Isolation and Identification of Enterotoxigenic *Escherichia coli* (ETEC) from Stools of Diarrheal Patients

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Dedicated
To
My **PARENTS**

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is the major cause of diarrhea in children in the developing countries. It colonize the small intestine where they secret toxins. ETEC possess colonization factors (CFs) that are binds with the epithelial cell in small intestine. To determine the dynamic changing pattern of ETEC infection, a large scale surveillance should be carried out which will give a complete picture about the prevalence of CFs and toxin types of ETEC strains. The correlation of ETEC infection with patient's age, also provide vital information about the ETEC pathogenicity. For these reason, a surveillance program was conducted, to determine the prevalence of CF and toxin and to the ETEC infection with the age of patients.

In this study, diarrheal stools collected on the routine 2% surveillance sampling carried out in the International Centre for Diarrheal Disease, Bangladesh (ICDDR, B) were tested for ETEC February to April 2008.

Toxin-positive isolates were detected by enzyme-linked immunosorbent assay (ELISA), and reconfirmed with the PCR technique. The CFs was detected by the immunodot-blot assay by using specific monoclonal antibodies (Mabs). About 19% of stools were positive for ETEC and only 3% are mix infection with ETEC and *V.cholerae*. Of these, 19% ETEC isolates 44% produced LT and ST, 30% produced LT and the rest of 26% was ST producing. LT and ST-producing ETEC were more frequently detected. Among the total ETEC isolated, highest isolation rate about 27% were isolated from children 2 to 5 years of age. About 16% of ETEC isolates expressed known CFs and only 3% were mix infection with ETEC and *V.cholerae* together.

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BSA	Bovine serum albumin
CF	Colonization factor
CFA	Colonization factor antigen
CS	Coli surface-associated antigen
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
ICDDR, B	International Centre for Diarrhoel disease Research, Bangladesh
IgG	Immunoglobulin G
LB	Luria-Bertoni broth
Mabs	Monoclonal antibodies
ST	Heat-stable enterotoxin

LT	Heat-labile enterotoxin
PCR	Polymerase chain reaction
PCFs	putative colonization factors
CT	Cholera toxin
LSD	Laboratory Science Division

Chapter1

INTRODUCTION

1.1. BACKGROUND

Theodore von Escherich, a German bacteriologist, discovered the bacterium *Escherichia coli* in 1885. The bacterium, commonly known as *E. coli*, can be found in the human intestinal tract and comes in multiple forms. *E. coli* is only two microns in length and one micron wide. *Escherichia coli* are gram-negative, non-sporulating, and rod-shaped with small pilli which is responsible for mobility. It is usually found in the intestinal tract of human of other animals, whereas as it is generally a harmless and useful inhabitants. *E.coli* was 1st suspected as being the causative agent of children's in 1945 when nursery epidemics of severe was reported (Bray, 1945). Infection by pathogenic *E.coli* is the main cause of l disease. In human there are five unique classes of *E.coli* that can cause inflammation of the stomach and bowels (gastroenteritis) and, therefore, termed enterovirulent.

Infection by pathogenic *E.coli* is the main cause of disease, and still remains a public health problem in many parts of the world including Bangladesh. In humans there are five important categories of *Escherichia coli*, which cause l disease (Nataro and kaper, 1998). There are the enteropathogenic *E. coli* (EPEC), can cause outbreak in newborn nurseries. enteroinvasive *E. coli* (EIEC) that invades (passes into) the intestinal wall to produce severe . Enterohemorrhagic *E. coli* (EHEC), a type of EHEC, *E.coli* 0157:H7 can cause bloody and the hemolytic uremic syndrome (anemia and kidney failure). Enterotoxigenic *E. coli* (ETEC) produces a toxin that acts on the intestinal lining, and is the most common cause of travellers. Enteroaggregative *E. coli* (EAggEC) can cause acute and chronic (long lasting) in children. Of these recognized dirrheagenic categories of *Escherichia coli*, ETEC is the most common, particularly in the developing world (WHO, 1999) and the most common cause of bacterial in children.

1.1.1. Enteropathogenic *E.coli* (EPEC):

The EPEC strains traditionally have been defined as member of specific *E.coli* serotypes that have been epidemiologically incriminated as causes of infantile and include the following somatic (O) serogroups: O44, O55, O86, O111, 0114, O119, O125, O126, O127, O128, O142,

and O158. More recently, EPEC has been defined according to specific virulence properties. Enter pathogenic *E.coli* strains adhere to intestinal mucosa and produce a characterstic diarrhoea in the gastrointestinal tract. Eteropathogenic *E. coli* does not produce enterotoxin and are not invasive. Epidemiologic studies indicate that EPEC is a common cause of in children less than 1 year of age, particularly in developing countries (Donnenberg MS, et al., 1992). EPEC induce a watery similar to ETEC, but they do not possess the same colonization factors and do not produce ST or LT toxins but there are reports that they produce an enterotoxin similar to that of *Shigella*. They produce a non fimbrial adhesin designated as intimin, an outer membrane protein that mediates the final stages of adherence (McKee ML et al., 1995). Other virulence factors may be related to those in *Shigella sp.*

The distinguishing characteristic of EPEC is their close association with the intestinal microvilli and the subsequent removal from the intestine during the course of the disease (Andrade JR, et al., 1989 and Jerse AE, et al., 1990). EPEC are also able to manipulate actin filaments near the epithelial cell membrane and cause the formation of a pedestal that these microbes 'sit upon' (Knutton S, et al., 1989) . EPEC strains are said to be moderately invasive and unlike ETEC or EAEC, they induce an inflammatory response. EPEC cause an acute that is common in infants in the developing world (Nataro JP, et al., 1998). The normally lasts for a short period of time (Donnenberg MS, et al., 1993) but there are cases of protected infection, with some reports of illness lasting as long as 120 days (M. J. Blaser PDS et al., 1995) .

1.1.2. Enterohemorrhagic *E.coli* (EHEC)

Hemorrhagic colitis is caused by *E.coli* 0157:H7 and, less frequently, by other serotypes of *E.coli* (026:H11) that produce cytotoxins resembling those found in shield dysenteriae, type 1. These toxins are referred to as shiga-like toxins or verotoxins. EHEC *E.coli* 0157:H7 has a bovine reservoir. Typically, EHEC is detectable in the feces of no less than 5% at any point in time. The agent has been detected at similar, or slightly higher, prevalence among cattle being held at saughter plants and on the external surface of the hides of recently slaughtered animal.

The distinguishing feature of EAEC strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent in young children. Like ETEC strains these bacteria adhere to the intestinal mucosa and cause non-bloody without causing inflammation, suggesting that the organisms produce a toxin of some sort (Bhan MK, et al., 1989). . After adherence these bacteria stimulate mucus secretion and the thick mucus layer results in a biofilm formation that becomes difficult to penetrate (Tzipori S et al., 1993) . The lack of tissue invasion also suggests a toxin might be involved in causing. The toxin produced by these bacteria is a heat stable enterotoxin (EAST 1) which is a 4.1-kDa protein (38 amino acids) encoded by the astA plasmid-associated gene (Savarino SJ 1993). A recent report suggests that EAEC may secrete yet another toxin capable of stimulating a severe acute intestinal inflammatory response in lighted rat intestinal loops and in infected children (Eslava C et al., 1993) .

