Isolation of Shiga Toxin Producing *Escherichia coli* O157:H7 from Bovine Samples of Dhaka, Bangladesh.

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

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DECLARATION

I hereby declare that the thesis project titled **"Isolation of Shiga Toxin Producing *Escherichia coli* O157:H7 from Bovine Samples of Dhaka, Bangladesh"** submitted by me has been carried out under the supervision of Ms. Namista Islam, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is based on actual and original work carried out by me. 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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Dedicated to…

My Parents

Abstract

Shiga toxin producing *Escherichia coli* (STEC) have recently emerged as important food-borne pathogens especially serotype O157:H7. Human diseases ranging from mild diarrhea to hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura can be caused by STEC, typically affecting children, elderly and immune-compromised patients. Bangladesh is considered as an endemic area for shiga toxin producing *E.coli* O157:H7. The study is conducted to isolate *E.coli* from bovine samples followed by genotyping identification using PCR. For this purpose bovine feces were collected around Dhaka city to isolate *E. coli*. The samples were first enriched in enrichment broth and then plated onto MacConkey agar. A total of 61 isolates from 7 samples were presumptively selected as *E. coli* from primary MacConkey plate. The isolates were subjected to detailed biochemical characterizations using Eosin Methylene Blue (EMB) agar medium, Indole production test, Methyl-red test, Voges-Proskauer's test, Citrate utilization test, Triple Sugar Iron test and fermentation test. Out of 35 samples analyzed, only 22 isolates, gave identical biochemical properties compared to a reference *E. coli* strain. Culturally and biochemically positive isolates were tested for *stx*1 and *stx*2 genes. From all these isolates, no *stx*1 gene was detected but 3 were detected for *stx*2. Therefore, this data showed the prevalence of *E. coli* in Bangladesh and demands for further study for the prevention of diseases.

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LIST OF ABBREVIATIONS

NA	Nutrient Agar
MAC	MacConkey Agar
EMB	Eosin Methylene Blue Agar
TSB	Trypticase Soy Broth
MR	Methyl Red
E.coli	*Escherichia coli*
STEC	Shiga toxin producing *E.coli*
IMViC	Indole, Methyl red, Voges-Proskauer's, Citrate
VP	Voges-Proskauer's
TSI	Triple Sugar Iron
stx	Shiga Toxin
^{o}C	Degree Celsius
mins	Minutes
hrs	Hours
BF	Bovine feces
MS	Milk sample
HS	Human sewage

Chapter 1

Introduction

1.1 About *Escherichia coli*

Escherichia coli (*E. coli*) is the most prevalent infecting organism in the family of gram-negative bacteria known as enterobacteriaceae. *E. coli* bacteria were discovered in the human colon in 1885 by German bacteriologist Theodor Escherich. Dr. Escherich also showed that certain strains of the bacterium were responsible for infant diarrhea and gastroenteritis, an important public health discovery. Although *E. coli* bacteria were initially called Bacterium coli, the name was later changed to *Escherichia coli* to honor its discoverer. *E. coli* is often referred to as the best or most-studied free-living organism. More than 700 serotypes of *E. coli* have been identified. The "O" and "H" antigens on the bacteria and their flagella distinguish the different serotypes. It is important to remember that most kinds of *E. coli* bacteria do not cause disease in humans. Indeed, some *E. coli* are beneficial, while some cause infections other than gastrointestinal infections, such as urinary tract infections. [i]

Table 1: Scientific Classification of *E.coli* [ii]

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*E. coli*

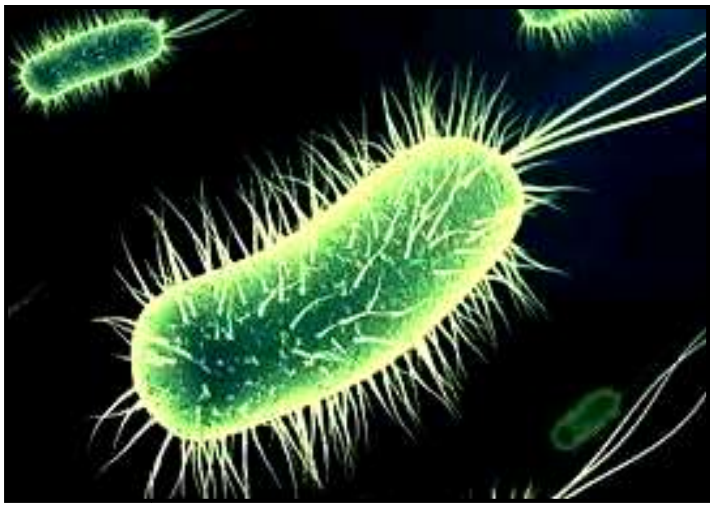


Figure 1: *Escherichia coli*

1.2 Description of the Organism

E. coli are Gram-negative, rod-shaped bacteria and are members of the family Enterobacteriaceae. Other species of the genus Escherichia include *E. adecarboxylata, E. blattae, E. fergusonii, E. hermanii* and *E. vulneris*. Pathogenic *E. coli* are classified into specific groups based on the mechanisms by which they cause disease and clinical symptoms. These categories include enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and diffusely adhering *E. coli* (DAEC). STEC are Shiga-toxin producing *E. coli*, also known as verocytotoxin-producing *E. coli* (VTEC). The STEC strains that cause haemorrhagic colitis (bloody diarrhoea) belong to the EHEC group of pathogenic *E. coli*. In developed countries EHEC is the most serious of the pathogenic *E. coli;* however, in developing countries EPEC is a major disease causing agent in children. Strains of E. coli can be characterized serologically based on the detection of specific O (somatic), H (flagella) and K (capsule) antigens. For most *E. coli* strains the O and H antigens are sufficient to identify the strain. For example, *E. coli* O157:H7 is the leading cause of STEC infections internationally [1,2]

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

The bacterium *Escherichia coli* O157:H7 has been reported as the predominant serotype of Shiga toxin producing *E. coli* (STEC) [3]. Cattles are considered to be the principal natural reservoirs of the organisms, excreting the bacteria in their feces [4]. Consumption of foods, particularly undercooked ground beef and raw milk has been associated with large food poisoning outbreaks, in which this organism was identified as the etiologic agent 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 [iii].

1.4 Virulence factors

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*, *stx2* and their variants are heterogeneous and immunologically non cross reactive. Stx1 is virtually identical to shiga-toxin produced by *Shigella dysenteriae* type 1, while *stx*2 shares only ~56% identity with *stx*1 [5]. Some of the STEC strains harbour a ~90kb plasmid encoding several virulence determinants which includes enterohemolysin (*ehxA*), bi-functional catalase-peroxydase (*katP*), secreted serine protease (*espP*) and type II secreting system (*etpD*) [6].

1.5 Reservoirs, Sources and Mode of Transmission of STEC

E. coli are ubiquitous in the intestines of warm-blooded vertebrates. Cattle are the best characterized reservoir species for *E. coli* O157:H7 and up to 50–80% of cattle herds (beef and dairy) may be colonized. The organism does not cause illness in bovines. There is no effective method to eradicate the organism from herds. Other potential sources of human infection include deer, elk, sheep, and goats. There are rare reports of *E. coli* O157:H7 being isolated from other species including dogs, horses, flies and seagulls. The reservoirs for non-O157 STEC are not well characterized [iv].

For *E. coli* O157:H7, common exposures are ingestion of contaminated food or direct contact with animals on farms or at petting zoos. Undercooked beef (especially hamburger), foods cross

contaminated from raw beef, and raw milk contaminated with cattle feces are the prototypical sources of common-source outbreaks. Venison is another potential source. Contaminated produce, including leafy greens, alfalfa sprouts, and unpasteurized apple cider are other recognized exposure sources. Person-to-person transmission can occur directly (households, child care centers, institutions) or indirectly (contaminated drinking or recreational water). In all of these modes of transmission, the infectious dose is very low.

1.6 Clinical Presentations

The incubation period for STEC is two to 10 days, usually three to four days. Though STEC infection may be asymptomatic, it typically begins with watery diarrhea associated with abdominal pain, and occasionally with nausea and vomiting. Fever is not a prominent symptom. The watery diarrhea may or may not progress to bloody diarrhea. A serious complication, Hemolytic Uremic Syndrome (HUS), occurs in two to 15% of STEC infection cases. HUS is more common in extreme of ages with children less than five years most frequently affected [7]. The principal organ affected in STEC-mediated HUS is the kidney. This is presumed to be the consequence of the high level of renal blood flow and abundant baseline expression and high degree of inducibility of the Shiga toxin glycolipid receptor, Gb3, within the glomerular microcirculation [8]. These factors are more pronounced in younger children, accounting, in part, for the heightened susceptibility of pediatric patients to this disease. The severity of renal injury varies in degree from urinary abnormalities such as hematuria and proteinuria to acute renal failure. Approximately 40% of patients with STEC-induced HUS require temporary dialysis support until they recover from the acute episode [9]. The second most important organ affected in the disease is the brain. Nearly all children manifest lethargy and irritability. However, more serious cerebral complications, including seizures, cortical blindness, and thrombotic strokes, occur in 5 to 10% of patients. These reflect a combination of factors such as vascular injury, hypertension, azotemia, hyponatremia, and hypocalcemia [10].

1.7 Treatment

The etiology of STEC-induced HUS was unknown for so long, this left a therapeutic void into which clinicians leapt in an effort to treat the disease. Unfortunately, as outlined in the following

section, none of these treatments have had any impact on the incidence and severity of STEC-induced HUS. This has led some clinicians to adopt a stance that there is no treatment for STEC-induced HUS except prevention. However, recent advances in the understanding of the pathobiology of *stx* in these circumstances will hopefully justify renewed attempts to ameliorate the disease, with, for instance, antibody-based *stx* treatment strategies [v].

1.8 Objectives

Bangladesh is considered as an endemic area for STEC O157:H7. The objective of this study is to investigate some of the common virulence factors of E.coli from bovine by PCR. Therefore the prevalence of E.coli O157:H7 in bovine of Bangladesh would be investigated by this project. Moreover no unique virulent factor has been identified so far that is specific to E.coli isolates from bovine mastitis. By this study there can be a possibility to isolate the virulent factor that would be distinctive for STEC.

Chapter 2

Materials and Method

2.1 Working Place

Overall research was performed in the Microbiology Specialized Research Laboratory, Department of Mathematics and Natural Sciences, BRAC University.

2.2.1 Sample Collection

Six individual samples were collected from different places around Dhaka city. The samples were collected in sterile plastic bags (for solid samples) and containers (for liquid samples).

