2 MATERIALS AND METHODS

2.1 Animals

Healthy male *Mastomys coucha* (30-40 gm) and jirds (*Meriones unguiculatus*, 30-40 gm) were used in the present study. All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. Throughout the study, the animals were housed in climate (23 ±2 °C; RH: 60%) and photoperiod (12 hr light-dark cycles) controlled animal quarters. They were fed standard rodent “maintenance diet” prepared in-house (QC analysis: carbohydrates 58.30%, protein 21.1%, fat 7.2%, crude fibre 6.6%, moisture 6.8%) supplemented with dried shrimps (*M. coucha*) and had free access to drinking water.

2.2 Parasite

For experimental purpose *B. malayi*, having many of the biological aspects similar to *W. bancrofti* was used in the study. It is a subperiodic strain of human filarial infection and has successfully been transmitted to various vertebrate hosts i.e. from small rodents (jird, *Mastomys coucha*, BALB/c mice, SCID mouse etc.) to big animals (cats, dogs etc.) including various species of monkeys: Rhesus (*Macaca mulata, Presbytis entellus, P. mellophos, P. cristata, Macaca radiata* etc). The infection is transmitted through black eyed susceptible strain of *Aedes aegypti* mosquitoes developed by McDonald (Liverpool School of Tropical Medicine and Hygiene, U. K.).

2.3 Animal models

Rodents are preferred model for laboratory studies throughout the world. ‘GRA’ strain (Germany) of *Mastomys coucha* (belonging to family-Muridae; Fig. 3.1A) is fully permissive/susceptible to *B. malayi* and is being maintained in the animal house of CSIR-Central Drug Research Institute since more than 40 years. It is a multimammate, prolific breeder with average litter size of 8 - 10 babies (Wilson & Reeder, 1993). *M. coucha* model is found amenable to perform chemotherapeutic and immunological investigations in experimental filariasis (Khan *et al.*, 2004; Mukhopadhyay *et al.*, 1996; Murthy *et al.*, 1983;
Immune responses of *M. coucha* is similar to human, so this animal was used as model for experimental purpose (Dixit *et al.*, 2006; Gaur *et al.*, 2008; Sahoo *et al.*, 2009; Verma *et al.*, 2013).

*M. coucha* is highly susceptible to *B. malayi* infection when infection is given via subcutaneous (s.c.) route but its peritoneal cavity (p.c.) is not conducive for development and establishment of L3 of the parasite (Gupta *et al.*, 2004; Murthy *et al.*, 1997). This is a very good model for *B. malayi*. However p.c. of the animals is expected to provide us opportunity to obtain precise quantitative data on worm burdens as a function of time (Rajan *et al.*, 2002). Moreover, this approach also helps to delineate the primary protective inflammatory responses to incoming L3 infection as well as the modulation of these responses by filarial parasite molecules (Dixit *et al.*, 2004).

Another rodent, the Mongolian gerbil (*Meriones unguiculatus*, family Gerbillinae, Fig. 3.1B) has proven to be an excellent permissive rodent model for the study of lymphatic filariasis using *B. pahangi* or *B. malayi* (McVay *et al.*, 1990). The animal (called ‘Jird’) was used for the propagation of *B. malayi* parasites in our institute’s animal house according to the method described by (McCall *et al.*, 1973). It has shown full development of L3 of *Brugia* specie given through both i.p. or s.c. route. The animal is being well maintained and used for various studies on filariasis. Its litter size is smaller than *M. coucha*.

![Fig. 3.1: Experimental Filarial models – *Mastomys coucha* (A), *Meriones unguiculatus* (B)](image-url)
2.4 Maintenance of *B. malayi* infection

*B. malayi* infection was cyclically transmitted *M. coucha* through experimentally reared and bred black eyed filaria susceptible mosquitoes *A. aegypti* (Fig. 3.7).

2.4.1 Rearing and breeding of *A. aegypti* colony

In the laboratory the mosquitoes were reared and bred in an insectarium maintained at controlled temperature (26 ± 1°C) and humidity (80 ± 5%). The adult mosquitoes were kept in nylon mesh cages and provided 10% glucose solution supplemented with vitamin B, soaked in cotton for feeding. From time to time female mosquitoes were fed on normal *M. coucha* blood to promote egg laying. A beaker containing water was kept in the cage for egg laying. The eggs are laid after about 40 hr blood feeding. These were filter separated and stored after drying at same temperature. Eggs can be preserved in such condition for 3 - 4 months. For maintenance of mosquito life cycle the eggs were transferred to enamel bowl containing water. The larvae hatch out in the water within 24 hrs and these were provided with feed containing dog biscuits and yeast powder. The larvae usually took about 8 - 10 days to become pupae, which ultimately developed into adult mosquitoes within 48 hr (Fig. 3.2).

![Fig. 3.2: Larva (A), Pupa (B), Mosquito feeding on host (C), Mosquitoes in cage (D)](image)

2.4.2 Feeding of mosquitoes on mf positive *M. coucha*

*B. malayi* infected *M. coucha* showing optimum level of mf (100 - 200 mf/10 μl) in circulating blood was used as donors. Before feeding on blood of infected *M. coucha* (donor) the mosquitoes starved for 2-3 hr, were kept inside the wire netting immobilized mosquito cage. These were then allowed to feed on the donor animals at 12.00 noon when plentiful of mf was present in the circulation (Gupta *et al.*, 1990). After 1 hr of feeding the donor animal was
removed and mosquitoes were provided with glucose solution as mentioned above (Fig. 3.3) In 9 - 10 days time the mf in the mosquitoes developed into L3.

![Image](image1)

**Fig. 3.3:** Feeding of mosquitoes on mf positive *M. coucha*

### 2.4.3 Isolation of L3 from mosquitoes

On day 9 or 10 post feeding, the mosquitoes were paralyzed and crushed gently in 4 - 5 ml of 0.6% insect saline (IS) and transferred to Baerman’s apparatus which consisted of glass funnel, Nytex cloth (Thermo Fisher Scientific Inc., USA), latex tubing (Gyan Scientific traders, Lucknow, India) with a pinch cock. The funnel was filled with lukewarm IS. Crushed mosquitoes were then put on the cloth and allowed to stand for half an hour with light provided from top the funnel using a table lamp. The L3 released from the mosquitoes move away from light, traverse through the cloth and settled down at the bottom of the tube (Fig. 3.4). These were collected by opening the pinchcock and washed with IS several times to remove the mosquito debris. The larvae (L3) were counted and used immediately for exposure to animals.

![Image](image2)

**Fig. 3.4:** Paralyzed mosquitoes (A), Crushing of mosquitoes (B), Baerman’s apparatus for L3 separation (C)
2.4.4 Inoculation of L₃ to M. coucha/jird and assessment of the infection

6 - 8 weeks old male M. coucha were inoculated with active and motile L₃, (100 per animal) subcutaneously (s.c.) (Fig. 3.5). Establishment of infection was examined in blood smear after day 90 post larval inoculation (p.l.i.) and thereafter monitored at regular intervals (Fig. 3.6). Animals showing desired levels of infection were used for transmission of the infection to healthy animals. Thus, the cycle was continually maintained.

Similarly in jirds of the same age group about 200 L₃ per animal were inoculated intraperitoneally (i.p.). The larvae develop into adult worms in about three months and can be harvested thereafter when required.

Fig. 3.5: Inoculation of L₃

Fig. 3.6: Stained blood smear showing microfilariae of B. malayi (A), Single microfilaria (B)
Materials and Methods

Fig. 3.7: Transmission and maintenance of *B. malayi* infection in *M. coucha* through *A. aegypti*
2.5 Cloning

2.5.1 Gene selection

The whole gene sequence of DIM-1 of *B. malayi* has been taken from NCBI database of *B. malayi* by blast search. The gene sequence is as follows:

```
ATGCCGGAGGGTAAGGCACCTCATTTCCCGCAGCAGCCGGTAGCTCGACAAAACGATGATG
GATCATTGGAACTGGAAGTGGTTTTTTAGAAAGCTCAACCAAGAGGGAAGCATGCTTACTCC
GCCATTTTACAAATCAAGATCTTGCTGAATAGTGAGTCTGAGCTAGTCTGATATTTGGAATAC
ACATGGAAAAGGAAAGCAGGAAATTTAAACCTTAAACTTACAGGATTCGCTCAACGGTCTTCGAG
AAACCACAATCTACTTCTCGAGCATGGGCAAGTATATGTTTATGGAATTCAGGGCGAAATCGATTC
TTAAACCTACCTTCTGCTGGCAAAAGGTGAAAGAATTCTGAGCTAATCGGATTCGAGTGGAAAATTGT
TCTACGAAGAAGCAAACATCAACATATTACGCTCTGCTGAAATCAAAGAGCGAAGAGAAAA
GGATGGGCCCAATTTTCGACACAGGAAAGCAATGCAAATACTGGAAAATACAGGAGCGATAG
AAATTTGGAGTGCCAAAGGAGCTCGCCGACCTTTACCCGTAACCAAGACAGATTAGTCAAAAACCTCAG
ATTCTGTTGATCCAGCTTATTGTTTTGATATTGGAATTCAGGATCTGACAAACCCAGGAGGCTATCTGG
TTAAATCCAAGGCGAAAGATGAGGAATCAAGCTCGTAATTTTTGGATATACACCTGAGGCT
GGTGCAACACGTCTACTGGAATTGAAACTGAAAAATTATAAGGAAAGGAGTAGGTGTAACATATA
CGTGCAATATTAAGGAGCTGTGGAAGGAAGCTGGAATTTGAGCATCGAACATCGGAAGAAGCATG
AGACGAGGAGCAGGATGACGACCGACGAGGCTG
```

2.5.2 Design of primer for the gene

A primer is a short synthetic oligonucleotide which is used in many molecular techniques from PCR to DNA sequencing. These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which we wish the primer to anneal. While designing the primers it is often necessary to make predictions about melting temperature (Tm) and propensity to form dimmers with itself or other primers in the reaction. Factors are taken into consideration as follow: (1) Primers with 17-28 bases in length. (2) Base composition was 40-60% (G+C). (3) Primers end at 3’ was in a G or C, or CG or GC. (4) Tm between 50-60 °C was preferred. (5) 3'-ends of primers was not complementary. (6) Primer self-complementary (ability to form 2° structures such as hairpins) was avoided. (7) Runs of three or more Cs or Gs at the 3’ -ends of primers were avoided (Dieffenbach *et al.*, 1993).
The oligo analyzer program was used to calculate both the Tm of the primers, as well as any undesirable pairings of primers. Primer Sequence was as follows:

**Primer for DIM-1**

FP- 5' CCC AAG CTT ATG CCG GAG GGT AAG GCA C 3'
RV- 5' CCC TCG AGT CAC GCC TCG CTG GCA T 3'

### 2.5.3 mRNA isolation

#### 2.5.3.1 Isolation of adult parasites

Adult parasites were harvested from peritoneal cavity of jirds (*M. unguiculatus*) having 5-6 months old *B. malayi* infection as described by (Dixit *et al.*, 2004; Murthy *et al.*, 1997). Highly motile adult worms were washed several times with sterile phosphate buffered saline (PBS; pH 7.2) and used for isolation of mRNA.

