Chapter 7
Heterologous Cloning and Expression of a Recombinant Chimeric Protein comprising of TDH and TRH Truncated Haemolysins of Vibrio parahaemolyticus

7.1 INTRODUCTION

Vibrio parahaemolyticus is a major food-borne pathogen that causes infections in humans through the consumption of raw, undercooked or contaminated seafood. Infections tend to occur during the summer or early fall when water temperatures is higher along the coastal marine and estuarine environments throughout the world. The infection by this bacterium includes acute gastroenteritis, wound infection and rarely septicemia. V. parahaemolyticus comprise many virulence factors among which haemolysins and Type III secretion systems are known to play major role during infections.

Haemolysin is the most widely distributed cytolsin among pathogenic Vibrios with various roles in the infection process (Iida and Honda 1997; Shinoda 1999). Four types of haemolysins are known to be elaborated by V. parahaemolyticus; the Thermostable Direct Haemolysin (TDH) produced by Kanagawa phenomenon (KP) positive strains, TDH- Related Haemolysin (TRH) produced by KP negative strains, Thermo Labile Haemolysin (TLH) and delta-VPH produced by all strains (Zhang and Austin., 2005). TDH is a homodimer protein with a molecular weight of 42 kDa determined by gel Filtration, which consist of two subunits of molecular weight of 21 kDa when separated in SDS-PAGE (Takeda et al., 1978, Iida and Honda 1997). The N-terminal region of the toxin is thought to be involved in the binding process, whereas the region near the C-terminal region has been implicated in post-binding
activities. The TRH has 67 percent similarity with amino acid sequence of TDH sharing some similar epitopes and is found to be immunologically related (Takahashi et al., 2000). Both the toxins show several similar features of variable affinity to different cell types including cardiac muscle cells, enterocytes and toxicity to many cultured cells like chloride leakage from human colonic cells (Takeda., 1983). On the other hand, the TLH is a non-pore forming toxin displaying phospholipase activity and hemolytic activity (Taniguchi et al., 1985, 1986); its role in pathogenicity is still not clearly instated. But it was found that the tlh gene is strongly upregulated when a genomic screen is performed under conditions meant to mimic the intestinal environment of the human host (Gotoh et al., 2010). Among haemolysins the presence of TDH invariably gets correlated to the virulence of *V. parahaemolyticus* even though the exact role of TLH which is present in all *V. parahaemolyticus* strain still needs to be assessed. There could be a possibility that TLH being a haemolysin, may be in association with TDH the other haemolysin, may have some role in the virulence. A strategy to detect both of these haemolysins therefore, can surely be useful in confirming the virulent strain of *V. parahaemolyticus*.

Presently, there are no licensed vaccines or toxoids approved or found suitable for use in humans/animals to provide protection against *V. parahaemolyticus* infections. The presence of multiple virulence factors in *V. parahaemolyticus* enables the pathogen to be a menace with respect to human infections. Till now, the treatment regimen followed for these infections involves antibiotics like ceftazidime (cephalosporins) and doxycycline or a fluoroquinolone alone (Han et al., 2007). With the emergence of antibiotic resistance genes coupled to lack of rapid and low cost
detection systems, a therapeutic inter-vention employing multi-component, nontoxic, antigenic domains of different toxin moieties could be a workable strategy.

In this study, therefore, an attempt was made to construct a novel chimeric gene, encompassing the truncated regions of TDH (predominant virulence factor) and TLH (species specific protein) to make use of it both for detection and protection capabilities. Hyper immune antisera raised against the recombinant fusion protein was evaluated and the results demonstrated specific detection of the wild-type toxins among the \textit{V. parahaemolyticus} strain and also an effective neutralization of their toxicities.

5.2 MATERIALS AND METHODS

(i) Bacterial strains, plasmids and media

All the bacterial strains used in this study are mentioned in Table 7.1. Reference strains are obtained from American Type Culture Collection (ATCC), USA; Institute for Microbial Technology (IMTECH), Chandigarh and National Institute of Cholera and Enteric Disease (NICED), Kolkata. \textit{V. parahaemolyticus} O3:K6 strain K11555 (NICED) was used as the source of chromosomal DNA for cloning and expression of recombinant chimeric protein Tlh/Tdh. \textit{Escherichia coli} plasmid, pRSET A (Invitrogen, India) controlled by the strong bacteriophage T7 promoter was used as the expression vector. The prokaryotic host strain for plasmid transformation was \textit{E. coli} BL21 (DE3) pLysS, which was grown in Luria Bertani (LB) broth (Hi-media, India) containing 35 µg/ml chloramphenicol.