The sources of the samples are mentioned below:

1. Bovine feces sample-1: Shanir Akhra, Dhaka
2. Bovine feces sample-2: Kamrangir Chor, Dhaka
3. Bovine feces sample-3: Mohakhali, Dhaka
4. Milk sample-1: Gulshan-1, Dhaka
5. Milk sample-2: Gabtoli, Dhaka
6. Milk Sample-3: Keraniganj, Dhaka
7. Human sewage sample: Sayedabaad, Dhaka.

Figure 2: Collection of bovine feces sample

2.2.2 Enrichment of microorganisms present in samples

Trypticase Soy Broth (TSB) was used for enrichment. Samples were inoculated into TSB broth in 1:10 ratio and incubated at 37 ^{o}C overnight

2.2.3 Culture of bacteria on specific agar plates

1. The enriched samples were diluted in different concentrations with 0.9% NaCl solution.
2. Individual NA and MAC plates were taken for different concentrations and 100µl of diluted solutions were spread in each plate.
3. All the plates were incubated for 24 hrs at 37 °C.
4. After 24 hrs the cultural characteristics were observed and numbers of colonies were counted of both types of media. The pink colonies on MAC were supposed to be of the desired *E.coli* strains.

2.3 Confirmation of desired microorganism

1. Single isolated pink colonies from MAC plates were obtained and streaked into EMB agar plate which is specific for the target organism.
2. The plates were kept in the incubator overnight at 37 °C.
3. The colonies showed green metallic sheen color were identified from MAC and streaked again in NA and incubated overnight at 37 °C.
4. The isolated grown colonies were tended to be *E.coli.*

2.4 Preparation of stock sample

Short-term preservation

1. T_1N_1 agar was prepared and taken in small vials.
2. Then the vials were autoclaved at 121 °C for 15 mins and kept at 4 °C for an hr to gelatinize.
3. T_1N_1 agar butt in a vial was inoculated by stabbing bacterial growth of each isolate from nutrient agar plate and incubated at 37 °C for 3-5 hrs.
4. After incubation the surface of the medium was covered with sterile paraffin oil and the vial was stored at room temperature.

Long-term preservation

For long-term preservation, 200µl of sterile glycerol was added in each vial and the medium was covered with sterile paraffin oil and the vial was stored at room temperature and at -20 °C as well.

2.5 Confirmation of the Isolated *E.coli* strains

2.5.1 Confirmation of plating bacteria

1. Stock culture were revived in NA plates and streaked into MAC and EMB agar those are specific for *E.coli.*
2. The plates were incubated overnight at 37 °C.
3. The pink and green sheen growth of bacteria on MAC and EMB respectively was the confirmation for *E.coli.*

2.5.2 Biochemical Identification

Biochemical tests were performed according to the methods described in Microbiology Laboratory Manual [Cappuccino *et al*., 2005]. The biochemical tests carried out were Indole production, Methyl-red, Voges- Proskauer's, Triple sugar iron, Carbohydrate fermentation and Citrate utilization test.

1. **Indole production Test**

- Colorless bacterial colonies were picked from the Nutrient agar plate and were inoculated in peptone water which contains amino acid tryptophan and incubated overnight at 37oC. Following incubation a few drops of Kovac's reagent were added.
- Detection of positive result would give a purple layer at the top or a negative result had a yellow or brown layer.

2. **Methyl red (MR) Test**

- The bacterium to be tested was inoculated into potassium phosphate broth (MR-VP broth), which contained dextrose, peptone and potassium phosphate and incubated at 37 °C for 24 hrs.

- Over the 24 hrs the mixed-acid producing organism might produce sufficient acid to overcome the phosphate buffer and remained acidic.

3. Voges-Proskauer's Test

- Bacterium to be tested was inoculated into potassium phosphate broth (MR-VP broth) and incubated for 24 hrs.
- Barritt's reagent A was added to the test broth and shaken.
- Barritt's reagent B was added and the tube was allowed to stand for 15 mins.
- Appearance of red color was taken as a positive test, negative tube might be held for an hour after addition of reagents.

4. Triple Sugar Iron (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.

5. Carbohydrate fermentation Test

- The Durham tubes should be inserted in an inverted position into all the tubes, fully filled with broth (Lactose, Dextrose and Sucrose)
- Each labeled carbohydrate broth (Lactose, Dextrose and Sucrose) was inoculated aseptically with each of the seven bacterial cultures.
- After inoculation into a particular sugar, the loop was sterilized in order to avoid cross contamination of the tube with other sugars.
- The tubes were incubated for 24 hrs at 37 °C.

6. **Citrate utilization Test**

- Colorless bacterial colonies were picked from the Nutrient agar plate by a straight wire and inoculated into the slope of Simmon's citrate agar and incubated overnight at 37 °C.
- If the organism had the ability to utilize citrate, the medium changed its color from green to Prussian blue; a negative slant would have no growth of bacteria and would remain green.

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

2.6.1 DNA Extraction

- 100µl distilled water was taken in each Eppendorf tube.
- A loop full of different bacterial strains were collected from freshly sub cultured NA plate and mixed with distilled water gently.
- All the tubes were heated in the water bath at 100 °C for 10 mins and then kept on ice for a min.
- After that all the tubes were centrifuged at 12000rpm for 10mins.
- Finally the supernatants (template DNA) were accumulated in different eppendorf tubes.

2.6.2 Polymerase Chain Reaction (PCR)

a) Master-mix preparation

The master-mix for each sample to run PCR was prepared according to the following measurement:

Table 2: Components of PCR master-mix

Components	Concentration	Amount
1. Taq buffer	10x	2µl
2. dNTPs	2mM	1µl
3. *stx1* forward primer	1µm	1µl
4. *stx1* reverse primer	1µm	1µl
5. *stx2* forward primer	1µm	1µl

6. *stx2* reverse primer	1µm	1µl
7. Taq polymerase	1unit/µl	1µl
8. Template DNA	_	1.5µl
9. $MgCl_2$	50mM	0.5µl
10. Nuclease free water	_	10µl
		Total: 20µl

Table 3: Primers used in the study

Target Gene	Primer	Sequence	Amplicon Size	Reference
stx1	LP30 (F)	5'-CAGTTAATGTGGTGGCGAAGG -3'	348 bp	[11]
	LP31 (R)	5'-CACCAGACAAATGTAACCGCTC -3'		
stx2	LP41 (F)	5'-ATCCTATTCCCGGGAGTTTACG -3'	584 bp	[11]
	LP42 (R)	5'-GCGTCATCGTATACACAGGAGC -3'		

b) PCR thermal cycle setup:

While conducting the PCR the thermal cycle was set up according to the following:

Table 4: Thermo cycling conditions for PCR

Step	Temperature	Time
Initial denaturation	95°C	5mins
35 cycles	94°C	1min
	54 °C (annealing)	1min
	72°C	1min
Final elongation	72°C	5mins
Hold	4°C	_

- Before conducting PCR the master-mix for individual samples were prepared in proportion to the measurement mentioned above.

- Then the thermal cycler was set to maintain 35 cycles and different time and temperature were assigned for different steps to be performed.
- After certain time period of accomplishment the PCR products were gone through gel electrophoresis to acquire the final result which would notify the presence of *stx1* and *stx2* gene in the sample strains.

2.6.3 Gel electrophoresis

- To carry out gel electrophoresis 1.5% conc. Agorose gel was prepared and comb was used afterwards to create wells for sample loading.
- 1µl of loading dye was mixed with each sample which was acquired after PCR.
- The sample loading was maintained by following order:

Table 5: Sequence of sample loading in Agorose gel

Lane	Sample	Amount
Lane 1	100 bp DNA ladder	3µl
Lane 2	Positive reference strain: STEC	5µl
Lane 3	HS-8	5µl
Lane 4	Negative reference strain: *E.coli* K12	5µl
Lane 5	BF-1	5µl
Lane 6	MS-1	5µl
Lane 7	BF-2	5µl
Lane 8	MS-2	5µl
Lane 9	BF-3	5µl
Lane 10	MS-3	5µl

- After organizing the gel it was kept in the gel apparatus and adequate TBE buffer was added to the apparatus.
- Then the gel apparatus was connected with the power supply and the process was carried out at 70V
- When the sample reached the positive end the gel was observed under UV light with the help of trans-illuminator to study the result.

Chapter 3

Results

3.1 Enrichment of bacteria

The collected samples were mixed with Trypticase Soy Broth (TSB) and after 24 hours turbidity was observed in the broth.

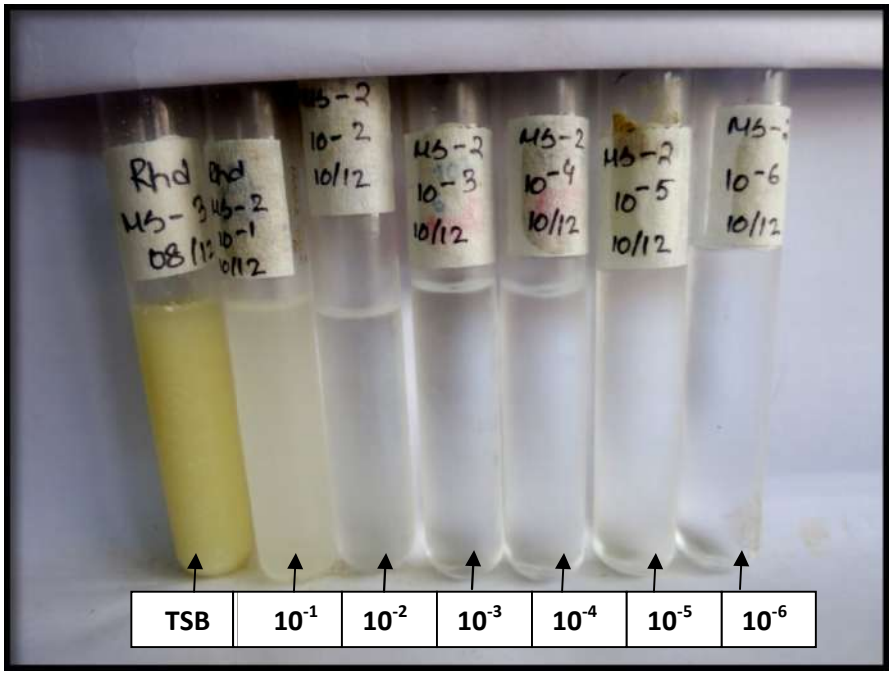


Figure 3: Enrichment in TSB and Serial dilation of the samples

3.2 Calculation of bacterial growth and isolation of *Escherichia coli (E.coli)* colonies

The samples were diluted in different concentrations and the bacteria were allowed to grow in both Nutrient Agar (NA) and MacConkey Agar (MAC). From MAC plates isolated colonies were obtained.

