Isolation of Shiga toxin producing *Escherichia coli* 0157:H7 from street food and raw vegetables in Dhaka City

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

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Declaration

I hereby declare that the thesis work titled "**Isolation of Shiga toxin producing *Escherichia coli* 0157:H7 from street food and raw vegetables in Dhaka City**" has been written and submitted by me, Afra Anjum without the use of other sources than those mentioned.

It is further asserted that this Bachelor's Thesis has never been submitted in the same or substantially similar version to any other examinations office. All explanations that have been adopted literally or analogously are marked as such.

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Dedication

To

My Precious Parents,
Borobhaiya,
Chotobhaiya,
Bhabi & Ayan

Acknowledgement

I would like to thank all of the people who contributed in some way to the work described in this study. First and foremost, I would thank the Almighty for His blessings thatgave me the strength to persevereagainst all odds and perform well &complete this thesis.

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ABSTRACT

E. coli O157:H7, a Shiga toxin producing microbe was first acknowledged as a virulent organism in 1982 during an analysis of an outbreak of haemorrhagic colitis associated with consumption of hamburgers from a fast food chain restaurant. An inspection of more than three thousand *E. coli* cultures collected between 1973 and 1982 showed only one isolate with a serotype O157:H7. In the following ten years, there were just about thirty outbreaks documented in the United States. It is assumed that there might have been more cases, but remained unreported since an outbreak could not be detected as the number of affected was not large enough to lead it into an investigation.

Ability of *E. coli* O157:H7 to induce injury in humans is a result of its ability to produce numerous virulence factors, most notably Shiga toxins Stx1 and Stx2, both of which constitute one of the most potent toxins known to man. Endothelial cells line the interior surface of blood vessels, and are known to be tremendously susceptible to *E. coli* O157:H7, which is cytotoxigenic to these cells. Besides, Shiga toxin, *E. coli* O157:H7 produces several other virulence factors, which include proteins which aid in the attachment and colonization of the bacteria in the intestinal wall and which can break down red blood cells and release iron to help support metabolism in *E. coli* . Virulence factors facilitate this organism's ability to cause intestinal and extra-intestinal diseases such as diarrhoea, haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS), urinary tract infections (UTI), septicaemia and neonatal meningitis.

Street foods and raw vegetables are infamous for the lack of hygiene and potentially hazardous microbial quality. Ingestion of these foods may lead to diarrhoea. In this study r, 6 samples from Dhaka city was collected, cultured in various media for enumeration, isolation and screening of *E. coli* colonies which were further analysed to check for the presence of *stx* genes using PCR and gel electrophoresis. The six samples collected were: Salad, Fruit Mix, Sugar Cane Juice, Betel leaves, Pulse (from Bhelpuri) and raw coriander. The samples collected initially were enriched in enrichment media overnight, followed by a 6 fold dilution series which were then used for spread plating on nutrient agar and MacConkey agar. Pink colonies screened from MacConkey agar plates were streaked on EMB for confirmation with the observation of metallic sheen. The confirmed *E. coli* isolates were later subjected to DNA extraction and amplification after which the bands for *stx* genes were observed and recorded. Out of the six samples tested for stx1 and stx2 genes, four showed the presence of stx2 genes, which in many papers have been mentioned as the more virulent gene out of the two. The presence of the *stx2* genes in regular food signifies how close we are to a large outbreak. Knowledge of processing such food or avoiding when possible may prevent occurrence of foodborne diseases.

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1 INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Escherichia coli is one of the most ubiquitous microorganisms in the world. It was first discovered by a German bacteriologist, Theodor Escherich in 1885. This bacteria was thus named after him.

E.coli includes a variety of strains which exhibit activities that range from pathogenic to beneficial. *E.coli* is a non-spore forming, gram negative facultative anaerobe. The cells are typically rod shaped and range in diameter from 0.2 to 1μm and are about 2μm long. Strains that have flagella are motile and the flagella are usually observed to have a peritrichous arrangement. It can be commonly found in animal faeces, lower intestines of mammals, and on the edges of hot springs. They grow best at 37°C. This bacteria predominantly subsists in the intestines of all warm-blooded animals. Thus it was categorised as an enteric organism, related to the Greek-root of its Family name, *Enterikos*, meaning "intestine". *E. coli* contributes to bowel functions in human large intestines, where it can help in digestion processes, food breakdown and absorption, and vitamin K production.

1.2 Biochemistry of *E. coli*

The complete genome sequence of *E.coli* was first published on September 1997. Knowledge of the genome sequence was expected to facilitate the deepening of our understanding of this bacteria and contributing to easier identification of different strains. The strain of *E. coli* used for the sequencing project was not a pathogen. The *E. coli* genome consists of about 4,600,000 base pairs and contains approximately 4,000 genes (National Human Genome Research Institute).

E. coli is a **chemoautotroph** signifying that the naturally occurring (wild-type) strain of *E. coli* doesn't require any growth factors. It can synthesize all 20 amino acids, all vitamins, nucleotides, and fatty acids, which it uses during growth and metabolism, from water, simple inorganic chemicals and an energy source. It can extract the energy it needs through the oxidation of organic compounds. *E coli* can grow and reproduce much faster if these compounds are provided. Under the right conditions and with a complete mix of growth factors, an *E. coli* population can double in size about every 20 minutes. In the colon, where *E. coli* has

to compete for growth factors with other intestinal bacteria and the host, it can take around 12 hours for the population to double.

E.coli exercises mixed acid fermentation under anaerobic conditions and hence generates lactate, succinate, ethanol, acetate and carbon dioxide. The bacteria can grow either under aerobic or anaerobic condition. . This occurs via redox reactions, that includes the oxidation of pyruvic acid, formic acid, hydrogen and amino acids, and the reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide.

E. coli has the propensity to transfer DNA via bacterial conjugation, transduction or transformation, which permits genetic material to spread horizontally throughout an existing population. These methods have aided in the spread of the gene encoding the shiga toxin from Shigella to *E. coli*. Only 20% of the genome is common to all strains (ECDC factsheets). *E. coli* is referred to as an ideal model organism. The reasons behind this include their major presence in the intestine and ability to survive temporarily outside of the body. The bacteria grows easily in vitro and its genetic structure is simple and can easily be manipulated, hence making it an ideal model for studying.

1.3 Types of *E. coli*

The majority of *E. coli* strains are harmless but when strains acquire certain genetic material, they can develop into pathogens. *E. coli* strains are among the most recurrent of bacterial causes of diarrhoea and are stratified by the clinical syndromes they produce. The characteristics of diarrheal illness caused by enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC, also called Shiga toxin-producing *E. coli* or STEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC or EAggEc) are as follows

1.3.1 Enteropathogenic *E. coli* (EPEC)

EPEC is a major cause of potentially fatal diarrhoea in infants in developing countries (Kaper, Nataro and Mobley, 2004). It is a causative agent of diarrhoea in humans, rabbits, dogs, cats and horses. EPEC lack fimbriae, ST and LT toxins. They contain an adhesin known as intimin which aids them to bind host intestinal cells.

EPEC consist of virulence factors that are analogous to those found in Shigella, and may produce a shiga toxin. Adherence to the intestinal mucosa results in a reshuffling of actin in

the host cells, causing considerable deformation. EPEC cells are not highly invasive. They enter host cell and cause inflammation.

1.3.2 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *Escherichia coli*, or ETEC, are a group of *E. coli* that produce special toxins which stimulate the lining of the intestines causing them to secrete excessive fluid, thus producing diarrhoea. ETEC employ fimbrial adhesins (projections from the bacterial cell surface) to attach enterocyte cells in the small intestine. ETEC can produce two proteinaceous enterotoxins: (i) LT a heat-labile enterotoxin, is the larger among the two, and resembles cholera toxin in structure and function while (ii) heat-stable toxin ST, is the smaller enterotoxin that causes cGMP build-up in the target cells followed by a secretion of fluid and electrolytes into the intestinal lumen. Although different strains of ETEC can produce either one or both of these toxins, the illness caused by each toxin is similar. ETEC is transmitted by food or water contaminated with animal or human faeces.

1.3.3 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) subsists only in humans. Infections resemble shigellosis in pathogenesis and symptoms include a dysentery-like diarrhoea with fever. EIEC infiltrate and proliferate within epithelial cells of the colon causing widespread cell destruction. They do not produce toxins, but damages the intestinal wall severely through mechanical cell destruction. EIEC strains are invasive organisms, the invasion usually does not overtake the submucosa layer.

1.3.4 Enteroaggregative *E. coli* (EAEC)

EAEC is found only in humans. EAEC was given its name due to the occurrence of fimbriae which aggregate tissue culture cells. They are non-invasive. EAEC binds to the intestinal mucosa to cause watery diarrhoea without causing inflammation. They make a haemolysin and an ST enterotoxin that is similar to that of ETEC.

1.3.5 Enterohaemorrhagic *E. coli* (EHEC)

EHEC is known as Verotoxin-producing *Escherichia coli* as EHEC causes the haemolytic-uremic syndrome (HUS) and sudden kidney failure. This group is also known by a number of other names, including shiga-like toxin-producing *E. coli* (STEC or SLTEC), haemolytic

uremic syndrome–associated enterohaemorrhagic *E. coli* (HUSEC) and verocytotoxin-orverotoxin-producing *E. coli* (VTEC).

EHEC uses bacterial fimbriae for attachment. It is moderately invasive and has a phage-encoded shiga toxin that can induce an intense inflammatory response. The toxin elucidates 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).*E. coli* (EHEC) is found to cause disease in humans, cattle, and goats. The most infamous member of this virotype is strain O157:H7, which causes bloody diarrhoea.

1.4 Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*

Virulence factors are produced by organisms that contribute to pathogenicity and aid bacteriain survival and growth. Adhesins, invasins, and antiphagocytic factors help to support and maintainthe colonization of the host. The factors that cause damage to the host comprise of toxins, haemolysins, and proteases.

Specific pathogens have a wide range of virulence factors. Some are encoded chromosomally and are essential to the bacteria. These include capsules and endotoxins, whereas others are gained from mobile genetic elements like plasmids and bacteriophages (some exotoxins). Virulence factors encoded on mobile genetic elements spread through horizontal gene transfer, and can convert harmless bacteria into dangerous pathogens. Bacteria like *Escherichia coli O157:H7* gained the majority of its virulence from mobile genetic elements.

1.4.1 Adhesion

Fimbrial adhesins

Fimbriae are short, fine appendages surrounding the cell. These protein projections aid in adhesion to surfaces of host cells. Fimbriae are prime factors in facilitating bacterial virulence since they enable the bacteria to colonize human epithelial cells (cells of mucous membranes). At the end of each fimbria are special proteins called adhesins. The explicit category of adhesins differs by type of bacteria, but in spite of the type, adhesins permit bacteria with fimbriae to adhere to host cells by docking, like a lock and key, with receptor proteins on the surface of host epithelial cells.

