A STUDY ON THE PROSPECT OF *Escherichia coli* ISOLATED FROM RAW BEEF SAMPLES AS A POTENTIAL RESERVOIR OF ANTIBIOTIC RESISTANCE.

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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Declaration

I, A.B.M. Rezwanul Kabir declare that this thesis and the work entitled **"A study on the prospect of *Escherichia coli* isolated from raw beef samples as a potential reservoir of antibiotic resistance"** submitted to the Department of Mathematics and Natural Sciences (MNS), BRAC University in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology is a record of work carried out by me under the joint supervision of my supervisors.

I further declare that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

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Dedicated to my Father

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviations		Descriptions
E. coli		*Escherichia coli*
EMB		Eosin Methylene Blue
MHA		Muller-Hinton Agar
MAR		Multiple Antibiotic Resistance
MIU		Motility, Indole, Urease test
MR		Methyl Red
VP		Voges-Proskauer
rpm		rotations per minute
µL		microliter
mL		milliliter
g/L		grams per liter

Abstract

With the consumption of beef reaching new heights each year all over the world, the prospect of *E. coli* isolated from raw beef samples as a potential reservoir of antibiotic resistance was investigated. A total of 17 *E. coli* isolates were obtained from raw beef samples collected from Mirpur area in Dhaka city. Antibiotic susceptibility test of these isolates showed that all the isolates were resistant to Penicillin and Oxacillin. The isolates also exhibited resistance to Cephalexin (94%), Erythromycin (71%), Streptomycin (24%), Tetracycline (18%), Amoxicillin (12%), Kanamycin (12%), Ampicillin (12%) and Levofloxacin (5.9%). The antibiotic susceptibility test also revealed that two isolates, S6 and S8, were resistant to 15 out of 20 (75%) antibiotics that they were tested upon. Analysis of the plasmid profiles of the isolates showed that the isolates harbored different number of plasmid that ranged from 1 to 3. The size of plasmids also varied, with plasmids as small as 2.0MDa to as big as 65MDa were observed. The two highest multi-drug resistant isolates, S6 and S8, were seen to share a common plasmid of 2.0MDa that might account for the high level of resistance. This 2.0MDa plasmid was seen have resemblance in size with the plasmids pOSAK1 and p4821 which were found to share homology with the core region of the antibiotic resistance plasmid NTP16 of *Salmonella typhimurium* strains. One of the two highest multi-drug resistant isolate, S6, was seen to harbor a 65MDa plasmid that shared resemblance in size to the plasmid pO157 which is characteristic to the Shiga toxin-producing *E. coli* O157:H7. Analysis of the plasmid profiles and antibiotic susceptibility tests revealed that a correlation between the plasmids and antibiotic resistance can be established.

Chapter 01: Introduction

CHAPTER 01: INTRODUCTION

1.1 Background of the study:

The world consumed about 129.5 billion pounds of beef in 2016 (Cook, R. 2016). In the United States, beef production totaled 25.3 billion pounds in 2016, up 6 percent from the previous year (USDA, 2017). Although numerical statistics on consumption of beef in Bangladesh was unavailable, it can logically be said that beef consumption is increasing day by day. As the fast food industry is blooming and the consumption of meat, which is left undercooked many a times, is increasing than ever before, food borne diseases are hitting high levels each year and so is the reported cases of foodborne illnesses. In United States in the year 2016, 24,029 people reported foodborne illnesses according to the Centers for Disease Control and Prevention's (CDC's) Foodborne Surveillance Network (FoodNet), and much of the incidents relates to a common source- meat (Kuehn, M. 2017). And when it comes to popularity, beef undoubtedly sits high on the list. Thus there is a growing concern over food safety especially on raw beef that can be a habitat for a number of microorganisms such as pathogenic strains of *E. coli*, many of which might be multi drug resistant.

Escherichia coli (E. coli) is often regarded as a model organism for scientific research. The rod shaped Gram negative coliform bacteria is the most studied microorganism. The fact that it can be grown and cultured easily and inexpensively in a laboratory setting makes it the ideal candidate for scientific research. In addition to that the genetic simplicity, favourable growth rates and ability to incorporate foreign DNA makes *E. coli* the perfect candidate for use in Biotechnology.

Despite all the advantages, a few strains of *E. coli* can be dangerous to people. *E. coli* O157:H7 is one strain of the dangerous strains and is shed in the manure of many warm-blooded animals including cattle such as cows. *E. coli* O157:H7 is harmless to most animals but is dangerous to humans, especially to those with an immature or weakened immune system, because it produces a toxin that can cause severe illness. People can become infected by consuming undercooked meats such as beef that is contaminated by *E. coli* O157:H7.

As much as the threat of *E. coli* O157:H7 is alarming, even more alarming is the issue of antibiotic resistance. According the World Health Organization (WHO), antibiotic resistance is one of the world's greatest health threats to date (Haddox, 2013). And because of its genetic simplicity, a wide spectrum of *E. coli* is becoming resistant to an increasing number of antibiotics. Uncontrolled usage of antibiotics in treatment of animals and their integration in animal feeds has been believed to account for the increase in antibiotic resistance (WHO, 2000; Galland et al., 2001).

While scientists are trying harder than ever before to design drugs that are more effective against the resistant bacteria, it is important to understand how these bacteria are becoming resistant in the first place. The biggest reason for a wide range of antibiotic resistance is Horizontal Gene Transfer (HGT) (Munita and Arias, 2016), which is the movement of genetic material between unicellular and/or multicellular organisms. And these resistant genes might either be present in chromosome or in plasmids, which are small, circular, double-stranded DNA molecules. Plasmids are of great interest to scientists as they are carry resistant genes and exchange of these resistant genes between donor and recipient cells can be achieved through plasmids more readily through conjugation (Wilkins, 1995).

Thus there is a need growing need to understand the relation between plasmids and antibiotic resistance and this study aimed to achieve that by studying *E. coli* isolates from raw beef. In doing so, the study also revealed the prospective of raw beef to be a potential reservoir of antibiotic resistance.

1.2 Objectives of the study

The specific aims and objectives of the study are as follows:

- To isolate and identify *E. coli* from raw beef samples from a particular area of Dhaka city.
- To study the antibiotic resistance pattern of the *E. coli* isolates.
- To analyze the plasmid profiles of the *E. coli* isolates
- To establish, if possible, a correlation between antibiotic resistance and plasmid profiles of the *E. coli* isolates.

1.3 Literature Review

1.3.1 Escherichia coli (E. coli):

Escherichia coli is a gram negative, facultative anaerobic, rod shaped, coliform bacteria (Oxford English Dictionary,. 2005). *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 (Marler, B. 2009).

E. coli is the most studied microorganism and although most of the strains are harmless, some of the strains like *E. coli* O157:H7 can be deadly. *E. coli* has been an important tool for Biotechnology as it has taught us about DNA, it has been used to make synthetic fuel, making medicine, fighting cancer and even making Bio-computers (Feltman, R. 2013).

E. coli that are responsible for the numerous reports of contaminated foods and beverages are those that produce Shiga toxin, so called because the toxin is virtually identical to that produced by Shigella dysenteria type 1 (Griffin and Tauxe, 1991). The best-known and also most notorious *E. coli* bacteria that produce Shiga toxin is *E. coli* O157:H7 (Lim et al. 2010).

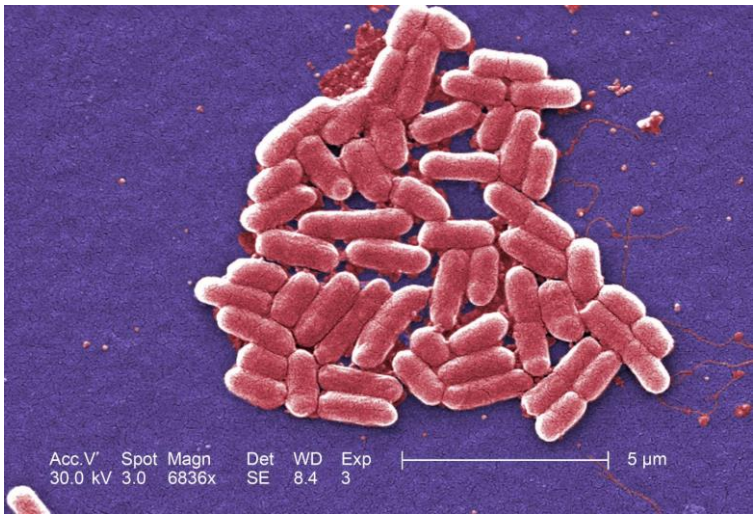

Fig 1.1 A cluster of colorized *E. coli* as seen under a scanning electron microscope (Retrieved from https://www.eurekalert.org/multimedia/pub/86125.php).

1.3.2 Antibiotic Resistance:

Antibiotic resistance occurs when microorganisms alter when they are exposed to antibiotics. These microorganisms that develop multiple antibiotic resistance are occasionally referred to as "superbugs". Hence, medicines become unproductive and infections stays back in the body and thus the risk of spread to others increases. (WHO, 2017).

Antibiotic resistance occurs naturally over time, typically through genetic variations. Nevertheless, the misuse and overuse of antibiotic is quickening this process. In numerous areas of the world, antibiotics are overused and misused in people and animals, and frequently applied without professional advice.Instances of misuse include when they are taken by people with viral infections like colds and flu, and when they are given as growth promoters in animals(WHO, 2017).

Antibiotic resistant-microbes are prevalent in people, animals, food, and the environment. They can spread between people and animals, and from person to person. Poor infection control, insufficient sanitary conditions and improper food-handling boost the spread of antibiotic resistance (WHO, 2017).

Antibiotic resistance is a worldwide issue. According the World Health Organization (WHO), antibiotic resistance is one of the world's greatest health threats to date (Haddox, 2013). New forms of antibiotic resistance can cross international boundaries and spread between continents easily (CDC, 2013).

