Comparative study of the presence of stx1 and stx2 genes from clinical and environmental samples of *Shigella*

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DECLARATION

I hereby declare that the thesis work titled "Comparative study of the presence of stx1 and stx2 genes from clinical and environmental samples of *Shigella*" has been written and submitted by me, Rocksher Annur 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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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.

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ABSTRACT

Shigellosis, a disease caused by *Shigella* spp. occur because of the presence of stx1 and stx2 genes. *Shigella*spp. Sometimes escapes detection by the traditional culture methods. Bacterial isolates were chosen on the basis of their structural, morphological and biochemical character. To get the most efficient result along with traditional method PCR technique was used. Antibiotic sensitivity testing was done to see the resistance of the isolates against Amoxicillin, Ampicillin, Tetracyclin, Chloramphenicol, Norfloxacinantibiotic.To detect stx1, Ka1F and Ka1R primers were used which yielded 348 bp fragment of stx1 gene. For stx2 gene Ka2F and Ka2R primers were used to get 584 bp fragment of stx2 gene. The thermal profile for 35 cycles were set using the following parameters 94°C for 10 minutes,94°C for 1 minute,55°C for 1 minute,72°C for 1 minute and 72°C for 7 minutes. This study was conducted to see the presence of *Shigella* spp. on the environment. Also, to find out if the environmental *Shigella* spp. has stx1 and stx2 gene required to cause disease.

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LIST OF ABBREVIATIONS

ShET	**Shigella toxin–producing E coli**
PMN	**Polymorphonuclear cell**
HUS	**Hemolytic-uremic syndrome**
NA	**Nutrient Agar**
XLD	**Xylose Lysine Deoxycholate**
SS- Agar	**Salmonella Shigella Agar**
MIU	**Motility test**
VP	**Voges–Proskauer test**
MR	**Methyl red (MR) test**
TSI	**Triple Sugar Iron**
AST	**Antibiotic sensitivity test**
icddr,b	**International Centre for Diarrheal Disease Research, Bangladesh**
OD	**Optical density**
MHA	**Muller-Hinton Agar**
PCR	**Polymerase chain reaction**

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Chapter 1: Introduction

1. INTRODUCTION

Shigella is a group of Gram-negative, facultative intracellular pathogens, on-spore-forming, non-motile, rod-shaped bacteria belonging to the Enterobacteriaceae family. They are 0.3 - 1μm in diameter and 1 - 6μm in length, appearing single, in pairs and in chains. Recognized as the etiologic agents of bacillary dysentery or shigellosis in the 1890s, Shigella was adopted as a genus in the 1950s.*Shigella* is estimated to cause nearly half a million illnesses each year in the United States, with more than 5,400 hospitalizations and 38 deaths. Shigella bacteria were discovered in 1897 by a Japanese Scientist named Kiyoshi Shiga.

Shigellosis, a disease caused by bacteria belonging to the genus Shigella, is an illness of humans and some primates characterized by cramps, painful defecation, fever, diarrhea and dysentery (Acheson and Keusch, 1995). Transmission occurs via the fecal-oral route, through direct person-to-person contact, or indirectly through contaminated food, water, or fomites. Since as few as 10 organisms can cause infection, shigellosis is easily transmitted and can be acquired during short-term travel. Only humans and higher primates carry Shigella. Food borne outbreaks have been linked to contaminated foods commonly consumed raw, as well as infected food handlers. Outbreaks have also been traced to contaminated drinking water, swimming in contaminated water, and sexual contact between men. Once ingested these survive gastric acidity and invade the colonic mucosa, resulting in mucosal abscesses and ulceration [Weir, 2002].

1.1.1Subgroup of *Shigella*

There are four species of *Shigella* and they are known as *Shigella dysenteriae*, Shigella *flexneri,Shigellaboydii and Shigella sonnei.* These species are classified into four serogroup based on their biochemical and serological characteristics.Serogroup means a group of bacteria that are antigenically closely related. Based on their O-antigen the serogroup are designated as A, B, C, and D respectively. Furthermore, these serogroups are again divided into serotypes. Serogroup A has 15 serotypes,serogroup B contains 6 serotypes ,serogroup C is comprised of 19 serotypes but group D has only 1 serotype. *Shigella dysenteriae* type 1 is associated with causing

bacillary dysenteriae which is endemic throughout the world and it is responsible for 165 million cases of diarrhea approximately every year. This particular species of *Shigella* is known to produce shiga toxin. Shiga toxin is known as the etiological agent of Shigellosis. *Shigella dysenteriae* is mainly found in places where there is poor sanitation and in refugee camps. It is the agent of epidemic shigellosis and is responsible for large-scale outbreaks in Central Africa, Southeast Asia, and the Indian subcontinent. In endemic areas it is isolated from up to 30% diarrheal patients [Hale, 1991].On the other hand *Shigella flexneri* are biochemically heterogeneous and antigenically complex.Among the four species of Shigella, S. flexneri is the most prevalent species in Bangladesh [Haider et al., 1989]. Shigella boydii is named after Boyd, who first described these strains from India (1931) [Parija, 2009].In comparison with other Shigella serogroups this species has been less often reported worldwide. S.boydii is typically related with people who have travelled to endemic areas but it is relatively rare in developed countries. Isolation rate of this species is less than 1–2% of the total Shigella isolates, except in the Indian subcontinent [Ranjbar et al., 2008]. Shigella sonnei is named after Sonne, who first described these strains from Denmark (1915) [Parija, 2009]. In most of the patients infected with S.sonnei, watery diarrhea occurs as a prodrome, or as the only clinical manifestation [Hale &Keusch, 1996]. However, some patients with Shigella infection- especially those with S.sonnei-never progress to the dysenteric phase, whereas others may develop dysentery without a prodrome [Bergelson et al., 2008].

1.1.2 Taxonomy

Table1. Taxonomy of *Shigella*

Domain	**Bacteria**
Phylum	**Proteobacteria**
Class	**Gammaproteobacteria**
Order	**Enterobacteriales**
Family	**Enterobacteriaceae**
Genus	**Shigella**

1.1.3 Pathogenesis

The host response to primary infection is characterized by the induction of an acute inflammation, which is accompanied by polymorphonuclear cell (PMN) infiltration, resulting in massive destruction of the colonic mucosa. Apoptotic destruction of macrophages in sub-epithelial tissue allows survival of the invading *Shigella*, and inflammation facilitates further bacterial entry.