#### 2.5.3.2 mRNA isolation by Trizol method

TRI reagent (Molecular Research Center, USA) used for the extraction, is a mixture of guanidine thiocyanate and phenol in a mono-phase solution that effectively dissolves DNA, RNA and protein on homogenization or lysis of tissue sample. When chloroform is added and mixture is centrifuged, the mixture separates into three phases, an aqueous phase containing the RNA, an interphase containing the DNA and an organic phase containing the proteins. The extraction was done according to manufacturer’s instructions (Molecular Research Centre Inc.) (Chomczynski & Sacchi, 1987).

The live and highly motile parasites were washed with Diethylpyrocarbonate (DEPC, Sigma-Aldrich, USA) treated water and approximately 60 adult parasites (male and female worms in 1:3 ratio) was transferred to 1 ml TRI reagent in a 1.5 ml sterile eppendorf tube and kept for 3 hr at room temperature (RT) or 4 °C for overnight. Parasites were homogenized using sterile homogenizer and allowed to stand for half an hour at RT. Tube was centrifuged at 12,000 g for 5 min at 4 °C in order to remove the debris and the supernatant was transferred to a fresh eppendorf tube. Chloroform (200 l) was added to 1 ml of TRI reagent and mixed vigorously, and incubated for 10 min at RT. Tube was centrifuged at 12,000 g for 15 min at 4 °C. The colorless aqueous phase was transferred to a fresh eppendorf tube and 500 l of isopropyl alcohol was added into it mixed properly and incubated for 10 min at RT. Tube was
Materials and Methods

Centrifuged at 12,000 g for 10 min at 4 °C, supernatant was discarded and the pellet was washed with 1 ml of 75% ethanol. Tube was then centrifuged at 7,500 g for 5 min at 4 °C and tube containing RNA pellet was kept for air drying. The pellet of RNA was dissolved in 20 μl of BPC (Biotechnology performance certified; Sigma-Aldrich, USA) water and then RNA was estimated using Nano-drop spectrophotometer (Thermo Scientific, USA).

➢ Estimation of RNA

1 μl of dissolved RNA sample in BPC water was taken and absorbance (OD) was read at 260 and 280 nm with BPC water as a blank. Ratio of 260/280 was calculated to check the purity of RNA. Range for RNA was found between 1.6 and 1.8. Finally the concentration of RNA was calculated.

2.5.4 cDNA synthesis

RevertAid® first strand cDNA synthesis kit (Fermentas Life Sciences, USA) was used for the synthesis of cDNA from RNA. This relies on a genetically engineered version of the Moloney Murin Leukemia Virus transcriptase with low RNase H activity. This allows the synthesis of full-length cDNA from long templates (up to 13 kb) (Schmidt et al., 2004).

The following reaction mixture was prepared in a PCR tube on ice for 20 μl reaction.

Total RNA 5 μg
Oligo (dT) primer 1 μl
DEPC treated water 12 μl

The mixture was mixed gently with quick spin at 2000 g for 1 min and incubated for 5 min at 70 °C on a thermocycler (Bio-rad, USA) followed by chilling on ice and centrifuged at 2000 g for 2 min at 4 °C.

5X reaction buffer 4 μl
Ribonuclease inhibitor 1 μl
10mM dNTP mix 2 μl

The tubes were placed on ice and then the reaction mixture was added to each tube. The contents of the tubes were mixed by centrifugation at 2000 g for 2 min at RT and incubated for 5 min at 72 °C on thermocycler (Bio-Rad, USA) and then the mixture was incubated at 42 °C for 60 min. The reaction was stopped by heating the tubes at 70 °C for 10 min. The contents in
tubes were chilled on ice and stored at -20 °C for further use. Synthesized first strand cDNA could be used as a template in the polymerase chain reaction (PCR) for amplification of desired gene.

**Gradient PCR**

A gradient PCR is often done to optimize a PCR and figure out what annealing temperatures work best. During the PCR, a temperature gradient, which can be programmed between 1 °C and 20 °C, is built up across the thermoblock. This allows the most stringent parameters for every primer set to be calculated with the aid of only one single PCR reaction (Weier & Gray, 1988).

In the fresh DEPC treated and autoclave PCR tube, all the components were mixed according to the standard protocol.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X PCR mastermix buffer)</td>
<td>80</td>
</tr>
<tr>
<td>Forward primer</td>
<td>10</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>10</td>
</tr>
<tr>
<td>cDNA (template)</td>
<td>15</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>45</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>160</strong></td>
</tr>
</tbody>
</table>

The above reaction mixture was equally divided into 8 PCR tubes each containing 20 µl, mixed well by quick centrifugation at 2000 g for 2 min at 4 °C and reaction was carried out in Thermocycler (Bio-Rad, USA).

Conditions used for amplification were:

- **Initial Denaturation**: 94 °C for 4 min
- **Denaturation**: 94 °C for 1 min
- **Primer Annealing**: 55-65.5 °C for 1.30 min
- **Re-naturation**: 72 °C for 1 min
- **Final Extension**: 72 °C for 10 min

Then amplified products were resolved in 1% agarose gel and visualized under U.V. light.
Agarose gel electrophoresis

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA (Kryndushkin et al., 2003). Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones (Sambrook & Russel, 2001). In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired “band” from a stained gel viewed with a UV transilluminator (Sharp et al., 1973).

Agarose gel electrophoresis provides multiple advantages that make it widely popular. For example, nucleic acids are not chemically altered during the size separation process and agarose gels could easily be viewed and handled. Furthermore, samples could be recovered and extracted from the gels easily for further studies. Still another advantage was that the resulting gel could be stored in a plastic bag and refrigerated after the experiment, there may be limits. Depending on buffer during electrophoresis in order to generate a suitable electric current and to reduce the heat generated by electric current could be considered as limitations of electrophoretic techniques (Lodge et al., 2007; Sharp et al., 1973).

Reagents

**Tris-acetic acid –EDTA (TAE) buffer:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>24.2 g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>5.71 ml</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8)</td>
<td>20 ml</td>
</tr>
<tr>
<td>TDW (final vol.)</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

Autoclaved at 15 lb and 121 °C for 15 min.

**Ethidium bromide (EtBr):** (Sigma, USA) (stock 10 mg/ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtBr</td>
<td>100 mg</td>
</tr>
<tr>
<td>TDW</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

100 mg EtBr dissolved in 10 ml TDW, stirred for several hours to ensure dye was dissolved and stored at 4 °C.
Agarose 0.3 g
1X TAE 30 ml
EtBr 2 µl

0.3 g of Agarose dissolved was in 30 ml of TAE by boiling. Into this 2 µl of EtBr was added.

Agarose gel was prepared by suspending dry agarose (Lonza, USA) in aqueous TAE buffer, then boiling the mixture until a clear solution is formed. The solution was cooled and EtBr was added. EtBr is a cyclic planer molecule that binds between the stacked base pairs of DNA. This agarose solution was poured into a caster, surrounded by wall of plastic frame and sealed with tape. Loading wells were formed by placing a comb in the poured gel solution. The comb was removed after setting of the gel. The gel with caster was placed in electrophoresis tank and covered with TAE buffer. Samples were prepared by mixing with loading dye and were loaded in to the wells of submerged gel. The molecular weight marker (Fermentas, USA) was also run simultaneously. The gel was run at constant voltage of 50 V. DNA bands were viewed under UV light in UV transilluminator (Spectroline, USA) which fluoresce orange-red due to binding of EtBr. The image was captured using a gel documentation system (Bio-Rad, USA) (Fig. 3.8).

Figure 3.8: Flow chart of agarose gel electrophoresis
2.5.5 PCR amplification of gene

PCR technique developed by (Mullis, 1990) is extremely powerful. PCR was used to make a huge number of copies of a gene. PCR was done on a thermocycler (Bio-Rad, USA), which could heat and cool the tubes with the reaction mixture in a very short time. PCR consisted of three defined sets of times and temperatures, termed steps, these were:

a) Denaturation
b) Annealing
c) Extension

Procedure

In the fresh PCR tube all the components were mixed according to the standard protocol.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X PCR mastermix (buffer)</td>
<td>20 μl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>3 μl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>3 μl</td>
</tr>
<tr>
<td>cDNA (template)</td>
<td>4 μl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>10 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>40 μl</strong></td>
</tr>
</tbody>
</table>

Then reaction mixture was then divided equally into two fresh PCR tubes (20μl) and then allowed to carry out in Thermocycler (Bio-Rad, USA).

Conditions used for amplification

<table>
<thead>
<tr>
<th>PCR Steps</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94 °C (4 min)</td>
</tr>
<tr>
<td>Final Denaturation</td>
<td>94 °C (1 min)</td>
</tr>
<tr>
<td>Annealing</td>
<td>58 °C for 1 min</td>
</tr>
<tr>
<td>Renaturation</td>
<td>72 °C ( 1 min)</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C (10 min)</td>
</tr>
<tr>
<td>Cycles of amplification</td>
<td>30</td>
</tr>
</tbody>
</table>

Then amplified product was resolved in 1.0% Agarose gel and visualized under U.V light.
2.5.5.1 Elution of amplified DNA (gene product) from the gel

The amplified PCR product was eluted from the gel using Quick Gel Extraction Kit (INVITROGEN, USA) according to manufacturer’s instructions. Bands were cut from the gel containing amplified DNA fragment under UV transilluminator (spectroline, USA) and suspended in tube containing 1 ml gel resolublisation buffer. Tube was incubated for 15 min at 50 ºC for dissolution of gel. An extraction column was placed into 2 ml wash tube and the dissolved gel was loaded in column. Tube was centrifuged at 12,000 rpm for 1 min at 4 ºC, the flow was discarded and the column was placed back into the wash tube (repeated the step 2-3 times). 700 µl of wash buffer containing ethanol was added to column and incubated for 5 min at RT. Tube was centrifuged at 12,000 rpm for 1 min at 4 ºC. Supernatant was discarded and emptied column with tube was centrifuged for 1 min so as to remove any residual wash buffer. The column was placed in 1.5 ml recovery tube and 30 µl of pre-heated (65-70 ºC) TE buffer was added to the center of column. Tube was incubated for 1 min at RT. Tube was centrifuged at 12,000 rpm for 2 min at 4 ºC. Purified DNA retained in the recovery tube and the column was discarded. Purified DNA was stored at -20 ºC (or 4 ºC for immediate use).

