Effect of different regulatory factors on prevalent virulence factors expressed by enterotoxigenic *Escherichia coli* - a comprehensive study

Thesis Submitted for the degree of
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in
Life Science & Bio-technology

By

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(Index No.: 78/19/Life Sc./26)

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2023

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Maa, Baba

& Didi

DECLARATION

I do, hereby, declare that the work embodied in this thesis entitled "**Effect of different regulatory factors on prevalent virulence factors expressed by enterotoxigenic *Escherichia coli* - a comprehensive study**" submitted for the award of Doctorate of Philosophy (Science) in Life Science and Bio-technology, is the completion of work carried out under the supervision of Dr. Nabendu Sekhar Chatterjee, Scientist – F, at the Division of Biochemistry, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata. Neither this thesis nor any part of it has been submitted for either any equivalent degree/diploma or any other academic award elsewhere.

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Place: Kolkata

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "Effect of different regulatory factors on prevalent virulence factors expressed by enterotoxigenic *Escherichia coli* – a comprehensive study" Submitted by Sri / ~~Smt.~~ INDRANIL MONDAL who got his / ~~her~~ name registered on 09.09.2019 .For the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of Dr. Nabendu Sekhar Chatterjee and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

NSChatterjee

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is an enteric pathogen isolated from diarrheal patients and accounts for nearly 1,00,000 deaths annually in recent years around the globe. The peak of incidence of ETEC mostly occurs in children below the age of 5 years. In developed nations, like The United States, ETEC is acknowledged as a major cause of foodborne disease. According to Global Burden of Diseases (GBD) 2015, in India, children aged less than 5 years, around 6% of all diarrheal deaths were due to ETEC. Pathogenesis of ETEC causes the release of electrolytes, water and finally watery diarrhea which occur due to the release of plasmid-encoded enterotoxins – heat-labile (LT) and/or heat-stable (ST). To release the enterotoxins, the bacteria first attach to the epithelium of the small intestine via antigenic fimbriae called colonization factors (CFs), a major virulence determinant for initiating pathogenesis. Besides this CFs and some Non- Classical Virulence factors (NCVFs) also aid to the pathogenesis.

With this aim in mind this thesis is focused on comprehending the distribution of virulence determinants as well as the pattern of expression of the ETEC strains of this region. Additionally, we also investigated to find any correlation with the antimicrobial response pattern. In this study we finally focused on effect of different regulatory factors on commonly expressed CFs.

Archived ETEC strains of total of 379 samples isolated between 2015 -2019 used for this study (Isolated fron stool specimens of diarrheal patients admitted at the Infectious Diseases and Beliaghata General Hospital (ID & BG Hospital, Kolkata) and Dr. B.C.Roy Post Graduate Institute of Pediatric Sciences during surveillance study).

Multiplex PCR were employed to identify virulence determinants followed by confirmatory singleplex PCR. For expression study, RNA isolation followed by quantitative real time PCR was used. To observe response of different antibiotics, the Kirby-Bauer method was used. For the detection and quantification of CS6, CS5, and EatA Real time q-RT PCR and ELISA was performed. To prepare isogenic mutants of HNS lambda Red recombinase mutagenesis system was followed. Promoter assay was performed by β-galactosidase assay. Cell adhesion assay was done on intestinal cell-line HT-29. In vivo study was done with Rabbit ileal loop assay recovered from New Zealand White Rabbit.

Out of the tested 379 strains, 46% strains harboured both the enterotoxins ST and LT, whereas 15% were LT only. Among the major colonization factors, CS6 (41%) was the most prevalent followed by CFA/I (35%) and CFA/III was the lowest (3%). Among the NCVFs, EatA (69%) was predominant. However expanding the panel of detection by including most of the discovered CFs in this study revealed 97% positivity. Still 3%(n=350) isolates did not tests for any virulence determinants, indicating that there is still same factor responsible for ETEC colonization that are yet to be discovered. The prevalent combination of virulence determinants was CS6+CS5 and EatA along with elt and or esth. Though CFA/I had emerged as another predominant CF but it had diverse CFs and NCVFs along with it. In our study we observed that not all ETEC strains expressed their respective virulence factors that they possess within their genome and plasmid. Our study also investigated the response of ETEC strains against different antibiotics and surprisingly we found that there were multidrug resistant (MDR) strains. Most common antibiotic combination against which resistance was found is Am+Azm+E+S+NA. This thesis focused on the effect of regulatory factors on prevalent colonisation factors taking multiple parameters into consideration in order to gain a better understanding of the pathogenic mechanisms of this heterogeneous enteropathogen. We also expect that our results will provide a better insight to understand the differential expression of the virulence genes in ETEC and how they relate during pathogenesis. This will help as a foundation for developing various intervention strategies in future.

ETEC encounters different signals within host intestine and it can trigger linked genetic and metabolic pathways. Our experiments with addition of different compounds in the growth media maybe indicative of change in the intestinal environment which sometimes acts advantageous as well as sometime deleterious to the bacteria. It is also important to remember that virulence regulation conditions examined in the lab may differ during human infection as we cannot totally mimic the gut microbiome and the physiological conditions.

We strongly believe that the only way to making an effective vaccine with broader efficacy should contain toxins as well as CS6, CS5 and EatA for this region as this is the only combination found prevalent for a long time in our years of study. Molecular details of expression studies will help in better understanding of the ETEC pathogenesis and this knowledge could be translated for effective and safe drug for disrupting regulators of ETEC pathogenesis. This study should reduce the existing knowledge gap in minimising ETEC infection.

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Contents

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Abbreviations

°C	*Degree Celsius*
A_{260}	*Absorbance at 260 nm*
A_{280}	*Absorbance at 280 nm*
AcOH	*Glacial acetic acid*
APS	*Ammonium Persulphate*
BSA	*Bovine serum albumin*
c.f.u	*Colony forming unit*
$CaCl_2$	*Calcium chloride*
cDNA	*Complementary DNA*
CF	*Colonization Factor*
CFA	*Colonization factor antigen*
CTAB	*Hexadecyltrimethylammonium bromide*
DMEM	*Dulbecco's modified eagle's medium*
DMSO	*Dimethylsulfoxide*
DNA	*Deoxy ribonucleic acid*
dNTP	*Deoxynucleoside triphosphate*
E.coli	*Escherichia coli*
EDTA	*Ethylenediaminetetraacetic acid*
ELISA	*Enzyme-linked immune sorbent assay*
ETEC	*Enterotoxigenic Escherichia coli*
FA	*Fluid accumulation*
FBS	*Fetal bovine serum*
Fe	*Iron*
GOI	*Gene of interest*
grm	*Grams*
HRP	*Horseradish peroxide*
HT29	*Human colorectal adenocarcinoma cell line*
IPTG	*Isopropyl β- d-1-thiogalactopyranoside*
kDa	*Kilo Dalton*
kV	*Kilo Volt*

LB	*Luria Bertani*
LT	*Heat-labile enterotoxin*
M	*Molar*
M9	*Minimal Media*
MeOH	*Methanol*
MeOH	*Methanol*
$MgCl_2$	*Magnesium chloride*
ml	*Millilitre*
mM	*Milli molar*
mm	*Millimeter*
N	*Normal*
Na_2CO_3	*Sodium carbonate*
NaCDC	*Sodium chenodeoxycholate*
NaCH	*Sodium cholate hydrate*
NaCl	*Sodium Chloride*
NaDC	*Sodium deoxycholate*
NaGCH	*Sodium glycocholate hydrate*
$NaHCO_3$	*Sodium bicarbonate*
NaOH	*Sodium hydroxide*
NBT	*Nitro Blue Tetrazolium*
NCVF	*Non-classical Virulence Factor*
ng	*Nanogram*
nm	*Nanometer*
OD	*Optical density*
PAGE	*Polyacrylamide gel electrophoresis*
PBS	*Phosphate buffered saline*
PCR	*Polymerase Chain Reaction*
pmole	*Pico molar*
PVDF	*Polyvinylidene difluoride*
qRT-PCR	*Real-Time Quantitative Reverse Transcription PCR*

RNA	*Ribonucleic acid*
RT	*Room temperature*
SDS	*Sodium dodecyl sulfate*
SEM	*Standard error mean*
SOB	*Super Optimal Broth*
SOC	*Super Optimal broth with Catabolite repression*
ST	*Heat-stable enterotoxin*
TAE	*Tris acetate EDTA*
TB	*Transformation Buffer*
TCA	*Taurocholic acid sodium salt hydrate*
TCA	*Trichloroacetic acid*
TE	*Tris EDTA*
TEMED	*Tetramethylethylenediamine*
Tm	*Temperature of Melting*
TMB	*3,3',5,5'-Tetramethylbenzidine*
UV	*Ultraviolet*
VF	*Virulence factor*
WT	*Wild type strain*
X-gal	*5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside*
β-ME	*β-mercaptoethanol*
µg	*Microgram*
µl	*Microlitre*
µM	*Micromolar*
µm	*Micrometre*

CHAPTER 1

Review of Literature

1.1. *Escherichia coli*

Escherichia coli, a versatile bacterial species comprising harmless commensal as well as different pathogenic variants with the ability to either cause intestinal or extraintestinal diseases in a vast variety of hosts like humans and other animals,belongs to the genus *Escherichia.* This broad spectrum of versatility made *E. coli* is a well-suited model organism to study related to bacterial dispersions and adaptation to different growth conditions and niches (Jang *et al.,* 2017).

1.2. Discovery

Dr. Theodor Escherich, a German-Austrian paediatrician, first described the bacterium in 1885 and gave it the name "*bacterium coli commune*". Later, Castellani and Chalmers coined the name *Escherichia coli* in 1919, which was officially accepted in 1958.

1.3. The bacterium *Escherichia coli*

E. coli is a rod-shaped, gram negative bacterium (***Figure 1.1***.) of the Enterobacteriaceae family. It can live in environments with or without air. *Escherichia coli* cells usually occur as single straight rods and typical dimensions are 1.1–1.5 μm broad by 2–6 μm in length.

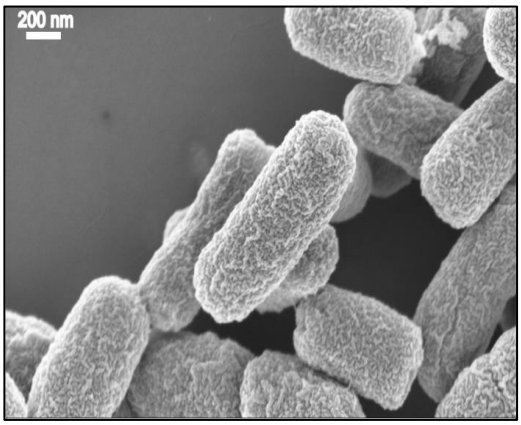


Figure 1.1.Scanning electron microscopic image of *Escherichia coli*. ***Source:*** *Duggal et al.,ACS Chemical Biology 2020*

They are either motile or non-motile, and mobility is due to lateral, rather than polar flagella. In addition to flagella, many strains have proteinaceous appendages or fibres extending outwards from the cell surface known as fimbriae or pili, which mainly plays a role for the bacteria for the initial binding to host tissues (Croxen *et al.,* 2013).This normally harmless commensal needs only to acquire a combination of mobile genetic elements to become a highly adapted pathogen capable of causing a range of diseases, from gastroenteritis to extra intestinal infections of the urinary tract, bloodstream and central nervous system. Pathogenic *E. coli* by acquired virulence factors via

plasmids, transposons, bacteriophages, and/or pathogenicity islands. Serogroups, pathogenicity mechanisms, clinical signs, and virulence factors are used to classify *E. coli* pathovars (Kaper *et al.,* 2004).

1.4. Evolution of pathogenic variants

There are a number of highly adapted *E. coli* clones that have developed particular virulence traits, giving them a greater capacity to adapt to different environments and the ability to cause a variety of diseases. These virulence traits are commonly encoded on genetic components that can be mobilised into various strains to produce novel virulence factor combinations or on genetic elements that may have previously been mobile but have now evolved to become "locked" within the genome (Kaper *et al.,* 2004).

The established view is that the pathogenic *E. coli* variants were evolved from commensal *E. coli* in humans or animals through horizontal transfer of virulence genes or HGT, resulted into various pathovars to cause clinically and epidemiologically distinctive diseases. Pathogenic bacteria have vast collections of virulence genes called pathogenicity islands (PAIs), which are present on plasmids or incorporated into the chromosome but absent in non-pathogenic bacteria. The features passed down through HGT enable the recipient bacteria to live in a different niche and endure harsh environments. Pathogenic *E. coli* genome can be up to 1 Mb larger than those of commensal isolates, mainly due to the acquisition or loss of PAIs and other genetic materials (Rasko *et al.,* 2008).In addition to gene transfer, genomic instability has recently gained a lot of attention. Various bacteria have been found to have genomic instability that helps them to colonize in host gut. To survive in the varying microenvironment within host organisms must adapt and facilitate beneficial mutations within their genome. Here genomic instability comes into play to achieve the goal of survivability. The plasticity of phylogenetic groupings indicates that important events like ancestral selective sweeps linked to beneficial mutations can represents *E. coli* evolution. Metabolic pathways seem to evolve in a phylogenetic order, with *E. coli* strains belonging to the same group sharing more pathways. Several *E. coli* pathovars have explicitly evolved in parallel at varying periods, mostly through the acquisition of mobile genetic elements bearing genes related to specific pathogenic behaviours (Chaudhuri *et al.,* 2012).

1.5. The *E. coli* genome

In 1997 for the first time whole genome sequence of *E. coli* was completed and the strain used was K-12 MG1655 strain of *E. coli.* Little or no genetic manipulation has been introduced to maintain the sequenced strain as a lab strain since then. There are 4,639,221 base pairs in the published genome (***Figure 1.2.***). Protein-coding genes account for 87.8% of the genome, stable RNAs account for 0.8%, and noncoding repeats account for 0.7%. Eleven percent of the genome is involved in gene expression regulation as well as other functions. A circular map of the *E. coli* genome is represented above (Blattner *et al.,* 1997).

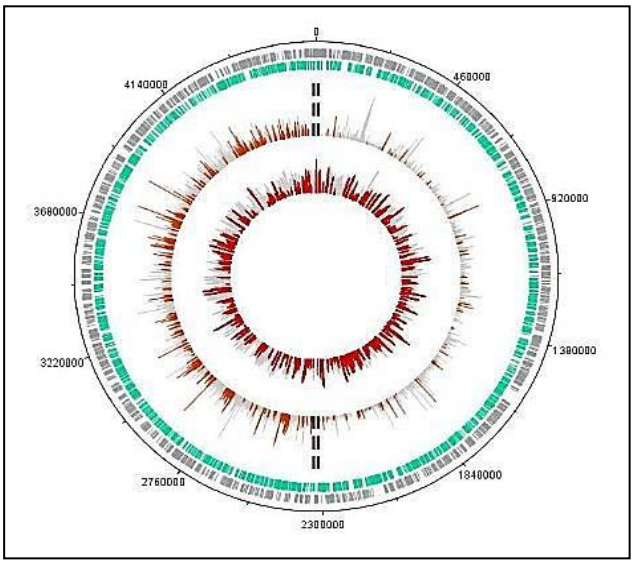


Figure 1.2.Distribution of functional sites in the genome of *E. coli* K12 MG1655. Two outer circles: gene map of the strain in both strands. Circle 3: relative number (N) of 10 – 11-nucleotide RNAs (left semi-circle) and ≥ 44 -nucleotide RNAs (right semicircle). ***Source:*** *Панюков & Panyukov, 2013*

1.6. The commensal *E. coli*

E. coli a facultative anaerobe is among the first gut colonizers that help to establish suitable environment for the gut to establish a commensal microbiota. Usually the host and *E. coli* co-operate mutually for decades where *E. coli* gets "food and shelter", and the host benefits from *E. coli* vitamin K and the colonization resistance. This colonization resistance is important for every host because it protects the host gut against colonization of pathogenic bacteria including pathogenic *E. coli* (Erjavec, 2019). Generally it has been found that five different commensal *E. coli* colonize human gut mucous layer at any given time (Apperloo-Renkema *et al.,* 1990).

1.7. The pathogenic *E. coli*

In 1940 *E. coli* strains with outbreaks of infantile diarrhea established the pathogenic form of *E. coli* (Bray and Bacteriology, 1945). Pathogenic *E. coli* mainly divided into two major groups: the intestinal pathogenic *E. coli* (IPEC), related to pathogenic effect in gastrointestinal tract, and the extraintestinal pathogenic *E. coli* (ExPEC), related to infections of extraintestinal anatomic sites. So to classify different pathovars of *E. coli* were identified pathogenic for humans and animals. Mechanisms of pathogenesis have also been studied. Urinary tract infections (UTIs), diarrheal disease, Gastroenteritis, sepsis are some common clinical symptoms that could be diagnosed from infection with either of these pathogenic variants (Kaper *et al.,* 2004).

Among the intestinal pathogenic *E. coli* there are six well-described categories: enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Pathogenic variant of extraintestinal pathogenic *E. coli* (ExPEC) are mainly Uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC).

1.7.1. Enterohaemorrhagic Escherichia coli (EHEC)

Enterohemorrhagic *Escherichia coli* (EHEC) is responsible for outbreaks of bloody diarrhea as well as hemolytic uremic syndrome (HUS) worldwide. Much of the morbidity and mortality associated with EHEC infection is triggered by use of conventional antimicrobials in response of EHEC infection. It promotes the release of the potent Shiga toxin. This is why it is also known as Verocytoxin-producing *Escherichia coli* (VTEC) or Shiga-toxin-producing *Escherichia coli* (STEC).Natural reservoir of EHEC are cattle, and approximately 75% of EHEC outbreaks can be linked back to the consumption of contaminated bovine-derived products. It travels through the bovine gastrointestinal (GI) tract that allow for its survival through the acidic environment of the distal stomachs, and ultimately colonization in the recto-anal junction (RAJ). There they destroy microvilli and below the bacterial attachment site a pedestal-like structure is formed that cups individual bacteria (***Figure 1.3.***) (Nguyen *et al.,* 2012).

1.7.2. Enteropathogenic E. coli (EPEC)

EPEC was the first *E. coli* strain to be identified in human disease in the 1940s and 1950s and the major cause of infantile diarrhea worldwide. On epithelial cells, the attaching and effacing (A/E) histopathology can be observed at microscopic levels which are the identifiable stage of EPEC infections leading to profuse watery, sometimes bloody diarrhea (***Figure 1.3.***) (Deborah Chen and Frankel, 2005).

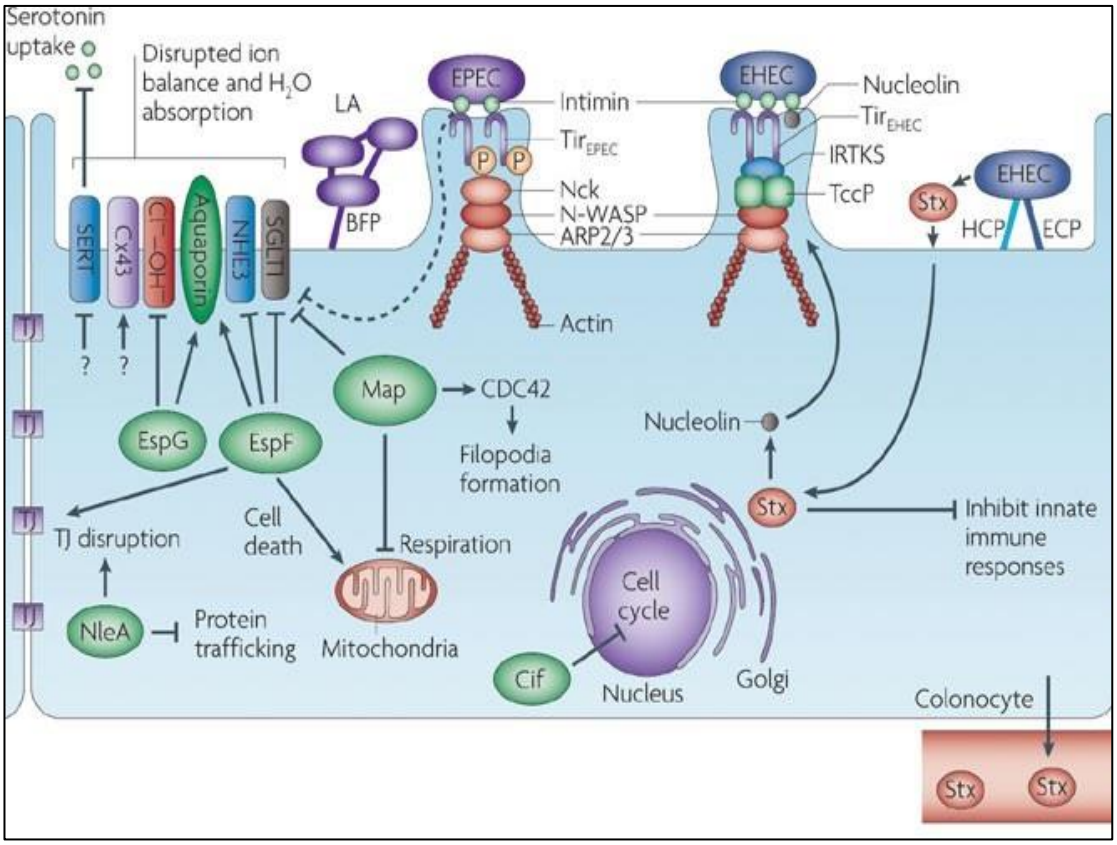


Figure 1.3.Mechanism of pathogenesis of EPEC and EHEC. ***Source:*** *Croxen et.al., Nat Rev Microbiol. 2009*

1.7.3. Enteroaggregative Escherichia coli (EAEC)

Enteroaggregative *Escherichia coli* (EAEC) is a fairly heterogeneous category of emerging enteropathogens, associated with cases of acute or persistent watery diarrhea in children as well as adults worldwide. Following ETEC, it is the prevalent cause of traveller's diarrhea. EAEC is classified as a diarrheal pathogen based on its distinctive aggregative adherence (AA) to HEp-2 cells in culture and formation of biofilm on the intestinal mucosa with a "stacked-brick" adherence phenotype, which

is connected to the presence of a 60 MDa plasmid (pAA) (***Figure 1.4.***) (Kaur *et al.*, 2010).

1.7.4. Enterotoxigenic Escherichia coli (ETEC)

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most widely recognized diarrheal pathogens in developing countries causing acute diarrheal infections in humans and animals. They are also a prevalent cause of travellers' diarrhea among people who travel from developed countries to the developing nations. ETEC primarily colonize in the small intestinal epithelia followed by secretion of enterotoxins, LT and ST, which produce a net secretion of electrolytes and water into the gut lumen and, as a result, cause severe watery diarrhea. (***Figure 1.4.***) (Croxen and Finlay, 2010).

1.7.5. Enteroinvasive Escherichia coli (EIEC)

EIEC infection is diagnosed by bloody, mucoid diarrhea. They are responsible for Shigella like dysentery. So it is generally accepted that EIEC and Shigella have the similar mechanisms of pathogenicity. EIEC have been shown to invade the colonic epithelium, a phenotype mediated by both plasmid and chromosomal loci. In addition, elaborate one or more secretory enterotoxins that may play roles in diarrheal pathogenesis. This pathovar differs from the other *E. coli* pathovars, because it includes obligate intracellular bacteria that have neither flagella nor adherence factors. Virulence is largely due to a 220 kb plasmid that encodes a T3SS on the Mxi–Spa locus that is required for invasion, cell survival and apoptosis of macrophages (Schroeder and Hilbi, 2008).

1.7.6. Diffusely adherent Escherichia coli (DAEC)

DAEC contains Afa-Dr adhesins or individually Afa, Dr, and F1845 adhesins encoded by the afa, dra and daa operons respectively, are capable of producing diarrhea. It has the capability to diffusely adhere to HeLa and HEp-2 cells. It can cause diarrhea in children between the age of 18 months and 5 years, as well as in recurring urinary tract infections (UTIs) in adults (***Figure 1.4.***) (Mansan-Almeida *et al.*, 2013).

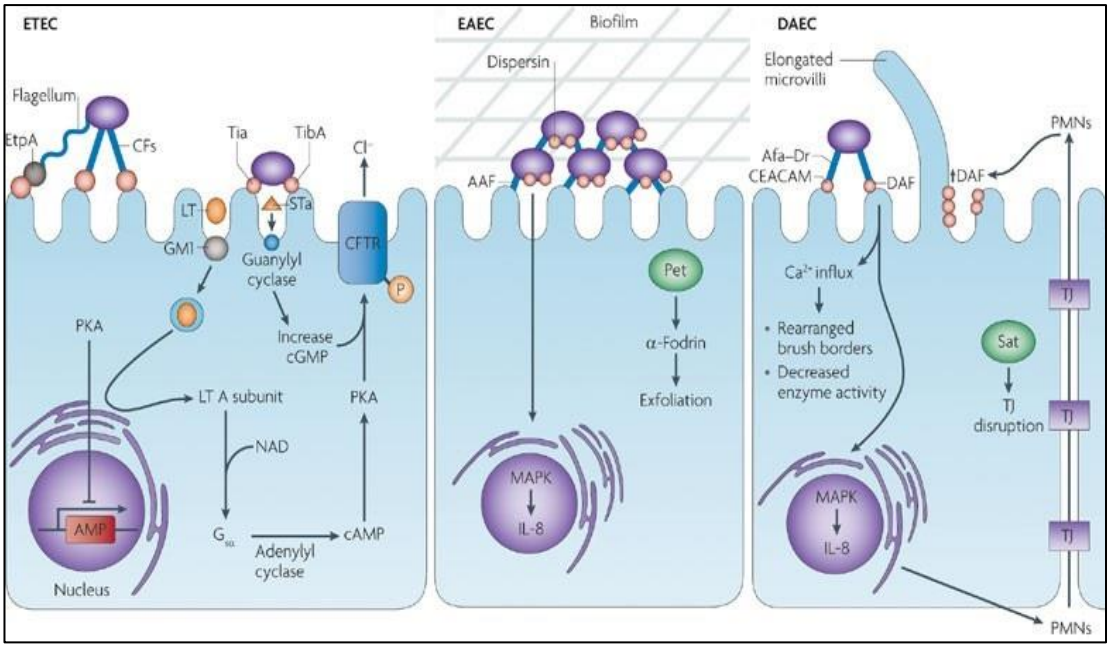


Figure 1.4. Mechanism of pathogenesis of ETEC, EAEC and DAEC. ***Source:*** *Croxen et.al., Nat Rev Microbiol. 2009*

1.7.7. Extraintestinal Pathogenic Escherichia coli (ExPEC)

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are responsible for a majority of human extraintestinal infections globally, resulting in enormous direct medical and social costs. ExPEC strains are comprised of many lineages, but only a subset is responsible for the vast majority of infections. ExPEC bacteria are classified as APEC (avian pathogenic *Escherichia coli*), UPEC (UroPathogenic *Escherichia coli*), and NMEC (non-pathogenic *Escherichia coli*). Most of urinary tract infections (UTIs) in young healthy women are caused by ExPEC. NMEC ExPEC is one of the leading causes of neonatal meningitis (Poolman and Wacker, 2016).

1.7.8. Hybrid Diarrheagenic E. coli

❖ EPEC/ETEC: A recent study reported the occurrence of hybrid strain of EPEC/ETEC isolated from a child with acute diarrhea. This strain possesses eae and elt virulent genes of EPEC and ETEC respectively (Dutta *et al.*, 2015).

❖ STEC/ETEC: According to a new study in Finland, 1% of the human STEC and 14% of the animal and environmental STEC possess genes typically present in ETEC. This hybrid STEC/ETEC strain harboured genes encoding both Shiga toxins

1 and/or 2 along with ETEC specific heat-stable (ST) enterotoxin (Nyholm *et al.*, 2015).

1.8. Enterotoxigenic *Escherichia coli* (ETEC)

Among *E. coli* pathovers, ETEC is the leading cause of diarrheal illness in children under 5 years of age and adults in the developing world. In developed nations, like The United States, ETEC is one of the major causes of foodborne disease. Enterotoxigenic *Escherichia coli* (ETEC) accounts for nearly 1,00,000 deaths annually in recent years around the globe. ETEC pathogenesis is attributable to its attachment to the host intestinal epithelial cells. For this ETEC express plasmid encoded fimbriae known as virulence factors. Upon attachment ETEC releases enterotoxins - heat-labile (LT) and/or heat-stable (ST) followed by water loss and diarrhea. However recent studies showed that Colonization factors are aided by chromosomally encoded virulence factors called as Non Classical Virulence factors (NCVFs).

1.9. Discovery of ETEC

ETEC was identified by S.N. De and his colleagues in 1965 in Kolkata. For the first time they observed a cholera-like illness but failed repeatedly in isolating *Vibrio cholerae*. Finally they isolated bacterium coli from faces in the same way as *Vibrio cholerae* and confirmed by Rabbit ileal loop (RIL) assay. Later in 1968, R.B. Sack confirmed those findings when he found presence of *E. coli* concentrations in diarrheal samples of patients admitted with cholera-like symptoms. Those findings strongly suggested *E. coli* as the etiological agent of this syndrome. Upon using the RIL assay, he was able to confirm that active cultures as well as culture filtrates, inflamed rabbit ileal loop and results into swelling. Later in 1967, veterinary scientists examined

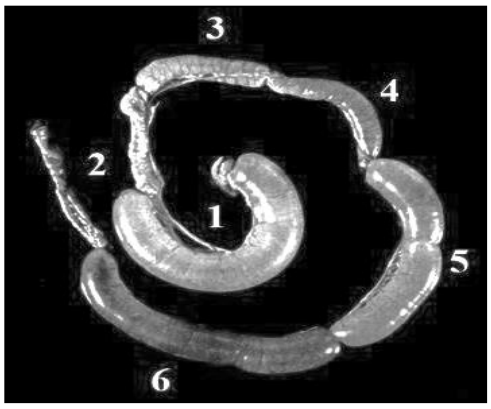


Figure 1.5. *E. coli* culture filtrates in the RIL assay. Loops 1 and 2 represent positive *V. cholera* enterotoxin and negative (saline) controls, respectively. Loops 6, 5, 4, and 3 show results obtained with increasing dilutions of the ETEC culture filtrates. ***Source:*** *RB Sack, Indian J Med Res, 2011.*

stool samples from animals with diarrhea using the ileal loop model in pigs, dogs, and rabbits and found *E. coli* as the main bacteria causing the symptoms and identified the enterotoxins LT and ST and colonization factors (***Figure 1.5.*)** (Sack, 2011). Those findings further strengthen the idea that ETEC strains from humans and animals are closely related and their effect should be studied with equal importance.

1.10. Evolution of ETEC

Enterotoxigenic *E. coli* strains show both phenotypic and genetic diversity relating to the fact that genes for both ST and LT are encoded on plasmids (Gyles *et al.*, 1974). Multiple evidences support the idea that ETEC have arisen through independent acquisition of the plasmids. Generally enterotoxigenic *E. coli* are represented by multiple H and O serotypes (Wolf, 1997). On the other hand some phylogenetic comparisons based on MLST (multi-locus sequence typing) suggest that ETEC strains are not highly conserved by the chromosomal background of (Turner *et al.*, 2006). Most extensive phylogenetic analysis of ETEC till date, considering over 1000 ETEC isolates, Steinsland *et al.* determined that strains divided into distinct clonal groups represented the majority of ETEC (Steinsland *et al.*, 2010). Their study suggested that the population of currently circulating ETEC strains likely emerged on several occasions from distinct established globally distributed lineages.

1.11. ETEC Infection

1.11.1. Sources

Due to inadequate clean water and poor sanitation ETEC remains as an under recognized but extremely important cause of diarrhea in the developing world. Several studies analysed surface water collected from different sources have found ETEC as one of the common source of contamination owing to spread of disease among the local as well as people traveling to those area (Ohno *et al.*, 1997). Apart from water contaminated foods also plays critical role in transmitting ETEC infection. Food sources include fresh fruits and vegetables (like lettuce), basil, parsley, shrimp, meat, crab, tuna pastes etc. Besides these contaminated seafood also acts as vehicle for infection transmission (Daniels, 2006). Outbreaks of ETEC infection has also been recorded in cruise ships owing to contaminated tap water

(O'Mahony *et al.,* 1986). In a study done by Sack *et al*. in 1977 found that ETEC found in animal sources food in US and among 8% of ETEC produced one or both LT and ST toxins. Diarrheal episodes were also recoded from Sweden caused by ETEC from contaminated weaning food (Sack *et al.,* 1977).

1.11.2. ETEC; cause of Travellers' diarrhea

Sack *et al*. reported in 1977 that episodes of diarrheal diseases were detected among American Peace Corps Volunteers (PCVs) during their first few weeks of stay in Kenya (Sack *et al.,* 1977). Study on travellers to Mexico revealed that ETEC strains producing both the toxins were the most commonly found pathogen among the diarrheal patients (Merson *et al.,* 1976). Studies involving patients in developed countries like USA, it has been established that Travelers' diarrhea (TD) among USA individuals remain a prevalent illness of those who visit developing countries, however most of those studies were focused in the context of short term travel. Sixty nine percent studies involved military personnel and long term non-military travellers were accounted for 33% of studies. This study showed that their travel to Middle East countries, Southeast Asia and Latin America relates to enteropathogenic infection causing diarrhea (***Figure 1.6.***). Among the pathogens most isolated pathogens were ETEC and EAEC with significant regional variability. The cases of TD were reported approximately in 37% of patients accounting for highest percentage among other diseases. Morbidity remain significant with 21% placed Sick in quarters (SIQ) while 15% received IV fluids and approximately 3% needed hospitalization (Olson *et al.,* 2019). A study over a period of 7 years from 2010-2016 revealed that for Travellers' Diarrhea ETEC was the most common etiological agent.

In TD ETEC pathogenic genotype also varies on a regional matter, e.g., 45% of ETEC isolates were found to be producing both LT and/or ST in patients travelling to India. LT-only ETEC was mostly found from visitors to Jamaica accounting for 58%. Among the studied 275 ETEC isoltaes it was found that 57% of strains produced Colonization factor Antigens (CFA) out of which approximately 50% isolates produces CS6 as the CFA (Jiang *et al.,* 2002). Incidents of TD due to ETEC were 36% in total identified causes in people travelling to Africa and 39% to the Middle East (Olson *et al.,* 2019).Seasonality also remained a key factor playing significant role in ETEC infection to the travellers.

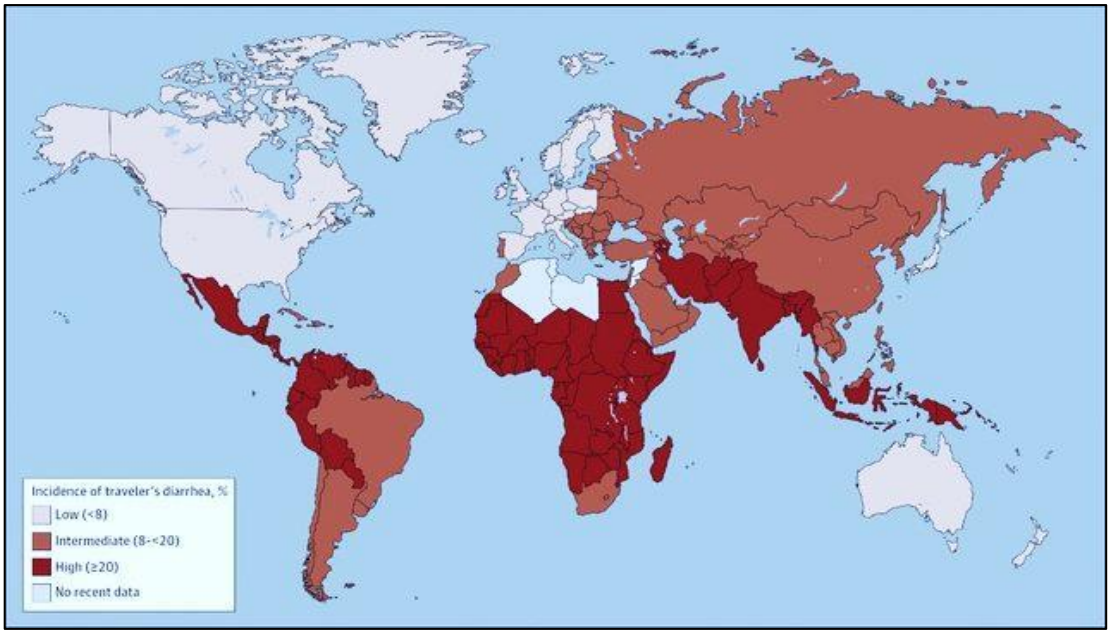


Figure 1.6. Incidence rates of travelers' diarrhea in the initial 2 weeks of stay in various regions of the world among visitors residing in industrialized countries, 2012-2014. ***Source:*** *Steffen et Al.,JAMA. 2015*

It was found that higher incidents of ETEC infection was recorded in warm seasons suggesting that travellers to the areas like Middle East, Africa, Southeast Asia are more prone to develop ETEC infection during warm period. Such seasonality may be induced by change of climate pertaining to increased growth of bacteria in suitable environmental temperature. Advent of rains after summer also aid to the cause as rain owes to higher chances of water contamination. Relating to seasonality different toxin phenotypes are also been observed as ST-ETEC become more frequent in summer season whereas LT toxin may be found all year along (Qadri *et al.*, 2005). Thus strains circulating particularly in an area with variations if growth during different season affects children as well as adult travellers to those areas. Data suggests that among all TD 20-40% of diarrheal illness is because of ETEC infection. Thus, ETEC seems to be the most frequent cause of TD in Europeans and North Americans visiting developing countries (Ericsson and medicine, 2013; Shaheen *et al.*, 2003).

1.11.3. Symptoms of ETEC infection

Initially ETEC was diagnosed as cholera like diarrheal illness in both adults as well as children. Later various studies observer a mild shift from cholera like illness and recorded a secretory diarrhea ranging mild to severe. Clinically diarrheal stool for ETEC infection is categorised as rice-watery stool leading to abnormalities of small

intestine with moderate to severe dehydration. Beside these other clinical symptoms include recurring vomiting, dehydration which are more or less similar to cholera. Research involving incubation with ETEC showed that in the proximal small bowel a concentration of 10^7-10^9 cfu/ml *E. coli* infection is needed and organisms belonged to one or two serotypes. Other than those severe symptoms less severe symptoms include quick pulse, decreased skin turgor, lowered blood pressure, dry mouth, muscle cramps etc. After the onset of first infection with the pathogen it usually appears after 1-3 days and last about 4-5 days if hydration is maintained properly. In severe dehydration cases in children it may be fatal if ignored (Qadri *et al.*, 2005).

1.11.4. Detection of ETEC infection

As ETEC infection shows similar symptoms as *V.cholerae*, *Shigella* and rotavirus actual data on episodes of ETEC infection is often misleading. When ETEC was first discovered identification of enterotoxins included physiological assays like RIL model assay for LT (De *et al.*, 1956). For detection of ST infant mouse model was used (Dean *et al.*, 1972). Later adrenal cells in tissue culture was applied for the detection of LT (Donta *et al.*, 1974). Further studies on more suitable and time saving method for detection of LT a new method of GM1 ganlioside ELISA was launched (Bäck *et al.*, 1979). The serotyping detection of ETEC was done by determining 'O' serogroups associated with the LPS and 'H' serogroups associated with flagella (Ørskov *et al.*, 1976). However studies by Stoll et.al. showed that clinical ETEC isolates may belong to large serotype which may change over time (Stoll *et al.*, 1982) . For detection of ST competitive ELISA was used where presence of ST in the filtrate inhibits the binding of monoclonal anti-ST Abs to solid-phase-bound ST gangliosides to the well (Svennerholm *et al.*, 1986). Later RPLA (reverse passive latex agglutination) assay was used to detect presence of LT/ST in culture filtrates by agglutination pattern on a microtire plate coated with Ab adsorbed latex particles (Pimbley and Patel, 1998). Further radiolabeled DNA probes encoding LT and ST was employed for detection of the enterotoxins and PCR method was used for rapid and direct detection of both the LT and ST enterotoxins (Olive, 1989).

1.12. Antibiotic treatment of ETEC and associated resistance

ETEC infection shows similar symptoms when infected with cholera or *Shigella* sp. making it somewhat difficult as well as slow process to detect infection of ETEC. This disguised nature makes ETEC an unsuitable candidate for early treatment with antibiotics. As antibiotic treatment is not an integral part of treatment for childhood diarrhea it has been difficult to study the effects of antibiotics on ETEC in case of children. However, antibiotics showed promising results in case of adults with travellers' diarrhea where ETEC infection is detected and symptoms are well characterized. Earlier when ETEC was first isolated it was seen that ETEC isolates were mostly sensitive to antimicrobial agents including tetracyclines and trimethoprim-sulfamethoxazole (Bradley Sack, 1990). Later further studies with effect of different antibiotics showed that erythromycin, ciprofloxacin, ofloxacin, azithromycin, norfloxacin showed promising result (Ericsson and medicine, 2013). But recent studies showed that ETEC evolution on the aspect of antibiotic resistance is far more aggressive and most of the antibiotics previously used for treatment are becoming less effective day by day. Many studies around the world showed that most of the ETEC stains isolates in recent times are resistant to one or more antibiotics and the isolates were multidrug-resistant (MDR) (Mondal *et al.*, 2022).

1.13. Pathogenesis and virulence factors of ETEC

ETEC follows similar pattern like other pathogenic bacteria for its infection strategy. Firstly these organisms adhere to host cells and multiply. Upon evasion of host defence their primary infection damage the host cells (Mims *et al.*, 2001). First after entering the host small intestine bacteria express proteinaceous appendages known as colonization factors and colonize. Upon colonization it releases enterotoxins followed by acute secretory diarrhea. These colonization factors as well as toxins are known as classical colonization factors (Yamamoto and Yokota, 1983). Some other colonization factors are also detected which further aid Classical colonization factors for adhesion to the epithelial cells; those are called Non Classical virulence factors (NCVFs). Primary Classical colonization factors are plasmid encoded which are taken up by ETEC to survive during their million years of co-adaptation within the host as well as environment.

1.13.1. Enterotoxins

Upon attachment to the host intestinal epithelial cells ETEC releases enterotoxins that binds to enteric receptors of the cells and cause de-regulation of the chloride channel CFTR, leading to increased secretion of chloride ions, bicarbonate and electrolytes followed by secretory diarrhea. Almost all ETEC strain produce either or both Heat Labile Toxin (LT) and Heat Stable toxin (ST).

- ❖ Heat-labile eneterotoxin

LT toxin was originally described as Heat-Sensitive enterotoxigenic factor. Later it was renamed as heat labile enterotoxin due to its structural disintegraty in high temperature. Structurally LT is closely related to CT or Cholera toxin. It belongs to multimeric AB_5 family of proteins where a single A subunit (LTA) is associated with five B subunit in a ring like structure. Apart from that the LTs can be subcategorised into two groups; LT-I and LT-II. Both these types are structurally quite similar but B subunit of LT-II share little sequence similarity with LT-I. Besides this it has been observed that LT-II variant is rarely detected in human ETEC isolates (Connell, 2007).

In terms of both structure and function, LT is closely related to cholera toxin (CT) from V. cholerae. Like CT, LT is a multimeric AB5 toxin, composed of a single A subunit (LTA) associated with a ring of five B subunits (LTB) (Hardy *et al.*, 1988). This 28kDa 'A' subunit possess a disulphide bridge connecting two domains; A1 and A2. A1 is the functional toxin and A2 is the helical part that links the subunit to B subunit (Guth, 2000) (***Figure 1.7.***). Though LTA subunit can retain its catalytic activity when free, the LTB subunit is required to enter within the host intestinal epithelium cells.

First the B subunit binds to the GM1 gangliosides expressed on the host cells and thereby triggers endocytosis of the holo-toxin. Some research has indicated that LTB can bind to gangliosides other than GM1 with low affinities. These gangliosides include those that lack a sialic acid residue (asialo-GM1) rather have a second sialic acid residue (GD1b) (McKenzie *et al.*, 2007).

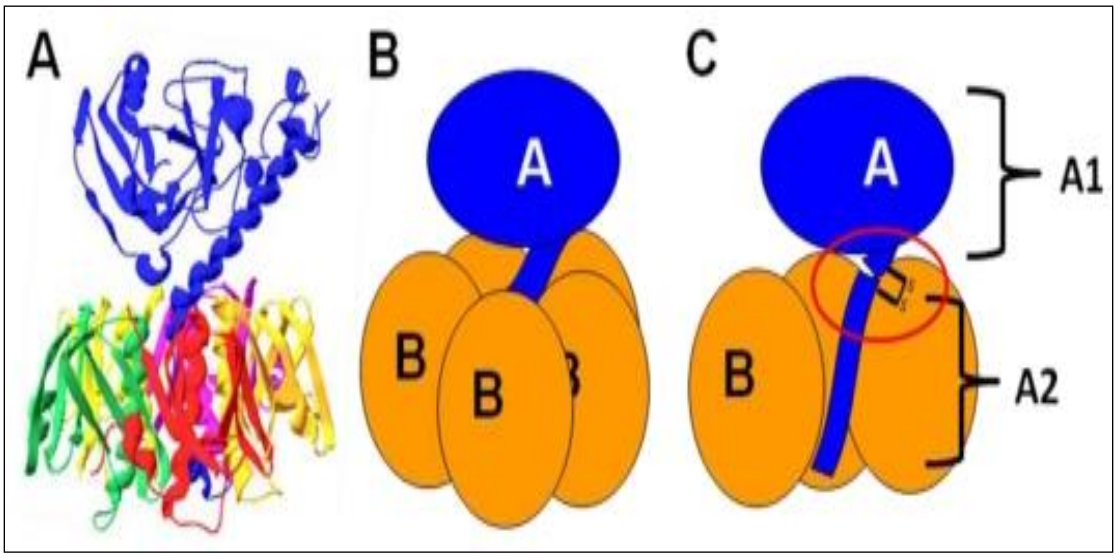


Figure 1.7. The structural organization of LT (A) The crystal structure of LT holotoxin, generated using Swiss-PDB viewer (version 4.0.1) using PDB number 1LTA. The globular A1 fragment and the helical A2 peptide can be seen in blue, along with a ring of five B subunits. (B) A schematic of the subunit organization of LT. (C) A schematic of LT showing a cutaway view of the toxin's central core. The location of the site of proteolytic processing ("nicking"), which is subtended by a disulfide bond, is circled. Nicking occurs after secretion of the toxin, and the disulfide is reduced inside the host cell, releasing the catalytically active A1 fragment.
Source: *Mudrak B et al., Toxins (Basel). 2010*

After binding to the ganglioside the A subunit is translocated through the intracellular membrane and allosterically interact with ADP rybosylating factors to ADP- ribosylate Gsα which is an guanine nucleotide protein (Tsai *et al.*, 1987). This binding inhibits GTPase activity followed by constitutive adenylate cyclase activity. This in turn increase intracellular cAMP levels anf stimulate CFTR channel resulting into loss of eletrolytes and water (***Figure 1.8.***) (Sears and Kaper, 1996).

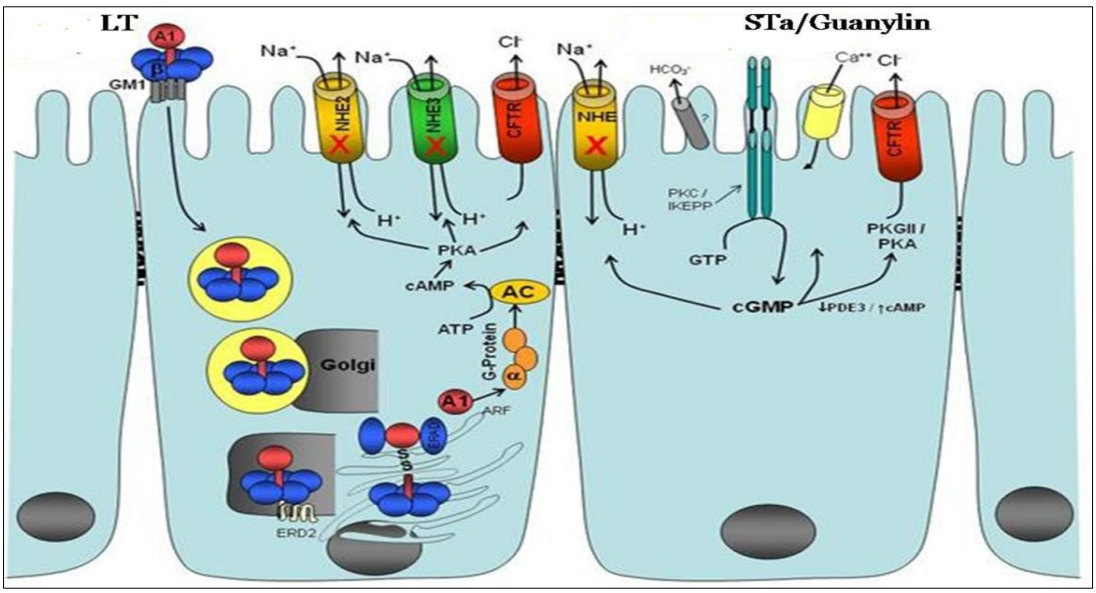


Figure 1.8. Mechanisms of action of the heat-labile (LT) and heat-stable enterotoxin (STa). LT binds to the monosialoganglioside GM1 receptor at the host mucosa surface and triggers endocytosis of the holotoxin. The A1 domain of the A subunit is transported through the Golgi complex and endoplasmic reticulum to activate the Gsα subunit of G-protein. This A1 domain interacts with ADP- ribosylating factors to ADP-ribosylate this Gsα subunit in order to activate G-protein and consequently adenylyl cyclase (AC). The AC cleaves ATP to cAMP and subsequently activates protein kinase A, which inhibits NaCl absorption (NHE transporters) and increases chloride secretion through the cystic fibrosis transmembrane regulator (CFTR). Peptide ligands of the extracellular domain of GC- C activate the intracellular catalytic domain of GC-C resulting in cGMP formation, which activates several pathways: a) inhibition of the NHE3 transporter, which decreases NaCl absorption; b) activation of CFTR, which leads to secretion of chloride; and c) increased calcium influx.
Source: *Lima et al., Braz J Med Biol Res, 2014.*

- Heat-stable enterotoxin

In 1970 it was observed that even after heat inactivation bacteria showed enterotoxigenic activity. This indicated that the bacteria possess some other toxin candidate other than LT. That toxin was named as Heat-stable enterotoxin (Smith and Gyles, 1970). Later it was found that structurally it is a peptitde with less than 50 amino acids and this small size and 3D structure was responsible for withstanding heat (Alouf *et al.,* 2005). These proteins were found to be cysteine rich and it can be divided into two structural, functional and antigenically distinct groups: the protease-resistant STa and the protease-sensitive STb.

STa belongs to a family of toxins consisting of a single peptide chain of 2kDa. Variants of STa are of two types, one is STaH (19aa) and another is STaP (18aa). STaH is solely found in ETEC isolates from human while STaP (Porcine variant) is

mainly found in isolates from pig, lambs, claves, chickens and sometimes human also (Nair and Takeda, 1998). Primarily STa toxins are synthesized as pre-pro-peptide of 72 aa that are later cleaved to form mature toxin. Disullfide bond involving 6 cystine residue is crucial for their full biological activity (Nair and Takeda, 1998; Yamanaka *et al.*, 1998).

STa binds to receptor on villus of ileum and jejunum brush border of epithelial cells. This binding is done through the extracellular domain GC-C. This inturn activates the intracellular domainof GC-C resulting into hydrolysis of GTP and cGMP accumulation within the cell (Akabas, 2000). Incresed level of cGMP activates cGMP-dependent protein kinase II (cGMPKII) and that results into phosphorylation of CFTR channel. Activated CFTR induces Cl- and HCO3- secretion from the cell resulting into loss of fluid into the lumen (Hug *et al.*, 2003). On the other hand elevated cGMP levels inhibit phosphodiesterase 3 (PDE3),that activates cAMP.

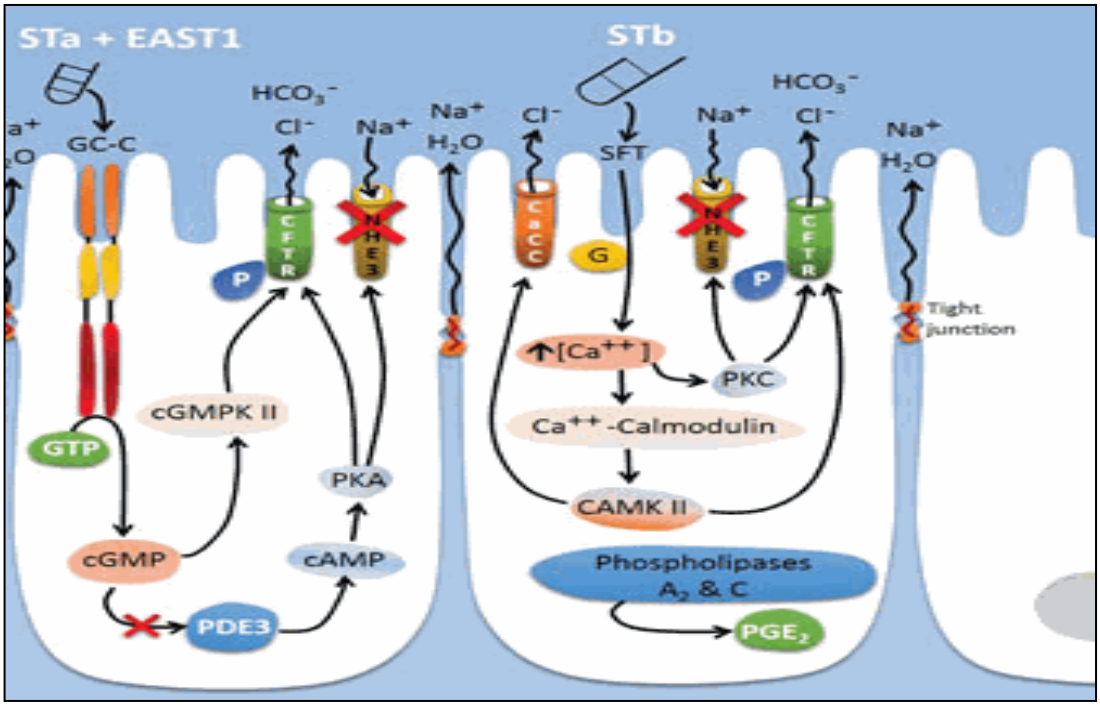


Figure 1.9. Mechanism of action of ETEC ST toxin on intestinal epithelial cells. Signaling leading to water and electrolyte loss through activation of ion channels and loosening of tight junctions by the various toxins is described. CFTR, cystic fibrosis transmembrane regulator; AC, adenylate cyclase; ARF, ADP-ribosylation factor; PKA, protein kinase C; PKC, protein kinase C; GM1, ganglioside GM1; GC-C, guanylate cycles C; SFT, sulfatide; ER, endoplasmic reticulum; Gsα, α component of an heterotrimeric G protein; NHE3, Na+/H+-exchanger 3; PDE3, phosphodiesterase 3; cGMPKII, cGMP-dependent protein kinase II; cAMPKII, calmodulin-dependent protein kinase II; CaCC, calcium-activated chloride channel; P, phosphorylation. ***Source:*** *Dubreuil et al., Ecosalplus.2016*

This cAMP, by activating protein kinase A (PKA) leads to activation of CFTR as well as Na+ reabsorption inhibits through phosphorylation of Na+/H+-exchanger 3 (NHE3). Though this effect of STa is reversible (Dubreuil *et al.*, 2016).

ETEC producing STb are almost exclusively detected in isolates from pigs, cattles etc. and very rarely associated with human ETEC isolates (Handl and Flock, 1992). STb type toxin is of 48 amino acid (aa) long with 4 cystine residues involved in disulphide bond. The precursor molecule is synthesized as a 71aa long sequence with a 23aa signal sequence (Picken *et al.*, 1983). The peptide spanning from Cys10 to Cys48 is crucial for its full activity. Acidic glycosphingolipid present on the cell membrane acts as the STb receptor (Chao *et al.*, 1997). Upon internalization STb activates GTP-binding regulatory protein resulting in increased Ca2+ level inside the cell and activates calcium/calmodulin-dependent protein kinase II. This chain of events ensures activation of CFTR resulting into loss of fluid (***Figure 1.9.***) (Yamanaka *et al.*, 1998).

1.13.2. ETEC virulence plasmids

Virulence plasmids are the extrachromosomal element with self-replicating property. During decades of co-habitation and evolution bacteria take up genetic material to survive and exert property to withstand harsh conditions. Naturally occurring plasmids promote a variety of traits including virulence, drug resistance, hydrocarbon biotransformations, as well as metabolism of rare substances. A large number of different types of plasmids occur among *E. coli* but it has been observed that plasmids encoding virulence traits falls under single incompatibility family known as IncF (Kaper *et al.*, 2004) . Some of these plasmids have lots of variation while some have similarity in terms of conserved domain for pathogenicity.

ETEC strains, to cause disease must colonize the hosts intestinal epithelia which is mediated by the fimbriae acting as adhesins (Nagy and Fekete, 2005; Turner *et al.*, 2006). These adhesins are known as Colonization factors (CFs). Another defining factor for ETEC infection is the release of enterotoxins which are also plasmid encoded. CFs of ETEC isolates from human can either be plasmid encoded or encoded by chromosome, especially by a polycistronic including the fimbrial genes, chaperons and ushers. However, human ETEC CFs encoded by virulence plasmids appears to have been acquired by flanking ISs and transposons. After undergoing

several evolutionary modifications these CFs genetically varied over time (Gaastra and Svennerholm, 1996). Recent studies have identified more than 25 distinct CFs with distinct genetical variation (Mondal *et al.,* 2022).

Completely sequenced first human ETEC CF-encoding plasmid was plasmid pCoo (Froehlich *et al.,* 2005). Plasmid pCoo isolated from human ETEC strain C921b-1, which was known to express CS1 and CS3 (Perez-Casal *et al.,* 1990). Sequencing of pCoo revealed its co-integrate nature, containing regions sharing homology with RepFIIA plasmid R100 from *Shigella* sp.(Nakaya *et al.,* 1960) and RepI1 plasmid R64 from *Salmonella enterica serovar Typhimurium* (Komano *et al.,* 2000) . The composite regions of pCoo are separated by IS100 associated direct repeats, suggesting that a recombination event occurring between regions of two different plasmids resulted in the formation of the co-integrate pCoo (Froehlich *et al.,* 2005). Remarkably the pCoo possess the polycistronic coo operon, containing four genes encoding the CS1 pilus and also EatA, which is a protein similar to the serine protease autotransporter (Patel *et al.,* 2004). The coo genes of pCoo fall within the RepI1- like portion whereas eatA is within the RepFIIA-like portion. Analysis of clinical CS1 isolates revealed that they contain both portions of pCoo suggesting that co-integrated plasmids are stable in circulation (Froehlich *et al.,* 2005).

Plasmid pH10407_95 is a RepFIIA plasmid which possess CFA/I encoding genes, indicates that the CS operons of ETEC have been acquired during multiple events on multiple plasmid backbones. Like pCoo, pH10407_95 contains a truncated F transfer region which makes these CF-encoding plasmids to rely on other plasmids for mobilization and co-transfer (Johnson *et al.,* 2009).

1.13.3. Colonization factors

First and foremost of the series of events during ETEC infection is the colonization in the host intestine and for that several proteinaceous surface structures expressed by ETEC know as colonization factors (CFs). At least 25 different CFs (***Table 1.1.***) have been described to date and most are plasmid-encoded. The genes for the structural subunits and the transport and assembly proteins required for the biogenesis of CFs are usually encoded in operons. These DNA fragments have a lower GC content and codon usage than normally associated with *E. coli* and are

generally flanked by insertion sequences and transposons (Gaastra and Svennerholm, 1996).

Table 1.1. **List of different CFs**

CFs	Morphology	Size (nm)	Gene	Protein encoded	Mw (kDa)
CFA/I	Fimbrial	7	*cfaB, cfaE*	CfaB, CfaE	15, 41
CS1	Fimbrial	7	*cooA*	CooA	16.5
CS2	Fimbrial	7	*cotA*	CotA	15.3
CS3	Fibrillae	2-3	*cstH*	CstH	15.1
CS4	Fimbrial	6	*csaB, csaE*	CsaB, CsaE	17, 40
CS5	Helical	5	*csfA, csfD*	CsfA, CsfD	21, 41
CS6	Non- Fimbrial	-	*cssA, cssB*	CssA, CssB	18, 15
CS7	Helical	3-6	*csvA*	CsvA	21.5
CS8 (CFA/III)	Fimbrial	7	*cofA*	CofA	20.5
CS10	Non- Fimbrial	-	-	-	16
CS11	Fibrillae	3	-	-	-
CS12	Fimbrial	7	*cswA, cswF*	CswA, CswF	19, 17.4
CS13	Fibrillae	-	*cshE*	CshE	27
CS14	Fimbrial	7	*csuA1*	CsuA1	15.5
CS15	Non- Fimbrial	-	*nfaA*	NfaA	16.3
CS17	Fimbrial	7	*csbA*	CsbA	17.5
CS18	Fimbrial	7	*fotA*	FotA	25
CS19	Fimbrial	7	*csdA*	CsdA	16
CS20	Fimbrial	7	*csnA*	CsnA	20.8
CS21	Fimbrial	7	*lngA*	LngA	22
CS22	Non- Fimbrial	-	*cseA*	CseA	15.7
CS23	Fimbrial	-	*aalE*	AalE	28
CS30	Fimbrial	7	*csmA, csmF*	csmA, csmF	18.5, 17.2

Source: *Boylan et al.,1956; Knutton et al.,1987, 1989;McConnell et al.,1989, 1990; Heuzenroeder et al.,1990; Aubel et al.,1992; Jordi et al.,1992; Giron et al.,1994; Froelich et al.,1994, 1995; Schmidt et al.,1995; Taniguchi et al.,1995; Gaastra et al.,1996; Valvatane et al.,1996; Wolf et al.,1997; Duthy et al.,1999; Pichel et al.,2000; Ludi et al.,2006; Von Mentzer et al., 2017.*

For the assembly of functional CFs genes clustered genes in operon found to be responsible. Those DNA fragments are flanked by ISs and Transposons. Structurally these operons are made up of genes that code for a chaperone gene, pillins and an

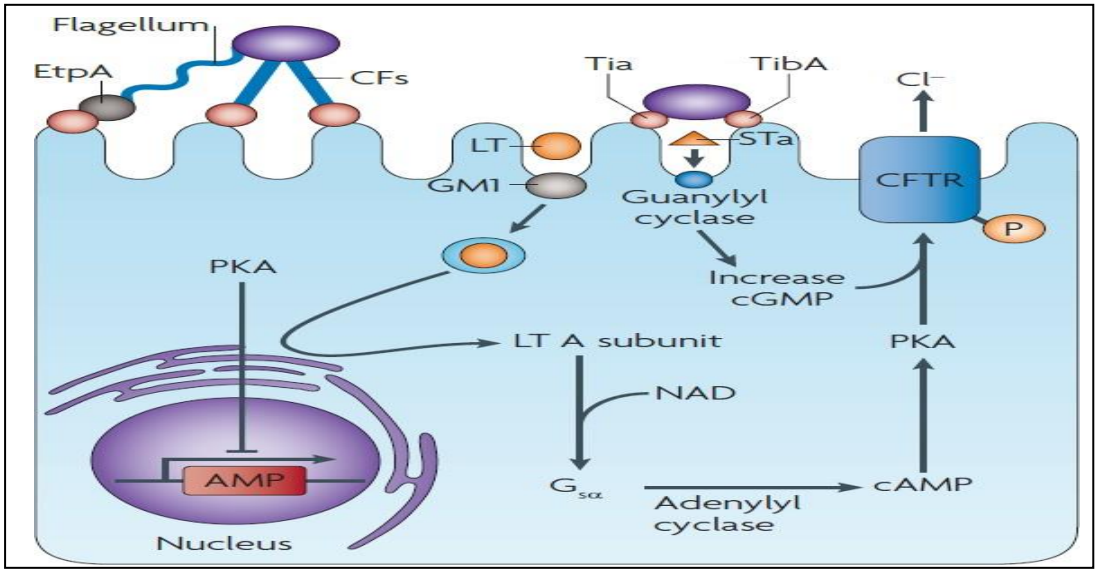


Figure 1.10. Enterotoxigenic *Escherichia coli* (ETEC) become anchored to enterocytes of the small bowel through colonization factors (CFs) and an adhesin that is found at the tip of the flagella (EtpA). Tighter adherence is mediated through Tia and TibA. Two toxins,heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST), are secreted and cause diarrhea through cyclic AMP (cAMP)- and cyclic GMP(cGMP)-mediated activation of cystic fibrosis trans membrane conductance regulator (CFTR). ***Source:*** *Croxen et al., Nat Rev, 2009*

usher gene. The CF operons include two or three pilin genes, one of which encodes a major pilin that makes up the majority of the pilus shaft and the others minor pilins that only make up a small percentage of the pilus structure (Wolf, 1997).