(ii) Extraction of genomic DNA

\textit{V. parahaemolyticus} O3:K6 strain K11555 was grown in APW containing 3 percent NaCl and was used as source of DNA. Genomic DNA was isolated from over-
Table 7.1: Standard strains used in cloning and expression studies of recombinant fusion TLH/TDH protein.

<table>
<thead>
<tr>
<th>Standard Bacterial strains</th>
<th>Source</th>
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<tbody>
<tr>
<td><em>V. parahaemolyticus</em> ATCC 17802</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> K11555 (03:K6)</td>
<td>NICED</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> E2253 (02:K3)</td>
<td>NICED</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> K21886 (01:KUT)</td>
<td>NICED</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> K12262 (01:K25)</td>
<td>NICED</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> E2270 (04:K8)</td>
<td>NICED</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> L10847 (04:K63)</td>
<td>NICED</td>
</tr>
<tr>
<td><em>V. cholerae</em> 6120/06</td>
<td>NICED</td>
</tr>
<tr>
<td><em>V. vulnificus</em> ATCC 27562</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>V. mimicus</em> VM1</td>
<td>NICED</td>
</tr>
<tr>
<td><em>V. furnissi</em> ATCC 35016</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>V. alginolyticus</em> ATCC 17749</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em> ATCC 35654</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>Pleisomonas shigelloides</em> MTCC 1737</td>
<td>IMTECH</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 10536</td>
<td>ATCC</td>
</tr>
</tbody>
</table>

Table 7.2: Primers used in cloning and expression studies of recombinant fusion TLH/TDH protein.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Restriction site</th>
<th>Accession number</th>
</tr>
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<tbody>
<tr>
<td><em>tlh</em></td>
<td>CTTCAGGAAGTGATCAGCAGCAAG</td>
<td>Xho I (229683-230939)</td>
<td>BA000032</td>
</tr>
<tr>
<td></td>
<td>CTGCAGAAACCACCTTTGTTGATTTGATCT</td>
<td>Pst I</td>
<td></td>
</tr>
<tr>
<td><em>tdh</em></td>
<td>CTGCAGCCATCTGCTCCCTTTTCTCTG</td>
<td>Pst I (1450828-1451397)</td>
<td>BA000032</td>
</tr>
<tr>
<td></td>
<td>AAGCTTTCTCATATGCTTCTACATTAACA</td>
<td>Hind III</td>
<td></td>
</tr>
</tbody>
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night pellet by standard phenol-chloroform extraction protocol with modifications according to the method of Tada and co-workers (1992) and stored in -20 °C until use.

(iii) PCR primers, construction of chimeric gene and recombinant plasmid

The complete nucleotide sequences for the *tlh* and *tdh* genes of *V. parahaemolyticus* were obtained from Genbank nucleotide database. The primers were designed complementarily to conserved portions of the *tlh* and C terminal region of *tdh* which had minimum codon bias with *E. coli* host and had selected restriction site overhangs at their 5’ ends (Table 7.2). Identical restriction site (*Pst I*) sequence was retained at the 5’ ends of the reverse primer of *tlh* and forward primer of *tdh*. The oligonucleotides used in this study were custom synthesized from MWG Biotech Pvt Ltd (Bangalore, India).

PCR was performed for each gene separately in a 50 µl reaction mixture, which contained 50 ng of genomic DNA, 1.5 mM of MgSO₄ in 1 X PCR buffer, 250 nM of each primer, 200 µM of dNTP mix and 1 U of *Pfu* DNA polymerase, in eppendorf master gradient (Hamburg, Germany). Amplification was carried out with initial denaturation for 5 min at 94 °C followed by 30 cycles of denaturation for 1 min at 94 °C, annealing at 58 °C for 1 min and extension at 72 °C for 3 min and a final extension of 72 °C for 10 min. The PCR products of *tlh* gene and *tdh* gene were purified by GenElute PCR Clean-Up Kit (Sigma, India).