2.5.6 Cloning of DNA in TA vector

TA cloning is a subcloning technique that doesn't use restriction enzymes and is easier and quicker than traditional subcloning. The technique relies on the ability of adenine (A) and thymine (T) (complementary basepairs) on different DNA fragments to hybridize and in the presence of ligase, become ligated together. PCR products are usually amplified using Taq DNA polymerase which preferentially adds an adenine to the 3' end of the product. Such PCR amplified inserts are cloned into linearized vectors that have complementary 3' thymine overhangs.

The insert was created by PCR using Taq DNA polymerase. This polymerase lacks 3’ to 5’ proofreading activity and, with a high probability, adds a single, 3’ adenine overhang to each end of the PCR product. It is best if the PCR primers have guanines at the 5' end as this maximizes probability of Taq DNA polymerase adding the terminal adenosine overhang (Protocol, 2009). Thermostable polymerases containing extensive 3’ to 5´ exonuclease activity should not be used as they do not leave the 3' adenine-overhangs (Manual, 2004) (Fig. 3.9).
The target vector is linearized and cut with a blunt-end restriction enzyme. This vector was then tailed with dideoxythymidine triphosphate (ddTTP) using terminal transferase. It was important to use ddTTP to ensure the addition of only one T residue. This tailing left the vector with a single 3’-overhanging thymine residue on each blunt end (Holton & Graham, 1991). Manufacturers commonly sell TA Cloning "kits" with a wide range of prepared vectors that have already been linearized and tagged with an overhanging thymine residue.

For cloning, PCR product of genes was ligated with pTZ57R/T which contains T overhangs.

![Vector map of pTZ57R/T](image)

**Fig. 3.9:** Vector map of pTZ57R/T (T/A vector)

### 2.5.6.1 Ligation of PCR product and TA vector

The PCR product with an overhang was mixed with TA vector in high proportion. The complementary overhangs of a TA vector and the PCR product hybridize. The result was a recombinant DNA, the recombination being brought about by DNA ligase.
In a 15 μl ligation reaction, following components were added in a microcentrifuge tube.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA Vector</td>
<td>1.5</td>
</tr>
<tr>
<td>PCR product</td>
<td>8</td>
</tr>
<tr>
<td>Ligation buffer</td>
<td>3</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>15</strong></td>
</tr>
</tbody>
</table>

The ligation mixture was incubated at 6 °C for overnight.

### 2.5.6.2 Transformation in DH5α competent strain of *E. coli*

Plasmid transformation into bacterial competent cells is a key technique in molecular cloning. It is the process to incorporate foreign DNA into host cell. Kurien and Scofield have described a quick and moderately efficient method of bacterial colony transformation (Kurien & Scofield, 1995). More recently, Chen has proposed an alternative convenient and rapid method for the genetic transformation of *E. coli* with plasmids. By mixing the recipient cells and plasmid DNA and spreading them directly on selective medium plates containing Ca2+, the so-called 'plate transformation' could achieve almost the same transformation efficiency as the classical transformation method with calcium (Chen et al., 2001).

There were three basic steps in the introduction of plasmid DNA into the cells.

**Preparation of competent cells:** There are two main methods for transformation of competent bacterial cells, the calcium chloride and the electroporation method (Dagert & Ehrlich, 1979; Maeda et al., 2004; Topcu, 2000). We preferred to opt calcium chloride method. The DH5α strain of *E. coli* colony was inoculated in a 5 ml LB vial and grown overnight at 37 °C without shaking. 250 μl of overnight grown culture was inoculated to 25 ml of LB broth for nearly 2 hours at 37 °C with vigorous shaking, till O.D. reached to 0.6. Chilled the culture in ice bath for 15 min. and harvested the cells by centrifugation at 4,500 rpm for 10 minutes at 4 °C. Resuspended the pre-chilled pellet in 20 ml CaCl$_2$ of 0.1 M and kept in ice for 20 min. Centrifuged at 6,000 rpm for 3-4 min. and discarded the supernatant. Resuspended the pellet in 2 ml pre-chilled CaCl$_2$ of same same molarity and added pre-
chilled 15% glycerol. The mixture was mixed and kept in aliquots (200 µl/ependorff tube) at -80 °C for 6 months.

- **Introduction of recombinant plasmid into the competent cells:** Ligation product in 15 µl volume was added into 200 µl of DH5α (*E. coli* strain) cells and incubated in ice for 15 min. Heat shock treatment was given for 90 sec at 42 °C and then immediately transferred in ice. 800 µl C-medium added to above culture and placed in shaker incubator at 37 °C for 1 hr. Briefly spined the mixture after 2 hr and 200 µl of lower part was taken and poured on petri dish containing ampicillin and X-gal and incubated at 37 °C for overnight (Sambrook & Russel, 2001).

- **Selection of transformants:** After incubation at 37 °C for overnight, two kinds of colonies grown on amp-X-gal plate. White colonies (positive) in which transformation of gene was occurred and blue colonies which did not transform. This is called as Blue-white screening for the rapid and convenient detection of recombinant bacteria. The method is based on the principle of α-complementation of the β-galactosidase gene (Ullmann *et al.*, 1967).

Positive colonies were picked up from ampicillin-X-gal plate and streaked on new ampicillin plate with marking of colony number. The master plate incubated at 37 °C for overnight and then the plate was stored at 4 °C for future use.

**2.5.6.3 Confirmation of positive colony by colony PCR**

Transformed DH5α cells picked up from master plate and diluted in 50 1 of BPC water/TE buffer in micro-centrifuged tube. Mixture was boiled for 8-10 min in water bath, centrifuged at 1,000 rpm for 5 min and supernatant was collected. Supernatant was used as template in PCR. PCR was done as describe above by using primers of gene. Amplified product was resolved in 1% agarose gel and visualized under U.V light (H Mirhendi, 2007).

**2.5.6.4 Sequencing of gene**

The nucleotide and coding amino acid sequences of 966 bp amplified product were compared with *B. malayi* draft genomes using BLASTn and the BLASTx search programs (Altschul *et al.*, 1997). Comparison with expressed sequence tag (EST) sequences was
performed using tBLASTn (NCBI) and multiple sequence alignment by CLUSTAL-W algorithm.

2.5.7 Sub cloning of DIM-1 TA clone in expression vector (pTriEx-4)

2.5.7.1 Isolation of plasmid DNA from transformed \textit{E. coli}

Positive colonies picked from master plate were inoculated in a 5 ml LB-amp tubes aseptically and incubated overnight at 37 °C in a shaker incubator with continuous shaking at 200 rpm. Plasmid was isolated using Quick Plasmid Mini Prep Kit (INVITROGEN) according to manufacturer’s instructions. The isolated plasmid was checked on 1% agarose gel and visualized under U.V light.

2.5.7.2 Expression vector (pTriEx-4)

pTriEx-4 contains flanking baculovirus sequences to permit the generation of recombinant baculoviruses using the BacVector™ System. Expression in \textit{E. coli} is regulated by the tightly controlled T7lac promoter. Expression can be induced in hosts such as NovaBlue by infecting with \(\lambda\)CE6, a phage that constitutively expresses T7 RNA polymerase from the \(\lambda\)pL and \(\lambda\)pI promoters. Alternatively, pTriEx recombinant plasmids can be transferred into a (DE3) pLacI host that allows IPTG based induction. Native protein can be expressed by cloning into the NcoI site, or native protein can be generated by cloning into the PshAI or SmaI sites and cleavage of the fusion protein with enterokinase or thrombin, respectively (Fig. 3.10).
2.5.7.3 Restriction digestion of plasmid of TA clone and plasmid of expression vector (pTriEx-4)

Restriction digestion technique is used for cleaving DNA molecules at specific sites, ensuring that all DNA fragments that contain a particular sequence have the same size; furthermore, each fragment that contains the desired sequence has the sequence located at exactly the same position within the fragment. The cleavage method makes use of an important class of DNA-cleaving enzymes isolated primarily from bacteria. These enzymes are called restriction endonucleases or restriction enzymes, and they are able to cleave DNA molecules at the positions at which particular short sequences of bases are present (Hartl et al., 2001).

For sub cloning of TA clone, expression vector pTriEx-4 was used for DIM-1. A restriction digestion is a procedure used to prepare DNA for analysis or other processing by digestion of DNA fragment with restriction enzymes. In each 15 μl digestion reaction following component were added in a microcentrifuge tube.
Materials and Methods

<table>
<thead>
<tr>
<th>Components</th>
<th>Plasmid of TA Clone</th>
<th>Plasmid of pTriEx-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE3 Buffer</td>
<td>1.5 μl</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>BamH1</td>
<td>1 μl</td>
<td>1 μl</td>
</tr>
<tr>
<td>XhoI</td>
<td>1 μl</td>
<td>1 μl</td>
</tr>
<tr>
<td>Template plasmid</td>
<td>8 μl</td>
<td>-</td>
</tr>
<tr>
<td>Expression vector</td>
<td>-</td>
<td>8 μl</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>3.5 μl</td>
<td>3.5 μl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>15 μl</td>
<td>15 μl</td>
</tr>
</tbody>
</table>

The both digested plasmid (plasmid of TA clone and plasmid of expression vector (pTriEx-4) was checked separately on 1% agarose gel and visualized under U.V light.

