Journal of Immunology and Allergy
ISSN: 2582-6549
Shuvra SK, 2023-J Immuno Allerg
Review Article

Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City

Samira Khandaker Shuvra*

Microbiologist, Biotechnologist, Assistant Teacher, 2554 Natta Blvd, Bellmore, NY11710

***Corresponding Author:** Shuvra SK, Microbiologist, Biotechnologist, Assistant Teacher, 2554 Natta Blvd, Bellmore, NY11710.

Received Date: 05-05-2023

Accepted Date: 13-06-2023

Published Date: 18-09-2023

Abstract

Escherichia 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. Ability of *Escherichia 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, *Escherichia 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 *Escherichia 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. In this study r, 7 samples from Dhaka city was collected, cultured in various media for enumeration, isolation and screening of Escherichia coli colonies which were further analysed 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 Skin 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 *Escherichia 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 the surrounding signifies how close is a large outbreak. Knowledge of processing such food or avoiding such environmental contacts or taking precautions when possible may prevent occurrence of diseases.

Keywords: Diarrhoea; *Escherichia coli*; Haemorrhagic colitis; Haemolytic uremic syndrome; Urinary tract infections; Septicaemia; Neonatal meningitis.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

Introduction

Escherichia 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 the bacteriologist.

Escherichia coli are found in the environment, foods, and intestines of people and animals. *Escherichia coli* are a large and diverse group of bacteria. Although most strains of *Escherichia coli* are harmless, others can make people sick. Some kinds of Escherichia coli can cause diarrhea, while others cause urinary tract infections, respiratory illness and free-living organisms.

More than 700 serotypes of *Escherichia coli* have *been* identified. The "O" and "H" antigens on the bacteria and the flagella distinguish the different serotypes.

Character and morphology

Escherichia 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*

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 the 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.

Serotype

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

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

- O antigen-a polymer of immunogenic repeating oligosaccharides (1-40 units)
- Core region-phosphorylated no repeating oligosaccharides
- Lipid A (endotoxin)

K antigen: A thick, mucous-like, layer of polysaccharide that surrounds some pathogen Escherichia 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 *Escherichia coli* to move. The H antigen group starts from H1 to H56 with some exceptions. These are encoded by the fliC gene.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

Types of *Escherichia Coli*

Escherichia coli consists of a diverse group of bacteria. Pathogenic *Escherichia coli* strains are categorized into pathotypes.

Six pathotypes are associated with diarrhea and collectively are referred to as diarrheagenic *Escherichia coli*.

- Shiga toxin-producing *Escherichia coli* (STEC): STEC may also be referred to as Verocytotoxin-producing *Escherichia coli* (VTEC) or enterohemorrhagic Escherichia 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 *Escherichia coli* (STEC). Cattles are considered to be the principal natural reservoirs of the organisms, excreting the bacteria in the 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.

- Enterotoxigenic *Escherichia coli* (ETEC): Enterotoxigenic *Escherichia coli*, or ETEC, are a group of *Escherichia coli* that produce special toxins which stimulate the lining of the intestines causing them to secrete excessive fluid, thus producing diarrhoea. 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.

- Enteropathogenic *Escherichia 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 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. 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.

- Enteroaggregative *Escherichia 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

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

and the hemolysin in virulence has not been proven.

- Enteroinvasive *Escherichia 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 damage the intestinal wall severely through mechanical cell destruction.

- Diffusely adherent *Escherichia coli* (DAEC): Some types of EPEC are referred to as diffusely adherent *Escherichia 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.

Shiga toxin producing *Escherichia coli* (STEC)

One particular type of *Escherichia coli* causes disease by making a toxin called Shiga toxin. The bacteria that make these toxins are called "Shiga toxin-producing *Escherichia 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. 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 the 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 sequelae. STEC is often sub-divided into two categories: *Escherichia coli* O157 and non-O157 STECs [2]. Most common type of STEC: The most commonly identified STEC infections in North America are Escherichia coli O157:H7 (often shortened to Escherichia coli O157 or even just O157).

- When you hear news reports about outbreaks of *Escherichia coli* infections, they are usually talking about *Escherichia Coli* O157.
- Other types of STEC: Other types of *Escherichia coli* in the STEC

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

pathotype are sometimes called "non-O157 STECs.

Virulence factor

Virulence factors are produced by organisms that contribute to pathogenicity and aid bacteria in survival and growth. Adhesions, invasives, and antiphagocytic factors help to support and maintain the colonization of the host. The factors that cause damage to the host comprise of toxins, hemolysins, 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 stx2 shares only ~ 56% identity with stx1.