The purified PCR products of both the haemolysin genes were initially digested using *Pst I* enzyme to allow in-frame cloning and separated in 1 percent agarose gel. The PCR band of predicted size is excised and purified using GenElute Gel Extraction Kit (Sigma, India). The resultant enzyme digested PCR products of amplified *tlh* and *tdh* genes were further subjected to ligation using T4 DNA ligase to
get a single chimeric gene \textit{tlh/tdh}. The chimeric gene was further PCR amplified using the forward primer \textit{tlh} and reverse primer of \textit{tdh} using the same PCR conditions as mentioned before. The resultant chimeric gene was finally digested using the enzymes \textit{Xho I} and \textit{Hind III} and ligated into the predigested plasmid pRSET A. The integrity of the insertion was ascertained by digesting the recombinant plasmid using \textit{Xho I} and \textit{Hind III} (Fig 7.1) and by PCR targeting degenerate T7 primers. The recombinant plasmid was sequenced to confirm the reading frame.

(iv) Expression and purification of recombinant chimeric protein

The ligated mixture of fusion gene \textit{tlh/tdh} and pRSET A plasmid were transformed into competent BL21(DE3) pLysS and were grown in LB media containing ampicillin (100 µg/ml) and chloramphenicol (35 µg/ml). The procedures of competent cell preparation and transformation were followed according to Sambrook and co-workers (1989). A 100 µl of overnight pre-culture of each chimeric gene positive strains were used for expression studies by growing them in 5 ml of LB broth containing antibiotics. Isopropyl β-D-thiogalactopyranoside (IPTG) is added at a final concentration of 1 mM, when cell concentration reached 0.4 - 0.6 at OD\textsubscript{600}. After inducing for 4-6 hrs at 37 °C with constant agitation, the bacterial cells were harvested by centrifuging at 4000 × g for 20 min. The presumptive colonies were finally confirmed for expression of the recombinant protein by examining in 12 percent Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS PAGE) as well as by western blotting, probing with anti- His antibodies in 1:5000 dilutions.

The recombinant protein was purified under denaturing conditions at room temperature by Ni\textsuperscript{2+}-NTA affinity chromatography. The pellet from 100 ml of culture are thawed to room temperature after freezing it at -20 °C or -80 °C for 1hr (minimum)
and resuspended in 10 ml lysis buffer containing 7 M Urea, 0.1 M NaH$_2$PO$_4$, 10 mM Tris-Cl and pH 8.0. Cells were constantly stirred at room temperature for 1 hr and the clear lysate produced was centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant containing 6X His tagged recombinant proteins was mixed with 1 ml Ni$^{2+}$-NTA slurry in Superflow resin columns that had been pre-equilibrated with the lysis buffer. This suspension was gently stirred for 1 hr at room temperature to mix the resin and the lysate. The columns are allowed to stand so that the resin settles down, followed by collection of the flow through. A wash step was performed with wash buffer containing 7 M urea, 0.1 M NaH$_2$PO$_4$, 10 mM Tris-Cl and pH 6.3 to remove contaminants, followed by elution step with 3 ml of eluting buffer containing 8 M urea, 0.1 M NaH$_2$PO$_4$, 10 mM Tris-Cl with pH 4.5. The final step of elution is repeated at least 4 times. Protein concentrations were estimated by Folin-Lowry’s method (1951) using BSA (Sigma) standards.

The crude extracts (induced and uninduced E. coli cells) and affinity-purified recombinant protein were compared and analyzed by separating on a 12 percent SDS-PAGE gel. 25 µg of crude extract and 5 µg of purified protein were loaded and separated on the gel and proteins were detected by staining with coomassie blue.

(v) Generation of polyclonal and monoclonal antibodies against recombinant fusion protein *tlh/tdh*

Female BALB/c mice (6-7 week old) were immunized at weekly intervals with 50 µg of recombinant protein by injecting subcutaneously and intramuscularly emulsified with an equal volume of Freund’s complete adjuvant (Sigma, India). Subsequent booster doses were given intramuscularly with Freund’s incomplete adjuvant (Sigma, India) at 7, 14, 21 and 28 days. The mice were bled one week after the last
immunization and the sera were collected after centrifuging the blood clot and stored at -20 °C in aliquots. Sera from these mice were evaluated in direct binding assays for antibody reactivity with the recombinant Tlh/Tdh fusion protein, with a negative control. Once an appropriate antibody titer was observed in ELISA, the animals were given a final booster injection with the recombinant protein four days prior to exsanguination and splenectomy. The recombinant protein Tlh/Tdh specific polyclonal antibodies were detected by using a standard plate ELISA format in which 20 µg of the antigen was coated to the solid phase. When the required titer was obtained, a final dose of 40 µg protein was injected intraperitoneally.