2.5.7.4 Elution of digested plasmid from the gel

The both digested plasmid, plasmid of TA clone and plasmid of vector (pTriEx-4) were cut from the gel containing digested fragments under U.V. transilluminator and put in 1.5 ml appendorff and DNA was subsequently eluted by Quick Plasmid Mini Prep Kit (INVITROGEN) according to manufacturer’s instructions.

2.5.7.5 Ligation of digested expression vector with amplified product

The digested eluted plasmid DNA of TA clone was ligated into the digested eluted DNA of Expression vector. In the 15 μl ligation reaction, following components were added in the microcentrifuge tube.

<table>
<thead>
<tr>
<th>Components</th>
<th>1.5 μl</th>
<th>8 μl</th>
<th>3 μl</th>
<th>1 μl</th>
<th>1.5 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested Expression vector DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digested TA Clone DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ligation buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclease free water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The ligation mixture was incubated at 4 °C for overnight.
2.5.7.6 Transformation

15 µl of ligated product was transformed in 200 µl of DH5α competent cells on amp-plate, similarly as described previously and incubated overnight at 37 °C.

2.5.7.7 Confirmation of clone

The positive colonies were screened and master plate was prepared on new amp-plate and incubated overnight at 37 °C. The colonies were then checked by colony PCR. The amplified product was resolved in 1% agarose gel and visualized under U.V. light, amplification denoted insertion of gene in the vector (positive result).

2.5.7.8 Isolation of plasmid from transformed DH5α cells

From the master plate the transformed cells were inoculated into 6 ml LB-amp vial aseptically which was incubated overnight at 37 °C in a shaker incubator at 200 rpm and plasmid was isolated from the culture. Isolation of plasmid using Quick Plasmid Mini Prep Kit (INVITROGEN) was same as described above.

2.5.8 Over expression of DIM-1 in expression cell (BL21-DE3 cells)

*E. coli* (BL21-DE3) cells are one of the most widely used expression hosts, and DNA is normally introduced in a plasmid expression vector. Over expression in *E. coli* are well developed and work by increasing the number of copies of the gene or increasing the binding strength of the promoter region so assisting transcription (Baneyx, 1999). Steps of over expression of the protein are as follows:

2.5.8.1 Transformation of isolated plasmid in BL21-DE3 cells

1.5 µl of isolated plasmid was finally transformed in 200 µl of BL21. Competent cells on amp-plate was incubated overnight at 37 °C. Master plate was prepared on new amp-plate and incubated overnight at 37 °C.

2.5.8.2 Confirmation of clones by colony PCR

The positive colonies were screened and master plate was prepared. The colonies were then checked by colony PCR (H Mirhendi, 2007). The amplified product was resolved in 1%
agarose gel and visualized under U.V. light. Amplification indicated transformation of plasmid in BL21 cells (positive result).

2.5.8.3 Expression of proteins from transformed E. coli BL21-DE3 cells

5 ml of overnight incubated primary culture of transformed BL21-DE3 cells was added into 250 ml of LB broth (secondary cultures) containing ampicillin followed by incubation in shaker incubator (200 rpm) at 37 °C till O.D. reached to 0.6-0.8. Secondary culture was induced with 1 mM IPTG (250 µl/250 ml culture, stock conc - 1M, pH 7.2) and further incubated in 200 rpm shaker incubator at 25 °C for 4 hr. The culture was then centrifuged in 50 ml tube at 5,000 rpm for 5 min in cold (4 °C).

2.5.9 Checking of protein solubility

The induced culture pellet was suspended in lysis buffer (50 mM Tris, 250 mM NaCl, pH 8.0) containing 10 mM imidazole, Triton-X and supplemented with 1000 uM protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Sigma–Aldrich, St. Louis, USA) followed by incubation of the suspension with 1 mg/mL lysozyme (Sigma–Aldrich, St. Louis, USA) for 30 min in ice. The bacterial cell suspension was pulse sonicated in ice for 10 x 20 sec with 10 sec intervals between two pulses and centrifuged at 2500 g for 30 min at 4 °C.

The above supernatant and pellet was collected. The 10 µl supernatant was loaded run on SDS-PAGE and remaining supernatant was loaded on Ni-column whereas pellet was further processed. Pellet was again resuspended in binding buffer and PMSF (1 mM) and lysozyme (100 µg /ml) was added into it. The pellet was again sonicated under chilled condition until clear solution was obtained and centrifuged at 15000 rpm for 15 min at 4 °C. Supernatant was transferred to a fresh tube and filtered with 0.45 µm membrane filter. Supernatant (10 µl) was resolved on SDS-PAGE and remaining supernatant was loaded on Ni-column following the method described by (Davis et al., 1999; Wilkinson & Harrison, 1991). In order to check whether express protein was cytosolic or membrane bound, both supernatant and pellet was resolved on 10% SDS-PAGE (Davis et al., 1999; Sambrook & Russel, 2001).
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide is a polymer of acrylamide cross linked by bis-acrylamide. The pore size of polyacrylamide gel matrix is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%, and the ratio of acrylamide to bis-acrylamide is approximately 3:1 ratios. The proteins get separated from each other on the basis of both size and charge as they move inside the gel matrix by the force exerted due to electrostatic field generated by two electrodes at opposite ends.

This variation of PAGE is used to separate proteins on the basis of their mass, by electrophoresis in a polyacrylamide gel in the presence of Sodium dodecyl sulphate (SDS), an anionic detergent. SDS \([\text{CH}_3 (\text{CH}_2)_{11}\text{OSO}_3\text{Na}]\) denatures the proteins and disrupts all non-covalent interactions in the native proteins i.e. all the proteins are reduced into their primary structure in linear form. In the presence of excess SDS the protein binds to SDS and become negatively charged. Protein-SDS complexes therefore move towards the anode during electrophoresis and owing to the molecular-sieving properties of the gel, their mobility (and therefore the distances they migrate in a given time) is inversely proportional to the \(\log_{10}\) of their molecular weights. The small molecules run first followed by bigger size of molecules. \(\beta\)-mercaptoethanol is added to reduce the disulphide bridges in the proteins.

The electrophoresis was carried out in between two glass plates sealed at the base. There are two types of gels in between the plates separating gel, and a small layer of stacking gel on top. The two gels differ in the concentration of acrylamide and their pH. The buffer used for electrophoresis was Tris-Glycine buffer. Differences in pH and composition between these two gels caused the samples concentrated into narrow bands before separation occurred during migration through the resolving gel (Fig 3.11).
Materials and Methods

Reagents

Solution 1 (30% Acrylamide solution)

- Acrylamide 30 g
- Bis-acrylamide 0.8 g

Both solutes were dissolved first separately (~45 ml each), then mixed together, filtered, made up the final volume to 100 ml and stored at 4 °C in dark bottle.

Solution 2 (0.75M Tris, pH 8.8)

- Tris 9.075 g

Tris was dissolved in 75 ml TDW, adjusted pH to 8.8 with 1 N HCl and made the final volume to 100 ml and then added 0.2 g SDS on the top without disturbing.

Solution 3 (1.25 M Tris, pH 6.8)

- Tris 15.142 g

Tris was dissolved in about 75 ml TDW, pH adjusted to 6.8 with 1 N HCl and made up the final volume to 100 ml, SDS 0.1 g was added on the top without disturbing and stored at 4 °C.

Fig. 3.11: Protein separation by SDS-PAGE
10% Ammonium persulphate (APS)

APS 100 mg

APS was dissolved in 1 ml TDW just before use.

Sample buffer (2x stock) 2 M Tris, pH 6.8

Tris 15.142 g

Dissolved the material in about 25 ml TDW, adjusted pH to 6.8 with 1 N HCl, made up the final volume to 30 ml.

Loading buffer (2x)

Stock sample buffer 3 ml
SDS 200 mg
β-Mercaptoethanol 0.5 ml
Glycerol 1 ml

Dissolved the ingredients separately, then mixed, filtered and made up the final volume to 5 ml, added a pinch of bromophenol blue.

Electrode buffer (10X)

Tris 12.129 g
Glycine 57.69 g
SDS 49 g

Dissolved the biochemicals separately, then mixed, filtered and made up the final volume to 400 ml.

Sample preparation

For culture: Pellet were mixed with loading buffer and heated in boiling water bath for 10 min. Mixture was centrifuged at 5000g for 5 min. Supernatant was loaded in well for separation.

For purified recombinant protein: The protein stock eluted through Ni-column was mixed with Sample Loading Buffer in 1:1 ratio and heated in boiling water bath for 10 min. The samples were then kept in ice till loading into the wells.
Procedure

Supernatant of culture and Purified protein was resolved in 10% gels (Laemmli, 1970). Preparative (10×10 cm slab) gels were prepared and extract was run in gel with a dual-gel electrophoresis chamber (MiniVE, Amersham Biosciences, NJ, USA). 200 µg of the sample was loaded per gel and molecular weight marker (SM0671; Fermentas Life Sciences, USA; Mol. Wt. range: 170-10 kDa with rDIM-1bm) was run simultaneously, alongside.

➤ Preparation of gel

The resolving and stacking gels were prepared according to the standard protocol. SDS PAGE was run using vertical gel electrophoresis. The wells were loaded with the test samples and allowed to run at 50 mA.

<table>
<thead>
<tr>
<th></th>
<th>Resolving gel (10%)</th>
<th>Stacking Gel (3.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1</td>
<td>3.4 ml</td>
<td>220 µl</td>
</tr>
<tr>
<td>Solution 2</td>
<td>4.5 ml</td>
<td>--</td>
</tr>
<tr>
<td>Solution 3</td>
<td>--</td>
<td>200 µl</td>
</tr>
<tr>
<td>APS</td>
<td>290 µl</td>
<td>66 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>TDW</td>
<td>1.2 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Total</td>
<td>9 ml</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

➤ CBB staining for visualization of proteins in gel

Reagents

Staining solution 0.5 gm Coomassie Brilliant Blue R-250 dissolved in 100 ml of destaining solution

Destaining solution 40% methanol and 10% acetic acid in TDW

The gel was kept in the staining solution for 3 hr with gentle shaking on the rocker and transferred to destaining solution for overnight or extended the time till each band of protein was clearly visible.
2.5.10 Purification of recombinant DIM-1 of *B. malayi* (rDIM-1bm) by Ni–NTA-column (affinity chromatography)

The protein purification system is based on remarkable selectivity of Ni–NTA (Nitrilotriacetic acid) resin for recombinant proteins carrying a small affinity tag consisting of 6 consecutive histidine residues. The high affinity of Ni–NTA resins for 6-x-His tagged proteins or peptides is due to both the specificity of the interaction between histidine residues and immobilized nickel ions and to the strength with which these ions are held to the NTA resin. NTA has a tetradequate chelating group that occupies four of six sites in the nickel coordination sphere and as a result the proteins are strongly bound to the resin (Fig. 3.12).