Different types of CFs are divided according to their antigenicity, N-terminal amino acid sequence of the major subunit, structural morphology, molecular weight etc. Worldwide CFA/I, CFA/II and CFA/IV groups are most prevalent CFs. CFA/I is a uniform rigid rod fimbrial structure composed of a single antigenic type of fimbria. CFA/II consists of CS3 alone or in combination with CS1 or CS2. CFA/IV consists of CS6 alone or in combination with CS4 and CS5 (Gaastra and Svennerholm, 1996). Besides this minor CF group include CS7, CFA/III (CS8), CS10, CS11, CS12, CS13, CS14, CS15, CS17, CS18, CS19, CS20, CS21, CS22, CS23 and CS30. Most of the CFs is fimbrial but only CS6, CS10 and CS15 are reported to be non-fimbrial in structure.

1.13.4. Non-Classical Virulence Factors

Apart from Classical colonization factors some other pathogenecity related factors are also found which aid to the pathogenesis. Those factors are Non-classical virulence factors or NCVFs which include tia, tibA, eatA, etpA , leoA, and several others.

- *Tia*, an ETEC adhesin/invasin

A chromosomally encoded adhesin, encoded on a 46-kb pathogenicity island (Tia-PAI) with lower GC content than the rest of the genome (Brown and Hardwidge, 2007). Tia, is a 25 kDa protein located to the outer membrane and functions as adhesion as well as invasin and binds to a specific receptor on HCT8 cells. Interestingly, a tia probe hybridized to ETEC, EPEC and EAEC strains, suggests that it may have a wider prospect in respect to *E. coli* pathogenesis (Mammarappallil *et al.,* 2000).

- *TibA*, a glycosylated adhesion

TibA is a chromosomally encoded protein which aid to the non-fimbrial adherence of ETEC to host intestinal cells (Elsinghorst *et al.,* 1992). It is a member of autotransporter family, 104 kDA glycosylated outer membrane protein. The glycosylated form directs bacteria to bind to specific receptors for invading the epithelial cells but they can't go deep with this (Lindenthal *et al.,* 1999). In addition to adherence to mammalian cells, TibA promotes aggregation of a bacterial population and the formation of a biofilm and this event is independent of TibA glycosylation. The ability to help in adhesion as well as invasion proves Tia to be an important part of bacterial pathogenesis (Lindenthal *et al.,* 2001).

- *EatA*, serine protease autotransporter

EatA (ETEC autotransporter A) is a 147.7 kDa protein encoded by pCS1 plasmid and secreted as a passenger protein fragment of EatA. EatA regulates bacterial adherence by degrading the adhesin EtpA and cause enhanced delivery of LT toxin (Patel *et al.,* 2004). EatA also degrade MUC2, found in the mucosal surface of the small intestine and as a result, facilitate bacterial access to cell surface receptors, toxin delivery (Kumar *et al.,* 2014).

- *EtpA*, a glycosylated adhesin

It is a 170 kDa glycosylated two-partner secretion (TPS) exoprotein encoded on the large virulence plasmid (Fleckenstein *et al.,* 2006). Recent studies suggest that EtpA functions as a molecular bridge, binding both to host cell receptors and to the tips of ETEC flagella, where it interacts with highly conserved regions of flagellin protein (Roy *et al.,* 2009). EtpA, interacts with flagellin, for its' optimal adhesion of H10407 in vitro, and for intestinal colonization in a murine model.

- *LeoA*, a GTPase virulence factor

It is thought to be the key structural element in the LT secretion pathway. Analysis of TPAI1 at the downstream of tia revealed the presence of an ORF where the gene product has similarity to the motifs of bacterial secretion apparatus. In frame mutation of these genes cause marked decrease in fluid accumulation due to decrease in LT concentration in the culture supernatant (Fleckenstein *et al.,* 2000).

1.14. Epidemiology of ETEC

First time in Calcutta in 1958 a cholera like illness was detected in which no etiologic agent could be identified. Later further study recognised that agent as enterotoxigenic *E. coli*. Since 1968, ETEC strains have been isolated frequently in Southeast Asian adults having cholera like illness. However during that time no data was there to recognise the spectrum of the disease severity as well as which age group was most vulnerable. In Chicago, a study of hospitalized children with diarrheal disease suggested that as high as 80% of children were infected with enterotoxigenic strains and this may be the first time when ETEC was detected in children (Sack, 1975).

Later several studies detected enterotoxigenic *E. coli* among children below 5 years of age making it a frequent cause of infantile diarrhea (Qadri *et al.,* 2005). The Global Enteric Multicenter Study (GEMS) conducted in Asia and Africa revealed that ETEC was one of the top four pathogens - rotavirus, shigella, enterotoxigenic *Escherichia coli* (ETEC), and Cryptosporidium sp. that causes approximatexly 70% of all diarrheal diseases in children below the age of 5 years (Kotloff *et al.,* 2013). Recent statistics showed that annually more than 1,00,000 deaths is directly

attributable to ETEC infection around the globe (Hosangadi *et al.*, 2019). Studies also indicated that in developing countries and among travellers from developed countries to developing regions suffer from diarrheal illness in which ETEC is the most commonly detected pathogen (Arduino and Dupont, 1993). The peak of incidence of ETEC mostly occurs in children below the age of 5 years. In developed nations like the United States, ETEC is acknowledged as a major cause of foodborne disease.

Disease burden estimation of ETEC of all ages has increased significantly in number of deaths from 59,200 in 2013 to 74,100 in 2015 (Hosangadi *et al.*, 2019). According to Global Burden of Diseases (GBD) 2015, in India, around 6% of all diarrheal deaths among children aged ≤ 5 years, was due to ETEC(Mondal *et al.*, 2022). GEMS study estimated that about 4% of all diarrheal deaths due to ETEC in children of ≤ 5 years (***Figure 1.11.)*** (Khalil *et al.*, 2018). Globally ETEC is detected frequently in Southeast Asian, African and Middle East regions of the world. During 2016-2017 ETEC has been detected almost 75% of food items consumed by human followed by diarrhea in Iraq (Taha and Yassin, 2019). In Bangladesh a study involving a cohort of 321 children monitored from birth to 2 years of age it was found that there was an incidence of0.5 episode/child/year in approximately in approximately 20% of cases (Qadri *et al.*, 2007). A hospital monitoring in the Nile River Delta detected ETEC in 20.7 % of children between September 2000 and August 2003 (Shaheen *et al.*, 2009). A study in Bolivian children revealed that about 87% of infantile diarrhea was attributed to ETEC (Gonzales *et al.*, 2013). In Egypt, over the course of the two-year trial among babies, ETEC was the most common cause of diarrheal illness accounting for about 20% of all cases (Mansour *et al.*, 2014). About 6-7% of all diarrheal children were detected positive for ETEC in the central region of Kenya (Mbuthia *et al.*, 2018). In a Guatemalan rural community, ETEC was responsible for 26% of severe diarrhea in children requiring hospitalization and 15% of diarrhea in the general population (Torres *et al.*, 2015).

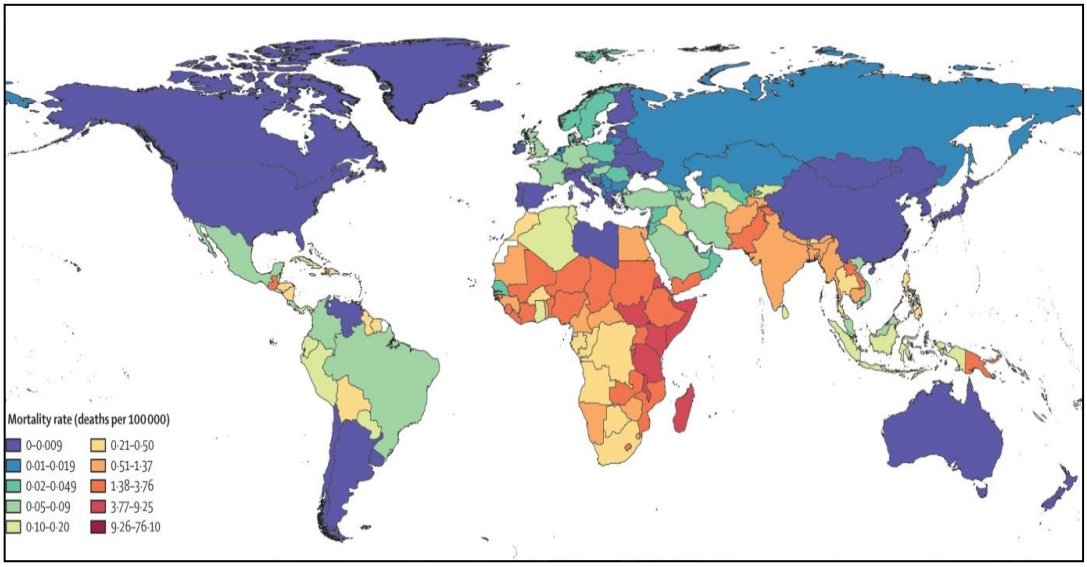


Figure 1.11. Eenterotoxigenic *Escherichia coli* diarrhea mortality rate per 100 000 people for all ages. ***Source:*** *Khalil et al., Lancet Infect. Dis. 2018*

In this eastern region of India, almost 5% of diarrheagenic *Escherichia coli* (DEC) was ETEC (Dutta *et al.*, 2013). Out of the detected diarrheagenic *Escherichia coli*, ETEC is the third most prevalent cause of pathogenesis (about 13.6%) between 2008-2012 in Karnataka, India(Singh *et al.*, 2019). Between September 2005 and November 2009 a surveillance among US military personnel in Egypt ETEC was found in approximately 50% of diarrheal isolates (Nada *et al.*, 2013). According to a recently published report, diarrhea accounted for 12.5% of deaths in Africa and 19.85% of deaths in South Asia among the persons of age group 5-14 years. Among persons >15 years of age, diarrhea mortality was 6.35% in Africa and 3.7% in South Asia. In 2010, there were approximately 4.5Lakhs diarrheal deaths in Africa and 4.8 lakhs diarrheal deaths in South Asia (Lamberti *et al.*, 2014). The incidence of ETEC infection increases in people of over 15 years of age and in addition about 25% of ETEC illness is seen in adult as reported by Qadri *et al.*, 2000 (Qadri *et al.*, 2000). Further analyses around the globe showed that the elderly patients (> 65 yr) are also susceptible to ETEC infections (13%) requiring hospitalization (Faruque *et al.*, 2004). The reason behind decrease in ETEC infections after infancy and increase at adulthood may be due to both environmental and after infancy and increase at adulthood may be due to both environmental and immunological factors (Qadri *et al.*, 2005).

1.14.1. Epidemiology of Virulence factors among ETEC isolates

1.14.1.1. Distribution of Enterotoxins

Based on findings aroud the globe involving ETEC infection in developing countries it has been found that toxin types and colonization factors (CFs) isolated from diarrheal patients varies according to the endemic regions. This region specificity makes the epidemiological study on ETEC enterotoxins more important. A community based study in Bangladesh revealed that 60% of the ETEC stains secreted LT/ST as their toxin irrespective of their age (Qadri *et al.,* 2005). Global Enteric Multicentre (GEMS) Study revealed that overall, 68% of patient isolates were ST-only or LT/ ST genotypes that were significantly associated to MSD in GEMS. In 35.2% of the cases, STh-only strains were discovered. The remaining 31.8% of case isolates were LT-only (Vidal *et al.,* 2019).A separate study revealed that globally LT toxin genes were present in approximately 60% of field ETEC strains associated with diarrheal incidents in humans, either LT alone (27%) or in combination with ST (33%) (Isidean *et al.,* 2011). In the event of a flood in Bangladesh , toxin distribution revealed that ST-only ETEC strain were more common (67%) than strains producing either LT-only(15%) or both LT and ST (20%) (Qadri *et al.,* 2005) . another study involving urban population of Bangladesh detected approximately half of the ETEC isolates expressed only ST followed by 30% expressing LT/ST and 21% expressed only LT as their toxin (Qadri *et al.,* 2007).

In Bali, Indonesia approximately 70% of ETEC strains expressed ST, whereas solely LT was discovered in 30% of isolates and lastly both LT/ST were found in on 1% of isolates (Subekti *et al.,* 2003).In clinical strains from Shenzhen, China, ST was the predominant toxin type (82%) in ETEC isolates (Li *et al.,* 2017). A prospective study in Israeli military detected LT-only ETEC was the most common type found in 49% of isolates followed by ST-only and LT+ST –ETEC (Cohen *et al.,* 2010) . Among Jakarta residents 72 % ETEC isolates produced ST, 23% LT, and 5 % of ETEC strains produced both ST and LT toxins (Oyofo *et al.,* 2001).

1.14.1.2. Distribution of CFs and NCVFs

Till date more than 25 Classical colonization Factors (CFs) and more than 5 Non Classical Virulence Factors (NCVFs) have been identified. Among major CFA group CFA/I consist only one CF, CFA/II group comprises of CS1, CS2 and CS3 and CFA/IV group consists of CS4, CS4 and CS6. Among the minor CFs CFA/III and CFs from CS7 to CS30 belong. Although only 6-8 CFs and 2-3 NCVFs are commonly isolated from ETEC strains recovered around the globe. Among the major CFA groups CFA/IV found in approximately 20% strains followed by CFA/I present in 17% of strains. CFA/I group has been detected among 9% of ETEC isolates. But the pattern of detection of major CFs significantly varies with the variation of region and climate. THE GEMS study found 32% strains having CFA/IV while CFA/I and CFA/II were encoded by 21% and 14% strain respectively. The only significant CF found in LT-only isolates was CS6-alone, which was found in 16.8%. Only1.2% of the LT-only strains encoded CFA/I or CFA/II (Vidal *et al.,* 2019).

A study in Bangladesh in 1996-1998 involving diarrheal patients, found that 56% of the total samples were positive for the tested 12 CFs. Among which CS4 with CS5/CS6 was the prevalent one detected in31% of isolates. Next predominant CF was CFA/I (24%) CS1 was found in 20% of strains. Among less common CFs CS7, CS14, CS12, CS8 or CFA/III were detected(Qadri *et al.,* 2000). Flood hit Bangladesh in 2004, observed a spike in diarrheal illness. During analysis cholera and ETEC diarrhea was mostly detected. In that study surprisingly almost 80% of the diarrheal patients were found to have ETEC bacteria as one of the diaarhea causing organisms. The most prevalent CF was CFA/I, followed by strains producing CS4 + CS6 or CS5 + CS6, and some minor CFs (Qadri *et al.,* 2005). Between 2007 and 2012 in Bangladesh, almost 50% of diarrheal patients were infected with ETEC pathogen. Among those ETEC population both CS5 and CS6 was found in 18% strains followed by CFA/I detected in 14% strains. Interestingly a major CF CS6 was found to be co-present with minor CF CS17 in 10% of strains. Nine percent strains were CS14 expressing ETEC (Begum *et al.,* 2014). Further study detected that most common CFs were CFA/I, CS5+CS6 and CS7 (Begum *et al.,* 2016).

ETEC isolated from Indonesia detected that approximately 11% of strains expressed CFA/I, 8% expressed CS6, 4% expressed CS1/CS3, 2% expressed CS5 and 1% expressed CS5/CS6 (Subekti *et al.,* 2003). In Shenzhen, China 54% strains were found to harbour one or more CFs among which CS6 was the prevalent one followed by CS21 (Li *et al.,* 2017). In rural Egypt, CFA/I was the prevalent CF expressing in 10% of ETEC strains followed by CS6 (9%) , CS14 in 6% of strains (Shaheen *et al.,* 2003). Another study in Egyptian children revealed that almost 75% of the strains expressed CFs of either CFA/I, CS6 or CS5+CS6 (Shaheen *et al.,* 2009). During 2005-2009 ETEC strains isolated from US military personnel in Egypt, revealed that almost 68% isolates had at least one CF of which CS6 was the most commonly detected CF in 47% of strains. Mostly CS6 was detected alone or in combination with CS4 or CS8 (Nada *et al.,* 2013). During study of ETEC isolated from diarrheal patients in Tunisia, CFA/I and CS6 were the most common CFs, with 44.6% of isolates showing no connection with either CFA (Al-Gallas *et al.,* 2007). CS21 was the prevalent CF found in children of Chile among 70% strains positive for CFs. IN addition among NCVFs eatA was the predominant one found in 71% strains followed by etpA and etpB (Del Canto *et al.,* 2011). Most recent study on the ETEC isolates from 2008-2015 in Kolkata, India revealed that CFA/I was the prevalent CF (37%) followed by CS6 detected in 36% of strains among 56% positive for any CFs. Whereas 59% strains were positive for NCVFs of which eatA was the predominant one followed by etpA. Overall 29% strains were found to be devoid of any CFs or NCVFs (Bhakat *et al.,* 2019).

1.15. ETEC infection and malnutrition

Among the top disease causing enteropathogens ETEC remains the most common cause of diarrhea among children below 5 years of age. Studies have revealed that exposure to enteropethogens during early stages of life associated with poor growth in areas where access to health system is not that good. Prior studies have detected LT-ETEC associated malnutrition in children. Several studies indicated that poor growth in child attributed to ETEC infection. Also it has been detected that LT toxin that mark the presence of certain CFs which may mediate the associated malnutrition (Platts-Mills *et al.,* 2017). ETEC infection found to be linked with stunted growth, cognitive impairment and overall malnutrition effects in areas of low resource

availability. In developing nations where adequate nutrient could not be afforded in many during severe diarrheal episodes leads to observable decrease in productivity during adult stage (Qadri *et al.,* 2005).On the other hand children suffering with malnutrition suffer more and effects of ETEC diarrhea was found to be more severe than children without nutrient deficiency, a study in India found (Mathur *et al.,* 1985). Recent study showed that infected of human intestinal epithelial cells with ETEC causes a significant inhibition of uptake in Vitamin B1or thiamin. This inhibition is mediated by LT toxin and is associated with a decrease in the expression of intestinal thiamin transporters. This demonstrates the negative effect of ETEC infection on the overall bioavailability of the essential micronutrient thiamin to the host (Ghosal *et al.,* 2013) . GEMS study found that ST-ETEC exhibit a shift towards moderate to severe diarrhea in children suffering malnutrition (Tickell *et al.,* 2020).

1.16. Distribution of CS6 among ETEC isolates

Research around the globe detected CS21, CS6, and CFA/I as major CFs of ETEC pathogens. In Indian subcontinent CS6 is still the predominant CF detected (Mondal *et al.,* 2022). Furthermore earlier studies showed that almost 30% of all CFs detected as CS6 with or without other CFs (Wolf, 1997). Generally CS6 exists either alone or in combination with CS4 and/or CS5. Studies have found that CS5 is more common to be co-present with CS6 as compared to CS4. When ETEC infection among traveller and non-traveller population was studied it has been found that CS6 was present in 20% of travellers and 6% of Non-travellers whereas CS6 and CS5 co-expression was detected almost 6% in both traveller and non-traveller population (Isidean *et al.,* 2011).

1.16.1. Genetic assembly of CS6

ETEC CFs are the most important factor for ETEC adhesion to the intestinal epithelial cells. Among the 27 antigenically different CFs, CS6 still remain the predominant CF circulating in developing countries. DNA sequencing of CS6 operon revealed that CS6 operon is a stretch of 4219bp long DNA which consists of four ORF (Open reading frame) namely cssA, cssB, cssC, cssD. The ORF cssA and cssB are structural subunit while cssC and cssD are periplasmic chaperone and molecular usher (***Figure 1.12.***) (Wolf, 1997).

The oligomers of CS6 made by cssAB form a (CssA-CssB)n complex in a spherical shape in an 1:1 stoichiometry, and forms hetero polyadhesin. This structure supports multivalent attachment to the host receptors (Roy *et al.*, 2012) (**Figure 1.13.**).

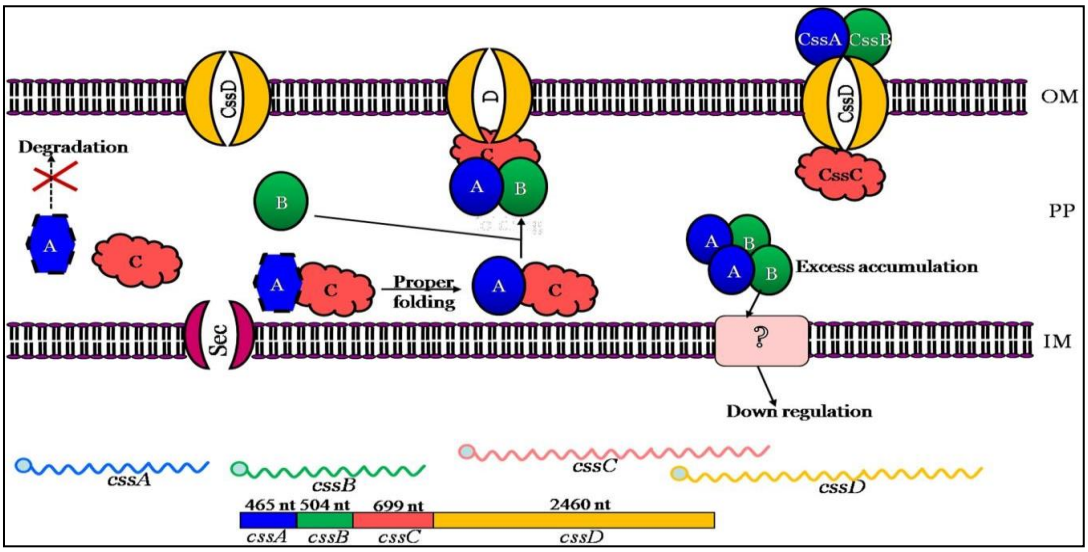


Figure 1.12. Schematic model of CS6 maturation. OM, outer membrane; PP, periplasm; IM, inner membrane; A, CssA; B, CssB; C, CssC; D, CssD; ?, unknown factors. ***Source:*** *Wajima et al., Microb Pathog, 2011.*

Different allelic variations rise due to several natural mutations in the structural genes, cssA and cssB. These allelic variants are reported as AI, AII, AIII, BI and BII producing 5 subtypes so far. Out if these subtype mainly AIBI and AIIIBI association is found in clinical ETEC isolates whereas, AIIBII subtype is found in asymptomatic ETEC isolates. When toxin related to subtype was investigated it was found that ETEC with ST alone or with LT harbours AIBI and AIIIBI subtype. ETEC with LT toxin found to harbour AIIBII subtype (Sabui *et al.*, 2012).

All four ORFs i.e;cssABCD include a signal sequence that uses "Sec" machinery to translocate protein across the inner membrane. The structural subunits cssA and cssB ,after synthesis translocated into the periplasm. If there cssA is not properly folded, rapidly get degraded by cssC. But stability of CssB doesn't depend on interaction with CssC (Tobias *et al.*, 2008).

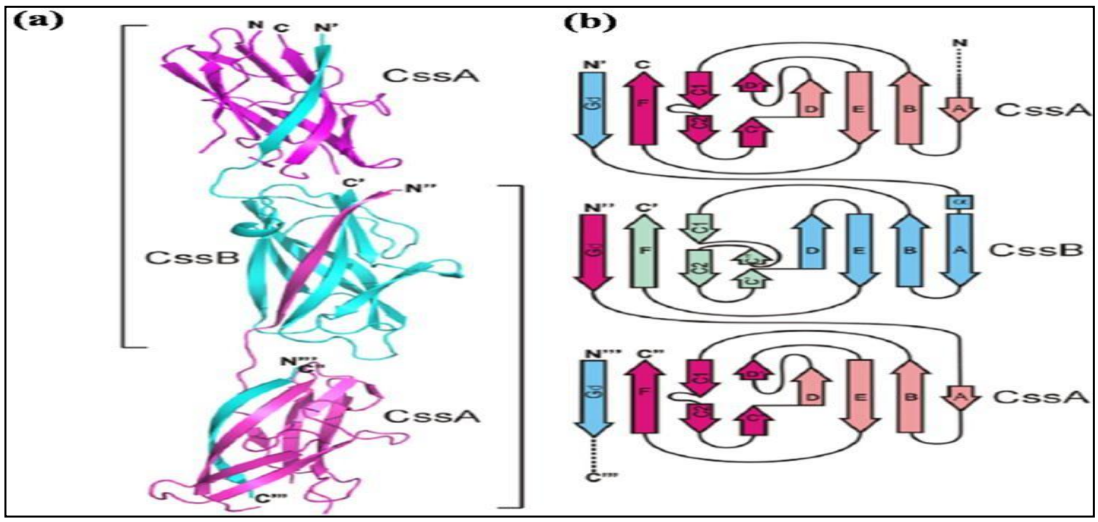


Figure 1.13. Model of the CS6 polymer. **(a)** 3D reconstruction of a CS6 polymer based on the crystal structures of the CssA and CssB subunits. CssA and CssB are shown in magenta and cyan respectively. Brackets indicate two distinct two-subunit repeats in the CS6 fibre. **(b)** Topology diagram of the CS6 fibre. Arrows indicate strands; the single a helix is shown as a rectangle. The strands in the same subunit are shown in slightly different colours to indicate two β-sheets of the β sandwich. ***Source:*** *Roy et al., Mol Microbiol, 2012.*

First cssA forms a complex with cssB and cssC. This trio complex cssA-cssB-cssC is recognised by cssD and finally cssA-cssB complex is transported across the outer membrane to the cell surface while cssC-cssD cannot pass through (Wajima *et al.*, 2011).

1.17. Regulatory factors of ETEC

The host-pathogen interaction is mainly reducible to two outcomes, first where the host doesn't suffer damage and secondly the conditions where host suffer damage in terms of tissue damage, homeostasis imbalance any many others. This interaction mainly depends on the strategy of pathogen to survive within host microenvironment. When gastrointestinal pathogens infect a host their first aim remains to colonize and sustain. To colonize pathogens devise different mechanisms which evolve with exposure to different environments for long time. From the perspective of pathogen it is crucial that they ensure successful colonization upon entry to the host for survival.

ETEC colonizes the host intestinal epithelia by the virtue of colonization factors and thereby release enterotoxins and cause disease to the host. So for successful colonization ETEC bacteria must adapt to the host microenvironment and response

accordingly for optimum colonization. In this regard different host factors also try to maintain homeostasis, and different factors affect different CFs differently. Host factors pH, temperature, osmolality plays as important physiological factors in successful colonization of ETEC.

Difference in external osmolarity triggers changes in osmotic pressure causing swelling or plasmolysis. Studies have revealed that elevated osmolarity involve a bi-phasic response where level of osmoregulatory ions increase followed by dramatic increase in the cytoplasmic concentration of osmoprotective compounds (Brown, 1976). The most rapid response against the osmotic up-shock, both in Gram-positive and Gram-negative bacteria, is a stimulation of Sodium (Na+) or potassium (K+) uptake followed by secondary response through Glycine betaine synthesis (Sleator and Hill, 2002). These changes in bacteria trigger bacterial adaption to the changes environment. These osmoregulatory shift also effect expression of different virulence factors as well as affects bacterial growth. As ETEC infection progresses rehydration therapy is the most obvious strategy to fight infection. Glucose and salt solutions in oral rehydration therapy may have an effect on ETEC virulence gene regulatory mechanism.

Other than those physiological factors different compounds present in host body also affects expression of ETC virulence factors. Different small compounds like Short chain fatty acids (SCFAs)also affect bacterial virulence as reported by different studies (Sun and O'Riordan, 2013). Principally acetate, propionate, and butyrate are regarded as Short chain fatty acids (SCFA). These SCFAs are fermentation end products of the intestinal microbiota that exerts nutritional, regulatory, and immunomodulatory functions. In common eneteric pathogens SCFA act as a signal for virulence gene regulation (Zhang *et al.,* 2020).

Another potential compound in regulating virulence gene expression in bacteria is sodium bicarbonate ($NaHCO_3$). Studies have revealed that sodium bicarbonate present in high concentrations in upper small intestine, a suitable place for colonization of many bacteria. In *V. cholerae* bicarbonate induces virulence gene expression by inducing Tox-T activity (Abuaita *et al.,* 2009). In other gram negative bacteria also, certain change in bicarbonate concentration negatively affects growth and virulence (Dobay *et al.,* 2018).

1.18. Aspects of vaccine development against ETEC infection

Diarrheal illness still is the second leading cause of death for children below 5years of age. ETEC continues to be the leading cause of childhood diarrhea as well as traveller's diarrhea. Since its devastating effects harm childhood and also sometimes fatal possess a great concern for child health and world science community to find an effective vaccine. Still there are no such vaccines in market that can cover the broad spectrum of this disease. The concept of a successful vaccine first came from the observation where immunity has been built in cases of ETEC-reinfection (Steinsland *et al.,* 2003). ETEC being a toxin induced diarrheal illness; it requires effective vaccination to prevent severity. Effective vaccine could help mitigate diarrheal mortality as well as morbidity which in turn will minimize the spread of diarrheal illness.

Major obstruction in the way of an effective vaccine is the geographic region specific diversity of ETEC CFs. Though few common ETEC CFs are common in most case still that varies in combination in different geographic settings. Therefore a feasible approach may be region specific vaccination where vaccine based upon most prevalence virulence factor in that region (Sommerfelt *et al.,* 1996). Since ETEC could express more than 25 CFs and NCVFs and also different toxins in different strains it is difficult to find an optimal challenge model to unambiguously evaluate efficiency of vaccine candidates. In this regard a multivalent vaccine candidate including CFA/I and CS1-CS6 could give broad coverage in terms of efficacy as in most geographic areas almost 50-80% of ETEC strains harbour those factors. Besides this inclusion of toxoid antigens can induce anti-toxin immunity against ETEC LT and ST toxins. But with increase in number of factors to be included in a vaccine the change also increases to make such an effective vaccine. So there may be a way to categorise vaccine based on their precursor candidate and can be grouped accordingly to apply in different settings. Vaccines that are under consideration are grouped into different types, such as toxin-based, live attenuated, inactivated whole-cell, hybrid, and fimbrial antigen vaccines (***Table 1.2.***) (Walker *et al.,* 2007).

Among the toxins LT shows to be strongly immunogenic. A study with transdermal patch of LT showed elevated immune response against the toxin. Nontoxic B

component LTB or a mutant variant of LT is under the lens for a protective high efficacy multivalent toxoid-CF vaccine. In addition LTB expressed in corn and potatoes showed the ability to induce development of IgG against LT in human volunteers when ingested. A phase II study involving transdermal patch of LT revealed that it can reduce the onsets of traveller's diarrhea in Guatemela and Mexican travellers (Frech *et al.*, 2008). Another study conducted on dmLT, a double mutant variant of LT as oral vaccine showed to enhance mucosal IgA responses (Akhtar *et al.*, 2021) . On the other hand some experiments on ST mutants which was conducted directly onsite using mutagenesis showed promising result in developing an immunogenic ST-containing vaccine (Zegeye *et al.*, 2018).

Table 1.2. Different vaccine candidates and stages of their development

Sl.No.	Candidate vaccine	Developer	Progress statue
1	Inactivated tetravalent whole cell supplemented with LTB–CTB hybrid toxoid; may include dmLT adjuvant (ETVAX)	PATH, Sweden biotechnology laboratory	Phase II clinical
2	Live attenuated cells by genes aroC, omp, omp F with adjuvant dmLT (ACE527)	PATH	Phase II clinical
3	ZH9 attenuated typhoid vaccine expressing LT-ST toxoid (Typhetec)	Prokarium Co.	Preclinical
4	Second generation of attenuated Shigella vaccine expressing CF/CS antigens and LT toxoid	CVD Vaccine Development Center	Preclinical
5	Anti-binding factor subunit vaccine	NMRC	Phase II clinical
6	Anti-binding factor subunit vaccine and toxoid (MEFA)	Kansas University	Preclinical
7	Fusion LT-ST and conjugated LTB and ST	International Consortium of Intestinal Vaccine	Preclinical
8	dmLT	PATH	Phase II clinical

*Novel toxoids and antigens are being explored by a number of investigators from: Washington University in St. Louis; University of Maryland; University of Virginia; University of Bergen; South Dakota State University; Kansas State University; the Sanger Institute; Johns Hopkins Bloomberg School of Public Health; and Antigen Discovery, Inc.

Source: *(Mirhoseini et al., 2018), A.L. Bourgeois et al. Vaccine 2016*

Several studies indicated that live attenuated or inactivated whole-cell vaccines may be viable to apply if safety issues can be mitigated. A study conducted by Clayton Harro from Johns Hopkins University, USA showed that a new multivalent ETEC vaccine (ACE527) have completed Phase IIb immunization challenge stage (Harro *et al.*, 2011). Inactivated whole cell vaccine also showed progress in recent studies. Study in Jan Holmgren, Goteborg University, Göteborg, Sweden observed that an inactivated whole cell ETEC vaccine consisting 4 *E. coli* strains with over-expressing CFA/I, CS3, CS5 and CS6 along with a LTB-CTB hybrid toxoid can induce strong intestinal and serum antibody response in a murine model (Svennerholm and Glenn, 2016). Also, a prototype conserved ETEC tip-adhesin (FTA)-based vaccine, delivered by transcutaneous immunization and intradermal routes was well tolerated and induced impressive levels of anti-tip adhesin ELISA and functional antibodies (Stephen Savarino, Naval Medical Research Centre, USA). Recent nonhuman primate studies demonstrated that intradermal immunization with FTA and mLT induced significant protection (84%) against challenge with a fully virulent ETEC strain.

Additional ETEC proteins may have vaccine potential, these novel proteins are categorized as ETEC-specific plasmid-based on EtpA and EatA and Chromosomal-based which may include Yghj, EaeH and Ag (Fleckenstein *et al.*, 2014).

Recent progress in vaccine trials on animal model and human volunteer challenge model has accelerated the course of producing an effective vaccine to a significant level. Besides these well designed field trials with a large sample size should help in development of a safe and effective vaccine with broader coverage.

CHAPTER 2

Objectives

2.1. From literature review we understood that ETEC virulence factors are highly diverse based on region. This led us to study further about the distribution of different virulence factors in this region and their regulation during pathogenesis. This thesis work is aimed to better understand region specific distribution of Classical and Non-Classical virulence factors in this region. Also we focused on response of archived ETEC strains against different classes of antibiotics. Further insight into the study of prevalent virulence factors has also been done in respect to their regulation by different regulatory factors that might affect their expression. This led to define our objectives as…..

Objective I: Understanding the distribution of common virulence factors in enterotoxigenic *Escherichia coli* using molecular methods

Objective II: Interpreting the antimicrobial resistance pattern and molecular characterization of enterotoxigenic *Escherichia coli*

Objective III: Unraveling the effect of regulatory factors on prevalent virulence factors of enterotoxigenic *Escherichia coli*

CHAPTER 3

Materials & Methods

3.1. Bacterial strains

Bacterial strains used in this study were isolated from stool samples collected from diarrhea patients admitted at the Infectious Diseases and Beliaghata General Hospital (ID & BG Hospital, Kolkata) and Dr. B.C. Roy Post Graduate Institute of Pediatric Sciences during the period of 2015-2019 under the hospital based surveillance project of the ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED). Well characterized clinical ETEC isolate IDH00469 and IDH04266 harbouring LT and ST genes were used as wild type strains whichever appropriate and when required. All other ETEC strains used in this study were collected from archive of Bacteriology Department as and when required. Laboratory strains E. coli BL21 (DE3) (Promega) and Top10 (Invitrogen) were used as control JM109 (Promega, USA), T7 shuffle were used as required.

3.2. Culture media and additives

For ETEC isolation stool samples were plated onto MacConkey agar (SRL, India) plates and incubated for 16-18 hours at 37°C. Colonies fermenting lactose on MacConkey agar plate having round and pink morphology were identified as ETEC. These strains were further plated on Luria Bertini (LB) agar (Sigma, Aldrich) plates. Culture from this non-selective medium was grown in LB and finally stored in 15% glycerol at -80°C for further use. The strains were grown routinely on broth and/or agar (BD Difco, USA) plates of LB or M9 minimal media (BD Difco, USA), at 37°C. Routinely 1.8% agar was used. All media were prepared according to manufacturer's protocol. Generally all culturing of bacteria was performed at 37°C. Modifications were made as and when required for experiments. Minimal medium, M9, was supplemented with 0.2% glucose as the primary carbon source. Media was prepared by adding appropriate concentrations of powder (W/V) in DNA/RNA-free water followed by autoclave or filter sterilization (0.2 μm).

Sodium Chloride (NaCl) (Merck), Sodium Acetate (CH3COONa) (Merck), Sodium Propionate (CH3CH2COONa) (Sigma), Sodium Butyrate (CH3CH2CH2COONa) (Sigma) and Sodium Carbonate (NaHCO3) (Merck) were used as medium additives. Bile component taurocholic acid sodium salt hydrate (TCA), sodium cholate hydrate (NaCH), sodium chenodeoxycholate (NaCDC), and sodium deoxycholate (NaDC)

(Sigma) was used as required. Additives were supplemented in M9, LB and CFA broths in different concentrations. Media with no additive added was used as control.

3.3. Extraction of DNA templates

3.3.1. Boil Lysis Method

Few colonies of ETEC from agar plate were taken in Tris-EDTA buffer (pH-6.8) and boiled for 10 minutes followed by immediate cooling in ice. Then the mix was centrifuged and an aliquot of the supernatant was used as DNA template for PCR.

3.3.2. Phenol-chloroform extraction method

This method was adopted from "Molecular cloning. A laboratory manual by T Maniatis, E F Fritsch and J Sambrook" (Wood, 1983). Overnight grown ETEC culture was pelleted down by centrifugation for 10 minutes at 10000 ×g. The cell pellet was resuspended in lysis buffer and incubated for 1 hour at 37°C. Then equal volume of phenol/chloroform (1:1) was added and mixed by inverting the tube until the phases are completely mixed. The lysate was centrifuged at 16,000 ×g for 15 minutes and the upper aqueous phase was transferred to a new tube. To precipitate the DNA, 3 volume of cold 100% ethanol was added and mix gently and the tube was kept at -20˚C for 30 minutes and centrifuged down at 16,000 ×g for 15 minutes at 4°C. The supernatant was discarded and the DNA pellet was rinsed with 70% ethanol. After centrifugation the supernatant was discarded and the DNA pellet was air dried. The DNA was resuspended DNA in TE buffer.

3.3.3. CTAB Protocol

Bacterial whole genomic DNA was isolated by using CTAB method as described by William *et al.,*(William *et al.,* 2012). Briefly, late log phase or early stationary cells were pellet down at 10,000 ×g for 10 minutes and the supernatant was discarded. The pellet was resuspended in TE buffer. 10% SDS and Proteinase K (10mg/ml) were added and incubated for 1-3 hours at 60°C. After the cells were lysed (as seen by cleared solution with increased viscosity) 5 M NaCl was added. CTAB (heated to 65°C) was added @ 100µl per 1 ml culture and incubated at 65°C for 1 hour with occasional shaking. Chloroform: Isoamyl alcohol (24:1) @ 500 µl per 1 ml was added and mixed for 20 minutes on ice. Centrifuged at 16,000 ×g for 10 minutes at room temperature and the aqueous phase was transferred to clean microcentrifuge tube to which phenol:chloroform:isoamyl alcohol (25:24:1) @ 500 µl per 1 ml was added and mixed well. Centrifugation at 16,000 ×g for 10 min at room temperature.

To the aqueous phase 0.6 volume isopropanol (-20°C) was added and incubate at -20°C for 2 hours. Centrifugation at 10000 ×g for 15 minutes at 4°C. The pellet was washed with cold 70% ethanol. Centrifugation at 10000 ×g for 5 minutes at 4°C. The supernatant was discarded and the pellet was air dried at room temperature. Then the pellet was resuspended in DNase free water. Then RNase I @ 10U/µl was added and m jnincubated at 37°C for 1 hour. The enzyme was heat inactivate at 70°C for 15 minutes followed by placing the tube in ice. The DNA was ethanol precipitated and finally the DNA was eluted in TE buffer and stored in –20˚C.

3.4. Phenol precipitation and quantification of DNA template

DNA and PCR products were ethanol precipitated. Briefly, the salt concentration of the DNA sample was adjusted by adding 1/10th volume of 3.2 M sodium acetate, pH 5.2 or an equal volume of 5 M ammonium acetate. Then, 2-2.5 volume 100% ice-cold ethanol was added and mixed well. The mixture was kept at -20˚C for 30 min and spun down at 16,000 ×g for 15 minutes at 4˚C. The supernatant was carefully separated and 70% ice-cold ethanol was added and mixed by vortexing. It was again centrifuged for 10 min at 16,000 ×g at 4˚C. The supernatant was discarded and the precipitated DNA was air dried. Finally the DNA was eluted in TE buffer.

- Estimation of DNA concentration

Concentration of DNA = [(A260× dilution factor× 50)/ 1000] µg ml^{-1}

Where, A260 is absorbance of the diluted DNA at 260 nm. For DNA, A260 of 1.0 corresponds to 50 µg ml^{-1} concentration.

3.5. Isolation of plasmid DNA

Plasmids were isolated using Wizard® plus Minipreps DNA purification system (Promega, USA). Briefly, 1-10 ml of overnight grown bacterial culture was pelleted by centrifugation at 1,400 ×g for 10 min. The bacterial pellet was dissolved in 400 µl of cell resuspension solution (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 µg ml^{-1} RNase A). Next, 400 µl of cell lysis solution (0.2 M NaOH; 1% SDS) was added and the cell suspension became clear. After addition of 400 µl of neutralization solution (1.32 M potassium acetate, pH 4.8) the lysate was centrifuged at 10,000 ×g for 15 min. The supernatant was mixed with 1 ml of resin and a vacuum of 15 inches of Hg was applied to pull the resin/lysate completely through the Minicolumn. The resin was then thoroughly washed with wash buffer (80 mM potassium acetate; 8.3 mM

TrisHCl, pH 7.5; 40 µM EDTA; 55% ethanol). Plasmid bound to the membrane was recovered with nuclease free water.

3.6. Primer Designing

The primers were produced by Integrated DNA Technologies, Inc. in the United States, and the sequences were analysed using the BLAST tool for degeneracy and cross-reactivity with other ETEC genes and CFAs. For multiplex PCR the primer sets were created using Tm values that were similar. The BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch) was followed. For molecular detection of virulence determinants, a total of 3 primer sets for toxin detection, 24 primer sets for colonization factors and 5 primer sets for Non-classical virulence determinants were used *(Table A.1.).* Using known strains, the specificity of the primer pairs was validated. To confirm the toxins, CFA/I, and all non-classical virulence factors amplicons, ETEC H10407 was employed. Strains used in previous research by Ghosal *et al.,* 2007 and Sabui *et al.,* 2010 were used for conformation of all other colonization factors (Ghosal *et al.,* 2007; Sabui *et al.,* 2012). During the confirmation of the primer sets, all amplicons were validated by sequencing on an automated DNA sequencer (ABI 3730 DNA Analyzer; Applied Biosystems)

3.7. Polymerase chain reaction

Polymerase chain reaction (Saiki *et al.,* 1985) throughout the study wherever required was done in 0.2 ml PCR tubes with the following reaction mixture as described in table 3.2. The reagents for PCR are from Promega, USA. Having confirmed the specificity of each primer set by single PCR, we performed multiplex PCR (Toma *et al.,* 2003) by combining different primer sets in different ratios and tested the control strains in several PCR cycling protocols (***Table 3.1.***). For detection of CFA genes Multiplex PCR was performed followed by confirmatory singleplex PCR. PCR was done by using thermal cycler (Eppendorf, Germany). The volume of primers and water for multiplex PCR were adjusted as per the numbers of primers used in each mixture.

Table 3.1. Composition of the reaction mixture of PCR

Components for PCR Mixture	Volume	Final concentration
5X Reaction Buffer	4 µl	1X
$MgCl_2$, 25 mM	2 µl	2.5µM
Deoxyneucleotide mix	0.8 µl	100µM
Taq Polymerase, 5U/µl	0.2 µl	0.05u/µl
Forward Primer, 10 µM	0.4 µl	0.25µl
Reverse Primer, 10 µM	0.4 µl	0.25 µl
DNA Template	2 µl	~10ng
Nuclease free Water	10.2 µl	
Final Volume	20 µl	

The melting temperature (Tm) of the primers was estimated by using the formula: Tm = 4 (G + C) + 2 (A + T). The final annealing temperature was set by checking the amplicons by gradient PCR. The PCR conditions were as follows:

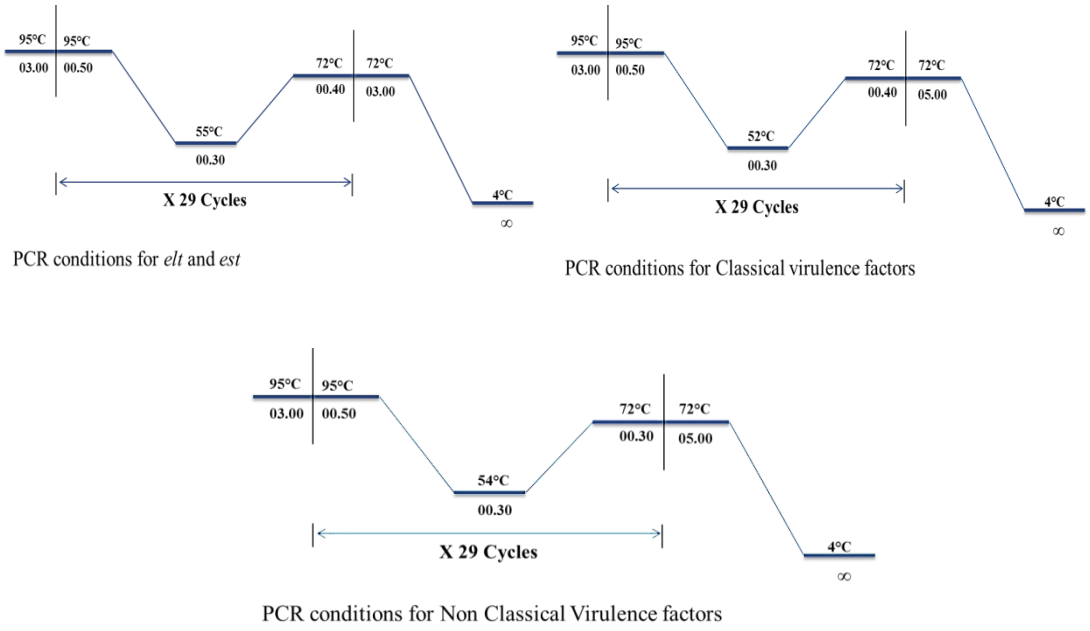


Figure 3.1. Schematic diagram of PCR conditions for *elt*, *est*, CFs and NCVFs

3.8. Agarose Gel Electrophoresis

To separate the PCR products according to their size agarose gel electrophoresis was employed. The size of the PCR products was assessed by comparing them to a DNA ladder (Thermo ScientificTM DNA Ladder) that ran alongside the PCR products on the gel. The amplified DNA fragments were resolved by 1.2% agarose gel electrophoresis. Briefly, agarose was heated to dissolve in 1X TAE. Temperature of the suspension was brought to approximately 55°C and poured in gel caster (AmpliSize; Bio-Rad Laboratories). Electrophoresis was performed and the amplified PCR products were visualized under UV transillumination after staining with ethidium bromide (EtBr). The images were captured using a gel documentation system (Biorad, USA).

3.9. Purification of PCR products

The PCR or gel-cut products were purified using Wizard SV Gel and PCR clean-up system (Promega, USA). Briefly, equal volume of membrane binding solution (4.5 M guanidine isothiocyanate; 0.5 M potassium acetate, pH 5.0) was mixed with PCR product. For gel-cut products membrane binding solution was added @ 1 µl mg^{-1} and heated at 65°C to dissolve the agarose gel. The mixture was poured into SV Minicolumn and incubated for 1 min at RT. Vacuum was applied (at least 15 inches of Hg) to pull the solution completely and the membrane was washed by adding 700 µl followed by 500 µl membrane wash solution (10 mM potassium acetate, pH 5.0; 80% ethanol; 16.7 µM EDTA, pH 8.0). To remove the residual amount of ethanol the Minicolumns were spin down at 16,000 ×g for 5 min. The membrane was incubated at RT with 30 µl nuclease free water for 1 min. The DNA was eluted by centrifugation at 16,000 ×g for 1 min in a fresh tube. The absorbance was measured at 260 nm to determine the concentration of the eluted product.

3.10. DNA sequencing

For DNA sequencing, at first, the desired gene or gene fragment was amplified by PCR and cloned in pGEM®-T Easy Vectors (Promega, USA). The cloning was done as per manufacturer's protocol. In short, ligation reaction was set and incubated for overnight at 4°C. Next day, the ligated product was transformed into JM109 High Efficiency Competent Cells and each transformation culture was plated onto

LB/ampicillin/IPTG/X-Gal plates. White colonies positive for the gene of interest was stored and used for plasmid isolation. The pUC/M13 Primers were used to sequence inserts cloned into the pGEM-T easy vectors. The Primer Sequences were Reverse (17mer): 5´-d(CAGGAAACAGCTATGAC)-3´ and Forward (24mer): 5´-d(CGCCAGGGTTTTCCCAGTCACGAC)-3´ from Promega, USA.

Specific primer of 1 µl of 3.2 pmole (either forward or reverse) was mixed with 2 µl of reaction mixture and 1 µl of 10X reaction buffer (Life technologies, NZ). The volume of water was depending on the template to make the final volume 10 µl. The amount of template (PCR purified) was adjusted depending on the amplicon size (***Table 3.2***). The sequencing PCR conditions were as shown in *Figure 3.2.*

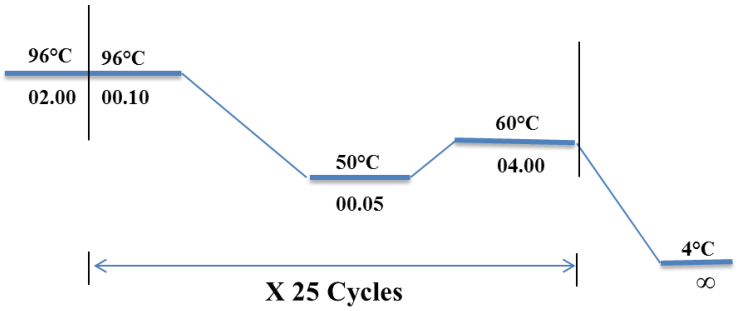


Figure 3.2. Schematic diagram of PCR conditions for sequencing

Table 3.2. Amount of DNA used for sequencing PCR

Template size	Amount to be added (ng)
100 – 200 bp	2 – 5
200 – 500 bp	5 – 10
500 – 1000 bp	10 – 20
1 – 2 kb	20 – 50
Greater than 2 kb	40 - 100

❖ Ethanol precipitation

After that the PCR products were ethanol precipitated. Briefly, the salt concentration of the DNA sample was adjusted by adding 1/10th volume of 3.2 M sodium acetate, pH 5.2 or an equal volume of 5 M ammonium acetate. Then, 2-2.5 volume 100% ice-cold ethanol was added and mixed well. The mixture was kept at -20°C for 30 min and spun down at 13,200 xg for 15 min under 4°C conditions. The supernatant

was carefully separated, 200 µl of 70% ice-cold ethanol was added and mixed by vortexing. It was again centrifuged for 10 min at 13,200 xg. The supernatant was discarded and the precipitated DNA was air dried. Before loading to automated ABI Prism 3200 DNA sequencer (Applied Biosystems, USA), the samples were snap-chilled in formamide to disrupt the secondary structures. The identities of the amplicons were confirmed by NCBI Blast program (http://www.ncbi.nlm.nih.gov/BLAST). Translation from genomic sequence to amino acid sequence was performed using Gene Runner Software. The CLUSTAL W program (https://www.genome.jp/tools-bin/clustalw) was used to alignment of the multiple sequences.

3.11. RNA purification

To isolate RNA, log phase bacterial cells were taken from broth culture and suspended in sterile PBS (pH 7.4). This suspension was washed twice with PBS and harvested by centrifugation at 8,000 ×g for 10 min at 4°C. The RNA was isolated using TRIzol reagent (Mono phasic solution of phenol and guanidine isothiocyanite). In brief, 500 µl of TRIzol was added to the pellet of 5 ml bacterial culture (1 OD at 600 nm) and mixed thoroughly. The suspension was then incubated at room temperature (RT) for 5 min. Chloroform was added @ 0.2 ml ml^{-1} TRIzol and was shaken vigorously for 15 sec, incubated at RT for 2-3 min. The suspension was then centrifuged at 12,000 ×g for 15 min at 4°C and the aqueous phase was transferred to a DNase/ RNase free microcentrifuge tube. Isopropanol was added @ 0.5 ml ml^{-1} TRIzol (Invitrogen, USA) and incubated for 10 min at RT. The RNA was precipitated by centrifugation at 12,000 ×g for 10 min at 4°C. The RNA pellet was washed with 500 µl of 75% ethanol (@ 1 ml ml^{-1} TRIzol), air dried and dissolved in nuclease free water. Next, 10 µg of RNA was treated with RNase-free DNase I (NEB, UK) following the supplier's protocol and stored at -80C until further use.

- Estimation of RNA concentration

Concentration of RNA = [(A260× dilution factor× 40)/ 1000] µg ml^{-1}

Where, A260 is absorbance of the diluted RNA at 260 nm. For RNA, A260 of 1.0 corresponds to 50 µg ml^{-1} concentration. Purity of RNA was determined from the

ratio of the absorbance at 260 and 280 nm. Ideally purest form of RNA has an A260/A280 ratio of 2.0.

3.11.1. DNase treatment

The Ambion® DNA-free™ DNase Treatment and Removal Reagents were used to remove contaminating DNA from RNA preparations. 0.1 volume of 10X DNase I Buffer and 1 µl rDNase I were added to the RNA sample and mixed gently. Then it was incubated at 37°C for 20–30 minutes. DNase Inactivation Reagent @ 0.1 volume was added and incubated for 2 minutes with occasional mixing. Then centrifuged at 10,000 ×g for 1.5 minutes and the RNA was transferred to a fresh tube. Conventional PCR for *parC* (a housekeeping gene) was used to confirm the removal of genomic DNA.

3.11.2. Reverse transcription (cDNA Synthesis)

The cDNA was prepared from 1 µg of RNA using Reverse Transcription (RT) System (Thermo Scientific Verso cDNA Kit) following the manufacture's protocol.

Table 3.3. cDNA Reaction mix preparation

Components	Volume	Final Concentration
5X cDNA synthesis buffer	4 µl	1X
dNTP Mix	2 µl	500 µM each
RNA Primer	1 µl	
RT Enhancer	1 µl	
Verso Enzyme Mix	1 µl	
Template (RNA)	1-5 µl	1 µg
Water, nuclease-free	To 20 µl	
Total volume	20 µl	

Briefly, RNA was heated at 70°C to denature the secondary structures and snap chilled. 1 µg of RNA was mixed with the reaction mixture ***(Table 3.3.)***. The reaction mixture of RT-PCR was incubated for 10 min at RT and then for 15 min at 42 °C.

Enzymes were heat inactivated by incubating for 5 min at 95°C and kept for at least 5 min at 4°C. The RT-PCR products were stored at -20°C until further use**.**

3.12. Expression study of Virulence genes

First, the RNA was isolated and RT-PCR was done to make their cDNA. Then qRT-PCR was performed to check the expression of the virulence genes using cDNA as templates. Gene specific forward and reverse primers were used for the same.

- ❖ Expression study of CS6, CS5 and EatA

Gene specific forward and reverse primers for the CS5 minor structural subunit gene, *csfD*; CS6 structural subunit gene, *cssB*; *eatA* passenger domain, and the *E. coli* housekeeping gene, *parC* was designed using PrimerQuest™ Tool of Integrated DNA Technologies, Inc., (IDT), USA and the primers were synthesized by IDT, USA (***Table A.2.***). The fluorescent dye SYBR Green was used for detection in ABI 7500 Real Time PCR system (Applied Biosystems). Fold increase or decrease was calculated by the comparative Ct method also referred to as the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). The house keeping gene, *parC* was used as internal control. Negative controls without reverse transcriptase were prepared in parallel with the cDNA from the same amount of RNA for all samples. The PCR conditions were used as follows:

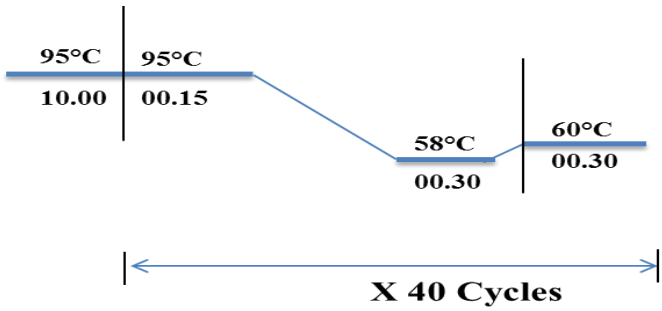


Figure 3.3.Schematic diagram of qRT-PCR conditions used

The real time RT-PCR assays were run in 20µl reactions using a maximum of 100ng cDNA (***Table 3.4.***).

Table 3.4. PCR Master Mix for cDNA preparation

Components	Volume	Final Concentration
2X SYBR® Green PCR Master Mix (Applied Biosystems)	10 µl	1X
Forward Primer	1 µl	100 nM
Reverse Primer	1 µl	100 nM
Template	Variable	100 ng
Water	To make 20 µl	
Total	20 µl	

3.13. Kirby-Bauer Disk Diffusion Susceptibility Test

Kirby-Bauer method was used for bacterial susceptibility towards antimicrobial agents. The antibiotic discs (Becton, Dickinson and Company, USA) used are as follows - Imipenem (Ipm)-10µg; Chloramphenicol (C) - 30µg; Cefapime (Fep) - 30µg; Doxycycline (D)- 30µg; Ceftriaxone (Cro)-30µg; Tetracycline (Te)-30µg; Norfloxacin (Nor)-10µg; Sulfamethoxalone W/Trim (Sxt); Ciprofloxacin (Cip)-5µg; Ampicillin (Am)-10µg; Azithromycin (Azm)-15µg; Nalidixic Acid (Na)-30µg; Erythromycine (E)-15µg; Streptomycine (S)-10µg;

The test was performed on the Mueller-Hinton (MH) agar plates following clinical and laboratory standards institute (CLSI) guidelines (Hsueh *et al.*, 2010). The isolates were inoculated in MH Broth and incubated at 37°C up to 0.5 McFarland standard turbidity. The culture was streaked thoroughly on to MH Agar plates using cotton swabs. After air-drying the plates, antibiotic discs were placed and after overnight incubation at 37°C, the zone of inhibition was measured as per manufacturer's protocol.

3.14. Competent cell preparation

A single colony was inoculated in LB broth and incubated at 37°C for overnight with shaking. Next day 100 µl of overnight grown culture was added to 10 ml of LB broth (1:100) and allowed to grow till the A600 reaches 0.4. The bacterial suspension was pelleted at 4,000 rpm for 15 min under 4°C conditions. The pellet was then dissolved in 100 mM ice-cold CaCl2 (filter sterilized) and incubated in ice for 45 min. The

suspension was again centrifuged at 4,000 rpm for 15 min at 4°C. Finally, after discarding the supernatant the pellet was dissolved in 1 ml ice-cold 85% 100 mM CaCl2 and 15% glycerol solution. 50 µl aliquots were preserved at -80°C for long term storage.

3.14.1. Ultra Competent E. coli Cells preparation

First SOB media was inoculated with *E. coli* cells and grown upto OD_{600} of 0.6-0.8 at 18˚C. After incubating in ice for 10 minutes the cells were centrifuged at 2500 x*g* for 10 minutes at 4˚C. The cells were resuspended in ice cold TB and inubated in ice for 10 minutes. The cells were centrifuged at 2500 x*g* for 10 minutes at 4˚C and the cells were dissolved in ice cold TB + 14% DMSO. After incubating in ice for 10 minutes the cells were dispensed in 50µl aliquots in pre-cooled1.5 mL micro-centrifuged tubes. The aliquots were stored at -80˚C (Sambrook and Russell, 2006).

3.14.2. Electrocompetent E. coli Cells preparation

Electrocompetent cells were prepared as per manufacturer's (NEB, USA) protocol. Fresh inoculum was given in SOB pre-warmed to 37°C from overnight culture and was grown at 37°C with shaking until the cultures reached an OD600 of 0.5-0.7. The cultures was placed on ice for 15 minutes and then centrifuged at 5000 xg for 10 minutes at 4°C. The cells were completely suspended in ice cold 10% glycerol while keeping the suspension on ice and again centrifuged. The pellet was again suspended in ice cold 10% glycerol. After centrifugation at 5000 xg for 10 minutes at 4°C, the pellet was dissolved in 1 ml ice cold 10% glycerol and 100µl was dispensed in cold microcentrifuge tubes on ice. Cells were used for electroporation or kept in -80°C freezer for storage.

3.15. Transformation

In 50 µl of competent cell suspension 5 µl (5 ng^{-1} µg) of plasmid DNA was added, mixed by swirling and incubated on ice for 30 min. Heat-pulse was given to the tubes in for 45 seconds at 42°C water bath and incubated on ice for 2 min. Pre-warmed 0.5 ml SOC broth (Invitrogen, USA) was added to the tube incubated for 1 h at 37°C with shaking at 225–250 xg. Finally, appropriate volume of transformation reactions were plated on LB agar plates with appropriate antibiotics and incubated overnight at 37°C for. Next day the colonies were screened by PCR to select the positive ones.

3.16. Construction of non-polar isogenic mutants and ETEC complement strains

Construction of isogenic non-polar gene-deletion mutants for genes CS5 and EatA were generated in ETEC wild-type strain, IDH00469 by the lambda Red recombination mutagenesis system (Datsenko and Wanner, 2000).The gene to be knocked-out is replaced with kanamycin resistance gene. The steps are:

Step1: Transformation of wild type ETEC strain IDH00469 with pKD46. The ETEC strain was made competent and transformed with pKD46 plasmid. After overnight growth on LB+ 100µg/mL Ampicillin plates at 30˚C, colonies were checked for pKD46 presence by PCR. Positive colonies were preserved at -80˚C.

