Isolation of stx1 and stx2 genes from clinical and environmental samples of *Escherichia coli*

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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March 2017

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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, Samira Khandaker Shuvra 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. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

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Dedication

To

My Father, My Mother and My younger Sister Dia

ACKNOWLEDGEMENT

The piece of work I accomplished in pursuance of my B.S. dissertation happens to be the first undertaking of this nature I have ever been exposed to. It may be a small step as such but for me it was a great leap. I needed help and encouragement not to be frustrated in the event of repeated failures in my experiments. Fortunately there were people around me who provided the needed supports.

My regards, gratitude, indebtedness and appreciation goes to my respected supervisor **Fahareen-Binta Mosharraf**, Assistant Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University for her constant supervision, constructive criticism, expert guidance, enthusiastic encouragement to pursue new ideas and a good sense of humor and never ending inspiration throughout the entire period of my research work.

I express my gratitude towards **Prof. A. A. Ziauddin Ahmad**, Chairperson, Department of Mathematics and Natural Sciences, BRAC University, for his kind cooperation, active support and constant supervision. **Prof. Naiyyum Choudhury,** coordinator of Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences, for his exemplary guidance, monitoring and constant encouragement throughout the Project. He has been my moral guide throughout my student life.

I would like to thank and express my deepest gratitude to **Dr. Mahboob Hossain**, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University who helped and guided me in my report writing and providing time to time suggestions regarding setting of experimental designs, interpretation of results and subsequent directions for the whole work without being a bit of impatient. It would have been impossible to submit my report without his cordial help.

I also express my heartiest gratitude to **Nahreen Mirza and Promon Khan**, Teaching Assistant, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University who were abundantly helpful and offered invaluable assistance, support and guidance. Without

their supervision, cooperation and valuable suggestions it would be very difficult to complete the research.

My departmental teachers often enquired about my progress of the work and I was encouraged to keep in touch with them to have their valuable advice. I thank them all for their kind and affectionate care.

I extend my special thanks to my seniors and batch mates in the laboratory, who provided me with good working environment by their advice and encouragement to make me feel at home in my hard times.

My sincere appreciation is extended to staffs of the department and my friend **Saria Farheen** and **Rocksher Annur**, who helped me even beyond their duty period to continue my research work.

Samira Khandaker Shuvra

March, 2017

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 hemorrhagic colitis associated with consumption of hamburgers from a fast food chain restaurant. 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. 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 diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), urinary tract infections (UTI), septicemia and neonatal meningitis. In this study r, 7 samples from Dhaka city was collected, cultured in various media for enumeration, isolation and screening of *E. coli* colonies which were further analyzed to check for the presence of *stx* genes using PCR and gel electrophoresis. The seven samples collected were: Door knob swab, tea water, bhel puri, kitchen pipe swab, vegetable water, Lake water and Rectal swab. The samples collected initially were enriched in enrichment media overnight, followed by a dilution series which were then used for spread plating on nutrient agar and MacConkey agar and EMB for confirmation with the observation of pink colonies and 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 seven samples tested for stx1 and stx2 genes, two showed the presence of stx1 genes and one showed the presence of stx2 gene. The presence of the stx1 and *stx2* genes in regular food and in our surrounding signifies how close we are to a large outbreak. Knowledge of processing such food or avoiding such environmental contacts or taking precautions when possible may prevent occurrence of diseases.

Contents

List of Tables

List of Figures

List of abbreviations

EMB	Eosin Methylene Blue Agar
IMVIC	Indole, Methyl red, Voges-Proskauer, Citrate
LB	Luria Bertani
MAC	MacConkey Agar
MR	Methyl Red
NA	Nutrient Agar
TSI	Triple Sugar Iron
icddr,b	International Center for Diarrheal Disease Research, Bangladesh

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Escherichia coli (*E. coli*) is the most prevalent infecting organism in the family of gram-negative bacteria known as enterobacteriaceae. It is a gammaproteobacterium commonly found in the lower intestine of warm-blooded organisms. It was first discovered by a German bacteriologist, Theodor Escherich in 1885 and was named after him.

Escherichia coli are found in the environment, foods, and intestines of people and animals. *E. coli* are a large and diverse group of bacteria. Although most strains of *E. coli* are harmless, others can make people sick. Some kinds of *E. coli* can cause diarrhea, while others cause urinary tract infections, respiratory illness and pneumonia, and other illnesses. *Escherichia coli (E. coli)* bacteria normally live in the intestines of people and animals. Most *E. coli* are harmless and actually are an important part of a healthy human intestinal tract. However, some *E. coli* are pathogenic, meaning they can cause illness, either diarrhea or illness outside of the intestinal tract. The types of *E. coli* that can cause diarrhea can be transmitted through contaminated water or food or through contact with animals or persons (Donnenberg, M. *Escherichia coli*: Virulence Mechanisms of a Versatile Pathogen. San Diego: Academic Press, 2002).

E. coli is often referred to as the best or most-studied free-living organism. More than 700 serotypes of *E. coli* have been identified. The “O” and “H” antigens on the bacteria and their flagella distinguish the different serotypes.

1.2 Character and Morphology

E. coli is a Gram-negative, facultative anaerobic, typically rod shaped bacterium which does not sporulate. Cells are about 2.0 micrometers (μm) long and 0.25–1.0 μm in diameter. Motile strains possess flagella in a peritrichous arrangement.

Domain and Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gamma Proteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: *Escherichia*
Species: *Escherichia coli* [Moder, 2008].

1.3 Habitat

Escherichia coli cycles between two principal habitats. The bacteria start its life in the intestines of warm-blooded animals and spend most of the time there. Then after excreted into the environment, they spend the rest of their life in water, sediment, and soil-that are shown to be quite distinct with respect to physical conditions and the spectrum and level of available nutrients [Savageau, 1983].

1.4 Serotype

Serotype is a subdivision system of bacteria based on major surface antigens. In case of *E. coli*, the antigens are:

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

1. O antigen - a polymer of immunogenic repeating oligosaccharides (1–40 units)

2. Core region - phosphorylated no repeating oligosaccharides

3. Lipid A (endotoxin)

K antigen: A thick, mucous-like, layer of polysaccharide that surrounds some pathogen *E. coli*, known as acidic capsular polysaccharide (CPS). There are two separate groups of K antigen, I and II. Group I is associated with capsular polysaccharides and II with extra intestinal diseases.

H antigen: The H antigen is flagellant, of the flagella that allow *E. coli* to move. The H antigen group starts from H1 to H56 with some exceptions. These are encoded by the fliC gene.

1.5 Types Of *E.coli*

E. coli consists of a diverse group of bacteria. Pathogenic *E. coli* strains are categorized into pathotypes. Six pathotypes are associated with diarrhea and collectively are referred to as diarrheagenic *E. coli*.