The presence of fimbriae on *E. coli* O157 is conflicting (Sherman, Cockerill, Soni, and Brunton.*1991)* and the role of the pO157 plasmid in adhesion is also controversial, with decreased adhesion, increased adhesion and no change in adhesion associated with pO157 loss (Junkins and Doyle 1989)

Intimin

Intimin facilitates intimate colonization of *Escherichia coli* O157:H7in the microvillus brush border of enterocytes forming a characteristic attaching and effacing lesion. After being expressed by the bacterial cell surface, it binds to the translocated intimin receptor (Tir). Along with over 25 other bacterial proteins, Tiris released from attaching and effacing *E. coli* directly into the cytoplasm of intestinal epithelial cells by a Type 3 Secretion System (T3SS). Once inside the cytoplasm of the host cell, Tir is introduced into the plasma membrane, enabling surface exposure and intimin binding.T3SS is a protein appendage that injects bacterial virulence proteins into eukaryotic cells to alter their physiology for the benefit of the pathogen.

Although fimbriae and OMPs have been identified which may be associated with STEC adhesion, most studies have concentrated on intimin as a potential adhesin. (Hicks, Frankel, Kaper, Dougan, and Phillips 1998).*In vivo* studies using calves and gnotobiotic piglets demonstrated that intimin-deficient *E. coli* O157 mutants were less virulent than the parent strain and were less able to colonize the intestine of these animals (Dean-Nystrom, Bosworth, Moon, and O'Brien *1998*).

1.4.2 Shiga toxins

There are two main antigenically distinct type of toxins, Stx1 and Stx2 (O'Brien and Holmes 1987). Stx1 and Stx2 are compound toxins consisting of an A subunit (32 kDa) and a pentameric B subunit (7.7 kDa monomer). The B subunits form a hollow ring and the C-terminus of the A subunit is inserted into this. The B subunits intercede binding to receptors in eukaryotic cell membranes and the receptors have been identified as globotriaosylceramide (Gb3).Toxin molecules are taken in by a receptor-mediated endocytic mechanismwhen the toxins are attached to the target cell membrane. Toxin-containing vesicles are formed which in some cases cause the vesicles to undergo fusion with lysosomes resulting in toxin degradation. In other cases, the A subunit is ‘nicked’ by a protease generating a catalytically active 27-kDa A1 fragment and a 4-kDa A2 subunit, after the processing in the Golgi apparatus and endoplasmic reticulum. The released A1 subunit has RNA N-glycosidase activity and cleaves

a specific bond in the 28S rRNA. This cleavage prevents binding of amino acyl-tRNA to 60s ribosomal units, inhibiting the peptide chain elongation step of protein synthesis which then leads to cell death (Sandvig and van Deurs 1996).

Although the majority of strains of *E. coli* O157 produce Stx2 only, amongst the non-O157 STEC ones the toxin phenotype is much more variable with isolates producing Stx1 alone occurring commonly. There is considerable epidemiological evidence to indicate that STEC isolates producing Stx2 are more commonly associated with serious diseases than isolates producing Stx1 or Stx1 and Stx2 (Kleanthous, Smith, Scotland, Gross, Rowe, and Taylor1990*)*.Although Stx1 and Stx2 have similar structures and modes of action their toxicities appear to be distinct. Stx2 was a 1000 times more cytotoxic than Stx1 towards human renal microvascular endothelial cells which is the putative target of Shiga toxins in the development of HUS (Louise and Obrig 1995). The increased toxicity of Stx2 was not related to a greater number of receptors for this toxin on the cell surface, indeed the reverse was true. In a mouse model, infection with an *E. coli*O157 isolate producing both Stx1 and 2 resulted in fatal cortical tubular necrosis. (Law, D. 2000) Death was prevented by passive immunization with a monoclonal antibody to Stx2 but not to Stx1. Challenging mice with a strain of *E. coli* K-12 carrying cloned Stx2 caused death, but *E. coli* K-12 carrying cloned Stx1 did not (Wadolkowski, Sung, Burris, Samuel, and O'Brien 1990). Tesh, Burris, Owens, Gordon, Wadolkowski, O'Brien (1993) went on to show that purified Stx2 had an approximately 400-fold lower LD_{50} for mice than Stx1. The association of Stx2 producing *E. coli* with the development of HUS may reflect a preferential ability of Stx2 to damage renal glomerular vasculature. Using transformed human intestinal microvascular endothelial cells Jacewicz, Acheson, Binion, West, Lincicome & Fiocchi(1999)showed that Stx2 was more toxic than Stx1 for these cells. The authors concluded that the increased toxicity of Stx2 to endothelial cells may be relevant to the preponderance of Stx2 producers in the pathogenesis of HC. The method via which renal damage done by Shiga toxins are produced in the bowel is not known yet.

1.4.3 Enterohaemolysin

Enterohaemolysin (Ehx) is identified by the production of small turbid zones of haemolysis after subsequent incubation on blood agar with washed erythrocytes for 18–24 hours. The precise role of Ehx in human infection is unknown and it is possible that the toxin may be of more relevance to infection in animals. However, the *ehx* operon is highly conserved among STEC strains suggesting that Ehx is under a strong selective pressure and plays an important

role in STEC survival (Boerlin Chen, Colbourne, Johnson, De Grandis & Gyles 1999). Ehx is not crucial for development of HUS and HC although it is recurrently linked with isolates causing severe disease. Its connection in human disease is unclear. The function of Ehx is therefore unknown, there is no convincing data of a role in human infections and the toxin may be more important in aiding infectionsin animals.

1.5 Serotype

Serotype or serovar are distinct serological variants, which differ from one another mainly or solely in the antigenic identity of their lipopolysaccharide, flagella, or capsule. Different serovars of enteric bacteria are often found to be linked with the ability to inhabit different hosts. Pathogenic *E.coli* strains can be categorised based on elements that can elicit an immune response in animals, namely:

1. O antigen: part of lipopolysaccharide layer
2. K antigen: capsule
3. H antigen: flagellin

1.5.1 O antigen

The outer membrane of an *E. coli* cell contains millions of lipopolysaccharide (LPS) molecules, which consist of:

1. O antigen, a polymer of immunogenic repeating oligosaccharides (1-40 units)
2. Core region of phosphorylated non-repeating oligosaccharides
3. Lipid A (endotoxin)

The O antigen is used for serotyping *E.coli* and these O group designations go from O1 to O181. The O antigen is encoded by the rfb gene cluster while the rol (cld) gene encodes the regulator of lipopolysaccharide O-chain length.

1.5.2 K antigen

The acidic capsular polysaccharide (CPS) is a thick, mucous-like, layer of polysaccharide that surrounds some pathogen *E. coli*. There are two separate groups of K-antigen groups, named group I and group II. The former consists of 100 kDa (large) capsular polysaccharides, while the latter is associated with extra-intestinal diseases and are under 50 kDa in size.

1.5.3 H antigen

It is the antigen in the flagella of motile bacteria and is important in serologic classification of enteric bacteria. The flagella allows *E. coli* to move. H antigens groups go from H1 to H56 with some exceptions.

1.6 Detection of *E. coli*

1.6.1 *E. coli* 0157:H7 Chromogenic Agar Base

Chromogenic culture media enable faster detection of specific microorganisms compared to classical culture media, based on the detection of specific enzyme activities. They also improve sensitivity and can reduce the need for sub-culturing and confirmatory tests. These chromogens are biochemical compounds that produce a visible and qualitative colour change when degraded by specific microbial enzymes. Rainbow chromogenic agar is a medium that contains chromogenic substrates that are specific for two *E. coli* associated enzymes: β-galactosidase (a blue-black chromogenic substrate) and β-glucuronidase (a red chromogenic substrate).Strain O157:H7 is typically glucuronidase negative so it forms unique and distinctive black or grey colonies.

Many other non-O157 toxigenic strains overproduce β-galactosidase relative to β-glucuronidase on this medium and consequently are typically coloured purple, violet or blue. Most non-pathogenic *E. coli* strains are glucuronidase positive, and range from pink to magenta with occasional purple or blue strains. Nearly all other bacterial species are either inhibited on this medium or they grow as white or cream-colored colonies.

1.6.2 Immunological Methods

Immunoassays, predominantly ELISA, have played a crucial part in the 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).

1.6.3 DNA-Based Methods

DNA probes may be used to detect a specific gene of *E.coli* O157. According to Dr. Michael A. Pfaller, DNA probes are “single-stranded pieces of nucleic acid, labelled with a specific tracer (isotope, enzyme, or chromophore), that will hydrogen bond (hybridize) with

complementary single-stranded pieces of DNA (or RNA) under the appropriate conditions of pH, temperature, and iconic strength."

DNA probe can be produced by use one of three methods which are (1) using a template DNA with the help of purified biological enzymes, (2) using automated DNA synthesizers and (3)allowing many copies of DNA probe to be obtained when virus or bacteria replicate and then labelled with a tracer or reporter molecule to allow detection in hybridization. Single stranded DNA binds on filters and is exposed to an excess of DNA probes but only one of these will hybridize. At the same time unbound DNA is detected by a variety of available methods using florescence and dye, etc.

1.7 Treatment

EHEC strains are usually prone to a variety of antibiotics. In a potential study, Proulx demonstrated a trend toward a lower incidence of HUS in those receiving antibiotics (Proulx et al., 1992). Another 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, Tanimura, Yoshino, Matsuda, Uchida and Ikeda, 1998). There are, however, retrospective studies which suggest that patients who received antibiotics may be at greater risk of developing HUS (Nataroand Kaper, 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 intra-colonic bacteria, thereby increasing the systemic absorption of toxin (James and Nataro, 1998).

1.7.1 Vaccines

Presently, there are no convenient vaccines available against EHEC, but a number of experimental vaccines are undergoing trials on animals. Vaccine development is 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). A parenteral vaccine specific for O157 EHEC has been developed based on O157 polysaccharide conjugated to protein carriers (KonaduRobbins, Shiloach, Bryla and Szu1994). 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. (James and Nataro, 1998).

1.8 General Objectives of the Study

Toxin producing *E. coli* is an important food borne and waterborne pathogen with worldwide distribution. Raw meat and vegetables are particularly likely to carry a 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,Afsah-Hejri, Rukayadi, Khatib, Lye, Loo, MohdShahril, Puspanadan,Kuan, Goh, John, Nakaguchi, Nishibuchi and Son 2013). Outbreak studies have revealed that *E. coli* is able to survive and develop in different types of minimally processed vegetables. It is also capable of surviving at refrigeration temperatures 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 been made to isolate and characterize *E. coli* from street food and raw vegetables. Street foods in Dhaka are infamous for the lack hygiene standards.

