CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

Chemicals of analytical grade were purchased from Sigma Chemical Company, St Louis, USA and Merck, Germany. Chemicals from HiMedia, Mumbai, India were used for media preparation. Restriction enzymes, T4 DNA ligase, Taq polymerase and Hybond membranes were procured from Amersham Pharmacia Biotech Asia Pacific, Hong Kong. Oligonucleotide primers were synthesised from Integrated DNA Technology Inc., (IDT), Lexington, USA. QIAquick PCR purification kit from Qiagen GmbH, Hilden, Germany was used to purify the PCR products and restriction enzyme digested plasmids and inserts. For small-scale purification of plasmids Wizard prep plasmid purification Kit was procured from Stratagene, USA. ABIprism dideoxy dye terminator cycle sequencing kit was purchased from Perkin Elmer, USA. C3 antigen detection kit was from Biocientífica S.A, Argentina.

2.2 BACTERIAL STRAINS, PLASMIDS

The T7 expression vector, pRSET A, and E.coli strains DH5α, BL21 (DE3)Plys S and BL21 were from Invitrogen, SanDiego, USA. The GST fusion vector pGEX 2T was from Amersham Pharmacia Biotech, UK. Genotypes of the E.coli strain used in the present study are given in Appendix 1. Maps of the plasmid vectors pRSET A and pGEX-2T are shown in Appendix 2.
2.3 ANIMALS USED

New Zealand white rabbits were obtained from King Institute, Chennai and were maintained under standard conditions with food and water *ad libitum*.

2.4 CULTURE MEDIA

Luria-Bertani (LB) broth was the most commonly used media for growing *E.coli*. To prepare LB broth, 10g of tryptone, 5g of sodium chloride and 5g of yeast extract were dissolved in 1L of distilled water and the pH was adjusted to 7.2 with 1N NaOH. 1.5% agar was added to the liquid broth to prepare solid medium. Media was supplemented with 100µg/ml of ampicillin. *Streptococcus pneumoniae* was grown in Todd Hewitt broth supplemented with 0.5% yeast extract.

2.5 BLOOD

5 ml of blood was collected with prior consent from patients attending a local hospital between the age groups 6 and 22. They were selected based on positive blood / csf cultures. The criteria for selection included fever with signs of meningitis, tachypnoea, inability to feed, respiratory distress and evidence of toxaemia. Relevant investigations carried out included microscopy, Gram stain, and culture. Anti-pneumolysin titres were detected by ELISA.

2.6 RECOMBINANT CLONES USED IN THE PRESENT STUDY

The recombinant clone pMSply1 expressing pneumolysin was cloned and characterised in our laboratory. This gene was amplified by PCR using pneumolysin gene specific primers designed using sequences available in GenBank and was cloned into plasmid pRSET A.
The domain 4 region of pneumolysin was cloned and expressed in the GST vector pGEX2T using insert specific primers. Map of the cloning vector pRSET A and pGEX2T are given in the Appendix 2. The sequence of the primers, their restriction sites and annealing temperature used for PCR amplification are shown in Table 2.1.

**Table 2.1 Sequence of the primers, restriction sites and annealing temperature**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 Forward</td>
<td>TAATACGACTCACTATAGGG</td>
<td>56°C</td>
</tr>
<tr>
<td>pRSET (Reverse)</td>
<td>TAGTTATTCAGCACGGGTGG</td>
<td>56°C</td>
</tr>
<tr>
<td>Ply1 (Forward)</td>
<td>CGCGGATCCATGGCAATAAGCA BamHI</td>
<td>52°C</td>
</tr>
<tr>
<td>Ply2 (Reverse)</td>
<td>CCCAAAGCTTATGCTATTTCTACCTTT HindIII</td>
<td>52°C</td>
</tr>
<tr>
<td>pGEX (Forward)</td>
<td>GGGCTGGCAAGCCACGTTGGTG</td>
<td>56°C</td>
</tr>
<tr>
<td>pGEX (Reverse)</td>
<td>CCGGGAGCTGATGTGTCAGAGG</td>
<td>56°C</td>
</tr>
<tr>
<td>Domain 4 (Forward)</td>
<td>CGCGGATCCAAACGGAGATTTCGCTGGGA BamHI</td>
<td>56°C</td>
</tr>
<tr>
<td>Domain 4 (Reverse)</td>
<td>CCGGAAATTCATGCTATTTTCTACCTATC EcoRI</td>
<td>56°C</td>
</tr>
</tbody>
</table>