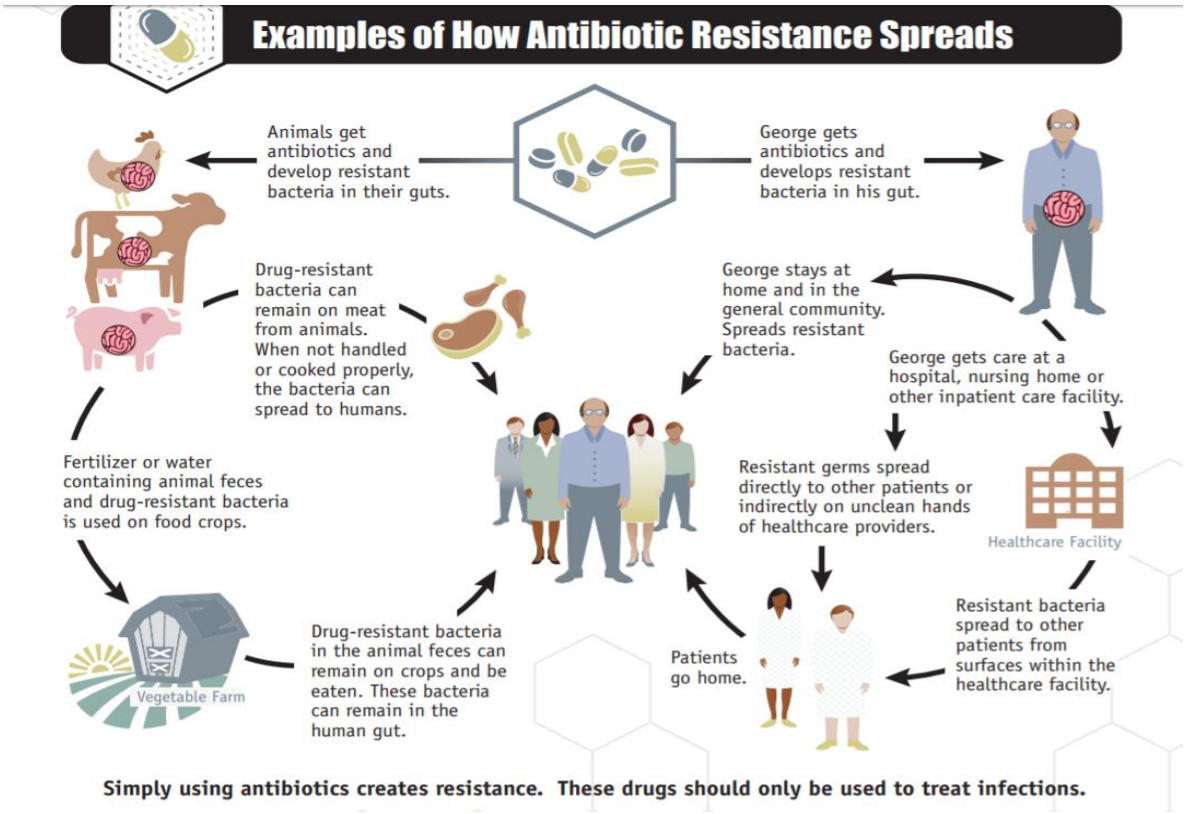

Fig 1.2 Example of how antibiotic resistance spreads (CDC, 2013).

1.3.3 Plasmids:

Plasmids are small circular pieces of DNA that replicate independently from the host's chromosomal DNA (Griffiths et al., 2000). They are mainly found in bacteria, but also exist naturally in archaea and eukaryotes such as yeast and plants. Plasmids allow functional benefits to the host like resistance to antibiotics or virulence. Plasmids are easy to modify and they have the ability to self-replicate (Lodish et al., 2000). These characteristics make them attractive tools for Genetic Engineering.

Scientists use plasmids to control gene expression in target cells (Alberts, B. 2002). Properties such as flexibility, versatility, safety, and cost-effectiveness allow scientists to make use of plasmids in order to carry out many applications. The fact that they are self-replicating, easy to work with and stable, plasmids have emerged as dynamic prospect in the field of Biotechnology.

Bacteria take up new plasmids from other bacterial cells during an event called conjugation (Low, K. 2001). As much as they can gain the, bacteria can also readily lose them, for example during cell division one of the daughter cells might miss out on getting a plasmid.

Even though plasmids were initially used to comprehend gene function, they are currently used for a range of studies used to examine promoters, small RNAs, or other genetic elements (Addgene, 2014).

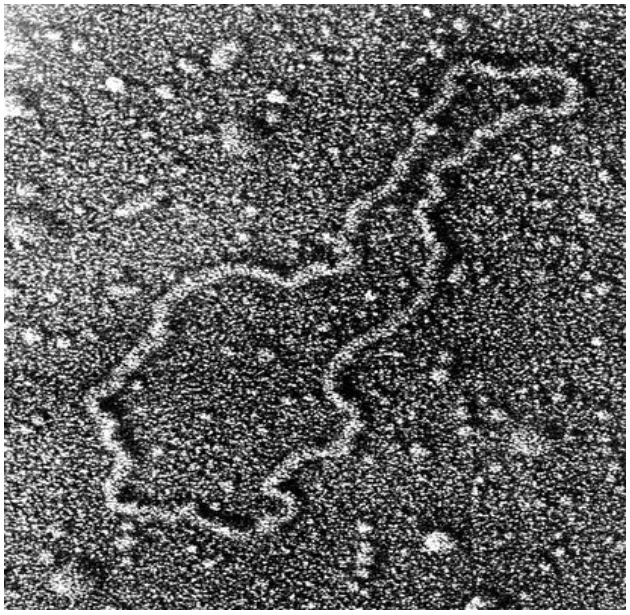

Fig 1.3 The electron microscope image shows DNA of the pSC101 plasmid (Cohen, N. 2013).

1.3.4 Horizontal Gene Transfer (HGT)

Horizontal Gene Transfer (HGT) is the exchange of genetic material between organisms that are not in a parent–offspring connection. It is a widely recognized mechanism for adaptation in bacteria and archaea. Antibiotic resistance and pathogenicity are frequently associated with HGT (Soucy et al., 2015).

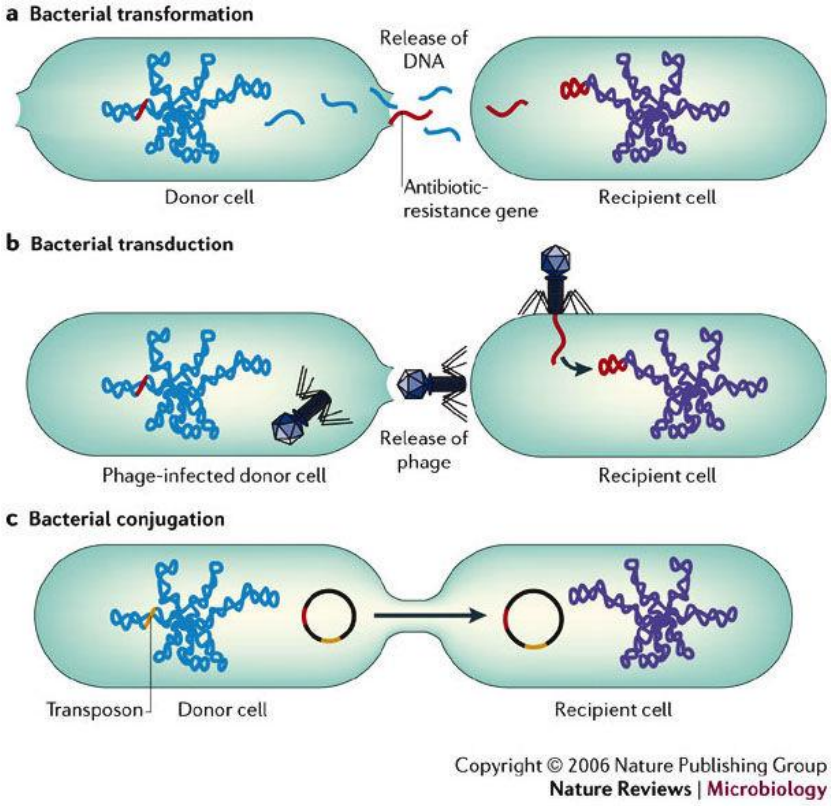

Fig 1.4 Horizontal gene transfer between bacteria. A) Bacterial transformation, b) Bacterial transduction c) Bacterial conjugation. (Retrieved from http://www.nature.com/nrmicro/journal/v4/n1/fig_tab/nrmicro1325_F2.html)

1.3.5 Plasmid Profiling

Plasmid profiling is a typing system that is used to study the distribution of plasmids. Plasmid profiling, also known as plasmid fingerprinting, is important because plasmids can contain genes for antibiotic resistance or virulence factors or genes required to activate virulence genes on the chromosome (Schroeder, M. 2017). In plasmid profiling methods, partially purified plasma deoxyribonucleic acid species are separated according to molecular size by agarose gel electrophoresis and viewed under UV illumination (Mayer, L W. 1998).

Chapter 02: Materials and Methods

CHAPTER 02: MATERIALS AND METHODS

2.1 Working place:

The entire study was carried out in the laboratory of the Department of Mathematics and Natural Sciences (MNS) at BRAC University.

2.2 Collection of samples:

20 raw meat samples was collected from different butchers shop from Mirpur area in Dhaka city. Each sample was collected from a different butchers shop. The samples were kept in sterile zipper bags and homogenized without minimum delay. Care was taken and hand gloves were worn at each steps while handling the samples to prevent contamination.

2.3 Homogenization:

The samples were then homogenized on 0.85% peptone salt solution using mortar and pestle. Once again care was taken while homogenization and it was sure that all the equipment were sterilized with 70% ethanol beforehand.

2.4 Incubation:

500 µl of the sample was then put into test tubes containing EC broth, which is a selective broth for the growth of coliforms and *E. coli* from food and environmental samples (Oxoid., 2010), and incubated at 37 ºC for 24 hours.

2.5 Growth on MacConkey agar:

A loop full of the broth culture was then streaked on to MacConkey agar and incubated at 37 ºC for 24 hours for the primary screening of *E. coli* colonies. MacConkey Agar is recommended for use as a selective and differential medium for the isolation of gram-negative bacilli (including coliform organisms and enteric pathogens), on the basis of lactose fermentation (Hardy Diagnostics, 2000).

2.6 Growth on Eosin Methylene Blue (EMB) agar:

Eosin methylene blue agar (EMB) is a selective and differential medium used to isolate fecal coliforms like *E. coli*, which produces a characteristic metallic green sheen. The isolated red-pink colonies from the MacConkey agar are then streaked onto EMB agar and at 37 ºC for 24 hours. The resulting colonies that produced themetallic green sheen were further cultured into new EMB agar plates and this step is triplicated in order to increase the chances of obtaining a pure culture of *E. coli*.

2.7 Culture Preservation:

17 *E. coli* isolates were obtained from the 20 samples and these were stored on MacConkey agar and subcultured every two weeks until the end of the study.

Also one loop-full of bacterial inoculum was transferred to 5 ml nutrient broth medium and incubated at 37°C for 24 hours. Then, 600 µl of broth was mixed with 400 µl autoclaved glycerol and stored in -20◦C refrigerator in 1.5ml centrifuge tubes.

2.8 Biochemical Tests

These 17 isolates were thought to be *E. coli* but a set of biochemical tests were performed in order to confirm that they were indeed *E. coli*. The following biochemical tests were performed:

- Gram Staining
- Methyl Red (MR) Test
- Voges–Proskauer (VP) Test
- Citrate Utilization Test
- Oxidase Test
- Catalase Test
- Indole Test
- Nitrate reduction Test
- Triple Sugar Iron (TSI) Test
- Urease test, Motility test and Indole test by MIU agar

2.8.1 Gram Staining

This is the test that differentiates between the two broad categories of bacteria: Gram positive and Gram negative. Gram positive bacteria gives a purple stain while Gram negative bacteria gives a pink stain. The morphology of the bacteria can also be checked using this method.

