Isolation, Identification and Molecular Characterization of Pathogenic Organisms Obtained from Meat samples (Cooked, Semi-cooked and Raw) of Different Areas of 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

Rabeya Tafsire Rudhy

ID: 13236007

June, 2017

Biotechnology Program

Department of Mathematics and Natural Sciences Department

BRAC University

Declaration by the researcher

This is to certify that the research work done by the title of “Isolation, Identification and Molecular Characterization of Pathogenic Organisms Obtained from Meat samples (Cooked, Semi-cooked and Raw) of Different Areas of Dhaka City” was done in the lab of Mathematics and Natural Sciences Department at BRAC University under the supervision of Kashmery Khan, Lecturer BRAC University. It is also certified that all the reach data and result are original and authentic.

Rabeya Tafsire Rudhy

Certified by

Kashmery Khan

Supervisor

Lecturer

Department of Mathematics and Natural Sciences

BRAC University

Acknowledgement

I am grateful to the almighty for letting me experience the wonderful journey of this life. I would like to give my gratitude towards Professor Dr. A. A. Ziauddin Ahmad, Chairperson, Mathematics and Natural Sciences Department (MNS), BRAC University and to Professor Naiyyum Choudhury, former coordinator of the biotechnology and microbiology program, Mathematics and Natural Sciences Department (MNS), BRAC University, Chairman, Bangladesh Atomic Energy Regulatory Authority (BAERA) for showing me a path through which I will build my life upon.

At this point I want to show my respect to Ms. Kashmery Khan, Lecturer MNS Department who was my thesis supervisor. From the beginning till end she showed me the ways to scrutinize a situation and how to solve it in innovative ways. At the same time Ms. Zubaida Marufee Islam, Lecturer, MNS Department gets the credit for allowing me the creative thinking and giving encouragement in each step.

I also want to thank my honorable course instructors Dr. Aparna Islam, Associate Professor and coordinator of postgraduate program, Ms. Romana Siddique, senior lecturer and current coordinator of undergraduate Biotechnology Program, Ms. Jebunnesa Chowdhury, Assistant professor and Dr. Mahboob Hossain, Associate Professor of Mathematics and Natural Science Department of biotechnology for their immense support and faith in me.

It would have been extremely difficult for me to complete the thesis without the help of Ms. Asma Binte Afzal and Ms. Shahana Chowdhury, Lab officers, BRAC University, Salman Khan Promon and Ms. Nahreen Mirza, Teaching Assistants, BRAC University. They corrected me in every step and enabled me to meet the desired result. Last but not the least, I am forever grateful to my parents and my two brothers for believing in me, who made me the person I am today and who see me as one of the prominent figures in the field of successful scientific community.

Rabeya Tafsire Rudhy

Abstract

Meat is the main source of protein now a days all over the world. From the people living under boarder line of poverty to high maintenance society, beef, mutton and chicken are taken as important source of proteins. Although beef and mutton are the most desirable ones; because of the availability and low price chicken is consumed even more than the rest. Due to improper handling and poor hygiene at the time of preparing the food, meat related foods are extremely unhealthy in Bangladesh. In this study 43 samples were taken from different areas of Dhaka city, the results supported the mentioned concern. Most of the meat samples were cheap and on the list of regular intake of students studying in these areas. Some of the samples showed almost 80-90% contamination with *Escherichia coli that* is a coliform bacterium and found in human excreta mostly. Most of the samples were cooked and processed; nonetheless they did not lack any less of organisms or contaminations. Chicken samples were collected in three states, which were cooked, semi-cooked and raw. Some of the cooked and most consumed samples showed presence of 6-7 organisms. The organisms identified so far are *Escherichia coli*, *Enterobacteor aerogens*, *Salmonella spp*, *Staphylococcus spp*, *Pseudomonas* and others. The purpose of this study is to make the authorities regulating food safety aware of such contamination and take necessary steps to avoid this sort of disorientation towards food related business. In our research biochemical tests such as streaking on EMB and MacConkey agar, MRVP, TSI etc. were done, in addition morphological characteristics of the single colonies of isolated microorganisms were also examined and interpreted. DNA from samples positive for *E. coli* were isolated and by gel electrophoresis their bands were examined where most of them gave positive bands for STEC, meaning positive for pathogenic *E. coli* strains.

Key words: Meat, chicken, pathogenic, coliform, stx1, multiplex PCR

1.1 Introduction

Meat and meat related consumption of each year is around 75 pounds around the world (Gould S. and Friedman L. F., 2015) In Bangladesh few people are vegetarian, other than them all solvent families and family members belong to them consume meat at least once daily as a key protein source. Many cuisines are available in our country such as Mughlai, indian, thai, Mexican and their main ingredient is meat. People love to eat biriyani, roast, burger, fried meat, sasliks etc. However, food borne disease is a pervasive 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). This is the urge of present time to determine how pathogenic these bacteria are and to what extent they can have effects in human body.

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. (Sharma and Chattopadhyay, 2015) most microorganism related contamination is due to zoonotic diseases. They also analyzed 200 meat samples of chicken and mutton from Kolkata and found similar types of bacteria such as *Escherichia coli, Pseudomonas, Klebsiella, Enterobacter aerogens* etc. (Sheikh *et al*, 2013) from three samples two were confirmed as *Escherichia coli* 0157:H7 by multiplex PCR. Samples were collected and incubated in buffered peptone water for further analysis (Matheson *et al*, 2004).

1.1 Meat

Different types of meat such as beef, chicken, mutton are consumed as main protein source from the beginning of human life. At the very beginning it was consumed raw, then after the discovery of fire it was consumed cooked. However, now-a-days meat is consumed in various ways, cooked, semi cooked or by other preparations as salads. Meat is composed of water, protein and fat. Meat production industry is one of the highest valuable one. Unprocessed meat can be

spoiled or rotten within hours or days and can cause infection at a quick rate. The infections can be caused by bacteria, fungi, dirt etc. In this thesis project we tried to identify the bacteria related with spoilage of meat samples collected.

1.2 Diarrhea

Diarrhea is caused by the increase of bowl frequency movements resulting in looseness of stool and mucous stool. It can also be a result of secretion of fluid into intestine or reduced absorption of fluid from the intestine. Symptoms associated with it are abdominal cramp, watery stool etc. There are two types of diarrhea, absolute and relative. Absolute diarrhea is the result of five or more bowel movements a day consisting liquid stool. On the other hand, relative diarrhea is the result of increase bowel movement than one individual's usual habit. Diarrhea can be acute or chronic; the complications include dehydration, loss of electrolytes, irritation in bowels and anus etc. Stool test are useful to examine the microorganisms or food poisons behind the diarrhea. Tests include examination of white blood cells, or enzymes produced by parasites or growing bacteria in culture plates (Marks, M. J., 2017)

1.3.1 Foods associated with Diarrhea

Certain foods may trigger diarrhea in some people. Some foods to avoid that may cause diarrhea include fried foods, foods with rich sauce, fatty cuts of meat, citrus fruit, artificial sugar, too much fiber, fructose etc.

1.3.2 Prevention

Dehydration can be treated with oral saline, home remedies. Absorbent, anti – motility medications, IV fluids can also be effective. In serious cases antibiotics if only culture proven bacteria are isolated and identified.

1.4 Isolation of Different Bacteria

Isolation of different bacteria was done by incubating the samples in enriched broth such as EC broth and then transferred to selective media. Spread plate technique was also done to identify

various bacteria origins from incubated samples. Most frequently found bacteria were *Eschericia coli, Pseudomonas, Streptococcus pyogenes, Staphylococcus epridermis, Staphylococcus aureus, Bacillus spps, Micrococcus lutues, Serretia mercenes, Salmonella typhi, Klebsiella, Shigella* etc. (Thanigivel and Anandhan, 2015)

1.4.1 *Escherichia coli*

Escherichia coli is a gram negative, facultative anaerobic, rod shaped, coliform bacteria. Most *E. coli* strains are harmless but some of them can cause serious food poisoning and food contamination. The harmless strains are part of normal flora of human gut were they help the host to produce vitamin K_2. These bacteria can be easily grown and cultured in laboratory. With the use of it many milestone in pharmaceutical and in medical zones were successful including the recombinant DNA technology. However, these bacteria are evolving and creating major health hazards in human body.

Scientific classification

Domain:	Bacteria
Kingdom:	Eubacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacterials
Family:	Enterobacteriaceae
Genus:	Escherichia
Species:	*Escherichia coli*

1.4.1.2 The role of *E. coli* in various inflammatory diseases

E. coli remain as normal flora in human body although while virulent, it can cause urinary tract infection, neonatal meningitis, hemorrhagic colitis etc. The signs and symptoms include vomiting, abdominal cramps, diarrhea and sometimes fever. It can also cause bowel tissue necrosis.

The most virulent strain of *E. coli* is *E. coli* 0157:H7 that produces toxins like shiga toxin which causes premature destruction of red blood cells. Otherwise, *E. coli* produce six types of infections. They are given below:

- Enterotoxigenic E. coli (ETEC)
- Enteropathogenic *E. coli* (EPEC)
- Enteroaggregative *E. coli* (EAEC)
- Enteroinvasive *E. coli* (EIEC)
- Diffusely adherent *E. coli* (DAEC)
- Uropathogenic *E. coli* (UPEC)

1.4.1.3 Enterotoxigenic *Escherichia coli* (ETEC)

ETEC is one of the leading bacterial causes of diarrheas in developing countries. It also causes travelers' diarrhea. It is produces a heat labile enterotoxin and a heat stable enterotoxin. It produces infection with profuse, watery diarrhea with no blood. It can be treated with rehydration therapy and antibiotics. ETEC uses fimbrial adhesins (projections from the bacterial cell surface) to bind enterocyte cells in the small intestine. ETEC can 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.

1.4.1.4 Enteropathogenic *E. coli* (EPEC)

EPEC also causes diarrhea but the molecular mechanisms of colonization and aetiology 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. (Theresa J. Ochoa and Carmen A. Contreras, 2011)

1.4.1.5 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.

1.4.1.6 Enteroinvasive *E. coli* (EIEC)

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

1.4.1.6 Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *Escherichia coli* (DAEC) have been considered a diarrheagenic 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 to virulence.

1.4.1.7 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 enterohemorrhagic *E. coli* (EHEC). This pathotype is the one most commonly heard about in the news in association with foodborne outbreaks.

