Comparison of genotypic and phenotypic method for detection of Enterotoxigenic *Escherichia coli* isolated from diarrheal patients

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Dedicated
To
My Parents

Acknowledgement

I begin in the name of Allah the most beneficial, the most merciful for blessings, protection and mental power in all aspects of my life. All the applauses to Allah to accomplish this thesis work.

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is one of the main causes of childhood diarrhea and in travelers in developing countries. ETEC expresses one or both of two different enterotoxin subtypes: heat-stable toxins (ST), heat-labile toxin (LT) and more than 25 different colonization factors (CFs) that mediate adherence to the intestinal cell wall. A total number of 3459 stool specimens were tested from patients enrolled in the 2% routine surveillance system in ICDDR, B during the period of March, 2009 to July, 2010 and 10% (n=364) ETEC strains were detected at the Immunology Laboratory. Genotypic, multiplex-PCR and phenotypic, GM1-enzyme-linked immunosorbent assay (GM1-ELISA) method were chosen to compare those isolated strains. Immuno Dot blot assay using specific monoclonal antibodies (MAbs) was done for the detection of colonization factors (CFs). Thirty strains have shown discrepancy in results among 364 ETEC strains. In which, 17% (n=5) was positive for heat stable toxin (ST), 10% (n=3) was positive for the heat labile toxin (LT), and 73% (n=22) was positive for both LT and ST toxin in case of PCR method but in ELISA, LT was found 17%, ST was 33% and LT/ST was 37%. About 67% of ETEC strains from stool specimens were colonization factor (CF) positive. Of these, 40% of LT toxin, 70% of ST toxin and 55% of LT/ST toxin producing ETEC strains were CF positive. Among different colonization factors CS5 + CS6 and CS14 were the predominant phenotypes, followed by CS6, CFA/I, CS17, CS1+CS3+CS21, CS2+CS3+CS21 in terms of occurrence whereas, CS2 + CS3 expressing ETEC strains were less frequently isolated from stool specimens. We did not find a very good similarity in results derived from genotypic and phenotypic methods. About 13% of PCR positive strains were negative by ELISA among those 30 strains. In PCR, ST was 17% whereas 33% found in ELISA and LT was 10% by PCR whereas 17% found in ELISA. In conclusion, we therefore did not find similar rates of LT and ST by the PCR method; it might due to low concentration of DNA in template that was prepared. It is possible that either the *eltB* or *estA* gene is present as a silent gene or, alternatively, that the levels of expression of the gene for these toxin are so low that the toxin was not detected by the ELISA method. Thus in future study, rRT-PCR can be carried out to evaluate genotypic and phenotypic toxin results specificity.

Abbreviations

ADP	Adenosine di phosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine mono phosphate
CF	Colonization factors
CFA agar	Colonization factor antigen agar
CS	Surface antigen
CTB	B- subunit of cholera toxin
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
g	Gram
GM1	Monosialosyl ganglioside
GMP	Guanosine monophosphate
GTP	Guanosine tri phosphate
GTPase	Guanosine tri phosphatase
HRP	Horse radish peroxidase
ICDDR,B	International Centre for Diarrheal Disease Research,Bangladesh
Ig G	Immunoglobin G
I.V.	Intra venous
Kd	Kilo Daltons
LB	Luria-Bertani
LT	Heat labile Toxin
LT/ST	Both LT and ST
µl	Microliter
mL	Mililiter
Mab	Monoclonal antibody

Abbreviations

NAD	Nicotinamide adenine dinucleotide
OPD	Ortho phenylene diamine
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
rCTB	Recombinant cholera toxin B subunit
rpm	Revolution per minute
rRT-PCR	Real-time reverse transcriptase PCR
ST	Heat stable Toxin
STh	Heat stable Toxin first isolated from human
STp	Heat stable Toxin first isolated from porcine
TBE	Tris Borate EDTA buffer
V. cholerae	*Vibrio cholerae*
WHO	World Health Organization

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Introduction

1.1 Introduction

In developing countries, each year roughly four billion episodes of acute diarrhea or approximately 3.2 episodes per child occur among children under five years of age [5]. The major etiologic agents that account for the estimated 1.5 million deaths per year are enterotoxigenic *Escherichia coli* (ETEC), rotavirus, *Vibrio cholerae*, and *Shigella* spp. [2, 1]; all are known to be endemic in essentially all developing countries. Diarrhea (from the Greek word) means "flowing through" [3]. It is the condition of having three or more loose or liquid bowel movements per day [4]. The loss of fluids through diarrhea can cause dehydration and electrolyte imbalances.

During diarrhea the body loses water and electrolytes (both of which are necessary for life) in the form of liquid stool and vomitus. If the water and salts are not replaced fast, the body gets dehydrated. If more than 10% of the body's fluid is lost death may occur. Children are more likely than adults to die from diarrhea because they become dehydrated more quickly. In Bangladesh waterborne diseases are common and some diseases have a characteristic seasonal pattern, increasing exponentially at certain predicted periods of the year but remaining endemic all year round [6].

1.2 Causes of diarrhea:

A few of the more common causes of diarrhea include the following:

- **Bacterial infections.** Several types of bacteria consumed through contaminated food or water can cause diarrhea. Common organisms include: *Vibrio cholerae*, enterotoxigenic *Escherichia coli* (ETEC), *Campylobacter, Salmonella, Shigella* spp.
- **Viral infections.** Many viruses cause diarrhea, including rotavirus, adenovirus,Norwalk virus, cytomegalovirus, herpes simplex virus, and viral hepatitis.
- **Parasites.** Parasites can enter the body through food or water and settle in the digestive system. Parasites that cause diarrhea include *Giardia lamblia, Entamoeba histolytica*, and *Cryptosporidium*.

- **Food intolerances.** Some people are unable to digest food components such as artificial sweeteners and lactose—the sugar found in milk.
- **Reaction to medicines.** Antibiotics, blood pressure medications, cancer drugs, and antacids containing magnesium can all cause diarrhea.

People who visit foreign countries are at risk for traveler's diarrhea, which is caused by eating food or drinking water contaminated with bacteria, viruses, or parasites. Traveler's diarrhea can be a problem for people visiting developing countries. In travelers however bacterial infections predominate [6].

1.3 Different types of diarrhea:

- Secretory diarrhea
- Osmotic diarrhea
- Exudative diarrhea
- Motility-related diarrhea
- Inflammatory diarrhea
- Dysentery

1.3.1 Secretory diarrhea:

In secretory diarrhea there is an increase in the active secretion, or an inhibition of absorption. There is little to no structural damage of lumen, intestinal brush boarder. Therefore, to maintain a charge balance in the lumen, sodium is carried with it, along with water. In this type of diarrhea intestinal fluid secretion is isotonic with plasma even during fasting [7].

1.3.2 Osmotic diarrhea:

Osmotic diarrhea occurs when too much water is drawn into the bowels. This can be the result of maldigestion (e.g., pancreatic disease or celiac disease), in which the nutrients are left in the lumen to pull in water. Osmotic diarrhea can also be caused by osmotic laxatives (which work to alleviate constipation by drawing water into the bowels). In healthy individuals, too much magnesium or vitamin C or undigested lactose can produce osmotic diarrhea and distention of the bowel [7].

1.3.3 Exudative diarrhea:

Exudative diarrhea occurs with the presence of blood and pus in the stool. This occurs with inflammatory bowel diseases, such as Crohn's disease or ulcerative colitis, and other severe infections such as *E. coli* or other forms of food poisoning [7].

1.3.4 Motility-related diarrhea:

Motility-related diarrhea occurs when the motility of the gastrointestinal tract (GI) is abnormal. This type of diarrhea may arise if the ingested food moves too quickly in the gastrointestinal tract and there is not enough contact time between the food and the intestinal membrane, which results in an insufficient absorbance of both the nutrients and water [7].

1.3.5 Inflammatory diarrhea:

Inflammatory diarrhea occurs when there is damage to the mucosal lining or brush border, which leads to a passive loss of protein-rich fluids, and a decreased ability to absorb these lost fluids. It can be caused by bacterial infections, viral infections, parasitic infections, or autoimmune problems such as inflammatory bowel diseases [7].

1.3.6 Dysentery :

Generally, if there is blood visible in the stools, it is not diarrhea, but dysentery. The blood is trace of an invasion of bowel tissue. Dysentery is a symptom of, among others, *Shigella*, enteroinvasive *E. coli.* and *Entamoeba histolytica* [7].

1.4 Transmission and symptoms of diarrhea:

Diarrheal diseases are transmitted through the fecal-oral route and are spread through contaminated food and drinking water or from person to person as a result of poor hygiene and sanitation. Infants who are not exclusively breast fed, young children, and adults who are malnourished or have weakened immune systems are at greatest risk [4]. Diarrhea may be accompanied by loose motion, cramping, abdominal pain, bloating, and nausea. Depending on the cause, a person may have a fever or bloody stools [8].

1.5 General features of *E.coli* :

Theodor Escherich first described *E. coli* in 1885, as *Bacterium coli commune,* which he isolated from the feces of newborns. It was later renamed *Escherichia coli,* and for many years the bacterium was simply considered to be a commensal organism of the large intestine. It was not until 1935 that a strain of *E. coli* was shown to be the cause of an outbreak of diarrhea among infants [14].

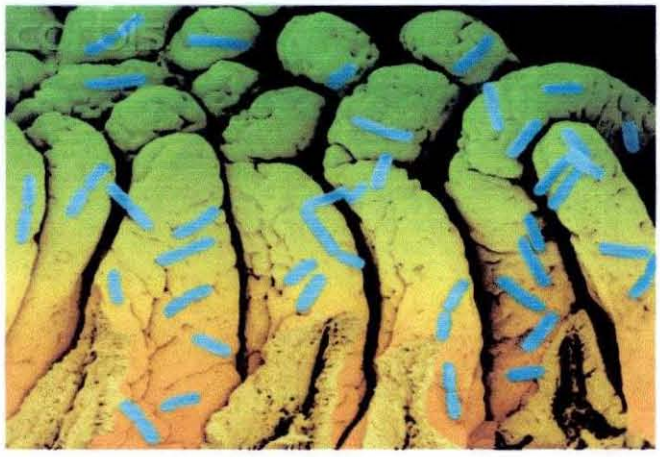

Figure 1.1: *E. coli* on the surface of human small intestine
Source: Visuals Unlimited/Corbis

The GI tract of most warm-blooded animals is colonized by *E. coli* within hours or a few days after birth. The bacterium is ingested in foods or water or obtained directly from other individuals handling the infant. The human bowel is usually colonized within 40 hours of birth [15]. *E. coli* can adhere to the mucus overlying the large intestine. Once established, an *E. coli* strain may persist for months or years. Resident strains shift over a long period (weeks to months), and more rapidly after enteric infection or antimicrobial chemotherapy that perturbs the normal flora [16, 17]. The basis for these shifts and the ecology of *Escherichia coli* in the intestine of humans are poorly understood despite the vast amount of information on almost every other aspect of the organism's existence. The entire DNA base sequence of the *E. coli* genome has been known since 1997 [18].

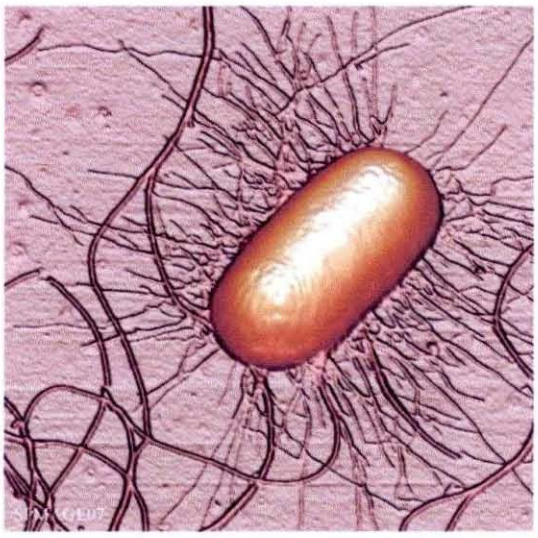

Figure 1.2: An *Escherichia coli* cell is imaged using tapping mode AFM under dry condition. Well preserved pili and flagella structures can be seen clearly. The size of the cell is about 1.9um long and 1um wide. The width of pili is about 20nm and flagellum is about 30nm. (**Source:**http://www.icmm.csic.es/spmage/spmageview)

E. coli is the head of the large bacterial family, *Enterobacteriaceae*, the enteric bacteria, which are facultatively anaerobic Gram-negative rods that live in the intestinal tracts of animals in health and disease. A number of genera within the family are human intestinal pathogens (e.g. *Salmonella*, *Shigella*, *Yersinia*). Several others are normal colonists of the human gastrointestinal tract (e.g. *Escherichia*, *Enterobacter*, *Klebsiella*), but these bacteria, as well, may occasionally be associated with diseases of humans [18].

The genome of *E. coli* consists of a single circular chromosome of about 4 to 5 million base pairs (bp), depending on the strain, and may include multiple plasmids of various sizes (4 kb to several hundred kb) that are dispersed within the cytoplasm. The DNA of several strains has been sequenced and the differences between these have helped explain some aspects of virulence and pathogenicity [19].