There are four steps involved in Gram staining. Firstly, a smear of bacteria is prepared onto a glass slide and heat treated. Gram's iodine is then added to the smear. If the bacteria are gram positive their thick peptidoglycan layers retain the purple color. Secondly, mordent is applied which stabilizes the purple stain. Thirdly, 95% ethanol is used to wash the stain; if the bacterium is negative its thin peptidoglycan layer is washed off. Finally, a counter dye safranin is added which is retained by gram negative bacteria. Lastly, the slide is viewed under a compound microscope to analyze the color and shape of the bacteria (Cappuccino and Sherman, 2005).

2.8.2 Methyl Red (MR) Test

Bacterial culture are inoculated MRVP broth in a clean test tube and incubated overnight at a temperature of 37°C for 24 hours. The following day, 5 drops of methyl red was added and the medium was observed for the immediate development of color. Appearance of a red colour indicates a positive result (Cappuccino and Sherman, 2005).

2.8.3 Voges–Proskauer (VP) Test

Bacterial culture are inoculated MRVP broth in a clean test tube and incubated overnight at a temperature of 37°C for 24 hours. Then 0.6ml (12 drops) of Barrit's reagent A and 0.2ml (4drops) of Barrit's reagent B was added and gently shake the medium to atmospheric oxygen. The tube was then allowed to remain still for 10-15mins and the solution was observed for color changes to determine whether the result is positive (pink-red) or negative (yellow) (Cappuccino and Sherman, 2005).

2.8.4 Citrate Utilization Test:

The citrate agar was prepared and 2 ml media was added to clean vials. The vials were autoclaved and then left to cool at a slanted position in order to create a butt and slant. An inoculating needle was used to pick up a single colony from a 24 hours fresh bacterial culture. The slant of the media was streaked from bottom to top using a zigzag motion with the needle. The vials were incubated at 37°C for 24 hours. The colour of the media was observed after incubation. Colour change to blue is considered to be positive and no colour change was considered to be negative (Cappuccino and Sherman, 2005).

2.8.5 Oxidase Test:

The oxidase test detects bacteria that produce cytochrome c oxidase, which is an enzyme of the bacterial transport system. All aerobic bacteria are oxidase positive. In positive cases, a deep blue or purple stain appears within 5-10 seconds. Organisms such as Pseudomonas, Campylobacter are oxidase positive organisms. In this procedure, Kovacs Oxidase Reagent was used. Its composition is 1% tetra-methyl-p-phenylenediamine dihydrochloride, in water (Cappuccino and Sherman, 2005).

2.8.6 Catalase Test:

A sterile microscopic slide was placed on a petri deish and a small amount of organism picked using a sterile inoculating loop. Then 1 drop of 3% H_2O_2 was placed on the organism on the microscopic slide by using a dropper. Finally, it was observed for the presence of bubbles of oxygen gas (Cappuccino and Sherman, 2005).

2.8.7 Indole Test:

For the indole test, tryptophan broth was inoculated with bacterial culture and incubated at 37°C for 24 hours. Then 0.5 ml of Kovac‘s reagent was added to the broth culture. Finally, it was observed for color changes to determine whether the result is positive (cheery red ring) or negative (yellow) (Cappuccino and Sherman, 2005).

2.8.8 Nitrate reduction Test:

The nitrate broth was made, boiled and 5 ml broth was added to each test tube using a glass pipette. The broth was autoclaved and left to cool. Using an inoculating loop, a single colony was picked from a 24 hours fresh bacterial culture and used to inoculate the broth in each test tube. The test tubes were incubated at 37°C for 24 hours. After incubation, 5 drops of each reagent A and reagent B was added to the test tubes respectively. Formation of a red colour was observed (Cappuccino and Sherman, 2005).

2.8.9 Triple Sugar Iron (TSI) Test:

The sugar utilization test or carbohydrate utilization test is basically used to detect the carbon source that is utilized by the organisms present. To conduct the test, a straight inoculating needle was used to pick an isolated colony and inoculated the TSI slant by first stabbing the butt down to the bottom, and then streaking the surface of the slant. The results were observed after 24 hours of incubation at 37°C. Interpretations were made based on the following:

- **Acid production**: Changes the medium into yellow color- organism ferments the given carbohydrate and produce organic acids there by reducing the pH of the medium into acidic.
- **Acid and Gas production**: Changes the medium into yellow color-organism ferments the given Carbohydrate and produce organic acids and gas. Gas production can be detected by the presence of small bubbles in the inverted Durham tubes.
- **The Absence of fermentation**: The broth retains the red color. The organism cannot utilize the carbohydrate but the organism continues to grow in the medium using other energy sources in the medium. (Cappuccino and Sherman, 2005).

2.8.10 Urease test, Motility test and Indole test by MIU agar:

For this test, the MIU was made, boiled and autoclaved. Some empty test tubes were autoclaved as well. After autoclave, the media was left to cool so that the temperature went down to 50°C. On a separate flask, 40% urea solution was made and filtered. To the cooled media, the urea solution was added and mixed. A sterile glass pipette was used to transfer 6ml of the media to the autoclaved test tubes. The media was left to cool down completely until it had a semi solid consistency. Using an inoculating needle, a colony from a 24 hours fresh bacterial culture was picked up and inoculated in the medium by stabbing the needle down into the media. The needle was then withdrawn and taken out in a vertical manner. The test tubes were incubated at 37°C for 24 hours. The appearance and colour of the media was observed after incubation (Cappuccino and Sherman, 2005).

2.9 Antibiotic Susceptibility Test

The antibiotic disk diffusion test is done to detect antimicrobial susceptibility in the bacterial isolates Kirby-Bauer antibiotic testing was used to test whether particular bacteria are susceptible to specific antibiotics.

In this study, the effect of 20 different commercially available antibiotics was determined. The list of antibiotics used is as follows:

Table 2.1 Antibiotic disks, their amount, and Zone of Inhibition size

		Disk diffusion diameter (in millimeter, mm)		
Antibiotics	**Amount per disk**	**Resistant mm or less**	**Intermediate**	**Sensitive mm or more**
Penicillin (PEN)	10 µg	10	11-21	22
Amoxicillin (AMX)	10 µg	13	14-17	18
Streptomycin (STR)	10 µg	10	11-14	15
Levofloxacin (LVX)	3 µg	15	14-18	19
Oxacillin (OXA)	1 µg	10	11-12	13
Kanamycin (KAN)	30 µg	13	14-17	18
Cephalexin (LEX)	30 µg	22	23-24	25
Ampicillin (AMP)	10 µg	13	14-16	17
Ciprofloxacin (CIP)	5 µg	19	20-21	22
Erythromycin (ERY)	15 µg	13	14-17	18
Tetracycline (TET)	10 µg	14	15-18	19
Chloramphenicol (CHL)	30 µg	12	13-17	18
Ceftazidime (CAZ)	30 µg	16	17-18	19
Ceftriazone (CRO)	10 µg	18	19-21	22
Nalidixic Acid (NAL)	30 µg	13	14-18	19

Table 2.1 Continued

		Disk diffusion diameter (in millimeter, mm)		
Antibiotics	**Amount per disk**	**Resistant mm or less**	**Intermediate**	**Sensitive mm or more**
Trimethoprim/Sulfamethoxazole (SXT)	25 µg	12	13-15	16
Nalidixic Acid (NAL)	30 µg	13	14-18	19
Imipenem (IPM)	10 µg	13	14-15	16
Amikacin (AMK)	30 µg	14	15-16	17
Clindamycin (CLI)	2 µg	14	15-20	21
Norfloxacin (NOR)	10 µg	12	13-16	17

In accordance to performance standards for Antimicrobial Disk Susceptibility Tests, CLSI (formerly NCCLS).

2.9.1 Preparation of inoculums of the bacterial isolates

The test tubes were labeled carefully with the number of the samples to be prepared. 85% saline solution was prepared and taken on the test tubes. Using a burnt inoculating loop, one or two colonies of the bacterial isolates were picked up from sub cultured nutrient agar plates and suspended on the saline .Then all the test tubes were vortexed properly to make the suspension homogenous

2.9.2 Comparison with the McFarland Solution

The inoculums were then compared with standard McFarland 1.0.

2.9.3 Inoculation on the MHA plates

MHA (Muller-Hinton Agar) plates with proper labeling of the samples were prepared. Autoclaved swab was dipped into the bacterial suspensions and rotated so that it is completely wet with the suspension. The test tubes having the bacterial suspension were vortexed before dipping the cotton swab. The swab was then streaked several times on the dried surface of the MHA plate to make a pure lawn ensuring the contact of the cotton of the swab with all the edges of the plate. The agar

plate was being rotated 90 degrees each time it was being streaked, to ensure the even distribution of the inoculums.

2.9.4 Placement of the antibiotic disks

A burnt sterile forceps was used to insert the antibiotic discs on the MHA plate. Antibiotic discs were placed on the surface of the inoculated MHA plates. Each of the discs was slightly pressed with the forceps on the MHA plate so that it sticks properly to the agar surface. The discs were not placed close to the edge of the plates was as the zones will not be fully round and lead to inaccuracy of the test as the measurement cannot be taken properly. The MHA plates were next inverted and incubated at 37ºC for 24 hours.

2.9.5 Measurement of the zone of inhibition

Following the incubation, the zone of inhibition for each of the antibiotics was observed on the MHA plate. The size of zones for each antibiotic were measured carefully in millimeters (mm) using a ruler. All the measurements were taken viewing the back of the Petri dish. The zone size was recorded on the recording sheet in a chart.

2.10 Plasmid Profiling

2.10.1 Plasmid isolation:

Plasmid was isolated according to the modified hot alkaline method by Kado & Liu (1981). 1.5 ml of 24 hours shaking bacterial culture was taken in micro centrifuge tubes. The tubes containing bacterial suspension were centrifuged at 14000 rpm for 5 minutes. After centrifugation, the supernatant was discarded as much as possible and the pellet was taken. 40µL Kado-I buffer was added to the pellet and was mixed properly by pipetting. Then 80 µL Kado-II buffer was added and was mixed by inverting the tubes (rolling 3 or 4 times). The tubes were placed in hot water bath at 55ºC for 1 hour. After 1 hour 250µL phenol chloroform mixture (1:1) was added to the tubes and was mixed well by upside down the tubes for 30 minutes. After 30 minutes the tubes were centrifuged at 14000 rpm for 5 minutes. Once the centrifugation is completed, 3 layers will be observed in the tubes. Top layer containing plasmid DNA, middle layer containing the protein debris and the bottom layer containing the phenol. Plasmid DNA is carefully removed from the

top layer by micropipette and is transferred to a new tube. The new tube containing plasmid DNA is then stored at -20ºC for using it further in gel electrophoresis.

2.10.2 Preparation of gel and Gel Electrophoresis

For gel electrophoresis, 0.525g agarose was added to 75ml of 1x TBE buffer to prepare a 0.7% agarose gel. Ethidium bromide (0.5 mg/mL) was then added to the gel with care. The gel was then carefully placed on to the electrophoresis tank and samples (12 µl of plasmid DNA and 3 µl of dye) were carefully loaded on to the wells. Gel run was then carried out with 1x TBE buffer at 50 volts.

After the gel run was completed, the gel was put in distilled water for 20 minutes for destaining. After destaining was done, the gel was viewed under UV illumination.