1.4.1.8 Phylogenetic tree of *E. coli* is given below:

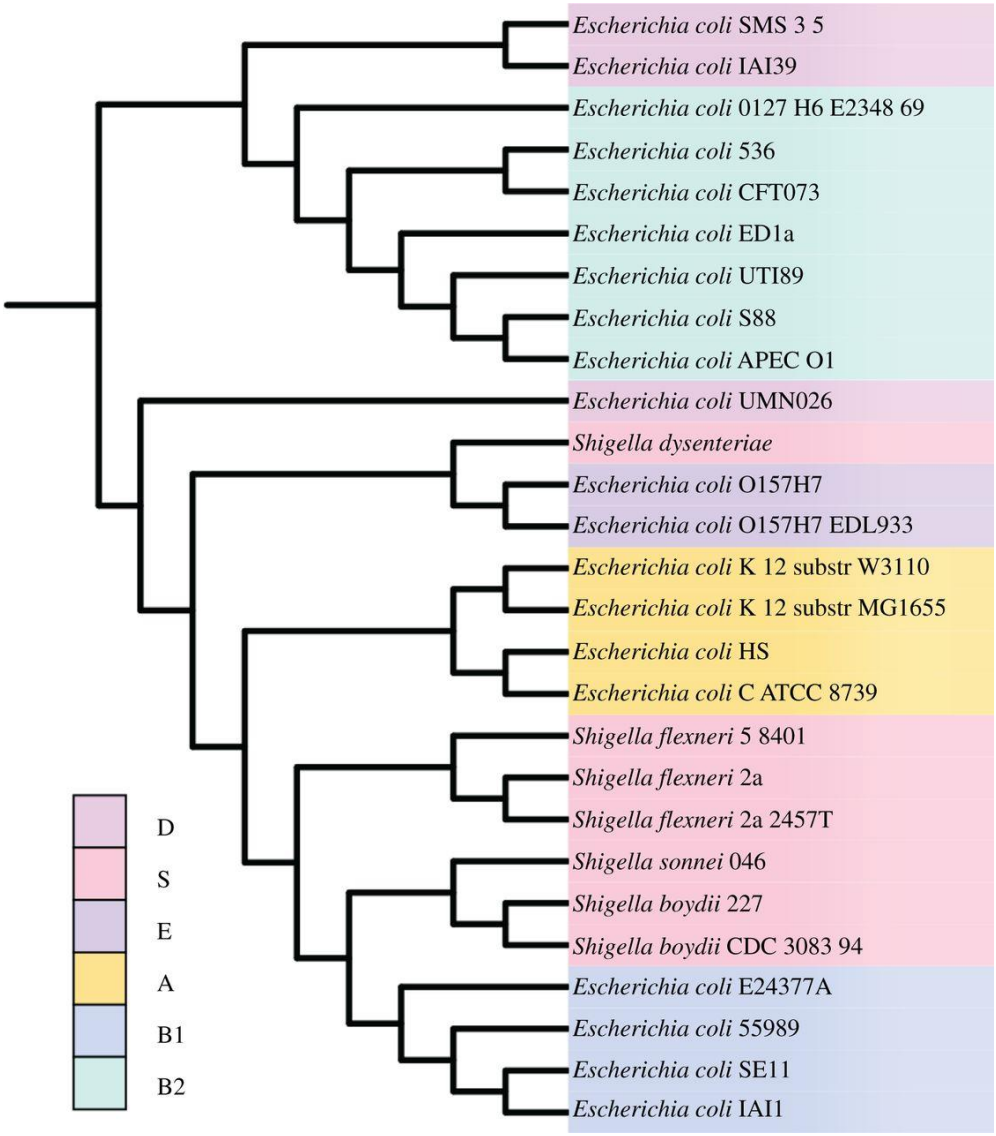

Figure: 1.4.1 Phylogenetic tree of *E .coli (*Elizabeth S., Mark A. R. ,2012)

1.4.1.9 Epidemiology of gastrointestinal infection

Transmission of *E. coli* occurs mostly via 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 reservoir of *E. coli* 0157:H7 and they can transfer it 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.

1.4.1.10 Antibiotic therapy and resistance

E. coli severe infections are usually treated with antibiotics but different strains response to different antibiotics. As gram negative bacteria *E. coli* responds to Amoxicillin, Aztreonam, Trimethoprim-slfamethoxazole, Ciprofloxacin etc. 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 (Papadopoulos D. *et al*, 1999). This may lead to a resistance strain, such as, MRSA.

1.4.1.11 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 lactamses. They are resistant to Penicillin and Cephalosporins. These strains are very difficult to treat and are thought to be one kind of superbug in process.

1.4.2 *Pseudomonas*

Pseudomonas is a gram negative aerobic bacterium. The most common and studied species of *Pseudomonas* are *P. aeruginosa, P. fluorescens, P. syringae* etc. It can be found in water, plant

seeds of dicots. They can form biofilms. They spoil foods and can generate a fruity odor. Most *Pseudomonas spp* are naturally resistant to penicillin and the majority of related beta lactam antibiotics. But they are mostly sensitive to Ticarcillin, Imipenem, Ciprofloxacin, Tobramycin, Gentamycin etc. *Pseudomonas* species give green color in differential media such as cetrimide media. They are common cause of spoilage of milk, cheese, meat and fish.

1.4.3 *Bacillus*

Bacillus is a gram positive rod shaped bacteria. They can be obligate aerobes or facultative anaerobes. They are ubiquitous in nature which makes them deadly. They can be living free or parasitic. They can form endospores in stressful environments but these are not true spores. They can remain dormant for a long period of time and can be viable while nourishment is available. Some important bacillus species are *Bacillus anthrax, Bacillus subtilis, Bacillus thurengiensis* etc. Among these *Bacillus anthrax* created endemic causing the disease anthracic in 2014 effecting 170 patients mostly from a district called Meherpur, who were in close contact of bovines (Robert H., 2014).

Other than this, *Bacillus* is also being used as model organisms sometimes. A portion of *Bacillus thuringiensis* genome was incorporated into cotton and corn crops resulting pest resistant GMOS. *Bacillus* are also able to produce large quantity of enzymes such as alpha amylase, protease subtilisin etc.

1.4.4 *Klebsiella*

Klebsiella is a non-motile, gram negative, rod shaped bacteria with a polysaccharide based capsule. It appears to be found everywhere in the nature such as in water, soil, plants, insects, animals and in humans. *Klebsiella* are facultative anaerobes, most strains can survive with citrate and glucose as their sole carbon source and ammonia as their sole nitrogen source. They produce a prominent capsule or slimy layer that can be used for their serological identification. They can be found in human mouth, nose, and gastrointestinal tract as normal flora but can be opportunistic pathogens in immuno compromised individuals. They can cause pneumonia, urinary tract infection, septicemia, meningitis, diarrhea etc.

1.4.5 *Serratia marcescens*

Serratia marcescens is a rod shaped gram negative bacteria. It is a human pathogen involved in hospital acquired infections. It can be found in respiratory tracts or urinary tracts of patients and in gastrointestinal system of children. It can be commonly found in bathrooms, shower, and toilet water line or in basins. It projects red color that can be easily confused with drop of blood. It also gives red pigmentations in agar such as nutrient agar. Once established it can be very hard to remove, however bleach based disinfectant can be useful. It is also called the flesh eating bacteria when contagious. Its pigmentation is due to a pigment called prodigiosin. It can also cause urinary tract infection, tear duct infection, keratitis, conjunctivitis, respiratory tract infection etc.

1.4.6 *Shigella*

Shigella is a gram negative, facultative anaerobe, non-spore forming, non-motile rod shaped bacteria. It is genetically closely related to E coli. It is the causative agent of shigellosis, which is a disease caused in only primates but not in other mammals. When it occurs typically dysentery is noticed, but diarrhea can also be an after effect. Shigellosis infection can be caused by ingestion; generally it invades epithelial lining of colon, causing severe inflammation and death of lining cells of colons. *Shigella* produces toxins such as ShET1 and ShET2, which may contribute to diarrhea. It can result is stool containing blood, mucous or pus. In rare cases, it can cause seizures in children.

1.4.7 *Micrococcus luteus*

Micrococcus luteus is a gram positive, nonmotile, coccus shaped pigmented bacteria. It is urease and catalase positive. It can be found in soil, dust, water, air as normal flora. It can colonize in human mouth, oropharynx or upper respiratory tract. It can be opportunistic in case of sick patients. It gives bright yellow colonies in nutrient agar. It can be easily mistaken with *Staphylococcus aureus*, therefore a bacitracin susceptibility test is performed. It has one of the smallest genomes of free living actinobacteria comprising a single circular chromosome.

1.4.8 *Streptococcus*

Streptococcus is a cocci shaped, gram negative bacteria. They are mostly facultative anaerobes. They can cause meningitis, bacterial pneumonia, endocarditis etc. Common *Streptococcus* are not pathogenic and residues in mouth, skin, intestine and upper respiratory tract. They are classified based on their hemolytic properties. Alpha-hemolytic species cause oxidization of iron in hemoglobin molecules within red blood cells, giving it a greenish color on blood agar. Beta-hemolytic species cause complete rupture of red blood cells. On blood agar, this appears as wide areas clear of blood cells surrounding bacterial colonies. Gamma-hemolytic species cause no hemolysis. *Streptococcus pneumonia* is alpha hemolytic strains whereas *Streptococcus pyogenes* are beta hemolysis strains. *S. pyogenes* contains streptolysin, an exotoxin, is the enzyme produced by the bacteria which causes the complete lysis of red blood cells. There are two types of streptolysin: streptolysin O (SLO) and streptolysin S (SLS).

1.4.9 *Staphylococcus*

*Staphylococcu*s is a gram positive, cocci shaped bacteria and form in grape like clusters. The genus *Staphylococcus* includes minimum 40 species but the most common one is *S. aureus*. All the *Staphylococcus* species can grow in the presence of bile salts. *S. aureus* can be found in nasal tract or skin in normal human. They also can be found in soil. A recent study indicates *Staphylococcus* can be more harmful than suspected as they are able to transfer gene horizontally with their mobile genetic elements. As a result they can grow antibiotic resistance rapidly and can produce antibiotic resistant strains in future.

1.4.9.2 Methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA is a superbug which is caused by Methicillin resistant *Staphylococcus aurues*. It has developed as the result of horizontal gene transfer that does not respond to most antibiotics which includes Penicillin, Methicillin, Oxacillin etc. They are prevalent in hospitals, prisons and in nursing homes. They invade open wounds, invasive devices such as catheters and attack individuals with weak immune system.

1.4.10 *Salmonella typhi*

Salmonella typhi is a motile, facultative anaerobe, rod shaped, gram negative bacteria. It causes typhoid fever in human and no other known natural reservoirs are found other than human. Worldwide, typhoid fever affects roughly 17 million people annually, causing nearly 600,000 deaths. It can be identified by the growth on Macconkey and EMB agar. As the bacteria are strictly non lactose fermenting it does not growth in them. It can also cause illnesses such as paratyphoid fever, and food poisoning (salmonellosis). *Salmonella typhi* contains an endotoxin which increases the virulence of the strain; it produces and excretes a protein known as invasion. It is able to inhibit the oxidative burst of leukocytes, making innate immune response ineffective. The entry of the bacteria is oral fecal route and the incubation period is 1-3 weeks.

1.5 Antibiotic susceptibility Test

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 Mullar Hington 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 Ampicillin, Amoxicillin, Ceftriaxone, Chloramphenicol, Erythromycin, Polymixin B, Nalidixic acid, Oxicillin etc. were used. (Bauer AW *et al*, 1997)

1.6 Molecular diagnostics

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

1.6.1 DNA

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. DNA is a molecule that carries the genetic information of an entire organism. It comprises with 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) Multiplex PCR

1.6.2 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 thermostable enzyme called Taq polymerase. To identify if any specific region of DNA segment of 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 small amount of desired DNA up to 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. We put forward and reverse primer for **stx1 gene** in the PCR procedure to detect **shiga toxin** in ***E. coli* samples**.