1. Shiga toxin-producing *E. coli* (STEC): STEC may also be referred to as Verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC). This pathotype is the one most commonly heard about in the news in association with food borne outbreaks. The bacterium *Escherichia coli* O157:H7 has been reported as the predominant serotype of Shiga toxin producing *E. coli* (STEC). Cattles are considered to be the principal natural reservoirs of the organisms, excreting the bacteria in their feces. The first outbreak of STEC O157:H7 was recorded in the United States in 1982 and other outbreaks occurred later in the United Kingdom, continental Europe, Africa, New Zealand and Japan over the next decade. STEC O157:H7 infections cause hemorrhagic colitis and hemolytic uremic syndrome (HUS), which includes thrombocytopenia and acute renal failure.

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 diarrhea. 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. ETEC is transmitted by food or water contaminated with animal or human faeces.

3. Enteropathogenic *E. coli* (EPEC): The first pathotype described was EPEC which is primarily associated with causing diarrhea in children in developing countries. It is a

causative agent of diarrhea 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. Adherence procedure is very complicated and has dramatic effects in the structure of the cells which results in rearrangements of actin. The phenomenon is sometimes called "attachment and effacing" of cells. EPEC strains are not as invasive as *Shigella*, and unlike ETEC or EAEC, they cause an inflammatory response.

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. EAEC strains have the ability to attach to tissue culture cells in an aggregative manner. The organisms produce an enterotoxin of some sort. Recently, a distinctive heat-labile plasmid-encoded toxin has been isolated from these strains, called the EAST (Entero Aggregative ST) toxin. They also produce a hemolysin. The role of the toxin and the hemolysin in virulence has not been proven.

5. Enteroinvasive *E. coli* (EIEC): There are no known animal reservoirs of EIEC. So the primary source for EIEC appears to be infected humans. 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.

6. Diffusely adherent *E. coli* (DAEC): Some types of EPEC are referred to as diffusely adherent *E. coli* (DAEC), based on specific patterns of adherence. DAEC strains are significantly associated with diarrhea in children and urinary tract infections in adults. They are an important cause of traveler's diarrhea in Mexico and in North Africa.

1.6 Shiga Toxin Producing *E.coli* (STEC)

One particular type of *E. coli* causes disease by making a toxin called Shiga toxin. The bacteria that make these toxins are called "Shiga toxin-producing *E. coli*," or STEC, for short. Shiga toxin-producing *Escherichia coli* (STEC) were first discovered in 1977 and first associated with the clinical syndrome hemolytic-uremic syndrome (HUS) in 1983. Shiga toxin-producing Escherichia coli (STEC) are important enteric pathogens worldwide, causing diarrhea with or

without blood visibly present and hemolytic uremic syndrome. STEC are unique among diarrheogenic E coli in producing Shiga toxin type 1 and type 2, the virulence factors responsible for bloody diarrhea and hemolytic uremic syndrome. Cattle and other ruminants are the natural reservoir of STEC as their normal intestinal flora. Humans become infected by consumption of foods contaminated with cattle feces. Approximately 5%–10% of people with STEC infection will develop hemolytic-uremic syndrome (HUS), ~10% of those who develop HUS will die or have permanent renal failure, and up to 50% of those who develop HUS will develop some degree of renal impairment. Important concepts in understanding the pathogenesis and prevention of STEC-associated HUS are emerging, although no specific therapy yet exists. Early diagnosis of STEC infection is important because of the contraindication for treating STEC using antimicrobial agents, and the intense supportive care needed if renal failure occurs. Optimal management of STEC infection includes intravenous hydration, avoidance of antimotility agents and antimicrobials, and monitoring for sequel. STEC is often sub-divided into two categories: *E.coli* O157 and non-O157 STECs (Kaper, J.B., and A.D. O'Brien. *Escherichia coli O157:H7 and Other Shiga Toxin-producing E.coli Strains.* Washington, DC: American Society for Microbiology Press, 1998)

- **Most common type of STEC:** The most commonly identified STEC infections in North America are *E. coli* O157:H7 (often shortened to *E. coli* O157 or even just O157). When you hear news reports about outbreaks of *E. coli* infections, they are usually talking about *E. coli*O157.
- **Other types of STEC:** Other types of *E. coli* in the STEC pathotype are sometimes called "non-O157 STECs."

1.7 Virulence factor

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

Multiple virulence factors contribute to the pathogenesis of STEC. Its main pathogenic property is production of Shiga toxin (*stx*), which inhibits the protein synthesis of the host cells leading to cell death. STEC has the ability to produce one or more *stx's* (*stx1*, *stx2* or variants). Stx1 is virtually identical to shiga-toxin produced by *Shigella dysenteriae* type 1, while *stx*2 shares only ~56% identity with *stx*1.

1.7.1 Shiga toxins

Shiga toxin (Stx) is one of the most potent bacterial toxins known. stx is found in some serogroups of *Escherichia coli* (called Stx1 in *E. coli*). In addition to or instead of Stx1, some *E. coli* strains produce a second type of Stx, Stx2, that has the same mode of action as Stx/Stx1 but that is antigenically distinct. The Shiga toxin (Stx) of EHEC cleaves ribosomal RNA, thereby disrupting protein synthesis and killing the intoxicated epithelial or endothelial cells. Stx1 and Stx2 are compound toxins consisting of an A subunit (32 kDa) and a pentameric B subunit (7.7 kDa monomer). The *stxs* bind to the glycosphingolipid Gb3, a molecule comprised of a lipid or ceramide component and a trisaccharide of (α-gal (1→4)-β-gal (1→4)-β-glc).

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.

In contrast to Stx, the active site of the A-subunit of Stx2 is accessible in the holotoxin, and a molecule of formic acid and a water molecule mimic the binding of the adenine base of the substrate. Further, the A-subunit adopts a different orientation with respect to the B-subunits in Stx2 than in Stx, due to interactions between the carboxyl termini of the B-subunits and neighboring regions of the A-subunit. Of the three types of receptor-binding sites in the B-pentamer, one has a different conformation in stx2 than in stx, and the carboxyl terminus of the A-subunit binds at another. Any of these structural differences might result in different mechanisms of action of the two toxins and the development of hemolytic uremic syndrome upon exposure to stx2.

1.8 Objectives of the study

The aim of the entire project was to isolate the specific organism *E.coli* from environmental and Clinical sample. After isolation of the samples, a specific gene, which is stx1 and stx2 were identified by PCR method. Comparison was done in between the environmental and clinical sample after the PCR result was observed.

2. MATERIALS AND METHOD

2.1 Working place

The laboratory works of this research study were carried out in the laboratory of Microbiology, of the Department of Mathematics and Natural Sciences of BRAC University.

