REVIEW OF LITERATURE
2.1 **CLASSIFICATION**

The strain *S. dysenteriae* is known to produce the Shiga toxin. It was for the first time described by Conradi in 1903. The Shiga family includes the Shiga toxin from *Shigella dysenteriae* type1 and Shiga-like toxins belonging to A-B class of bacterial toxins produced by enteropathogenic *Escherichia coli*, *Campylobacter jejuni*, *Salmonella typhimurium*, *Vibrio cholerae*, *Vibrio parahaemolyticus* (Strockb-ine et al., 1985) and in *Citrobacter freundii* and *Enterobacter cloacae* (Paton and Paton, 1997).

### 2.1.2 Classification of the A chain of the toxin from *Shigella dysenteriae* (Fraser 1994)

Since the enzymatic activity is localized in the A chain of the toxin, hence its classification is given below:

- **Lineage**: Shiga toxin
- **Class**: Alpha and Beta (A + B)
- **Fold**: Ribosome inactivating protein (RIP)
- **Superfamily**: Ribosome inactivating protein (RIP)
- **Family**: Shiga toxin, A chain
- **Protein**: Shiga toxin, A chain
- **Species**: *Shigella dysenteriae*
- **Shiga toxin A - chain (rRNA N-glycosidase)**
  (EC 3.2.2.22)
The Shiga toxin family is composed of bacterial proteins which
1) share the 1A: 5B holotoxin molecular structure.
2) are 28S rRNA N-glycosidases and inhibit protein synthesis.
3) use the neutral glycolipid globotriaosylceramide as the primary functional toxin receptor.

The prototype toxin of the family, Shiga toxin is produced by *Shigella dysenteriae* – type 1A. A few of the serotypes of *Escherchia coli* synthesize one or more toxins designated Shiga-like toxins (SLT's) or verocytotoxins (VT). SLT's are categorized on the basis of their antigenic nature into SLT-I and SLT-II types.

Polyclonal antibodies to Shiga toxin neutralize SLT-I. SLT-1 was found to be nearly identical to Shiga toxin with a difference of only one amino acid.

The SLT-II activity is not neutralized by the antisera to Shiga toxin and studies have shown that no cross-hybridization with SLT-I specific DNA probes occurs and their production is not iron regulated. Toxins of the SLT-II family have been sub-grouped into the prototype SLT-II toxin and its variants SLT-II i.e. SLT-II a, SLT-II c, SLT-II e or SLT-IIv.

The SLT-II c toxin exhibits only partial serological reactivity to SLT-II. The receptor for SLT-II c is Gb3.

A second sub-type of SLT-II family consists of SLT-II e which shares 93 % and 84 % amino acid homology to SLT-II A and B subunits of SLT-II respectively. SLT-II e is found in the porcine strains of *E. coli*.
and is associated with edema disease of pigs. The receptor for SLT-II e is \( \text{Gb}_4 \) (globotetraosylceramide).

A third SLT-II related toxin is SLT-II va which shares only 70% amino acid homology in the A and 84.3% in the B sub-unit of SLT-II (Russmann et al., 1994; Ramegowda and Tesh, 1996).

2.2 SHIGA TOXIN AND PATHOGENESIS

Keusch et al. (1972) found that the enterotoxin from *Shigella dysenteriae* – 1 is cytotoxic to intestinal epithelial cells *in vivo*. The enterotoxin was found to produce alterations and extrusions of villous epithelial cells as early as one hour after challenge. By six hours, the morphology of epithelial cells changed from columnar to cuboidal. The villi were shortened in this period along with the shortening of the villus to cryptic ratio.

Keusch and Jacewicz (1975) suggested that the enterotoxin and neurotoxin are closely related proteins that could be identical. The biological evidence that the neurotoxicity, enterotoxicity and cytotoxic activities are due to one entity was later given by Eiklid and Olsnes (1983).

There is a relationship between production of the cytotoxin and clinical features of shigellosis. Patients infected with strains of *Shigella* that produced greater amounts of the toxin were more likely to have fever and occult blood in their stools. The amount of cytotoxin determined in the study, however, did not correlate with the number of
stools passed, severity of abdominal pain, nausea or vomiting (Cleary et al., 1986).

The role of Shiga toxin in pathogenesis of bacillary dysentery was studied by Fontaine et al. (1988) using a Tox - mutant of Shigella dysenteriae - 1. Shiga toxin was found to have no effect on the rate of intracellular growth of bacteria or on the rapid killing of invaded host cells. The production of Shiga toxin correlated with the presence of blood in stools, a sharp drop in polymorphonuclear cells and histopathological alterations such as the destruction of capillary vessels within the connective tissue of the colonic mucosa, severe inflammatory vasculitis of the peritoneal mesothelium and major efflux of inflammatory cells. It was proposed that Shiga toxin could influence the severity of bacillary dysentery by inducing colonic vascular damage during the course of infection.