2.7 **POLYMERASE CHAIN REACTION**

The pneumolysin gene was amplified by PCR on a MJ Minicycler, Watertown, Massachusetts, USA for cloning in T7 expression system. The domain 4 region of pneumolysin was also PCR amplified for cloning into the GST vector.
PCR was performed as follows. A reaction mixture containing

i. 200µM of each dNTPs
ii. 1X PCR buffer (50mM KCl, 10mM Tris.Cl, pH8.3)
iii 2.5mM MgCl₂
iv 0.5mM of each primer
v 1 unit of Taq DNA polymerase and
vi 20ng of template

were mixed. PCR was performed on a MJ Minicycler, Watertown, Massachusetts, USA. The optimal annealing temperature of 56°C was used for all the primer sets. The PCR parameters used are as follows:

a) Initial denaturation 96°C 5 minutes
b) Denaturation 96°C 1 minute
Annealing 55°C/46°C 1 minute 35 cycles
Extension 72°C 1 minute
c) Final extension 72°C 10 minutes

The amplified PCR products were analysed by agarose gel electrophoresis. For further manipulations, the amplified PCR products were purified using QIAquick PCR purification columns (Qiagen, Hilden, Germany).

For screening transformants, a small portion of freshly grown transformed colony was picked using a sterile tooth pick and resuspended in 50µl of 0.1 X TE (1mM Tris and 0.1mM EDTA, pH 8.0) buffer. The cells were lysed by boiling for 10 minutes, snap-chilled on ice, centrifuged at 12000g for 10 minutes and 1µl of the supernatant was used as template in PCR.
2.8 RESTRICTION DIGESTION AND LIGATION OF DNA

Restriction enzyme digestions were performed using enzymes from Amersham Pharmacia Biotech Asia Pacific, Hong Kong in the manufacturer’s recommended buffers. Restriction enzyme digestions were performed as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (3-4μg)</td>
<td>5 μl</td>
</tr>
<tr>
<td>Buffer (10X)</td>
<td>2 μl</td>
</tr>
<tr>
<td>Enzyme (2-3 units/μg of DNA)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Water</td>
<td>12 μl</td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 37°C for 1 hr and the samples were analysed on a 1.2% agarose gel.

For cloning the restriction enzyme digested fragments were purified by QIAquick PCR purification kit and estimated by measuring the absorbance at 260 nm and stored at -20°C till use.

The ligation mixture consisted of

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligation buffer (10X)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Vector (~50ng)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Insert (20-50ng)</td>
<td>1 μl</td>
</tr>
<tr>
<td>10mM ATP</td>
<td>1 μl</td>
</tr>
<tr>
<td>T4 DNA ligase (10 Weiss units)</td>
<td>1 μl</td>
</tr>
</tbody>
</table>

The total reaction volume was made up to 10 μl with distilled water and ligations were performed overnight (16 hrs) at 4°C. Molar ratios of 3 – 6 : 1 of insert to vector were used in the ligation reactions.
2.9 CONFIRMATION OF RECOMBINANT CONSTRUCTS BY SEQUENCING

The nucleotide sequences of the cloned inserts in the recombinants pMSplyl and pMS2TD4 were sequenced using vector and insert specific primers. The nucleotide sequence and restriction enzyme sites were verified to check for errors.

