Detection of ETEC toxins and identification of molecular basis of extended spectrum beta-lactamase (ESBL) mediated resistance in a multidrug resistant *Enterobacter agglomerans* strain

A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in Partial Fulfillment of the Requirement for the Degree of Bachelor of Science in Microbiology

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DECLARATION OF AUTHENTICITY

This is to declare that the research work aggregating the results reported in this thesis entitled '**A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in Partial Fulfillment of the Requirement for the Degree of Bachelor of Science in Microbiology**' has been carried out by the undersigned under the joint supervision of Dr.Kaiissar Mannoor, Scientist and Head of ideSHi(institute of developing Science and Health initiatives) and Professor Dr.Naiyyum Choudhury, former Coordinator of Biotechnology program, Department of Mathematics and Natural Science of BRAC University. It is further declared that the research work presented here is original and submitted in the partial fulfillment for the degree of Bachelors of Science in Microbiology, BRAC University, Dhaka.

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Dedicated To

My Greatest Strength

‘My Beloved Family’

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ABSTRACT:

Enterotoxigenic *Esherichia coli* (ETEC) is one of the salient causative agents of diarrhea particularly in developing countries and renders a major health complication in travelers to endemic areas. Eventually, infections caused by Extended Spectrum Beta Lactamase (ESBL) producing bacteria have been emerging worldwide and to avoid the inefficacy of the initial antibiotic regimen, rapid detection of ESBLs in Enterobacteriaceae is now in utmost priority. The study aimed to differentiate toxin producing ETEC strains as well as to determine antimicrobial resistance spectrum in a set of ETEC isolates collected from diarrheal patient. A multidrug resistant *Enterobacter agglomerans* strain was also characterized both phenotypically and genotypically for ESBL production. Toxin ELISA and multiplex PCR were performed to detect ETEC toxins along with antimicrobial sensitivity towards 13 different antibiotics by disk-diffusion method. Combination disk test was done to characterize multi-drug resistant *Enterobacter agglomerans* strain phenotypically, whereas conventional PCR using gene specific primers and Sanger sequencing were conducted to identify the resistance genes. Among 50 ETEC strains, 14 were positive for LT, 20 for ST and 16 for both ST and LT type toxins. The most prevalent subtype of ST toxin was STh (72%) over STp (28%). According to resistance pattern, more than 80% of the strains were resistant to Ampicillin and Erythromycin. About 90 % of ETEC strains were resistant to more than two antibiotics. Among different ESBL types, $ESBL_A$ was phenotypically identified through combination disk test. Conventional PCR using specific primers found the existence of the $ESBL_A$ subtype CTX-M1 gene and the primer amplified product size was about 841bp. Nucleotide sequencing and subsequent nBLAST analysis revealed 99% similarity with homologous bacterial strains including *Klebsiella pneumoniae, Salmonella enterica, and Escherichia coli* harboring the resistance gene. Alarming escalation of antibiotic resistance impedes the treatment and prevention of infectious diseases, resulting in increased mortality and morbidity. Therefore, development of alternative strategies needs to be propagated for resolving the serious problems due to multi-drug resistance pathogens.

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Abbreviations:

ETEC	Enterotoxigenic *Escherichia coli*
TD	Travelers' diarrhea
WHO	World Health Organization
CFAs	Colonization-factor Antigens
LT	Heat-labile toxin
ST	Heat-stable toxin
AMP	Adenosine Monophosphate
GMP	Guanosine Monophosphate
GM1 ELISA	Ganglioside Enzyme-linked Immunosorbent Assay
AST	Antibiotic Sensitivity Testing
ESBL	Extended Spectrum beta-lactamase
MDR	Multi Drug Resistance
CTX-M	Cefotaximase - Munich
BLAST	Basic Local Alignment Search Tool
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
µL	Microliter
PCR	Polymerase chain reaction
UV light	Ultraviolet light
$MgCl_2$	Magnesium Chloride
TBE	Tris/Borate/EDTA
RNA	Ribonucleic Acid
BMRC	Bangladesh Medical Research Council
IEDCR	Institute of Epidemiology, Disease Control and Research

Chapter 1: Introduction

1.1 Detection of Enterotoxigenic *E.coli* (ETEC)

1.1.1 Background:

Escherichia coli (*E. coli*) is the most prevalent infecting organism among the gram-negative Enterobacteriaceae family. *E. coli* is a rod shaped bacterium that is often used in laboratory studies and is usually harmless, residing in the human digestive tract as part of the normal flora. *E. coli* bacteria were discovered in the human colon in 1885 by German bacteriologist Theodor Escherich. The bacteria naturally and harmlessly exist in the intestines of all warm-blooded animals, hence the Greek-related root of its family name, *Enterikos*, meaning "intestine". It has been stated that the average *E. coli* population in an adult's intestine is approximately 0.1 percent of the total bacteria. The bacteria are needed by the body to aid in the production of several vitamins, such as Vitamin K and the B-Vitamins. *Escherichia* organisms are gram-negative bacilli that exist singly or in pairs. *E coli* is facultatively anaerobic with a type of metabolism that is both fermentative and respiratory. They can be either non-motile, or, motile by peritrichous flagella.

E. coli O157:H7 is a pathogenic strain of *E. coli* that was first identified as a cause of disease in the United States in 1982, during an investigation into an outbreak of gastrointestinal illness. It is one of the most frequent causes of the many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), traveler's diarrhea and other clinical infections such as neonatal meningitis. Since 1885, *E. coli* has been recognized as both a harmless commensal and a versatile pathogen (Bower et al., 1999). *E. coli* consists of a diverse group of bacteria. Pathogenic *E. coli* strains are categorized into pathotypes. Six pathotypes are associated with diarrhea and collectively are referred to as diarrheagenic *E. coli*.

- Shiga toxin-producing *E. coli* (STEC): STEC may also be referred to as Verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC). This pathotype is the one most commonly associated with foodborne outbreaks.
- Enterotoxigenic *E. coli* (ETEC) : cause of traveler's diarrhea.
- Enteropathogenic *E. coli* (EPEC): cause of childhood diarrhea.
- Enteroaggregative *E. coli* (EAEC): persistent diarrhea in children in developing countries.

- Enteroinvasive *E. coli* (EIEC): causes a *Shigella* -like dysentery.
- Diffusely adherent *E. coli* (DAEC): cause of childhood diarrhea and traveler's diarrhea.

Estimates show that in developing countries the highest incidence of childhood diarrhea per year is 4.8 episodes occurring during the first year of life which by the age of 4 gradually decreases to 1.4 per year, while the average number of diarrheal episodes per year is 3.2. Furthermore, the highest age-mortality rate (8.5 children per1000/year) occurred in children under 1 year of age (Kosek et al.,2003). However, it remains the second most common cause of deaths in under 5 children according to the Global Burden Disease Report of the WHO.

1.1.2 Enterotoxigenic *E.coli* (ETEC):

ETEC is a major cause of traveler's diarrhea and is endemic in most underdeveloped countries with significant mortality rates in children. It is the major source of *E. coli*-mediated diarrhea in humans and livestock. ETEC infections cause more than 280 million annual episodes of diarrhea resulting in mortality numbers exceeding 550,000 deaths of under-5 children (Anders et al.,2016). The significant negative health and socio-economic impact of ETEC infection manifests itself mainly in the least developing nations with poor sanitation and inadequate supplies of clean water.

1.1.3 Seasonality:

In Bangladesh, ETEC follows a very characteristic biannual seasonality with two separate peaks; one at the beginning of the summer season, in late spring, and another one in the autumn months following monsoon, but it remains endemic all the year round. Such seasonality may be initiated by climate and spread by environmental factors.

The frequency of Travelers' diarrhea (TD) among international travelers travelling to tropical and semitropical regions of the developing world ranges from 10% to 60%. The highest rates of TD are seen in Latin America, Africa and the Indian subcontinent (DuPont et al.,2009). This can be attributed to high ambient temperature and rainfall in those regions favoring the growth and spread of bacteria that contaminate food and water. These changes may further evolve in response to current global climate changes.

1.1.4 Transmission:

ETEC diarrhea is typically initiated through intake of contaminated food or drinks and is associated with low socio-economic conditions and poor access to clean water and sanitation. In addition, ETEC is able to adhere firmly to fresh vegetables, which increases risk for transmission (Lucia et al.,2016). ETEC is transmitted when a person eats food, or drinks water contaminated with ETEC bacteria. Human and animal wastes are the main source of ETEC contamination.

1.1.5 Virulence factors:

ETEC possess specialized pili, antigenically unrelated to common pili that act as ligands to bind the bacterial cells to specific complex carbohydrate receptors on the epithelial cell surfaces of the small intestine. Since this interaction results in colonization of the intestine by ETEC with subsequent multiplication on the gut surface, these fimbriae are termed to as colonization-factor antigens (CFAs).

Genes coding for the production of CFAs reside on the ETEC virulence plasmids, usually on the same plasmids that carry the genes for one or both of the two types of *E. coli* enterotoxin including heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). ST is farther classified into two major genotypes which are STa and STb; typically, ETEC strains isolated from humans produce STa encoded by the *estA* gene, whereas STb is predominantly produced by animal ETEC strains. Two different subtypes of STa exist; STh and STp. These designations were chosen, since STh previously was considered to be mainly of human origin and STp of porcine origin. Several allelic forms of the *estA* gene exist, and both alleles encoding STp and STh have been found among human ETEC isolates. These two variants of STa are very similar in structure and function. STh consists of 18 amino acids and STp of 19 amino acids; both variants contain 6 cysteine residues, and accordingly, most epitopes in the ST molecule are conformational. Both STh and STp bind to specific receptors on intestinal brush border membrane of the small intestine to activate the guanylyl cyclase-cGMP system, which results in diarrhea (Biolin et al., 2006).

1.1.6 Pathogenesis:

ETEC adhesins are fimbriae which are species-specific. For example, the K-88 fimbrial Ag is found on strains from piglets; K-99 Ag is found on strains from calves and lambs; CFA I and CFA II are found on strains from humans. These fimbrial adhesins adhere to specific receptors on enterocytes of the proximal small intestine (Wellington and van Elsas., 1992).

Adhesion to the mucosal epithelial cells allows transfer of enterotoxins produced by ETEC bacteria which stimulate the release of liquid from the cells lining the intestinal walls. ETEC produce one or both of two enterotoxins; heat-labile (LT), and heat-stable (ST) that cause intestinal epithelial cells to secrete excess fluid. LT is a large (84,000 Da), immunogenic oligotoxin related to cholera toxin in sequence, structure, and mechanism. In contrast, ST is a small polypeptide (2,000 Da) which is non-immunogenic in its natural form. Epidemiological data suggest that strains secreting ST, with or without LT, induce the most severe disease among children of developing countries (Arne et al., 2010).

Both toxins have been found to disrupt intestinal fluid homeostasis and cause hypersecretion of fluids and electrolytes through activation of adenylate cyclase (by LT) or guanylate cyclase (by ST) in small intestinal mucosal cells(Zhang et al.,2006). This mechanism elevates either cyclic Adenosine Monophosphate (cAMP) or cyclic Guanosine Monophosphate (cGMP) levels in intestinal epithelial cells which results in stimulation of active chloride secretion. It also inhibits electro-neutral sodium chloride adsorption in the intestinal epithelium and subsequently causes unidirectional fluid secretion.

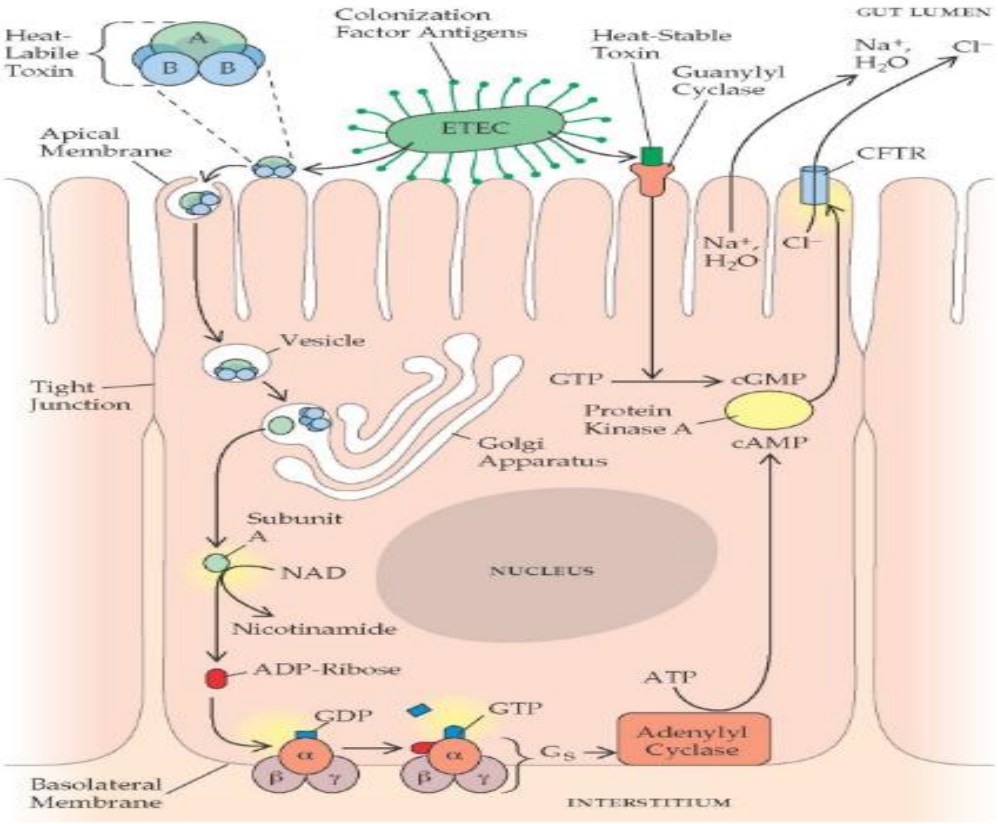

Figure 1.1: Cellular pathogenesis of *E coli* having CFA pili

ETEC infection results in the production of acute watery diarrhea and abdominal cramping. Other symptoms such as fever, vomiting, chills, headache, muscle aches, and bloating can also occur but are less common. Illness usually lasts 3-4 days following exposure to the bacteria but can persist for up to 3 weeks. Supportive measures including rehydration tend to be sufficient for recovery and hospitalization or antibiotics are usually not required.

According to a hospital-based study in Bangladesh, ETEC producing both LT and ST or ST alone were found to cause relatively more severe disease than that caused by LT-producing ETEC strains (Qadri et al., 2005) mentioned in the table.