Step 2: Substrate DNA designing and generation of fusion linear PCR product for transformation. First 500bp upstream and downstream sequence of the gene to be knockout was searched and primers were designed to get 2 linear fragments from upstream (fragment 1) and downstream (fragment 3) of the gene of interest (GOI) (Table A.3). Then primers were designed to get the kanamycin resistance gene (fragment 2) from pKD13 plasmid including the two FRT sites. Amplification of the 3 regions were done by Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific, USA) and fusion of the three fragments were created to get a single linear PCR fragment having sequence from upstream of GOI followed by Kanamycin resistance gene and sequence from downstream of GOI (fragment 1 fragment 2 fragment 3) (Shevchuk *et al.,* 2004). This fusion fragment was used for transformation. The steps for making the fusion of linear DNA fragments were (Figure 3.4):

a. Primers I and II were used to amplify fragments 1 and primers V and VI to amplify fragment 3 from ETEC genomic DNA. Using primers III and IV, the kanamycin resistance gene (fragment 2) was amplified from plasmid pKD13.
b. The fragments were gel-purified with extraction from agarose gel using Wizard® SV Gel and PCR Clean-Up System and their concentrations were recorded.
c. Triple reaction step A: No primer fusion. The PCR mix was 4 µl 5X Phusion™ HF Buffer; 0.4 µl 10 mMdNTP mix (Invitrogen); 100ng fragment 1; 100ng fragment 3; 20ng fragment 2; 0.2 µl Phusion™ High–Fidelity DNA Polymerase; water to make up the volume to 20µl. Cycling parameters: initial

denaturation 98°C 30 seconds, subsequent steps 98°C 10 seconds, annealing at 50°C 20 s, extension 72°C 1 minute, 10 cycles total, hold at 4°C.

d. Triple reaction step B. Primer fusion. The PCR mix was 4 µl 5X Phusion™ HF Buffer; 0.4 µl 10 mMdNTP mix (Invitrogen); 3µl of unpurified PCR product from step A; 0.2 µl primer I and 0.2 µl primer VI; 0.2 µl Phusion™ High–Fidelity DNA Polymerase; water to make up the volume to 20µl. Cycling parameters: initial denaturation 98°C 30 seconds, subsequent steps 98°C 10 seconds, annealing at 54°C 20 seconds, extension 72°C 1 minute 30 seconds, 30 cycles total, final additional extension 72°C 5 minutes, hold at 4°C.
e. The resulting fused PCR product was analyzed by electrophoresis in 1% agarose.

Step 3: Transformation of the PCR fused fragment by electroporation. The the ETEC strain IDH00469 (now maintaining pKD46) were grown at 30˚C in LB + Ampicillin up to $O.D_{600}$ 0.1and to it L-arabinose (SigmaAldrich, USA) at a final concentration of 10 mM was added. The cells were again incubated at 30˚C and continued to growth to OD_{600} = 0.4-0.5. The cells were then made electrocompetent and electroporated with linear PCR fragments generated in step 2 (Figure 3.5.).

Step 4: Removal of the kanamycin cassette. The kanamycin cassette was removed by FLP expression. The transformed colonies were transformed with pCP20 plasmid and grown at 30˚C. Positive colonies were screened by PCR and again incubated overnight at 37 ˚C. Colonies were screened by PCR for the successful removal of the kanamycin marker or alternatively the colonies was screened by streaking on two different LB agar plates, one conditioned with 30µg/ml kanamycin, and the other one without addition of antibiotics. Clones growing only on LB agar plates without kanamycin but no longer on kanamycin supplemented LB agar plates had successfully removed the selection marker by FLP recombination.

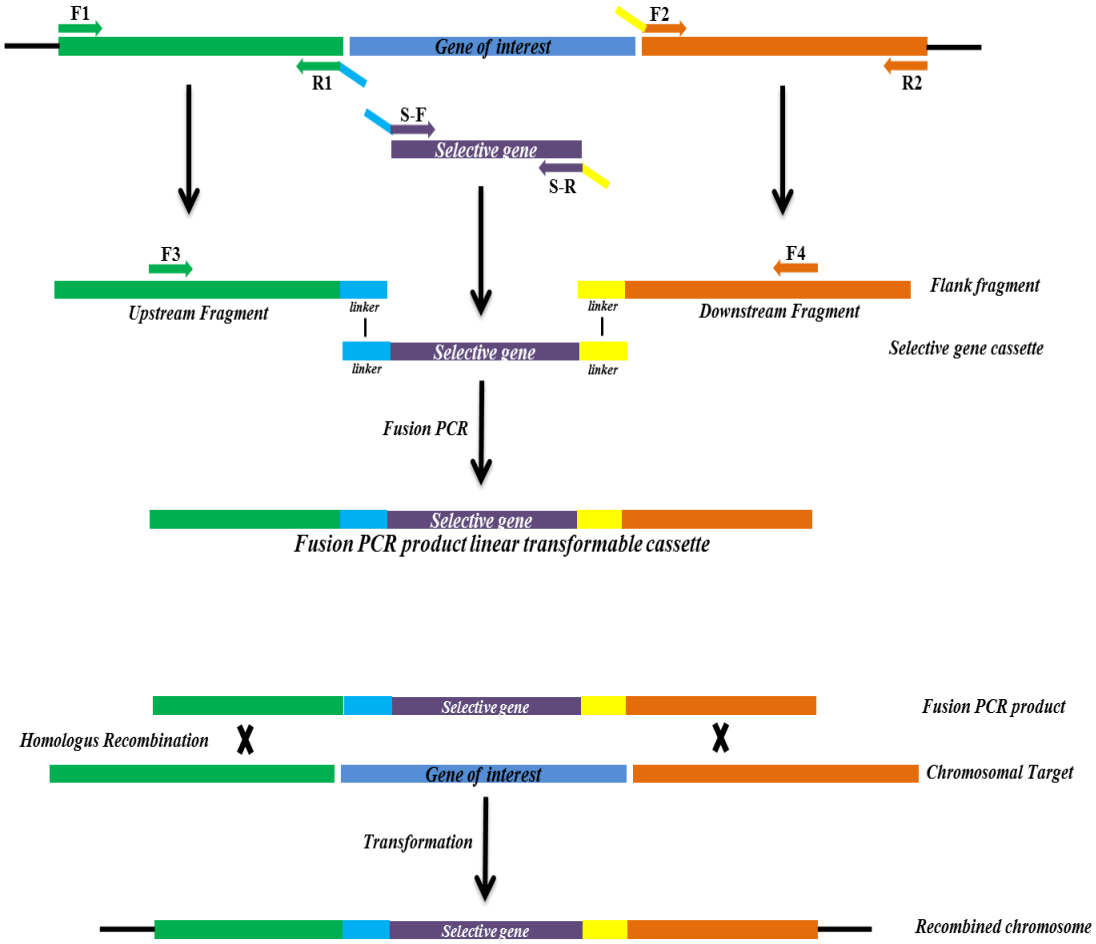


Figure 3.4. Schematic Diagram of triple fusion PCR followed by Transformation by elctroporation

3.16.1. Electroporation

The electroporation was done in BTX ECM 630 with BTX 1mm electroporation cuvettes. SOC media and LB-antibiotic plates were prewarmed at 37°C prior of starting the electroporation. The electrocompetent cells were thawed on ice for 10 minutes or freshly made cells were used. Sterile microcentrifuge tubes and electroporation cuvettes were placed on ice. To the cold microcentrifuge tubes, 50µl cells and DNA at varying concentrations were added and mixed by flickering the tubes. For control, pUC19 was added with Milli-Q water and followed for electroporation. The DNA-cell mixture was transferred to the cold cuvette and placed in the electroporation module. The electroporator was set at 1350 V, 5 ms pulse, resistance @ 600 Ohms and capacitance @ 10 µF. Immediately, after pulse, 950 µl SOC was added and transferred to a sterile culture tube. The tube was incubated at 37°C with moderate shaking. Aliquots of the transformed culture were

spreaded on LB plates supplemented with kanamycin 30 µg/mL and incubated overnight at 37°C. Next day colonies were screened for gene disruption by PCR and positive colonies were stocked and kept at -80°C.

3.16.2. Complementation of mutant

For complementation of the mutants pBAD-TOPO TA expression vector (Invitrogen) was used and manufacturer's protocol was followed. PCR primers (***Table A.4.***) were designed and the open reading frame of the gene was PCR amplified by using Platinum® Taq DNA Polymerase (Invitrogen) in a 20µL PCR reaction (***Table 3.6.***). The PCR fragment was cloned into pBAD-TOPO TA expression vector and the recombinant vector was transformed into One Shot® TOP10 *E. coli* competent cells. An Insert:Vector molar ratio of 3:1 was used in TOPO® Cloning reaction (***Table 3.5.***). The cloned vector was transformed into the mutant strain and the complemented strain was induced by 0.2% arabinose.

Table 3.5. pBAD-TOPO cloning mix

Reagent	Volume
Fresh PCR product	0.5 to 4 µl
Salt solution	1 µl
Sterile water	Add to a final volume of 5 µl
TOPO vector	1 µl
Final volume	6 µl

Table 3.6. Phusion PCR mix for linear fusion PCR fragments generation

Components for PCR Mixture	Volume
5X Phusion™ HF Buffer	4 µl
10 mM dNTPs	0.4 µl
Forward Primer	0.2 µl
Reverse Primer	0.4 µl
Phusion™ High–Fidelity DNA Polymerase	0.2 µl
DNA Template	2 µl
Nuclease free Water	To make up the volume
Final Volume	20 µl

3.17. Poly acrylamide gel electrophoresis

3.17.1. Protein estimation by modified Lowry method

The concentration of the protein sample was measured by modified Lowry method (Lowry, 1951).The following three types of reagents were used for the estimation method:

Reagent A: 2% Na2CO3 in 0.1 M NaOH containing 0.16% Na-K tartrate + 0.1% SDS

Reagent B: 4% CuSO4. 5H2O

Reagent C: 100:1= A:B (100 ml + 1ml)

At room temperature 200 µl of the protein sample was incubated with 600 µl of reagent C, at RT for 15 min. Then 1 N Folin reagent was added to the mixture and re-incubated at 37ºC for 30 min. the OD was measured at 660 nm. The concentration of the protein was estimated from the equation; concentration = OD/ 0.0026, which was obtained from a standard curve of BSA.

3.17.2. SDS-PAGE

The purity of the protein samples was determined by SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) (Raymond and Weintraub, 1959). The gel percentage was determined based on the protein sample size range and the appropriate separating/resolving gel containing the desired concentration of acrylamide ***(Table 3.7.).***The sample was heated with loading dye for 5 min at 100 °C before loading and the run was performed at 110 V in a gel running system (ATTO, Japan). After electrophoresis, the gel can be stained with Coomassie Brilliant Blue R-250 to see the separated proteins, or it can be processed further for western blotting. The proteins emerged as discrete bands within the gel after staining.

Table 3.7. Composition of SDS-PAGE gel

Reagents	Resolving Gel (pH 8.8)						Stacking Gel (pH 8.8)
	5%	7.5%	10%	12.5%	15%	20%	
Solution A (ml)	1.7	2.5	3.3	4.2	0.45	5	0.45
Solution B (ml)	2.5	2.5	2.5	2.5	2.5	2.5	-
Solution C (ml)	-	-	-	-	-	-	0.75
H_2O (ml)	5.8	5	4.2	3.33	2.5	0.8	1.8
APS (µl)	23	23	23	23	23	23	10
TEMED (µl)	5.5	5.5	5.5	5.5	5.5	5.5	7

3.17.3. Coomassie staining

Following the SDS-PAGE, the gel was first fixed in 50% methanol (MeOH) and 5% glacial acetic acid (AcOH) for 30 min and the gel was then incubated with staining solution (50% MeOH, 50% AcOH and 0.1% coomassie blue) at RT for 1 h. To visualize the protein bands, the gel was destained with 5% MeOH and 7.5% AcOH (Meyer and Lamberts, 1965).

3.18. Western Blotting

For western blotting, the electrophoretic transfer of proteins from SDS-PAGE to nitrocellulose or PVDF membrane was done. The membrane, gel, and filter pads were incubated in transfer buffer (48 mM Tris; 39 mM Glycine; 0.05 percent SDS; 20 percent MeOH) for 30 minutes at room temperature to transfer the proteins from polyacrylamide gel to nitrocellulose membrane, and then the transfer cassette was assembled for semi-dry transfer as shown ***(Figure 3.5.)*.** The run lasted 1 hour and was carried out at 200 mA. To block non-specific sites, the membrane was incubated in blocking buffer overnight at 4 °C after the transfer. The membrane was cleaned three times with TBS-T the next day and the membrane was dipped in blocking buffer in which the primary antibody with the appropriate dilution was added and was incubated for 2 hours at room temperature. After washing thrice, the secondary antibody was added to TBS-T that had been labelled with alkaline phosphatase (AP) or horseradish peroxidase (HRP). NBT (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl phoaphate) were used as substrates for AP conjugated antibody, and

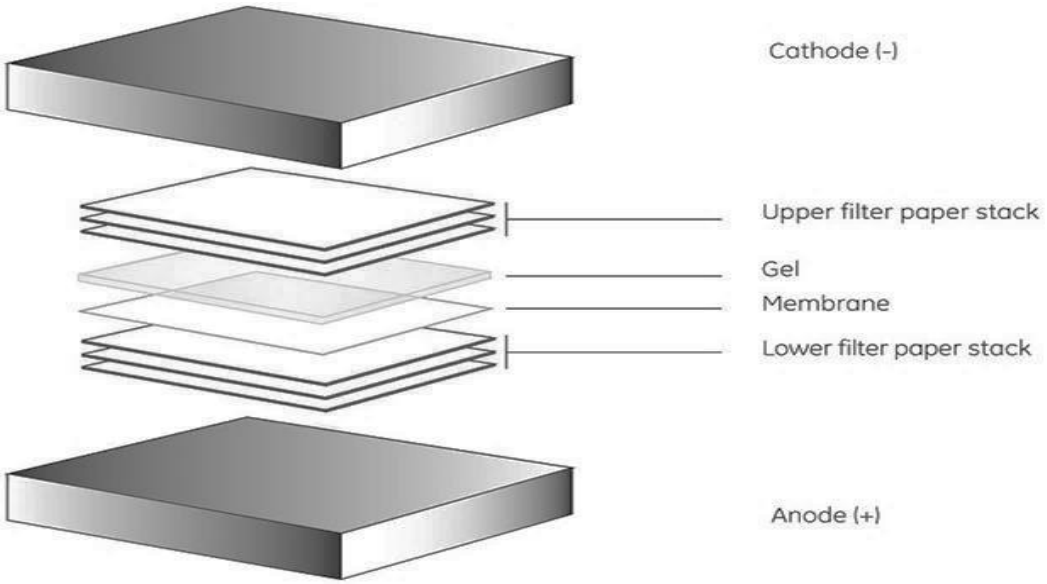


Figure 3.5. Construction of a gel-membrane sandwich in a semidry transfer setup is similar to wet transfer. All components are soaked in transfer buffer and placed in direct contact with the electrodes, compressing the sandwich.

luminal and peroxide were employed as substrates for HRP conjugated antibody. For alkaline phosphate, NBT solution (7.5 mg NBT dissolved in 175 l DMF, dimethylformamide, and 75 l water) and BCIP (3.75 mg BCIP dissolved in 250 l DMF) were freshly mixed with 24.5 ml bicarbonate buffer (210 mg Na2CO3 and 5.07 mg MgCl2, pH 9.8 in 25 ml water) and incubated with the membrane in the dark until the desired bands appeared. HRP conjugated antibody detection was done by chemiluminescence which was recorded by chemi doc imaging system, Biorad, USA.

3.19. β-galactosidase Reporter Assay

Beta-Galactosidase Assay (also known as the "Miller Assay") (Miller and Lee, 1984)was used to determine the promoter region of the bacteria by detecting their LacZ activity. Transformed ETEC strains were incubated in LB overnight at 37˚C and next day, bacteria were transferred to fresh M9 media with desired concentrations of additives and were grown to mid-log phase.

- Preparation of Cells

The cells were incubated on ice for 20 minutes to stop their growth. The cells were pelleted down by centrifuging for 10 minutes at 5000 x*g* at 4˚C. The cells were resuspended in same volume of chilled Z buffer and the $O.D_{600}$ of the suspension was measured (blank against Z buffer). The cells were diluted in Z buffer with 0.5

mL cells + 0.5 mL Z buffer to a final volume of 1 ml. The cells were permeabilized by adding 100µl chloroform and 50µl 0.1% SDS (w/v) and equilibrated in a 28˚C waterbath for 5 minutes.

❖ Assay

The reaction was started by adding 200µl o-nitrophenyl-β-D-galactoside (ONPG) and mixed well by vortexing. The time of addition was recorded. The cells were incubated at 28˚C for sufficient yellow colour to be developed. After that the reaction was stopped by adding 500µl of 1M Na_2CO_3. The time of addition was recorded. The suspension was centrifuged at 16000 x*g* for 5 minutes to remove debris and chloroform and O.D was measured at 420 nm and at 550 nm.

❖ Calculation of units of activity

The β-galactosidase activity was expressed as Miller units.

Miller Units = 1000 x $[(OD_{420} - 1.75 \times OD_{550})] / (T \times V \times OD_{600})$

OD_{420} and OD_{550} are read from the reaction mixture.

OD_{600} reflects cell density in the washed cell suspension.

T = time of the reaction in minutes.

V = volume of culture used in the assay in ml.

The units give the change in A_{420}/min/mL of cells/OD_{600}.

3.20. Cloning PCR Products with pGEM®-T Easy Vectors

The pGEM®-T Easy Vector Systems (Promega, USA) protocol was followed for cloning of PCR products. Briefly the ligation reaction was set up and mixed by pipetting (Table 3.8.).The reaction was set for 1 hour at RT or the reaction was incubated overnight at 4°C.

Table 3.8.Reaction set up for cloning in pGEM®-T Easy Vector Systems

Reagents	Standard Reaction	Positive Control	Background Control
2X Ligation Buffer	5µl	5µl	5µl
pGEM®-T Easy Vector	1µl	1µl	1µl
PCR Product	Xµl	-	-
Control Insert DNA	-	2µl	-
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
Deionized water to a final volume of	10µl	10µl	10µl

3.21. Protein purification

3.21.1. Purification of CS6 and CS5 from ETEC isolates

CS6 and CS5 were purified from the wild type ETEC strain by heat saline extraction method. For CS6 isolation (Ghosal *et al.,* 2009), bacteria were cultured in CFA broth overnight at 37 °C and suspended in 0.85% NaCl. The suspensions were then centrifuged at 8,000 *xg* for 20 minutes after being incubated at 60°C for 20 minutes with moderate shaking at 20 rpm. The heat saline extract was then precipitated with ammonium sulphate. It was first precipitated with 35% ammonium sulphate to remove impurities, and then precipitated with 60% ammonium sulphate saturation.

For CS5 isolation (Heuzenroeder *et al.,* 1989), bacteria were cultured in CFA broth overnight at 37°C and suspended in phosphate buffer, pH 7.5. The suspension was subjected to heat shock at 56°C for 20 min. This cell suspension was then centrifuged (3000 x*g* for 10 min) to remove cells. The supernatant was then precipitated with 5% ammonium sulphate.

After centrifugation the pellet of both CS6 and CS5 were dissolved in buffer A (20 mM Tris-HCl, pH 6.8).The protein sample was dialyzed in the same buffer overnight and placed onto a 3-ml Q-Sepharose FF column (GE Healthcare, Sweden) that had been pre-equilibrated in buffer A. A linear gradient of buffer B (20mM Tris-HCl and 1M NaCl, pH 6.8) was used to elute the protein attached to the anion exchange matrix at a flow rate of 1 ml min^{-1}. The fractions containing the desired proteins were combined and dialyzed in buffer A at 4°C overnight.Step elution in buffer B concentrated the dialyzed material on a 1ml UNO Q1column (Bio-Rad, USA). Peak fractions were fed at a flow rate of 0.5 ml min^{-1} onto a Superdex 200 (GE Healthcare, Sweden) gel filtration column that had been previously equilibrated with 0.15 M NaCl in buffer A. The UNO Q1 column was used to concentrate the protein-containing pooled fractions once again.

These proteins were subsequently used for antiserum production.

3.21.2. Purification of EatA from ETEC isolates

The passenger domain of EatA was purified by cloning. Using primers a 1,483-bp region of the *eatA* gene encoding a portion of the passenger domain was amplified by PCR (Table A.6.). The resulting amplicon was cloned into pET-22b, using the appropriate sites to create an expression plasmid, of pET-22b + *eatA* that encodes a

six-histidine tag-*eatA* fusion protein. To ensure that the insert of *eatA* was in-frame with the polyhistidine tag DNA sequencing of pET-22b + *eatA* was performed. The recombinant plasmid encoding a His-protein was over-expressed in BL21λDE3 cells by 0.5mM IPTG induction for 16 hours at 20°C and purified by nickel affinity chromatography. The affinity bead used was Ni-NTA Agarose and it was equilibrated in equilibration buffer (25mM Tris, 100mM- NaCl, 7M Urea, pH 7.5). The protein was eluted in elusion buffer (25mM Tris, 100mM NaCl, 7M Urea, 250 mM-Immidazole, pH 7.5) and stored in the same buffer. The eluted recombinant EatA fragment was used for generation of polyclonal antisera.

3.22. Antiserum preparation

Samples of both *cssB* and *csfD* were run on SDS–15% PAGE gels, and bands corresponding to these proteins were excised from the gel and were each emulsified with 0.5 ml of 1X phosphate-buffered saline (PBS). At day 0, each mouse was immunized intraperitoneally. Periodic boosters were given for 3 weeks. Each mouse was exsanguinated by cardiac puncture, and the serum obtained was stored at -20°C in the presence of 0.02% sodium azide. The specificities of the antiserum were examined by Western blot analysis. For *eatA* antisera generation, first the r*eatA* protein was run on 12.5% SDS-PAGE to check for purity and was used to immunize New Zealand White rabbits. Periodic boosters were given for 4 weeks with Freund's Incomplete Adjuvant. Final collection of Anti-Sera was done one week after 4th Booster dosage of immunogenic injections and the serum obtained was stored at -20°C in the presence of 0.02% sodium azide. Polyclonal rabbit antisera were then examined by western blotting.

3.23. Enzyme-linked immunosorbent assay (ELISA)

3.23.1. GM1-ELISA for LT

Ganglioside-GM1-ELISA was performed to detect LT (Ristaino *et al.*, 1983). ELISA microtiter (NUNC, USA) wells were coated with GM1 (0.5µg/ml in 0.06M sodium carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4˚C. Wells were washed thrice with PBS-T and blocked with blocking buffer for 2 hours. After washing with PBS-T, 100µl of diluted primary antibody was added to each well and incubated for 2 hours at room temperature. The wells were again washed thrice with PBS-T and 100µl of HRP-conjugated secondary antibody (Sigma-Aldrich, USA), diluted at the optimal concentration in blocking buffer was added to the wells and

incubated for 1-2 hours at room temperature. After washing TMB was added and incubated for optimum colour development at RT. Stopping solution was added and O.D was measured at 450nm. Standard curve was generated using 2 fold serial dilution of purified LT and this curve was used to get unknown LT concentration.

3.23.2. Indirect ELISA

For the evaluation of ST production, microplates (NUNC, USA) were coated with supernatant of the desired strain after required growth time diluted in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6 at 4 °C for overnight. At each step, plates were washed with PBS-T. Plates were then blocked with blocking buffer for 2 hours. 100µl of diluted primary antibody was added to each well and incubated for 2 hours at room temperature. Antigen-antibody reaction was detected by addition of HRP-conjugated secondary antibody (Sigma-Aldrich, USA) for 1 hour followed by addition of TMB. O.D at 450nm was measured after stopping the reaction after colour development (Rocha *et al.,* 2013).

3.23.3. Quantification of CS6, CS5 and EatA by indirect ELISA

Protein expression of genes was detected by indirect ELISA and protein was coated which was detected by their respective anti- polyclonal antibody. Briefly antigen was coated to microplate (Nunc-Immuno™ MicroWell™ 96 well solid plates, Sigma Aldrich, USA) by mixing the protein with coating buffer @ 1:1. The plates were incubated for 2 hours at RT or at 4°C overnight. After the incubation time the coating buffer was removed and the plates were washed thrice by PBS. The coated wells were blocked by adding 300µl blocking buffer and incubated for 2 hours at RT or at 4°C overnight. After incubation the wells were washed three times with PBS-T. Following that 100 µl of diluted primary antibody was added to each well and incubated for 2 hours at room temperature. The wells were again washed thrice with PBS-T. 100 µl of HRP-conjugated anti-rabbit IgG secondary antibody or HRP-conjugated anti-mice IgG secondary antibody (Sigma-Aldrich, USA), diluted at the optimal concentration (according to the manufacturer) in blocking buffer was added to the wells and incubated for 1-2 hrs at room temperature. The wells were washed thrice with PBS-T. For detection, 3, 3′, 5, 5′- tetramethylbenzidine (TMB) (BD OptEIA, USA) was used as a substrate and incubated for 15-30 minutes for optimum colour development. Equal volume of stopping solution was added and the optical density was measured at 450 nm by an ELISA reader (Bio-Rad, USA).

3.23.4. Whole-bacterial cell ELISA to quantify CS6 surface expression

The phenotypic expression of CS6 on the bacterial surface was quantified by ELISA assays as per Elder *et al.,* 1982 (Elder *et al.,* 1982). A 10^7 c.f.u. mid-log phase bacterial suspension was incubated with 0.15% SDS, washed with ethanol, and heated for 5 minutes at 100°C. After being processed, the bacterial pellet was diluted in 100µl carbonate buffer (pH 8.2) and used for coating ELISA plate to measure total CS6. To quantitate the surface expression, 100 µlof processed ETEC was added to the wells used for coating and incubated overnight at 4°C. Unbound bacteria were decanted and wells were washed thrice with PBS followed by blocking in 5% (w/v) non-fat skimmed milk in PBS. After washing, the bound CS6 was determined by anti-*cssB* polyclonal antibody as the primary antibody followed by HRP-conjugated secondary antibody. Using a standard curve obtained with purified CS6 the amount of protein was determined.

3.24. Scanning Electron Microscopy

Samples were primarily fixed with 3% cacodylate buffered glutaraldehyde for overnight followed by dehydration with ascending grades of ethanol (50% to 100%). Then samples were transferred from 100% ethanol into a 1:2 solution of Hexamethyldisilazane (HMDS): 100 % ethanol for 20 minutes. Next the samples were transferred to a fresh solution of 2:1 solution of Hexamethyldisilazane (HMDS):100%ethanol for 20 minutes. After that the samples were transferred into 100% HMDS for 20 minutes and this step is repeated. Finally the samples in 100%HMDS solution were kept overnight in a fumehood for drying with the lid loosely covered. Samples were mounted on stubs and transferred to a sputter coater and sputter coated with gold at 1.2 kV. Finally the samples are examined and imaging done in the SEM.

3.25. Tissue Culture

The HT-29 cell lines were cultured in Corning T-25 flasks using Dulbucco's Modified Eagle's medium (DMEM) at 37°C with 5% CO2 using a HERAcell 150 humidified incubator (Thermo Scientific, USA) to form a monolayer. The amount of sodium bicarbonate added was 3.7 g L^{-1} for DMEM and 1.2 g L^{-1} for HAM F-12 DMEM. The medium were supplemented with fetal bovine serum (FBS) (Himedia, India), 10% (v/v) DMEM. The other supplements were 1% non-essential (100X)

amino acids (MP Biomedical, USA), 1% (v/v) antibiotics (Penicillin 5000 IU ml^{-1} and Streptomycin 5 mg ml^{-1} (MP Biomedical, USA). The pH of the media was adjusted to ~ 7.4 and sterilized by filtration under aseptic conditions. The spent culture media was replaced with fresh one in alternate days and the monolayer was used after achieving ~90% confluency. The cells in T-25 flask were treated with trypsin-EDTA to detach the cells and then used for sub-culturing. Before the initiation of the experiment, the epithelial cells were serum starved for 18 h in appropriate medium with 0.5% FBS. The stock of the cultured epithelial cell were prepared by using 95% complete growth media and 5% DMSO (Sigma-Aldrich, USA) and kept in liquid nitrogen.

3.25.1. ETEC adherence with cultured epithelial cells by plate count method

Bacterial suspension at a concentration of 10^7 c.f.u. ml^{-1} was pelleted down and dissolved in culture medium. This suspension was added to epithelial cells grown on 12-well plates. After 3 hours of incubation at 37°C in 5% CO_2, cells were washed three or four times with PBS to remove unbound bacteria and detached by 0.1% Triton X-100 or by Trypsin-EDTA. Adhered bacteria were counted after serial dilution by plating on MacConkey agar plates for c.f.u counts.

3.26. Animal experiments

3.26.1. Ethics statement

All animal experiments were conducted following the standard operating procedure as outlined by committee for the purpose of supervision and control experiments on animals (CPCSEA), Government of India. The animal experiment protocol was approved by the Institutional Animal Ethics Committee of ICMR-National Institute of Cholera and Enteric Diseases (NICED/CPCSEA/68/GO/(25/294)/2016-IAEC/NSC-1).

3.26.2. Rabbit ileal loop assay and FA ratio

The rabbit ileal loop assay (De *et al.,* 1956) was performed in young New Zealand white rabbits. Rabbit ileal loop surgery was performed on 6-week-old New Zealand rabbits (male, weighing 1-2 kg) that had been fasted for 48 hours previous to the procedure. Intravenously, the general anaesthetic Ketamine-50 (35 mg kg^{-1} body weight) and the local anaesthetic Xylocaine 2% (5 mg kg^{-1} body weight) were given. The intestine was removed through a mid-line incision in the abdomen, and 10 cm

intestinal loops with 2 cm interloops on both sides were created. The desired strains of ETEC were grown in LB or CFA broth and in each loop, 10^7 c.f.u. bacterial cells were added. PBS-inoculated loop was used as a negative control. After 18 hours of incubation the fluid accumulation (FA) ratio was measured. Recovered ETEC was used for gene expression studies as required by qRT-PCR. Toxin levels were detected by ELISA in the accumulated fluid. Adherence assay was performed and tissue samples were collected for histological analysis

FA ratio = Fluid accumulation (in ml) / length of the loop (in cm)

3.26.3. Adherence assay

Adherence assay was performed in both rabbit and mice. The adhered bacteria was counted by homogenizing the intestinal section (for rabbit) or the whole intestine (for mice) in 1 ml PBS (taken as crude) after washing the intestinal section with PBS. Then serial dilution was done and plated onto MacConkey agar to enumerate the viable bound ETEC.

3.27. Statistical analysis

All the experiments were conducted at least in triplicates wherever applicable. All data will be expressed as mean±SE (Standard Error). Data obtained from all the experiments was analyzed by ANOVA with post hoc evaluations. A level of significance was considered at P value < 0.05.

3.28. Safety Statement

All experiments were conducted following the standard operating procedure as outlined by Department of Biotechnology, Government of India Handbook and was approved by the Institutional Bio Safety Committee (IBSC), ICMR- National Institute of Cholera and Enteric Diseases, Kolkata, India. (Approval No. NICED/BS/NSC- 004/2016 dated 27/04/2016).

CHAPTER 4

Results

Objective I

Understanding the distribution of common virulence factors in enterotoxigenic *Escherichia coli* using molecular methods

4.1. Background

Under this objective we had studied the distribution of different virulence factor genes along with their expression to understand their geographical distribution in this region. For this a total of 379 archived samples of ETEC were analysed in this study. Of 379 ETEC samples, 26% (n=97) strains were isolated along with other pathogens and 74 % (n=282) strains were isolated where ETEC was the sole pathogen.

4.1.1. Distribution of ETEC isolates in different age group over the years

Among the 379 archived ETEC samples cases of ETEC infection was highest in 2015 (106 cases) and gradually decreasing over the next 4 years, i.e.: in 2016 we had 101 cases, in 2017- 68 cases, in 2018- 54 cases and 2019- 50 cases. This distribution was compared with different age groups categorised as age group below 5 years, age group of 5-18 years of age and age group comprising above 18 years of age. Comparison with different age groups it was found that almost 50% of cases were in children of less than 5 years of age (<5 years). The remaining 50% cases were detected mostly from patients above 18 years of age and in all those years least number of ETEC cases (almost 6%) were recorded in age group of 5-18 years (***Table 4.1.1.***).

4.1.2. Toxin types on ETEC isolates

Among the isolated ETEC isolates 46% strains (n=175) were found to harbour both *elt* and *esth* toxin genes. This was followed by 37% (n=139) strains harbouring *esth* alone and 15% (n=56) of strains harbouring only *elt*as toxin gene. Among all the strains only 1% strain was found to have *elt* and *estp* toxin genes and only estp variant of ST toxin was found to be harboured by less than 1% of strains. Only 1 strain was detected positive for the presence of *elt, esth* and *estp* (***Table 4.1.2***.).

Table 4.1.1. Distribution of ETEC isolates in different age group over the years

Age group	Years				
	2015	**2016**	**2017**	**2018**	**2019**
<5 years	44	55	38	22	28
5-18years	4	6	5	5	1
>18 years	58	40	25	27	21
Total	106	101	68	54	50

Table 4.1.2. ETEC distribution over the years with toxin genes

Year	Toxin genes						
	elt[*]	*est*[†]	*elt*[*]+*esth*[†]	*elt*[*]+*estp*[‡]	*estp*[‡]	*elt*[*]+*esth*[†]+ *estp*[‡]	**Total**
2015	10	36	56	2	2	0	**106**
2016	34	33	34	0	0	0	**101**
2017	2	25	38	1	1	1	**68**
2018	5	22	27	0	0	0	**54**
2019	5	23	20	1	1	0	**50**
Total	**56**	**139**	**175**	**4**	**4**	**1**	**379**

[*] ***elt-*** heat labile toxin gene

[†] ***esth*** –heat stable toxin gene; human variant;

[‡] ***estp*** –heat stable toxin gene; porcine variant

Table 4.1.3. Occurrence of ETEC in different age group with toxin genes

Age	Toxin genes‡			
	elt§(%)	*est*†(%)	*elt+est*†(%)	P value
<5	33(17.8)	72(38.4)	80(43.2)	0.357
5-18	2(8.0)	10(43.4)	11(47.8)	
>18	21(12.2)	61(35.6)	89(52.0)	
Total	56	143	180	

§***elt-*** heat labile toxin gene
†***est*** –heat stable toxin gene; includes both human (*esth*) and porcine (*estp*) variant
‡**Toxin genes** were detected using PCR method. Please see Material and Methods for details

4.1.3. Distribution of toxin genes among ETEC isolates identified over time

In all the years from 2015-2019, *elt+esth* was the most frequently detected toxin gene combination among the ETEC isolates followed by *esth* only strains, making the *elt+ esth* toxin gene combination the most prevalent toxin type in this region of India. Interestingly among the ETEC isolates during these 5 years, the presence of *estp+elt* were observed in alternate years, i.e., in 2015, 2017, and 2019. The *estp* harbouring strains were also detected in these above-mentioned years only (***Table 4.1.2.***).

4.1.4. Age-wise distribution of toxin genes among ETEC isolates

Different toxin distribution among the ETEC isolates revealed that *elt+est* was the prevalent combination of toxin among all age groups i.e., <5 years, 5-18 years, and > 18 years of age. In age groups <5 years *elt+est* harbouring strains were 43%, in the age group 5-18 years 48% and of isolates from adults it was 51% whereas 18%, 9% and11% of strains harboured *elt* gene, respectively. Four ETEC isolates from children of ≤5 year's age had both *elt+ estp* as toxin gene and this combination has not been found in patients of more than 5 years of age. Two strains in the age group 5-18 years were found harbouring e*stp* gene only. Only 1 ETEC isolate harbouring *elt+ esth+ estp was* found in the patient of 5-18 years of age group. However, when analysed it was observed that distribution of toxin genes is not statistically significantly associated with age groups (p value: 0.357) (***Table 4.1.3.***).

4.1.5. Level of dehydration in comparison with toxin types

Firstly level of dehydration was categorized into three types, i.e. severe dehydration, some dehydration and none. When data previously recorded during collection and analysis it was found that 72% of patients experienced some level of dehydration. Severe dehydration was observed in the least number of patients (8%). Rest of the 20% patients had not experienced any dehydration during the infection.

Table 4.1.4. Dehydration level associated with toxin genes

Dehydration level	Toxin genes			Total	P value
	elt[§]	*est*[†]	*elt+est*[†]		
None	14	31	31	76	
Some	37	101	134	272	0.687
Severe	4	11	16	31	
Total	55	143	181	379	

[§]***elt-*** heat labile toxin gene

† ***est*** –heat stable toxin gene; includes both human (*esth*) and porcine (*estp*) variant

Further analysis including comparison with toxin types produced by ETEC isolates , it was revealed that among *elt+est* harbouring ETEC strains, 74% were from patients having 'some' level of dehydration followed by 17% patients with no dehydration and only 9% experienced 'severe' level of dehydration. In *elt* harbouring strains 67% were from patients experiencing 'some' level of dehydration followed by similar patter to that of *elt+est* harbouring ETEC causing dehydration. ETEC strains that harboured *est* was associated with 70% patients with 'some' level of dehydration. In every type it was found that instances of 'severe' level of dehydration were the lowest in occurrence (***Table 4.1.4.***).Statistical analysis revealed that there is no significant association between level of dehydration and toxin genes (p value>0.05).

4.1.6. Genotypic distribution of ETEC virulence factors

All the 379 ETEC isolates were tested for the presence of Classical virulence factors (CFs) and Non-Classical virulence factors (NCVFs). Among the ETEC isolates 369 strains were found to have at least one CF or NCVF or both as their virulence factor. It was observed that both the CF and NCVF were present in 69% of strains. Only classical CFs was detected in 22% of strains whereas strains harbouring only NCVF genes were 6% of total. Only 3% of total strains were found to be devoid of any virulence determinants studied in this project (***Figure 4.1.1A***).

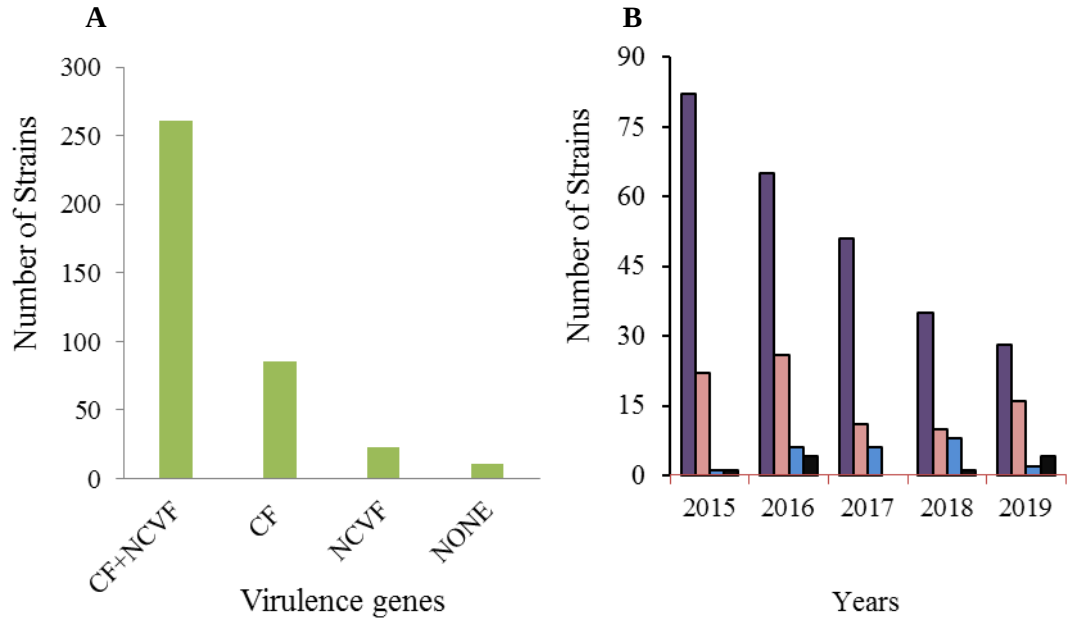


Figure 4.1.1. Distribution of ETEC virulence factors during the study period (2015–2019). **(A)** Shows overall distribution of virulence determinants. **(B)**( ) CFs; ( ) NCVF; ( ) CF+NCVF; and) None; represents year wise distribution of virulence determinants. A total of 379 clinical samples were screened for CFs – classical colonization factors; and NCVFs – non-classical virulence factors. CFs denotes the isolates positive only for the classical colonization factors; NCVFs represents strains positive only for the non-classical virulence factors; CF+NCVF represents strains positive for both the virulence factors; and NONE represents strains negative for both the virulence factors. We tested a total of 29 virulence factors (24 CFs and 5 NCVFs) and the results shown are based on the screened factors.

Analysis involving distribution of virulence factors over the years revealed that the isolates followed a similar pattern in terms of harbouring CFs and NCVFs. It has been found that both CF+NCVF as their virulence determinants and least being the isolates without any detectable virulence factors (***Figure 4.1.1B***).

When the distribution of virulence factors in ETEC isolates were compared to the presence of toxin genes in the isolates, it was revealed that among 46% strains harbouring CFs had both both the *elt+est* toxin genes, followed by 40% of strains having *est* toxin gene. Distribution of the toxin genes among NCVF harbouring ETEC isolates revealed that maximum strains (47%) had both the *elt+est* toxin genes (***Table 4***).

4.1.7. Distribution of Classical Virulence factors among ETEC isolates

PCR analysis showed that 354 strains among 379 ETEC strains harboured Classical colonization factors. Further we divided those CFs into two group, i.e. major CF group and minor CF group. Major CFs were group under CFA/I, CFA/II, CFA/III, and CFA/IV. Under these groups a total of 8 major CFs were included which are mostly found in ETEC of all regions. Remaining CFs were grouped under minor CFs. CFA/II group comprises of CS1, CS2 and CS3 while CFA/IV group comprises of CS4, CS5 and CS6. Remaining two groups contain single CF.

- ❖ Major CF distribution in relation to toxin genes

Among different major CF groups the CFA/IV group was detected in 43% of the ETEC isolates followed by the CFA/I, and CFA/II group. The CFA/III was detected in least number of isolates. When toxin type was compared to different CF groups it was found that approximately half of the strains harbouring CFA/IV CFs expressed LT+ST as their toxin type followed by 36% strains expressing ST as their toxin and strains expressing LT only was the least among them. This similar pattern of toxin expression is followed by other three major CF groups where LT+ST expressing strains remain prevalent (***Table 4.1.5.***).

Table 4.1.5. Occurrence of the most prevalent combination of CF with toxin genes

Classical Genes	Toxin			Total
	elt	*est*[*]	*elt+est*	
CS6	19	51	73	143
CFA/I	19	52	51	122
CS5	9	21	45	75
CS6+CS5	7	18	40	65
CFA/I+CS21	3	17	21	41
CS6+CFA/I	3	13	20	36
CS6+CS21	0	8	6	14

[†]***est***includes both human (*esth)* and porcine *(estp)* variant

[‡]Classical colonization factor (**CF**) genes, Classical CF genes studied here by PCR; please see Materials and Methods for details

In CF distribution analysis it was found that CS6 was the prevalent CF detected. Alongside this the occurrence of other CFs were also compared to the toxin type those ETEC strains expressed. Analysis revealed that in 41% of LT+ST ETEC strains CS6 was present. Among LT-ETEC isolates CS6 was detected in 34% of strains and similarly CS6 was also detected in equal frequency in ST-ETEC strains. When distribution of CFA/I with toxin type was analysed it was revealed that CFA/I was almost equally detected in both LT+ST ETEC strains and ST-only ETEC strains. Least number of CFA/I was detected in LT-only ETEC. In ST-only ETEC isolates, CFA/I was detected in almost the same number (37%) but in LT+ST strains it was detected in only 28% strains compared to 41% strains positive for CS6 (***Table 4.1.5.***). Following CS6 and CFA/I, CS5 is another major CF found commonly in 21% CF positive ETEC isolates. Most prevalent combination of Major CF was found to be CS6 with CS5 followed by CFA/I with minor CF CS21. Among the prevalent major CF combination, 61% of CS6+CS5 ETEC isolates were found to harbour *elt+est* as their toxin gene followed by ST-only CS6+CS5 isolates (27%) and only 10% of CS6+CS5 isolate harboured *elt* as their toxin gene. Whereas 55% of CS6+CFA/I isolate harboured *elt+est* as their toxin gene. In CFA/I+CS21 isolates 51% of strains harboured *elt+est* as prevalent toxin gene.

4.1.7.1. Major CFs distribution during the time period

Archived ETEC strains isolated between 2015-2019 were found to harbour CS6 as the predominant CF. In 2015 CS6 was detected in 35% of the isolates, 44% in 2016, 43% in 2018, 24% in 2018 and 34% of isolates harboured CS6 in 2019 (*Table 4.1.6.*). CFA/I was detected in most strains after CS6 in all those years. In 2016, CS6 and CFA/I were detected almost in similar abundance in the ETEC isolates. In 2016 and 2017, CS6 was detected in a higher percentage (44% and 43%, respectively) than that of 2015, 2018, and 2019. Among the rarely encountered CFs, CFA/III was detected in 3% of isolates only. CS5 was another major CF which was detected in 21% of strains. In 2016 CS5 was detected in 25% of strains followed by 24% in 2017. In 2015 and 2018 CS5 was detected in approximately 20% of strains. CS3 was another major CF found in ETEC strains after CS5. Interestingly CS3 was also detected in similar pattern over the years.

4.1.7.2. Minor CF among ETEC isolates in relation to toxin genes

Minor CFs were detected in approximately 75% of the total ETEC. Among these, 11%strains harboured *elt* toxin gene only, 42% harboured est toxin gene onlyand 47% harboured *elt+est* toxin gene. Among the minor CFs, CS21 was accounted for the highest presence (31%). In these strains, *elt+est* positive CS21 was maximum, whereas only 3 strains had the *elt* gene. In most cases minor CFs were detected in conjecture of any of the major CFs. Most common pattern of detecting major CF with minor CF was CS6+CS21, CFA/I+CS21 and some others (***Table 4.1.7.***).

Table 4.1.6. Major CF distribution over the years

Major CF Groups	CF‡	Years					Total	Percentage (%)
		2015	2016	2017	2018	2019		
CFA/I	CFA/I	32	42	16	17	15	122	34
CFA/II	CS1	5	17	6	3	6	37	10
CFA/III	CS2	8	5	4	3	4	24	7
	CS3	14	24	12	5	7	62	18
	CFA/III	1	3	4	1	1	10	3
CFA/IV	CS4	3	3	2	4	1	13	4
	CS5	22	25	16	7	5	75	21
	CS6	37	44	29	16	17	143	40

‡Classical colonization factor (**CF**) genes, Classical CF genes studied here by PCR; please see Materials and methods for details

Table 4.1.7. Minor CF distribution over the years

Minor CFs‡	Years					Total	Percentage (%)
	2015	2016	2017	2018	2019		
CS21	23	20	21	15	9	88	25
CS23	20	8	6	3	0	37	10
CS20	18	0	10	0	3	31	9
CS28	17	1	3	2	3	26	8
CS27	12	6	0	1	1	20	6
PCF071	4	5	7	3	1	20	6
CS22	13	0	0	1	0	14	4
CS12	8	2	1	0	0	11	3
CS18	2	2	7	0	0	11	3
CS14	7	2	1	0	2	12	3
CS17	6	5	2	0	2	15	4
CS15	4	0	0	3	5	12	3
CS19	0	0	0	0	0	0	0
CS13	0	0	0	0	0	0	0
CS26	0	0	0	0	0	0	0
CS30	0	0	0	0	0	0	0

‡Classical colonization factor (**CF**) genes, Classical CF genes studied here by PCR;

4.1.7.3. Minor CF among ETEC isolates over the years

During 2015-2019 period, CS21 was detected maximally amongst other minor CFs in every year, e.g., 21% isolates in 2015, 19% isolates in 2016, 30% isolates in 2017, 27% isolates in 2018, and 18% isolates in 2019 (***Table 4.1.7.***), This was followed by detection of CS23 in these years except in 2019, when no strains were found having CS23. CS18 and CS20 were almost detected in equal frequency in 2015 followed by ETEC strains of 2017. In 2016 and 2018 CS20 was not detected at all. Among other minor CFs, CS12 and CS18 was found in the least number of strains. Four minor CFs i.e. CS19, CS13, CS26 and CS30 were not detected in any of the ETEC isolates in our study.

4.1.8. Distribution of Non-classical virulence factor (NCVF) genes

Seventy five per cent of the ETEC strains isolated during 2015-2019 harboured NCVF genes. Out of the positive NCVFs strains, EatA were most commonly detected NCVF (69%) followed by EtpA (42%), tibA (18%) and tia (12%). The least detected NCVF in our study was LeoA (7%). Most of the NCVF harbouring ETEC isolates were in co-presence of at least one or more classical CFs. In this study, only 8% of isolates had NCVF genes as their sole virulence factors. EatA was mostly detected (42%) with prevalent classical CF CS6 and 6% of EatA genes were in ETEC isolates having no CF. While out of 379 ETEC isolates, almost 25% isolates were NCVF negative.

4.1.8.1. Distribution of NCVF genes in ETEC isolates in relation to toxin genes

When analysed in comparison with toxin genes it was obtained that LT+ST- ETEC isolates harboured maximum NCVF in 134 of 284 strains (47%) and LT only strains harboured least number of NCVF (11%). EatA, the predominant NCVF, found maximally in LT+ST strains (49%) and LT only EatA only was minimum (9%). Following EatA, EtpA also showed the same pattern of distribution accounting for half of the EtpA strains (50%) producing LT+ST toxins and almost equal percentage produced ST toxins *(**Table 4.1.8.**)*.

Table 4.1.8. Occurrence of ETEC NCVFs with toxin genes

NCVF[§]	Toxin genes			Total
	elt	*est*[†]	*elt+est*[†]	
EatA	17	82	97	196
EtpA	10	50	59	119
Tib	5	21	24	50
Tia	10	12	13	35
LeoA	3	8	9	20

[†] ***est*** includes both human (***esth)*** and porcine ***(estp)*** variant
[§] Non-classical virulence factor (**VF**) genes. Non-classical VF genes studied here by PCR

4.1.8.2. *Year-wise distribution of NCVF genes among ETEC isolates*

Analysis of year wise distribution revealed that EatA was the prevalent non-classical VF detected in our region followed by EtpA as the next common one. EatA was identified in highest number in 2015 whereas EtpA was most detected in 2016.In 2016 the least common NCVF, LeoA was identified only in one strain. NCVF genes were found to be an independent variable over time (*Table 11*).

Table 4.1.9. NCVF distribution with time

NCVF[§]	Years					Total	Percentage (%)
	2015	2016	2017	2018	2019		
EatA	64	46	41	29	16	196	69
EtpA	23	41	24	15	16	119	42
TibA	19	11	13	5	2	50	18
Tia	10	7	7	6	5	35	12
LeoA	12	1	4	2	1	20	7

[§]Non-classical virulence factor (**VF**) genes. Non-classical VF genes studied here by PCR; please see Materials and methods for details.

4.1.9. Expression pattern of virulence factors in relation to their presence

To delineate whether the ETEC strains express every single virulence determinants we applied real time PCR analysis. Interestingly it was observed that most of the ETEC strains expression only a few factors among those present.

4.1.9.1. Expression of Classical Virulence factors among ETEC strain

The strains that were positive for at least one CF were checked for expression of their virulence genes. Approximately 53% of strains harbouring CS6 as one of their CF, expressed their CF, followed by 50% of CS5-ETEC strains expressing CS5. The second most prevalent CF, CFA/I was expressed by 20% of the CFA/I-ETEC. Similar degree of expression was also detected in CS21 harbouring ETEC isolates. While CS3 was expressed in 35% of CS3 harbouring ETEC strains. The remaining CFs were expressed in a very low percentage whereas CFA/III, CS17 was not expressed by any strains at all (***Figure 4.1.2.***).

4.1.9.2. Expression of Non-Classical Virulence factors among ETEC strain

When expression of NCVFs were studied it was revealed that out of 196 strains having EatA, 45% strains expressed RNA. For EtpA it was 40% of the EtpA harbouring ETEC which express their RNA. Tia was expressed in 37% of the strains while expression of TibA and LeoA was among the lowest (***Figure 4.1.2.***).

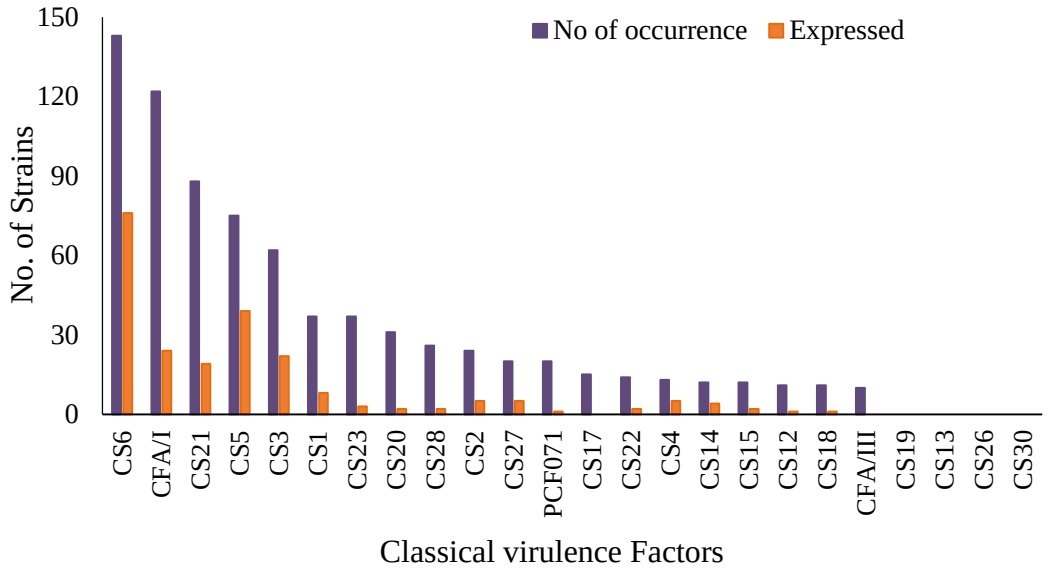


Figure 4.1.2. Distribution of presence and expression of different Classical virulence factors (**CFs**)

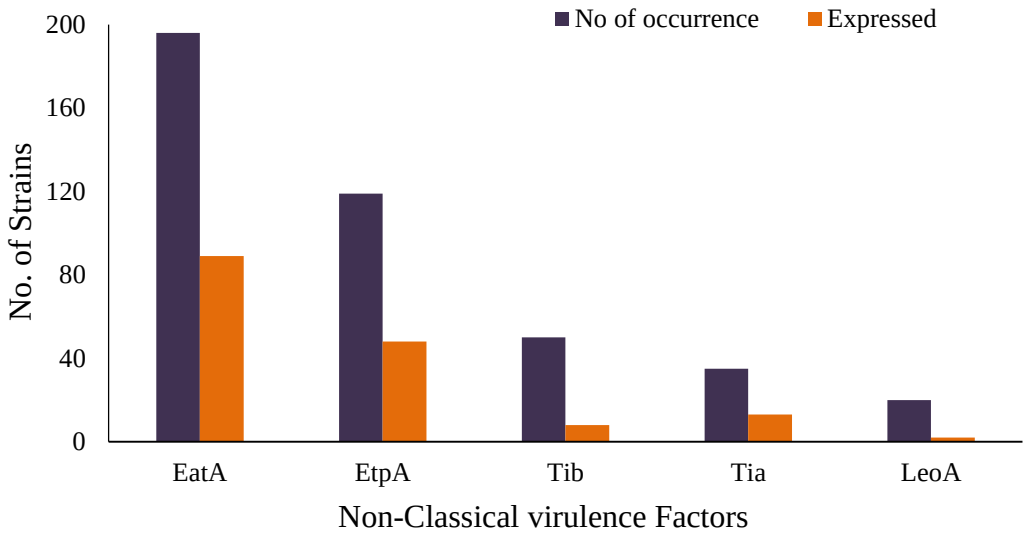


Figure 4.1.3. Distribution of presence and expression of different Non- Classical virulence factors (NCVFs)

4.1.9.3. Expression of CS6 in different strains

As CS6 was the prevalent virulence factor found in our study we focused our study to delineate level of expression of CS6 in different representative strains compared to lab reference strain (***Figure 4.1.4***). Experiments have revealed that not all strains expressed CS6 and also level of CS6 expression was different in different isolates. The strains which don't express their genes were confirmed by SDS-PAGE and ELISA.

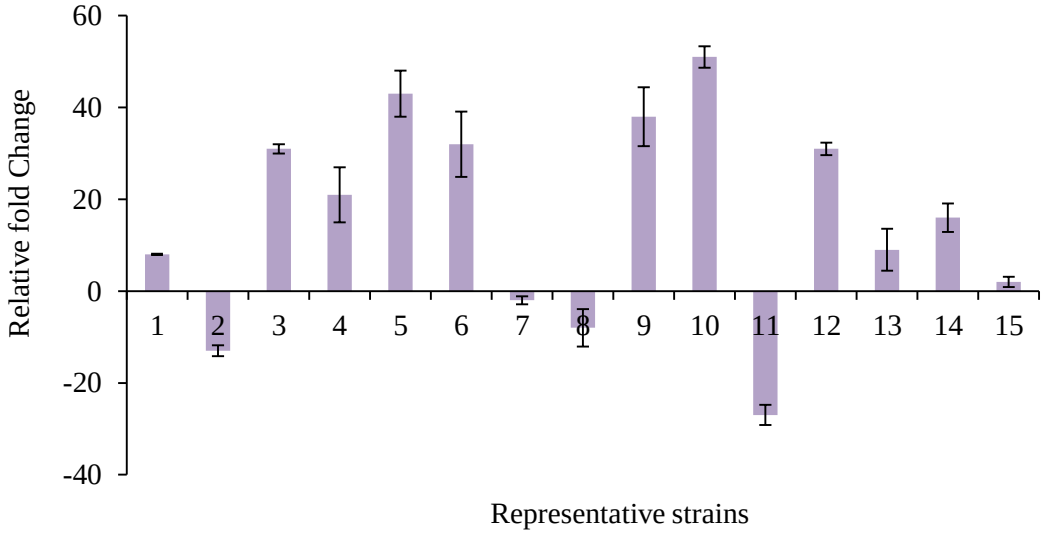


Figure 4.1.4. Differential expression level of CS6 in representative strains when compared to CS6 positive Lab reference strain. A wide rage in level of expression has been found as indicated.

Objective II

Interpreting the antimicrobial resistance pattern and molecular characterization of enterotoxigenic *Escherichia coli*

4.2. Background

Under this objective we have studied response of ETEC strains to different antibiotics. For this a total of 379 archived samples of ETEC were analysed in this study and 14 different antimicrobial agents (Antibiotics) were included to check the response. Antibiotics were selected from commonly used drug of choice in this region as well as antibiotics used in ETEC prevalent areas.

4.2.1. Response of ETEC strains to different antimicrobial agents

When ETEC strains were subjected to different antibiotics it was revealed that almost all strains showed resistance to more than one antibiotic. Imipenem (Ipm), a fourth generation drug, was the only against which no bacteria was found to be resistant followed by Chloramphenicol (C) against which only 12% strains showed resistance. We found resistance against Ceftriaxone (Cro) in 22%, Cefapime (Fep) in 28%, Doxycycline (D) in 36%, Norfloxacin (Nor) in 36%, Tetracycline (Te) in 42% and Sulfamethoxalone W/Trim (Sxt) in 46% strains. More than 60% of strains were resistant against Ciprofloxacin (Cip), Ampicillin (Am), Azithromycin (Azm), and Nalidixic Acid (NA). In our study we found that all strains were resistant against Erythromycine (E) and Streptomycine (S) *(**Figure 4.2.1.**)*.

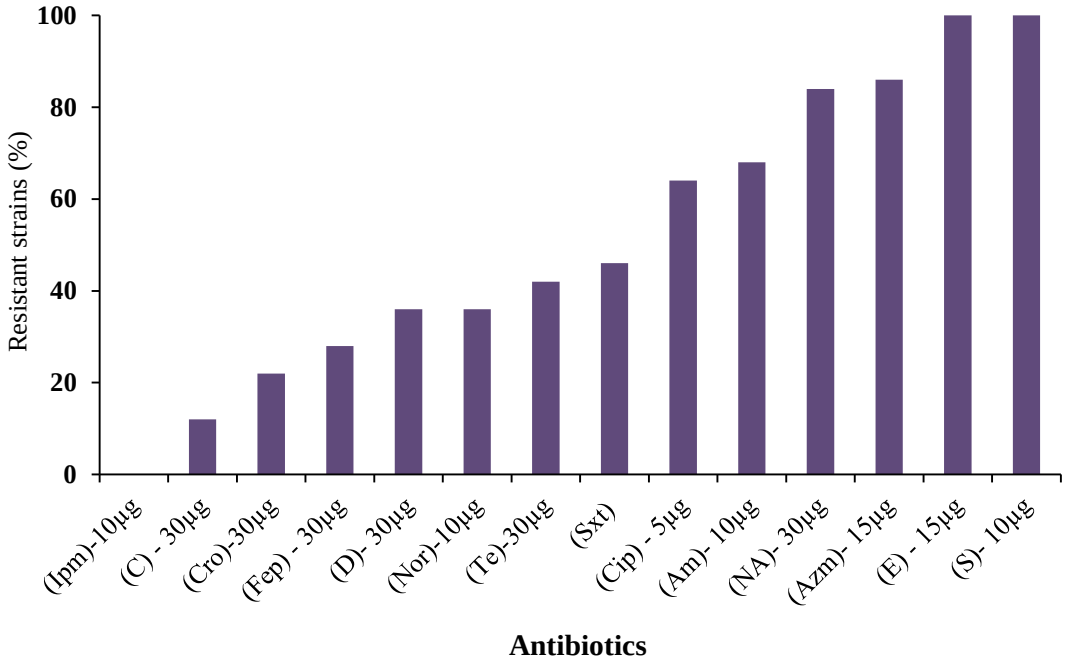


Figure 4.2.1. Antibiotic resistance pattern of ETEC isolates against different antibiotics

4.2.2. Antibiotic susceptibility of CS6-ETEC isolates

From this study, CS6 was identified as the prevalent CF during 2015-2019. The antibiotic susceptibility in the CS6-harbouring ETEC isolates (143) was tested for antibiotic response against 14 antimicrobial agents *(**Table 4.2.1.**)*. All CS6-harbouring isolates were resistant to one or more antimicrobial agents with the most frequent resistance found against 10 µg streptomycin (99.5%) and 15µg erythromycin (97.7%). More than 80% of strains were resistant to 30µg Nalidixic Acid and 15µg Azithromycin. All the ETEC isolates were sensitive to 10µg Imipenem. Majority of the strains were sensitive to 30µg Chloramphenicol.

Table 4.2.1. Response of CS6 harbouring ETEC isolates and ETEC isolates having no detectable virulence factors to antimicrobial agents

Antibiotics*	Number of CS6 Resistant strains [Percent (%)]	Number of None* Resistant strains [Percent (%)]
Imipenem (Ipm)-10µg	0 (0)	0 (0)
Chloramphenicol (C) - 30µg	8 (6)	2(20)
Cefapime (Fep) - 30µg	34 (24)	3 (30)
Doxycycline (D)- 30µg	39 (27)	2 (20)
Ceftriaxone (Cro)-30µg	46 (32)	2 (20)
Tetracycline (Te)-30µg	47 (33)	2 (20)
Norfloxacin (Nor)-10µg	51 (36)	5 (50)
Sulfamethoxalone W/Trim (Sxt)	58 (41)	3(30)
Ciprofloxacin (Cip) - 5µg	100 (70)	6 (60)
Ampicillin (Am)- 10µg	107 (75)	8 (80)
Azithromycin (Azm)- 15µg	120 (84)	7 (70)
Nalidixic Acid (NA)- 30µg	124 (87)	10 (100)
Erythromycine (E) - 15µg	140 (98)	10(100)
Streptomycine (S)- 10µg	142 (99)	10 (100)

To compare the results, we also tested 10 strains, in which no known virulence factors were detected. Among these isolates, almost the similar pattern of response to antimicrobial agents was found *(Table 4.2.1.)*.

4.2.3. Different combinations of antibiotics and their resistance against CS6-ETEC isolates

When combinations of different antibiotic resistance were analysed compared to the toxin types in CS6-harbouring strains, a very few strains showed a similar pattern of resistance to different antibiotic combinations *(Table 4.2.2.)*.

