Isolation, molecular characterization and antibiotic resistant of pathogenic bacteria obtained from raw meat in Dhaka city

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

Submitted by

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JULY 2018

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DECLARATION

I hereby declare that the thesis project titled 'Isolation, molecular characterization and antibiotic resistance of bacteria obtained from raw meat available in different areas of Dhaka city' has been written and submitted by me, Nurzahan Yasmin Tuly and has been carried out under the supervision of Kashmery Khan, Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared 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. All explanations that have been adopted literally or analogously are marked as such.

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ACKNOWLEDGEMENT

First and foremost, I would like to express my thanks to Almighty Allah because He has given me the opportunity and strength to finish this research. I am also thankful for His blessings to my daily life, good health and healthy mind.

I acknowledge my esteem to Professor **A F M Yusuf Haider**, Chairperson of MNS Department of BRAC University for giving me the opportunity to complete my undergraduate thesis. I am really grateful to Professor **A. A. Ziauddin Ahmad**, former Chairperson of MNS department of BRAC University Professor **Shah M. Faruque**, **Dr. Aparna Islam, Dr. Mahboob Hossain** of MNS Department of BRAC University for allowing me and encouraging me to complete my undergraduate thesis.

My regards, gratitude , respect and indebtedness goes to my respected supervisor **Kashmery khan**, Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, Brac University for her guidance, suggestions, constructive criticism, enthusiastic encouragement , providing new ideas throughout my thesis work.

I would like to thank **S M Rakib-Uz-Zaman**, Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, Brac University for his suggestions, encouragement, providing new ideas throughout my thesis work

I would like to thank and express my deepest gratitude toward **Salman Khan Promon** for guiding and helping me in my report writing and providing time to time suggestions regarding setting of experimental designs, interpretation of results and subsequent directions for the whole work without being a bit of impatient.

I would also like to thank **Nahreen Mirza** and **Asma Binte Afzal** for their time and support throughout my thesis work.

Abstract

A large number of people intake proteins from meat. But the problem is a number of bacteria are being identified during the several years from raw beef which cause different physical problems and sometimes turns into life-threatening. Improper handling and poor hygiene while handling and preparing meat causes different kinds of health problems in Bangladesh. Transmission of *E. coli* through the contact with animals and individual to individual via the fecal-oral route has become common. In this study, 24 samples of raw beef were collected from different areas of Dhaka city. After performing biochemical tests *Eschrerichia coli, Staphylococcus, Bacillus*, and *Enterobacteriaceae* were found in the selective media also. In this research biochemical tests such as streaking on EMB, Macconkey, XLD, and Nutrient agar medium were done. After that, antibiotic sensitivity testing was done by using 4 antibiotics; they are Kanamycin, Gentamycin, Erythromycin, and Streptomycin. Resistance was found against Erythromycin from 7 samples. Genomic DNA was isolated from samples. Then PCR was done with 16SrRNA and *STX* 1 separately to identify the presence of *STX 1* and *STX 2*. Gel electrophoresis results showed bands which correspond to the presence of 16SrRNA and *STX 1*,that signifies the presence of pathogenic bacteria in meat. DNA sequencing of 2 samples showed that they are *Enterobacter* and *Lactobacillus brevis*.

Chapter: 1
Introduction

Introduction

1.1 Background

In Bangladesh, the number of vegetarian people is very few. Most of the solvent family members consume at least 1 kg of meat per week. The foodborne disease is a great problem caused by consumption of contaminated food and water now a day. Most interestingly, it is already revealed that meat is a superior medium for many microorganisms to grow because it is rich in moisture, nitrogenous compounds (e.g. amino acids, peptides, and proteins) and plentifully, minerals and accessory growth factors (Thanigaivel and Anandhan, 2015). Recently diarrheal case studies are more frequent than previous records and it is suspected evolved bacteria are the reasons behind them (Thanigaivel and Anandhan, 2015). This is because of proper handling issues and improper cooking method (Noorang *et al*, 2009). Corresponding works have not been done in Bangladesh before in broad-spectrum yet but in India, Nepal, and Iran etc. Most microorganism related contamination is due to zoonotic diseases.

Meat is most popular amongst the most mainstream protein sources from the antiquated time. Beef is one of them. Beef can be consumed in various ways, cooked, semi-cooked or by other preparations salad. Beef is composed of water, protein, and fat. Unprocessed meat may get rotten within hours or days and can result in infection at a very fast rate. In the case of raw beef, these infections are mainly caused by bacteria and in most of the cases by Enterobacter (*E.coli).* A major problem in food hygiene is the fecal contamination of beef and chicken meat with Enterobacteriaceae such as *Escherichia coli*,

The 16S ribosomal RNA (rRNA) gene sequences are used to study bacterial phylogeny and taxonomy. There are a number of reasons for which this is used as the most common housekeeping genetic marker. These reasons include (i) this is present in almost all bacteria, often existing as a multigene family, or operons;(ii) the function of the 16SrRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1500 bp) is large enough for informatics purposes. (Patel 2001; Větrovský and Baldrian 2013). 16S rRNA gene is also used for confirmation of bacterial isolates along with biochemical and phenotypical methods of bacterial identification including *E. coli* and Shiga toxin-producing *E. coli*. Length of 16SrRNA gene sequence is about

approximately 1550 bp and comprises variable and conserved regions, which are important in characterizing bacteria. (Clarridge 2004).

In developed countries, *E. coli* O157: H7 infections are becoming a very threatening food-borne disease (Griffin and Tauxe 1991). In Asia, there have been isolation and report of *E. coli* of sero groupO157 in the countries, like China, Japan, and India. (Gupta *et al*, 1992; Yamada *et al*, 1993; Griffin 1995).Continuous monitoring of beef meat in Bangladeshi market is mandatory as there are severe chances of presence of pathogenic *E.coli*. Therefore, the current study is aimed to investigate the occurrence and characterization of *E. coli* and Shiga toxin-producing *E. coli* (STEC) pathogen in raw beef sold in Dhaka city.

1.2 Diarrhea

Diarrhea is a condition in which feces are discharged from the bowels frequently and in a liquid form. It is caused by increased secretion of fluid into the intestine, reduced absorption of fluid from the intestine or rapid passage of stool through the intestine. There are two types of diarrhea, absolute and relative. Absolute diarrhea is the result of five or more bowel movements a day consisting of liquid stool. On the other hand, relative diarrhea is the result of increase bowel movement than one individual's usual habit. Bacterial infections can cause severe symptoms, often with vomiting, fever and severe abdominal cramps or abdominal pain.

In the greater part of the cases, different gram negative and gram positive bacteria are responsible for different opportunistic infections in human.

1.3 Prevention from bacterial infections

In the case of diarrhea, dehydration can occur which can be treated by oral saline. Absorbent, anti–motility medications, IV fluids can work well against this. Antibiotics are powerful medicines which can fight against bacterial infections in serious cases. Taking an antibiotic as directed, even after symptoms disappear, is key to curing an infection and preventing the development of resistant Bacteria.

1.4 Microorganisms commonly found in raw beef

In my thesis, I have found gram-negative *Escherichia coli* in 50% of my samples and also found gram-positive *Lactobacillus brevis* in one of my samples. These microorganisms are discussed below. *E.coli* which is a member of the genus *Escherichia*, contains mostly motile Gram-negative, rod-shaped facultative anaerobic bacilli. Its serotyping is done based on surface antigens, specifically their somatic (O) and flagella (H) antigens. *E. coli* strains can be divided into commensal strains and pathogenic strains based on their clinical and genetic characters. The pathogenic strains are subdivided into intestinal pathogen (also termed enteric or diarrhoeagenic) strains and extra intestinal pathogen strains. (Russo and Johnson 2000). The six most recognized pathotypes of diarrhoeagenic are, enteroaggregative, enteropathogenic, enterohaemorrhagic, enteroinvasive (EIEC) and diffusely adherent *E.coli.* (Cennimo *et al* 2007). This division was based on distinct virulence factors and pathogenic features (Xia *et al*, 2010). *E. coli* serotype O157: H7 was shown to belong to a group of *E. coli* that produces some toxins which are similar to Shiga toxin produced by *Shigella dysenteriae* and distinct from previously described *E. coli* heat-stable and heat labile toxins. Though it was not until 1993, after a large multistate outbreak of *E.coli* O157: H7 resulting in more than700 illnesses and four deaths, that this organism was recognized as an important and threatening human pathogen. (MacDonald and Osterholm 1993; Bell *et al*,1994).