The table 1.1 illustrates an association of severity of disease with toxin types and presence of known colonization factors on ETEC in children up to 3 years of age in rural Bangladesh during 2-year surveillance:

CF and toxin type	No. (%) with symptoms that were			Total
	Mild	Moderate	Severe	
CF positive	142 (56)	90 (36)	20 (8)	252
CF negative	140 (64)	56 (26)	22 (10)	218
ST	131 (56)	78 (33)	24 (11)	233
ST/LT	69 (52)	54 (41)	10 (7)	133
LT	82 (79)	14 (13)	8 (8)	104

Table 1.1: Estimation of virulence factors of ETEC in Bangladesh in 2005

The genes encoding the LT, STp and STh toxins represent the targets for ETEC identification and detection. For genotypically determining the presence of three toxin genes, multiplex Polymerase Chain Reaction (PCR) is done in which genes for specific toxins are amplified using specific primers and results are obtained through separating the PCR products in agarose gel electrophoresis. After identification, the positive colonies are subjected to GM1 ganglioside enzyme-linked immunosorbent assays (GM1-ELISA) for phenotypic detection. Toxin ELISA is the method in which the absorbance of color is estimated for ascertaining the presence of LT and ST toxins in ETEC isolate.

1.1.7 Antimicrobial resistance:

Increasing antibiotic resistance is a global concern, especially in low and middle income countries. A study in Lima, Peru to determine antimicrobial susceptibility and resistance mechanism of 205 ETEC isolates obtained from children under-two years of age reported that ETEC isolates were resistant to ampicillin (64%), cotrimoxazole (52%), and tetracycline (37%); with 39% of the isolates being multidrug-resistant. When compared to isolates producing both heat-stable and heat-labile toxins (21%), isolates producing either Heat-stable toxin (48%), or,

heat-labile toxin (40%) expressed higher rates of multidrug resistance. Only 10% of isolates were resistant to nalidixic acid and none to ciprofloxacin or cefotaxime. Ampicillin and sulfamethoxazole resistance was most often associated with bla_{TEM} (69%) and *sul2* genes (68%), respectively. The prevalence of beta-lactamase enzyme in organisms is now inactivating the efficacy of the antibiotics (Anicia et al.,2015).

1.1.8 Treatment:

Individuals with diarrhea are treated with clear liquids to prevent dehydration and loss of electrolytes. Oral rehydration salts or premixed oral rehydration solutions are often used to treat dehydration. Zinc treatment is also given to speed up the recovery time.

Fluoroquinolones have been found to be effective in treating ETEC infection. Fluoroquinolones interfere with DNA supercoiling and promote DNA gyrase-mediated unwinding of double-stranded DNA. The aminoglycosides bind irreversibly to the 50S subunit of the 70S bacterial ribosomes (Allen et al.,1998). Although antibiotics can shorten the length of diarrheal disease, especially if given early, ETEC is frequently resistant to common antibiotics, including trimethoprim-sulfamethoxazole and ampicillin. Since antibiotic resistance is on the increase worldwide, the decision to treat ETEC diarrheal patients with an antibiotic should be carefully considered with regards to the severity of the illness. For this reason, an Antibiotic Sensitivity Testing (AST) should be observed for choosing the most effective antibiotic against ETEC isolates.

1.1.9 Vaccines:

No vaccines directed against ETEC bacteria are available at this time. Dukoral, an oral whole-cell/recombinant B-subunit vaccine directed against cholera, has been found to provide short term efficacy against ETEC diarrhea. Protective efficacy against cholera is 85 percent, while protection against the heat-labile toxin of ETEC reaches 67 percent (Steffen R et al., 2005).

1.2 Detection of ESBL (Extended Spectrum beta-lactamase) gene

1.2.1 Background:

Antibiotics were first used to treat serious infections in the 1940s.Since then; antibiotics have saved millions of lives and transformed modern medicine. Antibiotics are powerful drugs that are generally safe and very helpful in fighting disease, but there are times when antibiotics can actually be harmful. Antibiotics are used as veterinary and human medicines for treatment, control and prevention of infectious diseases. However, their repeated off-label over use can have un-anticipated adverse effects including development of antibiotic resistance in bacteria towards modern beta-lactam antibiotics. As antibiotic resistance grows, the antibiotics that are used to treat infections do not work as well or at all. The loss of effective antibiotics will undermine our ability to fight infectious diseases and manage the infectious complications common in vulnerable patients undergoing chemotherapy for cancer, dialysis for renal failure, and surgery, especially organ transplantation, for which the ability to treat secondary infections is crucial. If that ability is lost, the ability to safely offer people many life-saving and life-improving modern medical advantages will be lost with it. Efforts to prevent such threats should be built on the foundation for improving public health strategies which include immunization, infection control, protecting the food supply, antibiotic stewardship, and reducing person-to-person spread through screening and treatment education.

1.2.2 Multi Drug Resistance (MDR):

During the last few decades, the incidence of microbial infections has increased dramatically. Continuous deployment of antimicrobial drugs in treating infections has led to the emergence of resistance among the various strains of microorganisms. Multidrug resistance (MDR) is defined as insensitivity or resistance of a microorganism to the administered antimicrobial drugs. According to WHO, these resistant microorganisms (like bacteria, fungi and parasites) are able to combat attack by antimicrobial drugs, which leads to ineffective treatment resulting in persistence and spreading of infections. Although the development of MDR is a natural phenomenon, extensive rise in the number of immuno-compromised conditions like HIV-infection, diabetic patients, individuals who have undergone organ transplantation, and severe burn patients, makes the body an easy target for hospital acquired infectious diseases, thereby contributing to further spread of MDR (Tanwar et al.,2014).

1.2.3 Severity of MDR:

When first-line and second-line antibiotic treatment options are limited by resistance or are unavailable, healthcare providers are forced to use antibiotics that may be more toxic to the patients and frequently more expensive and less effective. Even when alternative treatments exist, research has shown that patients with resistant infections are often much more likely to die, and survivors have significantly longer hospital stays, delayed recuperation, and long-term disability.

Antimicrobial resistance is associated with high mortality rates and high medical costs and has a significant impact on the effectiveness of antimicrobial agents. MDR provokes obstruction in disease control by intensifying the possibility of spreading of resistant pathogens, thus, declining efficacy of treatment and, hence, resulting in prolonged time of infection in patients. Expansion of global trade and tourism leads to increased potential of MDR to spread all over the world and decrease in export and import of various products affecting the economy of developing countries.

1.2.4 Mechanisms of MDR:

The main mechanisms whereby the organism develop resistance to antimicrobial agents include enzymatic inactivation, modification of the drug targets, and reduction of intracellular drug concentration by changes in membrane permeability or by the over expression of efflux pumps. With respect to efflux pumps, they provide a self-defense mechanism by which antibiotics are actively removed from the cell. For anti-bacterials, these results in sub lethal drug concentrations at the active site that in turn may predispose the organism to the development of high-level target-based resistance. Antimicrobial drugs generally act on the microbes either by inhibiting a metabolic pathway like nucleotide synthesis which in turn leads to the inhibition of DNA/RNA synthesis and further protein synthesis and disruption of the cell membrane, or, by competing with the substrate of any enzyme involved in cell wall synthesis (Jadwiga et al.,2013).

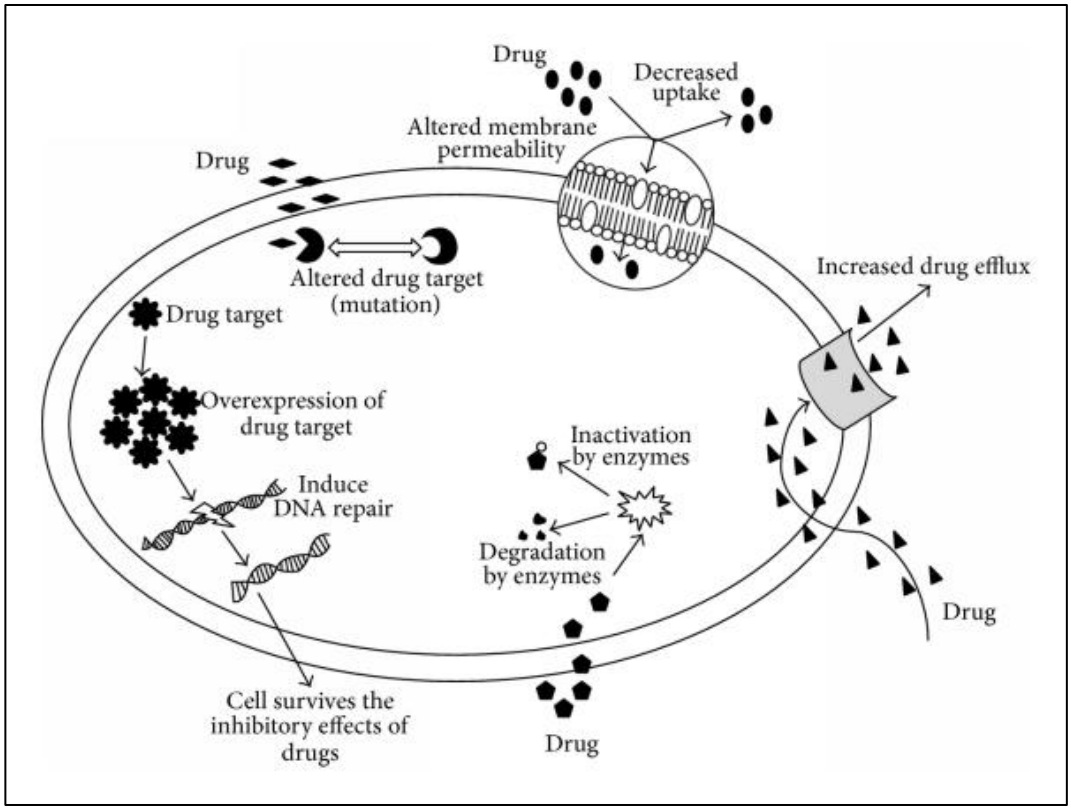

Figure 1.2: Mechanism of Multi-drug resistance

Microorganisms have evolved a multitude of mechanisms to overcome the effectiveness of drugs, thereby surviving exposure to the drugs. New forms of antibiotic resistance can cross international boundaries and easily spread between continents. World health leaders have described antibiotic-resistant microorganisms as "nightmare bacteria" that "pose a catastrophic threat" to people in every country in the world.

Among all of the bacterial resistance problems, gram-negative pathogens are particularly worrisome, because they are becoming resistant to nearly all drugs that would be considered for treatment. The most serious gram-negative infections occur in healthcare settings, and are most commonly caused by Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter*.

1.2.5 Extended Spectrum β-lactamase (ESBL):

One of the antibiotic resistance mechanisms in bacteria is the production of specific enzymes such as beta-lactamases to break down the four-atom beta-lactam ring of β-lactam antibiotics. When this ring is opened through hydrolysis by these enzymes, the antimicrobial properties of the antibiotics are completely lost. The extended spectrum beta-lactamases (ESBL) are rapidly

evolving plasmid-mediated group of beta-lactamases. ESBL can hydrolyse penicillins, first, second, and third-generation cephalosporins, and aztreonam (but not cephamycins or carbapenems). The antibiotic resistance leads to increased morbidity, mortality, and cost of treating infections, in particular, those caused by ESBL-producing bacteria (Tekiner et al.,2016).

The ESBLs are mainly encoded by plasmids and mobile genetic elements such as integrons, insertion sequences, transposons and plasmids. These genetic elements are easily transferable to other bacteria such as those belonging to Enterobacteriaceae. The widespread use of third generation cephalosporins and aztreonam has led to the emergence and dissemination of ESBL producing strains and their encoding genes, in particular, among Enterobacteriaceae associated with severe enteric illnesses (Ghafourian et al.,2014).

1.2.6 Antibiotic Inactivation:

ESBLs are capable of inactivating antibiotics in multiple ways. Exploiting biochemical features, ESBLs mediate resistance through targeting and cleaving hydrolytically susceptible chemical bonds. Infrequently, pathogenic bacteria can exploit oxidation and reduction of antibiotics which inhibits the efficacy of drugs. In addition, Chemical substitution can also result in demobilization of antibiotics. Modification of the target site of drugs is also one of the major resistance mechanisms which can render antibiotics impotent for binding properly.

Different mutational events and horizontal transfer of genetic materials are the two prevalent genetic mechanisms for acquiring resistance. Antibiotic resistance genes may be transferred between bacteria by different modes of conjugation, transformation or transduction. Over the last 15 years, β-lactamase enzymes that have an extended spectrum of activity (ESBL) against the majority of β-lactams, including cephalosporins but not carbapenemases, have evolved. One of these, CTX-M-15, initially found in *E. coli* but now found in other members of Enterobacteriaceae and frequently associated with a specific lineage, uropathogenic clone ST131, has spread worldwide (Sibhghatullah et al.,2015).

The risk of infection is particularly high in individuals in association with prolonged hospitalization, catheterization, nursing home residency, previous antibiotic treatment, underlying renal or liver pathology, and travel to high-risk areas.

1.2.7 SPREAD OF ESBL:

ESBL can be passed from person to person by contaminated hands if they have not been cleaned properly. ESBL can get onto the hands by touching contaminated surfaces or equipment. ESBL is not spread through the air by coughing or sneezing.

Preventing the spread of antibiotic resistance can only be achieved with widespread engagement, especially among leaders in clinical medicine, healthcare leadership, agriculture and public health. Although some people are at greater risk than others, none can completely avoid the risk of antibiotic resistant infections.

1.2.8 Risk factors for ESBL-producing bacterial acquisition include:

- Direct transfer from another hospital, nursing home, retirement home or other health care facilities, including between facilities in the same health care corporation.
- Any hospital, nursing retirement home or other health care facility admission in the past year.
- Patients receiving health care services or hemodialysis at home.
- Patient living in a communal setting (e.g. shelter, halfway house)
- Patients who had previous infection by an antibiotic-resistant organism (e.g., MRSA, VRE)

1.2.9 Classification of ESBL-producers:

For the detection and confirmation of Enterobacteriaceae resistant bacteria, ESBLs can be divided into three classes on the basis of β-lactamase enzyme producer which are farther subdivided based on functional group.

1.$ESBL_A$: CTX-M (Cefotaximase –Munich), SVH (sulfhydryl variable) ESBL,
TEM (Temoneira) ESBL, PER (*Pseudomonas* Extended Resistant)

2. $ESBL_{M-C}$: AmpC, OXA (Oxyimino beta lactam)

3. $ESBL_{Carba}$: Carba A: KPC (*Klebsiella pneumoniae* Carbepenemase)
Carba B: NDM (New Delhi Metallo β-lactamase),
VIM (Verona integron encoded Metallo β-lactamase)
Carba D: oxa-48

$ESBL_A$ producers are resistant to penicillins, Monobactam (Aztreonam) and cephalosporins; 1st generation cephalosporins (Cefadroxil, Cefradine, Cefazolin), 2nd generation cephalosporins (Cefuroxime), 3rd generation cephalosporins (Ceftazidime, Cefixime, Cefotaxime, Cefpodoxime, Ceftriaxone) and 4th generation cephalosporins (Cefepime, Cefozopran). $ESBL_A$ producers are susceptible to carbapenem antibiotics.