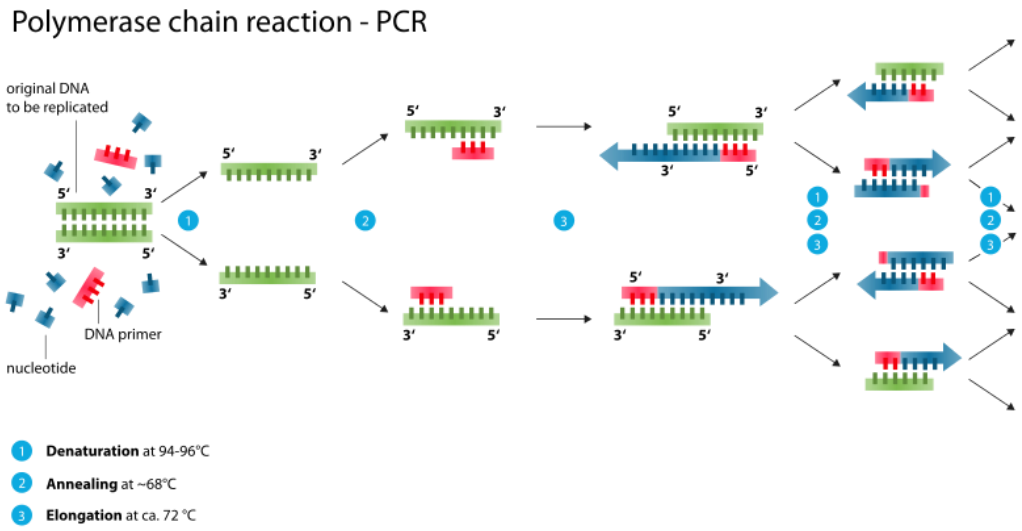

Figure 1.6.1 PCR cycles

1.6.3 Gel electrophoresis

Gel Electrophoresis is the method for viewing weather primers have bind 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.

1.6.4 Multiplex PCR

Multiplex PCR is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment. Multiplex PCR reactions can be subdivided into two categories; Single template and multiple template PCR reaction. The single template PCR reaction uses a single template to pair with several pairs of forward and reverse primers, whereas the multiple templates PCR use multiple templates and several primer sets. The applications of multiplex PCR are pathogen identification, High throughout SNP Genotyping, Mutation analysis, Gene Deletion Analysis, Template quantification, Linkage analysis, RNA detection, in forensic studies etc.

Advantages of Multiplex PCR

- Multiplex PCR is efficient than normal PCR and more accurate
- There is more chance to adjust the correct temperature and time without loss of raw materials.
- It can ensure the template quantity and quality at the very primary level.

1.7.1 Rationale Objective of the Study

Many bacteria that produce shigatoxin show similar kind of symptoms and after effects. It is important for us to inform people about the harmful effects that may become life threatening for them. To raise awareness and to make people follow strict rules to ensure proper health care system this study results must be taken into consideration.

1.7.2 Objective Of the study

The study was conducted to check for overall hygiene different meat samples around Dhaka city. Also to point out how many different types of bacteria are responsible for causing diarrheal disease in the population, especially in children was the concern of the study. So, finally the study aims towards the following objectives which are:

1. Determination of the level of pathogenicity of organisms found in the meat samples and how the bacteria react to specific antibiotics.
2. Finding specific antibiotic against the infectious diseases.

3. Devising specific method of meat processing so that no contamination occur.

1.7.3 Future Objectives

The study includes different types of meat with different preparation. As a result, what types of meat are better to consume are analyzed. The future objectives of the study are as follows:

- Increasing sample size to find out the overall scenario of meat market.
- Molecular analysis of different organisms, including pathotyping and serotyping.
- Examining different antibiotics for their efficacy.
- Identification and characterization of proteins involved in causing same kind of pathogenic symptoms and disease.
- Making general people aware of health hazards related to improper handling of meat processing and consumption.

The purpose of the study serves to grab attention of authority who supplies meat and meat related foods for huge population around Bangladesh and of those who control the quality of these food types.

2.1 Collection of samples

Samples were collected from different locations of Dhaka city, from famous restaurants to local markets. Samples were basically three types: raw, semi cooked and cooked. Samples were collected in sterile boxes and brought in the laboratory of BRAC University with minimum time wastage. Samples were occasionally mixed with other ingredients such as spices in case of curry items.

Raw chicken **Chicken curry** **Grilled chicken**

Figure2.1 collected samples in sterile boxes

2.2 Homogenization

Around 25 grams of meat sample were 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 on the previous basis.

Figure2.2 homogenization of samples

2.3 Dilution of samples

At first 5.5 grams of homogenized sample were put in 50 grams of EC broth. Here $1/10^{th}$ dilution was done. Then again, after 24 hours of incubation the sample showed growth in the EC broth. From this broth, 100 ml of solution was mixed with 900 ml of 0.9% saline solution. Here, $1/100^{th}$ dilution was done from the initial sample concentration.

2.4 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 additional 24 hours in total 48 hours.

Figure 2.3 Incubation of samples

2.5 Morphological characteristics

Spread plates of 100 ml from each incubated samples were done. After 24 hours of incubation in 37 degrees, the spread plate showed distinct colonies and morphological characteristics. From that spread plate, single colonies were separated to grow in selective media and identify organisms.

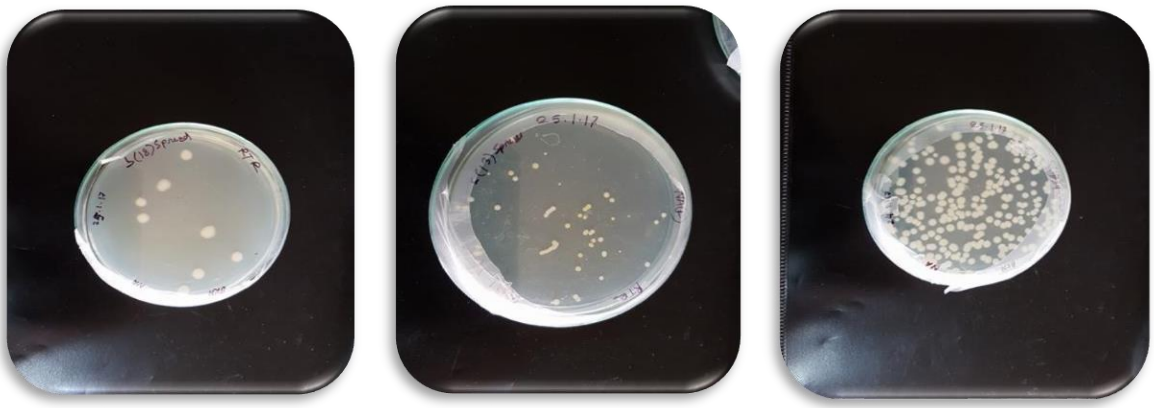

Figure 2.4 Spread plate containing single colonies

2.5 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 compound microscope and gram negative ones show 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.

2.6 Selective and differential media

2.6.1 Selective media allows the growth of 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.

Example: Mannitol salt agar or MSA is a commonly **used** selective and differential growth medium for detecting pathogenic Staphylococcus species. These organisms ferment the mannitol in the media and produce a byproduct that causes the pH indicator phenol red to turn the media color yellow.

2.6.2 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 coliform 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.

2.6.3 Cetrimide agaris the selective media is used in this experiment to isolate and characterize *Pseudomonas* species which are gram negative bacteria.It contains cetrimide, which is the selective agent against alternate microbial flora. Cetrimide also enhances the production of *Pseudomonas* pigments such as pyocyanin and fluorescein, which show a characteristic blue-green and yellow-green color, respectively.

2.6.4 Blood agaris an enriched medium for bacteria. It allows differentiating bacteria based on the hemolysis of blood cells present in it. There can be three types of hemolysis, Beta hemolysis, alpha hemolysis and gamma hemolysis. It consists of a base containing a protein source (e.g. Tryptones), soybean protein digest, sodium chloride (NaCl), agar and 5% sheep blood.

Figure 2.6 Bacterial colonies growing on Blood agar, MSA agar and Cetrimide agar respectively

Following selective and differential media were used to identify specific types of microorganisms:

Selective and differential media used to detect specific organisms	
For isolation of bacteria	**Media used**
For colliform bacteria	EMB agar
For lactose fermenting and non lactose fermenting	MACconkey agar
For S. typhi	XLD media
For pseudomonas spp.	Cetrimide media
For Stapyloccus aureus	Blood agar
For the isolation of pathogenic staphylococci	MSA agar

Table 2.1 Selective and differential media used to detect specific organisms

2.7 Biochemical tests

While characterizing and identifying microorganisms Biochemical tests are most frequently used. These tests differentiate bacteria from each other by showing their proper metabolism and fermentation pathway and utilization of carbon sources. They have become the essential procedures in case of identification of microorganisms these days.

Following biochemical tests were performed:

- TSI
- iMVC test
- Oxidase test
- Catalase test
- Nitrate reduction test
- Sugar utilization tests

- Urease test
- Motility test etc.

2.7.2 The Triple Sugar Iron (TSI) test is a biochemical test to detect if an organism produces acid as a byproduct, therefore the acid changes the pH of the medium and color changes shows definite results. In this process, microorganisms exhibit their ability to ferment sugars and sometimes may produce hydrogen sulfide. This biochemical test is very effective for microorganisms such as *Salmonella* and *Shigella*and is used as selective media for identification of these kinds of microorganisms.

The agar is set as a slant in a glass test tube that contains pH sensitive dye such as phenol red. It also contains 1% lactose, 1% sucrose, 0.1% glucose, sodium thiosulfate, ferrous sulfate or ferrous ammonium sulfate, agar etc. Organisms are incubated in the TSI slant for 24 hours and color change and gas/H_2S production is noticed.

TSI test can be interpreted in following ways:

Results (slant/butt)		**Symbol**	Interpretation
Slant	**Butt**		
Red	Yellow	K/A	Glucose fermentation only; Peptone catabolized
Yellow	yellow	A/A	Glucose and lactose and/or sucrose fermentation
Red	red	K/K	No fermentation; Peptone catabolized
Yellow	Yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation; Gas produced
Red	no color change	K/NC	No fermentation; Peptone used aerobically
Red	yellow with bubbles	K/A,G	Glucose fermentation only; Gas produced
Red	yellow with bubbles and black precipitate	K/A,G, H2S	Glucose fermentation only; Gas produced; H2S produced

Red	yellow with black precipitate	K/A, H2S	Glucose fermentation only; H2S produced
Yellow	yellow with black precipitate	A/A, H2S	Glucose and lactose and/or sucrose fermentation; H2S produced
No change	no change	NC/NC	No fermentation

*Here A=acid production; K=alkaline reaction; G=gas production; H2S=sulfur reduction

Table 2.2 TSI test result interpretation

Figure2.7 Results of TSI test

2.7.3 iMVC test It stands for four different types of test done 'I' for Indole test, 'M' for methyl red, 'V' for Voges-Proskauer and 'C' for citrate test.

2.7.3.1 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.

2.7.3.2 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.

2.7.3.3 Citrate test

Through this test, one can identify a microorganism that it a sole citrate user as a carbon source. The agar contains citrate and ammonium ions (nitrogen source) and bromothymol blue as an indicator. The citrate agar is green before inoculation, and turns blue as a positive test indicator, meaning citrate is utilized.