Three-day-old rabbits were inoculated intragastrically with high and low VT producers and VT negative E.coli to study the pathogenesis of diarrhea. The clinical symptoms correlated with the presence of detectable free VT in the colon. There was a vast increase in apoptosis in surface epithelium, increased mitotic activity in crypts, mucin depletion, a mild to moderate infiltration of neutrophils in the lamina propria and epithelium. Multiple foci of attached bacteria were seen in epithelial cells of gut-associated lymphoid tissue, cecum and colon. The studies led to
the conclusion that VT plays an important role in the pathogenesis of diarrhea caused by *E.coli* (Pai et al., 1986).

The neurological manifestations of shigellosis were found to occur without evidence of Shiga toxin production as the strains used in the study were lacked the structural genes for toxin production (Ashkenazi *et al.*, 1990). Low dilutions of the sonicates of *Shigella* isolates from children with neurological symptoms caused agglutination of neuroblastoma cells, but no cell killing was observed by Ashkenazi *et al.* (1994).

The interaction of VT2e with pig intestine was studied by Waddel *et al.* (1996). VT 2e was found to bind significantly to microvillus membranes from pig jujenum and ilium, but not to mucus. In the pig intestine, the receptors for VT 2e were found to be present, but their role in absorption of the toxin was not clear since intra-intestinal inoculation of pigs with large quantities of VT 2e did not result in edema disease.

### 2.3 Sensitivity of Various Cell Lines

Various cell lines were tested for their susceptibility to Shiga toxin. Several strains of HeLa (cervical carcinoma cells) were shown to be particularly sensitive (Elklid and Olsnes, 1980). Other sensitive cell lines known are KB, human liver, and monkey kidney (Vero) cells. The colon carcinoma cell line HT-29 is moderately sensitive, whereas WI-38, Henle-407, Chinese Hamster Ovary, BHK or human melanoma cells are not
sensitive unless delivered intracellularly by a live bacterium. Moyer et al. (1986) found only half of the primary cultures of human colonic and ileal epithelial cells to be sensitive to Shiga toxin.

Dividing human vascular endothelial cells (HUVEC) are more sensitive to Shiga toxin than quiescent cells in confluent monolayers (Obrig et al., 1988).

Shiga family toxins are directly cytotoxic to human glomerular endothelial cells (Obrig et al., 1988). There is evidence that verocytotoxins from E.coli bind to P blood group antigens of human erythrocytes in vitro (Bitzan et al., 1994).

The sensitivity of HUVEC to Shiga toxin is $10^6$ fold less than Vero cells (Tesh et al., 1991) but their sensitivity can be induced by pre-incubation with LPS or IL-IB and tumor necrosis factor (TNF) (Louise et al., 1991).

A comparison of the effects of SLT-I on cytokine butyrate treated HUVEC and human saphenous vein endothelial cells (HSVEC) was done by Klesch et al. (1996). Untreated HSVEC were found to be more susceptible than HUVEC to Stx-1.

Ramegowda and Tesh (1996) reported that undifferentiated THP-1 cells were sensitive to SLTs. Treatment of cells with differentiation factors resulted in an increase in resistance which was associated with a decrease in toxin receptor expression. U-937 cells and monocytes (PBMn) were resistant to Stx irrespective of the state of maturation.
In HeLa cells, the cytotoxic effect of Shigella toxin is related to the binding of the B sub-unit to N-linked glycoprotein on the cell surface that contains oligomeric B- 4- linked N-acetyl D-glucosamine (Glc NAC) (Donohue-Rolfe et al., 1986). The glycolipid was identified as Gb3 (globotriaosylceramide) (Jacewicz et al. 1986). In a subsequent study, these authors (Jacewicz et al., 1989) while determining the binding kinetics of Shiga toxin to HeLa cells found two classes of binding sites: one of low affinity and high capacity and another of high affinity and low capacity. The number of high affinity sites present on the cells correlated with their sensitivity to the toxin.

Sandvig et al. (1984) observed that cycloheximide, but not swainsonine sensitized Vero cells to Shiga toxin. The role of ST and SLT in mediating direct damage to cells was studied by Tesh et al. (1991). Confluent endothelial cells were sensitive to Shiga toxin, but less than Vero cells. Lipopolysaccharides (LPS) were not cytotoxic and did not augment Shiga toxin mediated toxicity. When co-incubated with tumor necrosis factor, lower doses of Shiga toxin were found to be cytotoxic.

Neuroblastoma cell lines LA-NI, LA-N5 and IMR were not susceptible to Shiga toxin. Pretreatment with TNF also did not affect the susceptibility of the neuroblastoma cells (Ashkenazi et al., 1994).

The effects of the toxin on the morphology and protein synthetic capability of HeLa cells in tissue culture were inhibited by brefeldin A.
It was reported that butyric acid sensitizes A-431 cells which otherwise bound and internalized the Shiga toxin, but were completely resistant to it. Cells not exposed to butyric acid were not affected by Shiga toxin. The protein synthesis in the treated cells was however, reduced to half. There was no effect of short incubation times with butyric acid. Pre-treatment of cells with LPS, IL-1β or TNF-L enhanced toxicity. Sodium butyrate was found to increase both Gb$_3$ content and toxin binding, but inhibited cytokine enhanced cytotoxicity (Keusch et al., 1996).