2.2 Materials

2.2.1 Sample collection

Seven samples were collected from different locations in Dhaka, mostly from Mohakhali, Mohammadpur and Dhanmondi. The first sample was collected from door knob of female toilet in second floor of BRAC University, Mohakhali, and Dhaka. Second sample was tea water from a local tea store in Mohammadpur, Dhaka. The third sample was bhel puri from a local vendor of Mohammadpur Bus stand. Bhel Puri is a common road side food in Bangladesh. Fourth sample comes from kitchen pipe of home (Mohammadpur). The fifth sample is lake water of Dhanmondi 32 Lake. Sixth sample is vegetable water of a local vendor in Mohakhali, near BRAC University. Finally the seventh and last sample is rectal swab of a patient admitted in National Institute of Disease Of The Chest And Hospital, Dhaka. The samples were collected from the month of August to September.

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

SOURCE	LOCATION	TIME	*E.coli* POSITIVE
Door knob (S-B)	Mohakhali	08:30 am	5 out of 7 isolates
Tea water (S-C)	Mohammadpur	07:00 am	2 out of 4 isolates
Bhel Puri (S-E)	Mohammadpur	11:00 pm	5 out of 8 isolates

Kitchen pipe swab (S-G)	Mohammadpur	11:00 pm	3 out of 5 isolates
Lake water (S-H)	Dhanmondi	06:00 pm	1 out of 3 isolates
Vegetable water (S-I)	Mohakhali	09:30 am	1 out of 4 isolates
Rectal swab (S-J)	National Institute of disease of the chest and Hospital, Mohakhali	12.30 pm	1 out of 2 isolates
Total			18 out of 33 isolates

2.3 Methods

Sterile Duran flasks were used for carrying liquid. All samples were inoculated within 30 minutes of collection except the ones collected at night and refrigerated overnight. Those samples were then cultured in an enrichment media in the morning.

2.3.1 Isolation of *E. coli* from Samples

- Enrichment
- Spread plating

2.3.2 Quantification and Isolation

The following day, the number of colonies on the nutrient agar plates was quantified and the colony forming unit was calculated. 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). All the plates for all seven sources showed TNTC count.

2.4 Stock and Subculture preparation

- **Stock:** Selected colonies that showed pink colonies on MacConkey agar and 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 inoculated T1N1 media were incubated at 37°C for about 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.
- **Subculture:** The selected colonies from nutrient agar were again cultured onto fresh NA plates for further stocking and biochemical tests.

2.5 Identification of *E. coli*

Some identification tests were done for confirmation of *E.coli* bacteria. This included spreading the raw sample in MacConkey and Eosin Methylene Blue (EMB) agar plates, Biochemical tests etc.

2.5.1 Selective Media:

One of the tests was spreading on Eosin Methylene Blue (EMB) and MacConkey media to ensure the presence of *E. coli* as they act as selective media for this gram negative bacillus.

a. Freshly prepared EMB and MacConkey plates were taken and labeled.
b. A glass rod was taken and flamed before pipeting sample from raw source. In case of swabs the cotton bar was used to do so.
c. Spreading was done on the plates.
d. Plates were incubated overnight at 37ºC.
e. Green sheen colonies should be seen in EMB plate and Pink colonies in MacConkey plates the next day.

MacConkey is a selective medium that inhibits the growth of Gram-positive bacteria due to the presence of crystal violet and bile salts. Gram-negative bacteria grow well on MAC. Gram-negative enteric bacteria like *E.coli* that grow on MacConkey agar are differentiated by their ability to ferment lactose. If the lactose is fermented by the bacteria, the production of the acid drops the pH of the media. The drop in pH is indicated by the change of neutral red indictor to pink**.** Strongly lactose fermenting bacteria produce sufficient acid which causes precipitation of the bile salts around the growth. It appears as a pink halo surrounding individual colonies or areas of confluent growth (Cappuccino and Sherman, 2011).

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).

2.5.2 Biochemical Identification

Biochemical tests were performed according to the methods described in Microbiology Laboratory Manual [Cappuccino *et al*., 2005]. The colonies sub cultured in nutrient agar are used here. The biochemical tests were Indole production test, methyl-red test, Voges-Proskauer test, citrate utilization test, triple sugar iron agar (TSI) test, Motility Indole urease (MIU) test, catalase test, Oxidase test and gram staining. 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.

a) Indole test: Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound Indole. [Cappuccino *et al*., 2005] The organism was grown in peptone water broth overnight. Kovac's reagent was added after incubation. Detection of positive result would give a purple layer at the top or a negative result had a yellow or brown layer.

b) Methyl-red test: When the culture medium turns red after addition of methyl red, because of a pH at or below 4.4 from the fermentation of glucose, it is called positive result. When the culture medium remains yellow, which occurs when less acid is produced (pH is higher) from the fermentation of glucose, it is called negative result. An orange color 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).

c) Voges-Proskauer test: Bacterium to be tested was inoculated into 3 ml dextrose phosphate broth (MR-VP broth) and incubated at 37^0C for 24 hours. To the aliquots of each broth cultures 10 drops of Barritt's reagent A was added and the cultures were shaken. Immediately, 10 drops of Barritt's reagent B was added and the cultures were shaken again. Cultures were then kept aside for 15 minutes for the reaction to occur. After 15 minutes, the colors of the cultures were examined and the results were recorded. Appearance of a red color was taken as a positive result. The absence of a rose color is a negative result (McDevitt, 2009).

d) Citrate utilization test: The shift in pH turns the bromothymol blue indicator in the medium from green to blue above pH 7.6 (Elmanama, 2009).

e) Triple sugar iron agar (TSI) test: To inoculate, colorless isolated colony from the Nutrient agar plate was picked with a cool, sterile needle, stabbed into the TSI containing dextrose, lactose and sucrose butt. Incubated with caps loosened at 37 °C for overnight and examined after 24 hrs for carbohydrate fermentation, CO_2 and H_2S production (Lehman, 2005). Yellow (acidic) color in the butt indicated that the organism being tested capable of fermenting all the three sugars, whereas red (alkaline) color in the slant and butt indicated that the organism being tested is non-fermented.

f) Motility Indole urease (MIU) tests: The colonies are stab-inoculated. Motile organisms show either diffused growth or turbidity extending away from stab inoculation line while nonmotile organisms grow along the stabline. Organisms that utilize urea produce

ammonia which makes the medium alkaline, showing pink-red color by change in the phenol red indicator. (Lehman, 2005).

g) Catalase test: To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculum of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production.

h) Oxidase test: This test is used to assist in the identification of all of which produce the enzyme cytochrome Oxidase.

i) Gram staining: Gram positive bacteria gives stain dark purple due to retaining the primary dye called Crystal Violet in the cell wall. Gram negative bacteria gives stain red or pink due to retaining the counter staining dye called Safranin.