1.4.1 *Enterobacter*

Enterobacter is a member of the Enterobacteriaceae family. *Enterobacter cloacae* are clinically the most important species. The colonies of Enterobacter strains may be slightly mucoid. Their fermentative activity is more limited than *Klebsiella.* The normal habitat of *Enterobacter* spp.is probably soil and water, but the organisms are occasionally found in human feces and the respiratory tract. Infection of hospital patients, notably of urinary the tract, occurs. *Enterobacter* spp is also an important cause of bacteremia. An outer membrane protein, termed OmpX, may be a pathogenic factor for strains. This protein appears to reduce production of porins, leading to decreased sensitivity to Beta-lactam antibiotics and might play a role in host cell division.

1.4.2 Scientific classification

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

1.4.3 *Enterobacter cloacae*

This is the most frequently isolated *Enterobacter* species from humans and animals. Its part has an increasing importance in hospital-acquired infections (ICU, emergency units, urology). *Enterobacter cloacae* are less susceptible to chlorination than *E.coli*. It may also be isolated from meat, hospital environment and on the skin of man as a commensal.

1.4.4 *Enterobacter hormaechei*

The name *Enterobacter hormaechei* is proposed for a new species of the family Enterobacteriaceae, formerly called enteric group 75, which consists of 23 strains, 22 of which were isolated from human. It was first identified as a unique species in 1989. It can cause nosocomial infections, including *Enterobacter hormaechei*, spread via horizontal transfer and is often associated with extended-spectrum beta-lactamase production. This increases the challenges associated with treatment by limiting therapeutic options. This strain can be an underreported cause of bacterial infection, especially in neonates.

1.4.5 *Enterobacter kobei*

The name of *Enterobacter kobei* sp.nov.is proposed for a group of the definition of the family organisms referred to as NIH Group 21 at the National Institute of Health Tokyo. The members of the species are gram-negative, motile rods conforming to the family Enterobacteriaceae. *Enterobacter kobei* is phenotypically most closely related to the species *Enterobacter cloacae.* Nosocomial urosepsis can be caused by this.

1.4.6 *Enterobacter xiangfangensis*

This strain was first isolated from traditional sourdough in Heilongjiang Province, China. It was Characterized by a polyphasic approach, which included 16SrRNA gene sequence analysis. It can be the leading cause of nosocomial infection across the world.

1.4.7 *Enterobacter cancerogenus*

Enterobacter taylorae is a Gram-negative bacterium formerly known as Enteric Group 19, and also known as *Enterobacter cancerogenus.* Strains of *Enterobacter taylorae* are positive for Voges-Proskauer, citrate utilization, arginine, and malonate utilization. They ferment D-glucose and also ferment D-mannitol, L-rhamnose, and cellobiose. It occurs in human clinical specimens, being isolated from blood and from spinal fluid. It is known to cause infections and not susceptible to penicillin’s nor cephalosporin’s.

1.5 *Escherichia coli*

Escherichia coli are facultative anaerobic bacteria. Their normal habitats are lower intestine in man and animals, although some toxin-producing strains can produce pathogenicity ranging from mild diarrhea to fatal complications. Shiga toxin producing *E.coli* is one of such toxigenic groups. Most of the strains of *E.coli* are not harmful but are the part of healthful bacterial flora in the human gut. However, some types can cause illness in humans, including diarrhea, abdominal pain, and fever and sometimes vomiting. It can also cause pneumonia and urinary tract infection. *E.coli* is one of the most familiar pathogens of Enterobacteriaceae. Some of the strains of *E.coli* can cause serious food spoilage. Possible sources of *E.coli* include undercooked ground beef, unpasteurized milk, juice or raw vegetables.

1.5.1 Scientific classification

Domain: Bacteria

Kingdom: Eubacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *Escherichia coli*

The most harmful life-threatening strain of *E. coli* is *E. coli* 0157: H7 that produces toxins like Shiga toxin which can cause the premature destruction of red blood cells. *E.coli* can produce six types of infections. They are:

1) Enterotoxigenic *E. coli* (ETEC)
2) Enteropathogenic *E. coli* (EPEC)
3) Enteroaggregative *E. coli* (EAEC)
4) Enteroinvasive *E. coli* (EIEC)
5) Diffusely adherent *E. coli* (DAEC)
6) Uropathogenic *E. coli* (UPEC)

1.5.2 Enterotoxigenic *Escherichia coli* (ETEC)

ETEC is one of the main bacterial causes of diarrheas in different countries. It also causes travelers' diarrhea. It produces a heat-labile enterotoxin and a heat stable enterotoxin. It can produce infection with profuse, watery diarrhea with no blood. It can be treated with rehydration therapy and antibiotics. ETEC uses fimbria adhesions (projections from the bacterial cell surface) to bind enterocyte cells in the small intestine. ETEC may produce two proteinaceous enterotoxins; the larger of the two proteins, LT enterotoxin, is similar to cholera toxin in structure and function. The smaller protein, ST enterotoxin causes cGMP accumulation in the target cells and a subsequent secretion of fluid and electrolytes into the intestinal lumen. ETEC strains are noninvasive, and they do not leave the intestinal lumen.

1.5.3 Enteropathogenic *E. coli* (EPEC)

EPEC also causes diarrhea but the molecular mechanisms of colonization and etiology are different. EPEC does not possess ST or LT toxin, but they contain an adhesion known as intimin which binds to the host intestinal cells. It produces similar symptoms like shigellosis. Because of The adhesion deformation of the host intestinal cell wall takes place. Causative agents of EPEC can be humans, rabbits, dogs, cats and horses.

1.5.4 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E.coli* can only be found in humans. They aggregate tissue cells as they bind to the intestinal mucosa and cause watery diarrhea. They do not cause fever and they are noninvasive. They also produce an agent called hemolysin and an ST enterotoxin similar to ETEC.

1.5.5 Enteroinvasive *E. coli* (EIEC)

EIEC infection causes a syndrome that is identical to shigellosis, with profuse diarrhea and high Fever.

1.5.6 Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *Escherichia coli* (DAEC) have been considered a diarrhoeagenic category of *E.coli* for which several potential virulence factors have been described in the last few years.

Despite this, epidemiological studies involving DAEC have shown inconsistent results. In this work, two different collections of DAEC possessing Afa/Dr genes, from children and adults, were studied regarding characteristics potentially associated with virulence.

1.5.7 Uropathogenic *E.coli* (UPEC)

UPEC is one of the main causes of urinary tract infections. It is part of Shiga toxin-producing *E.coli* (STEC)—STEC may also be referred to as Verocytotoxin-producing *E. coli* (VTEC) or Enterohaemorrhagic *E.coli* (EHEC). This pathotypes is the one most commonly heard about in the news in association with foodborne outbreaks.