Gross pathology consists of mucosal edema, erythema, friability, superficial ulceration, and focal mucosal hemorrhage involving the recto sigmoid junction primarily. Microscopic pathology consists of epithelial cell necrosis, goblet cell depletion, PMN infiltrates and mononuclear infiltrates in lamina propria, and crypt abscess formation.

Shigella bacteria invade the intestinal epithelium through M cells and proceed to spread from cell to cell, causing death and sloughing of contiguously invaded epithelial cells and inducing a potent inflammatory response resulting in the characteristic dysentery syndrome. In addition to this series of pathogenic events, only *S.dysenteriae* type 1 has the ability to elaborate the potent Shiga toxin that inhibits protein synthesis in eukaryotic cells and that may lead to extraintestinal complications, including hemolytic-uremic syndrome and death. Invasion of M cells, the specialized cells that cover the lymphoid follicles of the mucosa, overlying Peyer patches, may be the earliest event.

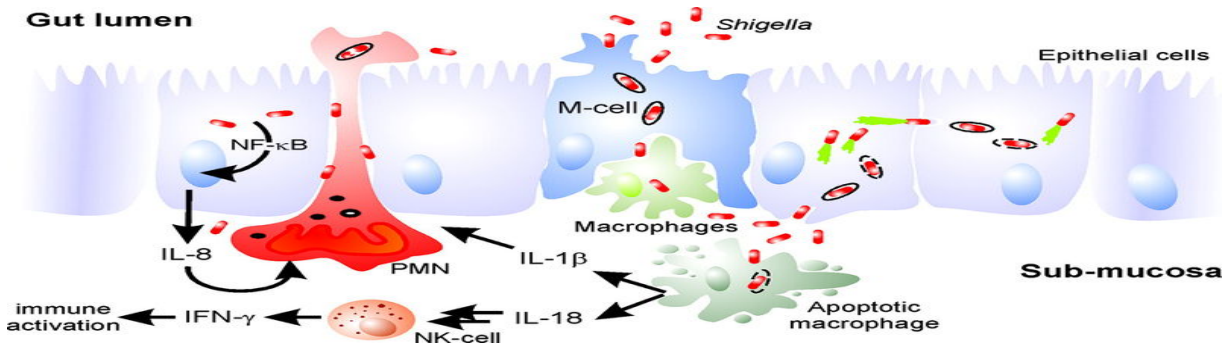


Figure no 1: Pathogenesis of *Shigella*

1.1.4 Shiga Toxin

Shigella produce a toxin, designated as Shiga toxin, which contributes to the development of necrotic lesions of the colon. The Shiga toxin was first described in 1903 by Conradi, who reported that intravenous inoculation of auto lysates of Shiga's bacillus (Shigella dysenteriae 1) paralyzedand killed rabbits. Although various animals exhibit different level of susceptibility to the lethal dose of shiga toxin, but only rabbit and mouse display neurological symptoms. Studies in the 1950s suggested that Shiga toxin does not act directly on neurons (i.e., is not actually a neurotoxin) but that it can cause secondary neurological disorders by its action on the vascular system of the brain and spinal cord.

The stx produced by *S. dysenteriae* 1 is a heterodimeric protein, consisting of five B binding subunits of 69 amino acids (Strockbine et al., 1988) and one A catalytic subunit of 293 amino acids (Seidah et al., 1986). The A subunit is nicked by a proteolytic enzyme and its disulphide bond reduced to form the enzymatically active A1 fragment, which acts by cleaving a specific adenine residue in the 60S ribosomal subunit of a eukaryotic cell and thus disrupting protein synthesis (Tesh and O'Brien, 1991). The genes encoding the toxin subunits have been sequenced (Seidah et al., 1986; Kozlov et al., 1988; Strockbine et al., 1988). The genes lie in an operon with stxA upstream of stxB and separated from it by 12 non-coding nucleotides. Both subunit genes are preceded by ribosome-binding sites; an iron-regulated promoter, under the control of the Fur regulatory protein, is 5′ to the stxA gene (Calderwood and Mekalanos, 1988; Strockbine et al., 1988). Transcription of stxB occurs both from this promoter as well as from an independent B subunit promoter (Habib and Jackson, 1992). The cloning of the toxin genes has allowed the creation of non-toxigenic derivatives of S. dysenteriae 1 by in vivo marker exchange (Fontaine et al., 1988).

Stx1 and Stx2 are both encoded by a bacteriophage inserted into the chromosome. Stx1 increases inflammatory cytokine production by human macrophages, which, in turn, leads to a burst of interleukin (IL)-8. This could be relevant in recruiting neutrophils to the lamina propriaof the intestine in hemorrhagic colitis and accounts for elevated levels of IL-8 in serum of patients with diarrhea-associated HUS.

1.1.5 Objectives of the study

The aim of the study was to isolate specific organism *Shigella* from the environment and clinical sample. After isolation of the samples, a specific gene which is stx1 and stx2 were identified by PCR technique. Comparison was done on clinical and environmental samples of *Shigella.*

Chapter 2: Materials and Methods

2.1.1 Working Place

All laboratory based works were conducted in Microbiology and Biotechnology laboratory, Department of Mathematics and Natural Sciences, BRAC University. The research has been conducted following a selected protocol for isolation of *Shigella* species from environmental sample and detection of stx1 and stx2 gene using PCR method and gel electrophoresis.

2.1.2 Sample Collection

Autoclaved sterile Duran flasks were used for collecting all the liquid samples. 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. Different types of environmental samples were collected from different sources from the month of August to September, 2016.The average temperature of Dhaka city during the month of August to September were between 37°C during the daytime. The average temperature dropped down to 28°C at night time.

Table no 2: Source of the environmental samples, location and date of collection

Environmental Sample	Location	Date
Fish Water	Mirpur	02-08-16
Vegetable Water	Motijhil	08-08-16
Salad	Mohakhali	14-8-2016
Lake Water	Gazipur	30-8-2016
Shrimp Water	Savar	01-09-2016
Drinking Water	Mohakhali	20-09-2016
Poultry Water	Mohamadpur	28-09-2016

2.1.3Isolation of Shigella from environmental samples

Enrichment Method

a) Luria-Bertani (LB) medium was used for enrichment.

b) 5 ml of LB broth was prepared into a screw capped test tube.

c) 1 ml of each liquid sample was inoculated into 10ml of LB broth

d) The inoculated LB medium was then incubated overnight at 37°C

Dilution and Spread plating

a) In a 9 ml saline solution 1 ml of the desired sample is added using a micro pipette. This is called the first dilution. Again serial dilution from 10^{-2} -10^{-3} was done following the same procedure.

b) 100 µl of solution from the dilution sample was taken with a micro pipette and added in NA, XLD, SS agar plates
c) The plates were inverted and put in the incubator at 37°C for 24 hours.
d) The count was considered between 30-300 colonies per plate. A count of more than 300 colonies is said to be 'too numerous to count' (TNTC) and that less than 30 colonies is said to be 'too few to count' (TFTC). All the plates for all ten sources showed TNTC count.