**Reagents**

Freshly prepared reagent was used for purification of proteins by Ni-column (affinity chromatography).

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
</table>
| Equilibrium Buffer/Lysis Buffer/Binding Buffer | 20 mM Tris (pH 7.5)  
50 mM NaCl  
5 mM imidazole |
| Washing buffer I                 | 20 mM Tris (pH 7.5)  
50 mM NaCl  
10 mM imidazole |
| Washing buffer II                | 20 mM Tris (pH 7.5)  
50 mM NaCl  
20-80 mM imidazole |
| Elution buffer                   | 20 mM Tris (pH 7.5)  
50 mM NaCl  
250 mM imidazole |
| Charging buffer                  | 50 mM NiSO₄  |
| Strip buffer                     | 20 mM Tris (pH 8)  
50 mM NaCl  
100 mM EDTA |
Materials and Methods

Approximately 5 ml of Ni\textsuperscript{2+} NTA slurry was filled in a 10 ml column fitted with filter disc. Allowed slurry to settle and wash column with 100 ml of water and equilibrated the column with 10 ml of binding buffer supplemented with protease inhibitor cocktail. The 10 ml protein sample was loaded on Ni-column and incubated for 1 hr. The flow of supernatant collected and added 10 ml washing buffer I (10 mM imidazole). The flow of washing buffer I was collected. The column washed with 10 ml volume of washing buffer II (50 mM imidazole) and collected the flow of washing buffer II. The protein eluted in 10 ml elution buffer (300 mM imidazole) and collected the flow of elution buffer as 1 ml fraction each. After elution, the column was then equilibrated with binding buffer and stored at 4 °C for reuse in future.

To reuse the column, the Ni-column (5 ml) was washed with 50% ethanol and then 100 ml of MQ was passed through the column in order to remove traces of ethanol from the column. The column was first of all cleaned with strip buffer and then 100 ml of MQ. 5-10 ml of charging buffer (50 mM NiSO\textsubscript{4}) was added to charge the column. The column was then equilibrated with binding buffer. Then the protein sample was loaded on Ni-column. Each fraction collected during purification was checked on 10% SDS-PAGE for presence, yield and level of purification of the protein (Sambrook & Russel, 2001; Wilson & Walker, 2005).
2.5.11 Protein estimation by Bradford method

An estimation of protein concentration is essential to be done rapidly and accurately in many fields of protein study. The Bradford assay has become the preferred method for quantifying protein (Bradford, 1976).

The Bradford assay relies on the binding of the dye Coomassie Blue G-250 to protein. The quantity of protein can be estimated by determining the amount of dye in the blue ionic form (Bradford, 1976). This is usually achieved by measuring the absorbance of the solution at 595 nm. In 96-well flat bottom plates, 10 µl of sample was poured followed by addition of 100 µl of Bradford reagent. O.D. was taken at 595nm in ELISA plate reader (PowerWave X, Bio-
Tek instruments Inc. USA). A set of standard solution of BSA at different concentration was prepared and put in the same manner alongside the test samples as shown in Fig. 3.1. The amount of protein was calculated by projecting the O.D. values in the standard curve. The graph was plotted from the O.D. obtained corresponding to various concentrations. The concentration of test sample was calculated by projecting the value on to the straight line.

<table>
<thead>
<tr>
<th>conc of BSA (mg/ml)</th>
<th>Mean O.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.018</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0285</td>
</tr>
<tr>
<td>0.4</td>
<td>0.049</td>
</tr>
<tr>
<td>0.6</td>
<td>0.073</td>
</tr>
<tr>
<td>0.8</td>
<td>0.102</td>
</tr>
<tr>
<td>1</td>
<td>0.1215</td>
</tr>
<tr>
<td>1.2</td>
<td>0.138</td>
</tr>
<tr>
<td>1.4</td>
<td>0.172</td>
</tr>
</tbody>
</table>

**Fig. 3.13:** Protein estimation using BSA as standard

### 2.5.12 Western blotting for transfer of resolved proteins on nitro cellulose paper (NCP)

This technique was originally developed for Southern DNA blotting. Proteins can be transferred by electrophoresis from various media, such as polyacrylamide gel to NCP or nylon under the influence of electric current. The proteins are immobilized on the membrane, providing a blot that can be probed by various means. Such a blot is called a Western blot. It permits the individual polypeptides in a complex mixture to be analyzed in various manners.

The resolved components of rDIM-1bm was transferred to NCP using a wet electroblotter (TE22, Amersham Biosciences, NJ, USA) with a constant current (50 mA) at 4°C (Towbin et al., 1979).
Reagents

Blotting buffer (pH 8.5)-(10X)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>30.30 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>144 g</td>
</tr>
<tr>
<td>SDS</td>
<td>10 g</td>
</tr>
</tbody>
</table>

Made up the volume to 1000 ml by TDW.

Working solution (Transfer buffer)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blotting buffer (10X)</td>
<td>30 ml</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>60 ml</td>
</tr>
</tbody>
</table>

Made up the volume upto 300 ml with TDW.

Procedure

- After electrophoresis the gel was equilibrated in transfer buffer.
- While the gel was equilibrating, a piece of nitrocellulose membrane was cut to the same dimensions as the gel. Wet the membrane was transfered by sliding it slowly at a 45° into the buffer and soaked.
- Two pieces of blotting paper were cut to the dimension of the gel. The paper was completely saturated by soaking in transfer buffer.
- Assembled the blotting paper, nitrocellulose membrane (NCP), and the gel in the transfer apparatus.
- Preparation of cassette for protein transfer.
  - Placed one piece of the presoaked blotting paper onto the scrub pad in transfer apparatus.
  - The gel was placed at cathode side and NCP on the anode side.
  - Placed the presoaked nitrocellulose membrane on top of the gel.
  - Removed the bubbles trapped between the NCP and gel by rolling glass pipette over it.
Materials and Methods

- Placed the second sheet of presoaked blotting paper on top of the NCP.
- Assembled the cassette and transferred onto the chamber filled with transfer buffer.
- ~50 mA was supplied for 3 hr.

Following completion of the transfer discarded the blotting paper and NCP was air dried on blotting paper and stored at −20 ºC for further use.

2.5.13 Confirmation of r-protein with anti-histidine antibody in western blot

NCP membrane with rDIM-1bm protein was incubated, with 3% BSA (Calbiochem, USA) prepared in Tris-buffered saline (TBS), at room temperature for 2 hr blocked. Thereafter the membrane was washed with TBS-Tween-20 (TBS-T20) and incubated with 1:2500 dilution of mouse anti-His antibody (Thermo scientific, USA) at 37 ºC for 90 min followed by washing and incubation, with 1:10,000 dilution of rabbit anti-mouse IgG-(H+L) conjugated with HRP (mp biomedicals, USA), at room temperature for 2 hr. Blot was developed with the substrate solution (citric acid and Na2HPO4) containing chromogenic substance 3-3-Diamino benzidine (DAB; Sigma, USA) prepared in TBS and H2O2 and bands visualized (Misra et al., 1993; Singh et al., 2009). The reaction was stopped by adding TDW. The strips were then dried, mounted on the drawing paper, and the images were captured by gel documentation system (Bio-Rad, USA). The molecular weight analysis was done using the software Quantity One® (Bio-Rad, USA) with the help of prestained molecular weight marker run alongside the gels. Single band of ~36 kDa represented the purified r-protein.

2.5.14 Bacterial endotoxins test for recombinant DIM-1

Bacterial endotoxin is the lipopolysaccharide (LPS) component of cell wall of Gram-negative bacteria. It is pyrogenic, and when injected into the mammalian body causes a variety of symptoms, the most recognizable of which is an increase in core body temperature.

The LAL test is a method of the Bacterial Endotoxin Test (BET), for detecting the level of Gram-negative bacterial endotoxin in a given sample or substance (Guy, 2003). The details of reactions in endotoxin assay are given below in Fig. 3.14.
Materials and Methods

The units of measurement for the LAL test are Endotoxin Units (EU). These are a measure of the activity of the endotoxin. Endotoxins differ in their biological activity or potency; the pyrogenicity or LAL reactivity of one endotoxin preparation may be very different from that of another of the same weight. The LAL test today is more robust, it remains open to a degree of variation (McCullough & Weidner-Loeven, 1992).

The purified rDIM-1bm was tested for presence of endotoxin using E-TOXATE® (Limulus Amebocyte Lysate) test kit (Sigma, USA) according to manufacturer’s instructions.

Reagents

- **E-toxate single test vial (Sigma, USA)**
  
  Dry concentrate from *Limulus polyphemus*. Sensitive from 0.05 to 0.1 endotoxin units (EU) per vial. Stored below 0 °C. Test material n solution form was added directly to dry vial.

- **Endotoxin free water (Autoclave triple distilled water)**
  
  Pyrogen free water stored at room temperature was used in order to avoid any contamination of exogeneous endotoxin.

Procedure

The assay was performed directly in the vial of E-TOXATE (Sigma, USA).

- Aluminium seal was removed and rubber stopper loosened after labeling.
Materials and Methods

- Added test sample (0.2 ml) and water (0.2 ml) to the test vials. By lifting rubber stopper carefully from each vial, solutions were added directly to the bottom of the tube and stopper was again placed.
- Vortex-mixed each vial briefly to ensure homogeneity. Excessive mixing and foaming was avoided. The tubes were kept undisturbed for 1 hr at 37 °C.

Reading

After 1-hr incubation, gently removed tubes/vials one at a time and slowly inverted at 180 degrees while observing the evidence of gelation. A positive test is the formation of a hard gel which permits complete inversion of the vial without disruption of the gel. All other results: soft gels, turbidity, increase in viscosity, clear liquid, are considered negative.

Interpretation of Results:  

(+) = HARD GEL  

(−) = Absence of HARD GEL

Our eluted proteins were found free from endotoxin as evidenced by very soft gels.