Table 4.2.2. Antibiotic resistance profile linked to toxin type of CS6-harbouring ETEC strains

No. of antibiotics	Antibiotic combinations	Toxin type of CS6 strains			Total
		LT+ST	ST	LT	
13	Am+Cro+D+Te+ Cip+Azm+E+S+NA+Sxt+C +Fep+Nor	1			1
11	Am+Cro+ Cip+Azm+E+S+NA+Sxt+C +Fep+Nor	1			1
	Am+Cro+D+Te+ Cip+Azm+E+S+NA+Fep+Nor	1			1
	Am+D+Te+ Cip+Azm+E+S+NA+Sxt+C +Nor	1			1
10	Am+D+Te+ Cip+Azm+E+S+NA+Sxt+Nor	1		1	2
	Am+Cro+ Cip+Azm+E+S+NA+Sxt+Fep+Nor	1			1
	Am+Cro+D+Te+ Cip+Azm+E+S+NA+Sxt	1			1
	Am+Te+ Cip+Azm+E+S+NA+Sxt +Fep+Nor	1			1
	Am+Cro+D+Te+ Cip+Azm+E+S+Sxt +Fep		1		1
	Am+D+Te+ Cip+Azm+E+S+NA+C +Nor		1		1
	Am+Cro+ Cip+Azm+E+S+NA+C +Fep+Nor	1			1
	Am+Cro+D+ Cip+Azm+E+S+NA+Fep+Nor			1	1
9	Am+Cro+ Cip+Azm+E+S+NA +Fep+Nor	5	1		6
	Cro+Te+ Cip+Azm+E+S+NA+Sxt+Fep		1		1
	Am+Te+ Cip+Azm+E+S+NA+Sxt+Nor	2	1		3
	Am+Cro+ Cip+Azm+E+S+NA+Sxt+Nor		1		1
	Am+D+ Cip+Azm+E+S+NA+Sxt+Nor	1			1
	Am+D+Te+ Cip+Azm+E+S+NA+Nor	2			2
	Am+D+Te+ Cip+E+S+NA+Sxt+Nor	1			1
	Am+D+Te+ Cip+Azm+E+S+NA+Sxt	2	1		3

	Am+Cro+Te+ Cip+Azm+E+S+NA+Sxt		1		1
	Am+Cro+D+Te+ Cip+Azm+E+S+Sxt	1			1
	Am+Cro+ Cip+Azm+E+S+NA+Sxt+Fep			1	1
	D+Te+ Cip+Azm+E+S+NA+Nor		2		2
	Am+ Cip+Azm+E+S+NA+Sxt+Nor	2	1		3
	Am+ Cip+Azm+E+S+NA+Sxt+Fep		1	1	2
	Am+Cro+ Cip+E+S+NA+Fep+Nor		1		1
	Am+Cro+ Cip+Azm+E+S+NA+Fep		1	1	2
	Am+Cro+D+Azm+E+S+NA+Fep	1			1
	Am+Cro+D+ Cip+Azm+E+S+NA	1			1
	Am+Cro+ Cip+Azm+E+NA+Fep+Nor		1		1
8	Am+Cip+Azm+E+S+NA+Sxt+C	1			1
	Am+Azm+E+S+NA+C +Fep+Nor	1			1
	Am+Cro+ Cip+Azm+E+S+NA+Nor			1	1
	Am+Cro+Te+ Cip+Azm+E+S+NA	1			1
	Am+D+Te+ Cip+Azm+E+S+Nor	1			1
	Am+Cro+Azm+E+S+NA+Sxt+Nor	1			1
	Am+Cro+ Cip+Azm+E+S+NA+Sxt		2		2
	Am+Te+ Cip+Azm+E+S+NA+Sxt+		1		1
	Am+D+Azm+E+S+Sxt+Fep+Nor	1			1
	Am+Te+ Cip+E+S+NA+Sxt+Nor		1		1
	D+Te+ Cip+E+S+NA+Sxt	1	1		2
	Am+ Cip+Azm+E+S+NA+Nor	5			5
	Am+Te+ Cip+Azm+E+S+NA		3		3
	Am+Cro+ Cip+Azm+E+S+NA	1	1	1	3
	Am+D+Cip+Azm+E+S+NA	3			3
	Am+Cro+ Cip+Azm+E+S+Sxt		1		1
	Am+D+ Cip+Azm+E+S+Sxt	1			1
7	Am+ Cip+Azm+E+S+NA+Sxt	3	1		4
	Am+Cro+ Cip+E+S+NA+Sxt		1		1
	Am+Te+Azm+E+S+NA+Sxt	1			1
	Am+Cro+Azm+E+S+NA+Fep	2	1		3
	Cro+ Cip+Azm+E+S+NA+Nor		1		1
	Cro+Azm+E+S+Sxt+Fep+Nor		1		1
	Cro+Cip+Azm+E+S+NA+C		1		1
	Am+Azm+E+S+NA+Sxt+Nor	1			1
6	Cip+Azm+E+S+NA+Nor	2			2
	Cip+E+S+NA+Sxt+Fep			1	1

	Am+Cip+Azm+E+S+NA	1	1	2	4
	Am+Azm+E+S+NA+Fep	1			1
	Am+Cro+Azm+E+S+Fep	1			1
	Am+Cro+ Cip+E+S+Fep		1		1
	Am+ Cip+Azm+E+S+Sxt	1		1	2
	D+Te+Azm+E+S+Sxt		1		1
	Te+ Cip+Azm+E+S+NA			1	1
	D+Te+Azm+E+S+NA		1		1
	Am+Te+ Cip+E+S+NA	1			1
	D+Te+ E+S+NA+Sxt	1	1		2
	Am+Te+E+S+NA+Sxt		1		1
	D+Te+ Cip+S+NA+Sxt		1		1
	Te+ Cip+E+S+NA+Sxt		1		1
	Cro+Azm+E+S+Fep+Nor		1		1
5	Cip+Azm+E+S+NA	3	1		4
	Am+Azm+E+S+NA	4	3	2	9
	Am+Cro+E+S+NA	1			1
	D+Te+Azm+E+S		2		2
	D+Te+ Cip+S+NA		1		1
	D+Azm+E+S+NA		1		1
	Am+E+S+NA+Sxt	2			2
	Am+Azm+E+NA+Sxt		1		1
	Am+Azm+E+S+Nor	1			1
4	Azm+E+S+NA	2	2		4
	Am+Azm+E+S			1	1
	D+Te+ E+S		1		1
	Cip+Azm+E+S			1	1
	Cip+E+S+NA			1	1
	Am+Cip+S+NA	1			1
3	E+S+NA			1	1

Objective III

Unravelling the effect of regulatory factors on prevalent virulence factors of enterotoxigenic *Escherichia coli*

4.3. Background

Following the entry into the host ETEC must pass through the acidic environment of the stomach; overcome the bactericidal effects of host effectors; transverse the mucous layer of small intestine before colonization and propagate an infection. Several enteropathogens are known to respond to different host regulatory factors in the gastrointestinal tract (e.g., bicarbonate, short chain fatty acids, NaCl , alkalinity etc.). Upon response differentially they are able to adjust their degree of virulence to the most favourable niche of infection (Joffre *et al.,* 2019).

As classical colonization factors are the key player in successful attachment and thereby colonization , which is aided by Non-Classical virulence factors, we focused our study on the effect of some host regulatory factors that modulate expression of prevalent Classical colonization factors like CS6 , CS5 and Non-Classical virulence factor EatA during pathogenesis. Different regulatory factor compounds were supplemented additives in M9, CFA and LB broths in different concentrations. Media without any additives was used as control for those experiments. Level of modulation in expression of CS6, CS5 and EatA was quantified by qRT-PCR and also modulation in protein levels were quantified by ELISA. Other than those compounds a genetic global regulator was also studied for its effect on prevalent colonization factor.

4.3.1. Effect of regulatory factors on the prevalent Classical virulence factor CS6 and CS5; Non-Classical virulence factor EatA

4.3.1.1. Effect of Sodium bicarbonate on CS6, CS5 and EatA

The influence of Sodium Bicarbonate on expression of CS6 was studied by growing ETEC under defined minimal media (M9) was examined with added supplementation with Sodium Bicarbonate ($NaHCO_3$) in a gradually increasing concentration. At concentration of 0.3% $NaHCO_3$ in M9, the expression of CS6 was upregulated by 7-fold in comparison to condition where no supplementation was added (Control) ***(Figure 4.3.1.)***. In 0.2% and 0.5% $NaHCO_3$ added condition showed induction in expression but the level of induction was not significant. In 0.1% $NaHCO_3$ added condition CS6 expression was almost similar to control.

As observed in RNA expression the level of protein induction was also similar. In 0.3% $NaHCO_3$ CS6 expressed highest among all other conditions with an increase of approximately 6-fold. In other 3 conditions like 0.1%, 0.2% and 0.5% the level of induction in protein expression was similar as in RNA expression (***Figure 4.3.1.***).

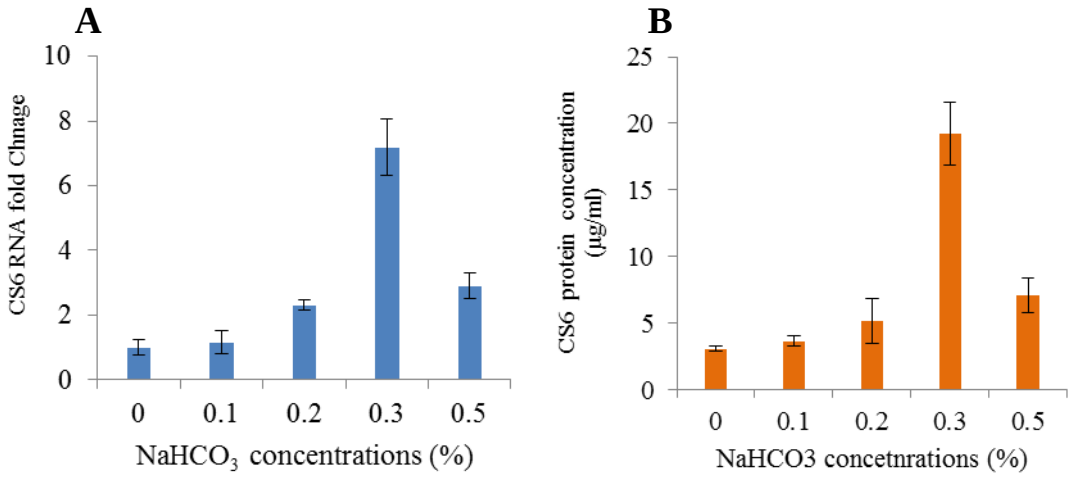


Figure 4.3.1. Effect of Sodium Bicarbonate on **CS6** expression. The effect on CS6 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

When expression of CS5 was observed it was found that in different concentrations of $NaHCO_3$ there was no significant modulation in CS5 expression in RNA level as well as in protein level (***Figure 4.3.2.***). In all other conditions increase in level of CS5 expression was almost 2-fold as compared to control.

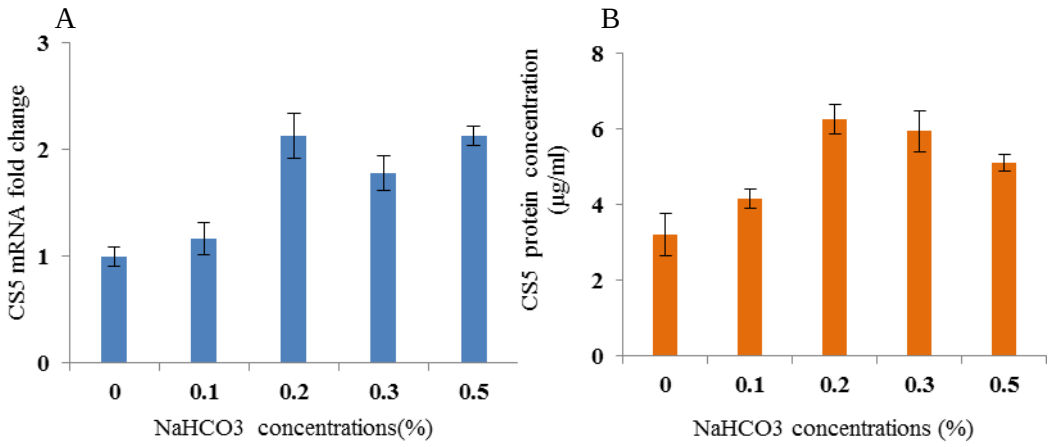


Figure 4.3.2. Effect of Sodium Bicarbonate on CS5 expression. The effect on CS5 expression was studied at (A) RNA level by quantitative RT-PCR, (B) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

Whereas eatA was modulated in similar conditions like CS6 i.e. in 0.3% $NaHCO_3$ level of expression of eatA was increased maximally by 9-fold. In ELISA where eatA protein was quantified it was found to in congruence with level of RNA expression as compared to control (***Figure 4.3.3)***.

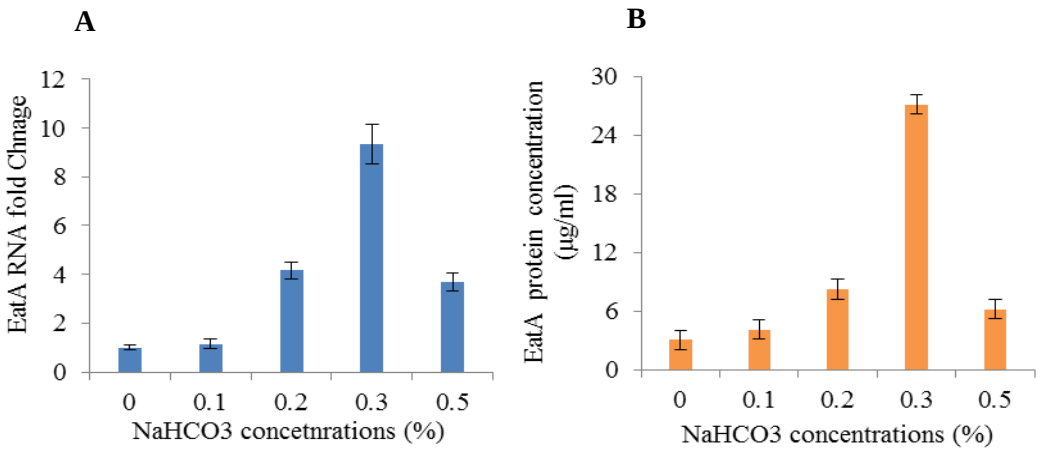


Figure 4.3.3. Effect of Sodium Bicarbonate on **eatA** expression. The effect on CS6 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

4.3.1.2. Effect of Short Chain Fatty Acids (SCFAs) on CS6, CS5 and eatA

Short-chain fatty acids (SCFA), primarily acetate, propionate, and butyrate, are produced by microbial fermentation of undigested carbohydrates and dietary fibres in the gut. The effects of SCFAs on virulence factors were evaluated in different concentrations pertaining to concentrations similar to small intestine. For experiments C-2: Acetic acid. C-3: Propionic acid. C-4: n-Butyric acid was used.

- ❖ Effect of Acetate on CS6, CS5 and eatA

In M9 broth at a concentration of 6-8mM acetate owes to the elevated CS6 expression by 8-10-fold approximately. Maximum expression was CS6 was observed in presence of 8mM acetate as compared to no acetated added condition (control) (***Figure 4.3.4.A***). However CS5 was maximally expressed in 6mM acetate condition as compared to control (***Figure 4.3.5.A***). EatA showed similar pattern of response as CS6. There was a 9-fold increase in eatA expression as compared to control (***Figure 4.3.6.A***). Expression of CS6 was not significantly increased in presence of 2mM, 4mM and 10mM acetate added condition. Whereas CS5

expression was significantly increased in 8mM as well as in 10mM when compared to control.

Similar to RNA expression CS6 protein expression was maximally increased by almost 11-fol in presence of 8 mM acetate in the media (***Figure 4.3.4.B***). In same condition expression of eatA was increased by 9-fold which was the maximum among other concentrations (***Figure 4.3.6.B***). However CS5 expression was highest in 6mM acetate added condition when compared to control. In 8 mM acetate the expression of CS5 protein was also close to maximum. In 2mM acetate CS5 was decreased as compared to control (***Figure 4.3.5.B***).

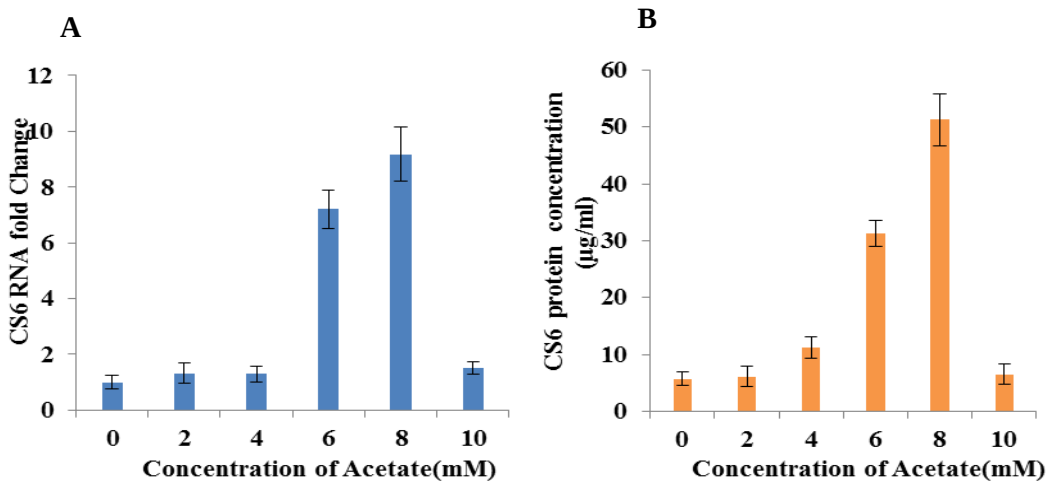


Figure 4.3.4. Effect of Acetate on **CS6** expression. The effect on CS6 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three

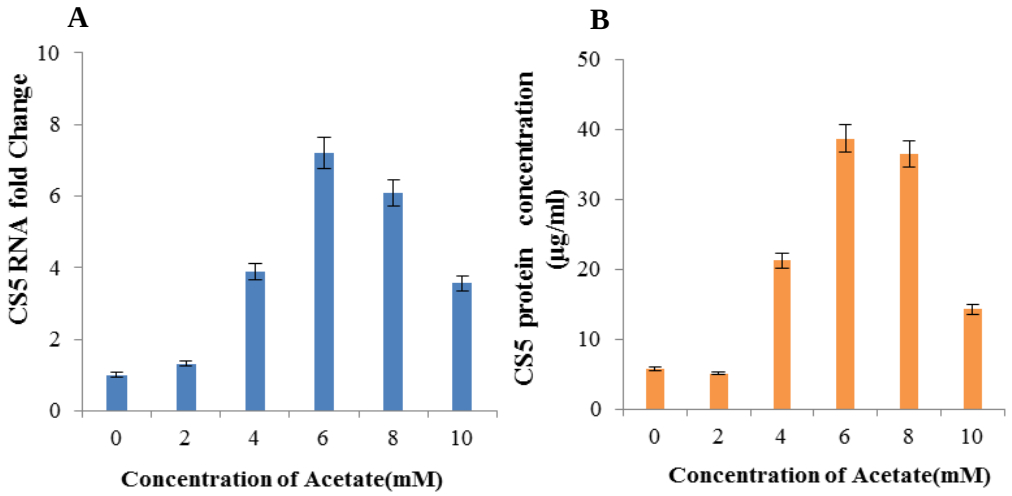


Figure 4.3.5. Effect of Acetate on **CS5** expression. The effect on CS5 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

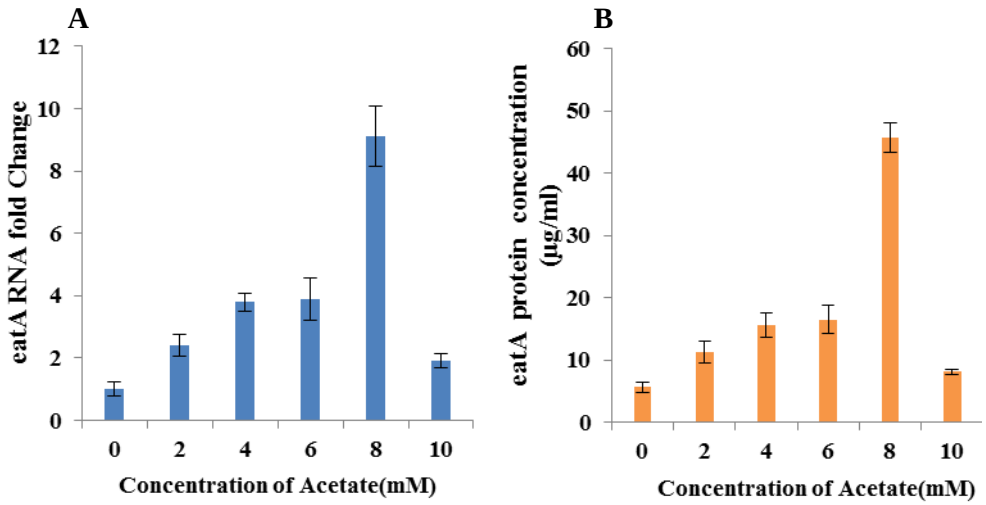


Figure 4.3.6. Effect of Acetate on **EatA** expression. The effect on eatA expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

- ❖ Effect of Propionate on CS6, CS5 and eatA

The influence of propionate on virulence factors was examined by adding different concentrations of propionate similar to intestinal conditions in different pathological circumstances. In M9 defined media CS6 expression gradually increased from 1.5mM to 2.5mM propionate supplemented condition as compared to control (No-propionate). But with increasing the concentrations further the expression of CS6 decreased gradually (***Figure 4.3.7.A***). CS6 was upregulated by 4-fold in presence of 2.5mM propionate. Expression of CS5 was highest in 2.5mM propionate similar to eatA in same concentration. In 2mM as well as 2.5mM propionate CS5 expression was upregulated by 3-fold (***Figure 4.3.7.A***). . In 1.5mM and 3.5mM CS5 and eatA expression was increased but not significantly.

The level of protein production by the induction of propionate was in accordance with RNA expression. CS6 protein was maximally expressed with an increase of 5-fold in 2.5mM condition as compared to control (***Figure4.3.7.B)***. In CS5 and eatA also 2.5mM concentration induced the expression by 5- fold and 14-fold respectively. With increasing concentrations of propionate the level of protein was decreased after 2.5mM showing a bell shaped curve.

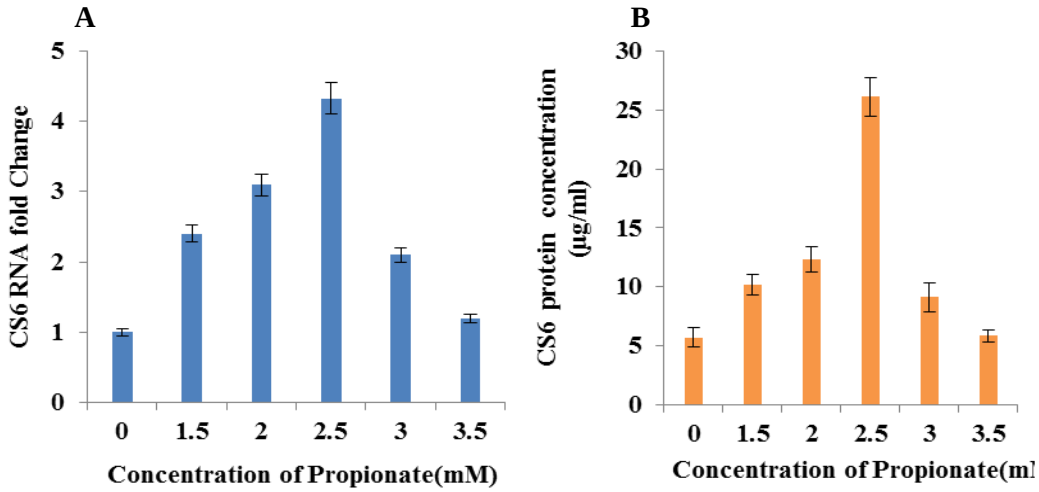


Figure 4.3.7. Effect of Propionate on **CS6** expression. The effect on CS6 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

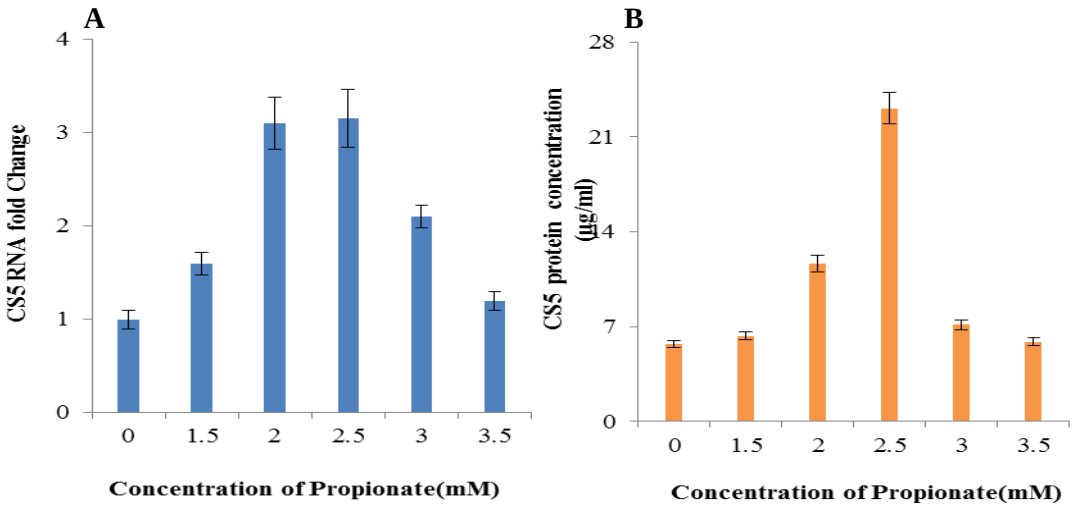


Figure 4.3.8. Effect of Propionate on **CS5** expression. The effect on CS5 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment

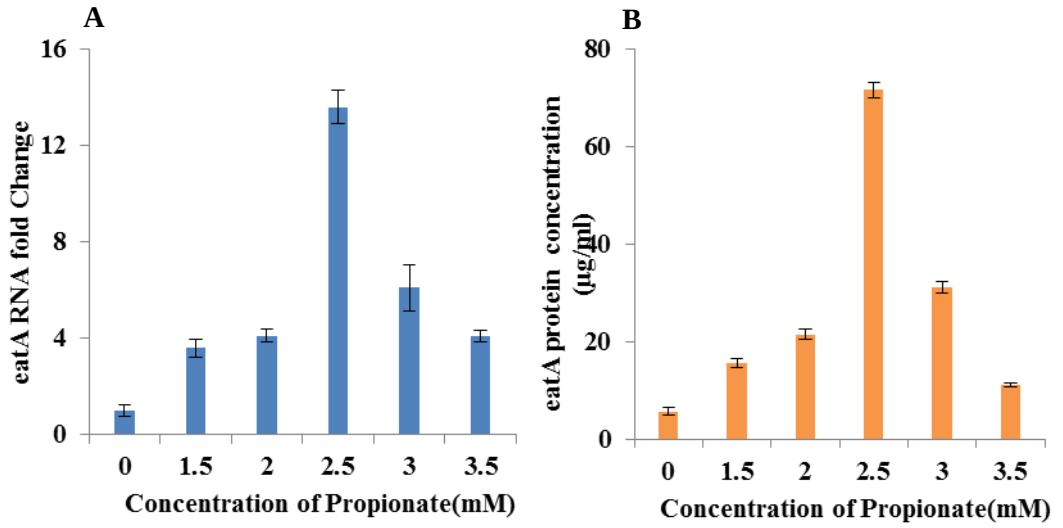


Figure 4.3.9. Effect of Propionate on *eatA* expression. The effect on eatA expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

❖ Effect of Butyrate on CS6, CS5 and eatA

Expression of CS6 was upregulated by ~9-fold in presence of butyrate, a compound of SCFA at 1.5mM concentration in M9 media (***Figure 4.3.10.A***). But upon increasing the concentration upto 4mM CS6 expression was downregulated as compared to control. CS5 expression was most at 2mM (***Figure 4.3.11.A***) and eatA expression was maximally increased at 1.5mM (***Figure 4.3.12.A***) butyrate added condition. In other concentrations of 0.5 mM, 1 mM and 1.5 mM expression of CS5 was almost same whereas eatA was increased in 1mM condition significantly.

CS6 protein was maximally produced at 1.5mM propionate whereas in higher concentration at 4mM expression of CS6 gets halved as compared to control (***Figure 4.3.10.B***). CS5 protein in presence of propionate was produced more than control in all concentrations with maximum at 2mM (***Figure 4.3.11.B***). In presence of 1mM and 1.5mM propionate eatA expression was upregulated maximally. With further increase in concentration expression of eatA was downregulated (***Figure 4.3.12.B***).

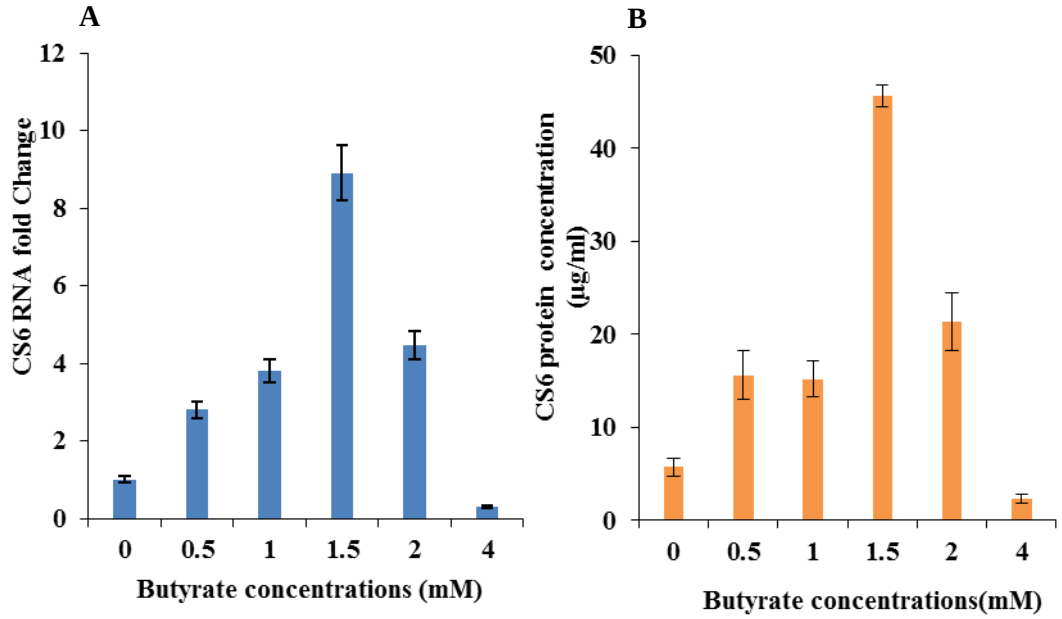


Figure 4.3.10. Effect of Butyrate on ***CS6*** expression. The effect on CS6 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

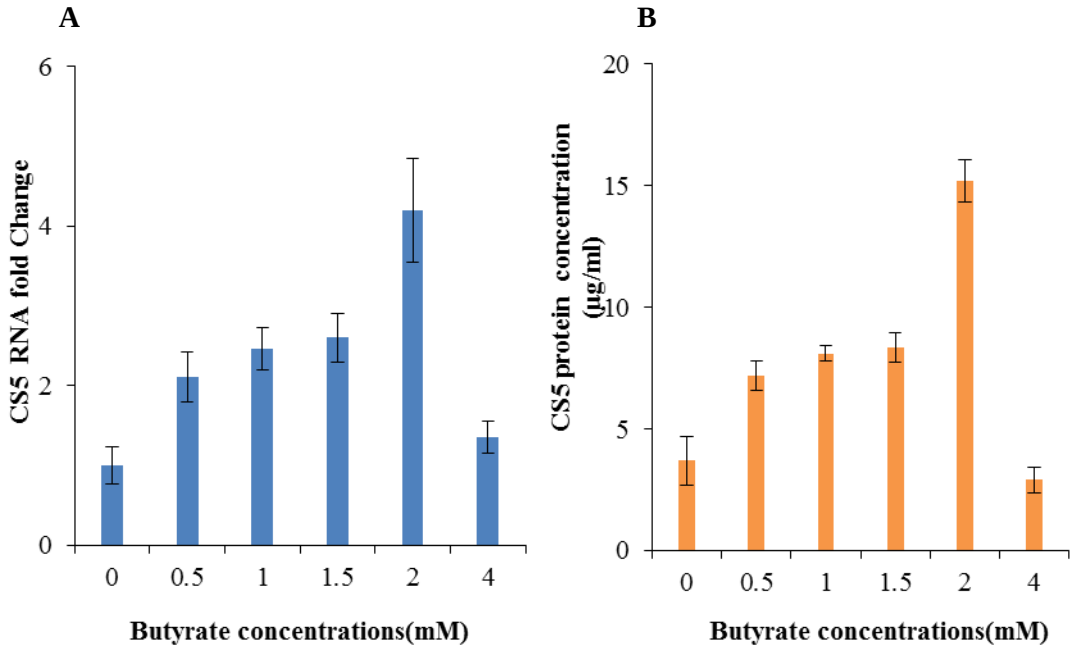


Figure 4.3.11. Effect of Butyrate on ***CS5*** expression. The effect on CS5 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

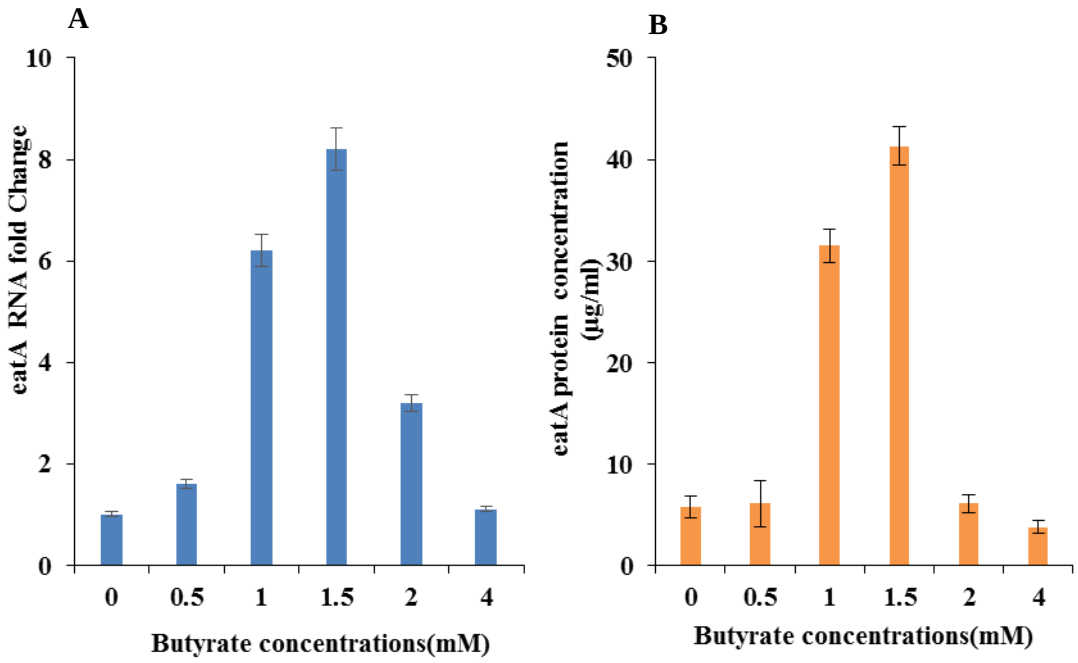


Figure 4.3.12. Effect of Butyrate on ***eatA*** expression. The effect on eatA expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

4.3.1.3. Effect of Sodium Chloride (NaCl) on CS6, CS5 and eatA

When 0.5% NaCl was added externally to the media, CS6 RNA expression was upregulated by 12-fold (***Figure 4.3.13.***) when compared to untreated ETEC in the media (Control). Similarly an upregulation of 8-fold was seen in CS5 expression at 0.5% NaCl (***Figure 4.3.14.***). EatA was upregulated by 3-fold in the same concentration as compared to control.

Production of CS6 protein was increased by 13-fold in presence of 0.5% NaCl whereas induction in CS5 expression was similar to that of RNA expression. In 0.5% NaCl EatA production was increased by approximately 4 fold (***Figure 4.3.15.***).

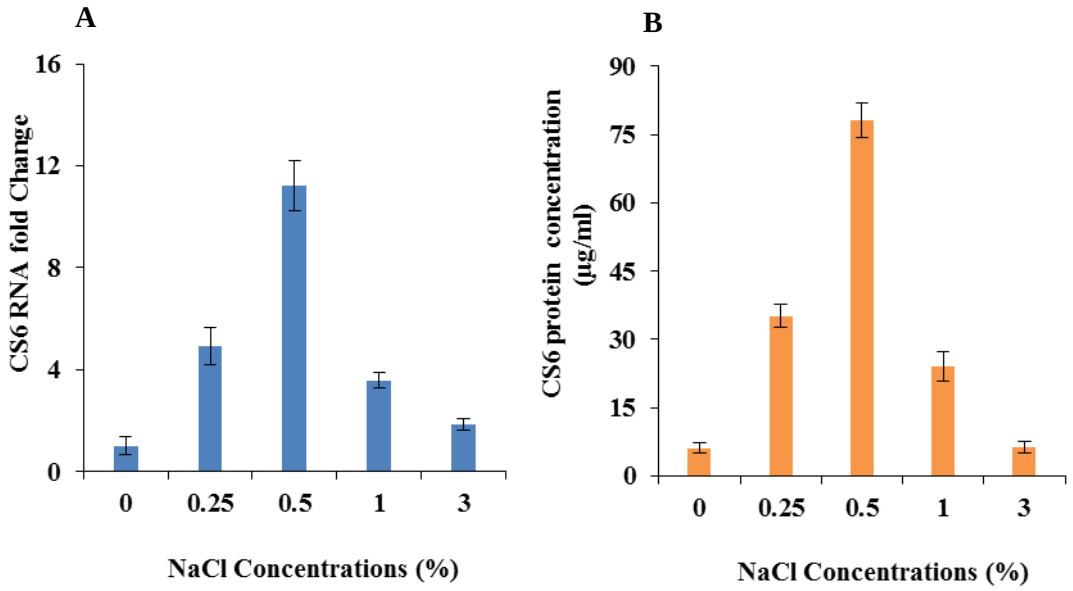


Figure 4.3.13. Effect of NaCl on ***CS6*** expression. The effect on CS6 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

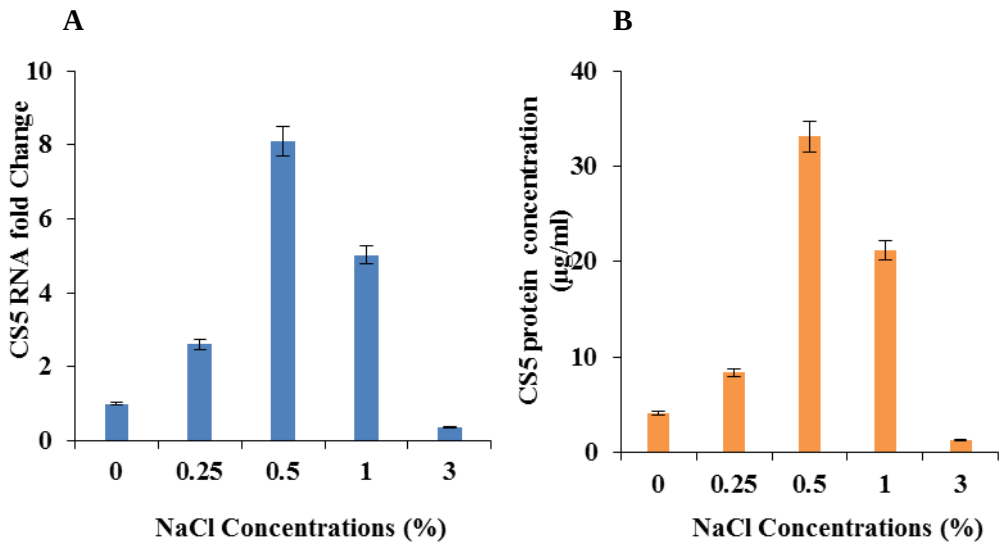


Figure 4.3.14. Effect of NaCl on ***CS5*** expression. The effect on CS5 expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

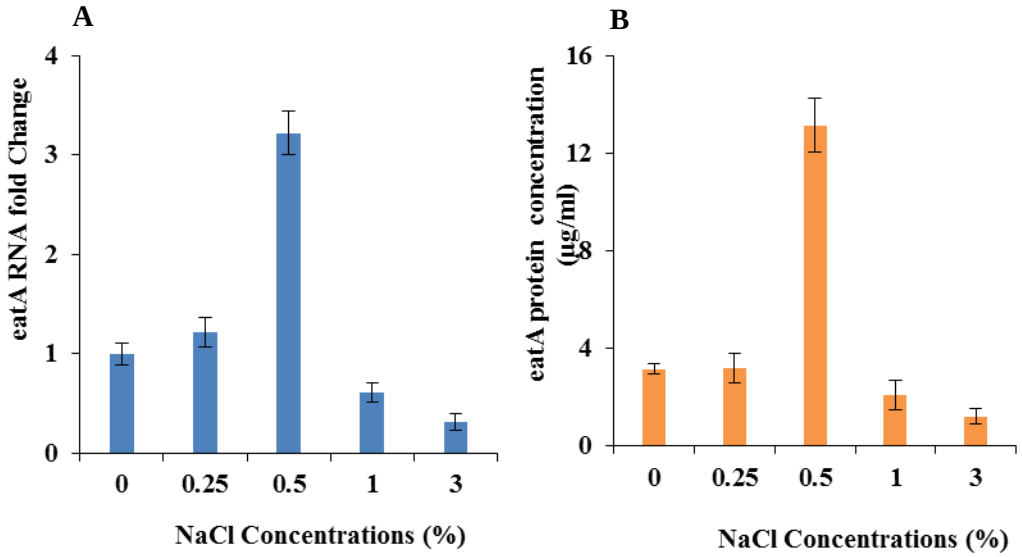


Figure 4.3.15. Effect of NaCl on ***eatA*** expression. The effect on eatA expression was studied at (**A**) RNA level by quantitative RT-PCR, (**B**) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

4.3.2. Modulation of prevalent colonization factor CS6, by Sodium Chloride (NaCl)

As NaCl was found to be one of the regulatory factors to induce CS6 and availability of NaCl is abundant in gut as well as body fluid we further investigated effect of NaCl on CS6 in detail.

A. Modulation of CS6 in representative strains by NaCl

We selected 10 representative strains including the lab reference strain to observe level of induction of CS6 expression in presence of 0.5% NaCl concentration in LB media. It was found that CS6 in all strains except one were induced significantly in presence of NaCl. Level of induction varied from 5-fold to 16-fold in some strains (*Figure 4.3.2.1.*).

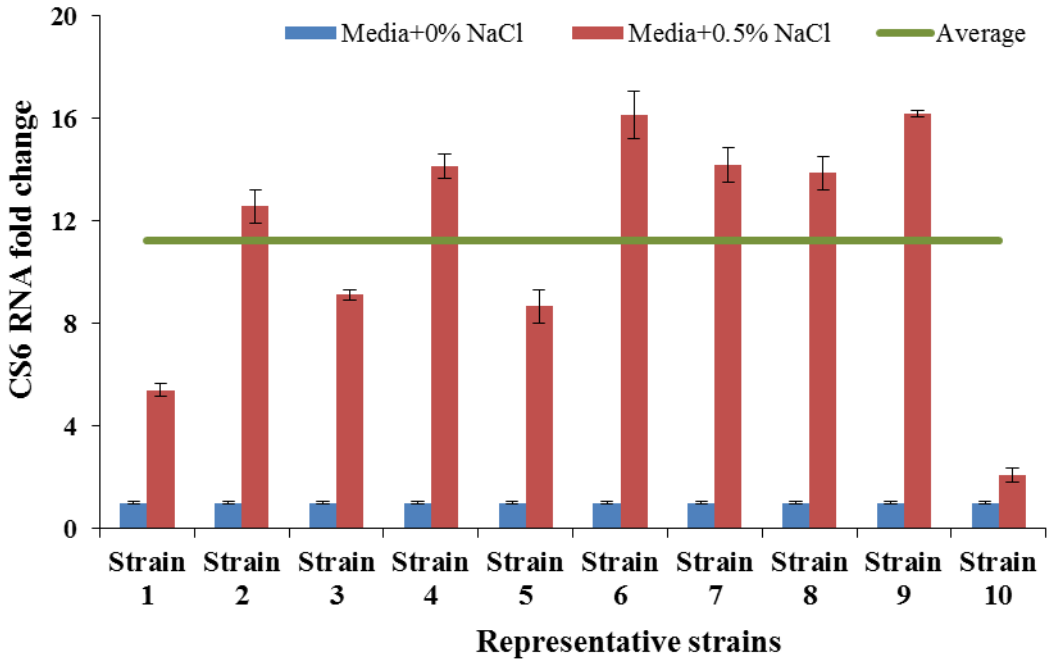


Figure 4.3.2.1. Effect of NaCl on **CS6** of representative strains. Here 0.5% NaCl was added to the media for experiment.

B. Phenotypic surface expression of CS6 is induced by NaCl

Surface expression of CS6 was increased almost 6-times in presence of optimum concentration of NaCl. However further increase in NaCL concentration was coupled with decrease in phenotypic expression of CS6 on ETEC surface (***Figure 4.3.2.2.***).

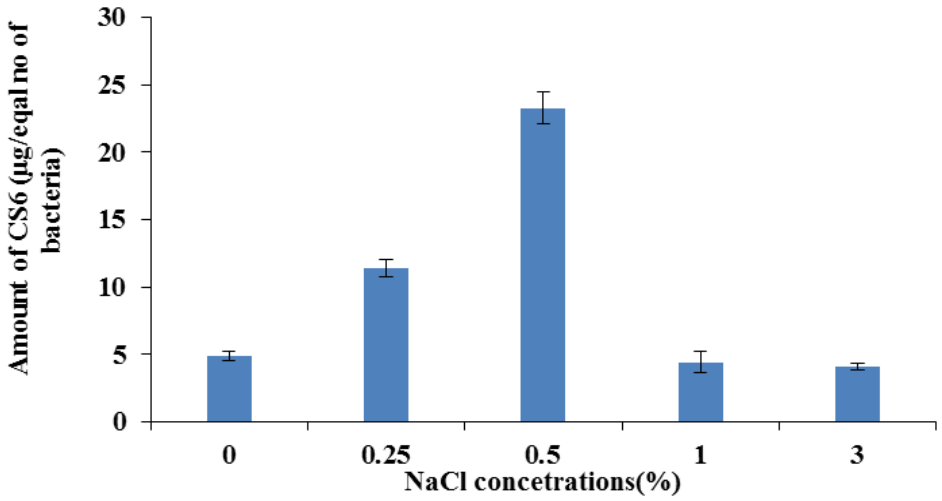


Figure 4.3.2.2. Phenotypic expression of CS6 on ETEC surface in presence of NaCl.

C. CS6 expression is induced post infection with HT-29 cell in presence of NaCl

For ETEC, adhesion to the epithelial layer of the small intestine is a prerequisite for pathogenesis. ETEC grown in LB media were used to infect HT-29 cell and bacteria was recovered post infection in different time point. It was observed that infection with 10^7 CFU/mL bacteria infected for 8 hours gave significant result compared to other time points. It was observed that CS6 from recovered bacteria was induced by 5-fold in presence of 0.5% NaCl. Whereas in 1% and 3% NaCl concentration CS6 expression was significantly reduced as compared to control where no NaCl was added **(*Figure 4.3.2.3.*).**

Further, we investigated whether ETEC infection to HT-29 cell in presence of NaCL induced adherence of bacteria to the cultured epithelial cell. We found that optimum level of NaCl significantly induced adherence of ETEC to the epithelial cell surface. Higher concentration of NaCL like 3% reduced the number of adhered bacteria to the HT-29 cell surface **(*Figure 4.3.2.4.*)**.

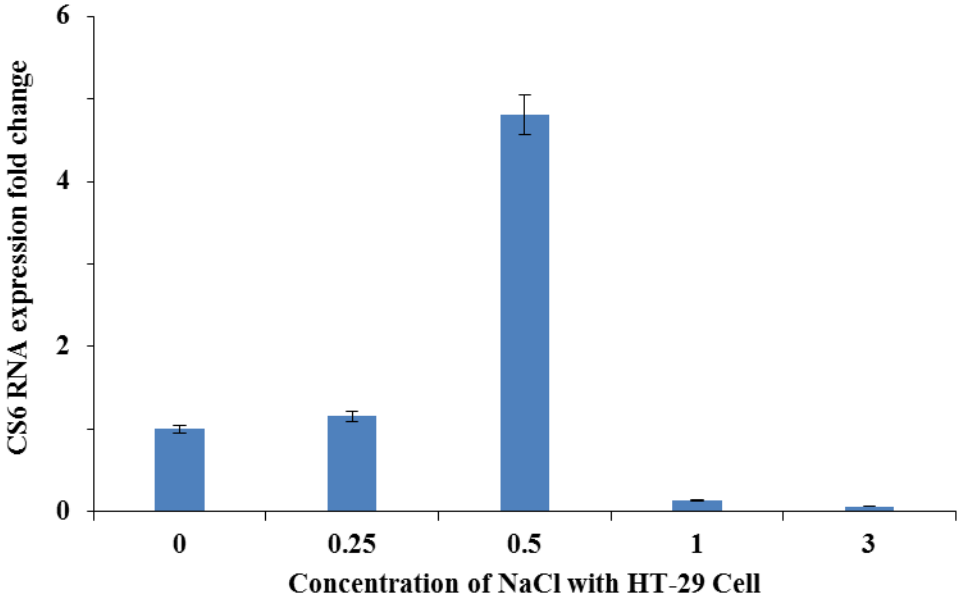


Figure 4.3.2.3. ***CS6*** expression in different NaCl concentrations post infection with HT-29 cell

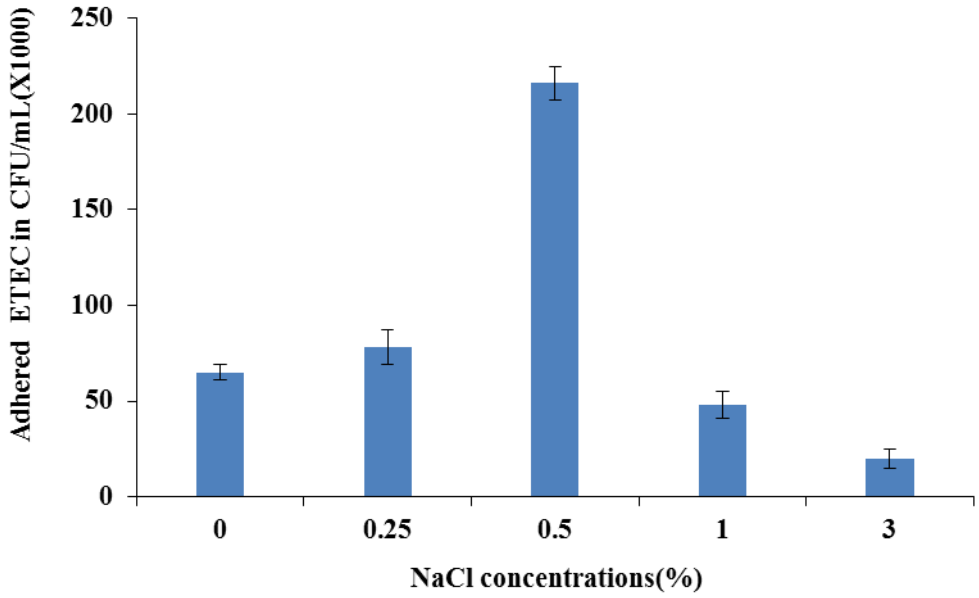


Figure 4.3.2.4. Adhered ETEC to epithelial cell in different NaCl concentrations post-infection with HT-29 cell

D. Effect of NaCL on CS6 promoter activity

Previous work in our lab predicted CS6 promoter using bioinformatics 'softberry bacterial promoter prediction'. Upon prediction, we cloned that part in a promoter-less plasmid, pTL61T and check for promoter activity. For negative control, the empty plasmid was introduced into strains. Deletion of the promoter was also prepared. The promoter constructs were transformed into ETEC test strains and relative β-Galactosidase activity expressed in miller units and was analysed for promoter activity. Promoter activity upon deletion estimated that the region -350bp to -255bp holds the key promoter sequence for the CS6 gene. Activity level of this region was investigated in samples of bacteria grown in presence of NaCl. It was found that CS6 promoter transcription was upregulated by 8-fold as compared to control in presence of 0.5% NaCl ***(Figure 4.3.2.5.)***.

Result calculated in miller units = (ETEC+CS6 promoter in Plasmid) – (ETEC+ Promoter less plasmid)

Control in miller units = ETEC promoter less plasmid

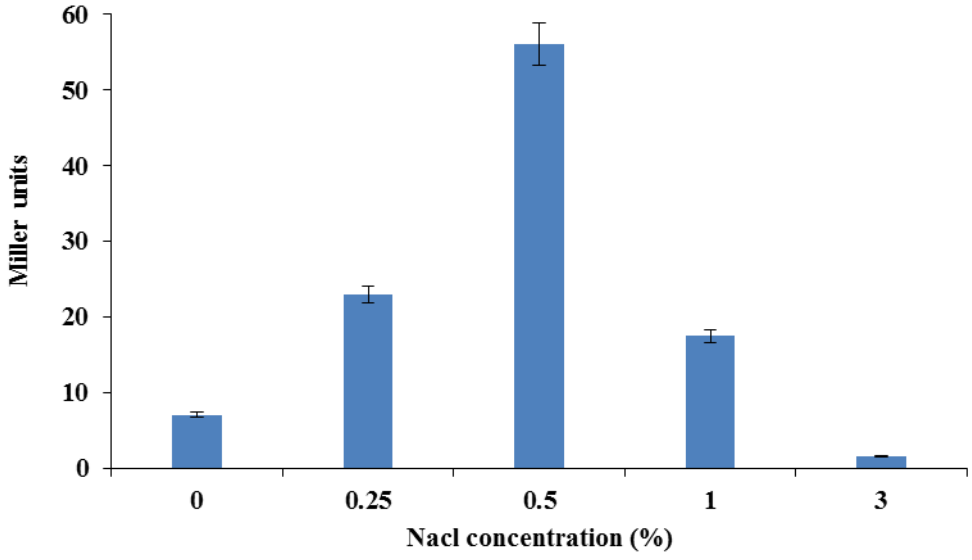
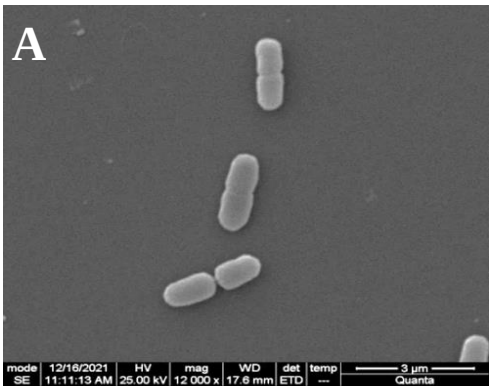
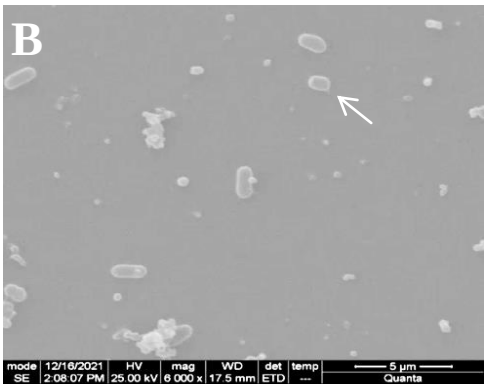


Figure 4.3.2.5. Expression of predicted ***CS6*** promoter in promoter-less plasmid by β-galactosidase assay

E. Morphological changes of ETEC due to NaCl

Whether the change in CS6 expression was attributed to morphological changes of ETEC was investigated by using Scanning Electron Microscopy (SEM). Structural integrity of ETEC was there when ETEC was grown in M9 minimal media. But with addition of NaCl the structure of bacterium changes to a more spherical shape rather that rod shape. Further increase in NaCl concentration lead to complete spherical shape of bacterium.





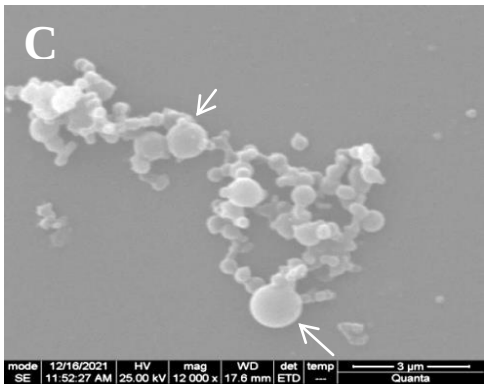


Figure A: Elongated ETEC cells seen under microscope without any external treatment.
Figure B: When cells were treated with **1%** NaCl few cells were seen with morphologically round in shape.
Figure C: Most of the cells became round in morphology when cells were treated with **3%** NaCl.
(↑ indicates ETEC bacterium)

F. Animal Model Experiments

In vivo adhesion of bacterial strain grown in presence of NaCl was evaluated in Rabbit ileal loop assay.

Rabbit ileal loop assay: In young New Zealand white rabbits, the Rabbit ileal loop assay (***Figure 4.3.2.6.***) was performed. ETEC strains grown in the presence and absence of varying concentrations of NaCl were inoculated in rabbit ileum. As negative control, a PBS-inoculated loop was employed. After incubation accumulated fluid was collected and FA ratio was calculated (***Figure 4.3.2.7.***).

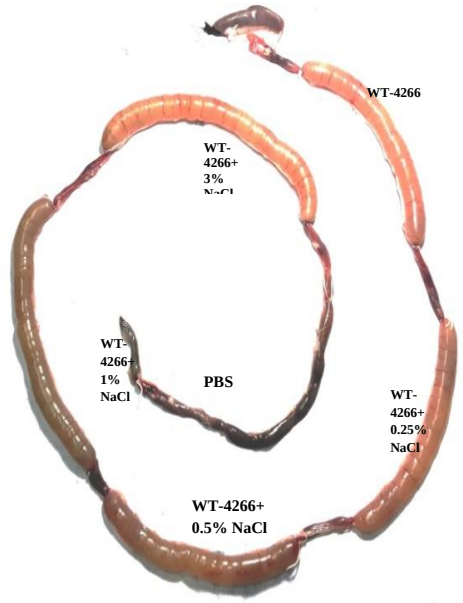


Figure 4.3.2.6. Rabbit ileal loop assay to evaluate enterotoxigenic activity of ETEC at different iron concentrations. Rabbit ileal loops were inoculated with 10^7 c.f.u/mL of ETEC strain (WT) and incubated for 18 h. PBS strain was used as control

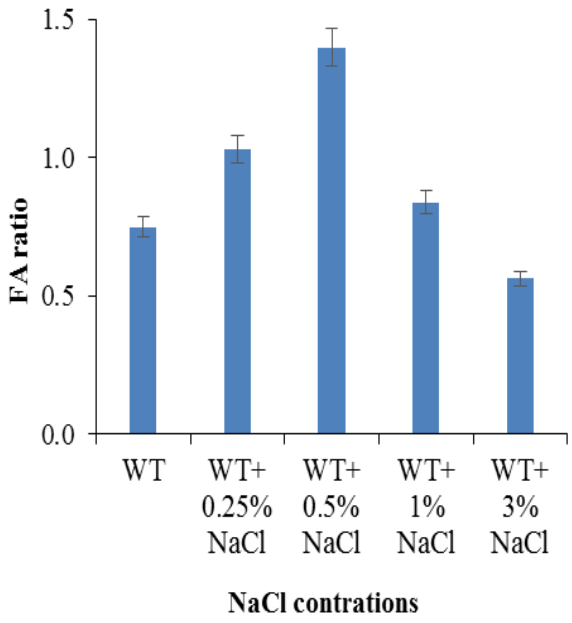


Figure 4.3.2.7. Analysis of fluid accumulation of WT ETEC strain in presence of different concentrations of NaCl in Rabbit ileal loop assay. The results are reported as fluid accumulation (FA) (in millilitres) per loop length (in centimeters). The mean ± SEM are shown; n = 3.

After 18 hours of incubation, and intestinal loop sections were washed three times in PBS, homogenised, and serially diluted in PBS. Introducing these bacterial cultures on MacConkey agar plates yielded the adhering bacterial count. When ETEC was grown in presence of 0.5% NaCl then adherence increased by 2 –fold whereas in

presence of 3% NaCl the adherence was almost equal in comparison to the untreated condition (***Figure 4.3.2.8.***).

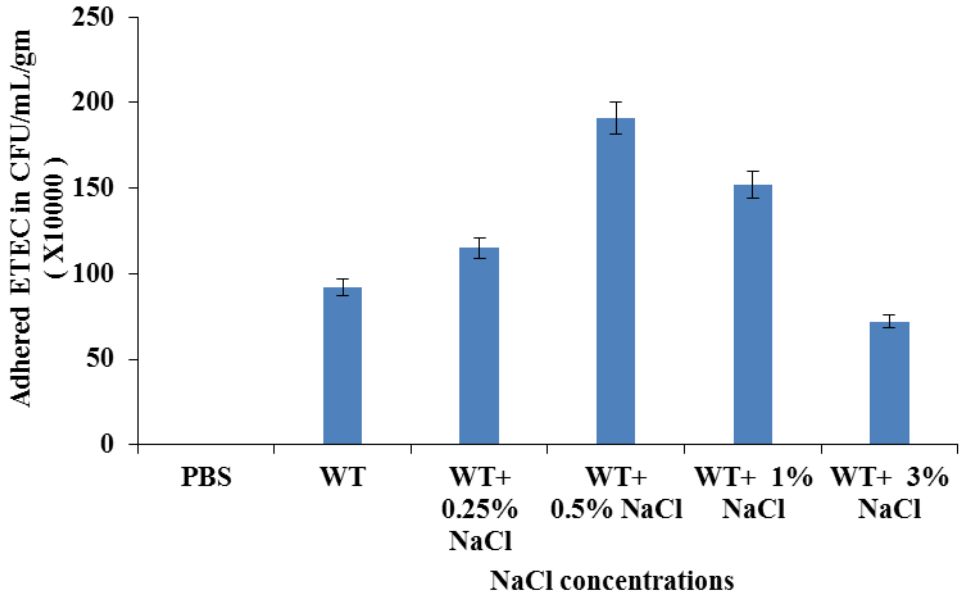


Figure 4.3.2.8. Effect of NaCL on ETEC adherence to the rabbit ileal epithelium by ligated rabbit ileal loop assay.

4.3.3. Effect of Global regulator H-NS on expression of CS6

The Histone-like Nucleoid Structuring (H-NS) factor is a component of bacterial nucleoprotein that has been incorporated into the virulence gene regulatory networks of many bacteria influencing gene expression on a global scale(Navarre *et al.*, 2006). So we investigated whether H-NS acts as a regulator in expression of CS6. For this we made knockout of H-NS gene by λ-Red recombination method from a strain that harboured CS6. Firstly we detected the time point where H-NS expressed the most. After 8 hour of infection CS6 was measured in WT as well as in ΔH-NS to compare with by q-RT PCR, We found that CS6 expression was increased by 16-fold in ΔH-NS condition compared to WT. This indicates that H-NS acts as a negative regulator of CS6.