2.5.3 Disc diffusion method for antibiotic susceptibility test

Kirby-Bauer antibiotic testing (also called KB testing or disk diffusion antibiotic sensitivity testing) uses antibiotic-containing wafers or disks to test whether particular bacteria are susceptible to specific antibiotics. Mueller Hinton Media is used for the antibiotic susceptibility test of bacteria. For antibiotic susceptibility test first off all MacFarlane solution was made.

One to two specific bacterial colonies were taken by a sterile loop from 24 hours of fresh subculture plate. Then it was inoculated into 0.8% physiological saline solution and mixed by vortexing. Next the turbidity of the saline solution was compared with 1% MacFarlane solution. Turbidity was observed by OD machine at 360nm. If the turbidity of the saline solution and MacFarlane solution becomes same then this saline solution containing bacteria can be used for the test. After taking turbidity, a cotton swab was dipped into the turbid saline solution and bacterial lawn was made on Muller Hinton agar media. Through a sterile forceps, specifics antibiotics were placed on the inoculated agar media and disks were slightly pressed on the agar to place it well. Then inoculated plates were incubated at 37ºC for 24 hours. After the incubation

period plates were observed and result were recorded. Results were taken by observing and measuring diameter of the clear zone around the antibiotics discs. According to the diameter of clear zone, it was determined whether the organisms were susceptible, intermediate or resistant to the antibiotics. No clear zone also indicates resistance to the antibiotic.

In this study, different types of antibiotics were used to see the antibacterial pattern of *Escherichia coli*. The list of antibiotics and their sensitivity level are given below:

Table 2: Antibiotics and their sensitivity level

Antibiotics name	**Resistant (mm)**	**Intermediate (mm)**	**Susceptible (mm)**
Amoxicillin (30µg)	≤ 13	14-17	≥ 18
Chloramphenicol (30µg)	≤ 12	≥ 18	13-17
Ciprofloxacin (5µg)	≤ 15	≥ 21	16-20
Tetracycline (30µg)	≤ 14	≥ 19	15-18
Streptomycin (10µg)	≤ 14	12-14	15-18

2.6 Characterization of *E.coli* by PCR and Gel electrophoresis

The polymerase chain reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. 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 conclude that the strains isolated from the environmental samples are indeed Shiga-toxin producing *E.coli*.

2.6.1 DNA Extraction

DNA isolation is a process of purification of DNA from sample using a combination of physical and chemical methods. By studying DNA, scientists can identify genetic disorders or diseases, and they can also possibly find cures for them by manipulating or experimenting with this DNA. DNA extraction is done in the following way, where the extracted DNA is known as template.

1. Transfer 2 ml of the overnight *E. coli* culture (grown in LB medium) to a 2 ml Eppendorf tube and centrifuge at max speed (13,500 rpm) for 3min to pellet the cells. Then discard the supernatant.
2. Resuspend the cell pellet in 600 µl lysis buffer and vortex to completely resuspend cell pellet.
3. Incubate 1 h at 37 °C.
4. Add an equal volume of phenol/chloroform and mix well by inverting the tube.
5. Spin at max speed for 5 min at RT. There is a white layer (protein layer) in the aqueous: phenol/chloroform interface.
6. Carefully transfer the upper aqueous phase to a new tube by using 1 ml pipetman.
7. To remove phenol, add an equal volume of chloroform to the aqueous layer. Again, mix well by inverting the tube.
8. Spin at max speed for 5 min.
9. Remove aqueous layer to new tube.
10. To precipitate the DNA, add 2.5 or 3 volume of cold 200 proof ethanol and mix gently.
11. Incubate the tube at -20 °C for 30 min or more.
12. Spin at max speed for 15 min at 4 °C.
13. Discard the supernatant and rinse the DNA pellet with 1 ml 70% ethanol (stored at RT).
14. Spin at max speed for 2 min. carefully discard the supernatant and air-dry the DNA pellet (tilt the tube a little bit on paper towel). To be faster, dry the tube at 37 °C incubator.
15. Resuspend DNA in TE buffer.

2.6.2 Master mix Preparation for PCR

Table 3: Master Mix preparation (per PCR tube)

Components	Volume
Master Mix	12.5µl
Nuclease free Water	5µl
Forward primer (1 µM)	2.5µl
Reverse primer (1 µM)	2.5µl
dsDNA template	2.5µl
Total	25µl

2.6.3 Primers used in the study

Table 4: Primer sequence

Target Gene	Primer	Sequence	Amplicon size
stx1	KalF	5'-GGGATAGATCCAGAGGAAGG-3'	622 bp
	KalR	5'-CCGGACACATAGAAGGAAACTC-3'	
stx2	Ka2F	5'-CTGGCGTTAATGGAGTTCAG-3'	381 bp
	Ka2R	5'-CCTGTCGCCAGTTATCTGAC-3'	

2.6.4 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 were set as given below (for 35 cycles):

a. 94^0C for 10 minutes
b. 94^0C for 1 minute
   55^0C for 1 minute
   72^0C for 1 minute
c. 72^0C for 7 minutes
d. 4^0C until further use

2.6.5 Gel electrophoresis

For the preparation of 40ml of a 2% Agarose solution, 0.8g Agarose was measured into a flask and 40ml of TE buffer was added to it. This solution was heated in a microwave oven until the Agarose dissolved and the solution became clear. Ethidium bromide was added to it after a while. 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 10-15 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 with TBE buffer. To prepare samples for electrophoresis, 2µl of gel loading dye was added for every 5µl of DNA solution. The PCR Master Mix (3µl) was loaded along with the dye. The gel was run at 80 volts and it took approximately 1 hour for the run to be complete. The gel was observed under UV light for band visualization.

Table 5: Sequence of sample loading in Agarose gel

Lane	Sample
Lane 1	100 bp DNA ladder
Lane 2	Positive reference strain
Lane 3	Sample B
Lane 4	Sample C
Lane 5	Sample E
Lane 6	Sample G
Lane 7	Sample H
Lane 8	100 bp DNA ladder
Lane 9	Positive reference strain
Lane 10	Sample B
Lane 11	Sample C
Lane 12	Sample E
Lane 13	Sample G
Lane 14	Sample H

3 Results

3.1 Isolation of *E. coli* from samples

Seven basic samples were collected from various places of Dhaka city. The samples were enriched in LB broth in some cases for 18 hours at 37°C. After enrichment; the culture broth was subjected to a twofold dilution. From appropriate dilutions, the broth cultures were spread plated onto nutrient agar to determine the number of colony forming units. MacConkey Agar and EMB Agar were 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 the plates. Approximately 33 isolates were obtained from primary isolation plates of nutrient agar and 18 were considered for further investigation.