1.6 Phylogenetic tree of *E. coli*:

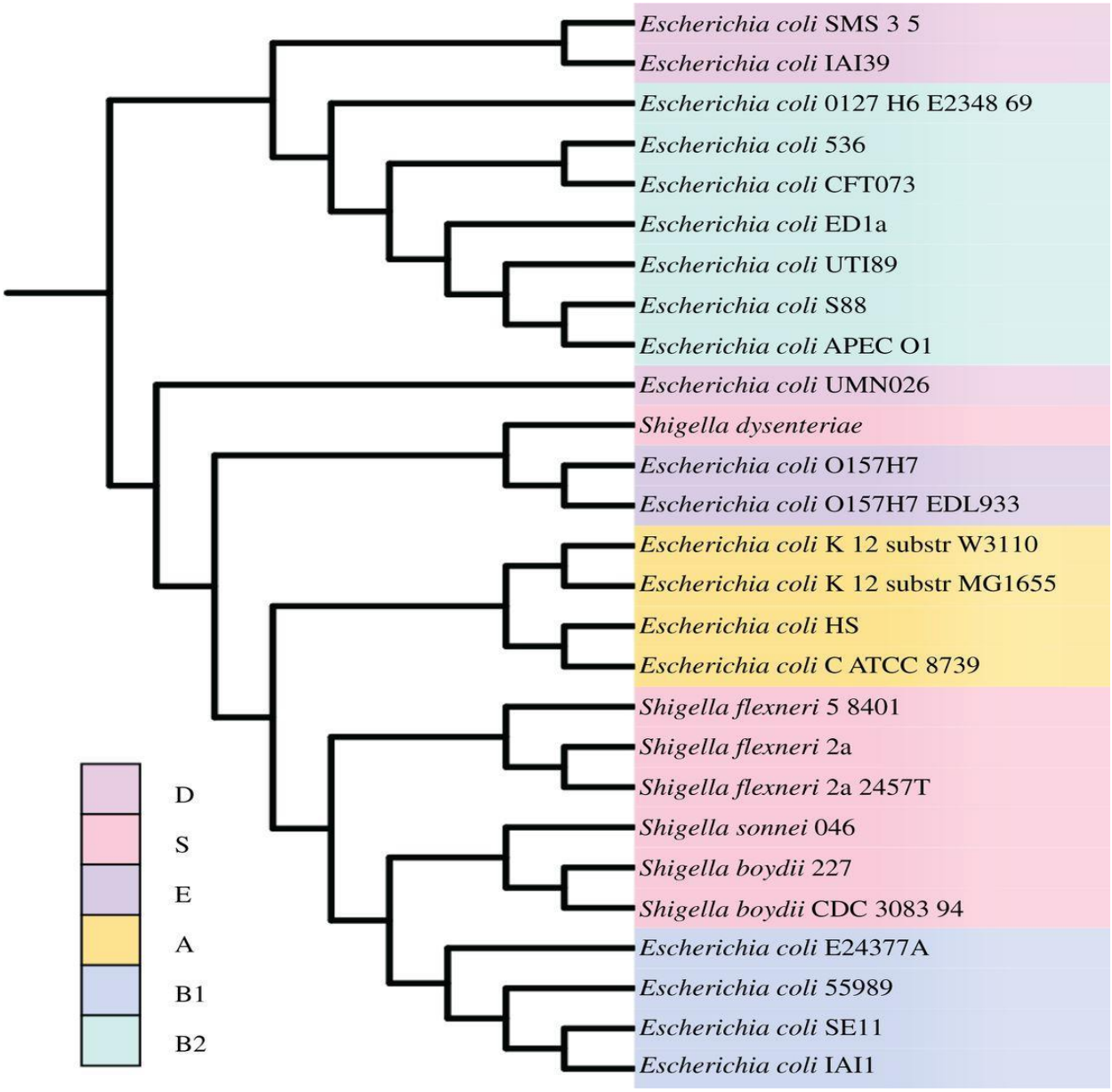

Figure 1.1: Phylogenetic tree of *E .coli*

1.6.1 Epidemiology of gastrointestinal infection

Transmission of *E. coli* occurs mostly via the fecal-oral route. Common causes include unhygienic food preparation, intake of contaminated water or sewage water, contact with manure of domestic animals. Dairy and beef are the primary reservoirs of *E. coli* 0157: H7 and they can asymptotically through shedding and thorough their faces. Cucumber, raw ground beef, raw spinach, raw milk, unpasteurized juice, and dairy products were responsible for previous outbreaks of *E. coli*0157: H7. According to experts, this cycle of transmission can be disrupted by proper cooking of food, introducing sanitization of food producers or workers with Gloves and pasteurizing liquid products. STEC has been reported also by transmitted by flies.

1.6.2 Antibiotic therapy and resistance

E. coli severe infections are usually treated with antibiotics but different strains response to Different antibiotics. Because of gram-negative bacteria, *E.coli* samples were found sensitive against Kanamycin, Gentamycin and Streptomycin and almost half of the samples were found resistant against Erythromycin. However, antibiotic resistance is a growing problem, this might be due to antibiotics are sued as growth promoters in animal feeds. A study in the Journal of Science (August 2007) said the rate of adaptive mutations in *E. coli* in happening at a 10-5 per genome per generations. This may lead to a resistance strain, such as MRSA.

1.6.3 Beta-lactamase strains

Some strains of *E. coli* have been identified to produce enzymes so that they can show resistance Towards the broad spectrum of beta-lactamases. They are resistant to Penicillin and Cephalosporin's. These strains are very difficult to treat and are thought to be one kind of Superbug in process.

1.6.4 *Lactobacillus brevis*

Lactobacillus brevis is a gram-positive, rod-shaped species of lactic acid bacteria which is heterofermentative, creating carbon dioxide and lactic acid during fermentation. There are approximately 16 different strains. It can be found in many different environments, such as fermented foods, and as normal micro biota. *L. brevis* is found in food such as sauerkraut and pickles. It is also one of the most common causes of beer spoilage. Ingestion has been shown to improve human immune function, and it has been patented several times. Normal gut micro biota *L. brevis* is found in human intestines, vagina, and feces. It works as a probiotic.

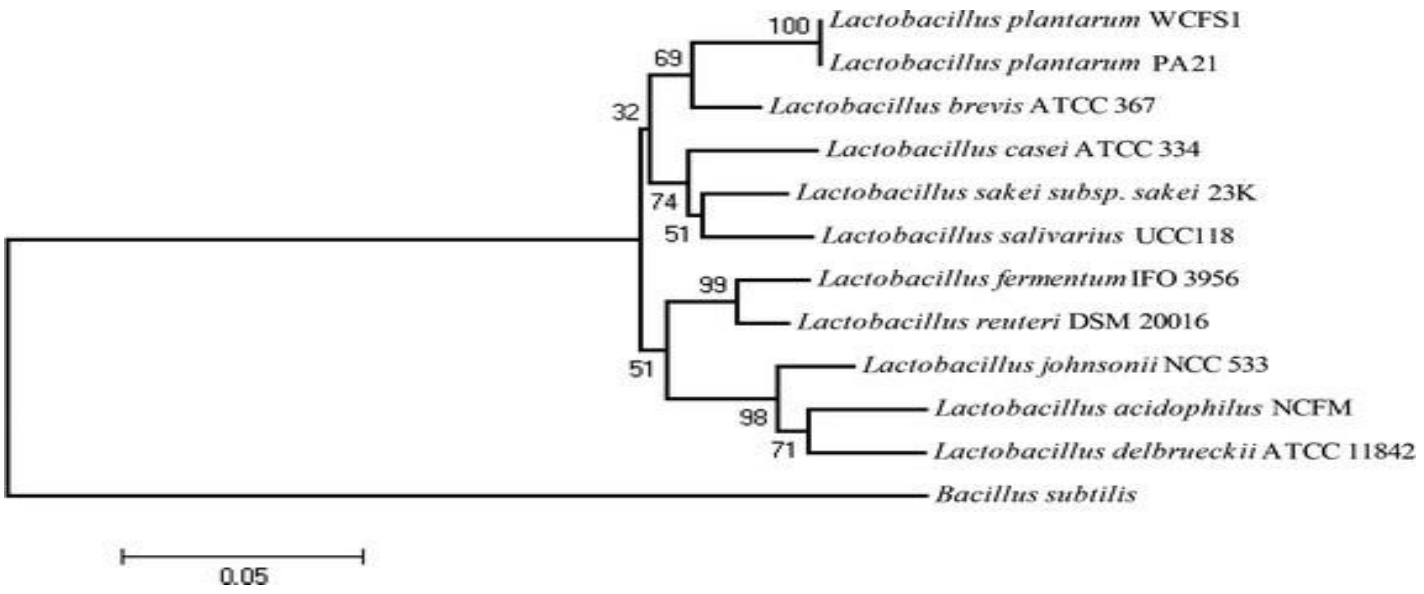

Fig 1.2: Phylogenetic Tree of *Lactobacillus brevis*

1.7 Antibiotic resistance

An antibiotic also is known as an antibacterial, is a type of antimicrobial drug used in the treatment and prevention of bacterial infections. They may either kill or inhibit the growth of bacteria. The above-mentioned organisms can cause infections in human that can be treated by antibiotics. Antibiotic sensitivity or antibiotic susceptibility is the susceptibility test of bacteria to specific antibiotics that are tested. Susceptibility can vary in case of every organism even within their different strains. Therefore, a range of susceptible acceptance is used to observe if an organism is susceptible or resistance or shows intermediate resistance to that particular antibiotic. For this method, antibiotic disks are used that are commercially available. For antibiotic susceptibility procedure, Muller Hinton Agar is primarily used where lawn culture of tested bacteria are done.

This procedure is called the Kirby-Bauer method. If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition will be demonstrated and if the bacteria are resistant to any particular antibiotic it will grow over the antibiotic disk. Sometimes bacteria gain resistance after a certain period of time and thus creating secondary zones of growth (*Bauer AW et al, 1997*). 50% of raw beef samples showed sensitivity to kanamycin, Gentamycin, and Streptomycin, and 50% showed resistance to Erythromycin. Bacteria are gradually acquiring resistance against the antibiotics used against those. They can secrete enzymes to degrade the antibiotics or change the structure of the active site where antibiotics would have worked. It is very important to find out microbes that are resistant to different antibiotics. Moreover, it is crucial to use the most suitable antibiotic to treat any infection for proper treatment. Therefore, antibiotic sensitivity testing must be done.