Selective Media

Xylose Lysine Deoxycholate (XLD) agar medium: It is a selected media for the isolation of Shigella species from samples. XLD agar medium has a pH which is approximately 7.4 and it appears red due to its phenol redindicator. *Shigella* is unable to ferment xylose it remains red. Shigella doesn't produce hydrogen sulfide thus it doesn't give any black color colonies.

Salmonella Shigella (SS) Agar: SS agar is used for the detection of Salmonella and some Shigella species.it depends on the fermentation of lactose and the absorption of neutral red as the bile salts precipitate in the acidic condition. Neutral red turns red in the presence of an acidic pH, thus showing fermentation has occurred. Shigella a non-lactose fermenting organisms appear as transparent or translucent colorless colonies on SS Agar.

Procedure:

a) Freshly prepared XLD and SS agar plates were taken and labeled.
b) 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.
c) 100 µl solution was taken on the plates and was spread.
d) Plates were incubated overnight at 37ºC.

2.1.4 Selection of Suspected Isolates

After 24 hours incubation at 37°C all the plates were checked and total number of colonies in per plate was recorded. This was done in all plates to deduce the overall heterotrophic and specific microbial load. The colonies that showed the characteristics of *Shigella* were labeled. And some of the isolates from the nutrient agar plate were streaked into new plates for subculture and storage.

2.2.1Biochemical Test for Confirmation of Isolates

Biochemical tests were used to detect the presence or absence of gram-negative or gram-positive bacteria. Biochemical tests were performed using a variety of methods and experimental techniques; the method of testing varied depending on whether researchers are testing for positive or negative bacteria. All the tests were done according to the methods described in the Microbiology Laboratory Manual (Cappuccino and Sherman, 2011).To identify *Shigella* spp. biochemical tests are done and these tests are :Gram staining ,Indole production test, Citrate utilization test, Oxidase test, Voges–Proskauer, Methyl red and TSI agar are done. Prior to these tests the isolates were grown in nutrient agar plates at 37°C.

Gram staining

a) The sample was first heat-fixed in a glass slide with the Bunsenburner. Then crystal violet was added in the smear and was let it stand for 1 minute.

b) After washing the slide with indirect stream of water it was flooded with the mordant: Gram's iodine. Then again the slide was washed with indirect stream of water.

c) Decolorizing, ethanol was added to flood the smear and then again washed with water.Counterstainsafari was added and was kept for 30 seconds to 1 minute.

d) Then the slide was again washed with indirect water stream.

e) After that the slides were air dried and result of the staining procedure was observed under oil immersion (100x) using a Bright field microscope.

Indole test

a) Bacterium to be tested was inoculated in 6 ml peptone water, which contains the amino acid tryptophan and incubated at 37°C for 24 hours.

b) Following overnight incubation, five drops of Kovac's reagent were added.

c) Formation of a rose red ring at the top indicates a positive result. A negative result can have a yellow or brown layer.

Motility test

a) Bacterium to be tested was taken with a sterile needle and added to MIU agar with a straight stab. This was incubated for 24 hours at 37°C.

b) Afterwards, the agar is observed; if movement is seen which travels from the stab to the agar the organism is motility positive. If organism does not grow beyond the stab, it is non-motile.

Citrate test

a) A single bacterial colony of each bacterium to be tested was picked up from eachnutrient agar plates by a needle and inoculated into the slope of Simmon'scitrate agar and incubated at 37°C for 24 hours.

b) All the media that changed their color from green to a Prussian blue, is taken as a positive result. A negative slant would have no growth of bacteria and would remain green.

Urease test

a) Bacterium to be tested was taken with a sterile needle and added to MIU agar with a straight stab. This was incubated for 24 hours at 37°C.

b) Afterwards, if the media turns pink the organism is urease positive. Negative result is no change in media color.

Oxidase test

a) Two drops of oxidase reagent (p- Aminodimethylaniline oxalate) were added onto the filter paper (Whatman, 1MM).A loop full of each bacterium to be tested were taken from nutrient agar plate and streaked onto the filter paper (Whatman, 1MM).

b) A positive reaction would turn the paper from violet to purple within 1 to 30 seconds. Delayed reactions should be ignored as that might give false positive result.

Voges–Proskauer test

a) Bacterium to be tested was inoculated into 3 ml dextrose phosphate broth (MRVP broth) and incubated at 37°C for 24 hours.

b) To the aliquots of each broth cultures 10 drops of Barritt's reagent A was added and the cultures were shaken. After a few minutes, 10 drops of Barritt's reagent B was added and the cultures were shaken again.

c) 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.

Methyl red (MR) test

a) Bacterium to be tested was inoculated into 3 ml dextrose phosphate broth (MRVP broth), which contained dextrose and a phosphate buffer and incubated at 37°C for 24 hours.

b) Over the 24 hours, the mixed-acid producing organism might produce sufficient acid to overcome the phosphate buffer and remain acidic.

c) The pH of the medium was tested by the addition of five drops of MR reagent.

d) Development of a red color was taken as a positive result. MR negative organism would give a yellow color.

Triple Sugar Iron (TSI) Test

a) A single bacterial colony of each bacterium to be tested was picked up from each nutrient agar plates by a needle and stabbed into the TSI containing dextrose, lactose and sucrose butt.

b) Caps of the tubes were loosened and incubated at 35°C for overnight and were examined after 18-24 hours for carbohydrate fermentation, CO_2 and H_2S production.

c) A yellow (acidic) color in the butt indicated that the organism being tested is capable of fermenting all the three sugars, whereas a red (alkaline) color in the slant and butt indicated that the organism being tested is a non-fermenter.

d) A black precipitation in the butt of the tube is the indication of H_2S production. Presence of bubbles, splitting and cracking of the medium is the indication of CO_2 gas production.