2.10 EXPRESSION OF THE RECOMBINANT PROTEINS IN E.COLI

For storage and maintenance purposes, the recombinant plasmids pMSplyl and pMS2TD4 were transformed in an E.coli host DH5α which lacks T7 RNA polymerase and hence does not express the protein. For expression studies the recombinant plasmids were transformed into E.coli strain BL2 l(DE3)Plys S and BL21 respectively. The T7 RNA polymerase gene is present as a chromosomal copy under the control of lac UV5 promoter in the E.coli strain BL21 (DE3), enabling the expression of the genes under the control of T7 promoter with the use of a gratuitous inducer like IPTG. Being a lon protease negative strain BL21 (DE3) helps in preventing the cleavage of the expressed heterologous proteins. The expected molecular weight of the expressed protein along with the N-terminal histidine fusion tag is 57kDa.

The plasmid pGEX2T contains the tac promoter followed by the fusion tag glutathione-S-transferase which is followed by the polylinker and translation termination codon TGA. Induction of the tac promoter with IPTG results in the synthesis of the soluble fusion protein of molecular weight 38 kDa.

2.10.1 Expression kinetics

The following protocol was used for expressing the proteins of interest.

a) E.coli BL21 (DE3) and BL 21 were transformed with the desired gene constructs.
b) A single recombinant *E. coli* colony from each construct (pMSplyl and pMS2TD4) was inoculated in 2.5 ml of LB containing Ampicillin (100μg/ml) and grown overnight at 37°C.

c) 50ml of fresh LB medium was inoculated with 10% of the overnight grown culture. The culture was grown at 37°C with vigorous shaking to an OD$_{600}$ 0.6.

d) 1ml aliquot of cells were removed prior to IPTG induction (Uninduced), centrifuged and stored at -20°C. This was taken as time zero sample. The culture was induced by the addition of IPTG to a final concentration of 1mM and the culture was grown for 4 hrs at 37°C.

e) Every one hour after induction 1ml of the culture was removed and centrifuged and the pellet was stored at -20°C for further analysis.

f) The cell pellets were solubilised in 1X SSB, electrophoresed on a 10% SDS-PAGE, followed by staining with Coomassie Brilliant Blue dye.

2.10.2 Expression of recombinant proteins for preparative purification

A single colony of the recombinant *E.coli* was grown in 20 ml of LB media overnight at 37°C. The overnight grown culture (10ml) was used to inoculate 200ml of LB media containing 100μg/ml of ampicillin, grown at 37°C with vigorous shaking. When the OD$_{600}$ of the culture reached 0.6, the expression of the recombinant protein was induced by the addition of IPTG (1mM IPTG, final concentration) and grown for a further period of 4 hrs at 37°C. The cells were harvested by centrifugation (4000g, 20 minutes) and stored at -20°C till further use. Similarly the domain 4 peptide was also expressed after induction with 0.1 mM IPTG and cells frozen at -20°C.
2.11 PURIFICATION OF THE RECOMBINANT PROTEINS

2.11.1 Recombinant pneumolysin

The T7 expression vector, pRSET A, contains six histidine residues as a N-terminal fusion tag. This His tag enables easy identification of the expressed recombinant protein using mouse anti-His antibodies. The affinity of the histidine residues to bind to metals could be exploited to purify the expressed fusion protein by metal affinity chromatography.

The cell pellet containing the recombinant protein was solubilised overnight at 4°C (with shaking) in denaturing buffer (6M Guanidium hydrochloride, 100mM NaH₂PO₄, 100mM Tris-Cl, pH 8.0). The sample was centrifuged at 13000g for 15 minutes and the supernatant was used for purification of the recombinant protein. The expressed recombinant protein was purified from crude cell lysate using Immobilised metal affinity chromatography (LKB Pharmacia, Uppsala, Sweden). NiNTA superflow resin was procured from Qiagen, Hilden, Germany and used as matrix. The flow rate of the column was set at 1ml/minute. The matrix was equilibrated with Urea buffer (8M Urea, 100mM NaH₂PO₄, 10mM Tris-Cl, pH 8.0) till the flow throughs’ absorbance at 280nm stabilised. The sample was loaded on to the column till the matrix was saturated. Then the matrix was washed with urea buffer till the absorbance value of the flow through reached an absorbance of 0.01 at 280 nm. The contaminating proteins bound to the matrix were washed with 15 column volumes of urea buffer containing 50mM, 100mM and 150mM of imidazole. The recombinant protein was finally eluted with urea buffer containing 500mM imidazole.