1.8 Molecular diagnostics

Molecular diagnostics are based on DNA or deoxyribonucleic acids of an organism, in this case. *E.coli.*

1.8.1 Genomic DNA

DNA is the abbreviation of deoxyribonucleic acid, the organic chemical of complex molecular structure which is present in all prokaryotic and eukaryotic cells and in many viruses. DNA codes genetic information for the transmission of inherited traits. DNA was first discovered in 1953 by James Watson, Francis Crick with the data acquired by Raymond Gosling who stole the data from his teacher Rosalind Franklin. It comprises four major types of macromolecules that are nitrogen-based – Cytosine(C), Guanine (G), Adenine (A), and Thiamine (T). It also contains a sugar called deoxyribose and a phosphate group. The nucleotides are joined together in a chain by covalent bonds between the sugar of one nucleotide and the phosphate of the next nucleotide. DNA molecules are coiled as a double helix with two strands. They store biological information, although a large part of them are non-coding. DNA can be of two types: chromosomal DNA and Plasmid DNA. Humans only have chromosomal DNA, but microorganisms such as E coli have plasmid DNAs. Plasmid DNAs can be more than hundreds in number per cell and therefore their medical and clinical application is huge. They contain extra chromosomal information and can multiply independently.

For molecular diagnostics, three tests procedure were performed. They are

1) PCR (polymerase chain reaction)
2) Gel electrophoresis
3) Sequencing

1.8.2 Polymerase chain reaction (PCR)

PCR is used to amplify any small amount of DNA by changing making the double-stranded DNA single-stranded and again double stranded while changing the temperature frequently and With a specific thermos table enzyme called Taq polymerase. To identify if any specific region of the DNA segment of the gene is present in the sample, two primers: forward and reverse primers are designed and put into the PCR mix along with DNA template and master mix. This is the most common method that is still used to amplify a small amount of desired DNA up to the present. Many up to date PCR methods that are more accurate and fast are invented such as real-time PCR. However, they are costly and hard to maintain. There are mainly three steps involved in PCR. In the first step, the two strands of the DNA double helix are physically separated by

applying high temperature such as 95◦ Celsius which is known as the denaturing stage. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase and amplify the target DNA. This step is called the annealing step and it involved temperature such as 50◦ -60◦ Celsius. The last step is called the elongation step where the double-stranded DNAs are elongated and multiplied in geometric series such as two DNA strands from one Strand, four from two strands and so on. These cycles are repeated over and over maximum to 35 cycles for this thesis purpose. I have used 16SrRNA gene sequences to study bacterial phylogeny and taxonomy. This is the most common housekeeping genetic marker for several reasons.

1.8.3 Gel electrophoresis

Gel Electrophoresis is a laboratory method used to separate mixtures of DNA, RNA or proteins according to molecular size. In gel electrophoresis, the molecules to be separated are pushed by an electrical field through a gel that contains small pores. The molecules travel through the pores in a speed that is inversely related to their lengths. This means that a small DNA molecule will travel a greater distance through the gel than will a larger DNA molecule. As DNA molecules are negatively charged when an electric field is applied they tend to move inside the agarose gel matrix and short molecules ran faster than the bigger and heavy ones.

1.8.4 Partial DNA Sequencing

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases-adenine, guanine, cytosine and thymine-in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery. DNA sequencing may be used to determine the sequence of individual genes, larger genetic regions of any organism. Information obtained using sequencing allows researchers to identify changes in genes, associations with diseases and phenotypes and identify potential drug targets. Among 14 *E.coli* Samples isolated from raw beef; 1 gram negative and 1 gram-positive sample were sent for partial sequencing to (Apical Scientific, Malaysia).

1.9 Objective Of the study

- Isolation and biochemical identification of different bacteria from raw beef
- Investigation the presence of pathogenic *E.coli* and their antibiotic resistance
- Molecular detection and characterization of bacteria through polymerase chain reaction.

Chapter: 2
Materials & Methods

2. Materials and Methods

This study was held in the biotechnology and microbiology laboratory of BRAC University by following all the required instructions and by abiding all the rules of the institution.

2.1 Sample Collection

For this research, I have collected raw beef from different shops in Dhaka city. One piece of the raw beef nearly 25 grams from each shop was collected in previously sterile packets. I have collected 24 samples from different butchers situated in the local market, such as Mohakhali, Notunbazar, Kuril, Banani Kacha bazaar, Kochukhet bazar. The surprising thing is samples collected from different departmental stores like Genius super shop in Bashundhara, KB super shop, Mehedi mart, were also containing gram-negative *E.coli.*

2.2 Homogenization

Around 25 grams of meat sample was put in sterile bags with 0.85% peptone salt solution. Then The samples were homogenized using mortar and pestle. All equipment was washed with 70% Ethanol.

Figure 1.3: homogenization of samples

2.3 Incubation

Samples were incubated by mentioned serial dilution above for 24 hours in 37 - degree Incubator. Few samples showed little growth, they were incubated for an additional 24 hours in total 48 hours.

2.4 Gram Staining

This is the procedure by which two main categories of Bacteria are separated: gram-positive and Gram-negative. By this method, gram-positive bacteria show their shapes and purple staining under a compound microscope and gram-negative ones show a pink color. There are four steps involved. First of all, a smear of bacteria is prepared onto a glass slide. Then it is heat treated and applied with grams iodine. 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.

2.5 Selective and differential media

2.5.1 Selective media

This media allows the growth of a certain type of organisms while inhibiting the growth of other organisms. This selective property can be achieved by adding certain dyes, antibiotics, salts or specific inhibitors that will affect the metabolism or enzymatic systems of the organisms.

2.5.2 Differential media

These are mostly used for differentiating closely related organisms with the presence of dyes and chemicals in the media. Example: Two of the most frequently used media in this thesis project was Eosin methylene blue agar (EMB) and Macconkey agar. EMB is a selective and differential media for isolating E.coli form bacteria. Eosin Y and methylene blue are pH indicators. In this

agar *E.coli* shows Green metallic sheen. On the other hand, Macconkey agar is used to isolate gram-negative enteric bacteria that can ferment lactose from the non-lactose fermenting ones.

2.5.3 Eosin Methylene Blue Agar

This medium is used to select and differentiate Gram-negative bacteria. It was prepared by adding 35.96gm of dry powder into 1000ml distilled water. It was put on the burner and stirred regularly to dissolve the powder completely. Later, the flask containing the medium was covered with aluminum foil and taken to the autoclave. After bringing the medium out of the autoclave, it was put on sterile Petri dishes to be plated and hardened. Later, organisms from different sources were streaked by following the previously mentioned streaking method.

2.5.4 Macconkey Agar

Macconkey was the first solid differential media to be formulated which was developed at 20th century by Alfred Theodore Macconkey. This agar is a selective and differential media used for the isolation and differentiation of non-fastidious gram-negative rods, particularly member of the family Enterobacteriaceae. It was prepared in the laboratory by adding 51.5 gm. of dry powder into 1000 ml distilled water. Then it was boiled and later autoclaved and then was put on Petri dishes to be plated.

2.5.5 Xylose Lysine Dextrose Agar

It is selective for Gram-negative organisms. This is prepared by adding 56.68gm of the powder to 1000ml distilled water. Later by boiling with heat, it is dissolved. After dissolving, it is plated on Petri dishes, hardened and streaked on.

2.5.6 Nutrient Agar (NA)

Nutrient agar is a basal growth medium for most non-fastidious organisms. In this study, it was used to isolate organisms from the saline suspension. For the preparation of NA, laboratory grade NA powder sold in correct composition was prepared. About 28gm of NA powder was added to 1000ml distilled water. It was then dissolved in a conical flask and boiled on the burner until bubbles appeared. Later, the flasks were covered in small pieces of aluminum foil and kept in an autoclave for sterilization. After being autoclaved, the liquid medium was put in sterile dry Petri dishes. For a large plate, the 30ml medium was used. The plating was done inside the laminar

airflow. Each plate was kept until the medium solidified. Later, the lids on the plates were put on and the plates were stored for upcoming use.

2.6 Biochemical tests

While characterizing and identifying microorganisms Biochemical tests are most frequently used Techniques for identification of microorganisms. For my study, I had to do indole test, citrate utilization test, methyl red (MR) and Voges-Proskauer (VP).