ESBL has generally been defined as transmissible β-lactamases that can be inhibited by Clavulanic acid, Tazobactam or Sulbactam, and which are encoded by genes that can be exchanged between bacteria. Currently the most common genetic variant of ESBL is CTX-M.

1.2.10 CTX-M:

CTX-M β-lactamases are found exclusively in the functional group and thought to originate from chromosomal ESBL genes found in *Kluyvera spp*, an opportunistic pathogen of the Enterobacteriaceae family found in the environment. The first CTX-M proteins were discovered in the late 1980s and today more than 100 variants have been sequenced. CTX-M ESBLs were acquired by horizontal gene transfer from other bacteria using genetic apparatuses such as conjugative plasmid or transposon (Sibhghatulla et al.,2015).

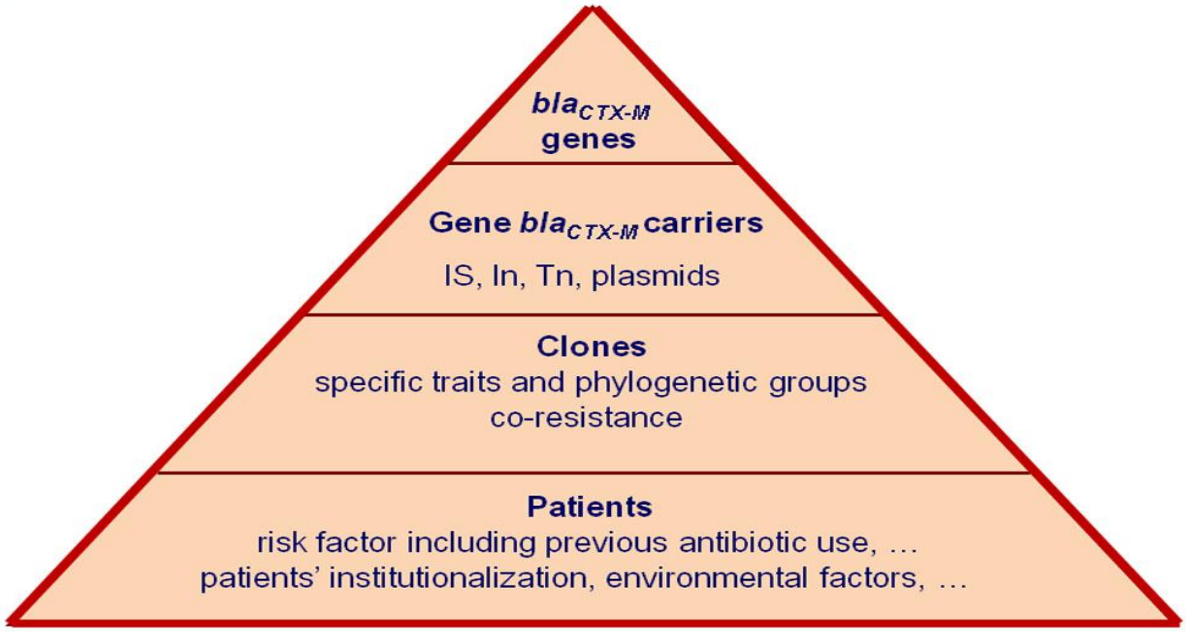

Figure 1.3: Factors fueling the emergence, maintenance, and spread of the CTX-M extended spectrum β-lactamases (ESBLs).

Unlike other ESBLs, CTX-M family constitutes a complex and non-homogeneous group of enzymes. The first view and alignment of the amino-acid sequences of the different CTX-M variants allowed to classify these enzymes in five clusters but recent reports added at least two more additional clusters. Phylogenetic analysis suggests that CTX-Ms were not originated by mutations from previous plasmid meditated enzymes, but through mobilization of chromosomal *bla* genes from *Kluyvera spp*, when they were incorporated into mobile genetic elements. These original mobilized *bla* CTX-M genes affected cefotaxime to a higher degree than ceftazidime. According to the evolutionary point of view, CTX-Ms as other ESBLs later diverged by punctual mutations probably as a consequence of antibiotic selective pressure once *Kluyvera spp*. *Bla* CTX-M genes were mobilized and were incorporated into mobile genetic elements. This also gave the CTX-M enzymes the opportunity to enhance the hydrolytic activity against ceftazidime (Rafael et al.,2012).

Aim of the Study:

1. To differentiate toxin producing *E. coli* strains (ETEC) according to the toxin types they produce by PCR amplification of a part of each toxin gene using toxin specific primers.

2. Determining the antibiotic sensitivity/resistance patterns of these ETEC strains using 13 different antibiotics.

3. Phenotypic and genotypic characterization of an ESBL producing multidrug resistant strain of *Enterobacter agglomerans* obtained from an independent study on acute respiratory infections.

Chapter 2: Methods and Materials

This study was conducted at institute for developing Science and Health initiatives (ideSHi). Ethical approval for the study was obtained from Bangladesh Medical Research Council (BMRC).

2.1 Microbiological Culture:

A total of 50 *Escherichia coli* strains isolated from diarrheal patients for another study, were preserved in -70°C freezer as glycerol stock. The strains were first subcultured on MacConkey Agar plates (BD Difco™; Cat no: 212123). Then, gram staining and colony morphology characterization were done to confirm and isolate only the specific *E. coli* colonies. The plates were incubated for 24 hours at micro-aerophilic (less oxygen present compared to the atmosphere) condition in a 37 °C incubator.

Then, the toxin producing colonies were identified using Toxin ELISA and Multiplex PCR.

Isolation of *E. coli* strains:

Differential staining was performed to characterize the isolated bacteria as gram-positive or gram-negative. Specific *E. coli* colonies were identified through observing gram staining properties and bacterial cellular morphology under microscope.

Gram staining for *E. coli* confirmation:

Firstly, isolated colonies along with a drop of normal saline were heat fixed on the center of a glass slide. The center was then flooded with crystal violet for 1 minute; excess dye was washed gently with deionized water. After applying the gram's Iodine for 1 minute, it forms complex structure with crystal violet which tightly binds with the cell wall of the bacteria. And again, the smear was washed gently with deionized water. Subsequently, Acetone was poured off for a minute and washed off. It acts as the decolorizing agent so that it dehydrates the peptidoglycan layer through shrinking. Large crystal violet cannot penetrate and remain trapped into the thick peptidoglycan layer of gram-positive bacteria.

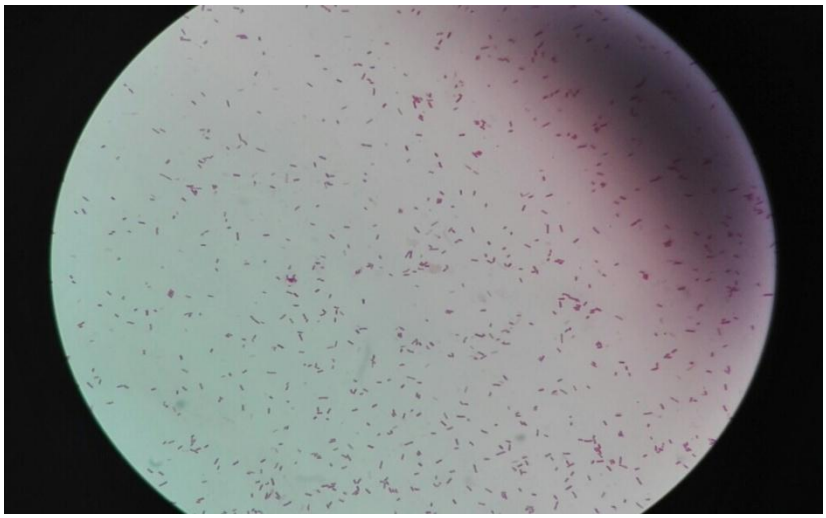

Fig2.1: *Escherichia coli* under light microscope after gram-staining

In case of gram-negative cell walls, the outer membrane could not retain crystal violet iodine complex and hence the color was lost. Afterwards, Safranin was applied for 30 seconds as counter stain which gives light red or pink color in the cell wall of gram-negative bacteria due to retention. Later, the glass slide was washed, drained and examined under Light microscope (Olympus, CX41, Japan). The *E. coli* bacteria were characterized as gram negative bacilli.

2.2 Detection of Enterotoxigenic *E. coli* (ETEC) by PCR:

ETEC Polymerase Chain Reaction (PCR) was done to detect LT (Heat-labile enterotoxin) and ST (Heat-stable enterotoxin; STh and STp) genotypes. Six individual lactose-fermenting colonies with deep pink color from MacConkey agar plate were randomly selected and tested for ETEC detection by Multiplex Polymerase Chain Reaction using LT, STh and STp gene specific primers.

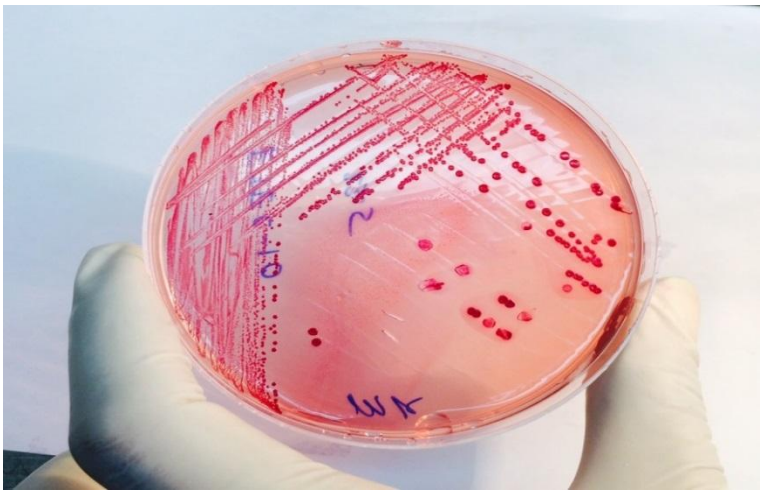

Figure 2.2: ETEC on MacConkey Agar Plate

2.2.1 Template DNA preparation:

An eppendorf tube was taken and 100µl of PBS (Phosphate Buffer Saline, pH~7.2-7.4) was poured to each tube. One loop of bacteria was taken from six different colonies on MacConkey agar plate and added into the individual tubes with PBS. The bacteria were suspended by vortex. Then, the suspension was heated at boiling temperature on water bath for 10 minutes. Tubes were then immediately transferred to ice, kept for one minute, and centrifuged at 12,000rpm (rotation per minute) for 10 minutes. The supernatant contains the template DNA which was ready to use in PCR.

2.2.2 Master Mix preparation:

The PCR master mix was prepared manually by adding *Taq* polymerase (TaKaRa, Code no: R001A), reaction Buffer, dNTPs, nuclease free water, the primers (Forward and Reverse), and template DNA in a PCR tube on following composition (Table 1):

Table 2.1: Reaction set up for PCR for 25 µl reaction volume

Components	Amounts
10X PCR buffer,with$Mgcl_2$	2.5 µl
dNTPs (2.5mM each)	4.0 µl
$MgCl_2$(25mM)	0.5 µl
Primer LT mixture(4pm/µl)	2 µl
Primer STh mixture(4pm/µl)	2 µl
Primer STp mixture(4pm/µl)	2 µl
Taq Polymerase(5U/µl)	0.15µl
Nuclease Free Water	10.35 µl
Template DNA	1.5 µl

The Primers, template DNA, and *Taq* Polymerase were added right before loading the sample in the PCR machine (Infinigen, USA). All the steps were performed on eppendorf pcr cooler rack.

Table 2.2: Toxin specific primers with sequence

Primer name	Sequence(5´→3´)
ST_h-F	TTCACCTTTCCCTCAGGATG
ST_h-R	CTATTCATGCTTTCAGGACCA
STp-F	TCTTTCCCCTCTTTTAGTCAG
STp-R	ACAGGCAGGATTACAACAAAG
LT-F	ACGGCGTTACTATCCTCTC
LT-R	TGGTCTCGGTCAGATATGTG

Optimized temperature conditions were used for PCR: pre-denaturation at 95°C for 5 minutes; 39 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The thermal cycling profile of the PCR is tabulated below (Table 2.3):

Table 2.3: Thermal cycling profile of ETEC PCR

PCR condition	Temperature	Time
Initial denaturation	95°C	5 minutes
Denaturation	94°C	30 seconds
Annealing	54°C	30 seconds
Extension	72°C	30 seconds
Final extension	72°C	5 minutes

39 cycles

2.2.3 Agarose Gel Preparation:

Amplified PCR products were analyzed by electrophoresis on 1% agarose gel. Calculated amount of Agarose (Ultrapure, Invitrogen, USA) was taken in conical flask and required amount of 1X TBE (Tris–borate EDTA) buffer was added. Then, the mixture was melted at high temperature in microwave oven for 2-3minutes and 2µl of Gel red (Biotium, Cat no: 41003, USA) was added to the gel. After mixing well, it was poured on a gel casting tray with the comb and finally kept for 15-20 minutes in room temperature for gel solidification. While pouring the melted gel mix solution into the gel tray, care was taken so that no bubbles were formed.

2.2.4 Detection of PCR product using agarose gel electrophoresis

About 6 µl aliquot of the PCR product along with 2 µl of loading dye (ThermoFisher, code no: R0611)was mixed and loaded into individual wells of the gel. A ladder of size 1kb plus (Generuler, ThermoFisher, USA) was also added into the same gel for estimating the molecular weight of linear, double-stranded PCR products which were estimated to be within 1,500 bp. Amplified PCR products were electrophoresed at 150 volts for 30 minutes. The separated DNA bands were observed on a Gel documentation system (Bio-Rad, USA) under Ultraviolet light.

Table 2.4: Size of specific toxin PCR products:

Toxin Name	Size
LT	300bp
STh	166bp
STp	100bp

2.3 Confirmation of specific ETEC colony by Toxin ELISA:

PCR positive samples were subjected to GM1 ELISA (Human Anti-Ganglioside M1 specific Enzyme Linked Immunosorbent Assay) to detect specific toxin producing colonies. For preparation of template DNA for PCR, six colonies were collectively used. As for PCR positive specimens, all the six colonies could be toxin producing or the positive PCR results could be attributed to any of six colonies. So in order to confirm only the toxin producing colonies or colony, toxin ELISA was performed where the marked individual colony (each of six colonies used for PCR) were added on individual well of ELISA plate. The steps are mentioned below:

1. About 100µl GM1 (120 Nano molar) ganglioside solution was added to each well of an ELISA plate and incubated at 37°C for four hours. (Plates for LT toxin test)
2. GM1-coated plates were washed twice with PBS (Phosphate Buffer Saline); the wells were blocked by the addition of 200µl of 0.1% BSA-PBS and then kept at 37°C for 30 minutes.
3. Later, it was washed once with PBS and about 100µl of supplemented LB Broth was added.
4. Six colonies from MacConkey agar plate which were used in ETEC PCR were identified and inoculated in the ELISA plate in individual wells (six colonies in six different wells of ELISA plate) using a wooden stick and the plates were covered with plastic film to prevent evaporation.

