Isolation and Characterization of *Escherichia coli* from Raw Vegetables

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

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Declaration

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled "***Isolation and Characterization of Escherichia coli from Raw Vegetables***" submitted by the undersigned, has been carried out under joint supervision of Professor Naiyyum Choudhury, Coordinator of Biotechnology Program, Department of Mathematics and Natural Science, BRAC University and Professor Chowdhury Rafiqul Ahsan, Department of Microbiology, University of Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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Contents

List of Tables

List of Figures

List of Abbreviations

MW	:	Molecular weight
KDa	:	Kilodalton
mg	:	Milligram
gm	:	Gram
kg	:	Kilogram
L	:	Liter
ml	:	Milliliter
µl	:	Microliter
mM	:	Millimolar
M	:	Molar
pmol	:	Picomole
mm	:	Millimeter
µm	:	Micrometer
nm	:	Nanometer
mA	:	Milliampere
V	:	Volt
PBS	:	Phosphate buffer solution
e.g.	:	For example
et al.	:	And others
pH	:	Negative logarithm of hydrogen ion concentration
BHK 21	:	Baby Hamster Kidney cell line
bp	:	Base pair
rpm	:	Rotation per minute
UV	:	Ultra violet
spp.	:	Species

Abstract

Escherichia coli is a genetically heterogeneous group of bacteria whose members are typically nonpathogens that are a part of the normal microflora of the intestinal tract of humans and animal. However, some strains have acquired virulence factors that enable them to cause important intestinal and extra intestinal diseases such as diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), urinary tract infections (UTI), septicemia and neonatal meningitis. *E. coli* has been reported to be responsible for the outbreaks linked to consumption of fresh but raw vegetables and fruits (such as lettuce, spinach, carrots, radish sprouts, alfalfa, unpasteurized apple cider, melon and berries etc.). These vegetables get contaminated with pathogenic *E. coli* while growing in the field or on application of fertilizer (cow dung) or during harvesting, transport, processing, storage and distribution. In Bangladesh, neither sporadic illness nor outbreak associated with *E. coli* from fresh raw vegetables has been reported so far. For this purpose raw vegetables were collected around Dhaka city to isolate *E. coli*. The samples were first enriched in enrichment broth supplemented with bile salt and then plated onto MacConkey agar. A total of 65 isolates from 60 raw vegetable samples were presumptively selected as *E. coli* from primary MacConkey plate. The isolates were subjected to detailed biochemical characterizations using eosin methylene blue (EMB) agar medium, indole production test, methyl-red test, Voges-Proskauer test, citrate utilization test, triple sugar iron test and fermentation test. Out of 60 samples analyzed, only 30 isolates, 11 from Lettuce, 6 from chopped vegetables, 5 from Capsicum, 4 from sprout, 3 from Broccoli and 1 from street salad gave identical biochemical properties compared to a reference *E. coli* strain. Culturally and biochemically positive isolates were tested for *stx*1 and *stx*2 genes. From all these isolates, only *stx*1 gene was detected from eight isolates. Live cells of all *stx*1 gene positive isolates were found to show strong enterotoxic activity in the rabbit ileal loop assay. However, culture filtrates prepared from these isolates gave mild cytotoxic and neurotoxic activity. Alpha hemolysin activity was recorded from these isolates. Antibiotic sensitivity test was performed. All the isolates were found to be sensitive to cefoxitin, chloramphenical, gentamycin, naladixic acid, nitrofurantoin, norfloxacin and sulphamethoxazole/trimethoprim. To our knowledge, this is the first possible report of isolation of Shiga toxin producing *E. coli* from raw vegetables and its findings emphasize the need for vigorous washing with safe running water before consumption to prevent human infection.

Chapter 1:

Introduction and Literature Review…

1. Introduction and Literature Review

1.1 Background

The genus *Escherichia coli* is named after Theodor Escherich who isolated the type species of the genus. It is a gram-negative bacillus occurring singly or in pairs. *Escherichia coli* is facultatively anaerobic with both a fermentative and respiratory type of metabolism. It is either nonmotile or motile by peritrichous flagella. *E. coli* is a major facultative inhabitant of the large intestine and ubiquitous in the human environment. It is one of the most frequent causes of the many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI) and 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, 1999).

1.2 Literature Review

1.2.1 *Escherichia coli*

Escherichia coli is a common bacterium that has been studied intensively by geneticists because of its small genome size, normal lack of pathogenicity and ease of growth in the laboratory.

The bacterium *Escherichia coli (E. coli)*, originally known as Bacterium coli commune, belongs to the Family *Enterobacteriaceae* and was first isolated and characterized in 1885 by the German scientist and pediatrician Theodore 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. *E. coli* is the major component of normal healthy stool. Some species of *E. coli* co-exist with us nicely; some species cause serious disease. Most of the *E. coli* causes no trouble to humans. Problems arise either if *E. coli* gets somewhere they shouldn’t be (such as the urinary tract) or if people are infected by one of the dangerous strains, such as *E. coli* O157:H7. *E. coli* is the major cause of diarrhea (some mild and some severe) (Bower, 1999). It is also a common friendly bacterial

inhabitant of the intestinal tract, aiding in the digestion of food. In scientific research, *E. coli* is a standard vector for rapid mass production of biological building blocks, from DNA to protein. Rare would be a biological researcher who has not used *E. coli*. The strain found in healthy intestines and used in the laboratory is different from the dangerous strain being reported in the news. *E. coli* 0157:H7 can be lethal. Its extra genes enable it to stick to the walls of the intestine and produce the virulent Shiga toxin, which injures cells in the intestinal walls as well as blood vessels in the intestine, leading to bleeding. In the bloodstream, these toxins damage other blood vessels, particularly in the kidneys, leading to renal failure.

1.2.2 Biochemistry of *E. coli*

E. coli, is a prokaryotic organism without a nuclear membrane, is a representative living material often used in laboratories and class rooms. *E. coli* reproduces rapidly (under optimal situation 0.5 hr/generation) such that results for a number of experiments can be quickly obtained. Certain mutants of *E. coli* have been defined that cannot express certain proteins at saturation growth and therefore die. *E. coli* was also the organism used to elucidate the regulation of the *lac* operon in genetics. Its ability to take up exogenous genetic material under the procedure known as DNA-mediated cell transformation has also made it a popular model for studies using recombinant DNA. Using recombinant DNA techniques, *E. coli* can be manipulated in research laboratories and in the classroom to produce any DNA, RNA or protein of interest. Also, it is easy to manipulate *E. coli* both genetically and biochemically. Most importantly, it shares fundamental characteristics, such as DNA and messenger RNA; with all other organisms. The value of *E. coli* in recombinant DNA makes it a good model organism to study the genetic material. An *E. coli* genome contains between 4200 and 5500 genes, with <2000 genes conserved among all strains of the species (the core genome). The bacterium's pan-genome (genetic repertoire of a given species) consists of almost 20,000 genes (Mora *et al*., 2011). Continuous gene flux occurs during *E. coli* divergence, mainly as a result of horizontal gene transfers and deletions. This genetic plasticity accelerates the adaptation of *E. coli* to varied environments and lifestyles, as it allows multiple gene combinations that result in phenotypic diversification and the emergence of new hypervirulent (STEC and EAECO104:H4-B1-ST678) and successful (ExPECO25b:H4-B2-ST131) strains that combine

both resistance and virulence genes, which in classical pathogenic *E. coli* strains traditionally have been mutually exclusive (Mora *et al*., 2011).

1.2.3 Types of *E. coli*

Certain isolates of *Escherichia coli* have been implicated in a wide range of diseases that affect either animals or humans worldwide. To date, eight pathovars and their mechanisms of disease have been extensively studied. These pathovars can be broadly classified as either diarrhoeagenic *E. coli* or extraintestinal *E. coli* (ExPEC). Six pathovars-enteropathogenic *E. coli* (EPEC), entero-haemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC; including *Shigella*), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) are diarrhoeagenic and two pathovars-uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) are the most common ExPEC isolates (Figure1.1). Other pathovars have been identified, but their mechanisms of pathogenesis are not as well defined (Croxen and Finlay, 2010).

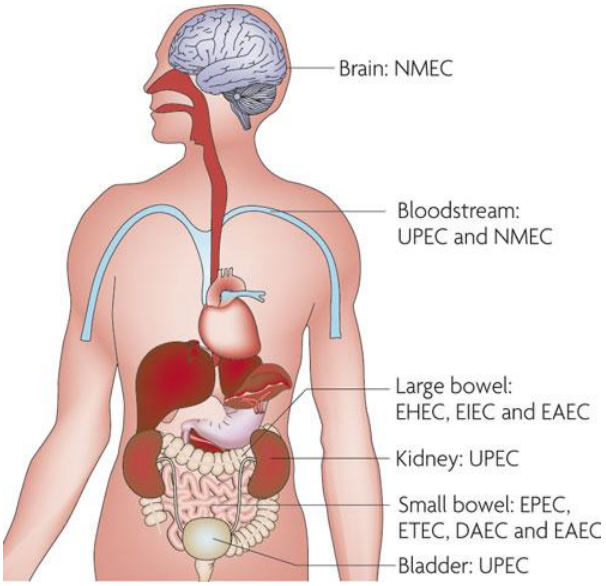


Figure1.1: Sites of pathogenic *E. coli* colonization

(Courtesy: Croxen and Finlay, 2010)

Enteropathogenic *E. coli* (EPEC)

EPEC is a major cause of potentially fatal diarrhea in infants in developing countries (Kaper *et al*., 2004). They do not possess any colonization factors and do not produce ST or LT toxins. They produce a non fimbrial adhesin designated intimin, an outer membrane protein that mediates the final stages of adherence. Although they do not produce LT or ST toxins, there are reports that they produce an enterotoxin similar to that of *Shigella*. Other virulence factors may be related to those in *Shigella* (Wellington and vanElsas, 1992). Adherence of EPEC strains to the intestinal mucosa is a very complicated process and produces dramatic effects in the ultra structure of the cells resulting in rearrangements of actin in the vicinity of adherent bacteria. The phenomenon is sometimes called "attaching and effacing" of cells. EPEC strains are said to be "moderately-invasive" meaning they are not as invasive as *Shigella*, and unlike ETEC or EAEC, they cause an inflammatory response. The diarrhea and other symptoms of EPEC infections probably are caused by bacterial invasion of host cells and interference with normal cellular signal transduction, rather than by production of toxins. They are an important cause of traveler's diarrhea in Mexico and in North Africa (Uttly *et al*., 1998).

Enterohemorrhagic *E. coli* (EHEC)

Cattle are a key reservoir for EHEC, which is a highly infectious A/E (human attaching and effacing) pathogen that colonizes the distal ileum and large bowel in humans and is often the causative agent of outbreaks of severe gastroenteritis in developed countries. Transmission to humans usually occurs through contaminated food and water. In North America, Japan and parts of Europe, most outbreaks are due to EHEC serotype O157:H7, whereas other serotypes are important health concerns in other developed countries. Adults and children infected with EHEC suffer from haemorrhagic colitis (bloody diarrhea) and further complications can lead to the potentially fatal haemolytic uraemic syndrome (HUS) (Kaper *et al*., 2004). EHEC are also considered to be "moderately invasive". Nothing is known about the colonization antigens of EHEC but fimbriae are presumed to be involved. The bacteria do not invade mucosal cells as readily as *Shigella* but EHEC strains produce a toxin that is virtually identical to the Shiga toxin. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of EHEC strains to cause HUS. The toxin is phage encoded and its production is enhanced by iron deficiency (Griffin, 1995; Samuel *et al.*, 1988)

Enterotoxigenic *E. coli* (ETEC)

ETEC is an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. The disease varies from minor discomfort to a severe cholera-like syndrome. ETEC is acquired by ingestion of contaminated food and water and adults in endemic areas evidently develop immunity. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid-encoded. 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. Enterotoxins produced by ETEC include the LT (heat-labile) toxin and/or the ST (heat-stable) toxin, the genes for which may occur on the same or separate plasmids. The LT enterotoxin is very similar to cholera toxin in both structure and mode of action. It is an 86 kDa protein composed of an enzymatically active (A) subunit surrounded by 5 identical binding (B) subunits. It binds to the same identical ganglioside receptors that are recognized by the cholera toxin (i.e., GM1) and its enzymatic activity is identical to that of the cholera toxin (Wellington and van Elsas, 1992).

Enteroinvasive *E. coli* (EIEC)

EIEC closely resembles *Shigella* in its pathogenic mechanisms and the kind of clinical illness they produce. EIEC penetrates and multiplies within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes a dysentery-like diarrhea with fever. EIEC apparently lacks fimbrial adhesions but does possess a specific adhesion that as in *Shigella* and is thought to be an outer membrane protein. Also, like *Shigella*, EIEC strains are invasive organisms. They do not produce LT or ST toxin and unlike *Shigella*, they do not produce the *Shigella* toxin (Wellington and van Elsas, 1992).

Enteroaggregative *E. coli* (EAEC)

The distinguishing feature of EAEC strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhea without invading or causing inflammation. This suggests that the organisms

produce a toxin of some sort. Recently, a distinctive heat labile plasmid encoded toxin has been isolated from these strains, called the EAST (EnteroAggregative ST) toxin. They also produce a hemolysin related to the hemolysin produced by *E. coli* strains involved in urinary tract infections. The role of the toxin and the hemolysin in virulence has not been proven. The significance of EAEC strains in human disease is controversial (Wellington and van Elsas, 1992).

Diffusely adherent *E. coli* (DAEC)

DAEC is a heterogenous group that generates a diffuse adherence pattern on HeLa and HEp-2 cells. This pattern is mediated by proteins encoded by a family of related operons, which includes both fimbrial (for example, Dr and F1845) and afimbrial (Afa) adhesins, collectively designated Afa–Dr adhesins (Servin, 2005). DAEC isolates that express any of the Afa–Dr adhesins (which are referred to as Afa–Dr DAEC) colonize the small bowel and have been implicated in diarrhea in children between the ages of 18 months and 5 years, as well as in recurring urinary tract infections (UTIs) in adults (Servin, 2005).