1.6 Pathogenesis of *E. coli*:

Nowadays, particularly for diarrheagenic strains (those that cause diarrhea) pathogenic *E. coli* are classified based on their unique virulence factors and can only be identified by these traits.

Hence, analysis for pathogenic *E. coli* usually requires that the isolates first be identified as *E. coli* before testing for virulence markers. Pathogenic strains of *E. coli* are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis) [22]. Different pathotypes and different virulence determinants account for pathogenesis of infection caused by *E coli* [20].

A) The seven recognized pathotypes for diarrheagenic *E coli* are as follows:

1. Enterotoxigenic *E. coli* (ETEC)

2. Enteropathogenic *E. coli* (EPEC)

3. Enteroaggregative *E. coli* (EAEC)

4. Enterohemorrhagic *E. coli* (EHEC, also known as Shiga toxin–producing *E. coli* [STEC]

5. Enteroinvasive *E. coli* (EIEC)

6. Diffusely adherent *E. coli* (DAEC)

7. Cytolethal distending toxin producing *E. coli* (CDT-EC)

B) Extra-intestinal *E. coli* (ExPEC)

1. Neonatal meningitis *E. coli* (NMEC)

2. Uropathogenic *E. coli* (UPEC)

3. Avian pathogenic *E. coli* (APEC)

Over 700 antigenic types (serotypes) of *E. coli* are recognized based on O, H, and K antigens. At one time serotyping was important in distinguishing the small number of strains that actually cause disease. Thus, the serotype O157:H7 (O refers to somatic antigen; H refers to flagellar antigen) is uniquely responsible for causing HUS (hemolytic uremic syndrome) [18-21].

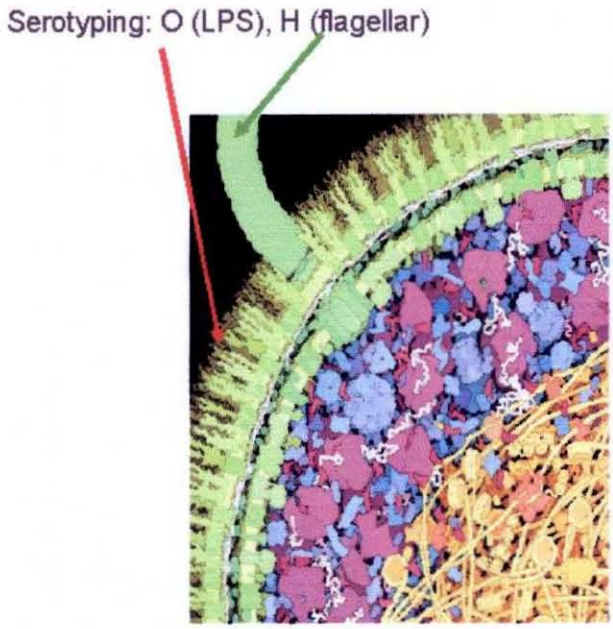

Figure 1.3: *E. coli* with O and H serotype

Source: http://mgl.scripps.edu/people/goodsell/illustration/public

Some pathogenic *E coli* strains produce cytotoxic enterotoxins (encoded on plasmid or bacteriophage DNA) that induce watery diarrhea without causing substantial tissue damage. Other strains harbor plasmid-encoded invasion factors that allow invasion of the mucosa or plasmid or bacteriophage-encoded cytotoxic enterotoxins that can cause tissue damage [21, 23].

Diarrheagenic *E. coli*: virulence determinants and characteristics of disease are as follows:

1. **EIEC** (Enteroinvasive *E. coli*)

-Nonfimbrial adhesins, possibly outer membrane protein
-Invasive (penetrate and multiply within epithelial cells)
-Does not produce shiga toxin
-Dysentery-like diarrhea (mucous, blood), severe inflammation, fever
-Causes a syndrome with profuse diarrhea and high fever.

2. EPEC (Enteropathogenic *E. coli*)

-Non fimbrial adhesin (intimin)
-EPEC adherence factor (EAF) enables localized adherence of bacteria to intestinal cells
-Moderately invasive (not as invasive as Shigella or EIEC)
-Does not produce LT or ST; some reports of shiga-like toxin
-Usually infantile diarrhea; watery diarrhea with blood, some inflammation, no fever; symptoms
-Probably result mainly from invasion rather than toxigenesis

3. EAEC (Enteroaggregative *E. coli*)

-Adhesins not characterized
-Non invasive
-Have fimbriae which aggregate tissue culture cells
-Produce ST-like EAST (EnteroAggregative ST) toxin and a hemolysin
-Persistent diarrhea in young children without inflammation or fever
-The significance of EAEC strains in human disease is controversial

4. EHEC (Enterohemorrhagic *E. coli*)

-Adhesins not characterized, probably fimbriae
-Moderately invasive
-Bind tightly to cells
-Does not produce LT or ST but does produce shiga toxin
-Pediatric diarrhea, copious bloody discharge (hemorrhagic colitis), intense inflammatory response, may be complicated by hemolytic uremia
-Characterized by the production of verotoxin or Shiga toxins (*Stx*)
-Although *Stx1* and *Stx2* are most often implicated in human illness, several variants of *Stx2* exist

5. DAEC (Diffusely adherent *E. coli)*

-Produce a fimbrial adhesin or a related adhesin [18].

-Patients infected with DAEC had watery diarrhea without blood or fecal leukocytes induce a cytopathic signal transduction effect.

6. CDT-EC (Cytolethal distending toxin producing *E. coli*)

-Latest class of diarrheagenic *E. coli.*

-CDT-producing *E. coli* additionally possessed the virulence factors of EPEC or EAEC.

-Some species of campylobacter also produce a cytolethal-distending toxin related to that of CDT-producing *E. coli* and which also causes distension and eventual disintegration of cells of certain lines [24].

7. ETEC (Enterotoxigenic *E. coli*)

-Can produce two proteinaceous enterotoxins: LT and/or ST toxin

-Fimbrial adhesins e.g. CFA I, CFAII, K88. K99 to bind enterocyte cells in the small intestine

-Non invasive

-Watery diarrhea in infants and travelers; no inflammation, no fever

-They do not leave the intestinal lumen

-Virulence genes carried on plasmids; ST genes on transposon

In Table 1.1 there is a short comparison of the pathogenic mechanisms of diarrheagenic *E. coli*

Table 1.1: Comparison of the pathogenic mechanisms of diarrheagenic *E. coli*

***E. coli* class**	**Main virulence factor**
EIEC (Enteroinvasive *E. coli)*	Plasmid-mediate invasion genes
EPEC (Enteropathogenic *E.coli*)	Bundle-forming pili, intimin, Tir
EAEC (Enteroaggregative *E. coli)*	Enteroaggregative heat-stable enterotoxin (EAST1)
EHEC (Enterohemorrhagic *E.coli*)	Verocytotoxin / Shiga-like toxin

DAEC (Diffusedly adherent *E. coli)*	Hemolysin and cytotoxic necrotising factor 1 (CNF1)
CDT-EC (Cytolethal distending toxin (CDT)- producing *E. coli)*	Cytolethal distending toxin (CDT)
ETEC (Enterotoxigenic *E. coli)*	Heat-stable (ST) and heat-labile (LT) enterotoxins and colonization factors (CFs).

1.7 Historical aspects of *E. coli.* infection:

The history of enterotoxigenic *E. coli* begins in 1956 in Calcutta [10]. De and his colleagues injected live strains of *E. coli*, isolated from children and adults with a cholera-like illness, into isolated ileal loops of rabbits and found that large amounts of fluid accumulated in the loops, similar to that seen with *Vibrio cholerae*. However, they did not test the filtrates of these cultures to determine whether they produced an enterotoxin. These findings were not followed up until 1968, when Sack reported studies, also in Calcutta, of adults and children with a cholera-like illness, who had almost pure growth of *E. coli* in both stool and the small intestine [11]. These *E. coli* isolates were found to produce a strong cholera-like secretory response in rabbit ileal loops, both as live cultures and as culture filtrates [12]. The patients were also found to have antitoxin responses to the heat-labile enterotoxin produced by these organisms [13].

1.8 Enterotoxigenic *E. coli* (ETEC) in diarrheal disease:

ETEC is defined as the *E. coli* that contains at least one member of two defined groups of enterotoxins: ST and LT [25]. It is the most common among the seven diarrheagenic *E. coli* strains and is an important human and animal pathogen [26]. ETEC infection occurs when a person eats foods or drinks water contaminated with ETEC. Specific virulence factors differentiate ETEC from other categories of diarrheagenic *E. coli* such as enterotoxins and colonization factors (CFs) [25, 27]. ETEC belongs to a heterogeneous family of lactose fermenting *E. coli*, belonging to a wide variety of O (somatic), K (capsular) and H (flagellar) antigenic types, which produce enterotoxins [27]. Characteristically, ETEC colonize the small

intestine by adhering to the epithelium and induce secretion by elaborating toxins without invasion of or damage to cells [26].

1.9 Epidemiology of ETEC infection:

Infants and children up to 5 years of age are more susceptible to ETEC diarrhea [28-30]. This susceptibility of young children has been observed due to poor public health and hygiene conditions as well lack of immunity and prior exposure [28, 29]. The incidence increases again in those over 15 years of age [30, 31]. Studies from India and Bangladesh first described ETEC to be a cause of adult diarrhea resembling cholera in the severity of infection [31, 32]. Studies in Bangladesh showed that the incidence of mixed infections increase with age [33]. In cases of mixed infections in children, rotavirus is the most common, followed by other bacterial enteropathogens, e.g., *V. cholerae*, *Shigella* spp. [34-36]. In traveler's diarrhea, enteroaggregative *E. coli* and *Campylobacter* spp. have been common pathogens together with ETEC [37, 38]. The incidence of ETEC infections in developing countries decreases after 5 years of age with a decrease of infections between the ages of 5 to 15 years [31].

1.10 Clinical features of ETEC diarrhea patients:

Diarrheal disease caused by ETEC was first recognized as a cholera-like illness in both adults and children [39]. The diarrhea produced by ETEC is of the secretory type: the disease begins with a sudden onset of watery stool (without blood or inflammatory cells) and often vomiting, which lead to dehydration from the loss of fluids and electrolytes (sodium, potassium, chloride, and bicarbonate) in the stool [39, 40]. The loss of fluids progressively results in a dry mouth, rapid pulse, lethargy, decreased skin turgor; decreased blood pressure, muscle cramps, and eventually shock in the most severe forms [40].

1.11 ETEC virulence factors:

Pathogenesis of ETEC strains is mediated by two major virulence factors, enterotoxins and colonization factor antigen (CFA). The ability of ETEC to adhere to the intestinal epithelium of the host is an important virulence determinant, and adhesion is mediated by pertinacious surface appendages called colonization factors (41) are designated by colonization factor antigen (CFA),

CS (coli surface antigen), or putative colonization factor (PFA). ETEC produces many defined colonization factors (pili/fimbrial or nonfimbrial), under plasmid control [42, 43]. After colonization, ETEC produces one or two enterotoxins called heat labile toxin (LT) and heat stable toxin (ST) and strains may express either or both [10].

1.12 Toxins:

There are two types of toxins secreted by ETEC to cause diarrhea. The toxins are heat labile toxin (LT) and heat stable toxin (ST). They are further classified according to their property and the host they colonize.

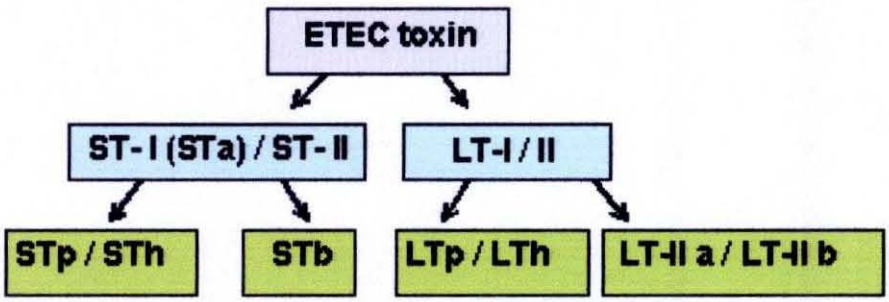

Figure 1.4: Classification of ETEC enterotoxins

1.12.1 Heat-stable toxin (ST):

Characteristics of ST toxin are as follow:

Heat stable enterotoxin is a small, monomeric, single-peptide toxin that includes two unrelated classes: STa and STb, which differ in both structure and mechanism of action. STs are so named because they are stable at 100° C for 15 min. Only toxins of the STa class have been associated with human disease. The STb toxin is associated with animal disease [44-47].