5. Thereafter, the LT plates were incubated with shaking at 150-250 rpm overnight at 37°C.The wells which lack visible growth(turbidity) were excluded.
6. New GM1 coated plates (Plates for ST toxin test) for each LT-plate was washed twice with PBS. These ST plates were blocked with 200µl of 0.1% BSA-PBS and then kept at 37°C for 30 minutes.
7. Afterwards, the ST plates were washed once with PBS and about 100µl of recombinant ST-CTB (0.5 µg/mL) conjugate solution was added; then it was incubated at room temperature for 60 minutes.
8. Again, the ST plates were washed thrice with PBS. About 50µl from the LT plates was transferred to the corresponding wells in the ST-plates. Then, 50µl of anti-ST MAb and ST in a ratio of 1:3 were added to solution immediately after transfer. Later, the ST plates were shaken gently and incubated standing still at room temperature for 90 minutes.
9. In case of LT plates, the plates were washed thrice with 0.05% PBS-Tween and 100µl of anti-LT MAb was added. The LT plates were also incubated at room temperature for 90 minutes.
10. Finally, both LT and ST plates were washed thrice with PBS-Tween and 100µl of anti-Ig enzyme conjugate was added. Both plates were then incubated at room temperature for 90 minutes.
11. After incubation, plates were again washed thrice with PBS-Tween prior to substrate addition. Before measuring the absorbance, 100µl of substrate solution was added to each well.
12. For LT plates, it was incubated for 20 minutes after substrate addition, while for ST plates the incubation period was 10-15 minutes. The absorbance was measured at 450nm using a microplate reader (Titertek).

Data interpretation:

In case of LT plates, a positive result is an A450 nm value of ≥mean background+0.1;
Where the background is defined as the mean absorbance determined for LT-negative control strains. For ST plates, the 50% inhibition concentration value (IC50) according to the equation below-

IC_{50}= Mean absorbance for ST-negative control strains ÷2;

Here, the background is defined as the mean of the absorbance at 450 nm of the ST-negative controls.

2.4 Antimicrobial Sensitivity Test (AST):

To determine the antibiotic susceptibility of these ETEC strains, disk-diffusion method was used. Specified ETEC colonies from MacConkey Agar Plate were picked by a sterile cotton swab and suspended in saline solution. The turbidity of the inoculum was adjusted to 0.5 McFarland standards by adding more organisms or more saline, thus achieving a log phase containing approximately $1.5x10^8$organisms/ml. A sterile cotton swab was immersed into the bacterial suspension and the excess suspension was removed by rotating the swab with a firm pressure against the inner side of the tube above the fluid levels. The inoculum was then lawned evenly on the entire surface of a Mueller- Hinton agar plate in three different planes (by rotating the plates approximately 60° angle each time) to get a uniform distribution of the organism.

The inoculum was then allowed to dry at room temperature with lids closed. The antibiotic discs (Oxoid, UK) were then placed 15 mm away from the edge of Petri dish on the lawned surface of the Mueller- Hinton Agar using a sterile needle while keeping at least 25 mm gap in between the discs. The discs were gently pressed down to ensure contact. The disks were applied within 15 minutes of inoculating the plates. The plates were then incubated at 37°C in an aerophilic incubator (Memmert, Germany) for 22-24 hours. Following overnight incubation, each plate was examined and the diameter of complete inhibition zone measured with the help of a slide calipers placed beneath the surface of the petri dish without opening the lids. Zone of inhibition was measured in mm in two directions at right angle to each other through the center of each disc and the average of the two readings was taken.

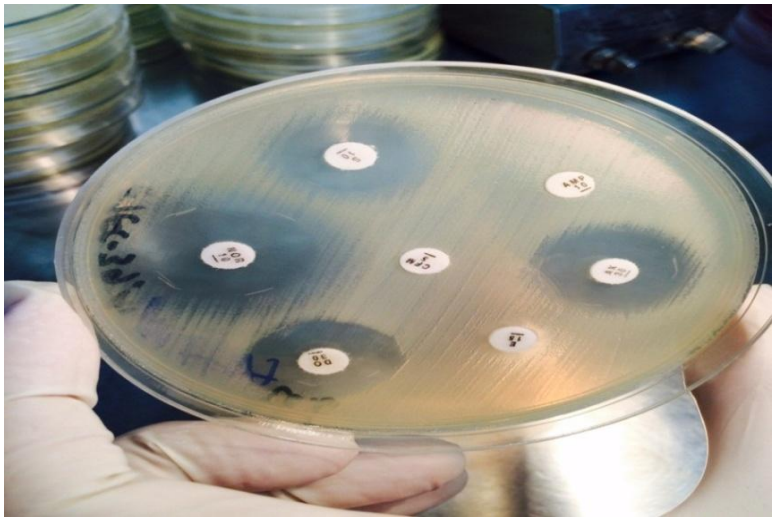

Figure 2.3: Antimicrobial sensitivity test (AST) using disk-diffusion method

In this study, 13 different commercially available antibiotics were used. The antibiotics were selected for the Antimicrobial Sensitivity Test as those are specified for the ETEC toxins according to CLSI (Clinical and Laboratory Standards Institute) protocol. Thirteen antibiotic disks which were used for AST along with their zone diameter for ETEC are given below:

Table 2.5: Zone Diameter interpretation chart for Enterobactericeae *E coli (*ETEC)

Antibiotic Name	**Abbreviation**	**disk potency**	**Result Interpretation**		
			Sensitive	**Intermediate**	**Resistant**
Ampicillin	AMP	10µg	≥22	19-21	≤18
Ciprofloxacin	CIP	5 µg	≥21	16-20	≤15
Ceftriaxone	CRO	30 µg	≥23	20-22	≤19
Doxycycline	DO	30 µg	≥14	11-13	≤10
Nalidixic Acid	NA	30 µg	≥19	14-18	≤13
Norfloxacin	NOR	10 µg	≥17	13-16	≤12
Streptomycin	S	10 µg	≥15	12-14	≤11
Sulfomethoxazole	SXT	25 µg	≥16	11-15	≤10
Tetracycline	TE	30 µg	≥15	12-14	≤11
Cefixime	CFM	5 µg	≥19	16-18	≤15
Amikacin	AK	30 µg	≥17	15-16	≤14
Azithromycin	AZM	15 µg	≥18	14-17	≤13
Erythromycin	E	15 µg	≥23	14-22	≤13

Ref: Clinical and Laboratory Standards Institute, Zone Diameter for Enterobacteriaceae, M100-S24 Performance Standards for antimicrobial Susceptibility Testing; Twenty-Fourth Information Supplement.

2.5 Multidrug resistant *Enterobacter agglomerans*

For identifying extended spectrum beta-lactamase (ESBL) mediated resistance, a multi-drug resistant strain of *Enterobacter agglomerans* which showed resistance against all the tested antibiotics was selected. The *Enterobacter agglomerans* strain was taken from a cryopreserved bacterial stock which had been isolated from an acute respiratory infection surveillance project at ideSHi. This specific microorganism belonging to the Enterobacteriaceae family was chosen as it was considered a suitable suspect for harboring ESBLs.

2.6 Phenotypic detection of ESBL gene:

Disk diffusion susceptibility testing was employed and zone diameters were recorded according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) recommendation. Combination Disk Strip test was performed for susceptibility testing. The disk strip consists of two disks and the method utilizes the antibiotic alone and in combination with inhibitor. These depend on comparing the zones given by discs containing an extended-spectrum cephalosporin with clavulanate with those for identical discs without this inhibitor. If an ESBL is present, the zones are enlarged by the presence of the inhibitor. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. In combined disk test, diameter differences of ≥5mm between the two discs were considered as the indicative of class A -ESBL producer.

Detection Criteria for ESBL$_A$ Producer: (CTX-M1)

The key characteristic that is used to discriminate enzymes belonging to different classes is responsiveness to specific inhibitors; like class A enzymes are inhibited by Clavulanic acid.

Combination disk: CPD/CPD+CV
CPD= Cefpodaxime
CV= Clavulanic acid (inhibitor)
Positive: ratio ≥5 mm.

Or, **Combination disk:** CAZ/ CAZ+CV
CAZ: Ceftazidime
Positive: Ratio≥ 8

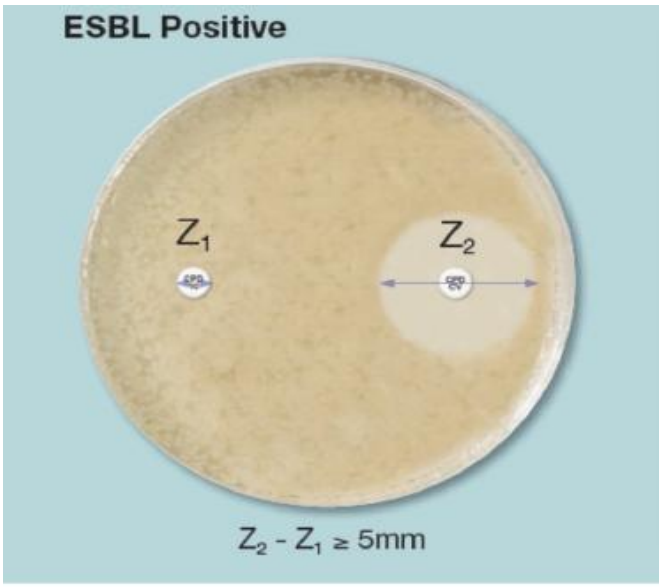

Figure 2.4: Combination disk test for phenotypic detection of ESBL$_A$ producer

Detection Criteria for ESBL$_{carba}$A producer: (KPC)

Meropenem (MR)/meropenem + boronic acid (MR+BO)

Ertapenem (IMP)/ertapenem + boronic acid (IMP+BO)

Positive test: Ratio ≥8

Detection Criteria for ESBL$_{carba}$B producer: (NDM, VIM)

Meropenem (MR)/meropenem + DPA (Dipicolinic acid)

Imipenem (IMP)/imipenem + DPA (Dipicolinic acid)

Positive test: Ratio ≥8

Detection Criteria for ESBL$_{carba}$D producer: (OXA-48)

Carbapenem/ Carbapenem + NaCl

Positive test: Ratio ≥8

Mueller-Hinton Agar (MHA) medium is used for the routine susceptibility testing of non-fastidious microorganisms. As the strain *Enterobacter agglomerans* belong to the Enterobacteriaceae family, it was grown on non-selective and non-differential MHA medium. The inoculum suspension was prepared which was equivalent to a 0.5 McFarland standard and well isolated colonies were selected from overnight growth. Through using a sterile loop, morphologically similar *Enterobacter agglomerans* colonies were dipped into the suspension to

produce an even, visible turbidity. After the plates have reached to the room temperature, the inoculum was lawned evenly over the entire surface by inoculating in three directions. The disk containers were allowed to warm at room temperature for avoiding condensation of water on disks. Afterwards, the antibiotic disks were put in firm, even contact with the surface of the medium. Within 15 minutes of disk placement, the plates were inverted and incubated at 37°C incubator for approximately (16-20) hours.

2.7 Genotypic detection of ESBL gene:

Genotypic detection for the presence of ESBL (Extended Spectrum beta-lactamase) genes in the resistant strain of *Enterobacter agglomerans* was performed using gene-specific Polymerase Chain Reaction.

The DNA sequences of 5 ESBL genes were retrieved from the nucleotide database of National Center for Biotechnology Information (NCBI). The retrieved FASTA sequence was then used to design five sets of ESBL gene specific primers. The specificity of the designed primers was confirmed by further analysis using the primer BLAST tool of NCBI (Stephen et al., 1990) to prevent any non-specific binding and amplification.

The name and sequence of the primers specific to the ESBL genes along with their melting temperature (Tm), and guanine-cytosine (GC %) content are given below:

Primers for KPC gene:

Oligo	Sequence(5′→3′)	Tm	MW	GC content
KPC-1A	CTGTCTTGTCTCTCATGGCC(20)	59.4	6026	55%
KPC-1B	CCTCGCTGTGCTTGTCATCC(20)	61.4	6011	60%

Primers for Oxa-48 gene:

Oligo	Sequence (5′→3′)	Tm	MW	GC content
Oxa48-F	GCGTGGTTAAGGATGAACAC(20)	57.3	6206	50%
Oxa48-R	CATCAAGTTCAACCCAACCG(20)	57.3	6015	50%

Primers for VIM gene:

Oligo	Sequence(5′→3′)	Tm	MW	GC content
VIM1F	AGTGGTGAGTATCCGACAG(19)	56.7	5893	52.6%
VIM1R	ATGAAAGTGCGTGGAGAC(18)	53.7	5613	50%

Primers for NDM gene:

Oligo	Sequence(5′→3′)	Tm	MW	GC content
NDM-F	GGTTTGGCGATCTGGTTTTC(20)	57.3	6161	50%
NDM-R	CGGAATGGCTCATCACGATC(20)	59.4	6102	55%

Primers for CTX-M1 specific gene:

Oligo	Sequence(5′→3′)	Tm	MW	GC content
CTX-M1-3F	AATCACTGCGCCAGTTCACGCT(22)	62.1	6655	54.5%
CTX-M1-R2	AGCCGCCGACGCTAATACA(19)	58.8	5767	57.9%

Table 2.6: Desired PCR product size of ESBL genes

CTX-M1	841bp
KPC	795bp
NDM	621bp
VIM	241bp
oxa-48	400bp

2.7.1 ESBL gene detection using conventional PCR:

PCR was performed with the enlisted primers using a T_{100} ™ thermal cycler (Bio-Rad, USA). The final reaction volume was 10µl containing 1 µl 10X PCR buffer with $MgCl_2$salt (Clontech, code no:R001A),0.25µl $MgCl_2$(25Mm), 1µl of dNTPs mixture (2.5 mM),0.5µl of each of forward and reverse primers,0.1µl of TaKaRaTaq™ DNA polymerase (Clontech, code no: R001A) and 3 µl of template DNA. Total volume was made 10µl with Nuclease Free Water.

Table 2.7: PCR master mix for ESBL detection PCR

Components	Amounts
2.5 mMdNTPs	1µl
10X buffer	1µl
25mM$MgCl_2$	0.25 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
*Taq*polymerase	0.1 µl
Nuclease free water	3.75 µl
Template DNA	3 µl

The thermal cycling profile that was followed for the sample is given below:

Table 2.8: Thermal cycling profile for ESBL PCR

PCR condition	Temperature	Time
Initial denaturation	95°C	5 minutes
Denaturation	95°C	30 seconds
Annealing	58°C	30 seconds
Extension	72°C	1 minute
Final extension	72°C 10°C	6 minutes ∞

35cycles

Annealing Temperature and the duration of elongation was modified for the various ESBL genes. The PCR was carried out for 35cycles. Afterwards, the amplicon was stored at +4°C for further analysis.