Chapter 03: Results

CHAPTER 03: RESULTS

3.1 Growth and Appearance of the isolates on MacConkey and EMB Agar.

Both MacConkey and EMB Agar are selective and differential media for *E. coli*. Upon 24 hour incubation period, 17 isolates from the 20 samples produced pink-red colonies on MacConkey agar. All of these 17 isolates also produced metallic green sheen when further cultured on EMB agar. All of these are characteristic for *E. coli*. However, sample 01, 07 and 17 didn't produce any pink-red colonies and neither green sheen when cultured on MacConkey agar and EMB agar respectively. Hence the samples 01, 07 and 17 were discarded from the investigation.

Table 3.1. Growth and Appearance of the 20 samples on MacConkey and EMB Agar

Isolate	Appearance on MacConkey Agar	Appearance on EMB Agar	Assumption
S1	No Pink-Red colonies were observed	No colony produced Metallic green sheen.	*E. coli* is not present
S2	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S3	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S4	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S5	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S6	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S7	No Pink-Red colonies were observed	No colony produced Metallic green sheen	*E. coli* is not present
S8	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S9	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S10	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S11	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S12	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S13	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S14	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S15	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S16	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S17	No Pink-Red colonies were observed	No colony produced Metallic green sheen	*E. coli* is not present

Table 3.1 Contd.

Isolate	Appearance on MacConkey Agar	Appearance on EMB Agar	Assumption
S18	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S19	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present
S20	Pink-Red colonies	Metallic Green sheen	*E. coli* might be present

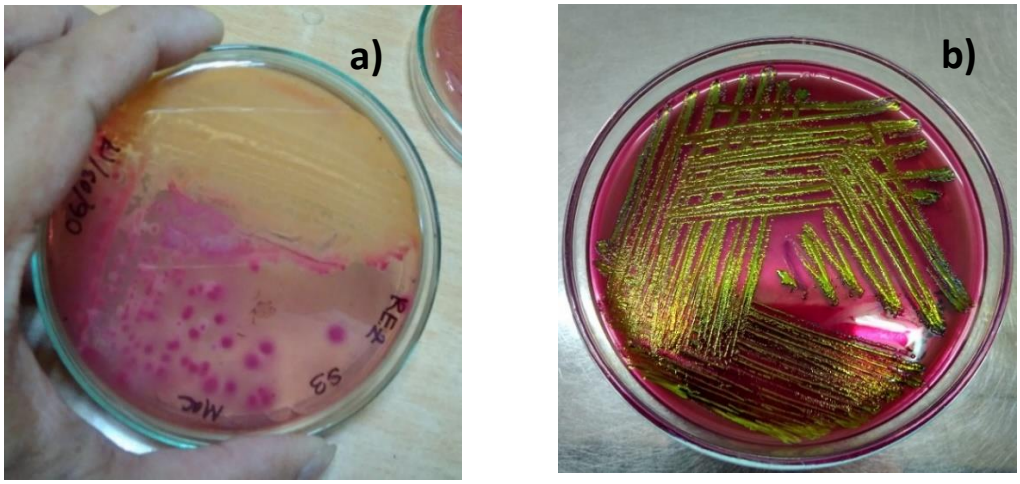

Fig 3.1 Growth and Appearance on Mackonkey and EMB Agar a) Growth on MacConkey Agar, E.coli has characteristic pink-red colonies b) Subsequent growth of the pink-red colonies on EMB Agar, *E. coli* has distinct metallic green sheen.

3.2 Biochemical Tests:

These 17 isolates were thought to be *E. coli* but a set of biochemical tests were performed in order to confirm that they were indeed *E. coli*.

3.2.1 Gram Staining:

All the 17 isolates which were believed to be *E. coli* stained pink after Gram staining was performed. In addition to that, the morphology of the isolates were checked under the microscope and all of them were seen to be rod shaped. Hence, it was conclude that all the 17 isolates were rod shaped Gram-negative bacteria.

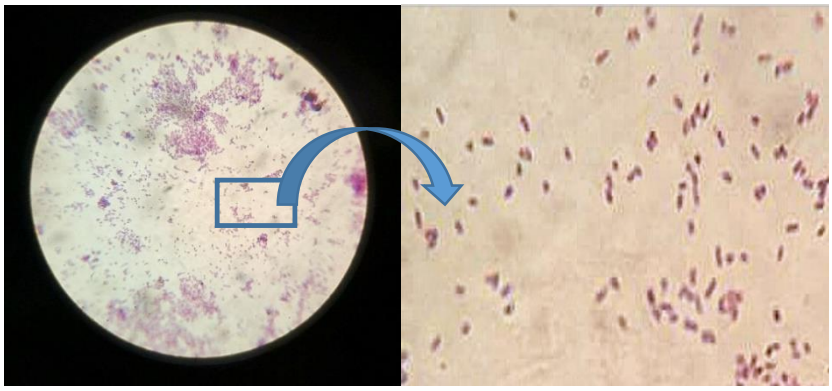

Fig 3.2 Gram staining of one of the isolate showing the pink stain characteristic of Gram-negative bacteria and rod shaped morphology

3.2.2 Methyl Red (MR) and Voges–Proskauer (VP) Tests:

- **Methyl Red (MR):**

All the 17 isolates gave red colour upon addition of a few drops of Methyl red. Hence all the isolates gave positive result for *E. coli*.

Fig 3.3 Appearance of red colour upon addition of Methyl Red indicating a positive result

- **Voges–Proskauer (VP):**

All the 17 isolates gave a negative result for the VP test. Upon addition of the solution containing alpha-naphthol and potassium hydroxide, no apparent colour change was observed.

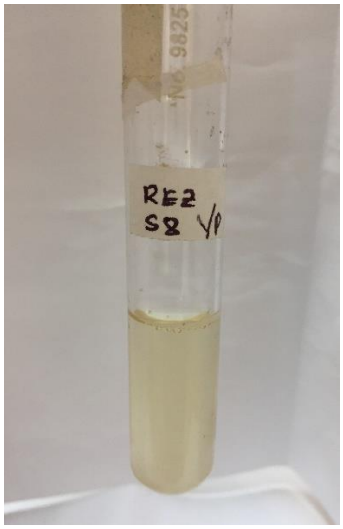

Fig 3.4 Negative result for the VP test showing no colour change upon addition of alpha-naphthol and potassium hydroxide

3.2.3 Citrate Utilization Test:

All the 17 isolates showed negative result for the citrate utilization test. The green colour of the citrate didn't change to blue upon incubation for 24 hours.

Fig 3.5 Negative result for citrate utilization test. No colour change from green to blue occurred.

3.2.4 Oxidase Test:

For the oxidase test, all the 17 isolates gave a negative result upon addition of Kovac's reagent.

3.2.5 Catalase Test:

All the 17 samples were positive for the catalase test. Upon addition of hydrogen peroxide (H_2O_2), bubbles of oxygen gas was produced.

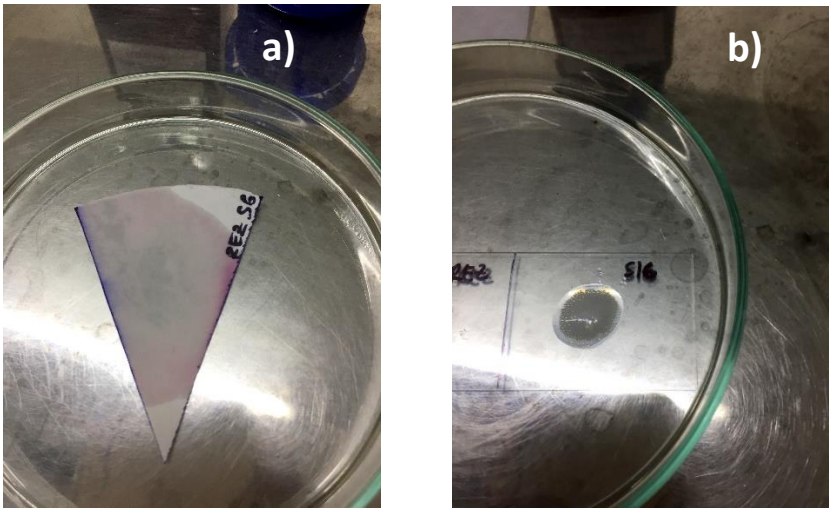

Fig 3.6 Result for catalase test a) Negative result for oxidase test b) Positive result for catalase test showing bubbles of oxygen gas.

3.2.6 Indole Test:

All the 17 isolates showed positive result for the indole test and a red/pink ring was formed at the top of the solution.

Fig 3.7 Result for Indole test. The red/pink ring indicates a positive result for the indole test.

3.2.7 Nitrate reduction Test:

All the 17 isolates showed positive result for the nitrate reduction test meaning that they reduced nitrate (NO_3) to nitrite (NO_2).

Fig 3.8 Series of steps showing that nitrate (NO3) has been reduced to nitrite (NO2).

3.2.8 Triple Sugar Iron Test (TSI):

For the TSI test, all the 17 isolates produced yellow slants and butt. They also produced a gas. However, hydrogen sulphide (H_2S) gas was not produced by any of the 17 isolates.

Fig 3.9 Results of the TSI Test showing yellow slants and butts with control.

3.2.9 Urease test, Motility test and indole test by MIU agar:

In the MIU test, all the 17 isolates showed signs of motility. All the isolates were urease negative.

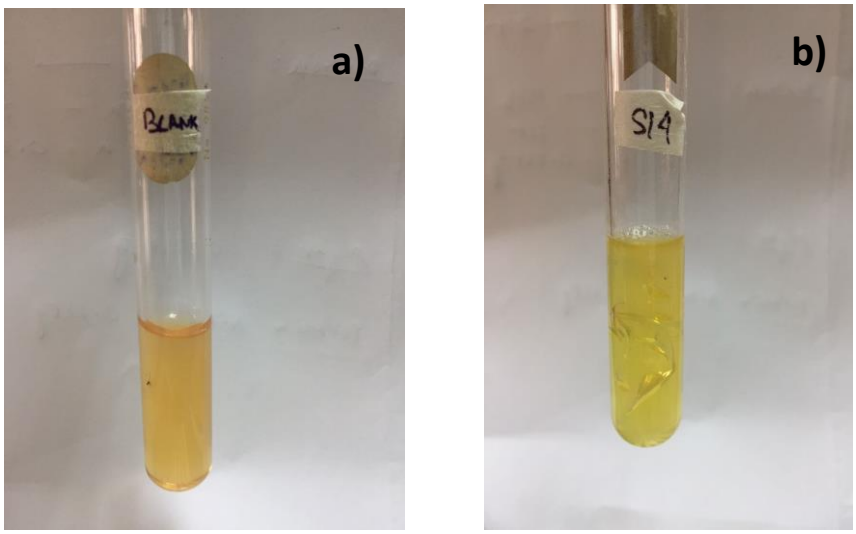

Fig 3.10 MIU Test a) MIU agar with no bacteria b) MIU agar inoculated with sample S14 showing motility positive and urease negative result.