Uropathogenic *E. coli* (UPEC)

UPEC infections account for roughly 80% of all UTIs, causing cystitis in the bladder and acute pyelonephritis in the kidneys. UPEC has the challenge of moving from the intestinal tract to establish an infection in the urinary tract, where it uses peptides and amino acids as the primary carbon source for fitness (Alteri *et al*., 2009). The ability to ascend the urinary tract from the urethra to the bladder and kidneys reflects exceptional mechanisms for organ tropism, evading innate immunity and avoiding clearance by micturition. Several highly regulated virulence factors contribute to this complex pathogenesis, including multiple pili, secreted toxins (for example Sat and vacuolating autotransporter toxin (Vat)), multiple iron acquisition systems and a polysaccharide capsule (Wiles *et al*., 2008).

Neonatal meningitis *E. coli* (NMEC)

NMEC, a common inhabitant of the gastrointestinal tract, is the most frequent cause of Gram-negative associated meningitis in newborns. Fatality rates can approach 40 % (Kaper *et al*., 2004) and survivors are usually burdened with severe neurological sequelae. The pathogenesis of

NMEC is complex, as the bacteria must enter the bloodstream through the intestine and ultimately cross the blood–brain barrier into the central nervous system, which leads to meningeal inflammation and pleocytosis of the cerebrospinal fluid. Recently, a lambdoid phage that encodes O acetyltransferase was discovered, which acetylates the O antigen to provide phase variation and diversity to the capsule (Deszo *et al*., 2005) and may therefore hide the bacteria from host defenses.

1.2.4 Virulence Factors

Adhesion/colonization

Pathogenic *E. coli* strains possess specific adherence factors that allow them to colonize sites that *E. coli* does not normally inhabit, such as the small intestine and the urethra (Table1.1). Most frequently these adhesins form distinct morphological structures called fimbriae (also called pili) or fibrillae, which can belong to one of several different classes. Fimbriae are rod-like structures of 5–10 nm diameters that are distinct from flagella. Fibrillae are 2–4 nm in diameter and are either long and wiry or curly and flexible (Cassels and Wolf, 1995). The Afa adhesins that are produced by many diarrhoeagenic and uropathogenic *E. coli* are described as afimbrial adhesins, but in fact seem to have a fine fibrillar structure that is difficult to visualize (Keller *et al*., 2002). Adhesins of pathogenic *E. coli* can also include outer-membrane proteins, such as intimin of UPEC and EHEC, or other non-fimbrial proteins. Some surface structures trigger signal transduction pathways or cytoskeletal rearrangements that can lead to disease. For example, the members of the Dr family of adhesins that are expressed by DAEC and UPEC bind to the Decay-Accelerating Factor (DAF also known as CD55), which results in activation of phosphatidylinositol 3-kinase (PI-3-kinase) and cell-surface expression of the major histocompatibility complex (MHC) class I-related molecule MICA (Tieng *et al*., 2002). The IcsA protein of EIEC nucleates actin filaments at one pole of the bacterium, which allows it to move within the cytoplasm and into adjacent epithelial cells on a ‘tail’ of polymerized actin (Golderg *et al*., 1995). Even surface structures that are present on commensal *E. coli* strains can induce signaling cascades if the organism encounters the appropriate receptor. The LPS of *E. coli* and other Gram-negative bacteria binds to Toll-like receptor 4 (TLR4), triggering a potent cytokine cascade that can lead to septic shock and death (Tapping *et al*., 2000). Flagellin, the main

component of flagella, can bind to TLR5, thereby activating interleukin (IL)-8 expressions and an inflammatory response (Hayashi *et al*., 2001).

Table 1.1: *E. coli* virulence factors: colonization and fitness factors

Factor	Pathotype	Activity/effect
IcsA (VirG)	EIEC	Nucleation of actin filaments
Intimin	EPEC, EHEC	Adhesin, induces TH1 response; 10 variants described
Dr adhesions	DAEC, UPEC	Adhesin, binds to decay-accelerating factor (DAF), activates PI-3-kinase, induces MICA; >10 Dr adhesins described
P (Pap) fimbriae	UPEC	Adhesin; induces cytokine expression
CFAs	ETEC	Adhesin, >20 different factors designated CFA, CS or PCF
Type-1 fimbriae	All	UPEC adhesin; binds to uroplakin
F1C fimbriae	S fimbriae	Adhesin
S fimbriae	UPEC, MNEC	Adhesin
Bundle-forming pilus (BFP)	EPEC	Type IV pilus
Aggregative adherence fimbriae	EAEC	Adhesin; >4 subtypes
Paa	EPEC, EHEC	Adhesion
ToxB	EHEC	Adhesin
Efa-1/LifA	EHEC	Adhesin
Long polar fimbriae (LPF)	EHEC, EHEC	Adhesin
Saa	EHEC	Adhesin
OmpA	MNEC, EHEC	Adhesin
Curli	Various	Adhesin; binds to fibronectin
IbeA, B, C	MNEC	Promotes invasion
AslA	MNEC	Promotes invasion
Dispersin	EAEC	Promotes colonization; aids mucous penetration
K antigen capsules	MNEC	Antiphagocytic; >80 K types
Aerobactin	EIEC	Iron acquisition, siderophore
Yersiniabactin	Various	Iron acquisition, siderophore
IreA	UPEC	Iron acquisition, siderophore receptor
IroN	UPEC	Iron acquisition, siderophore receptor
Chu (Shu)	EIEC,UPEC, MNEC	Iron acquisition, haem transport
Flagellin	All	Motility; induces cytokine expression through TLR5; >50 flagella (H) serotypes
Lipopolysaccharide	All	Induces cytokine expression through TLR4; >180 O types

Toxins

More numerous than surface structures that trigger signal transduction pathways are secreted toxins and other effector proteins that affect an astonishing variety of fundamental eukaryotic

processes (Table 1.2). Concentrations of important intracellular messengers, such as cyclic AMP, cyclic GMP and Ca2+, can be increased, which leads to ion secretion by the actions of the heat-labile enterotoxin (LT), heat-stable enterotoxin a (STa) and heat-stable enterotoxin b (STb), respectively, all of which are produced by different strains of ETEC (Sears & Kaper,1996). The Shiga toxin (Stx) of EHEC cleaves ribosomal RNA, thereby disrupting protein synthesis and killing the intoxicated epithelial or endothelial cells (Melton-Celsa and O'Brien, 998). The cytolethal distending toxin (CDT) has DNaseI activity that ultimately blocks cell division in the G2/M phase of the cell cycle (De Rycke and Oswald, 2001). Another toxin that blocks cell division in the same phase, called Cif (cycle-inhibiting factor), does not possess DNaseI activity, but might act by inhibition of Cdk1 kinase activity (Marches *et al*., 2003). The cytotoxic nectrotizing factors (CNF 1 and CNF 2) deaminate a crucial glutamine residue of RhoA, Cdc42 and Rac, thereby locking these important signaling molecules in the 'on' position and leading to marked cytoskeletal alterations, multi nucleation with cellular enlargement, and necrosis (Lerm*etal*.,1999). The Map protein of EPEC and EHEC has at least two independent activities stimulating Cdc42-dependent filopodia formation and targeting mitochondria to disrupt membrane potential in these organelles (Kenny *et al*., 2002). The various toxins are transported from the bacterial cytoplasm to the host cells by several mechanisms. LT is a classic A–B subunit toxin that is secreted to the extracellular milieu by a type II secretion system (Tauschek *et al*., 2002). Several toxins, such as Sat, Pet and EspC are called autotransporters because part of these proteins forms a β-barrel pore in the outer membrane that allows the other part of the protein extracellular access (Henderson *et al*., 1998). The SPATEs (serine protease auto transporters of enterobacteriaceae) are a subfamily of serine protease autotransporters that are produced by diarrhoeagenic and uropathogenic *E. coli* and *Shigella* strains. EPEC, EHEC and EIEC contain type III secretion systems, which are complex structures of more than 20 proteins forming a 'needle and syringe' apparatus that allows effector proteins, such as Tir and IpaB to be injected directly into the host cell (Hueck *et al*., 1998). The UPEC haemolysin is the prototype of the type I secretion mechanism that uses TolC for export from the cell (Balakrishnan *et al*., 2001). No type IV secretion systems have been described for pathogenic *E. coli*, with the exception of the type IV-like systems that are involved in conjugal transfer of some plasmids.

Table 1.2: *E. coli* virulence factors: toxins and effectors

Factor	Pathotype	Toxin class	Target	Activity/Effect
Heat-labile enterotoxin (LT)	ETEC	AB subunit, type II	Gs	ADP ribosylates and activates adenylate cyclase resulting in ion secretion
Shiga toxin (Stx)	EHEC	AB subunit	Rrna	Depurinates rRNA, inhibiting protein synthesis; induces apoptosis
Cytolethal distending toxin (CDT)	Various	ABC subunit	DNA	DNaseI activity, blocks mitosis in G2/M phase
Shigella enterotoxin 1 (ShET1)	EAEC, EIEC	AB subunit	-	Ion secretion
Urease	EHEC	ABC subunit	Urea	Cleaves urea to NH3 and CO2
EscP	EPEC	Autotransporter	-	Serine protease; ion secretion
EspP	EHEC	Autotransporter	-	Serine protease; cleaves coagulation factor
Haemoglobin-binding protease (Tsh)	ExPEC, APEC	Autotransporter	Haem	Degrades haemoglobin to release haem/iron
Pet	EAEC	Autotransporter	Spectrin	Serine protease; ion secretion; cytotoxicity
Pic	UPEC, EIEC, EAEC	Autotransporter	-	Protease, mucinase
Sat	UPEC	Autotransporter	-	Vacuolation
SepA	EIEC	Autotransporter	-	Serine protease
SigA	EIEC	Autotransporter	-	Ion secretion
Cycle-inhibiting factor (Cif)	EPEC, EHEC	Type III effector	-	Blocks mitosis in G2/M phase; results in inactivation of Cdk1
EspF	EPEC, EHEC	Type III effector	-	Opens tight junctions, induces apoptosis
EspH	EPEC, EHEC	Type III effector	-	Modulates filopodia and pedestal formation
Map	EPEC, EHEC	Type III effector	Mitochondria	Disrupts mitochondrial membrane potential
Tir	EPEC, EHEC	Type III effector	Nck	Nucleation of cytoskeletal proteins, loss of microvilli, GAP-like activity
IpaA	EIEC	Type III effector	Vinculin	Actin depolymerization
IpaB	EIEC	Type III effector	Caspase 1	Apoptosis, IL-1 release; membrane insertion
IpaC	EIEC	Type III effector	Actin	Actin polymerization, activation of Cdc42 and Rac
IpaH	EIEC	Type III effector	Nucleus	Modulates inflammation
IpgD	EIEC	Type III effector	Ptdlns (4,5)P2	Inositol 4-phosphatase, membrane blebbing
VirA	EIEC	Type III effector	Tubulin	Microtubule destabilization, membrane ruffling
StcE	EHEC	Type II effector	C1-esterase inhibitor (C1-INH)	Cleaves C1-INH, disrupts complement cascade
HlyA	UPEC	RTX toxins	Erythrocytes, Leukocytes	Cell lysis
Ehx	EHEC	RTX toxins	Erythrocytes, Leukocytes	Cell lysis
Cytotoxicnecrotizing factors (CNF-1,-2)	MNEC, UPEC, NTEC		RhoA, Cdc4, Rac	Altered cytoskeleton, necrosis
Lifa/Efa	EPEC, EHEC		Lymphocytes	Inhibits lymphocyte activation, adhesion
Shigella enterotoxin 2(ShET2)	EPEC, EHEC		-	Ion secretion
Heat-stable enterotoxin a (STa)	ETEC	Heat-stable enterotoxins	Guanylate cyclase	Activates guanylate cyclase resulting in ion secretion
Heat-stable enterotoxin b (STb)	ETEC	Heat-stable enterotoxins	-	Increase intracellular calcium resulting in ion secretion
EAST	Various	Heat-stable enterotoxins	Guanylate cyclase	Activates guanylate cyclase resulting in ion secretion

The versatility of the *E. coli* genome is conferred mainly by two genetic configurations: virulence related plasmids and chromosomal pathogenicity islands. All six categories of diarrheagenic *E. coli* have been shown to carry at least one virulence related property upon a plasmid. EIEC, EHEC, EAEC and EPEC strains typically harbor highly conserved plasmid families, each encoding multiple virulence factors (Hales *et al*., 1992; Nataro *et al*., 1987; Wood *et al*., 1986). McDaniel and Kaper have shown recently that the chromosomal virulence genes of EPEC and EHEC are organized as a cluster referred to as a pathogenicity island (McDaniel *et al*., 1995; McDaniel and Kaper, 1997). Such islands have been described for uropathogenic *E. coli* strains (Donnenberg and Wech, 1996) and systemic *E. coli* strains (Bloch and Rode**,** 1996) as well and may represent a common way in which the genomes of pathogenic and nonpathogenic *E. coli* strains diverge genetically. Plasmids and pathogenicity islands carry clusters of virulence traits, yet individual traits may be transposon encoded (such as ST) (So and McCarthy, 1980) or phage encoded (such as Shiga toxin) (O'Brien *et al*., 1992).

1.2.5 Serotyping and Phylogeny

E. coli can be serotyped based on their O (somatic), H (flagellar) and K (capsular) surface antigen according to the modified Kauffman scheme. Till now a total of 170 different 'O' antigen which is defined as serogroup has been recognized. The O antigens are quiet stable to heat (can withstand heat), whereas K and H antigens are heat labile. Because the flagellar protein are less heterogeneous than the carbohydrate side chains that make up the O groups, considerably fewer H antigenic type exist (around 30). *E. coli* of specific serogroup can associated with certain clinical syndrome but it is not in general the serologic antigens that determine virulence. The serotypes and serogroups serve as readily identifiable chromosomal marker that correlate with specific virulent clone (Nataro and Kaper, 1998).