1.1.3. Enteroinvasive *E.coli* (EIEC):

EIEC are rare causes of throughout the world. EIEC closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. After colonization, EIEC penetrate and multiply within epithelial cells of the colon causing widespread cell destruction results in severe inflammation, bloody, mucus-laden and fever (Goldberg MB, et al., 1993, Sansonetti PJ 1992). EIEC apparently lack fimbrial adhesins but do possess a specific adhesin that as in *Shigella* is thought to be an outer membrane protein. The organisms also share similarity at the DNA sequence level to *Shigella* (Goldberg MB et al., 1993). EIEC do not produce heat labile toxin (LT) or heat stable toxin (ST) and unlike *Shigella*, they do not produce the shiga toxin either. Enteroininvasive *E.coli* strains include specific serotypes of *E.coli* (O28, O112, O115, O124, O136, O143, O144, O147, O152, O164 and, O167) that are different from EPEC serotypes. The EIEC strains can invade intestinal epithelial cells. EIEC can cause dysentery, similar to that caused by shield, characterized by fever, vomiting, crampy abdominal pain, and tenesmus. Stools often contain blood and leukocytes. However, most patients experience watery without blood or mucus.

1.1.4. Enteroaggreative *E.coli* (EAggEC):

Enteroaggreative *E.coli* is defined by their characteristic adherence pattern in tissue-culture-based assays. Unlike EPEC which demonstrates localized adherence, these strains exhibit a "stacked brick" adherence pattern on HEP-2 or HeLa cells. An additional group of *E.coli* that adheres diffusely to these cell lines needs further clarification. The distinguishing feature of EAEC strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent in young children. Like ETEC strains these bacteria adhere to the intestinal mucosa and cause non-bloody without causing inflammation, suggesting that the organisms produce a toxin of some sort (Bhan MK et al., 1989). After adherence these bacteria stimulate mucus secretion and the thick mucus layer results in a biofilm formation that becomes difficult to penetrate (Tzipori S et al., 1992). The lack of tissue invasion also suggests a toxin might be involved in causing. The toxin produced by these bacteria is a heat stable enterotoxin (EAST- 1) which is a 4.1-kDa protein (38 amino acids) encoded by the *astA* plasmid-associated gene (Savarino SJ et al., 1993) . A recent report suggests that EAEC may secrete yet another toxin capable of stimulating a severe acute intestinal inflammatory response in lighted rat intestinal loops and in infected children (Eslava C et al., 1993).

Table 1: Classification of *Escherichia coli* associated with Diarrhea

E.coli	Epidemiology	Diarrhea	Mechanism
EHEC	Hemorrhagic colitis and hemolytic uremic syndrome in all ages and thrombotic thrombocytopenic purpura in adults.	Bloody or non bloody	Cytotoxin production and adherence
EPEC	Acute and chronic endemic and epidemic in infants	watery	Adherence effacement
EIEC	with fever in all ages	Bloody or not	Adherence, invasion of mucosa
EAggEC	Chronic in infants	watery	Adherence,
ETEC	Infantile in developing countries and traveler's	watery	Adherence, enterotoxin production.

(Louisiana Office of public Health- Infectious Disease Epidemiology section- Infectious Disease Control).

1.1.6. **Enterotoxigenic** *E.coli* **(ETEC):**

Enterotoxigenic *Escherichia coli* (ETEC) has been reported to be a major cause of diarrhea in humans throughout the world (Sack, 1975; Albert et al., 1995; Gaastra & Svennerholm, 1996), especially in developing countries (F.Qadri, 2005). It is a cause of morbidity and mortality in children up to 5 years of age in developing countries (Black, 1993). It has also been found to be the most common cause of traveller's in all countries where surveys have been conducted (Black, 1990). Even adult in these setting may develop various illnesses and die as a consequence of infection with enteric pathogens, Such as rotavirus, *Vibrio cholerae*, enterotoxigenic *Escherichia coli (*ETEC) and *shigelll adysenteria* type. Children in more developed countries also suffer from infectious leading to costly hospitalization and sometimes death (Glass et al.1991). Furthermore,

travellers from developing countries have a high incident of caused by enteric pathogens (Black, 1990). It has been suggested that nearly half of the people traveling to Africa, Asia and America develop dirrheal infection (Svennerholm et al., 1997). ETEC is defined as the *E. coli* strains that contain at least one member of two defined groups of enterotoxins: ST and LT (Levine MM et al 1989) . It is the most common among the five diarrheagenic *E. coli* (Black RE et al., 1993) strains and is an important human and animal pathogen (Huilan S et al., 1991). It is also the most common cause of infectious (Black RE et al., 1993) , especially in hot and humid climates, where contaminated water is readily available (Wolf MK, 1997). It is a worldwide problem that is responsible for approximately 400,000 to 800,000 deaths per year (Gilligan PH, 1999). caused by ETEC has much in common with cholera: both result from ingestion of rather large inocula of gram-negative bacteria (Wolf MK, 1997). Enterotoxigenic *E. coli* infection is characterized by its abrupt onset and relatively short incubation period of 14 to 50 hours. The diseases vary from minor discomfort to a severe cholera-like syndrome. Symptoms include watery without blood, and the disease can vary in severity (Hyams KC et al., 1991). ETEC possess specialized pilli, antigenically unrelated to common pilli, which act as ligands to bind the bacterial cells to the specific complex carbohydrate receptors on the epithelial surfaces of the small intestine (Evans DG et al., 1978). It is generally thought that after these noninvasive bacteria are ingested; the bacteria adhere to the epithelia of the small intestine through colonization (Helander A 1997; Wolf MK1997). The enterotoxins, heat-labile enterotoxin (LT) and heat-stable toxin (ST), are then secreted into the gut lumen and attached to specific gut receptors, resulting in aberrations in the epithelial cells' fluid homeostasis mechanisms (Levine MM 1983, Wolf MK 1999).

1.1. PATHOGENIC MECHANISMS

The pathogenicity of ETEC strains is attributed to their ability to colonize the small intestine and to produce one or both of the two types of enterotoxins. The toxins activate the enterocyte guanylate cyclase (cGMP) and the adenylate cyclase (cAMP) systems, leading to a net secretion of electrolytes and water into the intestinal lumen.

ETEC possesses a variety of surface located adhesions that anchor the bacteria to intestinal mucosal receptors. This anchorage enables the bacteria to resist the flushing action of the intestinal peristalsis and delivered in close proximity to the entrecotes.