Table 6: Results of colony counting calculation on NA and MAC agar plates

Sample	Concentration	Medium	Number of Colonies
1. BF-1	10^{-1}	NA	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		TNTC
	10^{-5}		TNTC
	10^{-6}		118
	10^{-1}	MAC	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		TNTC
	10^{-5}		TNTC
	10^{-6}		38
2. BF-2	10^{-1}	NA	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		TNTC
	10^{-5}		187
	10^{-6}		49
	10^{-1}	MAC	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		121
	10^{-5}		57
	10^{-6}		11
3. BF-3	10^{-1}	NA	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC

	10^{-4}		TNTC
	10^{-5}		35
	10^{-6}		7
	10^{-1}	MAC	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		243
	10^{-5}		37
	10^{-6}		3
4. MS-1	10^{-1}	NA	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		TNTC
	10^{-5}		TNTC
	10^{-6}		104
	10^{-1}	MAC	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		TNTC
	10^{-5}		87
	10^{-6}		41
5. MS-2	10^{-1}	NA	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		TNTC
	10^{-5}		TNTC
	10^{-6}		61
	10^{-1}	MAC	TNTC
	10^{-2}		TNTC

	10^{-3}		TNTC
	10^{-4}		21
	10^{-5}		9
	10^{-6}		5
6. MS-3	10^{-1}	NA	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		TNTC
	10^{-5}		86
	10^{-6}		65
	10^{-1}	MAC	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		18
	10^{-5}		11
	10^{-6}		4
7. HS	10^{-1}	NA	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		TNTC
	10^{-5}		TNTC
	10^{-6}		131
	10^{-1}	MAC	TNTC
	10^{-2}		TNTC
	10^{-3}		TNTC
	10^{-4}		TNTC
	10^{-5}		37
	10^{-6}		18

In both culture plates the growth was observed but the number of colonies varied due to different concentrations. For highest concentrations the growth became TNTC and the colonies were counted easily in lower concentrated plates.

Table 7: Cultural characteristics of bacterial colonies on NA and MAC agar plates

Sample	Conc.	Medium	Cultural Characteristics					
			Size	**Margin**	**Elevation**	**Form**	**Pigment**	**Consistency**
1. BF-1	10^{-1}	NA	Pinpoint	Entire	Flat	Punctiform	1. White 2. Yellow	Rough
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	1. White 2. Yellow	Rough
	10^{-3}		Pinpoint	Entire	Flat	Punctiform	1. White 2. Yellow	Rough
	10^{-4}		Pinpoint	Entire	Flat	Punctiform	1. White 2. Yellow	Rough
	10^{-5}		Pinpoint	Entire	Flat	Punctiform	1. White 2. Yellow	Rough
	10^{-6}		Small	Entire	Flat	Circular	1. White 2. Yellow	Smooth
	10^{-1}	MAC	Pinpoint	Entire	Flat	Punctiform	Dark pink	Mucoid
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	Dark pink	Mucoid
	10^{-3}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-4}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-5}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy

	10^{-6}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy
2. BF-2	10^{-1}	NA	Pinpoint	Entire	Flat	Punctiform	White	Rough
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	White	Rough
	10^{-3}		Pinpoint	Entire	Flat	Punctiform	White	Rough
	10^{-4}		Small	Entire	Flat	Circular	White	Smooth
	10^{-5}		Small	Entire	Flat	Circular	White	Smooth
	10^{-6}		Moderate	Entire	Flat	Circular	White	Smooth, creamy
	10^{-1}	MAC	Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-3}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-4}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-5}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy
	10^{-6}		Large	Entire	Raised	Circular	Dark pink	Smooth, creamy
3. BF-3	10^{-1}	NA	Pinpoint	Entire	Flat	Punctiform	White	Smooth
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	White	Smooth
	10^{-3}		Pinpoint	Entire	Flat	Punctiform	White	Smooth
	10^{-4}		Moderate	Entire	Raised	Circular	White	Smooth, creamy
	10^{-5}		Large	1. Entire 2.filamentous	1.Raised 2.Umbonate	1. Circular 2. Rhizoid	1. White 2. Yellow	Smooth, creamy
	10^{-6}		Large	1. Entire	1.Raised	1. Circular	1. White	Smooth,

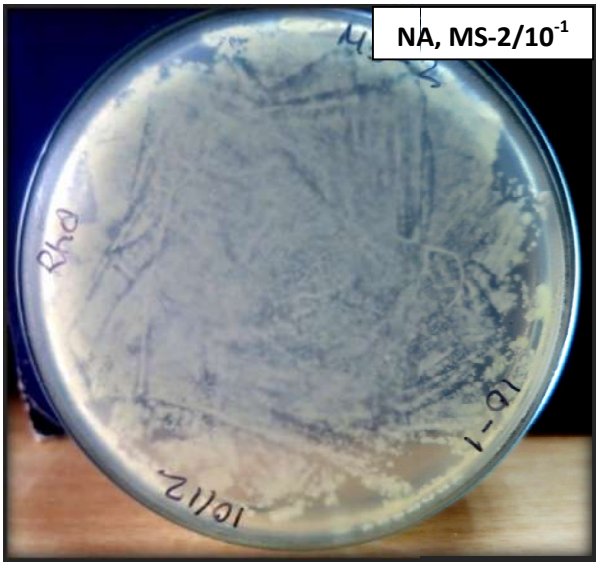
				2.filamentous	2.Umbonate	2. Rhizoid	2. Yellow	creamy
	10^{-1}	MAC	Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-3}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-4}		Moderate	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-5}		Large	Entire	Raised	Circular	Dark pink	Smooth, creamy
	10^{-6}		Large	Entire	Raised	Circular	Dark pink	Smooth, creamy
4. MS-1	10^{-1}	NA	Pinpoint	Entire	Flat	Punctiform	White	Rough
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	White	Rough
	10^{-3}		Pinpoint	Entire	Flat	Punctiform	White	Rough
	10^{-4}		Small	Entire	Flat	Circular	White	Smooth, creamy
	10^{-5}		Small	Entire	Raised	Circular	White	Mucoid
	10^{-6}		Small	Entire	Raised	Circular	White	Mucoid
	10^{-1}	MAC	Pinpoint	Entire	Flat	Punctiform	1.Dark pink 2. Light pink	Rough
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	1.Dark pink 2. Light pink	Rough
	10^{-3}		Small	Entire	Flat	Circular	1.Dark pink	Smooth, creamy

							2. Light pink	
	10^{-4}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-5}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy
	10^{-6}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy
5. MS-2	10^{-1}	NA	Pinpoint	Entire	Flat	Punctiform	White	Smooth, creamy
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	White	Smooth, creamy
	10^{-3}		Moderate	Entire	Flat	Circular	White	Smooth, creamy
	10^{-4}		Moderate	Entire	Raised	Circular	White	Smooth, creamy
	10^{-5}		Moderate	Entire	Raised	Circular	White	Smooth, creamy
	10^{-6}		Moderate	Entire	Raised	Circular	White	Smooth, creamy
	10^{-1}	MAC	Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-3}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-4}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-5}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy

	10^{-6}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy
6. MS-3	10^{-1}	NA	Pinpoint	Entire	Flat	Punctiform	White	Smooth, creamy
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	White	Smooth, creamy
	10^{-3}		Moderate	Entire	Flat	Circular	White	Smooth, creamy
	10^{-4}		Large	Entire	Raised	Circular	White	Smooth, creamy
	10^{-5}		Large	Entire	Raised	Circular	White	Smooth, creamy
	10^{-6}		Large	Entire	Raised	Circular	White	Smooth, creamy
	10^{-1}	MAC	Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-3}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-4}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy
	10^{-5}		Large	Entire	Raised	Circular	Dark pink	Smooth, creamy
	10^{-6}		Large	Entire	Raised	Circular	Dark pink	Smooth, creamy
7. HS	10^{-1}	NA	Pinpoint	Entire	Flat	Punctiform	White	Rough
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	White	Rough
	10^{-3}		Small	Entire	Flat	Circular	White	Rough
	10^{-4}		Small	Entire	Flat	Circular	White	Smooth,

								creamy
	10^{-5}		Moderate	Entire	Raised	Circular	White	Smooth, creamy
	10^{-6}		Moderate	Entire	Raised	Circular	White	Smooth, creamy
	10^{-1}	MAC	Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-2}		Pinpoint	Entire	Flat	Punctiform	Dark pink	Smooth, creamy
	10^{-3}		Small	Entire	Flat	Circular	Dark pink	Smooth, creamy
	10^{-4}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy
	10^{-5}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy
	10^{-6}		Moderate	Entire	Raised	Circular	Dark pink	Smooth, creamy

In most of the NA plates the colony characteristics are observed similar so that in MAC plates. NA plates represented white colonies and MAC plates sumbolized as pink colonies. By observing pink colonies further confirmation tests are to be performed to get positive result about *E.coli.*



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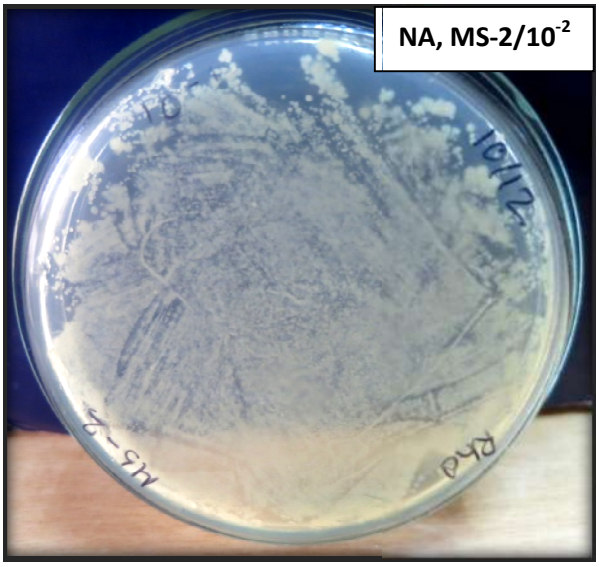