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 *Escherichia coli*). In addition to or instead of Stx1, some *Escherichia 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 *Escherichia 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.

Materials and method

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.

Materials

Sample collection: Seven samples were collected from different locations in Dhaka, mostly from Mohakhali, Mohammadpur and Dhanmondi. The first sample was collected

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

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. Bhelpuri 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 skin 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.

Source	Location	Time	*Escherichia coli* Positive
Door knob (S-B)	Mohakhali	8:30 AM	5 out of 7 isolates
Tea water (S-C)	Mohammadpur	7:00 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	6:00 PM	1 out of 3 isolates
Vegetable water (S-I)	Mohakhali	9:30 AM	1 out of 4 isolates
Skin 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

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

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.

Isolation of *Escherichia coli* from Samples

- Enrichment
- Spread plating

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.

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, the stocks would be discarded. The colonies that showed a green sheen for the second time were kept, and the 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.

Identification of *Escherichia coli*

Some identification tests were done for confirmation of *Escherichia coli* bacteria.

This included spreading the raw sample in MacConkey and Eosin Methylene Blue (EMB) agar plates, Biochemical tests etc.

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

- Freshly prepared EMB and MacConkey plates were taken and labeled.
- A glass rod was taken and flamed before pipetting sample from raw source. In case of swabs the cotton-bar was used to do so.
- Spreading was done on the plates.
- Plates were incubated overnight at 37°C.
- Green sheen colonies should be seen in EMB plates and pink colonies in MacConkey plates the next day.

MacConkey's 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 *Escherichia coli* that grow on MacConkey agar are differentiated by the 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 [3]. 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 *Escherichia coli*.

The *Escherichia coli* colonies are black colonies with a metallic green sheen caused by the large quantities of acid that is

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

produced and that precipitates out the dyes onto the growth's surface [3].

Biochemical Identification

Biochemical tests were performed according to the methods described in Microbiology Laboratory Manual [3]. 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 *Escherichia 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: Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound indole [3]. 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.
- 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 [4].
- Voges-Proskauer test: Bacterium to be tested was inoculated into 3 ml dextrose phosphate broth (MR-VP broth) and incubated at 370C 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 [4].
- Citrate utilization test: The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6.
- 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. 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.
- 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.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

Organisms that utilize urea produce ammonia which makes the medium alkaline, showing pink-red color by change in the phenol red indicator.

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

Disc diffusion method for antibiotic susceptibility test

Kirby-Bauer antibiotic testing (also called KB testing 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 of all, MacFarlane solution was made (www.microbiologyinfo.com).

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.

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
Tetracyclin (30µg)	≤ 14	≥ 19	15-18
Streptomycin (10µg)	≤ 14	14-Dec	15-18

Table2: Antibiotics and the sensitivity level.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

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 results 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.

Characterization of *Escherichia 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 *Escherichia coli*.

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.

- Transfer 2 ml of the overnight *Escherichia 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.
- Resuspend the cell pellet in 600 µl lysis buffer and vortex to completely resuspend cell pellet.
- Incubate 1 h at 37 °C.
- Add an equal volume of phenol/chloroform and mix well by inverting the tube.
- Spin at max speed for 5 min at RT. There is a white layer (protein layer) in the aqueous: phenol/chloroform interface.
- Carefully transfer the upper aqueous phase to a new tube by using 1 ml pipette.
- To remove phenol, add an equal volume of chloroform to the aqueous layer. Again, mix well by inverting the tube.
- Spin at max speed for 5 min.
- Remove aqueous layer to new tube.
- To precipitate the DNA, add 2.5 or 3 volume of cold 200 proof ethanol and mix gently.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

- Incubate the tube at -20 °C for 30 min or more.
- Spin at max speed for 15 min at 4 °C.
- Discard the supernatant and rinse the DNA pellet with 1 ml 70% ethanol (stored at RT).
- 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.
- Resuspend DNA in TE buffer.

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

Table 3: Master mix preparation (per PCR tube).

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):
    1. 94°C for 10 minutes
    2. 94°C for 1minute 55°C for 1 minute 72°C for 1 minute
    3. 72°C for 7 minutes
    4. 4°C until further use

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.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

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.

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'	

Table 4: Primer sequence.