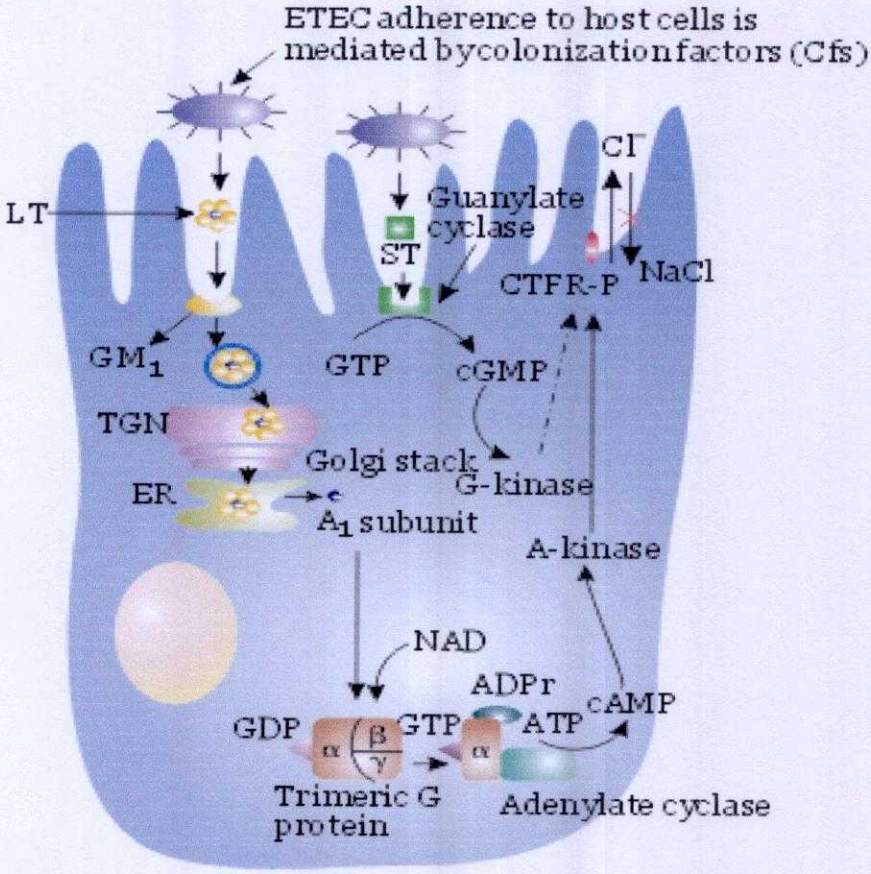

Figure 1: Mechanism of LT and ST

1.2.1. TOXINS

The enterotoxins produced by ETEC are distinguished on the basis of their thermo stability into heat-labile enterotoxins (LT) and heat-stable enterotoxins
(ST) (Holgren, 1985; Cohen and Giaella, 1995).

1.2.2. **Heat- labile enterotoxin (LT)**

The definition of LT is some what arbitary. Most LT preparation is destroyed considerably at temperature less than 60°C for 30 minutes (Sack, 1975). LT produced by strains of human origin, although not identical, is clearly related immunologically to that produced by porcine strains (Honda et al., 1984); the two forms of LT have been called LTh and LTp. LT is closely related to cholera toxin (CT) produced by strains of *V. cholerae* in its structure, antigenically and activity (Gill and woolkalis, 1985; Homgren, 1985). LT and CT are both protein complexes consisting of one polypeptide A-subunit and five B-subunits. Subunit A and B have molecular weight of 25,000 and 11,500 and are synthesized with single peptide of 18 and 21 amino acid, respectively. LT is located in periplasmic space and only a small amount is released into culture fluid; in this respect, it differs from CT, which is secreted extracellularly. In both CT and LT, the B-subunits are responsible for binding of the toxin to the epithelial cells. Both toxins can bind to the GM1 ganglioside, but there appears to be additional receptors for LT in the rabbit intestine, these have been identified as galactoprotein. Subunit A of LT is usually isolated as a single unique polypeptide but can be activated to yield two fragments, A_1 and A_2.

After translocation across the membrane of intestinal epithelial cells, A_1 catalyzes the NAD-dependent activation of adenylate cyclase to cause an increase in the concentration of cyclic AMP (cAMP). In the intestinal epithelial cells, cAMP inhibits the adsorptions of sodium and therefore, of chloride and water. In the crypt cells, it increases sodium secretion and cause loss of chloride water. The resulting water electrolyte outflow in to the lumen of small intestinal leads to profuse electrolyte rich osmotic diarrhea.

1.2.3. Heat-stable enterotoxin (ST)

Generally the enterotoxins that withstand 100°C for 15 minutes are considered as heat-stable. Heat-stable enterotoxins are secretory peptides produced by some bacterial strains, such as enterotoxigenic *Escherichia coli* (Ghanekar Y et al., 2003). These peptides keep their 3D structure and remain active at 100°C. Different STs recognize distinct receptors on the cell surface and thereby affect different intracellular signaling pathways. For example, STa enterotoxin binds and activates membrane-bound guanylate cyclase, (Hasegawa M et al., 2005) which leads to the intracellular accumulation of cyclic GMP and downstream effects on several signaling pathways. These events lead to the loss of electrolytes and water from intestinal cells. Members of heat-stable enterotoxin B family assume a helical secondary structure, with two alpha helices forming a disulfide cross-linked alpha-helical hairpin. The disulfide bonds are crucial for the toxic activity of the protein, and are required for maintenance of the tertiary structure, and subsequent interaction with the particulate form of guanylate cyclase, increasing cyclic GMP levels within the host intestinal epithelial cell (Rizo J et al., 1995). The ST group is comprised of small, nonimmunogenic polypeptides. Depending on methanol solubility, ST is divided into two classes that are designated as ST_a and ST_b. The ST_a is methanol soluble and active in neonatal piglets and infant mice, but inactive in weaned pigs. The ST_a class consists of two chemically simillar toxins, the ST_aI (STI_a or ST_p) and ST_a11 (STI_b or ST_b). The ST_a1 having molecular weight 1970 is produced by human and porcine isolates of ETEC, while ST_aII having molecular wight 2040 is produced by human isolates only (Aimoto et al., 1982; Moseby et al; 1983; Takao et al, 1983; Yamamoto et al; 1987). ST_b in methanol is insoluble and active in weaned pigs but inactive in infant mice. It is only exceptionally produced by ETEC isolates from human and its pathogenic role is uncertain. Since the bilogical activity of ST in intestinal loops can be readilt reversed by rinsing (Evans et al., 1973), it would appear that binding occurs with no internalizatio of a fragment with enzymatic activity. The binding of ST is likely similar in nature to many hormones. However, nothing is known about the receptors for ST on microvillus membrane. ST stimulates guanylate cyclase activity. There appears to be no effect of the toxin on cyclic GMP phosphodiesterase since, in intact mucosa, theophylline increases cyclic GMP concentration about three-fold in presence as in the absence of ST. It seems very likely;

therefore, the cyclic GMP is another intracellular second messenger for secretion and, in particular, mediates the secretory response to ST. Most guanylate cyclase in the small intestine is located in villus cells and much of it is associated with the brush border membrane. Cyclic GMP inhibits Na^+ coupled Cl^- uptake into epithelial cells.

Table2: Characteristics of human ETEC CFs (Gaastra et al., 1997)

CF	CS	Morphology	Size (Kd)	Toxins
CFA/1 –like Group				
CFA/1	CFA/1	F 7 nm	15.0	ST+LT,ST
CS1	CS1	F 7 nm	16.5	ST+LT
CS2	CS2	F 7 nm	15.3	ST+LT
CS4	CS4	F 6 nm	17.0	ST+LT
PCF0166	CS14	F 7 nm	15.5/17.0	ST
CS17	CS17	F 7 nm	17.5	LT
CS19	CS19	F 7 nm	16.0	ST+LT
CS5 group				
CS5	CS5	H 5 nm	21.5	ST
CS7	CS7	H 3-5 nm	21.5	ST+LT
PCF09	CS13	f	27.0	LT
CS20	CS120	F 7 nm	20.0	ST+LT
PCF020	CS18	F 7 nm	25.0	ST+LT
Type-1V-like				
CFA/111	CS8	F 7 nm	18.0	LT
Longus	CS21	F 7	22.0	ST+LT,ST,LT
8786	CS15	nF	16.3	ST
Distinct				
CS3	CS3	F 2-3	15.1	ST+LT,
CS6	CS6	nF	14.5/16.0	ST+LT, ST,LT
PCFO148	CS11	f 3 nm		ST+LT
PCF0159	CS12	F 7 nm	19.0	ST+LT
2230	CS10	nF	16.0	ST