4.3.4. Modulation of the expression of CS6 in a dysregulated condition of CS5 and eatA in the presence of NaCl, Glucose and components of bile

In our study we found CS6 as the prevalent colonization factor in this region and mostly CS6 occur with CS5 and eatA. So we investigated whether these virulence

factors effect expression of CS6 in presence of regulatory factors like NaCl, Glucose and components of bile. We knockout CS5 and eatA gene by λ-Red recombination method from a strain that harboured CS6, CS5 and eatA and prepare the following mutant strains –ΔCS5, ΔeatA and ΔCS5ΔeatA strain harbouring CS6. We further tested the effect of regulatory factors in those strains for production of CS6 by qRT-PCR. For the experiments, HT 29 cells were infected with ETEC wild type (WT) strains (strain harbouring CS6, CS5 and EatA) and mutants at 10^7 cfu/ml and different additives. We compare the conditions at which we get the maximum expression to the condition closely resembling the normal human physiological concentrations.

- ❖ Effect of NaCL

In WT as well as in all mutant strains expression of CS6 was upregulated in when 0.5% NaCL was added. In ΔCS5 (CS6+eatA) condition , the expression of CS6 was almost similar to that of WT but in ΔeatA (CS6+CS5) condition CS6 expression downregulated by 3-fold and in double mutant condition or ΔCS5ΔeatA (CS6 only) condition CS6 expression was the lowest. Similar pattern of Change in expression of CS6 was seen in protein lysate analysis. Here also wild type strain showed maximum expression of CS6 as compared to all other mutant condition (***Figure 4.3.4.1.)***.

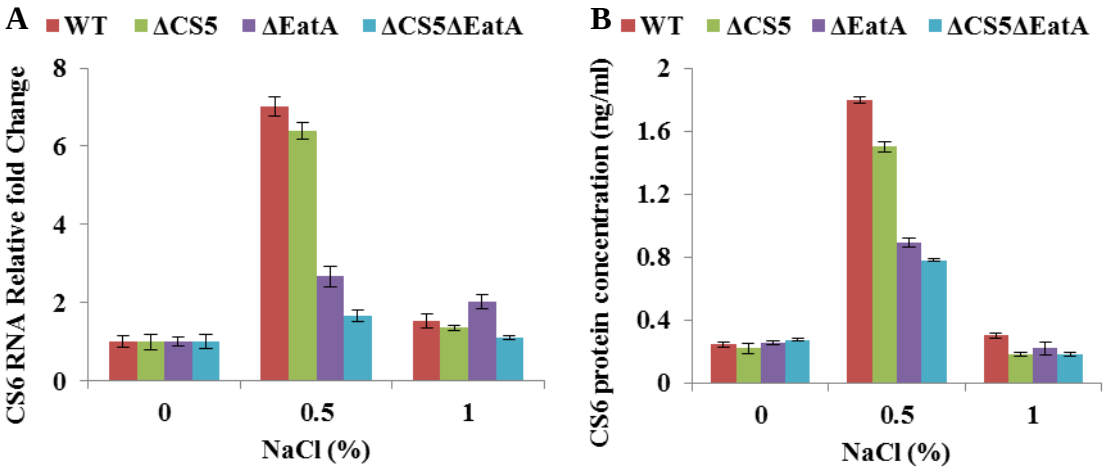


Figure 4.3.4.1. Effect of NaCL on CS6 expression in WT (wild-type) strain and different mutant strains. The effect on CS6 expression was studied in HT-29 tissue culture at (A) RNA level by quantitative RT-PCR, (B) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

❖ Effect of Glucose

In presence of 0.1% glucose in medium CS6 expression was the maximum in ΔeatA condition in comparison with wild type and other mutant conditions. In the double mutant condition, CS6 expression was the lowest (***Figure 4.3.4.2.***)The protein production coincides with the RNA expression results.

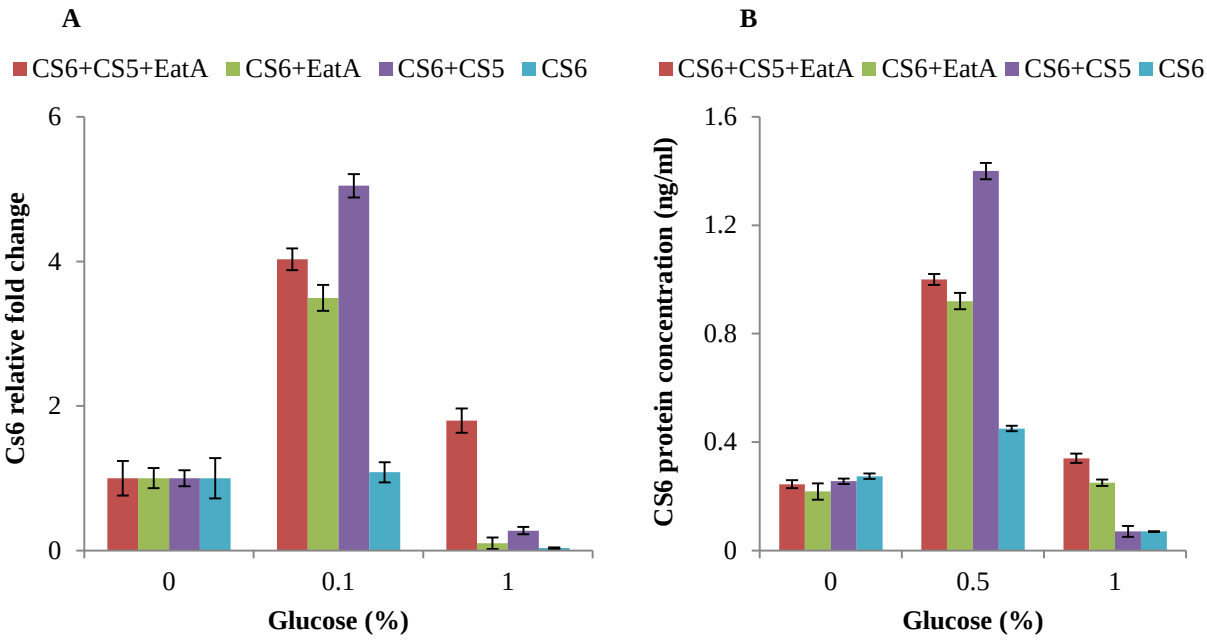


Figure 4.3.4.2. Effect of Glucose on CS6 expression in WT (wild-type) strain and different mutant strains. The effect on CS6 expression was studied in HT-29 tissue culture at (A) RNA level by quantitative RT-PCR, (B) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

❖ Effect of Bile components

As CS6 expression is independent of bile we investigated modulation of CS6 expression in presence of different bile components like Taurocholic acid sodium salt hydrate (TCA) salt and Sodium chenodeoxycholate (NaCDC) salt.

A. Effect of Taurocholic acid sodium salt hydrate (TCA) salt

CS6 expression in WT strain was maximum in presence of 0.1% TCA salt, a component of bile as compared to other mutant conditions. Whereas in ΔCS5, ΔEatA, and double mutant ΔCS5ΔEatA condition expression of CS6 gradually decreases. In protein expression also similar pattern of CS6 expression was observed (***Figure 4.3.4.3.)***.

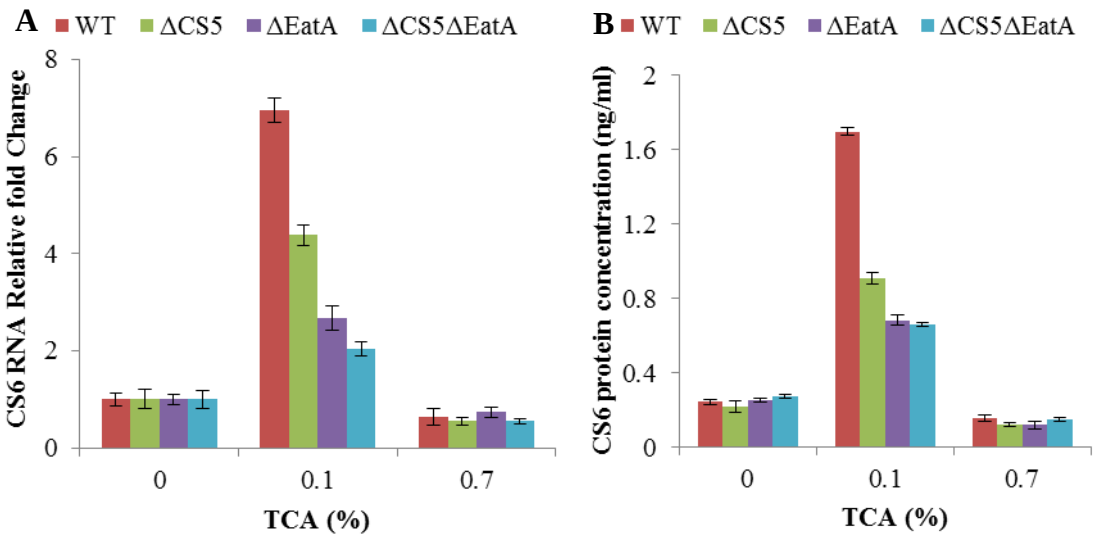


Figure 4.3.4.3. Effect of TCA on CS6 expression in WT (wild-type) strain and different mutant strains. The effect on CS6 expression was studied in HT-29 tissue culture at (A) RNA level by quantitative RT-PCR, (B) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

B. Effect of Sodium chenodeoxycholate (NaCDC) salt

In wild type strain, CS6 expressed highest in presence of 0.1% Sodium chenodeoxycholate (NaCDC) salt, a component of bile. In the dysregulated condition of CS6 like ΔCS5 ΔEatA CS6 expression was decreased by 3.5 fold (***Figure 4.3.4.3.***).

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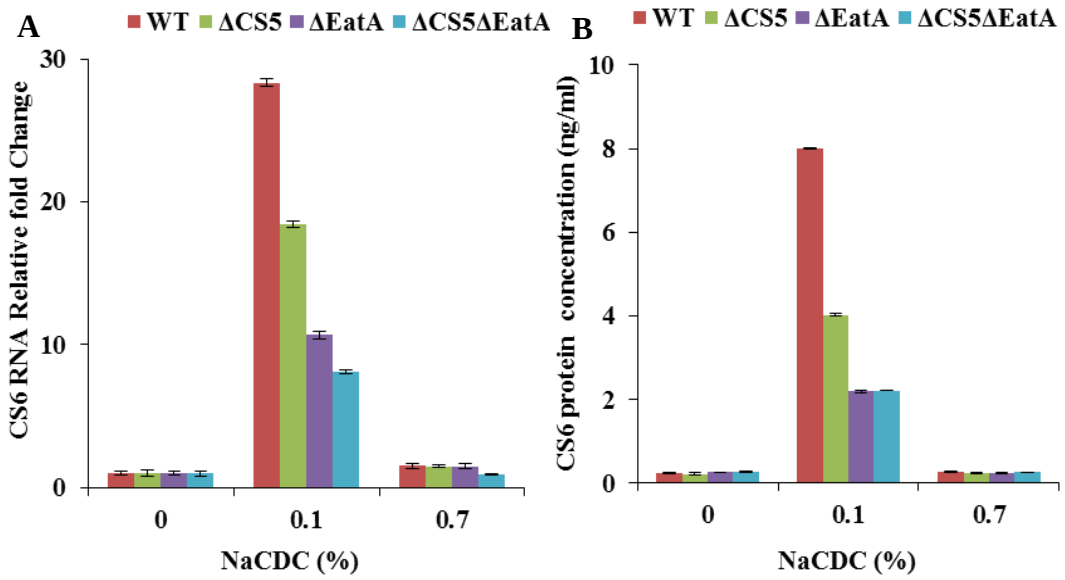


Figure 4.3.4.3. Effect of NaCDCon CS6 expression in WT (wild-type) strain and different mutant strains. The effect on CS6 expression was studied in HT-29 tissue culture at (A) RNA level by quantitative RT-PCR, (B) protein level by ELISA. The data was provided as mean ± SEM after each experiment was conducted three times (n=3).

CHAPTER 5

Discussion

Among *Escherichia coli* pathotypes, enterotoxigenic *E. coli* (ETEC) is the most common cause of diarrheal illness in children under 5 years of age and adults in the developing world and in travellers to these areas (Black, 1990). According to WHO reports ETEC is attributable to 280-400 million diarrheal episodes in children below the age of 5 years and 74,100 deaths annually. Despite the fact that mortality has decreased in recent decades, morbidity has not (Hosangadi *et al.*, 2019). Additionally, the bacterium also causes 44 million cases and 89,000 deaths in older children & adults. ETEC causes 8.5 million Disability Adjusted Life Years (DALYs) or one million Years Lived with Disability (YLDs) (Arduino and Dupont, 1993).

Pathogenicity of ETEC depends on the presence of several virulence factors such as heat-labile and heat-stable enterotoxins, as well as colonization factors (CFs). So far, there are more than 25 CFs identified in ETEC (Qadri *et al.*, 2005). Colonization is mediated by the interaction of CFs with their cognate receptors on the intestinal epithelium to enable the bacteria to withstand intestinal peristalsis and initiate the infection. This is followed by release of enterotoxins thereby initiating pathogenesis.

Generally oral rehydration therapy is the most common mode of treatment of ETEC diarrhea when the infection is mild to moderate. Oral medication as well as hospitalization is also needed in severe dehydration cases. Even in some cases among children the severe dehydration is followed by sad untimely demise. On the other hand vaccination may be a feasible and more accurate course of action for prevention of the disease prior to the onset of severity. However variability among ETEC virulence factors made this approach more evidence based and also limited the fast progress. To overcome this problem proper knowledge of distribution of virulence factor is needed the most.

In order to find the distribution of such virulence determinants, ETEC isolates from 2015-2019 were screened for their toxin genes, Classical virulence factors (CF) and Non- Classical virulence factors (NCVFs). When toxin genes in clinical ETEC strains were investigated we observed that presence of both the toxin genes, i.e., LT+ST ETEC strains the most common one followed by ST only strains and LT only strains. This pattern was comparable to a study in Bangladesh where presence of both the toxin genes were the maximum (Begum *et al.*, 2014). Previous study in our

lab also indicated the similar pattern of toxin presence in clinical ETEC isolates (Bhakat *et al.,* 2019). In contrast globally LT-ETEC is found more frequently than ST alone or in combination with LT. A study revealed that 60% of the strains expressed LT where LT alone was in 27% of strains or in combination with ST (33%) (Isidean *et al.,* 2011).However a study in Kenya reported higher frequency of ST alone strains followed by LT-only and LT+ST strains (Kipkirui *et al.,* 2021). This result was also in accordance to the findings of Indonesia where ST toxin was the most commonly isolated toxin type (Subekti *et al.,* 2003). Another study in Shanghai found ST was the major type of toxin followed by LT and least common was LT+ST harbouring ETEC (Tang *et al.,* 2015). Global enteric multicentre study (GEMS) on Asia and Africa reported 68% were either ST only or LT/ST ETEC strains (Vidal *et al.,* 2019). In contrast study in Colombia revealed LT as the prevalent toxin followed by ST and both LT and ST (Guerra *et al.,* 2014). This result is also close to findings in Peruvian Children where 52% strains harboured LT toxin followed by ST-only strains (25%) (Rivera *et al.,* 2010). The variation between our findings and other studies can be attributable to different geographical region, target population and sample volume.

Studies related to ETEC strains are often done from children below the age of 5 years as evident from GEMS study conducted in children <59 months in years in Asia and Africa (Kotloff *et al.,* 2013). Similar to this in our study we found more ETEC cases in children below 5 years of age. A higher number of patients with ETEC infection belonging to age group >5 years was also found in a study in Shenzen, china (Li *et al.,* 2017). A previous study by Bhakat et al showed the presence of ETEC is almost similar in <5 years of age and >5 years of age group (Bhakat *et al.,* 2019). Though globally ETEC is mainly associated with <5years of age group, higher age groups are also vulnerable to this disease. We also observed that lower age group patients with ETEC were mostly associated with no diarrhea whereas higher age groups were associated with moderate to severe diarrhea. Though medications are mainly targeted towards ETEC infection in children, adults should also be kept in consideration for proper medication for a broader aspect of immunity against this pathogen.

In our study we found the result trends for presence of at least one CF present in 91% of strains which can be compared to the frequencies of 23-94% isolates positive

for CF reported in a systematic review done by Isidean *et al.,* 2011 (Isidean *et al.,* 2011). In Bangladesh, 56% of the isolates tested positive for the CFs (Qadri *et al.,* 2000). Another study in Bangladesh revealed that 49% of the ETEC isolates were positive for CF (Begum *et al.,* 2014). Contemporary another study on Nicaraguan children reported this frequency at 50% of ETEC strains (Vilchez *et al.,* 2014). In 2017 a study in Shenzhen, China, found that 54% of ETEC isolates had one or more CF present in ETEC strains (Li *et al.,* 2017). In 53.3 % of ETEC strains isolated from children, CF genes were found and 21.1% in controls from Teresina/PI, Brazil (Nunes *et al.,* 2011). Previous study in our lab also detected 56% of ETEC strains with at least one CF present (Bhakat *et al.,* 2019). In Peruvian children with ETEC, CFs were detected in 64% of diarrheal samples and 37% of control samples (Rivera *et al.,* 2013). The high presence of CF in ETEC isolates in our study could be due to inclusion of 24 CFs which accounted for most of the strains detected for CFs.

In our study CS6 was distributed predominantly among the ETEC isolates followed by CFA/I and CS21. Similarly an earlier analysis, our findings from Kolkata strains between 2008-2014, CS21, and CS6 were the predominant CFs (Bhakat *et al.,* 2019). Previous study in Kolkata region detected CS6 as the most common CF (Dutta *et al.,* 2013; Ghosal *et al.,* 2007). A similar trend in the presence of CS6 was also observed in China and Guatemala (Li *et al.,* 2017; Torres *et al.,* 2015). CS6 was also the prevalent CF detected from ETEC cases for traveller's diarrhea (Rivera *et al.,* 2013). Our revelations are also in accordance with GEMS discovery from studies conducted on Asian and African countries where CFA/I as well as CS1-CS6 are some major CF antigens (Kotloff *et al.,* 2013). In rural Egypt, most of the ETEC strains had CFA/I, followed by CS6 as the predominant CF (Shaheen *et al.,* 2004). A study in Zambia also was in accordance with our result where CS6 is the prevalent CF (Simuyandi *et al.,* 2019). Whereas study in Bali, Indonesia indicated CFA/I as the predominant CF (Subekti *et al.,* 2003). In accordance with our findings, a study on the ETEC population in Nepal revealed that CS21 (62.6%) and CS6 (30.2%) were the most prevalent CFs (Margulieux *et al.,* 2018). A study in Bangladesh detected CS5, CS6, and CS1 as predominant CFs (Begum *et al.,* 2014). In contrast ETEC isolated from Chilean newborns, CS21 was found to be the most common CF (Montero *et al.,* 2017). In our study we mainly found CS6 expressed alone or in combination with CS5 and/or CS4 which is in accordance with findings of Gaastra *et al.,* 1996 and

Wolf *et al.,* 1997 (Gaastra and Svennerholm, 1996; Wolf, 1997). Similarly co-occurrence of CS6 and CS5 was previously reported from our region (Ghosal *et al.,* 2007; Sabui *et al.,* 2012).

Analysis of the distribution of non-classical virulence factors (NCVFs) revealed that three-fourth of the strains contained at least one or more of these factors. Among these EatA was the predominant one followed by EtpA. This pattern was observed in clinical ETEC isolates of Northern Colombia, South America(Guerra *et al.,* 2014). Generally EatA was distinguished in strains harbouring colonization factors (CFs), recommending a significant part to advance intestinal colonization. Similar trend of presence of EatA and colonization factors were also found in Chilean ETEC isolates (Del Canto *et al.,* 2011). However association between EtpA and colonization factors was not found. This might be because of cooperation of EtpA with highly conserved flagellin and accordingly elevating adherence to the intestinal wall (Roy *et al.,* 2009). Other than these two TibA, Tia and LeoA were found to in low frequency in our setting. However, two strains with LeoA were negative for any classical colonization factor genes. This outcome repudiates with the examination from Chile, Colombia, and Guatemala where the greater part of the LeoA strains were negative for any classical genes (Del Canto *et al.,* 2011; Torres *et al.,* 2015).

Among the remaining one-fourth of strains negative for NCVFs, only 10 strains were negative for known CFs. There were only 4 % of CF negative strains that were positive for NCVF i.e.: Tia, TibA, and EtpA. So, despite inclusion of most of the discovered CFs and NCVFs, we were unable to detect any factor responsible for colonization in 10 strains suggesting that there may be additional colonization determinants yet to be perceived. In contrast only 56% of ETEC isolates during a 2-year study period in Bangladesh were positive for the CFs included in that study (Qadri *et al.,* 2000). Similarly in Shenzen, China, 46% of strains lacked any evaluated CFs(Li *et al.,* 2017). In Chilean study classical and non-classical adhesins were found to be negative in 16% of ETEC strains (Del Canto *et al.,* 2011). This disparity in results may be attributed to inclusion of more number of CFs in study as well as population of ETEC cases.

Overall we found CS6 and CS5 as the most prevalent combination of virulence factors along with EatA. This combination of virulence factors are found to be

consistent with the years as it was found in our previous study with the ETEC isolates from 2008 to 2014. This trend indicates that we should track distribution of these factors for successful vaccine design with a broader coverage.

However the trend of presence of virulence factor genes doesn't imply with expression similarly. When we looked into the expression of those genes we found that not all genes present were expressed. We found that 53% strains harbouring CS6, expressed their gene. The GEMS study reported that among the CS6-only isolates only 38.5% were positive for phenotypic expression by dot-blot(Vidal *et al.,* 2019). We also found CS6 to be co expressed with CS5 more frequently which is in accordance with a study on Egyptian children where CS5 and CS6 were expressed commonly (Shaheen *et al.,* 2009). Phenotypic expression of CS6 depends on the presence of a functional chaperone component IV which protects the structural subunit of CS6 from degradation in the periplasm during translocation to the bacterial surface (Wolf, 1997). This lack of expression in comparison to the number of CS6 present in ETEC isolates may be due to in-vitro growth conditions that may not trigger the expression to the full extent. This was also evidenced from experiments where certain external trigger through supplementation in the media led to the expression of non-expressive CS6 in ETEC isolates. Single nucleotide polymorphisms, minor alterations in structural or chaperone genes, or lower copy number plasmids may all allow PCR amplification but diminish or abolish CF expression in isolates that are PCR positive but expression negative. This notion is supported by the fact that a single point mutation in the untranslated region upstream of the chaperone encoding gene caused CS6 phenotypic expression to be negative in CS6 genotypically positive ETEC strains. The function of this mutation is unknown, however it could be involved in the chaperone subunit's ribosome binding and subsequent translation (Tobias *et al.,* 2008).

Characterization of the antibiotic susceptibility profile of ETEC revealed a high rate of antimicrobial resistance to frequently used antibiotics such as ampicillin (68%), Azithromycin (86%) and Ciprofloxacin (64%). One striking revelation was that almost all strains were resistant to one or more drugs included in this study. A similar study in Bolivia reported higher frequency of resistance against ampicillin (Rodas *et al.,* 2011). Our result also coincided with a study on Peruvian children that showed 64% of ETEC strains were resistant to ampicillin (Medina *et al.,* 2015). In

this investigation, we observed that more than 60% of CS6 harbouring ETEC isolates of this region showed resistance towards nalidixic acid, azithromycin, and tetracycline which was also observed in ETEC strains of Bangladesh showing resistance to azithromycin (78.26%), nalidixic acid (73.19%) tetracycline (65.21%), ampicillin (60.86%) (Rahman *et al.,* 2020). Among all the antibiotics we found imipenem as the sole antibiotic against which no bacteria was resistant. Similar result was observed in a study in Odisha, India where only 2% strains were resistant to imipenem constituting the lowest frequency among other antibiotics (Moharana *et al.,* 2019). Due to the increased AMR, new antimicrobial agents such as azithromycin have been used as the first-line agent for ETEC infection treatment. azithromycin is a broad-spectrum macrolide antimicrobial agent against several bacterial species, and is very effective for Enterobacteriaceae infection treatment (Gomes *et al.,* 2019). In contrast almost 86% strains showed resistance against azithromycin. Similar to our result high frequency of azithromycin resistant ETEC strains were detected in a study in Sanghai, China (Xiang *et al.,* 2020). Whereas a low frequency of 10-30% azithromycin resistant strains were detected in a recent study in 2019 (Guiral *et al.,* 2019). Our results are in accordance with a previous study in India where ETEC resistance to ciprofloxacin was reported (Chakraborty *et al.,* 2001). Our result also suggests that resistance to quinolones and third-generation cephalosporins has increased in ETEC strains of this region. A similar observation was also revealed by Guiral *et al.,* 2019. High resistance against nalidixic acid was evident in a study in Bangladesh which is in accordance with our result (Begum *et al.,* 2016). In summary our results strengthen the message that multidrug resistant ETEC strains are rising in every parts of the world. This is may be due to variation of food choices available worldwide as well as increase in over the counter drug use. This also indicates that treatment with antibiotics should be well studied before effective administration and region based variation as well as reduced susceptibility to many drugs of choice made the study of antimicrobial resistance profiling more important than ever.

ETEC bacterium may contain multiple virulence factors but all it need is one expressive virulence factor and toxin to successfully exert pathogenesis. So it becomes so important to answer how the virulence factors are regulated in the microenvironment of the site of infection. ETEC is often called as sister organism of

vibrio cholerae which has been thoroughly studied (Lee *et al.,* 1999; Sánchez and Holmgren, 2005) but there is abundant knowledge gap when it comes to regulation of virulence factors of ETEC genes. On the basis of those observations our thesis aimed to unravel effect of different regulatory factors on colonization factors CS6 and CS5, and non-classical virulence factor EatA which are most common for pathophysiological analysis of ETEC in this region with a special emphasis on CS6 as it holds the position for most prevalent virulence factors in more than 10 years of epidemiological study in this region. Host microenvironment is set up by healthy gut microbiota and host immune system, as well as environmental factors (Sekirov *et al.,* 2010). So the interaction between host and pathogen becomes important and the effect of different factors as well as identifying more regulatory factors becomes crucial in diagnosing the extent of pathogenesis. In this study we identified several factors assuming that they might have an effect on virulence factors of ETEC. This study showed that CS6, CS5 and EatA expression is modulated by bicarbonate, different SCFAs and NaCl.

ETEC enters the human body through contamination of water and other food substances. Upon entering they encounter a range of environmental changes. To begin with these change a shift in temperature of 37°C, the acidic environment in stomach. After evasion of those changes, the main play begins in the small intestine where they colonize for successful pathogenesis. Here the acidic pH is neutralized by sodium bicarbonate dissolved in a solution secreted by pancreas and intestinal mucosa (Feldman *et al.,* 2020). In our study we found sodium bicarbonate to significantly modulate expression of CS6 but not CS5. This non induction of CS5 expression is consistent with another study done in Bangladesh (Nicklasson *et al.,* 2012). Sodium bicarbonate has shown to induce virulence associated genes in *V. cholerae* as well as in some other bacteria (Abe *et al.,* 2002; Abuaita *et al.,* 2009). In our study we also found EatA, an associated protein for virulence, is enhanced by bicarbonate. Bicarbonate ions (HCO_3^-) in the intestinal tract contributes to the buffering of extracellular fluids in equilibrium with CO_2, the concentration of the ion in the ileum is greater than that in the jejunum, and thus the HCO_3^- ion concentration increases along the intestinal tract (Wrong, 1981). Consistent with this notion our results of a varied activity of virulence genes indicate the effect of

bicarbonate modulating expression of CS6. From this study it may be concluded that expression of CS6 is more closely associated with EatA than CS5.

However despite the fact that different host and environmental factors regulate virulence expression in ETEC, no unique positive regulator of CS6 has been detected (Nicklasson *et al.,* 2012). Gut microbiota are the collective community of billions of bacteria residing within human gut, also sometimes called as virtual organ of human body(Bull; Rath and Dorrestein, 2012). Collectively they produce thousands of metabolites, which sometimes replace many host functions that in turn influence the host fitness phenotype, and health (Vyas *et al.,* 2012). Available non-digestible carbohydrates (NDC), endogenous intestinal mucus serves as food to the gut microbiota. The primary end products of fermentation of NDCs are the Short Chain Fatty Acids (SCFAs) (Wong *et al.,* 2006). SCFAs are mainly produced by saccharolytic fermentation of fibres (e.g., resistant starch, polysaccharides and simple sugars), which escape digestion and absorption (Topping and Clifton, 2001). The discovery that SCFA appear to be the natural ligands for free fatty acid receptor 2 and 3 (FFAR 2/3), found on a wide range of cell types, including enteroendocrine and immune cells, has led to renewed interest in the role of SCFA in human health (Le Poul *et al.,* 2003; Nilsson *et al.,* 2003). Main SCFAs produced are Acetate, Propionate and Butyrate are found to be playing major role in maintaining healthy gut(Nogal *et al.,* 2021). Among the SCFAs in our study we found that Acetate, Propionate and Butyrate modulates virulence factor expression in different concentration which may be comparable to the modulation of LT toxin in ETEC (TAKASHI *et al.,* 1989). Acetate in our experiment found to be responsible for modulation of CS6 and EatA expression in ETEC which may indicate that it affects the colonization of bacteria followed by hampering the growth. This result seems to be in accordance with a study on bacterial growth inhibition (Pinhal *et al.,* 2019). Prohaszka observed an anti-bacterial effect of SCFAs in enteric *Escherichia coli*-infections of rabbits (Abrams and Bishop, 1966; Prohaszka, 1980). SCFAs also found to regulate the growth and virulence of enteric pathogens, such as enterohemorrhagic *E. coli* (EHEC), *Klebsiella* and *Salmonella* (Zhang *et al.,* 2020). In comparatively low concentrations butyrate found be effective on CS6 expression which indicates that butyrate remain more active than acetate and propionate when produced. A similar result has been observed in as study where protein and

transcriptomic analysis showed expression of virulence genes in EHEC strain Sakai is strongly induced by sodium butyrate but not by sodium acetate or sodium propionate (Nakanishi *et al.,* 2009). Recent studies shows that SCFA propionate and acetate are involved in regulating growth, colonization and virulence of AIEC (Elhenawy *et al.,* 2019; Ormsby *et al.,* 2020). Utilization of acetate has also been proved to enhance colonization of invasive *E. coli* in mice (Elhenawy *et al.,* 2019). In our study we found that propionate at ≥3 mM and acetate ≥10mM supressed expression of virulence factor genes. Similar observation has been reported where growth and virulence of AICE was diminished (Zhang *et al.,* 2020). Results from our study raise the speculation that loss of commensal bacteria due to inflammation would chance luminal microenvironment concurrently. These changes may favour colonization of harmful bacteria as well as associated growth and virulence which in turn could initiate further inflammation in the intestine.

Generally ETEC bacteria senses a variety of environment stimuli such as temperature, pH, salt concentrations, oxygen levels, carbon source and several other compounds (Bachmann *et al.,* 1996; Mekalanos, 1992), and use these cue to regulate their gene expression based on their surroundings. Within the host body the intestinal microenvironment provides the bacteria with different cues and thereby influences pathogenesis. Among these cues sodium chloride (NaCl) has been an important regulatory factor controlling virulence in several organisms. In our study we found NaCl in 0.5% induce virulence expression of CS6, CS5 as well as EatA. This result is in accordance with another study done on *V. cholerae* where expression of cholera toxin, Tcp pili and other virulence factors were found to be affected by osmolarity (Miller and Mekalanos, 1988). We also studied the effect of NaCl ion CS6 in different stages of virulence *in-vitro* and *in-vivo.* We found that 0.5% NaCL concentration induce CS6 expression in both RNA and protein level. Also phenotypic expression of CS6 is induced. Behind this high level of expression of virulence factor gene replication may have some role as bacterial replication is a key factor for host colonization, resource exploitation, and between-host transmission (Rohmer *et al.,* 2011). A similar study using environmentally transmitted bacterial fish pathogen *Flavobacterium columnare* as a model organisms revealed that abundant nutrients in environment could act as a selective pressure for higher virulence and faster evolutionary rate (Kinnula *et al.,* 2017). Another study showed

that nutrient deficiency can diversify population structure and virulence strategies in opportunistic pathogens (Sundberg *et al.*, 2014). Higher concentration of NaCl in our study revealed that it induces the morphological change of ETEC and thereby reduce virulence as well as growth. A study showed that similar result where osmolar condition had negative influence on *E. coli* growth (Li *et al.*, 2021). Another study showed that in 0.5% concentration *E. coli* attainted maximum growth similar to the report in our study (Abdulkarim *et al.*, 2009). Other studies also showed that high level of salinity modulated genes related to virulence factors, capsid proteins, drug resistance genes etc. in *Burkholderia pseudomallei,* which indicated the interlink between bacterial virulence and envioronmental cues (Rubiano-Labrador *et al.*, 2015). A study on *Vibrio alginolyticus* showed thatsalinity stress facilitated the virulence potential of the marine bacterium (Dayma *et al.*, 2015).

ETEC possesses a myriad of regulatory factors controlling its virulence. Among them H-NS (histone-like nucleoid structuring) is considered to be one the global regulatory factors (Mellies and Barron, 2006; Yang *et al.*, 2005). H-NS is a pleiotropic regulator, which binds AT-rich DNA regions blocking the interaction of the RNA polymerase, silencing transcription of housekeeping, and virulence genes (Dorman, 2007). In our study we found that ΔH-NS condition of ETEC produce CS6 in higher level in comparison to WT ETEC indicating role of H-NS as a silencer for CS6 gene in ETEC. This result is in accordance with a study on CS3 where similar effect of H-NS was observed (Ares *et al.*, 2019).

Previous study in our lab showed that the expression of CS6 of WT ETEC harbouring CS5, CS6 and EatA was maximum as compared to CS6 in ΔCS5 and/or ΔEatA condition. This indicates that CS6 expression is influenced by other virulence factors like CS5 and EatA. We further put an insight on how different host compounds affect expression of CS6 in host mutant conditions. We found that in all the compounds like glucose, NaCDC, TCA and NaCl the expression of CS6 was highest in wild type strains despite the changes in supplement condition as compared to mutant condition This result indicate that level CS6 expression is dependent upon presence of other commonly co-occurred CF CS5 and NCVF EatA.

CHAPTER 6

Conclusion & Significance

➢ Conclusion

Our study is aimed to unravel the distribution of most prevalent combination of classical and non-classical virulence factors among clinical ETEC isolates to better understand probable vaccine candidate in this eastern region of India and their relatedness towards antimicrobial response against different antibiotics. Besides this our study focused on effect of different host factors like SCFAs, Bile Salts, Glucose and Salts on the prevalent colonization factors during pathogenesis. Our conclusions obtained from this study are:

- ❖ This study further strengthens the established notion of variation of colonization factors among ETEC isolates.
- ❖ Although children are prone to ETEC infection our results showed that adults also suffer from ETEC infection therefore it is important to include both the age groups, children and adults for management of the infection.
- ❖ The prevalent combination of virulence determinants was CS6+CS5 and EatA along with *elt* and or *esth*. Though CFA/I had emerged as another predominant CF but it had diverse CFs and NCVFs along with it.
- ❖ Previous study in our lab detected 29% isolates (n=350) with no virulence determinants. However expanding the panel of detection by including most of the discovered CFs in this study revealed 97% positivity. Still 3%(n=350) isolates did not tests for any virulence determinants, indicating that there is still same factor responsible for ETEC colonization that are yet to be discovered.
- ❖ Other than CS6, CFA/I also emerged as one of the most prevalent CF in with or without combinations of different CFs and NCVFs.
- ❖ To our understanding, no non-classical virulence gene investigation has ever been conducted in this zone. Our findings suggested that non-classical virulence variables should be included in the ETEC vaccine candidate.
- ❖ ETEC isolates exhibit a wide range of toxins, CFs, and non-classical virulence factors. For successful vaccine development, a mixture of these elements must be considered.
- ❖ In our study we observed that not all ETEC strains expressed their respective virulence factors that they possess within their genome and plasmid.

- We found that genotypic presence of CS6 does vary with its phenotypic expression. This indicates that for a fruitful epidemiological study we should consider genotypic as well as phenotypic approach to completely cover the spread in infectivity of ETEC.
- Vaccine development should also consider more phenotypic approach as it will be the ultimate antigen that ETEC uses to progress infection.
- This work integrates a large amount of experimental and epidemiological data into a genetic and phenotypic perspective, and it serves as a prototype for future diagnostic and intervention efforts.
- Our study also investigated the response of ETEC strains against different antibiotics and surprisingly we found that there were multidrug resistant (MDR) strains.
- This thesis focused on the effect of regulatory factors on prevalent colonization factors taking multiple parameters into consideration in order to gain a better understanding of the pathogenic mechanisms of this heterogeneous enteropathogen. We also expect that our results will provide a better insight to understand the differential expression of the virulence genes in ETEC and how they relate during pathogenesis. This will help as a foundation for developing various intervention strategies in future.
- ETEC encounters different signals within host intestine and it can trigger linked genetic and metabolic pathways. Our experiments with addition of different compounds in the growth media maybe indicative of change in the intestinal environment which sometimes acts advantageous as well as sometime deleterious to the bacteria.
- ETEC has to adapt to the different conditions of the gut that it encounter and has to optimize its virulence mechanisms in a multifactorial process. It is also important to remember that virulence regulation conditions examined in the lab may differ during human infection as we cannot totally mimic the gut microbiome and the physiological conditions.

➢ Significance of this study

We strongly believe that the only way to making an effective vaccine with broader efficacy should contain toxins as well as CS6, CS5 and EatA for this region as this is the only combination found prevalent for a long time in our years of study. Details of the effect of different factors should provide better understanding of the ETEC pathogenesis and how our general daily food intake affects pathogenesis. Molecular details of expression studies will help in better understanding of the ETEC pathogenesis and this knowledge could be translated for effective and safe drug for disrupting regulators of ETEC pathogenesis. This study should reduce the existing knowledge gap in minimising ETEC infection.

CHAPTER 7

References

Abdulkarim, SM, AB Fatimah, JG ;J Journal of Food Agriculture Anderson and Environment 2009. Effect of salt concentrations on the growth of heat-stressed and unstressed *Escherichia coli*. 7: 51-54.

Abe, Hiroyuki, Ichiro Tatsuno, Toru Tobe, Akiko Okutani, Chihiro ;J Infection Sasakawa and immunity 2002. Bicarbonate ion stimulates the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic *Escherichia coli* O157: H7. 70: 3500-3509.

Abrams, Gerald D and Jane E ;J Journal of Bacteriology Bishop 1966. Effect of the normal microbial flora on the resistance of the small intestine to infection. 92: 1604-1608.

Abuaita, Basel H, Jeffrey H ;J Infection Withey and immunity 2009. Bicarbonate induces Vibrio cholerae virulence gene expression by enhancing ToxT activity. 77: 4111-4120.

Akabas, Myles H ;J Journal of Biological Chemistry 2000. Cystic fibrosis transmembrane conductance regulator: structure and function of an epithelial chloride channel. 275: 3729-3732.

Akhtar, Marjahan, Nuder Nower Nizam, Salima Raiyan Basher, Lazina Hossain, Sarmin Akter, Taufiqur Rahman Bhuiyan, Firdausi Qadri and Anna ;J Frontiers in immunology Lundgren 2021. dmLT adjuvant enhances cytokine responses to T cell stimuli, whole cell vaccine antigens and lipopolysaccharide in both adults and infants. 12: 654872.

Al-Gallas, Nazek, Olfa Bahri, Aida Bouratbeen, Assia Ben Haasen, Ridha Ben ;J American Journal of Tropical Medicine Aissa and Hygiene 2007. Etiology of acute diarrhea in children and adults in Tunis, Tunisia, with emphasis on diarrheagenic *Escherichia coli*: prevalence, phenotyping, and molecular epidemiology. 77: 571-582.

Alouf, Joseph E, Daniel Ladant and Michel R Popoff 2005. The comprehensive sourcebook of bacterial protein toxins: Elsevier.

Apperloo-Renkema, HZ, BD Van der Waaij, D ;J Epidemiology Van der Waaij and Infection 1990. Determination of colonization resistance of the digestive tract by biotyping of Enterobacteriaceae. 105: 355-361.

Arduino, Roberto C and Herbert L ;J Baillière's clinical gastroenterology Dupont 1993. Travellers' diarrhea. 7: 365-385.

Ares, Miguel A, Judith Abundes-Gallegos, Diana Rodríguez-Valverde, Leonardo G Panunzi, César Jiménez-Galicia, Ma Dolores Jarillo-Quijada, María Lilia Cedillo, Marìa D Alcántar-Curiel, Javier Torres and Jorge A ;J Frontiers in microbiology Girón 2019. The coli surface antigen CS3 of Enterotoxigenic *Escherichia coli* is differentially regulated by H-NS, CRP, and CpxRA global regulators. 10: 1685.

Bachmann, BJ ;J *Escherichia coli*, Salmonella: cellular and molecular biology 1996. *Escherichia coli* and Salmonella: cellular and molecular biology. 2: 2460-2488.

Bäck, E, AM Svennerholm, J Holmgren and R ;J Journal of Clinical Microbiology Möllby 1979. Evaluation of a ganglioside immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. 10: 791-795.

Begum, Yasmin A, KA Talukder, Ishrat J Azmi, Mohammad Shahnaij, A Sheikh, Salma Sharmin, A-M Svennerholm and Firdausi ;J PloS one Qadri 2016. Resistance pattern and molecular characterization of enterotoxigenic *Escherichia coli* (ETEC) strains isolated in Bangladesh. 11: e0157415.

Begum, Yasmin Ara, Nabilah Ibnat Baby, Abu SG Faruque, Nusrat Jahan, Alejandro Cravioto, Ann-Mari Svennerholm and Firdausi ;J PLoS neglected tropical diseases Qadri 2014. Shift in phenotypic characteristics of enterotoxigenic *Escherichia coli* (ETEC) isolated from diarrheal patients in Bangladesh. 8: e3031.

Bhakat, D, A Debnath, R Naik, G Chowdhury, AK Deb, AK Mukhopadhyay and NS ;J Journal of applied microbiology Chatterjee 2019. Identification of common virulence factors present in enterotoxigenic *Escherichia coli* isolated from diarrheal patients in Kolkata, India. 126: 255-265.

Black, Robert E ;J Reviews of infectious diseases 1990. Epidemiology of travelers' diarrhea and relative importance of various pathogens. 12: S73-S79.

Blattner, Frederick R, Guy Plunkett III, Craig A Bloch, Nicole T Perna, Valerie Burland, Monica Riley, Julio Collado-Vides, Jeremy D Glasner, Christopher K Rode and George F ;J science Mayhew 1997. The complete genome sequence of *Escherichia coli* K-12. 277: 1453-1462.

Bradley Sack, R ;J Reviews of infectious diseases 1990. Travelers' diarrhea: microbiologic bases for prevention and treatment. 12: S59-S63.

Bray, John ;J Journal of Pathology and Bacteriology 1945. Isolation of Antigenically Homogeneous Strains of Bact. coli neapolitanum from Summer Diarrhea of Infants. 57: 239-247.

Brown, AD413986 ;J Bacteriological reviews 1976. Microbial water stress. 40: 803-846.

Brown, Eric A and Philip R ;J Microbiology Hardwidge 2007. Biochemical characterization of the enterotoxigenic *Escherichia coli* LeoA protein. 153: 3776-3784.

Bull, Part ;J Integr. Med 1: the human gut microbiome in health and disease. 17.

Chakraborty, Subhra, JS Deokule, Pallavi Garg, SK Bhattacharya, RK Nandy, G Balakrish Nair, S Yamasaki, Yamasaki Takeda and T ;J Journal of clinical microbiology Ramamurthy 2001. Concomitant infection of enterotoxigenic *Escherichia coli* in an outbreak of cholera caused by Vibrio cholerae O1 and O139 in Ahmedabad, India. 39: 3241-3246.

Chao, Kinlin L, Lawrence A ;J Infection Dreyfus and Immunity 1997. Interaction of *Escherichia coli* heat-stable enterotoxin B with cultured human intestinal epithelial cells. 65: 3209-3217.

Chaudhuri, Roy R, Ian R ;J Infection Henderson, Genetics and Evolution 2012. The evolution of the *Escherichia coli* phylogeny. 12: 214-226.

Cohen, Dani, Joshua Tobias, Anya Spungin-Bialik, Tamar Sela, Raid Kayouf, Yael Volovik, Miri Yavzori, Moshe ;J Foodborne Pathogens Ephros and Disease 2010. Phenotypic characteristics of enterotoxigenic *Escherichia coli* associated with acute diarrhea among Israeli young adults. 7: 1159-1164.

Connell, Terry D ;J Expert review of vaccines 2007. Cholera toxin, LT-I, LT-IIa and LT-IIb: the critical role of ganglioside binding in immunomodulation by type I and type II heat-labile enterotoxins. 6: 821-834.

Croxen, Matthew A and B Brett ;J Nature Reviews Microbiology Finlay 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. 8: 26-38.

Croxen, Matthew A, Robyn J Law, Roland Scholz, Kristie M Keeney, Marta Wlodarska and B Brett ;J Clinical microbiology reviews Finlay 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. 26: 822-880.

Daniels, Nicholas A ;J Clinical Infectious Diseases 2006. Enterotoxigenic *Escherichia coli*: traveler's diarrhea comes home. In Enterotoxigenic *Escherichia coli*: traveler's diarrhea comes home, 335-336: The University of Chicago Press.

Datsenko, Kirill A and Barry L ;J Proceedings of the National Academy of Sciences Wanner 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. 97: 6640-6645.

Dayma, Paras, Ishan H Raval, Nidhi Joshi, Neha P Patel, Soumya Haldar and Kalpana H ;J Aquatic Living Resources Mody 2015. Influence of low salinity stress on virulence and biofilm formation potential in Vibrio alginolyticus, isolated from the Gulf of Khambhat, Gujarat India. 28: 99-109.

De, SN, K Bhattacharya, JK ;J Journal of pathology Sarkar and bacteriology 1956. A study of the pathogenicity of strains of Bacterium coli from acute and chronic enteritis. 71: 201-209.

Dean, Andrew G, Yi-Chuan Ching, Ronald G Williams and Lewis B ;J Journal of infectious diseases Harden 1972. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. 125: 407-411.

Deborah Chen, Huiwen and Gad Frankel 2005. Enteropathogenic *Escherichia coli*: unravelling pathogenesis. FEMS Microbiology Reviews 29: 83-98. doi: 10.1016/j.femsre.2004.07.002 ;J FEMS Microbiology Reviews

Del Canto, Felipe, Patricio Valenzuela, Lidia Cantero, Jonathan Bronstein, Jesús E Blanco, Jorge Blanco, Valeria Prado, Myron Levine, James Nataro and Halvor ;J Journal of clinical microbiology Sommerfelt 2011. Distribution of classical and nonclassical virulence genes in enterotoxigenic *Escherichia coli* isolates from Chilean children and tRNA gene screening for putative insertion sites for genomic islands. 49: 3198-3203.

Dobay, Orsolya, Krisztina Laub, Balázs Stercz, Adrienn Kéri, Bernadett Balázs, Adrienn Tóthpál, Szilvia Kardos, Pongsiri Jaikumpun, Kasidid Ruksakiet and Paul M ;J Frontiers in microbiology Quinton 2018. Bicarbonate inhibits bacterial growth and biofilm formation of prevalent cystic fibrosis pathogens. 9: 2245.

Donta, Sam T, Harley W Moon and Shannon C ;J Science Whipp 1974. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. 183: 334-336.

Dorman, Charles J ;J Nature Reviews Microbiology 2007. H-NS, the genome sentinel. 5: 157-161.

Dubreuil, J Daniel, Richard E Isaacson and Dieter M ;J EcoSal Plus Schifferli 2016. Animal enterotoxigenic *Escherichia coli*. 7.

Dutta, Sanjucta, Sucharita Guin, Santanu Ghosh, Gururaja P Pazhani, Krishnan Rajendran, Mihir K Bhattacharya, Yoshifumi Takeda, G Balakrish Nair and Thandavarayan ;J PLoS One Ramamurthy 2013. Trends in the prevalence of diarrheagenic *Escherichia coli* among hospitalized diarrheal patients in Kolkata, India. 8: e56068.

Dutta, Sanjucta, Gururaja P Pazhani, James P Nataro and Thandavarayan ;J International Journal of Medical Microbiology Ramamurthy 2015. Heterogenic virulence in a diarrheagenic *Escherichia coli*: evidence for an EPEC expressing heat-labile toxin of ETEC. 305: 47-54.

Elder, Bonnie L, David K Boraker and Paula M Fives-Taylor 1982. Whole-bacterial cell enzyme-linked immunosorbent assay for Streptococcus sanguis fimbrial antigens. Journal of clinical microbiology 16: 141-144.

Elhenawy, Wael, Caressa N Tsai, Brian K ;J Cell Host Coombes and Microbe 2019. Host-specific adaptive diversification of Crohn's disease-associated adherent-invasive *Escherichia coli*. 25: 301-312. e305.

Elsinghorst, Eric A, DJ ;J Infection Kopecko and immunity 1992. Molecular cloning of epithelial cell invasion determinants from enterotoxigenic *Escherichia coli*. 60: 2409-2417.

Ericsson, Charles D ;J Principles and practice of travel medicine 2013. Travellers' diarrhea. 197-208.

Erjavec, Marjanca Starčič ;J The Universe of *Escherichia coli* 2019. Introductory Chapter: The Versatile *Escherichia coli*. 3.

Faruque, Abu SG, Mohammed A Malek, Ashraful I Khan, Sayeeda Huq, Mohammed A Salam and David A ;J Scandinavian journal of infectious diseases Sack 2004. Diarrhea in elderly people: aetiology, and clinical characteristics. 36: 204-208.

Feldman, Mark, Lawrence S Friedman and Lawrence J Brandt 2020. Sleisenger and Fordtran's gastrointestinal and liver disease: pathophysiology, diagnosis, management: Elsevier health sciences.

Fleckenstein, James M, Luther E Lindler, Eric A Elsinghorst, James B ;J Infection Dale and immunity 2000. Identification of a gene within a pathogenicity

island of enterotoxigenic *Escherichia coli* H10407 required for maximal secretion of the heat-labile enterotoxin. 68: 2766-2774.

Fleckenstein, James M, Koushik Roy, Julia F Fischer, Michael ;J Infection Burkitt and immunity 2006. Identification of a two-partner secretion locus of enterotoxigenic *Escherichia coli*. 74: 2245-2258.

Fleckenstein, James, Alaullah Sheikh and Firdausi ;J Expert review of vaccines Qadri 2014. Novel antigens for enterotoxigenic *Escherichia coli* vaccines. 13: 631-639.

Frech, Sarah A, Herbert L DuPont, A Louis Bourgeois, Robin McKenzie, Jaime Belkind-Gerson, Jose F Figueroa, Pablo C Okhuysen, Norma H Guerrero, Francisco G Martinez-Sandoval and Juan HM ;J The Lancet Meléndez-Romero 2008. Use of a patch containing heat-labile toxin from *Escherichia coli* against travellers' diarrhea: a phase II, randomised, double-blind, placebo-controlled field trial. 371: 2019-2025.

Froehlich, Barbara, Julian Parkhill, Mandy Sanders, Michael A Quail and June R ;J Journal of bacteriology Scott 2005. The pCoo plasmid of enterotoxigenic *Escherichia coli* is a mosaic cointegrate. 187: 6509-6516.

Gaastra, Wim and Ann-Mari ;J Trends in microbiology Svennerholm 1996. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). 4: 444-452.

Ghosal, Abhisek, Rudra Bhowmick, Rajat Banerjee, Sandipan Ganguly, S Yamasaki, T Ramamurthy, T Hamabata, Nabendu Sekhar ;J Infection Chatterjee and immunity 2009. Characterization and studies of the cellular interaction of native colonization factor CS6 purified from a clinical isolate of enterotoxigenic *Escherichia coli*. 77: 2125-2135.

Ghosal, Abhisek, Rudra Bhowmick, Ranjan Kumar Nandy, T Ramamurthy and Nabendu Sekhar ;J Journal of clinical microbiology Chatterjee 2007. PCR-based identification of common colonization factor antigens of enterotoxigenic *Escherichia coli*. 45: 3068-3071.

Ghosal, Abhisek, Nabendu S Chatterjee, Tristan Chou and Hamid M ;J American Journal of Physiology-Cell Physiology Said 2013. Enterotoxigenic *Escherichia coli* infection and intestinal thiamin uptake: studies with intestinal epithelial Caco-2 monolayers. 305: C1185-C1191.

Gomes, Cláudia, Lidia Ruiz-Roldán, Judit Mateu, Theresa J Ochoa and Joaquim ;J Scientific reports Ruiz 2019. Azithromycin resistance levels and mechanisms in *Escherichia coli*. 9: 1-10.

Gonzales, Lucia, Samanta Sanchez, Silvia Zambrana, Volga Iñiguez, Gudrun Wiklund, Ann-Mari Svennerholm and Åsa ;J Journal of clinical microbiology Sjöling 2013. Molecular characterization of enterotoxigenic *Escherichia coli* isolates recovered from children with diarrhea during a 4-year period (2007 to 2010) in Bolivia. 51: 1219-1225.

Guerra, Julio A, Yesenia C Romero-Herazo, Octavio Arzuza and Oscar G ;J BioMed research international Gómez-Duarte 2014. Phenotypic and genotypic characterization of enterotoxigenic *Escherichia coli* clinical isolates from northern Colombia, South America. 2014.

Guiral, Elisabet, Milene Gonçalves Quiles, Laura Muñoz, Javier Moreno-Morales, Izaskun Alejo-Cancho, Pilar Salvador, Miriam J Alvarez-Martinez, Francesc Marco, Jordi ;J Antimicrobial agents Vila and chemotherapy 2019. Emergence of resistance to quinolones and β-lactam antibiotics in enteroaggregative and enterotoxigenic *Escherichia coli* causing traveler's diarrhea. 63: e01745-01718.

Guth, Beatriz Ernestina Cabilio ;J Memórias do Instituto Oswaldo Cruz 2000. Enterotoxigenic *Escherichia coli*-an overview. 95: 95-97.

Gyles, Carlton, Magdalene So and Stanley ;J Journal of infectious Diseases Falkow 1974. The enterotoxin plasmids of *Escherichia coli*. 130: 40-49.

Handl, Carina E and Jan-Ingmar ;J Journal of diarrheal diseases research Flock 1992. STb producing *Escherichia coli* are rarely associated with infantile diarrhea. 10: 37-38.

Hardy, SJ, Jan Holmgren, Susanne Johansson, Joaquin Sanchez and Timothy R ;J Proceedings of the National Academy of Sciences Hirst 1988. Coordinated assembly of multisubunit proteins: oligomerization of bacterial enterotoxins in vivo and in vitro. 85: 7109-7113.

Harro, Clayton, David Sack, A Louis Bourgeois, R Walker, Barbara DeNearing, Andrea Feller, Subhra Chakraborty, Charlotte Buchwaldt, Michael J ;J Clinical Darsley and Vaccine Immunology 2011. A combination vaccine consisting of three live attenuated enterotoxigenic *Escherichia coli* strains expressing a range of colonization factors and heat-labile toxin subunit B is well tolerated and

immunogenic in a placebo-controlled double-blind phase I trial in healthy adults. 18: 2118-2127.

Heuzenroeder, MW, BL Neal, CJ Thomas, R Halter and PA ;J Molecular microbiology Manning 1989. Characterization and molecular cloning of the PCF8775 CS5 antigen from an enterotoxigenic *Escherichia coli* 0115: H40 isolated in Central Australia. 3: 303-310.

Hosangadi, Divya, Peter G Smith, David C Kaslow and Birgitte K ;J Vaccine Giersing 2019. WHO consultation on ETEC and Shigella burden of disease, Geneva, 6–7th April 2017: Meeting report. 37: 7381-7390.

Hsueh, Po-Ren, Wen-Chien Ko, Jiunn-Jong Wu, Jang-Jih Lu, Fu-Der Wang, Hsueh-Yi Wu, Tsu-Lan Wu, Lee-Jene ;J Journal of Microbiology Teng, Immunology and Infection 2010. Consensus statement on the adherence to Clinical and Laboratory Standards Institute (CLSI) Antimicrobial Susceptibility Testing Guidelines (CLSI-2010 and CLSI-2010-update) for Enterobacteriaceae in clinical microbiology laboratories in Taiwan. 43: 452-455.

Hug, Martin J, Tsutomu Tamada and Robert J ;J Physiology Bridges 2003. CFTR and bicarbonate secretion to epithelial cells. 18: 38-42.

I**sidean, SD, MS Riddle, SJ Savarino and CK** ;J Vaccine Porter 2011. A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression. 29: 6167-6178.

Jang, Jeonghwan, H-G Hur, Michael J Sadowsky, MN Byappanahalli, Tao Yan and Satoshi ;J Journal of applied microbiology Ishii 2017. Environmental *Escherichia coli*: ecology and public health implications—a review. 123: 570-581.

Jiang, Zhi-Dong, Brett Lowe, MP Verenkar, David Ashley, Robert Steffen, Nadia Tornieporth, Frank von Sonnenburg, Peter Waiyaki and Herbert L ;J The Journal of infectious diseases DuPont 2002. Prevalence of enteric pathogens among international travelers with diarrhea acquired in Kenya (Mombasa), India (Goa), or Jamaica (Montego Bay). 185: 497-502.

Joffre, Enrique, Matilda Nicklasson, Sandra Álvarez-Carretero, Xue Xiao, Lei Sun, Intawat Nookaew, Baoli Zhu and Åsa ;J Scientific reports Sjöling 2019. The bile salt glycocholate induces global changes in gene and protein expression and activates virulence in enterotoxigenic *Escherichia coli*. 9: 1-14.

Johnson, Timothy J, Lisa K ;J Microbiology Nolan and Molecular Biology Reviews 2009. Pathogenomics of the virulence plasmids of *Escherichia coli*. 73: 750-774.

Kaper, James B, James P Nataro and Harry LT ;J Nature reviews microbiology Mobley 2004. Pathogenic *Escherichia coli*. 2: 123-140.

Kaur, P, A Chakraborti and A2 ;J Interdisciplinary perspectives on infectious diseases Asea 2010. Enteroaggregative *Escherichia coli*: an emerging enteric food borne pathogen. 2010.

Khalil, Ibrahim A, Christopher Troeger, Brigette F Blacker, Puja C Rao, Alexandria Brown, Deborah E Atherly, Thomas G Brewer, Cyril M Engmann, Eric R Houpt and Gagandeep ;J The Lancet infectious diseases Kang 2018. Morbidity and mortality due to shigella and enterotoxigenic *Escherichia coli* diarrhea: the Global Burden of Disease Study 1990–2016. 18: 1229-1240.

Kinnula, Hanna, Johanna Mappes, Janne K Valkonen, Katja Pulkkinen and Lotta-Riina ;J Evolutionary Applications Sundberg 2017. Higher resource level promotes virulence in an environmentally transmitted bacterial fish pathogen. 10: 462-470.

Kipkirui, Erick, Margaret Koech, Abigael Ombogo, Ronald Kirera, Janet Ndonye, Nancy Kipkemoi, Mary Kirui, Cliff Philip, Amanda Roth, Alexander ;J Tropical Diseases Flynn, Travel Medicine and Vaccines 2021. Molecular characterization of enterotoxigenic *Escherichia coli* toxins and colonization factors in children under five years with acute diarrhea attending Kisii Teaching and Referral Hospital, Kenya. 7: 1-7.

Komano, Teruya, Tetsu Yoshida, Koji Narahara and Nobuhisa ;J Molecular microbiology Furuya 2000. The transfer region of IncI1 plasmid R64: similarities between R64 tra and Legionella icm/dot genes. 35: 1348-1359.

Kotloff, Karen L, James P Nataro, William C Blackwelder, Dilruba Nasrin, Tamer H Farag, Sandra Panchalingam, Yukun Wu, Samba O Sow, Dipika Sur and Robert F ;J The Lancet Breiman 2013. Burden and aetiology of diarrheal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. 382: 209-222.

Kumar, Pardeep, Qingwei Luo, Tim J Vickers, Alaullah Sheikh, Warren G Lewis, James M ;J Infection Fleckenstein and immunity 2014. EatA, an

immunogenic protective antigen of enterotoxigenic *Escherichia coli*, degrades intestinal mucin. 82: 500-508.

Lamberti, Laura M, A Louis Bourgeois, Christa L Fischer Walker, Robert E Black and David ;J PLoS neglected tropical diseases Sack 2014. Estimating diarrheal illness and deaths attributable to Shigellae and enterotoxigenic *Escherichia coli* among older children, adolescents, and adults in South Asia and Africa. 8: e2705.

Le Poul, Emmanuel, Cécile Loison, Sofie Struyf, Jean-Yves Springael, Vincent Lannoy, Marie-Eve Decobecq, Stéphane Brezillon, Vincent Dupriez, Gilbert Vassart and Jo ;J Journal of Biological Chemistry Van Damme 2003. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. 278: 25481-25489.

Lee, Sang Ho, David L Hava, Matthew K Waldor and Andrew ;J Cell Camilli 1999. Regulation and temporal expression patterns of Vibrio cholerae virulence genes during infection. 99: 625-634.

Li, Fen, Xue-Song Xiong, Ying-Ying Yang, Jun-Jiao Wang, Meng-Meng Wang, Jia-Wei Tang, Qing-Hua Liu, Liang Wang and Bing ;J Frontiers in Microbiology Gu 2021. Effects of NaCl Concentrations on Growth Patterns, Phenotypes Associated With Virulence, and Energy Metabolism in *Escherichia coli* BW25113. 12.

Li, Yinghui, Qiang Luo, Xiaolu Shi, Yiman Lin, Yaqun Qiu, Dongyue Lv, Yixiang Jiang, Qiongcheng Chen, Min Jiang, Hanwu ;J Foodborne pathogens Ma and disease 2017. Phenotypic and genotypic characterization of clinical Enterotoxigenic *Escherichia coli* Isolates from Shenzhen, China. 14: 333-340.

Lindenthal, Christoph, Eric A ;J Infection Elsinghorst and immunity 2001. Enterotoxigenic *Escherichia coli* TibA glycoprotein adheres to human intestine epithelial cells. 69: 52-57.

Lindenthal, Christoph, Eric A ;J Infection Elsinghorst and immunity 1999. Identification of a glycoprotein produced by enterotoxigenic *Escherichia coli*. 67: 4084-4091.

Livak, Kenneth J and Thomas D ;J methods Schmittgen 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. 25: 402-408.

Lowry, Oliver H ;J J biol Chem 1951. Protein measurement with the Folin phenol reagent. 193: 265-275.

Mammarappallil, Joseph G, Eric A ;J Infection Elsinghorst and immunity 2000. Epithelial cell adherence mediated by the enterotoxigenic *Escherichia coli* Tia protein. 68: 6595-6601.

Mansan-Almeida, Rosane, Alex Leite Pereira and Loreny Gimenes ;J BMC microbiology Giugliano 2013. Diffusely adherent *Escherichia coli*strains isolated from children and adults constitute two different populations. 13: 1-14.

Mansour, Adel, Hind I Shaheen, Mohamed Amine, Khaled Hassan, John W Sanders, Mark S Riddle, Adam W Armstrong, Ann-Mari Svennerholm, Peter J Sebeny and John D ;J Journal of clinical microbiology Klena 2014. Pathogenicity and phenotypic characterization of enterotoxigenic *Escherichia coli* isolates from a birth cohort of children in rural Egypt. 52: 587-591.

Margulieux, Katie R, Apichai Srijan, Sirigade Ruekit, Panida Nobthai, Kamonporn Poramathikul, Prativa Pandey, Oralak Serichantalergs, Sanjaya K Shrestha, Ladaporn Bodhidatta, Brett E ;J Antimicrobial Resistance Swierczewski and Infection Control 2018. Extended-spectrum β-lactamase prevalence and virulence factor characterization of enterotoxigenic *Escherichia coli* responsible for acute diarrhea in Nepal from 2001 to 2016. 7: 1-7.