The objective of this study was to investigate the prevalence of STEC in street foods and raw vegetables and their correlation to disease. The aim of this study was to determine the incidence of STEC and evaluate the quality of raw vegetables and street food in Dhaka city.

1.8.1 Specific Objectives include

- Isolation of *E. coli* from raw vegetables and street food by selective enrichment of the samples followed by selective plating.
- Biochemical tests, for confirmation of being *E. coli*, of the isolated colonies.
- Genotypic characterizations of the isolates to check for presence of*stx*1 and *stx*2 genes by polymerase chain reaction (PCR) and gel electrophoresis.

2 MATERIALS AND METHODS

2.1 Sample collection and Identification

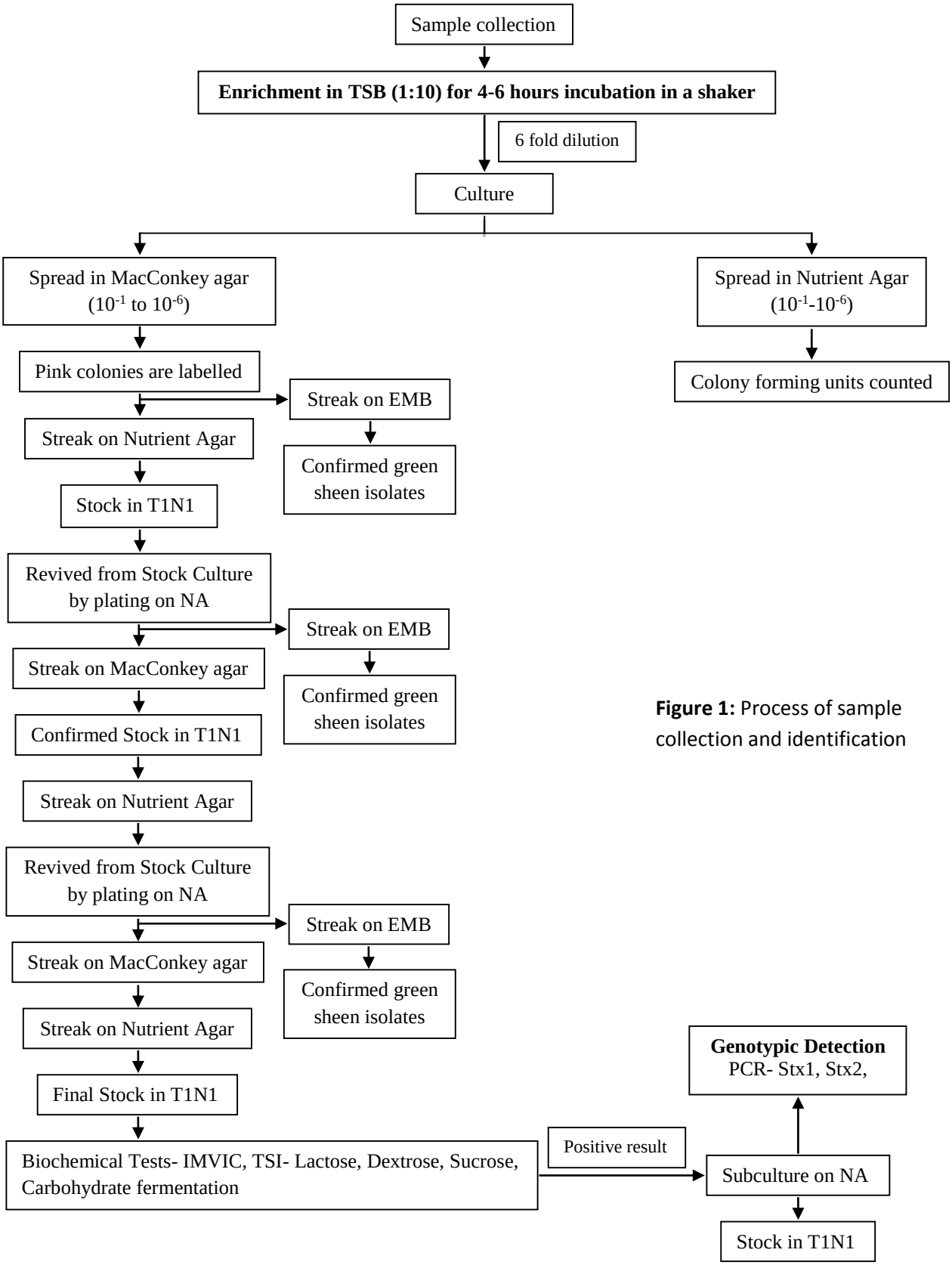


Figure 1: Process of sample collection and identification

2.2 Materials

2.2.1 Samples

Six samples were collected from different locations in Dhaka, mostly from Mohakhali near BRAC University. The first sample, Salad, was collected from the Mohammadpur Bihari Camp. Secondly, a fruit Mix was collected from Mohakhali, in front of BRAC University. The 'Amra' Mix was bought from a street vendor that has a reputation for selling hand mixed fruit that caused diarrhoea among 3 people with one being very severe. The third sample was Mashed Pulse which is usually served with bhelpuri, a common street food in Dhaka that was also collected in front of BRAC University in the following week. Sugar cane juice was the fourth sample that was collected outside Mohakhali due to lack of availability in the Mohakhali Area. The reason for finding less sugarcane juice was due to the change of season. The fifth sample was betel, which is usually eaten raw by people, traditionally with shupari and tobacco leaves. This sample was collected from Mohakhali. Finally the sixth sample was coriander, a favourite addition to many dishes, either cooked or raw (salad).

Table 1: Sample Collection: Source, Time, Temperature, Humidity and number of *E. coli* Isolates

Source	Location	Date	Time	Temperature	*E.coli* positive	*EHEC* Positive
Salad	Bihari camp	17.10.14	7pm	28°C	3/12	
Amra Mix	Mohakhali	12.11.14	10am	29°C	2/10	
Mashed Pulse	Mohakhali	25.11.14	10am	27°C	1/12	
Sugar Cane Juice	Mirpur	25.11.14	10am	28°C	2/16	
Coriander	Mohakhali	29.11.14	2pm	28°C	1/15	
Betel	Mohakhali	30.11.14	2pm	29°C	1/8	

2.2.2 Reference Strains

A positive strain from the previous study, Isolation and Characterization of *Escherichia coli* from Raw Vegetables, was taken as the positive control. *K12* was used as the negative control for PCR. All these above mentioned organisms were preserved in several T1N1 agar media.

The strains were sub-cultured onto nutrient agar every week to prevent contamination and ensure viability.

2.2.3 Media for Culture-Based Methods

Different media were used during this experiment. Tryptic Soy Broth was used for enrichment of bacteria present in the sample. Nutrient agar was used for quantification of the total number of bacteria in the sample and to subculture *E.coli*. T1N1 was used for preservation. Two types of media were specifically used for isolating *E. coli*. These are MacConkey agar and Eosin Methylene Blue agar.

- **MacConkey Agar Medium**

  MacConkey agar was used for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting gram-negative bacteria from the non-fermenting ones. When *E. coli* ferment lactose, it gives pink coloured colonies on MacConkey agar.

- **Eosin-Methylene Blue Agar Medium**

  Eosin-Methylene Blue agar is used to select gram-negative bacteria from the gram-positive ones. The bacteria which ferment lactose in the medium form coloured colonies, while those that do not ferment lactose appear as colourless colonies. *Escherichia coli* colonies grow with a metallic sheen with a dark centre. Eosin Y and Methylene Blue are indicators.

2.3 Methods

2.3.1 Collection of Samples

Sterile zip bags (wiped with 70% ethanol) were used for carrying solid foods, while sterile flasks were used for carrying liquid. All samples were inoculated within 30 minutes of collection except for the salad sample, which was collected at night and refrigerated overnight. The sample was then cultured in an enrichment media in the morning.

Figure 2: Preparation of Sugar

Figure 3: Betel leaves

Figure 4: Amra Mix (Fruit sample)

Figure 5: Bhelpuri (Pulse + salad)

Figure 6: Coriander

2.3.2 Isolation of *E. coli* from Samples

- **Enrichment**

  Tryptic Soy Broth was used for enrichment. Individual samples were inoculated into two separate broths in the ratio of 1:10. For the liquid sample Sugar-cane juice, 1 ml sample was inoculated into 10ml of TSB broth. The inoculated TSB broth was then incubated overnight at 37°C (16-18 hours).

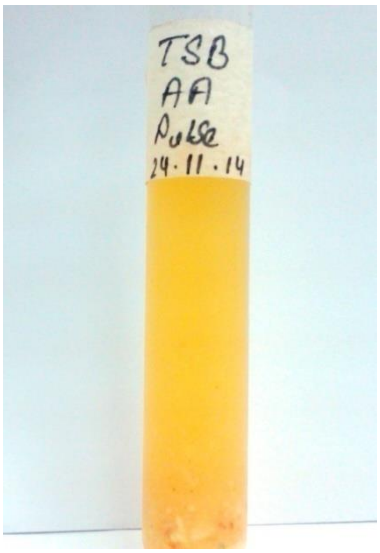


Figure 7: Enrichment media TSB-containing Pulse sample (Bhelpuri)

- **Dilution and Spread Plating**

  1ml of enriched broth was subjected to a 10-fold dilution series from 10^{-1} to 10^{-5} in 9ml of normal saline. From each dilution test tube, 0.1ml of suspension was spread plated onto nutrient agar and MacConkey agar plates respectively. The plates were then incubated at 37°C for 16-18 hours.

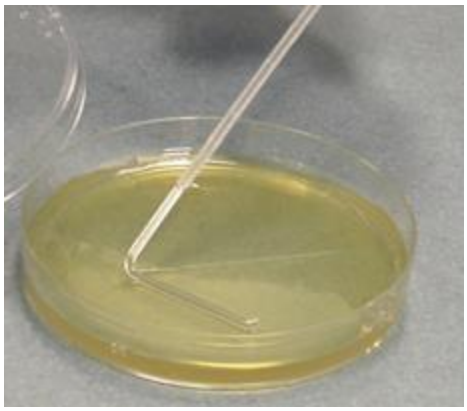


Figure 8: Spread plate method using a glass rod

2.4 Quantification and Isolation

The following day, the number of colonies on the nutrient agar plates were quantified and the colony forming unit was calculated. According to the standard colony counting method, the number of colonies is counted only when there are between 30-300 colonies per plate. If a dilution shows growth of more than 300 colonies, it is said to be 'too numerous to count' (TNTC) and if a dilution shows less than 30 colonies it is said to be 'too few to count' (TFTC).