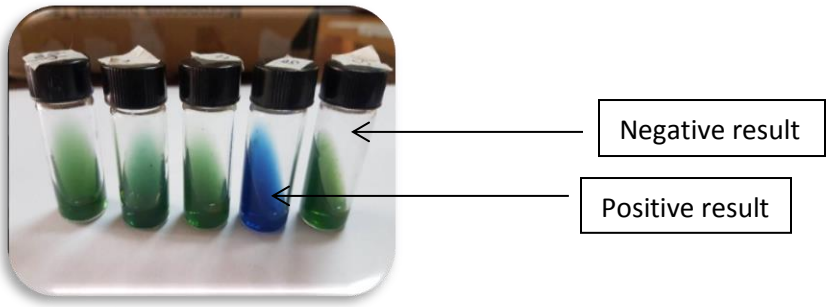

Figure 2.8 Citrate test result

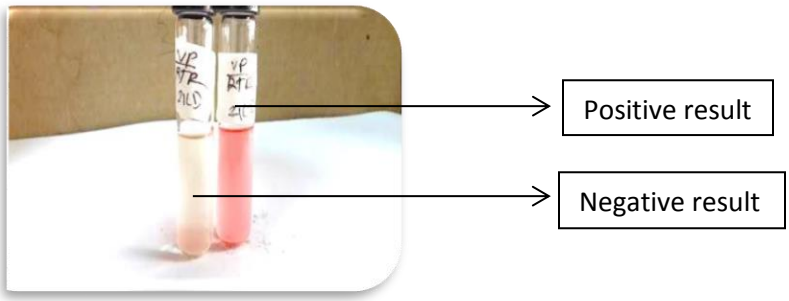

Figure2.9 VP test result

Figure 2.10 MR Test result

2.7.4 Oxidase test: This 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.

2.7.5 Catalase test: In this test the presence of catalase enzyme is detected with hydrogen peroxide. If any organisms are oxidase positive it produces bubbles releasing oxygen from hydrogen peroxide (H_2O_2). For this test 24 hours old culture of tested organism is needed.

$$2H_2O_2 \xrightarrow[\text{catalase}]{} 2H_2O + O_2 \text{ (gas bubbles)}$$

Bacteria thereby protect themselves from the lethal effect of Hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism. This test can be used to

identify microorganisms such as *Clostridium, Mycobacterium tuberculosis, Enterobacteriaceae* etc.

2.7.6 Nitrate reduction test by this test the presence of nitrate ion in solution is detected. Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO3) to nitrite (NO2) using the enzyme nitrate reductase. Nitrate broth contains nutrients and potassium nitrate as a source of nitrate. After incubating the nitrate broth, a dropperfull of sulfanilic acid and α-naphthylamine is added.

If the organism has reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. When sulfanilic acid is added, it will react with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α-naphthylamine to form a red-colored compound. If the medium turns red after the addition of the nitrate reagents, it is considered a positive result for nitrate reduction.

If the medium does not turn red after the addition of the reagents, it can mean that the organism was unable to reduce the nitrate, or it could mean that the organism was able to denitrify the nitrate or nitrite to produce ammonia or molecular nitrogen. Therefore, another step is needed in the test. If the tube turns red after the addition of the zinc, it means that unreduced nitrate was present. Therefore, a red color on the second step is a negative result. The addition of the zinc reduced the nitrate to nitrite, and the nitrite in the medium formed nitrous acid, which reacted with sulfanilic acid. The diazotized sulfanilic acid that was thereby produced reacted with the α-naphthylamine to create the red complex.

If the medium does not turn red after the addition of the zinc powder, then the result is called a positive complete. If no red color forms, there was no nitrate to reduce. Since there was no nitrite present in the medium, either, that means that denitrification took place and ammonia or molecular nitrogen were formed.

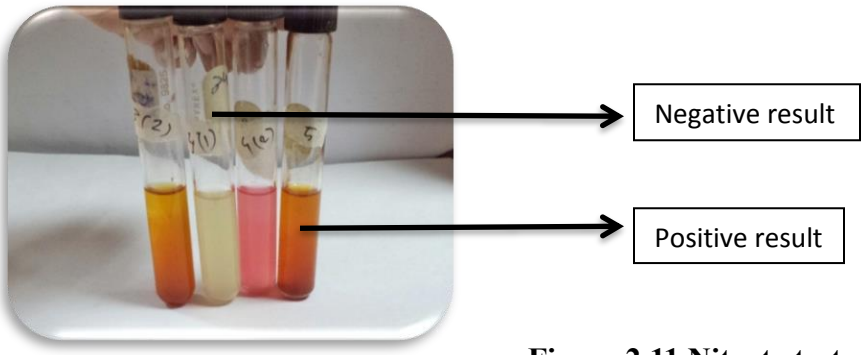

Figure 2.11 Nitrate test results

2.7.7 Sugar utilization tests

The sugar utilization test or carbohydrate utilization test is basically used to detect the carbon source that is utilized by the organisms present. In this thesis procedure four sugars were used as carbon sources. There are:

- Lactose
- Fructose
- Dextrose
- Arabinose

Some organisms also produce gas while using the carbon sources. This gas production can be detected through the gas accumulation in the Durham tubes that was placed inside the test tubes containing sugar bases beforehand. A pH indicator is also involved in this process such as phenol red. So when the organisms used up the sugars and produce acid, the pH indicator phenol red changes the color and turns the solution into a bright yellow. Gas that is produced while utilization of sugars takes place can be hydrogen or carbon dioxide.

Expected Results

1. **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.

2. **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.
3. **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.

.

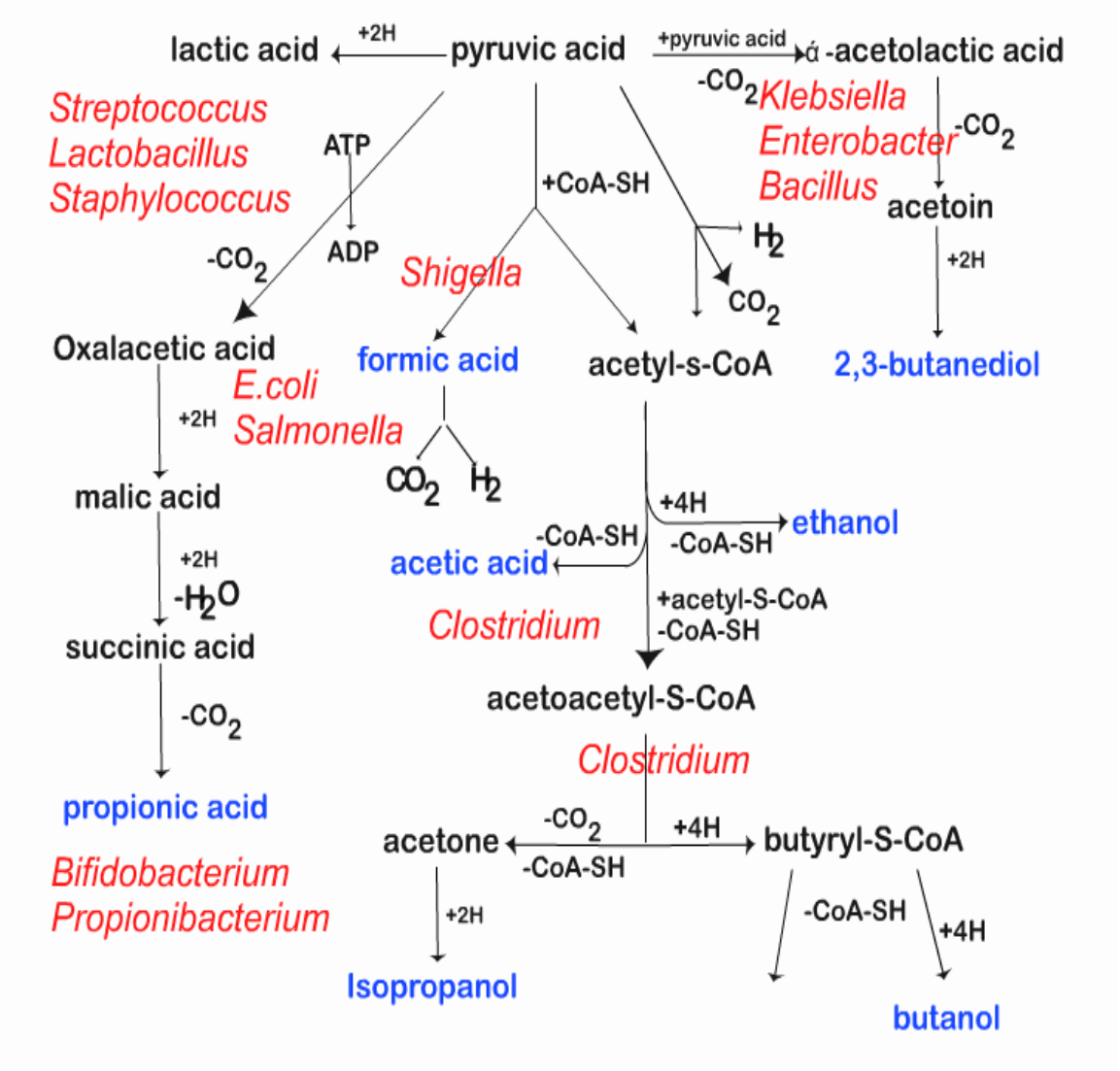

Figure 2.12 Pathway of carbohydrate utilization by various organisms

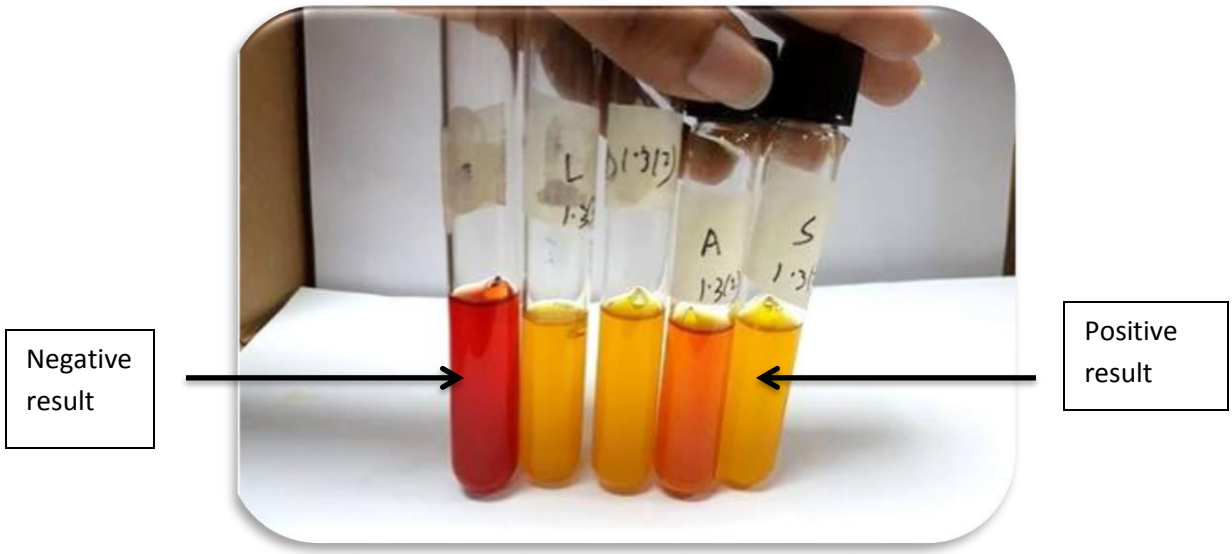

Figure 2.13 Sugar utilization test results

2.7.8 Urease test, Motility test and indole test by MIU agar

MIU medium is a semi solid agar that is used to detect if an organism is motile/ non motile. It also contains urea that can only be used by organisms that can produce the enzyme urease. It also contains the pH indicator phenol red that converts the media color into the bright red when the organism can produce the enzyme urease. After 24 hours incubation if the media is unchanged the organism is motile negative and also urease negative. If a single pink ring appears at the upper part of the media then it is described that the organism is indole positive. This Test is very effective as with a single procedure three test results can be obtained.