Phylogenetic analysis has shown that *E. coli* is composed of four main phylogenetic groups (A, B1, B2 and D). ExPEC mainly belong to phylogroups B2 and D and AIEC to phylogroup B2; diarrheagenic *E. coli* normally belongs to phylogroups A, B1 and D and the non-pathogenic commensal strains to phylogroups A and B1 (Mora *et al*., 2011).

1.2.6 Detection of *E. coli*

1.2.6.1 Culture-Based Methods

1.2.6.1.1 Tryptic Soy Broth

Tryptic Soy Broth is used for the cultivation of a wide variety of microorganisms. Enzymatic Digest of Casein and Enzymatic Digest of Soybean Meal are nitrogen sources in TSB. Dextrose is the carbon energy source that facilitates organism growth. Sodium Chloride maintains osmotic balance; Dipotassium Phosphate is a buffering agent (Cunnif, 1995).

1.2.6.1.2 MacConkey Agar

The agar medium most commonly used for the isolation of *E. coli* is MacConkey agar. It is a selective media which contains lactose as sugar, peptone, sodium chloride, bile salt; inhibits the growth of other gram positive Enterobacteriaceae, crystal violet and neutral red is also used to understand the nature of fermentation.

MacConkey Agar is recommended for the detection and isolation of Gram-negative organisms from clinical, dairy; food, water, pharmaceutical and industrial sources. Enzymatic digest of gelatin, enzymatic digest of casein, and enzymatic digest of animal tissue are the nitrogen and vitamin sources in MacConkey Agar. Lactose is the fermentable carbohydrate. During Lactose fermentation a local pH drop around the colony causes a color change in the pH indicator, Neutral Red and bile precipitation. Bile salts mixture and Crystal Violet are the selective agents, inhibiting Gram-positive cocci and allowing Gram-negative organisms to grow (Holt and Krieg, 1994). Sodium Chloride maintains the osmotic environment. Agar is the solidifying agent.

1.2.6.1.3 Chromogenic Media

Chromogenic culture media provide a rapid and accurate method of isolating and enumerating target microorganisms based on the detection of specific enzyme activities. Not only do chromogenic media enable faster detection of specific microorganisms compared with classical culture media, they also improve sensitivity and can reduce the need for subculture or confirmatory tests. These chromogens are biochemical compounds that produce a visible and qualitative color change when degraded by specific microbial enzymes. For rapid screening of

E. coli MUG (4-methylumbelliferyl-β-D-glucuronide) is used (Thompson *et al*., 1990). Another widely used chromogenic media is X-gal or BCIG (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Frampton et al., 1988).

1.2.6.2 Phenotypic Method

1.2.6.2.1 HEp-2 Adherence Assay

One of the most useful phenotypic assays for the diagnosis of diarrheagenic *E. coli* is the HEp-2 adherence assay. The method has recently been reviewed in detail (Donnenberg and Nataro, 1995). This assay was first described in 1979 by Cravioto *et al.* (Cravioto *et al*., 1979) and remains the "gold standard" for the diagnosis of EAEC and diffusely adherent *E. coli* (DAEC). Although, it has been modified often since its first description, including variations in extending the incubation time to 6 h or changing the growth medium during the incubation, collaborative studies have shown that the assay performed as first described provides the best ability to differentiate among all three adherent diarrheagenic categories (EPEC, EAEC and DAEC) (Vial *et al*., 1990). The HEp-2 adherence assay entails inoculating the test strain onto a semi confluent HEp-2 monolayer and incubating it for 3 h at 37°C under 5% CO2. After this incubation time, the monolayer is washed, fixed, stained and examined by oil immersion light microscopy. The three patterns of HEp-2 adherence, localized adherence (LA), aggregative adherence (AA) and diffuse adherence (DA) can be differentiated reliably by an experienced technician. However, the authors have found some strains which yield equivocal results reproducibly in HEp-2 assay.

1.2.6.3 Immunological Methods

Immunoassays, particularly ELISA, have played a vital role in screening of *E. coli* from food samples and stool specimens. For this, antibodies against particular *E. coli*, outer membrane antigens of that *E. coli* strain and Shiga toxins are often used (Paton and Paton, 1998). Latex agglutination assay for specific *E. coli* antigens have greatly facilitated (Nataro and Kaper, 1998). An immunomagnetic separation system (Dynal, Oslo, Norway) has recently been developed to reduce the time and improve the sensitivity of detection of *E. coli*. Cytotoxic activity of *E. coli* present in stool on Vero cell line is also an extremely sensitive method to

detect the current or recent presence of toxin producing *E. coli*, but this method is time consuming (Khan *et al.*, 2003).

1.2.6.4 DNA-Based Methods

DNA probes and PCR assay are two principal DNA based methods for detecting *E. coli*. DNA based methods have the potential for rapid and sensitive detection of pathogen because they can target unique genetic sequences, such as virulence genes of microorganisms. DNA fragment probes have been used in research studies for nearly 10 years to detect different strains of *E. coli*. Nucleic acid-based probes can be of two types: oligonucleotide or polynucleotide (fragment probes). DNA fragment (polynucleotide) probes may be derived from genes that encode a particular phenotype or may instead be empirical probes which, through extensive testing, are found to be linked with the presence of a phenotype. Although empirical probes have generated useful results (Baudry *et al*.,1990;Wood *et al*.,1986), probes which represent the virulence genes themselves are generally superior (Giro'n *et al*.,1993). Various PCR primers have been developed successfully for several of the categories of diarrheagenic *E. coli*. In multiplex PCR techniques incorporating primers for genes encoding attaching and effacing (*eae*), β-glucuronidase (*uidA*), enterohaemolysin *(ehxA*) and flagellar H7 antigen (*fliC*) to allow specific detection of *E. coli* O157:H7 and other EHEC (Nataro and Kaper, 1998; Khan *et al.*, 2003).

1.2.7 Strain Subtyping

A variety of techniques have been used to differentiate strains of pathogenic *E. coli* for epidemiological studies.

1.2.7.1 Plasmid Profiling

Plasmid profiles have been used to distinguish strains of *E. coli* on the basis of profiles produced when intact plasmids are size separated by gel electrophoresis. Ratnam *et al*. (1988) found three basic plasmid profiles: profile I- characterized by plasmids of 68.7 and 4.3 MDa, profile II-characterized by plasmids of 66.2 and 1.8 MDa and profile III-characterized by a 62.5 MDa plasmid. Plasmid profiles are only useful as epidemiological markers in fresh bacterial isolates that carry a number of different plasmids. Problems can arise in interpretation when plasmids are

lost, gained or transferred, conformational changes of the plasmid or strand nicking. In addition, plasmids of the same molecular weight may not share the same DNA sequence. Thus, plasmid profiling is not a particularly discriminatory subtyping technique.

1.2.7.2 Biotyping and Antimicrobial Susceptibility Testing

Biotyping classifies isolates based on their ability to produce different enzymes, colony morphology and utilization of carbohydrates, amino acid and growth on different media. Antimicrobial susceptibility testing determines the pattern of resistance to selected antibiotics.

1.2.7.3 Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis uses a suitable DNA probe (λ DNA or stx) in southern hybridization of digested DNA. The genomic DNA is cleaved using restriction enzymes such as *Pst*I, *Pvu*II, *EcoR*I or *Hind*III. Probing the restriction fragments with λ DNA is reportedly more discriminatory than probing with rRNA (ribotyping) or stx fragments (Samadpour *et al.*, 1993). Analysis of STEC strains using RFLP is a sensitive and stable method, easy to perform and can reliably identify outbreak strains from sporadic cases. RFLP has been applied to study the molecular epidemiology of a number of food-borne outbreaks of STEC (Khan *et al.*, 2003).

1.2.7.4 Ribotyping

Ribotyping comprises restriction enzyme digestion of the genomic DNA, which is probed using a plasmid containing the *E. coli* rRNA operon (Grimont *et al.*, 1986). This detects DNA polymorphisms in or flanking the genes encoding the ribosomal RNA (Martin *et al.*, 1996). However, success varied with the restriction enzyme used.

1.2.7.5 Pulsed Field Gel Electrophoresis (PFGE)

PFGE has been used by several groups to investigate the molecular epidemiology of *E. coli* infections where macro restriction analysis of the genome is carried out using an infrequent cutting restriction enzyme (*Xba* I) which cuts the genome into 10 to 20 fragments ranging in size from 20 to 700 kb. These larger DNA molecules are separated by agarose DNA electrophoresis. In 1995, the Centres for Disease Control and Prevention (CDC) set up a national electronic

database of PFGE subtypes known as "Pulsenet" to facilitate recognition of outbreaks (Lingwood, 1996).

1.2.7.6 Random Amplification of Polymorphic DNA (RAPD)

RAPD is more efficient and discriminatory than ribotyping and is quicker and less technically demanding than PFGE. Like in PFGE, the dendrograms can be made by RAPD products. Other PCR based subtyping methods such as repetitive DNA element PCR (rep-PCR), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) or amplified fragment length polymorphism (AFLP) are available presently to subtype the EHEC strains (Khan *et al.*, 2003)

1.2.8 Treatment

Treatment of EHEC disease is limited largely to supportive care. Although EHEC strains are generally susceptible to a variety of antibiotics, there are no prospective studies showing conclusively that the use of antibiotics alters the outcome of disease. In a prospective study, Proulx demonstrated a trend toward a lower incidence of HUS in those receiving antibiotics (Proulx *et al.*, 1992). Consistent with this study, a retrospective study conducted during the 1996 outbreak in Japan indicated that early treatment with one specific antibiotic, fosfomycin, was associated with a reduced risk of HUS (Takeda *et al.*,1998). There are, however, retrospective studies which suggest that patients who received antibiotics may be at greater risk of developing HUS (Nataro *et al.*, 1998); since these were not prospective, randomized trials, it could be that the patients who were most severely ill were more likely to receive antibiotics.

The use of antibiotics may be harmful for two potential reasons: first, lysis of bacteria by some antibiotics leads to increased release of toxin, at least in vitro; second, antibiotic therapy could kill other intracolonic bacteria, thereby increasing the systemic absorption of toxin. While there is controversy about the use of antibiotics, the use of antimotility agents such as loperamide is definitely not indicated in the management of disease due to EHEC; there is evidence that the use of such agents can increase the risk for development of HUS, possibly by delaying intestinal clearance of the organism and thereby increasing toxin absorption (Cimoli *et al.*, 1994).

Treatment of renal disease due to EHEC is primarily supportive, except for some experimental therapies currently being evaluated in clinical trials. Current treatment regimens may include

dialysis, hemofiltration, transfusion of packed erythrocytes, platelet infusions and other interventions as clinically indicated. Severe disease may require renal transplant. A promising therapy now being evaluated in clinical trials is Synsorb-Pk, which consists of a chemically synthesized analog of Gb3, the receptor for Shiga toxin, coupled to diatomaceous earth. This compound would be ingested by patients with bloody diarrhea in the hope that it could absorb toxin from the intestine and prevent the development of HUS. Initial phase I trials have been promising and phase III trials to assess efficacy are in progress (Armstrong *et al.*, 1995).

1.2.9 Vaccines

There are no currently available vaccines to prevent disease due to EHEC, but a number of experimental approaches are being investigated in animals. Vaccine development has been severely hampered by the lack of an appropriate animal model wherein animals challenged orally with EHEC will develop HUS. A crucial antigen in any potential vaccine is the Shiga toxin (Stx). Parenteral Stx toxoid vaccines have shown protective effects in rabbits (Bielaszewsha *et al.*, 1994) and pigs (Bosworth *et al.*, 1996). Attenuated *Vibrio cholerae* (Nataro *et al.*, 1998) and *Salmonella typhimurium* (Tzschaschel *et al.*, 1996) vaccine strains that express StxB have been constructed. The *V. cholerae* constructs have been administered orally to rabbits and have engendered neutralizing serum antibodies and partial protection from the enterotoxic effects of Stx (Acheson *et al.*, 1996). The intestinal adherence factor intimin has also been expressed in attenuated *V. cholerae* strains (Nataro *et al.*, 1998). A parenteral vaccine specific for O157 EHEC has been developed based on O157 polysaccharide conjugated to protein carriers (Konadu *et al.*, 1994). An ideal broad-spectrum EHEC vaccine should probably engender both systemic immunity against Stx and local intestinal immunity against intimin and other intestinal colonization factors.

1.2.10 Toxin Production and Mode of Action

E. coli produces various toxigenic activity including enterotoxicity, cytotoxicity, neurotoxicity and hemolysin. Enterotoxin production leads to fluid accumulation in the ileum of Rabbit. Cytotoxicity of *E. coli* has various effects on Vero cell line or Hela cell line. Neurotoxicity of *E. coli* brings remarked changes in mice; it might cause paralytic effect or mice leathality.

1.2.10.1 Enterotoxin

An enterotoxin is a protein exotoxin released by a microorganism that targets the intestines. Its target site is indicated within its name.

Enterotoxins are chromosomally encoded exotoxins that are produced and secreted from several bacterial organisms. They are often heat stable and are of low molecular weight and water soluble. Enterotoxins are frequently cytotoxic and kill cells by altering the apical membrane permeability of the mucosal (epithelial) cells of the intestinal wall. They are mostly pore forming toxins (mostly chloride pores), secreted by bacteria that assemble to form pores in cell membranes. This causes the cells to die.

Enterotoxin and exotoxin are two classes of toxin that are produced by bacteria.

An exotoxin is a toxin that is produced by a bacterium and then released from the cell into the surrounding environment. The damage caused by an exotoxin can only occur upon release. As a general rule, Enterotoxin tends to be produced by gram positive bacteria rather than Gram-negative bacteria. There are exceptions, such as the potent enterotoxin produced by *Vibrio cholerae.*

In contrast to Gram positive bacteria, many Gram-negative species possess a molecule called lipopolysaccharide. A portion of the lipopolysaccharide, called the lipid A, is a cell associated toxin or an endotoxin.