2.4.1 Screening for Suspected Isolates on EMB Plates

Figure 9: Pink colonies (Lactose Fermenters) are labelled with numbers and streaked onto NA and EMB maintaining their respective labels

Suspected *E. coli* isolates from fresh MacConkey plates were labelled and streaked onto Eosin Methylene Blue (EMB) agar plates using sterile inoculation loops for presumptive confirmation of *E. coli*. Same colonies were also cultured onto nutrient agar plates for storage and for subcultures. EMB agar medium contains lactose and the dyes eosin and methylene blue that permit differentiation between enteric lactose fermenters and non-fermenters as well as identification of the gram negative 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).

2.4.2 Stock

Selected colonies that showed a metallic green sheen on EMB were selected and colonies from respective nutrient agar (NA) were sub-cultured in T1N1 media for stock purpose. For each isolate, two stocks were prepared. The selected colonies from NA were again cultured onto fresh NA plates for further stocking and biochemical tests. The inoculated T1N1 media were incubated at 37°C for 3-4 hours. Following growth, colonies from T1N1 were cultured on NA. After overnight incubation of the colonies, they were again sub-cultured on Mac and EMB for further confirmation of appearance of a green sheen. If the colonies did not show any green sheen, their stocks were discarded. The colonies that showed a green sheen for the second

time were kept, and their respective colonies were selected from respective NA plates for inoculation into T1N1 Agar. The second stock was called ‘Confirmed Stock’.

2.4.3 Biochemical Identification

Biochemical tests were performed with metallic green sheen producing isolates according to the methods described in the 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. Colony isolates which showed results that are expected of *E.coli* were selected and fresh stocks were made again. The stock that was made after the confirmation of biochemical tests was called ‘Final Stock’.

- **Indole Test**

  This test was used to determine the production of indole by deamination and hydrolysis of the amino acid tryptophan by the activity of the tryptophanase enzyme. The organism was grown in peptone water broth overnight. If the bacteria produced the enzyme, tryptophan was converted to an indole molecule, pyruvate and carbon dioxide. Kovac’s reagent was added after incubation. Indole (if present) reacted with the aldehyde in the reagent to give a red colour. An alcoholic layer concentrated the red colour as a ring at the top of the tube, which indicated a positive result.

- **Methyl Red (MR) Test**

  The MR test was applied to analyse the bacterial ability to produce stable acid end products by means of a mixed-acid fermentation of glucose. Generally, *Escherichia coli* ferments sugar by the mixed acid pathway resulting in a low ratio of CO_2 to H_2 produced by fermentation. The large quantity of acids produced cause a significant decrease in the pH of the culture medium. The MR broth contains glucose, peptone, and a phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decrease in pH results. The pH indicator methyl red (p-dimethylaminoaeobenzene-O-carboxylic acid) has been found to be suitable to measure the concentration of hydrogen ions between pH 4.4 (red) which indicates a positive result and 6.0 (yellow) which indicates a negative result. An orange colour indicates an intermediate

pH and would be considered as a negative result due to insufficient accumulation of acids (*Methyl Red and Voges-Proskauer Test Protocols*. Sylvia McDevitt).

- **Voges-Proskauer (VP) Test**

  It identifies organisms that are able to produce acetoin from the degradation of glucose during 2,3-butanediol fermentation. The Voges-Proskauer test determines the capability of some organisms to produce non acidic or neutral end products, such as acetyl methyl carbinol, from the organic acids that result from glucose metabolism. The end products of glucose metabolism, pyruvic acid, is further metabolized by using the Butylene glucol pathway to produce neutral end product such as acetoin and 2,3-butanediol. When Barrit's reagent A (40% KOH) and Barrit's reagent B (5% solution of alpha naphthol) are added, it will detect the presence of acetoin, the precursor of 2,3- butanediol synthesis. Acetoin, in the presence of oxygen and Barrit's reagent, is oxidized to diacetyl where alpha naphthol acts as a catalyst. Diacetyl then reacts with guanidine components of peptone. As a result, a pink complex is formed, imparting a rose colour to the medium. Development of a deep rose colour in the culture 15 minutes following the addition of Barritt's reagent is indicative of the presence of acetoin and represents a positive result. The absence of a rose colour is a negative result (McDevitt, 2009).

- **Citrate Utilization**

  The Citrate Utilization Test was applied to verify the ability of an organism to use citrate as the sole source of carbon. Simmons Citrate agar is a medium containing citrate as the sole carbon source and ammonium salts as the sole nitrogen source. Organisms that metabolize citrate utilize the ammonium salts releasing ammonia and increasing the pH of the medium. Bromothymol blue is present in the medium as the indicator dye. It is green in colour at neutral pH and turns a deep blue above a pH of 7.6 (Elmanama, 2009).

- **Triple Sugar Iron (TSI) Agar**

  Triple sugar iron (TSI) agar is a differential medium used to determineH_2S production and the type of carbohydrate fermentation. Gas from carbohydrate metabolism can also be detected. Bacteria can metabolize carbohydrates aerobically (with oxygen) or fementatively (without oxygen). TSI differentiates bacteria based on their fermentation of lactose, glucose and sucrose and on the production of hydrogen sulphide. TSI is most frequently used in the

identification of the *Enterobacteriaceae* family, although it is useful for other gram-negative bacteria as well. TSI contains three carbohydrates: glucose (0.1%), sucrose (1%), and lactose (1%).

When any of the carbohydrates are fermented, the drop in pH will cause the medium to change from reddish-orange (the original colour) to yellow. A deep red colour indicates alkalization of the peptones. Sodium thiosulfate in the medium is reduced by some bacteria to hydrogen sulphide (H_2S), a colourless gas. The hydrogen sulphide will react with ferric ions in the medium to produce iron sulphide, a black insoluble precipitate (Lehman, 2005).

- **Carbohydrate Fermentation Test**

  The Carbohydrate Fermentation test uses Phenol Red Broth to test for the fermentation of different sugars. Phenol red broth is a general purpose fermentation media which includes the pH indicator phenol red and a series of test tubes each with a different sugar. Glucose, lactose and sucrose fermentations were observed. When the bacteria ferments the sugar (glucose, lactose or sucrose) an acid will build up, changing the colour of phenol red. Sugars such as glucose undergo "fermentation" when it acts as an electron donor, such as in glycolysis, and one of its metabolic products (such as pyruvate) acts as an electron acceptor in the fermentation reaction. In reality, most sugars other than glucose are said to undergo 'fermentation' when it is either hydrolysed into glucose or converted into glucose or both which then is fermented. The second step in the process is the fermentation reaction that generates end products such as different acids, ethanol, hydrogen gas, carbon dioxide gas, and other organic compounds, depending on the specific reactions. Which reactions will occur depends on the species of bacteria.

  Each test tube also contains an inverted and filled Durham tube which will allow one to visualise the production of gas. Phenol red broth also contains protein that may allow for the detection of the degradation of peptones and their resulting alkaline end products. These alkaline end products change the broth colour to pink, but this is often only observed at the very top of the tube (where oxygen is most abundant) when the tubes are totally undisturbed. Generally, the colour of the broth changes from red to yellow, inferring the breakdown of the sugar, hence a positive result.

2.5 Amplification of DNA from the Isolates

Polymerase chain reaction (PCR) is a technique that is used to amplify trace amounts of DNA. PCR amplification is only part of the identifying test, however. In this study, PCR is used to increase the amounts of these unique sequences so they can then be used to determine, with a very high probability, the identity of the source. In a nutshell, the aim was to determine the presence of stx1 and stx2 genes such that it can concluded that the strains isolated from the environmental samples are indeed Shiga-toxin producing *E.coli*.

2.5.1 Template Preparation

The DNA templates of the different sample isolates are prepared in the following way. The colony isolates are taken from the T1N1 stock and streaked onto nutrient agar plates and incubated at 37°C overnight. If growth is insufficient, the samples are sub-cultured onto a new nutrient agar plate and again kept overnight in the incubator.

Eppendorf tubes were labelled with colony isolate numbers or initials. 100µl of distilled water was pipetted into these Eppendorf tubes. An inoculation loop was used to scoop bacteria from a freshly sub-cultured nutrient agar plate which were then mixed with the water in the labelled tubes. The suspensions were then vortexed. The suspensions were kept at -20°C for an hour to adapt to the cold temperature. After an hour, the tubes were transferred to the water bath (100°C) and kept for 10 minutes. The tubes were centrifuged at 12,000 rpm, for 10 minutes. The supernatant from each tube was transferred to a fresh Eppendorf tube, and was stored at -20°C for later use.

2.5.2 Preparation of the Reaction Mixture

Table 2

Components	Volume (per sample)
Buffer (10X)	2µl
dNTP (2 µM)	1µl
Forward primer (1 µM)	1µl
Reverse primer (1 µM)	1µl
Taq Polymerase (1 unit/µl)	1µl
Water	13µl
dsDNA template	1µl
Total	20µl

2.5.3 Primer Sequences used for PCR

Table 3

Target Gene	Primer	Sequence	Amplicon Size
stx1	LP30	5'-CAGTTAATGTGGTGGCGAAGG -3'	348 bp
	LP31	5'-CACCAGACAAATGTAACCGCTC -3'	
stx2	LP41	5'-ATCCTATTCCCGGGAGTTTACG -3'	584 bp
	LP42	5'-GCGTCATCGTATACACAGGAGC -3'	

2.5.4 PCR Conditions

The Polymerase Chain Reaction (PCR) consists of 3 main steps:

- Melting→ Denaturing of the DNA duplex template at a high temperature to yield single stranded DNA
- Annealing→ Primers anneal to the single stranded target DNA sequence
- Elongation→ DNA polymerase extends the primers by adding dNTPs to the phosphate backbone

The parameters of the thermal cycle for PCR was set as given below:

i. 95°C for 5 minutes (Initial Denaturation)

ii. 94°C for 30 seconds (Denaturation)
    54°C for 30 seconds (Primer annealing)
    72°C for 30 seconds (Elongation)

iii. 72°C for 5 minutes (Final Extension Step)

iv. 4°C until further use

2.6 Electrophoretic Analysis of Amplified DNA Product

For the preparation of 100ml of a 1.5% agarose solution, 1.5g agarose was measured into a flask and 100ml of 1X buffer was added to it. This solution was heated in a microwave oven until the agarose dissolved and the solution became clear. The solution was poured into the gel tray and the comb set close to one end of the gel. The gel was left undisturbed at room temperature for about 10 minutes to allow for uniform solidification.