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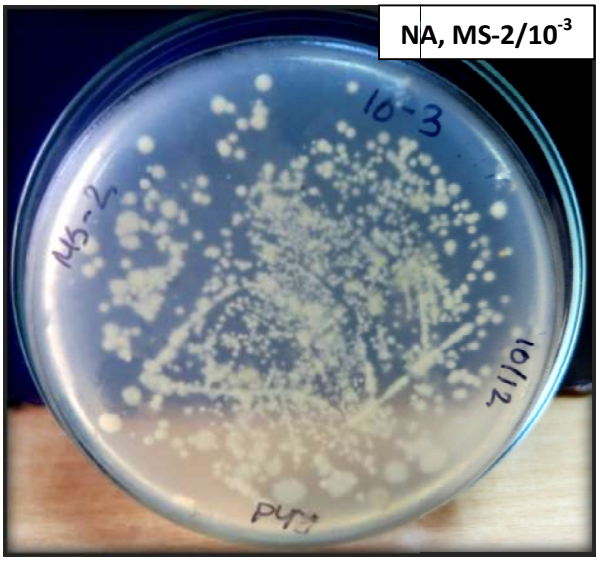

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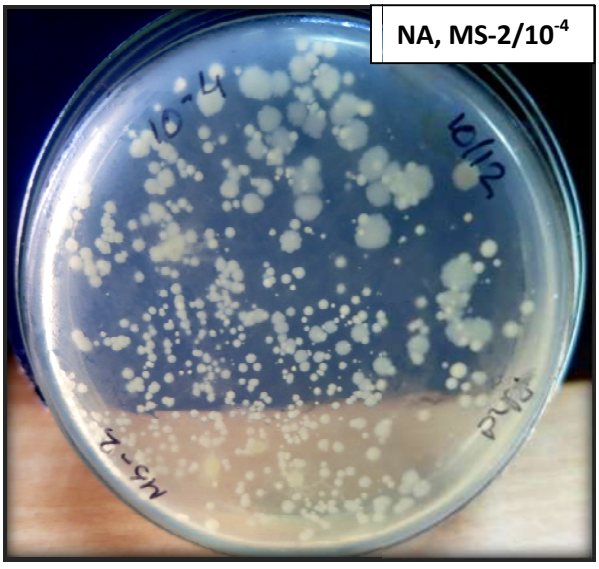
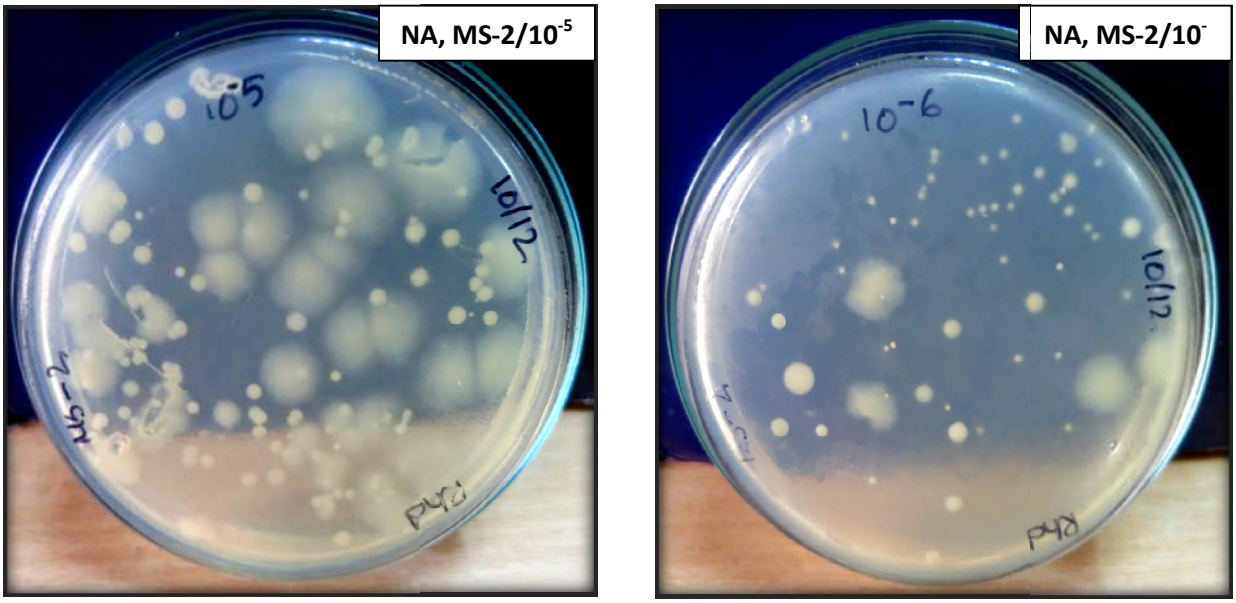
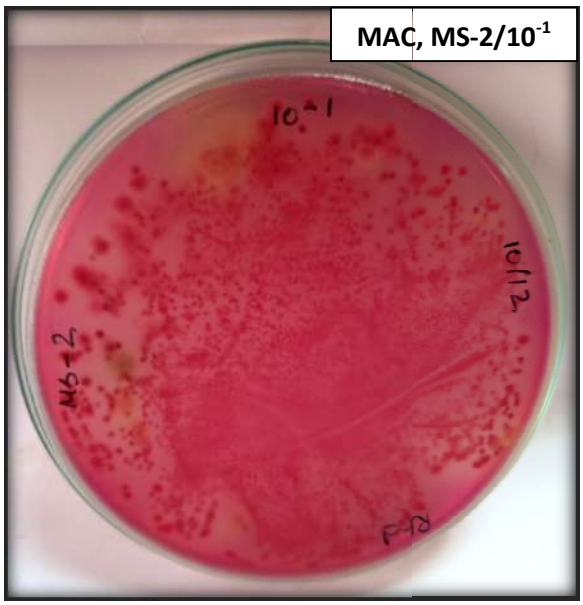


d)

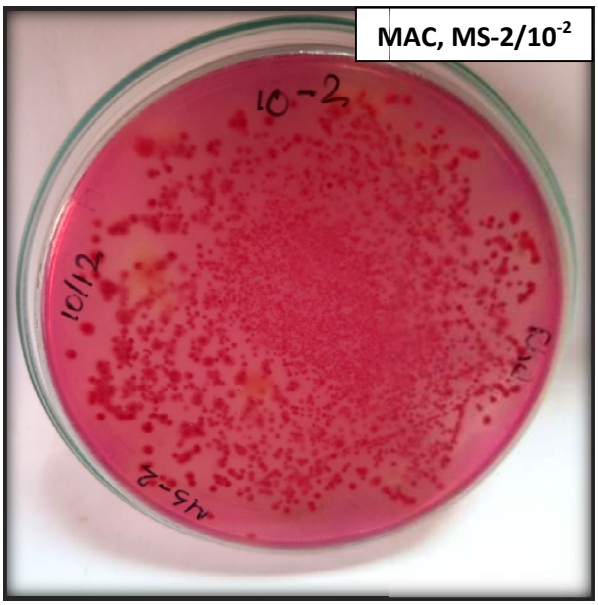


e) **f)**

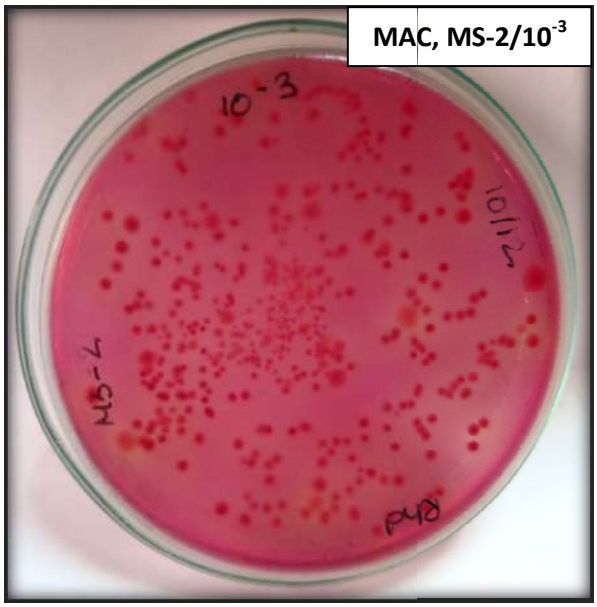
Figure 4: Cultural characteristics of bacterial colonies on NA plates a) conc. 10^{-1}, b) conc. 10^{-}, c) conc. 10^{-3}, d) conc. 10^{-4}, e) conc. 10^{-5}, f) conc. 10^{-6}.


MAC, MS-2/10-1
10-1
10/12
MS-2
PYO

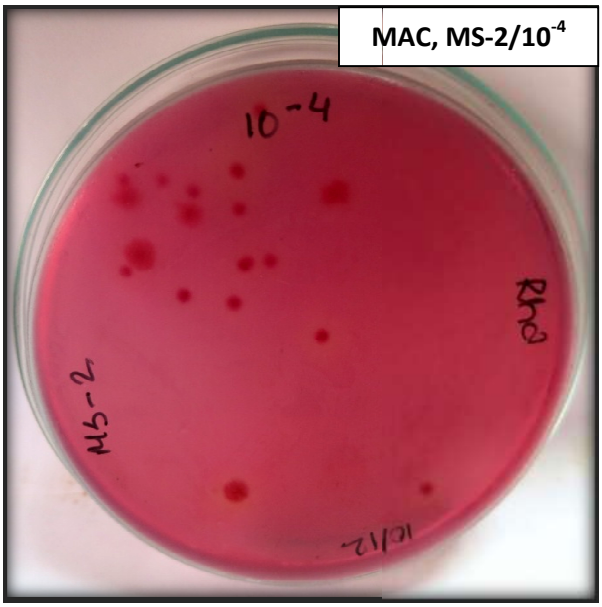
a)


MAC, MS-2/10-2
10-2
10/12
MS-2

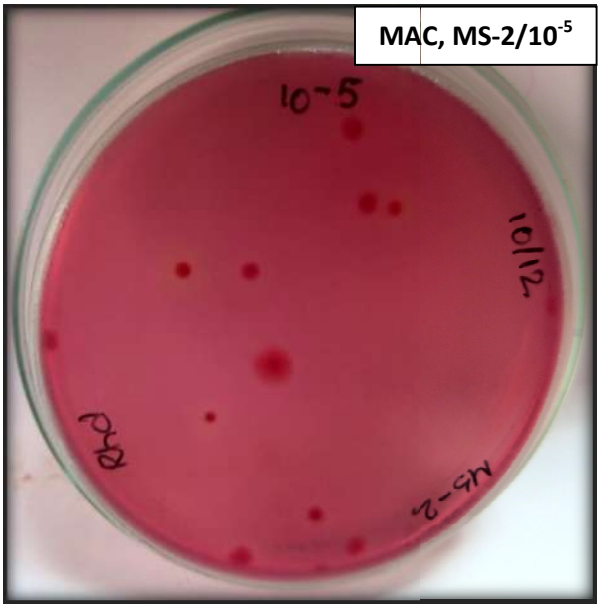
b)