An enterotoxin is a type of exotoxin that acts on the intestinal wall. Another type of exotoxin is a neurotoxin. This type of toxin disrupts nerve cells.

1.2.10.1.1 Mode of Action

Enterotoxins have a particularly marked effect upon the gastrointestinal tract, causing vomiting, diarrhea and abdominal pain. The action of enterotoxins leads to increased chloride ion permeability of the apical membrane of intestinal mucosal cells. These membrane pores are activated either by increased cAMP or by increased calcium ion concentration intracellularly. The pore formation has a direct effect on the osmolarity of the luminal contents of the intestines. Increased chloride permeability leads to leakage into the lumen followed by sodium and water

movement. This leads to a secretory diarrhea within a few hours of ingesting enterotoxin. Several microbial organisms contain the necessary enterotoxin to create such an effect.

1.2.10.2 Cytotoxin

Treating cells with the cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they loose membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death (apoptosis).

Cells undergoing necrosis typically exhibit rapid swelling, lose membrane integrity, shutdown metabolism and release their contents into the environment. Cells that undergo rapid necrosis *in vitro* do not have sufficient time or energy to activate apoptotic machinery and will not express apoptotic markers.

Apoptosis is characterized by well defined cytological and molecular events including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation and cleavage of DNA into regularly sized fragments.

Cells in culture that are undergoing apoptosis eventually undergo secondary necrosis. They will shut down metabolism, lose membrane integrity and lyse.

Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. Compounds that have cytotoxic effects often compromise cell membrane integrity.

Vital dyes, such as trypan blue or propidium iodide are normally excluded from the inside of healthy cells; however, if the cell membrane has been compromised, they freely cross the membrane and stain intracellular components (Riss and Moravec, 2004).

Alternatively, membrane integrity can be assessed by monitoring the passage of substances that are normally sequestered inside cells to the outside.

In this study Baby Hamster Kidney 21 (BHK 21) cell line was used to observe to cytolytic effect toxin.

BHK21 cells are one of the few lines that can be grown both in suspension as well as anchorage dependent. Both culture system offer distinction advantages. Suspension cultures are very easy to scale up and there is no need to dislodge the cells for subculture. The main difficulty is media exchange and adaptation to persuasion systems. Cell metabolism is also affected by cell morphology. Recombinant Chinese Hamster Ovary (CHO) cells have been reported to have a ten times lower specific productivity. These differences make micro carrier culture an attractive alternative. CultiSpher-G eliminates the major difficulties associated with micro carrier culture; scale up difficulties and low harvest yields. The large growth span obtained through macro porosity makes it possible to use scale up steps of more than 50 times. As CultiSpher-G is based on highly purified gelatin, enzymes that are very specific can be used for harvesting. This results in high cell yield with almost 100% viability.

1.2.10.3 Neurotoxin

Neurotoxin is derived from the (nevron/neuron) meaning "nerve" (derived from neuro: "cord") and Latin toxicum meaning "poison" (derived from Greek toxikon pharmakon, meaning "arrow poison"). They are extensive class of exogenous chemical neurological insults (Spencer *et al*., 2000) which can adversely affect function in both developing and mature nervous tissue (Olney, 2002).

The term neurotoxic is used to describe a substance, condition or state that damages the nervous system and/or brain, usually by killing neurons. The term is generally used to describe a condition or substance that has been shown to result in observable physical damage.

1.2.10.3.1 Examples of Neurotoxin

Though neurotoxins are often neurologically destructive but ability to specifically target neural components is important in the study of nervous system (Kiernan *et al.,* 2005).

Common examples of Neurotoxins include:

Chemicals: lead, ethanol, glutamate, nitric oxide.

Biological: botulinum toxin (Rosales *et al.,* 1996), tetrodotoxin (Kiernan *et al.,* 2005).

1.2.10.3.2 Mode of action

A neurotoxin is a substance which inhibits the functions of neurons. Neurons are found throughout the brain and nervous system, and the function of these unique cells are critical for a variety of tusks, ranging from automatic nervous system jobs like swallowing to higher level brain function.

Neurotoxins can work variety of ways, with the danger of exposure varying depending on the neurotoxin involved and the dosage.

Neurotoxin activity can be characterized by the ability to inhibit neuron control over on concentrations across the cell membrane (Kiernan *et al.,* 2005) or communication between neurons across a synapse (Arnon *et al.,* 2001). Local pathology of neurotoxin exposure often includes neuron excitotoxicity or apoptosis (Dilkranian, 2001) but can also include glial cell damage (Deng *et al.,* 2003).

In some cases, neurotoxins simply severely damage neurons so that they cannot function. Others attack the signaling capabilities of neurons, by blocking releases of various chemicals or interfering with the methods of reception for such transmission and sometimes telling neurons to send false signals. A neurotoxin may also destroy neurons altogether.

1.2.10.4 Hemolysin

Hemolysins are certain proteins and lipid that cause lysis of red blood cells by damaging their cell membrane. Although the lytic activity of some microbial hemolysins on red blood cells may be important for nutrient acquisition or for causing certain conditions such as anemia, many hemolysin producing pathogens do not cause significant lysis of red blood cells during infection. Although hemolysins are able to lyse red blood cells in vitro, the ability of hemolysins to target other cells, including white blood cells, often accounts for the effects of hemolysins during infection. Most hemolysins are proteins but others such as rhamnolipids are lipid biosurfactants (Stipcevic *et al.,* 2005).

Hemolysins can be identified by their ability to lyse red blood cells *in vitro*. Not only the erythrocytes are affected by hemolysins but there are also some effects among other blood cells,

such as leucocytes (white blood cells). *E. coli* hemolysin is potentially cytotoxic to monocytes and macrophages, leading them to autolysis and death.

1.2.10.4.1 Classification

Visualization of hemolysis of red blood cells in agar plates facilitates the categorization of hemolysin.

Depending upon production of hemolysin microbes can be classified into 3 categories:

a) Alpha (α) hemolytic: partial hemolysis of RBC
b) Beta (β) hemolytic: complete hemolysis of RBC
c) Gamma (γ) hemolytic: no hemolysis

1.2.10.4.2 Mechanism of Action

One way hemolysin lyses erythrocyte is by forming pores in phospholipid bilayers (Chalmeau *et al.*, 2011; Bhakdi *et al.*, 1988)

Other hemolysins lyse erythrocytes by hydrolyzing the phospholipids in the bilayer.

1.2.11 Antibiotic Sensitivity

Antibiotic sensitivity testing is used to determine the susceptibility of bacteria to various antibiotics. This standardized test is used to measure the effectiveness of a variety of antibiotics on a specific organism in order to prescribe the most suitable antibiotic therapy (Quintiliani and Courvalin, 1994; Francois *et al*., 1997).

1.2.11.1 Antibiotic Resistance of *E. coli*

Since 1940s, researchers have discovered numerous naturally occurring and synthetic antibiotics. Bacteria that are resistant to all of these antibiotics have been observed. In many cases, a given bacterium may exhibit multiple drug resistance; that is, it is resistant to several different antibiotics. Often, these antibiotic resistance genes are located on plasmids and can be transferred from one cell to another and even from one bacterial species to another. As a result,

it is not unusual for normal bacteria isolated from the body to exhibit resistance to one or more commonly used antibiotics (Mayer *et al*., 1995).

1.2.12 Vegetables as Source of *E. coli*

Over past two decades consumption of fresh vegetables has increased for various reasons; these are considered as important sources of vitamins, nutrients and fiber (Olaimat and Holley, 2012), consumers' awareness of health aspects of vegetables and fruits have increased and consumers are more concerned about healthy eating and staying healthy.

Outbreaks study showed that *E. coli* was able to survive and grow in different types of minimally processed vegetables and fruits (Abadias *et al*., 2012). The large nation-wide spinach outbreak in US (2006) was linked to the environmental *E. coli* O157:H7 from the field (Grant*etal*., 2008). In 2006, *E. coli* contamination of lettuce and spinach resulted in 81 and 199 cases (3deaths) in US (CDC2006; Sela *et al*., 2009; CDC, 2011). In 2008, *E .coli* contamination of lettuce resulted in 134 cases in Canada (Warriner and Namvar, 2010). Most of the outbreaks were related to the packaged product. Products had been washed and disinfected with hypochlorite before packaging but the process was insufficient to eliminate *E. coli* O157:H7 and prevent infections (Pérez-Rodríguez *et al*., 2011).

In this study raw vegetable, packaged sprout, street salad and processed chopped vegetable were selected as they often are eaten raw without much further processing. *E. coli* and other resistant bacteria colonize these vegetables for a number of reasons; the direct use of antibiotics during cultivation, use of contaminated fertilizer or irrigation water or unknown human selection pressure (Osterblad *et al*., 1999).

1.2.12.1 *E. coli* from Raw Vegetables in Bangladesh

Similar to many of the developing and underdeveloped countries, the hygienic conditions in Bangladesh are severely compromised and living with domestic animals within the same premises is a common practice among the Bangladeshi population. High prevalence of STEC in animal reservoirs appears to have a little impact on public health, considering the lack of STEC-associated infections among the Bangladeshi population (Islam *et al*., 2007). Nevertheless, to

determine the underlying risk of infection with STEC among the Bangladeshi population, it is important to know the occurrence and transmission of these organisms in the human food chain.

In previous studies occurrence of Shiga toxin producing *E. coli* in Bangladeshi food chain have been reported. *E. coli* O157:H7 have been isolated from raw meat, raw milk and street vended juice (Islam *et al*., 2010). To our knowledge, this is going to be the first report of *E. coli* isolated from raw vegetables in Bangladesh.

1.3 Objective of the Study

1.3.1 General Objectives

Toxin producing *E. coli* is an important food borne and waterborne pathogen with worldwide distribution. Raw meat and vegetables are particularly likely to carry large number of Bacteria including toxin producing *E. coli.* This organism has been reported to be responsible for outbreaks linked to the consumption of fresh vegetables such as lettuce, spinach, carrots, sprouts, alfalfa (Chang *et al*., 2013). Outbreaks studies have shown that *E. coli* can survive and grow in different types of minimally processed vegetables. It also has the ability to survive at refrigeration temperature and under harsh environmental conditions. In this study, raw vegetables have been selected as they are often eaten raw without any heat treatment, sometimes without washing and peeling. Therefore, in this study an attempt has made to isolate and characterize *E. coli* from raw vegetables.

1.3.2 Specific Objectives

- Isolation of *E. coli* from raw vegetables by selective enrichment of the samples followed by selective plating.
- Genotypic characterizations of the isolates by *stx*1 and *stx*2 genes by polymerase chain reaction (PCR).
- Phenotypic characterizations of the isolates by investigation of enterotoxicity, cytotoxicity, neurotoxicity, hemolysin activity.
- Antimicrobial susceptibility of the isolates.

Chapter 2:

Materials and Methods...

2. Materials and Methods

2.1 Materials

2.1.1 Samples

Sixty raw vegetable samples were collected from various super shop, local bazaar and street shop around Dhaka city.

Table 2.1: Area of sampling, number and types of samples

Place	Type Of Market	Types Of Samples (Number Of Samples)
Uttara	Local Bazaar	Lettuce(3), Broccoli(2), Capsicum(1), Baby Corn(1), Celery(1), Zucchini(1)
Gulshan	Local Bazaar	Lettuce(3), Broccoli(2), Chinese Leaf(1)
Elephant Road	Local Bazaar	Lettuce(2), Broccoli(2)
Motijheel	Local Bazaar	Cucumber(1), Lettuce(2), Capsicum(1), Chinese Leaf(1)
Bashundhara	Local Bazaar	Lettuce(1), Chinese Leaf(1)
Malibag	Local Bazaar	Lettuce(1)
New Market	Local Bazaar	Lettuce(1), Capsicum(1), Mint(1), Tomato(1), Spring Onion(1), Carrot(1)
Banani	Local Bazaar	Mint(1), Lettuce(1), Capsicum(1), Carrot(1)
Poribag	Local Bazaar	Cucumber(1)
Mohakhali	Local Bazaar	Cucumber(1), Lettuce(2)
Kachukhet	Local Bazaar	Lettuce(1)
Kawran Bazar	Local Bazaar	Coriander(1)
Kakrail Bazar	Local Bazaar	Coriander(1)
Departmental store,Gulshan	Super Shop	Lemon Grass(1), Sprout(1)
Departmental store, Uttara	Super Shop	Sprout(2)
Departmental store, Banani	Super Shop	Chopped Vegetables(2), Broccoli(1), Sprout(4), Baby Corn(1), Asparagus(1), Celery(1), Zucchini(1), Carrot(1)
Total 16		**60**

2.1.2 Reference Strain

One American Type Culture Collection strain of *E. coli* O157:H7 (ATCC-12079) was used as reference strain for comparison. The reference strain was streaked on MacConkey agar plate and incubated overnight at 37^{o}C. Biochemical, serological and molecular tests according to the standard procedure reconfirmed the strain.

Pseudomonas aeruginosa is used as negative control for biochemical test.

E. coli 25922 was also used in antibiogram susceptibility test for comparison.

V. cholerae 569B was also used in rabbit ileal loop test for comparison.

All these above mentioned organisms were preserved in several T_1N_1 agar media.

2.1.3 Media

Two types of media used for isolation of *E. coli*. These are MacConkey Agar and enrichment broth.

2.1.3.1 MacConkey Agar Medium

MacConkey agar is used for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria. As *E. coli* can ferment lactose it gives pink colour colony on MacConkey agar.

2.1.3.2 EC Broth

EC broth is selective medium to enumerate the number of desired Bacteria. Along with other growth promoting substances which is supplemented with Bilesalt (No.3) inhibits Gram positive and non-enteric bacteria.