The cytotoxicity of culture supernatants of strains encoding SLTs incubated with mouse and human intestinal mucus treated cells was increased. This increased toxicity after mucus treatment was probably not due to cleavage of the A sub-unit into A$_1$ and A$_2$ sub-units, but due to direct activation of SLT by mucus (Melton-Celsa et al., 1996).

Karpman et al. (1997) found that mice inoculated with *E. coli* 0157: H7 developed gastrointestinal, neurologic and systematic symptoms, necrotic foci in the colon, glomerular and tubular histopathology. Mice inoculated with SLT-II positive strains developed severe neurotoxic and a higher frequency of systematic symptoms along with glomerular pathology as compared to SLT-II negative strains. Anti-SLT-II antibodies protected against the disease.
According to Fernandez – Prada et al. (1997) infection of human monocyte derived macrophages in vitro and with virulent *S. flexneri* resulted in cell death involving rupture of plasma membrane by swelling, disintegration of ultrastructure and general karyolysis. These features resembled oncosis and were in striking contrast to observation on mouse macrophages where infection by virulent *Shigella* caused death through apoptosis. Patterns of intracellular survival of *Shigella* strains reflected difference between *Shigella* serotypes, and also between the two macrophage cell lines.

Louise et al. (1997) examined individual signal transduction components involved in the sensitization of HUVEC to Stx. Their results demonstrated that class I and class II protein kinase isoenzymes (PKC) are required for sensitization of HUVEC to Stx. HUVEC responded to some, but not all the sensitizing agents tested. NF-\(\kappa\)B activation did not appear to be involved in the sensitization of HUVEC to Shiga toxin by LPS, TNF, IL-1 on PMA.

The role of second messengers in Stx induced fluid secretion was evaluated by Kaur et al. (1997). It was observed that PKC, intracellular calcium stores and prostaglandins play the same role. The extracellular calcium pool, however, had no significant role in the process of Stx induced enteritis.
2.4 **TOXIN AS THE ANTIGEN**

O'Brien et al. (1977) while studying the serological relationship of the toxins from *S. dysenteriae*-1 and *S. flexneri*-2 A, observed a serological relatedness between the two toxins as shown by cross-neutralization tests.

Two toxin-converting phages from *E. coli* 0157:H7 strain 933 encoded antigenically distinct toxins with similar biological activities. These were designated as SLT-I and SLT-II. Strains of *E. coli* produced either SLT-I or SLT-II or both the toxins (Stockbrine et al., 1986).

The antigenic relationship that exists between the VT-2 and SLT-II toxins was examined by Head et al. (1988) using homologous and heterologous antisera. It was indicated that small but significant differences exist between VT-2 and SLT-II. A site-directed mutagenesis was carried out by Ito et al. (1991) to find out antigenic differences between VT-2 and VT-2 vh. The results suggested the difference in antigenicity due to difference in the B subunit of the two toxins. This difference was attributed to dissimilarity in the amino acid residue at position 24 in the B sub-unit.

The antigenic relationship existing among the major outer membrane proteins (MOMP) produced by the four species of *Shigella* was studied (Roy et al., 1994). The MOMP from *S. dysenteriae*-1 cross-reacted with MOMPs of the other three species of *Shigella*. This indicated that the proteins were antigenically related in the different species.
Synthetic peptides of Shiga toxin B sub-unit induced antibodies that neutralized its biological activity (Harari et al., 1988). Serological response to it of SLT-I and its peptide fragments indicated that it is a vaccine candidate to counter the action of the toxin (Boyd et al., 1991).

Beilaszewska et al. (1997) studied the localization of intravenously administered verocytotoxins (SLT) 1 and 2 in rabbits immunized with homologous and heterologous toxoids and toxoid sub-units. It was seen that in vivo there is cross neutralization of VT-1 and VT-2. This in vivo cross neutralization was found to be a function mainly of antibodies directed towards the A-subunits. It was suggested that VT-1A or VT-2A might be a suitable immunogen for immunizing humans against systemic VT-mediated disease.

Monoclonal antibodies against SLT from E. coli neutralized the cytotoxicity of SLT as well as its lethal action and enterotoxicity. (Stockbriene et al., 1985).

2.5 CULTURE CONDITIONS AND TOXIN INDUCING FACTORS

van Heyningen and Gladstone (1953) found that iron inhibits the production of Shiga toxin. As many as 40-330 atoms of iron were needed to inhibit the production of one molecule of Shiga toxin. Similar effect was caused by reduced growth temperature. Iron, however, did not suppress toxin synthesis in E. coli strains carrying the cloned genes. It also did not decrease β-galactosidase production in the strains harboring operon fusion plasmids. (Weinstein et al. 1988)
The effects of culture conditions: incubation time, culture media, incubation temperature, starting pH, aeration, static culture, anaerobic conditions, carbon sources, amino acids, antibiotics, and mitomycin C on the yield of SLT-II v from *E. coli* were studied by McLeod and Gyles (1989). It was shown that SLT-II v is primarily cell associated and that incubation at 37°C for 24 hours with shaking in syncase broth adjusted to pH 8.5 resulted in best yield. Addition of various carbon sources or amino acids did not increase the yield of the toxin. The yield of the toxin from strain TB-1 (pCG-6) was significantly increased upon addition of mitomycin C to the culture medium.