Afterwards the comb was gently removed and the gel tray with the gel was placed in the electrophoresis chamber and covered (until all wells were submerged) with electrophoresis buffer (TBE buffer, that is, the same buffer which was used to prepare the agarose). To prepare samples for electrophoresis, 1.5µl of gel loading dye was added for every 5µl of DNA solution. The PCR Master Mix was loaded along with the dye. The gel was run at 60 volts and it took approximately 1 hour for the run to be complete. The gel was stained in 0.5µg/ml ethidium bromide solution for band visualisation under short wave UV light.

2.7 Preservation of *E. coli* Isolates

For short-term preservation, an inoculation needle was used to scoop bacteria from each isolate's nutrient agar plate. The needle was then used to stab 2ml of T1N1 agar medium in glass vials repeatedly. Then the vials were incubated at 37°C for 6 hours. After incubation, the surface of the medium was covered with sterile paraffin oil and the vial was stored at room temperature.

3 RESULTS

3.1 Isolation of *E. coli* from samples

Six street samples were collected from various local bazaar and super shops around Dhaka city. The samples were enriched in TSB for 18 hours at 37°C. After enrichment; the culture broth was subjected to a six fold dilution. From appropriate dilutions, the broth cultures were spread plated onto nutrient agar to determine the number of colony forming units, while MacConkey Agar was used for isolation of *E.coli*. Both types of agar media were incubated at 37°C overnight. After incubation, different types of colonies were observed on MacConkey agar plates (Figure 3.2). Colonies showing typical morphological characteristics of *E. coli* (Table 3.1) were sub-cultured onto EMB plates. Over 60 isolates were taken from primary isolation plates and considered for further investigation.

Table 4: Salad Sample

Media	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**Cfu**
Nutrient Agar	TNTC	TNTC	TNTC	TNTC	181	40	$1.81*10^{9}$
MacConkey	TNTC	120	2	0	0	0	$1.2*10^{6}$

Table 5: Fruit Sample

Media	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**Cfu**
Nutrient Agar	TNTC	TNTC	TNTC	TNTC	75	5	$7.5*10^{8}$
MacConkey	TNTC	TNTC	TNTC	TNTC	58	4	$5.8*10^{8}$

Table 6: Sugar Cane Juice

Media	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**Cfu**
Nutrient Agar	TNTC	TNTC	79	16	7	1	$7.9 *10^{6}$
MacConkey	TNTC	85	38	5	0	0	$3.8 *10^{6}$

Table 7: Pulse

Media	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**CFU**
Nutrient Agar	TNTC	TNTC	TNTC	TNTC	137	33	$1.37 *10^{9}$
Mac Conkey	TNTC	TNTC	TNTC	126	40	3	$1.26 *10^{8}$

Table 8: Betel Leaves

Media	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**CFU**
Nutrient Agar	TNTC	TNTC	112	82	24	14	$8.2 * 10^{7}$
MacConkey	156	51	8	0	0	0	$1.56 * 10^{5}$

Table 9: Coriander

Media	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**CFU**
Nutrient Agar	TNTC	TNTC	TNTC	TNTC	TNTC	195	$1.95 * 10^{10}$
MacConkey	TNTC	TNTC	TNTC	TNTC	TNTC	159	$1.59 * 10^{10}$

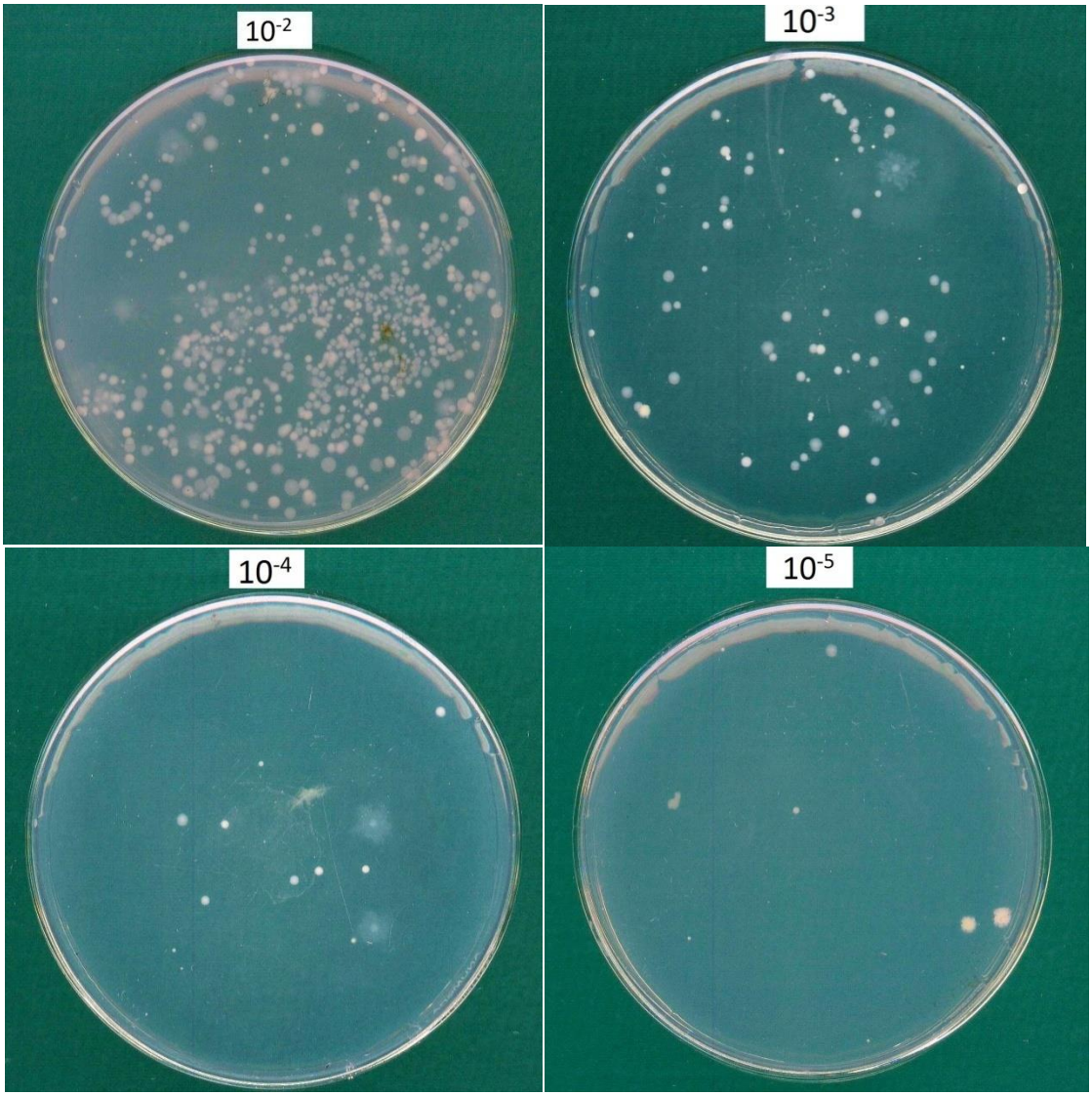


Figure 10: Nutrient Agar plates showing bacterial growth from sugar cane juice. Each photo represents a different dilution factor

Dilution Factor of the original sample

Initially 1ml of the sample was added to 9ml of enrichment broth.

Therefore, if Dilution Factor $= \frac{Final\ Volume}{Sample\ Volume} = \frac{10}{1} = \mathbf{10}$

For calculating the number of colonies from sugar cane juice in nutrient agar, the dilution factor was taken as $\mathbf{10^3}$

So, Total Dilution Factor$= 10 \times 10^3 = 10^4$

So CFU/ml$= \frac{(No.of\ colonies) \times Dilution\ Factor}{Volume\ of\ culture\ plate} = \frac{79 \times 10^4}{0.1} = \mathbf{7.9 \times 10^6}$

Table 10: Comparison of total CFU from NA and Mac plates of different samples

Samples	CFU in Nutrient Agar	CFU in MacConkey Agar
Salad	$1.81*10^{9}$	$1.2*10^{6}$
Fruit	$7.5*10^{8}$	$5.8*10^{8}$
Pulse	$1.37*10^{9}$	$1.26*10^{8}$
Sugar Cane Juice	$7.9*10^{6}$	$3.8*10^{6}$
Betel	$8.2*10^{7}$	$1.56*10^{5}$
Coriander	$1.95*10^{10}$	$1.59*10^{10}$