Catalase Test

a) Using a sterile loop a small amount of colony was added in a clean glass slide.
b) Then few drops of hydrogen peroxide was added on the colony
c) Then the slide was observed to see the production of oxygen bubbles. If there are bubbles it means that the sample is catalase positive and incase of no bubbles it is catalase negative.

2.2.2 Preparation of Stock Sample

Short term preservation

3 ml of T_1N_1 agar butt in each vial was inoculated by stabbing isolates from nutrient agar plates. Then the vials were incubated at 37°C (SAARC) for 6 hours. After incubation, the surface of the medium was covered with 200 µl sterile paraffin oil and the vials were appropriately labeled and stored at room temperature.

Long term preservation

For long-term preservation, 500 µl of bacterial culture grown in trypticase soy broth at 37°C for 6 hours was taken in a sterile cryovial. Then 500 µl of sterile glycerol was added to the broth culture and the cryovial was stored at -20°C.

2.3.1AntibioticSensitivityTest

An antibiotic sensitivity or susceptibility test is done to help choose the antibiotic that will be most effective against the specific types of bacteria. It is the susceptibility of bacteria to antibiotics. Susceptibility can vary within a species as some strains are more resistant than the other. Resistance arises through one of three ways: natural resistance in certain types of bacteria; genetic mutation; or by one species acquiring resistance from another .Testing for antibiotic sensitivity is often done by the Kirby-Bauer method.

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 vortexed. 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. Using 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. Also if there is no clear zone then the antibiotic is considered resistant.

2.3.2 List of Antibiotic Disk Used

Table no 3: Antibiotics used for sensitivity testing.

Antibiotic	Disk Indication Number
Amoxicillin	AMC 30
Ampicillin	AMP 10
Tetracyclin	TE30
Chloramphenicol	C10
Norfloxacin	NOR10

2.4.1 DNA Extraction of *Shigella*

DNA isolation is a process of purification of DNA from sample using a combination of physical and chemical methods. Isolation of DNA is needed for genetic analysis, which is used for scientific, medical, or forensic purposes. Presence of proteins, lipids, polysaccharides and

some other organic or inorganic compounds in the DNA preparation can interfere with DNA analysis methods, especially with polymerase chain reaction (PCR). They can also reduce the quality of DNA leading to its shorter storage life.

For the positive control *Shigelladysenteriae* sample was taken from icddr,b. Along with the environmental samples the DNA from the clinical samples was also extracted and sent for PCR and gel electrophoresis.

2.4.2 DNA Extraction Protocol

a) 2ml of overnight *Shigella* culture in LB broth was transferred to a 2ml eppendrof tube and centrifuged at 13500 rmp for 3 min to pellet the cells.

b) The supernatant were discarded without disrupting the layer.

c) The pellet was re-suspended in 600µl lysis buffer and vortexed to completely suspend the cells.

d) Then it was incubated 1hour at 37°C

e) Then 600 µl of phenol:chloroform were added and mixed well to completely mix the phases.

f) Then it was spinned at 13500 rmp for 5 minute at 37°C.

g) Then carefully the aqueous phase was transferred into a new tube.

h) To remove phenol an equal volume of chloroform was added into the aqueous layer.And it was mixed by inverting the tubes.

i) Then at 13500 rmp for 5 minute it was again spinned.

j) To precipitate the DNA 2.5 or 3 volume of cold 200 proof ethanol was added to the tubes.

k) Then at -20°C it was incubated for 15 minute for 30 minute.

l) Then again spinned at 13500 rmp for 15 minute at 4°C.

m) Then the supernatant were discarded and the DNA pellet was rinsed with 1 ml 70% ethanol.

n) Then again spinned for 2 min at 13500 rmp speed.

o) Then 25 µl of TE buffer were re-suspended into the DNA.

p) Isolated genomic DNA was observed on an agarose gel.

2.4.3Mastermix Preparation for PCR

Table no 4: Master Mix preparation

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

2.4.4 Primer

Table no 5: Primers for stx1 and stx2 genes

Target Gene	Primer	Sequence	Amplicon size
stx1	KalF	5'-GGGATAGATCCAGAGGAAGG-3'	348 bp
	KalR	5'-CCGGACACATAGAAGGAAACTC-3'	
stx2	Ka2F	5'-CTGGCGTTAATGGAGTTCAG-3'	584 bp
	Ka2R	5'-CCTGTCGCCAGTTATCTGAC-3'	

2.4.5 PCR Conditions

The Polymerase Chain Reaction (PCR) mainly follows the below procedure:

a) Initialization step: This step consists of heating the reaction to a temperature of 94–96 °C.
b) Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
c) Annealing step: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific.
d) Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °Cand commonly a temperature of 72 °C is used with this enzyme.

The parameters for 35 thermal cycle for PCR was set using the following conditions:

i. 94^0C for 10 minutes
ii. 94^0C for 1 minute
iii. 55^0C for 1 minute
iv. 72^0C for 1 minute
v. 72^0C for 7 minutes
vi. 4^0C until further use

2.4.6 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 no 6:Sequence of sample loading in Agarose gel

Well number	Sample number
Well -1	100 bp ladder
Well -2	Positive clinical sample
Well -3	Sample A-2
Well -4	Sample A-4
Well -5	Sample E-1
Well -6	Sample E-2
Well -7	Sample E-5
Well -8	100 bp ladder
Well -9	Positive clinical sample
Well-10	Sample A-2
Well – 11	Sample A-4
Well -12	Sample E-1
Well -13	Sample E-2
Well -14	Sample E-5

Chapter 3: Results

3.1.1 Isolation of *Shigella* species from samples

Seven samples were collected from various part of Dhaka city. Those samples were first grown in LB broth for enrichment purposes at 37°C for 18-24 hours. After the enrichment process was done those samples were diluted from 10^{-1}-10^{-2} depending on their sources and concentration. After that the broth samples were cultured in agar plates to see the colony forming unit. When raw sample was used in nutrient agar media they gave TNTC result. After dilution the colony forming number decreased. After that the samples were cultured in selective media. From those selective media 15 isolates were taken for biochemical test for further investigation.