2.6.1 Indole test*:*

In this case of this test, organisms are grown in peptone water broth that contains tryptophan, which is converted into an indole molecule, pyruvate, and ammonium when the organisms grown in the broth produces enzyme tryptophanase. To test the broth for indole production, Kovac's reagent is added after incubation. A positive result shows a pink/red layer forming on top of the liquid.

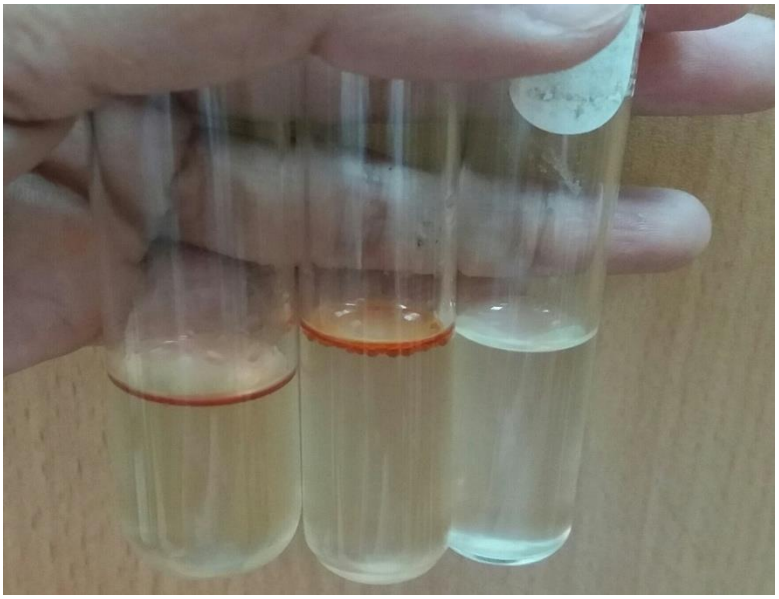

Figure 1.4: a positive result in the middle, positive control in the left corner and negative control in the right

2.6.2 Methyl red and Voges–Proskauer test

These tests both use the same broth for bacterial growth. The broth is called MRVP broth. After Growth, the broth is separated into two different tubes, one for the methyl red (MR) test and one For the Voges-Proskauer (VP) test. For Methyl red test organisms show best results after 24 hours of incubation while for Voges-Proskauer test organisms show best results after 48 hours of incubation. In MR test 5-6 drops of Methyl red is added and without shaken left for 10-15 Minutes. If red color appears then the test is positive. Here methyl red detects the production of acids which is the result of metabolism of various acids fermentation pathway using pyruvate as substrate. When the acid can lower the pH than 4.2 the media turns red, on the other hand when The pH is 6.2 or above it is an indication that butanediol fermentation has taken place.

The VP test uses alpha-naphthol and potassium hydroxide to test for the presence of acetylmethylcarbinol (acetoin), an intermediate of the 2, 3-butanediol fermentation pathway. After adding both reagents, the tube is shaken vigorously then allowed to sit for 5-10 minutes. A pinkish-red color indicates a positive test, meaning the 2; 3-butanediol fermentation pathway is used.

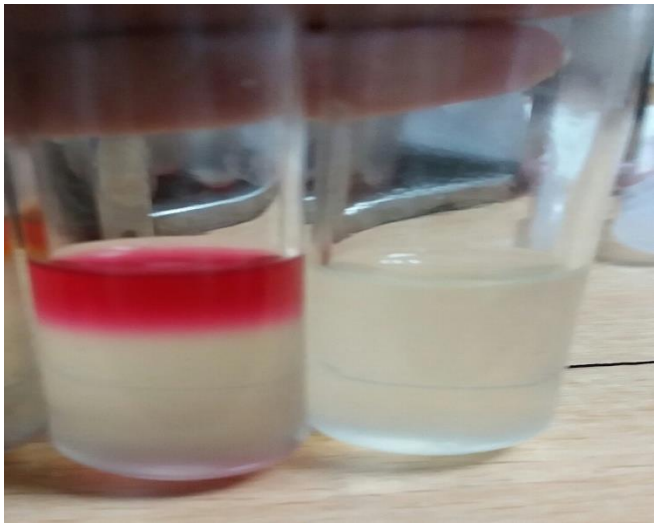

Figure 1.5: Left one shows VP positive and right one shows VP negative

Figure 1.6: Left one is MR positive and right one is negative

2.6.3 Citrate test

Simmons citrate agar was boiled in distilled water and then poured into small vials. After being autoclaved, it was kept at an angled position to create a slant. After the slants hardened, an overnight culture of the isolates was streaked on the slant. It was kept at an incubator for overnight. On the next day, the plants were observed for color change. Blue color indicated a positive result; green color indicated a negative result.

Figure 1.7: Blue color shows a positive result and green color shows a negative result

2.6.4 Storing of the isolates in T1N1

The isolates were stored in T1N1 vials. The T1N1 was prepared by adding 1gm of tryptone casein digest, 1gm of NaCl and 0.6gm of agar powder. The mixture was boiled and poured into glass vials. Later, the vials were autoclaved and allowed to solidify. After it solidified, bacterial inoculum was taken on a sterile needle and stabbed on the media. It was kept in the incubator for overnight. On the next day, 300ul of sterile paraffin oil was added on top of the agar. The vials were tightly capped and stored in room temperature.

2.6.5 Antibiotic Sensitivity Test

Antibiotic sensitivity or antibiotic susceptibility is the susceptibility test of bacteria to specific Antibiotics those are tested. Susceptibility can vary in case of every organism even within their different strains. Therefore a range of susceptible acceptance is used to observe if an organism is susceptible or resistance or shows intermediate resistance to that particular antibiotic. For this Method, antibiotic disks are used that are commercially available. For antibiotic susceptibility procedure, Muller Hinton Agar is primarily used where lawn culture of tested bacteria are done. This procedure is called Kirby-Bauer method. If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition will be demonstrated and if the bacteria is resistant to any particular antibiotic it will grow over the antibiotic disk. Sometimes bacteria gain resistance after a certain period of time and thus creating secondary zones of growth. For this thesis purpose, Antibiotic disk such as Kanamycin, Streptomycin, Gentamycin and Erythromycin were used.

2.7 Antibiotic Mechanism

Erythromycin: inhibits bacterial protein synthesis by binding to bacterial 50S ribosomal Subunits.

Kanamycin: work by binding to the bacterial 30S ribosomal subunit.

Streptomycin: This is a protein synthesis inhibitor. It binds to small 16r RNA of the 30s subunit of the bacterial ribosome.

Gentamycin: It works by irreversibly binding the 30S subunit of the bacterial ribosome interrupting protein synthesis.

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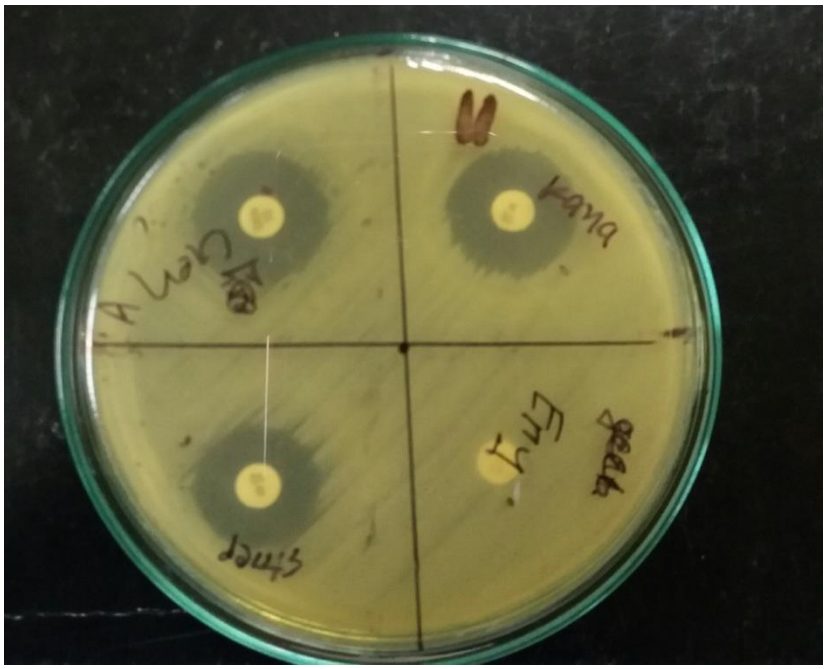

Figure: 1.8: Antibiotic sensitivity and resistance test results in case of *E.coli*

2.8 DNA Extraction

The samples were grown overnight in nutrient broth and then were used to isolate the DNA. The Wizard® Genomic DNA Purification Kit was used in to obtain genomic DNA from the potential experimental bacterial isolate. From the overnight culture, 1 ml from the broth was added to a 1.5 ml micro centrifuge tube. Then it was centrifuged (Centrifuge; Eppendorf, Germany) at 13,500 RPM for 2 minutes to separate the cells. The bacterial pellet was resuspended, and DNA was extracted according to the protocol provided with the Wizard® Genomic DNA Purification Kit. The DNA was stored at -20◦C until use.