Table 6: Total CFU from Nutrient Agar plates of different samples

Samples	CFU in Nutrient Agar
Door Knob swab (S-B)	$6.8*10^5$
Tea Water (S-C)	$4.5*10^5$
Bhel Puri (S-E)	$5.1*10^5$
Kitchen Pipe swab (S-G)	$6.2*10^5$
Lake Water (S-H)	$8.6*10^5$
Vegetable Water (S-I)	$8.9*10^5$
Rectal Swab (S-J)	$5.5*10^5$

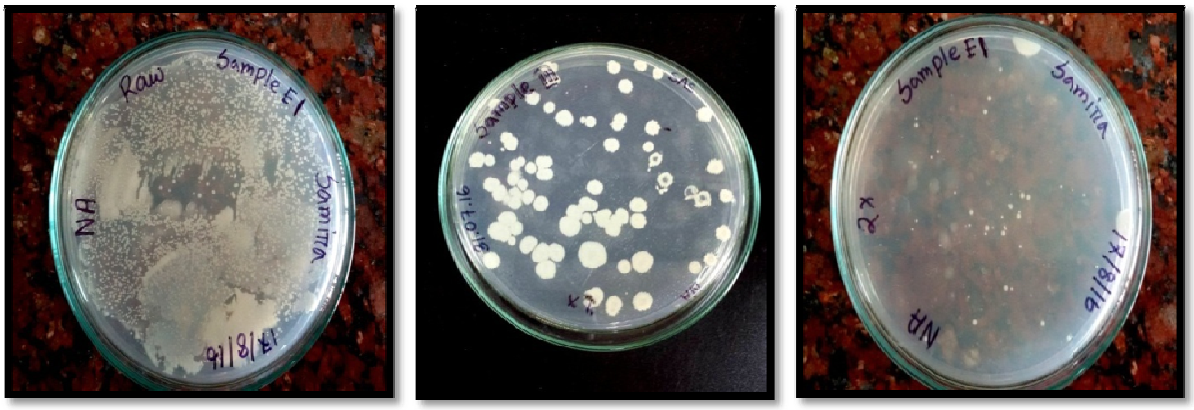

Figure 1: Nutrient Agar plates showing bacterial growth from different samples

3.2.1 Identification of suspected *E. coli* isolates on MacConkey and EMB plates

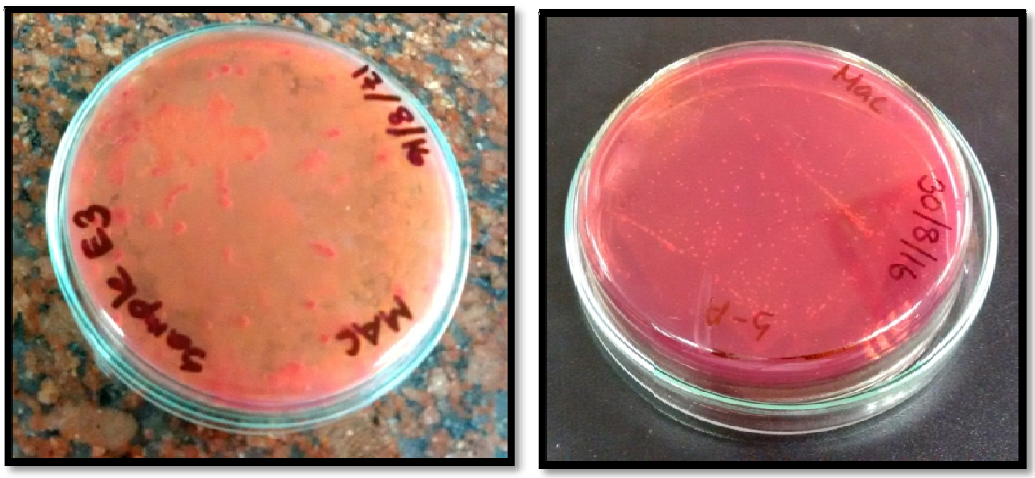

Figure 2: MacConkey agar plates showing growth of lactose fermenting bacteria from Bhel Puri and Door knob samples.

After being cultured on EMB Agar and incubated overnight, the *E.coli* was grown as green metallic sheen form. Other bacteria would be grown as pink or dark purple form.

Table 7: Cultural characteristics of colony isolates on EMB media

Sample	Colony isolate	Cultural characteristics on EMB agar
S-B	B-1	Green metallic sheen
	B-2	Green metallic sheen
	B-3	Dark purple colony
	B-4	Green metallic sheen
	B-5	Pink colony
	B-6	Green metallic sheen
	B-7	Green metallic sheen
S-C	C-1	Dark purple colony
	C-2	Green metallic sheen
	C-3	Green metallic sheen
	C-4	Pink colony
S-E	E-1	Green metallic sheen
	E-2	Green metallic sheen
	E-3	Green metallic sheen
	E-4	Pink colony
	E-5	Green metallic sheen
	E-6	Dark purple colony
	E-7	Pink colony
	E-8	Green metallic sheen
S-G	G-1	Pink colony
	G-2	Green metallic sheen
	G-3	Dark purple colony
	G-4	Green metallic sheen
	G-5	Green metallic sheen
S-H	H-1	Pink colony

	H-2	Green metallic sheen
	H-3	Dark purple colony
S-I	I-1	Pink colony
	I-2	Dark purple colony
	I-3	Green metallic sheen
	I-4	Pink colony
S-J	J-1	Green metallic sheen
	J-2	Dark purple colony

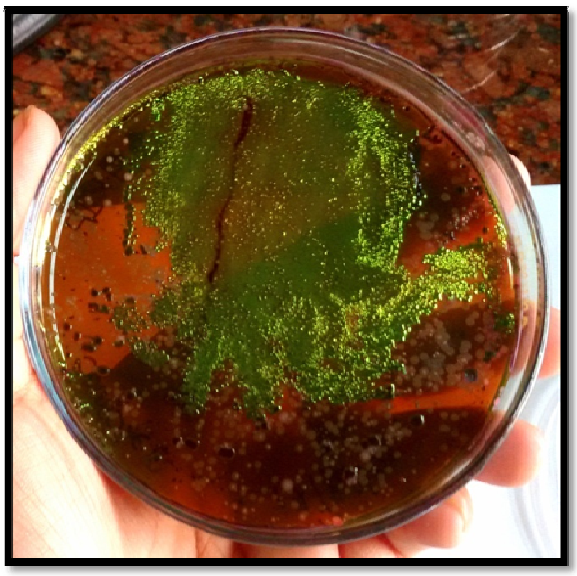

Figure 3: EMB subculture plate showing growth from various samples.

3.2.2 Subculture

After the positive isolates were determined, they were subculture in Nutrient agar for further use.