2.11.2 Recombinant domain 4

The GST fusion system is an integrated system for the expression and purification of fusion proteins produced in E. coli. Fusion proteins are easily purified
from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B (Amersham Pharmacia, Sweden).

A single transformed colony containing recombinant pGEX2T domain 4 was inoculated into 100ml LB medium and was grown at 37°C till the OD_{600} reached 0.6. The expression of recombinant domain 4 protein was induced by adding 0.1mM IPTG final concentration. The culture was incubated for an additional 3 hours with shaking. Cells were centrifuged at 7000g for 10 min and the pellet was resuspended in 5 ml of ice cold 1X PBS. The cells were freeze thawed repeatedly for lysis and the cells were then spun at 13000g for 10 min to remove insoluble materials.

2 ml of 50% slurry of glutathione Sepharose 4B was equilibrated with 1X PBS. 5 ml of the culture supernatant was added to the slurry and mixed well, and incubated at room temperature for 10 min. The matrix was washed with 10 bed volumes of 1X PBS and the column was drained completely. This was repeated twice. The fusion protein was eluted by the addition of 1 ml of Glutathione elution buffer (10mM reduced Glutathione in 50mM Tris-Cl, pH8.0) per ml of bed volume. The elution was repeated two more times and all the 3 eluates were pooled and analysed by SDS-PAGE.

2.12 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The levels of pneumolysin specific IgG and isotypes of IgG in the serum samples were determined by ELISA (Jalonen et al 1989). A checkerboard titration using various antigen concentrations (10μg, 1μg, 500ng and 100ng) and dilutions (1:500, 1:200, 1:100) of antibodies was used to determine the optimal antigen concentration to be coated and the antibody dilution to be used. Optimal antigen concentration of 1μg/ml protein was used in all further concentrations. Anti-human IgG alkaline phosphate conjugate and anti-mouse alkaline phosphate conjugate were used at dilutions recommended by the manufacturer Sigma, St. Louis, USA.
Wells of microtitre plates (Immulon II, Dynatech) were coated with 1μg/ml of pneumolysin in carbonate buffer. Plates were incubated overnight at 4°C. Wells were washed after this step and all subsequent washes with PBS containing 0.2% Tween-20. Plates were blocked with PBS containing 3% bovine serum albumin for 1 hour at 37°C. 100μl of 1:100 dilution of sample sera in PBS was added in duplicate and incubated at 37°C for 2 hours. The plates were washed 4 times with PBS Tween. 100μl of goat anti-human-alkaline phosphatase conjugated antibody at 1:20,000 dilution in PBS-1%BSA was added and incubated at 37°C for 1 hour and the plates were washed with PBS Tween. 100 μl of substrate p-nitro phenyl phosphate (1mg/ml) in substrate buffer (100mM Tris-Cl, pH 9.5, 100mM NaCl and 5mM MgCl₂) was added and color was developed. The reaction was arrested after 20 minutes by addition of 100 μl of 3M NaOH per well. Absorbance was determined using an ELISA reader (EL311SX Biotek instruments, Inc., VT, USA) at 405nm. The absorbance of wells coated with PBS alone was subtracted from all values. Control wells containing all reagents except serum were also used in each plate.

For isotype ELISA, following the addition of human sera, respective monoclonal antibody (mAb) were added and incubated at 37°C for 2 hours. The plates were washed and incubated with alkaline phosphatase conjugated anti-mouse IgG for 1 hour at 37°C. The list of monoclonal antibodies and the dilution used are given in Table 2.2.