Table no 7: Total colony-forming units for all the environmental samples

Samples	CFU
Fish water	TNTC
Vegetable water	$6.8*10^{-5}$
Salad water	$5.2*10^{-5}$
Lake water	$6.6*10^{-5}$
Shrimp water	TNTC
Drinking water	$4.5*10^{-5}$
Poultry water	$6.1*10^{-5}$

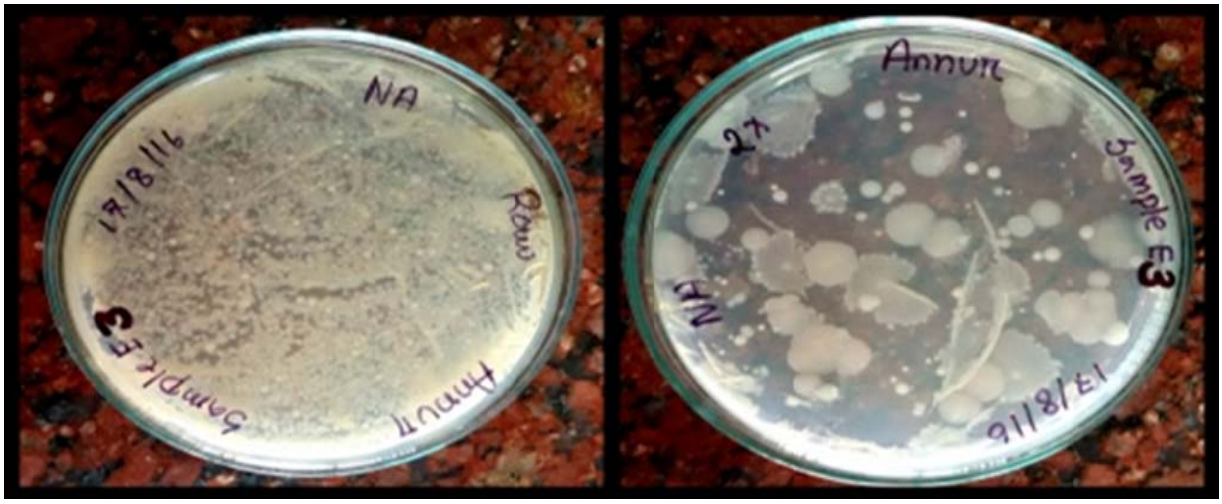


Figure no 2: Nutrient Agar showing bacterial growth of shrimp water sample.

Figure no 3: Nutrient Agar showing bacterial growth of fish water sample.

3.1.2 Identification of *Shigella* from the environmental samples

Table no 8: Cultural Characteristics of environmental samples in XLD agar and SS agar

Samples	Colony isolates	Cultural characteristics	
		XLD Agar	**SS Agar**
Fish Water	A-1	Yellow colonies with slightly red colonies	Pink colonies
	A-2	Smooth, red colonies	Colorless with black center colonies
	A-3	Red colonies with black center	Colorless with black center colonies
	A-4	Smooth ,red colonies	Colorless with black center colonies, colorless colonies
	A-5	Red colonies along with few black center	Colorless colonies
Vegetable Water	B-1	Red colonies with black center	Colorless with black center colonies
Vegetable water	B-2	Red colonies with black center	Colorless with black center colonies
	B-3	Yellow colonies	Pink colonies
	B-4	Yellow colonies with slight red	Pink colonies
	B-5	Red colonies	Colorless colonies
	B-6	Red colonies also with some red colonies with black center	Colorless, colorless and black center
Salad water	C-1	Red colonies with black center	Colorless and black center
	C-2	Yellow colonies	Pink colonies
	C-3	Yellow colonies	Pink colonies

	C-4	Red colonies black center	Colorless and black center
	C-5	Red colonies	Colorless colonies
Lake water	D-1	Grey with black center	Pink colonies
	D-2	Yellow colonies	Pink colonies
	D-3	Pink colonies with black centers	Colorless black center colonies
	D-4	A few red colonies along with yellow colonies	Pink colonies
Shrimp water	E-1	Red colonies	Colorless colonies
	E-2	Red colonies with slightly yellow colonies	Colorless colonies ,pink colonies
	E-3	Red colonies ,yellow colonies	Pink and colorless colonies
	E-4	Red colonies along with some red and black center colonies	Colorless colonies, colorless and black center colonies
	E-5	Red colonies	Colorless colonies
Drinking water	F-1	Yellow colonies	Pink colonies
	F-2	Yellow colonies with no growth in some parts on the agar plate	Pink colonies
	F-3	Few red colonies with pink and yellow colonies	Colorless colonies
	F-4	Yellow and pinkish colonies	Pink colonies
Poultry water	G-1	Red colonies with black center	Colorless black center colonies
	G-2	Red and black center colonies mixed	Colorless colonies few and black center few colonies
	G-3	Yellow colonies with pink and black center	Pink colonies along with colorless black center colonies
	G-4	Yellow and pink colonies	Pink colonies
	G-5	Yellow colonies	Pink colonies

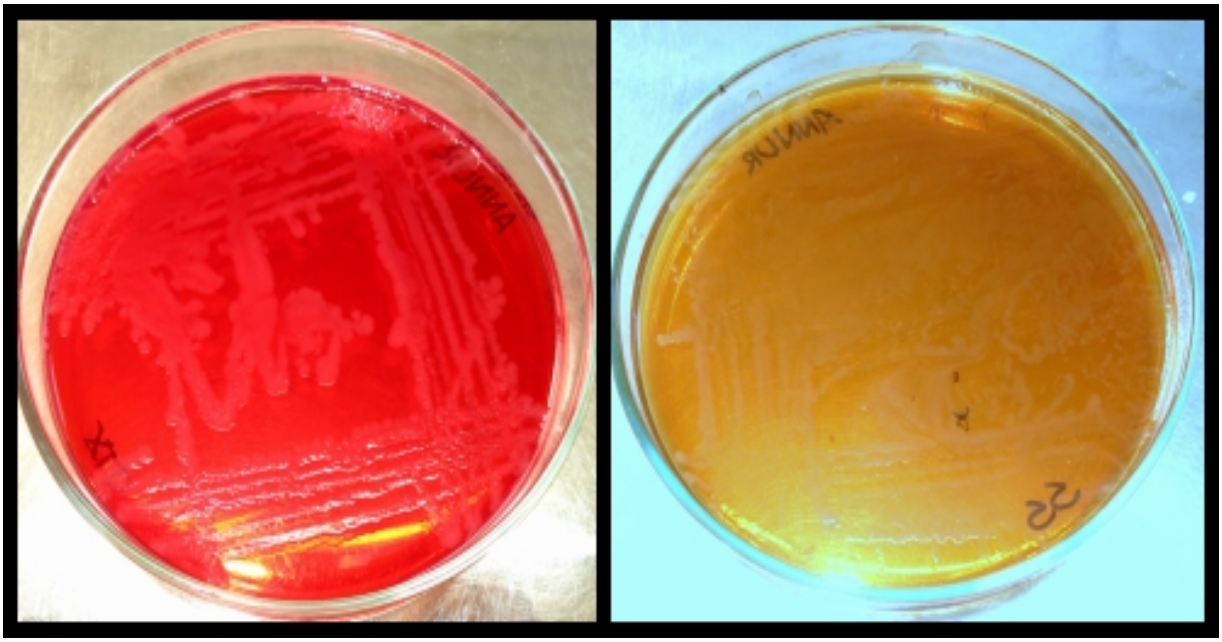


Figure no 4: Environmental sample of *Shigella* grown in XLD agar and SS agar.