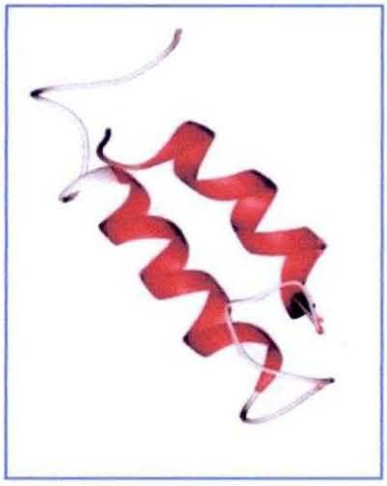

Figure 1.5: Molecular structure of ST

(**Source:** OPM database)

Structural features:

Low-molecular-mass polypeptides that contain multiple cysteine residues forming disulfide bonds that is essential for their heat stability and biologic activity [45]. STa toxin is a ~2-kDa peptide containing 18 or 19 amino acid residues [47].
STb toxin is a 48-amino-acid peptide containing two disulphide bonds. There are two variants of STa, designated STp (ST porcine or STIa) and STh (ST human or STIb) after their initial discovery in strains isolated from pigs or humans, respectively. Both variants can be found in human ETEC strains [27].

Functions:

STa: activates guanylate cyclase resulting in ion secretion [45] and STb: increase intracellular calcium resulting in ion secretion [46].

Mechanism:

STa binds to extracellular ligand-binding domain of guanylate cyclase C (GC-C) in the brush-border membrane of intestine epithelial cells, resulting in activation the guanylate cyclase

activity of this receptor, leading to increased intracellular cGMP levels. Na^+ and Cl^- absorption is inhibited in villus tip cells, whereas secretion of Cl^- is stimulated in intestinal crypt cells. The pathway involved may include cGMP-dependent kinase and cAMP-dependent kinase [47]. The intestinal receptor for STb has not been identified. STb leads to the release of serotonin, production of prostaglandin E_2, and increased free cytosolic Ca^{2+} concentration [46, 47].

1.12.2 Heat-labile toxin (LT):

Characteristics of LT toxin are as follow:
Two antigenically distinct types : LT-I and LT-II. LT-I is plasmid-encoded [50]. LT-I is expressed by *E. coli* strains that are pathogenic for both humans and animals [44]. LT-II only produced by strains isolated from animals [50]. The LT-II serogroup of the LT family shows 55 to 57% identity to LT-I and CTX in the A subunit but essentially no homology to LT-I or CTX in the B subunits [49-51].

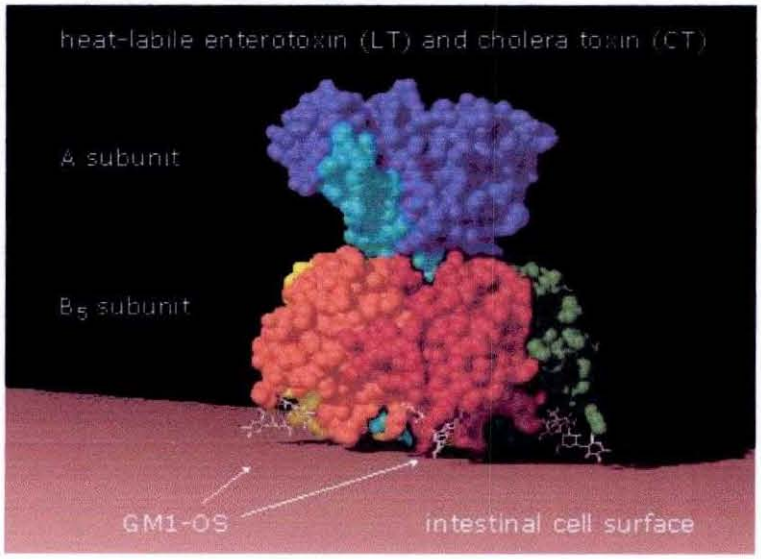

Figure 1.6: Architecture of the cholera toxin (CT) AB5 heterohexamer which is very closely related to the heat labile enterotoxin (LT) from enterotoxigenic *E. coli* .
(**Source:** http://skuld.bmsc.washington.edu/raster3d/examples/examples.html)

Introduction

Structural features:

Structurally and functionally similar to cholera enterotoxin. LT-I is an oligomeric toxin of 86 KDa composed of one A subunit (28-KDa) and five identical B subunits (11.5-KDa) [48]. The B subunits are arranged in a ring or "doughnut" form [51.52]. The B subunit binds ganglioside GM1 in the host cell membrane. The A subunit is proteolytically nicked to form the A_1 and A_2 subunits that remain associated by virtue of a disulfide bond. The A_1 peptide functions enzymatically as an ADP-ribosyltransferase [50].

LT-II is associated primarily with animal-specific ETEC strains, subgrouped into two antigenic variatants: LT-IIa and LT-IIb. The A subunit of LT-II acts in the same manner as that of LT-I. B subunit of LT-IIa binds ganglioside GD_{1b}, while LT-IIb binds GD_{1a} [52-54].

Functions:

ADP ribosylates and activates adenylate cyclase resulting in ion secretion

Mechanism:

LT transfers the ADP-ribose from NAD to arginine residue 201 on the alpha subunit of GTP_s, blocking their GTPase activity, so adenylate cyclase permanently activated, leading to increased intracellular concentrations of cAMP. cAMP activates the cAMP-dependent protein kinase A (PKA). This activation leads to phosphorylation of ion channels in the apical membrane of intestine epithelial cells. A principal target is CFTR (cystic fibrosis transmembrane conductance regulator). Thus there is increased secretion of Cl^- from intestinal crypt cells and decreased absorption of Na^+ and Cl^- by villus tip cells [53, 54].

1.12.3 Mechanism of pathogenesis:

ETEC strains cause diarrhea through the action of the enterotoxins LT and ST. These strains may express an LT only, an ST only, or both an LT and an ST.

-> After binding to the host cell membranes, the LT toxin is endocytosed and translocated through the cell in a process involving trans-Golgi vesicular transport [54].

-> The cellular target of LT is adenylate cyclase located on the basolateral membrane of epithelial cells.

-> The A_1 peptide of LT toxin acts by transferring an ADP-ribosyl moiety from NAD to the alpha subunit of the GTP-binding protein, G_S, which stimulates adenylate cyclase activity. ADP-ribosylation of the $G_{S\alpha}$ subunit results in adenylate cyclase being permanently activated, leading to increased levels of intracellular cyclic AMP (cAMP).

-> cAMP-dependent protein kinase (A kinase) is thereby activated, resulting in secretion of anions (predominantly Cl^- by a direct effect, and HCO_3^- indirectly) by crypt cells and a decrease in absorption of Na^+ and Cl^- by absorptive cells [55].

-> ST is thought to act by binding the ST membrane receptor, GC-C. Activation of GC-C results in increased levels of intracellular cGMP.

-> cGMP exerts its effects in increasing chloride secretion and decreasing NaCl absorption [56, 57]. The increased luminal ion content draws water passively, resulting in a net water loss into the gut lumen.

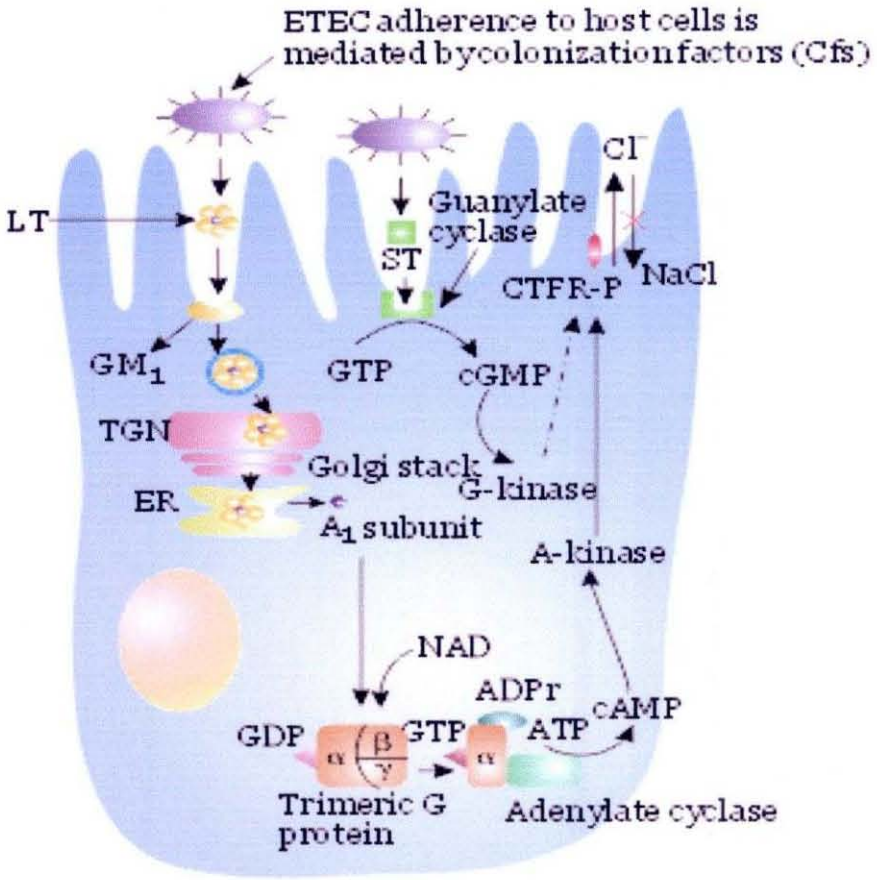

Figure 1.7: Classic mechanisms of action of ETEC toxins

1.13 Colonization factors (CFs):

To cause diarrhea, ETEC strains must adhere to small bowel enterocytes, an event mediated by colonization factors (CFs). The colonization factors are mainly fimbrial or fibrillar proteins, although some colonization factors are not fimbrial in structure [58]. More than 22 colonization factors (CFs) have been recognized among human ETEC and many more are about to be characterized [43]. Three major morphologic varieties of CFs exist:

- rigid rods,
- bundle-forming flexible rods, and
- thin flexible wiry structures.

For example, the prototype CFA/I, has rigid rod-shaped fimbria. It is a single fimbrilar structure [59]. A nomenclature for the CFs designating them as coli surface antigen (CS) was introduced in the mid-1990s [43]. Some of the better-characterized CFs can be subdivided into different families, i.e., the colonization factor I-like group (including CFA/I, CS1, CS2, CS4, CS14, and CS17) [43], and the coli surface 5-like group (with CS5, CS7, CS18, and CS20) [60], and those that are unique (CS3, CS6, and CS10 to CS12).

CFA/II is composed of three distinct coli surface antigens CS1, CS2, and CS3 respectively. The fimbrillar stucture CS3 could be expressed alone or together with either the fimbrial CS1 or CS2. CFA/III is a bundle forming pilus with homology to the type 4 fimbrial family. Similarly, CFA/IV was shown to consist of the non- fimbrial CS6 alone or in combination with either CS4 or CS5 fimbriae [61]. Some more characterized CFs was called putative colonization factors (PCFs) combined with the serogroups of the strains from which the fimbriae were first identified. Putative CFs are found with varying frequencies such as CS14 (PCFO166) and CS12 (PCFO159). Newly described CF named Longus has been found on some human ETEC stains [62].

Table 1.2: Past and present designations for colonization factors of ETEC

Nomenclature		Type of antigen
Old	**New**	
CFA/I	CFA/I	F
CS1	CS1	F
CS2	CS2	F
CS3	CS3	f
CS4	CS4	F
CS5	CS5	H
CS6	CS6	nF
CS7	CS7	H
CFA/III	CS8	F
2230	CS10	nF
PCF0148	CS11	f
PCF0159	CS12	F
PCF09	CS13	f
PCF0166	CS14	F
8786	CS15	nF
CS17	CS17	F
PCFO20	CS18	F
CS19	CS19	F
CS20	CS20	F
Longus	CS21	F
CS22	CS22	f

Abbreviations: CS, Coli Surface antigen; CFA, Colonization Factor Antigen; PCFO, putative colonization factor; F, fimbrial; f, fibrillae; nF, nonfimbrial; H, helical. All except CFA/I have the CS designation [42].

Correlation between presence of colonization factors and toxin of ETEC is also important, which is presented in Table 1.3.

Table 1.3: Combination of CFs and toxin of ETEC

CS	Morphology	Molecular weight(Kd)	Enterotoxins
CFA/I	F	15	ST + LT
CS1	F	16.8	ST + LT
CS2	F	15.3	ST + LT
CS3	f	15.1	ST + LT
CS4	F	17	ST + LT
CS5	H	21	ST + LT
CS6	nF	14.5/ 16.0	ST + LT
CS7	H	21.5	ST + LT
CS8	F	18	LT
CS10	nF	16	ST
CS12	F	19	ST + LT
CS14	F	15.5/ 17.0	ST + LT
CS17	F	15.5/ 17.5	LT

Abbreviations: CS- Coli Surface antigen, CFA- Colonization Factor Antigen, F- fimbrial, f- fibrillae, nF- non- fimbrial, H- helical, KD- kilo Dalton, LT- heat labile toxin, ST- heat stable toxin.

There are cross-reactive epitopes that have been considered as candidates for vaccine development within each of these families [63]. Of the wide range of CFs, the most commonly present on diarrhoeagenic strains and those seen on 50 to 80% of isolates include CFA/1, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS14, CS17 and CS21 [42]. Other ETEC fimbriae, known as longus serogroups has also been found on a large proportion of human ETEC [64, 65]. However, CFs has not been detected on all ETEC and roughly on 40-50% of all strains worldwide not known colonization factors have been detected. This may be probably due to absence of adhesins or because of lack of specific antibodies or molecular methods for their detection [10].