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

Table 5: Sequence of sample loading in Agarose gel.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

Results

Isolation of *Escherichia 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 colonies forming units. MacConkey Agar and EMB Agar were used for isolation of *Escherichia 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.

Samples	CFU in Nutrient Agar
Door Knob swab (S-B)	6.8*105
Tea Water (S-C)	4.5*105
Bhel Puri (S-E)	5.1*105
Kitchen Pipe swab (S-G)	6.2*105
Lake Water (S-H)	8.6*105
Vegetable Water (S-I)	8.9*105
Skin Swab (S-J)	5.5*105

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

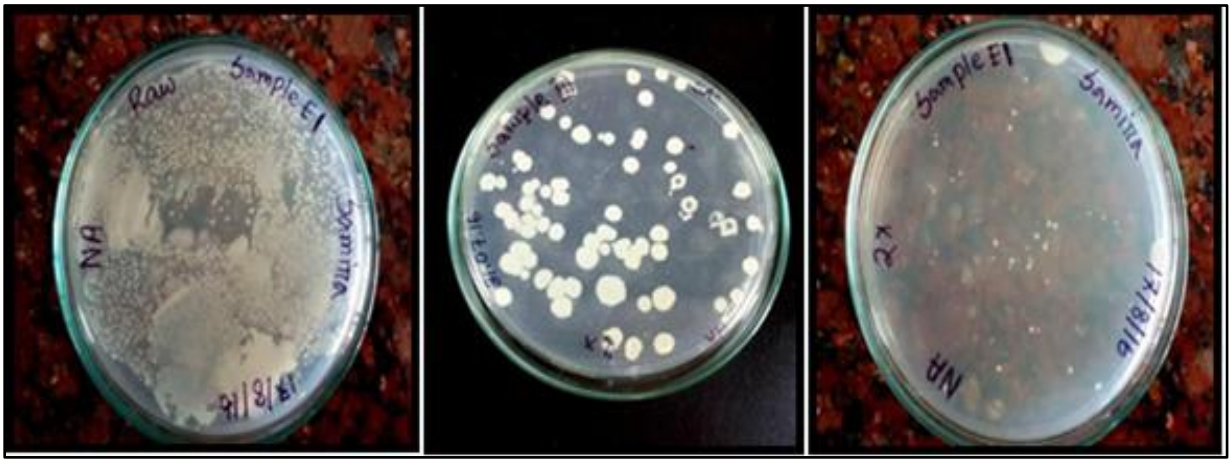

Figure 1: Nutrient Agar plates showing bacterial growth from different samples.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

Identification of suspected *Escherichia coli* isolates on McConkey and EMB plates

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.

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

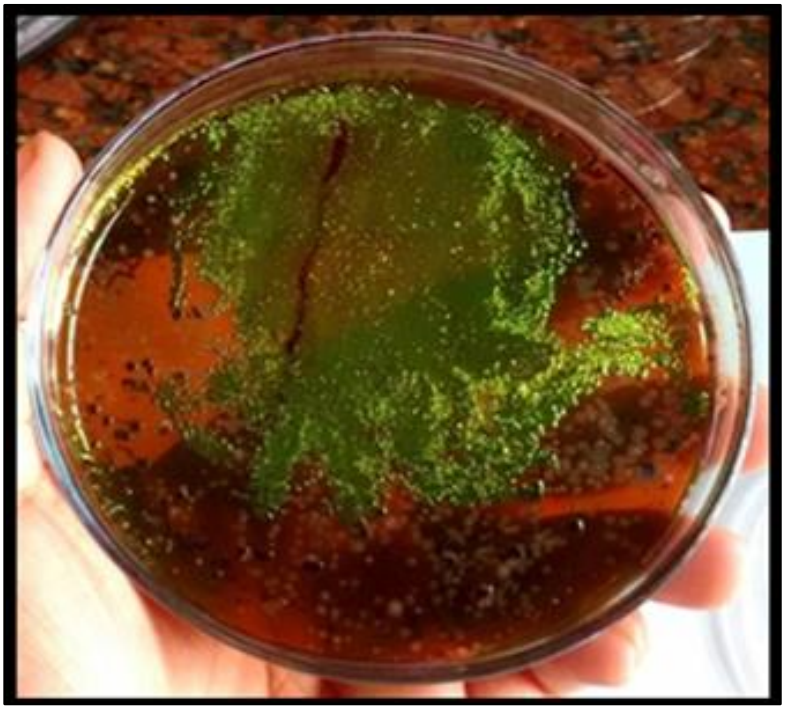

Figure 3: EMB subculture plate showing growth from various samples.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