2.7.2Agarose gel preparation and electrophoresis:

Amplified PCR products were analyzed by agarose gel electrophoresis by the same procedure as described in section 2.2.3, except for different lengths of PCR products, appropriate concentration of agarose gel was used.

2.7.3 Gel extraction of PCR products:

After observing the presence of the desired DNA in the PCR amplicon, DNA was purified from the agarose gel using QIAGEN gel extraction kit. Before purification, approximately 30µl of PCR products along with the loading dye were loaded and electrophoresis was done. For getting smoother separation of DNA bands, the gel was run at 80 volts for 1.5 hours.

Firstly, the DNA bands from the agarose gel were excised carefully with sharp scalpel and then the excised gel weighed in an Electronic balance machine (Mega, Japan standard). To dissolve the gel, QG buffer was added. To dissolve the gel properly, it was incubated at 50°C for 10 minutes with periodic vortexing. Then, 1 gel volume of isopropanol was added to increase the yield of DNA fragments. Seven hundred µl of sample solution was transferred into QIA quick column and centrifuged at 17,900g for 1 minute. After discarding the flow- through, QG buffer was added and centrifuged to remove all the traces of agarose. Later, for washing 750µl of PE buffer was added to the QIAquick column and centrifuged for 1 minute at 17,900g. It was centrifuged twice and the flowthrough was discarded to remove the whole of Ethanol as it will precipitate the DNA. The QIAquick column was placed into a clean 1.5ml micro-centrifuge tube. To elute DNA, 50µL of nuclease free water was added to the center of the QIAquick membrane which was then centrifuged for 1 minute at maximum speed. The column was then discarded and the supernatant containing the extracted PCR product was stored at +4°C for downstream use.

2.7.4 Measurement of DNA concentration and purity:

DNA concentration was measured with EON spectrophotometer (BioTek, USA) using Take 3 plate. Approximately 2µl Nuclease Free water was used as blank and 2µl of DNA sample was loaded on the Take 3 plate and OD (optical density) was measured spectrophotometrically. The

concentration was measured in ng/µl and the purity was checked using OD ratio at 260 nm/280 nm reading. The result was analyzed with Gen5 Software.

2.8 DNA sequencing: (Sanger method)

Fluorescence-based automated cycle sequencing entails a DNA template, a sequencing primer (forward or reverse), a thermostable DNA polymerase, deoxynucleoside triphosphate (dNTPs), sequencing buffer and fluorescent dye-labeled 2′,3′-dideoxynucleotide triphosphates (ddNTPs).

All the components are mixed and subjected to cycles of denaturation, annealing, and extension in a thermal cycler. DNA polymerase incorporates either a dNTP (A, C, G, or T) or the corresponding ddNTPs (nucleotide base analogs that lack the 3′-hydroxyl group essential for phosphodiester bond formation by DNA polymerase) at each step of chain extension depending on the relative concentrations of both molecules. When a dNTP is added to the 3′ end, chain extension is continued. However, when a ddNTP terminator (ddA, ddC, ddG, or ddT each tagged with different fluorescent dye) is added to the 3′ end, chain elongation is terminated, forming labeled extension products of various lengths.
The DNA samples were sequenced at IEDCR (Institute of Epidemiology, Disease Control and Research). The calculation of cycle sequencing was obtained according to the measured template concentration.

Table2.9: Master cycle components with amount

Component	Amount
5X PCR sequencing buffer	2.0µl
Big dye(2.5X)	0.50µl
primers	0.2 µl
Template	1-10 ng
water	up to 10 µL

The tubes containing the template were spun, and 10-20 ng/µL (depending on the concentration) of each of the purified PCR products were added to the 8-tube PCR strip. Then nuclease free water was added to the mixture to make the total volume 10 µL. The PCR tubes were centrifuged

at 4000 rpm for 3 minutes. Then, the PCR strip was placed in the Mastercycler® gradient (Cat. No. 4095-0015, USA Scientific) Thermal Cycler and subjected to following thermal cycling profile: pre-denaturation at 95°C for 10 minutes; 25 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 5 seconds and extension at 72°C for 4 minutes; and a final extension at 72°C for 6 minutes (Table 2.11).

Table 2.10: Thermal cycling profile for cycle sequencing

PCR condition	Temperature	Duration	
Initial denaturation	95˚C	**10 minutes**	
Denaturation	**95** ˚C	**10 seconds**	**25 cycles**
Annealing	**55** ˚C	**5 seconds**	
Extension	**72** ˚C	**4 minutes**	
Final extension	**72** ˚C	**6 minutes**	
	10 ˚C	∞	

After completion of cycle sequencing, the reaction plate was centrifuged at 4100 rpm for 2 minutes. Then, 45µl of SAM solution and 10 µl of X-terminator (Applied Biosystems, USA) were added per 10µl volume. Both of the solution aid in removal of impurities by desalting as salts interfere with electro-kinetic injection, and elimination of remaining labeled ddNTPs, thus, minimizing background noise produced by dye blobs in the sequencing results. Before addition, the X-terminator solution was vortexed properly at maximum speed for at least 30 seconds, until it became homogenous. As it was difficult to pipette the highly dense X-terminator solution out from the bottom of its container, wide bore micropipette tips were used. Later, the reaction plate was sealed and vortexed for half an hour. The mixture was then centrifuged at 4100 rpm for 2 minutes and the supernatant was collected for capillary electrophoresis. Ten µl of supernatant was transferred to a fresh sequencing tube. Before placing the sequencing tubes into the capillary electrophoresis instrument, it was covered with Septa mat. Rest of the supernatant was stored at +4°C for later use.

Sequence analysis:

Sequencing data were analyzed by Chromas Lite 2.4 software to identify the sequence alignments for showing identity and detecting mutations. The obtained sequence was subjected to further analysis using Basic Local Alignment Search Tool (BLAST) for finding sequence similarity with sequences already reported in online databases.

Chapter 3: Results

3.1 Enumeration of three specific ETEC toxins:

A total of 50 ETEC strains were used. Multiplex PCR and GM1 ELISA were performed for phenotypic and genotypic characterization of enterotoxins expressed by the ETEC strains. Combined results from both PCR and Toxin ELISA were taken into consideration on the basis of three toxin profiles which include: LT, ST and combined LT/ST profile. The bar-graph (figure 3.1) below represents the toxin profile of 50 ETEC isolates.

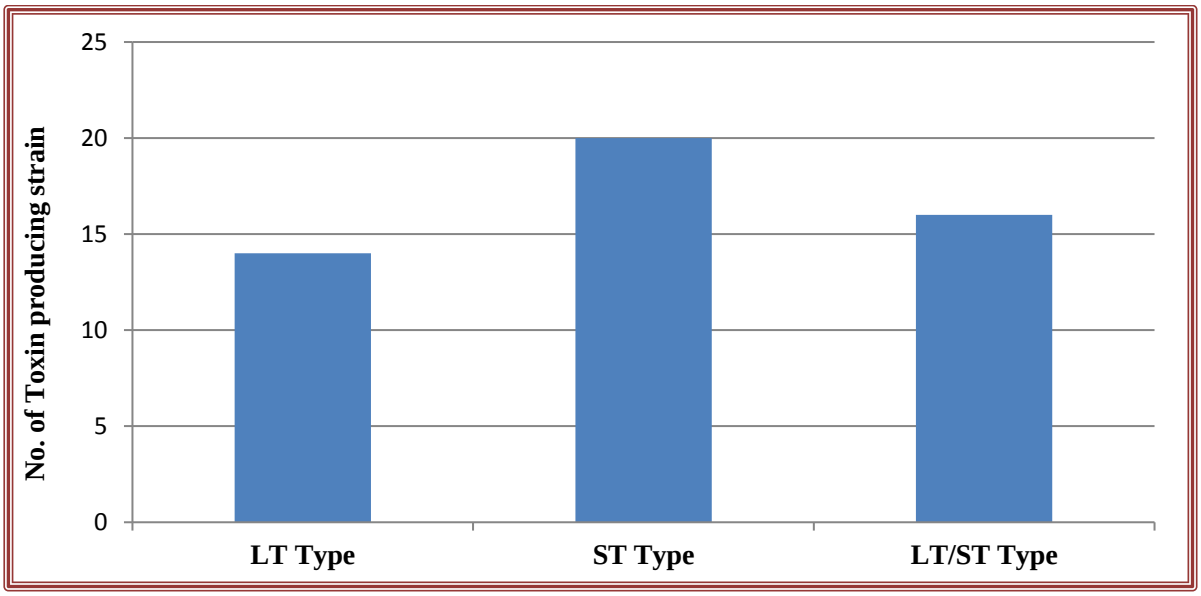

Figure3.1: Combined Toxin profile from PCR and Toxin ELISA. Heat-stable (ST) toxin possessing ETEC strains were substantially more common than Heat-labile(LT) toxin possessing ETEC strains among the fifty ETEC isolates collected from diarrheal patient.

Among 50 ETEC strains, LT and ST producing strain were 14 and 20, respectively. The rest 16 of 50 strains were detected as both LT/ST producing. Heat-stable (ST) toxin expressing ETEC strains were substantially more common than Heat-labile(LT) toxin expressing ETEC strains among the fifty ETEC isolates collected from diarrheal patients.

For defining the prevalence of specific subtype of ST toxin, the ETEC strains were analyzed statistically through pie-chart representation. The pie chart depicts the percentage of the two subtypes of ST toxin which are STp and STh.

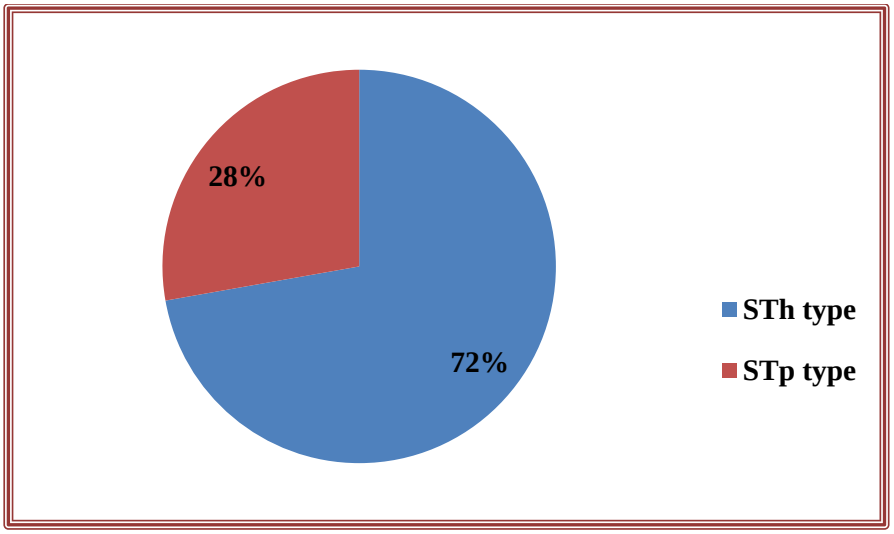

Figure 3.2: The Percentages of the two subtypes of ST enterotoxin. STh subtype toxin was approximately three times more frequent than STp subtype.

As can be seen from the chart (figure 3.2), STh type toxin was more prevalent (72%) compared to the STp toxin.

Alarming rates of multidrug resistance inflated the need for monitoring of resistance. To ascertain the antibiotic resistance pattern, Antimicrobial Susceptibility Tests were performed on the fifty ETEC strains. Antibiotic Sensitivity spectrum of the isolated ETEC strains against 13 different antibiotics is depicted in figure: 3.3.

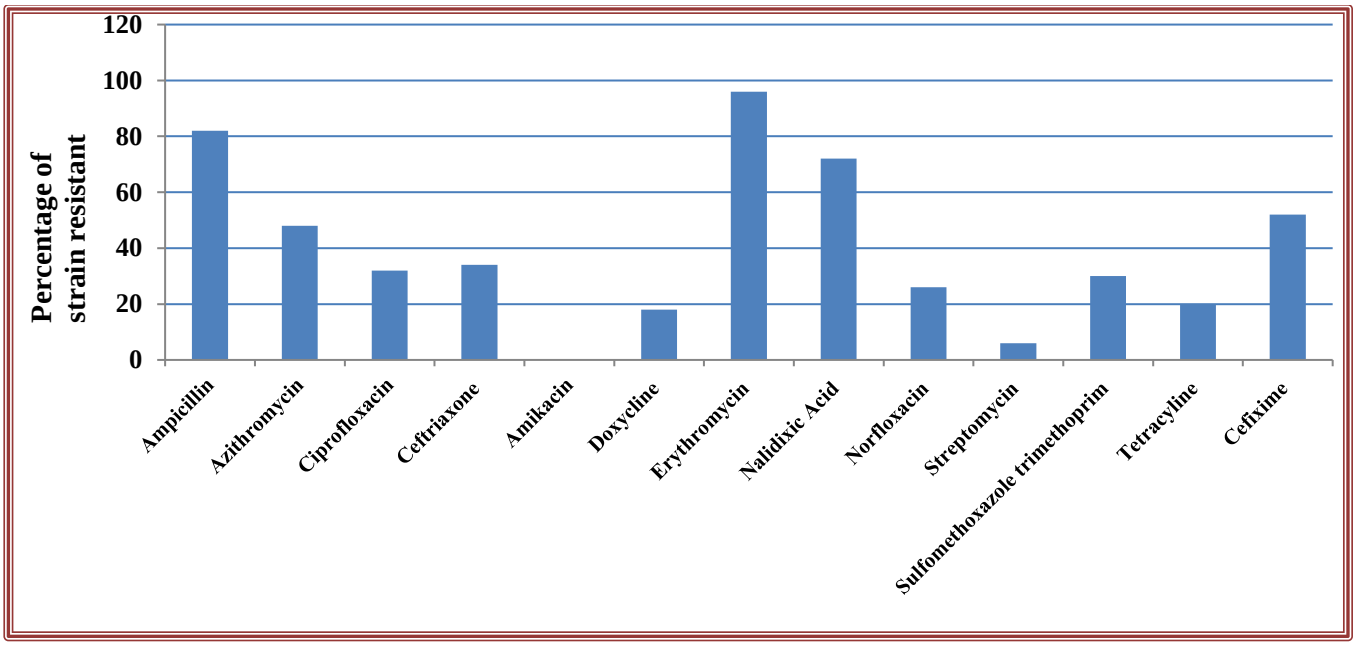

Figure 3.3: Antibiotic susceptibility profile of the enlisted ETEC samples. The maximum resistance was observed against Erythromycin, whereas the susceptibility of the ETEC strains was 100% for Amikacin.