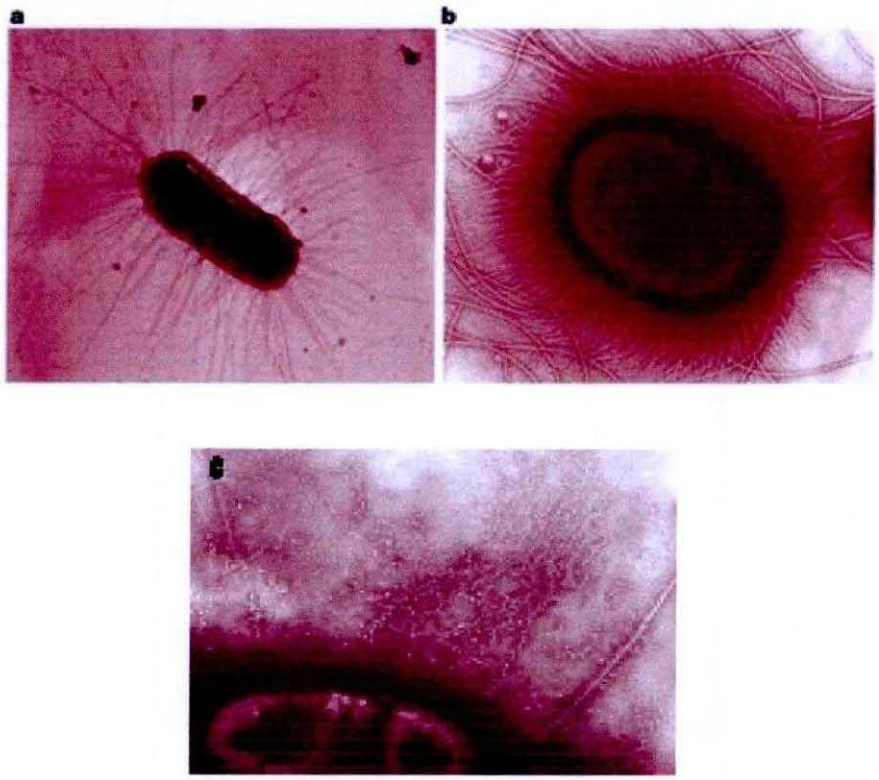

Figure 1.8: CF expressing ETEC. *E. coli* produce a variety of colonization factors, many of which are hair-like structures of various morphologies called fimbriae (also called pili) or fibrillae. **a)** Long, straight colonization factor antigen (CFA)/III fimbriae of ETEC (5–7 nm in diameter) protruding peritrichously from the bacterial surface. **b)** Abundant long, straight CFA/I fimbriae (5–7 nm) of ETEC contrasting with thicker, wavy flagella. **c)** Thin (2–3 nm), flexible,

wiry CS3 fibrillar structures produced by ETEC that extend several micrometres from the cell surface.

Source: Nature Reviews Microbiology volume: *2*, 123-140 (February 2004).

1.14 Seasonality of ETEC:

In Bangladesh, ETEC follows a very characteristic biannual seasonality with two separate peaks, one at the beginning of the hot season, that is, the spring, and another peak in the autumn months, just after the monsoons, but it remains endemic all year [38, 66, 62]. Such as seasonality may be initiated by climate and spread by environmental factors. Seasonality for the different toxin phenotypes has also been suggested, with ST-producing ETEC strains being more common in the summer (30, 67) whereas LT-producing ETEC strains are present all year round and do not show any seasonality.

1.15 ETEC infection and malnutrition:

As for other diarrheal diseases, preexisting malnutrition can lead to more severe enteric infections, including those caused by ETEC, possibly due to the immunocompromised nature of the host that also predisposes these individuals to a greater bacterial load on the mucosal surfaces of the gut than the well-nourished child [68]. Micronutrient deficiency such as vitamin A and zinc is quite common in developing countries and generally increases the morbidity due to diarrheal illnesses [69, 70].

1.16 Protection through breast feeding:

Breast feeding is known to reduce overall diarrhea and mortality [71, 72]. Since secretory immunoglobulin A antibodies to CFs and enterotoxin are present in breast milk samples from mothers in developing countries [73, 74, 75], it would be natural to assume that breastfed infants should be protected from ETEC diarrhea. This effect is short term and does not last long after infancy, and an overall protection is not seen in the crucial first 2 to 3 years of life [30, 67, 76]. The limited capacity of breast milk to protect against ETEC diarrhea in developing countries can also be attributed to other social and behavioral factors.

1.17 Treatment and management:

The treatment for ETEC diarrhoea includes rehydration strategies and antimicrobial therapy. The correction and maintenance of hydration is always most important. Antimicrobials are useful only when the diagnosis or suspicion of ETEC-related diarrhea is made.

1.17.1 Rehydration:

Intravenous fluids (such as Ringer's lactate) are required initially for all patients with severe dehydration. Supplement of fluid and minerals in the form of oral saline (ORS) is the easiest way of rehydration until the diarrhea ceases.

1.17.2 Antimicrobials:

Diarrhea in children is caused not only by ETEC but also by other bacterial and viral agents. The antimicrobial treatment of traveler's diarrhea has changed over the years because of increasing antimicrobial resistance [77]. When ETEC were first recognized, the bacteria were usually highly sensitive to tetracyclines and trimethoprim-sulfamethoxazole [78]. However, with time, antibiotic resistance emerged; antimicrobials that have been used for the treatment include doxycycline, trimethoprim-sulfamethoxazole, erythromycin, norfloxacin, ciprofloxacin, ofloxacin, azithromycin, and rifamycin [77, 78].

1.18 Aim of the study:

Acute diarrheal diseases are a major health problem in developing countries and ETEC is the major bacterial pathogen contributing to the disease burden in children less than 5 years of age. The objective of the study was to determine the prevalence of enterotoxigenic *Escherichia coli* (ETEC) infection among 2% systemic surveillance study of the ICDDR, B. and analyzed the data for understanding the phenotypic and genotypic difference of the pathogen.

The specific objectives of the present study were:

- Determination and confirmation of enterotoxigenic *E. coli* in surveillance patients by stool culture method.
- Rapid detection of ETEC toxin (LT, ST or both) by molecular technique (PCR).
- Characterization of ETEC by immunochemical methods for phenotypic determination by ELISA.
- To determine the presence and distribution of colonization factors in these strains.

Chapter Two
Methods and Materials

2.1 Methods and materials

This study is carried out at International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B) in Dhaka. Stool samples were collected from diarrheal patients enrolled in the 2% routine surveillance at the Laboratory Science Division (LSD), ICDDR, B. In the surveillance system every 50th patient attending the hospital is screened for ETEC. This study is approved by the Research Review Committee (RRC) and Ethical Review Committee (ERC) of the centre.

2.2 Samples collection and screening:

Stool or rectal swab samples were collected in sterile plastic containers from every 50th patient attending the hospital. These specimens were then screened for ETEC, PCR and ganglioside GM1-ELISA techniques were performed to confirm genotypic and phenotypic detection of ETEC toxin. Later, ETEC toxin positive colonies were tesed by dot-blot method to detect the colonization factors. The overview of the study plan to detect Enterotoxigenic *Escherichia coli* (ETEC) is shown in the following flow chart (2.1):

2.3: Over view of this study plan:

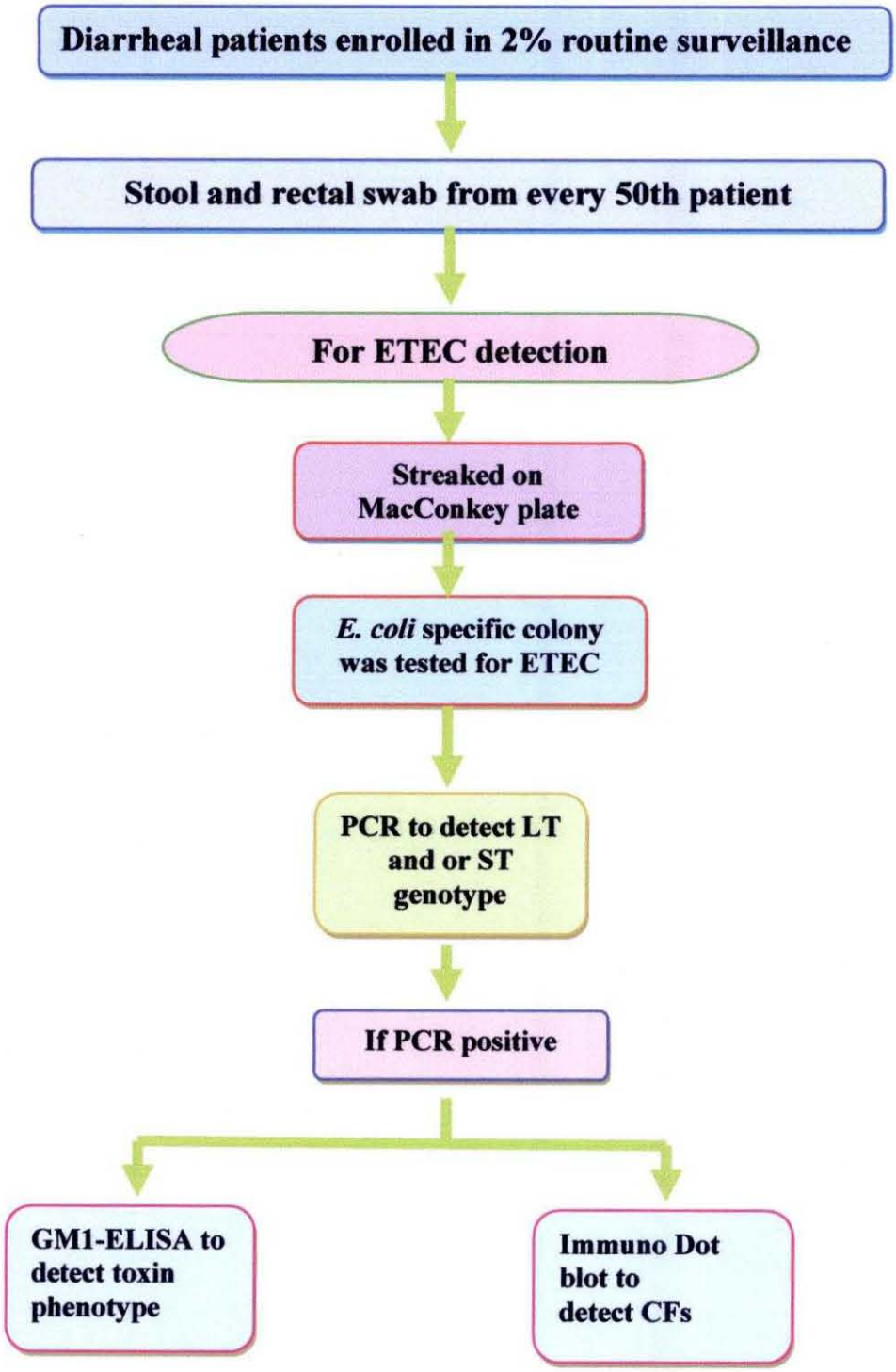

Figure 2.1: Flowchart for detection of ETEC

2.4: Identification of ETEC:

E. coli was recovered easily from clinical specimens on MacConkey agar, on the basis of their morphology. To confirm detection of ETEC freshly collected stool samples were plated on to MacConkey agar plates and incubated at 37°C overnight. Six individual lactose-fermenting colonies with deep pink colour from each clinical sample were tested. MacConkey agar plates with *E.coli* colonies store at 4-8°C for ≤1 week The enterotoxins were detected by PCR method and ganglioside GM1-ELISA method.

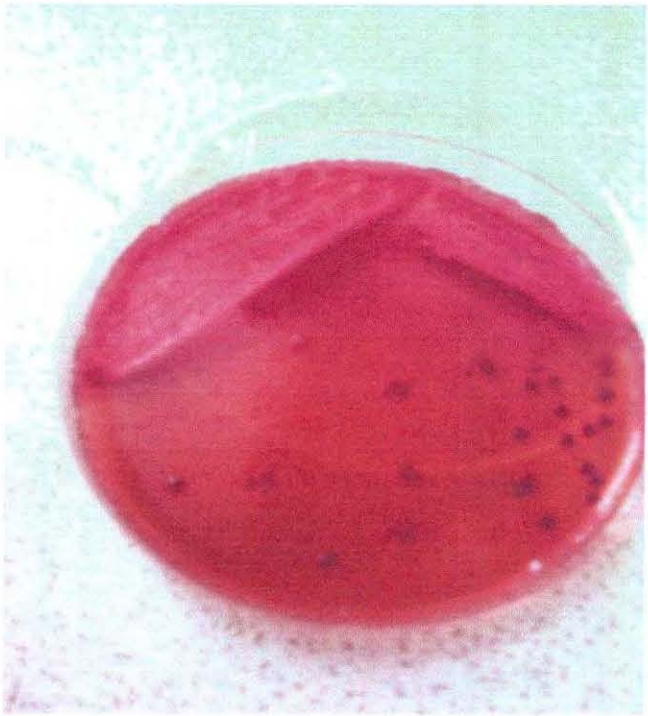

Figure 2.2: *E. coli* colonies growing on MacConkey agar plate

2.4.1: Detection of LT and/or ST by Polymerize Chain Reaction:

- Template Preparation:

100 µL of PBS was poured to each Eppendorf tube.
↓
One loop of bacteria was taken in each tube (from a pool of six colonies).
↓
Heated at 100 ^{0}C on water bath for 10 minutes.
↓
Transferred the tubes on ice and kept for a minute.
↓
Centrifuged the tubes at 12,000 rpm for 10 minutes.
↓
The supernatant was the template, ready to use.