2.6 Sequence and phylogenetic analysis

The sequence of amplified product was analyzed with Basic Local Alignment Search Tool (BLASTx) against non redundant protein sequences in NCBI (Altschul et al., 1997; Gish & States, 1993). Sequence of rDIM-1bm was translated in possible reading frames using ExPASy translation tool (http://web.expasy.org/translate/) to determine the corresponding amino acid sequence, molecular weight of protein and pI value (Gasteiger et al., 2003). Protein sequences of DIM-1 from the different nematodes available in NCBI database (B. malayi: XM_001899486.1; Caenorhabditis elegans: NP_001024421.1; Loa loa: XM_003137552.1; A. suum: ADY47722.1) were aligned using Clustal W software. The phylogenetic trees were constructed based on the protein sequence alignments with Maximum Likelihood (ML) Method using PROML programs of PHYLIP package version 3.69 with global rearrangements and randomized input order options. Sequence was submitted to SMART server (http://smart.embl-heidelberg.de/) for domain prediction.

2.7 In silico modeling studies

Initially blastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) against protein data bank was carried out to search for putative templates. Crystal structure (pdb-id-3dmk) of
Down syndrome cell adhesion molecule (DSCAM) of *Drosophila melanogaster* (Sawaya *et al*., 2008) showed 24.42% sequence identity with DIM-1bm and selected as template to build homology model. Modeller9v10 (Eswar *et al*., 2007) was used to build 20 models based on pair wise Clustal W alignment of query and template. Considering its low sequence identity with available templates, we also used fold recognition server Phyre² (http://www.sbg.bio.ic.ac.uk/Phyre²/) for better prediction of 3D structure of DIM-1bm. At very low sequence identity, protein model can be constructed and may be used for further *in silico* analysis (Matsuse *et al*., 2013 ; Rabbani *et al*., 2012). Constructed models were submitted to structural analysis and verification server (http://nihserver.mbi.ucla.edu/SAVES/) for assessment of stereo-chemical qualities.

### 2.8 ImmunoLocalization of DIM-1 in *B. malayi* Adult Parasite by Confocal Microscopy

Over the past ten years, confocal microscopy has developed from a technique limited to specialists in microscopy into a standard research tool in cell biology. Initiated by the discovery of green fluorescent protein (GFP) in the early 1960s, this enabled investigators to apply molecular cloning methods, fusing the fluorophore to various proteins in order to monitor cellular processes in living systems, confocal microscopy gained importance to become an essential technique. The most common application of confocal microscopy is to compare the localisation and behaviour of relevant signaling molecules in one single cell using either fixed cells or live cell imaging. Such studies have been made possible by the development of confocal microscopes that are capable of efficiently collecting multiple colours of fluorescence and developing new dyes beside GFP that have extended the useful spectrum of fluorescence microscopy.

To determine DIM-1 distribution in the parasites by immunolocalization, adult parasites were harvested from peritoneal cavity of *B. malayi* infected jirds (Dixit *et al*., 2004; Murthy *et al*., 1997). Highly motile adult worms were washed several times with sterile phosphate buffered saline (PBS; pH 7.2) containing antibiotics (penicillin 100 U/ml; streptomycin: 100 μg/ml). Adult worms were fixed over night in 4% phosphate-buffered formaldehyde (PBF; pH 7.2) at room temperature. The fixed worms were dehydrated in series of graded alcohols (70%, 95% and 100%), cleared in xylene and embedded in paraffin wax. The paraffin blocks were sectioned by microtome and 3 μm thick sections were used for localization of the protein. Slide
containing sections were treated with xylene to remove paraffin wax and sections were washed 4-5 times with TDW to remove xylene.

For immunofluorescence, Slide containing sections were washed in washing/blocking solution (0.02% NaN₃, 3% BSA, 0.5% w/v glycine in PBS; pH 7.2) and left in blocking solution for 2-3 hr at RT. Sections were incubated with primary antibody (raised in mouse against rDIM-1bm) at dilution (1:20 in blocking solution) for overnight at 4 °C. Sections were washed with washing solution for 4-5 times properly. Further, secondary antibody alexa flour 488 dye-conjugate (Invitrogen, USA; 1:1000 in blocking solution) was added to the sections and incubated for 2 hr. In order to avoid background staining the sections were washed thoroughly with washing solution for 5-7 times to remove unbound secondary antibody. Sections were then mounted in DPX and the images were captured with a Zeiss LSM 510 META (Zeiss, Jena, Germany) confocal laser scanning microscope equipped with a plan-apochromat 20X objective. For excitation of alexa flour 488 dye-conjugated secondary antibody, argon laser lines of 488 nm were used. For control sections, the same procedure was applied by using primary antibody from non-immunized animals (Vieira et al., 2012).

2.9 Reactivity of antibodies to rDIM-1bm with resolved fractions of mf, L₃, L₄ and adult worm stages by immunoblotting

To find out the presence of DIM-1 in various life-stages of B. malayi, the crude extracts derived from adult worms, L₄, L₃ and mf were run on 10% SDS-PAGE followed by Western blotting.

2.9.1 Isolation of parasites (adult and L₄) and preparation of parasite extract

B. malayi adult worms and L₄ were isolated from p.c. of jirds harbouring 5 - 6 months and 22 days old infection, respectively (Dixit et al., 2004; Murthy et al., 1997). Soluble extract of B. malayi adult worm (BmA) and L₄ stage of parasite was then prepared as described by (Murthy et al., 2008; Tandon et al., 1988) with some modifications. Briefly, the parasites were washed 3 - 4 times in sterile PBS (pH 7.2) containing antibiotics (penicillin 100 U/ml; streptomycin: 100 µg/ml) and homogenized in a porter Elvejhm tissue grinder (A. Thomas Scientific, Philadelphia, PA) at 4 °C. The homogenate was sonicated (by Soniprep 150, UK)
Materials and Methods

for 10 cycles of 30 sec each at 10 micron amplitude with intermittent gap of 1 min at 4 °C. Protein content was measured by the method of (Bradford, 1976).

2.9.2 Isolation of L₃ and preparation of parasite extract

L₃ extract was prepared according to the method described by (Murthy et al., 1983). Briefly, L₃ of B. malayi were isolated from laboratory bred A. aegypti mosquitoes which were fed on infected M. coucha showing high microfilaraemia. Parasites were thoroughly washed in PBS and homogenized (5000 rpm) for 5 min followed by pulsed sonication (8 pulses of 15–20 sec each at 1–2 min intervals) at 10kc for 2–3 min at 4 °C. Protein content was measured by the method of (Bradford, 1976).

2.9.3 Isolation of mf and preparation of mf extract

B. malayi mf were isolated from p.c. of jirds harbouring 5 - 6 months old infection (Murthy et al., 1997). Mf were washed 3 - 4 times in sterile PBS (pH 7.2) containing antibiotics and homogenized as above. The homogenate was then sonicated (by Soniprep 150, UK) for 10 cycles of 10 sec each at 10 micron amplitude with intermittent gap of 1 min at 4 °C. Protein content was measured by the method of (Bradford, 1976).

2.9.4 Fractionation of different stages of B. malayi by SDS-PAGE

SDS-PAGE was carried out as described by (Laemmli, 1970). Briefly, the extracts were mixed in sample buffer [Bromphenol Blue (10 mg/ml), SDS (4.4%), Tris-Cl (0.5 M), beta mercaptoethanol (300 mM)] and heated at 95 °C for 5 min followed by resolved in 10% SDS-PAGE. Prestained molecular weight marker (SDS7B; Sigma, USA) was also run simultaneously.

2.9.5 Immunoblotting of resolved component of mf, L₃, L₄ and BmA with sera of rDIM-1bm immunized M. coucha

Resolved proteins of different stages of parasite extracts were transferred to nitrocellulose membrane (NCP, porosity: 0.22µ; Millipore, India) using a wet electroblotter according to the method described by (Towbin et al., 1979). NCP membrane carrying the resolved proteins of adult, L₄, L₃ and mf extract was cut vertically into 4-mm wide strips and blocked with 3% BSA in 10 mM Tris-buffered saline TBS (pH 7.4) at 4 °C for overnight. The
blots were then washed with TBS-T$_{20}$ (0.05%) and incubated with sera (1:10 in BSA-TBS-T$_{20}$) of rDIM-1bm immunized and non-immunized animals at 37 °C for 90 min. The blots were then washed and incubated with anti-mouse IgG-conjugated with HRP (Sigma, USA; 1:1000). Substrate solution (citric acid and Na$_2$HPO$_4$) containing chromogenic reagent 3-3-Diaminobenzidine (DAB) prepd in TBS and H$_2$O$_2$ was added and the bands were visualized. The reaction was stopped with TDW; the strips were dried and analyzed. The molecular weight range of SDS-PAGE resolved proteins and immunoreactive bands in western blots were determined with the help of standard molecular weight marker (SDS 7B, Sigma) using Quantity One® (Biorad, USA) software in gel documentation system (Bio-Rad, USA) (Joseph et al., 2011).

2.10 Immune responses of rDIM-1bm immunized animals

2.10.1 Grouping and immunization of animals

In this study, 12 groups of animals were divided into 3 different immunization schedules. Each schedule consisted of 4 groups, and each group comprised of 5-6 animals in two experiments.

2.10.1.1 Immunization and experimental schedule

Immunization of animals of various groups and experimental schedules are described in Table 2.1.

(i) 3-Dose immunization schedule (standard immunization protocol for vaccination)

Two Groups of *M. coucha* received 3 injections of the rDIM-1bm (50 g/animal) or PBS through subcutaneous (s.c.) route. The first injection was in Freund’s complete adjuvant (FCA) on day 0. The two booster doses were half the amount of protein mixed with Freund’s incomplete adjuvant (FIA) and administered on days 14 and 21.

To see effect of immunization with rDIM-1bm on subsequently introduced infection, two groups of animals, immunized (rDIM-1bm+FCA/FIA) and non-immunized/control (PBS+FCA/FIA) received 100 L$_3$ on day 28 post first immunization (p.f.i.) through s.c. route (Table 3.1).
(ii) 3-Week repeated immunization schedule (non-standard procedure)

Four groups of *M. coucha* were included in this group. Two groups received rDIM-1bm (10 μg r-protein) or PBS at 3 or 4 days interval (i.e. twice a week) for 3 weeks. The first and subsequent doses were administered as described above. One group each from immunized (rDIM-1bm+FCA/FIA) and non-immunized/control (PBS+FCA/FIA) (Table 3.1).