From the bar chart (figure 3.3), it can be deduced that Amikacin (Ak) would be the most effective antibiotic against ETEC isolates as these showed least resistance against the drug. On the contrary, ETEC strains showed greater percentile of resistance to certain antibiotics which were Erythromycin (92%), Ampicillin (82%) and Nalidixic acid (75%).

3.2 Extended Spectrum Beta Lactamase (ESBL)

3.2.1 Confirmation of multidrug resistance of an *Enterobacter agglomerans* strain:

Before phenotypic and genotypic characterization of ESBL in the multidrug resistant *Enterobacter agglomerans* strain, its antibiotic susceptibility was tested following Clinical Laboratory Standard Institute (CLSI) guidelines (Figure-3.4). The *Enterobacter agglomerans* strain has shown resistance to all of 13 antibiotics tested (Table-3.1). This confirmed the multidrug resistance of the *E. agglomerans* strain. The resistance pattern of the strain was further analyzed both phenotypically and genotypically.

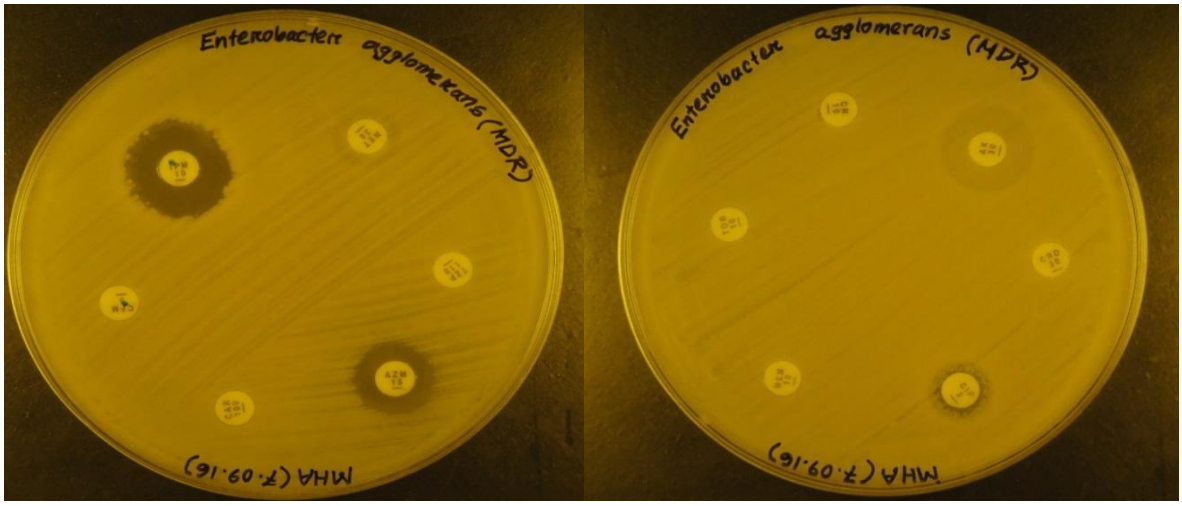

Figure 3.4: AST (Antimicrobial Susceptibility testing) through disk-diffusion method. According to the zone diameter interpretation chart of CLSI guidelines, the strain had shown resistance to thirteen antibiotics (Gentamycin, Ciprofloxacin, Ceftriaxone, Tobramycin, Imipenem, Meropenem, Netilmicin, Pipercilin+Tazobactam, Carbenicillin, Cefixime, Amikacin, Azithromycin, and Polymyxin B).

Table 3.1: Resistance pattern observation of *Enterobacter agglomerans* against 13 antibiotics:

Antibiotics Name	Short Name	Concentration	Interpretation(mm)			Results	
			Sensitive	Intermediate	Resistant	Zone of inhibition (mm)	Findings
Gentamycin	CN	10µg	≥15	13-14	≤12	6	Resistant
Ciprofloxacin	CIP	5 µg	≥21	16-20	≤15	7	Resistant
Ceftriaxone	CRO	30 µg	≥23	20-22	≤19	6	Resistant
Tobramycin	TOB	30 µg	≥14	11-13	≤10	6	Resistant
Imipenem	IMP	10 µg	≥23	20-22	≤19	*16*	Resistant
Meropenem	MEM	10 µg	≥17	13-16	≤13	6	Resistant
Netilmicin	NET	30 µg	≥15	13-14	≤12	6	Resistant
Pipercilin+ Tazobactam	TZP	100 µg	≥21	18-20	≤17	6	Resistant
Carbenicillin	CAR	100 µg	≥23	20-22	≤19	6	Resistant
Cefixime	CFM	5 µg	≥19	16-18	≤15	6	Resistant
Amikacin	AK	30 µg	≥17	15-16	≤14	6	Resistant
Azithromycin	AZM	15 µg	≥18	14-17	≤13	12	Resistant
Polymyxin B	PB	15 µg	≥14	12-13	≤11	12	Resistant

Reference: Clinical and Laboratory Standards Institute, Zone Diameter for *Enterobacteriaceae*, M100-S24 Performance Standards for antimicrobial Susceptibility Testing; Twenty-Fourth Information Supplement.

3.2.2 Combination disk test for phenotypic detection:

As some of the drugs were beta-lactam ring containing antibiotics, combination disk test was performed for phenotypic confirmation of whether the multidrug resistant *Enterobacter agglomerans* strain was harboring beta-lactamase. An inoculum of the resistant MDR *E. agglomerans* was lawned on a MHA plate and Cefpodaxime, and Cefpodaxime+clavulonic acid discs positioned on the lawn. The confluent lawn of growth was observed which defines the correct inoculum. Zone edges were read from the point of complete inhibition judged by the bare eyes. Afterwards, the zone diameters were measured with a slide caliper while keeping the MH plates against a dark background illuminated with reflected light.

No zone of inhibition was observed around the Cefpodaxime disk indicating resistance towards Cefpodaxime. This defines zero efficacy of the drug alone as it allows the growth of the organism. On the other hand, the Cefpodaxime+clavulanate disk produced inhibition zone indicating susceptibility. This zone of inhibition points towards the action of Clavulanic acid which acts as an inhibitor of $ESBL_A$. This outcome indicated the presence of ESBL in the *Enterobacter agglomerans* strain and phenotypically characterized the beta lactamase to belong to the class $ESBL_{A.}$

3.2.3 Genotypic detection:

Under the class $ESBL_A$, there are several subclasses of ESBL such as TEM beta-lactamases, SHV beta-lactamases, CTX-M beta-lactamases etc. For genotypic detection of the specific sub-classes of ESBL present in the strain, polymerase chain reaction (PCR) with gene specific primers was employed. The PCR assays revealed the presence of CTX-M1 gene (figure-3.5).

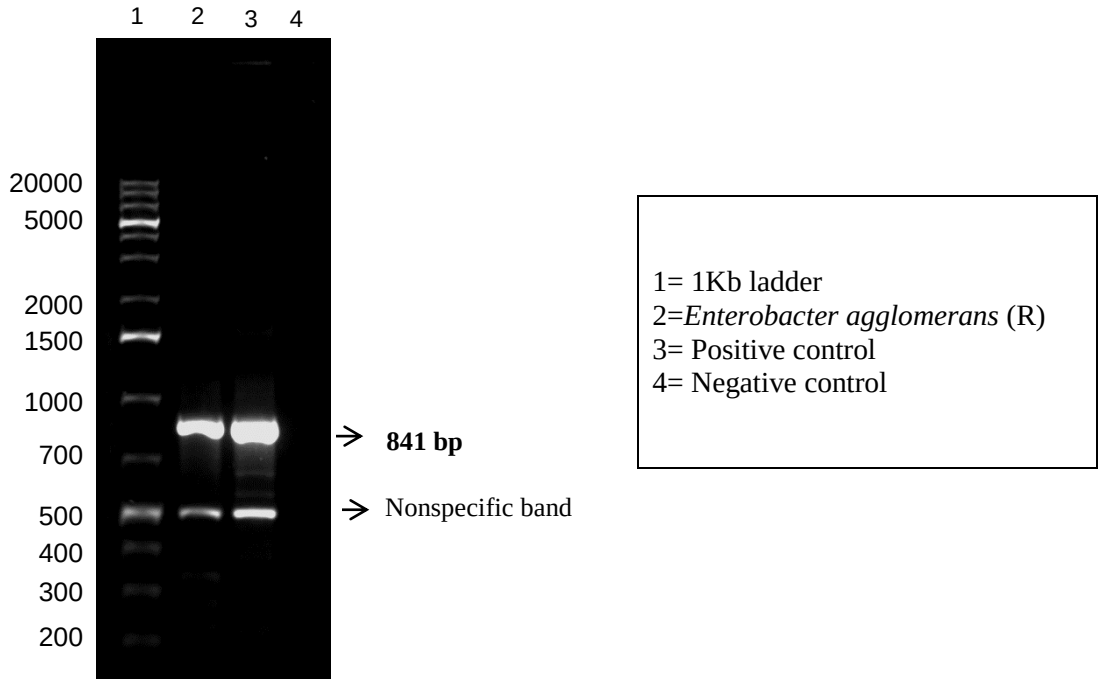

Figure 3.5: PCR result for the presence of *CTX-M1*genein *Enterobacter agglomerans*. After extraction of the total bacterial genome and amplification using specific primers, the bands were resolved through 1% agarose gel electrophoresis. The desired band size (*CTX-M1* gene) for the gene specific primer is 841 bp. In the figure, lane 1: 1kb plus ladder; Lane 2: *Enterobacter agglomerans***;** Lane 3: positive control and lane 4: (negative control).

Purity and amount of purified PCR product:

After purification of PCR products by spin column-based method, the purity and concentration of the DNA sample was measured spectrophotometrically. The ratio of OD at 260 nm and 280 nm of the extracted DNA was found to be 1.88 and the concentration 29.2ng/µl.

3.2.4 Sequencing results:

A positive PCR result for CTX-M1 gene necessitated the final step which is known as gene sequencing for genotyping. Purified PCR product was subjected to sequencing and sequencing result presented in figure-3.6 to figure-3.10.

In figure-3.6, the chromatogram depicts the two-dimensional plot with the ordinate axis giving concentration in accordance to detector response (figure-3.6). Sequencing data were analyzed by Chromas Lite 2.1 tool which generated a four color chromatogram showing the result of sequencing run. Different bases are represented in different colors that are defined below:

1. Adenosine=green
2. Guanine=black
3. Cytosine=blue
4. Thymine=red

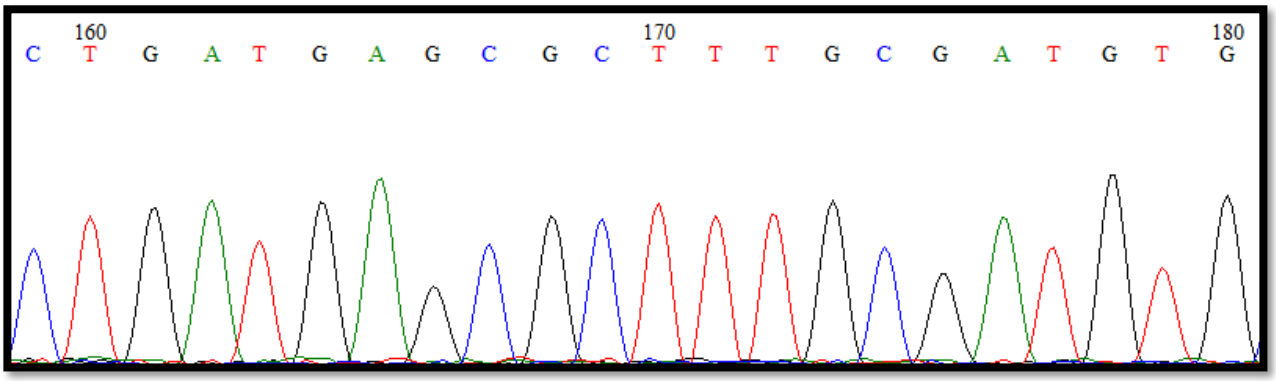

Figure 3.6: Diagrammatic representation of a part of the chromatogram of sequence.
Well-formed and distinctive single colored peaks and lack of background noises were observed, indicating refined sequencing with proper concentration of template and primer. In the chromatogram, the area under the peak is considered as the measure of component concentration.

Nucleotides are demonstrated using single letter codes in text based format which is defined as FASTA format. The specific format is documented through using Bioedit software. Sequencing data for the bacterial isolate containing the CTX-M1gene is shown below in Figure3.7.

>*Enterobacter Agglomerans*_CTXM_1
CGWGGCGMGGCACCGTCACGCTGTTGTTAGGAGTGTGCCGCTGTATGCGCAAACGGCGGA
CGTACAGCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATT
GATTAACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTG
CAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAA
TCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGC
GGAAAAGCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTA
CAGCGATAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCAC
CGCGTTCGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTT
AAACACCGCCATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAAC
TCTGCGGAATCTGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGAC
ATGGATGAAAGGCAATACCACCGGTGCAGCGAGCATTCAGGCTGGACTGCCTGCTTCCTG
GGTTGTGGGGGATAAACCGGCAGCGGTGGCTATGGCACCACCAACGATATCGCGGTGATC
TGGCCAAAGATCGTGCGCCGCTGATTCTGGTCACTTACTTCMCCCAGCCTCAACCTAAGC
AGAAAGCCGTCGCGWGAT

Figure3.7: Sequencing data obtained using CTX-M1 specific forward primer; consisting of 798 nucleotide bases.

The gene sequence was analyzed with NCBI Nucleotide Basic local alignment search tool (BLAST) and a list of sequences which were most similar to the query sequence was acquired.