3.2 Identification of suspected *E. coli* isolates

3.2.1 Presumptive identification on EMB plate

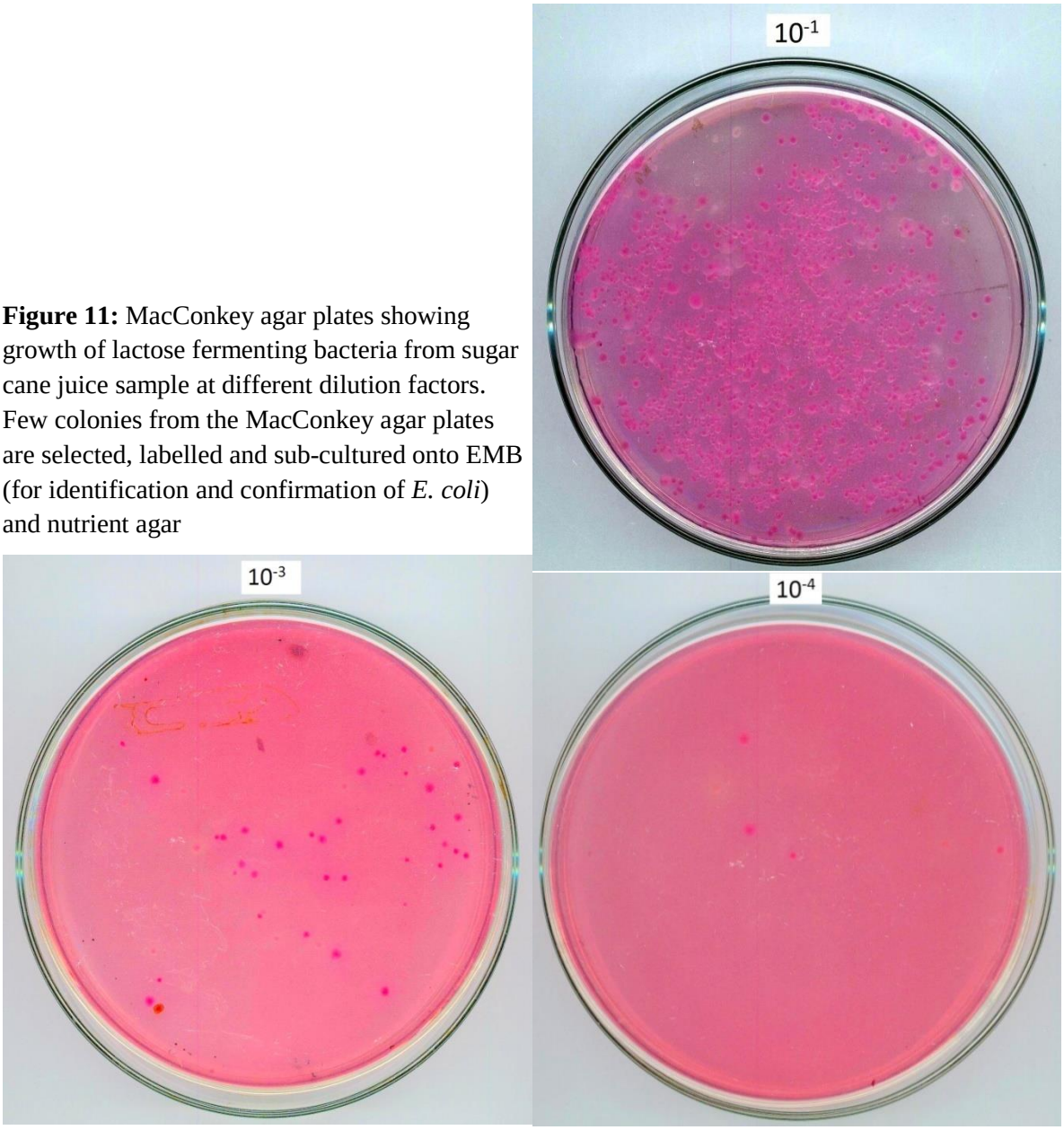


Figure 11: MacConkey agar plates showing growth of lactose fermenting bacteria from sugar cane juice sample at different dilution factors. Few colonies from the MacConkey agar plates are selected, labelled and sub-cultured onto EMB (for identification and confirmation of *E. coli*) and nutrient agar

Figure 12: EMB subculture plates showing growth from various samples. From left, Sugar sample colony isolates S-1 to S-8, Fruit sample isolates F-7 to F-10 (F-9 showing green sheen) and Pulse sample isolates shown in the last two pictures. PS-3 and PS-4 out of the total 8 samples showed green sheen and hence are concluded as *E. coli.*

3.2.2 Biochemical identification

Table 11

Colony Isolate	Indole	Methyl red	Vogespros kauer	Citrate	TSI	Fermentation		
						Dextrose	Lactose	Sucrose
CI 1	+	+	-	-	+	+	+	+
CI 2	+	+	-	-	+ (gas)	+	+	-
CI 3	+	+	-	-	+	+	+	+
F 6	+	+	-	-	+	+	+	-
F 9	-	+	-	-	+ (gas)	+	+	-
PS 3	+	+	-	-	+	+	+	-
PS 4	-	+	+	-	+ (gas)	+	+	+
S-3	+	+	-	-	+	+	+	+
S-11	+	+	-	+	+	+	+	-
B 12	+	+	-	-	+	+	+	+
C 6	+	+	-	-	+	+	+	+
C 10	-	+	+	+	+	+	+	+

Legend: + indicates a positive result; **–**indicates a negative result; (gas) indicates gas production

Salad Sample- CI 1, 2, 3

Fruit Sample: F-6 and F-9

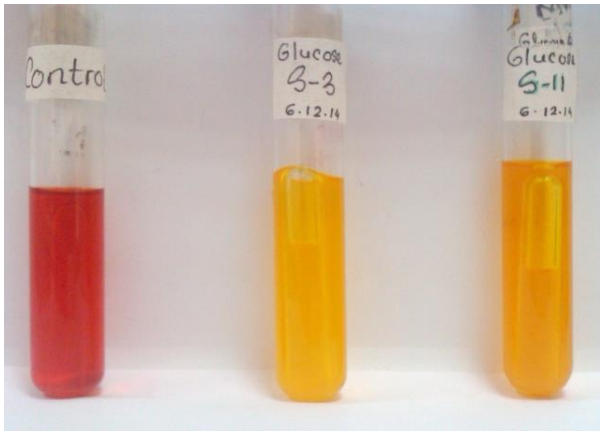
Pulse Sample: PS-3 and PS-4

Sugar Cane Juice: S-3 and S-11

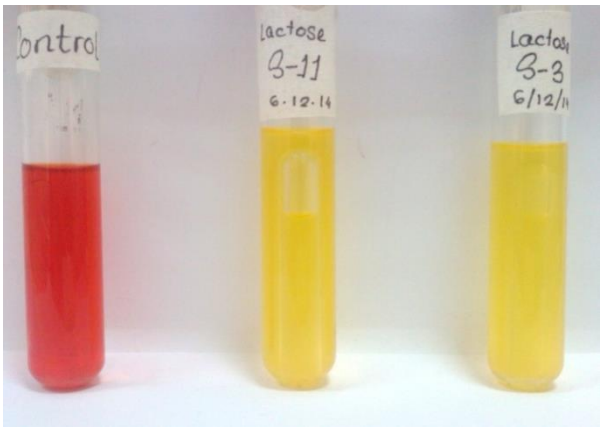
Betel Leaves: B-12

Coriander: C-6 and C-10

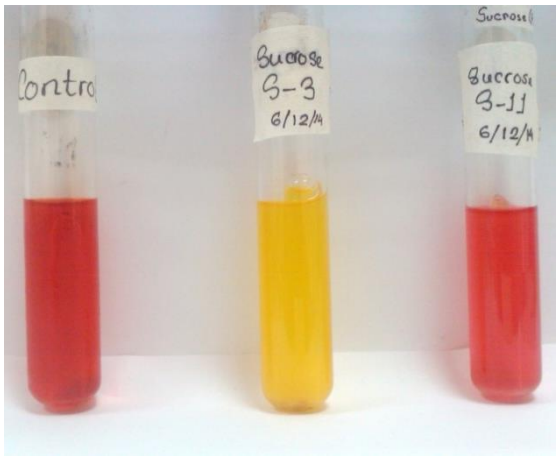
Figure 13: Carbohydrate Fermentation Tests



Glucose Fermentation of Isolates S-3 and S-11 showing positive results

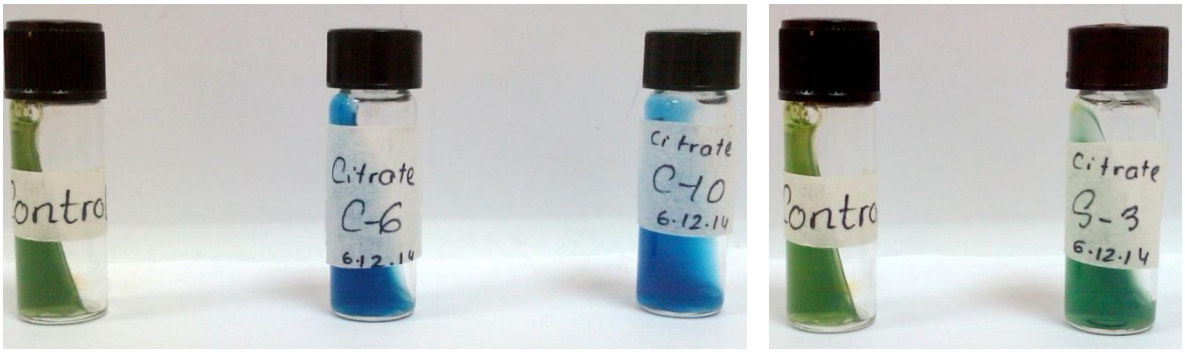


Lactose Fermentation of Isolates S-3 and S-11 showing positive results



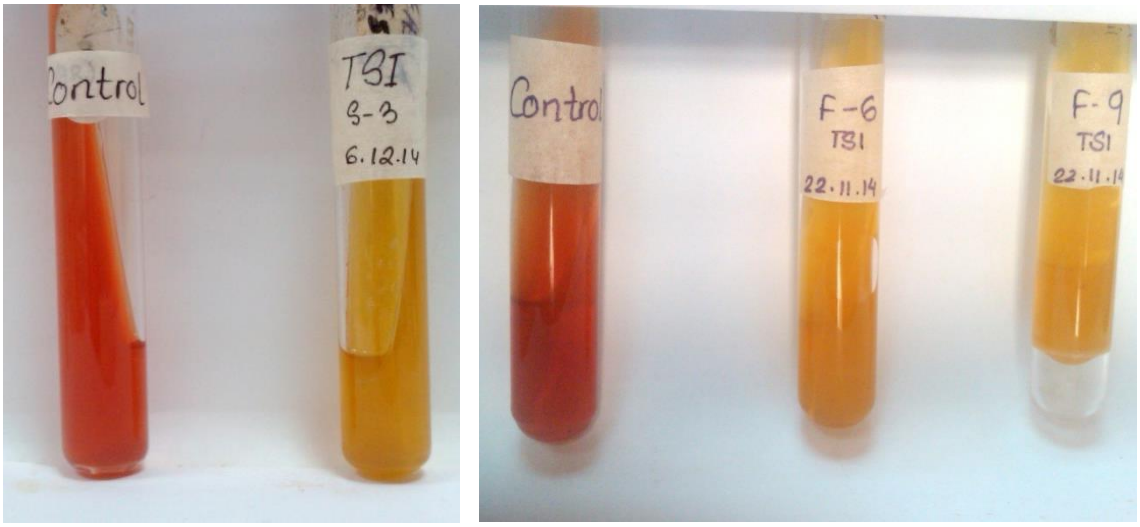
Sucrose Fermentation of Isolates: S-3 is positive and S-11 is showing negative results

Figure 14: Citrate Utilization



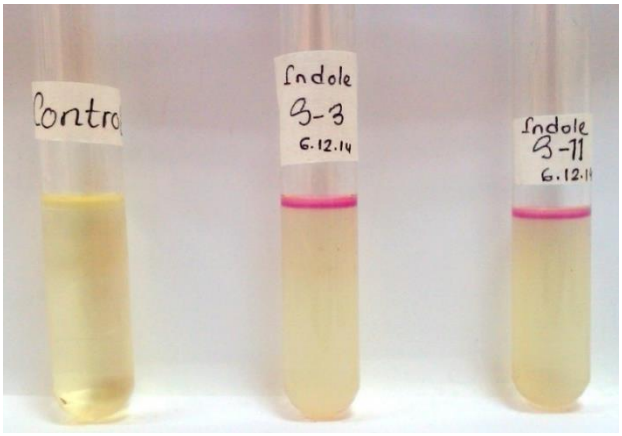
C-6 and C-10 from the coriander sample showing positive results, and S-3 from sugar cane juice showing a negative result