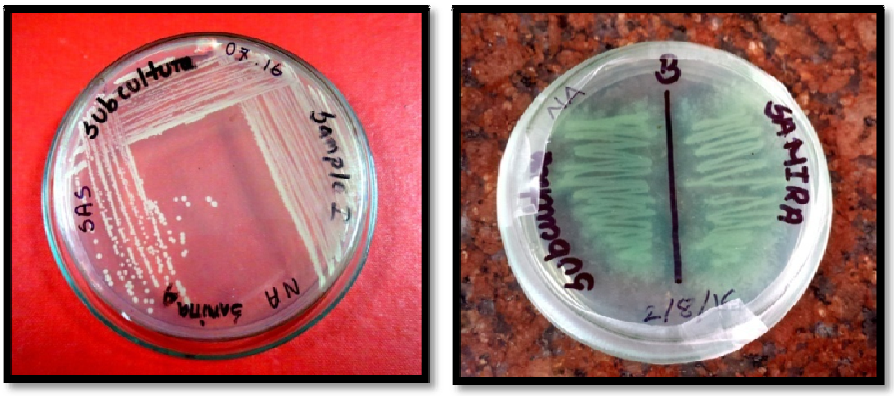

Figure 4: Subcultures of sample I and sample B

3.3 Biochemical identification

Nine biochemical tests were done for further confirmation. They are Indole test, MR test, VP test, Citrate test, TSI test, MIU test, Catalase test, Oxidase test and Gram Staining. After this 7 isolates were confirmed out of 18 isolates. 3 isolates showed intermediate result. They were re-tested and they showed negative results.

Table 8: Biochemical test results

Colony Isolate	Indole	MR	VP	Citrate	TSI	MIU	Catalase	Oxidase	Gram Staining
S-B1	+	-	-	+	+	-	-	-	Gram -
S-B2	+	+	-	-	+(gas)	+	+	-	Gram -
S-B4	-	-	-	+	-	-	-	-	Gram +
S-B6	+	+	-	-	+(gas)	+	+	-	Gram -
S-B7	-	-	-	+	-	-	-	-	Gram +
S-C2	+	+	-	-	+(gas)	+	+	-	Gram -
S-C3	-	-	-	+	-	-	-	-	Gram -
S-E1	+	+	-	-	+(gas)	+	+	-	Gram -
S-E2	-	-	-	+	-	-	-	-	Gram +
S-E3	+	+	-	-	+(gas)	+	+	-	Gram -

S-E5	-	-	-	+	-	-	-	-	Gram +
S-E8	+	+	-	+	+	-	-	-	Gram -
S-G2	-	-	-	+	-	-	-	-	Gram -
S-G4	+	+	-	-	+(gas)	+	+	-	Gram -
S-G5	-	-	-	+	-	-	-	-	Gram +
S-H2	+	+	-	-	+(gas)	+	+	-	Gram -
S-I3	-	-	-	+	-	-	-	-	Gram +
S-J1	+	-	-	-	+	-	-	-	Gram -

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

Reconfirmation:

Biochemical test results for S-B1, S-E8 and S-J1

Colony Isolate	Indole	MR	VP	Citrate	TSI	MIU	Catalase	Oxidase	Gram staining
S-B1	-	-	-	+	-	-	-	+	Gram -
S-E8	-	-	-	+	-	-	-	+	Gram -
S-J1	-	-	-	+	+	-	-	+	Gram-

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

Figure 5: Indole test results and citrate result showing control, positive result and negative result

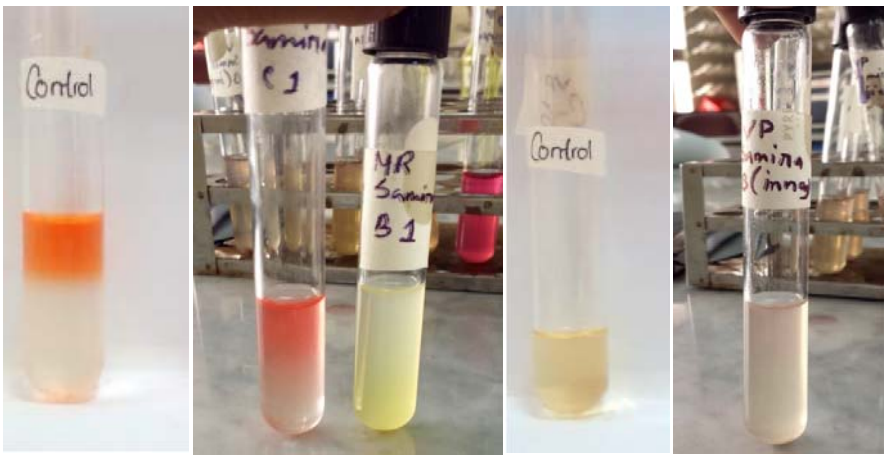

Figure 6: Methyl red test and VP test result showing control, positive and negative results

Figure 7: TSI test result showing control, positive result with gas production, positive result with H_2S production and negative result and MIU test result

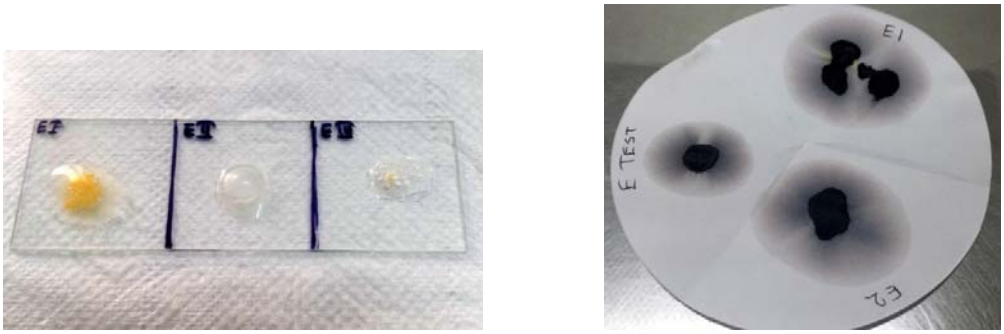

Figure 8: Oxidase test result on a slide showing bubble production as positive result and no bubble production as negative result and catalase test showing negative result

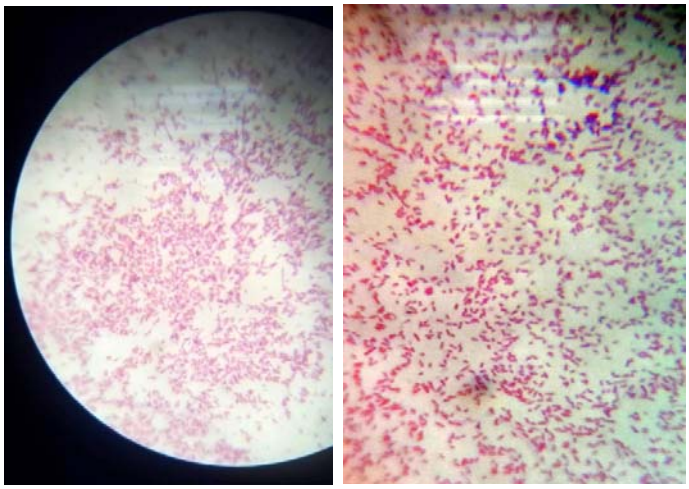

Figure 9: Gram negative bacteria seen under microscope

3.4 Antibiotic result

The zone of inhibition for around each antibiotic disc is measured and it is determined whether they are sensitive, resistant or intermediate towards the sample. The result was compared with the ideal chart and 5 out of 7 samples were concluded as positive.