Temperature and medium dependent secretion of proteins by Shiga toxin producing *E. coli* was studied by Ebel *et al.* (1996). Growth of the bacteria in Luria broth resulted in secretion of polypeptides of 104, 55, 54, and 37 kDa. Growth in serum free tissue culture medium, however, resulted in production of p104 and p37 and additional peptides of 25 and 22 kDa in the supernatant fluid. Production of these polypeptides depended upon the growth temperature. It was induced in cultures grown at 37°C.

Karch *et al.* (1986) found that sub-inhibitory concentration of trimethoprim - sulphamethazole increased the total yield of Shiga like toxin produced by *Shigella dysenteriae* – 1 and by *E. coli*. Al-Jumaili *et al.* (1992) reported an enhancement in the production of verotoxin by *E. coli* by treatment with mitomycin. The treatment enhanced the
production upto a 100 fold. The mucus from mouse intestine and human colon induced SLT-II c expression in E. coli 091: 421 (Melton-Celsa et al., 1996). However, treatment of EHEC strains, namely E. coli 0157, 0111 and 026 with imipenum induced much lower levels of release of SLT and endotoxin than treatment with ceftazidime (Takabashii et al., 1997).

The role of Rec A and environmental factors was studied for their regulatory effect on SLT-II operon by construction of a plasma pADR-28 carrying genes for the promoter proximal portions of SLT-IIa and the gene for bacterial alkaline phosphatase. The mechanism was rec A dependent. A slight impact of growth temperature on SLT-II was observed. Osmolarity, pH, oxygen tension, acetates, iron levels or carbon source did not have any impact of SLT-II expression (Muhldorfer et al., 1996).

2.6 ENTRY AND TRANSPORTATION OF THE TOXIN

The sensitivity of Vero and HeLa cells to Shiga toxin was strongly dependent on the presence of divalent cations (Sandvig and Brown, 1987). It was shown that Ca^{2+} transport through physiologically present channels was required for entry of the Shiga toxin into cells.

It was seen by fluorescence spectroscopy that receptor bound B chain largely remained on the membrane surface without penetrating into the lipid bilayer Surewicz et al. (1989). The A-chains however, interacted directly with the receptor-free lipid bilayer.
The transport of the cytotoxin from *S. dysenteriae-1* to the cytosol is possibly through endocytosis (Sandvig et al., 1989). Inhibition of endocytosis by ATP depletion of the cells prevented toxin uptake. Exposure of the cells to the toxin at low pH, which inhibits translocation, but not endocytosis, allowed toxin to accumulate in a compartment that protected it from antibodies. Sandvig *et al.* (1994) showed that in butyric acid treated A-431 cells endocytosed Shiga toxin was not only transported to the trans-Golgi network, but also to all Golgi stacks, to the endoplasmic reticulum and to the nuclear envelope. Furthermore, it was found that butyric acid sensitized the cells to Shiga toxin. This was consistent with the possibility that retrograde transport is required for the translocation of toxin to the cytosol. It was further observed that shiga toxin is internalized predominantly or exclusively via clathrin coated pits (Sandvig and vanDeurs, 1994). Once internalized, it is delivered to endosomes. There is retrograde transport of both ST and B subunit of the toxin from the Golgi complex to the ER (Sandvig *et al.*, 1994). It is regulated by butyric acid and cAMP. The process of retrograde transport could possibly be regulated by signals imposed on the cells in situ.

To investigate the retrograde transport pathway, a recombinant B fragment that was carboxy-terminal modified by addition of an N-glycosylation site and the KDEL peptide was constructed (Johannes *et al.*, 1997). A significant part of this chimeric protein was found to be
glycosylated after incubation with HeLa cells. The B-glyc KDEL was found to be delivered from the plasma membrane to the endoplasmic reticulum via the Golgi apparatus through the trans-Golgi network. There could possibly be a continuous pathway from the plasma membrane to the endoplasmic reticulum.

According to Acheson et al. (1996), biologically active Stx-I is capable of moving across intact polarized intestinal epithelial cells without apparent cellular disruption probably via a transcellular pathway.

While studying the role of the disulphide bond in Shiga toxin A-chain for toxin entry into cells, Garred et al. (1997) suggested that the disulphide bond in the A-chain was not required for rapid intoxication of cells, but was important for toxicity for a long incubation time.

2.7 RELEASE OF TOXIN

To release the toxin from the bacterial cells, polymyxin treatment was given by Griffin and Gemski (1983). The amount of toxin released was found to be dependent upon polymyxin concentration and the time of incubation. Similar observations were made by Donohue-Rolfe and Keusch (1983).

2.8 PURIFICATION

There has been a gradual evolution of various schemes leading to the purification of the toxins of shiga family. van Heyningen and
Gladstone (1953) produced and purified the neurotoxin from *Shigella shigae*. The toxin obtained was electrophoretically homogeneous.

Using French-press lysates, affi-gel blue chromatography and chromatofocussing, O'Brien and LaVeck (1983) purified the toxin from an enteropathogenic *E. coli* to apparent homogeneity.