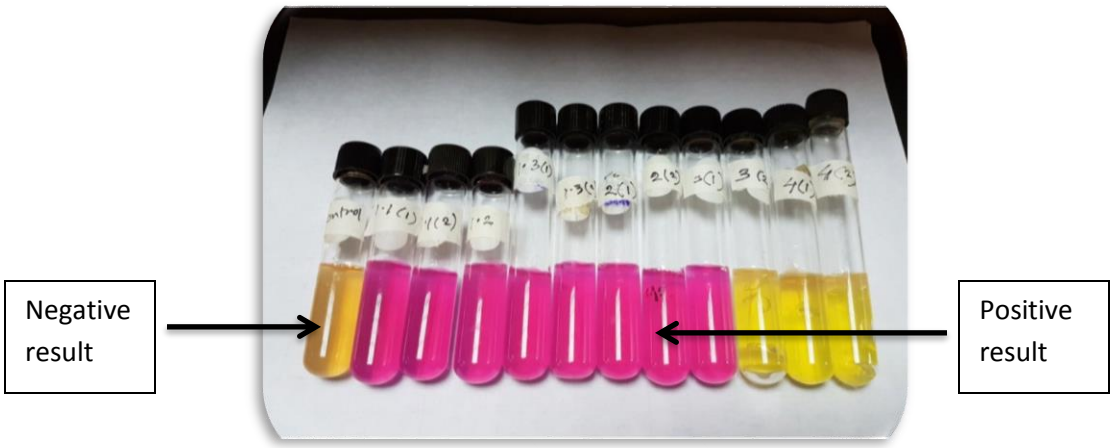

Figure 2.14 MIU test results

2.8.1 Antibiotic Sensitivity Test

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, Mullar Hington 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 Ampicillin, Amoxicillin, ceftriaxone, Chloramphenicol, Erythromycin, Polymixin B, Nalidixic acid, Oxicillin etc. were used.To check the results, four disks were placed in the agar plate while a control that contains no antibiotic was placed in the middle of the plate.

2.8.2 Antibiotic used in this project:

Antibiotic	Mechanism
Ampicillin	lysis the cell wall of bacteria
Amoxicillin	impairs the bond that holds the cell wall of bacteria thus creates holes in cell wall and kills the bacteria
Azithromycin	inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome
Ciprofloxacin	functions by inhibiting DNA gyrase, and a type II topoisomerase, topoisomerase IV, necessary to separate bacterial DNA, thereby inhibiting cell division
Chloramphenicol	prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome
Clindamycin	inhibits bacterial protein synthesis by binding to bacterial 50S ribosomal subunits
Ceftatrioxone	works by inhibiting the nucleopeptide synthesis in the bacterial cell wall
Imipenem	acts as an *antimicrobial* through the inhibition of cell wall synthesis of various gram-positive and gram-negative bacteria
Kanamycin	"irreversibly" binds to specific 30S-subunit proteins and 16S rRNA
Doxycycline	works by preventing bacteria from reproducing through the inhibition of protein synthesis
Nalidixic acid	inhibits a subunit of DNA gyrase and topoisomerase IV and induce formation of cleavage complexes
Levofloxacin	inhibits the two type II topoisomerase enzymes, namely DNA gyrase and topoisomerase IV, acts as bacteriacide
Polymixin B	binds to the cell membrane and alters its structure, making it more permeable.
Erythromycin	inhibits bacterial protein synthesis by binding to bacterial 50S ribosomal subunits

Rifampicin	inhibits bacterial RNA polymerase
Vancomycin	acts by prevention of cell-wall biosynthesis of bacteria
Tobramycin	binds irreversibly to one of two aminoglycoside binding sites on the 30 S ribosomal subunit, inhibiting bacterial protein synthesis

Table 2.3 List of antibiotics and their function

Figure 2.15 Antibiotic susceptibility results in case of *Pseudomonas* Species

Figure 2.16 Antibiotic sensitivity test results in case of *E. coli* samples

2.9.1 Molecular diagnostics

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

- PCR(polymerase chain reaction)
- Gel electrophoresis
- Multiplex PCR

2.9.2 Isolating chromosomal DNA with boiling method: For this method ample amount of colonies from isolated bacteria culture plate was inoculated into 200 µl of dH_2O. Then the samples were kept in -20◦ c temperature for 1 hour. After that the samples were heated into 95◦ C temperature water for 10 minutes. As a result, due to temperature shock the chromosomal DNA came out of the bacterial cell. The samples were then centrifuged at 13500 rpm for 10 minutes. Supernatants were collected into another microphage tube. Then the samples were kept in -20◦ C for further storage.

2.9.3 Polymerase chain reaction (**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 thermostable 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.

Cycles of PCR with Temperature	Time
94◦ Celsius	5 minutes
95◦ Celsius	30 seconds
56◦ Celsius	30 seconds
72◦ Celsius	30 seconds
4◦ Celsius	Until storage

Table 2.3 cell cycles of PCR

Components of PCR	
Components	**Amount**
Master mix	12.5 µl
Forward Primer	2.5 µl
Reverse Primer	2.5 µl
Template	5 µl
Nuclease free water (Q H_2O)	2.5 µl
Total	**25 µl**

Table 2.4 components of PCR for each sample

2.9.4 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 TBE buffer as best results were obtained from this combination. For comparison a known band size called ladder was also added to the gel base to interpret specific results.

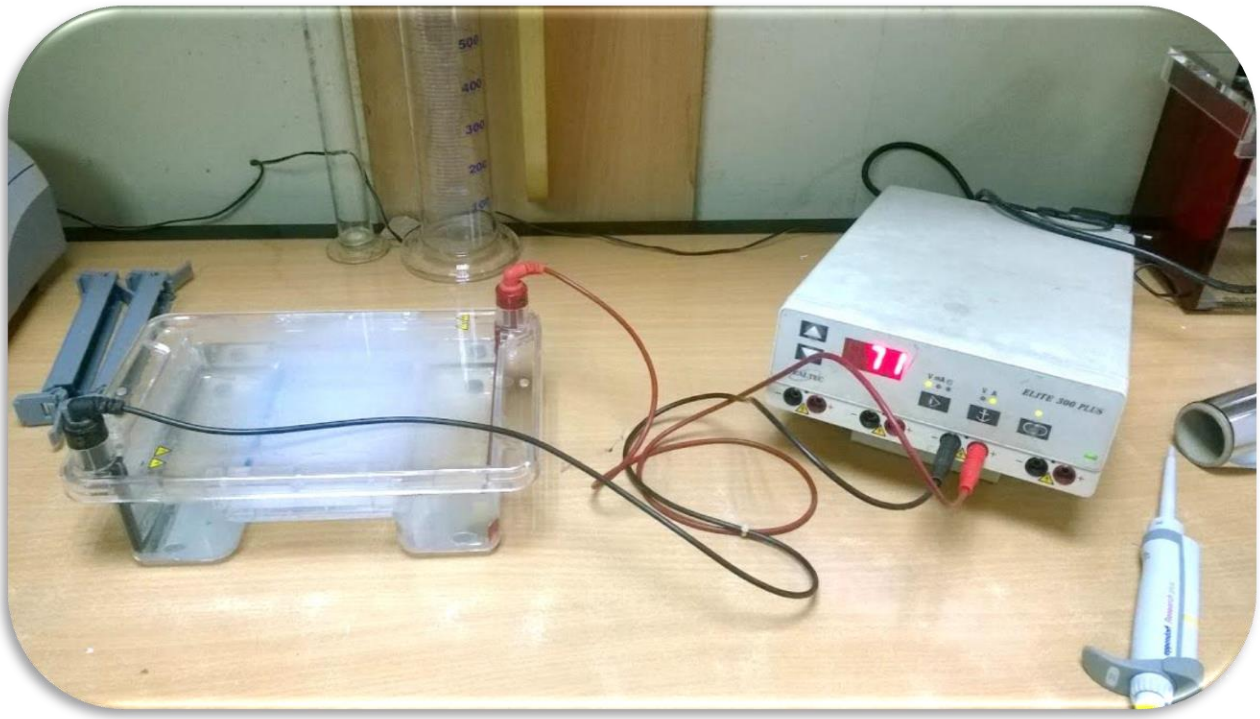

Figure2.17 Gel electrophoresis apparatus

2.9.5.1 Multiplex PCR

Multiplex-PCR is one kind of PCR where pools of genes are given in the same PCR cycle that will contain multiple primer sets but in a single PCR mixture. It is used to detect multiple genes in a short amount of time. This procedure is less time consuming and accurate for pathogenic gene detection. For this method, we went to ICDDRB, in their Food laboratory, where they work with pathogens obtained from food and related to food. They checked for total eight genes. Those genes were bfpA, eaeA, astA, st, it, ehxA.

2.9.5.2Genes detected in Multiplex PCR

Pathogenic gene tested	Results	Comment
Lt	-	All isolates were positive for stx1 and stx2 genes only
stx1	+	
stx2	+	
Bfp	-	
eae	-	
aaiC	-	
aat	-	
ipaH	-	
ial	-	

Table 2.5 Genes demonstrated in multiplex PCR

2.10 Storage of samples and organisms

The incubated samples and organisms were stored in respectively -20◦ Celsius temperature freeze and in T_1N_1 Media for further analyzing and molecular diagnostic purpose.

Figure 2.19 Storage of incubated samples

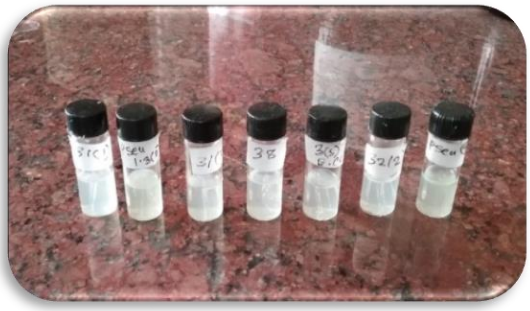

Figure2.20 Storage of isolated bacterial samples in T_1N_1 Media

3. Results

3.1 Sample collection:

All the samples were collected within sterile boxes and then transferred to BRAC laboratory without any human touch. The samples show numerous contamination except sample 25, this one showed no bacterial growth in spread plate method or in any differential media (Table 3.1).