F = Fimbrial; f = Fimbrilae; nF = Nonfimbrial; H = Helical, two fimbrils arranged in a helix ; CFs = Colonization factors ; LT = Heat-labile enterotoxins ; ST = Heat-stable enterotoxin

1.2.4. Genetic control of toxin production

Various studies have demonstrated that both ST and LT are genetically controlled by plasmids. Two general types of plasmids have been described, a homogenous group that controls LT and ST production and a heterogeneous group that controls ST production alone (Gyles et., 1974; Yamamoto and Yokota, 1983; Skerman et al., 1972). These plasmids exist in low copy number. Plasmids carrying these virulence genes are non-self transmissible but may be transferred to other bacterial cells via a plasmid-mediated conjugal transfer system (Reis et al; 1980; Yamamoto and Yokota, 1983). The genes encoding ST_aI, ST_aII, LT_h and LT_p have been subcloned and sequenced (Dallas and Falkow, 1979a; Sc and McCarthy, 1980; spicer and Noble, 1982; Moreley et al, 1983; Yamamoto and Yokota, 1983; Yamamoto et al; 1987).

1.2.5. Colonization factors

The ability of ETEC to adhere to the intestinal epithelium of the host is an important virulence determinant, and adhesion is mediated by proteinaceous surface appendages called colonization factors. Infection caused by enterotoxigenic *Escherichia coli* (ETEC) possesses a serious health problem to children in developing countries. Colonization of the small intestinal mucosa by ETEC strains is mediated by antigenically specific fimbriae, also known as colonization factor antigens (CFA). The importance of this study arises from reports that active and passive immunization with ETEC strains harboring CFAs induced protective immunity against in animal models with preformed antibodies. In humans, ETEC containing CFA/I, II, III and IV have been identified. In the two decades following this initial discovery, a number of candidates CFs have been identified. At present, there are over 25 colonization factors have been recognized among human ETEC and many more are being characterized (Gaastra and Svennerholm, 1996; Qadri et al., 2005b). These are mainly protein fimbril and fimbrillar, which are made up of several identical subunits. Some more characterized CFs were called putative colonization factors (PCFs) combined with the serogroups of the stains from which the fimbriae were first identified can be subdivided into two families, the colonization factor I-like group (including CFA/I, CS1, CS2, CS4, CS14, CS17 (Gaastra and Svennerholm, 1996). No adhesins have yet been detected

that occur both on strains of human and animal origin (De Graaf and Gaastra, 1994). However, CFs has not been detected on all ETEC and roughly on 40-50% of all strains worldwide on known colonization factors have been detected.

1.3. GEGRAPHICAL DISTRIBUTION

The proportion of ETEC expressing the different adhesions varies considerably from study to study. Thus, in Thailand and in Bangladesh, CFA/I produces were found in large proportions (~25%) (McConnell et al., 1985). A high percentage of the Bangladeshi strains produced CFA/II (~25%), while the CFA/II as uncommon among the isolates from Thailand. CFA/IV, as well as CFA/I, are common colonization factors of ETEC. Similarly, CFA/II^{+} ETEC seems to be present in most strain collections, *albeit*, in a somewhat lower frequency (5-15%) than CFA/I and CFA/IV, although in a recent cohort study of children in Chile, CFA/II as the most common CF (27%). There is still a high proportion of ETEC, particularly those producing LT only, in which no CF has yet been identified. This suggests that additional CFs may exist.

AIMS OF THE STUDY

The aims and objective of the study were:

1. Detect enterotoxigenic Escherichia *E.coli* (ETEC) in stool samples collected in the 2% routine surveillance system at the hospital of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B).
2. Determine the prevalence of 13 different colonization factors (CFs) on fresh ETEC isolates.
3. Understand the prevalence of ETEC as well as the different CFs in stool from diarrheal patients.

Chapter 2

MATERIAL AND METHODS

2.1. Place of Period of study

The present study was carried out in Immunology Laboratory, Laboratory Science Division (LSD); International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B), during the three month study period from February to April 2008.

2.2. SPECIMEN COLLECTION

Stool samples were obtained from 2% routine surveillance system at the ICDDR, B.
Fresh stools were streaked onto MacConkey agar plates (Appendix-III) and incubated at 37°C for overnight.
Six discrete, pinkish and morphologically identified *E.coli* colonies from MacConkey agar plates were selected and tested for the detection of both LT and ST producing isolates.

2.3 DETECTION OF LT-PRODUCING ETEC ISOLATES

The procedure for detection of LT is based on binding of the B-subunit of the toxin to GM1 ganglioside (Appendix-I) as described below.

The procedure used was as follows:

1) Polystyrene ELISA plates (NuncF), coated with GM1(0.3 nmol), Diluted in phosphate buffer saline (PBS) (10 mM, pH 7.2) (Appendix-III) and kept at room temperature for 12-14 hours .These GM1 plates can be used for 7 days when stored at 4°C

2) Plates were washed with PBS for two times and blocked with 0.1% BSA PBS (200µl/well). After adddition of blocking agent, the plates were incubated at 37°C for 30 minutes.

3) Wash once with PBS and add 100µl off supplemented Luria-Bertani (LB) broth (Appendix-III).

4) Approximately half of a bacterial colony was inoculated into wells of microtitre plates. Cover the plates with plastic film (to prevent evaporation) and incubate with shaking at 150-200 rpm overnight at 37°C.

5) After overnight incubation, 50µl of bacterial culture was transferred from the LT plates to the corresponding wells in the ST-plates. Rest of the culture discarded.

6) Washed the LT-plates three times with PBS containing 0.5% Tween (PBS Tween) (Appendix-I). Monoclonal antibody-specific for LT (Mab-LT39) (Appendix-II) was diluted in 0.1% BSA-PBS-Tween (dilution 1:100), and added to each well (100µl/well). After addition of Mab-LT, the plates were incubated at room temperature for 90 minutes.

7) Washed the plates with PBS-Tween for three times and add 100µl of anti- IgG-enzyme conjugate. (anti-mouse Ig GHP, 1:1000 dilution in BSA-PBS) (Appendix-I). The plates were incubated plate at room temperature for 90 minutes.

8) Washed the plates with PBS-Tween for three times. Add 100µl/well of substrate solution (H_20_2–OPD, Orthohpenylene diamine, Appendix-I) and incubated at room temperature.

9) Measure the absorbance at 450nm in a Titertek spectrophotometer. Absorbance value of 0.2 or more was considered as positive result.

For detection of LT-positive strain, each assay was carried out with ETEC strains positive for LT (ETEC strains 286C2) (Appendix-IV) and negative for LT (ETEC strain ST6411) (Appendix-IV) were the interval positive and negative control, respectively.

2.4 DETECTION OF ST-PRODUCING ETEC USING INHIBITION ELISA

The pure ST in *E.coli* strain was determined by its ability to inhibit the binding of Mab-ST to the GM1 and ST-CTB conjugates (Appendix-III).
The procedure is as follows:

1. Microtitre plates were coated with GM1 (0.3 nano mole) diluted in PBS and incubated at room temperature for about 12-14 hours.