A simple method of purification of ST or SLT from *S. dysenteriae* and *E. coli* 0157: H7 using immunoaffinity column chromatography was developed by Kongmuang *et al.* (1987). This method gave a high recovery rate.

Purification of a Shiga toxin A sub-unit –CD4 fusion protein that was cytotoxic to HIV infected cells was carried out by Al-Jaufy *et al.* (1995).

A system for production and rapid purification of large amounts of Shiga toxin / SLT-IB sub-unit was developed by construction of a plasmid expression vector (pEBC 32) encoding the B sub-unit under control of *trc* promoter (Calderwood *et al.*, 1990). Over producing clones of the SLT-IB cistron were used to purify the SLT-IB sub-unit by Ramotar *et al.* (1990).

To purify Shiga toxin from *E. coli* HB-101 containing SHT-23 was used by Kozlov *et al.* (1993). The holotoxin was purified by Blue Sepharose and chromatofocussing column chromatography.

Purification of recombinant Shiga-like toxin type 1-B subunit by using plasmid vector SBC-32 was described by Austin and Hovde (1995).
2.9 CHARACTERIZATION

Shiga toxins from various sources have been characterized. van Heypingen and Gladstone (1953) studied the properties of the Shiga neurotoxin. The toxicity was found to be 750 LD50/mg/kg in mouse and 115 x 10^4 /mg/kg in rabbit.

The SLT from *E. coli* H30 was characterized (O’Brien and Holmes, 1983). The Mr of the toxin was 48,000 while that of strain Shiga-60R was 58,000. They had comparable biological properties. The molecular weights of the sub-units were 31,500 and 4,000-15,000 in the absence of β-mercaptoethanol while in its presence an additional band was seen at Mr. 27000 was observed.

A major band of 35,000 and a minor band of <14000 was observed in verocytotoxin-2. Non-reducing PAGE revealed a single band possessing high activity and its CD50 was 1.39 x 10^6 units / mg protein in Vero cells (Petrie et al., 1987). A pl of 6.5 was obtained.

SLT-II variant had the following molecular weight subunits—33,000, 27,500 and 7,500 corresponding to A, A1, and B subunits respectively (MacLeod and Gyles, 1990). The CD50 on Vero cells was 0.5 ng and LD50 was 0.9 pg.

In gels containing 0.5 % SDS, the B subunit dissociated into monomers (Ramotar et al., 1990). It had the same glycolipid receptor specificity as the SLT-I holotoxin. The B subunit purified by Calderwood
et al. (1990) was biologically active in blocking binding of Shiga holotoxin to HeLa cells.

2.10 SUBUNITS

Shiga toxin from *Shigella dysenteriae* and Shiga like toxins of *E. coli*, are bipartite comprising an enzymatically active A sub-unit which is non-covalently associated with five receptor binding B sub-units (Olsnes *et al.*, 1981). The A-subunits were nicked by a bacterial protease to give two disulphide linked fragments designated $A_1$ which possessed the enzymatic activity and $A_2$ which was the bridge between the $A_1$ polypeptide and the pentamer (Kongmuang *et al.*, 1988).

The $A_1$ fragment had a molecular weight of 27,000 and the $A_2$ fragment was an entity having a molecular weight of 4000. The A-subunit had a molecular weight of 32,000 Daltons (Donohue-Rolfe *et al.*, 1984).

The $B$ sub-unit consisted of 69 amino acid residues, while the A subunit was a polypeptide of 297 residues (Seidah *et al.*, 1986). The $B$ sub-units of Shiga toxin, Shiga-like toxins 1 and 2 recognized the receptor globotriposylceramide ($Gb_3$) though the binding of Stx-II was less than Stx-I. The receptor for Stx-IIe, however, was globotetraosylceramide ($Gb_4$) which gave the toxin a different range of effected cells.
2.11 ASSEMBLY / HOLOTOXIN

Preliminary X-ray analysis of the B sub-unit was performed by Boodhoo et al. (1991) and crystallization was done by Stein et al. (1992). The studies established the doughnut shaped pentamer of the B sub-unit of the toxin. The individual B sub-units were composed of anti-parallel B-sheets and a \( \alpha \)-helix. The cleft formed by the interaction of B-sheets in adjacent monomers resulted in five carbohydrate binding sites per pentamer.

Haddad and Jackson (1993) identified a stretch of nine non-polar amino acid residues near the C-terminus of Shiga toxin A subunit required for sub-unit association. Biological and physical evidences indicated that the \( A_2 \) fragment of Shiga like toxin type-I was required for holotoxin integrity (Austin et al., 1994). The three amino acid residues in the B sub-unit of Shiga toxin and Shiga-like toxin-II essential for holotoxin integrity were identified by Perera et al., (1991). According to Hovde et al. (1988), glutamic acid 167 was an active-site residue of Shiga toxin and was critical for its activity.

Using hybrid cytotoxins, Weinstein et al. (1989) found that the B sub-unit dictated the cytotoxic specificity and localization of the toxin within the bacterial cell.