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

Table 7: Cultural characteristics of colony isolates on EMB media.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

Subculture

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

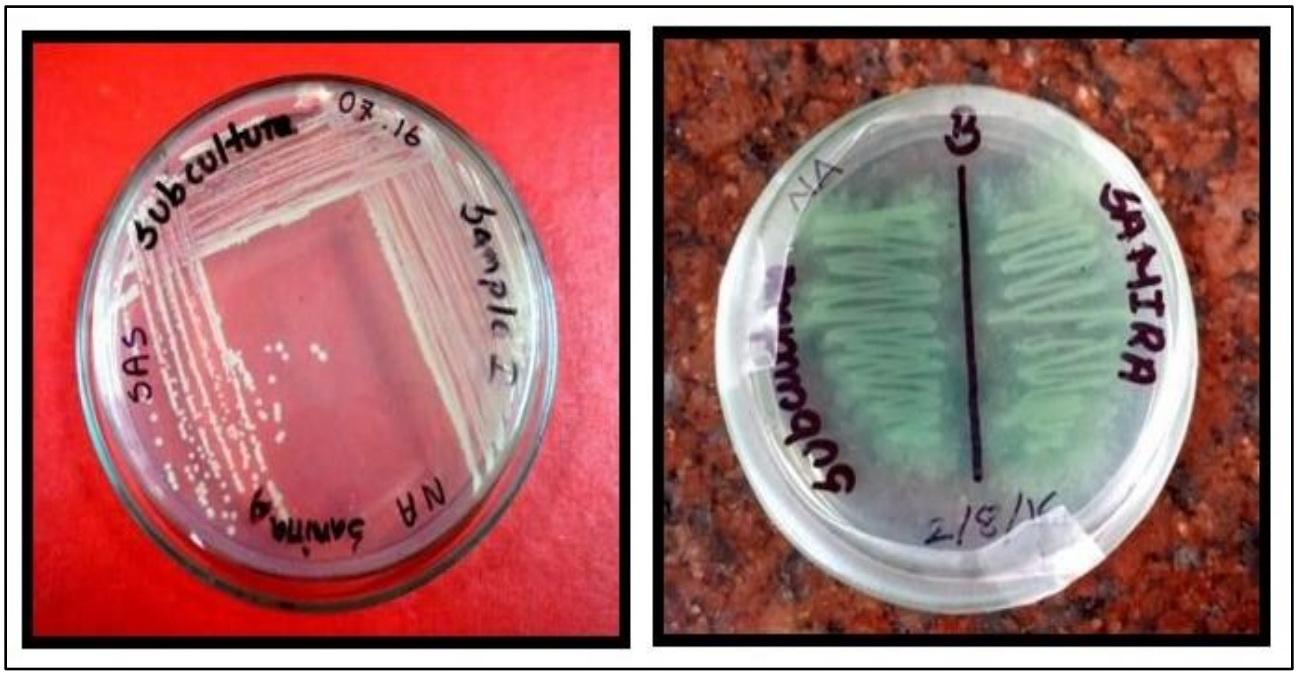

Figure 4: Subcultures of sample I and sample B.

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.