2.9 Polymerase chain reaction

This is used to amplify any small amount of DNA by changing making the double-stranded DNA single-stranded and again double stranded while changing the temperature frequently and with a specific thermos table enzyme called Taq polymerase. To identify if any specific region of DNA segment of the gene is present in the sample, two primers: forward and reverse primers are designed and put into the PCR mix along with DNA template and master mix. There are mainly

three steps involved in PCR. These cycles are repeated over and over maximum to 35 cycles for this thesis purpose

Name	Quantity (u L)
Master mix	12
Forward primer	2
Reverse prime	2
DNA sample	2
Nuclease-free water	7
Total	25

Table 1: List of reagents used in PCR

Firstly

For the PCR amplification, the universal 16SrRNA primer was used. The sequence of the forward primer is AGAGTTTGATCCTGGCTCAG and the sequence of the reverse primer is GGTTACCTTGTTACGACTT (Lane, 1991).

The PCR condition and timing were:

Table2: Duration and temperature for PCR.

Process	Temperature	Time
Initial denaturation	94	5 min
35 cycles		
Denaturation	94	1 min
Annealing	55	1 min
Elongation	72	1 min
Final extension	72	5 min

After doing the PCR with 16SrRNA, stx1 primer was also used to find out the presence the Shiga toxin-producing *E.coli.*

2.10 Gel electrophoresis

Gel Electrophoresis is the method for viewing weather primers have bound to the specific binding site. It is mainly used for the separation of DNA molecules based on their size. Gel electrophoresis can be different types; agarose-based gel electrophoresis is mainly done to analyze DNA bands using the dye Ethidium Bromide. In this procedure, electricity is passed through a gel and using the charges of DNA molecules different sizes of DNA bands are separated. As DNA molecules are negatively charged when an electric field is applied they tend to move inside the agarose gel matrix and short molecules ran faster than the bigger and heavy ones. For this method, the Agarose gel base is made with TE buffer and the gel is submerged into the buffer as best results were obtained from this combination. 1% agarose gel was made and was run at 80v for 40 minutes. For comparison, a known band size called ladder was also added to the gel base to interpret. The PCR products were loaded into the gel with 6x loading dye specific results before starting the run. The results were observed using a UV transilluminator.

Figure 1.9: Gel electrophoresis apparatus

Chapter: 3
Results

3. Results

All the samples were collected within sterile boxes and then transferred to BRAC laboratory Without any human touch. Among 24 samples, colonies of 14 were growing on EMB, Macconkey, XLD and nutrient agar.

Table 1: List of isolates

Sample Number	Name of Isolates	Sample Number	Name of Isolates
1.	Family needs in Bashundhara	16.	An open store at Bashundhara
2.	Banani bazaar	17.	Sayed Ali market at Jagonnathpur
3.	Gulshan bazaar	18.	Kuril bazar
4.	TB gate bazar Mohakhali	19.	Agora, Dhanmondi
5.	Shawpno at Bashundhara	20.	Dhali Bari bazaar, Solmaid
6.	Bengal Meat at Bashundhara	21.	Dumni bazaar
7.	Agora, Gulshan 2	22.	(Mohakhali)near BRAC University(an open store)
8.	Dhali super shop, Gulshan	23.	Vatara bazaar
9.	Genius shop, Uttara	24.	Ashkona bazaar
10.	An open store at TB Gate		
11.	An open store at Nikunja		
12.	Shawpno, Nikunja		
14.	Meena Bazar, Banani		
15.	Roadside shop of Mirpur 14		

E.coli was found in case of sample 2, 5, 6, 10, 11, 15, 16, 17, 18, 20, 21, 23, 24. Among all these sample 2, 6, 17, 18 and 22 were selected for further molecular identification.

3.1 Biochemical tests

Biochemical tests showed different types of bacterial isolates compared in this study. After the subculture on selective media, the isolates were taken for biochemical tests. After growing in the selective media and taking for biochemical tests, 14 samples showed a positive result. The ImVic test was done to see their potential to produce several organic compounds. Most of the samples were gram-negative and they were either coccus or rod-shaped. The highest number of gram-negative bacteria was found in Mohakhali area.

Sample number	Gram staining	MRVP				
		Methyl red	Voges-Proskauer	Citrate	Indole	Probable bacteria
1.Family needs, Bashundhara	Rod	-	+	+	-	Enterobacteriaceae
2.Banani Bazar	Rod	+	-	-	+	*E.coli*
3.Gulshan Kacha Bazar	Cocci	+	-	+	-	*Staphylococcus*
4.TB Gate Bazar	Rod	+	+	+	-	Enterobacteriaceae
5.Swapno at Bashundhara	Rod	+	+	+	-	Enterobacteriaceae
6.Bengal Meat, Bashundhara	Rod	+	+	-	+	*E.coli*
7.Agora, Gulshan 2		-	+	-	+	N/A
8.Dhali Super shop, Gulshan	Rod	+	+	+	-	Enterobacteriaceae
9.Genius shop, Uttara	Rod	+	+	+	-	Enterobacteriaceae
10.An open store at TB gate	Cocci	+	-	+	-	*E.coli*
11.An open store at Nikunja	Rod	+	-	-	+	*E.coli*

12.Swapno,Nikunja	Rod	-	+	+	-	Enterobacteriaceae
13.Meena bazaar ,Dhanmondi	Cocci	+	-	+	-	*Staphylococcus*
14.Meena Bazar, Banani	Rod	-	+	+	-	Enterobacteriaceae
15.Roadside shop at Mirpur 14	Rod	+	-	-	+	*E.coli*
16.An open store at Bashundhara	Cocci	+	-	+	-	*E.coli*
17.Open store at Kochukhet	Rod	+	-	-	+	*E.coli*
18.Kuril Bazar	Rod	+	-	-	+	*E.coli*
19.Sayed Ali store at Bashundhara	Cocci	+	-	+	-	*Staphylococcus*
20.Solmaid Bazar	Rod	+	-	-	+	*E.coli*
21.Agora ,Dhanmondi	Rod	+	-	-	+	*E.coli*
22.Mohakhali behind Brac university(an open store)	Cocci	-	-	-	-	*Bacillus*
23.Vatara Bazar	Rod	+	-	-	+	*E.coli*
24.Ashkona Bazar	Rod	+	-	-	+	*E.coli*

Figure 1.10: Bacterial colonies growing on EMB Agar, Macconkey Agar, XLD Agar, and Nutrient agar

3.2 Results of Gram Staining

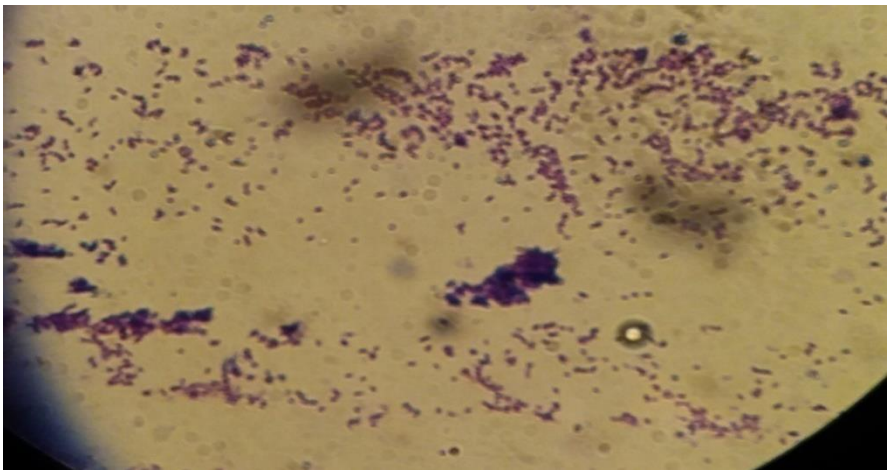

Figure 2.1: Cocci shaped bacteria

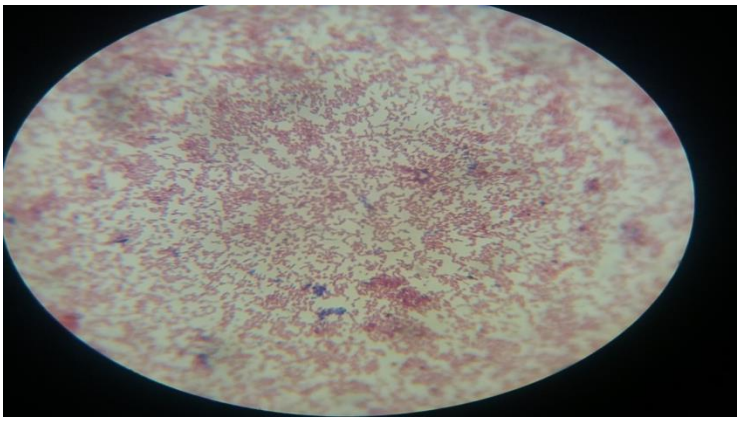

Figure 2.2: Rod-shaped bacteria

Most of the samples were gram-negative, whereas very few were gram-positive.