The amino acid sequence of the B sub-unit was determined by Seidah et al. (1986). It revealed a 69 amino acid peptide with a single disulphide bridge and predicted its molecular weight to be 7691. A
distant homology with the 103 residues B chain of cholera and *E. coli* enterotoxins was also revealed. Mutations affecting the activity of SLT-IA chain were studied by Deresiewicz et al. (1992). SLT-IA was placed under the control of an inducible promoter in *S. cerevisiae*. Induction was found to be lethal to the host. This lethality was used as the basis for selection of an alternated mutant of SLT-IA changed at residue 77, a locus known to be conserved in the ricin family. This gave a new insight into the geometry of the SLT-IA active site. Tyrosine-114 had a significant role similar to Y-77 in the enzymatic activity of Shiga like toxin-IA chain (Deresiewicz et al., 1993).

It has been indicated that proteolytic cleavage at arginine residues within the hydrophilic disulphide loop of the SLT-I a subunit is not essential for cytotoxicity (Burgess and Roberts, 1993).

### 2.15 CRYSTALLIZATION

Crystallization and preliminary X-ray crystallographic analysis of the Verotoxin B sub-unit was done by Boodhoo (1991). The crystals were found to grow over a wide pH range with a number of precipitants. The crystal structure of the B-oligomer of verotoxin-1 isolated from *E. coli* determined by Stein et al. (1992) was found to resemble the B-oligomer of cholera toxin and heat-labile enterotoxin from *E. coli* despite the absence of detectable sequence similarity between the proteins. Hart *et al.* (1991) crystallized the Shiga-like toxin-1 from *E. coli* in a form suitable for X-ray analysis. The rotation function using three-
dimensional diffraction suggested that the asymmetric unit was a tetramer.

The toxin from *S. dysenteriae* has been crystallized by Kozlov *et al.* (1993). Two crystals were grown—one as fine needles, hexagonal in cross-section and the other as thin plates.

### 2.16 GENE SEQUENCING

Calderwood *et al.* (1987) determined the nucleotide sequence of the Shiga-like genes of *E. coli*. They reported that genes of A and B subunits of SLT apparently constitute an operon with only 12 nucleotides separating the coding regions. The peptide sequence of A sub-unit of SLT was homologous to the A- subunit of plant toxin ricin.

The primary structure of the operon codings for *Shigella dysenteriae* toxin and SLT from temperate phage 30 was determined by Kozlov *et al.* (1988). The coding sequence of SHT and SLT genes differed in 4 nucleotides with a 1 nucleotide change which was responsible for one amino acid replacement.

The *E. coli* SLT-II variant gene was cloned and its nucleotide sequence determined by Gannon *et al.* (1990).

### 2.17 PROPERTIES

The mode of action of the Shiga toxin on peptide elongation was studied by Obrig *et al.* (1987). Elongation factor-1 dependent GTPase activity and enzymic binding of (3H) phenylalanine-tRNA to reticulocyte
ribosomes were found to be inhibited. 200 nM of toxin was required to inhibit 50% of the elongation factor 2-dependent translation of aminoacyl tRNA on ribosomes. It was thus a less potent inhibitor of EF-2 dependent reaction.

The site of action of verotoxin-2 on eukaryotic ribosomes was detected by Endo et al. (1988). Treatment with the toxins caused the release of a fragment of 400 nucleotides from 28S rRNA on treatment of the rRNA with alanine. This was accompanied by inhibition of protein synthesis and of EF-1 dependent aminoacyl tRNA binding to ribosomes. An adenine base at position 4324 (A-4324) was absent in toxin treated 28S rRNA. It was indicated that VT-2 and ST inactivate the 60S ribosomal sub-units by cleaving the N-glycosidic bond at position A-4324 in the 28S rRNA.

The RNAase activity was shown to be associated with 60S ribosomal inactivation by ricin, phytolaccin and Shiga toxin proteins (Obrig et al.,1985). The ST, SLT-II variant and ricin were single site RNA N-glycosidases of 28S RNA when microinjected into Xenopus oocytes (Saxena et al., 1989).

2.18 CLONING AND EXPRESSION OF ST AND SLT

The cloning and expression of a 1.7 kb Bal I-Bgl II fragment from the genome of bacteriophage H19B into pUC18 was carried out by Huang et al. (1986). The fragment specified the two VT sub-unit peptides having masses 31 and 5.5 kDa. The structural genes for SLT-II v were
cloned from *E. coli* S1191 and the nucleotide sequence compared with the other members of Shiga family by Weinstein *et al.* (1988). The structural genes for ST and SLT-I were found to be essentially identical, differing by only three nucleotides in the A-subunit genes resulting in a single amino acid difference at threonine position 45. In contrast, the structural genes for ST and SLT-I shared only 57-60 % nucleotide sequence homology with SLT-II. SLT-II v and SLT-II were highly homologous (79 %). SLT-II v genes were, however, found to be only distantly related (55-60 %) to the genes for Shiga toxin.

The primary structure of the operons coding for *Shigella* toxin and SLT from temperate phage H30 was determined by Kozlov *et al.* (1988). He deduced amino acid sequence of the A-chains of the toxins that was highly homologous to the A chain of plant toxin ricin.