The monoclonal antibodies (MAbs) were produced by following the method of Kohler and Milstein (1975) with minor modifications. Three days after the final immunization, the splenocytes of the mice were isolated and fused with Sp2/o-Ag 14 myeloma cell line at a ratio of 5:1 with 40 percent polyethylene glycol. The resulting hybridoma cells were selected and grown in Dulbecco’s modified Eagle’s medium (Sigma, India) supplemented with L-glutamine hypoxanthine aminopterin thymidine (HAT) and 10 percent of fetal bovine serum (Gibco, India). Hybridomas producing the antibodies of interest were selected by screening for specific antibody binding to the recombinant protein Tlh/Tdh by plate ELISA. The positive hybridoma cells were further subjected to recloning twice by limiting dilution for stabilization of the clones.

(v) Western blotting

The purified recombinant protein TLH/TDH as well as the concentrated culture supernatant which was predicted to include native haemolysins from V. parahaemolyticus were separated by 12 percent Poly Acrylamide Gel Electrophoresis (PAGE) and were blotted on nitrocellulose sheet (Gila, India). The native
**Fig 7.1:** Construction of recombinant plasmid for the expression of TLH/TDH fusion protein.
haemolysins were extracted from supernatant of 6 h grown culture by ammonium sulphate precipitation (65% saturation).

SDS-PAGE resolved proteins were transferred onto the nitrocellulose sheet membrane were subjected to the standard immunodetection procedure using hyperimmune sera or MAbs. The sheet was immersed in PBS containing 5 percent milk at 4 °C overnight to saturate protein-binding site. The antigen electroblotted onto the nitrocellulose membranes was incubated either with anti-sera (1:1000 dilution) or anti TLH/TDH mouse monoclonal antibody. Horseradish peroxidase-conjugated anti-mouse (Sigma, India) secondary antibody was used at a dilution of 1:1000. The bands were developed using Diaminobenzidine tetrahydrochloride (DAB) (Sigma, India) and H₂O₂ (0.03%) at room temperature.

(vi) Isotyping of murine immunoglobulins

Typing of the polyclonal and monoclonal antibody was carried out by plate ELISA using horseradish peroxidase labeled antimouse type sub-isotyping kit (Sigma, India), following the manufacturer’s recommendations.

(vii) Dot ELISA

The Specificity of monoclonal antibodies was tested by dot ELISA against various bacterial species mentioned in Table 7.1 as well as V. parahaemolyticus seafood isolates. The concentrated culture supernatant from different bacterial strains were mixed with carbonate-bicarbonate buffer (pH 9.6) and deposited onto nitrocellulose membrane. The membranes were blocked with 5 percent skimmed milk powder in PBS solution. The samples were incubated with MAb supernatant for 1 hr at 37 °C and detected with 1:1000 dilution of peroxidase-conjugated anti-mouse IgG
developed with the substrate DAB. The reactions were stopped by soaking the blots in distilled water.

(vii) Analysis of neutralizing activity

Polysera (1:1000 dilution) and monoclonal antibodies were incubated with concentrated supernatants containing native TLH and TDH in PBS for 1 hr at 37 °C before the washed human erythrocytes were subsequently added to a final concentration of 5 percent and incubated for another 1 hr at 37 °C. Haemolysis was measured at an OD of 545 nm. The positive and negative controls included toxins only and PBS, respectively. Each experiment was repeated three times and the mean±SD values were plotted in a graph.

5.3 RESULTS

(i) Design of chimeric gene tlh/tdh

The partial tlh and tdh genes which were used to facilitate the construction of chimeric gene were PCR amplified with respective primers containing restriction sites as overhangs to yield a PCR amplicon of size 357 bp and 426 bp, respectively. Fusion of tlh and tdh gene with the aid of restriction enzymes and ligation delivered a single PCR amplicon of 777 bp which is incorporated into the plasmid vector (Fig 7.2). The primers were designed in such a way that both the genes were in the proper open reading frame with themselves as well as with the N-terminal tag of the pRSET A plasmid. Restriction digestion of recombinant plasmid revealed a 2.9 kb linearized vector and 0.77 kb insert. Sequencing of the plasmid consisting of the chimeric fusion gene revealed proper open reading frame.

(ii) Cloning and expression of recombinant chimeric protein TDH/TLH
The expression of the cloned recombinant fusion protein was assessed by SDS-PAGE. The resolved proteins were stained with Coomassie blue and a band of increasing intensity in the expected size range for the recombinant protein was observed. The IPTG-induced recombinant protein was expressed at approximately 36 kDa region by SDS-PAGE (Fig 7.3), which was further confirmed by western blot using anti-His antibody (Fig 7.4). Protein expression and purification results showed that TLH/TDH was highly expressed. The affinity-purified recombinant protein extract had 2.8 mg/ml of concentration (Fig 7.5).