Mathur, R, V Reddy, AN Naidu and KA ;J Human Nutrition. Clinical Nutrition Krishnamachari 1985. Nutritional status and diarrheal morbidity: a longitudinal study in rural Indian preschool children. 39: 447-454.

Mbuthia, Oliver Waithaka, Scholastica Gatwiri Mathenge, Micah Ongeri Oyaro and Musa Otieno ;J Pan African Medical Journal Ng'ayo 2018. Etiology and pathogenicity of bacterial isolates: a cross sectional study among diarrheal children below five years in central regions of Kenya. 31.

McKenzie, Robin, A Louis Bourgeois, Sarah A Frech, David C Flyer, Arlene Bloom, Kazem Kazempour and Gregory M ;J Vaccine Glenn 2007. Transcutaneous immunization with the heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC): protective efficacy in a double-blind, placebo-controlled challenge study. 25: 3684-3691.

Medina, Anicia M, Fulton P Rivera, Maria J Pons, Maribel Riveros, Cláudia Gomes, María Bernal, Rina Meza, Ryan C Maves, Luis Huicho, Elsa ;J Transactions of the Royal Society of Tropical Medicine Chea-Woo and Hygiene

2015. Comparative analysis of antimicrobial resistance in enterotoxigenic *Escherichia coli* isolates from two paediatric cohort studies in Lima, Peru. 109: 493-502.

Mekalanos, JOHN J ;J Journal of bacteriology 1992. Environmental signals controlling expression of virulence determinants in bacteria. 174: 1-7.

Mellies, Jay L and Alex MS ;J EcoSal Plus Barron 2006. Virulence gene regulation in *Escherichia coli*. 2.

Merson, Michael H, George K Morris, David A Sack, Joy G Wells, John C Feeley, R Bradley Sack, Walton B Creech, Albert Z Kapikian and Eugene J ;J New England journal of medicine Gangarosa 1976. Travelers' diarrhea in Mexico: a prospective study of physicians and family members attending a congress. 294: 1299-1305.

Meyer, TS and BL ;J Biochimica et biophysica acta Lamberts 1965. Use of coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide-gel strips. 107: 144-145.

Miller, JH and KY Lee 1984. Experiments in molecular genetics. In Experiments in molecular genetics: Yi Hsien Pub. Co.

Miller, Virginia L and John J ;J Journal of bacteriology Mekalanos 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. 170: 2575-2583.

Mims, Cedric A, Anthony A Nash and John Stephen 2001. Mims' pathogenesis of infectious disease: Gulf Professional Publishing.

Mirhoseini, Ali, Jafar Amani and Shahram ;J Microbial pathogenesis Nazarian 2018. Review on pathogenicity mechanism of enterotoxigenic *Escherichia coli* and vaccines against it. 117: 162-169.

Moharana, Sonam S, Rakesh K Panda, Muktikesh Dash, Nirupama Chayani, Priyanka Bokade, Sanghamitra Pati and Debdutta ;J BMC Infectious Diseases Bhattacharya 2019. Etiology of childhood diarrhea among under five children and molecular analysis of antibiotic resistance in isolated enteric bacterial pathogens from a tertiary care hospital, Eastern Odisha, India. 19: 1-9.

Mondal, Indranil, Debjyoti Bhakat, Goutam Chowdhury, Asis Manna, Sandip Samanta, Alok Kumar Deb, Asish Kumar Mukhopadhyay and Nabendu Sekhar ;J Journal of Applied Microbiology Chatterjee 2022. Distribution of

virulence factors and its relatedness towards the antimicrobial response of enterotoxigenic *Escherichia coli* strains isolated from patients in Kolkata, India. 132: 675-686.

Montero, David, Maricel Vidal, Mirka Pardo, Alexia Torres, Eduardo Kruger, Mauricio Farfán, Miguel O'Ryan, Qingwei Luo, James Fleckenstein, Felipe ;J Infection Del Canto, Genetics and Evolution 2017. Characterization of enterotoxigenic *Escherichia coli* strains isolated from the massive multi-pathogen gastroenteritis outbreak in the Antofagasta region following the Chilean earthquake, 2010. 52: 26-29.

Nada, Rania A, Adam Armstrong, Hind I Shaheen, Isabelle Nakhla, John W Sanders, Mark S Riddle, Sylvia Young, Peter ;J Diagnostic Microbiology Sebeny and Infectious Disease 2013. Phenotypic and genotypic characterization of enterotoxigenic *Escherichia coli* isolated from US military personnel participating in Operation Bright Star, Egypt, from 2005 to 2009. 76: 272-277.

Nagy, Béla and Péter Zs ;J International journal of medical microbiology Fekete 2005. Enterotoxigenic *Escherichia coli* in veterinary medicine. 295: 443-454.

Nair, GB and Y ;J Microbial pathogenesis Takeda 1998. The heat-stable enterotoxins. 24: 123-131.

Nakanishi, Noriko, Kosuke Tashiro, Satoru Kuhara, Tetsuya Hayashi, Nakaba Sugimoto and Toru ;J Microbiology Tobe 2009. Regulation of virulence by butyrate sensing in enterohaemorrhagic *Escherichia coli*. 155: 521-530.

Nakaya, Rintaro, Akiko Nakamura, Yukio ;J Biochemical Murata and biophysical research communications 1960. Resistance transfer agents in Shigella. 3: 654-659.

Navarre, William Wiley, Steffen Porwollik, Yipeng Wang, Michael McClelland, Henry **Rosen, Stephen J Libby and Ferric C** ;J Science Fang 2006. Selective silencing of foreign DNA with low GC content by the H-NS protein in Salmonella. 313: 236-238.

Nguyen, Y, Vanessa ;J Frontiers in cellular Sperandio and infection microbiology 2012. Enterohemorrhagic E. coli (EHEC) pathogenesis. 2: 90.

Nicklasson, Matilda, Åsa Sjöling, Astrid Von Mentzer, Firdausi Qadri and Ann-Mari ;J PloS one Svennerholm 2012. Expression of colonization factor CS5 of enterotoxigenic *Escherichia coli* (ETEC) is enhanced in vivo and by the bile component Na glycocholate hydrate. 7: e35827.

Nilsson, Niclas E, Knut Kotarsky, Christer Owman, Björn ;J Biochemical Olde and biophysical research communications 2003. Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. 303: 1047-1052.

Nogal, Ana, Ana M Valdes and Cristina ;J Gut microbes Menni 2021. The role of short-chain fatty acids in the interplay between gut microbiota and diet in cardio-metabolic health. 13: 1897212.

Nunes, MRCM, FJ Penna, RT Franco, EN Mendes and PP ;J Journal of applied microbiology Magalhaes 2011. Enterotoxigenic *Escherichia coli* in children with acute diarrhea and controls in Teresina/PI, Brazil: distribution of enterotoxin and colonization factor genes. 111: 224-232.

Nyholm, Outi, Jani Halkilahti, Gudrun Wiklund, Uche Okeke, Lars Paulin, Petri Auvinen, Kaisa Haukka and Anja ;J PLoS One Siitonen 2015. Comparative genomics and characterization of hybrid Shigatoxigenic and enterotoxigenic *Escherichia coli* (STEC/ETEC) strains. 10: e0135936.

O'Mahony, Mary C, ND Noah, B Evans, D Harper, B Rowe, JA Lowes, A Pearson, B ;J Epidemiology Goode and Infection 1986. An outbreak of gastroenteritis on a passenger cruise ship. 97: 229-236.

Ohno, Akira, Ayumi Marui, Ernest Sanzetenea Castro, AA Reyes, Daniel Elio-Calvo, Husako Kasitani, Yoshikazu Ishii, Keizo ;J The American journal of tropical medicine Yamaguchi and hygiene 1997. Enteropathogenic bacteria in the La Paz River of Bolivia. 57: 438-444.

Olive, D Michael ;J Journal of Clinical Microbiology 1989. Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA **polymerase. 27: 261-265.**

Olson, Scott, Alexis Hall, Mark S Riddle, Chad K ;J Tropical diseases Porter, travel medicine and vaccines 2019. Travelers' diarrhea: update on the incidence, etiology and risk in military and similar populations–1990-2005 versus 2005–2015, does a decade make a difference? 5: 1-15.

Ormsby, Michael J, Síle A Johnson, Nuria Carpena, Lynsey M Meikle, Robert J Goldstone, Anne McIntosh, Hannah M Wessel, Heather E Hulme, Ceilidh C McConnachie and James PR ;J Cell reports Connolly 2020. Propionic acid promotes the virulent phenotype of Crohn's disease-associated adherent-invasive *Escherichia coli*. 30: 2297-2305. e2295.

Ørskov, Frits, Ida Ørskov, Doyle J Evans, R Bradley Sack, David A Sack, Torkel ;J Medical microbiology Wadström and immunology 1976. Special*Escherichia coli* serotypes among enterotoxigenic strains from diarrhea in adults and children. 162: 73-80.

Oyofo, Buhari A, Decy S Subekti, Ann-Mari Svennerholm, Nunung N Machpud, Periska Tjaniadi, TS Komalarini, Budhi Setiawan, James R Campbell, Andrew L Corwin, Murad ;J The American journal of tropical medicine Lesmana and hygiene 2001. Toxins and colonization factor antigens of enterotoxigenic *Escherichia coli* among residents of Jakarta, Indonesia. 65: 120-124.

Patel, Seema K, Jimmie Dotson, Kenneth P Allen, James M ;J Infection Fleckenstein and immunity 2004. Identification and molecular characterization of EatA, an autotransporter protein of enterotoxigenic *Escherichia coli*. 72: 1786-1794.

Perez-Casal, JOSE, JOHN S Swartley, JUNE R ;J Infection Scott and immunity 1990. Gene encoding the major subunit of CS1 pili of human enterotoxigenic *Escherichia coli*. 58: 3594-3600.

Picken, RN, AJ Mazaitis, WK Maas, M Rey, H ;J Infection Heyneker and immunity 1983. Nucleotide sequence of the gene for heat-stable enterotoxin II of *Escherichia coli*. 42: 269-275.

Pimbley, DW and PD ;J Journal of Applied Microbiology Patel 1998. A review of analytical methods for the detection of bacterial toxins. 84: 98S.

Pinhal, Stéphane, Delphine Ropers, Johannes Geiselmann and Hidde ;J Journal of bacteriology De Jong 2019. Acetate metabolism and the inhibition of bacterial growth by acetate. 201: e00147-00119.

Platts-Mills, James A, Mami Taniuchi, Md Jashim Uddin, Shihab Uddin Sobuz, Mustafa Mahfuz, SM Abdul Gaffar, Dinesh Mondal, Md Iqbal Hossain, M Munirul Islam and AM Shamsir ;J The American journal of clinical nutrition Ahmed 2017. Association between enteropathogens and malnutrition in children aged 6–23 mo in Bangladesh: a case-control study. 105: 1132-1138.

Poolman, Jan T and Michael ;J The Journal of infectious diseases Wacker 2016. Extraintestinal pathogenic *Escherichia coli*, a common human pathogen: challenges for vaccine development and progress in the field. 213: 6-13.

Prohaszka, L ;J Zentralblatt für Veterinärmedizin Reihe B 1980. Antibacterial effect of volatile fatty acids in enteric E. coli-infections of rabbits. 27: 631-639.

Qadri, Firdausi, Swadesh Kumar Das, ASG Faruque, George J Fuchs, M John Albert, R Bradley Sack and Ann-Mari ;J Journal of clinical microbiology Svennerholm 2000. Prevalence of toxin types and colonization factors in enterotoxigenic *Escherichia coli* isolated during a 2-year period from diarrheal patients in Bangladesh. 38: 27-31.

Qadri, Firdausi, Amit Saha, Tanvir Ahmed, Abdullah Al Tarique, Yasmin Ara Begum, Ann-Mari ;J Infection Svennerholm and immunity 2007. Disease burden due to enterotoxigenic *Escherichia coli* in the first 2 years of life in an urban community in Bangladesh. 75: 3961-3968.

Qadri, Firdausi, Ann-Mari Svennerholm, ASG Faruque and R Bradley ;J Clinical microbiology reviews Sack 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. 18: 465-483.

Rahman, Md Masuder, Parvez Ahmed, Antora Kar, Nazmus Sakib, Abu Zaffar Shibly, Fatama Tous Zohora, Md Nazmul ;J Foodborne Pathogens Hasan and Disease 2020. Prevalence, antimicrobial resistance, and pathogenic potential of enterotoxigenic and enteropathogenic *Escherichia coli* associated with acute diarrheal patients in Tangail, Bangladesh. 17: 434-439.

Rasko, David A, MJ Rosovitz, Garry SA Myers, Emmanuel F Mongodin, W Florian Fricke, Pawel Gajer, Jonathan Crabtree, Mohammed Sebaihia, Nicholas R Thomson and Roy ;J Journal of bacteriology Chaudhuri 2008. The pangenome structure of *Escherichia coli*: comparative genomic analysis of E. coli commensal and pathogenic isolates. 190: 6881-6893.

Rath, Christopher M and Pieter C ;J Current opinion in microbiology Dorrestein 2012. The bacterial chemical repertoire mediates metabolic exchange within gut microbiomes. 15: 147-154.

Raymond, Samuel and Lewis ;J Science Weintraub 1959. Acrylamide gel as a supporting medium for zone electrophoresis. 130: 711-711.

Ristaino, Polly A, Myron M Levine and Charles R ;J Journal of Clinical Microbiology Young 1983. Improved GM1-enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. 18: 808-815.

Rivera, FP, TJ Ochoa, RC Maves, M Bernal, AM Medina, R Meza, F Barletta, E Mercado, L Ecker and AI ;J Journal of clinical microbiology Gil 2010.

Genotypic and phenotypic characterization of enterotoxigenic *Escherichia coli* strains isolated from Peruvian children. 48: 3198-3203.

Rivera, Fulton P, Anicia M Medina, Edelweiss Aldasoro, Anna Sangil, Joaquim Gascon, Theresa J Ochoa, Jordi Vila and Joaquim ;J Journal of Clinical Microbiology Ruiz 2013. Genotypic characterization of enterotoxigenic *Escherichia coli* strains causing traveler's diarrhea. 51: 633-635.

Rocha, Letícia B, Christiane Y Ozaki, Denise SPQ Horton, Caroline A Menezes, Anderson Silva, Irene Fernandes, Fabio C Magnoli, Tania MI Vaz, Beatriz EC Guth and Roxane MF ;J Toxins Piazza 2013. Different assay conditions for detecting the production and release of heat-labile and heat-stable toxins in enterotoxigenic *Escherichia coli* isolates. 5: 2384-2402.

Rodas, Claudia, Rosalía Mamani, Jorge Blanco, Jesus Eulogio Blanco, Gudrun Wiklund, Ann-Mari Svennerholm, Åsa Sjöling and Volga ;J The Brazilian Journal of Infectious Diseases Iniguez 2011. Enterotoxins, colonization factors, serotypes and antimicrobial resistance of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from hospitalized children with diarrhea in Bolivia. 15: 132-137.

Rohmer, Laurence, Didier Hocquet and Samuel I ;J Trends in microbiology Miller 2011. Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. 19: 341-348.

Roy, Koushik, George M Hilliard, David J Hamilton, Jiwen Luo, Marguerite M Ostmann and James M ;J Nature Fleckenstein 2009. Enterotoxigenic *Escherichia coli* EtpA mediates adhesion between flagella and host cells. 457: 594-598.

Roy, Saumendra P, Mohammad M Rahman, Xiao Di Yu, Minna Tuittila, Stefan D Knight and Anton V ;J Molecular microbiology Zavialov 2012. Crystal structure of enterotoxigenic E scherichia coli colonization factor CS 6 reveals a novel type of functional assembly. 86: 1100-1115.

Rubiano-Labrador, Carolina, Céline Bland, Guylaine Miotello, Jean Armengaud and Sandra ;J PloS one Baena 2015. Salt stress induced changes in the exoproteome of the halotolerant bacterium Tistlia consotensis deciphered by proteogenomics. 10: e0135065.

Sabui, Subrata, Sanjucta Dutta, Anusuya Debnath, Avishek Ghosh, T Hamabata, K Rajendran, T Ramamurthy, James P Nataro, Dipika Sur and Myron M ;J Journal of clinical microbiology Levine 2012. Real-time PCR-based mismatch amplification mutation assay for specific detection of CS6-expressing

allelic variants of enterotoxigenic *Escherichia coli* and its application in assessing diarrheal cases and asymptomatic controls. 50: 1308-1312.

Sack, DA, DC Kaminsky, RB Sack, IA Wamola, F Orskov, I Orskov, RC Slack, RR Arthur and AZ ;J The Johns Hopkins medical journal Kapikian 1977. Enterotoxigenic *Escherichia coli* diarrhea of travelers: a prospective study of American Peace Corps volunteers. 141: 63-70.

Sack, R Bradley ;J Annual review of microbiology 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. 29: 333-354.

Sack, R Bradley ;J Indian Journal of Medical Research 2011. The discovery of cholera-like enterotoxins produced by *Escherichia coli* causing secretory diarrhea in humans. 133: 171-178.

Saiki, Randall K, Stephen Scharf, Fred Faloona, Kary B Mullis, Glenn T Horn, Henry A Erlich and Norman ;J Science Arnheim 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. 230: 1350-1354.

Sambrook, Joseph and David W ;J Csh Protoc Russell 2006. The inoue method for preparation and transformation of competent E. coli:"ultra-competent" cells. 2006: 10.1101.

Sánchez, Joaquín and Jan ;J Current opinion in immunology Holmgren 2005. Virulence factors, pathogenesis and vaccine protection in cholera and ETEC diarrhea. 17: 388-398.

Schroeder, Gunnar N and Hubert ;J Clinical microbiology reviews Hilbi 2008. Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion. 21: 134-156.

Sears, Cynthia L and James B ;J Microbiological reviews Kaper 1996. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. 60: 167-215.

Sekirov, Inna, Shannon L Russell, L Caetano M Antunes and B Brett ;J Physiological reviews Finlay 2010. Gut microbiota in health and disease.

Shaheen, HI, IA Abdel Messih, JD Klena, A Mansour, Z El-Wakkeel, TF Wierzba, JW Sanders, SB Khalil, DM Rockabrand and MR ;J Journal of clinical microbiology Monteville 2009. Phenotypic and genotypic analysis of enterotoxigenic *Escherichia coli* in samples obtained from Egyptian children presenting to referral hospitals. 47: 189-197.

Shaheen, Hind I, Karim A Kamal, Momtaz O Wasfy, Nemat M El-Ghorab, Brett Lowe, Robert Steffen, Neville Kodkani, Lorenz Amsler, Peter Waiyaki and John C ;J International journal of infectious diseases David 2003. Phenotypic diversity of enterotoxigenic *Escherichia coli* (ETEC) isolated from cases of travelers' diarrhea in Kenya. 7: 35-41.

Shaheen, Hind I, Sami B Khalil, Malla R Rao, Remon Abu Elyazeed, Thomas F Wierzba, Leonard F Peruski Jr, Shannon Putnam, Armando Navarro, Badria Z Morsy and Alejandro ;J Journal of clinical microbiology Cravioto 2004. Phenotypic profiles of enterotoxigenic *Escherichia coli* associated with early childhood diarrhea in rural Egypt. 42: 5588-5595.

Shevchuk, Nikolai A, Anton V Bryksin, Yevgeniya A Nusinovich, Felipe C Cabello, Margaret Sutherland and Stephan ;J Nucleic acids research Ladisch 2004. Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. 32: e19-e19.

Simuyandi, Michelo, Roma Chilengi, Sean B Connor, Joseph B Voeglein, Natasha M Laban, Katayi Mwila-Kazimbaya, Caroline C Chisenga, John Mwaba, David A Sack, Subhra ;J Archives of Microbiology Chakraborty and Immunology 2019. Enterotoxigenic *Escherichia coli* toxins and colonization factors among Zambian children presenting with moderate to severe diarrhea to selected health facilities. 3: 173-184.

Singh, Pankaj, Sharda C Metgud, Subarna Roy and Shashank ;J Journal of laboratory physicians Purwar 2019. Evolution of diarrheagenic *Escherichia coli* pathotypes in India. 11: 346-351.

Sleator, Roy D and Colin ;J FEMS microbiology reviews Hill 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. 26: 49-71.

Smith, H Williams and CL ;J Journal of medical microbiology Gyles 1970. The effect of cell-free fluids prepared from cultures of human and animal enteropathogenic strains of *Escherichia coli* on ligated intestinal segments of rabbits and pigs. 3: 403-409.

Sommerfelt, Halvor, Hans Steinsland, Harleen MS Grewal, Gloria Inés Viboud, Nita Bhandari, Wim Gaastra, Ann-Mari Svennerholm and Maharaj K ;J Journal of Infectious Diseases Bhan 1996. Colonization factors of enterotoxigenic *Escherichia coli* isolated from children in north India. 174: 768-776.

Steinsland, Hans, David W Lacher, Halvor Sommerfelt and Thomas S ;J Journal of clinical microbiology Whittam 2010. Ancestral lineages of human enterotoxigenic *Escherichia coli*. 48: 2916-2924.

Steinsland, Hans, Palle Valentiner-Branth, Håkon K Gjessing, Peter Aaby, Kåre Mølbak and Halvor ;J The Lancet Sommerfelt 2003. Protection from natural infections with enterotoxigenic *Escherichia coli*: longitudinal study. 362: 286-291.

Stoll, Barbara J, Roger I Glass, M Imdadul Huq, MU Khan, James E Holt and Hasina ;J Br Med J Banu 1982. Surveillance of patients attending a diarrheal disease hospital in Bangladesh. 285: 1185-1188.

Subekti, DS, M Lesmana, P Tjaniadi, N Machpud, JC Daniel, WK Alexander, JR Campbell, AL Corwin, H James Beecham III, C ;J Diagnostic microbiology Simanjuntak and infectious disease 2003. Prevalence of enterotoxigenic *Escherichia coli* (ETEC) in hospitalized acute diarrhea patients in Denpasar, Bali, Indonesia. 47: 399-405.

Sun, Yvonne and Mary XD ;J Advances in applied microbiology O'Riordan 2013. Regulation of bacterial pathogenesis by intestinal short-chain fatty acids. 85: 93-118.

Sundberg, Lotta-Riina, Heidi MT Kunttu and E Tellervo ;J BMC microbiology Valtonen 2014. Starvation can diversify the population structure and virulence strategies of an environmentally transmitting fish pathogen. 14: 1-6.

Svennerholm, AM, M Wikström, M Lindblad and J ;J Journal of clinical microbiology Holmgren 1986. Monoclonal antibodies against *Escherichia coli* heat-stable toxin (STa) and their use in a diagnostic ST ganglioside GM1-enzyme-linked immunosorbent assay. 24: 585-590.

Svennerholm, Ann-Mari and Gregory ;J New generation vaccines Glenn 2016. Vaccines against enterotoxigenic *Escherichia coli*. 742-750.

Taha, ZM and NA ;J Iranian Journal of Veterinary Research Yassin 2019. Prevalence of diarrheagenic *Escherichia coli* in animal products in Duhok province, Iraq. 20: 255.

TAKASHI, Katsuo, Itsuki FLUITA and Kazumine ;J Japanese journal of pharmacology KOBARI 1989. Effects of short chain fatty acids on the production of heat-labile enterotoxin from enterotoxigenic *Escherichia coli*. 50: 495-498.

Tang, Hong, Yong Li, Pingping Zhang, Jiayin Guo, Zheng Huang, Hao Xu, Qi Hou, Chuanqing Wang, Mei Zeng and Huiming ;J Zhonghua liu Xing Bing xue

za zhi= Zhonghua Liuxingbingxue Zazhi Jin 2015. Surveillance for diarrheagenic *Escherichia coli* in Shanghai, 2012-2013. 36: 1263-1268.

Tickell, Kirkby D, Rumana Sharmin, Emily L Deichsel, Laura M Lamberti, Judd L Walson, ASG Faruque, Patricia B Pavlinac, Karen L Kotloff and Mohammod J ;J The Lancet Global Health Chisti 2020. The effect of acute malnutrition on enteric pathogens, moderate-to-severe diarrhea, and associated mortality in the Global Enteric Multicenter Study cohort: a post-hoc analysis. 8: e215-e224.

Tobias, Joshua, Michael Lebens, Susanne Källgård, Matilda Nicklasson and Ann-Mari ;J Vaccine Svennerholm 2008. Role of different genes in the CS6 operon for surface expression of enterotoxigenic *Escherichia coli* colonization factor CS6. 26: 5373-5380.

Toma, Claudia, Yan Lu, Naomi Higa, Noboru Nakasone, Isabel Chinen, Ariela Baschkier, Marta Rivas and Masaaki ;J Journal of clinical microbiology Iwanaga 2003. Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. 41: 2669-2671.

Topping, David L and Peter M ;J Physiological reviews Clifton 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides.

Torres, OR, W González, O Lemus, RA Pratdesaba, JA Matute, G Wiklund, DA Sack, AL Bourgeois, AM ;J Epidemiology Svennerholm and Infection 2015. Toxins and virulence factors of enterotoxigenic *Escherichia coli* associated with strains isolated from indigenous children and international visitors to a rural community in Guatemala. 143: 1662-1671.

Tsai, Su-Chen, Masatoshi Noda, Ronald Adamik, Joel Moss and Martha ;J Proceedings of the National Academy of Sciences Vaughan 1987. Enhancement of choleragen ADP-ribosyltransferase activities by guanyl nucleotides and a 19-kDa membrane protein. 84: 5139-5142.

Turner, Susan M, Anthony Scott-Tucker, Lisa M Cooper and Ian R ;J FEMS microbiology letters Henderson 2006. Weapons of mass destruction: virulence factors of the global killer enterotoxigenic *Escherichia coli*. 263: 10-20.

Vidal, Roberto M, Khitam Muhsen, Sharon M Tennant, Ann-Mari Svennerholm, Samba O Sow, Dipika Sur, Anita KM Zaidi, Abu SG Faruque, Debasish Saha and Richard ;J PLoS neglected tropical diseases Adegbola 2019.

Colonization factors among enterotoxigenic *Escherichia coli* isolates from children with moderate-to-severe diarrhea and from matched controls in the Global Enteric Multicenter Study (GEMS). 13: e0007037.

Vilchez, Samuel, Sylvia Becker-Dreps, Erick Amaya, Claudia Perez, Margarita Paniagua, Daniel Reyes, Felix Espinoza and Andrej ;J Journal of medical microbiology Weintraub 2014. Characterization of enterotoxigenic *Escherichia coli* strains isolated from Nicaraguan children in hospital, primary care and community settings. 63: 729.

Vyas, Usha, Natarajan ;J Gastroenterology research Ranganathan and practice 2012. Probiotics, prebiotics, and synbiotics: gut and beyond. 2012.

Wajima, Takeaki, Subrata Sabui, Megumi Fukumoto, Shigeyuki Kano, Thandavarayan Ramamurthy, Nabendu Sekhar Chatterjee and Takashi ;J Microbial pathogenesis Hamabata 2011. Enterotoxigenic *Escherichia coli* CS6 gene products and their roles in CS6 structural protein assembly and cellular adherence. 51: 243-249.

Walker, Richard I, Duncan Steele, Teresa Aguado and Ad Hoc ETEC Technical Expert Committee ;J Vaccine 2007. Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic E. coli (ETEC) disease. 25: 2545-2566.

William, S, Helene Feil and A ;J Sigma Copeland 2012. Bacterial genomic DNA isolation using CTAB. 50.

Wolf, Marcia K ;J Clinical microbiology reviews 1997. Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. 10: 569-584.

Wong, Julia MW, Russell De Souza, Cyril WC Kendall, Azadeh Emam and David JA ;J Journal of clinical gastroenterology Jenkins 2006. Colonic health: fermentation and short chain fatty acids. 40: 235-243.

Wood, EJ 1983. Molecular cloning. A laboratory manual by T Maniatis, EF Fritsch and J Sambrook. pp 545. Cold Spring Harbor Laboratory, New York. 1982. $48 ISBN 0-87969-136-0. In Molecular cloning. A laboratory manual by T Maniatis, EF Fritsch and J Sambrook. pp 545. Cold Spring Harbor Laboratory, New York. 1982. $48 ISBN 0-87969-136-0: Wiley Online Library.

Wrong, OM 1981. Carbohydrates. In The Large Instestine: Its Role in Mammalian Nutrition and Homeostasis, Wrong OM, Edmonds CJ, Chadwick VS. In

Carbohydrates. IN The Large Instestine: Its Role in Mammalian Nutrition and Homeostasis, Wrong OM, Edmonds CJ, Chadwick VS: Halsted Press, New York.

Xiang, Ying, Feng Wu, Yinghui Chai, Xuebin Xu, Lang Yang, Sai Tian, Haoran Zhang, Yinxia Li, Chaojie Yang and Hongbo ;J BMC microbiology Liu 2020. A new plasmid carrying mphA causes prevalence of azithromycin resistance in enterotoxigenic *Escherichia coli* serogroup O6. 20: 1-9.

Yamamoto, T and Takeshi ;J Journal of bacteriology Yokota 1983. Sequence of heat-labile enterotoxin of *Escherichia coli* pathogenic for humans. 155: 728-733.

Yamanaka, Hiroyasu, Tomohiko Nomura, Yoshio Fujii and Keinosuke ;J Microbial pathogenesis Okamoto 1998. Need for TolC, an*Escherichia coli*outer membrane protein, in the secretion of heat-stable enterotoxin I across the outer membrane. 25: 111-120.

Yang, Ji, Marija Tauschek, Richard Strugnell and Roy M ;J Microbiology Robins-Browne 2005. The H-NS protein represses transcription of the eltAB operon, which encodes heat-labile enterotoxin in enterotoxigenic *Escherichia coli*, by binding to regions downstream of the promoter. 151: 1199-1208.

Zegeye, Ephrem Debebe, Morten Larsen Govasli, Halvor Sommerfelt, Pål ;J Human Vaccines Puntervoll and Immunotherapeutics 2018. Development of an enterotoxigenic *Escherichia coli* vaccine based on the heat-stable toxin.

Zhang, Shiying, Belgin Dogan, Cindy Guo, Deepali Herlekar, Katrina Stewart, Ellen J Scherl and Kenneth W ;J Antibiotics Simpson 2020. Short chain fatty acids modulate the growth and virulence of pathosymbiont *Escherichia coli* and host response. 9: 462.

CHAPTER 8

Appendix

Reagents and Solutions

1. Luria-Bertani (LB) broth

Reagents	Amount (g L^{-1})
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	10.00
Final pH 7.0 ± 0.2	

2. LB-agar

Reagents	Amount (g L^{-1})
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	10.00
Agar	18 - 20
Final pH 7.0 ± 0.2	

3. MacConkey agar

Ingredients	Amount (g L^{-1})
Peptide digest of animal tissue	17.00
Agar	13.50
Lactose	10.00
Sodium chloride	5.00
Bile salts	1.50
Proteose peptone	3.00
Neutral red	0.03

Crystal violet	0.001
Final pH at 25˚C : 7.1 ± 0.2	
Additional 0.8% agar was added if and when required.	

4. CFA broth

Ingredients	Percentage
Cassamino acid	1%
Yeast extract	0.15%
Magnesium sulphate	0.005%
Manganese chloride	0.0005%
Final pH 7.4 ± 0.2	

5. M9 Minimal Salts

Ingredients	Amount (g L^{-1})
Disodium Phosphate	6.78
Monopotassium Phosphate	3.00
Sodium Chloride	0.5
Ammonium Chloride	1.0
Glucose 20%	20 ml
1M $MgSO_4$	2 ml
1M $CaCl_2$	0.1 ml
Final pH 7.4 ± 0.2	

6. SOB medium

Reagent	Amount (for 100 mL)
Tryptone	2.0 g
Yeast extract	0.5 g
1M NaCl	1 mL
1M KCl	0.25 mL
2M Mg^{2+} stock (filter sterilized)	1 mL

7. SOC medium

SOB + 20 mM glucose (filter sterilized)

8. Phosphate Buffered Saline (PBS)

Reagent	Amount (g L^{-1})
Di-sodium hydrogen phosphate	2.67
Potassium dihydrogen phosphate	1.088
Potassium chloride	0.1998
Sodium chloride	7.946
Volume adjusted to 1L with double distilled water	
Final pH 7.4	

9. Tris Acetate EDTA (TAE) buffer

Reagent	Final Concentration (mmolar L^{-1})
Tris Base	40
Glacial acetic acid	20
EDTA	1
Volume adjusted to 1L with double distilled water	
Final pH 8.0	

10. Tris-EDTA Buffer

Reagent	Final Concentration (mmolar L^{-1})
Tris-HCl (1M); pH 7.4-8	40
Glacial acetic acid	20
EDTA	1
Volume adjusted to 1L with double distilled water	
Final pH 8.0	

11. S-Buffer

CTAB solution: 10% in 0.7M NaCl

4.1 g NaCl was dissolved in 80 mL MilliQ water. While stirring, 10 g CTAB was added. To dissolve, the solution was heated at 65ºC. The final volume was adjusted to 100 mL with MilliQ water. Final pH 8.0 was made with EDTA.

12. 2M Mg^{2+} stock

Reagent	Amount (grams per 100 mL)
Magnesium chloride	20.33
Magnesium sulfate	24.65

13. 10X DNA loading dye

Reagent	Amount
Glycerol	3.9 mL
10% SDS	0.5 mL
0.5M EDTA	0.2 mL
Bromophenol Blue (BB)	25 mg
Xylene Cyanol (XC)	25 mg
Volume adjusted to 10 mL with double distilled water	
Store at -20°C	

14. Nutrient agar Stab

Reagent	Amount (g L^{-1})
Nutrient Broth	8.00
Agar	8.00
Sodium Chloride	5.00

15. Dulbecco′s Modified Eagle′s Medium (DMEM) Complete media

Reagent	Amount
DMEM	13.5 grams per Litre
Sodium Bicarbonate	3.7 grams per Litre
Fetal Bovine Serum (FBS)	100 mL
Antibiotics	10 mL
Non-Essential Amino Acids	10 mL
Volume adjusted to 1L with MilliQ water	
Final pH 7.4	

16. Dulbecco′s Modified Eagle′s Medium (DMEM) Incomplete Media

Reagent	Amount
DMEM	13.5 grams per Litre
Sodium Bicarbonate	3.7 grams per Litre
Fetal Bovine Serum (FBS)	5 mL
Non-Essential Amino Acids	10 mL
Volume adjusted to 1L with MilliQ water	
Final pH 7.4	

17. 5X SDS Gel-loading dye

Reagent	Amount
Tris-HCl pH6.8	100 mM
SDS	4% (w/v)
Bromophenol blue	0.2% (w/v)
Glycerol	20% (w/v)
B-mercaptoethanol	200 mM
Volume adjusted to 1L with MilliQ water	
Final pH 7.4	

18. Buffer for polyacrylamide gel electrophoresis

Reagent	Final Concentration
Solution A	
Acrylamide	29.2%
Bis acrylamide	0.8%
The solution was stored in light resistant container.	
Solution B	
Tris HCl (pH 8.8)	1.5 M
SDS	0.4%
Solution C	
Tris HCl (pH 6.8)	0.5 M
SDS	0.4%

19.SDS-PAGE Running buffer

Reagent	Final Concentration
Tris base	0.025 M
Glycine	0.192 M
SDS	0.1%
The pH will be 8.3. No need for pH adjustment.	

20.Staining solution

Reagent	Final Concentration
Methanol	50%
Acetic acid	50%
Coomassie Brilliant blue	0.1%

21.Distaining solution

Reagent	Final Concentration
Methanol	5%
Acetic acid	7.5%
dH_2O	87.5%

22.Transfer buffer (for Western blot)

Reagent	Amount ($g\ L^{-1}$)
Tris	5.8
Glycine	2.9
SDS	0.05

23.X-Gal (2ml)

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactose, 100 mg) was dissolved in 2 ml of N,N´- dimethyl formamide to make a stock of 50 mg/ml X-Gal. The tube was covered with aluminium foil and stored in -20°C until use.

24. IPTG stock (0.1 M)

24 mg of Isopropyl-β-D-thiogalactopyranoside (IPTG) was dissolved in 1 ml of N, N´-dimethylformamide. The solution was filter sterilized and the tube was covered with aluminium foil. The solution was stored in 4 °C until use.

25. LA plate with antibiotics/IPTG/X-Gal

LA plate with appropriate antibiotic(s) was incubated at 37 °C for 30 minutes and 100 µl of 0.1M IPTG and 20 µl of 50 mg/ml X-Gal were spreaded on the LA plate. The plate was incubated for 30 minutes at 37 °C.

26. Cell lysis buffer

Reagent	Final Concentration
Potassium phosphate Buffer, pH 7.8	50 mM
NaCl	400 mM
KCl	100 mM
Glycerol	10%
Triton X-100	0.5%
Imidazole	10 mM

Prepare the potassium phosphate buffer (pH 7.8) by mixing 0.3 ml KH_2PO_4 and 4.7 ml K_2HPO_4 from their respective 1 M stock.

27. Plasmid DNA Purification Reagents

Reagent	Final Concentration
Cell Resuspension Solution	
Tris-HCl (pH 7.5)	50 mM
EDTA	10 mM
RNase A	100 µg/ml
Cell Lysis Solution	
NaOH	0.2 M
SDS	1%
Neutralization Solution	
Potassium Acetate (pH 4.8)	1.32 M
Column Wash Solution	
Potassium Acetate	80mM
Tris-HCl (pH 7.5)	8.3mM
EDTA	40µM
95% Ethanol	55%

28. Transformation Buffer

Reagent	Final Concentration
PIPES	10 mM
$CaCl_2 \cdot 2H_2O$	15 mM
KCl	250 mM
Adjust pH to 6.7 with 10 M KOH	
Then add, $MnCl_2 \cdot 4H_2O$	1.09 g for 100 mL
Filter sterilize	

29. Antibiotic Stock

Unless otherwise indicated, the antibiotic powder was dissolved in autoclaved MilliQ water and filter sterilized. The stock solutions aliquots were stored at -20ºC.

30. Purification of DNA from Agarose gel

Reagent	Final Concentration
Membrane Wash Solution	
Potassium Acetate, pH 5.0	10 mM
95% Ethanol	80 %
EDTA (pH 8.0)	16.7µM
Membrane Binding Solution	
Guanidine Isothiocyanate	4.5 M
Potassium Acetate, pH 5.0	0.5 M

31. Reagents for ELISA

A. Blocking Buffer

5% skimmed milk in PBS- T (0.05% Tween 20 in PBS, pH 7.4)

B. Wash Buffer

0.05% Tween 20 in PBS, pH 7.4

C. Coating Buffer

50 mM sodium carbonate, pH 9.6 and 20 mM Tris-HCL, pH 8.5

D. Primary/Secondary Antibody Solution

Primary/secondary antibody was diluted in 1x blocking buffer

E. Stopping Solution

2 M H_2SO_4

32.Z buffer

Reagent	Final Concentration
Sodium hydrogen phosphate	0.06 M
Sodium dihydrogen phosphate	0.04 M
Potassium chloride	0.01 M
Magnesium sulfate	0.001 M
B -mercaptoethanol (BME)	0.05 M
Adjust pH to 7.0 and store at 4˚C	

33. Phosphate buffer, 0.1 M

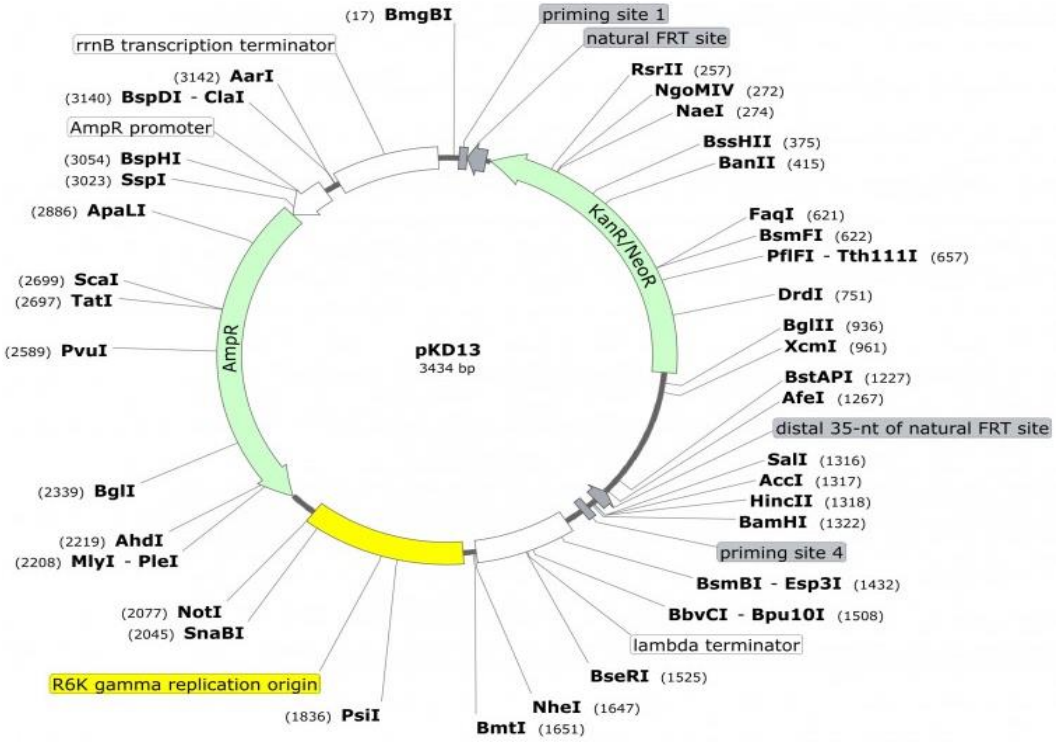
Reagent	Final Concentration
Sodium hydrogen phosphate	0.06 M
Sodium dihydrogen phosphate	0.04 M
Adjust pH to 7.0	
Phosphate buffer is stable at room temperature and does not need to be made fresh each time.	

34. ONPG (Ortho-Nitrophenyl-ß-galactoside)

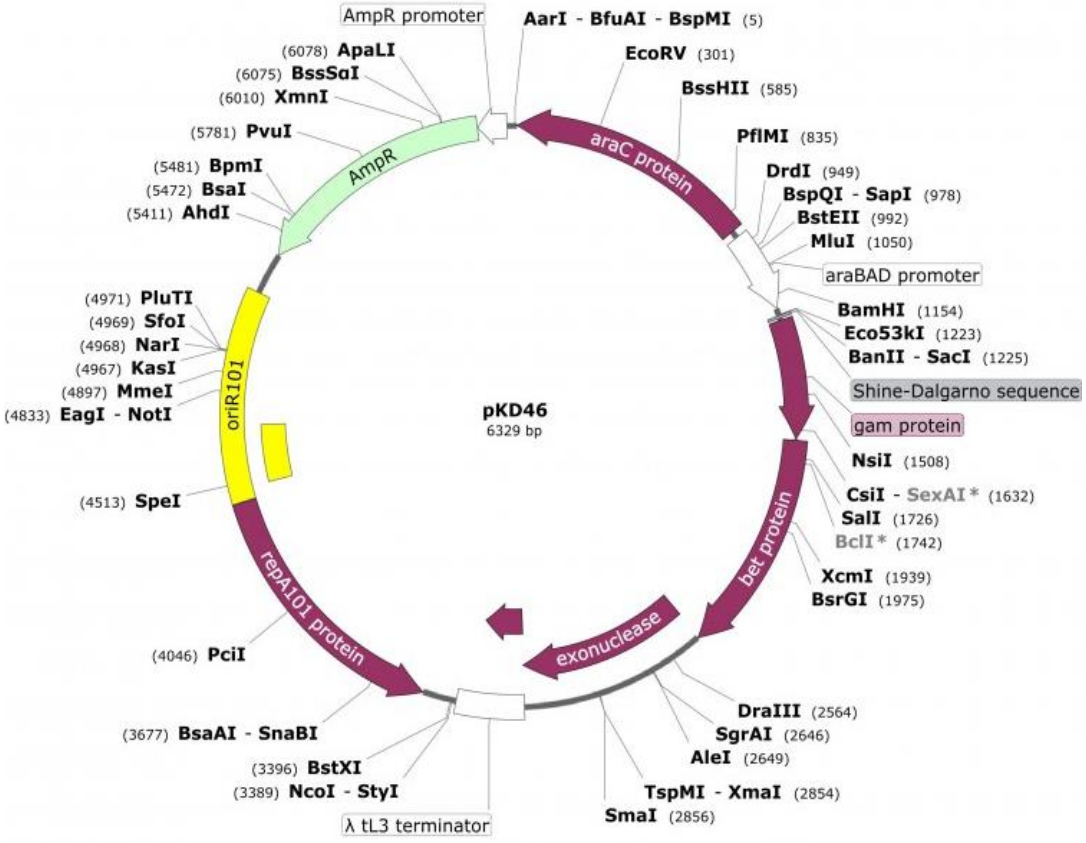
The ONPG was dissolved to a final concentration of 4mg/mL in 0.1M phosphate buffer pH 7.0.It should be prepared fresh each day prior to the experiment.

Vectors used

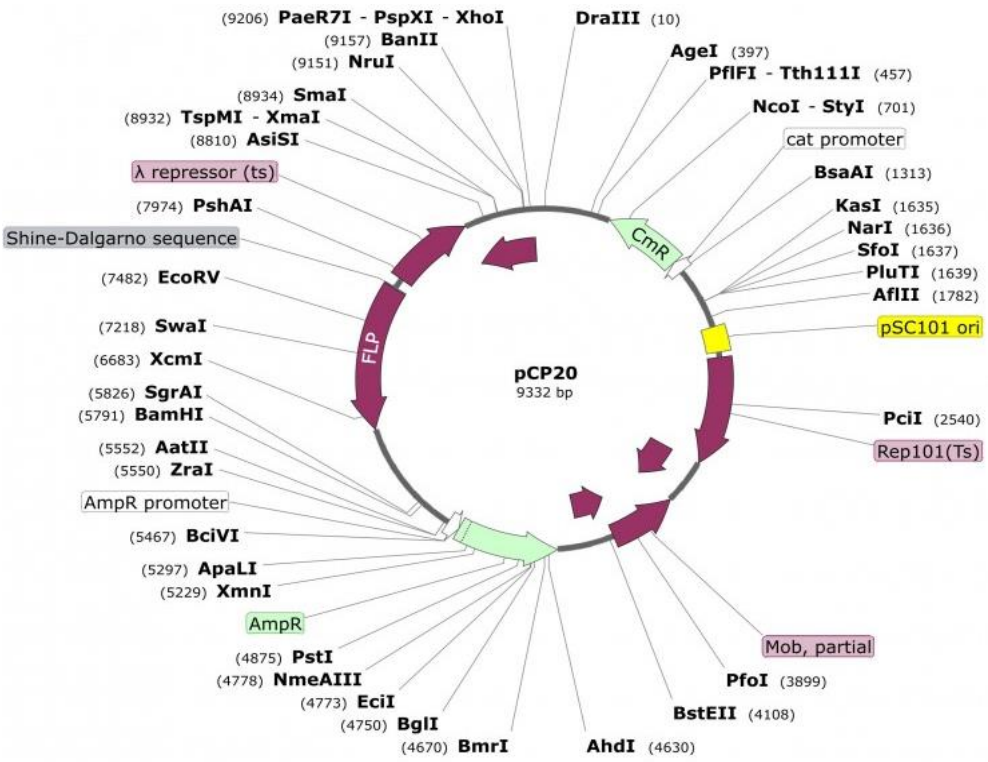
pKD13



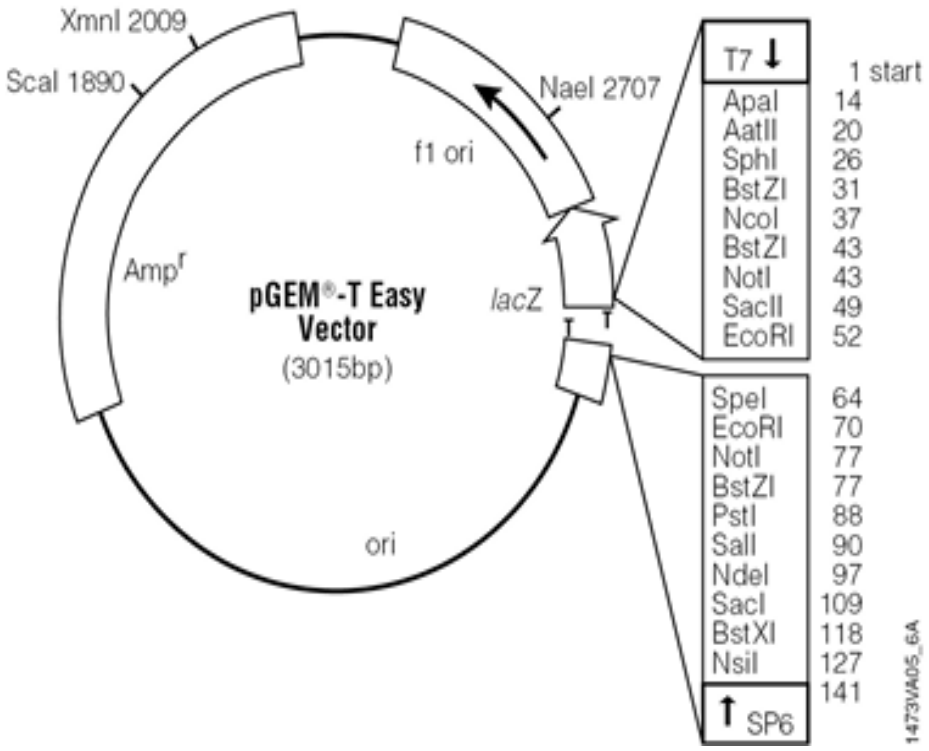
pKD46



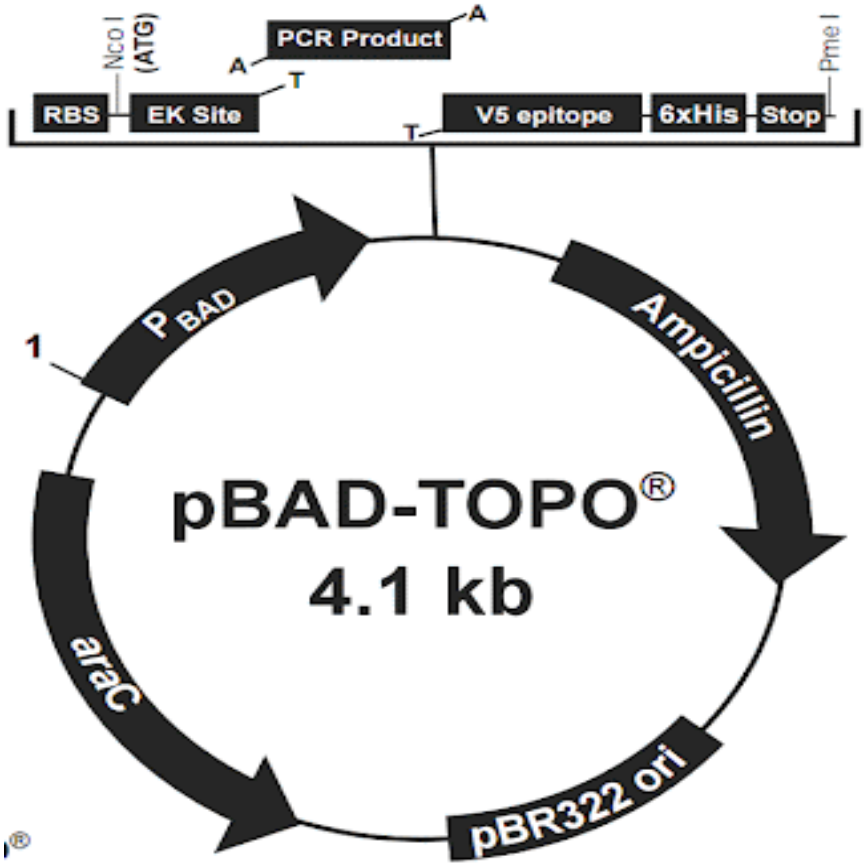
pCP20



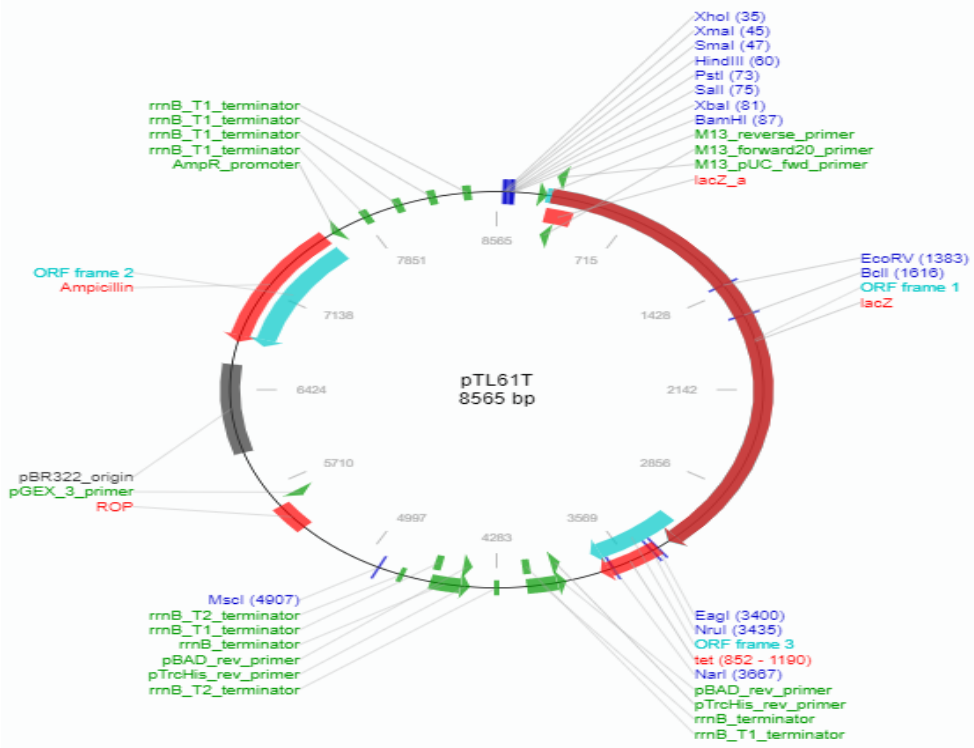
pGEM®-T Easy Vector



pBAD-TOPO TA expression vector



pTL61T Vector



pET-22b(+)

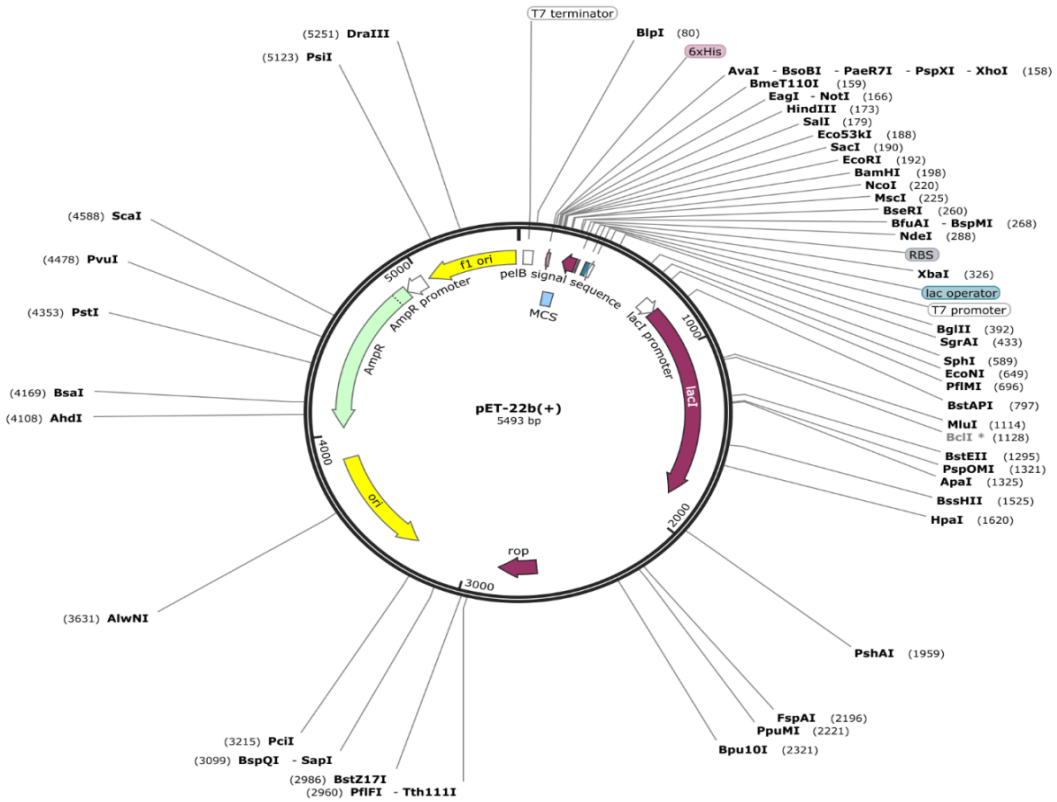

T7 terminator
BlpI (80)
6xHis
AvaI - BsoBI - PaeR7I - PspXI - XhoI (158)
BmeT110I (159)
EagI - NotI (166)
HindIII (173)
SalI (179)
Eco53kI (188)
SacI (190)
EcoRI (192)
BamHI (198)
NcoI (220)
MscI (225)
BseRI (260)
BfuAI - BspMI (268)
NdeI (288)
RBS
XbaI (326)
lac operator
T7 promoter
BglII (392)
SgrAI (433)
SphI (589)
EcoNI (649)
PflMI (696)
BstAPI (797)
MluI (1114)
BclI * (1128)
BstEII (1295)
PspOMI (1321)
ApaI (1325)
BssHII (1525)
HpaI (1620)
PshAI (1959)
FspAI (2196)
PpuMI (2221)
Bpu10I (2321)
(5251) DraIII
(5123) PsiI
(4588) ScaI
(4478) PvuI
(4353) PstI
(4169) BsaI
(4108) AhdI
(3631) AlwNI
(3215) PciI
(3099) BspQI - SapI
(2986) BstZ17I
(2960) PflFI - Tth111I
pET-22b(+)
5493 bp
f1 ori
pelB signal sequence
MCS
lacI promoter
lacI
AmpR promoter
AmpR
ori
rop
5000
1000
2000
3000
4000

Table A.1. List of primers used in this study

Target Gene	Primer Name	Oligonucleotide Sequences (5' → 3')	Product Size (bp)	Gene Bank Accession No.
***CS1**	Forward	TTGACCTTCTGCAATCTGA	324	M58550
	Reverse	CATCTGCATGGATTGAAAG		
***CS2**	Forward	TAACTGCTAGCGTTGATCC	368	Z47800
	Reverse	ATTAGTTTGCTGGGTGCTT		
***CS3**	Forward	GGTGGGTGTTTTGACTCTT	264	M35657
	Reverse	TGTTCGTTACCTTCAGTGG		
***CS4**	Forward	TTTTGCAAGCTGATGGTAG	250	X97493
	Reverse	TCTGCAGGTTCAAAAGTCA		
[†]**CS5**	Forward	TCCGCTCCCGTTACTCAG	226	X63411
	Reverse	GAAAAGCGTTCACACTGTTTATATT		
***CS6**	Forward	ATCCAGCCTTCTTTTGGTA	321	U04844
	Reverse	ACCAACCATAACCTGATCG		
[‡]**CS7**	Forward	TGCTCCCGTTACTAAAAATAC	203	AY009095.1
	Reverse	TAGATGTCGTATCACTACGT		
[§]**CS12**	Forward	TTACGTCTCTGATCATGG	449	AY009096.1
	Reverse	TAAAGCCGTAGCACCATC		
[§]**CS13**	Forward	TTGATGTGATGGTTATCGC	370	X71971.1
	Reverse	TTGGTAGCAAACTCCACC		
***CS14**	Forward	ACTGTGACAGCCAGTGTTG	394	X97491
	Reverse	AAACGACGCCTTGATACTT		
[§]**CS15**	Forward	TTCTTCAGTTACGGCAATG	282	X64623.1
	Reverse	TTCGCATTTTGGAAGGCG		
***CS17**	Forward	AACCTATTCTTCGGCTTCA	190	X97495
	Reverse	GCGCAGTTCCTTGTGTG		
[§]**CS18**	Forward	TTTGCTGCACTGCCTGC	354	U31413.1
	Reverse	TGGTGTCACTTTATCACC		
[§]**CS19**	Forward	AGCTCCAGCAGTTCTGTC	184	AY288101.1
	Reverse	AGCAGCTTCTGCATGAATC		
[§]**CS20**	Forward	ATTCCAGTCAGGCCACAC	313	AF438155.1
	Reverse	ACAGGAGTACCTTTACTGG		
***CS21**	Forward	ACAGTCCGCGTAGCAAT	400	EU107092.1
	Reverse	GTAAAACAGTTGTAGAGG		
[§]**CS22**	Forward	TTCTTCAGTTCAGGCAATG	245	AF145205.1
	Reverse	TATTCCCAGCGTCTGGC		
[§]**CS23**	Forward	AGACTCTGATTGCACTGG	249	JQ434477.1
	Reverse	TCGTAGTACGAATAGCCAG		

§CS26	Forward	AGGTTACCACCACAGTTC	296	HQ203050.1
	Reverse	AAGAGCCTTGTTCTCTGC		
§CS27	Forward	AAAGGTAAAGCGCAGAGC	208	HQ203047.1
	Reverse	TCTTTGTACCAGTGCTCG		
§CS28	Forward	ATTTGGGTGAGGTATCCA	379	HQ203046.1
	Reverse	AACTCATTTGCATCAACCG		
§CS30	Forward	ATAACGATGCAAGTAAGGC	429	LT174529.1
	Reverse	TTTGAGTACCAATGTAGGC		
§PCF071	Forward	AACCATGGGAGCATCTGC	421	AY513487.1
	Reverse	TTGACCAGCTGTTAGTCC		
***CFA/I**	Forward	GCTCTGACCACAATGTTTG	364	S73191
	Reverse	TTACACCGGATGCAGAATA		
***CFA/III (CS8)**	Forward	GCCTTCTGGAAGTCATCAT	438	D37957
	Reverse	CTGCCACATACTCCCAG		
***tia**	Forward	CTCTGGCTGATGAGAGC	172	U20318.1
	Reverse	TCATAGCCCACTGCAAG		
***etpA**	Forward	CAGACAGCTACACCAAC	221	AY920525.2
	Reverse	CGATTGAGTCGTCTCAG		
***leoA**	Forward	AGGCGAATCTGAAAGGC	315	AF170971.1
	Reverse	CATTCTTCTCGACAAAGG		
***tibA**	Forward	GTGCTTAACAATACTGGG	396	AF109215.1
	Reverse	TTCCAGCAGCATATTGAC		
***eatA**	Forward	CTGTAAATGGCGCTTATC	465	AY163491.2
	Reverse	TTAATGTTCCCACTCCTG		
***elt**	Forward	CACACGGAGCTCCTCAG	324	M17874.1
	Reverse	CAAACTAGTTTTCCATACTG		
***esth**	Forward	GCTAAACCAGTAGAGTC	149	M34916.1
	Reverse	CACCCGGTACAAGCAGG		
§*estp*	Forward	AGTCAGTCAACTGAATCAC	228	V00612.1
	Reverse	ATTTTCTCAGCACCAATAC		

*****Primers were designed as per Bhakat *et al.*, 2018
§ Primers were designed by using BLAST method (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for this study.
† Primers for CS5 Ref: Vidal *et al.*, 2019.
‡ Primers for CS7 Ref: Del Canto *et al.*, 2011

Table A.2: List of Primers used for qRT-PCR

Target Gene	Primer Name	Oligonucleotide Sequences (5' → 3')	Product Size (bp)	Gene Bank Accn. No.
csfD (CS5)	Forward	ACCCTGAGTACCGCAGTAGA	93	X63411.1
	Reverse	AACTGTTGGACTAGCGCTCC		
cssB (CS6)	Forward	CGGTTGAGATGACGATCCCT	90	U04844.1
	Reverse	GCTTTCCGATCTGCTGTCCA		
eatA	Forward	GGGCCTCTTGCAAACTATCT	103	AY163491.2
	Reverse	CTGATGACAGAACGCCAACTA		
parC	Forward	ATCCGCTGGTTGATGGTCAG	92	EU561348.1
	Reverse	TTTCGACAGACGGGATTCGG		

Table A.3: Primers for isogenic non-polar mutants and ETEC complement strains

Target Gene	Primer Number	Primer Name	Oligonucleotide Sequences (5' → 3')	Product Size (bp)	Gene Bank Accn. No.
CS5 (A)	I	Forward	AGTGAATGCAGCAGGTC	438	AJ224079.2
	II	Reverse	ATCGCTCAAGACGTGTAATTCTCATCCTTCTTTGTCT		
pKD13(B)	III	Forward	TTACACGTCTTGAGCGAT	1362	AY048744.1
	IV	Reverse	AACTATTCCGGCAAAGGAATTCACTGATCAGTGATAAG		
CS5 (C)	V	Forward	TCCTTTGCCGGAATAGTT	372	AJ224079.2
	VI	Reverse	ATCATTCAGTGTATCTGGG		
CS5 IP	VII	Forward	GGGCTTTTGTGAACGGATT	402	AJ22407

	VIII	Reverse	CTGCCTTGGCATTCATATC		9.2
CS5 complement	IX	Forward	ATGAAGAAAAATTTACTGATAACT	612	AJ224079.2
	X	Reverse	TTAAATATCAACAGCAAATGTTA		
eatA (A)	I	Forward	TCGGGAGGATAATTAGGG	381	FN649418.1
	II	Reverse	ATCGCTCAAGACGTGTAAAGCTATCCAACATAATGCG		
pKD13 (B')	III	Forward	TTACACGTCTTGAGCGAT	1362	AY048744.1
	IV	Reverse	GTACAAGGAGCTATCTATTATTCACTGATCAGTGATAAG		
eatA (C)	V	Forward	AATAGATAGCTCCTTGTAC	351	FN649418.1
	VI	Reverse	ATATAGACAAACCAAACGC		
eatA IP	VII	Forward	AACGCTATGTCCTGATAG	262	FN649418.1
	VIII	Reverse	AAGCGAAACATCGCATCG		
eatA complement	IX	Forward	ATGAATAAAGTGTTCTCTCTT	4092	FN649418.1
	X	Reverse	GAAATAATAACGGAAGTTAGC		
pKD46	1	Forward	ATCACCACTCTTCGCCAG	235	AY048746.1
	2	Reverse	TACAGGATTCATTGTCCTG		

Table A.4: Primers for Promoter Study and deletion of promoter regions

Target Gene	Primer Number	Primer Name	Oligonucleotide Sequences (5' → 3')	Product Size (bp)	Gene Bank Accn. No.
CS6 promoter	I	Forward	TCCCCCCGGGATAACTAACTGAAAAACAATG	573	NC022333.1
	II	Reverse	GCTCTAGATATAACACCATTACTATTGCT		
CS5 promoter	I	Forward	TCCCCCCGGGACAATCATTATACTATATTGAC	207	NZCP023347.1
	II	Reverse	GCTCTAGATTTATTCTCATCCTTCTTTG		
EatA promoter	I	Forward	TCCCCCCGGGCTGTTTAAATTCGGGAGG	406	FN649418.1
	II	Reverse	GCTCTAGATTTTGATAGCTGCGCAGC		
CS6 promoter	1 prCS6 (-573)	Forward	TCCCCCCGGGTATAACTAACTGAAAAACAATG	597	NC022333.1
	2	Forwa	TCCCCCCGGGACAAGCCGCAG	538	

prCS6 (-512)	rd	CCGCC	
3 prCS6 (-456)	Forwa rd	TCCCCCCGGGAATGCGCGGTGG AAATG	480
4 prCS6 (-350)	Forwa rd	TCCCCCCGGGACTACGATGGTC AGGTTG	374
4A prCS6 (-335)	Forwa rd	TCCCCCCGGGTTGCGGAGGCTA TGGC	359
4B prCS6 (-320)	Forwa rd	TCCCCCCGGGCCCTGGTACGAG CGCT	344
4C prCS6 (-305)	Forwa rd	TCCCCCCGGGTGAACAAAATG ACGAAAGC	329
4D prCS6 (-290)	Forwa rd	TCCCCCCGGGAAGCAGGTATGC CTGAAA	314
4E prCS6 (-275)	Forwa rd	TCCCCCCGGGAAAGCGTGCGTA TTGCC	299
4F prCS6 (-260)	Forwa rd	TCCCCCCGGGCCTGAAAACACA ACCCG	284
5 prCS6 (-255)	Forwa rd	TCCCCCCGGGAAACACAACCC GCTACG	279
6 prCS6 (-78)	Forwa rd	TCCCCCCGGGTCAATACGGTGC TCCG	102
7 prCS6 (-23)	Forwa rd	TCCCCCCGGGTATAGCAATAGT AATGGTGTT	47
prCS6 (+25)	Rever se	GCTCTAGAAATTAAACCAATTG TTTTCTTC	

Table A.5: Primers used for cloning of eatA in pet22b vector

Target Gene	Primer Number	Primer Name	Oligonucleotide Sequences (5' → 3')	Product Size (bp)	Gene Bank Accn. No.
eatA	r*eatA* I	Forward	TTTTT*GGATCC*CTACGATA AGAATGGAGT	1483	FN649418.1
	r*eatA* II	Reverse	TTTGG*CTCGAG*CGACCGTA CGCCTTTGATT		

CHAPTER 9

Publications

&

Conferences

Publications

- **Mondal I,** Bhakat D, Chowdhury G, Manna A, Samanta S, Deb AK, Mukhopadhyay AK, Chatterjee NS. Distribution of virulence factors and its relatedness towards the antimicrobial response of enterotoxigenic *Escherichia coli* strains isolated from patients in Kolkata, India. *J Appl Microbiol*. 2021 Jul 9. doi: 10.1111/jam.15206. PMID: 34242448.