The sequence of a variant SLT-I operon of *E. coli* 0111:H7 differed from SLT-I by 5bp resulting in two amino acid changes. It was determined to be more closely related to the gene encoding the sub-unit of ST from *S. dysenteriae-1* from which it differed by 3 bps resulting in one amino acid change (Paton *et al.*, 1993).

Haddad and Jackson (1993) identified the B sub-unit gene promoter in the Shiga toxin operon of *S. dysenteriae-1*. It was proposed that independent transcription of the *Stx B* gene regulates the overproduction of Stx B polypeptides and the 1-A: 6B subunit stoichometry of the holotoxin. A study conducted by Muhldorfer *et al.*
(1996) led to the conclusion that bacteriophages exhibit a regulatory impact on toxin production by the following two mechanisms:

1) Replication of the bacteriophages brought about an increase in toxin production due to concomitant multiplication of the toxin gene copies.

2) An influence of a phage-encoded regulatory molecule was demonstrated to be dependent on phage induction by the use of a translational gene fusion between the promoter and the structural gene for alkaline phosphatase (\textit{pho A}).

Butteron \textit{et al.} (1997) used \textit{V. cholereae} as a vector to deliver two distinct antigens that are -

1) known virulence factors,

2) immunogenic

3) antibodies to it are protective against disease caused by these organisms. A chromosomal EHEC fragment containing \textit{eae A} was added to the construct downstream of \textit{Stx B}. The resultant vector strains were analyzed. They were unstable, produced low levels of \textit{Stx B-I} and did not evoke anti-eae A antibody responses \textit{in vivo} following oral immunization.

2.19 \textbf{STABILITY OF TOXIN GENES}

Upon sub-culturing, there is a frequent loss of Shiga-like toxin genes in clinical isolates of \textit{E. coli} (Karch \textit{et al.} 1992). According to Schmidt \textit{et al.} (1993) there was a loss of Stx genes in five out of seven
Citrobacter freundii strains tested after the first subculturing on agar plates or after two passages in broth culture. The remaining two strains, however, retained the genes upon repeated subculture. Paton and Paton (1997) found the Shiga toxin type 2 gene from Enterobacter cloacae to be unstable. This was consistent with their observations on Stx-producing E. coli strains other than 0157 and 011 that also lost their toxin genes after sub-cultivation.

In contrast, the B subunit genes of SLT-II variant in E. coli 0157 strains were highly conserved (Russmann et al., 1994).

2.20 PRODUCTION OF TOXINS BY OTHER SPECIES

It has been suggested that S. flexneri and S. sonnei could be producing a toxin substance that is antigenically related to Shiga toxin (Keusch and Jacewicz, 1973). S. flexneri was found to produce a Shiga like toxin at very low levels. This toxin was lethal to mice, enterotoxic for ileal loops and cytotoxic to HeLa cells. A serological relatedness between the S. flexneri-2a toxin and S. dysenteriae-1 toxin was shown (Thomson et al., 1976).

The purification and physiochemical properties of a unique Vero cell cytotoxin from E. coli was reported by Padhye et al. (1986). This toxin had a molecular weight of 64,000 and was without subunits. It had a pI of 5.2 and was lethal to mice, but was not neutralized by rabbit antiserum to Shiga toxin.
Barlett et al. (1986) studied the production of Shiga toxin and other cytotoxins by serogroups of *Shigella*. Their results showed that in *Shigella* strains other than *S. dysenteriae*-1, the production of cytotoxins immunologically distinct from Shiga toxins was more common than the production of Shiga toxin.

Some strains of *S. sonnei* and *S. flexneri* associated with neurological symptoms produced a cytotoxin distinct from ST, SLT-I or SLT-II. Partial characterization showed its active component to be a protein with molecular mass of ~100-125 kDa. This study was performed by Askenazi et al. (1990).

Two novel enterotoxins elaborated by *Shigella* species have been reported (Nataro et al., 1995; Fasano et al., 1995). *Shigella* enterotoxin-1 (SLET-1) was found to be a chromosomally encoded iron-regulated 55 kDa complex identified in *S. flexneri-2a* strains, while ShET-2 was reported to be a 62.8 kDa single moiety protein discovered in enteroinvasive *E.coli* and located in the large invasiveness plasmid. The prevalence of ShET-1 in clinical isolates of diverse *Shigella* serogroups was studied by Noriega et al. (1995). Screening of Set-1 by DNA colony hybridization and PCR showed it to be present in all the *S. flexneri-2a* strains, but was rare in other *Shigella* serogroups.

Nataro et al. (1995) have identified and cloned a novel plasmid-encoded enterotoxin. DNA hybridization of the gene (designated *sen*) was found in 75% of EIEC strains and 83% of *Shigella* strains. The gene
was cloned and its product was a protein with a molecular mass of 63 kDa, which elicited enterotoxic activity in ussing chambers.

Two strains of Shiga-like toxin producing organisms were isolated by PCR and colony blot hybridisation by Paton and Paton (1996). They identified the two strains as *E. coli OR:49* and *Enterobacter cloacae*. Both the isolates were highly cytotoxic to Vero cells. Nucleotide sequence analysis showed the toxin encoded by *E. cloacae* SLT-II related gene to be very similar to SLT-II variants of *E. coli* differing from the most closely related toxin by 3 residues in the A chain.