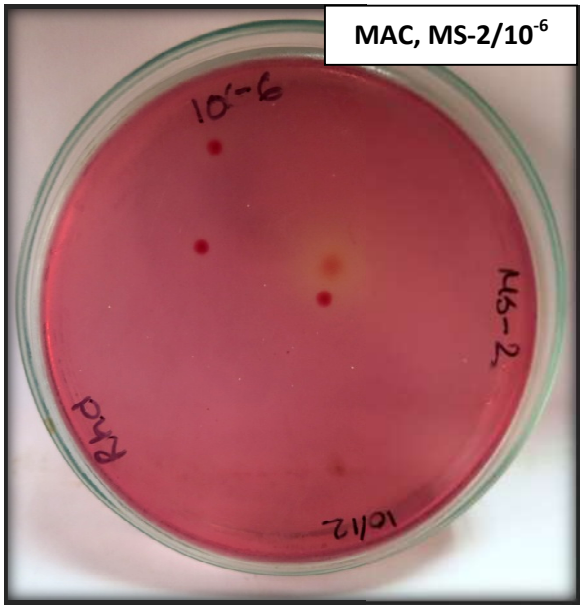

MAC, MS-2/10-3
10-3
10/12
MS-2
PYO

c)


MAC, MS-2/10-4
10-4
RHO
MS-2

d)





e) **f)**

Figure 5: Cultural characteristics of bacterial colonies on MAC agar plates a) conc. 10^{-1}, b) conc. 10^{-2}, c) conc. 10^{-3}, d) conc. 10^{-4}, e) conc. 10^{-5},f) conc.10^{-6}.

3.3 Identification of *E.coli* colonies on Eosin Methylene Blue (EMB) agar

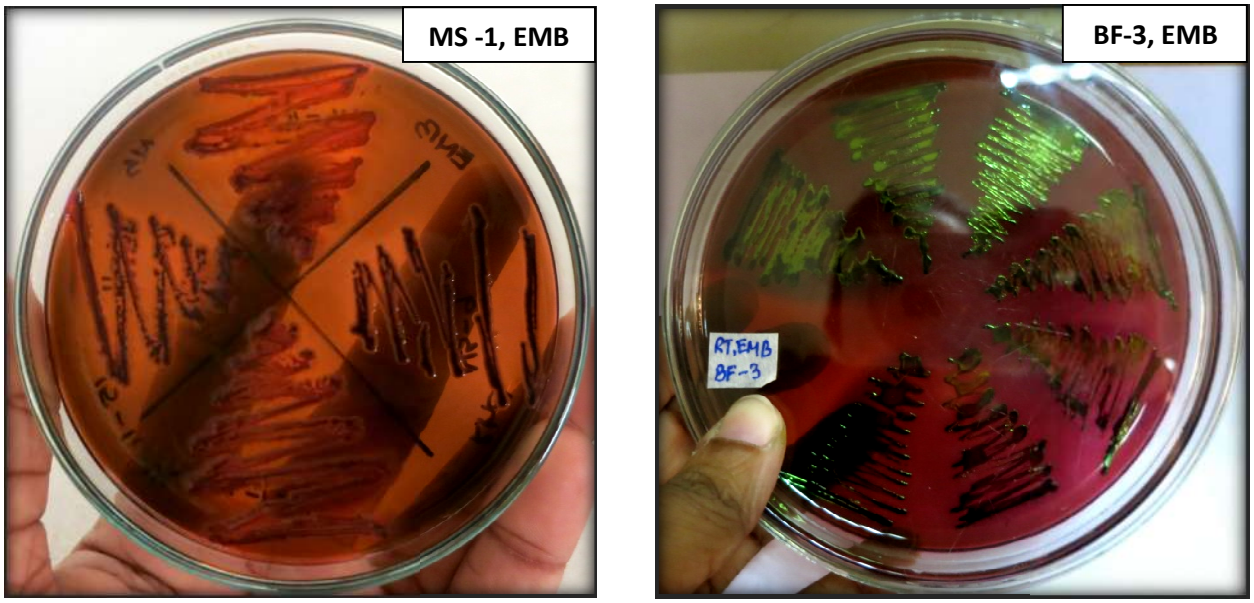
From the lowest concentrations of MAC agar plates some pink colonies were selected and cultured in EMB agar. The *E.coli* was grown as green metallic sheen form. Other bacteria would be grown as pink or dark purple form.

Table 8: Cultural characteristics of colony isolates on selective media of *E.coli*

Sample	Colony Isolates	Cultural characteristics on EMB agar
1. BF-1	I-1	Green metallic sheen
	I-2	Green metallic sheen
	I-3	Pink colony
	I-4	Pink colony
	I-5	Pink colony
	I-6	Dark purple colony
	I-7	Dark purple colony
	I-8	Pink colony
	I-9	Green metallic sheen
	I-10	Green metallic sheen
	I-11	Green metallic sheen
	I-12	Pink colony
	I-13	Green metallic sheen
	I-14	Pink colony
	I-15	Pink colony
	I-16	Green metallic sheen
2. BF-2	I-1	Dark purple colony
	I-2	Green metallic sheen
	I-3	Green metallic sheen
	I-4	Green metallic sheen
	I-5	Green metallic sheen
	I-6	Green metallic sheen

	I-7	Green metallic sheen
	I-8	Green metallic sheen
3. BF-3	I-1	Pink colony
	I-2	Green metallic sheen
	I-3	Green metallic sheen
	I-4	Dark purple colony
	I-5	Dark purple colony
	I-6	Dark purple colony
	I-7	Dark purple colony
	I-8	Dark purple colony
	I-9	Green metallic sheen
	I-10	Green metallic sheen
	I-11	Green metallic sheen
	I-12	Green metallic sheen
4. MS-1	I-1	Pink colony
	I-2	Pink colony
	I-3	Pink colony
	I-4	Pink colony
	I-5	Green metallic sheen
	I-6	Pink colony
5. MS-2	I-1	Green metallic sheen
	I-2	Green metallic sheen
	I-3	Pink colony
	I-4	Dark purple colony
	I-5	Dark purple colony
6. MS-3	I-1	Green metallic sheen
	I-2	Green metallic sheen
	I-3	Pink colony
	I-4	Dark purple colony
7. HS	I-1	Green metallic sheen

	I-2	Green metallic sheen
	I-3	Green metallic sheen
	I-4	Green metallic sheen
	I-5	Green metallic sheen
	I-6	Green metallic sheen
	I-7	Green metallic sheen
	I-8	Green metallic sheen
	I-9	Green metallic sheen
	I-10	Green metallic sheen



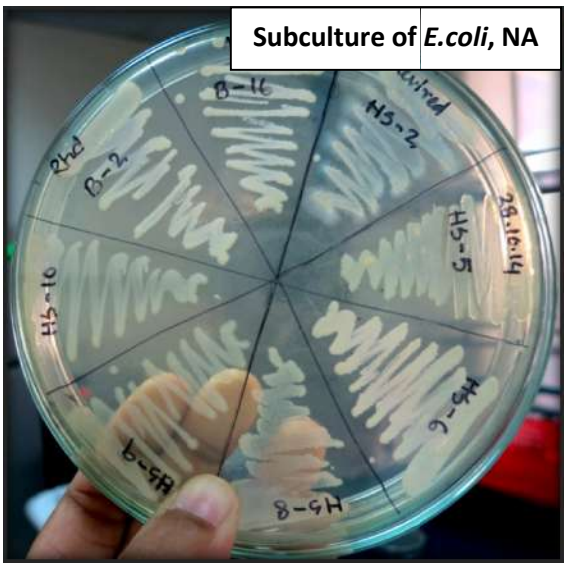
a) b)

Figure 6: Cultural characteristics on EMB agar: a) MS-1 isolates no 1,2,3,4 non *E.coli*

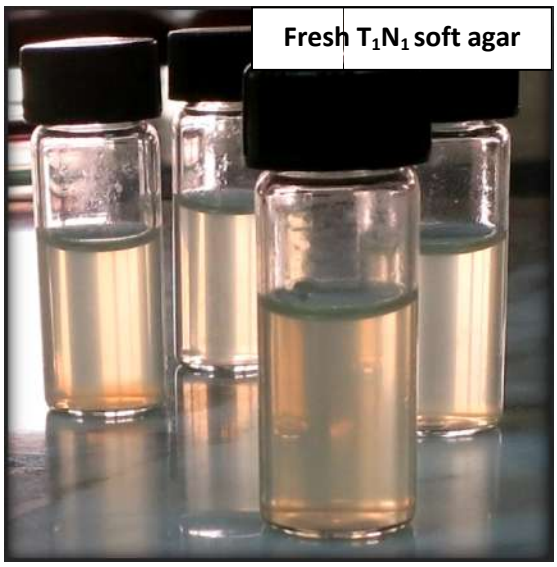
b) BF-3 isolates no 1-7 *E.coli*

3.4 Preservation of segregated *E.coli* colony isolates

The segregated *E.coli* colony isolates were grown in NA plates and from there they were preserved in T_1N_1 soft agar by 3-5 hours incubation at 37°C. The T_1N_1 vials became turbid after the bacterial growth.



a)



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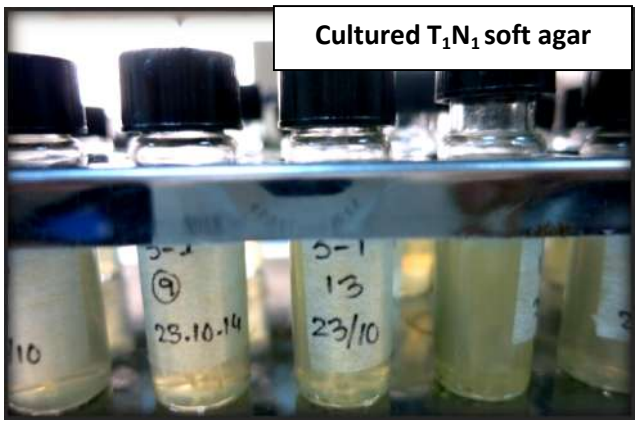


c)