2.2 Methods

2.2.1 Collection of Samples

Raw vegetable samples were aseptically collected from local market into sterile plastic bags and immediately transported to the laboratory and analyzed in the same day.

2.2.2 Isolation of *E. coli* from Samples

Twenty five gram of selected raw vegetable was measured and finely chopped aseptically. This was added to 225 ml of enrichment broth and homogenized and incubated at 37^{o}C for 16-18 h. Then 1 ml of enriched broth was subjected to 10-fold dilution series from 10^{-1} to 10^{-4} in 9 ml of PBS. From each10^{-1} to 10^{-3} dilution tubes, 0.1 ml of suspension was spread plated in duplicate onto MacConkey agar plates. The plates were then incubated at 37^{o}C for 16-18 h.

2.2.3 Identification of *E. coli* Isolates

2.2.3.1Cultural Properties on MacConkey Agar Plate

Morphological characteristics of colonies (size, shape, elevation, form, pigmentation and opacity) developed after incubation on MacConkey agar plate were carefully studied and recorded. Five to ten suspected *E. coli* colonies were picked up from the plate and subcultured onto fresh MacConkey plate to get pure culture.

2.2.3.2 Screening of Suspected Isolates on EMB Plate

Suspected *E. coli* isolates from the fresh MacConkey plate were streaked onto eosin methylene blue (EMB) agar plate for presumptive confirmation as *E. coli*. EMB agar medium contains lactose and the dyes eosin and methylene blue that permit differentiation between enteric lactose fermenters and non-fermenter as well as identification of the colon bacillus *E. coli*. The *E. coli* colonies are black colonies with a metallic green sheen caused by the large quantities of acid that is produced and that precipitates out the dyes onto the growth's surface (Cappuccino and Sherman, 2011).

2.2.3.3 Biochemical Identification

Biochemical tests were performed with green metallic sheen producing isolates according to the methods described in Microbiology Laboratory Manual (Cappuccino and Sherman, 2011). The biochemical tests were indole production test, methyl-red test, Voges-Proskauer test, citrate utilization test, triple sugar iron agar test and carbohydrate fermentation (lactose, dextrose, sucrose) test.

2.2.3.3.1 Indole Production Test

The amino acid tryptophan is a component of nearly all proteins and is therefore available to microorganisms as a result of protein breakdown. Some bacteria are able to produce an enzyme called tryptophanase that catalyzes the removal of the indole residue from tryptophan. Indole accumulates in the culture media while the rest of the tryptophan molecule (pyruvate and NH_3) is used to satisfy nutritional needs. The production of indole from tryptophan by microorganisms can be detected by growing them in a medium rich tryptophan. The accumulation of indole in the medium can be detected by adding Kovac's reagent, which reacts with indole, giving a water-insoluble bright red compound on the surface of the medium.

2.2.3.3.2 Methyl Red Test

The methyl red test is used to detect a specific type of fermentation called mixed acid fermentation. Some bacteria ferment glucose and produce large quantities of acidic end products that lower the pH of the medium below 5.0. The addition of pH indicator methyl red is used to detect this acidity (methyl red is red at pH 4.4 or below and yellow at pH 6.2 or above). A mixed acid fermentation is detected by growing bacteria in MR-VP medium and then after an incubation period, adding some methyl red reagent to the culture medium. If mixed acid fermentation occurred, a yellow color will develop.

2.2.3.3.3 Voges-Proskaure Test

The Voges-Proskaure test is used to detect a specific organism that carry out 2, 3-butanediol fermentation. When bacteria ferment sugars producing 2, 3-butanediol as a major end product, they accumulate this compound in the medium. The addition of 40% KOH and a 5% solution of alpha-naphthol in absolute ethanol will reveal the presence of acetoin (acetyl methyl carbinol), a precursor in the synthesis of 2, 3-butanediol. The acetoin, in the presence of KOH, will develop a pink color imparting a rose color to the medium. The reaction will occur in the presence of alpha-naphthol catalyst and a guanidine group that is present in the peptone of the MR-VP medium. Aeration increases the rate of oxidation of 2, 3-butanedio to acetoin.

2.2.3.3.4 Citrate Utilization Test

The citrate test determines the ability of microorganisms to use citrate as the sole source of carbon and energy. Simmon's citrate agar, a chemically defined medium with sodium citrate as the carbon source, NH_4^+ as a nitrogen source and the pH indicator bromophenol blue, is commonly used for this test. When microorganisms utilize citrate, they remove the acid from the medium, which raises the pH and turns the pH indicator from green to blue. A color change in the medium from green to blue indicates that the microorganisms tested can utilize citrate as its only carbon source.

2.2.3.3.5 Triple Sugar-Iron (TSI) Agar Test

The triple sugar-iron (TSI) agar test is designated to differentiate among the different groups or genera of the Enterobacteriaceae and to distinguish the Enterobacteriaceae from other Gram-negative intestinal-bacilli. This difference is made on the basis of difference in carbohydrate fermentation patterns and hydrogen sulfide production by the various groups of intestinal organisms. TSI agar medium contains lactose and sucrose in 1% concentrations and glucose in a concentration of 0.1%. To facilitate observation carbohydrate utilization patterns, TSI agar medium is made with slant and butt. TSI agar medium also contains sodium thiosulfate and ferrous sulfate for detection of hydrogen sulfide production, which is indicated by blackening of medium. Three types of results are observed in TSI test:

Reactions	Results
(a) Alkaline slant (red) and acid butt (yellow) with and without gas production(breaks in the agar butt)	Small amount of acid production from glucose fermentation and peptone utilization have caused alkaline reaction on the slant surface. In the butt, acid reaction is maintained due to reduced condition and slow bacterial growth
(b) Acid slant (yellow) and acid butt (yellow) with and without gas production	Large amount of acid production from lactose and/or sucrose fermentation has caused acid reaction on the slant surface.
(c) Alkaline slant (red) and alkali butt (red) or no change (orange-red) butt	No carbohydrate fermentation has occurred. Instead peptone has catabolized, resulting in alkali reaction.

2.2.3.3.6 Carbohydrate Fermentation

Carbohydrate fermentation is done to determine the fermentation capability of the organism. It is done under anaerobic condition in a fermentation broth tube containing Durham tube for detection of gas production .Specific carbohydrate serves as substrate and phenol red as pH indicator. The critical nature of fermentation reaction and the indicator make it an imperative that all culture has to be observed within 48 hours. Upon incubation carbohydrates that have been fermented with the production of acid and gas will cause the phenol red to turn yellow.

2.2.4 Genotypic Characterizations of the Isolates

2.2.4.1 Polymerase Chain Reaction (PCR) Using *stx*1 and *stx*2 Gene Primers

PCR is an enzymatic method of making multiple copies of a pre-selected segment of DNA. The amplification process is achieved with two synthetic oligonucleotide primers, a thermostable DNA polymerase (*Taq* polymerase) and four deoxyribonucleoside triphosphates acting at on the template DNA. There are three major steps in a PCR, which are repeated 25-40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

- Denaturation: This usually occurs at 94^{o}C, which breaks the base pairs and releases single-stranded DNA to act as templates for the next round of DNA synthesis.
- Annealing: At this stage, the primers attach to the templates. The temperature is estimated by determining the melting temperature of primer-template hybrid.
- Extension: At this stage, DNA synthesis occurs. The temperature is usually set at 72^{o}C, just below the optimum temperature for *Taq* polymerase.

2.2.4.1.1 Preparation of Template DNA

Each isolate was inoculated into 5 ml of Luria Bertani (LB) broth and incubated overnight at 37^{o}C. The cell pellet was harvested by centrifuging 500 µl of broth culture at 10,000 rpm for 5 min. The supernatant was discarded and the pellet was washed with 500 µl of phosphate buffered saline (PBS) by centrifuging at 10000 rpm for 5 min. The cell pellet was resuspended in 200 µl of Tris-EDTA (TE) buffer (pH 8.0) and then kept in a boiling water bath for 10 min. After

cooling on ice for 5 min, the suspension was centrifuged at 10,000 rpm for 5 min and 2 µl of the supernatant was used as template DNA.

2.2.4.1.2 Preparation of Reaction Mixture

PCR was carried out for several genes specific for virulence properties of *E. coli*. The reaction mixture for specific PCR was prepared for all the isolates simultaneously by mixing the components using the volumes mentioned in the Table 2.2. After mixing the reaction mixture with the template DNA and primers in the Table 2.3, the PCR tubes were placed in a thermal cycler (Applied Biosystems, Germany).

Table 2.2: PCR reaction mixture for *stx*1 and *stx*2 genes (25 µl vol)

Components	Volume(µl)
PCR grade water	12.375
10 x Ex buffer	2.5
50 mM $MgCl_2$	1.5
dNTPs mixture(2.5 mM each)	2.5
Taq polymerase(5U/µl)	0.125
Primer-1	0.5
Primer-2	0.5
Template	5
Total	25

Table 2.3: Primer sequences used for PCR

Target Gene	Primer	Sequence	Amplicon Size
*stx*1	LP30	5'-CAGTTAATGTGGTGGCGAAGG -3'	348 bp
	LP31	5'-CACCAGACAAATGTAACCGCTC -3'	
*stx*2	LP41	5'-ATCCTATTCCCGGGAGTTTACG -3'	584 bp
	LP42	5'-GCGTCATCGTATACACAGGAGC -3'	

2.2.4.1.3 PCR Conditions

PCR tubes containing the appropriate mixtures were heated at 94˚C for 5 min in the thermal cycler to ensure the complete denaturation of DNA templates. The PCR was then continued with the following program:

For *stx*1and *stx*2 PCR

94˚C, for 1 min 30 sec

55˚C, for 1 min

72˚C, for 1 min 30 sec

Twenty five cycles of these segments were repeated with a final extension of 10 min at 72˚C. After this, PCR tubes were stored at -20˚C until further analysis.

2.2.4.1.4 Electrophoretic Analysis of Amplified DNA Product

The amplified products were examined by resolving the PCR productsin1% agarose gel in TBE buffer (pH 8.2). 5µl of PCR product was mixed with 2µl of gel loading dye and loaded into the slots of the gel with the aid of a micropipette. Electrophoresis was performed with 60 V in horizontal electrophoretic apparatus by submerging the gel into TBE buffer for 50 min. Thenthe gel was stained with staining solution [10µl EtBr (0.5 µg/ml) in 100 ml TBE buffer] for 20 min and destained with distilled water for 10 min. The EtBr stained DNA bands were observedona UV transilluminator. Photographs were taken using Gel Documentation machine attached to a computer. The PCR product sizes were estimated using the 100 bp marker (Invitrogen, USA).

2.2.5 Phenotypic Characterizations of the Isolates

2.2.5.1 Enterotoxicity Test

This test was performed by rabbit ileal loop assay using the isolates AS1(7), AS1(8), UFL(14), UFL(7), ML2(3), ML2(7) and MBCV2(12), MBCV2(16). Live cells of the isolates were used as inocula.

2.2.5.1.1 Preparation of Live Cells as Inoculum

Ten ml of tryptic soy broth (TSB) was inoculated with 5-6 colonies from pure culture of the Selected isolate on MacConkey agar. The broth was shaken at 100 rpm and incubated at 37^{o}C for 4 h. Then 1 ml of broth culture was used as inoculum. Similar preparation was made with a known enterotoxigenic *V. cholerae*.

2.2.5.1.2 Rabbit Ileal Loop Assay

Two adult albino rabbits (New Zealand strain) of 1.5-2.0 kg body weight were starved for 24 h allowing only water. After proper anesthesia with a lower dose of sodium pentobarbital (0.5 ml/kg body weight, intravenous), the intestine was exposed and loops of 6-8 cm in length with 2-3 cm intervals between each were made using nylon thread. One ml of sample was inoculated into one loop. The animals were sacrificed after 18 h with excess sodium pentobarbital. The length of each loop and the volume of fluid accumulated were measured to determine the amount of fluid accumulation per unit length of gut. The loop was considered positive if the fluid accumulation is 0.4 ml/cm of loop, otherwise the loop was considered negative.

2.2.5.2 Cytotoxicity Test

2.2.5.2.1 Seed Culture Preparation

Ten ml screw cap test tube containing 5 ml of tryptic soy broth were inoculated with 3-4 colonies of the pure cultures of each isolates and incubated at 37^{o}C for 4 h with shaking (120rpm).

2.2.5.2.2 Preparation of Culture Filtrate

Seed cultures were taken out and added to 20 ml of TSB in 100ml conical flask. This was incubated at 37^{o}C for 24 hr with shaking(120rpm).The cultures were centrifuged at 10,000 rpm for 10 min at 10^{o}C and each supernatant was filtered through a Millipore membrane (0.45 µm pore diameter) and preserved at -20^{o}C. These culture filtrates were used for cytotoxicity and indirect neurotoxicity assays.

2.2.5.2.3 Growth Media

10% DMEM (Dulbecco's Modified Eagles' medium)

For 500 ml media:

FBS	50ml
Penicillin + Streptomycin	5ml(10000µg/ml)
Gentamycin	1ml(10mg/ml)
Amphoterin B 100µl	
Media	Rest

2.2.5.2.4 Cytotoxicity Assay

BHK-21, a baby hamster kidney fibroblast cell line, was maintained in DMEM. Cells ($3.2x10^4$/450µl) were selected onto 24 –well plates and incubated at 37ºC+5%CO_2. Next day, 50 µl of cell free extract was added each well. Cytotoxicity was examined under an inverted light microscope after 18hr incubation. Duplicate wells were used for each isolate along with *E. coli* O157:H7 as positive control and BHI medium as negative control.

2.2.5.3 Indirect Neurotoxicity Test

One ml culture filtrate of the *E. coli* isolates were injected intra peritoneal by duplicate mice. Mice were kept under observation for 5 days. Any changes in their movement (i.e. paralysis of muscle) were recorded.