(iii) 6-Week repeated immunization schedule (non-standard procedure)

The 6-week immunization schedule followed the same protocol as described above for 3-week immunization schedule (Table 3.1) except that in this repeated injections were given for 6 weeks.

2.10.2 Assessment of infection status

(i) Microfilaraemia

Microfilaraemia was monitored in L₃ exposed groups on day 90 post larval (L₃) inoculation (p.l.i.) and continued till day 205 p.l.i. Blood (10 μl) was drawn from animals between 12.00 noon and 1.00 p.m. and smears prepared, air dried for overnight, dehemoglobinized with tap water and stained with leishman stain. The stained smears were then examined under microscope for the presence of mf and counted (Murthy *et al.*, 1983).

(ii) Worm burden

On day 205 p.l.i. all the infected animals were killed; heart, lung, and testes from all the animals were isolated. Tissues were teased gently to avoid any damage to the parasites. Parasites were counted and examined under microscope for physical status of the worms to find out if there is any change in motility, cell adherence on their surface, death or calcification of the worms and recorded. All the surviving females were teased individually in a drop of saline to examine condition of intrauterine mf stages of the parasite (Gaur *et al.*, 2007). Number of worms recovered from the immunized infected animals was compared with that of control animals and percent alteration of worms calculated. Percent sterilization of female worms was determined over total live female worms recovered from treated or control animals.
### Table 3.1: Grouping of animals and experiment schedule

#### i. 3-Dose immunization schedule (standard immunization protocol for vaccination)

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization (1st dose: 50μg/animal)</th>
<th>Interval</th>
<th>Immunization (2nd dose: 25μg/animal)</th>
<th>Interval</th>
<th>Immunization (3rd dose: 25μg/animal)</th>
<th>Interval</th>
<th>L₃ exposure by</th>
<th>Killed on day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Im</td>
<td>PBS + FCA</td>
<td>14 Days</td>
<td>PBS+FIA</td>
<td>7 Days</td>
<td>PBS+FIA</td>
<td>7 Days</td>
<td>No infection</td>
<td>7 p.l.a.</td>
</tr>
<tr>
<td>rDIM-1bm-Im</td>
<td>rDIM-1bm+ FCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No infection</td>
<td>7 p.l.a.</td>
</tr>
<tr>
<td>Non-Im+L₃</td>
<td>PBS + FCA</td>
<td>3 Days</td>
<td>PBS+FIA</td>
<td></td>
<td>PBS+FIA</td>
<td>100/animal</td>
<td>205 p.l.i.</td>
<td></td>
</tr>
<tr>
<td>rDIM-1bm-Im+L₃</td>
<td>rDIM-1bm+ FCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### ii. 3-Week repeated immunization schedule (non-standard procedure)

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization (1st dose: 10μg/animal)</th>
<th>Interval</th>
<th>Immunization Subsequent doses (5μg/animal) at every 3rd or 4th day till 3 weeks</th>
<th>L₃ exposure by</th>
<th>Killed on day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Im</td>
<td>PBS + FCA</td>
<td>3 Days</td>
<td>/subsequent doses (5μg/animal) at every 3rd or 4th day till 3 weeks</td>
<td>No infection</td>
<td>3 p.l.a.</td>
</tr>
<tr>
<td>rDIM-1bm-Im</td>
<td>rDIM-1bm+ FCA</td>
<td></td>
<td></td>
<td>No infection</td>
<td>3 p.l.a.</td>
</tr>
<tr>
<td>Non-Im+L₃</td>
<td>PBS + FCA</td>
<td></td>
<td></td>
<td>100/animal</td>
<td>205 p.l.i.</td>
</tr>
<tr>
<td>rDIM-1bm-Im+L₃</td>
<td>rDIM-1bm+ FCA</td>
<td></td>
<td></td>
<td>100/animal</td>
<td>205 p.l.i.</td>
</tr>
</tbody>
</table>

#### iii. 6-Week repeated immunization schedule (non-standard procedure)

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization (1st dose: 10μg/animal)</th>
<th>Interval</th>
<th>Immunization Subsequent doses (5μg/animal) at every 3rd or 4th day till 6 weeks</th>
<th>L₃ exposure by</th>
<th>Killed on day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Im</td>
<td>PBS + FCA</td>
<td>3 Days</td>
<td>/subsequent doses (5μg/animal) at every 3rd or 4th day till 6 weeks</td>
<td>No infection</td>
<td>3 p.l.a.</td>
</tr>
<tr>
<td>rDIM-1bm-Im</td>
<td>rDIM-1bm+ FCA</td>
<td></td>
<td></td>
<td>No infection</td>
<td>3 p.l.a.</td>
</tr>
<tr>
<td>Non-Im+L₃</td>
<td>PBS + FCA</td>
<td></td>
<td></td>
<td>100/animal</td>
<td>205 p.l.i.</td>
</tr>
<tr>
<td>rDIM-1bm-Im+L₃</td>
<td>rDIM-1bm+ FCA</td>
<td></td>
<td></td>
<td>100/animal</td>
<td>205 p.l.i.</td>
</tr>
</tbody>
</table>

**Group abbreviation:** Non-Im (Non-Immunized); rDIM-1bm-Im (rDIM-1bm-Immunized); Non-Im+L₃ (Non-Immunized +BmL₃ infected); rDIM-1bm-Im+L₃ (rDIM-1bm-Immunized+BmL₃ infected); PBS: phosphate buffered saline; s.c.: subcutaneous; p.l.a.: post last administration of antigen; p.l.i.: post L₃ inoculation.
2.10.3 Weekly body weight

Body weight of all the animals of groups (immunized and non-immunized with or without L₃ under each schedule) was taken weekly fortnightly interval. Weight of animals was taken by analytical balance (Contech instrument pvt. Ltd., India).

2.10.4 Rectal temperature

Body temperature is a basic physiological parameter, which is often measured in awake rodents by rectal probe thermometry (Lomax, 1966).

During the testing time, animals from all the groups were exposed to rectal probing procedures. The rectal measurement procedure was executed by lifting the tail and inserting a rectal probe thermometer (Olex thermometer, India), with minimal restraint achieved by holding base of the tail. This positioning allowed to obtain rectal temperature values from the same body location. Approximately 20 s were required to position the mouse for rectal probe insertion. After insertion, the probe remained in the rectum for 40 s, allowing for temperature stabilization (Bae et al., 2007). The rectal temperature measurement procedure was executed over one minute.

2.10.5 Hematology (at autopsy)

2.10.5.1 Collection of Blood

Blood was collected from heart of animals through cardiac puncture. This method was approved by the Institute animal ethics committee. Cardiac puncture is recommended for collection of good quality and large volume of blood from the experimental animals. During blood collection, animals were killed by anesthesia (Sodium pentothal). 5 ml syringe was used for blood collection. Blood samples were taken from the heart, preferably from the ventricle slowly to avoid collapsing of heart. Blood was transferred in a tube containing anticoagulant Ethylenediaminetetra-acetic Acid (EDTA, 1.2 mg of the anhydrous salt per ml of blood) (Paulose & Dakshinamurti, 1987; Yoburn et al., 1984).
2.10.5.2 Total leukocyte count (TLC)

The total white blood cell or leukocytes count is expressed as an absolute number (Dacie & Lewis, 2011). They are cells of the immune system involved in defending the body against both infectious disease and foreign materials. WBCs consist of different and diverse types of cells: Granulocytes or polymorphonuclear leukocytes (Neutrophil, Eosinophil, Basophil, Granulocyte, and Monocyte), Agranulocytes or mononuclear leukocytes.

2.10.5.3 Differential leukocyte count (DLC)

Routine DLC was done according to Dacie & Lewis (2011). The differential count were, expressed as the percentage of each type of cell, related to the total leukocyte count and the results were reported in absolute numbers \((\times 10^9/l)\) (Dacie & Lewis, 2011).

2.10.5.4 Packed cell volume (PCV)

The hematocrit also known as packed cell volume (PCV) or erythrocyte volume fraction (EVF), is the volume percentage \(\%\) of red blood cells in blood (Dacie & Lewis, 2011). It is considered an integral part of complete blood count results with hemoglobin concentration, white blood cell count, and platelet count.

2.10.5.5 Erythrocyte sedimentation rate (ESR)

The method for measuring the ESR was recommended by the International Council for Standardization in Haematology (ICSH). ESR is the measurement of the sedimentation of red cells in diluted blood after standing for 1 h in an open-ended glass tube of 30 cm length mounted vertically on a stand (Dacie & Lewis, 2011).

2.10.5.6 Haemoglobin concentration

The haemoglobin (Hb) concentration of a solution is estimated by measurement of its colour, by its power of combining with oxygen or carbon monoxide or by its iron content.

All the hematological experiments were carried out on the MS9 analyzer (Melet Schloesing Laboratoires, USA) and the results were presented as means and standard deviations (SD), and subjected to statistical analysis.
2.10.6  Humoral mediated immune response

Collection of serum

Animals were killed by sodium pentothal and blood was collected from heart and transferred into tubes. Blood in tubes were centrifuged at 1200 rpm for 10 min. Serum (supernatant) was transferred into another tube and repeated the same process and transferred into fresh tubes and stored at -20/-40 °C (Dacie & Lewis, 2011).

2.10.6.1 Determination of IgG and its subclasses

Filaria specific IgG and its subtypes were detected in sera of animals as described by the manufacturer and partly followed the method of (Sahoo et al., 2009). The ELISA plates (NuncMaxisorp, Denmark) were coated with rDIM-1bm (optimum conc.: 1µg/ml) in carbonate buffer (0.06 M; pH 9.6) overnight at 4 °C. After overnight incubation the antigen was drained out and washed twice with PBS (0.01 M; pH 7.4) and then blocked with 3% BSA in PBS (0.01 M; pH 7.4) for 2 hr. The optimally diluted sera of M. coucha were used (IgG and IgG its subclasses 1:100) and incubated at 37 °C for 90 min. After 3 washings with PBS-0.01% Tween 20 (T-20) the plates were incubated at 37 °C for 90 min with anti-mouse IgG (1:50.000), IgG1, IgG2a, IgG2b and IgG3 conjugated with HRP (BD Pharmingen, USA) at 1:1000 in diluent [PBS-BSA(1.5%)-T-20(0.01%)]]. After 3-4 washings as above, the reactions were developed by using OPD substrate (O-phenylene diamine dihydrochloride, Sigma-Aldrich, USA) prepared in Citrate buffer [Citric acid (0.1M) + Na₂HPO₄ (0.2 M) in equal volumes, pH 5.0; 0.08% w/v OPD and 0.08% v/v H₂O₂] and Color developed within 15 - 20 min was stopped by adding 2.5 M H₂SO₄ and absorbance was measured at 492 nm using ELISA reader (Power Wave X-Bio-Tek., USA).