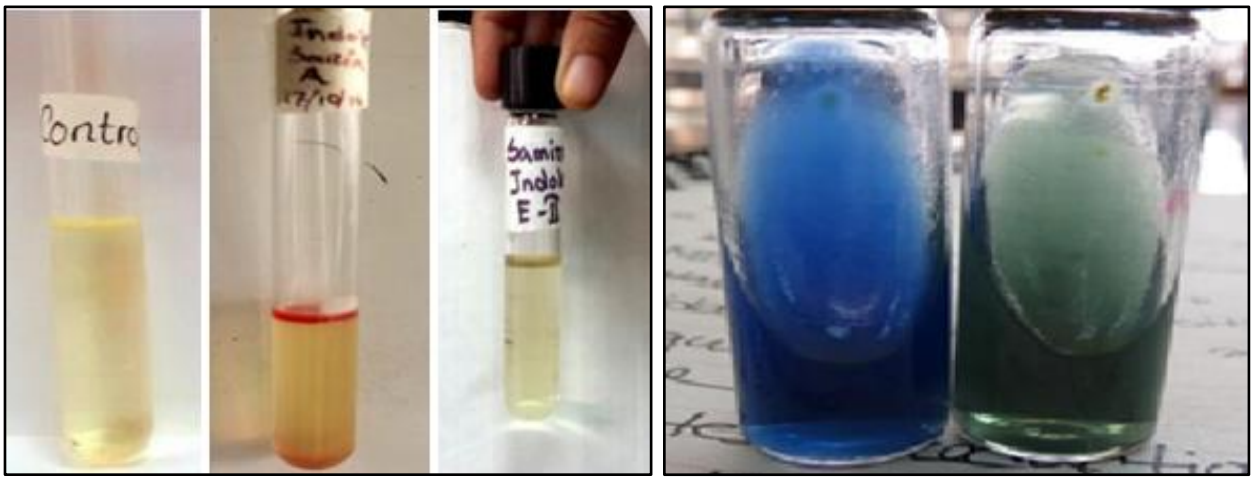

Figure 5: Indole test results and citrate result showing control, positive result and negative result.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

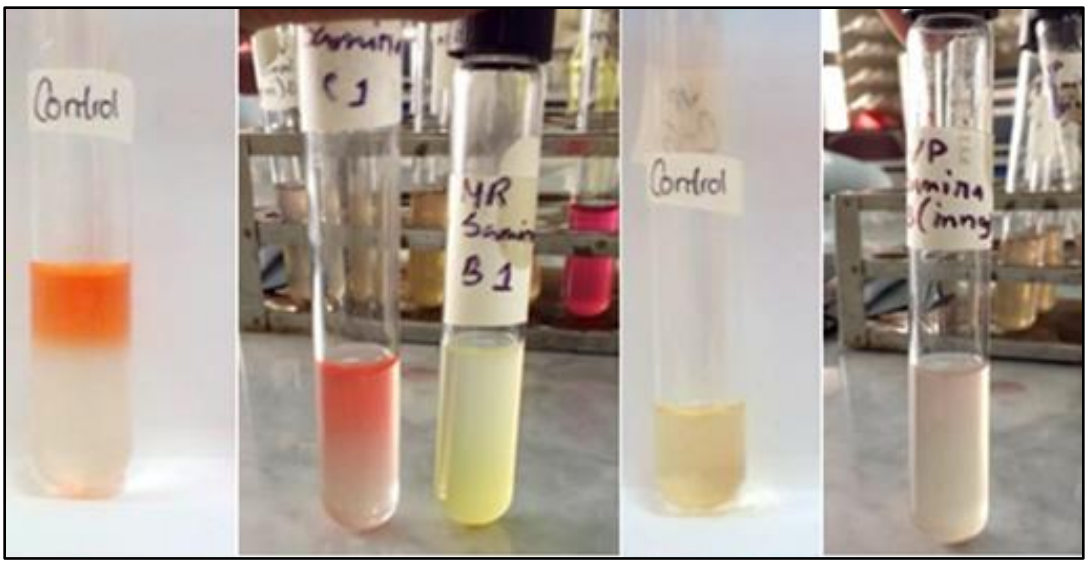

Figure 6: Methyl red test and VP test result showing control, positive and negative results.