Table 9: Zone of inhibition for different antibiotics

Sample	Tetracycline	Amoxicillin	Streptomycin	Chloramphenicol	Ciprofloxacin	Result
S-B2	18 mm	4 mm	17 mm	18 mm	24 mm	+
S-B6	12 mm	16 mm	4 mm	10 mm	16 mm	-
S-C2	26 mm	0 mm	16 mm	32 mm	32 mm	+
S-E1	16 mm	2 mm	16 mm	20 mm	22 mm	+
S-E3	10 mm	14 mm	22 mm	16 mm	10 mm	-
S-H2	18 mm	4 mm	15 mm	18 mm	24 mm	+
S-G4	18 mm	4 mm	12 mm	20 mm	28 mm	+

Legend+ indicates a positive result and – indicates a negative result

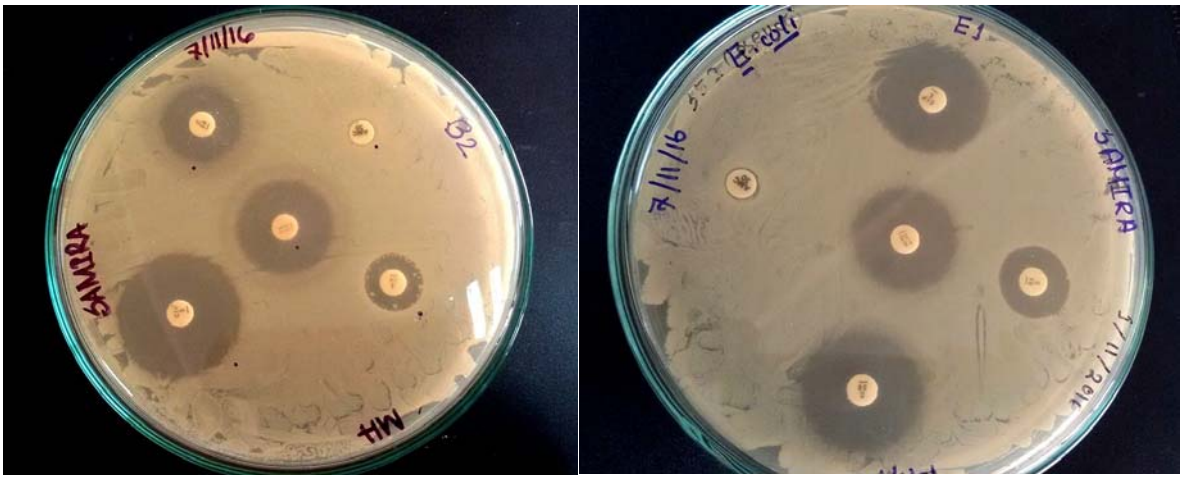

Figure 10: Zone of inhibition created by S-B2 and S-E1 for different antibiotics

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

Template DNA was prepared from cellular biochemically identified isolates 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 S-E1 and S-H2 were positive for *stx*1 but were negative for *stx*2 and isolate S-B2 was positive for s*tx2* and negative for the other, 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.