(iii) Production of polyclonal and monoclonal antibodies against the recombinant protein

Polyclonal antibodies were generated against the recombinant chimeric TLH/TDH of *V. parahaemolyticus* by immunization of female BALB/c mice. The polysera generated against the recombinant protein could simultaneously react with native TLH (43 kDa), TDH (21 kDa) and TRH (23 kDa) of *V. parahaemolyticus* (Fig 7.6) and was found to comprise of diverse isotypes of antibodies (Fig 7.7). The antibody of type IgG1 was found to be produced higher than other types of antibodies. Hybridomas were developed for the production of monoclonal antibodies (MAbs) against the recombinant protein. Out of four hybridoma cell lines generated, only one could be stabilized. Monoclonal antibodies secreted by this clone exhibited reaction not only for recombinant from TDH/TLH with protein size of 36 kDa, but also specifically at 21 kDa corresponding to native TDH protein of *V. parahaemolyticus* strain (Fig 7.8). This monoclonal antibody was found to be IgG1 in nature, which is the predominant MAb isotypes found in the polysera developed against the recombinant TDH/TLH. The Reactivity of these MAb was further assessed with other
organisms by examining reaction of MAb, VPA1 against the supernatant of cell culture by Dot-ELISA. There was no cross-reaction of the MAb, VPA1, and the specificity was found only to the TDH haemolysin of \textit{V. parahaemolyticus} (Fig 7.9).

(iv) Protection studies

Analysis of polyclonal antisera and monoclonal antibody for neutralizing activity of the haemolysis exhibited by the native exotoxins present in the supernatant revealed significant reduction in haemolysis of the human erythrocytes (Fig 7.10 and 7.11). The polysera could relegate the toxicities of TLH, TDH and TRH concurrently whereas the MAb, VPA1 could effectively neutralize TDH toxicity.

7.4 DISCUSSION

\textit{V. parahaemolyticus} infects to a wide range of hosts in marine ecosystem, including fishes, mollusks, crustaceans and cephalopods and is the major concern for the economic losses around the world. The pathogen causes food-borne acute gastroenteritis in humans, often associated with the consumption of raw or undercooked seafood. Clinical symptoms of \textit{V. parahaemolyticus} infections include watery diarrhea, abdominal cramps, nausea, vomiting, headaches, fever and chills (Shimohata, and Takahashi., 2010). Beside TDH role of TDH haemolysin in virulence has also been well documented. On M- Generuler 1kbDNA ladder SM0311 (MBI Fermentas, India), 1-\textit{tlh} gene, 2-\textit{tdh} gene, 3. \textit{tlh/tdh} gene the other hand the TLH so far has found application only as species specific protein. TDH and TRH are known to induce cytotoxicity in Vero cells and enterotoxicity in CaCo-2 cells, respectively. The role of TLH in the pathogenicity of the organism during gastroenteritis and septicaemia infections still remains uncertain. In the present study, a novel chimeric gene encompassing C-terminal region of TDH and a conserved region of TLH was
Fig 7.2: Construction of chimeric gene *tlh/tdh*.

Fig 7.3: Expression of recombinant fusion protein TLH/TDH.
Fig 7.4: Reactivity of anti-His antibody with recombinant clone TLH/TDH.

Lane: 1. Pageruler prestain protein ladder SM0671 (MBI Fermentas, India), 2. E. coli strain with Recombinant plasmid

Fig 7.5: Purification of recombinant fusion protein TLH/TDH.
Fig 7.6a: Reactivity of antisera with TLH⁺, TRH⁺ and TDH⁺ *Vibrio parahaemolyticus*.

Lane- M. Pageruler prestain protein ladder SM0671 (MBI Fermentas, India), 1. VPF1, 2. *V. parahaemolyticus* ATCC 17802, 3. *V. parahaemolyticus* K11555 (O3:K6).
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Fig 7.6b: Clustal W analysis of TDH and TRH haemolysins of *Vibrio parahaemolyticus*.

Fig 7.7: Isotyping of antisera raised against recombinant TLH/TDH chimeric fusion protein.
Fig 7.8: Reactivity of MAb VPA1 with recombinant TLH/TDH protein and native TDH haemolysin.


Fig 7.9: Specificity of MAb VPA1 by Dot-ELISA.