Figure 15: Triple Sugar Iron (TSI) Agar Tests



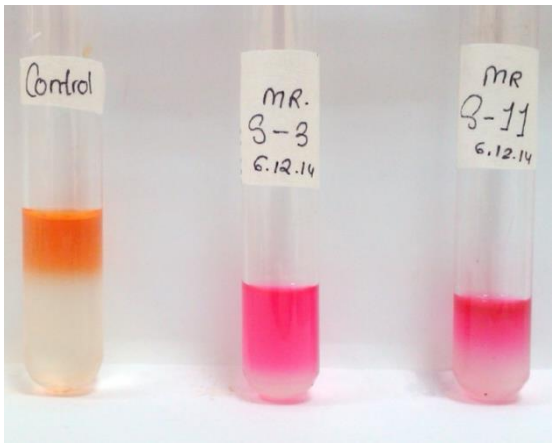
S-3 and F-6 showing positive results (media turned orange to yellow) but did not form gas. F-9 showed both a colour change and gas formation (media is displaced)

Figure 16: Indole Test



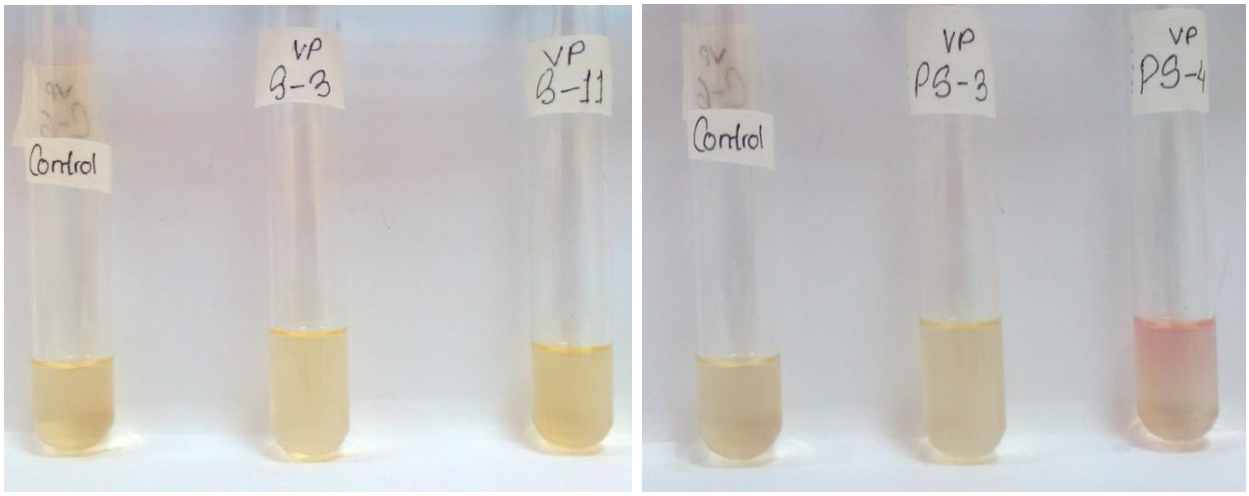
S-3 and S-11 showing positive result by formation of a pink ring (production of indole)

Figure 17: Methyl Red (MR) Test



S-3 and S-11 showing a positive result as they gave a red colour upon addition of methyl red

Figure 18: Voges-Proskauer (VP) Test



S-3, S-11 and PS 3 showed no colour change and hence are VP negative. PS-4 showed positive result by formation of slightly red colour

3.3 Genotypic Characterizations of the Isolates

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

Template DNA was prepared from cellular biochemically identified isolates by the boiling method and 1µl of template DNA was subjected to PCR to increase the chances of detecting *E. coli* specific virulent genes *stx1* and *stx2* using specific primers. Isolates that gave bands of expected size were suspected to carry these genes in their chromosomes. The results showed that isolates HS-2, C-10, S-3, PS-3 and CI 2 were positive for *stx*2 but were negative for *stx*1 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 and illustrated in the figure.

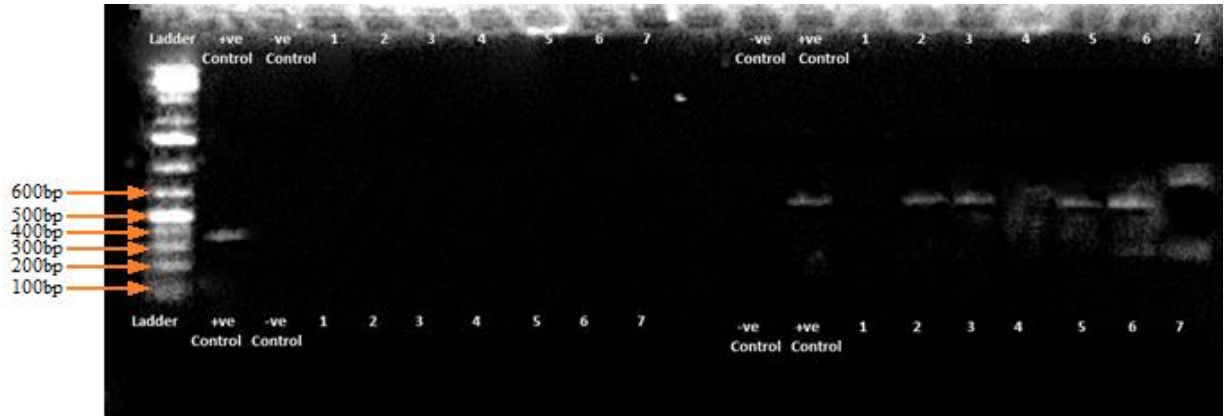


Figure 19: Gel Electrophoresis of *E. coli* samples

Lane 1: **100bp marker**

Lane 2: **Positive Control→ Reference Strain**

Lane 3: **Negative Control→ K12**

Lane 4: S-3

Lane 5: C-10

Lane 6: Be-12

Lane 7: F-6

Lane 8: C-12

Lane 9: PS-3

Lane 10: HS-2

Lane 11: Blank

Lane 12: **Negative Control→ K12**

Lane 13: **Positive Control→ Reference Strain**

Lane 14: S-3

Lane 15: C-10

Lane 16: Be-12

Lane 17: F-6

Lane 18: C-12

Lane 19: PS-3

Lane 20: HS-2

Table 12

Samples	Stx1	Stx 2
Salad (CI 2)	**-ve**	**+ve**
Fruit (F-6)	**-ve**	**-ve**
Pulse (PS-3)	**-ve**	**+ve**
Sugar Cane Juice (S-3)	**-ve**	**+ve**
Betel (Be-12)	**-ve**	**-ve**
Coriander (C-10)	**-ve**	**+ve**
Human Sewage (HS-2)	**-ve**	**+ve**

4 DISCUSSION

It is apparent that STEC are decidedly pathogenic and that virulence is not dependent on a single gene or gene product but is a multifactorial process. According to the study, it is safe to infer that the organism can survive in water, dusty dry environment and a wide range of foods including acidic products and then remain viable after transit through the acidic environment of the stomach. Colonization of the bowel, mediated by one or more of a range of potential adhesins, then occurs. Following colonization, several toxins and other proteins are produced which may assist with survival and multiplication in the intestinal environment. Amplification of potent Shiga toxins into the gut lumen causes intestinal damage and eventually systemic complications. The production of Shiga toxins, in particular s*tx2*, is a prerequisite and the capacity to adhere to the bowel mucosa is likely to be important. These two factors alone may be sufficient for an organism to cause disease, a theory supported by the data of Boerlin *et al*. (1999) which showed that these factors were highly related to disease in humans.

Shiga toxin-producing *Escherichia coli* (STEC) are estimated to cause more than 265,000 illnesses each year in the United States, with more than 3,600 hospitalizations and 30 deaths (Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, 2011) STEC infections often cause diarrhea, sometimes bloody. Some patients with STEC infection develop hemolytic uremic syndrome (HUS), a severe complication characterized by renal failure, haemolytic anemia, and thrombocytopenia that can be fatal. Most outbreaks of STEC infection and most cases of HUS in the United States have been caused by STEC O157.

Although all STEC infections are nationally notifiable, for several reasons many cases are likely not recognized (Scallan, Jones, Cronquist, Thomas, Frenzen, & Hoefer, 2006). Not all persons ill with STEC infection seek medical care, healthcare providers may not obtain a specimen for laboratory diagnosis, or the clinical diagnostic laboratory may not perform the necessary diagnostic tests. Accounting for under-diagnosis and under-reporting, an estimated 96,534 STEC O157 and 168,698 non-O157 infections occur each year (Scallan et al, 2011). STEC transmission occurs through consumption of contaminated foods, ingestion of contaminated water, or direct contact with infected persons (e.g., in child-care settings) or animals or their environments.

The current research study has been performed to isolate, identify, investigate and apprehend

the microbial quality of foods collected from street side stalls, mid-level restaurants and raw vegetables. The 6 collected food samples were handled properly to prevent contamination of the food before the analysis was performed. The six samples included the Salad Sample, Fruit Sample, Pulse from Bhelpuri, Sugarcane juice, Betel Leaves and Coriander. In addition to isolation and identification of the gram negative *E. coli*, the genes for toxicity to detect the strain of *E.coli* were analysed. The genetic detection of Shiga toxin producing *E. coli* 0157:H7 was done by using PCR for the amplification of the DNA of the Isolates, and primers *stx1* and *stx2* were used to check for the presence of the shiga toxin genes. Out of the six samples, 4 sample isolates contained the *stx2* genes which, according to Louise and Obrig 1995, are a 1000 times more cytotoxic than *stx1* towards human renal microvascular endothelial cells, the putative target of Shiga toxins in the development of HUS. The four samples that gave positive results were Salad (CI 2), Pulse (PS-3), Sugar Cane Juice (S-3) and Coriander (C-10).

Based on these results it can be inferred that the salad, pulse (from bhelpuri), sugar cane juice and raw coriander should not be ingested without proper processing. The salad was collected from a street side stall, and it is assumed that they were not processed or washed properly. Salad items require to be washed with clean water to remove the presence of bacteria. Bhelpuri sellers sometimes use old pulse in their bhelpuri which might allow the growth of STEC. Abstaining from bhelpuri might prevent one from getting infected since the traditional dish is not heated before eating. Sugar cane is a high source of carbohydrates, as a result growth of various organisms is expected. Street vendors cannot maintain appropriate hygiene standards of the machines since these are sold on street, and contamination is highly likely. Coriander was collected from a street vendor, since it was not washed, it contained a high number of organisms. Coriander needs to be washed several times since it contains dust particles from the open street, and it is known that the microbes cling to the dust particles.

If the residents of Dhaka city are not made aware of these findings, chances are high that there might be an outbreak of *E. coli* 0157:H7. Gradual increase in awareness regarding hygiene and prior knowledge of the disease and how it occurs can save the residents of the city from such an outbreak.