2.2.5.4 Determination of Hemolysin Activity

Hemolysis means the lysis of eryhtrocyes by bacterial enzyme, hemolysin. Hemolytic activity is identified with a blood agar medium. There are three types of hemolytic reactions:

1. (α) Alpha-hemolysis, an incomplete form of hemolysis, produces a green zone around the colony.
2. (β) Beta-hemolysis, a complete destruction of red blood cells, exhibits a clear zone around the colony.

3. (γ) Gamma-hemolysis is the indicative of the absence of any hemolysis (Cappuccino and Sherman, 2011). *E. coli* isolates were screened for hemolysis activity by streaking on sheep blood agar plate followed by incubation at 37^{o}C for 18 h.

2.2.6 Susceptibility of Antimicrobial Agents

Antimicrobial susceptibility testing was done by Kirby Bauer agar disk diffusion method according to the Clinical and Laboratory standard Institute (CLSI) formerly National Committee for Clinical Laboratory Standards (NCCLS). Minimum inhibitory concentrations (MICs) were determined isolates using the following antibiotics disc Amoxicillin/Clavulanicacid(30µg), Ampicillin(10µg), Cefoxitin(30µg), Cefuroxime/Sodium(30µ), Chloramphenical(30µg), Ciprofloxacin(5µg), Gentamycin(10µg), Kanamycin(30µg), Nalidixicacid(30µg), Nitrofurantoin(10µg), Norfloxacin(10µg), Streptomycin(10gµ), Sulphamethoxazole/Trimethoprim19:1(25µg), Tetracycline(30µg). *E. coli* ATCC25922 was used as control organism. Using NCCLS guidelines, each organism was classified either resistant or susceptible to the antimicrobials agent.

2.2.7 Preservation of *E. coli* Isolates

For short-term preservation, 2 ml of T_1N_1 agar medium in a vial was inoculated by stabbing bacterial growth of each isolate from MacConkey agar plate. Then the vial was incubated at 37^{o}C for 6 h. After incubation, the surface of the medium was covered with sterile paraffin oil and the vial was stored at room temperature.

For long-term preservation, 500 ml of bacterial culture grown in TSB at 37^{o}C for 6 h was taken in a sterile cryovial. Then 500 µl of sterile glycerol was added to the broth culture and the cryovial was stored at -20^{o}C.

Chapter 3:

Results...

3. Results

This present study was aimed to isolate and characterize *E. coli* from raw vegetables sources. Enrichment, selective plating, biochemical tests and molecular based methods have been applied for isolation and identification of *E. coli* from collected samples. Then the confirmed isolates were genotypically and phenotypically characterized.

3.1 Isolation of *E. coli* from Sample

Sixty raw vegetable samples were collected from various local bazaar and super shops around Dhaka city. The samples were enriched at 37^{o}C for 18h in enrichment broth containing Bile Salt No.3 (Figure 3.1). After enrichment; the culture broth was subjected to ten fold dilution series. From appropriate dilutions, the broth cultures were spread plated onto MacConkey Agar plates and the plates were incubated at 37^{o}C for 18h. After incubation, different types of colonies were observed on MacConkey agar plates (Figure 3.2). Colonies showing typical colony characteristics of *E. coli* (Table 3.1) were subcultured onto MacConkey plates. Over 65 isolates were taken from primary isolation plates and considered for further investigation.

Figure 3.1: Selective enrichment of various samples

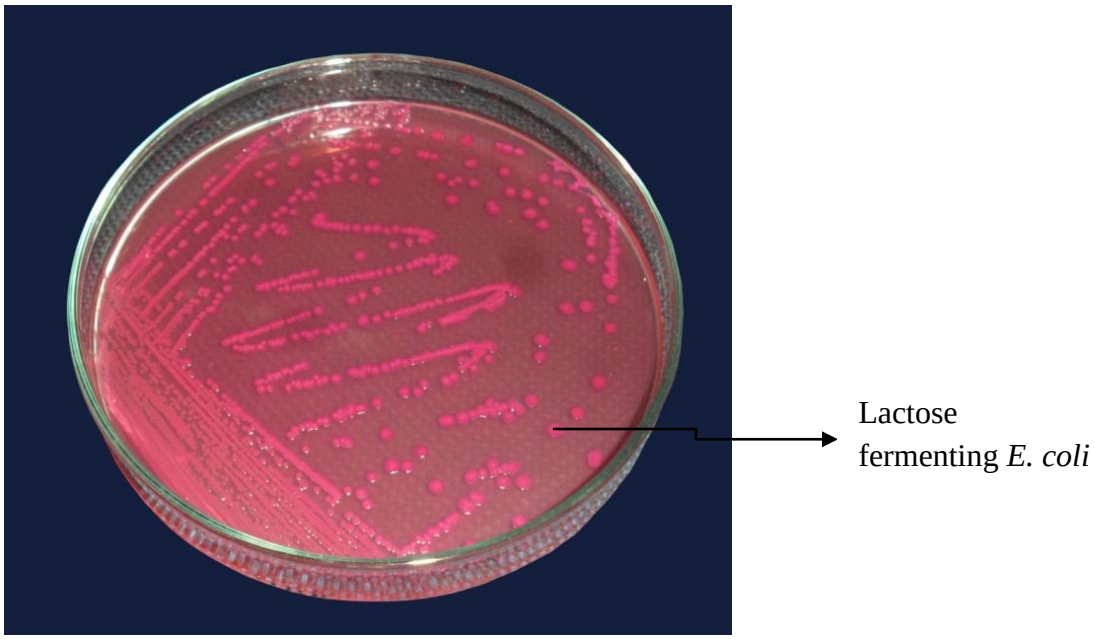


Figure 3.2: MacConkey agar plate showing typical pink lactose fermenting colonies of *E. coli*

Table 3.1: Colony characteristics of *E. coli* on MacConkey plate

Colony characteristics	MacConkey plate
Size	Moderate (1-2 mm)
Shape	Round
Elevation	Raised
Margin	Entire
Color	Deep pink Color
Opacity	Opaque

3.2 Identification of Suspected *E. coli* Isolates

3.2.1 Presumptive Identification on EMB Plate

From MacConkey plates, suspected *E. coli* isolates were picked up using needle and streaked onto EMB agar plates. After incubation, isolates showed green metallic sheen growths were selected for further identification (Figure 3.3) and others were discarded. Of 65 lactose fermenting suspected *E. coli* isolates, 30 produced typical green metallic sheen growths on EMB plates. The further identification was carried out only for these isolates.

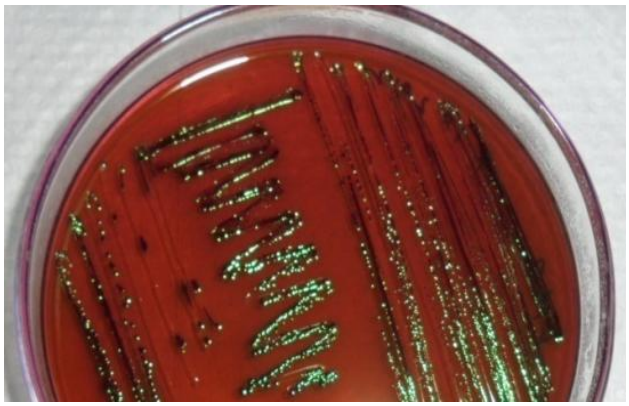


Figure: 3.3 EMB agar plate showing typical green metallic sheen growths of presumptive *E. coli* isolates.

3.2.2 Biochemical Identification

Presumptive isolates which gave metallic green sheen were subjected to different biochemical tests. Isolates showed pattern of biochemical reactions typical for *E. coli* as mentioned in (Table 3.2) were selected for further identification and confirmation. Figures 3.4 and Figures 3.5 are showing the results of biochemical tests of culture positive isolates.

Table 3.2: Typical results of biochemical tests of *E. coli* isolates

Tests	Results
Indole production	Positive
Methyl Red (MR)	Positive
Voges Proskauer (VP)	Negative
Citrate utilization	Negative
Triple Sugar Iron (TSI)	Acid butt, acid slant, Gas
Dextrose	Acid ,Gas
Sucrose	Acid, Gas
Lactose	Acid, Gas

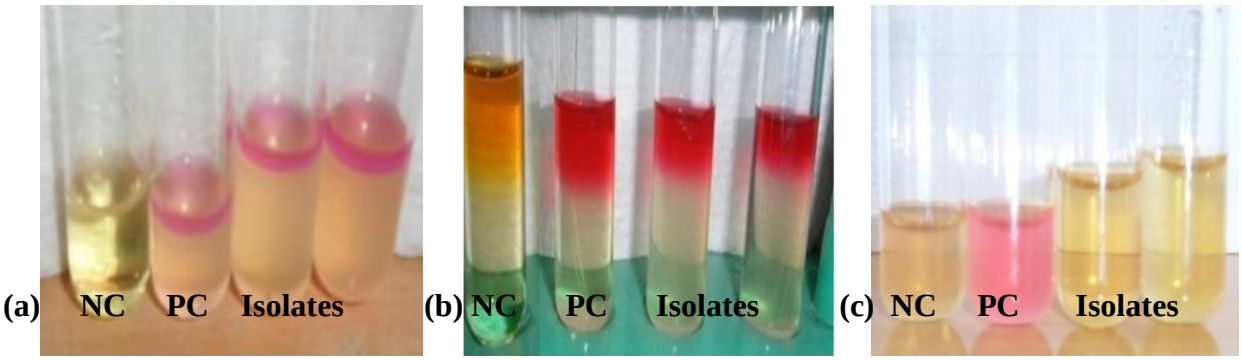


Figure 3.4: Test tubes showing biochemical tests for (a) Indole production, (b) Methyl red and (c) Voges -Proskauer test (Negative control, Positive control, Isolates)

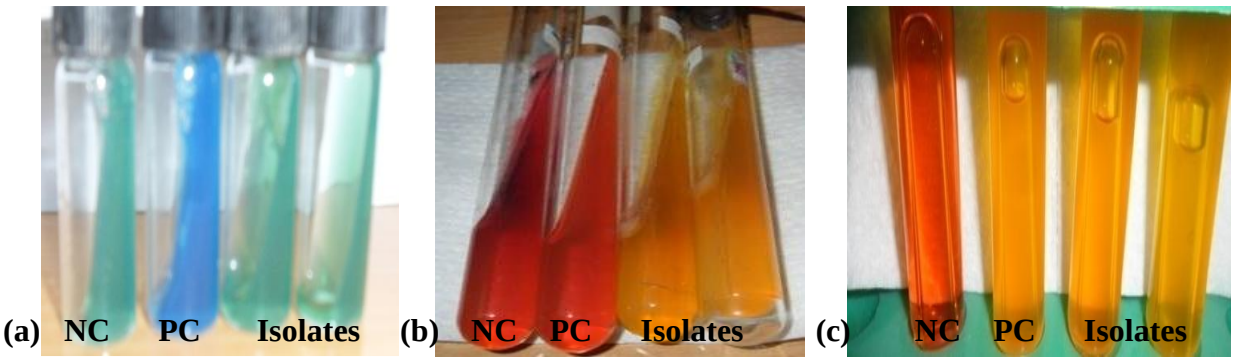


Figure 3.5: Test tubes showing biochemical tests for (a) citrate utilization, (b) TSI and (c) Fermentation test (Blank, Negative control, Positive control, Isolates)

By following the above procedures thirty isolates from various samples were culturally and biochemically identified as *E. coli* (Table 3.3).

Table 3.3: List of culturally and biochemically identified *E. coli* isolates

Isolate no.	Source	Area of sampling
MBS2(4)	Sprout	Banani
ML1(6)	Lettuce	Motijheel
UFL(14)	Lettuce	Uttara
AS1(7)	Sprout	Gulshan
UFB(18)	Broccoli	Uttara
MBS2(5)	Sprout	Banani
UC2(16)	Capsicum	Uttara
UFL(17)	Lettuce	Uttara
MLI(4)	Lettuce	Motijheel
UL2(6)	Lettuce	Uttara
ML1(1)	Lettuce	Motijheel
MBCV2(12)	Chopped vegetables	Banani
UC2(2)	Capsicum	Uttara
UC2(3)	Capsicum	Uttara
MKL1(5)	Lettuce	Mohakhali
ML2(3)	Lettuce	Motijheel
ERL2(3)	Lettuce	Elephant Road
UC2(10)	Capsicum	Uttara
AS1(8)	Sprout	Gulshan
UC2(4)	Capsicum	Uttara
ML2(7)	Lettuce	Motijheel
UFB(12)	Broccoli	Uttara
MCV1(20)	Chopped Vegetables	Banani
EB1(3)	Broccoli	Elephant Road
MCV1(10)	Chopped Vegetables	Banani
MCV1(4)	Chopped Vegetables	Banani
MCV2(16)	Chopped Vegetables	Banani
MCV1(18)	Chopped Vegetables	Banani
UL3(7)	Lettuce	Uttara
MoSAL(10)	Cucumber	Mohakhali

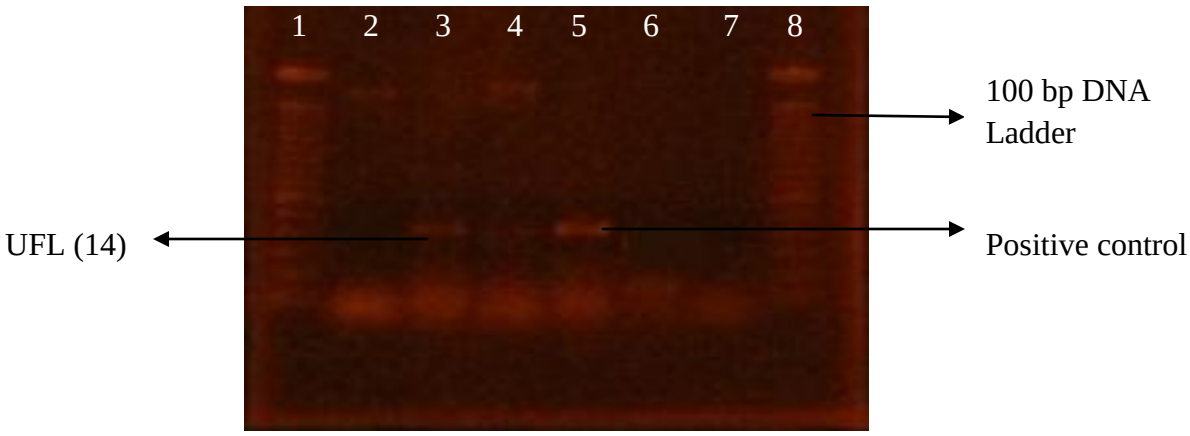
3.3 Genotypic Characterizations of the Isolates

Detection of *E. coli* O157:H7 Specific virulence Genes by PCR

Template DNA was prepared from cellular DNA of biochemically identified isolates by boiling method and 5 µl of template DNA was subjected to PCR for the detection of *E. coli* specific virulent genes *stx*1 and *stx*2 using specific primers. Isolates that gave bands of expected size were suspected to carry these genes in their chromosomes. The result revealed that isolate AS1(7), AS1(8), UFL(14), UFL(17), ML2(3), ML2(7), MBCV2(12) and MBCV2(16) were positive for *stx*1 but were negative for *stx*2 whereas the reference *E. coli* O157:H7 strain was positive for both *stx*1 and *stx*2 genes. The PCR results are presented in the Table 3.4 and illustrated in the Figure 3.6.