2. The GM1-coated plates were washed with PBS for two times and blocked with 0.1% BSA-PBS. After blocking, the plates were kept at 37 °C for 30 minutes.

3. After incubation, the blocking solution was discarded and the plate was washed with PBS. Following this, the specific reagent ST conjugate to CTB (ST-CTB) was diluted in 0.1% BSA-PBS by 1:300 dilutions and added to the plates and incubation carried out for 60 minutes at room temperature.

4. The plates were washed with PBS for three times and 50µl bacterial culture (from plate which was used for detecting LT-producing ETEC isolation) was added to the plate. Thereafter, Mab-spesific for ST (ST 1:400 dilution with 0.1% PBS) (Appendix-II) was added to the wells (50µl/well). After addition of Mab-ST, the plates were placed in shaker (50 rpm) at room temperature for 90 minutes.

5. After incubation, the plates were washed with PBS-Tween for three times and with PBS once. The conjugate anti-mouse IgG (Appendix-I) was added to each well (diluted 1:1000 dilution in BSA-PBS).

6. The plates were washed with PBS-Tween for three times and with PBS-Tween for three times and with PBS once. The cromogen-substrates, orthophenylene diamine

and hydrogen peroxide were added to the wells of ST-plates. Incubation was carried out at room temperature for 20 minutes.

7. After 20 minutes, the absorbance value was taken at 450 nm in Titertek spectrophotometer. An isolate was considered positive for ST if there was 50% or more inhibition of the absorbance as compared to the absorbance of the negative reference strains.

Each plate contained internal positive (ETEC strain ST6411) and negative control strains (ETEC strain 286C2) (Appendix-IV).

2.5. DETECTION OF ETEC BY POLYMERASE CHAIN REACTION (PCR) METHOD

DNA template preparation

1) For this method, 6 individual colonies were taken from MacConkey agar plate and suspended in an eppendorf tube containing 100µl of phosphate buffer saline (PBS) for the detection of LT and/or ST by PCR.

2) The suspension was boiled for 10 minutes and then kept in ice for a further 10 minutes. After this the suspension was centrifuged at 12,000 rpm for 10 minutes.

The resulting supernatant containing template DNA was used for PCR reaction. Two µl of supernatant was used for each PCR reaction.

Primer sets for LT and ST

Two gene specific primer sets were used for detection of LT and ST genes in *E. coli* strains. Sequence of primers specific for LT and ST genes were given in table1.

Table 3: Primers used for the identification of LT and ST genes

Toxins	Primers	Primer Sequence
LT	Forward	5' – GGCGACAGATTATACCGTGC – 3'
	Reverse	5' – CGGTCTCTATATTCCCTGTT – 3'
ST	Forward	5'– ATTTTTMTTTCTGTATTRTCTT– 3'*
	Reverse	5' – CACCCGGTACARGCAGGATT – 3'

*[M=A/C & R=A/G]

PCR reaction mixture

Table 4: The compositions of PCR reaction mixture (Master mix)

PCR buffer, without MgCl2 (10X)	**5.0 µl**
Primer LT F (2 pm/µl)	2.0 µl
Primer LT R (2 pm/µl)	2.0 µl
Primer STh F (2 pm/µl)	2.0 µl
Primer STh R (2 pm/µl)	2.0 µl
Primer STp F (2 pm/µl)	2.0 µl
Primer STp R (2 pm/µl)	2.0 µl
MgCl2 (25 mM)	1.0 µl
dNTPs (2.5 mM each)	0.8 µl
Taq. Polymerase (5U/µl)	0.3 µl
Deionized water	21.7µl
Total	48.0µl

Two reactions were carried for each sample, one for the detection of LT and other for the detection of ST. Five µl of LT primer (2 pmol) in LT marked PCR tube and 5 µl of ST primer (2 pmol) in ST marked PCR tube was taken and 13 µl of the master mix was added to each tube. Then 2 µl of previously prepared template DNA was added into both tubes in a total volume of 20 µl. PCR reaction was performed with a PTC-200 thermal cycler (Peltier Thermal Cycler, MJ Research) for 25 cycles.

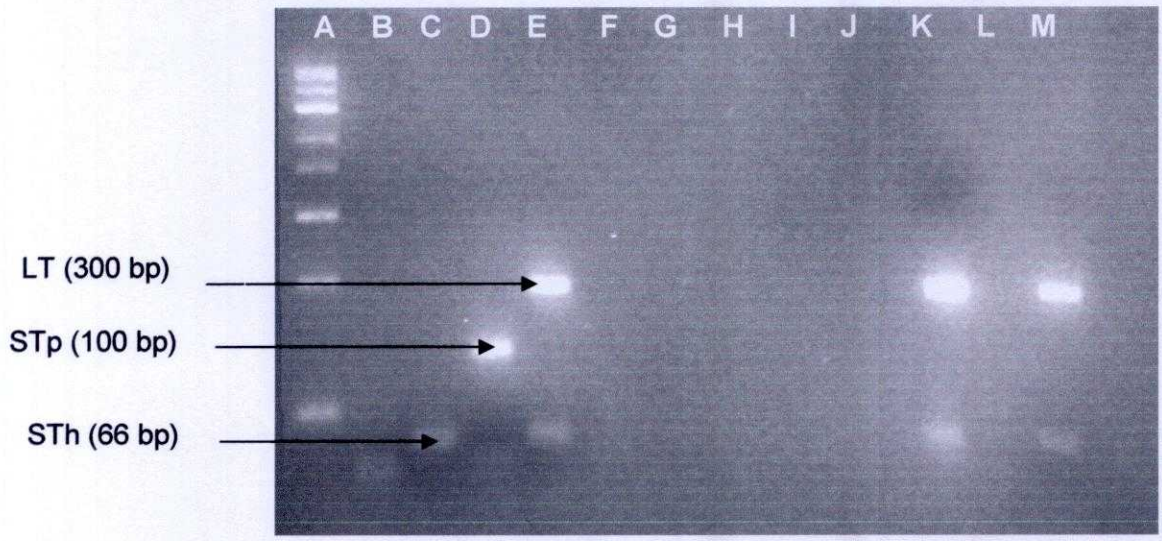

Figure **2:** Agarose gel electrophoresis for the detection of LT/ST genes of *Escherichia coli* isolated from stool. Lane A- DNA ladder and lane B represent negative control for LT/ST and, lane C-E are for control strains. Lane F-M is study samples. All strains are negative except strains of L and M lanes that are LT/ST positive ETEC.

Thermal cycle

First step 95°C for 5 minutes (initial denaturation)

Second step 94°C for denaturation

53°C for prime annealing {25 cycles}

72°C for elongation

Third step 72°C for 7 minutes (final extension step)

Agarose Gel (2%) electrophoresis

Amplified products were analyzed by agarose gel electrophoresis on 2% agarose gel was prepared by adding 2 g of agarose in 100 ml TBE (1X) buffer and melting at high temperature for 3-4 minutes after which it was allowed to cool. Four µl of ethydium bromide was added to the gel, mixed well and poured on a gel casting. Three µl of loading dye was mixed with 10 µl of PCR product and poured into the gel wells. Amplified PCR products were separated at 200 volt for 20 minutes. The band was then observed on a Gel documentation system (BIORAD) under UV light.