Lane: 1- A. *V. parahaemolyticus* K11555 (03:K6), B. *V. parahaemolyticus* K12262 (01:K25), C. *V. parahaemolyticus* K21886 (01:KUT), 2- A. *V. parahaemolyticus* ATCC 17802, B. *V. parahaemolyticus* E2253 (02:K3), C. *V. parahaemolyticus* E2270 (04:K8), 3- A. *V. cholerae* 6120/06, B. *V. vulnificus* ATCC 27562, C. *V. mimicus* VM1, 4- A. *V. furnissi* ATCC 35016, B. *V. alginolyticus* ATCC 17749, C. *V. parahaemolyticus* VPF2 (isolate), 5- A. *Aeromonas hydrophila* ATCC 35654, B. *Pleisomonas shigelloides* MTCC 1737, C. Positive control (recombinant fusion protein), 6- A. *Escherichia coli* ATCC 10536, B & C- Negative controls.
Fig 7.10: Protection studies using antisera raised against recombinant chimeric TDH/TLH protein.

Fig 7.11: Protection studies using MAb VPA1 raised against recombinant chimeric TDH/TLH protein.
constructed, cloned, expressed in a heterologous bacterial host. The recombinant protein was used to immunize female BALB/c mice and the hyperimmune polysera was tested for its potential in detecting and differentiating virulent strains of *V. parahaemolyticus* from avirulent strains. The antiserum was also utilized in neutralizing the toxicities of native wild type toxins.

The conserved regions of the TLH and TDH haemolysins were chosen after thorough bioinformatic studies utilizing BLASTn and BLASTp tools. TLH, being a species-specific marker for *V. parahaemolyticus* was chosen as one of the components in the fusion protein with an aim of generating antibodies for specific detection of all strains of *V. parahaemolyticus*. TDH and TRH are highly homologous structurally and immunologically. ClustalW alignment of the protein sequences of both the toxins revealed a high similarity (Fig: 7.6b). Worldwide TDH was observed to be associated with pandemic strains of the bacterium, thus emphasizing the relevance of this toxin in infectivity. Hence, the C terminal region of TDH which is elucidated to be functional in initiating the post binding activities during the toxicity was targeted as the second component of the fusion protein. The recombinant fusion protein retained the immunogenicity of both the native toxins as demonstrated by the reactivity of the hyperimmune sera to wild type toxins in Western blot.

Various techniques such as gene detection assays and immunological assays are available for detecting these virulence factors or their genes. Molecular methods often need costly equipment and reagents with trained personnel. Even though they are more sensitive in detection of protein related genes, they cannot confirm the expression of the protein by the bacteria. Nevertheless, Immunological assays could overcome this problem and are most convenient for clinical diagnostic laboratories.
Majority of the immunological assays developed till date incorporated either one or many monoclonal antibodies in combination for the simultaneous detection of TDH, TRH and TLH (Honda et al., 1989; Sakata et al., 2012). The Western blot developed in the current study could be one such strategy where a repertoire of TDH specific, TDH and TRH cross reactive, TLH specific antibodies occur naturally in the generated hyperimmune polysera can simultaneously and specifically detect all the three major haemolysins of *V. parahaemolyticus* strains. Alternatively, the MAb generated against the recombinant protein showed reactivity to the native TDH of *V. parahaemolyticus* alone and did not show cross-reactivity with any other *Vibrio* species or bacterial strains. This specific MAb can be utilized in detection of pandemic strains of *V. parahaemolyticus*.

Safety of the immunogen is of prime importance when a recombinant protein is to be immunized to murine hosts. The recombinant fusion protein developed here is non-toxic as demonstrated by its incapability to cause hemolysis on blood agar plate as well as for the purified mouse erythrocytes. High levels of specific serum immunoglobulins IgG\(_1\), IgG\(_{2a}\), IgG\(_{2b}\), IgG\(_3\) and IgM were found to be produced when the hyperimmune sera against the recombinant protein was isotyped after the immunization schedule. The antisera effectively relegated the toxicities of wild type TLH, TDH and TRH.

In conclusion, the recombinant fusion protein encompassing *V. parahaemolyticus* TDH and TLH was a remarkable antigen with no toxicity and the antibodies developed against the antigen could specifically identify TDH, TLH as well as TRH. The antisera against the immunogen effectively neutralized all the three wild-type toxins from clinical virulent strain *in vitro*. This study provides the basis for
determining the *in vivo* immunological correlates of the protective immunity demonstrated here.