Table 3.4: Virulence genes in the isolates and reference strain

Isolates	Polymerase Chain Reaction resutls	
	stx1	***stx2***
AS1(7)	+	-
AS1(8)	+	-
UFL(14)	+	-
UFL(17)	+	-
ML2(3)	+	-
ML2(7)	+	-
MBCV2(12)	+	-
MBCV2(16)	+	-
E. coli O157:H7ATCC 12079	+	+



Lane 1: 100 bp marker | Lane 2: Blank

Lane 3: UFL (14) | Lane 4: ML2 (3)

Lane 5: Positive control (*E. coli* O157:H7 12079) | Lane 6: Negative control

Lane 7: Blank | Lane 8: 100 bp marker

Figure 3.6: Agarose gel electrophoresis showing 348 bp PCR amplification products of the *stx*1 gene.

3.4 Phenotypic Characterizations of the Isolates

3.4.1 Enterotoxicity Test

In ligated rabbit ileal loop model, live cells prepared from the isolates all showed strong enterotoxic activity (Figure3.7)

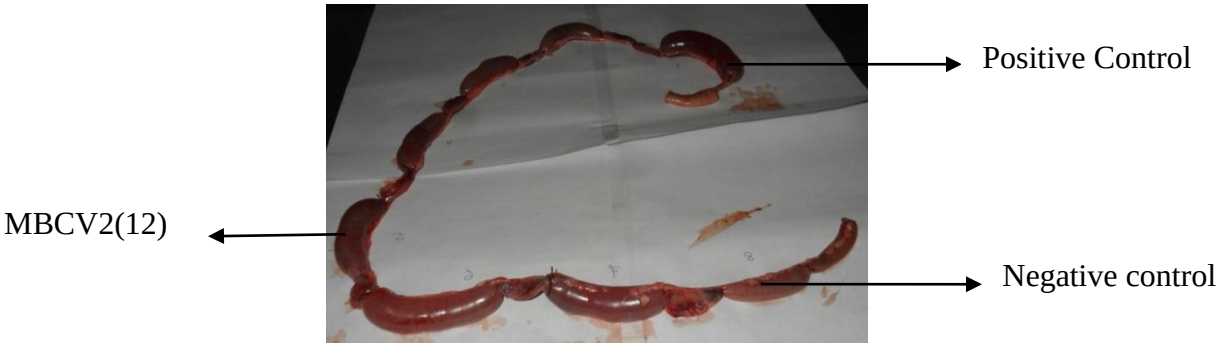


Figure 3.7: Ligated rabbit ileal loop after autopsy (Positive control=known toxigenic *Vibrio cholerae*, Negative control= Sterile culture broth)

3.4.2 Cytotoxicity Test

Culture filtrate of all *stx*1 positive isolates including positive control *E. coli* O157:H7 were tested for cytotoxicity assay using BHK 21 cell line. 50µl of filtrate was added with 450µl of cell thus making it 10 fold dilutions.

It was observed that comparing with the control (only cell) if considered as 100% (no dead cell) cell free extract of all 8 isolates shows near about 15- 20% killing of cell .

So the culture filtrates of the isolates show cytotoxicity in comparison with control (Figure 3.8).

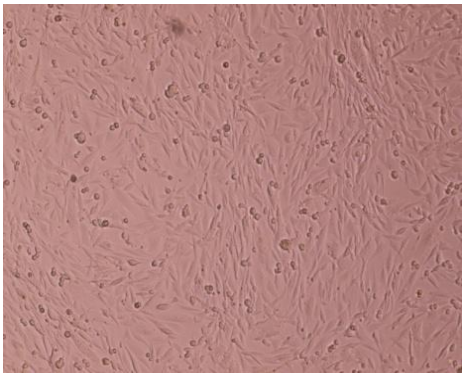
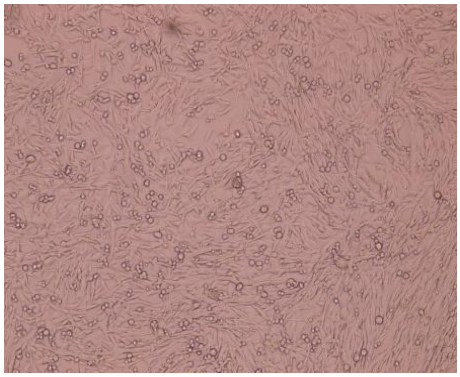
(a)  **(b)** 

Figure 3.8: BHK 21 appearances treated with *E. coli* isolates (a) control cell (b) cell treated with bacteria free extract.

3.4.3 Indirect Neurotoxicity Test

Mouse Lethality Test

Culture filtrate of all the isolates showed no lethal activity due to neurotoxicity though some short of dizziness & paralysis were observed after delivery of culture filtrate. To be more precise all the mice showed partial paralysis of hind legs for 3 days and later recovered. All the control mice died within 3-4 days.

3.4.4 Determination of Hemolysin Activity

With all the isolates hemolysin activity was determined on sheep blood agar plate and all of them showed alpha hemolysis around the growth which indicated that isolates were able to produce partial hemolysis (Figure 3.9).

Figure 3.9: Test of hemolytic activity

3.5 Antimicrobial Susceptibility

Antimicrobial susceptibility pattern of all 30 *E. coli* isolates is given below (Table3.5)

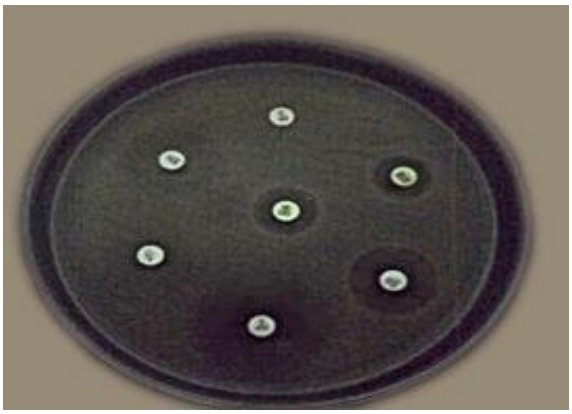


Figure3.10: Antibiogram of *E. coli* isolate

Table 3.5: Antimicrobial susceptibility pattern of *E. coli* isolated from raw vegetables

SL No	Antimicrobial Agent	Symbol	Disc Content (µg)	Susceptibility (n = 30)		
				Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
1	Amoxicillin/Clavulanic Acid	AMX	30	15 (50)	0 (0)	15 (50)
2	Ampicillin	AMP	10	15 (50)	0 (0)	15 (50)
3	Cefoxitin	FOX	30	0 (0)	0 (0)	30 (100)
4	Cefuroxime/Sodium	CXM	30	10 (33)	0 (0)	20 (67)
5	Chlorampenicol	C	30	0 (0)	0 (0)	30 (100)
6	Ciprofloxacin	CIP	5	10 (33)	0 (0)	20 (67)
7	Gentamycin	CN	10	0 (0)	0 (0)	30 (100)
8	Kanamycin	K	30	0 (0)	10 (33)	20 (67)
9	Nalidixic Acid	NA	30	0 (0)	0 (0)	30 (100)
10	Nitrofurantoin	F	300	0 (0)	0 (0)	30 (100)
11	Norfloxacin	NOR	10	0 (0)	0 (0)	30 (100)
12	Streptomycin	S	10	15 (50)	8 (27)	7 (23)
13	Sulphamethoxazole/ Trimethoprim(19:1)	SXT	25	0 (0)	0 (0)	30 (100)
14	Tetracycline	TE	30	10 (33)	0 (0)	20 (67)

Chapter 4:

Discussion…

4. Discussion

The consumption of fresh produce has increased over last two decades due to an important source of vitamins, nutrients and fiber (Olaimat and Holley, 2012). Moreover consumers' awareness of health aspects of fresh vegetables and fruits have increased and consumers are more concerned about correct eating habits and staying healthy. But at the same time outbreaks of food-borne illness related to fresh produced have increased (Warriner *et al*., 2009)

Numerous studies have investigated the potential sources of produce contamination in the supply chain both at the pre-harvest (in the field) and post-harvest stages. During the pre-harvest phase, pathogen populations can establish themselves on growing crops. The risk can be amplified after harvest either by further direct contamination or by proliferation of existing pathogen populations during processing and post harvest handling procedures. Water is likely to be an important source of contamination in the field. Possible sources are run-off from nearby animal pastures and irrigation from a contaminated source. The risk associated with using water from a range of sources that vary in microbiological quality for irrigation of produce has been assessed and the need for improved guidelines recognized (Hamilton *et al*., 2006; Tyrrel *et al*.,2006). While studies have found no internalization of *E. coli* O157:H7 in spinach plants through contaminated soil, uptake and internalization was found in spinach leaves after contaminated water was dropped on the leaves (Mitra *et al*., 2009). This suggests a lower likelihood of transmitting pathogen from contaminated water through drip irrigation versus a higher likelihood through overhead sprinkler systems. However, irrigation is not the only reported route of contamination linked to water. Use of water in postharvest processing has also played a role. In the US outbreak of *E. coli* O157 infections associated with pre-packaged spinach, trace-back and environmental investigations determined that one ranch in California's Salinas Valley was the likely source of the outbreak. The patterns produced by pulsed-field gel electrophoresis (PFGE) and multi locus variable number tandem repeat (MLVNTR) analysis from the strains involved in the outbreak matched those from isolates recovered from local feral swine and cattle feces (Jay *et al*., 2007). However, the manner in which the spinach became contaminated was not determined.

Insects are also a possible source of contamination. In laboratory conditions, contaminated flies have been shown to directly transfer bacteria to plant leaves or fruits (Iwasa *et al*., 1999; Sela *et al*., 2005; Talley *et al*., 2009). Studies have implicated flies in contamination of leaves by *E. coli* O157:H7 (Iwasa *et al*., 1999). Large numbers of flies belonging to the *Muscidae* and *Calliphoridae* families which were found in production fields adjacent to rangel and habitats occupied by cattle were shown to carry *E. coli* O157:H7 (Talley *et al*., 2009). Post harvesting processes, ranging from storage and rinsing to cutting, are also possible sources of contamination (Wachtel and Charkowski, 2002). The use of inadequately decontaminated water in hydrocoolers, which are used to store and process large quantities of fresh produce, can lead to contamination of an entire lot (Gagliardi *et al*., 2003).

Many studies have been conducted on the behavior and survival of human pathogens on plants. Most have focused on *E. coli* (largely *E. coli* O157:H7). Shiga toxin-producing *E. coli* (STEC) is a zoonotic pathogen colonizing mainly cattle and small ruminants. Although cattle products, principally beef, are the most commonly recognized sources of *E. coli* O157 infections, fruits and vegetables consumed raw are also an important source (Rangel *et al*., 2005). Three leaf attachment mechanisms have been described in *E. coli* O157. It adheres strongly to tomato skin, spinach leaves and roots of alfalfa sprouts. Adhesions to these surfaces are mediated by curli (Jeter and Matthysse, 2005). Expression of curli on the surface of non-pathogenic *E. coli* was shown to be sufficient to enable bacterial attachment to alfalfa roots, but deletion of curli genes in *E. coli* O157 did not abolish adhesion, suggesting that other attachment factors were involved (Jeter and Matthysse, 2005). Shaw and colleagues (2008) have shown that adhesion of *E. coli* O157, as well as the related enteropathogenic *E. coli* (EPEC), to a variety of salad leaves is mediated by the filamentous type III secretion system (T3SS), which is composed of EspA filaments (Knutton, 1995). Xicohtencatl-Cortes *et al*. have shown that flagella also play a role in *E. coli* O157 leaf attachment as deletion of *fliC* encoding flagellin reduced the level of adhesion (Xicohtencatl-Cortes *et al*., 2009). All this suggests that *E. coli* O157 uses multiple mechanisms to colonize plants and are well adapted to biosphere.

The phyllosphere is not the only ecological niche in which human pathogens are associated with plants. Whereas no evidence of uptake of *E. coli* O157 from soil to internal plant tissue was observed (Jablasone *et al*.,2005; Sharma *et al*., 2009), reports suggest that plant roots could be colonized by *E. coli* O157 and *Salmonella* (Wachtel *et al*., 2002; Islam *et al*., 2004 a,b; Jablasone *et al*., 2005; Jeter and Matthysse, 2005).