- Specific primer sequence used:

Toxins	Primers	Primer Sequence
LT	Forward	5' – ACGGCGTTACTATCCTCTC – 3'
	Reverse	5' – TGGTCTCGGTCAGATATGTG – 3'
STh	Forward	5' – AGTGGTCCTGAAAGCATG– 3'
	Reverse	5' – TACAAGCAGGATTACAACAC – 3'
STp	Forward	5' – TCTTTCCCCTCTTTT– 3'
	Reverse	5' – ACAGGCAGGATTACAAAG – 3'

- Master mixture preparation:

Table 2.1: Preparation of master mixture

No	Reagent	Amount per 1 sample reaction
1	PCR buffer (with $MgCl_2$)	5.0 µL
2	dNTP mix	8.0 µL
3	Primer LT mixture	4.0 µL
4	Primer STp mixture	4.0 µL
5	Primer STh mixture	4.0 µL
6	$MgCl_2$	1.0 µL
7	Taq polymerase	0.3 µL
8	Distilled water	21.7 µL
Total		48.0 µL

- ❖ 2 µL DNA templates added in each tube.

- Thermal cycle:

First step	94°C for 5 minutes (initial denaturation)	
Second step	94°C for denaturation-30 seconds	40 cycles
	54°C for prime annealing-30 seconds	
	72°C for elongation -30 seconds	
Third step	72°C for 5 minutes (final extension step)	
	4 °C until used.	

- Agarose Gel Preparation (2%):
  - Ultra pure agarose of 2 g was mixed in 100 ml 1X TBE buffer (Invitogen, ultra pure).
  - Heated in micro oven for four minutes and poured on specific gel- tray.
  - 4µL ethidium bromide added in the gel.
  - Comb fixed and allowed 30-40 minute for gel formation.

- Gel Electrophoresis:
  - Stained with loading dye. (4 µL dye with 25 µL PCR product)
  - Electophrosis run for 30 minutes at 150V
  - Gel photographed under UV light.

2.4.2: Detection of LT and/or ST toxin producing isolation by ELISA:

The procedure for detection of LT is based on the binding of the B-subunit of the toxin to GM1 ganglioside and the detection of ST is based on its ability to inhibit the binding of ST to the GM1 and ST-CTB conjugates by ELISA technique.

Methods and Materials

- Procedure
    - For coating purpose 100 µL of ganglioside GM1 solution, 0.5 µg/mL, was added to each well of an ELISA plate and incubated at room temperature overnight (these plates could be stored at +4°C for about 2 weeks until used).
    - GM1-coated plates were washed twice with PBS and then a concentrated solution of non-interacting protein; bovine serum albumin (BSA) (0.1% BSA-PBS, 200 µL /well) is added to all plate wells and incubated at 37°C for 30 minutes. This step is known as blocking, because the serum proteins block non-specific adsorption of other proteins to the plate.
    - Plates were washed once with PBS and 100 µL of Luria-Bertani (LB) broth containing 45 µg/mL of lincomycin was added to each well of the plate.
    - Six individual colonies (for one sample) from MacConkey plates (using which PCR was done) were inoculated, single bacterial colony/well, with wooden sticks. The outside rows and columns of the ELISA plate were excluded to avoid background.
    - The plates were covered with a plastic film (to prevent evaporation) and incubated with shaking at 250 rpm overnight at 37°C. These plates were used for the detection of LT toxin.
    - Another GM1 coated ELISA plate (for each to be tested for LT) was washed twice with PBS and blocked with 200 µL 0.1% BSA-PBS at 37°C for 30 minutes. These plates were used for the detection of ST.
    - The plates were washed once with PBS and then 100 µL of recombinant ST-CTB conjugate solution was added. Plates were incubated at room temperature for 60 min.
    - The plates were washed three times with PBS and then 50 µL volumes of the overnight cultures from the plates for detection of LT were transferred to the corresponding wells in the plates used for the detection of ST. Then

50 µL of anti-ST MAb ST (1:3 dilution) was immediately added and incubated at room temperature for 90 min.

- Following this the plates for the detection of LT were washed three times with PBS contained 0.05% Tween and 100 µL of anti-LT MAb LT 39:13:1 was added immediately and incubated at room temperature for 90 min.
- Both types of plates (LT and ST) were washed three times with PBS-Tween.
- After that 100 µL of conjugate (anti-mouse IgG-HRP, 1:1000 dilution in BSA-PBS-Tween) was added into each well and incubated at room temperature for 90 minutes.
- All plates were washed three times with PBS-Tween.
- The substrate solution (prepared immediately before use) was added at 100 µL /well and incubated at room temperature. Optical density was measured at 450 nm after incubation for about 20 minutes (maximal absorbance should not exceed 1.5) in a Titertek microplate reader.
- For each plate LT-positive, ST-positive, LT and ST positive, LT and ST negative strains were used as control to interpret the result and control the experiment.

2.4.3: Interpretation of results:

- **For detection of LT:** The background was defined as the mean absorbance determined for LT-negative control strains. A positive result was an absorbance at 450 nm value of ≥ mean background value was used as the cut off level for positive samples.
- **For detection of ST:** The background was defined as the mean of the absorbance at 450 nm of the ST-negative control strains. The 50%

inhibition concentration value (IC50) was calculated according to the equation below:

$$IC50 = \frac{\text{mean absorbance for ST - negative control strains } (E.coli\ E34420C \text{ and } 286C_2)}{2}$$

- The results were calculated as ≥50% inhibition of the absorbance value measured as compared to the absorbance value obtained with the negative reference strains.
- LT positive result: Absorbance value of ≥0.1 above background.
- An isolate was considered enterotoxin positive if ≥1 colony showed a positive reaction in ST- and/or LT ELISA.
- The test was invalid if the reference strain gave a result that was not consistent with its assigned toxin profile.

2.5: Phenotypic detection of Colonization factors (CFs):

The presence of CFs on the ETEC isolates was detected by rapid dot blot assay using specific monoclonal antibodies for the different antigens. Toxin positive colonies on MacConkey agar plate were plated on to colonization factor antigen agar (CFA agar) with bile salts and plates were incubated at 37°C, overnight. For testing for CS21 only, colonies were cultured on Trypticase Soy agar (TSA) plate containing 5% sheep blood. From each sample, enterotoxin positive *E. coli* colonies from CFA plus bile, and TSA plates are tested for the expression of CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CS12, CS14, CS17 and CS21 by a dot blot assay using monoclonal antibodies specific for the different CS antigens.

Methods and Materials

- Procedure:

- Nitrocellulose membrane sheets or strips were cut, approx. 5x5mm/dot, one sheet or strip for each CF-type to test. Before blocking the nitrocellulose membranes gloves and forceps were used.

- Strips of nitrocellulose filter paper (0.45 µm, Sigma, St. Louis, MO) were soaked in phosphate buffered saline (PBS, 10 mM, pH 7.2) and allowed to dry for 5-30 minutes.

- Two µL of bacterial suspension (corresponding to 4-10 McFarland standards) was applied on the strips as a dot on the membrane using a micropipette and allowed to dry for at least five minutes (the membranes could be stored refrigerated at 4-8°C for about 1 week).

- The membrane was blocked in 1% BSA-PBS for 20 minutes at room temperature (18-25°C) on a platform rotator (Heidolph Rotamax 120) with slow rocking (approximately at 3 x g). An incubation tray (BioRad) was used for nitrocellulose strips.

- After the blocking liquid was discarded, the same volume of antibody solution monoclonal antibodies diluted 1:30-1:50 was added and incubated for two hours in a humid chamber at room temperature on the rotary shaker. Antibody solution was prepared in 0.1% BSA-PBS with 0.05% Tween [20].

- After washing 3 times with PBS-0.05 % Tween[20] the same volume of the enzyme conjugate diluted in 0.1% BSA-PBS-0.05 % Tween (goat anti-mouse IgG HRP Jackson, dilution 1:1500) was added and incubated for two hours in a humid chamber at room temperature on the rotator.

Methods and Materials

- Strips were washed 3x5 minutes with PBS-0.05%Tween and once with PBS and substrate solution (4-chloro-1-naphtol-H_2O_2 in TBS) was added to develop color for about fifteen minutes.

- The membranes were thoroughly washed with tap water and dried.

2.5.1: Interpretation of results:

Black, bluish or gray dots on the strips represented positive reactions. The color intensity was compared with positive controls on each strip. For the test to be valid the control strains all gave positive results for assigned CFs.

Chapter Three
Results

3.1: Results

From March, 2009 to July, 2010, 3459 stool specimens were tested for Enterotoxigenic *Escherichia coli* (ETEC). About 10 % (n=364) of ETEC was seen among the patients enrolled in 2% surveillance study, in the Immunology Laboratory at ICDDR, B.

3.2: Characterization of study subjects:

3.2.1 Toxin profiles of ETEC strains:

Enterotoxigenic *E. coli* isolates from the stool samples of the patients with acute diarrhea were tested for toxin production. Heat labile enterotoxin (LT) and heat stable enterotoxin (ST) gene specific primers were used to identify the toxin genes using the PCR method (Figure: 3.1).

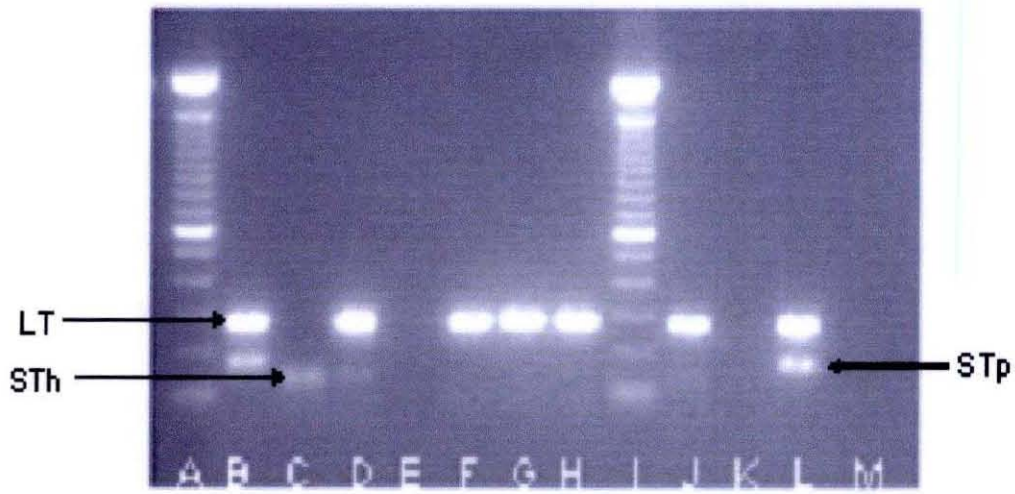

Figure 3.1: Agarose gel electrophoresis for the detection of LT/ST genes of *Escherichia coli* isolated from diarrheal stool specimens. Lane A and I- DNA ladder, lane B-D are for control strains and lane E represent negative control for LT/ST. Lane F-H and J-M are study samples. All strains are positive except strains of lane K and M. Lane F-H and lane J of strains represent LT toxin and lane L of strain represents LT/STp toxin

Results

PCR results confirmed ETEC positive strains. Strains were found to express LT was 10% and 17% strains expressed ST whereas 73% ETEC strains expressed LT and ST simultaneously.