Sample	Gram Staining	Methyl Red (MR)	Voges–Proskauer	Citrate	Oxidase	Catalase	Triple Sugar Iron (TSI)				MIU			Nitrate Reduction	Organism Interpretation
							Slant	Butt	Gas	H2S	Motility	Indole	Urease		
S2	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S3	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S4	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S5	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S6	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S8	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S9	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S10	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S11	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S12	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S13	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S14	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S15	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S16	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S18	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S19	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***
S20	"-" ve	+	-	-	-	+	**Y**	**Y**	✓	×	+	+	-	+	***Escherichia coli***

Table 3.2 Biochemical test results of the 17 isolates and their interpretation.

3.3 Prevalence of *E. coli* among the 20 samples:

After the biochemical tests were performed, it was confirmed that all the 17 isolates were indeed *E. coli*. Hence, the prevalence of *E. coli* among the 20 samples was 85%.

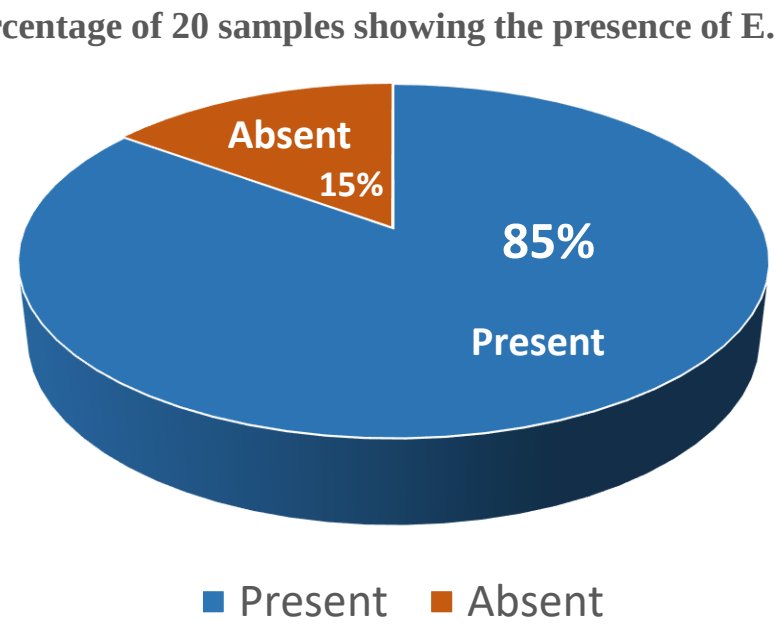

Fig 3.11 Percentage of *E. coli* present among the 20 samples after confirmation with Biochemical Tests.

3.4 Antibiotic Susceptibility Test:

3.4.1 Antibiotic susceptibility tests for all the 17 isolates:

The antibiotic susceptibility test was carried out on the 17 isolates using 12 antibiotics and the results showed that all the isolates were fully resistant to Penicillin and Oxacillin. They produced no zones of inhibition. All except isolate S15 showed resistance to Cephalexin. 12 isolates were resistant to Erythromycin, 4 isolates to Streptomycin (24%), 3 isolates to Tetracycline (18%), 2 isolates to Kanamycin (12%), 2 isolates to Amoxicillin (12%), 2 isolates to Ampicillin (12%) and 1 isolate was resistant to Levofloxacin (5.9%) respectively. No isolate was resistant to two antibiotics which were Ciprofloxacin and Chloramphenicol. The isolates were most susceptible to Chloramphenicol (n=16 with 1 isolate as intermediate), followed by Levofloxacin (n=16 with one isolate as resistant), Ciprofloxacin (n=14 with 3 isolates as intermediate), Tetracycline (n=14 with 3 isolates as resistant), Streptomycin (n=13), Amoxicillin (n=10), Kanamycin (n=9), Ampicillin (n=8) and Cephalexin (n=1). It was also noted that no isolate was susceptible to Penicillin, Oxacillin and Erythromycin. Sample S6 and S8 showed maximum resistance (n=8) out of the 12 antibiotics that were tested.

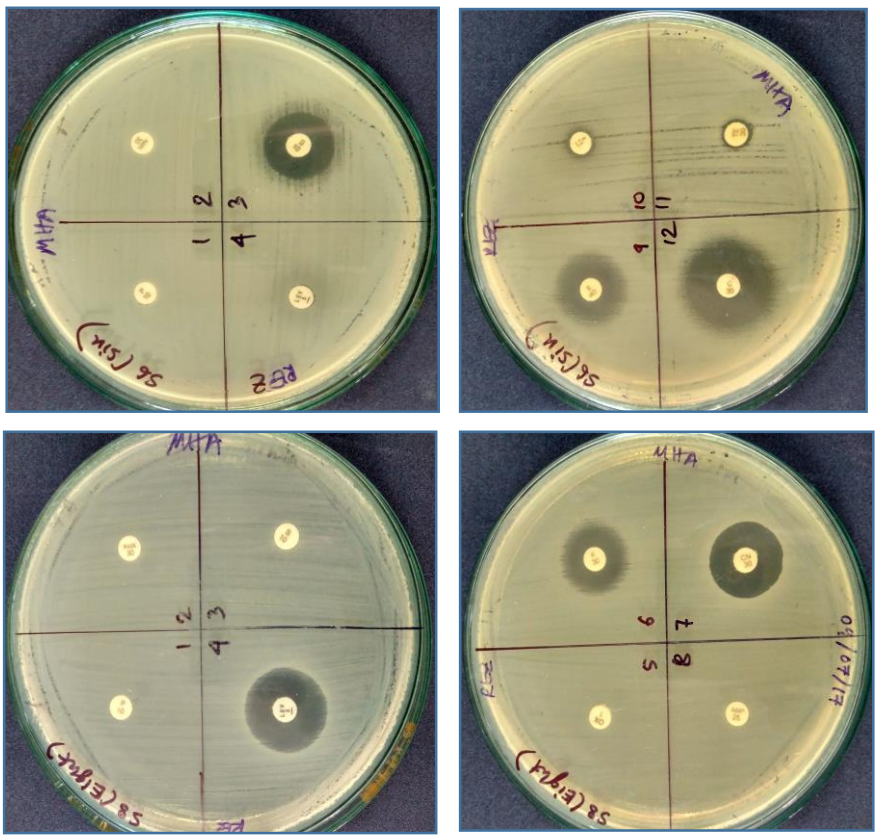

Fig 3.12 MHA plates showing the antibiotic susceptibility patterns of S6 and S8.

Antibiotic (Abbreviation)	S2	S3	S4	S5	S6	S8	S9	S10	S11	S12	S13	S14	S15	S16	S18	S19	S20
Penicillin (PEN)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Amoxicillin (AMX)	19	20	17	18	0.0	0.0	16	16	16	18	15	20	24	19	23	20	21
Streptomycin (STR)	20	20	17	17	17	0.0	14	12	15	15	13	17	17	18	21	09	18
Levofloxacin (LVX)	34	34	30	29	0.0	20	23	20	24	23	22	29	32	35	32	39	35
Oxacillin (OXA)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Kanamycin (KAN)	22	20	20	16	18	16	14	13	16	15	12	14	18	20	21	20	20
Cephalexin (LEX)	19	22	20	19	19	19	17	15	20	18	17	17	25	18	22	21	20
Ampicillin (AMP)	21	21	16	18	0.0	0.0	15	16	15	17	15	16	24	15	19	18	17
Ciprofloxacin (CIP)	37	34	31	29	18	20	21	25	29	17	23	25	30	38	36	43	35
Erytromycin (ERY)	10	09	07	08	05	05	06	05	05	05	04	05	16	17	16	15	17
Tetracycline (TET)	21	21	22	21	05	06	18	17	20	20	20	19	18	20	20	0.0	18
Chloramphenicol (CHL)	26	23	23	22	22	19	17	19	20	20	19	20	28	29	31	30	27

Table 3.3 Results of the Antibiotic Susceptibility Test of the 17 isolates.

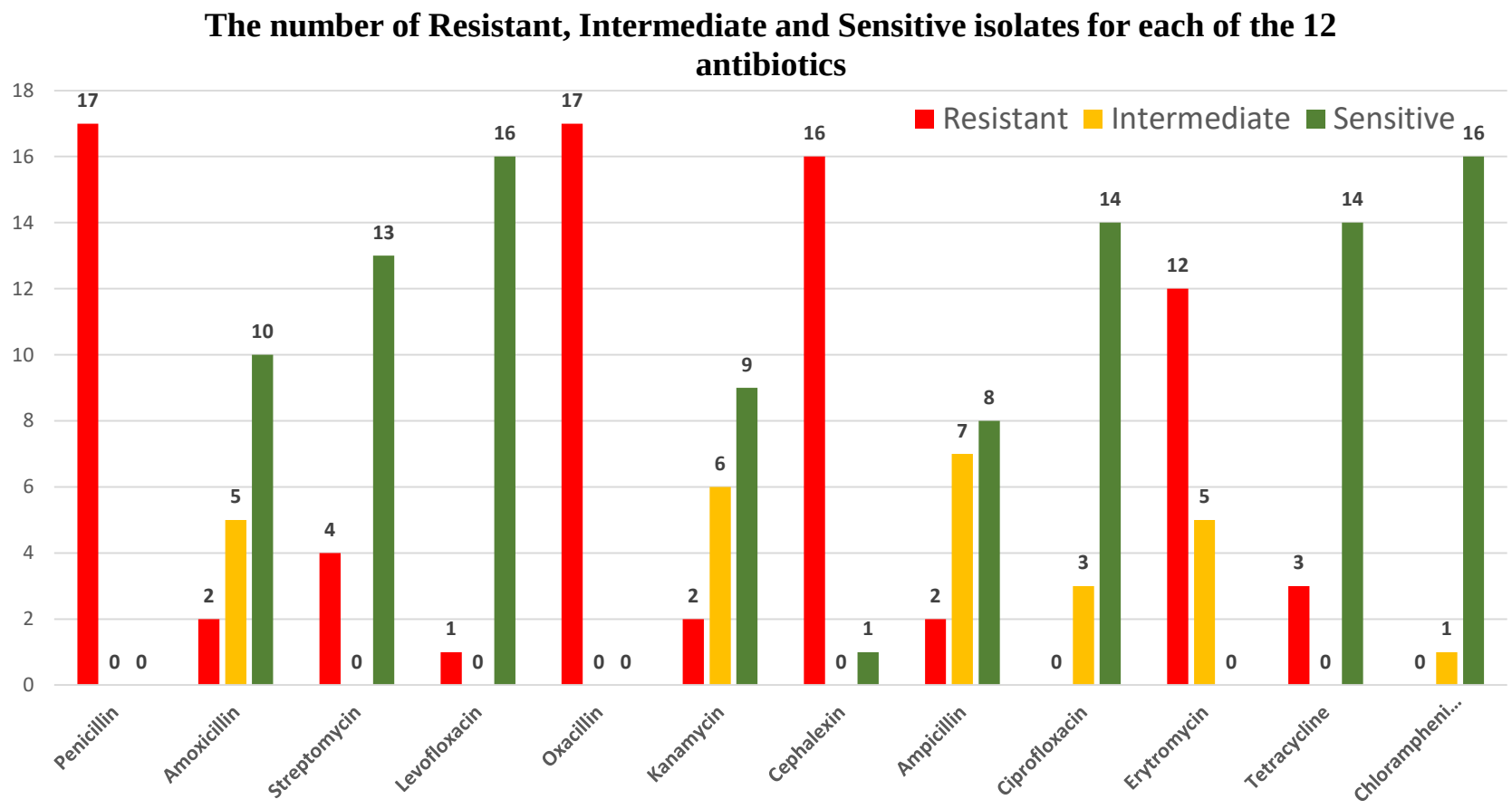

Fig 3.13 The number of Resistant, Intermediate and Sensitive isolates for each of the 12 antibiot