2.6. DETECTION OF DIFFERENT COLONIZATION FACTORS (CFs) ON ETEC ISOLATES

The presence of CFs on the ETEC isolates was detected by rapid dot blot assay using specific monoclonal antibodies for different antigens. Toxin positive colonies on MacConkey agar plate were plated on to colonization factor antigen agar (CFA agar) with and without bile salts and plates were incubated at 37°C, overnight. For testing for CS21 testing, colonies were cultured on Trypticase Soy Agar (TSA) containing 5% sheep blood. From each sample, enterotoxin positive E. coli colonies from CFA, CFA plus bile, and TSA plates were 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 different CS antigens.

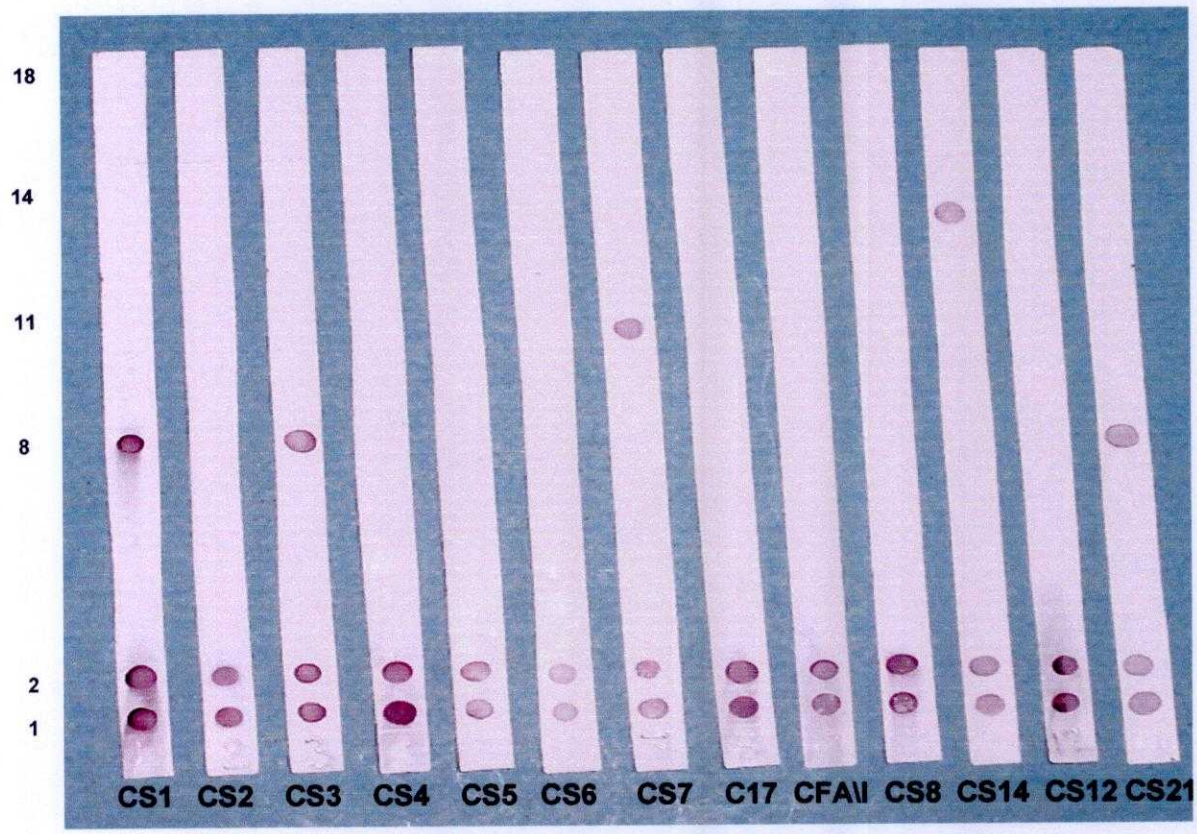

Figure 3: Colonization factors of ETEC detected by using immuno dot blot assay. The spots number 1 and 2 represent positive control for each specific colonization factor against specific CFs. Spots 8, 11 and 14 are positive strains for (CS1+CS3+CS21), CS7 and CS12 respectively. Rests of the strains are negative for all 13 colonization factors.

The procedure used was as follow:

Nitrocellulose membrane sheets or stripes 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.

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

2) 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).

3) 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 15 rpm). An incubation tray (BioRad) was used for nitrocellulose strips.

4) 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.

5) 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.

6) Stripes were washed 3 times 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.

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 (Fig.3).

2.7. STRAGE OF SAMPLES

Every toxin-positive isolates were stored at -70°C in CFA broth containing 20% glycerol. Sample was also stored at room temperature in CFA ager (Appendix-III).

Chapter 3

RESULT

During the three month of study period, about 19% (109/589) of the stool specimens were positive for ETEC. Among these isolates, about 30% (33/109) were LT-producing, 26% (28/109) were ST-producing and about 44% (48/109) were both LT and ST-producing (Table-5).

Specimens were collected from patient at different age groups. The ETEC were isolated from children <6-12 months of age was 15% and about 16% from more than 5 years of the age. Maximum isolation rate was in the age group 2 to 5 years about 27%. On the other hand, the minimum isolation rate was 15% (19/124) from < 6-12 months of the age. About 25% (22/87) and 20% (9/46) ETEC were isolated from patients of <12-24 months and the lowest age (<6 months) groups respectively (Table-6).

Total 109 ETEC were tested and 16% was CF positive. Among this, about 16% (5/32) was LT-producing, 14% (7/49) was both LT-and ST-producing and the rest of 18% (5/28) was ST producing (Table-7).

In different toxin types, different types of CF were also detected. In this study period CS13 (4) was the highest isolation and then CS7 (3), CS6 (3), CS5+CS6 (2), CS4+CS6 (2), CFA/I (2), CS6+CS8 (1) (Table-8).

The copathogen isolated from ETEC positive sample were obtained from children more than 5 years of age and adults. Among the pathogen in highest frequencies about 19 %(109/589) was ETEC and about 8% (50/589) was copathogen. The copathogen frequencies of *V.cholerae* Ogawa about 6%, and *V.cholerae* Inaba, (3%) (Table-9). In total 19% ETEC were isolated and only 3% samples were mixed infection with ETEC and *V.cholerae*.

Table- 5. Isolation of ETEC from the specimens of the diarrheal patients in 2% surveillance system

Total no. of sample tested	No.(%) of ETEC positive	Toxin Types, n. (%)		
		LT	ST	LT/ST
589	109 (19%)	33 (30%)	28 (26%)	48 (44%)

Table- 6. Isolation of ETEC from the study patients at different age groups.

Age group	ETEC positive isolates	
	Number	percentage
< 6 months (n = 46)	9	20
< 6-12 months (n = 127)	19	15
< 12-24 months (n = 81)	22	25
2 to5 years (n = 57)	12	27
≥ 5 years (n = 280)	47	16

Table 7. Detection of CF-positive ETEC isolates of different toxin types.

Toxin types	CFs (+ve) No. (%)	CFs (-ve) No. (%)
LT (n =32)	5 (15.62%)	27 (84.37%)
ST (n =28)	5 (17.85%)	23 (82.14%)
LT/ST (n =49)	7 (14.28%)	42 (85.71%)

Out of 109 ETEC isolates, 17 (16%) were CF positive.

Table 8. Correlation between toxin types and colonization factor of ETEC isolates.