Figure 7: a) Growth of *E.coli* isolates on NA, b) T_1N_1vials, c) T_1N_1vials with bacterial culture

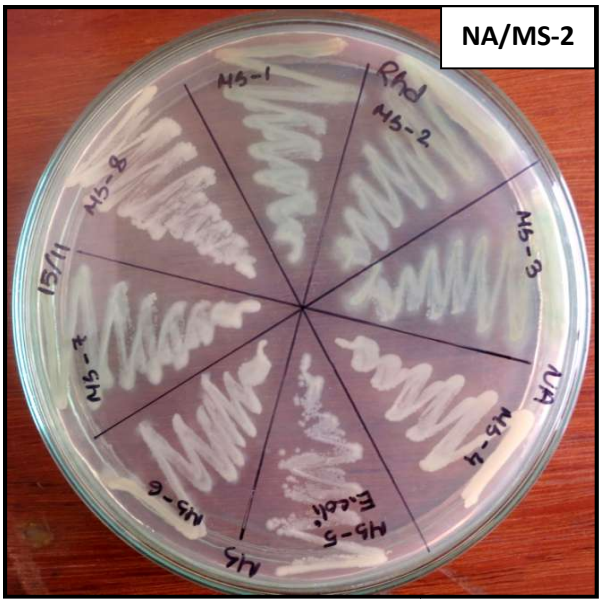
3.5 Confirmation of *E.coli* colony isolates

The *E.coli* colony isolates were revived in NA and then again grown on MAC and EMB agar plates where *E.coli* were formed as pink colony and green sheen respectively. For further confirmation several biochemical tests were performed to identify *E.coli* isolates like IMViC, TSI fermentation and Carbohydrate fermentation tests and *E.coli* showed the positive results accordingly.

Table 9: Cultural characteristics of colony isolates preserved in T_1N_1 soft agar

Sample	Colony Isolates	Cultural characteristics on MAC	Cultural characteristics on EMB agar
1. BF-1	I-1	Pink	Green metallic sheen
	I-2	Pink	Green metallic sheen
	I-9	Pink	Green metallic sheen
	I-10	Pink	Green metallic sheen
	I-11	Pink	Green metallic sheen
	I-13	Pink	Green metallic sheen
	I-16	Pink	Green metallic sheen
2. BF-2	I-2	Pink	Green metallic sheen
	I-3	Pink	Green metallic sheen
	I-4	Pink	Green metallic sheen
	I-5	Pink	Green metallic sheen
	I-6	Pink	Green metallic sheen
	I-7	Pink	Green metallic sheen
	I-8	Pink	Green metallic sheen
3. BF-3	I-2	Pink	Green metallic sheen
	I-3	Pink	Green metallic sheen
	I-9	Pink	Green metallic sheen
	I-10	Pink	Green metallic sheen

	I-11	Pink	Green metallic sheen
	I-12	Pink	Green metallic sheen
4. MS-1	I-1	Pink	Green metallic sheen
5. MS-2	I-1	Pink	Green metallic sheen
	I-2	Pink	Green metallic sheen
6. MS-3	I-1	Pink	Green metallic sheen
	I-2	Pink	Green metallic sheen
7. HS	I-1	Pink	Green metallic sheen
	I-2	Pink	Green metallic sheen
	I-3	Pink	Green metallic sheen
	I-4	Pink	Green metallic sheen
	I-5	Pink	Green metallic sheen
	I-6	Pink	Green metallic sheen
	I-7	Pink	Green metallic sheen
	I-8	Pink	Green metallic sheen
	I-9	Pink	Green metallic sheen
	I-10	Pink	Green metallic sheen



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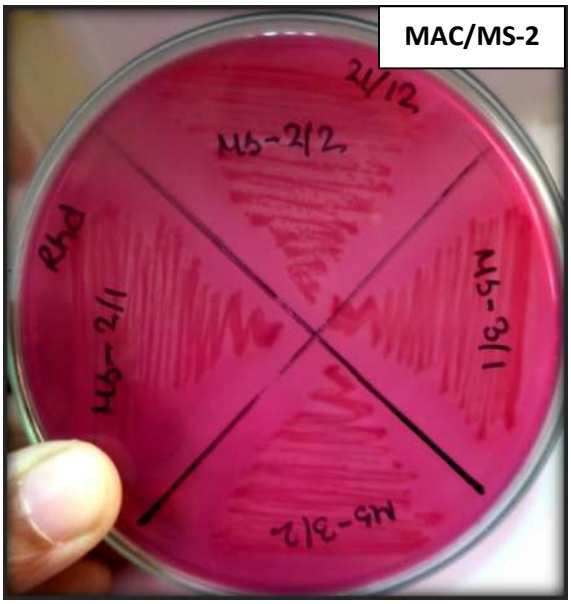

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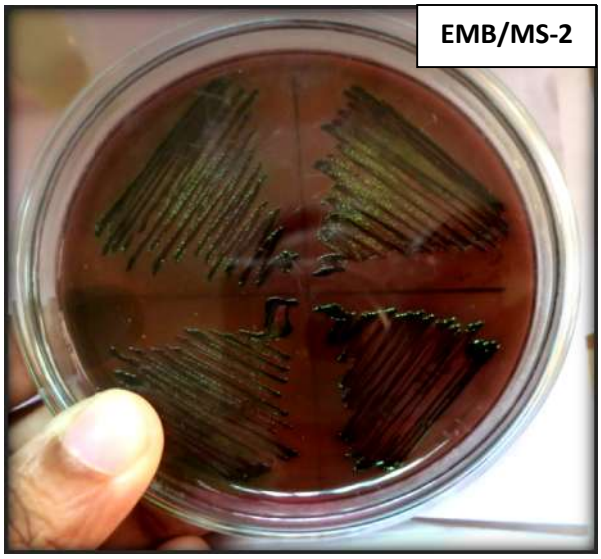


Figure 8: Confirmation tests of *E.coli* colony isolates a) Revived *E.coli* colony isolates from T1N1 soft agar, b) Growth of isolates as pink colony on MAC agar c) Growth of isolates as green sheen on EMB agar.

3.6 Further confirmation by Biochemical Tests

For more verification of the results various biochemical tests were accomplished and under this IMViC test, TSI fermentation test, Carbohydrate fermentation test and Citrate utilization test are conducted. E.coli gives positive results for Indole production, Methyl red reaction, TSI and Carbohydrate tests and shows negative results for Voges- Proskauer's reaction test and Citrate utilization test. For Indole production pink ring has been observed. Positive isolates have converted the color from transparent to red in methyl red reaction as acid is produced. For TSI they have changed the color of the media from red to yellow due to pH change from basic to acidic and can produce CO_2. *E.coli* can utilize glucose, sucrose and lactose as carbohydrates and here have given positive result by changing the color from red to yellow because of pH change. The E.coli isolates have kept the Voges- Proskauer's broth unchanged and done similar for Simmon's citrate agar as they do not utilize citrate.

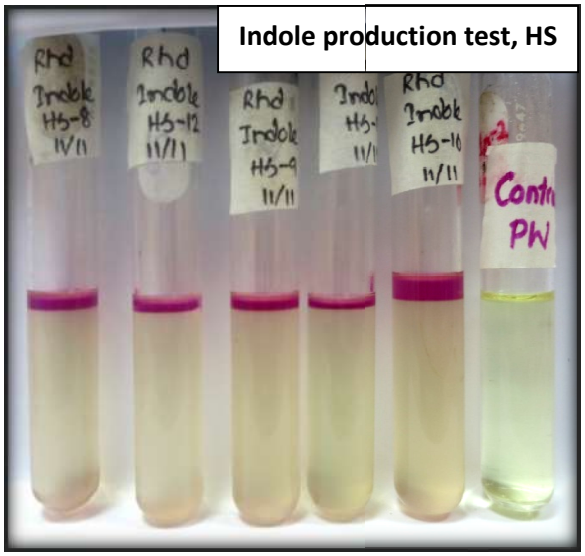
Table 10: Biochemical Test results

Sample	Colony Isolates	Biochemical Tests										
					TSI fermentation test				Carbohydrate fermentation test			
		Indole production test	Methyl Red reaction test	Voges- Proskauer's reaction test	Slant	Butt	CO_2	H_2S	Glucose	Sucrose	Lactose	Citrate Utilization Test
1. BF-1	I-1	+	+	-	A	A	-	-	AG	AG	AG	-
	I-2	+	+	-	A	A	-	-	AG	AG	AG	-

	I-9	+	+	-	A	A	-	-	AG	AG	AG	-
	I-10	+	+	-	A	A	-	-	AG	AG	AG	-
	I-11	+	+	-	A	A	-	-	AG	AG	AG	-
	I-13	+	+	-	A	A	-	-	AG	AG	AG	-
	I-16	+	+	-	A	A	-	-	AG	AG	AG	-
2. BF-2	I-2	+	+	-	A	A	-	-	AG	-	AG	-
	I-3	+	+	-	A	A	-	-	AG	-	AG	-
	I-4	+	+	-	A	A	-	-	AG	-	AG	-
	I-5	+	+	-	A	A	+	-	AG	-	AG	-
	I-6	+	+	-	A	A	-	-	AG	-	AG	-
	I-7	+	+	-	A	A	-	-	AG	-	AG	-
	I-8	+	+	-	A	A	+	-	AG	AG	AG	-
3. BF-3	I-2	+	+	-	A	A	-	-	AG	-	AG	-
	I-3	+	+	-	A	A	-	-	AG	AG	AG	-
	I-9	+	+	-	A	A	-	-	AG	-	AG	-
	I-10	+	+	-	A	A	-	-	AG	AG	AG	-
	I-11	+	+	-	A	A	-	-	AG	AG	AG	-
	I-12	+	+	-	A	A	-	-	AG	AG	AG	-
4. MS-1	I-1	-	+	-	A	A	+	-	AG	AG	AG	-
5. MS-2	I-1	-	+	-	A	A	-	-	AG	AG	AG	+
	I-2	+	+	-	A	A	-	-	AG	AG	AG	-
6. MS-3	I-1	+	+	-	A	A	-	-	AG	AG	AG	-
	I-2	+	+	-	A	A	-	-	AG	-	AG	-
7. HS	I-1	+	+	-	A	A	+	-	AG	AG	AG	-
	I-2	+	+	-	A	A	+	-	AG	AG	AG	-
	I-3	+	+	-	A	A	+	-	AG	AG	AG	-
	I-4	+	+	-	A	A	+	-	AG	AG	AG	-
	I-5	+	+	-	A	A	+	-	AG	AG	AG	+
	I-6	+	+	-	A	A	+	-	AG	-	AG	-

	I-7	+	+	-	A	A	+	-	AG	AG	AG	-
	I-8	+	+	-	A	A	+	-	AG	AG	AG	-
	I-9	+	+	-	A	A	+	-	AG	-	AG	-
	I-10	+	+	-	A	A	+	-	AG	AG	AG	-

KEY: A= acidic condition, K=alkaline condition, + = positive, - = negative, AG=both acid and gas production.