- Bhakat, D., **Mondal, I**. and Chatterjee, N.S., (2020). EatA, a non-classical virulence factor, of Enterotoxigenic *Escherichia coli* (ETEC) is modulated by the host factors during pathogenesis. *International Journal of Infectious Diseases, 101* (supplement 1), pp.3-4.

- Bhakat, D., **Mondal, I.,** Mukhopadhyay, A. K., & Chatterjee, N. S. (2021). Iron influences the expression of colonization factor CS6 of enterotoxigenic *Escherichia coli*. Microbiology, 167(9), 001089.

Conferences

- Poster presented in 31st ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases ,**Title**: Revealing the region-specific prevalence and expression of virulence factors present in enterotoxigenic *Escherichia coli*

  I.Mondal , D. Bhakat , A.K. Mukhopadhyay and N.S. Chatterjee
- Oral presentation at International e-Conference - 2021 on "Covid-19: Challenges and Opportunities in Pharmaceutical Research".**Title:** Interpreting the Expression profile of prevalent colonization factor CS6 of Enterotoxigenic *E. coli* in Kolkata region.

  Indranil Mondal , Debjyoti Bhakat , Asish K Mukhopadayay, Nabendu S Chatterjee

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ORIGINAL ARTICLE

Distribution of virulence factors and its relatedness towards the antimicrobial response of enterotoxigenic *Escherichia coli* strains isolated from patients in Kolkata, India

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Abstract

Aim: Enterotoxigenic *Escherichia coli* (ETEC) is one of the most widely recognized diarrhoeal pathogens in developing countries. The advancement of ETEC vaccine development depends on the antigenic determinants of the ETEC isolates from a particular geographical region. So, the aim here was to comprehend the distribution of virulence determinants of the clinical ETEC strains of this region. Additionally, an attempt was made to find any correlation with the antimicrobial response pattern.

Methods and results: Multiplex PCR was employed to identify virulence determinants followed by confirmatory singleplex PCR. For observation of antibiotic response, the Kirby-Bauer method was used. Out of 379 strains, 46% of strains harboured both the enterotoxins ST and LT, whereas 15% were LT only. Among the major colonization factors (CFs), CS6 (41%) was the most prevalent followed by CFA/I (35%) and CFA/III was the lowest (3%). Among the minor CFs, CS21 (25%) was most prevalent, while CS15 showed the lowest (3%) presence. Among the non-classical virulence factors, EatA (69%) was predominant. ETEC strains harbouring CS6 showed resistance towards the commonly used drug Ciprofloxacin (70%).

Conclusion: CS6 and *elt+est* toxin genes co-occurred covering 51% of the isolates. CS21 was found in most strains with *est* genes (43%). EatA was found to occur frequently when ST was present alone or with LT. CS6-harbouring strains showed an independent correlation to antimicrobial resistance.

Significance and Impact of the Study: This study would aid in identifying the commonly circulating ETEC isolates of Kolkata, India, and their prevalent virulence determinants. Knowledge of antibiotic resistance patterns would also help in the appropriate use of antibiotics. Furthermore, the study would aid in identifying the multivalent antigens suitable for region-specific ETEC vaccines with maximum coverage.

KEYWORDS

antimicrobial agents, colonization factor, Enterotoxigenic *Escherichia coli*, prevalence, virulence factors

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is an enteric pathogen estimated to cause about 400 million cases of diarrhoea and 157,000 deaths in children <5 years of age. Additionally, the bacteria also cause 44 million cases and 89,000 deaths in older children & adults. ETEC causes 8.5 million Disability Adjusted Life Years (DALYs) or 1 million Years Lived with Disability (YLDs) (Bourgeois, Wierzba & Walker, 2016). ETEC is the leading cause of diarrhoea in developing countries and among travellers from developed countries to these endemic regions (Arduino & Dupont, 1993). In developed nations like the United States, ETEC is acknowledged as a major cause of foodborne disease.

ETEC disease burden estimation of all ages has increased significantly in the number of deaths from 59,200 in 2013 to 74,100 in 2015 (Hosangadi, Smith, Kaslow, et al., 2019). Globally ETEC is detected frequently in Southeast Asian, African, and Middle East regions of the world. In Iraq, ETEC was detected in almost 75% of food items consumed by humans followed by diarrhoea during 2016–2017 (Taha & Yassin, 2019).

Global Enteric Multicentric Study (GEMS) indicated that ETEC was one of the four important pathogens related to diarrhoeal illnesses among children <5 years old in Africa and South Asia (Anderson IV et al., 2019). Although, it was previously thought that ETEC burden was lower, GEMS and MAL-ED molecular re-analysis data indicated the opposite spectrum. ETEC was among the top four pathogens causing diarrhoea-associated deaths among children <5 years old as estimated by CHERG (Hosangadi, Smith, & Giersing, 2019).

In the central region of Kenya, about 6%–7% of all diarrhoeal children were detected positive for ETEC (Mbuthia et al., 2018). In India, according to the global burden of diseases (GBD) 2015, around 6% of all diarrhoeal deaths in children aged less than 5 years were due to ETEC. In this eastern region of India, almost 5% of diarrheagenic *Escherichia coli* (DEC) was ETEC (Dutta et al., 2013). Out of the detected DEC, ETEC was the third most prevalent cause of pathogenesis (about 13.6%) between 2008 and 2012 in Karnataka, India (Singh et al., 2019). Surveillance study suggested that about 4% of clinical cases were ETEC among all diarrhoea infected patients hospitalized in Kolkata (Nair et al., 2010).

Pathogenesis of ETEC causes the release of electrolytes and water during watery diarrhoea. This was due to the release of plasmid-encoded two enterotoxins—heat-labile (LT) and heat-stable (ST). LT, encoded by *elt* gene, is an 84-kDa protein consists of an AB5 multimeric structure, where a pentamer of B chains has a membrane-binding function and the A chain is responsible for toxin activity. Structurally and functionally, LT is the mirror of cholera toxin. ST, encoded by *est* gene, is a non-antigenic low-molecular-weight peptide of 18–19 amino acids. It is of two variants, STp and STh, named after their discovery from porcine and humans respectively (Rao, 1985). ETEC strains produce either ST or LT or both; however, the ratio of different types of toxin-producing ETEC varies in different geographic regions (Qadri et al., 2005).

To release the enterotoxins, the bacteria first attach to the epithelium of the small intestine through different colonization factors (CF), also known as antigenic fimbriae called colonization factor antigens (CFA), a major virulence determinant for initiating pathogenesis (Gaastra & Svennerholm, 1996). CFAs encoded by ETEC are mainly divided into four major groups—CFA/I, CFA/II, CFA/III, and CFA/IV. These CFA groups are further subdivided according to their antigenic properties as coli surface antigens (CS). CFA/I is a single member of the first group. CFA/II group encodes CS3 alone or in combination with CS1 and CS2. CFA/III or CS8 is also a single member of this group. CFA/IV group encodes CS6 alone or in combination with CS4 and CS5. Rigid fimbriae CS1, CS2, CS4, CS5 and CFA/I are ~6 nm in diameter, whereas flexible fibrialle CS3 is ~3 nm in diameter (Vidal et al., 2019). Afimbrial CS6 is an assembly of two subunits, CssA and CssB associated tightly in an equal (1:1) stoichiometry (Sabui et al., 2016). Beside these main CF groups, there also exists minor CF which also helps in adherence of ETEC to the intestinal epithelium. Minor CFs include CS7, CS12, CS13, CS14, CS15, CS17, CS18, CS19, CS20, CS21, CS22, CS23, CS26, CS27, CS28, CS30 and PCF071 (Del Canto et al., 2012; Gaastra & Svennerholm, 1996; Mentzer et al., 2017). With the identification of CS30, there are 23 well-characterized CFs to date (Bhakat et al., 2018). Two more CFs are known in addition to these strains making the total number of CFs 25. The CFs together with the toxins are considered classical virulence factors. Out of the known CFs, only a few are prevalent such as CS6, CS5, CFA/I. The CFs like CS19, CS13, CS18 and CS15 are generally less common globally.

Besides the CFs, studies have indicated that other putative factors playing a role in ETEC pathogenesis, and these are known as 'non-classical virulence factors (NCVFs)'. EatA, EtpA, LeoA, TibA and Tia are the most commonly found NCVF in ETEC strains worldwide. EatA is a serine protease autotransporter that alleviates the delivery of LT toxin (Patel et al., 2004). EtpA is a secreted 170-kDa glycoprotein that plays a role in adherence of the bacteria to the intestinal mucosa (Fleckenstein et al., 2006). LeoA, a cytoplasmic protein, acts to maximize LT secretion and invasion of the epithelial cells by acting as a molecular bridge (Fleckenstein et al., 2000). TibA and Tia are the invasins which are autotransporters. They are predicted to promote adherence and promote the formation of biofilm in the host (Fleckenstein et al., 2010). More than 50% of the strains had EatA and EtpA as their NCVFs isolated from ETEC isolates in Bangladesh between 1998 and 2011 (Luo et al., 2015). In Bolivian children positive for ETEC, ClyA was the most commonly detected NCVF followed by EatA (Gonzales et al., 2013).

Until now, there is no effective licensed efficacious ETEC vaccine available and it allows indiscriminate use of antibiotics to curb the disease. As ETEC is prevalent throughout the globe and its variants are geo-region specific, so it is mandatory to know the disparity in the antibiotic response profile (Medina et al., 2015). About 20%–50% of all ETEC cases in developing countries emerge due to the wide range of antimicrobial resistance among the strains (Ericsson, 2003). Along with this, the emergence of multidrug-resistant ETEC is making the use of antibiotics unavailing against these pathogens (Diemert, 2006). Hence, unwrapping the antibiotic response of clinical ETEC isolates becomes a matter of immense importance.

According to WHO, the development of a practical and effective vaccine against ETEC is of high priority, especially for low- to middle-income countries which can be achieved by including prevalent toxins, CFs and NCVFs harboured by clinical strains. The strategy of developing a vaccine includes attenuated ETEC expressing CFs, multi-epitope fusion antigens, inactivated fimbriated ETEC, ST toxoids. As well as triggering intestinal secretory IgA antibodies to counter CFs and prevent ETEC from colonizing the digestive system is critical for effective immunization (Vidal et al., 2019).

The prevalence of these CFs and NCVFs is geographically based and changes with time. So, information about the prevalence of ETEC virulence determinants over a longer period of time is important for designing an effective ETEC vaccine with broader coverage. Therefore, monitoring the prevalence of various CFs and NCVFs changes with time turns out to be fundamental for defining the multivalent ETEC vaccine that would work over a wide scope of the topographical areas and effective for the long term.

In this study, the aim was to identify the commonly circulating virulence determinants of ETEC isolates from Kolkata, India. The antimicrobial response pattern among the strains having the most predominant virulence determinant was also studied. This study is expected to improve the knowledge about the strains from this region of India and their antimicrobial response pattern that would help to prescribe antibiotics for diarrhoeagenic patients with ETEC infection.

MATERIALS AND METHODS

Isolation and Identification of ETEC

In the hospital-based epidemiological surveillance study at the National Institute of Cholera and Enteric Diseases (NICED), stool specimens were collected from diarrhoeal patients admitted at the Infectious Diseases and Beliaghata General Hospital (ID & BG Hospital, Kolkata) and Dr. B.C. Roy Post Graduate Institute of Pediatric Sciences during 2015–2019. During this period, a total of 379 strains were positive for ETEC using the PCR-based analysis and collected for further investigation from the archive of NICED.

ETEC strains were plated onto MacConkey agar (SRL) plates and incubated for 16–18 h at 37°C. Colonies fermenting lactose on MacConkey agar plate having round and pink morphology were reconfirmed as ETEC and later used for experiments. These strains were further plated on Luria Bertini (LB) agar (Sigma Aldrich) plates. The culture from this nonselective medium was grown in LB and finally stored in 15% glycerol at −80°C for further use.

Extraction of DNA templates

DNA templates were prepared by the boil lysis method. Few colonies of ETEC from agar plate were taken in Tris-EDTA buffer (pH-6.8) and boiled for 10 min followed by immediate cooling in ice. Then the mix was centrifuged and an aliquot of the supernatant was used as a DNA template for PCR.

Detection of virulence determinants by multiplex PCR

For molecular detection of virulence determinants, a total of three primer sets for toxin detection, 24 primer sets for CFs and five primer sets for Non-classical virulence determinants were used (The details of primers are given in Table S1). The PCR reaction was carried in a total volume of 20 μl under the following condition-denaturation at 95°C for 10 min, denaturation at 94°C for 1 min, annealing at 52°C for 30 s, extension at 72°C for 60 s/kb of amplicon and a final extension at 72°C for 10 min. The virulence determinants tested positive in multiplex PCR was confirmed by simplex PCR under the same condition using respective primers for each of the genes.

Antimicrobial susceptibility test

The Kirby–Bauer method was used for bacterial susceptibility towards antimicrobial agents. For the antimicrobial susceptibility test, representative isolates were selected on the basis of having predominant CF. The antibiotic discs (Becton, Dickinson and Company) used are as follows: Imipenem (Ipm)-10 μg; Chloramphenicol (C)-30 μg; Cefapime (Fep)-30 μg; Doxycycline (D)-30 μg; Ceftriaxone (Cro)-30 μg; Tetracycline (Te)-30 μg; Norfloxacin (Nor)-10 μg; Sulfamethoxazole W/Trim (Sxt); Ciprofloxacin (Cip)-5 μg; Ampicillin (Am)-10 μg; Azithromycin (Azm)-15 μg; Nalidixic Acid (Na)-30 μg; Erythromycin (E)-15 μg; Streptomycin (S)-10 μg.

The test was performed on the Mueller–Hinton (MH) agar plates following Clinical and Laboratory Standards Institute (CLSI) guidelines (Hsueh et al., 2010). The isolates

were inoculated in MH Broth and incubated at 37°C up to 0.5 McFarland standard turbidity. The culture was streaked thoroughly onto MH Agar plates using cotton swabs. After air-drying the plates, antibiotic discs were placed and after overnight incubation at 37°C, the zone of inhibition was measured as per the manufacturer's protocols.

Statistical analysis

For comparison of two variables, a chi-square test was used. A $p < 0.05$ was considered statistically significant.

RESULTS

Toxin types of ETEC isolates

Archived isolates of ETEC were analysed in this study. Of 379 ETEC isolates, 38% ($n = 142$) strains were yielded along with other pathogens and 62% ($n = 237$) strains were isolated where ETEC was the sole pathogen. All the 379 strains screened were positive for toxin genes. Among the ETEC isolates, 175 strains harboured both *elt* + esth toxin genes (46%) followed by the strains harbouring *esth* gene alone (37%) and strains harbouring the only *elt* as toxin gene (15%). Only 1% of strains harboured *elt* + estp toxin gene and the frequency of *estp* only strains was also 1%. Only one strain was detected positive for the presence of *elt* + esth + *estp* (Table 1).

Distribution of toxin genes among ETEC isolates identified over time

ETEC strains isolated over 2015–2019 showed that the cases of ETEC infection were highest in 2015 (Table 1). The cases gradually decreased over the next 4 years. In all these years, *elt*+esth was the most frequently detected toxin gene combination among the ETEC isolates followed by *esth* only strains, making the *elt*+esth toxin gene combination the most prevalent toxin type in this region of India. The *elt* gene toxin was detected almost three times more in 2016 ($n = 34$) than in 2015 ($n = 10$). Among the isolates during these 5 years, the presence of *estp*+elt was observed in alternate years, that is, in 2015, 2017 and 2019. The *estp* harbouring strains were also detected in these above-mentioned years only (Table 1).

Age-wise distribution of toxin genes among ETEC isolates

Among the archived isolates, 49% of ETEC strains were isolated from patients younger than 5 years of age and 45% were above the age of 18 years (Table 2). The rest 6% were in between the age of 5–18 years.

When the distribution of toxin genes was studied in these three different age groups, that is, <5 years, 5–18 years, and >18 years of age, it was found that in all age groups, *elt*+est harbouring strains were more than *elt* only or *est* only ETEC isolates (Table 2). In age groups <5 years *elt*+*est* harbouring strains were 43%, in the age group 5–18 years 48% and of isolates from adults it was 51% whereas 18%, 9% and 11% of strains harboured *elt* gene respectively. Four ETEC isolates from children of ≤5 year's age had both *elt*+ *estp* as toxin gene and this combination has not been found in patients of more than 5 years of age. Two strains in the age group 5–18 years were found harbouring e*stp* gene only. Only 1 ETEC isolate harbouring *elt*+ *esth*+ *estp was* found in the patient of 5–18 years of age group. However, when analysed it was observed that distribution of toxin genes is not statistically significantly associated with age groups ($p = 0.357$).

Level of dehydration in comparison with toxin types

When recorded data of ETEC patients were collected on the level of dehydration they had during infection, it was observed

TABLE 1 ETEC distribution over the years with toxin genes

	Toxin genes						
Year	***elt***	***est***	***elt+esth***	***elt+estp***	***estp***	***elt+esth+estp***	**Total**
2015	10	36	56	2	2	0	106
2016	34	33	34	0	0	0	101
2017	2	25	38	1	1	1	68
2018	5	22	27	0	0	0	54
2019	5	23	20	1	1	0	50
Total	56	139	175	4	4	1	379

Toxin genes were detected using PCR method. Please see Material and Methods for details.

elt—heat labile toxin gene; *esth*—heat stable toxin gene; human variant; *estp*—heat stable toxin gene; porcine variant.

TABLE 2 Occurrence of ETEC in different age group with toxin genes

	Toxin genes			
Age	***elt* (%)**	***est* (%)**	***elt+est* (%)**	***p* value**
0–5 ($n = 185$)	33(17.8)	72(38.4)	80(43.2)	0.357
>5–18 ($n = 23$)	2(8.0)	10(43.4)	11(47.8)	
18+ ($n = 171$)	21(12.2)	61(35.6)	89(52.0)	

Toxin genes were detected using PCR method. Please see Material and Methods for details.

elt—heat labile toxin gene; *est*,—heat stable toxin gene; includes both human (*esth*) and porcine (*estp*) variant.

TABLE 3 Dehydration level associated with toxin genes

Dehydration level	Toxin genes				
	elt	***est***	***elt+est***	**Total**	***p* value**
None	14	31	31	76	0.687
Some	37	101	134	272	
Severe	4	11	16	31	

elt—heat labile toxin gene; est—heat stable toxin gene; includes both human (*esth*) and porcine (*estp*) variant.

that 72% of patients with some dehydration were maximum in number (Table 3). Severe dehydration was observed in the least number of patients (8%). The rest of the 20% of patients had not experienced any dehydration during the infection.

When this data of level of dehydration was compared to the type of toxin produced by ETEC strains, it was revealed that among *elt*+est harbouring ETEC strains, 74% were from patients having 'some' level of dehydration followed by 17% patients with no dehydration and only 9% experienced 'severe' level of dehydration. In *elt*, *est* and *elt+est* harbouring strains similar patterns of dehydration levels were observed with a maximum number of isolates with some level of dehydration and the least with a 'severe' level of dehydration. Statistical analysis revealed that there is no significant association between the level of dehydration and toxin genes ($p > 0.05$). The absence of association may be due to the small sample size and unavailability of data about if the patients had taken any over the counter drug before admission to the hospital.

Genotypic distribution of ETEC virulence factors

Among the ETEC isolates, both the classical CF and NCVF were present in 69% of the ETEC strains. Only CF genes were detected in 22% of the strains. Six percent of strains showed the presence of NCVF genes alone as their virulence determinants. No virulence determinants could be detected in 3% of the ETEC isolates in this study (Figure 1a).

The distribution of virulence factors over the years followed a similar pattern with isolates harbouring both CF+NCVF as their virulence determinants and least being the isolates without any detectable virulence factors (Figure 1b).

When the distribution of virulence factors in ETEC isolates was compared to the presence of toxin genes in the isolates, it was revealed that among 46% of strains harbouring CFs had both the *elt*+est toxin genes, followed by 40% of strains having *est* toxin gene. Distribution of the toxin genes among NCVF harbouring ETEC isolates revealed that maximum strains (47%) had both the *elt*+est toxin genes (Table 4).

Distribution of major CFs in ETEC isolates

The application of PCR enabled us to detect the presence of different CFs in this study as well as the prevalence of those CFs among ETEC isolates. Eight major CFs were targeted for detection grouped in CFA/I, CFA/II, CFA/III and CFA/IV (Table 5). The CFA/IV group was detected in 43% of the ETEC isolates followed by the CFA/I, and CFA/II group. The CFA/III was detected in the least number of isolates.

Major CF distribution in relation to toxin genes

Within the CFA/IV group, comprising of CFs CS4, CS5 and CS6, the CS6 was maximally detected (Table 6). CS6 harbouring ETEC isolates were detected in 41% of LT+ST strains. Among both LT-only and ST-only strains, CS6 detected almost in equal frequency, 34% and 36% respectively. Alongside CS6, in ST-only isolates, CFA/I was detected in almost the same number (37%) but in LT+ST strains it was detected in only 28% strains compared to 41% strains positive for CS6 (Table 6). Following CS6 and CFA/I, CS5 is another major CF found commonly in 21% CF positive ETEC isolates. The most prevalent combination of Major CF was found to be CS6 with CS5 followed by CFA/I with minor CF CS21. Among the prevalent major CF combination, 61% of CS6+CS5 ETEC isolates were found to harbour *elt+est* as their toxin gene followed by ST-only CS6+CS5 isolates (27%) and only 10% of CS6+CS5 isolate harboured *elt* as their toxin

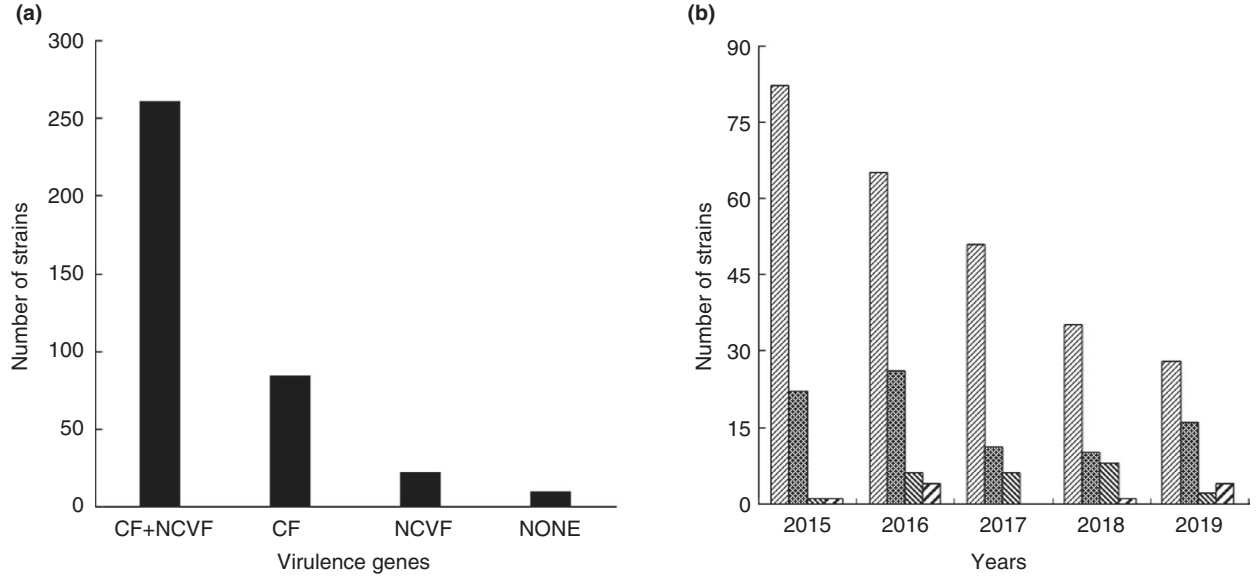

FIGURE 1 Distribution of ETEC virulence factors during the study period (2015–2019). (a) Shows overall distribution of virulence determinants. (b) (▩) CFs; (▨) NCVF; (▨) CF+NCVF; and (◪) None; represents year wise distribution of virulence determinants. A total of 379 clinical samples were screened for CFs—classical colonization factors; and NCVFs— NCVFs. CFs denote the isolates positive only for the classical colonization factors; NCVFs represents strains positive only for the NCVFs; CF+NCVF represents strains positive for both the virulence factors; and NONE represents strains negative for both the virulence factors. We tested a total of 29 virulence factors (24 CFs and 5 NCVFs) and the results shown are based on the screened factors

TABLE 4 ETEC Virulence factors distribution with toxin genes

Virulence factors	Toxin genes			
	elt	*est*	*elt+est*	Total
CF	48	143	163	354
NCVF	31	119	134	284
None	2	2	6	10

est includes both human (*esth*) and porcine (*estp*) variant.

Classical colonization factor (CF) genes studied here by PCR; please see Materials and Methods for details.

Non-classical virulence factor (VF) genes studied here by PCR; please see Materials and Methods for details.

No tested virulence factor detected.

TABLE 5 ETEC CF distribution with toxin genes

Major CF groups	Toxin genes			
	elt	*est*	*elt+est*	Total
I	19	52	51	122
II	6	22	55	83
III	4	3	3	10
IV	23	59	81	163

est includes both human (*esth*) and porcine (*estp*) variant.

Classical colonization factor (CF) genes, Classical CF genes studied here by PCR; please see Materials and Methods for details.

TABLE 6 Prevalent CF distribution with toxin genes

Prevalent major CFs	Toxin genes			
	elt	*Est*	*elt+est*	Total
CS6	19	51	73	143
CFA/I	19	52	51	122
CS5	9	21	45	75

est includes both human (*esth*) and porcine (*estp*) variant.

Classical colonization factor (CF) genes, Classical CF genes studied here by PCR; please see Materials and Methods for details.

TABLE 7 Occurrence of the most prevalent combination of CF with toxin genes

Classical genes	Toxin			
	elt	*est*	*elt+est*	Total
CS6+CS5	7	18	40	65
CFA/I+CS21	3	17	21	41
CS6+CFA/I	3	13	20	36
CS6+CS21	0	8	6	14

est heat stable toxin gene; includes both *esth* and *estp*.

gene. Whereas 55% of CS6+CFA/I isolate harboured *elt+est* as their toxin gene. In CFA/I+CS21 isolates, 51% of strains harboured *elt+est* as prevalent toxin gene (Table 7).

Distribution of major CFs over the years

During 2015–2019, CS6 was detected as the predominant CF over other CFs in every year, for example, 35% isolates in 2015, 44% isolates in 2016, 43% isolates in 2017, 24%

TABLE 8 Major CF distribution over the years

Major CF groups	CF	Years					Total	Percent
		2015	2016	2017	2018	2019		
CFA/I	CFA/I	32	42	16	17	15	122	34
CFA/II	CS1	5	17	6	3	6	37	10
	CS2	8	5	4	3	4	24	7
	CS3	14	24	12	5	7	62	18
CFA/III	CFA/III	1	3	4	1	1	10	3
CFA/IV	CS4	3	3	2	4	1	13	4
	CS5	22	25	16	7	5	75	21
	CS6	37	44	29	16	17	143	40

Classical colonization factor (CF) genes, Classical CF genes studied here by PCR; please see Materials and methods for details.

isolates in 2018, and 34% isolates in 2019 (Table 8). The detection of CFA/I in the ETEC isolates in these years were after CS6. In 2016, CS6 and CFA/I were detected almost in similar abundance in the ETEC isolates. In 2016 and 2017, CS6 was detected in a higher percentage (44% and 43% respectively) than that of 2015, 2018, and 2019. Among the rarely encountered CFs, CFA/III was detected in 3% of isolates only.

Minor CF among ETEC isolates

Seventy-five percent of the total ETEC strains were positive for harbouring at least one minor CF. Among these, 11%strains harboured *elt* toxin gene only, 42% harboured est toxin gene only and 47% harboured *elt*+est toxin gene. Among the minor CFs, CS21 was accounted for the highest presence (31%). In these strains, *elt*+est positive CS21 was maximum, whereas only three strains had the *elt* gene.

During the 2015–2019 period, CS21 was detected maximally over other minor CFs in every year, for example, 21% isolates in 2015, 19% isolates in 2016, 30% isolates in 2017, 27% isolates in 2018, and 18% isolates in 2019 (Table 9), This was followed by detection of CS23 in these years except in 2019, when no strains were found having CS23. In minor CFs, CS15 was found in the least number of strains found only. Four minor CFs, CS7, CS19, CS13, CS26 and CS30 were not detected in any of the ETEC isolates in our study.

Genotypic distribution of NCVF genes in ETEC isolates in the present study

The non-classical VFs were screened and found to be present in 75% of the ETEC strains during the study period 2015–2019. Out of these positive NCVFs strains, EatA (69%) was most commonly detected followed by EtpA (42%), tibA (18%), and tia (12%) (Table 10). LeoA gene (7%), was least detected NCVF in our study. Most of the NCVF harbouring ETEC isolates were in co-presence with at least one or more classical CFs. In this study, only 8% of the ETEC isolates had NCVF genes alone. EatA was mostly detected (42%) along with prevalent classical CF CS6. Six percent of EatA gene was detected in ETEC isolates having no detectable CFs. Out of all the ETEC isolates, 25% isolates were NCVF negative.

Distribution of NCVF genes in ETEC isolates in relation to toxin genes detected

When analysed in comparison with toxin genes, it was observed that *elt*+est-ETEC isolates harboured maximum NCVF in 47% strains (47%). In *elt* only strains, least number of NCVF (11%) was found. EatA, the predominant NCVF, found maximally in *elt*+est- strains (49%), and *elt* only strains, EatA was minimum (9%). Following EatA, EtpA also showed the same pattern of distribution accounting for half of the EtpA strains (50%) produced LT+ST toxins and almost equal percentage produced ST toxins (Table 10).

Year-wise distribution of NCVF genes among ETEC isolates

During the span of study from 2015 to 2019, EatA was the prevalent non-classical VF detected in our region followed by EtpA as the next common one. EatA was identified in the highest number in 2015, whereas EtpA was most detected in 2016. In 2016, the least common NCVF, LeoA was identified only in one strain. NCVF genes were found to be an independent variable over time (Table 11).

TABLE 9 Minor CF distribution over the years

	Years						
Minor CFs	**2015**	**2016**	**2017**	**2018**	**2019**	**Total**	**Percent**
CS21	23	20	21	15	9	88	25
CS23	20	8	6	3	0	37	10
CS20	18	0	10	0	3	31	9
CS28	17	1	3	2	3	26	8
CS27	12	6	0	1	1	20	6
PCF071	4	5	7	3	1	20	6
CS22	13	0	0	1	0	14	4
CS12	8	2	1	0	0	11	3
CS18	2	2	7	0	0	11	3
CS14	7	2	1	0	2	12	3
CS17	6	5	2	0	2	15	4
CS15	4	0	0	3	5	12	3
CS19	0	0	0	0	0	0	0
CS13	0	0	0	0	0	0	0
CS26	0	0	0	0	0	0	0
CS30	0	0	0	0	0	0	0

Classical colonization factor (CF) genes, Classical CF genes studied here by PCR; please see Materials and methods for details.

TABLE 10 Occurrence of ETEC NCVFs with toxin genes

	Toxin genes			
NCVF	***elt***	***est***	***elt+est***	**Total**
EatA	17	82	97	196
EtpA	10	50	59	119
Tib	5	21	24	50
Tia	10	12	13	35
LeoA	3	8	9	20

est includes both human (*esth)* and porcine *(estp)* variant.

Non-classical virulence factor (VF) genes. Non-classical VF genes studied here by PCR; please see Materials and methods for details.

Antibiotic susceptibility of ETEC isolates

From this study, CS6 was identified as the prevalent CF during 2015–2019. The antibiotic susceptibility in the CS6-harbouring ETEC isolates were tested for antibiotic response against 14 antimicrobial agents (Table 12). All CS6-harbouring isolates were resistant to one or more antimicrobial agents with the most frequent resistance found against 10 μg streptomycin (99.5%) and 15 μg erythromycin (97.7%). More than 80% of strains were resistant to 30 μg Nalidixic Acid and 15 μg Azithromycin. All the ETEC isolates were sensitive to 10 μg Imipenem. The majority of the strains were sensitive to 30 μg Chloramphenicol. When combinations of different antibiotic resistance were analysed compared to the toxin types in CS6-harbouring strains, a very few strains showed a similar pattern of resistance to different antibiotic combinations (Table S3).

To compare the results, we also tested 10 strains, in which no known virulence factors were detected. Among these isolates, almost a similar pattern of response to antimicrobial agents was found (Table 12).

DISCUSSION

Examining the presence of toxin genes in clinical ETEC strains during this study period, it was observed that the *elt+est* harbouring ETEC strains were most common, and *elt* only strains were found in the lowest number. This result is comparable with the findings from studies conducted in Bangladesh (Begum et al., 2014). Findings from a previous study in this region of Kolkata also indicated the same pattern of the presence of toxin genes in ETEC isolates (Bhakat et al., 2018). Global enteric multicenter study (GEMS) on Asia and Africa reported 68% were either ST only or LT/ST ETEC strains (Vidal et al., 2019). In contrast, the global prevalence of *elt* toxin gene is much higher than *est*, either alone or together with *elt* (*est+elt*). A study revealed that LT toxin genes are present in approximately 60% of the field ETEC strains associated with diarrheal incidents in humans, either LT alone (27%) or in combination with ST (33%) (Isidean et al., 2011).

Results presented here trends for the presence of at least one CF are present in 91% of ETEC isolates. This can be

TABLE 11 NCVF distribution with time

	Years						
NCVF	**2015**	**2016**	**2017**	**2018**	**2019**	**Total**	**Percent**
EatA	64	46	41	29	16	196	69
EtpA	23	41	24	15	16	119	42
TibA	19	11	13	5	2	50	18
Tia	10	7	7	6	5	35	12
LeoA	12	1	4	2	1	20	7

Non-classical virulence factor (VF) genes. Non-classical VF genes studied here by PCR; please see Materials and methods for details.

TABLE 12 Response of CS6 harbouring ETEC isolates and ETEC isolates having no detectable virulence factors to antimicrobial agents

Antibiotics	**Number of CS6 resistant strains (%)**	**Number of none resistant strains (%)**
Imipenem (Ipm)—10 μg	0 (0)	0 (0)
Chloramphenicol (C)—30 μg	8 (6)	2(20)
Cefapime (Fep)—30 μg	34 (24)	3 (30)
Doxycycline (D)—30 μg	39 (27)	2 (20)
Ceftriaxone (Cro)—30 μg	46 (32)	2 (20)
Tetracycline (Te)—30μg	47 (33)	2 (20)
Norfloxacin (Nor)—10μg	51 (36)	5 (50)
Sulfamethoxalone W/Trim (Sxt)	58 (41)	3(30)
Ciprofloxacin (Cip)—5 μg	100 (70)	6 (60)
Ampicillin (Am)—10 μg	107 (75)	8 (80)
Azithromycin (Azm)—15 μg	120 (84)	7 (70)
Nalidixic Acid (Na)—30 μg	124 (87)	10 (100)
Erythromycine (E)—15 μg	140 (98)	10(100)
Streptomycine (S)—10 μg	142 (99)	10 (100)

None refers to those ETEC isolates in which no virulence factors were detected by our tested virulence factors by PCR method.

Antibiotic disks were used (BD, USA) for this study using Kirby–Bauer Disc diffusion method.

compared to the frequencies of 23–94% isolates positive for CF reported in a systematic review done by Isidean et al., 2011. In Bangladesh, 49% of the ETEC isolates were positive for at least one CF (Begum et al., 2014). A study on Nicaraguan children reported that at least 50% of ETEC strains were positive for one CF (Vilchez et al., 2014). In Shenzhen, China, 54% of ETEC isolates had one or more CF present in ETEC strains (Li et al., 2017). The high presence of CF in ETEC isolates in our study (Table S2) was most probably due to the inclusion of 24 CFs in the detection protocol.

During an earlier analysis, findings from Kolkata strains between 2008 and 2014 showed CS21 and CS6 were the predominant CFs (Bhakat et al., 2018). In this study, the distribution of CS6 is predominant followed by CFA/I, rather than CS21. A similar trend in the presence of CS6 was also observed in China and Guatemala (Li et al., 2017; Torres et al., 2014). In the Kolkata region of India, CS6 was mostly detected in the clinical ETEC isolates (Dutta et al., 2013; Ghosal et al., 2007). The CS6 was the predominant CF in ETEC isolates from Zambia (Simuyandi et al., 2019), Guatemala (Torres et al., 2014). A study in Bali, Indonesia indicated CFA/I as the predominant CF (Subekti et al., 2003). Our revelations are also in accordance with GEMS discovery from studies conducted on Asian and African countries where CFA/I as well as CS1-CS6 are some major CF antigens. In rural Egypt, most of the ETEC strains had CFA/I, followed by CS6 as the predominant CF (Shaheen et al., 2004). CFA/I and CS17 are the most common virulence determinant detected in Bolivia (Rodas et al., 2011). In accordance with our findings, a study on the ETEC population in Nepal revealed that CS21 (62.6%) and CS6 (30.2%) were the most prevalent CFs (Margulieux et al., 2018). Another study in our laboratory earlier detected CS21 as the prevalent CF followed by CS6 among clinical ETEC isolates from Kolkata (Bhakat et al., 2018). In Bangladesh, frequently detected CFs were

CS5, CS6 and CS1 (Begum et al., 2014). The co-occurrence of CS5 and CS6 in ETEC strains was found in our study. This trend was also similar to the previous study (Bhakat et al., 2018). This is in accordance with the study that stated CS6 is expressed alone or in combination with CS5 or CS4 (Gaastra & Svennerholm, 1996; Wolf, 1997). This study found only three ETEC strains showing co-occurrence of CS6 and CS4.

In our study, we detected most of the ETEC strains having 1–5 CFs except a few strains having more than the average number of CFs (Table S2). So, there might be a possibility of having mixed strains in archived stock. This might suggest that during stock preparation of archived strains there could be some technical limitations which might give rise to mixed strains.

Analysis with respect to the presence of non-classical virulence determinants showed that three-fourth of the ETEC strains contained at least one or more of these factors. Among these, EatA was the most predominant one followed by EtpA. A study on ETEC clinical isolates of Northern Colombia, South America also indicated EatA as one of the most prevalent NCVF detected (Guerra et al., 2014). EatA was generally identified in strains having the presence of CFs, recommending a significant part to advance intestinal colonization. A similar trend of the presence of EatA and CFs were also found in Chilean ETEC isolates (Del Canto et al., 2011). However, any noteworthy connection of conjunction of EtpA and CFs was not found. This might be because of the cooperation of EtpA with highly conserved flagellin and accordingly elevating adherence to the intestinal wall (Roy et al., 2009). The other three NCVFs Tia, TibA and LeoA were found in low recurrence. Among these, LeoA was found in the most minimal recurrence and two strains with LeoA were negative for any classical CF genes. This outcome repudiates with the examination from Chile, Colombia and Guatemala where the greater part of the LeoA strains were negative for any classical genes (Del Canto et al., 2011; Guerra et al., 2014; Torres et al., 2014).

Among the remaining one-fourth of strains negative for NCVFs, only 10 strains were negative for known CFs. There were only 4% of CF negative strains that were positive for NCVF, that is: Tia, TibA and EtpA. So, despite the inclusion of most of the discovered CFs and NCVFs, we were unable to detect any factor responsible for colonization in 10 strains suggesting that there may be additional colonization determinants yet to be perceived.

Globally, reports of ETEC isolates negative for any virulence factors other than toxins are common. In Bangladesh, 51% of ETEC strains were negative for any CFs (Begum et al., 2014). In Thailand, 41% of ETEC isolates were negative for CFs (Puiprom et al., 2010). Similarly, in 46% of strains, no CFs were detected in Shenzen, China, and 33% in Iranian children (Li et al., 2017; Nazarian et al., 2014). This study identified just 3% of ETEC isolates negative for any virulence determinants. This was possible with the inclusion of more CF in the study design. This is an indication that for maximum coverage on the epidemiological survey we should follow the broad consideration of epidemiologic data about ETEC Classical and Non-Classical virulence determinants. This should be an important aspect in tracking the diversity of ETEC isolates in this region of India and prioritize the choice of the potential vaccine candidate, explicitly for this geographic realm of India.

This study on the antimicrobial response pattern of CS6-harbouring ETEC isolates towards common antimicrobial agents used for the treatment of ETEC-related diarrhoea, demonstrated that almost all these strains were resistant to early-generation drug erythromycin and streptomycin. More than 70% of CS6 harbouring ETEC isolates were ampicillin-resistant which is comparable to maximum resistance to ampicillin in Bolivian children (Rodas et al., 2011). A similar observation was reported in a study on Peruvian children that showed 64% of ETEC strains were resistant to ampicillin (Medina et al., 2015). The results obtained here are in accordance with a previous study in India where ETEC resistance to ciprofloxacin was reported (Chakraborty et al., 2001). In this investigation, it was observed that more than 60% of CS6-harbouring ETEC isolates of this region showed resistance towards nalidixic acid, azithromycin and tetracycline which were also observed in ETEC strains of Bangladesh showing similar resistance to the above antibiotics (Rahman et al., 2020). A study in Eastern Odisha, India revealed that more than 50% of ETEC strains were resistant to ceftriaxone and 2.4% of those isolates were resistant to imipenem (Moharana et al., 2019), whereas our investigation showed only 6% strains were resistant to ceftriaxone and no strains were resistant to imipenem. In this study, the absence of major similarity in resistance towards different combinations of antibiotics shows that there is a huge genetic diversity and acquiring pattern of resistance is also very diverse among the CS6-positive isolates.

WHO recommended using the prevalent CF around the world which includes CFA/I, CS1-CS6 along with LT-B of ETEC for developing vaccine candidates (Bourgeois et al., 2016). From our examination, it may be presumed that strains holding both the enterotoxins LT and ST, alongside classical CF CS6, CS5, CFA/I and CS21 along with NCVF EatA are the probable combination of virulence determinates coursing in this region.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

IM, DB and NSC conceptualized the study. IM, DB and GC performed experiments. IM, DB, AKM and NSC prepared the original draft of the manuscript. AM, SS, AKD and AKM collaborated in this study. All authors did data analysis, draft review, editing and approval.

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REFERENCES

Anderson, J.D., Bagamian, K.H., Muhib, F., Amaya, M.P., Laytner, L.A., Wierzba, T. et al. (2019) Burden of enterotoxigenic *Escherichia coli* and shigella non-fatal diarrhoeal infections in 79 low-income and lower middle-income countries: a modelling analysis. *The Lancet Global Health*, 7(3), e321–e330.

Arduino, R.C. & Dupont, H.L. (1993) Travellers' diarrhoea. *Baillière's Clinical Gastroenterology*, 7(2), 365–385.

Begum, Y.A., Baby, N.I., Faruque, A.S.G., Jahan, N., Cravioto, A., Svennerholm, A.-M. et al. (2014) Shift in phenotypic characteristics of enterotoxigenic *Escherichia coli* (ETEC) isolated from diarrheal patients in Bangladesh. *PLoS Neglected Tropical Diseases*, 8(7), e3031.

Bhakat, D., Debnath, A., Naik, R., Chowdhury, G., Deb, A.K., Mukhopadhyay, A.K. et al. (2018) Identification of common virulence factors present in enterotoxigenic *Escherichia coli* isolated from diarrhoeal patients in Kolkata, India. *Journal of Applied Microbiology*, 126(1), 255–265.

Bourgeois, A.L., Wierzba, T.F. & Walker, R.I. (2016) Status of vaccine research and development for enterotoxigenic *Escherichia coli*. *Vaccine*, 34(26), 2880–2886.

Chakraborty, S., Deokule, J.S., Garg, P., Bhattacharya, S.K., Nandy, R.K., Nair, G.B. et al. (2001) Concomitant infection of enterotoxigenic *Escherichia coli* in an outbreak of cholera caused by *Vibrio cholerae* O1 and O139 in Ahmedabad, India. *Journal of Clinical Microbiology*, 39(9), 3241–3246.

Del Canto, F., Botkin, D.J., Valenzuela, P., Popov, V., Ruiz-Perez, F., Nataro, J.P. et al. (2012) Identification of coli surface antigen 23, a novel adhesin of enterotoxigenic *Escherichia coli*. *Infection and Immunity*, 80(8), 2791–2801.

Del Canto, F., Valenzuela, P., Cantero, L., Bronstein, J., Blanco, J.E., Blanco, J. et al. (2011) Distribution of classical and nonclassical virulence genes in enterotoxigenic *Escherichia coli* Isolates from Chilean Children and tRNA gene screening for putative insertion sites for genomic Islands. *Journal of Clinical Microbiology*, 49(9), 3198–3203.

Diemert, D.J. (2006) Prevention and self-treatment of traveler's diarrhea. *Clinical Microbiology Reviews*, 19(3), 583–594.

Dutta, S., Guin, S., Ghosh, S., Pazhani, G.P., Rajendran, K., Bhattacharya, M.K. et al. (2013) Trends in the prevalence of diarrheagenic *Escherichia coli* among hospitalized diarrheal patients in Kolkata, India. *PLoS One*, 8(2), e56068.

Ericsson, C.D. (2003) Travellers' diarrhoea. *International Journal of Antimicrobial Agents*, 21(2), 116–124.

Fleckenstein, J.M., Hardwidge, P.R., Munson, G.P., Rasko, D.A., Sommerfelt, H. & Steinsland, H. (2010) Molecular mechanisms of enterotoxigenic *Escherichia coli* infection. *Microbes and Infection*, 12(2), 89–98.

Fleckenstein, J.M., Lindler, L.E., Elsinghorst, E.A. & Dale, J.B. (2000) Identification of a gene within a pathogenicity island of enterotoxigenic *Escherichia coli* H10407 required for maximal secretion of the heat-labile enterotoxin. *Infection and Immunity*, 68(5), 2766–2774.

Fleckenstein, J.M., Roy, K., Fischer, J.F. & Burkitt, M. (2006) Identification of a two-partner secretion locus of enterotoxigenic *Escherichia coli*. *Infection and Immunity*, 74(4), 2245–2258.

Gaastra, W. & Svennerholm, A.M. (1996) Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends in Microbiology*, 4(11), 444–452.

Ghosal, A., Bhowmick, R., Nandy, R.K., Ramamurthy, T. & Chatterjee, N.S. (2007) PCR-based identification of common colonization factor antigens of enterotoxigenic *Escherichia coli*. *Journal of Clinical Microbiology*, 45(9), 3068–3071.

Gonzales, L., Sanchez, S., Zambrana, S., Iniguez, V., Wiklund, G., Svennerholm, A.-M. et al. (2013) Molecular characterization of enterotoxigenic *Escherichia coli* isolates recovered from children with diarrhea during a 4-year period (2007 to 2010) in Bolivia. *Journal of Clinical Microbiology*, 51(4), 1219–1225.

Guerra, J.A., Romero-Herazo, Y.C., Arzuza, O. & Gómez-Duarte, O.G. (2014) Phenotypic and genotypic characterization of enterotoxigenic *Escherichia coli* clinical isolates from Northern Colombia, South America. *BioMed Research International*, 2014, 1–11.

Hosangadi, D., Smith, P.G. & Giersing, B.K. (2019) Considerations for using ETEC and Shigella disease burden estimates to guide vaccine development strategy. *Vaccine*, 37(50), 7372–7380.

Hosangadi, D., Smith, P.G., Kaslow, D.C. & Giersing, B.K. (2019) WHO consultation on ETEC and Shigella burden of disease, Geneva, 6–7th April 2017: meeting report. *Vaccine*, 37(50), 7381–7390.

Hsueh, P.R., Ko, W.C., Wu, J.-J., Lu, J.-J., Wang, F.-D., Wu, H.-Y. et al. (2010) Consensus statement on the adherence to Clinical and Laboratory Standards Institute (CLSI) Antimicrobial Susceptibility Testing Guidelines (CLSI-2010 and CLSI-2010-update) for Enterobacteriaceae in Clinical Microbiology Laboratories in Taiwan. *Journal of Microbiology, Immunology, and Infection*, 43(5), 452–455.

Isidean, S.D., Riddle, M.S., Savarino, S.J. & Porter, C.K. (2011) A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression. *Vaccine*, 29(37), 6167–6178.

Li, Y., Luo, Q., Shi, X., Lin, Y., Qiu, Y., Lv, D. et al. (2017) Phenotypic and genotypic characterization of clinical Enterotoxigenic *Escherichia coli* isolates from Shenzhen, China. *Foodborne Pathogens and Disease*, 14(6), 333–340.

Luo, Q., Qadri, F., Kansal, R., Rasko, D.A., Sheikh, A. & Fleckenstein, J.M. (2015) Conservation and immunogenicity of novel antigens in diverse isolates of enterotoxigenic *Escherichia coli*. *PLoS Neglected Tropical Diseases*, 9(1), e0003446.

Margulieux, K.R., Srijan, A., Ruekit, S., Nobthai, P., Poramathikul, K., Pandey, P. et al. (2018) Extended-spectrum β-lactamase prevalence and virulence factor characterization of enterotoxigenic *Escherichia coli* responsible for acute diarrhea in Nepal from 2001 to 2016. *Antimicrobial Resistance & Infection Control*, 7(1), 1–7.

Mbuthia, O.W., Mathenge, S.G., Oyaro, M.O. & Ng'ayo, M.O. (2018) Etiology and pathogenicity of bacterial isolates: a cross sectional study among diarrheal children below five years in central regions of Kenya. *Pan African Medical Journal*, 31(1), 88.

Medina, A.M., Rivera, F.P., Pons, M.J., Riveros, M., Gomes, C., Bernal, M. et al. (2015) Comparative analysis of antimicrobial resistance in enterotoxigenic *Escherichia coli* isolates from two paediatric cohort studies in Lima, Peru. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 109(8), 493–502.

Mentzer, A., Tobias, J., Wiklund, G., Nordqvist, S., Aslett, M., Dougan, G. et al. (2017) Identification and characterization of the novel colonization factor CS30 based on whole genome sequencing in enterotoxigenic *Escherichia coli* (ETEC). *Scientific Reports*, 7(1), 1–11.

Moharana, S.S., Panda, R.K., Dash, M., Chayani, N., Bokade, P., Pati, S. et al. (2019) Etiology of childhood diarrhoea among under five children and molecular analysis of antibiotic resistance in isolated enteric bacterial pathogens from a tertiary care hospital, Eastern Odisha, India. *BMC Infectious Diseases*, 19(1), 1–9.

Nair, G., Ramamurthy, T., Bhattacharya, M., Krishnan, T., Ganguly, S., Saha, D. et al. (2010) Emerging trends in the etiology of enteric pathogens as evidenced from an active surveillance of hospitalized diarrhoeal patients in Kolkata, India. *Gut Pathogens*, 2(1), 4.

Nazarian, S., Gargari, S.L.M., Rasooli, I., Alerasol, M., Bagheri, S. & Alipoor, S.D. (2014) Prevalent phenotypic and genotypic profile of enterotoxigenic *Escherichia coli* among Iranian children. *Japanese Journal of Infectious Diseases*, 67(2), 78–85.

Patel, S.K., Dotson, J., Allen, K.P. & Fleckenstein, J.M. (2004) Identification and molecular characterization of EatA, an autotransporter protein of enterotoxigenic *Escherichia coli*. *Infection and Immunity*, 72(3), 1786–1794.

Puiporm, O., Chantaroj, S., Gangnonngiw, W., Okada, K., Honda, T., Taniguchi, T. et al. (2010) Identification of colonization factors of enterotoxigenic *Escherichia coli* with PCR-based technique. *Epidemiology and Infection*, 138(4), 519–524.

Qadri, F., Svennerholm, A.-M., Faruque, A.S.G. & Sack, R.B. (2005) Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clinical Microbiology Reviews*, 18(3), 465–483.

Rahman, M.M., Ahmed, P., Kar, A., Sakib, N., Shibly, A.Z., Zohora, F.T. et al. (2020) Prevalence, antimicrobial resistance, and pathogenic potential of enterotoxigenic and enteropathogenic *Escherichia coli* associated with acute diarrheal patients in Tangail, Bangladesh. *Foodborne Pathogens and Disease*, 17(7), 434–439.

Rao, M.C. (1985). Toxins which activate guanylate cyclase: heat-stable enterotoxins. Ciba Foundation Symposium 112 – Microbial Toxins and Diarrhoeal Disease, 112, 74–93.

Rodas, C., Mamani, R., Blanco, J., Blanco, J.E., Wiklund, G., Svennerholm, A.-M. et al. (2011) Enterotoxins, colonization factors, serotypes and antimicrobial resistance of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from hospitalized children with diarrhea in Bolivia. *Brazilian Journal of Infectious Diseases*, 15(2), 132–137.

Roy, K., Hamilton, D., Ostmann, M.M. & Fleckenstein, J.M. (2009) Vaccination with EtpA glycoprotein or flagellin protects against colonization with enterotoxigenic *Escherichia coli* in a murine model. *Vaccine*, 27(34), 4601–4608.

Sabui, S., Debnath, A., Ghosal, A., Wajima, T., Hamabata, T., Ramamurthy, T. et al. (2016) Characterization of oligomeric assembly of colonization factor CS6 from enterotoxigenic *Escherichia coli*. *Microbiology*, 162(1), 72–83.

Shaheen, H.I., Khalil, S.B., Rao, M.R., Abu Elyazeed, R., Wierzba, T.F., Peruski, L.F. et al. (2004) Phenotypic profiles of enterotoxigenic *Escherichia coli* associated with early childhood diarrhea in rural Egypt. *Journal of Clinical Microbiology*, 42(12), 5588–5595.

Simuyandi, M., Chilengi, R., B Connor, S., B. Voeglein, J., M. Laban, N., Mwila-Kazimbaya, K. et al. (2019) Enterotoxigenic *Escherichia Coli* toxins and colonization factors among zambian children presenting with moderate to severe diarrhea to selected health facilities. *Archives of Microbiology & Immunology*, 3(4), 173–184.

Singh, P., Metgud, S.C., Roy, S. & Purwar, S. (2019) Evolution of diarrheagenic *Escherichia coli* pathotypes in India. *Journal of Laboratory Physicians*, 11(4), 346–351.

Subekti, D.S., Lesmana, M., Tjaniadi, P., Machpud, N., Sriwati, Sukarma et al. (2003) Prevalence of enterotoxigenic *Escherichia coli* (ETEC) in hospitalized acute diarrhea patients in Denpasar, Bali, Indonesia. *Diagnostic Microbiology and Infectious Disease*, 47(2), 399–405.

Taha, Z.M. & Yassin, N.A. (2019) Prevalence of diarrheagenic *Escherichia coli* in animal products in Duhok province, Iraq. *Iranian Journal of Veterinary Research*, 20(4), 255–262.

Torres, O.R., González, W., Lemus, O., Pratdesaba, R.A., Matute, J.A., Wiklund, G. et al. (2014) Toxins and virulence factors of enterotoxigenic *Escherichia coli* associated with strains isolated from indigenous children and international visitors to a rural community in Guatemala. *Epidemiology and Infection*, 143(8), 1662–1671.

Vidal, R.M., Muhsen, K., Tennant, S.M., Svennerholm, A.-M., Sow, S.O., Sur, D. et al. (2019) Colonization factors among enterotoxigenic *Escherichia coli* isolates from children with moderate-to-severe diarrhea and from matched controls in the Global Enteric Multicenter Study (GEMS). *PLoS Neglected Tropical Diseases*, 13(1), e0007037.

Vilchez, S., Becker-Dreps, S., Amaya, E., Perez, C., Paniagua, M., Reyes, D. et al. (2014) Characterization of enterotoxigenic *Escherichia coli* strains isolated from Nicaraguan children in hospital, primary care and community settings. *Journal of Medical Microbiology*, 63(Pt_5), 729–734.

Wolf, M.K. (1997) Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. *Clinical Microbiology Reviews*, 10(4), 569–584.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Conclusion: HPAI H5 viruses bearing the N6 subtype neuraminidase and mammalian adaptation markers in the polymerase genes are capable of causing lethal infection in ferrets as well as transmitting to uninfected animals in a direct contact setting. The heterogeneity in mammalian virulence among H5N6 viruses underscores the necessity of in vivo evaluation of H5Nx viruses as they continue to evolve and pose a threat to human health.

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0004

Correlation of host immune response to internalized and extracellular bacterial strains of streptococcus pneumoniae of various serotypes

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Background: Innate and adaptive immune response against pneumococcal infection is important for bacterial clearance. Pneumococcal cell wall plays an important role in host pathogen interaction. On the host, Toll-like receptors (TLRs) identifies the molecular components on pathogens which activates the host immune responses. It also triggers other pathogen specific responses and signalling molecules to inhibit internalization and colonization of this organism. The aim of this study was to investigate the regulation of genes encoding adherence and internalization to host immune response.

Methods and materials: *S. pneumoniae* strains of six serotypes (1, 3, 5, 19F, 23F, 14) were used. Their extracted cell walls were challenged against A549 human lung epithelial cell line. Expressions of 84 genes associated to host immune response were performed by RT2 Profiler PCR Array. Fold differences in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. Bacterial adherence was also measured simultaneously upon infection and expression of neuraminidases (NanA and NanB) were quantified.

Results: Bacterial adhesion assay showed strain of serotype 14 had the highest capability of adherence while serotype 23F has the weakest ability to adhere. In comparison to the host immune response of these strains, it was observed that strains of serotype 23F had significant upregulation of pro-inflammatory cytokines; CCL2,IL-8,IL-1B,IL-6, and CLEC4E. Other downstream TLR signalling molecules, IRAK1,IRAK2 were also up-regulated in this strain. Generally, regulation of the TLRs, pro-inflammatory cytokines (IL1A/B IL6, IL8), other downstream signalling molecules and adaptors showed serotypes 1, 3, 5 and 23F to be upregulated whilst serotype 14 showed downregulated expression.