Toxin types	No. of strains	Colonization factor
LT	5	CS6 (n =1), CS7 (n=2), CS6+CS8 (n =1), CS4+CS6 (n =1)
ST	5	CFA/1 (n=2), CS6 (n=2), CS14 (n=1)
LT/ST	7	CS7 (n=1), CS14 (n=3), CS5+CS6(n=2), CS4+CS6 (n =1)

Table 9. Isolation of copathogen during the study period.

Total sample tested	Pathogen isolated	No. of isolates	Percentage
595	ETEC	109	18
	V. cholerae (Inaba)	19	3
	V. cholerae (Ogawa)	31	6

Chapter 4

DISCUSSION

This study was carried out to understand the relative prevalence of enterotoxigenic *E.coli* from the diarrheal patients. Fresh stool samples were collected in the 2% systemic surveillance system of ICDDR, B.

The other important aspect of the study was to test specific immunoreagents and monoclonal antibodies (Mabs) that have been made available for detection of enterotoxigenic *E.coli.* However, monoclonal antibodies have now been produced to a number of different adhesions of ETEC. We have used a battery of 13 different specific Mabs for the purpose of assessing the prevalence of CFs.

Of the ETEC strains isolated worldwide, approximately 35% express both heat labile and heat stable enterotoxin, 35% expressed only ST and the rest (30%) expressed only LT (Gaastra and Svennerholm, 1997). In our study, 19% of the diarrheal stool samples were positive for ETEC. Approximately 44% expressed both heat labile and heat stable enterotoxin, 30% expressed only LT and rest 26% expressed only ST. In a previous study in Bangladesh, it was shown that 74% isolates were positive for both type of toxin, 2% for only LT and 24% for only ST (Gothefars et al., 1985). However, in that study, stool samples were studied in which initial stool cultures were negative for *V.cholerae*, *Shigella* spp. and *Salmonella* spp. However, in the present study all the diarrheal stool samples collected in the 2% surveillance system were included. In the earlier studies, ST was detected by the infant mouse assay whereas in the present study, ST was detected by the GM1 inhibition ELISA technique. In our study the toxin profile of all ETEC isolates have been tested and reconfirmed by PCR technique. The toxin profile varies from region to region. In several studies such as in a prospective cohort study of infant diarrhea in Nicaragua, 32% isolates were ST-producing isolates and 19% were only LT-producing. About 49% were both LT-and ST-producing (Panigua et al., 1997).

Another study in Bangladesh (Gothefers et al., 1985), have shown that, about 75% ETEC isolates were positive for CF. In that study, the incidence was higher than in other published studies. This may be an overestimation since the presence of the CFs was based on the mannose-resistant haemagglutinating properties of the strains. This may be due to the fact that they might have found a rather lower number of LT-producing isolates which rarely produce CFs, thus allowing

for high prevalence of CFs. About 16% ETEC isolates were positive for CF in the present study. The percentage of adhesion-positive ETEC was lower than in previous studies in Bangladesh. This may be because in a previous study only 2% of total ETEC isolates were LT-producing, whereas in the presents study about 29% were LT-producing. In a study carried out in Nicaragua in a birth cohort (Paniagua et al., 1997), about 60% ETEC isolates were positive for CF. A study carried out in North India showed that (Sommerfelt et al., 1996), about 76% of toxin positive ETECs was positive for CFs. About 46%ETEC isolates were positive for CFs in army personnel with traveler's diarrhoea in the Philippines, Nepal and Thailand (Serichantabergs et al., 1997). Which were more or less similar with the results of this study. In summary, theses result were also confirm that LT-producing were isolates are less commonly positive for CF. But our study LT/ST-producing were less commonly positive for CFs. In this study, stool sample were obtained from the 2% routine surveillance system. Those of age more than 5 years were also tested for ETEC. Among these, both LT/ST producing strains were isolated at a higher rate than either ST or LT producing isolates. This is to be expected since LT-producing isolates are found as commonly in healthy controls as in patients (Gaastra and Svennerholm, 1996).

In the present study, information has been obtained of the other pathogens present with ETEC in stool samples. About 19% of stools positive for ETEC were also positive for other pathogens, such as *V.cholerae* Inaba and *V.cholerae* Ogawa. In previous study (F.Qadri 2000) 17 %(381 of 2,251 stool samples were tested) of stools positive for ETEC were also positive for other pathogens, such as *V.cholerae*, *C.jejuni*, *Aeromonas* spp., *Shigella* spp. It was interesting to observe that about 93% of stool samples collected from individuals older than 5 years of age were positive for ETEC as well as other enteric pathogens. From this data, it cannot be concluded that the main causative agent for diarrhea, in these patients was due to ETEC infection.

ETEC along with other copathogen can also cause dehydrating diarrhea. However, in the rest 7% cases, it can be confirmed that diarrhea occurred due to ETEC infection. But in the lower age groups, such as children from more than 6 months to 12 months of age, only 18% samples positive for ETEC were also positive for other enteric pathogens. So, from this observation it can be concluded that was the main causative agent for diarrhea in these children (F.Qadri 2000).

From the above discussion it can be concluded that ETEC as sole pathogen can cause diarrhea in infants and younger children than in older children and adults.

In these studies the frequency of mixed infections generally increased with the age of the patients in the following order: less than 6 months, 23%; >6 months to less than 1 year, 27%; >1 to 2 years, 42%; >2 to 5 years, 44%; above 5 years, 70%. Thus, in the 0- to 1-year-old age group, ETEC was the sole diarrheal pathogen in approximately 75% of the children, and in the 0- to 3-year-old age group, ETEC was the sole diarrheal pathogen in about 70% of the children. In patients with mixed infections, rotavirus was most common copathogen with ETEC (15.6%), followed by *V. cholerae* serogroups O1 and O139 (13%), *Campylobacter jejuni* (8.4%), *Shigella* spp. (2.2%), and *Salmonella* spp. (1.5%). The detection of ETEC together with rotavirus occurred mostly in younger children (peaking at 6 to 12 months of age), whereas mixtures of *V. cholerae* and ETEC were common mostly in older children and adults (F.Qadri 2000). In our study the patient with mixed infections *V.cholerae ogawa* 0nly 3% and we saw the mixed infection generally with the age of more than five years of age (including adults).

Diarrheal disease due to ETEC continues to be a major health problem among children in the developing countries. Although the disease can be effectively managed by oral or intravenous replacement of fluid and electrolytes, prevention is preferable. An easy way of prevention would be to practice good sanitary habit and access to clean food and water. However, this may be available in developing countries in the near future, thereby necessitating the search for additional means of prevention.

It is imperative that protection from ETEC diarrhea be provided because these diseases lead to malnutrition and morbidity due to repeated infections. Clearly, the most promising possibility would be the development of cheap, efficacious and easily administratable ETEC vaccine. It is well known that ETEC colonization factors are important virulence factors and are excellent immunogens and have a central role in inducting anti-colonization immunity, thus making their inclusion in an ETEC vaccine candidate highly attractive. However, the considerable antigenic heterogeneity necessitates the inclusion of CFs in vaccine that prevails on ETEC in target population. For this reason it is necessary to conduct a large scale surveillance of the prevalence

of potential vaccine candidates such as CF and toxin prevalence in different time reflect the dynamic nature of ETEC evolution and its complex Ecology.