Sample Number	Sample type	Obtained from	Isolated samples
1.1	Raw beef	Local market	*E. coli, E aerogens*
1.2	Semi cooked beef	Retailer shop	*Pseudomonas spp*
1.3	Cooked beef	Restaurant	*E aerogens*
2	Raw minced beef	Meena Daily	*S aureus, klebsiella*
3	Raw cube chicken	Meena daily	*E. aerogens, E. coli*
4	Cooked chicken	Cart near BRAC	*Salmonella typhii, E coli, Pseudomonas spp*
5	Semi cooked chicken	Cart near BRAC	*E coli, Pseudomonas spp*
6	Chicken Kebab	Cart near BRAC	*E. coli*
7	Chicken Tandoori	Bismillah cafe	*Klebsiella*
8	Chicken Shwarma	Solna	*Bacillus spp, E. aerogens*
9	Chicken pie	Solna	*Micrococcus spp, shigella. Klebsiella,*

			S. aureus, Serratia marcesens
10	Beef curry	Khana pina restora	*Micrococcus spp, E aerogens, E aaureus, klebsiella*
11	Chicken curry	Khana pina restora	*Shigella*
12	Chicken Shawrma	Restaurant near BRAC	*Micrococcus spp, S aureus,*
13	Chicken Sandwich	Restaurant near BRAC	*S. typhi, S. aureus, Pseudomonas spp*
14	Beef curry	Baburchi restaurant	*S. typhi, Pseudomonas spp*
15	Chicken saslick	Belpeper restaurant	*klebsiella*
16	Mongolian chicken	Food cart	*S. epidermis*
17	Chicken samosa	Food cart	*Bacillus*
18	Beef bun	Food cart	*Bacillus*
19	Chicken nugget	CP	*S. typhi, klebsiella*
20	Raw beef	Local market	*Klebsiella*
21	Beef kebab	Food cart	*Klebsiella*
22	Beef bhuna	Food cart	*S. typhi*
23	Beef curry	Food cart	*S. epidermis*
24	Beef cooked	Restaurant	*S. epidermis*
25	Chicken fry	Restaurant	*N/A*
26	Frozen chicken Nugget	Super shop	*S. typhi, S. epidermis*
27	Kalo beef bhuna	Restaurant	*E. coli, klebsiella*
28	Cooked beef		*Klebsiella*
29	Raw chicken	Local bazar	*Shigella, Salmonella*
30	Chicken	Shop	*Klebsiella*

	sandwich		
31	Chicken Balls	CP	*Micrococcus lutes, S.aureus, Serretia mercenes*
32	Chicken Burger	chillox	*E. coli, Shigella, Bacillus*
33	Chicken soup	Restaurant	*Shigella, Bacillus, S. epidermis*
34	Beef curry	Ghoroya	*Bacillus, klebsiella*
35	Beef semi cooked	Food Cart	*S. aureus*
36	Beef cooked	Food Cart	*Bacillus*
37	Raw beef	Local market	*E aerogens, Bacillus*
38	Chicken Burger	Takeout	*Khlebsiella*
39	Beef burger	Takeout	*E. coli, S aurues*
40	Beef bacon	Takeout	*E. coli, E aerogens*

Table 3.1 Details about collected sample

3.2 Biochemical tests

Biochemical tests showed different types of bacterial isolates compared in this study.

Prevalence of organism in different meat samples

Isolates no.	Oxidase test	Catalase test	Indole	MIU			MRVP		Gelatin	Nitrate reduction	Simmon's citrate	Casein hydrolysis	Starch hydrolysis	Blood agar hemolysis	Eosin methylene blue agar	Cetrimide agar	TSI						Organism Interpretation
				Motility	Indole	Urease	Methyl Red	VogesProskauer									Slant/ Butt	Glucose	Lactose	Sucrose	H_2S production	Gas production	
1	-	+	-	+/-	+	+	+	-	-	+/-	-	-	+	-	-	+	R/Y	+	-	-	-	-	*Staphylococcus aureus*
2	-	+	-	-	-	-	-	+	-	+	+	+	-	-	+	-	Y/Y	-	+	+	-	-	*Klebsiella spp.*
3	-	+	-	+	-	+/-	+	-	-	+	+	-	+	+	-	-	R/B	-	-	-	+	+	*Shigella spp.*
4	+	+	-	+	+	+/-	+	-	-	+	+	+	-	-	+	+	B/B				+	+	*Salmonella spp.*
5	-	+	-	+	-	+/-	+	-	-	+	-	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli*
6	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	R/R	-	-	-	-	-	*Pseudomonas spp.*
7	-	+	-	+	+	+/-	-	+	-	-	+	+	+	+	+	-	(B) R/Y	+	-	-	+	-	*Staphylococcus spp.*
8	-	+	-	+	-	-	-	-	+	+	+	-	-	+	+	-	R/Y	+	-	+	-	-	*Serretia marcasens*
9	-	+	-	-	+	+	+	+	-	+	+	-	-	-	-	-	Y/Y	+	+	+	-	-	*Micrococcus luteus*
10	-	+	-	-	+	+	+	-	-	+	-	+	+	+	+	-	Y/Y	+	-	-	-	-	*Bacillus spp*

'+' = positive, '- ' = negative; 'Glu' = Glucose; 'Lac' = Lactose, 'Suc' = Sucrose, Y= Yellow, R= Red, B= Black,

Table 3.2 Biochemical test results of Different bacteria

With the results of different biochemical test organisms were identified. Isolated organisms are *E. coli, Staphylococcus epidermis, Staphylococcus aureus, Pseudomonas spp, Serretia marcasens, Micrococcus luteus, Bacillus spp, Salmonella typhi, Shigella spp and Klebsiella spp.*(Table 3.2).

3.3 Prevalence of organism in Chicken samples

Sample Types	Sources of sample	Sample category	Organism found	Organism Numbers	Organism prevalence
Chicken	Super mall, Local market, Food stalls, Branded packaged items, Food carts, Local food shops, restaurants	Raw	***E. coli***	**1**	**16.67%**
			Enterobacter aerogenes	1	16.67%
			Staphylococcus Epidermis	1	16.67%
			Salmonella typhirium	2	33.33%
			Shigella	1	16.67%
		Semi cooked	***E. coli***	**2**	**50%**
			Pseudomonas aereginosa	1	25%
			Klebsiella	1	25%
		cooked	*Bacillus spp*	4	11.76%
			Serretia marcasens	2	5.88%
			Micrococcus luteus	3	8.82%
			Klebsiella	5	14.70%
			Pseudomonas aeruginosa	3	8.82%
			Shigella spp	4	11.76%
			S aureus	4	11.76%
			Salmonella typhi	4	11.76%
			Staphylococcus epidermis	2	5.88%
			E. coli	**2**	**5.88%**
			Enterobacter aerogenes	1	2.94%

Table3.3 Prevalence of organism in Chicken samples

From the table 3.3 we can conclude that mostly prevalent bacteria in chicken samples were *klebsiella* spp, followed by *E coli*, *Salmonella* and *Shigella* species. *Stapyloccus aureus* were found in less number. However, it is observed that in cooked samples pathogenic organisms such as *Pseudomonas aeruginosa,, E. coli* are noticed more frequently than in the raw and semi cooked samples, which is a very alarming fact.

3.4 Prevalence of organism in beef samples

Sample Types	**Sources of sample**	**Sample category**	**Organism found**	**Organism Number**	**Organism prevalence**
Beef	Super mall, Local market, Food stalls, Branded packaged items, Food carts, Local food shops, restaurants	Raw	***E. coli***	**1**	**12.5%**
			Enterobacter aerogenes	2	25%
			Staphylococcus aureus	1	12.5%
			Bacillus spp	2	25%
			Klebsiella spp	2	25%
		Semi cooked	*Pseudomonas aeruginosa*	1	16.67%
			Klebsiella spp	1	16.67%
			Staphylococcus aureus	2	33.33%
			Enterobacter aerogenes	1	16.67%
			E. coli	**1**	**16.67%**
		cooked	*Pseudomonas aeruginosa*	3	15.79%
			Staphylococcus aureus	1	5.26%
			Salmonella typhi	1	5.26%
			Enterobacter aerogenes	2	10.52%
			Micrococcus luteus	1	5.26%
			Klebsiella spp	4	21.05%
			Bacillus spp	3	15.79%
			Staphylococcus epidermis	2	10.52%
			E. coli	**2**	**10.52%**

Table 3.4 Prevalence of organism in beef samples

From the table (3.4) we can conclude that just like chicken samples cooked beef samples contained more *E. coli* and *Pseudomonas* in numbers rather than in raw and semi cooked samples. This again proves that how unhygienic and carelessly foods are prepared in Dhaka city and we consume them in regular manner. However, other organisms isolated in the study from beef samples are *Staphylococcus aureus, Salmonella typhi, Enterobacter aerogenes, Micrococcus luteus, Klebsilla spp, Bacillus spps, Staphylococcus epidermis etc.*

3.5 Results of Staining

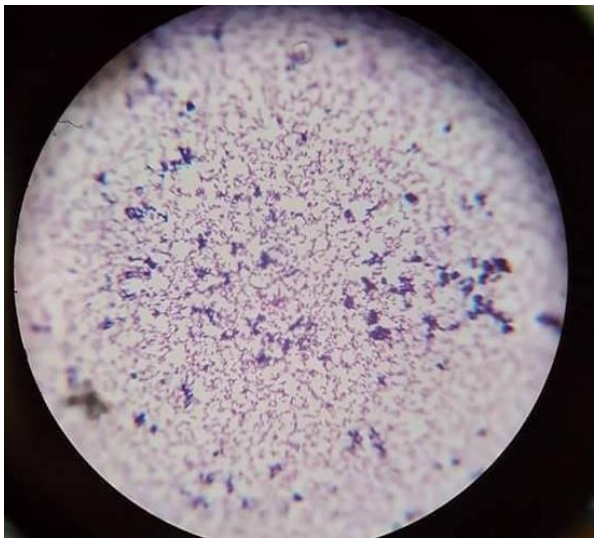

Figure3.1 Gram positive Bacteria

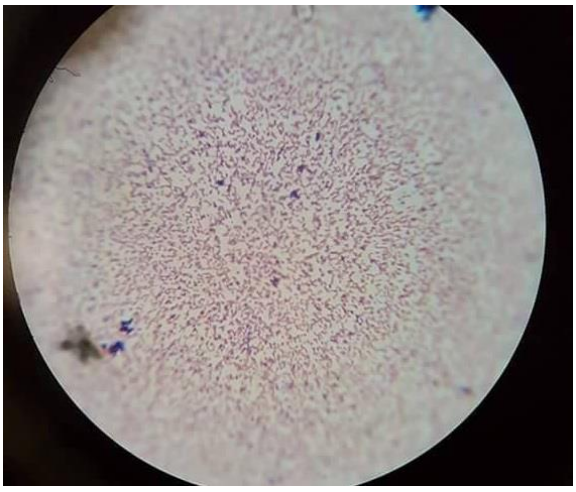

Figure3.2 Gram negative bacteria

Bacteria	Gram positive/ negative
E. coli	-
Pseudomonas	-
Bacillus	+
Klebsiella	-
Serretia marcescens	-
Shigella	-
Micrococcus	+
Streptococcus	-
Staphylococcus	+
Salmonella	-

Table 3.5 Gram staining results based on different bacteria

Among the bacteria *E coli, Pseudomonas spp, Klebsiella spp, Shigella, streptococcus* and *Salmonella* were gram negative and *Bacillus, micrococcus, Staphylococcus* were gram positive.

3.6.1 Antibiotic susceptibility for *E coli* isolates

Antibiotic list	Range (mm)			Iso1	In	Iso2	In	Iso3	IN	Iso4	In	Iso5	In	Iso6	In	Iso7	In	Iso8	In
	S	I	R																
Amoxicillin	≥20	6-19	≤5	19	I	19	I	19	I	18.5	I	19	I	18	I	18.5	I	19	I
Ampicillin	≥17	14-16	≤13	16	I	18	I	18	I	17	I	16	I	14	I	15	I	18	I
Chloramphenicol	≥18	13-17	≤12	23	S	23	S	22	S	24	S	23	S	22	S	23	S	23.5	S
Ciprofloxacin	≥22	17-21	≤16	26	S	27	S	25	S	24	S	23	S	24	S	25	S	25	S
Imipenem	≥20	13-19	≤12	28	S	27	S	26	S	26	S	27	S	25	S	26	S	28	S
Kanamycin	≥20	15-19	≤14	17	I	16	I	19	I	17	I	17	I	18	I	18	I	18	I
Nalidixic Acid	≥19	14-18	≤13	22	S	21	S	17	I	17	I	18	I	17	I	17	I	18	I
Polymixin B	≥6	4.5-5	≤4	13	S	13	S	12	S	12	S	12	S	12	S	12	S	12	S
Clindamycin	≥21	13-20	≤14	-	R	-	R	-	R	-	R	-	R	-	R	-	R	-	R
Doxycycline	≥9	2-8	≤7	20	S	18	S	20	S	18	S	17	S	17	S	20	S	18	S
Levofloxacin	≥9	7-8	≤6	26	S	22	S	22	S	22	S	22	S	22	S	22	S	26	S
Rifampicin	≥20	18-19	≤16	9	R	8	R	9	R	-	R	9	R	8	R	8	R	9	R
Erythromycin	≥23	14-22	≤13	-	R	-	R	-	R	-	R	-	R	-	R	-	R	-	R
Vancomycin	≥12	10-11	≤9	-	R	-	R	-	R	-	R	-	R	-	R	-	R	-	R
Tobramycin	≥9	7-8	≤7	14	S	5	S	18	S	18	S	17	S	17	S	18	S	18	S

**Interpretation: (-) noted samples showed full resistance towards the antibiotic disk

Table 3.6 results of Antibiotic susceptibility for *E coli* isolates

From the results it can be deducted that almost all antibiotics work against the isolates of *E. coli* except vancomycin, erythromycin and clindamycin. Most strains were susceptible towards imipenem, ciprofloxacin, amoxicillin and chloramphenicol.