3.3 Antibiotic sensitivity test

Table 4: Result of antibiotic sensitivity testing done on the isolates

Serial	Kanamycin		Gentamycin		Erythromycin		Streptomycin	
	ZS	Int	ZS	Int	ZS	Int	ZS	Int
2	21	S	20	S	0	R	18	S
5	18	S	20	S	7	S	20	S
6	21	S	20	S	10	S	19	S
10	20	S	19	S	7	S	19	S
11	19	S	20	S	O	R	19	S
15	20	S	20	S	O	R	19	S
16	20	S	20	S	0	R	1	S
17	18	S	20	S	0	R	15	S
18	21	S	21	S	0	R	20	S
20	10	S	15	S	0	R	10	S
21	20	S	21	S	10	S	20	S
22	20	S	20	S	0	R	10	S
23	20	S	21	S	7	S	19	S
24	10	S	20	S	0	R	15	S

S=Sensitive R=Resistant

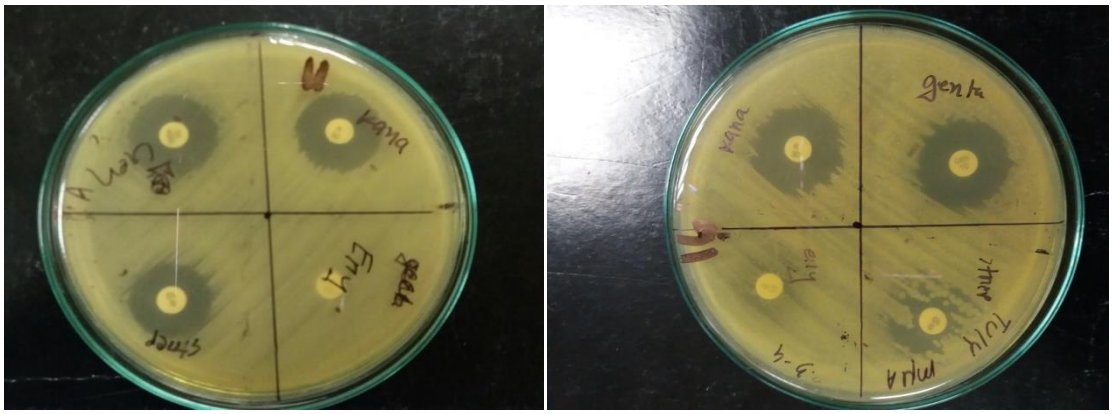

Figure 2.3: Antibiogram test

3.4 Polymerase chain reaction and electrophoresis results:

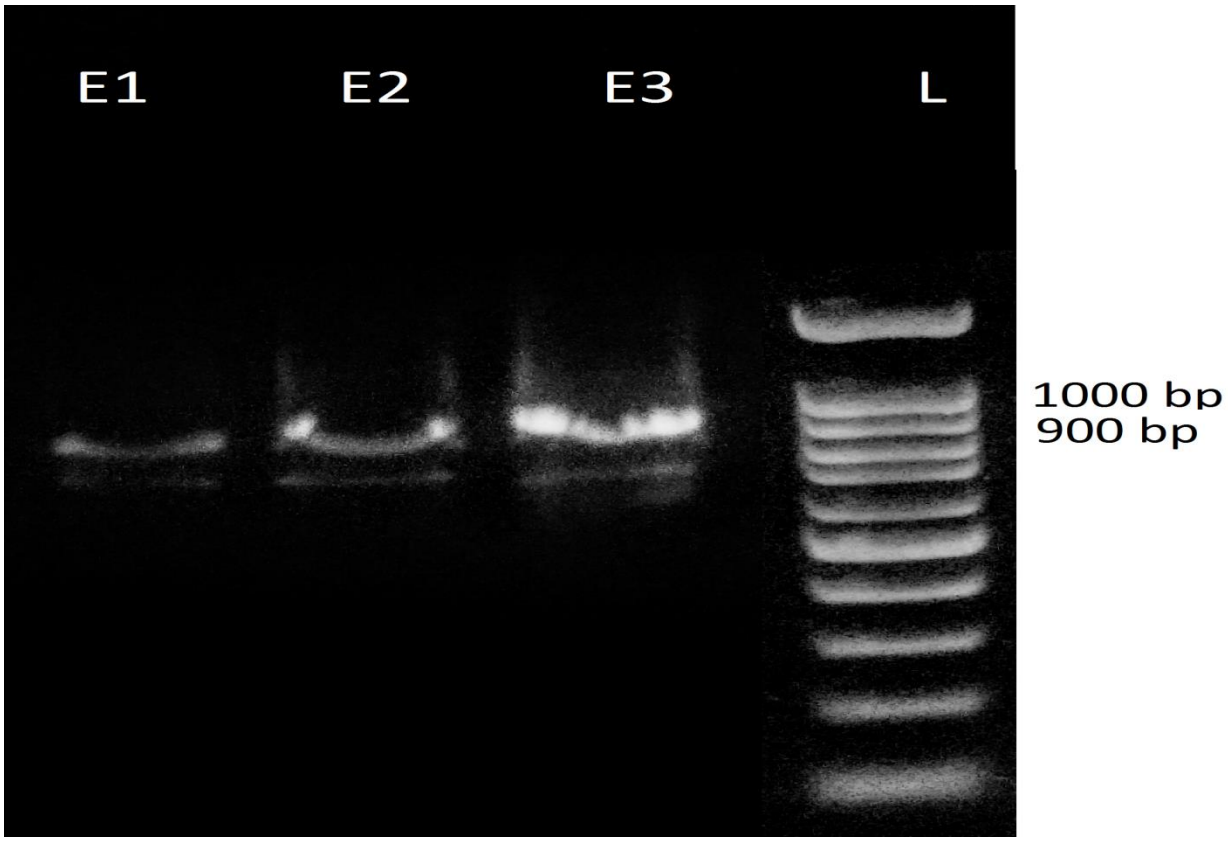

Figure 2.4: Presence of 16srRNA gene in sample 2, 18 and 22

These reasons for which 16SrRNA has been used are (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1500bp) is large enough for informatics purposes (Patel 2001; Větrovský and Baldrian2013). Aside from biochemical and phenotypical methods of bacterial identification including E. coli and Shiga toxin-producing E. coli, 16S rRNA gene is also used for confirmation of bacterial isolates. The isolates which were suspected as pathogenic were taken for PCR with *stx1*and 16SrRNA. The bands found for STX 1 and 2 were not that much clear. But the bands for the 16SrRNA gene came out well according to their expected band size. Moreover, for construction of phylogenetic tree to compare evolutionary relationship, 16s rRNA sequence is considered highly effective. After getting the 16s rRNA region,

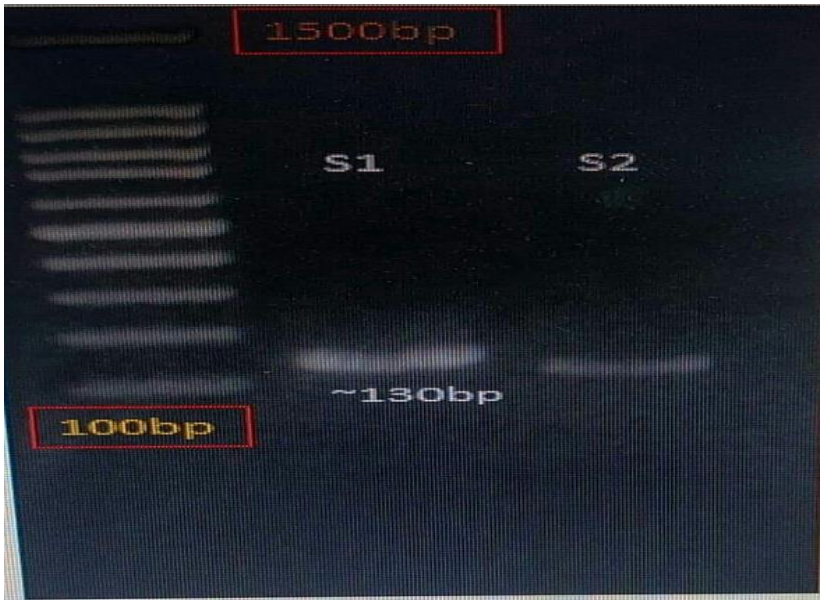

Figure 2.5: the presence of STX 1 gene under UV light (sample 2 and 6)

After that 2 of the samples were sent for sequencing to find out the strain exactly.