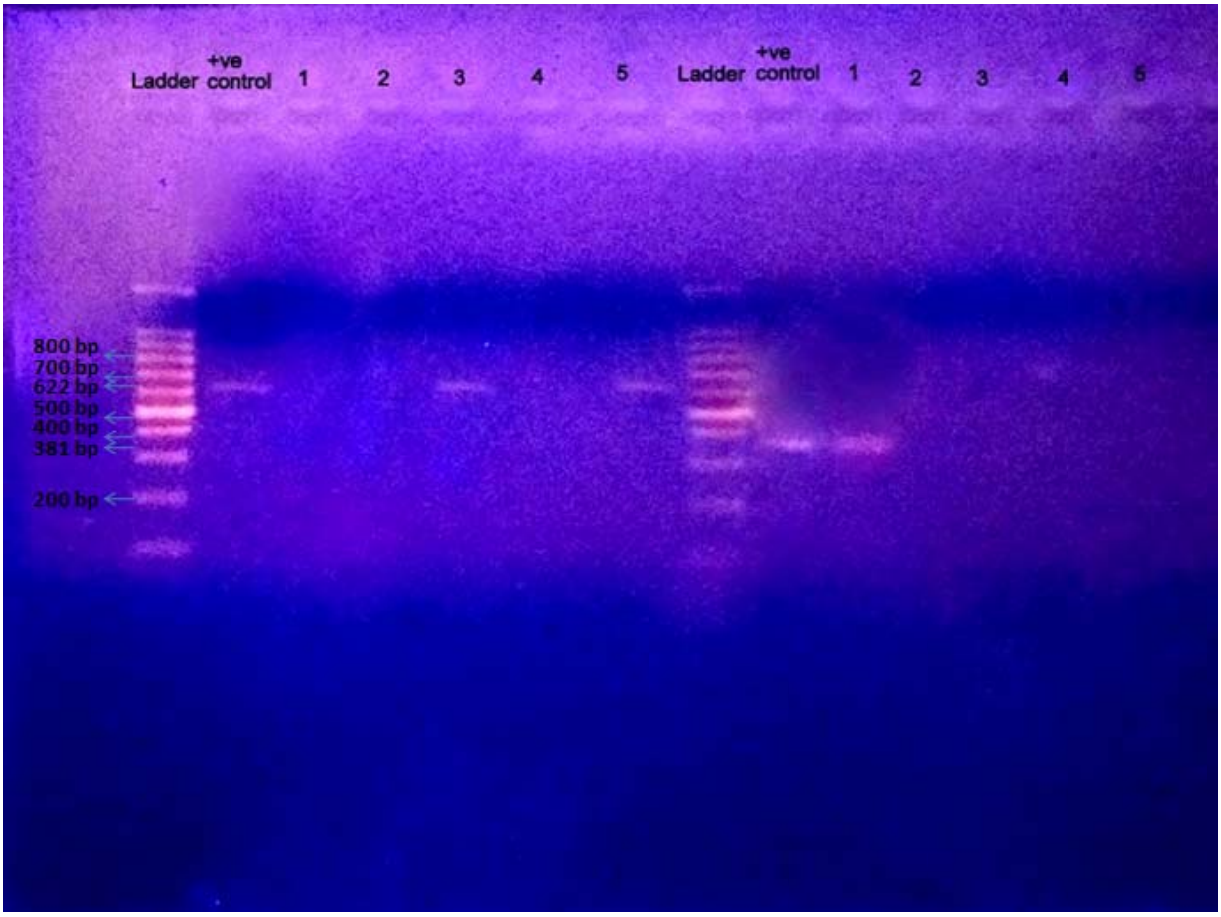

Figure 11: Gel Electrophoresis of *E. coli* samples

Lane 1: 100bp Marker

Lane 2: +ve control

Lane 3: S-B2

Lane 4: S-C2

Lane 5: S-E1

Lane 6: S-G4

Lane 7: S-H2

Lane 8: 100bp Marker

Lane 9: +ve control

Lane 10: S-B2

Lane: 11: S-C2

Lane 12: S-E1

Lane 13: S-G4

Lane 14: S-H2

Table 10: PCR results

Samples	Stx1	Stx2
Door Knob (S-B2)	-ve	+ve
Tea Water (S-C2)	-ve	-ve
Bhel Puri (S-E1)	+ve	-ve
Kitchen Pipe (S-G4)	-ve	-ve
Lake water (S-H2)	+ve	-ve

4. Discussion and Conclusion

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. Around 5–10% of those who are diagnosed with *E. coli* O157 infection develops HUS (Scallan, Jones, Cronquist, Thomas, Frenzen, & Hoefer, 2006).

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). Most Shiga toxin producing *E. coli* cases notified in Australia are sporadic infections. Infected individuals usually present with bloody diarrhea and some may experience kidney failure due to HUS. HUS may occur in 5-10% of individuals during EHEC outbreaks and is more likely to occur among children or the elderly. HUS carries a 12% risk of death or end stage renal disease with 25% of survivors suffering long-term renal consequences (Vally H, Hall G, Dyda A, et al. Epidemiology of Shiga toxin producing *Escherichia coli* in Australia, 2000-2010. *BMC Public Health* 2012; 12:63-74.) 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.

During the study total of 33 isolates from 7 samples of door knob, tea water, bhel puri, kitchen pipe, lake water, vegetable water and rectal swab were presumptively identified as *E. coli* from primary MacConkey plate and EMB plate. It showed that the samples which were assumed as the source of enteric bacteria were rightfully assumed so. The isolates were subjected to detailed biochemical characterizations like Indole production test, Methyl-red test, Voges-Proskauer test, Citrate utilization test, Triple Sugar Iron test, Motility Indole Urease test, Citrate test, Oxidase test, Gram staining and antibiotic test. Out of 18 samples analyzed, only 7 isolates, gave identical biochemical properties. It means the rate of spread of *E.coli* from these sources is approximately 38%. Culturally and biochemically positive isolates were tested for *stx*1 and *stx*2 genes. Total five isolates were selected. From all these isolates, two *stx*1 genes were detected and one was detected for *stx*2. So the surveillance of *stx* gene is 60% Therefore, this data showed the prevalence of *E. coli* in Bangladesh and demands for further study for the prevention of diseases.

Based on these results it can be said that the door knobs of toilets, bhel puri and lake waters are not safe.

After coming out of toilet, though we wash our hands properly, but chances are there that the door knobs might also be contaminated. So pure hygiene should be confirmed after coming out from the entire toilet area. The toilet are should also be cleaned regularly and properly to avoid infection. Bhel puri which is a daily choice of people in Dhaka city is also not safe according to the laboratory tests. Bhel puri contains several ingredients like potato, coriander etc. The contamination can spread from any of the component. Even the environment in which they are sold is not clean enough. This can cause severe illness among the people of Dhaka city. For this reason this sort of roadside food like bhel puri should be avoided. Lake waters cannot be used for drinking or washing purposes as it can also be a source of illness.

Both environmental and Clinical samples were used for the study and it was seen that the clinical sample which was a rectal swab, didn't contain any stx gene. The clinical sample collected was rectal swab from patient admitted in National Institute of disease of the chest and Hospital, Mohakhali. The patient didn't have any such disease. Usually the chances of getting *E.coli* having stx gene in rectal sample is average. According to a study, Escherichia coli O157:H7 was isolated from faeces (4.7%), skin swabs (8.7%) and carcasses before washing (8.1%) and after washing (8.7%) and on water samples (4.2%). The proportion of carcasses contaminated with E. coli O157:H7 was strongly associated with those recovered from faecal and skin samples (Mersha G, Asrat D, Zewde BM, Kyule M. Occurrence of Escherichia coli O157:H7 in faeces, skin and carcasses from sheep and goats in Ethiopia.2009). In human this bacteria is usually found in colon and anus and thus the infection sites related to them. But due to safety measures and lack of any such infection, this might not be available. On the other hand this seems available in environmental samples. The normal habitat of *E.coli* in environment can be a major reason behind their presence in the environmental samples.

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.

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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 121^0C for 15 min.

1. Nutrient Media (Himedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	5.0
Beef Extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

2. Nutrient Broth (Oxoid, England)

Ingredients	Amount(g/L)
Lab-lemcopowder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

3. T1N1 soft agar

Ingredients	Amount(g/L)
Tryptone	0.6 g
Sodium chloride	0.3g
Agar	0.42 g

4. 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

5. Eosin 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

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

Ingredients	Amount(g/L)
Magnesium sulfate	0.2
Ammonium dihydrogenphosphate	0.2
Ammonium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0

Agar	15.0
Bactobromthymolblue	0.08

7. MR-VP broth

Ingredients	**Amount(g/L)**
Peptone	7 g
Dextrose	5 g
Potassium phosphate	5 g

Appendix-II

Kovac's reagent

5 g of para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4ºC.

Methyl red reagent

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4ºC.

Barritt's reagent

Solution A

5 g of alpha-naphthol was dissolved in 95% ethanol. This solution was covered with aluminum foil and stored at 4ºC.

Solution B

40 g 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. This solution was covered with aluminum foil and stored at4ºC.

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) was 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.

Appendix-III

Instruments

The important equipments used through the study are listed below:

Serial Number	Name of Item	Specification
1	Autoclave, Model No: WAC-47	Korea
2	Sterilizer, Model No: NDS-600D	Japan
3	Balance(Core series): Adam	UK
4	Centrifuge, Model No: Code: 5433000.011	Eppendorf, Germany
5	Digital Homogenizer (Wise Tis)	Korea
6	Freezer (-20°C)	Siemens Germany
7	Gel Documentation System: Major Science	Taiwan
8	Horizontal Gel Electrophoresis Unit	Wealtec Corporation, USA
9	Incubator	UK
10	Laminar Airflow Cabinet	UK
11	Micropipettes	Eppendorf, Germany
12	Oven (Universal drying oven) Model: LDO-060E	Labtech, Singapore
13	Thermal Cycler, Model No: 2720	Applied Biosystems, USA
14	Refrigerator, Model: 0636	Samsung
15	Vortex Mixture	VWR International