3.6.2 Percentage of susceptibility against antibiotics of *E. coli* isolates

Antibiotics	Short Form	Resistance	Sensitive	Intermediate
Amoxicillin	**AMC30**	**0%**	**0%**	**100%**
Ampicillin	**AMP10**	**0%**	**0%**	**100%**
Chloramphenicol	**C30**	**0%**	**100%**	**0%**
Ciprofloxacin	**CIP5**	**0%**	**100%**	**0%**
Imipenem	**IMI10**	**0%**	**100%**	**0%**
Kanamycin	**K30**	**0%**	**0%**	**100%**
Nalidixic Acid	**NA30**	**0%**	**28.57%**	**71.43%**
Polymixin B	**PB300**	**0%**	**100%**	**0%**
Clindamycin	**DA2**	**100%**	**0%**	**0%**
Doxycycline	**DO30**	**0%**	**100%**	**0%**
Levofloxacin	**LE5**	**0%**	**100%**	**0%**
Rifampicin	**RD5**	**100%**	**0%**	**0%**
Erythromycin	**E15**	**100%**	**0%**	**0%**
Vancomycin	**VA30**	**100%**	**0%**	**0%**
Tobramycin	**TOB10**	**0%**	**100%**	**0%**

Table 3.7 Percentage of susceptibility against antibiotics of *E. coli* isolates

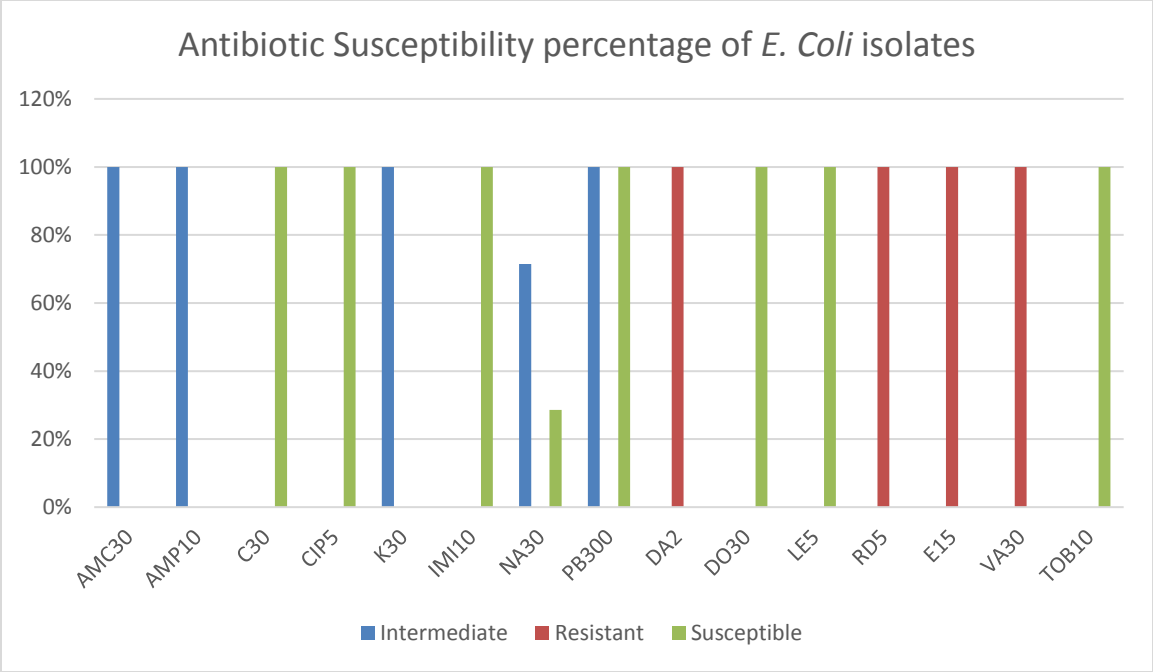

Table 3.8Bar graph of overall percentage of susceptibility against antibiotics of *E. coli* isolates

3.6.3 Antibiotic susceptibility for *Pseudomonas* spp. Isolates

From the results it can be concluded that Nalidixic acid, Gentamycin, Imipenem, Polymixin B, Levofloxacin and Tobramycin were effective against *Pseudomonas* spices, On the other hand Ciptofloxacin,Azithromycin and Amikacin showed poor performance against the bacteria. Chloramphenicol, Clindamycin, kanamycin, Vancomycin, Erythromycin were not able to show any activity against the *Pseudomonas* Species (from Table 3.9).

Antibiotic list	Range(mm)			Isolate1	In	Isolate2	In	Isolate3	In	Isolate4	In	Isolate5	In
	S	I	R										
Amikacin	≥15	9-14	≤8	12	I	14	I	14	I	12	I	12	I
Chloramphenicol	≥18	13-17	≤12	6	R	7	R	7	R	6	R	7	R
Ciprofloxacin	≥21	17-20	≤16	15	R	20	I	15	I	18	I	20	I
Imipenom	≥8	7-7.5	≤6	14	S	13	S	15	S	14	S	16	S
Kanamycin	≥9	7.5-8	≤7	5	R	4	R	5	R	6	R	6	R
Nalidixic Acid	≥19	14-18	≤13	20	S	20	S	15	S	18	S	15	S
Polymixin B	≥6	4.5-5	≤4	7	S	7	S	6	S	6	S	10	S
Clindamycin	≥21	13-20	≤14	-	R	-	R	8	R	9	R	-	R
Azithromycin	≥9	7-8	≤7	10	S	6	S	10	S	10	S	6	R
Levofloxacin	≥9	7-8	≤6	14	S	16	S	16	S	14	S	15	S
Gentamycin	≥10	8-9	≤7	10	S	12	S	12	S	11	S	11	S
Erythromycin	≥23	14-22	≤13	-	R	-	R	-	R	-	R	-	R
Vancomycin	≥12	10-11	≤9	-	R	-	R	-	R	-	R	-	R
Tobramycin	≥9	7-8	≤7	10	S	10	S	10	S	10	S	10	S

**Interpretation: (-) noted samples showed full resistance towards the antibiotic disk

Table 3.9 Results of Antibiotic susceptibility for *Pseudomonas* isolates

3.6.4 Percentage of susceptibility against antibiotics of *Pseudomonas isolates*

Antibiotics	Short Form	Resistance	Intermediate	Sensitive
Amikacin	**AN30**	**0%**	**100%**	**0%**
Chloramphenicol	**C30**	**100%**	**0%**	**0%**
Ciprofloxacin	**CIP5**	**80%**	**20%**	**20%**
Imipenom	**IMI10**	**0%**	**0%**	**100%**
Kanamycin	**K30**	**100%**	**0%**	**0%**
Nalidixic Acid	**NA30**	**0%**	**0%**	**100%**
Polymixin B	**PB300**	**0%**	**0%**	**100%**
Clindamycin	**DA2**	**100%**	**0%**	**0%**
Azithromycin	**AZM15**	**20%**	**0%**	**80%**
Levofloxacin	**LE5**	**0%**	**0%**	**100%**
Gentamycin	**GEN10**	**0%**	**0%**	**100%**
Erythromycin	**E15**	**100%**	**0%**	**0%**
Vancomycin	**VA30**	**100%**	**0%**	**0%**
Tobramycin	**TOB10**	**0%**	**0%**	**100%**

Table 3.10 Results of Antibiotic susceptibility for *Pseudomonas* isolates

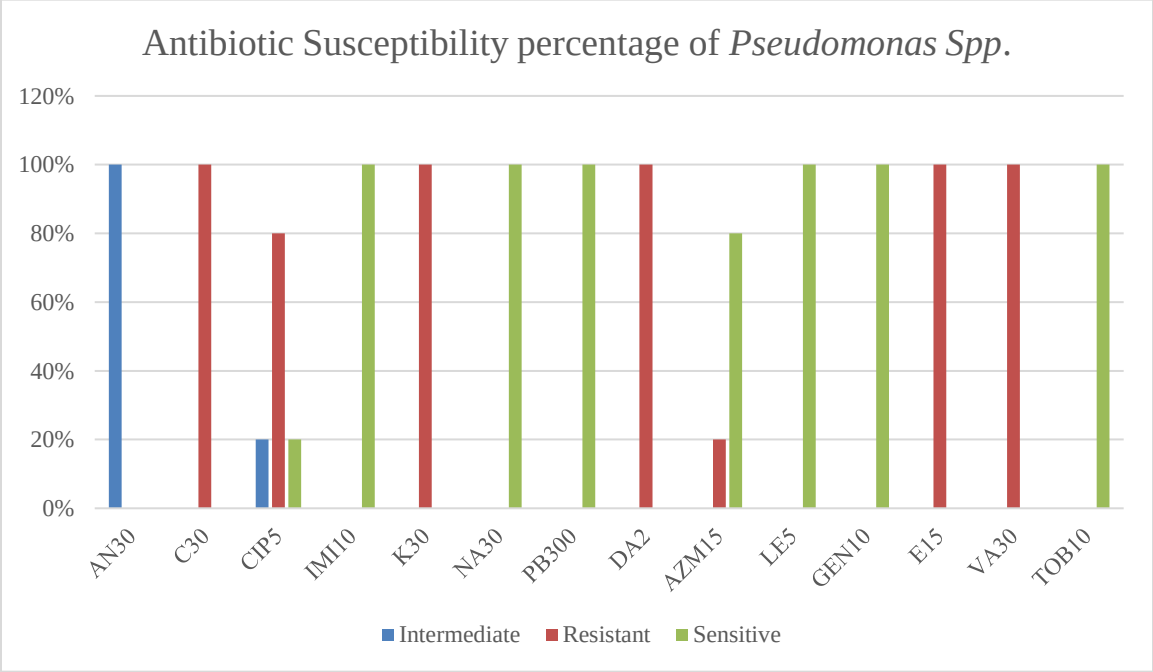

Table 3.11 Bar graph of overall percentage of susceptibility against antibiotics of *Pseudomonas* isolates