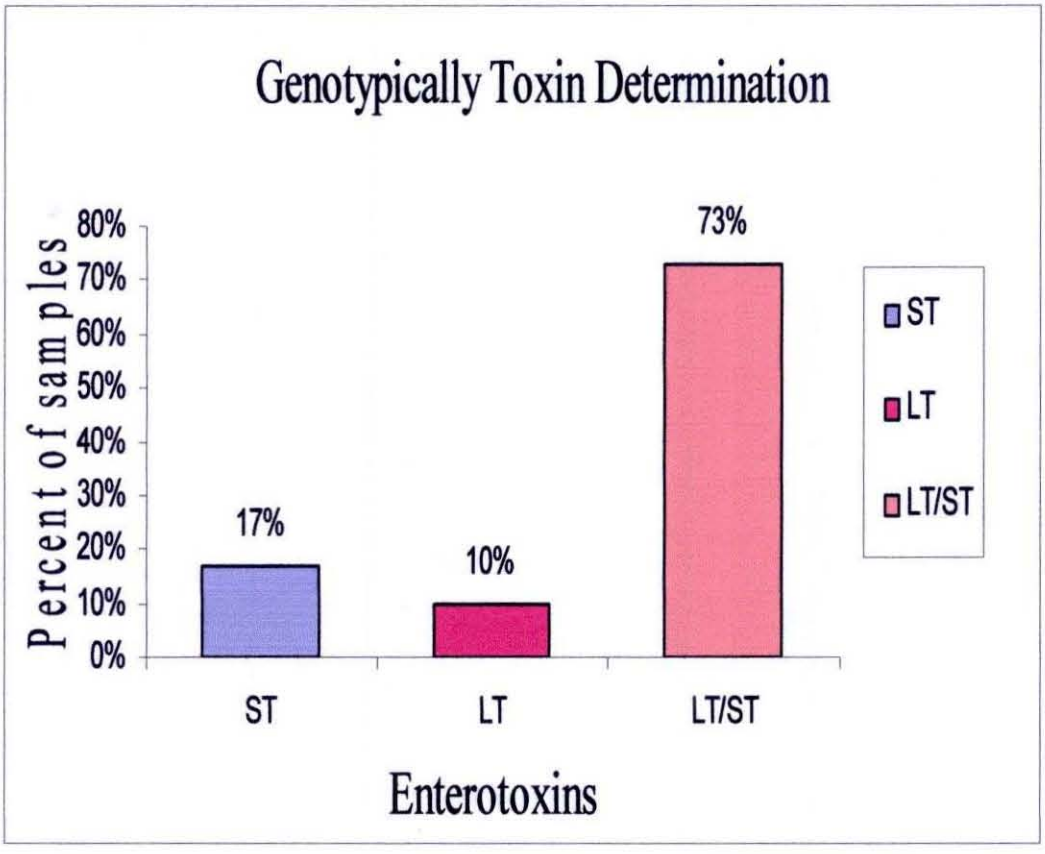

Figure 3.2: Distribution of enterotoxins in ETEC isolated from the stool samples are detected by Genotypic method (PCR).

More specifically, in case of ST toxin, STh was detected as 60% and STp represents 40% of the ST toxin. Additionally, in case of LT/ST toxin, 91% strains were positive for LT/STh while only 9% was positive for LT/STp.

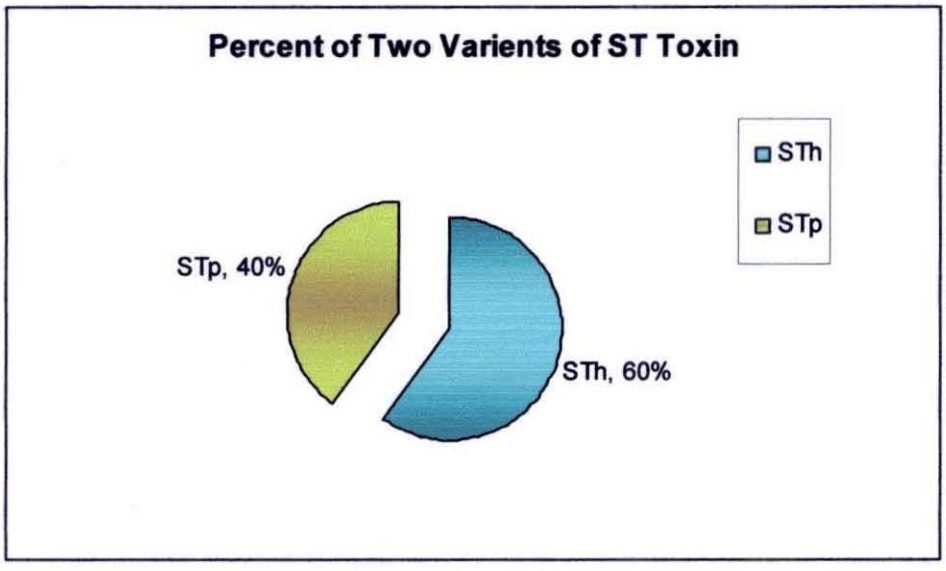

Figure.3.3: Two variants of ST toxin.

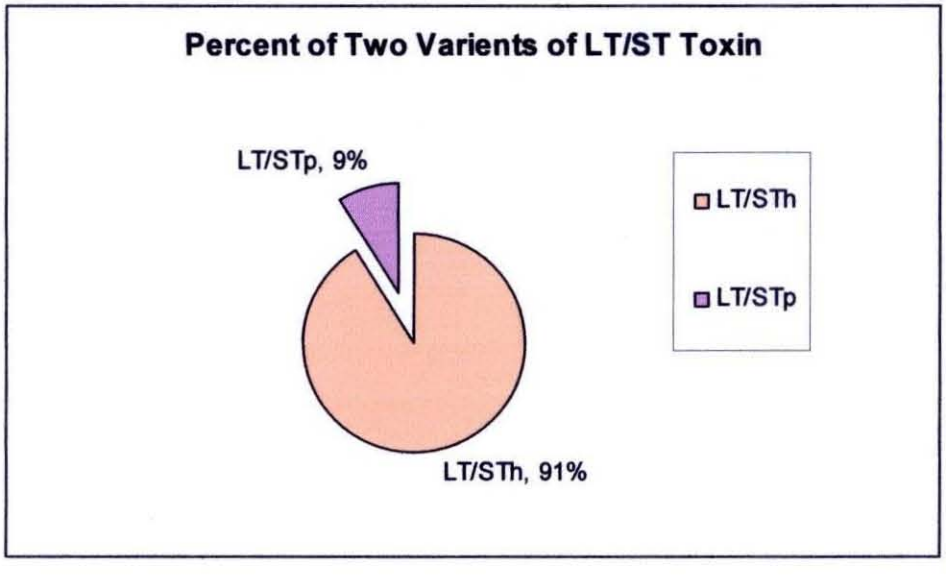

Figure.3.4: Two variants of LT/ST toxin.

3.2.2: Toxin phenotype:

After detection of the toxins genotypically, the positive colonies were subjected to ELISA for the phenotypic detection. Of the 30 ETEC strains collected from stool specimens; 33% (n =10) were only ST producers, 17% (n=5) were only LT producers and 37% (n =11) were LT and ST producers. In all the age groups, the ST only-producing isolates were the most prevalent, followed by LT- and ST-producing ETEC isolates and LT only-producing ETEC isolates (Fig. 3.5).

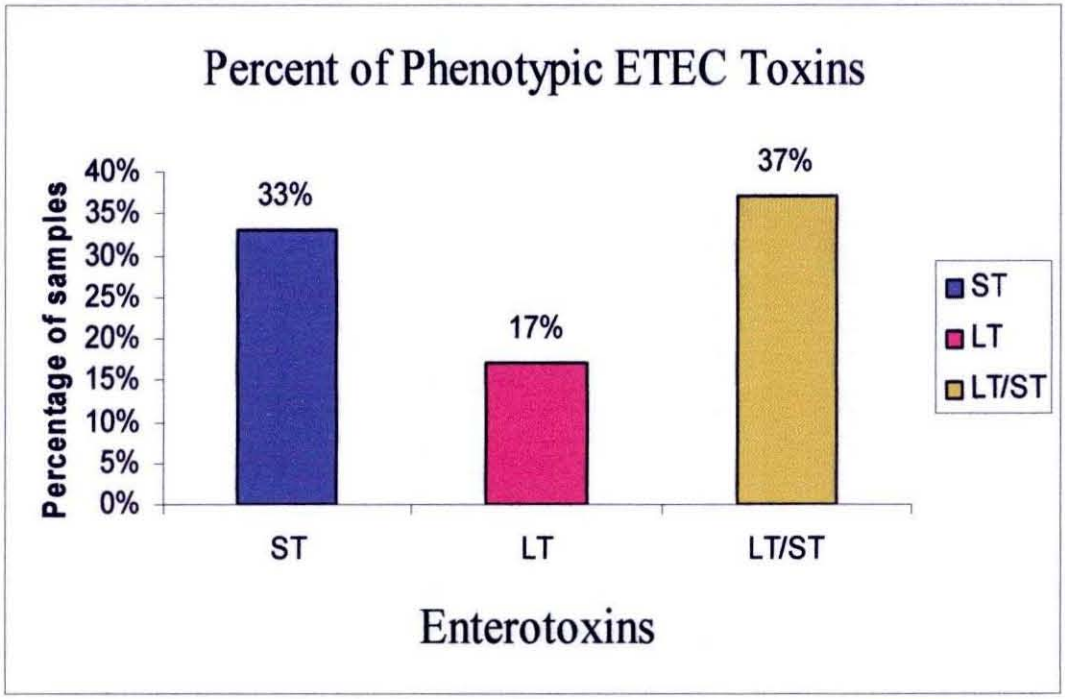

Figure 3.5: Distribution of Phenotypic enterotoxins (by ELISA) in ETEC isolated from the stool samples.

3.3: Immuno Dot blot assay for CFA characterization:

Phenotypic characterization of colonization factors of the 30 ETEC isolates were also carried out by immuno dot-blot assay applying 13 different colonization factors specific monoclonal antibodies. (Figure: 3.6)

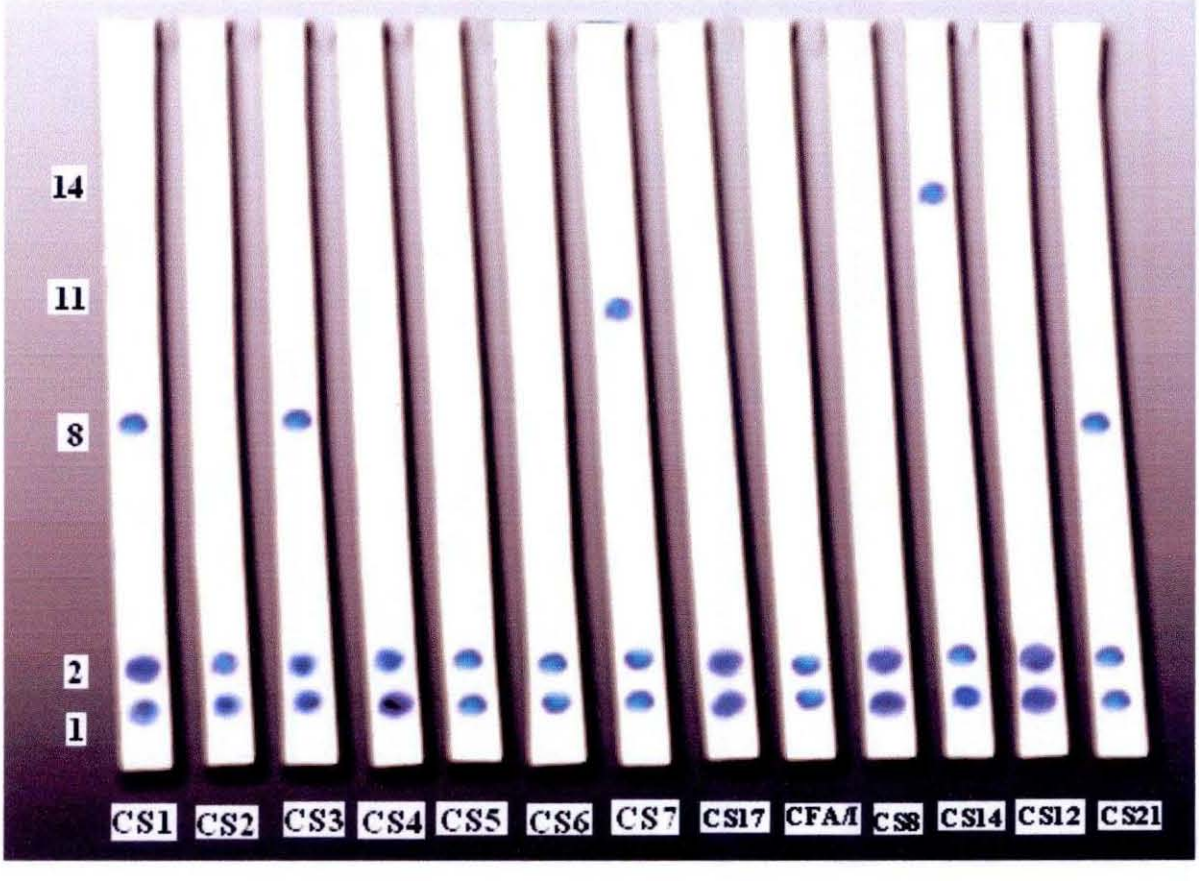

Figure 3.6: Colonization factors detected on ETEC using immuno dot blot assay. The spots number 1 and 2 represent positive control for each specific colonization factor against specific CFs. Spots 8, 11 and 14 are positive strains for (CS1+CS3+CS21), CS7 and CS14 respectively. Rest of the strains was negative for all 13 colonization factors.

.

3.3.1: Percentage of CF positive and CF negative strains in case of different toxins:

Among the 30 toxin positive ETEC isolates 67% (n= 20) ETEC strains were found to be colonization factor (CF) positive for 1 or more of the 13 CFs and remaining 33% (n= 10) strains were considered as CF negative as they did not show any positive spot on the nitrocellulose strips.

ETEC-ST strains showed a higher frequency of detectable CFs then ETEC-LT strains. For LT toxin, 40% (2 of 5) strains were CF positive; 70% (7 of 10) strains were CF positive for ST toxin and 55% (6 of 11) strains were CF positive for LT/ST toxin. The following figures show percentage of CF positive and negative strains in case of different toxins.