Indole production test, HS

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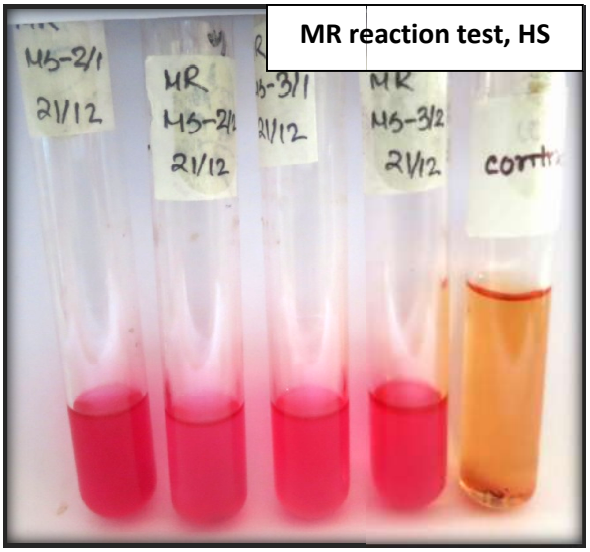
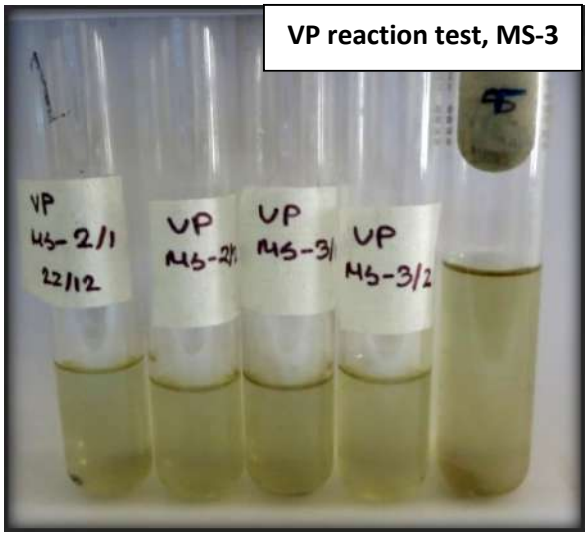
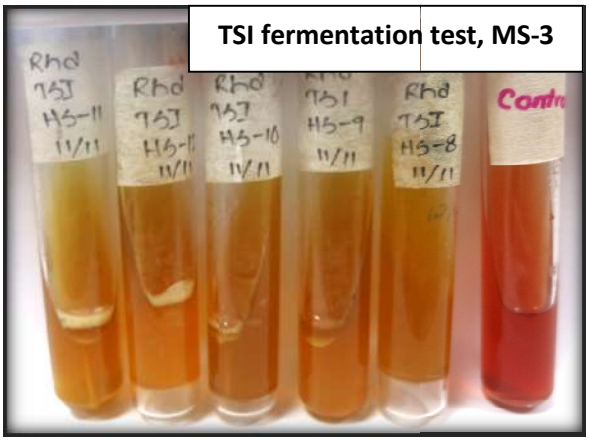

MR reaction test, HS

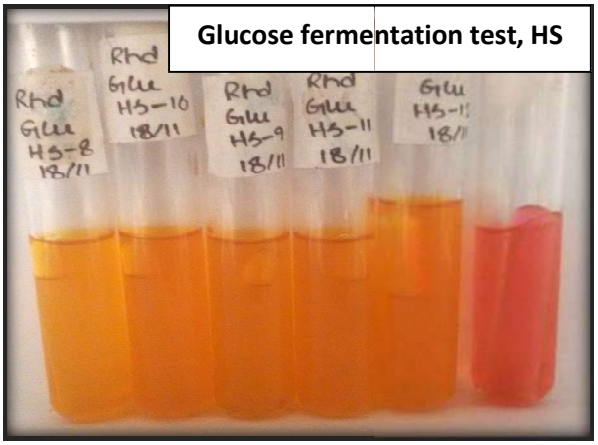
b)


VP reaction test, MS-3

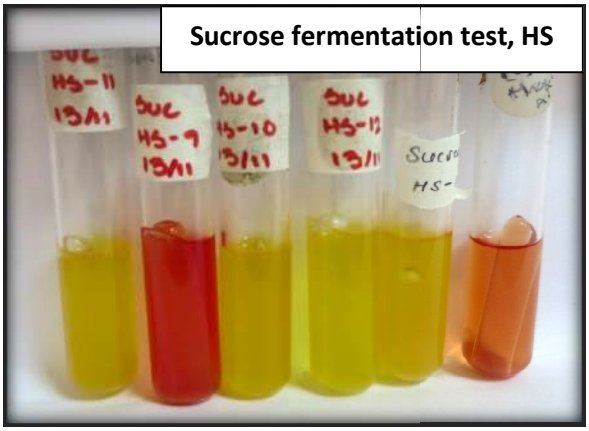
c)


TSI fermentation test, MS-3

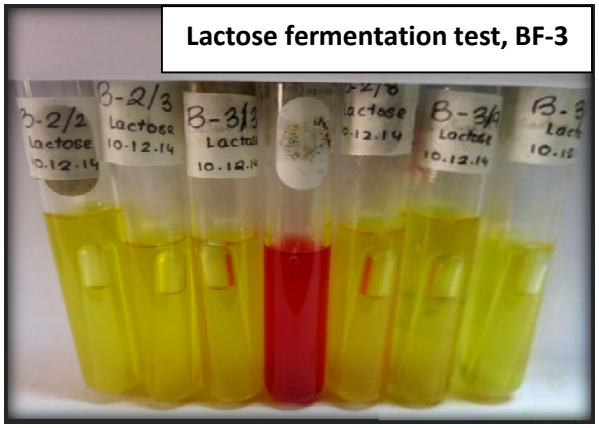
d)


Glucose fermentation test, HS

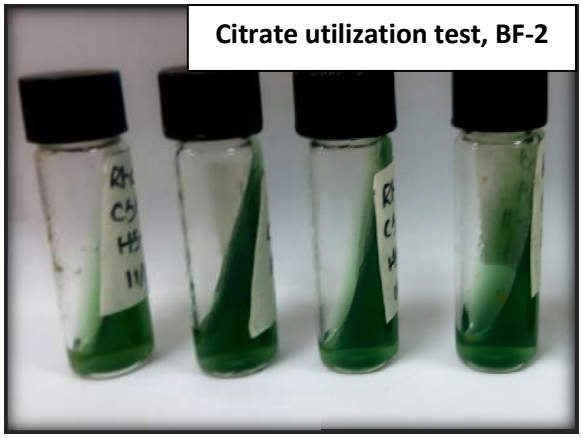
e)


Sucrose fermentation test, HS

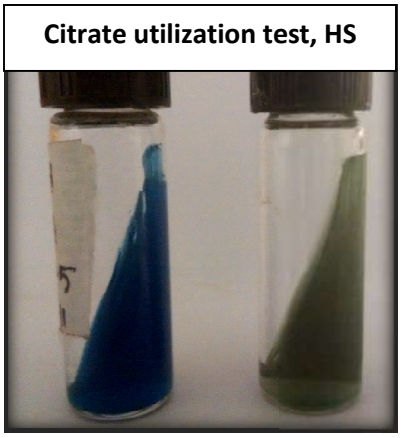
f)


Lactose fermentation test, BF-3

g)



h)



i)

Figure 9: Biochemical tests confirmation a) Indole production test, b) Methyl red reaction test, c) Voges- Proskauer's reaction test, d) TSI fermentation test, e) Glucose fermentation test, f) Sucrose fermentation test, g) Lactose fermentation test, h) Citrate utilization test, i) Citrate utilization test with positive result.

3.7 Gene characterization by Agarose gel electrophoresis of the PCR products

For gene characterization the isolates are gone through PCR for gene amplification. To confirm STEC *stx*1 and *stx*2 these genes are detected from the *E.coli* isolates. 348 bp is the ideal size of *stx*1 gene and no result is shown for *stx*1 except the reference STEC.

From 7 isolates of different 7 samples 3 isolates have showed positive result for presence of *stx*2 gene the ideal band size is 584 bp and isolates from HS, BF-1 and MS-1 have proven the presence of *stx*2 gene along with the reference STEC strain. The negative reference strain has not shown any positive result for both stx1 and *stx*2.

Table 11: Detection of *stx*1 gene in the PCR products

Sample	Band size	Result
STEC	348bp	+ve
HS-8	none	-ve
K12	none	-ve
BF-1	none	-ve
MS-1	none	-ve
BF-2	none	-ve
MS-2	none	-ve
BF-3	none	-ve
MS-3	none	-ve