Conclusion: The adherence assay reflects the bacteria found on the cell surface and not the internalized bacteria. It suggest that cell wall of *serotype* 23F induces greater pro-inflammatory host response as compared to cell wall of serotype 14. This finding suggests that serotype 23F internalize better compared to serotype 14, thus evoke a stronger signalling cascade as compared to serotype 14. This data suggest serotype 23F to be more invasive compared to serotype 14. This is supported with the increased expression of neuraminidase NanA in serotype 23F.

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0005

High virulence of ST 238 *Leptospira* interrogans isolated from small mammal captured in human leptospirosis suspected area in Selangor, Malaysia

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Background: Leptospirosis is a zoonotic and re-emerging disease caused by the *Leptospira* spp. Human leptospirosis presents with a wide range of symptoms and often being misdiagnosed with other diseases. It is well-known that the broad clinical presentations and severity of leptospirosis depend on the individual's age and immune status. However, the *Leptospira* species also contribute to the vast variation in clinical presentations. Hence, this study aimed to evaluate the virulence of three pathogenic (ST 238 *L. interrogans*, ST 242 *L. weillii*, ST 143 *L. borgepetersenii*) and six novel *Leptospira* spp. (*L. semungkisensis, L. fletcheri, L. congkakensis, L. jelokensis, L. perdikensis, L. langatensis*) isolated from small mammals and environments respectively in Malaysia in the hamster model.

Methods and materials: A four to six weeks hamsters were intraperitoneally injected with leptospires and observed up to 14 days for environment isolates and up to 21 days for small mammal isolates. Any moribund animals and the surviving animals were humanely euthanized and the kidney, liver and lungs were harvested for detection of leptospires and histopathology examination.

Results: ST 238 *L. interrogans* showed high virulence as four animals infected with this isolate died within the seven days of infection and gross finding showed haemorrhage in the lung. ST 242 *L. weillii* induced a chronic infection in the kidney as all animals survived up to 21st days though all animals infected with this isolate showed inactivity and weight loss after 6th days of infection. Animals infected with ST 143 *L. borgepetersenii* and the six environmental isolates showed no clinical symptoms.

Conclusion: Altogether, these data showed that local isolate of *L. interrogans* is highly pathogenic and as this strain was isolated from small mammal in human leptospirosis suspected area, a precautionary measure should be considered when coming in contact with this rodent and when visiting the place. The three pathogenic *Leptospira* spp. induced different level of virulence in the hamster model and future study through comparative genomic is needed to infer the virulence determinants of each isolate.

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0006

EatA,a non-classical virulence factor,of Enterotoxigenic *Escherichia coli* (ETEC) is modulated by the host factors during pathogenesis

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Background: Enterotoxigenic *Escherichia coli* (ETEC) is one of the leading causes of watery diarrhoea in developing countries, particularly among children less than five years and in travellers. Transmission is caused by contaminated food and water and is established in the small intestine via colonization factors (CFs).Recently it is shown that the pathogenicity of ETEC also

depends on the presence of additional virulence factors termed as non-classical virulence factors (NCVF). One of the most common NCVF isolated world-wide is EatA. EatA, an auto transporter, is important for ETEC to adhere to epithelial cells and takes part in intestinal colonization by digesting EtpA, secreted by ETEC. During infection pathogenic bacteria use specific host factors to modulate virulence and stress responses. In the continuing fight against diarrhoeal diseases, studies on ecological niches and their influence on survival and virulence of ETEC are important to generate new knowledge to inhibit or control the disease.

Methods and materials: Well-characterized ETEC strain harboring *est* and *elt* genes along with non-classical virulence, EatA was primarily used for this study. For the experiments, ETEC strain was grown in minimal media (M9) supplemented with 0.2% glucose; relevant additives were supplemented when required. RNA was isolated by using trizol and qRT-PCR was done to check the expression.

Results: Our experiment showed that individual bile component sodium chenodeoxycholate, sodium cholatehydrate, sodium taurodeoxycholate modulates EatA expression and was upregulated by 19-fold, 10-fold and 7-fold respectively. EatA expression was most at 0.0125% mucin concentration. EatA expression was positively upregulated by 40-fold when supplemented with 0.2 mM iron salt. In presence of NaCl, expression of EatA is upregulatedby 3-fold. EatA expression is downregulated in presence of glucose. EatA expression is most at pH 6. Maximal expression of EatA was seen at 37 °C. In rabbit model,the expression of EatA was most at the highest cell density ($1x10^8$ cells/ml). In in-vivo condition lower cell density did not produce increased fluid.

Conclusion: EatA expression depends on the host's intestinal factors. Understanding the pathogenesis of ETEC in different intestinal conditions generate important information that can be exploited towards developing methods of controlling infection and a strategy for vaccine development.

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0007

Cytopathological changes associated with the experimental inoculation of Brucella Abortus in laboratory animal (*cavia porcellus*)

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Background: Brucellosis is a zoonotic disease caused by Brucella Abortus which is a gram negative facultative intracellular pathogen, affecting both humans and animals. Transmission of bacteria has usually been through occupational hazards as well as through oral route where it lodges in the mucosa, beneath the sub mucosa these bacteria are ingested by phagocytes. It has been observed that the disease induces hepatomegaly and measurable splenomegaly related to accelerated lymphohistiocytic cells inside the spleen with predominant increases in the share of splenic macrophages, but it may also produce other degenerative and necrotic changes in other vital organs and lymph nodes. The intracellular life of Brucella limits touch to the host innate and adaptive immune responses, protects the organism from the effects of some antibiotics, and drives the only features of pathology in infected hosts which is normally separated into three wonderful levels; the incubation, acute and the extension phase.

Methods and materials: The study comprised of 3 male, 3 female and 3 control animals. Among these, the male and female animals were inoculated with doses of inoculum 1×10^8 CFU, 3×10^7 CFU and 1×10^7 CFU respectively. The reason of different doses was meant to check out extent of pathological lesions in the target laboratory animal. The bacterial strain isolate was obtained from the spleens of inoculated mice cultured in broth C (BBLTM, USA).

Results: Grossly, lymph nodes and mesenteric network were severely hyperemic and congested while, heart, liver, spleen, kidneys, lungs and heart were also congested, enlarged with pale mucous fluid present all over the dorsal surface. Intestines were swollen while strauss reaction and orchitis and anemic uterus were observed in male and female animals respectively. Microscopically, sloughing of germinal epithelium of seminiferous tubules, and infiltration of inflammatory cells were observed in epididymus. Emphysematous alveoli and thickened alveolar walls in lungs, increased bowman's space in kidneys, fragmentation in heart muscle fibers, lymphohistiocytic cells in spleen and vacuolation changes were observed in these cells.

Conclusion: The bacterium also induces specific gross and microscopic changes in all body organs apart from liver and spleen which were considered as the main predilection site of the bacteria.

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0008

Effect of Haskap (*Lonicera caerulea*) on *streptococcus pneumoniae* infected aged-mouse

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Background: Pneumonia is a high fatal disease in the world today. As *streptococcus pneumoniae* is most popular in bacterial pneumonia, improvement of pneumococcal pneumonia is considered to be one of the important factors in the longevity of the elderly. Haskap (*Lonicera caerulea*) is a fruit of a plant which has been eaten by the Ainu people (indigenous people in Hokkaido: northern part of Japan) as a fruit of longevity. However, there have been no scientific evidences of the Haskap as longevity so far. In this study, we examined whether Haskap fruit extract (HKP) is effective in aged-mouse infected with *S. pneumoniae.*

Methods and materials: 72 weeks old BALB / c female mice were orally administered HKP. On the second day of oral administration, mice were infected with *S. pneumoniae* ATCC 49619 strain intranasally. After that murine general condition such as survival rate were evaluated until 5 days. Lung tissue was aseptically isolated from mice at the time of death or 5 days after oral administration, and the number of colonies was counted after culturing on a blood agar medium at 37 °C under 5% CO_2 for 24 hours.Further, alveolar macrophage were collected from murine lung tissue treated with the HKP for 4 days. As a phagocytic assay, both alveolar macrophage and *S. pneumoniae* were mixed for 1 hour. Thereafter, the mixed samples were cultured on a blood agar medium, and then the number of colonies was evaluated.

Results: Although all untreated mice died up to 5 days, all HKP treated mice survived. Moreover, in the lung, the number of bacterial colonies from HKP treated mouse was decreased compared to untreated mouse. In the phagocytic assay, the number of bacteria

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Iron influences the expression of colonization factor CS6 of enterotoxigenic *Escherichia coli*

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is a major pathogen of acute watery diarrhoea. The pathogenicity of ETEC is linked to adherence to the small intestine by colonization factors (CFs) and secretion of heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST). CS6 is one of the most common CFs in our region and worldwide. Iron availability functions as an environmental cue for enteropathogenic bacteria, signalling arrival within the human host. Therefore, iron could modify the expression of CS6 in the intestine. The objective of this study was to determine the effect of iron availability on CS6 expression in ETEC. This would help in understanding the importance of iron during ETEC pathogenesis. ETEC strain harbouring CS6 was cultured under increasing concentrations of iron salt to assess the effect on CS6 RNA expression by quantitative RT-PCR, protein expression by ELISA, promoter activity by β-galactosidase activity, and epithelial adhesion on HT-29 cells. RNA expression of CS6 was maximum in presence of 0.2 mM iron (II) salt. The expression increased by 50-fold, which also reduced under iron-chelation conditions and an increased iron concentration of 0.4 mM or more. The surface expression of CS6 also increased by 60-fold in presence of 0.2 mM iron. The upregulation of CS6 promoter activity by 25-fold under this experimental condition was in accordance with the induction of CS6 RNA and protein. This increased CS6 expression was independent of ETEC strains. Bacterial adhesion to HT-29 epithelial cells was also enhanced by five-fold in the presence of 0.2 mM iron salt. These findings suggest that CS6 expression is dependent on iron concentration. However, with further increases in iron concentration beyond 0.2 mM CS6 expression is decreased, suggesting that there might be a strong regulatory mechanism for CS6 expression under different iron concentrations.

INTRODUCTION

In developing countries, acute diarrhoea is among the top major pathogenic agents responsible for deaths in children under the age of 5 years and also among all age groups according to the 2015 Global Burden of Disease Study [1]. Enterotoxigenic *E. coli* (ETEC) is the major etiological agent for acute watery diarrhoea in these countries and is estimated to cause 400 million episodes of diarrhoea and approximately 380000 deaths annually among children <5 years [2]. ETEC is also the causative agent for traveller's diarrhoea to people travelling to these endemic areas and is frequently exported to the developed countries [3].

Diarrhoea by ETEC is caused due to the release of the heat-labile (LT) and/or heat-stable (ST) enterotoxin. Both the toxins stimulate chloride secretion causing water and electrolytes to release into the intestinal lumen and subsequently, watery diarrhoea occurs [2]. For the toxins to be released, ETEC must adhere to the small intestine to withstand intestinal peristalsis. Different colonization factors, the major virulence determinant for initiating pathogenesis, mediates the adhesion or colonization of ETEC onto the surface of the small intestine [4]. With the recent discovery of CS30 [5], there are more than 27 different colonization factors, 24 are well-characterized [2, 4]. Among the colonization factors, CS6 is prevalent globally and it is the predominant colonization factor in this region [6–8]. In contrast to the other ETEC CFs that are fimbrial, CS6 is afimbrial. CS6 consists of two tightly-associated subunits CssA and CssB, organised in an equal (1:1) ratio, to form an external array of higher-order oligomers [9].

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Keywords: CS6; enterotoxigenic *Escherichia coli*; expression; ETEC; iron.

Abbreviations: CFs, colonization factors; ETEC, enterotoxigenic *Escherichia coli*; LT, heat labile enterotoxin; RT-PCR, real time - polymerase chain reaction; ST, heat stable enterotoxin.

Three supplementary figures are available with the online version of this article.

Table 1. Strains used in the study

ETEC strain	Culture on MacConkey agar	Other microorganism isolated along with ETEC	CF and toxin profile	Year of isolation
ETEC 4266*	*E. coli*	No	CS6; STh +LT	2008
ETEC 0001	*E. coli*	No	CS6; STh +LT	2008
ETEC 0002	*E. coli*	No	CS6; STh +LT	2009
ETEC 0003	*E. coli*	No	CS6; STh +LT	2010
ETEC 0004	*E. coli*	No	CS6; STh +LT	2012
ETEC 0005	*E. coli*	No	CS6; STh +LT	2014
ETEC 0006	*E. coli*	Yes	CS6; STh +LT	2008
ETEC 0007	*E. coli*	Yes	CS6; STh +LT	2009
ETEC 0008	*E. coli*	Yes	CS6; STh +LT	2010
ETEC 0009	*E. coli*	Yes	CS6; STh +LT	2012
ETEC 0010	*E. coli*	Yes	CS6; STh +LT	2014

*Reference strain.

When ETEC infects the host, it experiences an assortment of host environmental factors which may impact virulence gene expression by sensing the proper microenvironment for the appropriate expression of the virulence factors. Iron is one such essential element in the intestinal environment. In pathogenic bacteria the expression of virulence-associated properties is frequently involved with iron availability [10]. It has been demonstrated that the freely available iron increases the bacterial virulence in at least 18 different bacterial species [11].

Worldwide iron deficiency is one of the most prevalent nutritional disorders affecting infants, young children and women in developing countries and causes significant health consequences for example infection [12]. However, studies have also shown that iron supplements had adverse effects causing an exaggeration in enteric infections and fatality in young children [13, 14]. Numerous pathogens in animal models demonstrated increased virulence due to the administration of excess iron [15].

For the survival of a pathogen in the host, its ability to regulate gene expression throughout an infection is vital. Iron securing is vital for bacterial survival, and furthermore assumes a significant function in bacterial pathogenesis. The knowledge about the regulation of colonization factor CS6 in the human gut is limited and in this study, we attempted to determine the impact of iron accessibility on CS6 expression in ETEC. We found that CS6 expression increased up to 0.2 mM in iron concentration and beyond this concentration, CS6 expression decreased. Similar observations were made for ETEC adhesion and growth suggesting that there might be a strong regulatory mechanism for CS6 expression under different iron concentrations. Results obtained here would help in understanding the importance of iron during ETEC pathogenesis.

METHODS

Bacterial strains used

All the ETEC isolates (Table 1) used in this study were isolated from patients with acute watery diarrhoea admitted to the Infectious Diseases Hospital, Kolkata, and Dr. B C Roy Post Graduate Institute of Paediatric Sciences as routine surveillance system. A well-characterized clinical ETEC strain ETEC4266 harbouring ST-LT gene and colonization factors CS6 was primarily used as a wild-type strain in this study [16]. Other ETEC strains were used from the strain archive of Bacteriology as and when required. The strains used in this study were CS6-only expressing strains. The strains were verified for L5 lineage but the strains were CS5 negative. The strains were a STh producing variant. All the bacterial strains were maintained at −80 °C in Luria-Bertani broth (BD Difco, USA) containing 15% (v/v) glycerol as cryoprotectant.

Bacterial growth and additives

For the routine maintenance and growth, strains were grown on MacConkey Agar plates. For the experiments, Minimal Media (M9) (BD Difco, USA), Luria Bertani Broth (BD Difco, USA), and Colonization Factors expressing (CFA) media were used; minimal media (M9) was supplemented with glucose (Merck, Germany) (0.2% w/v) as carbon source. Relevant iron salts, ferrous sulphate heptahydrate (Sigma-Aldrich, USA), ferrous chloride (Sigma-Aldrich, USA), ferric chloride (Sigma-Aldrich, USA), and ferric nitrate nonahydrate (Sigma-Aldrich, USA) as additives were supplemented for the experiments when required. The iron-chelating agent desferrioxamine mesilate salt or deferoxamine (Sigma-Aldrich, USA) was added to the medium when required.

Table 2. Primers used for PCR and Real-time Reverse Transcriptase PCR (rt RT-PCR)

Gene	Direction	Primers (5′→3′)	Product size (in bp)	GenBank accession no.
cssB (CS6)	Forward	CGGTTGAGATGACGATCCCT	90	U04844
	Reverse	GCTTTCCGATCTGCTGTCCA		
parC	Forward	ATCCGCTGGTTGATGGTCAG	92	EU561348
	Reverse	TTTCGACAGACGGGATTCGG		
cssB (CS6)	Forward	ATCCAGCCTTCTTTTGGTA	321	U04844
	Reverse	ACCAACCATAACCTGATCG		
elt (LT)	Forward	CACACGGAGCTCCTCAG	324	M17874
	Reverse	CAAACTAGTTTTCCATACTG		
esth (STh)	Forward	GCTAAACCAGTAGAGTC	149	M34916
	Reverse	CACCCGGTACAAGCAGG		
CS6 promoter	Forward	TCCCCCCGGGATAACTAACTGAAAAACAATG	573	NC022333
	Reverse	GCTCTAGATATAACACCATTACTATTGCT		
fur	Forward	GATACCAGCGTCGTCAAACT	104	NC017633
	Reverse	ACGTCAGTGCGGAAGATTTAT		

RNA extraction

Cells were collected at the exponential phase by centrifugation and each culture processed individually. RNA was isolated using trizol (Invitrogen, Thermo Fisher Scientific) extraction method as per manufacturer's protocol. Briefly, pelleted cells were suspended in trizol reagent followed by the addition of chloroform, and the mixture was centrifuged at 4 °C to allow for phase separation. The aqueous phase containing the RNA was transferred to a new tube. Next isopropanol was added to the aqueous phase and incubated followed by centrifugation. The pellet was resuspended in 75% (v/v) ethanol and then centrifuged and air-dried after that. Then it was resuspended in RNase free water. The RNA was then subjected to Turbo DNase (Ambion) treatments, followed by inactivation of the reagent and precipitation. RNA concentrations were measured using the Optizen UV-Vis spectrophotometer. Agarose gel electrophoresis was done to check RNA quality and absence of contaminating DNA before use in subsequent experiments. All RNA samples were stored at −80 °C.

cDNA synthesis and quantitative reverse transcription PCR

cDNA was synthesized by Verso cDNA Synthesis Kit (Thermo Scientific) as per user manual. Controls for DNA contamination without reverse transcriptase (-RT) were prepared simultaneously with the synthesized cDNA from the same amount of total RNA. cDNA and controls were stored at −20 °C. The real-time RT-PCR assays were performed on ABI 7500 (Applied Biosystems, Foster City, CA) using SYBR Green I (Applied Biosystems) as the detector. Primers for the CS6, structural subunit gene *cssB* and the *E. coli* housekeeping gene p*arC* were designed by NCBI Primer designing software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 2). The relative expression of the target transcripts was calculated according to Livak method [17] using *parC* as an internal control. *cssA* and *cssB* are expressed equally and in equal stoichiometry. Therefore, measuring any one would be representative of CS6 expression. It is *cssB* transcripts that are being measured here in this study. However, it can be noted in case of any defective assembly process, c*ssA* itself is unstable in *E. coli* and that ETEC may possess a unique system to degrade *cssA* when expressed alone. According to Wajima *et al*., 2011, [18] stable expression of *cssA* is dependent on *cssB* but not vice versa, and *cssA* is more unstable in the ETEC genetic background than in laboratory strains. Therefore, focusing on *cssA* may produce confusing results in different experiments as our strains are clinical isolates. So, we focused on *cssB* for measuring the changes for CS6.

Enzyme-linked immunosorbent assay (ELISA)

The phenotypic expression of CS6 on the bacterial surface was quantified by ELISA assays as per Elder *et al*., 1982 [19]. To quantitate the surface expression, 10^7 c.f.u. ml^{-1} of ETEC was used for coating and incubated overnight at 4 °C. Unbound bacteria were decanted and wells were washed with PBS followed by blocking in 5% (w/v) non-fat skimmed milk in PBS. After washing, the bound CS6 was determined by anti-CssB polyclonal antibody as the primary antibody followed by HRP-conjugated secondary antibody. Using a standard curve obtained with purified CS6 the amount of protein (μg) was determined.

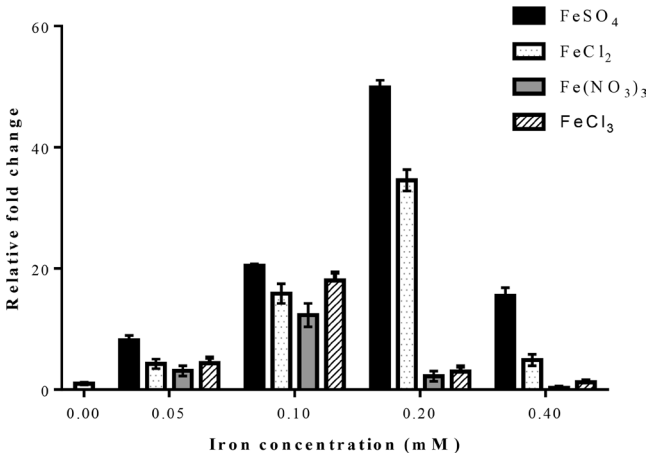

Fig. 1. Effect of iron salt on CS6 RNA expression in ETEC. CS6 RNA expression was quantified by RT-PCR using *parC* as the internal control. The strain was cultured in M9 minimal media with 0.2% glucose as carbon source and supplemented with varying concentrations of different iron salts [$FeSO_4$; $FeCl_2$; $Fe(NO_3)_3$; $FeCl_3$]. The bars show means and the standard error of the means (SEM) of three separate experiments. The results were significant when comparing the expression in presence of iron salts supplementation condition with the media only condition with no supplementation of iron (P<0.05).

LT was quantified by a GM1-based ELISA assay, using an anti-LTB Ab and GM1 as coating antigen [20]. STh was detected by indirect ELISA and protein was coated, which was detected by Anti-ST MAb [21].

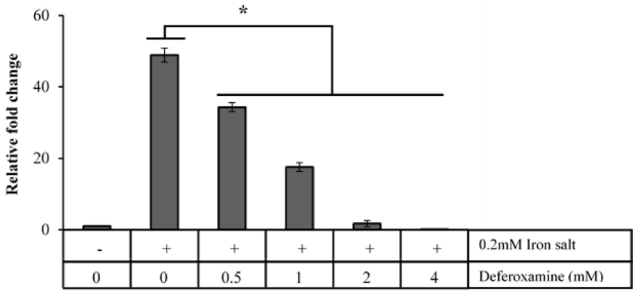

Fig. 2. Effect on CS6 expression in ETEC presence of iron chelation compound. The effect of 0.2 mM iron salt on the expression of the CS6 gene was studied in the presence of deferoxamine (Def) in dose-dependent concentration. The ETEC strain was grown in M9 minimal media with 0.2% glucose as carbon source with/without 0.2 mM $FeSO_4$ in the presence and absence of deferoxamine separately treated as indicated. Then the expression of CS6 was analysed by RT-PCR. The internal control used for the experiment was *parC*. Bars show means and standard error of the means of three separate experiments. *, Indicates significance (P=<0.05), when the expression in presence of iron salts and deferoxamine was compared to the expression only in presence of 0.2 mM iron.

Recombinant DNA techniques and promoter assay

All DNA manipulations were performed using standard molecular biology-based methods. For the promoter activity assay, we used the β-galactosidase expressing promoter-less plasmid pTL61T. We cloned CS6 promoter genes in this plasmid. In short, PCR amplified genomic DNA by using the promoter primers (Table 2) were cloned into plasmid pTL61T to generate plasmid-borne fusions by using the restriction enzymes Xho1 and Xba1 (New England Biolabs). The fusion plasmid was then transformed into the strains of ETEC (Table 1). The LacZ activity of the transcriptional reporter strains was measured by Miller assays [22]. In short, ETEC strains were incubated in LB overnight at 37 °C. Next, bacteria were transferred to M9 media containing desired concentrations of iron and were grown to mid-log phase and then placed on ice for 30 min. Then 200 µl of the culture was resuspended in 'Z' buffer. To this, 30 µl 0.1% (w/v) SDS and 60 µl of chloroform were added to each sample and vortexed. Next, 200 µl of ONPG (o-nitrophenyl-β-galactoside) solution, was added and incubated at 28 °C and the reaction was stopped after getting the desired yellow colour by adding 500 µl of 1M Na_2CO_3. The β-galactosidase activity was expressed as Miller units.

HT-29 cell culture and adhesion assay

The HT-29 epithelial cell lines were cultured in Corning 6-wells plate using DMEM (Dulbecco's Modified Eagle Medium) at 37 °C with 5% CO_2 using a Heracell 150i CO_2 Incubator (Thermo Scientific, USA) to form a monolayer. Sodium bicarbonate, 3.7 g was added to per litre of the media. Foetal bovine serum (FBS) (PAN-Biotech, Germany) 10% (v/v), 1% (v/v) non-essential amino acids (100X) (MP Biomedical, USA) and 1% (v/v) antibiotics (Penicillin 5000 IU per ml and Streptomycin 5 mg per ml; MP Biomedical, USA) were supplemented. The pH of the media was adjusted to ~7.2 and sterilized by filtration under aseptic conditions. The used media was replaced in alternate days and when the monolayer reached ~90% confluency it was used. The cells were treated with trypsin-EDTA to detach the cells and then used for sub-culturing. The stock of the cultured epithelial cells was prepared by using 95% complete growth media and 5% (v/v) DMSO (Sigma-Aldrich, USA) and kept in liquid nitrogen.

Before the adhesion experiments, the epithelial cells were serum-starved for 18 h in an appropriate medium with 0.5% (v/v) FBS. ETEC strains were pre-inoculated in M9 media supplemented with varying concentrations of iron salts and grown up to mid-log phase at 37 °C. Then 10^7 c.f.u. ml^{-1} of bacterial suspension was pelleted down and dissolved in the incomplete culture medium. This suspension was added to epithelial cells and after 3 h of incubation at 37 °C in 5% CO_2, cells were washed with PBS and detached by 0.1% Triton X-100 or by Trypsin-EDTA. The adhered bacteria were counted after serial dilution by plating on MacConkey agar plates.

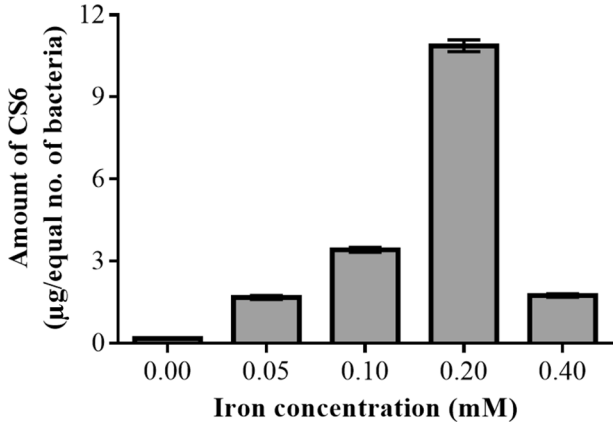

Fig. 3. Phenotypic expression of CS6 on ETEC surface in presence of iron. The level of CS6 on the bacterial surface and total CS6 protein in each strain was determined using 10^7 c.f.u. ml^{-1} of bacteria. CS6 was detected by anti-*cssB* antibody followed by incubation with HRP-conjugated secondary antibody. The amount of CS6 (µg) was determined from the standard curve. The bars show means and the standard error of the means (SEM) of three separate experiments. The results were significant when comparing the expression in presence of iron salts supplementation condition with the media only condition with no supplementation of iron (P<0.05).

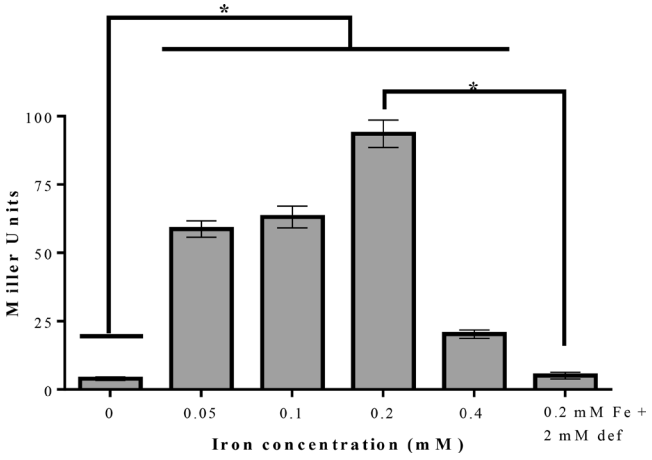

Fig. 4. CS6 promoter activity in response to 0.2 mM iron concentration. The effect of iron on the expression of CS6 promoter was measured by β-galactosidase activity assay as per Miller and Lee protocol, 1984 [22]. Promoter constructs were made in no-promoter pTL61T plasmid and transformed into strains used for this study. The transformed strain was grown in M9 minimal media with 0.2% glucose as carbon source and $FeSO_4$ was added at the indicated concentrations with or without 2 mM deferoxamine (Def). Assays were performed in triplicate, and bars show the mean and standard error of the means. *, Indicates significance (P=<0.05), when the promoter activity in the presence of iron salts was compared to the expression under no iron supplementation and also the decrease in the promoter activity when deferoxamine had been added in the presence of iron.

Rabbit ileal loop assay

In vivo adhesion of bacterial strains were also evaluated in the rabbit intestinal lumen. For this rabbit ileal loop assay was performed in young New Zealand white rabbits as described previously [23]. ETEC strains grown in M9 broth with or without iron salts were inoculated in rabbit ileum at different concentrations. The PBS-inoculated loop was used as a negative control. Intestinal loop sections recovered 18 h after the rabbit ileal loop experiment was washed in PBS three times, homogenized, and serially diluted in PBS. The adherent bacterial count was determined by plating these bacterial cultures on MacConkey agar plates.

Ethical approval

All the ETEC strains were isolated from patients following the approved institutional (ICMR-NICED) ethical guidelines (No. A-1/2009-IEC and No. A-1/2015-IEC). All animal experiments were conducted following the standard operating procedure as outlined by committee for the purpose of supervision and control experiments on animals (CPCSEA), Government of India and protocols were approved by the Institutional (ICMR-NICED) Animal Ethics Committee (NICED/CPCSEA/68/GO/(25/294)/2016-IAEC/NSC-1).

Statistical analysis

All the experiments were repeated at least three times. Data were expressed as mean±standard error of means (SEM). One-way ANOVA was used to analyse the data wherever applicable. A P value of <0.05 was considered statistically significant.

RESULTS

RNA expression of CS6 is influenced by iron concentration

The expression of CS6 in ETEC was investigated *in vitro* in the presence of different iron salts by quantitative real-time PCR. It was found that CS6 RNA expression increased significantly in a dose-dependent manner in the presence of iron salts (Fig. 1). The tested concentrations were from 0 to 0.4 mM iron. CS6 RNA expression increased by ~8-fold at 0.05 mM, 20-fold at 0.01 mM, 50-fold at 0.2 mM and 20-fold at 0.4 mM of $FeSO_4$ when compared to RNA expression in untreated conditions. It was observed that the increase in CS6 RNA was maximal in the presence of $FeSO_4$. In the presence of 0.2 mM $FeCl_2$, a maximum increase of 35-fold was observed in respect to untreated conditions. All the data with the supplement of iron salts were significantly modulated (P<0.05) when compared to the control or untreated condition with no supplement of iron. A significant increase in the CS6 expression can be seen in 0.1 mM for all iron salts and most significant increase in presence of 0.2 mM iron (II) salts when compared to the control condition with no supplementation of iron salts. There had been a significant decrease in the expression of CS6 in presence of 0.4 mM iron (II) salts when compared to the expression of CS6 at 0.2 mM iron (II) salts. Whereas there had been a significant decrease in the expression of CS6 when iron (III) salts were supplemented at a concentration of 0.2 and

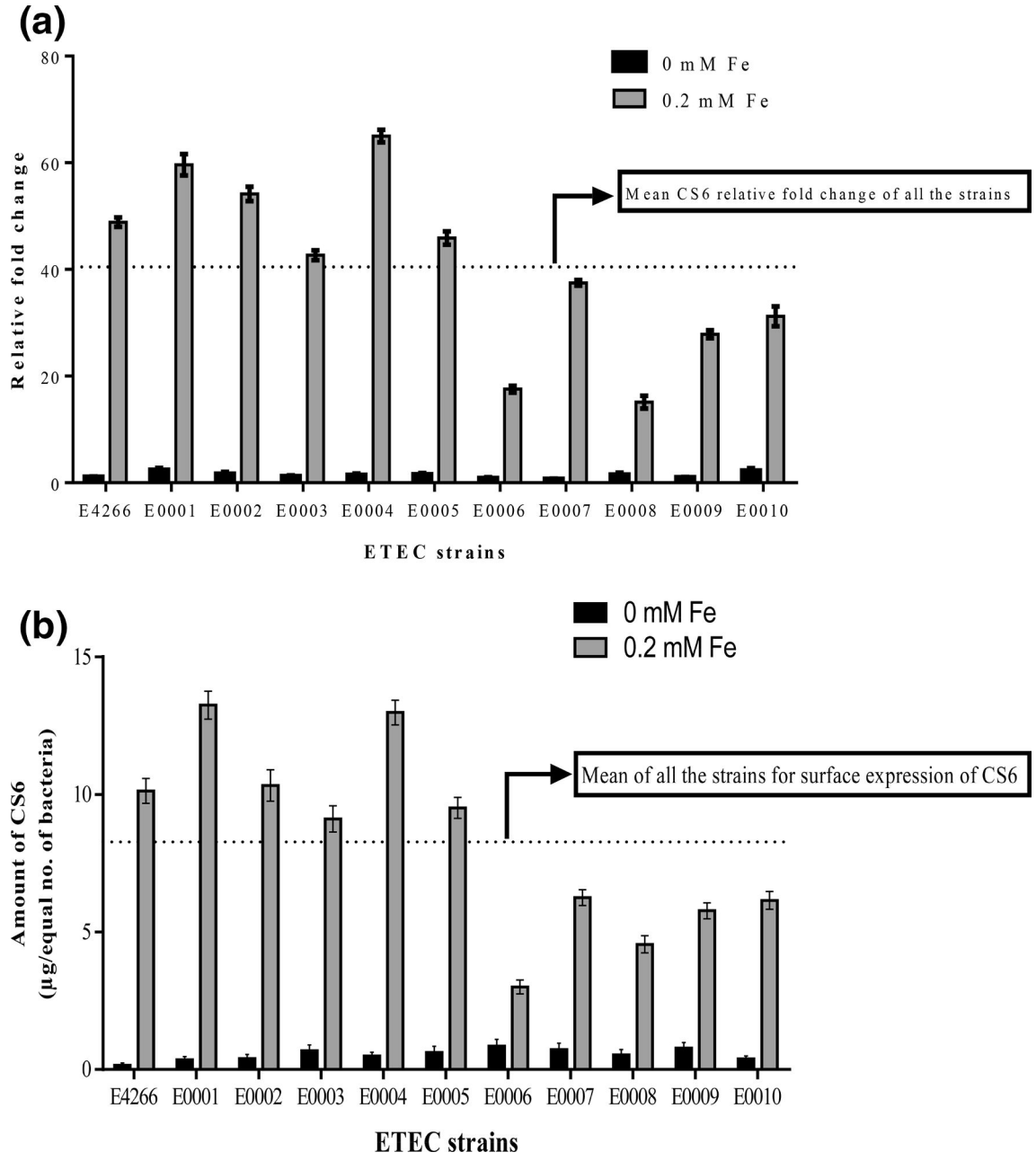

Fig. 5. CS6 expression in different ETEC strains in presence of iron salts. (a) Expression of CS6 in M9 minimal media (supplemented with 0.2% glucose as carbon source) supplemented with iron salts standardized to the level of transcription in cultured in M9 minimal media with 0.2% glucose as carbon source. RNA expression was measured by RT-PCR with *parC* as the internal control. (b) The level of CS6 on the bacterial surface was measured by anti-*cssB* antibody from the standard curve. In both the figures, bars show means and standard error of the means of three separate experiments. Dotted line represents the mean of all the strains.

0.4 mM when compared to the expression of CS6 at a 0.1 mM iron (III) concentration. The increase in presence of Fe (III) was not as much as Fe (II)-mediated induction and was maximal at 0.1 mM salt (12-fold and 18-fold for Fe $(NO)_3$ and $FeCl_3$, respectively) when compared to untreated RNA expression.

In *E. coli* it is already reported that *fur* expression is auto-regulated in response to iron. So for providing a control gene that is expressed under iron limitation we have checked the expression of *fur* in presence of different concentrations of iron ($FeSO_4$) by quantitative real-time PCR. It was found that *fur* RNA expression decreased significantly ($P<0.05$) in a dose-dependent manner in the presence of iron salts except for an increase of 0.2-fold of *fur* RNA in presence of 0.05 mM $FeSO_4$ (Supplementary file 1, Fig. S1, available in the online version of this article).

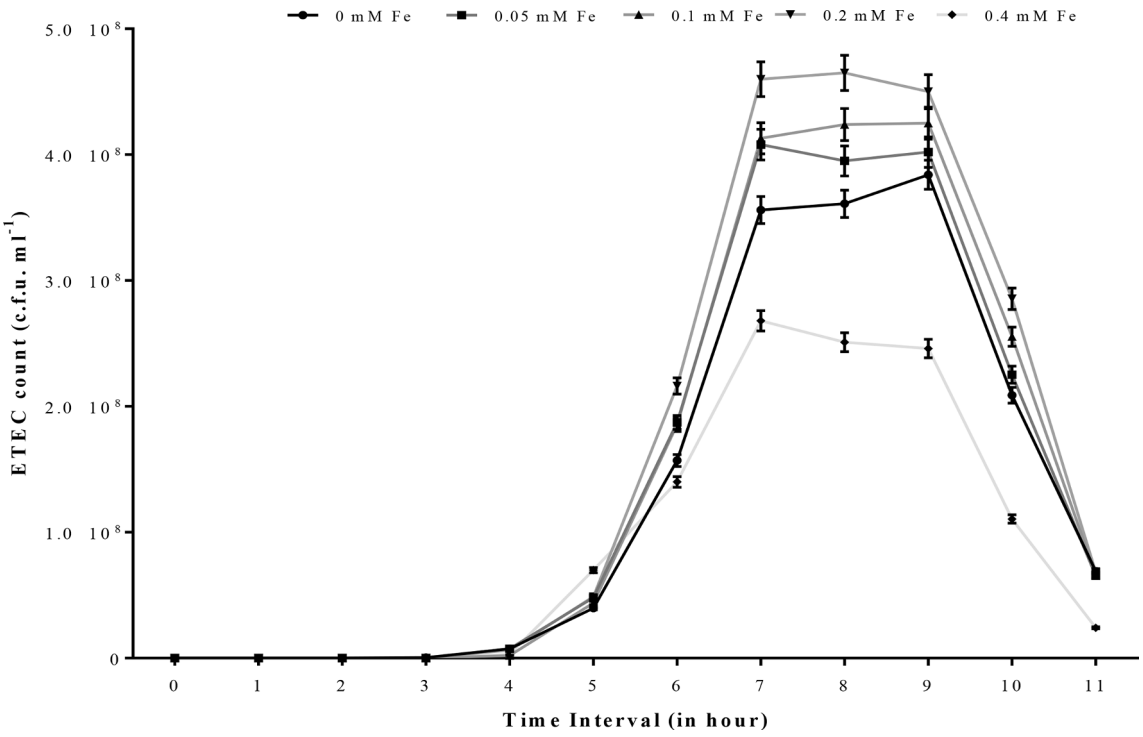

Fig. 6. Effect of iron salt on ETEC growth. ETEC growth was measured by colony counting method. The strain was cultured in M9 minimal media with 0.2% glucose as carbon source and supplemented with varying concentrations of iron salts ($FeSO_4$). The bars show mean and the standard error of the mean (SEM) of three separate experiments.

Expression of CS6 is reduced under iron starvation

To investigate the effect of iron depletion on CS6 expression M9 media, we examined the iron chelator deferoxamine (Def) in a concentration gradient manner along with the supplementation of iron salt at the concentration of 0.2 mM. Real-time PCR confirmed that sequestering of free iron by the addition of deferoxamine (Def) decreased the levels of CS6 expression. On addition of 0.5 and 1 mM deferoxamine, the RNA expression of CS6 was decreased by 1.5-fold and 3-fold respectively, when compared to no-deferoxamine treated condition and in presence of 0.2 mM iron. At 2 mM deferoxamine concentration, the RNA expression of CS6 is nearly the same as without iron salts and at 4 mM deferoxamine concentration the RNA expression of CS6 is downregulated by five-fold with respect to CS6 level in absence of iron. (Fig. 2). RNA fold change of CS6 expression gradually decreased in the presence of increasing concentrations of deferoxamine when compared to no deferoxamine added condition (P<0.05). These results indicated that iron availability increases CS6 expression as iron chelation reduces the expression of CS6.

Phenotypic expression of CS6 is induced in the presence of iron

The surface expression of CS6 was studied in the presence of 0.2 mM $FeSO_4$ salt by ELISA. (Fig. 3). The surface expression of CS6 increased gradually by 10-fold, 20-fold and 60-fold in presence of 0.05, 0.1 and 0.2 mM iron salt, respectively, when compared to CS6 grown without iron. However, the surface expression of CS6 decreased by six-fold when the concentration was further increased to 0.4 mM as compared to CS6 of ETEC grown in presence of 0.2 mM iron. The increase in phenotypic expression of CS6 with the addition of iron (II) salts was significant (P<0.05) when compared with the untreated condition. The result suggests that the optimum concentration for maximum CS6 expression is 0.2 mM iron (II) concentration.

CS6 promoter activity is upregulated in presence of iron

Since the expression of CS6 was regulated by iron availability, the modulation of the CS6 promoter was evaluated as a measure of β-galactosidase activity in the presence and absence of iron salts. The promoter activity of CS6 was increased in the presence of 0.05 and 0.1 mM iron concentration by ~15-fold and ~16-fold when compared to the promoter activity under untreated conditions. The promoter activity was elevated maximally by 23-fold at 0.2 mM iron concentration. The increase in the promoter activity on addition of iron was significant (P<0.05) when the promoter activity was compared with the untreated condition. However, it decreases with further increases in iron salt. Results showed that the decrease was five-fold in presence of 0.4 mM iron salt concentration when compared to the promoter activity under 0.2 mM condition (Fig. 4). The CS6 promoter activity

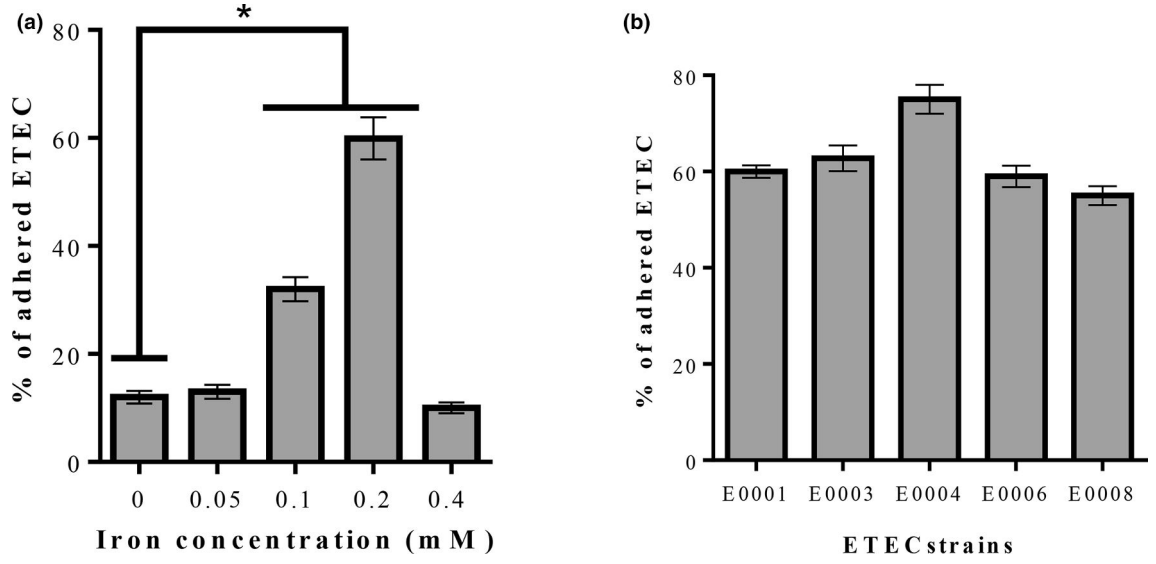

Fig. 7. Effect of iron on ETEC adherence to the epithelial cells. Adherence assays were performed for 3 hours in McCoy's 5A medium with 10% of FBS with or without iron salts. Percent of bacteria adhered to HT-29 cells for (a) reference strain in absence of Fe and the presence of a varying concentration of Fe(II), and (b) different CS6 harbouring ETEC strains in the presence of 0.2 mM iron salt. Assays were performed in triplicate, and bars show the mean and standard error of the means. *, Indicates significance (P=<0.05), when the adherence levels in presence of iron salt were compared with the no iron supplementation condition. The percentage of adhered bacteria was calculated as described in Methods.

was repressed in the presence of 0.2 mM iron salt and deferoxamine by 18-fold when compared to the promoter activity under 0.2 mM condition. This decrease in the promoter activity on the addition of deferoxamine under the iron supplementation condition is significant (P<0.05).

Expression of CS6 RNA in different ETEC strains harbouring CS6 is upregulated in presence of iron

To investigate whether the induction of CS6 RNA was strain-specific, the effect of iron on different ETEC strains harbouring CS6 was tested under the influence of 0.2 mM of iron salt. The experiment revealed that the RNA expression of CS6 had increased when supplemented with iron in the growth media with respect to untreated conditions in all the 11 strains tested (Fig. 5a). The mean increase in RNA expression of all the strains was 40-fold, being 15-fold and 65-fold as the minimum and maximum increase in expression, respectively. CS6 surface expression was also in accordance with the RNA expression data (Fig. 5b). CS6 surface expression was also increased significantly in different strains of ETEC at 0.2 mM iron (II) concentration.

Growth of ETEC is iron salt concentration-dependent

Next, the effect of iron salt on the growth of ETEC strain was examined. The experiment revealed an effect of iron salts on the growth of ETEC in a concentration dependent manner (Fig. 6). It was observed that in the presence of 0.05 mM iron concentration, the ETEC strain grew more by one-fold with respect to the untreated condition. At 0.1 mM iron concentration, the growth of ETEC increased by 1.5-fold. Maximum growth was observed in the presence of 0.2 mM iron which is two-fold higher as compared with the untreated condition. The growth was retarded by two-fold in the presence of 0.4 mM concentration of iron salt. Regression analysis showed that these differences in growth are not statistically significantly different. We had also checked the growth of ETEC in the presence of other iron salts. The results were similar as there was an increase in growth at iron concentrations from 0.05 mM to 0.2 mM for ferrous chloride and the growth was inhibited at higher iron concentrations (Fig. S2a). For ferric salts the growth was enhanced at iron concentrations of 0.05 and 0.1 mM and on addition of higher concentrations of iron salts the growth was retarded (Fig. S2b, c).

Adhesion of ETEC to epithelial cells is increased in presence of iron

Adhesion or colonization to the epithelial layer of the small intestine is a prerequisite for ETEC pathogenesis. To evaluate bacterial adhesion due to effect of iron salts, HT-29 cells were incubated with ETEC strain in the presence and absence of iron salts. It was observed that at 0.05 mM iron concentration, the ETEC adhesion to HT-29 cells was similar to the untreated condition. At 0.1 mM iron concentration, the adhesion of ETEC to HT-cells increased by three-fold. At 0.2 mM iron concentration, the adhesion increased maximally by five-fold compared to the untreated condition. Iron essentially improved the ability of ETEC cells to adhere to HT-29 cells contrasted with

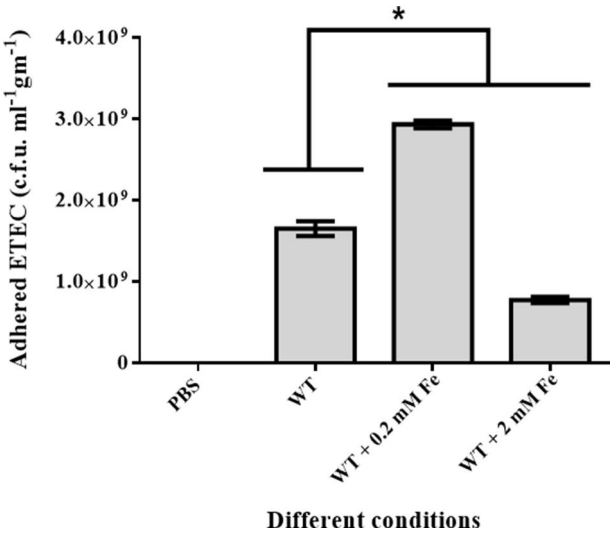

Fig. 8. Effect of iron on ETEC adherence to the rabbit ileal epithelium. Intestinal adherence or colonization was done in rabbit ileal loop. The ileal was divided into loops, each between 8–10 cm in length and interloop was present between loops. Loops were inoculated with 10^7 c.f.u. wild-type ETEC isolate expressing CS6 with or without iron. At 18 h of infection, the rabbit was sacrificed, and 2–3 small sections each of 2 cm from each loop were homogenized in 1 ml of PBS and ETEC adherence was measured in c.f.u. gm^{-1}. *, Indicates significance (P=<0.05), when the adherence levels in presence of iron salt were compared with the no iron supplementation condition.

untreated ETEC. However, at 0.4 mM iron concentration, the adhesion decreased by six-fold compared to the 0.2 mM iron condition. (Fig. 7a). When the adherence level in presence of iron salts were compared to the adherence level without iron salts it was found that the results were significant (P<0.05) at 0.1 mM and 0.2 mM iron supplementation condition. Therefore, it was observed that the adherence of ETEC increased in a dose-dependent manner with increasing concentrations of iron up to 0.2 mM, after which it started decreasing. The adhesion was further studied with other clinical ETEC strains harbouring CS6 in presence of 0.2 mM iron concentration. The adhesion of these five ETEC strains to HT-29 cells were about five-fold more with respect to untreated conditions (Fig. 7b). This also showed that the adhesion of ETEC was not strain-specific.

ETEC harbouring CS6 isolates were tested here for its adherence to rabbit ileal epithelial cells when supplemented with iron salts. The result suggested that when ETEC was grown in the presence of 0.2 mM iron salts, adherence increased by two-fold, whereas in the presence of 2 mM iron, the adherence was decreased by half in comparison to the wild-type ETEC grown without iron supplementation (Fig. 8). The adherence level increased significantly on addition of 0.2 mM iron salts whereas it decreased significantly when 2 mM iron salts had been supplemented (P<0.05).

DISCUSSION

In this study, the regulation of CS6 by iron was investigated and the analysis showed that iron acts as a modulator of CS6 expression. The expression of CS6 was enhanced in the presence of iron salts up to 0.2 mM iron and decreased thereafter with increased iron concentrations of 0.4 mM or more, suggesting that the optimum enhancement could be achieved under 0.2 mM. Though iron is critical for almost all living organisms, in high concentrations it is extremely toxic [24].

Uptake of both forms of iron, Fe(II) and Fe(III) by Feo system and by siderophores respectively are the different iron acquisition systems used by pathogens to obtain iron from the host [25]. Iron fundamentally happens in both of two oxidation states in biological systems as Fe(II) or ferrous, supported by low pH and low oxygen and Fe(III) or ferric state, which is the predominant form of iron under aerobic conditions [10]. Fe(III) is reduced to Fe(II) for subsequent incorporation into haem- or iron-containing proteins [26]. In this study, it was also observed that both forms of iron, ferrous and ferric, induced the expression of CS6 RNA. The modulation of CS6 in the presence of iron was not strain specific; maximum enhanced expression of CS6 was observed under 0.2 mM iron condition in other CS6-positive clinical ETEC strains, followed by decreased RNA expression with further increases in iron concentration. In comparison, under iron starvation conditions, the yield of the CFA/I fimbriae was enhanced in ETEC H10407 [27].

The effect CS6 RNA modulation by iron was reflected on the phenotypic expression of CS6. The expression of virulence factors had shown to be regulated by iron availability in an earlier study [28]. A rise in the expression of flagellin production with additional iron was also seen for extraintestinal pathogenic *Escherichia coli* [29]. Iron increases the synthesis and surface placement of adhesins in trichomonas [30]. On the contrary, a previous study had shown that in cases of colonization factors CS1, CS3 and CFA/I, iron starvation induced their expression [27].

The modulation CS6 expression could be attributed to the upregulated activity of the CS6 promoter in presence of iron up to 0.2 mM. The decrease in CS6 expression might also be due to downregulation of the promoter activity beyond the optimum iron concentration. It is possible that intracellular levels of free iron had reached toxic levels. Iron had been reported to control the promoters involved in *fur* regulation and upon interaction with iron *fur* becomes repressed [10] and our promoter analysis by bioinformatics using 'softberry bacterial promoter prediction tool' (Fig. S3) revealed the presence of *fur* region and RNA analysis showed downregulation of the *fur* gene in presence of 0.2 mM iron salts. However, in case of CFA/I, it was observed that its promoter activity was induced under iron starvation condition [27, 31].

The ability to adhere to the intestinal mucosal layer is a primary requirement for ETEC to initiate pathogenesis. In this current study, it was observed that with the increased CS6 expression in the presence of increased iron, adherence

of ETEC to intestinal HT-29 cells and rabbit ileal epithelium also increased. With an increase of iron concentration beyond 0.2 mM, there was decreased adherence due to decreased CS6 levels. These results suggested that iron may contribute to modulate ETEC colonization of the intestinal epithelial layer. A study demonstrated that iron enhanced the adhesion of enteric pathogen *S. typhimurium,* but in contrast, iron showed no effect or even reduced the adhesion of *E. faecalis* and *L. plantarum* [32]. Lactoferrin, an iron chelator, had appeared to disable the capacity of surface-expressed virulence factors and thereby in this way restraining the adherence of ETEC to epithelial cells [33].

Results presented here showed that addition of iron up to the optimum concentration of 0.2 mM resulted in a boosting growth of ETEC. Iron concentration beyond the optimum iron concentration affects ETEC growth. These results are in accord with the finding that when a lot of unabsorbed dietary iron enters the colon enteric microbes can possibly grow out. In infants, there was a significant increase in the sum of pathogenic *E. coli* through iron fortification [34]. It was observed that when the culture medium was supplemented with an excess of iron, the *Shigella* shiga toxin was produced in lesser amounts but the growth of bacteria was enhanced [28]. Free iron may lead to the rapid bacterial growth by abolishing the natural defensive bactericidal mechanisms in tissue fluids [11].

It has been shown that iron sequestering by lactoferrin inhibits the growth of enteric pathogens [33]. In pathogens like *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli* and *Enterobacter,* deferoxamine had a bacteriostatic effect as it depleted available iron and created an iron-scarce environment, as the bacteria could not use iron for their multiplication [35]. Chelating of iron by deferoxamine reduced the expression of CS6, both at RNA and protein levels. Iron chelation therapy has worked successfully to control infection against *S. aureus*, *Plasmodium falciparum, Yersinia enterocolitica, Vibrio vulnificus, Klebsiella pneumonia,* and *Aeromonas hydrophila* [36]. It was previously demonstrated that mice were less prone to infection with *Salmonella typhimurium* when serum iron levels were reduced. However, when normal serum iron levels was restored by injecting sufficient iron, infection increased [28]. These perceptions on the impacts of iron deprivation in biological systems propose the significance of iron to the microbes.

The LT and ST toxins of ETEC showed a different expression pattern compared to CS6 expression in the presence of iron. We found that iron richness had inhibited the secretion of the toxins (Fig. S4). Previous work with Shiga-like toxin I of enterohaemorrhagic *E. coli* showed toxin induction at iron starvation. Toxins such as shiga toxin, *Pseudomonas aeruginosa* A toxin, hemolysin and SLT of *E. coli* and diphtheria toxin were reduced in the presence of an increased iron concentration [28]. In contrast, it was found that LT secretion was inhibited in ETEC H10407 strain producing CFA/I fimbriae under iron starvation [27]. As a part of innate defence, the host limits iron availability by employing iron-binding proteins such as transferrin and lactoferrin so that invading pathogens cannot survive in low iron conditions and this low iron level acts as a signal to the pathogens for induction of virulence [37].

CONCLUDING REMARKS

In summary, our data showed that the free iron availability acts as a modulator for the expression of ETEC colonization factor CS6 which supports the hypothesis that iron can increase the virulence and growth of enteric pathogens. Our study suggests that ETEC is capable of expressing CS6 in consonance with the concentration of iron salts.

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Author contributions
D.B., I.M., N.S.C., conceptualized the study. D.B., I.M., performed experiments. All authors prepared the original draft of the manuscript and did data analysis, draft review, editing and approval.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Hosangadi D, Smith PG, Kaslow DC, Giersing BK. WHO consultation on ETEC and Shigella burden of disease, Geneva, 6–7th April 2017: Meeting report. *Vaccine* 2019;37:7381–7390.
2. Qadri F, Svennerholm A-M, Faruque ASG, Sack RB. Enterotoxigenic *Escherichia coli* in developing countries: Epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev* 2005;18:465–483.
3. Black RE. Epidemiology of travelers' diarrhea and relative importance of various pathogens. *Rev Infect Dis* 1990;12 Suppl 1:S73-9.
4. Gaastra W, Svennerholm A-M. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends Microbiol* 1996;4:444–452.
5. von Mentzer A, Tobias J, Wiklund G, Nordqvist S, Aslett M, *et al*. Identification and characterization of the novel colonization factor CS30 based on whole genome sequencing in enterotoxigenic *Escherichia coli* (ETEC). *Sci Rep* 2017;7:12514.
6. Sabui S, Dutta S, Debnath A, Ghosh A, Hamabata T, *et al*. Real-time PCR-based mismatch amplification mutation assay for specific detection of CS6-expressing allelic variants of enterotoxigenic *Escherichia coli* and its application in assessing diarrheal cases and asymptomatic controls. *J Clin Microbiol* 2012;50:1308–1312.
7. Begum YA, Baby NI, Faruque ASG, Jahan N, Cravioto A, *et al*. Shift in phenotypic characteristics of enterotoxigenic *Escherichia coli* (ETEC) isolated from diarrheal patients in Bangladesh. *PLoS Negl Trop Dis* 2014;8:e3031.
8. Bhakat D, Debnath A, Naik R, Chowdhury G, Deb AK, *et al*. Identification of common virulence factors present in enterotoxigenic *Escherichia coli* isolated from diarrhoeal patients in Kolkata, India. *J Appl Microbiol* 2019;126:255–265.
9. Sabui S, Debnath A, Ghosal A, Wajima T, Hamabata T, *et al*. Characterization of oligomeric assembly of colonization factor CS6 from enterotoxigenic *Escherichia coli*. *Microbiology (Reading)* 2016;162:72–83.
10. Andrews SC, Robinson AK, Rodríguez-Quiñones F. Bacterial iron homeostasis. *FEMS Microbiol Rev* 2003;27:215–237.

11. Bullen JJ, Rogers HJ, Spalding PB, Ward CG. Iron and infection: The heart of the matter. *FEMS Immunol Med Microbiol* 2005;43:325–330.
12. WHO U. Focusing on anaemia: Towards an integrated approach for effective anaemia control: *Joint statement by the World Health Organization and the United Nations Children's Fund*. 2004.
13. Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, *et al*. Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: Community-based, randomised, placebo-controlled trial. *The Lancet* 2006;367:133–143.
14. Oppenheimer SJ. Iron and its relation to immunity and infectious disease. *J Nutr* 2001;131:616S-635S.
15. Skaar EP. The battle for iron between bacterial pathogens and their vertebrate hosts. *PLoS Pathog* 2010;6:e1000949.
16. Ghosal A, Bhowmick R, Nandy RK, Ramamurthy T, Chatterjee NS. Pcr-based identification of common colonization factor antigens of enterotoxigenic *Escherichia coli*. *J Clin Microbiol* 2007;45:3068–3071.
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–408.
18. Wajima T, Sabui S, Fukumoto M, Kano S, Ramamurthy T, *et al*. Enterotoxigenic Escherichia coli cs6 gene products and their roles in cs6 structural protein assembly and cellular adherence. *Microb Pathog* 2011;51.
19. Elder BL, Boraker DK, Fives-Taylor PM. Whole-bacterial cell enzyme-linked immunosorbent assay for streptococcus sanguis fimbrial antigens. *J Clin Microbiol* 1982;16.
20. Sjöling A, Wiklund G, Savarino SJ, Cohen DI, Svennerholm A-M. Comparative analyses of phenotypic and genotypic methods for detection of enterotoxigenic *Escherichia coli* toxins and colonization factors. *J Clin Microbiol* 2007;45:3295–3301.
21. Rocha LB, Ozaki CY, Horton DSPQ, Menezes CA, Silva A, *et al*. Different assay conditions for detecting the production and release of heat-labile and heat-stable toxins in enterotoxigenic *Escherichia coli* isolates. *Toxins (Basel)* 2013;5:2384–2402.
22. Miller J, Lee K. *Experiments in Molecular Genetics*. Yi Hsien Pub. Co, 1984.
23. De SN, Bhattacharya K, Sarkar JK. A study of the pathogenicity of strains of *Bacterium coli* from acute and chronic enteritis. *J Pathol Bacteriol* 1956;71:201–209.
24. Sousa Gerós A, Simmons A, Drakesmith H, Aulicino A, Frost JN. The battle for iron in enteric infections. *Immunology* 2020;161:186–199.
25. Andrews S, Norton I, Salunkhe AS, Goodluck H, Aly WS, *et al*. Control of iron metabolism in bacteria. In: *Metallomics and the cell*. Springer, 2013. pp. 203–239.
26. Schaible UE, Kaufmann SHE. Iron and microbial infection. *Nat Rev Microbiol* 2004;2:946–953.
27. Haines S, Arnaud-Barbe N, Poncet D, Reverchon S, Wawrzyniak J, *et al*. ISCR regulates synthesis of colonization factor antigen I fimbriae in response to iron starvation in enterotoxigenic *Escherichia coli*. *J Bacteriol* 2015;197:2896–2907.
28. Payne SM, Neilands I. Iron and virulence in the family Enterobacteriaceae. *Crit Rev Microbiol* 1988;16:81–111.
29. Magistro G, Magistro C, Stief CG, Schubert S. The high-pathogenicity island (HPI) promotes flagellum-mediated motility in extraintestinal pathogenic *Escherichia coli*. *PloS one* 2017;12:e0183950.
30. Alderete JF, Nguyen J, Mundodi V, Lehker MW. Heme-iron increases levels of AP65-mediated adherence by *Trichomonas vaginalis*. *Microb Pathog* 2004;36:263–271.
31. Karjalainen TK, Evans DG, Evans DJ Jr, Graham DY, Lee C-H. Iron represses the expression of CFA/I fimbriae of enterotoxigenic *E. coli*. *Microbial Pathogenesis* 1991;11:317–323.
32. Kortman GAM, Boleij A, Swinkels DW, Tjalsma H. Iron availability increases the pathogenic potential of *Salmonella* typhimurium and other enteric pathogens at the intestinal epithelial interface. *PloS one* 2012;7:e29968.
33. Ochoa TJ, Cleary TG. Effect of lactoferrin on enteric pathogens. *Biochimie* 2009;91:30–34.
34. Jaeggi T, Kortman GAM, Moretti D, Chassard C, Holding P, *et al*. Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in kenyan infants. *Gut* 2015;64:731–742.
35. van Asbeck BS, Marcelis JH, Marx JJ, Struyvenberg A, van Kats JH, *et al*. Inhibition of bacterial multiplication by the iron chelator deferoxamine: Potentiating effect of ascorbic acid. *Eur J Clin Microbiol* 1983;2:426–431.
36. Carver PL. The battle for iron between humans and microbes. *CMC* 2018;25:85–96.
37. Sritharan M. Iron and bacterial virulence. *Indian J Med Microbiol* 2006;24:163–164.

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