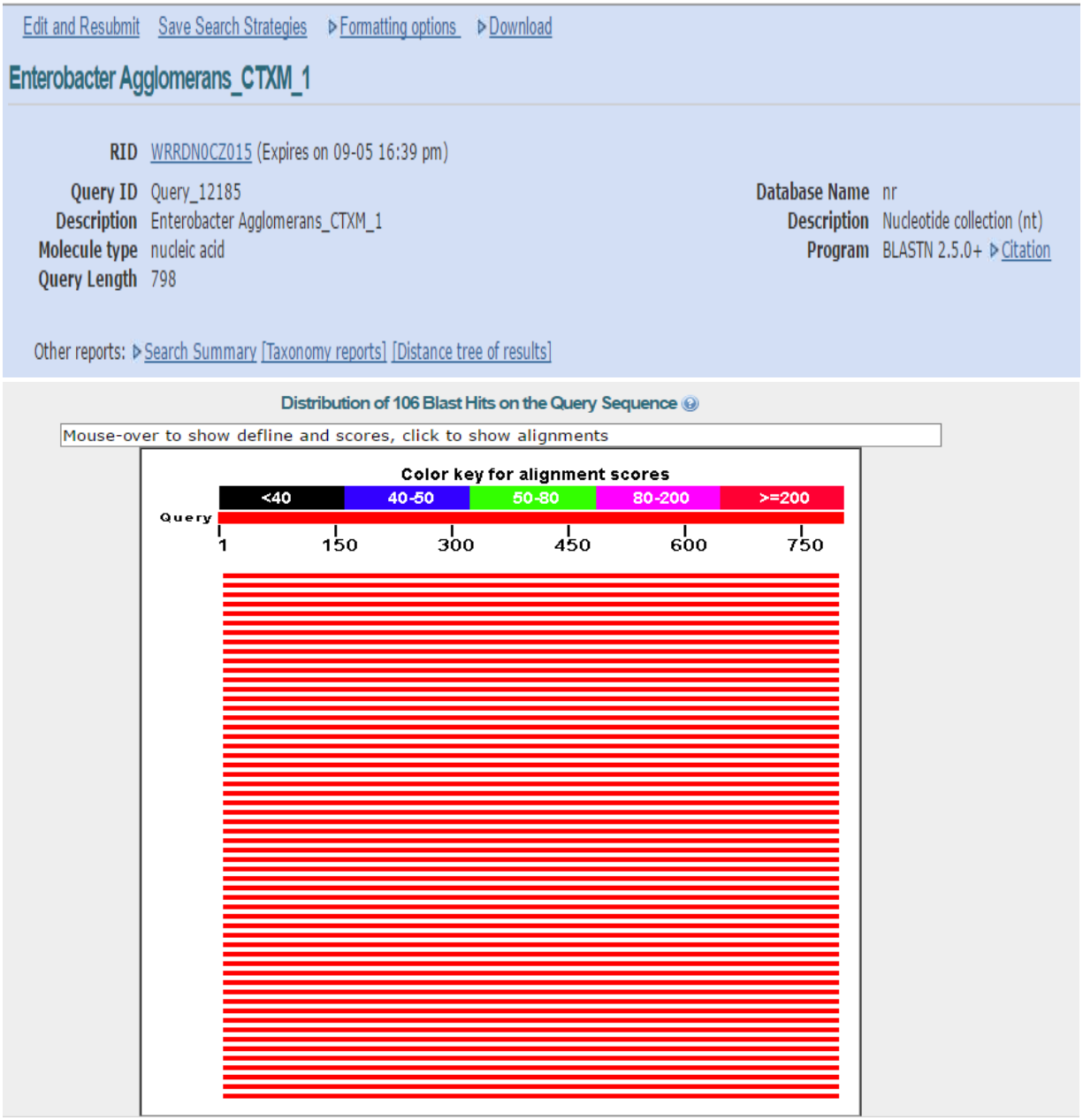

Figure 3.8: BLASTn result using the sequence of PCR product amplified by the CTX-M1 specific forward primer of the resistant bacteria.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Klebsiella pneumoniae isolate 23 plasmid pCTXM15_DHQP1400954, complete sequence	1424	1424	98%	0.0	99%	CP016925.1
Klebsiella pneumoniae isolate 11 plasmid pIncR_DHQP1300920, complete sequence	1424	1424	98%	0.0	99%	CP016919.1
Salmonella enterica strain FORC_019 plasmid pFORC19, complete sequence	1424	1424	98%	0.0	99%	CP012397.1
Klebsiella pneumoniae strain DHQP1002001, complete genome	1424	4274	98%	0.0	99%	CP016811.1
Escherichia coli strain K-15KW01, complete genome	1424	1424	98%	0.0	99%	CP016358.1
Escherichia coli isolate E. coli RL465 genome assembly, chromosome: RL465_chromosome	1424	1424	98%	0.0	99%	LT594504.1
Escherichia coli O25b:H4 plasmid, complete sequence	1424	1424	98%	0.0	99%	CP015086.1
Escherichia coli str. 690 plasmid pRCS57, complete genome	1424	1424	98%	0.0	99%	LO017738.1
Escherichia coli str. 3249 plasmid RCS105_pI, complete genome	1424	1424	98%	0.0	99%	LO017737.1
Escherichia coli str. 473 plasmid pRCS52, complete genome	1424	1424	98%	0.0	99%	LO017736.1
Escherichia coli blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-15, complete CD	1424	1424	98%	0.0	99%	NG_048935.1
Escherichia coli 1125509 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-163, co	1424	1424	98%	0.0	99%	NG_048948.1
Klebsiella pneumoniae KK08 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-156	1424	1424	98%	0.0	99%	NG_048940.1
Escherichia coli TN03 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-28, comple	1424	1424	98%	0.0	99%	NG_048977.1
Escherichia coli CTX-M-15 resistance cassette, isolate RS371	1424	1424	98%	0.0	99%	LN868277.1
Escherichia coli CTX-M-15 resistance cassette, isolate RS288	1424	1424	98%	0.0	99%	LN868276.1

Figure 3.9: Top 16 similar sequences resulted after alignments with the sequence of resistant bacterial strain.

After BLASTn analysis it was found that the resistant bacterial sequence matched with CTX-M1 gene (figure-3.10).

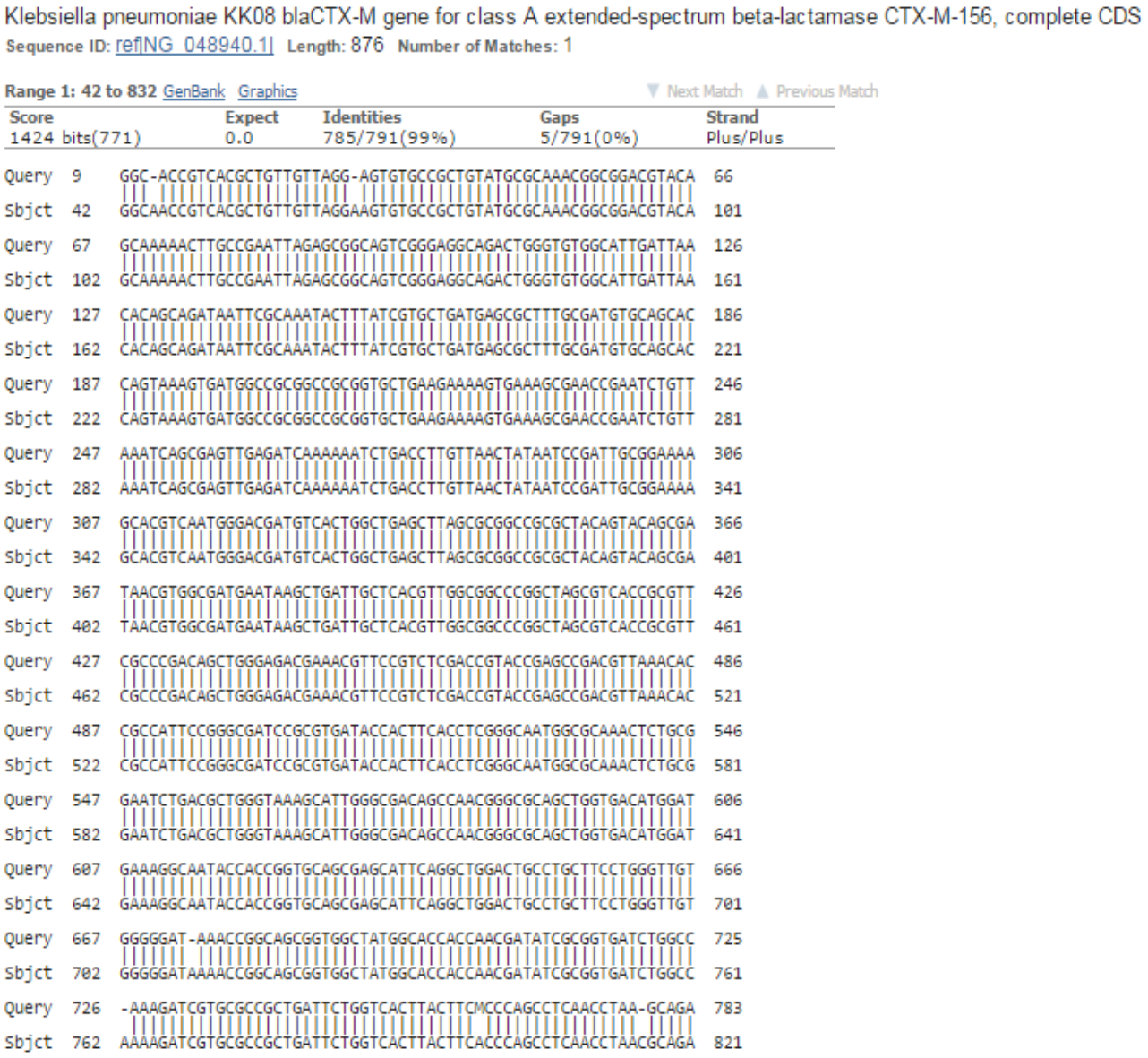

Figure 3.10: One of the matched sequences among sixteen with blaCTX-M gene for class (A) extended-spectrum beta-lactamase.

Maximum identity and lower e-value shown in the BLASTn results indicated that the amplified sequence belongs to the CTXM subclass of $ESBL_A$. Figure-3.10 illustrates the BLAST result of homologous sequences annotated as *Klebsiella pneumoniae, Salmonella enterica and Esherichia coli* harboring the resistance gene, indicating that the primer pair had amplified the gene of interest. In this study, β-lactamase producing gene was detected from the resistant strain of *Enterobacter agglomerans*. The BLAST analysis suggests that the same ESBL gene was found in two different bacterial strains, which may indicate a horizontal transfer of resistance genes as ESBLs are often plasmid encoded.

Chapter 4: Discussion

DISCUSSION:

There has been definite evidence on how diarrheal diseases has impacts on both economic and health care systems of a particular society. To elaborate, a child with an ongoing diarrheal disease can carry the effects to his/her future growth and cognitive development. Also it is a known fact that diarrheal diseases are one of the major causes of morbidity and mortality not only in the developing countries but also throughout the world. Studying the manifestation of ETEC-induced diarrheal diseases not only can help to reduce the death percentage in a particular population but also to develop different prevention strategies.

Moreover, as ETEC-induced diarrhea is mostly seen in new born or in children, a proper diagnosis and analysis of this infection is required for an effective treatment. To add up, the nature and conditions at which the ETEC toxins are more functional can be found out too. There are a variety of different genotypic and phenotypic methods available for the detection of ETEC toxins in clinical isolates. In this study, multiplex Polymerase Chain Reaction (PCR) was employed for detecting the LT, STp and STh enterotoxins using toxin specific primer sets. According to some other previous studies, multiplex-toxin PCR is recommended for the identification of ETEC, due to the high level of sensitivity and specificity of this method and its ability to detect both expressed and putatively silent genes, which ensures that no ETEC strains are, missed (A.sjoling. 2007). For this reason, multiplex PCR was conducted for the detection of two variants (STp and STh) of ST enterotoxin. This genotypic method was done for ETEC strains grown on MacConkey agar that allows differentiation of lactose fermenting *E. Coli* from other bacteria present in the stool. Then, the toxin positive isolates were analyzed through Ganglioside GM1 Enzyme linked Immunosorbent Assay (ELISA) method which differentiates the LT and ST toxins phenotypically. In case of multiplex PCR, any of the six colonies can be accounted for the positive toxin result as all six colonies were collectively used during template DNA preparation. On the contrary, the marked individual colony is inoculated into each well of ELISA plate through scrutinizing the distinct colonies. That's why, GM1 ELISA was followed after PCR to detect specific toxin producing colony for further confirmation. Although toxin ELISA can identify individual toxin producing colony, it cannot differentiate between the ST subtypes STh and STp. On the other hand, multiplex PCR can detect STh/STp type but as mentioned above it cannot detect individual toxin producing colony. Six colonies can be used to

prepare template DNA individually for six different reactions which would overcome the drawback but it would be much more costly than combination of single PCR reaction and toxin ELISA. So the combined method is both economical and highly sensitive which makes it a suitable approach for ETEC detection in developing countries.

One of the surveillance reports of Bangladesh from 2007-2012 manifested the overall rate of ETEC isolation where 43% were LT/ST, 27% LT and 30% ST positive (Begum et al., 2014). In this study, the percentages of ST and LT expressing ETEC strains were 40% and 28%, respectively, while both LT/ST toxin expression rate was 32%. Although this study was done using 50 strains only, the findings are quite comparable to the surveillance report. The higher prevalence of ETEC strains that express ST over LT is a noticeable finding as LT expressing ETEC strains are considered to be less pathogenic than those expressing ST. The ETEC strains that express both toxins simultaneously are often isolated from severe diarrheal infections. A study in Bolivia in 2010 evaluated the relation between virulence gene profiles and severity of disease. Their study showed that only ST type and both LT/ST type ETEC strains were significantly more often isolated from children with diarrhea, whereas only LT producing ETEC strains were more frequent in the control group, suggesting that LT type ETEC strains might be less important as pathogens (Gonzales et al., 2013).

When LT and ST are both present, they work synergistically, increasing the movement of fluid into the intestine over and above the levels observed with either toxin alone (Lisa et al., 2014). Data also demonstrate that the levels of inflammatory cytokines produced by intestinal epithelial cells in response to LT were significantly reduced in animals exposed to both enterotoxins. It suggests that there may be complex differences between ETEC strains expressing both LT/ST and strains that express LT or ST only in epithelial cell intoxication and potential differences in the secretory outcomes. But, ST production may reduce the hosts' ability to mount an effective innate or adaptive immune responses to infecting organisms. Here, the percentile of simultaneous expression of both toxins was 32% which portrays the severity of diarrhea in Bangladesh. This can be due to the poor sanitation and limited access to the clean water along with increasing antibiotic resistance.

In this study the higher incidence of STh (72%) variant compared to STp (28%) variant of ST toxin was found. Studies in Egypt and Guatemala have shown that there is a high frequency of STp compared to STh positive ETEC strains isolated from diarrheal stools of children (Bolin et al., 2006). However, STh producing ETEC is much higher than the STp type in Bangladesh (Yasmin et al., 2014). Low frequency of STp can be attributed to less consumption of porcine meat and products in the Bangladeshi settings.

Antibiotics have revolutionized the treatment of common bacterial infections and play a crucial role in reducing mortality. Antimicrobial therapy should be used in severe cases of diarrheal disease to reduce the duration of illness and may be used to prevent traveler's diarrhea. However, the progressive increase in antibiotic resistance among enteric pathogens is assumed to be a natural phenomenon now. When antibiotics fail to kill the bacteria effectively under therapeutic levels, it is understood that certainly this is an outcome of development of resistance by the bacteria which can be acquired in a couple of ways. When the susceptible bacteria are inhibited or killed by a dosage of a particular antibiotic, it exerts a selection pressure for the endurance levels of the resistant strains of the same bacteria. It is also a known fact that antibiotics are the secondary products of bacteria themselves, and hence it is not amusing at all that bacteria which can produce such antibiotics use these products against other bacteria in a competitive habitat. This triggers natural selection in low levels leading to the development of antibiotic resistance. On the contrary, the usage of antibiotic has been exploited over the decades and in such a manner that it can be termed as antibiotic abuse (Obire et al., 2009).