**Table 2.2 Monoclonal antibodies and the dilutions**

<table>
<thead>
<tr>
<th>IgG subclass</th>
<th>Clone</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>HP6001</td>
<td>1:3000</td>
</tr>
<tr>
<td>IgG2</td>
<td>HP6002</td>
<td>1:5000</td>
</tr>
<tr>
<td>IgG3</td>
<td>HP6047</td>
<td>1:2000</td>
</tr>
<tr>
<td>IgG4</td>
<td>HP6023</td>
<td>1:2000</td>
</tr>
</tbody>
</table>
2.14 HAEMOLYTIC ASSAY

The haemolytic activity of the proteins was assayed as follows (Mitchell et al 1989). Serial two fold dilutions of 50μl of the solution to be analysed were made in PBS in a microtitre plate. Sheep erythrocytes were washed well with PBS and 50μl of a 1.5% (v/v) suspension in PBS was added to each well. The plate was incubated at 37°C for 30 minutes. The haemolytic activity was defined as the inverse of the dilution of the 50μl of the sample protein that caused 50% haemolysis. This was read visually or after spinning the plate and reading the absorbance of the supernatant at 415nm.

2.15 COMPLEMENT C3 ASSAY

The levels of C3 in serum before and after treatment with pneumolysin and domain 4 of pneumolysin were checked using radial immunodiffusion plates (RID), Biocientifica SA, Argentina (Mitchell et al 1989). The procedure consists of an immunoprecipitation in agarose between an antigen and its homologous antibody. A quantitative relationship exists between ring diameter and antigen concentration. The plate was removed to room temperature to allow any condensation to evaporate. The wells were filled with 5μl of serum treated with D4 and untreated serum was also used. A piece of wet cotton was placed in the centre of the plate to avoid dehydration. The plate was left at room temperature overnight and was read the next day. The rings were measured to 0.1mm precision using a ruler. The values were plotted on a logarithmic graph paper and the values were read directly of the reference table provided with the kit.

2.16 LYMPHOCYTE PROLIFERATION

Peripheral blood mononuclear cells were prepared from blood of healthy volunteers by low density centrifugation in Ficoll-Hypaque medium (density 1.007, Pharmacia, Sweden) (Ferrante et al 1984). Cells were cultured in RPMI 1640 medium
with 10% AB serum. 2 X 10^5 million cells were cultured in wells of microtitre plates in the presence or absence of mitogen Phytohemagglutinin (PHA) and with and without pneumolysin. The total volume per well was 0.2ml and the cultures contained 10% AB. The cells were incubated at 37°C for 72 hours in an atmosphere of 5% CO2 at a high humidity and were pulse labelled with 1μCi of ^3H Thymidine 16 hours before being harvested. The cells were harvested by aspiration onto glass fiber filters with a multi-sample cell harvester (Insel Cell Harvester, CH3, England) and the radioactivity was measured by liquid scintillation spectrometry. PHA was used at an optimal concentration of 10μg/ml. Pneumolysin was used at concentrations ranging from 100 ng and 100 fg.

2.17 REVERSE TRANSCRIPTASE PCR

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by standard Ficoll-Hypaque density gradient centrifugation (Pharmacia). The mononuclear cells were suspended in RPMI-1640 media (Sigma) supplemented with 10% heat inactivated Fetal calf serum (Sigma) at a concentration of 4 x 10^6 cells per ml, cultured in 24 well plates and incubated for 6 hours at 37°C in the presence of 5% CO2. Pneumolysin (500ng) per ml of cells was added to the cultures and were incubated. Purified pneumolysin preparation was essentially free of contaminating bacterial endotoxin as determined by LAL assay. The RNA was extracted by the following procedure (Chomczynski and Sacchi 1987).

1. Cells were collected from the 24 well tissue culture plate in 1.5 ml eppendorf tubes and washed in RPMI medium and spun at 800g for 10 min. The supernatant was discarded.