3.4.2 Additional Antibiotic susceptibility tests for S6 and S8:

Sample S6 and S8 showed maximum resistance (n=8) out of the 12 antibiotics that were tested. Hence, their effect on antibiotics were further checked by further eight antibiotics- Ceftazidime, Ceftriazone, Trimethoprim/Sulfamethoxazole, Nalidixic Acid, Imipenum, Amikacin, Clindamycin and Norfloxacin

Additional Antibiotic Susceptibility test of the isolates S6 and S8 with Ceftazidime, Ceftriazone, Trimethoprim/Sulfamethoxazole, Nalidixic Acid, Imipenum, Amikacin, Clindamycin and Norfloxacin showed that they were both resistant to 7 out of the 8 antibiotics that were tested. Both showed no zone of inhibition when tested with Clindamycin. Sample 6 showed no zone of inhibition with Trimethoprim/Sulfamethoxazole and Nalidixic Acid as well. Both isolate S6 and S8 were sensitive to the antibiotic Imipenum only. Isolate S6 showed no zone of inhibition in 8 antibiotics and was considered to be highly resistant.

Table 3.4 Results of the Phase Two of the Antibiotic Susceptibility test with isolates S6 and S8.

	***E. coli* isolate**	
Antibiotic	**S6**	**S8**
Ceftazidime (CAZ)	**15**	**15**
Ceftriazone (CRO)	**17**	**16**
Trimethoprim/Sulfamethoxazole (SXT)	**0.0**	**12**
Nalidixic Acid (NAL)	**0.0**	**06**
Imipenem (IPM)	**17**	**17**
Amikacin (AMK)	**14**	**12**
Clindamycin (CLI)	**0.0**	**0.0**
Norfloxacin (NOR)	**10**	**12**

3.4.3 Multiple Antibiotic Resistance (MAR) Index and the Level of Antibiotic Resistance:

Multiple antibiotic resistance (MAR) index is calculated as the ratio of number of antibiotics to which organism is resistant to total number of antibiotics to which organism is exposed (Sandhu et al., 2016).

The Multiple Antibiotic Resistance (MAR) Indexes of the isolates showed that both S6 and S8 had a MAR Index of 0.75. S13 was the only isolate which had a MAR index of 0.5.S9, S10 and S19 had a MAR Index of 0.42.S2, S3, S4, S5, S11, S12 and S14 had a MAR Index of 0.33.S16, S18 and S20 had a MAR Index of 0.25. S15 had the least MAR Index of 0.17. 17.6% of the isolates had a High level of Resistance. 58.9% and 23.5% of the isolates had a Medium level of Resistance and Low level of Resistance respectively.

Table 3.5 MAR Index and Level of Resistance of the 17 isolates.

	Number of Antibiotics			
***E. coli* isolates**	**Resistant (a)**	**Tested (b)**	**MAR Index (a/b)**	**Level of Resistance**
S2	4	12	0.33	MEDIUM
S3	4	12	0.33	MEDIUM
S4	4	12	0.33	MEDIUM
S5	4	12	0.33	MEDIUM
S6	15	20	0.75	HIGH
S8	15	20	0.75	HIGH
S9	5	12	0.42	MEDIUM
S10	5	12	0.42	MEDIUM
S11	4	12	0.33	MEDIUM
S12	4	12	0.33	MEDIUM
S13	6	12	0.50	HIGH
S14	4	12	0.33	MEDIUM
S15	2	12	0.17	LOW
S16	3	12	0.25	LOW
S18	3	12	0.25	LOW
S19	5	12	0.42	MEDIUM
S20	3	12	0.25	LOW

3.5 Plasmid Profiling:

3.5.1 Gel Electrophoresis:

Gel electrophoresis of the plasmids of the 17 isolates showed bands of plasmid DNA of various sizes of the isolates and the control strain *E.coli* V517 was observed when viewed under UV illumination. Also, a horizontal lane of bands of chromosomal DNA was observed.

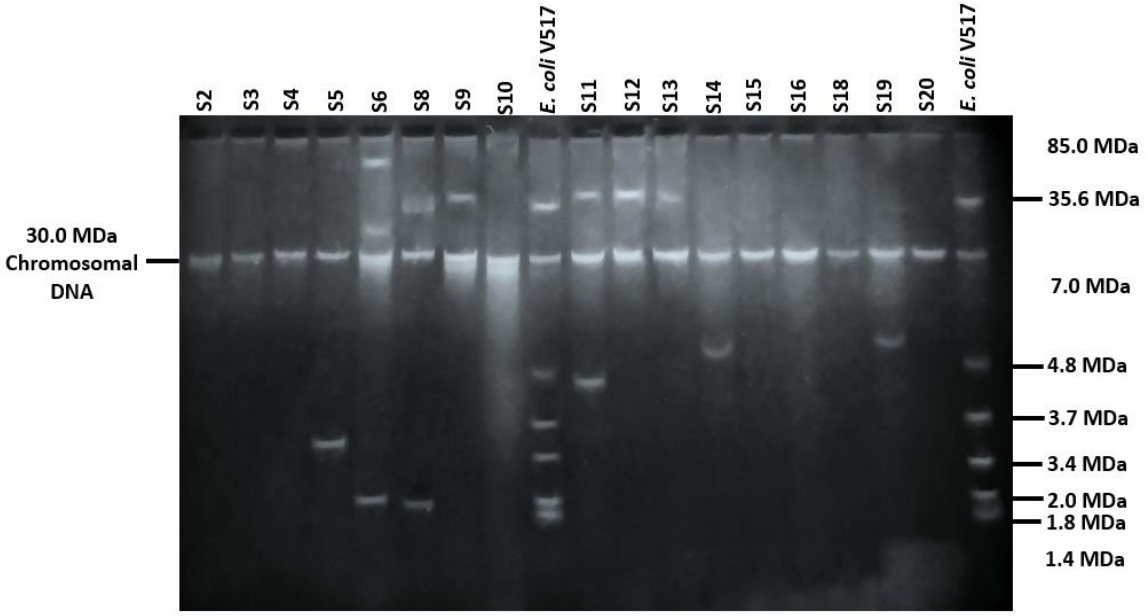

Fig 3.14 Plasmid Profiles of the isolates. Lane 1, 10 and 20 represent the plasmid profile of the control strain *E. coli* V517. Lane 2-9 and Lane 11-19 represent plasmid profiles of the 17 isolates.

3.5.2 Plasmid Profiles of the 17 isolates:

The plasmid profiles of the 17 isolates showed that 53% (9 of 17) of the isolates were seen to harbor plasmids. The isolates were seen to harbor different number of plasmids ranging from as low as 1 to as high as 3. Also, 8 (S2, S3, S4, S10, S15, S16, S18 and S20) isolates were seen to harbor no plasmids while 6 isolates (S5, S9, S12, S13, S14 and S19) contained only one plasmid, 2 isolates, S8 and S11 were seen to harbor two plasmids. Furthermore, isolate S6 contained three plasmids which was highest among the plasmid containing isolates.

Analysis of the profiles also showed that of the plasmids varied, with as big as approximately 65MDa to as small as 2.0 MDa. The most common plasmid was the plasmid with a size of 35.6MDa and was seen to present among 5 isolates: S8. S9, S11, S12, S13. A considerable portion of the plasmids (53.9%; 7 of 13) were large plasmid (> 20MDal). The isolate S8 and S9 contained a common plasmid which had a size of about 2.0 MDa.

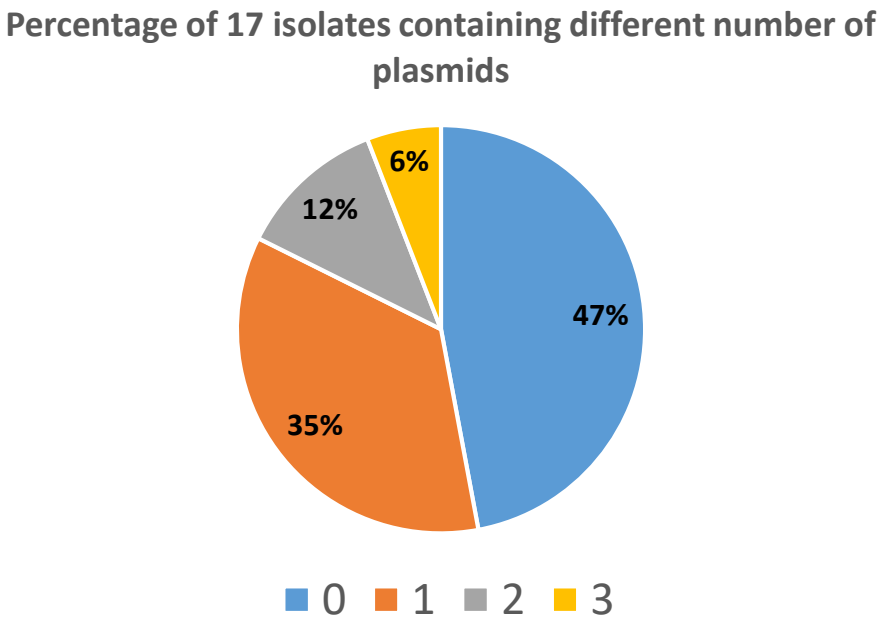

Fig 3.15 Percentage of 17 isolates containing different number of plasmids. As shown, 47% of the isolates didn't have any plasmid at all, 35% of the isolates contained one plasmid, 12% of the isolates harbored two plasmids and 6% of the isolates contained three plasmids.

Table 3.6 Plasmid profiles of the 17 samples.

E. coli Isolate	Lane in Fig	Plasmid Content (MDa) Approx.	Number of Plasmid
S2	2	-	0
S3	3	-	0
S4	4	-	0
S5	5	3.5	1
S6	6	65, 32, 2	3
S8	7	35.6, 2	2
S9	8	35.6	1
S10	9	-	0
S11	11	35.6, 4.2	2
S12	12	35.6	1
S13	13	35.6	1
S14	14	5.0	1
S15	15	-	0
S16	16	-	0
S18	17	-	0
S19	18	5.5	1
S20	19	-	0

3.6 Analysis of Plasmid Profiling and Antibiotic Resistance:

When the plasmid profiles and the corresponding antibiotic susceptibility were analyzed, it was found that the presence of plasmids accounted for resistance to higher number of antibiotics. The analysis showed that non-plasmid carrying isolates were resistant to a few number of antibiotics but plasmid carrying isolates were resistant to more number of antibiotics. The isolate S8 contained 2 plasmids and was resistant to 15 antibiotics out of 20 antibiotics while the isolate S6 contained 3 plasmids and was resistant to 15 antibiotics out of 20 antibiotics as well. However, S6 was fully resistant to 8 antibiotics and produced no zone of inhibition when tested with them. S6 and S8 has a common plasmid of size 2.0MDa, which might account for the similarities in the results of the antibiotic susceptibility test.