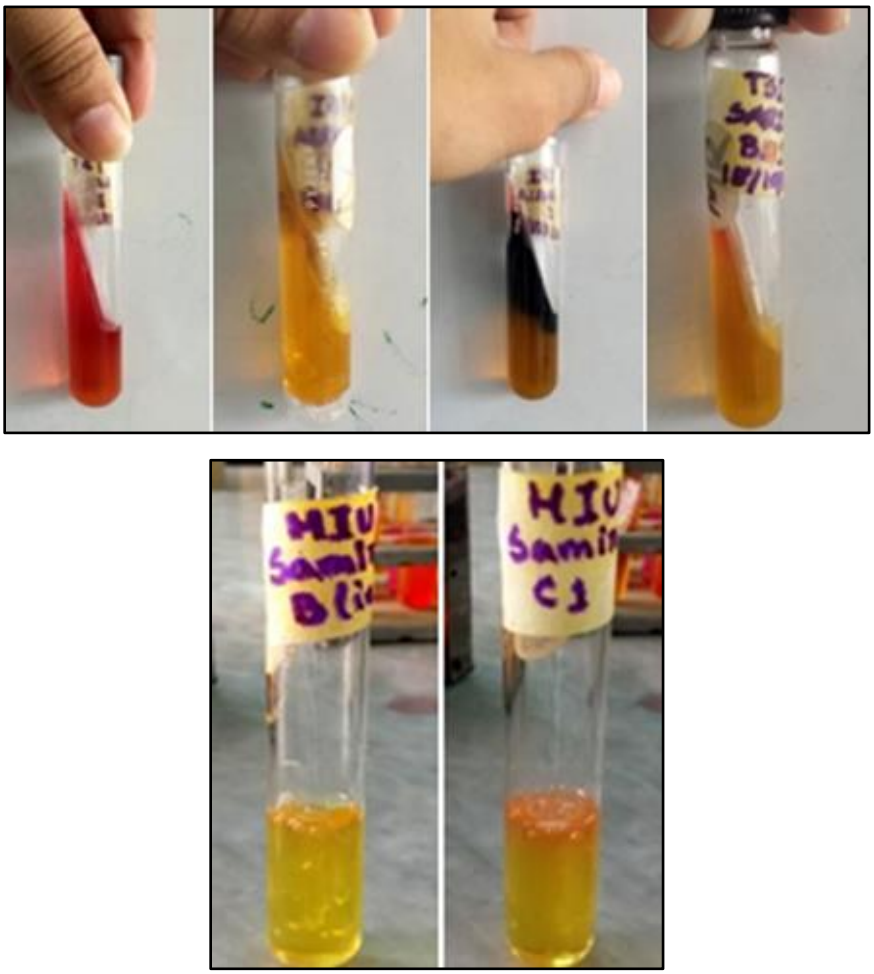

Figure 7: TSI test result showing control, positive result with gas production, positive result with H2S production and negative result and MIU test result.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

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 -

Table 8: Biochemical test results. Legend: + indicates a positive result; –indicates a negative result; (gas) indicates gas production.

Reconfirmation

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

Table 9: Biochemical test results for S-B1, S-E8 and S-J1. Legend: + indicates a positive result; –indicates a negative result; (gas) indicates gas production.

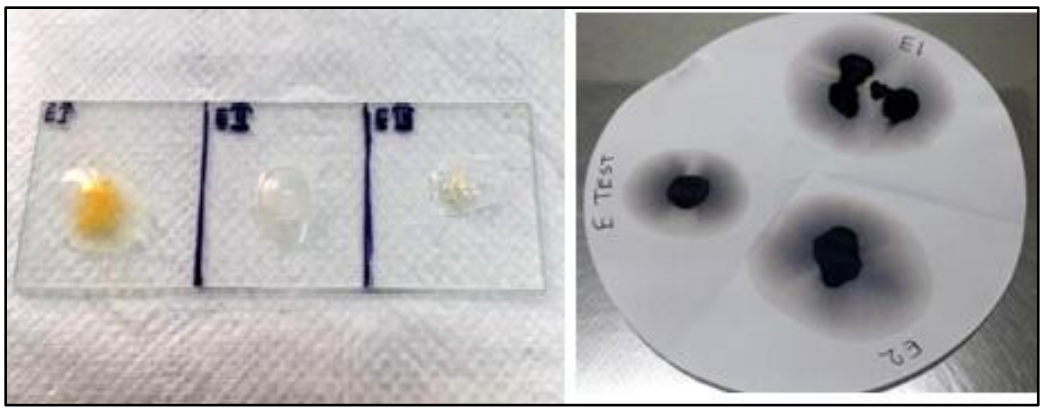

Figure 8: Oxidase test result on a slide showing bubble production as positive result and no bubble production as negative result and catalase test showing negative result.

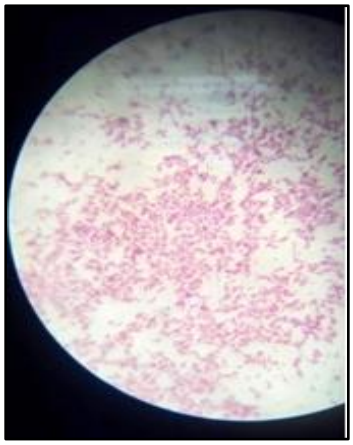

Figure 9: Oxidase test result on a slide showing bubble production as positive result and no bubble production as negative result and catalase test showing negative result.

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 considered positive.