2. 400 μl RNA zol was added to the eppendorf tubes containing the cells and RNA was solubilized by passing the lysate through the pipette tip.
3. 0.1 volume of chloroform was added and vortexed for 15 sec and kept on ice for 15 min.

4. Chloroform extraction was carried out by spinning the tubes at 12000g for 15 min at 4 °C.

5. The aqueous phase was aspirated into another tube and one volume of isopropanol was added. The RNA was precipitated by incubating the tubes at -20°C for two hours.

6. The tubes were then spun at 12000 rpm for 15 min at 4°C to pellet the RNA. The RNA pellet was washed with 75% ethanol twice and then spun at 12000g for 15 min at 4°C.

7. The RNA pellet was dried in a speed vac and dissolved in 20μl DEPC treated water.

All glassware were soaked and rinsed in a solution of 0.1% DEPC, baked at 160°C for 3 hours before use.

2.17.1 Reverse transcription reaction

Total RNA was resuspended in 9μl DEPC treated water and heated at 70°C for 5 min, after the addition of 100 pmoles of random hexamer (Amersham Pharmacia, UK), in a total volume of 10μl and then chilled on ice. Reverse transcription was performed in a final volume of 20μl containing 0.25mM mix of four dNTPs, 1X reverse transcriptase buffer (50mM Tris-HCl pH8.3, 75mM KCl, 3mM MgCl₂, 8mM DTT, 20 U Rnasein (Promega) and 200U Maloney Murine Leukemia virus reverse transcriptase (MMLV) (Life Technologies, Gaithersburg, MD), and followed by incubation of the tubes at 37°C for 60 min. MMLV is a RNA dependent DNA polymerase that has Rnase H activity. The reverse transcription reaction was stopped by heating the tube at 90°C for 5 min. The final reaction volume was diluted in 1:3 using DEPC treated water and stored at -20°C until use.
2.17.2 Primer Selection

Oligonucleotide primer pairs for each gene amplification were designed based on published cDNA sequences. The primer sequences were chosen from two exons of the studied genes in order to differentiate cDNA amplification products from any contaminating genomic DNA products. The following are the sequences of the oligonucleotides used (Table 2.3).

Table 2.3 Sequences of Oligonucleotide primers

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name</th>
<th>Sense / Antisense</th>
<th>Primer Sequence</th>
<th>Product size (bp)</th>
<th>Tₘ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Human IL-8</td>
<td>Sense</td>
<td>5'-ATGACTTCCAAGCTGGCCGTG-3'</td>
<td>289</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>5'-TCTCACCCCTCTTCAAAAACCTTCTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Human TNF-α</td>
<td>Sense</td>
<td>5'-CGGGACGTCGAGCTGGCCGAGGAG-3'</td>
<td>355</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>5'-CACCAGCTGGTTATCTCTCGACT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Human IFN-γ</td>
<td>Sense</td>
<td>5'-CAGCTCTGCATCGTTTTGGGTTCT-3'</td>
<td>458</td>
<td>60</td>
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<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>5'-TGCTCCTTCGACCTTGAAGCAGCAT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Human iNOS</td>
<td>Sense</td>
<td>5'-TGCTTTGTGCGGAGTGTCAG3'</td>
<td>800</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>5'-AGATGCTGTAACTCTTCTGG3'</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>Human IL10</td>
<td>Sense</td>
<td>5'-ATGCACAGCTCAGGCTGTCT3'</td>
<td>536</td>
<td>55</td>
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<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>5'-TCAGTTTCGTATCTTTAGTGCTA3'</td>
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<tr>
<td>6</td>
<td>Human GAPDH</td>
<td>Sense</td>
<td>5'-CCACCACATGGCAAATTCCTATGGCA3'</td>
<td>600</td>
<td>55</td>
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<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>5'-TCAGAGACGGCAGGTCCAGTCCA3'</td>
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</tr>
</tbody>
</table>
2.17.3 Polymerase Chain Reaction (PCR)

PCR was performed under the following conditions. To 2μl cDNA the following components were added.