In Bangladesh, no *E. coli* infection from raw vegetables has been reported yet. Although *E. coli* O157:H7 has been isolated from raw meat, raw milk, and street vended juice (Islam *et al*., 2010). The reasons might be (1) the lack of proper surveillance, (2) the presence of very low number of bacteria in environmental and food samples, (3) relatively few infections due to acquired immunity in the population and (4) cooking practices that effectively eliminate the pathogen and (5) tendency of not having enough fresh vegetables (Islam *et al*., 2007).

For this purpose, we examined over 60 raw vegetable samples to isolate *E. coli* and to determine the phenotypic and genotypic characteristics of the isolates. The organism is present in low number in food samples and contains many other bacteria in high number besides the target one and the desired bacterium may be lost by direct plating. Therefore, the present investigation suggests that food-borne organisms prefer enrichment media prior to isolation on selective plate. Enrichment medium used in the present study for the isolation of *E. coli* was enrichment broth which contains bile salt as selective agent to suppress the growth of Gram-positive and other non-enteric bacteria.

After overnight selective enrichment, the diluted broth culture was plated onto MacConkey (MAC) agar medium which is the most commonly used selective medium for the isolation of *E. coli*. The medium contains bile salts (to inhibit most Gram-positive bacteria), crystal violet dye (which also inhibits certain Gram-positive bacteria), neutral red dye (which stains microbes fermenting lactose), lactose and peptone. In this agar media *E. coli* appears as specific deep pink colony.

Over 65 suspected *E. coli* isolates were inoculated onto EMB agar plates. After incubation, a total of 30 isolates produced green metallic sheen growths on EMB plates. All the EMB positive isolates were subjected to biochemical testing that are widely used to differentiate bacteria of enterobacteriaceae family. The tests included indole production, MR-VP reaction, citrate

utilization, TSI and fermentation test. Thirty isolates gave positive reaction pattern similar to that of reference *E. coli* O157:H7 strain.

After isolation and biochemical identification, all isolates were subjected to detailed genotypic and phenotypic characterizations. The genotypic characterizations were carried out by the detection of virulence genes. The most important components of virulence in *E. coli* infection are production of one or more Shiga toxins (encoded by *stx*1 and *stx*2) which inhibit protein synthesis of host cells leading to death and production of intimin protein which is responsible for intestinal colonization and development of attaching-effacing lesions. The *stx*1 gene is virtually identical to Shiga toxin produced by *Shigella dysenteriae*, while *stx*2 has only 56% identity to *stx*1. The use of PCR to amplify *stx* genes serves as a highly specific and sensitive method to detect virulent *E. coli*. The isolates AS1(7), AS1(8), UFL(14), UFL(17), ML2(3), ML2(7) MBCV2(12) and MBCV2(16) showed positive results for *stx*1 PCR where reference *E. coli* O157:H7 strain showed positive PCR results for both the genes.

As 8 isolates out of 30 isolates were positive to *stx*1 gene, we may conclude that these are STEC or Shiga toxin producing *E. coli*.

The phenotypic characterizations of the isolates were carried out by enterotoxicity, cytotoxicity test, neurotoxicity test and hemolytic activity. The enterotoxicity test was performed in ligated rabbit ileal loop model. The live cells of the isolates were found to be stongly enterotoxic. The reason might be that the isolates produced Shiga-like toxin which had potential to cause watery diarrhea like toxigenic *Vibrio cholerae* in the rabbit gut. Therefore, fluid was accumulated in the ileal loop segment. In each experiment one of the rabbits was found to be dead which might be because of over toxin production of Shiga-like toxin. More investigation is required to find out the causative agent of much toxin production in the rabbit intestine.

Neurotoxicity assay was done to find out the toxigenic property of the isolates. Neurotoxin is not an inherent property of *E. coli* but the test was performed to ensure that these are not neurotoxigenic. The mice were found to show some symptoms of paralysis after 72 hours of injection. But later these mice were found to be active like earlier. Death is the final stage of neurotoxin due to inhibition of autonomic nervous system. From this observation it is clear that the toxin produced was not lethal.

Cytotoxicity assay was done to find out the capability of producing any toxin that is lytic to cells. BHK 21 cell line was used for this purpose. Cytotoxicity can be measured by the membrane integrity. All these *E. coli* isolates were capable of producing cytotoxin.

Antimicrobial susceptibility tests were done where different groups of antibiotics were used. Antibiogram was done using Macfarland standard. Antibiogram was done to understand the susceptibility pattern of these isolates. There are few reports of MDR (multi drug resistant) *E. coli* isolated from raw vegetables and fresh fruit. Isolates were 100% sensitive to cefoxitin, chloramphenicol, gentamycin, nalidixicAcid, nitrofurantoin, norfloxacin and sulphamethoxazole. Although isolation of *stx*1 gene positive *E. coli* is very alarming for us but as these are sensitive to many antimicrobials agent if the infection is identified early it is fully curable.

The lack of an effective antimicrobial treatment at any step from planting to consumption means that pathogens introduced at any point may be present on the final food product. Washing and rinsing some types of fruits and vegetables prolong shelf-life by reducing the number of microorganisms on the surfaces. Use of a disinfectant can enhance efficiency of removal up to 100 fold, but chemical treatments administered to whole and cut produce typically will not reduce populations of pathogens by more than 2 to 3 $\log_{10}$ CFU/g (Beuchat *et al*., 1998).

The elimination of bacteria from seeds by chemical or physical treatment is critical for reducing the risks of sprout borne disease outbreaks. Thermotherapy (e.g., hot water treatment) is another option that has been explored for seed disinfestation (Grondeau *et al*., 1994). There have been many reports describing the efficacy of chemical seed treatments for sprout seed including chlorine compounds (e.g., calcium and sodium hypochlorite), ethanol, hydrogen peroxide, calcium EDTA, 4-hydroxybenzoic acid, ozonated water, and commercial disinfectants (Beuchat *et al*., 1997; Beuchat *et al*., 1998; Lang *et al*., 2000; Taormina *et al*., 1999). Treatment of sprout seed with gaseous chemicals has also been evaluated (Delaquis *et al*., 1999). Ionizing radiation has been employed but failed to completely eliminate bacteria like *E. coli* O157 unless very high dose of radiation is used.

Conventional methods to reduce the rate of contamination include good agricultural practices in the seed production field, adherence to good manufacturing practices during minimal processing, proper harvesting and storage, seed testing, and antimicrobial treatments.

Concluding remarks

The present study was aimed at isolating *E. coli* from raw vegetables by using selective enrichment broth and selective media and characterizations of the isolates. For this purpose, raw vegetable samples, street salad and chopped vegetable samples were tested. Out of 60 selected samples 30 *E. coli* isolates were recovered. The recovery rate of Shiga toxin producing *E. coli* was found to be 20% from raw vegetable samples and 6.7% from chopped vegetables. Biochemical behaviors of all 30 isolates were identical to that of the reference strain. With these isolates molecular characterizations were carried out. Eight isolates were found to carry *stx*1 gene. In ligated rabbit ileal loop model, live cells of the representative isolates gave strong enterotoxic activity. On the other hand, culture filtrate of the representative isolates showed neurotoxic activity to some extent and were capable of producing cytotoxin. These isolates were also capable to partially lyse erythrocyte by the production of hemolysin. All isolates showed varying range of resistance to different antibiotics used. To our knowledge, this is probably the first report on isolation of Shiga toxin producing *E. coli* from raw vegetables in Bangladesh. The isolates were found to be highly pathogenic as they carried virulence gene *stx*1, which is responsible for hemolytic colitis and hemorrhagic uremic syndrome. Based on the risk of the presence of shiga toxin producing *E. coli* from raw vegetables people need to wash the raw vegetables thoroughly before taking and chopped vegetables before cooking. Contamination of *E. coli* might be due to watering these vegetables with contaminated water, lack hygiene while transport, in few cases while cultivation (lettuce). Prevention strategies may include the use of hand washing sanitizer, controlled and supervised handling of raw and chopped vegetables and clear separation of food-related activities from the areas of housing animals. All these may help to reduce the risk of transmission of this pathogen.

Chapter 5:

References…

5. References

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APPENDIX-I

Media composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121^{o}C for 15 min.

1. **MacConkey agar (Oxoid, England)**

Ingredients	Amount (g/L)
Peptone	20.0
Lactose	10.0
Bile salt no. 3	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
pH	7.1±0.2

2. Eosine methylene blue agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	10.0
Lactose	10.0
Di-potassium hydrogen phosphate	2.0
Eosin Y	0.4
Methylene blue	0.06
Agar	15.0
Final pH	6.8±0.2

3. Simmon's citrate agar (Oxoid, England)

Ingredients	Amount (g/L)
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto brom thymol blue	0.08

4. Triple sugar iron agar

Ingredients	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulfate	0.2
Sodium thiosulfate	0.2
Phenol red	0.0125
Agar	13.0
pH	7.3

5. MR-VP broth

Ingredients	Amount (g/L)
Peptone	7 g
Dextrose	5 g
Di-potassium hydrogen phosphate	5 g
Final pH	6.9

6. Tryptic soy broth (Oxoid, England)

Ingredients	Amount (g/L)
Casein peptone	17.0
Soya peptone	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5
Final pH	7.3±0.2

7. Peptone water

Ingredients	Amount (g/L)
Peptone	10 g
Sodium chloride	5 g

8. T_1N_1soft agar

Ingredients	Amount (g/L)
Tryptone	10 g
Sodium chloride	10 g
Agar	6 g

9. Luria Bertani broth

Ingredients	Amount (g/L)
Tryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g

10. Blood agar

Ingredients	Amount (g/L)
Lab lemco powder	10
Peptone	10
NaCl	5
Agar	15
Sterile defibrinated blood	70 ml
Final pH	7.3±0.2

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11. Muller hinton agar

Ingredients	Amount (g/L)
Beef Extract	3.0
Acid Hydrolysis of Casien	17.5
Starch	1.5
Agar	15
Distilled Water	1L

12. EC broth

Ingredients	Amount (g/L)
Tryptone	20.0
Lactose	5.0
Bile salt(No.3)	1.12
NaCl	5.0
K_2HPO_4	4.0
KH_2PO_4	1..5
pH	6.7±0.2

13. Carbohydrate fermentation (phenol red broth)

Ingredients	Amount (g/L)
Tryptone	10.0
NaCl	5.0
Sugar	5.0
Phenolred	0.2
Distilled water	1L
pH	7.3

14. Brain heart infusion (Oxoid, England)

Ingredients	Amount (g/L)
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium Chloride	5.0
Di-sodium phosphate	2.5
Final pH	7.4±0.2

15. Nutrient agar

Ingredients	Amount (g/L)
Peptone	5.0
NaCl	5.0
Beef extract	3.0
Agar	15
Distilled water	1L
pH	7.0

APPENDIX-II

Buffers and reagents

Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na_2HPO_4 and 2.0 gm of KH_2PO_4 in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 liter by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

0.5 M EDTA

18.61 gm of $Na_2EDTA.2H_2O$ (disodium ethylene diamine tetra-acetic acid) was dissolved in 80 ml of distilled water and the pH was adjusted to 8.0 with pellets of NaOH. Thefinal volume was made up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

10 x TBE (pH 8.3)

54.0 gm of Tris-base, 27.5 gm of boric acid and 20ml of 0.5 M EDTA (pH 8.0) were taken and distilled water was added to the mixture to make 500 ml.The buffer was stored at room temperature.

Sodium pentobarbital solution

250 ml Sodium pentobarbital solution contains 16.19 gm of sodium pentobarbital, 25 ml of absolute ethyl alcohol, 50 ml of propylene glycol and 5 ml of benzyl alcohol.

Gel loading buffer

10 x concentrated loading buffer consisted of 800µl of 20% Ficoll 400,400µl of 0.1 M EDTA (pH 8.0), 10 µl of 0.25% bromophenol blue and 200µl of 1% SDS in 590 µl of distilled water. It was stored at 4^oC in 1 ml aliquot.

Ethidium bromide solution

2.5 mg of ethidium bromide (Sigma, USA) was dissolved in 5 ml of distilled water at a concentration of 0.5 mg/ml. This solution was covered with aluminum foil and stored at room temperature.

Kovac's reagent

1.25 gm of para-dimethylaminobenzaldehyde was dissolved in 18.75 ml of amylalcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4^{o}C.

Methyl red reagent

0.01 gm of methyl red was dissolved in 30 ml of 95% ethanol.Then distilled water was added to make the final volume 50 ml. This reagent was covered with aluminum foil and stored at 4^{o}C.

Barritt's reagent

Solution A

1.25 gm of alpha-naphthol was dissolved in 95% ethanol with constant stirring to make 25 ml solution.This solution was covered with aluminum foil and stored at 4^{o}C.

Solution B

10 gm of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added to adjust the final volume to 25 ml.This solution was covered with aluminum foil and stored at 4^{o}C.

McFarland turbidity standrand No.5

Sulfuric acid	0.18M
Barium chloride	0.048M
Distilled Water	1000ml

APPENDIX-III

Instruments

The important equipment used through the study are listed below

- Autoclave, Model no: WAC-47 :Korea
- Sterilizer, Model no: NDS-600D :Japan
- Balance(Core series) :Adam,UK
- Centrifuge, Model no: Code: 5433000.011 :Eppendorf Germany
- Digital Homogenizer(Wise Tis) :Korea
- Freezer(-20°C) :Siemens ,Germeny
- Gel Documentation System :Major Science, Taiwan
- Horizontal Gel Electrophoresis Unit :Wealtec Corporation,USA
- Incubator :UK
- Laminer airflowcabinet :UK
- Millipore filter,045 μm :UK
- Micropipettes :Eppendorf, Germany
- Oven(Universal drying oven)Model:LDO-060E, :Labtech,Singapore
- Thermal Cycler, Model no: 2720 :Applied Biosystems,USA
- Refrigerator ,Model-0636 :Samsung,
- Shaking Incubator , Model-WIS-20R, :Korea
- UV Transluminator :Wealtec Corporation,USA
- Vortex Mixture :VWR International