Table 3.7 Analysis of plasmid profiling and antibiotic resistance.

		Antibiotics to which Isolates were resistant	
***E. coli* isolates**	**Number of plasmids**	**Numbers**	**Antibiotics**
S2	0	4	PEN, OXA, LEX, ERY
S3	0	4	PEN, OXA, LEX, ERY
S4	0	4	PEN, OXA, LEX, ERY
S5	1	4	PEN, OXA, LEX, ERY
S6	3	15	PEN, AMX, LVX, OXA, LEX, AMP, ERY, TET, CAZ, CRO, SXT, NAL, AMK, CLI, NOR
S8	2	15	PEN, AMX, STR, OXA, LEX, AMP, ERY, TET, CAZ, CRO, SXT, NAL, AMK, CLI, NOR
S9	1	5	PEN, OXA, STR, LEX, ERY
S10	0	5	PEN, OXA, KAN, LEX, ERY
S11	2	4	PEN, OXA, LEX, ERY
S12	1	4	PEN, OXA, LEX, ERY
S13	1	6	PEN, OXA, STR, KAN, LEX, ERY
S14	1	4	PEN, OXA, LEX, ERY
S15	0	2	PEN, OXA
S16	0	3	PEN, OXA, LEX
S18	0	3	PEN, OXA, LEX
S19	1	5	PEN, STR, OXA, LEX, TET
S20	0	3	PEN, OXA, LEX

Chapter 04: Discussion

CHAPTER 04: DISCUSSION

4.1 Discussion:

Meat has been a prominent source of *Escherichia coli (E. coli)*. And when it comes to the most common cattle on Earth, cow undoubtedly stands on top of the list. Likewise, raw beef is a prime source of *E. coli* and in Bangladesh where decapitation, handling and processing of meat are poorly managed, it was predicted that E.coli will be abundant in raw beef. During this investigation, 20 raw beef samples were collected from different butcher shops situated in Mirpur area of Dhaka city. Out of the 20 samples, *E. coli* was isolated from 17 samples, with a prevalence of 85% for this investigation. The high prevalence of *E. coli* in raw beef corresponds to findings obtained by Islam *et al.*, 2010, where the team isolated Shiga toxin (Stx)–producing *Escherichia coli* (STEC) from raw meat, raw milk, and street vended juices in Bangladesh. In their investigation, 66% (n = 41) of raw beef samples were found to be positive for Shiga toxin (Stx)–producing *Escherichia coli* (STEC). In addition to that, the prevalence of *E. coli* was found to be fairly high even in developed cities like the Greater Washington, D.C. In an investigation carried out by Zhao *et al.*, (2001), 19.0% of the beef samples were positive for *E. coli* obtained from 59 stores of four supermarket chains during 107 sampling visits in the Greater Washington, D.C. Hence, it can be said that raw beef is a prominent source of *E. coli*.

The isolates were then subjected to a set of Biochemical Tests according to Cappuccino, & Sherman. (2005) for confirmation. Once it was confirmed that the isolates were *E. coli*, the next step of the investigation was to check their antibiotic susceptibility. The antibiotic susceptibility test yielded that 100% (n=17) of the isolates were fully resistant to Penicillin and Oxacillin. It was also noted that all the isolates produced no zone of inhibition when tested with Penicillin and Oxacillin. The results also revealed that the isolates exhibited resistance to Cephalexin (94%), Erythromycin (71%), Streptomycin (24%), Tetracycline (18%), Amoxicillin (12%), Kanamycin (12%), Ampicillin (12%) and Levofloxacin (5.9%). No isolate was resistant to two antibiotics namely Ciprofloxacin and Chloramphenicol. These developments of antibiotic resistance can be deadly to both humans and other animals. Uncontrolled usage of antibiotics in treatment of animals and their integration in animal feeds has been believed to account for the increase in antibiotic resistance (WHO, 2000; Galland et al., 2001).

In the investigation, it was found that the resistance of all the 17 *E. coli* isolates to Penicillin was 100% with no zone of inhibition. According to Sabir *et al*., (2014), resistance of *E. coli* to Penicillin group of antibiotics have been on higher side and is increasing day by day in different parts of the world but there are only few reports which indicates 100% resistance to penicillin. On the other hand, the resistance to Oxacillin was found to be higher than in the investigation carried out by Reuben and Owuna (2013) where the percentage was 84.2%.

The antibiotic susceptibility test also revealed that the isolates were most susceptible to Chloramphenicol (94%), followed by Levofloxacin (94%), Ciprofloxacin (82%), Tetracycline (84%), Streptomycin (76%), Amoxicillin (59%), Kanamycin (53%), Ampicillin (47%) and Cephalexin (5.9%). It was noted that no isolate was susceptible to Penicillin, Oxacillin and Erythromycin. This trend in antibiotic susceptibility was in line with Altalhi *et al*., (2010) where they isolated *E. coli* form retail raw chicken meat. Chloramphenicol, which was the most efficient antibiotics against the isolates, prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome (Wisseman *et al*., 1953) while Levofloxacin, which was also highly effective against the isolates, inhibits the two type II topoisomerase enzymes, namely DNA gyrase and topoisomerase IV and acts as bacteriacide. Similarly, Ciprofloxacin functions by inhibiting DNA gyrase, and a type II topoisomerase, topoisomerase IV, necessary to separate bacterial DNA, thereby inhibiting cell division and Tetracyline works by inhibiting the binding of aminoacyl-tRNA to the mRNA-ribosome complex. They do so mainly by binding to the 30s ribosomal subunit in the mRNA translation complex (Chopra and Roberts, 2001). Also Streptomycin binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit (Luzzatto *et al*., 1968). Therefore, by looking at the mechanism of action of the effective antibiotics against the 17 isolates, it can then be said that the antibiotics which prevents the protein synthesis mechanisms are likely to most efficient against *E. coli* and this finding is consistent with Orelle *et al*., (2013) who worked on tools for characterizing bacterial protein synthesis inhibitors.

The most striking finding of the antibiotic susceptibly test was that two isolates, S6 and S8, showed resistance to 8 out of 12 (66.7%) of the antibiotics that they were tested upon. One notable fact of this finding was that both the isolates produced no zone of inhibition for 5 of the 8 (62.5%) antibiotics they were resistant to. This result led to a further antibiotic susceptibility test for these two isolates where they were tested with eight more antibiotics- Ceftazidime, Ceftriazone, Trimethoprim/Sulfamethoxazole, Nalidixic Acid, Imipenem, Amikacin, Clindamycin and

Norfloxacin. This particular antibiotic susceptibility test led to an alarming finding. It was observed that both the isolates, S6 and S8, were resistant to 7 out of 12 (87.5%) of the antibiotics. The only antibiotic that they were susceptible to was Imipenem where both produced a zone of inhibition of 17mm. Hence, the isolates S6 and S8 were considered to be highly resistant. The isolate S6 in particular was fully resistant to three antibiotics- Trimethoprim/Sulfamethoxazole, Nalidixic Acid and Clindamycin and produced no zone of inhibition. On the other hand, the isolate S8 was fully resistant to Clindamycin only. This results suggests that Imipenem seems to effective against *E. coli* that are highly resistant to multiple antibiotics and this observation coincides with results obtained by Jafri et al., (2014) where they investigated the antibiotic resistance of *E. coli* isolates from urine samples of Urinary Tract Infection (UTI) patients in Pakistan and also earlier by Bonten et al., (1990).

With increasing resistance to β-lactam antibiotics, scientists began developing β-lactamase inhibitors. Imipenem, being an example of such a drug, binds irreversibly to the β-lactamases produced by many bacteria, thereby inactivating the enzymes and leaving the microorganisms sensitive to β-lactamase susceptible antibiotics (Moellering R.C., 1991). In clinical practice, the β-lactamase inhibitors are often administered in combination with β-lactam antibiotics to extend the spectrum of antibacterial activity of the antibiotics (Jafri et al., 2014). Hence, Imipenem and other β-lactamase inhibitors such as Clavulanic acid, Sulbactam and Tazobactamu can be the drug of choice to treat serious *E. coli* infections or kill highly resistant *E. coli*.

Collective data from the antibiotic susceptibility test were collected and Multiple Antibiotic Resistance (MAR) Index was calculated. The values of the MAR Index ranged from as low as 0.17 (isolate S15) to as high as 0.75(isolate S6 and S8). Using the value of the MAR index, the Level of Resistance was checked and it was found that 17.6% of the isolates had a High level of Resistance while 58.9% of the isolates had a Medium level of Resistance and 23.5% of the isolates were of Low level of Resistance. Therefore, raw beef can be considered as a potential source for the threat of acquiring and being infected with multiple antibiotic resistant *E. coli*.

The next step of the investigation was to isolate plasmid from the isolates and create a plasmid profile. Plasmid was isolated according to the modified hot alkaline method by Kado & Liu (1981), electrophoresed in a 0.7% agarose gel containing ethidium bromide (0.5 mg/mL) and observed under UV illumination. *E. coli* V517 was used as control strain for the extraction method and plasmid size marker. Jahan et al., (2016) previously followed a similar conditions where they studied the plasmid

diversity of intestinal non- *Escherichia coli* community. The plasmid profiles of the isolates revealed an interesting result.

The gel electrophoresis results revealed that 53% (9 of 17) of the isolates were observed to harbor plasmids. Isolates were seen to harbor different number of plasmids ranging from as low as 1 plasmid to as high as 3 plasmids. This pattern was in line with to a study previously carried out by Chigor et al., (2010) who studied the multidrug resistance and plasmid patterns of *Escherichia coli* O157 and other *E. coli* isolated from diarrhoeal stools and surface waters from some selected sources in Zaria, Nigeria. Eight isolates (47%) were seen to harbor no plasmids. The distribution pattern of the plasmids showed that six isolates- S5, S9, S12, S13, S14 and S19 contained only 1 plasmid. Two isolates, S8 and S11, were seen to harbor 2 plasmids. Isolate S6 contained three plasmids which was highest among the plasmid containing isolates. Also, a variety of plasmid sizes were obtained for the result of the gel electrophoresis. Plasmid sizes ranged from big as approximately 65MDa to as small as 2.0 MDa. It was observed that the most common plasmid was the plasmid with a size of 35.6MDa and was seen to present among 5 isolates: S8. S9, S11, S12, and S13. A considerable portion of the plasmids (53.9%; 7 of 13) were large plasmid (> 20MDal).