- 0.25 mM dNTP mix
- 1X PCR buffer (50 mM KCl, 10 mM Tris-Cl pH 8.0, and 1.5 mM MgCl₂)
- 0.2 μM sense and antisense primers
- 0.15 U Taq Polymerase (Genei, India).

PCR was carried out using the following conditions, denaturing for 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1.30 min at 55°C, and 2 min at 72°C. PTC150 MJ Research PCR system was used for performing the above reaction.

2.18 PCR IN CLINICAL SAMPLES

For diagnosis of clinical samples, total genomic DNA from whole blood was extracted using a modified protocol of Lahiri and Numberger (1991). Briefly, whole blood was collected in a vacutainer tube, containing 100 μl of 15% EDTA. 200 μl of the blood was transferred to an eppendorf tube, and washed with 9 volumes of 0.17M NH₄Cl for red blood cell lysis. The pellet was washed twice with PBS and resuspended in TKM 1 buffer (Tris-HCl, pH 7.6, 10mM KCl, 10mM MgCl₂ and 2mM EDTA). 25μl of Nonidet P40 was added to lyse the cells and mixed well by inversion several times and centrifuged at 2200g for 10 min at RT. The supernatant was slowly poured off and to the pellet 200 μl of TKM II buffer (TKMI + 0.4M NaCl) was added and left in RT for 5 minutes. 50μl of 10% SDS was added and the whole suspension was mixed thoroughly by pipetting back and forth several times and the tubes were incubated at 55°C for 15 min. 300μl of 6N NaCl was added, mixed well and centrifuged at 12000g for 5 min. The supernatant containing the DNA was precipitated with 2.5 volumes of ethanol for 30 min at -20°C and spun down at 14000g for 15 min. The pellet was washed with 70% ethanol to remove the salts and the tubes.
centrifuged at 12000g for 15 min at RT and resuspended in 30μl of good quality water. 2μl of the DNA was used for PCR amplification.

2.19 GENERAL METHODS

Molecular biology methods such as agarose gel electrophoresis and SDS-PAGE, transformation and western blotting used in this study were adopted from Sambrook et al (1989) with minor modifications as described below.

2.19.1 Bacterial Genomic DNA Extraction

Bacterial genomic DNA was extracted as described by Dillard and Yother (1994). Briefly, bacterial cultures were grown in 100ml Todd-Hewitt Broth and centrifuged at 4000g for 10 min. The pellet was resuspended in 2.5ml TE (10mM Tris, 1mM EDTA pH 8.0). SDS was added to 1% and the cells were lysed at 65°C for 15 min. One fifth volume of 5M potassium acetate (pH 8.0) was added and incubated at 65°C for 15 min, followed by incubation on ice for 60 min. Cell debris was removed by centrifugation at 11000g for 10 min. Two volumes of ethanol was added to the supernatant and the DNA was removed with a glass rod. The DNA was dried, resuspended in TE, and used for PCR.

2.19.2 Agarose Gel Electrophoresis

Submerged horizontal 1-1.2% agarose gels were used to separate the DNA fragments. TBE buffer of pH 8.3 (98mM Tris, 89mM Boric acid and 2mM EDTA) was used for casting and running the gels. The electrophoresis was performed at 5-8 V/cm at room temperature. The gel loading buffer contained 20% glycerol with 0.01% orange G dye in TE buffer (50mM Tris, 10mM EDTA, pH 8.0). After electrophoretic separation of the DNA fragments, the agarose gels were stained with ethidium bromide (0.5 μg/ml) and visualised on a UV-Transilluminator (Fotodyne,
Photographs were taken with Tracktel GDS2 gel documentation system, Vision system, Germany using gelatin filter.