2.10.6.2 Determination of specific IgE

Filaria specific IgE were detected in sera of animals following the method described by manufacturer (Bethyl Laboratories Inc., USA) with some modifications to suit our conditions (Ottesen et al., 1981). ELISA strips (NuncMaxisorp, Denmark) were coated with rDIM-1bm (optimum conc.: 0.5 µg/ml) prepared in carbonate buffer (0.06 M; pH 9.6) and kept for overnight at 4 °C. After overnight incubation the unbound antigen was drained out and washed twice with PBS-T₂₀ (0.01M; pH 7.4). After blocking with 1% BSA-PBS (150
µl/well), the wells were washed thrice with PBS-T20 (washing solution) and then sera (100 µl/well; 1:4 dilution) diluted in BSA-T20 were added and incubated for 90 min at 37 °C followed by 3 - 4 washes with PBS-T20 and again incubated with antimouse IgE conjugated with HRP (1: 1000, 100 µl/well) for 90 min at 37 °C. The plates were washed thrice with PBS-T20. The substrate containing OPD and H2O2 was then added. Color developed within 15 - 20 min was stopped by adding 2.5 M H2SO4 and absorbance was measured at 492nm using ELISA reader (Power Wave X-Bio-Tek., USA).

2.10.6.3 Determination of specific IgM

Filaria specific IgM were detected in sera of animals following the method described by manufacturer (Bethyl Laboratories Inc., USA) with some modifications to suit our conditions. The procedure was exactly same as above. Optimum conc. of rDIM-1bm, dilutions of sera and antimouse IgM conjugated with HRP used were 2 ug/ml, 1: 1:50 and 1:1000, respectively.

2.10.6.4 Determination of specific IgA

Filaria specific IgA were detected in sera of animals (Sahu et al., 2008). The procedure was exactly same as above. Optimum conc. of rDIM-1bm, dilutions of sera and antimouse IgA conjugated with HRP used were 1 ug/ml, 1: 1:50 and 1:1000, respectively.

2.10.7 Cell mediated immune response

2.10.7.1 Nitric oxide production by macrophages

➤ Principle

Nitrite is a stable end product of the auto-oxidation of nitric oxide in aqueous solution, nitrite combine with acidic Griess Reaction. The presence of nitrite in the media was quantified using the method based on the chromophore formation from the diazotization of Sulphanilamide by acidic nitrite followed by coupling with cyclic amines such as Napthylene diamine dihydrochloride (NEDD) (Thomas et al., 1997).
Materials and Methods

- **Isolation and stimulation of peritoneal macrophages of *M. coucha***

  Animals were killed by an overdose of anaesthesia. Peritoneal macrophages were isolated under aseptic condition. A small cut was made in the skin of middle abdominal part with scissors. The underlying peritoneum was left intact. The peritoneum surface was cleaned with 70% ethanol and then 4-5 ml of incomplete DMEM containing 0.005% EDTA was introduced by using 10 ml disposable syringe. Peritoneal fluid was collected and centrifuged. To lyse the RBC, if any, the suspension was treated with 0.87% NH₄Cl for 1-2 min at 4 °C and centrifuged at 800-1000 rpm for 5 min. The cells were then washed thrice with medium, suspended in complete DMEM medium containing 10% FBS and counted in haemocytometer. The viability of the cells was checked by 0.1% trypan blue exclusion method and counted using haemocytometer. The final concentration of cells required for experiment was adjusted to 2 x 10⁶/ml and dispensed in 48 well culture plate, (Nunclon™, Denmark) each well contained 0.5 million cells. After overnight incubation at 37 °C in 5% CO₂ atmosphere, the cells were examined under inverted microscope (Nikon Eclipse TS 100) to ascertain whether the cells were adhered on the surface of the wells and stretched, the medium was removed and replenished with fresh medium (500 µl/well). Macrophages were stimulated *in vitro* with rDIM-1bm (1 µg/ml) and LPS (1 µg/ml) and incubated for 48 hr at 37 °C in 5% CO₂ atmosphere (Sahoo et al., 2009).

- **Procedure of NO determination**

  NO determination was carried out as described by (Sahoo et al., 2009). 100 µl of 48 hr post culture supernatant were dispensed in 96 well plates (Nunclon™, Denmark). An equal volume of the freshly prepared Griess reagent consisted of mixture of 1% Sulphanilamide + 0.1% NEDD prepared in 2.5% H₃PO₄ was mixed with the culture supernatants and incubated at 37 °C for 30 min. Standard was also run simultaneously to calculate the concentration of nitrite. Standard NaNO₂ solution of 100 mM concentration was serially diluted to 0.5 mM NaNO₂. Absorbance was read at 550 nm and recorded.
2.10.7.2 Cellular proliferation (lymphocyte transformation test; LTT)

Lymphocyte transformation test (LTT) was used to assess cellular proliferative response. The test used for the study was according to the method described by (Joseph et al., 2011). On autopsy, spleen was collected from animals, teased in sterile RPMI-1640 medium containing antibiotics (streptomycin: 100 µg/ml and penicillin: 100 U/ml) and passed through 70 µM cell strainer (BD Falcon) to get cell suspension. The suspension was haemolyzed osmotically by treating with 0.87% NH₄Cl for 1-2 min at 4 °C, washed thrice and suspended in complete RPMI supplemented with 10% FBS (GIBCO). Viability of the cells was checked by 0.1% trypan blue exclusion method, counted using haemocytometer and final cell concentration adjusted to 2 x 10⁶ cells /ml. The splenocytes thus obtained were used for lymphocyte transformation test and cytokine stimulation assays.

Lymphocyte transformation test was carried out by using spleen cells. Cell suspension was dispensed (200 µl/well) in 96 well tissue culture plate (Nunclon™, Denmark). Wells (in triplicate) received rDIM-1bm (1 µg/ml) or Concanvalin A (Con A, 10 µg/ml) for in vitro stimulation of cells. Unstimulated cells were kept to serve as control. The plate was then kept in incubator (ThermoForma) at 37 °C in 5% CO₂ atmosphere. After 72 hr culture 1 µci/well ³H-Thymidine (BRIT, Mumbai) was added to the media followed by 16-18 hr incubation at the same atmosphere. The cells were harvested, suspended in scintillation fluid and emission of β-ray was quantified by scintillation counter (LS Analyzer, Beckman Inc.). Results were expressed as counts per minute (CPM).

2.10.7.3 Cytokine determination by ELISA

Splenocytes were isolated from various groups of animals as described above in LTT procedure. The cells (2 x 10⁶ cells/ml) were dispensed (500 µl per well) in 48 well tissue culture plates (Nunclon™, Denmark). rDIM-1bm (1 µg/ml) or Lipopolysachharide (LPS, 1 µg/ml) were used for in vitro stimulation. Unstimulated wells were kept to serve as control. The cells were then incubated at 37 °C in 5% CO₂ atmosphere for 48 hr. Various cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, IL-13 and IL-17) were assayed in 48 hr post stimulated (PS) culture supernatant using sandwich ELISA.
Materials and Methods

For the assay mouse monoclonal antibodies to TNF-α, IL-2, IL-4, IL-6, IL-10, IL-13 and IL-17 (BD Biosciences, USA) and IFN-γ (Pierce Endogen, Rockford, USA) were used in a paired antibody sandwich ELISA method following the manufacturer’s instructions with some modifications to suit our conditions (Murthy et al., 2000). Triplicates of wells for each sample were run. The concentration of the cytokines was calculated using O. D. readings for standards (suitable for the paired antibodies obtained from the above source).

2.10.7.4 Macrophage function test

Macrophage function of experimental *M. coucha* (receiving r-protein) as well as controls (not receiving r-protein) was assessed. Phagocytosis assay was performed broadly following the method described by (Liu et al., 1999). Peritoneal macrophage cells were harvested from peritoneal lavage using incomplete RPMI-1640 media containing antibiotics (100 U penicillin/ml, 100 µg streptomycin/ml) and 1% FBS. The cells so obtained were washed twice with the medium and re-suspended in complete medium fortified with 10% FBS and plated at 3x10^5 cells per well in 24 well tissue culture plates (Nunclon™, Denmark). Cells were incubated for 3 hr at 37 °C and 5% CO₂ atmosphere and thereafter non-adherent cells were removed by repeated washing with incomplete medium. 0.5µl fluorescent latex beads (in 0.5 ml of complete culture medium) were added to each well followed by incubation for 2 hr as above. After incubation the cells were washed with PBS to remove extracellular beads. Cells were detached by repeated pipetting in PBS and fixed in 4% formaldehyde. Fixed cells were then analyzed by Flow Cytometer and percent fluorescent cells were measured at 543 nm (excitation) and 610 nm (emission). Total phagocytic capacity was calculated as: % positive cells x mean channel fluorescence.

2.11 Histopathology of lymph nodes

Immediately after killing of animals (*M. coucha*) at the termination of experiments the superficial, inguinal and axillary lymph nodes were dissected out and fixed in 4% phosphate-buffered formaldehyde (PBF) at room temperature. The tissues were dehydrated in series of alcohols (70%, 95% and 100%), cleared in xylene and embedded in paraffin wax. The paraffin blocks were sectioned by microtome and sections (3 µm thick) stained with Celestin blue-eosin and Toluidine blue for general histology and mast cells respectively. The total area
of section and the number (per mm$^2$) and granularity of mast cells in lymph nodes were determined using CCD camera-acquired microscopic images of sections and a computer image analysis program (Scion Image) (Dixit et al., 2004).

2.12 Statistical analysis

Results were presented as mean ± S.D. of 5-6 animals/group in two experiments and the data were analyzed in GraphPad Prism 3.03 using Student’s ‘t’ test and Tukey’s multiple comparison tests. Differences with P<0.05 were considered significant.