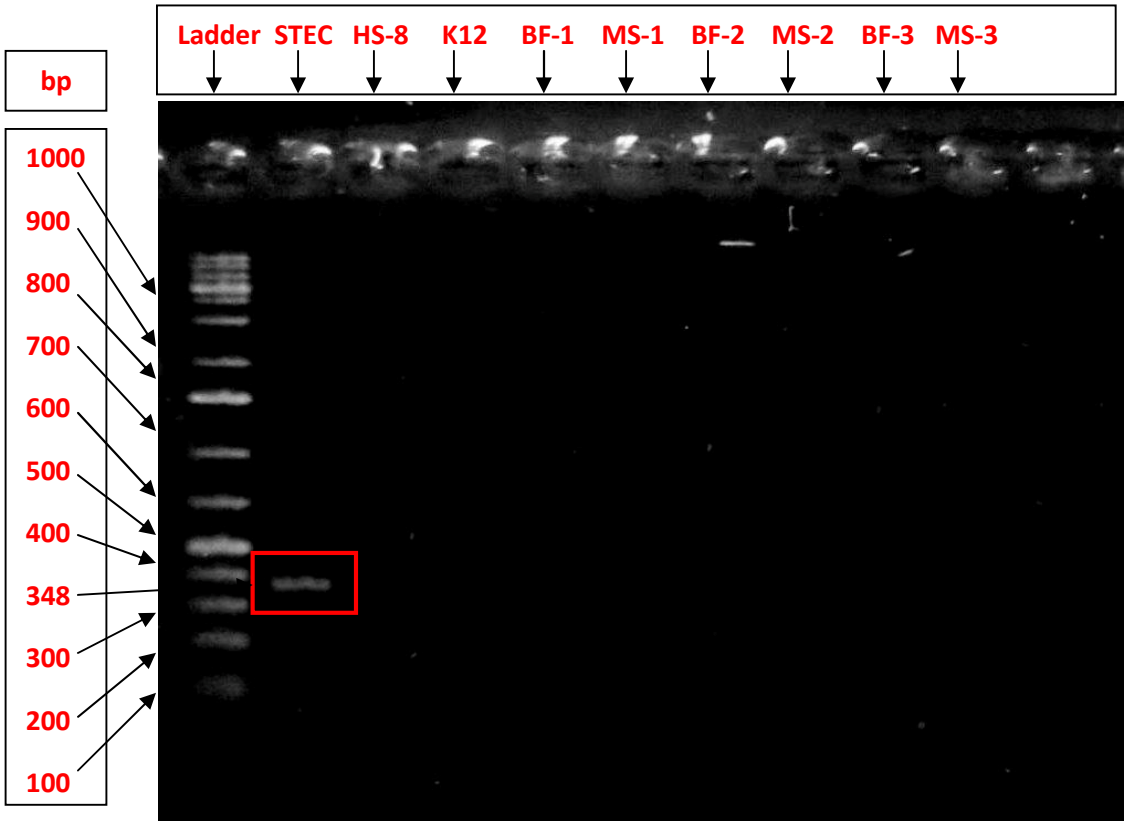


Figure 10: Detection of *stx*1 gene in PCR products

Table 12: Detection of *stx*2 gene in the PCR products

Sample	Band size	Result
STEC	584bp	+ve
HS-8	584bp	+ve
K12	none	-ve
BF-1	584bp	+ve
MS-1	584bp	+ve
BF-2	none	-ve
MS-2	none	-ve
BF-3	none	-ve
MS-3	none	-ve

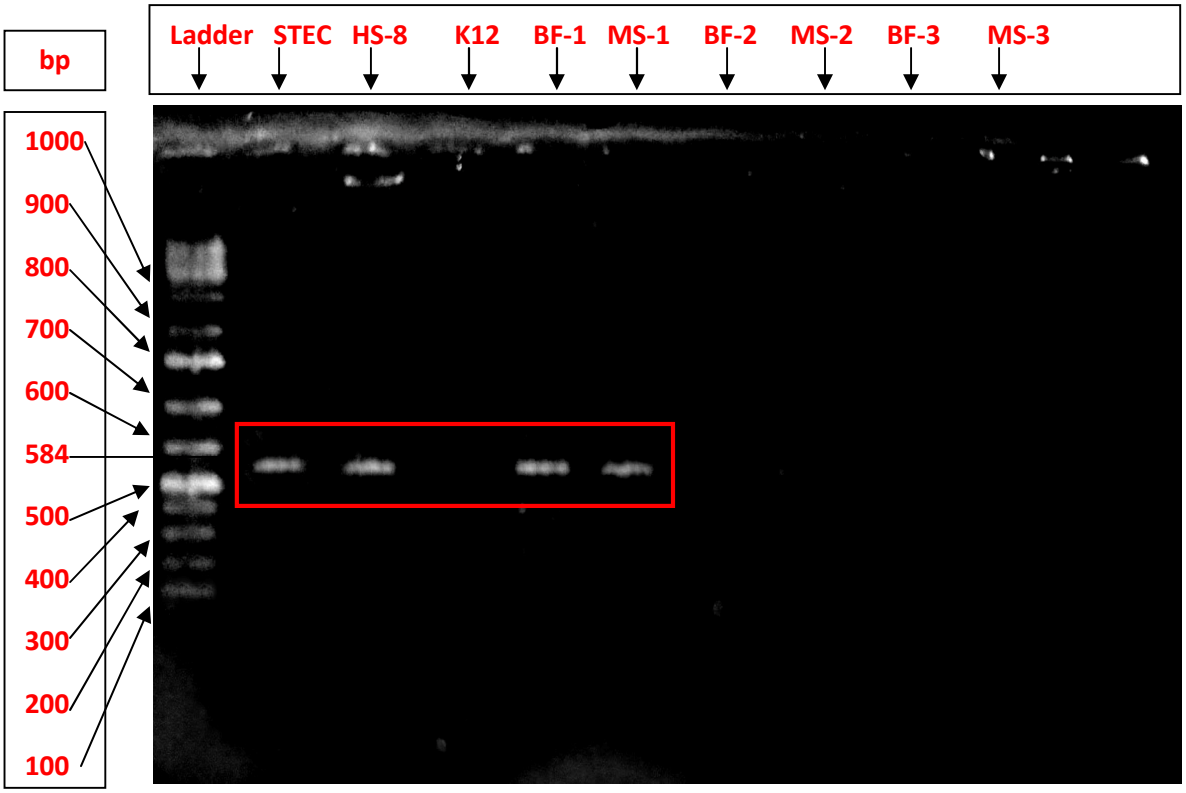


Figure 11: Detection of *stx*2 gene in PCR products

Chapter 4

Discussion

During the study total of 61 isolates from 7 samples of bovine feces, milk and human sewage were presumptively identified as *E. coli* from primary MacConkey 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 using Eosin Methylene Blue (EMB) agar medium, Indole production test, Methyl-red test, Voges-Proskauer's test, Citrate utilization test, Triple Sugar Iron test and fermentation test. Out of 35 samples analyzed, only 22 isolates, gave identical biochemical properties compared to a reference *E. coli* strain. It means the rate of spread of E.coli from these sources is approximately 63%.

Culturally and biochemically positive isolates were tested for *stx*1 and *stx*2 genes. Total seven isolates were selected from each sample. From all these isolates, no *stx*1 gene was detected but three were detected for *stx*2. So the surveillance of *stx* gene is 42% Therefore, this data showed the prevalence of *E. coli* in Bangladesh and demands for further study for the prevention of diseases. Thus cattle can be subjected as the reservoir of STEC in Bangladesh.

The *stx* genes have been detected by applying common genotypic tool PCR. If the gene sequencing method could be applied then the outcome might be remarkable. Besides *stx* genes the other virulence factors like *eae, hly, katP, espP* genes characterization could have been more significant.

In the current research work, a genotypic study demonstrated no differences in virulence factors between human and cattle isolates and both of these isolates carried *stx*2 genes. This finding suggested that a bloody diarrhea and urinary tract infection of humans in the present study, may be due to virulence *E.coli* O157:H7 strains that zoonotically transmitted from cattle [12].

STEC could not be implicated as a major causal agent of diarrhea. The presence of *E. coli* O157:H7 suggested that this enteropathogen may be of public health concern. Hence, routine screening of diarrheagenic stool samples for STEC may be useful [13].

The results of the study suggest that the bovine STEC O157:H7 isolates have potential to cause disease in Bangladesh which might also lead to any future outbreak in our country. Further studies are required with large number of isolates from various sources for better understanding of the virulence potential of local STEC O157:H7. In addition, more virulence characteristics should be analyzed by applying other assays like enterotoxicity assay, cytotoxicity assay, mouse lethality assay etc.

Chapter 5

Conclusion

5. Conclusion

Shiga toxin producing *E. coli* (STEC) are food-borne pathogens that cause hemolytic colitis and a serious sequel, HUS. The largest outbreaks of STEC are due to a single *E. coli* serotype, O157:H7 although STEC non-O157:H7 serotypes also cause similar diseases.

In this study STEC was isolated from bovine feces and milk samples and then characterized. The results showed that isolates from both samples represented the same genetic combinations.

Moreover, these isolates were clustered into the same molecular typing group, indicating that bovine feces and milk are reservoirs of *E. coli* O157:H7. Thus, it is important to control food contamination with *E. coli* O157:H7 on farms and in abattoirs to reduce the incidence of food borne infections in humans.

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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 121oC for 15 min.

1. Nutrient Agar (Himedia,India)

Ingredients	Amounts (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)
Pancreatic digest of gelatin	20.0
Magnesium chloride hexahydrate	1.4
Potassium sulfate anhydrous	10.0
Cetrimide	0.3
Agar-Agar	13.0

3. T1N1 soft agar

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

4. Trypticase soy broth, (Oxoid, England)

Ingredients	Amount (g/L)
Pancreatic digest of Casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Di-basic potassium phosphate	2.5
Glucose	2.5

5. MacConkey agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

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

Ingredients	Amount (g/L)
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	0.2
Ammonium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bacto brom thymol blue	0.08

7. Peptone Water

Ingredients	Amount (g/L)
Peptone	10.0
Sodium chloride	5.0

8. MR-VP broth

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

9. Triple sugar iron agar (Himedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	10.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.20
Sodium thiosulfate	0.30
Casein enzymatic hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0

10. Eosin methylene blue agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	10.0
Sucrose	5.0
Lactose	5.0
Di-potassium phosphate	2.0
Eosin Y	0.14
Methylene blue	0.065
Agar	13.50

APPENDIX-II
Buffers and reagents

1. Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4 and 2.0 g of KH2PO4 in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 liter by distilled water. The solution was sterilized by autoclaving and was stored.

2. 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 aluminium foil and stored at 4^{o} c.

3. 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.

4. 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 4 °C.

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 at 4 °C.

5. TBE Buffer

For 1 litre 108g Tris base, 55mg of Boric acid and40 ml of 0.5M EDTA (pH 8.0) is mixed and autoclave for 20mins.

APPENDIX-III

Instruments

The important equipments used through the study are listed below:

Thermal cycler	Applied biosystems 2720
Hot plate & stirrer	JSR
Thermo circulator	LabTech
Laminar air flow	SAARC
Horizontal gel electrophoresis unit	LabTech
Power supply	Wealtec
UV transilluminator	Wealtec
Centrifuge machine	Eppendorf,Germany
Vortex machine	Digisystem Vm-2000
Incubator	SAARC
Refrigerator	Toshiba
Freezer	Siemens
Microwave oven	Panasonic
Autoclave machine	Wise clave
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200µl)	Eppendorf, Germany
pH meter	Shanghai Ruosuaa Technology company, China

Appendix IV

PCR Reagents

1x loading dye, DNTPs, Taq polymerase, DNA ladder: Thermoscientific

Primers: Invitrogen

$MgCl_2$: Invitrogen