3.1.3 Subcultures

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

Figure no 5: Subcultures of Shrimp water and Fish water sample.

3.1.4 Biochemical identification

Ten biochemical tests were done for further confirmation. They are Indole test, MR test, VP test, Citrate test, TSI test, MIU test, Catalase test, Urease test, Oxidase test and Gram Staining.

Table no 9: Biochemical tests of environmental samples.

Isolate	Gram staining test	Indole production test	Motility test	Urease utilization test	TSI Fermentation				Voges-Proskauer reaction test	Methyl red reaction test	Citrate utilization test	Catalase activity	Oxidase activity
					Slant	Butt	H_2S	CO_2					
A-1	-	+	+	-	K	A	+	-	-	+	-	+	-
A-2	-	+	-	-	K	A	-	-	-	+	-	+	-
A4	-	+	-	-	K	A	-	-	-	+	-	+	-
A-5	-	+	-	-	K	A	-	-	-	+	-	+	-
B-4	-	-	+	+	K	A	+	-	-	+	-	+	-
B-5	-	+	-	-	K	A	-	-	-	+	-	+	-
B-6	-	+	-	-	K	A	-	-	-	+	-	+	-
C5	+	+	+	-	A	K	-	+	-	+	+	+	-
D-4	-	+	-	-	K	A	-	-	-	+	-	+	-
E-1	-	+	-	-	K	A	-	-	-	+	-	+	-
E-2	-	+	-	-	K	A	-	-	-	+	-	+	-
E-3	-	-	+	-	K	A	+	-	-	+	-	+	-
E-4	-	-	+	-	K	A	+	-	-	+	-	+	-
E-5	-	+	-	-	K	A	-	-	-	+	-	+	-
G-2	-	-	+	-	K	A	-	+	-	-	+	-	-

KEYS: A= acidic condition, K= alkaline condition, + = positive, - = negative, AG= both acid & gas production