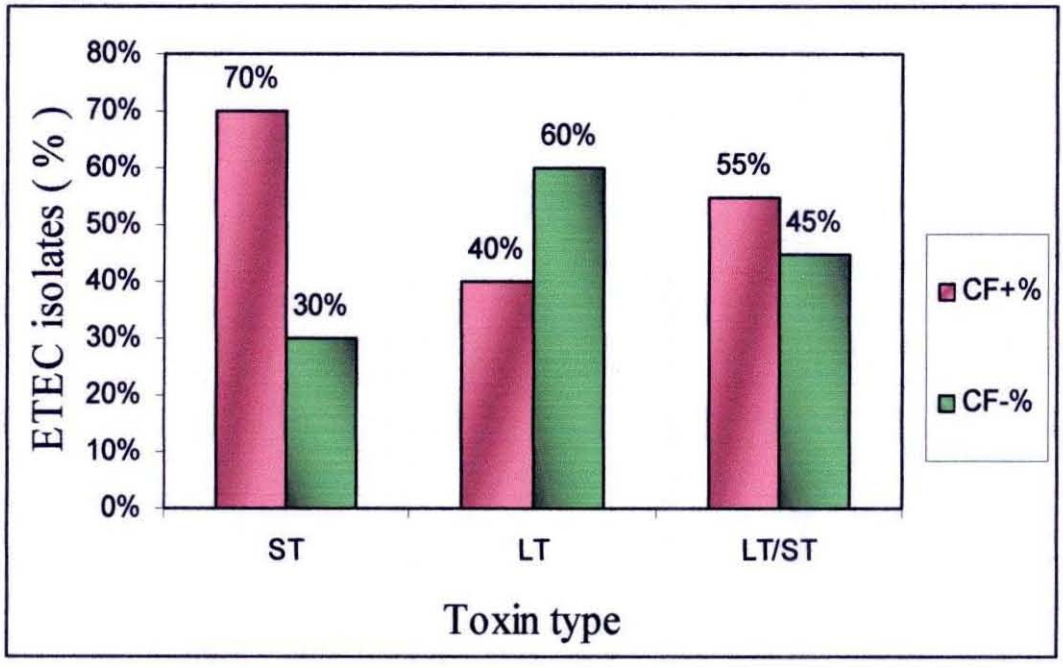

Figure 3.7: Distribution of colonization factors in different ETEC isolates

3.3.2: Overall percentage of different Colonization factors:

Again, overall percentage of 13 detected CFs are shown in the figure (Fig3.8). The coli surface (CS) antigen CS5+CS6 and CS14 (PCFO166) of the colonization factor antigen (CFA) were most prevalent, followed by CS5+CS6 and PCFO166 were 20%. Additionally, other detected CF decreased to 15% which were CS6. However CFA/I, CS1+CS3+CS21, CS2+CS3+CS and CS17 were 10% and CS+CS3 were 5%

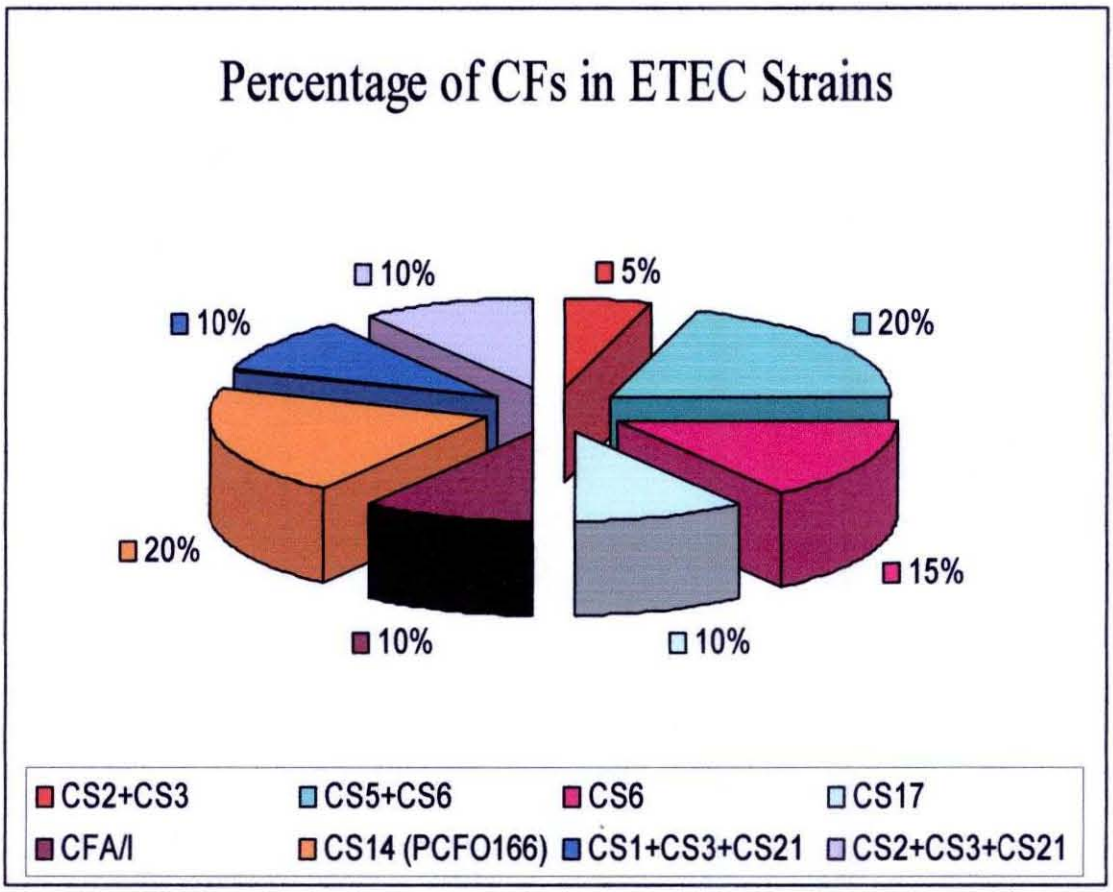

Figure 3.8: Percentage of CFs in ETEC isolates.

Results

For different ETEC toxin type specific colonization factor expression has been observed. CS5+CS6, CS17, CS14(PCFO166), CS2+CS3+CS21 (Longus) were expressed in ST,LT and LT/ST; CS6 were expressed in LT and LT/ST; CS1+CS3+CS21 (Longus), CS2+CS3 were expressed only in ST and CFA/I was expressed in LT/ST. Distribution of CFs among different toxin expressing ETEC is summarized in figure: 3.9.
A comparison of the toxin pattern and the expression of different CFs was carried out which is shown in Table 3.1

Table 3.1 Association of toxins and CFs in ETEC isolates

Toxin produced	Total no. of ETEC isolates	CF types (s) produced	No. (% of) isolates
ST	12	CS17	1 (8)
		CS2+CS3	1 (8)
		CS5+CS6	2 (17)
		CS6	3 (25)
		CS14(PCFO166)	2 (17)
		CS1+CS3+CS21	2 (17)
		CS2+CS3+CS21	1 (8)
LT	6	CS5+CS6	1 (17)
		CS17	1 (17)
		CS14(PCFO166)	3 (50)
		CS2+CS3+CS21	1 (17)
LT/ST	9	CS5+CS6	2 (22)
		CFA/I	1(11)
		CS17	1 (11)
		CS6	1 (11)
		CS2+CS3+CS21	2 (22)
		CS14(PCFO166)	2 (22)

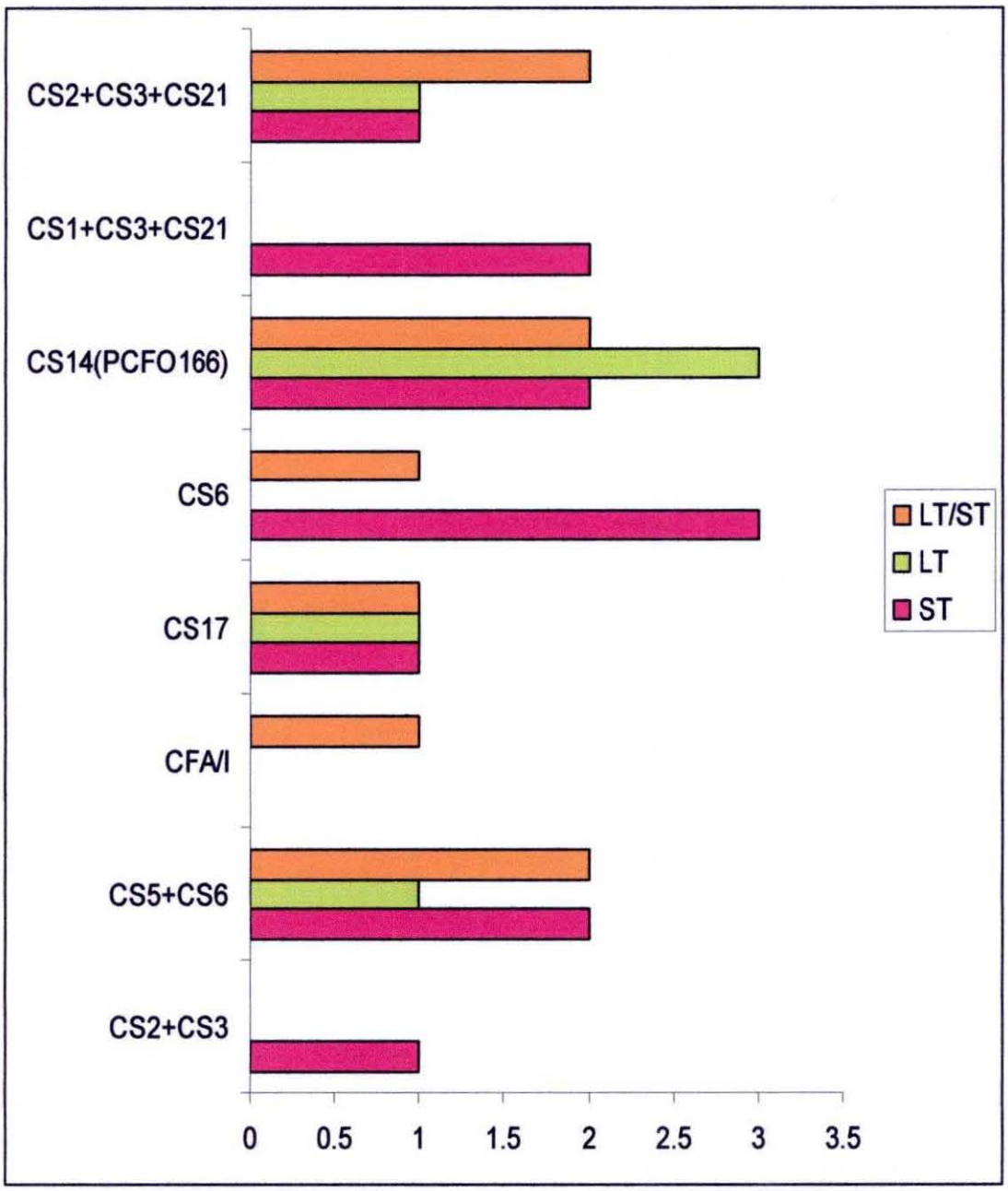

Figure 3.9: Distribution of Colonization factors among different toxin expressing ETEC

3.4: Comparism of Genotypic (PCR) and Phenotypic (ELISA) method:

PCR showed that 17% of all strains were ETEC-ST, 10% were ETEC-LT and 73% were ETEC-LT/ST. However, only 37% were positive for both toxin types by GM1-ELISA.

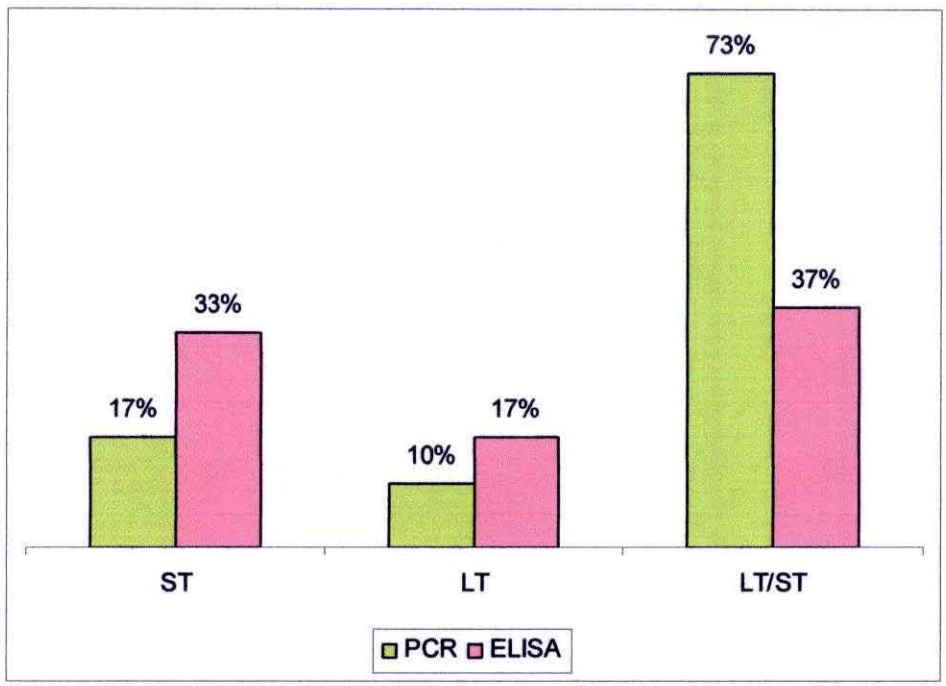

Figure 3.10: Percentage ETEC toxin between PCR and GM1-ELISA.