One of the important reasons that the ETEC diarrhea should be properly diagnosed is because in most cases it can be misdiagnosed as Cholera. As the clinical symptoms of both the diseases are more or less same, it can be mistreated too. For instance, rehydration can be a useful treatment for both cholera and ETEC diarrhea, whereas the spectrum of antibiotics used for both the diseases should be different. Antibiotics are found in a wide range and this has both positive and negative effects in the treatment of enteric diseases. Usually AST (Antibiotic sensitivity test) is conducted for research purposes, such as to find out the prevalence of a particular pathogen within a pathogen and thus prescribe a suitable antibiotic that is coherent with the data. Also it is very important to understand how the concentrations of the suggested antibiotics can affect both the pathogen and the host. A drug in a particular concentration which has the most effect on

pathogens and least effects on the hosts is the most desirable one. Moreover, it can be possible that many individuals are allergic to certain antibiotics or many antibiotics can have long time side effects; in order to avoid such issues, antibiotic sensitivity test should be done before any drug can be prescribed to a patient. It can help to detect whether a narrow spectrum or a broad spectrum antibiotic should be useful along with duration of the course.

In this study, Antibiotic susceptibility profiling was performed through disk-diffusion method to determine the resistance spectrum of the ETEC strains. All the ETEC isolates were susceptible to Amikacin which suggests that, Amikacin (Ak) would be the most effective antibiotic against ETEC organisms. The result also reported the highest resistance to Erythromycin (92%) followed by Ampicillin (82%), Nalidixic acid (75%) and Cefixime (55%). Multiple resistance rates were also high (about 90%). Ciprofloxacin (CIP) is a broad spectrum antimicrobial agent used nowadays for the treatment of diarrhea. But here, the ETEC isolates showed resistance of about 35%. The high level of resistant organism is narrowing down the number of effective antibiotics for treatment which in turn will have an adverse effect on health system. There were significant β-lactam (Ampicillin 82%, Cefixime 52% and Ceftriaxone 34%) resistances among the isolates and this poses health complications as these are the drugs of choice for the management of numerous bacterial infections.

Therefore, the need for vaccine is now in utmost priority. Epidemiological data suggest that ETEC strains which secrete heat-stable toxin (ST), alone or in combination with heat-labile toxin (LT) induce the most severe disease among children in developing countries. This makes ST an attractive target for inclusion in ETEC vaccine (Arne et al., 2010).

Antibiotic resistance is a public health problem of increasing magnitude, and finding effective solutions to address this problem is a critical focus. There are several ways of acquiring antibiotic resistance. One of the most fascinating way in which bacteria can acquire resistance is through spontaneous mutations in their genetic makeup. It is believed that the mutations inactivate the potency of the antibiotic by either eliminating the target portion of the cell which the antibiotics tend to attack, or by making the bacteria release chemicals that can inactivate the antibiotic itself. In many cases bacteria are able to change, or, seal the entry ports so that the antibiotic fails to enter the cell. In either of the way, such bacteria are naturally acquiring the resistance and hence induce the threat of spreading resistance. Another way in which bacteria

can acquire resistance is via conjugation, where plasmids from resistant bacteria are transferred to sensitive bacteria, resulting in the development of resistance and hence the entire population slowly acquires the phenomenon. A single bacteria population can acquire resistance to multiple antibiotics at once. Horizontal gene transfer is known to be the ultimate mechanism via which bacteria can spread the resistance.

Extended spectrum beta-lactamase family enzymes which are produced by certain resistant bacteria, hydrolyze extended-spectrum cephalosporins with an oxyimino side chain as well as the oxyimino-monobactam. The extended spectrum beta-lactamase producing bacteria are posing a huge threat to the world since its initial emergence in Germany in 1983. Patients admitted to hospitals are being widely affected by such bacteria, which includes ESBL producing *Klebsiella pneumoniae* and *Escherichia coli* (Knothe *et al*., 1983). Due to alarming elevation of their numbers, it is of utmost important that these bacteria are detected and researched on as much as possible in order to understand their mechanism of pathogenesis and tackled with proper elimination methods, such as antibiotics. ESBL producing bacteria are multidrug resistant. Discovering methods to eliminate them is of a deep scientific concern (Shaikh et al., 2014). Gene detection method is a modern approach in researching about these bacteria. ESBL are encoded by genes that can be exchanged between one bacterium to the other. The increased pathogenicity of bacteria carrying these genes enhances the mortality rate and health problems in the community.

Antibiotic-induced antimicrobial resistance can be acquired through random mutation, horizontal gene transfer and amplification by selective pressure. Administration of antibiotics may inhibit the target causative agent along with their perturbing effect to other competitive flora of the host body. Or else, the indiscriminate use of antibiotics might also be the reason of physiological or other changes of normal flora. This certain modification has impacts on the adaptability of the bacteria which leads to high prevalence of antimicrobial resistance. Acquisition of extended spectrum beta-lactamase encoding genes by opportunistic sensitive bacteria such as *Enterobacter agglomerans* from other resistant bacteria species by conjugation process can be a threat to public health. *Enterobacter agglomerans*, gram-negative bacilli of the Enterobacteriaceae family, is occasionally reported to cause opportunistic infection in immuno-compromised patients causing wound, blood, and urinary-tract infections. Antimicrobial Susceptibility Test through

disk-diffusion method revealed an *Enterobacter agglomerans* strain exhibiting resistance to 13 antibiotics which included both first and second line of antibiotics. According to the hypothesis of Goren et al (2003), horizontal gene transfer is facilitating antimicrobial resistance through transformation, mostly by antibiotic induced cell wall deficient bacteria. Beta-Lactam and glycopeptide antibiotics damage bacteria by inhibiting cell wall murein synthesis. During the process, cell-wall-deficient forms are generated before the bacteria die. These cell-wall-deficient forms have an increased ability to uptake DNA by transformation. As few as a single antibiotic resistant gene is taken up by the cell wall deficient form; it will develop into a resistant clone, despite most of the other bacteria being killed by the antibiotic. Since bacteria occupying the same ecological niche, such as the lower gastrointestinal tract is common, bacteria are often incubated with foreign DNA encoding resistance coming from the administration of antibiotics or other bacteria that have undergone lysis unrelated to antibiotic-induced killing.

Presence of multidrug resistance in this opportunistic bacterium makes it target for further analysis. As among the 13 antibiotics 7 contain beta lactam ring, combination disk test was executed for phenotypic detection of ESBL producers. Test results revealed presence of ESBL gene as the bacteria showed resistant against cefpodaxime but was sensitive to combination of cefpodaxime and clavulanic acid. However, detection in the molecular level is always preferable over phenotypical detection methods. This is because the types of ESBL cannot be distinguished using phenotypic methods which are essential since helpful information about epidemiology can be obtained from their determination. In addition, detection of ESBL producing bacteria using routine susceptibility tests in clinical laboratories sometimes produce false positive results by indicating ESBL bacteria to be susceptible to third generation cephalosporins, thus failing to identify them properly (Fouzia and Damle., 2015). Identification of various ESBL genes by gene detection methods provides better understanding of the ESBL production. Another study revealed that out of 246 samples tested, 58.1% were detected as ESBL positive by genotypic methods which reflects its greater sensitivity when compared to phenotypic methods (44.3%) (Yazdi et al, 2012). So, finally molecular approach was adopted to confirm the ESBL gene. Polymerase chain reaction and sequencing for ESBL gene revealed presence of CTX-M1 beta-lactamase in multi-drug resistance *Enterobacter agglomerans.* Among the five ESBL genes, the existence of CTX-M1 $ESBL_A$ producer was determined by PCR followed by Sanger sequencing and the sequencing result upon BLAST depicted the homologous bacteria harboring the

resistance gene. The top 16 BLAST results showed the existence of similar sequence in *E. coli*, *Salmonella enterica* and *Klebsiella pneumoniae* strain, all of which are known pathogenic bacteria and antibiotics are very commonly administered against those pathogens. The same ESBL genes found in different inter-species strain isolates, which may indicate a horizontal resistance gene transfer as ESBL are often plasmid encoded. One of the case studies reported the presumable transfer of a multidrug resistance plasmid from *Klebsiella pneumoniae* to *E. coli* in the gastrointestinal tract of a patient. Before treatment with carbapenem group of antibiotics, no carbapenem-resistant *E. coli* was found from the patient while after treatment with carbapenem, resistant strain was isolated from the same individual. This resistant *E. coli* had the *Klebsiella pneumoniae* plasmid, *bla*KPC-3 which conferred resistance to cephalosporins, monobactams, and carbapenems. The KPC-3 plasmid was identical in isolates of both species. The patient's gut flora contained a carbapenem-susceptible *E. coli* strain isogenic with the KPC-3–producing isolate, which suggests horizontal interspecies plasmid transfer (Goren et al., 2010).

CTX-M β-lactamases have emerged as the most prevalent ESBL enzyme in humans. The $bla_{\text{CTX-M}}$ gene encodes a 291 amino acid enzyme. A single amino acid change in $bla_{\text{CTX-M}}$ constitutes a new CTX-M type. The active sites of these enzymes are not enlarged to accommodate the drug, instead point substitutions lead to specific interactions which may be responsible for the enhanced hydrolytic activity against the antibiotic compounds (Naseer et al., 2011). CTX-M1 cluster is believed to have originated from the chromosomal *bla* genes of *Kluyvera* species following mobilization into plasmids. Some CTX-M producers have been found to coproduce VIM-1, NDM-1 or OXA-48 Carbapenemase. The resistant bacteria tend to become even more resistant by acquiring Multidrug resistance phenotypes which is termed as genetic capitalism of resistant bacteria (Canton et al., 2012).

Due to continuous increase in resistance of ESBL producing bacteria to different drugs, it is in demand that new drugs are formulated and invented instantly which is potent enough to work against these bacteria. This is quite a challenge but the method of ESBL gene detection and sequencing might provide a way in which such antibiotics can be designed. Detection of the changes and mutations of the genes of ESBL bacteria may provide a better insight of how the drug can be designed which will not be affected by mechanism ESBL enzyme uses. Bacterial pathogenesis and virulence factors can be understood in depth by genome analysis. Antibiotics

should be designed that have novel mechanisms of action. Antimicrobial drugs used presently target only a small portion of the bacterial genome. In order to make a drug work successfully, a good drug target is essential and they should be specific only to certain bacteria by being notably dissimilar to the orthologous human genes. In addition, the key target of the antibiotics should be present in the pathogenic bacteria (Donkor, 2013). All of these aspects can be developed only when there is proper information about the genetic makeup of the bacteria.

Gene detection techniques can provide a major breakthrough for screening, tracking and spread of ESBL. The technique has already greatly advanced our understanding about the mechanisms of pathogenesis and increased antibiotic resistance of ESBL producing bacteria. New drug targets have also been identified via gene sequencing which has helped to design different antibiotics against these bacteria. However, further research and in depth analysis is still required in order to fully understand and tackle such bacteria.

In summary, CTXM-1 gene in *Enterobacter agglomerans* rendered it resistant to multiple antibiotics. This is an alarming issue as sensitive opportunistic microorganisms are turning into multiple drug resistant strains. It is high time to take measure to prevent spread of antibiotic resistant genes from resistant to sensitive bacteria by increasing awareness about health and hygiene and also by imposing restriction to random use of antibiotics.

There is little doubt that emerging antibiotic resistance is a serious global problem. Arising multidrug resistance will hinder the therapeutic options; hence monitoring resistance is of paramount importance. Appropriate antimicrobial drug use has unquestionable benefit but physicians and the public in particular of developing countries like Bangladesh frequently use these agents inappropriately. The indiscriminate use and misuse of antibiotics should therefore be discouraged. The future usefulness of the drugs will however depend on the proper selection and halting spread of resistance among enteric organisms.

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Appendices:

Appendix-I

Media composition

All the media was autoclaved at 121°C for 15 minutes. The composition of the media used in the present study has been given below:

MacConkey Agar (Difco™):

Ingredients	Amount (g/L)
Peptone	17.0
Proteose Peptone	3.0
Lactose	10.0
Bile Salts No. 3	1.5
Sodium Chloride	5.0
Agar	13.5
Neutral Red	0.03
Crystal Violet	0.001

Luria-Bertani Broth:

Ingredients	Amount (g/L)
Peptone (Himedia,India)	5.0
Yeast Extract (Himedia,India)	2.5
NaCl (Sigma, Germany)	5.0

Mueller Hinton Agar (Oxoid, England):

Ingredients	Amount (g/L)
Beef, dehydrated infusion form	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar (Himedia, India)	15.0

Appendix-II: Buffers and Reagents

Tris Boric Acid EDTA (TBE) Buffer (500 ml):

To 500 ml distilled water, 5.4 g Tris HCL powder, 2.75 g boric acid and 0.5M EDTA of 2 mlwere dissolved. The pH of the buffer was adjusted to 8, autoclaved and stored at room temperature.

Phosphate Buffer Saline (PBS):

The composition along with amount is mentioned below:

1X PBS; 10 mMPhosphate (pH 7.2-7.4)

Reagents	For 100 ml
Sodium chloride(NaCl) (0.136M)	0.80
Di-sodium hydrogen Orthophosphate dodecahydrate $Na_2HPO_4.12H_2O$	0.137
Potassium phosphate monobasic (KH_2PO_4) (2mM)	0.0275
Potassium Chloride (KCl) (2.68mM)	0.02

Appendix-III
Instruments:

The equipment used throughout the study are listed below:

Instruments	Manufacturer
Autoclave	WiseClave
Refrigerator	Electra, Samsung (+4°C)- to store bacteria; Vestfrost (+4°C)- to store bacterial medium
Freeze	Vestfrost (-20°C) to store stock antibiotics; ESCO (-80°C) to store stock bacteria.
Incubator	Memmert
Shaking Incubator	WiseCube
Oven	WiseVen
Water bath	WiseBath
Micropipette	(2-20µl)- Gilson and Costar® (20-200µl)- Gilson and Costar® (200-1000µl)- Gilson
Bio-Safety Cabinet	ESCO Class-II Type-A2 Labculture® Biological Safety Cabinet
Vortex Mixture Machine	WiseMix
Weighing Machine	OHAUS®
Weighing Paper	Fisherbrand®
Spectrophotometer	Eon™ BioTek®
96-Well Plate	Nunc™ 96F Microwell Plate
Centrifuge Machine	Thermo SCIENTIFIC
Light Microscope	OLYMPUS CX41
T 100 ™ thermal cycler	Infinigen
Gel documentation machine	Bio-Rad
Take 3 plate	Bio-Tek
Antibiotic disks	Oxoid