Figure no 6: TSI fermentation test for environmental samples

Figure no 7: MIU test for environmental samples

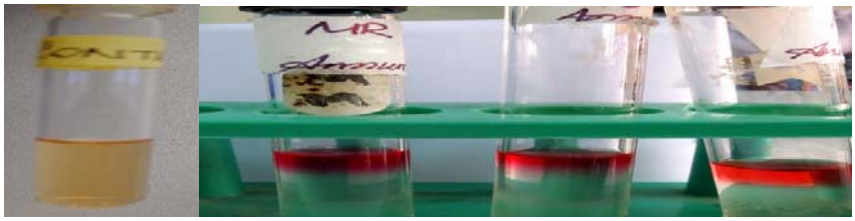


Figure no 8: Methyl red reaction test for environmental samples

Figure no 9: Oxidase test for environmental samples

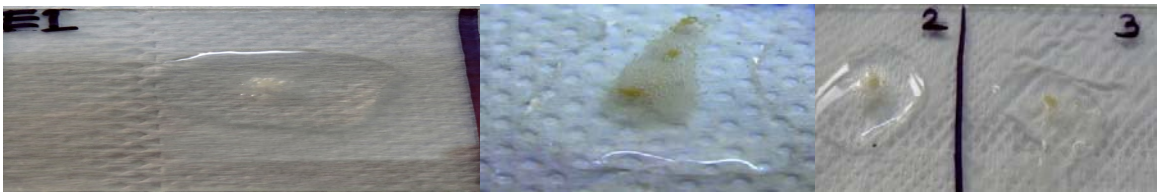


Figure no 10: Catalase test for environmental samples

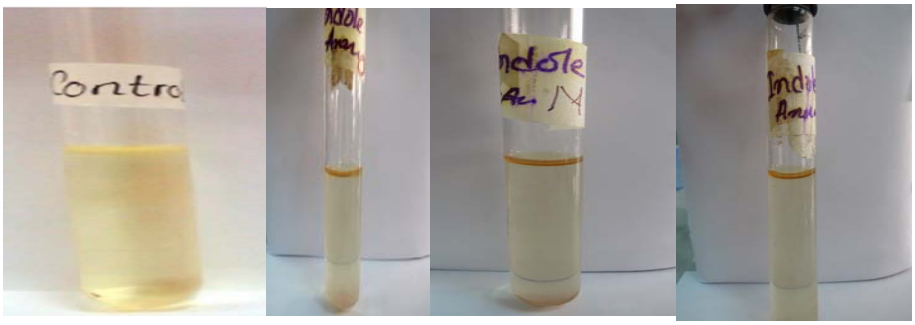


Figure no 11: Indole production test for environmental samples

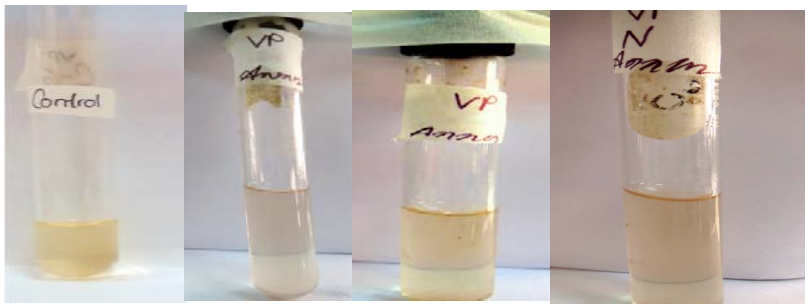


Figure no 12: Voges-Proskauer reaction test for environmental samples

Figure no 13: Citrate utilization test for environmental samples

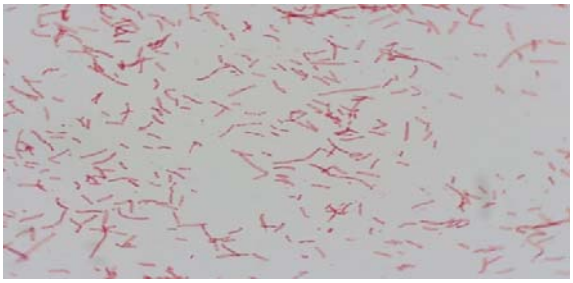


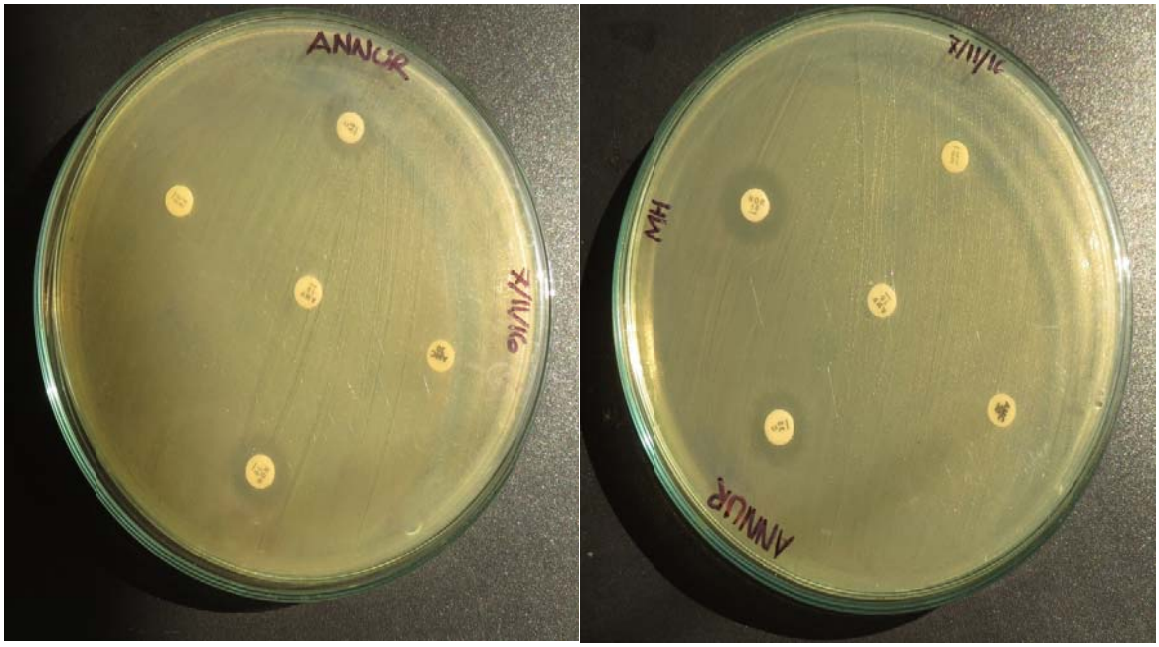
Figure no 14: Gram staining for environmental samples

3.1.5Antibiotic susceptibility of positive isolates against different antibiotics.

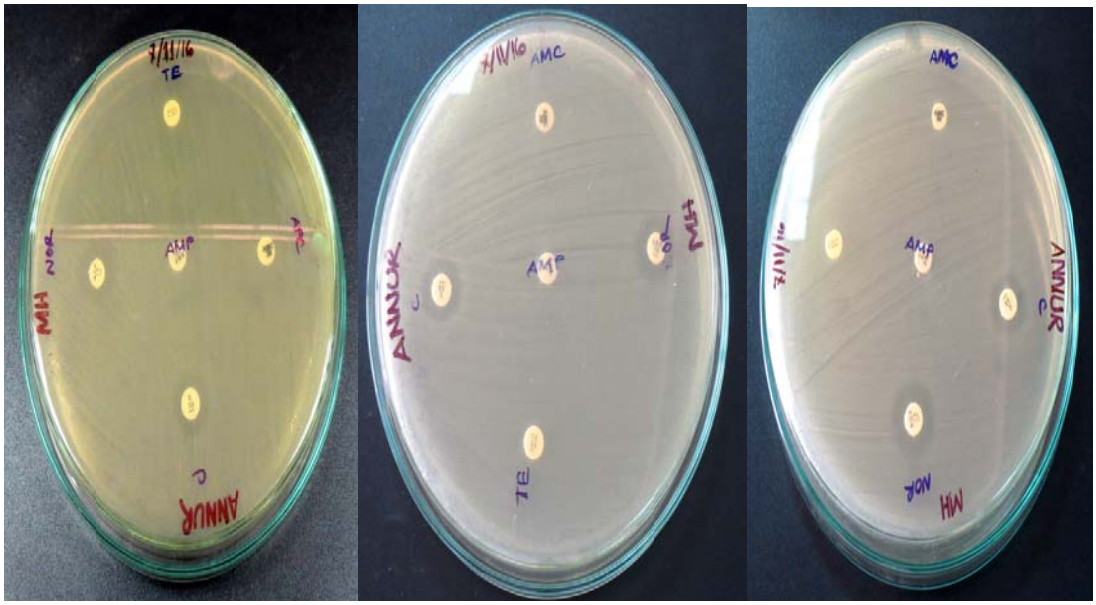
For antibiotic resistant test the samples that showed positive result for *Shigella spp* in biochemical test were taken. The samples were mostly from fish water, shrimp water. From there only sample A-2, A-4, E-1, E-2 E-5 were taken for PCR.

Table no 10: Antibiotic sensitivity test

Isolate	Antibiotic Name				
	Amoxicillin (AMC 30)	Ampicillin (AMP 10)	Tetracyclin (TE30)	Chloramphenicol (C10)	Norfloxacin (NOR10)
A-2	0 mm [R]	0 mm [R]	0 mm [R]	13 mm [I]	13 mm [I]
A-4	0 mm [R]	0 mm [R]	0 mm [R]	13 mm [I]	15 mm [I]
E-1	0 mm [R]	0 mm [R]	0 mm [R]	15 mm [I]	14 mm [I]
E-2	0 mm [R]	0 mm [R]	0 mm [R]	15mm [I]	13 mm [I]
E-5	0 mm [R]	0 mm [R]	0 mm [R]	14 [I]	15 mm [I]



a) Sample A-2 b) Sample A-4



c) Sample E-1 d) Sample - E-2 e)Sample E-5

Figure 15: Antimicrobial susceptibility of a) sample A-2 b) sample A-4 c) Sample E-1 d) sample E-2 e) Sample E-5

3.1.6Detection of *Shigella* Specific virulence Genes by PCR

For the positive isolates of Shigella template DNA was prepared and 1µl of template DNA was added to PCR to increase the chances for detecting specific virulent genes stx1 and stx2 using specific primers. Isolates having the specific genes will illuminate the band at desire position. After conducting PCR and gel electrophoresis none of the isolates showed any bands for stx1 and stx2 genes. PCR results are elaborated by the figure and table showing the result.