Chapter Four
Discussion

4.1 Discussion

Enterotoxigenic *Escherichia coli* (ETEC) is an important pathogen that causes diarrheal diseases. Of the seven recognized diarrheagenic categories of *Escherichia coli* [27], ETEC is the most common, particularly in the developing world [80]. There are a variety of different genotypic and phenotypic methods available for detection of ETEC toxins and colonization factors in clinical isolates. We compared multiplex PCR and GM1-ELISA to evaluate whether these methods gave similar results or not.

A total number of 3459 stool specimens were tested from patients enrolled in the 2% routine surveillance system in ICDDR, B during the period of March, 2009 to July, 2010 and 10% (n=364) ETEC strains were detected at the Immunology Laboratory. From those 30 discrepant ETEC strains were again analyzed to determine genotypic and phenotypic differences. PCR showed high levels of sensitivity and specificity and took less time to perform. There were three groups of enterotoxins isolated in the ETEC positive isolates. Among 30 discrepant strains LT was detected in 10%, ST was 17% and LT/ST was 73% by PCR. In case of ELISA, LT was in 17%, ST in 33% and LT/ST in 37% of strains thus almost 90% of the ETEC strains expressed LT, either LT alone or in combination with ST. Rest 13% were not detected by ELISA method.

It was observed that 40% of the LT only-producing ETEC isolates, 70% of the ST only- and 55% of the LT- and ST-producing ETEC isolates expressed CFs.

In the present study different types of colonization factors were also observed. Within the 30 discrepant ETEC isolates, CS5 + CS6 and CS14 were the predominant phenotypes, followed by CS6, CFA/I, CS17, CS1+CS3+CS21, CS2+CS3+CS21 whereas, CS2+CS3 expressing ETEC strains were less frequently isolated from stool specimens.

Among the strains, the most prevalent colonization factor CS14 was found in LT, ST and LT/ST toxin expressing ETEC strains. The antigen, CFA/I was detected in ST positive

ETEC strains. In combination with other colonization factors CS21 was present on all types of toxin producing ETEC. CS1+CS3+CS21 and CS2+CS3 were found in ST toxin producing ETEC.

For this purpose, two laboratory techniques PCR and ELISA were used. The isolates that were positive for PCR were analyzed by ELISA test to see whether the PCR results for toxins matches with the results of the ELISA test. PCR is a genotypic procedure in which gene for specific toxins are amplified using toxin specific primers and the results are seen in a gel under UV light on the other hand ELISA is a toxin phenotype detection procedure in which color intensity accounts for detection of toxin in ETEC isolates. Specificity and sensitivity of PCR result can be much more sensitive which is why for determination prevalence of ETEC emphasis was given to PCR results.

As the same sample strains were also subjected to the ELISA test, the ELISA result could match with the results of PCR. But we did not find a good correlation between the results of PCR and ELISA for the detection of ETEC toxins. We know that PCR is a more sensitive method than ELISA. But for detecting toxin expression by PCR, there must be a minimum level of DNA concentration in the template that is prepared. In PCR, ST was 17% whereas 33% found in ELISA and LT was 10% by PCR whereas 17% found in ELISA. We therefore did not find similar rates of LT and ST expression by the PCR method. Since prior to PCR, DNA concentration in the template was not measured, low DNA level might account for this discrepancy. At the same time, ELISA is less sensitive but the specific colonies negative for PCR gave a positive result in ELISA. In ELISA test, the colonies were subjected to overnight growth with shaking at 37°C, that's why the low LT and ST toxin producing strains might have multiplied in larger numbers to produce toxins that were detectable by ELISA. For this reason, PCR negative LT and ST strains were positive by ELISA. We did not check the broth grown bacteria also by PCR and this should be done in future studies.

About 13% of PCR positive strains were negative by ELISA. It is possible that either the *eltB* or *estA* gene is present as a silent gene or, alternatively, that the levels of expression of the gene for these toxin are so low that the toxin are not detected by the ELISA reader. The probability of phenotypic silencing may increase even more during storage and re-cultivation [79].

When we established the multiplex-toxin PCR, we particularly aimed to develop a robust PCR optimized for use with DNA prepared by the rapid-boil technique to allow examination of large strain collections without the need for labor intensive DNA preparations and for use in less-well-equipped laboratories, e.g., in developing countries. The assay was found to be robust, allowed for accurate identification of the three toxin genes with DNA prepared by simple boiling of the bacteria, and allowed rapid identification (less than 24 hrs) of ETEC from *E. coli* strains derived from cultures of diarrheal stool samples when the method was tested in the laboratory. It would also be possible to extract DNA directly from diarrheal stool samples and test for the presence of ETEC by the PCR method. However, this approach would require DNA extraction with a commercial kit to avoid PCR inhibitory factors present in the stool.

These discrepancies of results can be explained by Real time- PCR (RT-PCR). Less copy number of the toxin genes might justify the loss of toxin expression in ELISA, sometimes when the PCR method is used. Thus in future study, rRT-PCR can be carried out to evaluate genotypic and phenotypic toxin results specificity.

The choice of method for any specific laboratory is dependent on equipment, resources, and time constraints, but we suggest initial analysis of ETEC colonies from clinical isolates by using PCR. Subsequent analyses for CFs could be performed on one to two toxin-positive colonies from each original isolate, using either Dot blot or PCR. This study shows that both genotypic and phenotypic methods can be used for detection of ETEC. The PCR method can be used for large scale analysis of *E. coli* strains although concurrences with phenotypic methods are needed for further verification.

Chapter Five
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Appendix A

A. Reagents

1. **Bovine serum albumin (BSA)**
   Sigma chemical Co., St. Louis, MO, USA

2. **Low binding ELISA microtiter plates Nunc**
   Roskilde, Denmark

3. **Ganglioside GM1**
   Sigma chemical Co., St. Louis, MO, USA

4. **Goat anti- mouse IgG HRP conjugate**
   Jackson Immuno Research, West Grove, P. A., USA

5. **H_2O_2** (Hydrogen peroxide) (30%)
   Merck, Darmstadt, Germany

6. **Lincomysin**
   Sigma chemical Co., St. Louis, MO, USA

7. **Monoclonal antibodies**
   LT 39: 13: 1, mouse anti - LTB (IgG1)
   ST 1:3, mouse anti – ST (IgG1)
   From Ann – Mari Svennerholm, University of Goteborg, Sweden

8. **Orthophenylenediamine (OPD)**
   Sigma chemical Co., St. Louis, MO, USA

9. **ST – CTB conjugate**
   From Ann – Mari Svennerholm, University of Goteborg, Sweden

10. **Tween 20** (polyoxietylensorbitanmonolaurat)
    Sigma chemical Co., St. Louis, MO, USA

11. Nitrocellulose membrane, 0.45 micron
Sigma chemical Co., St. Louis, MO, USA

12. 4 – chloro – 1 napthol
Sigma chemical Co., St. Louis, MO, USA

13. Methanol (99.9%)
Riedel-de – Haen

14. Rabbit anti – mouse immunoglobulin horseradish peroxidase
Dakopatts

15. Agarose
Bio-Rad Laboratory, Richmond, CA, USA

16. Ethydium Bromide
Invitrogen, Scotland, UK

Appendix B

- Preparation of different media:

1. MacConkey agar plate (1000 mL)

Reagent	Amount
Peptone	20.0 g
Lactose	10.0 g
Nacl	5.0 g
Bile salts	1.5 g
Neutral red	0.05 g
Crystal violet	1.0 g
Bacto agar	15.0 g

pH =7.2

2. Colonization factor antigen (CFA) agar (1000 mL)

Reagent	Amount
Casamino acid	10.0 g
Yeast extracts	1.5 g
MgSO4	0.05 g
$MnCl_2$	0.005 g
Bacto agar	15 g
Distilled water	1000mL

pH =7.4

3. Colonization factor antigen (CFA) broth (1000 mL)

Reagent	Amount
Casamino acid	10.0 g
Yeast extracts	1.5 g
MgSO4	0.05 g
$MnCl_2$	0.005 g
Bile salts	1.5 g
Distilled water	1000mL

4. Colonization factor antigen (CFA) agar with bile (1000 mL)

Reagent	Amount
Casamino acid	10.0 g
Yeast extracts	1.5 g
MgSO4	0.05 g
$MnCl_2$	0.005 g
Bacto agar	15 g
Bile salts	1.5 g
Distilled water	1000mL

pH =7.4

5. Luria Bertani Broth (LB) 1000 mL

Reagent	Amount
Bacto tryptone	10.0 g
Nacl	10.0 g
Bacto yeast extracts	5.0 g
Distilled water	1000 mL

pH =7.4

Appendix C

1.Reference strains for PCR

Strains	Toxin type
E. coli ST 64111	ST_h^+
E.coli 286C2	LT^+
E.coli 195	STp
E. coli VM 75688	LT^+, ST_h^+
E. coli E34420C	ST^-, LT^-

2.Reference strains for ELISA

Strains	Toxin type
E. coli ST 64111	ST_h^+
E.coli 286C2	LT^+
E. coli VM 75688	LT^+, ST_h^+
E. coli E34420C	ST^-, LT^-

3. Reference strains for Dot blot assay

E. coli Strains	CF type
E. coli 258909-3	CFA/I
E 1392-75	CS1, CS3
278485-2	CS2, CS3
E 11881/9	CS4, CS6
VM 75688	CS5, CS6

Appendix D

D. Solutions

1. Phosphate buffer saline (PBS) (10x) (1 Litre)

Reagent	Amonut
KCl (2.68 mM)	2.0 g
$Na_2HPO_4.12\ H_2O$ (7.7 mM)	27.5 g
NaCl (0.136M)	80.0 g
KH_2PO_4 (2 mM)	2.75 g

pH 7.2-7.4
For PBS- Tween 0.05% Tween 20 was added in 1X PBS.

2. 0. 1M Sodium Citrate (1000 mL)

Reagent	Amount
Trinatrium Citrate ($Na_3C_6H_5O_4.2H_2O$)	29.4 g
H_2O (Deionized)	1000 mL

pH=4.5

3. Orthophenylene Diamine (OPD)-Hydrogen peroxide (Substrate) (10 mL)

Reagent	Amount
OPD	10.0 mg
0.1 M Sodium Cictrate	10.0 mL
30% H_2O_2	4.0 mL

4. Tris Buffer Saline (TBS) (1000 mL)

Reagent	Amount
Tris (0.02M)	4.2g
NaCl (0.5M)	29.2g

pH=7.5

5. 4- Chloro-1-Napthol-H_2O_2 Substrate (10 mL)

Reagent	Amount
4- Chloro-1-Naptho, 3 mg/ml in 99.9% methano	1.7 mL
Tris Buffer Saline	8.3 mL
30% H_2O_2	5.0 µl

6. Tris-Borate EDTA buffer (1X) (1Litre)

Reagent	Amount
Trizma (tris)	12.1gm
Boric acid	6.0 gm
EDTA Sodium	0.74 gm

7. Loading Dye Composition (6X) (10 ml)

Reagent	Amount
0.25% BPB (Bromo Phenol Blue)	0.025 g (Stock 1%BPB 2.5 ml)
0.05% XC (Xyelene Cyanol FF)	0.5 ml (Stock 1%XC 0.5 ml)
100 mM EDTA	2.0 ml (stock 0.5 mM 2.0 ml)
50% Glycerol	5.0 ml (stock 100% Gly 5.0 ml)

Appendix E

E. Antibodies for toxin ELISA and Dot blot assay:

- Monoclonal antibodies specific for LT; (MAb LT-39, mouse anti-LT IgG1)
- Monoclonal antibodies specific for ST; (MAb ST-1:3, mouse anti-ST IgG1)
- Monoclonal antibodies specific for CS1; (MAb CS1,12:4)
- Monoclonal antibodies specific for CS2; (MAb CS2,10:3)
- Monoclonal antibodies specific for CS3; (MAb CS3,10:2a)
- Monoclonal antibodies specific for CS4; (MAb CS4,4:6)
- Monoclonal antibodies specific for CS1; (MAb CS5,4:5)
- Monoclonal antibodies specific for CS6; (MAb CS6, 2A:4)
- Monoclonal antibodies specific for CS7; (MAb CS7, 5:2)
- Monoclonal antibodies specific for CS17; (MAb CS17, 8:1)
- Monoclonal antibodies specific for CFA/I; (MAb CFA/I, 1:6)
- Monoclonal antibodies specific for CFA/III; (MAb CFA/III, 3:3)
- Monoclonal antibodies specific for PCFO159; (MAb PCFO159, 5:1)
- Monoclonal antibodies specific for PCFO166;(MAb PCFO166, 1:6)
- Synthetic ST-CTB conjugate