The diarrheagenicity of cytolethal distending toxin and the suckling mouse response to the *cdt ABC* genes of *Shigella dysenteriae* was examined. This toxin was implicated in diarrhea caused by strains of *E. coli* and *Shigella* species. It induced unique morphological changes in Chinese hamster ovary cells, but its association with diarrhea remained unclear (Okuda *et al.*, 1997).

### 2.2.1 assay / detection

Several methods for assay and detection of the toxin have been reported in literature.

van Hey-ingen and Gladstone (1953) described a semi-micro method for the flocculation assay of the toxin. The method used the toxin, its anti-toxin, a dye and this mixture was transferred into capillaries which were then visualized under a binocular stereomicroscopy to observe flocculation, if any.
Assay for the enterotoxic activity of the toxin using ligated small bowel loops in white New Zealand rabbits has been described by Keusch et al. (1975). These authors have also described an assay for its neurotoxic activity using Swiss-Webster mice to determine LD\textsubscript{50} and its paralytic-lethal effect.

Immunoprecipitation using iodolabelled toxin and anti-toxin was described by Kessler (1975). Immunoprecipitation was done by using a fixed protein A-positive \textit{Staphylococcus aureus} and its radioactivity measured.

Indirect ELISA method for measurement of toxin antigen was described by Donohue-Rolfe et al. (1984). The method involved the use of a sub-unit specific mouse, monoclonal ascities fluid antibody 4D3. This assay gave reproducible results with as little as 10 pg toxin / well.

A method for the isolation of verotoxigenic \textit{E. coli} from human and food samples by colony ELISA was developed by Milley and Sekla (1993). The method utilized monoclonal antibodies directed against verotoxins and was sensitive to all verotoxin 1 and 2 producing strains.

Using PCR and DIG- labeled probes, a technique for the identification of Shiga-like toxin producing \textit{E. coli} was developed by Begum et al. (1993). PCR amplified the SLT gene sequences from whole bacterial colonies. The DIG- labeled probe identified the PCR products in a spot hybridization assay. The sensitivity and specificity of the assay was 99%. Tyler et al. (1991) identified the verocytotoxin type-2 variant B...
genes in *E. coli* using PCR and RFLP analysis. In this study, PCR was used to determine the presence or absence of VT2 variant target genes sequences and RFLP analysis permitted the prediction of the genotypes for VT2 and VT2 variant genes harboured by the VT2 producing *E. coli* strains. An improvement in PCR to detect *E. coli* SLT-II gene from clinical isolates was made by Parma *et al.* (1996). The modification involved the concentration of DNA, primer and magnesium chloride. This modified method allowed a rapid, sensitive and specific screening of clinical samples.

To detect SLT producing strains of *E. coli*, a colony blot assay was described by Karch *et al.* (1986). The method involved the measurement of the diameter of the zone formed around bacterial colonies using monoclonal antibodies to SLT.

The interaction of *Shigella* toxin with globotriaosylceramide receptor containing membrane by fluorescence spectrophotometry was done. Binding of the B subunit to the receptor resulted in a strong enhancement of fluorescence intensity (Surewicz *et al.*, 1989).

An assay for *in vitro* inhibition of protein synthesis was described by Lingrel, - (1972) on reticulocyte lysate. The method measured the incorporation of a radioactive amino acid that was indicative of protein synthesis.

The cytotoxicity assay involved the use of a susceptible mammalian cell line and measured the detachment of dead cells from
the monolayer by cell counting (Keusch et al., 1972). Alternately, viable firmly attached cells could be used for counting the percentage of surviving cells. Gentry and Dalrymple (1980) developed a spectrophotometric method for the assessment of proportion of surviving cells by a dye uptake method. The method was simple, sensitive and reproducible.

A radioactive tissue culture assay measured the effect of the toxin in causing inhibition of amino acid incorporation by pulsing HeLa cells with [14C] leucine (Reisbig et al. 1981). The reaction was terminated by TCA and the washed proteins were counted in a scintillation counter. The use of Shiga toxin-resistant Vero cells for the easy identification of the cytotoxin was done by Kongmuang et al. (1988). A neutral red assay was described by Valdivieso-Garcia et al. (1993). The assay quantitatively measured the Vero cell cytotoxicity of E. coli.

The spectrophotometric method of Gentry and Dalrymple (1980) remains the most popular method of assay of the toxin because of its sensitivity and ease of performance. It has therefore been used in the present study.

It is evident from the survey of literature that the genus Shigella are the causal organisms of the disease shigellosis. The species S. dysenteriae elaborates a toxin that may contribute to the exacerbation of the disease symptoms.
The Shiga toxin from the species \textit{S.dysenteriae} and Shiga -like toxins from \textit{E.coli} have been the subject of several studies which have led to the complete picture of the toxin molecule, its subunits, its structure, mode of action, interaction with cells and toxic effects etc.

The species \textit{S.flexneri} is of particular importance because it is the most common causative agent of the disease Shigellosis yet its toxin has not been characterized previously. The purpose of the present study was therefore to isolate, purify and characterize the toxin from this species and also the toxicities and protein profiles of all the four species was carried out for comparison.