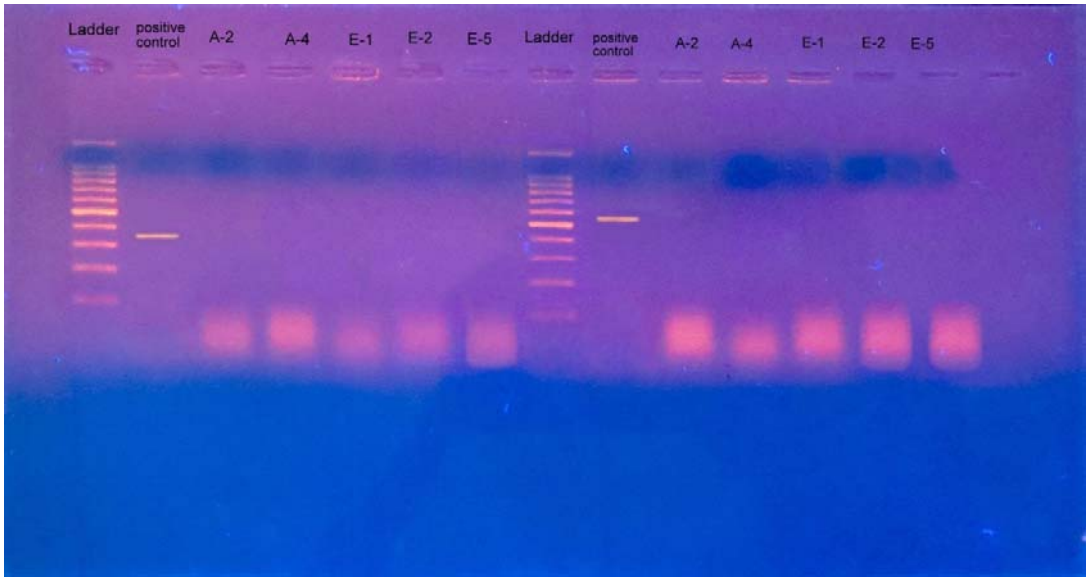


Figure no 16: Gel Electrophoresis of positive Shigella environmental sample for stx1 and stx2 genes

Table no 11: Gel Electrophoresis result interpretation

Sample	Stx1	Stx2
A-2	-	-
A-4	-	-
E-1	-	-
E-2	-	-
E-5	-	-

Keys: + = positive result, - = negative result

Chapter 4: Discussion and Conclusion

Shigella species are responsible for a substantial proportion of cases of bacillary dysentery, resulting in endemic disease and sporadic epidemics in developing countries. In Bangladesh, shigellosis causes considerable of morbidity and mortality particularly in young children. It has been estimated that more than 95 000 children <5 years of age die of shigellosis annually in Bangladesh. One of the major problems in combating shigellosis is the increasing frequency of antibiotic resistance in *Shigella* spp.

The main focus of this study was to isolate *Shigella* species from environmental samples and to identify stx1 and stx2genes and compare them with the clinical *shigella*sample, which is the causative agent of bacillary dysentery. Identification of Shigella from environmental sample is hard as the number of organism is small and it lacks suitable environment. For this reason the samples were enriched and underwent culture method along with several biochemical tests for detection of Shigella species. Furthermore PCR was used to amplify the DNA and stx gene was visualized under ultraviolet radiation.

During the process to isolate *Shigella* bacteria from environmental samples a lot of samples showed positive result. But after undergoing biochemical test only five showed all the criteria of being *Shigella*. Further investigation was done by doing an antibiotic sensitivity test to find out if the samples were resistant to certain antibiotics After PCR and agarose gel electrophoresis, it was seen that none of the isolates had stx genes present in their DNA.

Shigella dysenteriae serotype 1 is the only one of the four *Shigella* species that has been recognized as producing Shiga toxins (stx), potent cytotoxins that produce hemorrhagic colitis which can lead to the serious hemolytic uremic syndrome (HUS).We can come to a hypothesis that the isolated Shigellasamples did not have stx gene present as it might have been other subgroup of *Shigella*species. In lane 2 and 9 there were clinical samples of *Shigella dysenteriae* which were taken from icddr, b and they showed the band for stx1gene and stx 2 gene encoding shiga toxin. One of the reason might be that most of the samples were in the environment for a very long time and they could have lost the gene for causing shigellosis. Sometimesloss of

stxgenes provide advantages to the bacteria for a better adaptation to the human hosts as causing severer disease offers little benefit to the organisms for long term survival.

By going through the results of the environmental samples we can conclude that the samples indeed have *Shigella* samples but did not contain stx1 and stx2 genes. This means the samples were nonpathogenic.

Though we didn't find any stx1 and stx2 genes in the environmental sample doesn't mean that there aren't any pathogenic *Shigella* present in the environment. The sample number for the study was not ample, it can be another reason for not finding the desired result from the environment. Further study can be done by doing the study in two phase or over a gap of time to see if different climate shift has anything to do with the result.

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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
Yeastextract	2.0
Peptone	5.0
Sodiumchloride	5.0

3. T1N1 soft agar

Ingredients	Amount(g/L)
Tryptone	0.6 g
Sodiumchloride	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. Eosine methylene blue agar (Oxoid, England)

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

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

Ingredients	Amount(g/L)
Magnesiumsulfate	0.2
Ammonium dihydrogenphosphate	0.2
Ammoniumphosphate	0.8
Sodiumcitrate	2.0

Sodiumchloride	5.0
Agar	15.0
Bactobromthymolblue	0.08

7. MR-VP broth

Ingredients	**Amount(g/L)**
Peptone	7 g
Dextrose	5 g
Potassiumphosphate	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) were taken and distilled water was added to the mixture to make 500 ml. The buffer was stored at room temperature.

Gel loading buffer

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

Ethidium bromide solution

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

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