More extensive studies should be done on microbiological analysis of commonly consumed street foods; According to CDC the following approaches should be followed. Clinical diagnostic laboratories should strongly consider including STEC O157 in their routine bacterial

enteric panel. If bacterial culture for STEC O157 is not performed in parallel with Enzyme Immuno Assay, Stx-positive broths should be inoculated to a selective isolation medium, such as SMAC agar, and any resulting sorbitol-negative colonies should be tested with O157 antiserum or latex reagent. All confirmed and presumptive STEC O157 isolates and Stx-positive broths that do not yield STEC O157 should be forwarded to a public health laboratory as soon as possible for confirmatory testing and further genetic characterization. STEC O157 isolates should be confirmed, characterized, and tested by PFGE, and the pattern promptly entered into the PulseNet database. At the public health laboratory, the broth should be sub-cultured to selective agar and a representative sample of sorbitol positive and negative colonies tested by Stx EIA or PCR for *stx1* and *stx2* genes.

To assist analysis of possible outbreaks, clinicians should notify health departments about clusters of patients with bloody diarrhea or HUS, and clinical diagnostic laboratories should follow recommended procedures for identification of STEC. Screening stool specimens by clinical diagnostic laboratories for *stx* using PCR techniques is generally done. In addition to the detection of *stx* genes, the pathogenic organisms may need to be tested for other virulence factors- *intimin, and Ehx*. With this coordinated approach, accurate laboratory data can be combined with epidemiologic information to ensure prompt diagnosis and treatment of STEC O157 infections, improved diagnostic accuracy, and improved detection of outbreaks caused by non-O157 STEC.

Concluding Remarks

The detection of factors which are vital for the cause of disease may allow the development of improved analytical tools, allowing us to detect and isolate only those organisms with disease-causing potential. Investigation of pathogenicity factors also provides insights into possible therapeutic and immunization strategies. Various trials are under way looking at vaccine development in animals and humans. In particular, intimin has potential value as a cattle vaccine, other vaccines may be based on Tir or other secreted proteins, such as EspA, EspB and EspD, as these proteins are highly immunogenic and antibodies are produced during the course of infection in humans.

It has been over 15 years since strains of *E. coli* O157 emerged as a human pathogen. Although immense advancements have been made in our knowledge of these organisms, their toxins and accessory virulence factors there is still a great deal to learn before we can fully understand the processes leading to disease.

The latest progress in molecular techniques and the identification of the genetic loci encoding many of the alleged virulence properties will be tremendously constructive in identifying the contribution of the various factors to pathogenicity. The sequencing of the *E. coli* O157 genome and its comparison with that of *E. coli* K-12 will be crucial in identifying novel virulence attributes. *Escherichia coli* O157 mutants deficient in one or more factors, or producing distinct forms of Shiga toxins, can be assessed in both *in vitro* and *in vivo* models. Such mutants can also be compared with well-characterized non-O157 STEC, with known combinations of phenotypic properties, e.g. intimin, Ehx, EspP, catalase/peroxidase and haemoglobin utilization.

5 BIBLIOGRAPHY

Acheson, D.W.K., Lincicome, L., Jacewicz, M.S., Keusch, G.T. (1998) Shiga toxin interaction with intestinal epithelial cells. In*Escherichia coli O157:H7 and Other Shiga-Toxin-Producing E. coli Strains* ed. Kaper, J.B. and O'Brien, A.D. pp. 140 147. Washington, DC: American Society for Microbiology.

Boerlin, P., Chen, S., Colbourne, J.K., Johnson, R., De Grandis, S., Gyles, C. (1998) Evolution of enterohemorrhagic *Escherichia coli*hemolysin plasmids and the locus for enterocyte effacement in Shiga-like toxin producing *E. coli*. *Infection and Immunity* **66**, 25532563.

Chang WS, Afsah-Hejri L, Rukayadi Y, Khatib A, Lye YL, Loo YY, Mohd Shahril N, Puspanadan S,Kuan CH, Goh SG, John YHT, Nakaguchi Y, Nishibuchi M and Son R. 2013. Quantification of *Escherichia coli* O157:H7 in organic vegetables and chickens. *Int Food Res J.* **20:**1023-1029

Dean-Nystrom, E.A., Bosworth, B.T., Moon, H.W., O'Brien, A.D. (1998) Escherichia coli O157:H7 requires intimin for enteropathogenicity in calves. Infection and Immunity 66, 4560 4563.

General Microbiology Laboratory. Dr. Abdelraouf A. Elmanama, 2009. Web. 5 Jan. 2015.

Hicks, S., Frankel, G., Kaper, J.B., Dougan, G., Phillips, A.D. (1998) Role of intimin and bundle-forming pili in enteropathogenic*Escherichia coli* adhesion to pediatric intestinal tissue in vitro. *Infection and Immunity* **66**, 1570 1578.

Jacewicz, M.S., Acheson, D.W.K., Binion, D.G., West, G.A., Lincicome, L.L., Fiocchi, C. et al. (1999) Responses of human intestinal microvascular endothelial cells to Shiga toxins 1 and 2 and pathogenesis of hemorrhagic colitis. *Infection and Immunity* **67**, 14391444.

James P. Nataro, J. (1998). Diarrheagenic Escherichia coli. *Clinical Microbiology Reviews*, *11*(1), 142. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC121379/

Johnson, R.P., Clarke, R.C., Wilson, J.B., Read, S.C., Rahn, K., Renwick, S.A. et al. (1996) Growing concerns and recent outbreaks involving non-O157:H7 serotypes of verotoxigenic Escherichia coli. Journal of Food Protection 59, 1112 1122. Web of Science®

Junkins, A.D. & Doyle, M.P. (1989) Comparison of adherence properties of Escherichia coli O157:H7 and a 60-megadalton plasmid-cured derivative. Current Microbiology 19, 21 27.

Louise, C.B. & Obrig, T.G. (1995) Specific interaction of *Escherichia coli* O157:H7-derived Shiga-like toxin II with human renal endothelial cells. *Journal of Infectious Diseases* **172**, 1397 1401.

Kaper JB, Nataro JP and Mobley HL. 2004. Pathogenic *Escherichia coli*. *Nature Rev Microbiol.* **2:**123-140.

Kleanthous, H., Smith, H.R., Scotland, S.M., Gross, R.J., Rowe, B., Taylor, C.M. et al. (1990*)* Haemolytic uraemic syndromes in the British Isles. 1985–8: association with verocytotoxin producing *Escherichia coli*. 2. Microbiological aspects . *Archives of Disease in Childhood* **65**, 722 727.

Konadu E, Robbins JB, Shiloach J, Bryla DA and Szu SC. 1994. Preparation, characterization, and immunological properties in mice of *Escherichia coli* O157 O-specific polysaccharide-protein conjugate vaccines. *Infect Immun*. **62:**5048-5054.

Law, D. (2000). Virulence factors of Escherichia coli O157 and other Shiga toxin-producing E. coli. *J Appl Microbiol*, *88*(5), 729-745. doi:10.1046/j.1365-2672.2000.01031.x

O'Brien, A.D. & Holmes, R.K. (1987) Shiga and Shiga-like toxins. *Microbiology Reviews* **51**, 206 220.

Paton, A.W., Bourne, A.J., Manning, P.A., Paton, J.C. (1995) Comparative toxicity and virulence of *Escherichia coli* clones expressing variant and chimeric Shiga-like toxin type II operons. *Infection and Immunity* **63**, 2450 2458.

Pfaller, M. (1991). Diagnostic Applications of DNA Probes. *Infection Control And Hospital Epidemiology*, *12*(2), 103-110. doi:10.2307/30147054

Proulx F, Turgeon JP, Delage G, Lafleur L and Chicoine L. 1992. Randomized, controlled trial of antibiotic therapy for *Escherichia coli* O157:H7 enteritis. *J Pediatr*. **121:**299-303.

Methyl Red and Voges-Proskauer Test Protocols. Sylvia McDevitt, 08 Dec. 2009. Web. 5 Jan. 2015.

Nataro JP and Kaper JB. 1998. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev.***11:**142-201.

Sandvig, K. & Van Deurs, B. (1996) Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. *Physiological Reviews* **76**, 949 966.

Scotland, S.M., Willshaw, G.A., Smith, H.R., Rowe, B. (1990) Properties of strains of *Escherichia coli* O26: H11 in relation to their enteropathogenic or enterohemorrhagic classification. *Journal of Infectious Diseases* **162**, 1069 1074.

Sherman, P., Cockerill, F. III, Soni, R., Brunton, J. (1991) Outer membranes are competitive inhibitors of Escherichia coli O157:H7 adherence to epithelial cells. Infection and Immunity 59, 890 899.

Stevens, J. et al. (2006). "Actin-dependent movement of bacterial pathogens". *Nature Reviews Microbiology* 4: 91–101

Takeda T, Tanimura M, Yoshino K, Matsuda E, Uchida H and Ikeda N. 1998. Early use of antibiotics for STEC O157 infection reduces the risk of hemolytic uremic syndrome, pp 163-182. In *Escherichia coli O157:H7 and other Shiga toxin-producing E. coli* (Kaper JB and O‟Brien AD eds), ASM Press, Washington DC, USA.

Tesh, V.L., Burris, J.A., Owens, J.W., Gordon, V.M., Wadolkowski, E.A., O'Brien, A.D. et al. (1993) Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infection and Immunity* **61**, 3392 3402.

Todar K. Pathogenic E. coli. Available from: http://textbookofbacteriology.net/e.coli_2.html

Triple Sugar Iron Agar Protocols. Donald Lehman, 30 Sept. 2005. Web. 5 Jan. 2015.

Wadolkowski, E.A., Sung, L.M., Burris, J.A., Samuel, J.E., O'Brien, A.D. (1990) Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin type II. *Infection and Immunity* **58**, 3959 3965.

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 121oC 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. T1N1soft 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. 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

11. 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

10 x TBE (pH 8.3)
54.0gm of Tris-base, 27.5gm 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.

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 590µl of distilled water. It was stored at 4°C in 1ml aliquot.

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

Kovac's reagent
1.25gm 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°C.

Methyl red reagent
0.01gm 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 4oC.

Barritt's reagent
Solution A
1.25 gm of alpha-naphthol was dissolved in 95% ethanol with constant stirring to make 25ml solution. This solution was covered with aluminum foil and stored at 4°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°C.

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 Germany
Gel Documentation System: Major Science	Taiwan
Horizontal Gel Electrophoresis Unit	Wealtec Corporation, USA
Incubator	UK
Laminar Airflow Cabinet	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
Vortex Mixture	VWR International