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APPENDICES

Appendix-I

REAGENT

1. Ganglioside GM1
   Sigma Chemical Co., St.Louis, MO, USA,
   Cat No.G-7641

2. Bovin serum albumin
   Sigma Chemical Co., St.Louis, MO, USA,
   Cat No.A-4503

3. Tween-20(polyxyethylene-sorbitan mono-laurate)
   Sigma Chemical Co., St.Louis, MO, USA,
   Cat No.P-1379

4. Rabbit anti mouse immunoglobulin horseradish
   Peroxidase
   Dakopatts
   Cat.No.P-0260

5. Orthophenylene diamine(OPD)
   Sigma Chemical Co., St.Louis, MO, USA,
   Cat No.P-9029

6. 30% hydrogen peroxide
   Fisher Scientific Company
   Cat No.H-325

7. Lincomysin
   Sigma Chemical Co., St.Louis, MO, USA,
   Cat No.H-6004

8. Nitrocellulose membrane, 0.45
   Sigma Chemical Co., St.Louis, MO, USA,
   Cat No.HAHY-15150

9. .4-chloro-1-naphtol
   Sigma Chemical Co., St.Louis, MO, USA,
   Cat No.C-8890

10. 99.9% methanol
    Riedel-de-Haen
    Cat No.32213

Appendix-II

1. Monoclonal antibodies specific for LT
   (Mab LT-39, mouse ant-LT IgG1)

2. Monoclonal antibodies specific for ST
   (Mab ST-1:3, mouse ant-ST I

3. Monoclonal antibodies specific for CSI
   (Mab CSI, 12:4)

4. Monoclonal antibodies specific for CS2
   (Mab CS2, 10:3)

. 5. Monoclonal antibodies specific for CS3
   (Mab CS3, 10:2a)

6. Monoclonal antibodies specific for CS4
   (Mab CS4, 4:6)

7. Monoclonal antibodies specific for CS5
   (Mab CS5, 4:5)

8. Monoclonal antibodies specific for CS6
   (Mab CS6, 2A:4)

9. Monoclonal antibodies specific for CS7
   (Mab CS7, 5:2)

10. Monoclonal antibodies specific for CSI7
    (Mab CSI7, 8:1)

11. Monoclonal antibodies specific for CFA/1
    (Mab CFA/1, 1:6)

12. Monoclonal antibodies specific for CFA/111
    (Mab CFA/111 3:3)

13. Monoclonal antibodies specific for PCFO159
    (Mab PCFO159, 5:1)
14. Monoclonal antibodies specific for PCFO166
    (Mab PCFO166, 1:6)
15. Synthetic ST-CTB conjugate

All antibodies were obtained from Prof.Ann-Mari Svennerholm, University of Goteborg,Goteborg, Sweden

Appendix -III

Reference strains

E.coli VM75668 LT/ST +ve strain was use as positive control for both LT and ST.

E.coli E34420C LT/ST-ve strain was used as negative control for both LT and ST

Loading dye composition (6X)

0.25%BPB (Bromo phenol Blue)	: 0.025g	(Stock 1% BPB	2.5ml)
0.05% XC (Xylene Cyanol FF)	: 0.5ml	(Stock 1%XC	0.5ml)
100 mM EDTA	: 2 ml	(Stock 0.5mM	2.0ml)
50% Glyceron	: 5ml	(Stock 100% Gly	5.0 ml)
Distilled water	: Rest of water		Nil

For 10ml 1

Composition of TBE of buffer:

Name of regent	1X	10X (10 Litter)
Trizma (Tris)	12.1 gm	121gm
Boric acid	06.0 gm	60gm
EDTA Sodium	00.74gm	7.4 gm

Appendix-1V

CLONIZATION FACTORE (CFA) AGAR (to make one liter)

Casamino acid	...	10.0 g
Yeast extracts	...	1.5 g
$MnSO_4$	...	0.05 g
$MnCl_2$	...	0.005 g
Bacto ager	...	20.0 g
Distilled water	...	1000.0 ml

pH = 7.4

COLONIZATION FACTORE ANTIGEN (CFA) BROTH (to make liter)

Casamino acid	...	10.0 g
Yeast extracts	...	1.5 g
$MnSO_4$	...	0.05 g
$MnCl_2$	...	0.005 g
Bile salt	...	1.5 g
Distilled water	...	1000.0 ml

pH = 7.

COLONIZATION FACTORE ANTIGEN (CFA) AGER WITH BILE

Casamino acid	...	10.0 g
Yeast extracts	...	1.5 g
MnSO4	...	0.05 g
MnCl2	...	0.005 g
Bacto ager	...	20.0 g
Bile salt	...	1.5g
Distilled water	...	1000.0 ml

pH = 7.4

LURIA-BERTANI BROTH (LB) (to make one liter)

Bacto trypton	...	20.0 g
NaCl	...	10.0 g
Bacto yeast extracts	...	5.0 g
Distilled water	...	1000.0 g

MacCONKEY AGER (to make one liter)

Pepton	...	20.0 g
Lactose	...	10.0 g

Nacl	...	5.0 g
Bile salt	...	1.5 g
Neutral red	...	0.05 g
Crystal violet	...	1.0 g
Ager	...	15.0 g

pH = 7.2

PHOSPHAT BUFFER SALINE (PBS) (pH 7.2) and PBS- TWEEN

NaCl	...	80.0 g
Na_2PO_4	...	11.5 g
KH_2PO_4	...	2.0 g
KCl	...	2.0 g
Deionized water	...	1000.0 g

The concentrated solution (10X PBS) was diluted ten times and was used as working solution. For PBS –Tween, 0.05% Tween-20 was added in 1X PBS.

PHOSPHAT BUFFER SALINE (PBS) (pH 7.4) (for dot-blot)

NaCl	...	0.8 g/100 ml
Na_2PO_4	...	0 .115 g/100 ml

KH_2PO_4 ... 0.02 g/100 ml

The concentrated solution was diluted 14 times and used as working solution. For PBS-Tween, 0.05% Tween, 0.05% Tween -20 was added in diluted PBS.

ORTHOPHENYLENE DIAMINE (OPD)-HYDROGEN PEROXIDE SUBSTRATE (to make 10 ml)

OPD ... 10.0 mg

0.1 M solution citrate (pH 4.5) ... 10.0 ml

30 % H_2O_2 ... 4.0 ml

0.1M SODIUM CITRATE BUFFER (Ph 4.5) (to make one liter)

Trinatrium citrate ($Na_3C_6H_5.2H_2O$) ... 29.4 g

H_2O (deionized) ... 1000.0 ml

pH adjusted by 6 M HCI

4-CHLORO-1-NAPHTHOL-H2O2 SUBSTRATE

4- choloro- 1 –napthol ... 1.7 ml
3 mg/ml in 99.9 % methanol

Tris buffer saline ... 8.3 ml

30% H_2O_2 ... 5.0 µl

TRIS BUFFER SALINE (TBS) (pH 7.5)

Tris (0.02 M)	...	1.21 g/0.5 L
NaCl (0.5 M)	...	14.61 g/ 0.5 L

pH adjusted by 6 M HCl

APPENDIX -V

Reference ETEC STRAINs used as positive controls for detecting CFs

ETEC strains	CFs
E1392-75	CS1, CS3
350CIA	PCF0159
258909-3	CFA/1
E20738A	CS17
E34420A	CFA/111
E291011A	CS7
278485-2	CS2
E11881/9	CS4, CS6
E747476A	PCF0166
VM75688	CS5, CS6

Reference ETEC strains used as positive controls for detecting toxi

ETEC strains	Toxin
286 C2	LT
ST6411	ST

All strains were obtained form prof. Ann- Marri Svennerholm, University of Goteborg, Sweden.