The next step of the study was to compare the results of the plasmid profiling with the results of the antibiotic sensitivity test. The comparison revealed that plasmid containing isolated showed resistance to more number of antibiotics. Also, the study showed that there can be a correlation between the number of plasmids and antibiotic resistance as it was found that a greater number of plasmids showed greater resistance to antibiotics. A study carried out by Huang et al., (2012) on characteristics of plasmids in Multi-Drug-Resistant Enterobacteriaceae reported similar findings as their results demonstrated that MDR *E. coli* isolates harbored significantly more (≥3) plasmids compared to their non-MDR counterparts, which carried ≤2 plasmids. Also similar results were reported in Icgen et al. (2002) and Nsofor and Iroegbu (2013). Also Umolu et al., (2006) reported that *E. coli* isolates with high multi-drug resistance profiles were found to possess multiple plasmids. However, it can be suggested that not all the antibiotic resistance genes are located in plasmids. This is because 8 isolates didn't carry any plasmid at all but yet showed resistance to some antibiotics namely Penicillin, Oxacillin, Cephalexin and Erythromycin. Hence, it can be suggested that theses resistances were probably chromosomally mediated. However, no relation between the size of the plasmids and antibiotic resistance could be obtained from the study.

A striking finding of the study was that both of the highly resistant isolate, S6 and S8, were seen to carry a common plasmid. The size of this plasmid was observed to be approximately 2.0MDa. No other isolate was seen to carry this particular plasmid. This led to a suggestion that the 2.0MDa plasmid might account for the high antibiotic resistance of S6 and S8. The 2.0MDa (≈3.3-kb) plasmid might carry antibiotic resistant genes that are responsible for the high antibiotic resistance of the two isolates S6 and S8. The 2.0MDa (≈3.3-kb) plasmid was not seen to be present in any of the other isolates. Also a 35.6MDa plasmid present is S8 was also observed in S9, S11, S12 and S13 but none of them was as highly resistant as S8. S6 also had a 65.0 and a 32.0MDa plasmid but it wasn't present in S8 thus strengthening the fact that the 2.0MDa plasmid might be responsible for the high antibiotic resistance of S6 and S8. A resemblance in the 2.0MDa (≈3.3-kb) plasmid was found by Makino et al., (1998) in their work to determine the complete nucleotide sequences of 93-kb and 3.3-kb plasmids of an enterohemorrhagic *Escherichia coli* O157:H7 derived from Sakai outbreak. The 3.3-kb plasmid called pOSAK1 possesses a ColE1-like replication system (Makino et al., 1998). In another study carried out by Haarmann et al., (1998), it was found that the plasmid p4821 (molecular size 3.3-kb; 3307 bp) had similarity (>98%) to the core region of the antibiotic resistance plasmid NTP16 of *Salmonella typhimurium* strains. However, they found that the lack of tra genes indicated that the plasmid is nonconjugative. Non-conjugative plasmids are incapable of initiating conjugation, but they can be transferred only with the assistance of conjugative plasmids. So there is a chance that this particular 2.0MDa (≈3.3-kb) might be pOSAK1 or p4821. Therefore, there is a need to isolate this particular plasmid and sequence it to look for antibiotic resistance genes which in turn will surely provide new insights to the understanding of plasmid-mediated antibiotic resistance.

In addition to that, another striking finding of the study was the presence of an approximately 65MDa (98.8-kb) plasmid in the isolate S6. The size of this particular plasmid shows resemblance to the pO157 (range size from 92 to 104-kb) plasmid which is a highly conserved plasmid for the deadly *E. coli* O157:H7 (Lim et al., 2010). Enterohemorrhagic *Escherichia coli* O157:H7 is a major foodborne pathogen causing severe disease in humans worldwide. *Escherichia coli* O157:H7 causes hemorrhagic colitis and hemolytic-uremic syndrome in humans, and its major reservoir is healthy cattle (Sheng et al. 2007). Plasmid pO157 is found in 99 to 100% of clinical O157:H7 isolates from humans (Levine et al., 1987; Ostroff et al., 1989; Ratnam et al., 1988). Hence, there is possibility that the isolate S6 might be *E. coli* O157:H7. Further analysis of the plasmid of size 65.0MDa (98.8-kb) and also identification tests should be done for the isolate S6 to check if it is *E. coli* O157:H7.

The study was particularly important because food-borne illness and diseases are very common these days and reports of such cases are increasing day by day. With the advent of fast food, beef has been an immensely important ingredient ever since. With beef products coming in various forms, much of it can be left undercooked and leaves the possibility of infection very high. Also the butcher's shops from which the raw beef were collected are poorly maintained and meat processing is carried out in the open and in unhygienic conditions, often with vegetable shops nearby. This leaves the possibility of cross contamination of the vegetables, many of which are consumed raw e.g. cucumber, lettuce, carrots and many more. The fact that makes *E. coli* infections really dangerous is that many of them may be pathogenic *E. coli* or STEC and if these turn out to be highly resistant, patients may even die. Also, this study revealed that antibiotic resistance might be plasmid-mediated and if it does, it leads to the high risk of Horizontal Gene Transfer (HGT), which might lead to an even greater population of highly resistant bacteria. Once again, *E. coli* poses a specific threat because *E. coli*, being a Gram-negative bacteria can share plasmids through conjugation being both donor and recipient, more readily resulting in more and more bacteria possessing antibiotic resistance genes. Besides that, excessive use of antibiotic use will lead to the emergence of bacteria resistant to the antibiotic and it is sensible to investigate procedures that will minimize their impact. If it can be known whether antibiotic resistance is plasmid-mediated or chromosomally mediated, strategies against the resistant bacteria and drug designing will be more efficient.

The findings of the study have great prospects if further research is carried out. The isolation of *E. coli* can be carried out from raw beef samples collected from different areas of Dhaka city and assessments can be made accordingly. In addition to that, other parameters can also be added to bring in varieties of interpretations. Plasmid curing, which is the elimination of plasmid from a cell culture by treatment with different curing agents, followed by screening with the same or additional antibiotics can be made to find whether antibiotic resistance is plasmid-mediated or chromosomally mediated. Polymerase Chain Reaction (PCR) can also be carried out to look for the presence of pathogenic genes. Furthermore, additional research can be carried out on the 2.0MDa plasmid that was found to be common in both of the highly resistant isolates in this study.

To sum up, *E. coli* is being considered as a model organism and has been used as an efficient tool in the field of science let alone Biotechnology. With the advent of Genetic Engineering and Biotechnology, plasmids of *E. coli* has been used to produce many products like insulin, a lifesaving drug, and has been a constant model for various types of researches. As much helpful *E. coli* can be, it can also turn out to be pathogenic and can act agents of Horizontal Gene Transfer (HGT). So the importance of working with *E. coli* to observe its potential as a reservoir of antibiotic resistance is immense. Therefore, this study lays down the foundation for further research that would definitely add new insights to the never-ending prospects of Biotechnology.

4.2 Conclusion:

To conclude, this study has led to the strengthening of the fact that raw meat samples, especially raw beef, is a prominent source of *E. coli* and a notable portion of these isolates can be resistant to many of the antibiotics that are used in day-to-day life, often through animal feeds. The study has also led to the isolation of two highly resistance isolates, S6 and S8, of *E. coli* that are resistant to a large number of antibiotics. In addition to that, the study provided a picture of the plasmid profiles of the isolates and a relationship to the antibiotic resistance that they demonstrate. Furthermore, the study also led to the detection of a common plasmid that the two highly resistant isolate obtained from this investigation harbors and that there is a resemblance to the plasmids pOSAK1 and p4821.Last but not the least, an isolate S6 was seen to contain a plasmid that resembles in size to the pO157 which is highly conservative to the deadly *E. coli* O157:H7. So, the prospect of *E. coli* isolated from raw beef samples as a potential reservoir of antibiotic resistance is very high. Thus the objectives that were set during the beginning of the study were well met.

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Appendix – I

Media composition

The following media was used during the study. All components were autoclaved at 121°C, 15 psi for 15 minutes unless mentioned otherwise

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Luria Bertani Broth

Component	Amount (g/L)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	10.0

Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

Tryptophan Broth

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

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

Methyl Red Voges- Proskauer (MRVP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Motility Indole Urease (MIU) Agar

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Nitrate Reduction Broth

Component	Amount (g/L)
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

EC Broth

Component	gm/litre
Tryptone	20
Lactose	5.0
Bile salts No. 3	1.5
Di-potassium phosphate	4.0
Mono-potassium phosphate	1.5
Sodium chloride	5.0

Appendix II

Reagents

The following reagents were used throughout the study:

1. Barritt's reagent

Solution A: 5 g alpha-naphthol was dissolved in 95% ethanol. The reagent was covered in aluminum foil and stored at 4°C.

Solution B: 40 g KOH was dissolved in distilled water. Once the mixture cooled, creatine was added. Final volume was adjusted with distilled water and the reagent covered with aluminum foil was stored at 4°C.

2. Crystal violet Stain (2%)

2 g of crystal violet was dissolved in 20 mL of 95% ethyl alcohol. 0.8 g ofammonium oxalate monohydrate was next dissolved in 80 mL distilled water. The two solutions were mixed and filtered into sterile reagent bottle.

3. Iodine solution (Gram's)

6.7 g potassium iodide was dissolved in 100 mL of distilled water. To this, 3.3 g of iodine was added, stirred, and the solution made up to 1 liter with distilled water. The reagent bottle was covered in aluminium foil and stored at room temperature.

4. Kovac's reagent

5 g para-dimethylaminobenzaldehyde was dissolved in 75 mL amyl alcohol. To this, hydrochloric acid (1M) was added to make up the final volume of 25 mL. The reagent bottle was covered with aluminium foil and stored at 4°C.

6. Malachite green (0.5%)

0.5 g malachite green was dissolved in 100 mL distilled water. The solution was stored at room temperature by covering the reagent bottle with aluminium foil.

7. Methylene blue solution (1%)

1 g of methylene blue was dissolved in 75 mL of distilled water, and then diluted to make 100 mL. The solution was filtered out and stored in reagent bottle.

8. Methyl red reagent

0.1 g methyl red was dissolved in 300 mL of 95% ethyl alcohol. To this, distilled water was added to make up the final volume of 500 mL. The reagent was covered with aluminum foil and stored at 4°C.

9. Oxidase reagent

100 mg of N, N, N^1, N^1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 mL distilled water. The solution was covered with aluminum foil and stored at 4°C.

10. Safranin

0.1 g of safranin was dissolved in 75 mL of distilled water. The solution was diluted to 100 mL, filtered and stored in clean reagent bottle.

Appendix III

Instruments

Instrument	Company
Autoclave	SAARC
Cellulose filter paper (9.0 cm)	Whatman
Colorimeter, ISO 9001	Labtronics, India
Freeze (-20°C)	Siemens
Incubator	SAARC
Hotplate stirrer	LabTech
Micropipette (10-100 µL)	Eppendorf, Germany
Micropipette (100-1000 µL)	Eppendorf, Germany
Microscope	Optima
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Pipette (5 mL, 10 mL)	Eppendorf, Germany
Refrigerator (4 ^{o}C), Model: 0636	Samsung
Safety cabinet Class II Microbiological	SAARC
Surgical Millipore syringe filter (0.22µm)	Millex-GS
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Weighing balance	ADAM EQUIPMENT™, United Kingdom