining the importance of DEAD box domain in the enzyme. The *C. elegans* glh-1 and cgh-1 genes also contain regions of conserved sequence present amongst RNA Helicases and are predicted to be a single copy gene correlated with gametogenesis and protection from physiological germ line apoptosis (Deborah and Karen, 1993). Various studies have shown that Helicases are indispensable enzymes and in yeast the loss of one DEAD-box gene cannot be supplemented by over expression of another family member, which further suggests that each Helicase gene is independently essential (Tuteja, 2007).

The cDNA clone BmL3-Helicase was successfully expressed in bacterial expression system using host BL21 (DE3) *E. coli* cells and pET28a expression vector giving a highly soluble His-tag fusion translation product of ~50 kDa. Purification was carried out by binding of 6xHis affinity tag to Nickle-Nitrilotriacetic acid (Ni-NTA) column called ‘immobilized-metal affinity chromatography (IMAC)’ to obtain the enzyme in native functional form. The enzyme BmL3-Helicase also showed strong reactivity with sera of various categories (asymptomatic microfilaraemic carriers, symptomatic with or without mf) of target human lymphatic filarial parasite *W. bancrofti* as also with *B. malayi* infected microfilaraemic mastomys sera. Sera of endemic normal persons staying in filaria-endemic area with no parasitological or clinical signs of lymphatic filariasis (5 year follow-up) also reacted very strongly with this protein exhibiting the implication of BmL3-Helicase in immune protection studies too. The non-endemic normal human serum and uninfected mastomys serum did not give any reactivity with the BmL3-Helicase demonstrating the specificity of protein.

It has been observed earlier that the ATPase activity of so-called DEAD/DExH box of ATPases is polynucleotide-dependent (Youliang and Zhi-Ren, 2002). In present study also it was found that the ATP hydrolysis by BmL3-Helicase is substrate dependent. The amount of ATP hydrolysis increased with addition of RNA to BmL3-Helicase as compared to BSA and
BmL3-Helicase alone. An increase in ATP hydrolysis in presence of other substrates like dsDNA and ssDNA was also seen.

Further to elucidate the antigenic property of BmL3-Helicase, a polyclonal serum was raised in BALB/c mice and high antibody titres were observed in ELISA further indicating the presence of antigenic epitopes in the expressed protein. The stage-specificity of recombinant protein was seen by immunoblot and it was found that BmL3-Helicase was highly expressed in all the major life cycle stages of *B. malayi* i.e. L₃, both sexes of adult stages and microfilariae revealing that an immune response to *B. malayi* helicase is developed once the infective larvae are inoculated into the vertebrate host and the response is maintained with further larval development and transformation into subsequent life forms demonstrating further importance of this enzyme as diagnostic antigen.

The findings in present report thus suggest that BmL3-Helicase is a novel and unexplored enzyme of *B. malayi* and further characterization is needed to reveal its role in parasite biology.
Figure 1 Immunoscreening of Larval cDNA library of *Brugia malayi*. Arrows denote the positive plaques picked up for further purification. Seven immunoreactive clones were obtained which were then individually purified by secondary and tertiary screening.

Figure 2 Restriction map and sub-cloning of 1.16 kb BmL3-Helicase cDNA clone into expression vector pET28a (Novagen). The cDNA clone in pBluescript vector was double digested with *EcoRI* and *XhoI* restriction enzyme to obtain an insert of 1.16 kb. This insert was sub-cloned into expression vector pET28a after in-framing the Open reading frame.
Figure 3 Multiple sequence alignment of the deduced amino acid sequence of *B. malayi* with proteins of other species using Clustal-W software. Alignment of Bm-L3-Helicase with *B. malayi* (EDP36872), *Caenorhabditis briggsae* (CAE62876) and *C. elegans* (AAB71200). The putative DEAD box and SAT domains involved in Helicase activity are underlined. Regions of identity (*), strong similarity (:), and weak similarity (.) are indicated.
Figure 4 Expression, purification and localization of recombinant His-tag BmL3-Helicase as observed on 10% SDS-PAGE after Coomassie blue staining. The BmL3-Helicase/pET28a+ construct transformed into BL21 (DE3) E. coli cells for expression studies. [A] bacterial cell lysate before IPTG induction (lane 1), bacterial cell lysate after 0.5 mM IPTG induction (lane 2), uninduced recombinant bacterial lysate (lanes 3), lysate containing recombinant bacterial protein from IPTG-induced bacterial cells (lane 4), and Protein molecular weight marker (Fermentas, size in kDa) is shown on right side, [B] Localization of recombinant BmL3-Helicase in soluble (supernatant) and insoluble (pellet) fractions. The induced BL21 (DE3) cells were pelleted, sonicated, run on gel showed the presence of ~80% recombinant Helicase in soluble fraction, [C] BmL3-Helicase purified by Ni-NTA affinity chromatography: fractions of protein eluted with 250 mM imidazole; protein molecular weight marker (lane 9), size in kDa shown on the right.

Figure 5 Western blot analysis of bacterially expressed His-tag BmL3-Helicase by anti-His antibody. The Western blot experiments were carried out with 300 ng of purified 6xHis-tag BmL3-Helicase, anti-His antibody was diluted to 1:1,000 and developed with DAB (3', 3' Diaminobenzidine).
Figure 6 Cross-reactivity of BmL3-Helicase with pooled (10 patients each) human bancroftian sera belonging to various categories in western blots. Endemic normal (lane 1), microfilaric asymptomatic (lane 2), amicrofilaric symptomatic (lane 3), microfilaric symptomatic (lane 4), pooled (5 animals) B. malayi infected mastomys (lane 5), irradiated L3 immunized mastomys (lane 6), anti-His antibody (lane 7), prestained molecular weight protein marker (lane 8), normal mastomys serum (lane 9) and non-endemic control (lane 10).

Figure 7 Presence of BmL3-Helicase protein in various life-cycle stages of B. malayi. Soluble extracts (10 µg/lane) from microfilarae, L3, adult (male and female) stages of the parasite were resolved on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with mouse anti-BmL3-Helicase antibody. HRP labelled goat anti-mouse IgG (Sigma, USA) was used as the secondary antibody and the reactive bands were detected by DAB substrate. Recombinant BmL3-Helicase was used as positive control.
Figure 8 ATPase activities of the recombinant His-tag BmL3-Helicase. The ATPase activity is expressed as μM (concentrations) of released inorganic phosphate in the 50 μl of reaction volume. [A] ATPase activity is measured in the presence of 1 μg of yeast total RNA (Fermentas). The ATP hydrolysis reactions were carried out at room temperature for 30 min containing, 4 mM ATP alone (A); 4 mM ATP and 2.5 μg of BSA (B); 4 mM ATP and 2.5 μg of His tag BmL3-Helicase (C); 4 mM ATP and 2.5 μg of His tag BmL3-Helicase with RNA (D); 4 mM of dATP (E), dTTP (F), dCTP (G) and dGTP (H) with 2.5 μg of His-tag BmL3-Helicase. [B] Dependence of ATPase activity of BmL3-Helicase on the nucleic acids. Assays were carried out with 4 mM ATP and 10 μl of helicase (2.5 μg) in 50 μl reactions containing 0.5 μg of Yeast RNA (ss-RNA), 0.1 μg of synthetic dsRNA, 0.2 μg of 24-nt synthetic DNA oligonucleotides (ssDNA), and 1 μg of pBluescript linearized by EcoRI (dsDNA).