2.19.3 SDS-Polyacrylamide Gel Electrophoresis

Proteins were analysed by the method of Laemmli (1970) by SDS-PAGE with certain modifications. The various buffers used are as follows:

a) Acrylamide solution (30%): 29.2% acrylamide and 0.8% N,N-Methylene bis Acrylamide in distilled water. The solution was filtered through Whatman filter paper and stored in brown bottles at 4°C.

b) Separating gel buffer: 1.5 M Tris-Cl, pH 8.3
c) Stacking Gel buffer: 0.5M Tris-Cl, pH 6.8
d) Electrophoresis buffer: 0.025M Tris-Cl, 0.192 M Glycine, 0.1% SDS, pH 8.3
e) Sample solubilisation buffer (5X): 10% SDS, 10% (v/v) β-Mercaptoethanol, 50% sucrose, 0.025% Bromophenol blue in stacking buffer.

The proteins were separated on a 10% SDS-PAGE containing 5% stacking gel. Stacking gels were approximately 1/5 of the separating gel. Protein estimation was performed by Bicinchoninic acid (BCA) assay and equal amounts of protein were loaded in each well. A constant current of 20mA was used for the stacking of proteins and was resolved in the separating gel using 30mA. Gels were stained with Commassie Brilliant Blue (0.25% in 45% methanol and 10% acetic acid) for 2-3 hours at room temperature. The gels were destained overnight in 45% methanol, 10% acetic acid solution till a clear background was obtained. Photographs were taken with Tracktel GDS2 gel documentation system, Vision system, Germany.
2.19.4 Western Transfer of Proteins

After electrophoresis of the proteins, the SDS PAGE gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS) for 10 minutes. Nitrocellulose membrane (NCP) and filter paper were cut to exact size of the separating gel and soaked in transfer buffer. The NCP was overlaid on the gel and sandwiched between filter paper and scotch brite pads taking care that no air bubbles were trapped in between. Electrophoretic transfer was carried out using a semi dry transfer apparatus at 150 milliamps for 90 min using a Amersham Pharmacia transfer apparatus. After transfer, the molecular weight marker lane in the NCP was cut and stained with amido black (100 mg in 45% methanol, 10% acetic acid). The rest of the NCP was stained with Ponceau-S (0.2% Ponceau-S, Sigma, USA in 1% acetic acid) to ensure that protein has been transferred. Membrane was washed with PBS and then blocked overnight in 3% BSA in PBS. The NCP was washed with PBS containing 0.05% Tween-20 three times, each wash of 5 minutes duration and then incubated with appropriately diluted primary antibody for 2 hours at 37°C. After washing, the membrane was incubated with the appropriate secondary antibody at the recommended dilution. After five more similar washes the membrane was soaked for 10 minutes in detection buffer (100mM Tris pH 9.5, 100mM NaCl, 5mM MgCl₂). The colour development was carried out by the addition of 66 μl of 5-bromo 4-chloro-3-indolyl phosphate (BCIP, 50 mg/ml in diethyl formamide) and 33μl of Nitro Blue Tetrazolium (NBT, 50 mg/ml in 70% diethyl formamide) in 10 ml of detection buffer. The reaction was stopped after 15 minutes by the addition of 10mM EDTA.

2.20 PROTEIN ESTIMATION

Protein Estimation was carried out using the Micro BCA Protein Estimation kit (Pierce, Rockford, IL, USA) as described by Smith et al (1985).
1. 150µl of each standard or unknown sample was added to appropriate wells in a microplate. 150µl of diluent was used as blank.

2. 150µl of the working reagent was added to each well, mixed well on a plate shaker for 30 seconds.

3. The plate was covered and left at 37°C for 2 hours.

4. The plate was cooled to room temperature after incubation.

5. The absorbance was measured at 562 nm on a plate reader.

6. The average 562nm reading of blank wells was subtracted from the readings for each standard or unknown and the standard curve was plotted.

7. The values of the unknown were read from the standard curve.

2.21 STATISTICAL ANALYSIS

The Mann–Whitney U test was applied to analyze the humoral immune response data between normals and samples. Wilcoxon Signed Rank test was carried out to analyze the difference in the isotype levels within the groups. A probability value of p < 0.05 was considered statistically significant. Statistical analyses were performed using StatView 4.0.