	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	+

Table 9: Zone of inhibition for different antibiotics.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

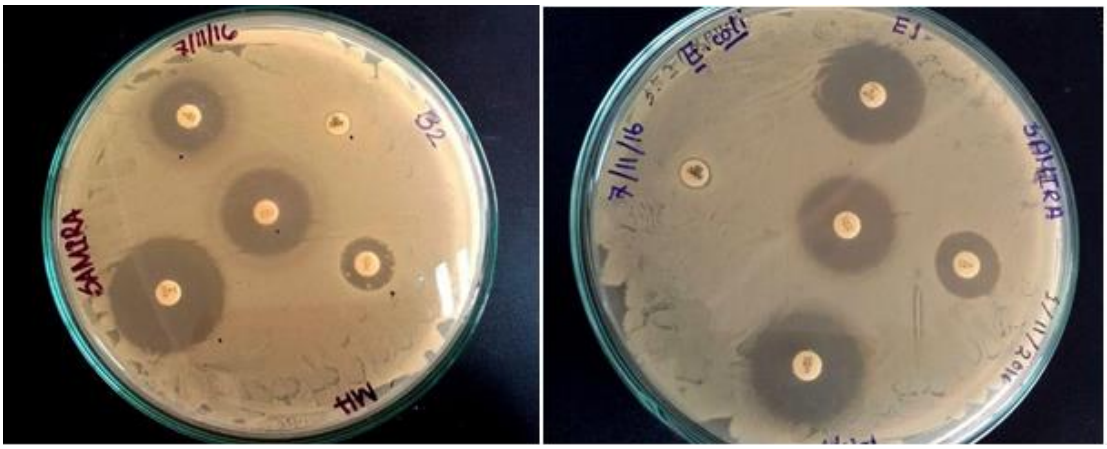

Figure 10: Zone of inhibition created by S-B2 and S-E1 for different antibiotic.

Detection of *Escherichia 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 *Escherichia coli* specific virulent genes stx1 and stx2 using specific primers. Isolates that gave bands of expected size were suspected to carry these genes in the chromosomes.

The results showed that isolates S-E1 and S-H2 were positive for stx1 but were negative for stx2 and isolate S-B2 was positive for stx2 and negative for the other, whereas the reference *Escherichia coli* O157:H7 strain was positive for both stx1 and stx2 genes.

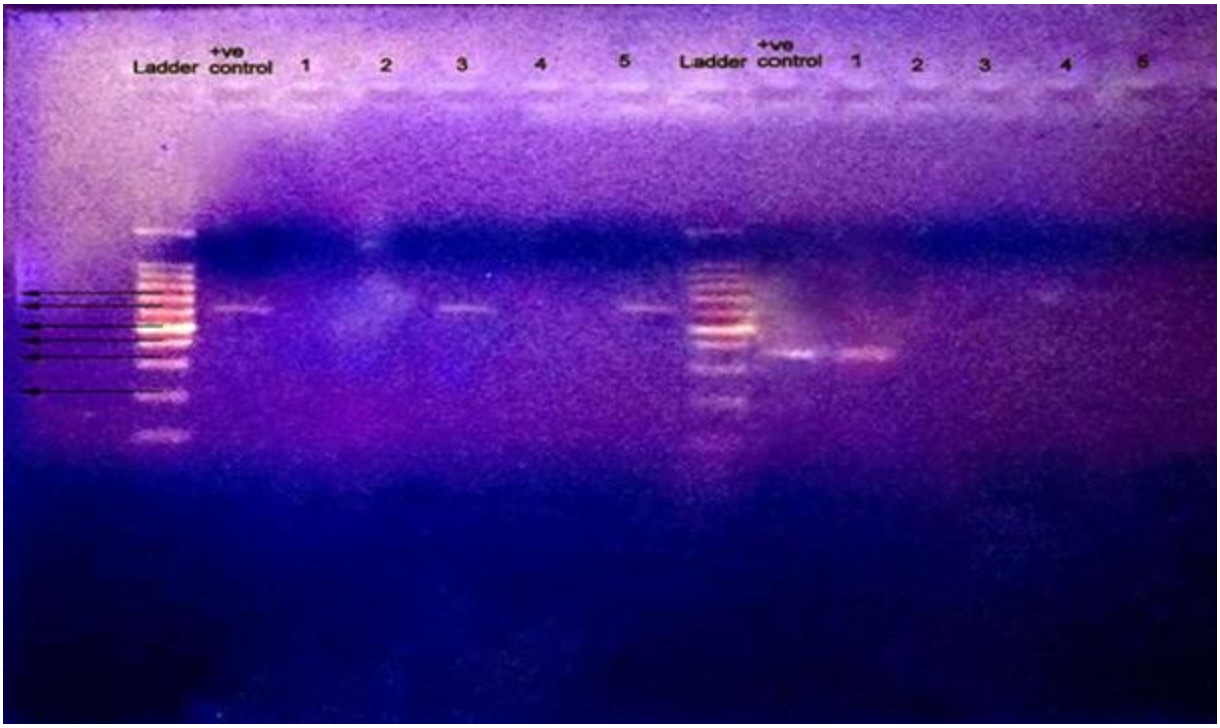

Figure 11: Gel Electrophoresis of *Escherichia coli* samples.