3.5 Partial Sequencing

Among the 3 samples containing 16SrRNA gene sequencing were done 2 samples from Invent Technologies (Apical Scientific, Malaysia). The sequence came out well. Though there was some noise. Later work was done with the 2 sequences.

The sequences of the 2 samples were copied to BLAST software ⇨ Nucleotide BLAST ⇨ Paste to FASTA and then click BLAST.

Among 2 of the samples, the sequence of sample 1 was similar to different novel *Enterobacter* strain like *Enterobacter xiangfangensis s, Enterobacter cloacae, complete genome, Enterobacter hormaechei strain, Enterobacter kobei strain, Enterobacter cancerogenus* etc.

The sequence of sample 2 was similar to *Lactobacillus brevis.*

Chapter: 4
Discussion

4. Discussion

The aim of the study was to find out gram-negative *E.coli* and other enteric bacteria in raw beef that can cause potential health problems as they are harmful. There have been many studies on meat like chicken, pork, semi-cooked, cooked chicken and beef and foods made from meat. These included searching for *Enterobacter aerogenes, Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella spp, and Lactobacillus spp* and so on. While the organisms like *E. coli* and *Salmonella* are enteric bacteria, gram-positive *S. aureus* or *B. cereus* are found on the skin and soil. These organisms can cause massive health damage that can vary from toxic shock syndrome to diarrhea.

However, there was no research work done on raw beef in Bangladesh. Beef is frequently consumed and very popular in this country. A study on the microbial contamination of raw beef is highly important.

Preparing fast foods and other types of meals with meats especially raw beef is very common nowadays. The alarming threat is that while handling raw beef sellers never follow any hygiene which they should. Another Thing is, there are some bacteria like *E.coli*, which has become a very common bacterium present in contaminated raw beef. In my research, I have collected 24 beef samples from different shops, including departmental stores. After that those samples were taken to Brac university biotechnology and microbiology laboratory and followed all the steps of isolation of bacteria in aseptic condition. The threatening fact is that among 24 samples I have found 13 gram-negative bacteria which include *E.coli*, different novel strains of *Enterobacter* and 1 gram-positive bacterium which is *Lactobacillus brevis.*

In the study of Christophe Mollet, Michel Drancourt and Didier (1994), where they analyzed that among some taxa such as *Enterobacteriaceae*, variation within this gene does not allow confident species identification. They have proved that rpoB is more useful and effective instead of universal primer 16SrRNA. Maybe this is the reason for which the sample which I have identified as *E.coli* came out as Enterobacteriaceae after sequencing. Another thing is, I have found out the partial sequences, not the whole genome sequences.

In Bangladesh, sellers are very careless about food hygiene. So, they don't bother to keep the area inside and outside of their shop cleaned. They even keep the raw beef whole day without freezing even sometimes till night. Some of the butchers were found to sell one-day raw beef on

next day. Another reason behind this contamination is the carelessness of people at home. When people handle raw beef at home most of the people do not follow any hygiene. Like, keep touching the other ready foods without washing their hands. And some don't refrigerate the beef properly like the ready to eat foods and raw beef are placed in nearby place of the refrigerator so the bacteria can easily be transmitted to foods.The samples containing gram negative and positive bacteria were confirmed through growing on different media at first. Then the biochemical test was done to further confirmation and after that more pathogenicity containing novel organisms, genomic DNA was isolated with Promega kit following the given steps.

Antibiotic sensitivity testing was also done using kanamycin, Gentamycin, Streptomycin and Erythromycin. 50% of the samples were sensitive against kanamycin, gentamycin, and streptomycin .But 7 samples showed resistance to Erythromycin, which is a matter of concern. Gradually the rate of antibiotic resistance is increasing and therefore the rate of severity is also increasing.

The extracted DNA s were gone through PCR with 16SrRNA Gene. Stx1 primers were also used to find the gene during PCR. 1 sample showed probable bands for Stx 1. The 3 samples which were containing 16srRna (Including 1 Stx) were set for further sequencing. It was done from Apical Scientific, Malaysia.According to Osaili *et al,* (2013), cattle are the main asymptomatic reservoir of Shiga toxin-producing *E. coli* that are capable of causing illness in human. In a recent study by Osaili *et al,* (2013), it was observed that 7.8%of beef samples were contaminated with Shiga toxin-producing *E.coli*. Also, the incidence of Shiga toxin-producing *E. coli* in this study was slightly higher when compared to a study carried out in South Africa, which indicates an incidence rate of 74.5 % (Vorster et al. 1994; Radu *et al*. 1998). The global increase in meat consumption and the consequential food-borne disease outbreaks call for continuous monitoring of raw meat and meat processing plants for the presence of *E. coli* and Shiga toxin-producing *E. coli*. In a recent study in China, 3 (0.59 %) of the 510 samples tested were positive for *E. coli* O157: H7 (Dong *et al*, 2015).

The use of both Shiga toxin genes stx1 common for the molecular characterization of Shiga toxin-producing *E. coli* (Fode Vaughan *et al*, 2003; Oporto *et al*, 2008; Sahilah *et al*, 2010; Tahamtan *et al*, 2010). According to Beutin (2006), these originated from S. dysenteriae and similar to Shiga toxigenic group of *E. coli* (STEC), serotype O157: H7 and other enterohaemorrhagic *E.coli*. These genes have also been found in Shiga toxin-producing *E.coli*

from other sources such as cattle fecal materials, dairy products, sheep, swine and environmental samples (Oporto *et al*, 2008; Tanaro *et al*, 2010; Ahmed and Shimamoto 2014; Ayaz *et al*, 2014; Bai *et al*, 2015).

The sequences of 2 of the bacterial samples came out good. Partial sequences were placed in BLAST software. By following the provided steps the strains which the samples were similar nearly 99% were found. Sample 1 contains 5 different novel strains of Enterobacter which are proved harmful in different ways for a human. Sample 2 contains gram-negative bacteria. The sequence of sample 2 was 99% similar to gram-negative *Lactobacillus brevis.* The research paper was found which showed the presence of *Lactobacillus* in pork meat products. Though, it was supposed to be *E.coli* strain. As we did partial sequencing but the presence of *Lactobacillus brevis* in raw beef is new. *Lactobacillus brevis* can be used as probiotic. But how isolated lactobacillus can work as probiotic about this fact further research is needed.

These results show that the ribosomal RNA sequencing is a powerful technique for bacteria identification, and the results in this study are in line with the previously developed by Fattahi *et* al, (2013) as a PCR target for *E. coli* detection in rainbow trout. Patel (2001) further reported that the use of 16S rRNA gene sequence in the study of bacterial taxonomy is widely for some reasons, which include the following: (a) its presence in almost all bacteria, often existing as a multigene family or operons; (b) the fact that the 16S rRNA gene (1500 bp) is large enough for informatics purposes; and (c) the fact that the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are more accurate measure of time (evolution). In conclusion, the use of 16S rRNA gene in this study for the PCR detection and characterization is still in place as 16S rRNA is a conserved and good site in bacteria especially *E.coli* for PCR detection and characterization as mentioned above study by Patel (2001); this researcher was able to successfully use 16S rRNA gene sequencing in the clinical laboratory for bacterial pathogen identification. Another researcher, Woo *et al,* (2008), also uses 16S rRNA gene sequencing in clinical microbiology laboratories for bacterial identification.

Chapter: 5
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