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

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

Table 10: PCR results.

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

Lane14:S-H

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 *Escherichia coli* O157 infection develops HUS [5].

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 [6]. Most Shiga toxin producing *Escherichia coli* cases notified in Australia are sporadic infections. Infected individuals usually present with bloody diarrhoea 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 [7]. 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 the 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 skin swab were presumptively identified as Escherichia 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-

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

Proskauer's 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 *Escherichia coli* from these sources is approximately 38%. Culturally and biochemically positive isolates were tested for stx1 and stx2 genes. Total five isolates were selected. From all these isolates, two stx1 genes were detected and one was detected for stx2. So, the surveillance of stx gene is 60% Therefore, this data showed the prevalence of *Escherichia coli* in Bangladesh and demands for further study for the prevention of diseases.

Based on these results it can be said that the doorknobs of toilets, bhelpuri and lake waters are not safe. After coming out of toilet, though hands are washed 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 should also be cleaned regularly and properly to avoid infection. bhelpuri which is a daily choice of people in Dhaka city is also not safe according to the laboratory tests. Bhelpuri 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 bhelpuri should be avoided. Lake water 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 skin swab, didn't contain any stx gene. The clinical sample collected was skin swab from patient admitted in National Institute of disease of the chest and Hospital, Mohakhali. The patient didn't have any skin disease. Usually, the chances of getting *Escherichia coli* having stx gene in skin sample are really less. 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 *Escherichia coli* O157:H7 was strongly associated with those recovered from faecal and skin samples [8]. But this result is for goat skin, not for human skin. In humans, the bacteria are usually found in colon and anus and thus the infection sites related to them. In case of any wound also they might be available, but not in normal skin. In case of skin diseases like SSTIs, they can be available [9]. But normal skin swab doesn't usually contain *Escherichia coli*. Still due to any contamination or environment around patient, the strain can be present, but the chances of getting stx gene are rare. On the other hand, this seems available in environmental samples. The normal habitat of *Escherichia coli* in environment can be a major reason behind the 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 *Escherichia 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.

References

1. Donnenberg MS. *Escherichia coli*: Virulence Mechanisms of a Versatile Academic Pathogen. Academic Press. 2002.
2. Frankel G, Phillips AD, Rosenshine I, Dougan G, Kaper JB, Knutton S. Enteropathogenic and Enterohaemorrhagic *Escherichia coli*: More Subversive Elements. Mol Microbiol. 1998;30(5):911-21. PubMed | CrossRef

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045

3. Cappuccino JG, Sherman N. Instructor Guide for Microbiology: A Laboratory Manual. Benjamin/Cummings Pub. Co.; 1996.
4. McDevitt S. Methyl red and Voges-Proskauer Test Protocols. Am Soc Microbiol. 2009;8.
5. Scallan E, Jones TF, Cronquist A, Thomas S, Frenzen P, Hoefer D, et al. Factors Associated with Seeking Medical Care and Submitting a Stool Sample in Estimating the Burden of foodborne Illness. Foodborne Pathog Dis. 2006;3(4):432-8. Pubmed | CrossRef
6. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy S, et al. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis. 2011;17(1):7. PubMed | CrossRef
7. Vally H, Hall G, Dyda A, Raupach J, Knope K, Combs B, et al. Epidemiology of Shiga Toxin Producing *Escherichia coli* in Australia, 2000-2010. BMC public health. 2012;12:1-2. PubMed | CrossRef
8. 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. Lett Appl Microbiol. 2010;50(1):71-6. PubMed | CrossRef
9. Swartz MN. Cellulitis. N Engl J Med. 2004;350(9):904-12. PubMed | CrossRef

Citation: Shuvra SK. Isolation of Shiga Toxin Producing *Escherichia coli* 0157:H7 from Environmental and Clinical Samples in Dhaka City-A Review. J Immuno Allerg. 2023;4(2):40-62.
DOI: https://doi.org/10.37191/Mapsci-2582-6549-4(2)-045