3.7 Gel electrophoresis result

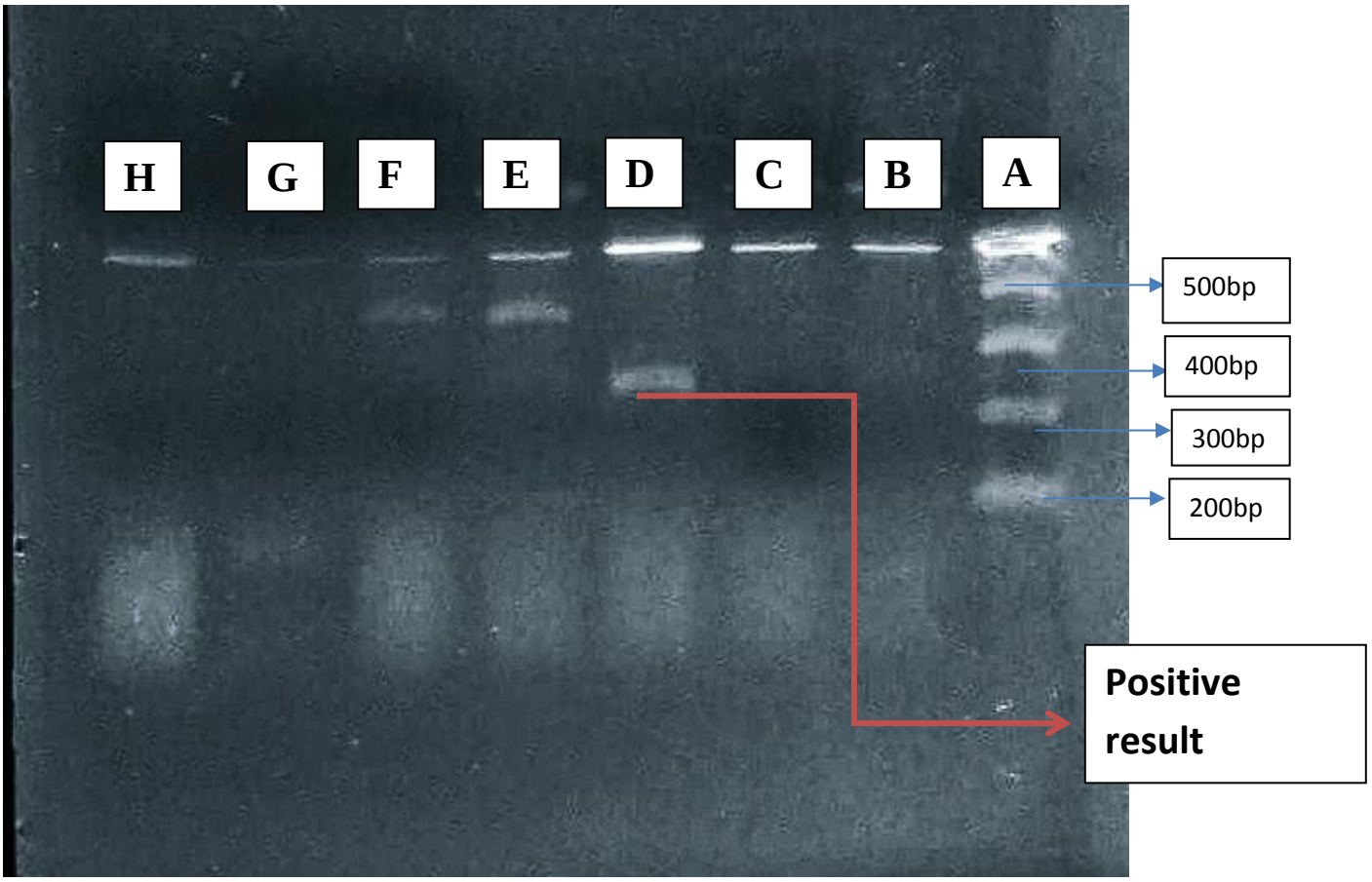

Figure 3.3 Results of Gel electrophoresis

In the gel electrophoresis there are eight lanes from A – H. The lane A contains ladder and from lane B – H different *E. coli* samples isolated from meat samples were run through. After doing PCR with the primer of stx1 gene and doing a gel run, the gel electrophoresis showed a band near 350 base pair in Lane D, which is a positive result for stx1 gene, therefore it can be concluded that that *E. coli* sample contained stx1 pathogenic gene.

3.8 Multiplex PCR results

Multiplex PCR was done in icddrb, where they looked for nine genes in total; they are lt, stx1, stx2, bfp, ear, aaiC, aat, ipaH, ial. Among them stx1 and stx 2 gene was positive in the two samples that were given to analyze (From Table 3.12).

Pathogenic gene tested	Results	Comment
lt	-	All isolates were positive for stx1 and stx2 genes only
stx1	+	
stx2	+	
bfp	-	
eae	-	
aaiC	-	
aat	-	
ipaH	-	
ial	-	

Table 3.12 Results of multiplex PCR

4. Discussion

Both beef and chicken samples used in the present study showed heavy contamination, with the exception of a single sample of fried chicken. Raw Chicken samples showed 16.67% contamination with *Escherichia coli*, 16.67% contamination with *Enterobacter aerogenes,* 16.67% contamination with *Staphylococcus epidermis,* 33.33% contamination with *Salmonella typhirium,* 16.67% contamination with *Shigella spp,*. However, in semi cooked samples *E. coli* was found in 50% cases**,** *Pseudomonas aeruginosa* was found in 25% cases and *Klebsiella spp* was found in 25% cases. Whereas in cooked chicken samples Bacillus spp. was found in 11.76% cases, *Serretia marcasens* was found in 5.88% cases, *Micrococcus luteus* was found in 8.82% cases, *Klebsiella spp*. was found in 14.70% cases, *Pseudomonas aeruginosa* was in found 8.82% cases, *Shigella spp* was found in 8.82% cases, *S. aureus* was found in 11.76% cases, *Salmonella typhi* was found in 11.76% cases, *Staphylococcus epidermis* was found in 5.88% cases, *E. coli* was found in 5.88% and *Enterobacter aerogenes* was found in 2.94% cases.

All above data shows similarity to the findings of a study carried out in India by Thanigivel and Anandhan (2015). They collected raw meat from mutton and chicken and subsequently isolated bacteria, such as, *Escherichia coli*, *Salmonella spp., Staphylococcus aureus* and *Pseudomonas.* However, it must be mentioned that they also isolated molds and fungi, such as, *Mucor, Aspergillus niger* and *Aspergillus fumigates* which were not found in the current study.
Present research showed chicken samples to be prevalently contaminated with *Klebsiella spp and Escherichia coli*, followed by *Salmonella typhi, Shigella spp* and *Enterobacter aerogenes*. However, it is very alarming that the most *Escherichia coli* contamination was seen in cooked chicken samples. This indicates improper handling and preparation of processed chicken- foods that are available in low cost market areas around Dhaka city. In another study done in Nigeria by Adeyanju and Ishola (2014) had been seen heavy bacterial contamination in poultry chicken with *E. coli* and *Salmonella spp*.

On the other hand, the raw beef samples showed 12.5% contamination with *Escherichia coli*, 25% contamination by *Enterobacter aerogenes*, 12.5% contamination with *Staphylococcus aureus*,., 25% contamination with *Bacillus spp*. However, the semi cooked beef samples showed 16.67% contamination with *Pseudomonas aeruginosa*, 16.67% contamination with *Klebsiella spp,* 33.33% contamination with *Staphylococcus aureus,* 16.67% contamination with

Enterobacter aerogenes and 16.67% contamination with *Escherichia coli*. Furthermore, the cooked beef samples showed 15.97% contamination with *Pseudomonas aeruginosa*, 5.26% contamination with *Staphylococcus aureus,* 5.26% contamination with *Salmonella typhi,* 10.52% contamination with *Enterobacter aerogenes,* 5.26% contamination with *Micrococcus luteus,* 21.05% contamination with *Klebsiella spp,* 15.79% contamination with *Bacillus spp,* 10.52% contamination with *Staphylococcus epidermis and* 10.52% contamination with *Escherichia coli.* In Ethiopia, where beef consumption is high, comparable microbial load were noticed in cooked and raw beef samples. (Gebeyehu *et al,* 2015).

It was seen in the present experiment that the beef samples are heavily contaminated with *Staphylococcus aureus and Klebsiella spp.* Gradually *Bacillus* and *Enterobacter aerogenes* were the next two organisms to create contamination followed by *E. coli*. Previous studies of Tewari *et al.*, (2013) showed *Bacillus cereus* to be a common pathogen found in meat samples, in amounts very parallel to those found in the present work. However, *Psedomonas aeruginosa* was also found in raw beef samples that showed resistance towards antibiotics such as Chloramphenicol, Kanamycin, and Clindamycin etc. Raw beef showed more contamination with *Enterobacter aerogenes,* which are related to contagious skin diseases. *Enterobacter spp.*is responsible for hospital infections such as bloodstream infections, which can be lethal (Reichley*et al*, 2007). Other semi-cooked and cooked beef samples were collected from food carts and restaurants; these also showed heavy contamination with bacteria such as *E. coli* and *pseudomonas*. This indicates that restaurants and food carts do not maintain proper hygiene or food processing techniques, which should raise a high level of caution.

A study was done in Kathmandu Valley where processed meat samples from chopping board, knife etc. were investigated by Acharya *et al, (*2016) and *Salmonella, Shigella* and *Staphylococcus* species were obtained in ample amount. This result indicates the resemblance between studies carried out in Dhaka city and the Kathmandu valley in terms of raw meat processing.

In the study by Sharma and Chattopadhyay (2015), where they analyzed raw meat samples *E. coli, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas*

spp., Salmonella spp., Bordetella, Klebsiella pneumoniae, Enterococcus aerogenes, Micrococcus spp., Citrobactor spp., Proteus spp., Klebsiella oxytoca and *Providencia spp.* were found. Their results also showed similar prevalence of the microorganisms in of the present study. All the data confirms that the current study was carried out in a manner supported by previously published literature to isolate bacteria responsible for causing diarrheal diseases in Dhaka city. However, after all the studies done with proper evidences, without proper steps taken from the government no outcome will come of it. In addition, people should be more concerned and aware of such issues while consuming food from local shops and markets.

The current research has shown that anti biotics such as Rifampicin, Vancomycin, Erythromycin and Clindamycin were not able to suppress the *E. coli* isolates; however Tobramycin, Levofloxacin, Polymixin B, Imipenem, Ciprofloxacin, Amoxacillin and Chloramphenicol were able to resist *E. coli* growth. In a work done by Kibert and Abera (2011), it had been noticed that *E. coli* is resistant against Erythromycin, but it is susceptible against Ciprofloxacin, resembling result was found in this study also.

From the results it can be concluded that Nalidixic acid, Gentamycin, Imipenem, Polymixin B, Levofloxacin and Tobramycin were effective against *Pseudomonas* spices, On the other hand Ciptofloxacin, Azithromycin and Amikacin showed poor performance against the bacteria. Chloramphenicol, Clindamycin, kanamycin, Vancomycin, Erythromycin were not able to show any activity against the *Pseudomonas* Species. According to Thanigivel and Anandhan (2015), *Pseudomonas* species are becoming multidrug resistant. This is also visible in the present research findings where all antibiotics tested were not very effectively able to suppress or halt the bacterial growth. Antibiotics such as Imipenem, Chloramphenicol, and Ciprofloxacin showed prominent barrier against the isolated bacteria till now. In works done previously,*Pseudomonas* species demonstrated marked resistance against monotherapy of Penicillins, Cephalosporins, Fluoroquinolones, Tetracyclines and Macrolides. Compared to this a study done byJaviya*et al*, (2008) it was exposed that combination drugs like Ticarcillin + Clavulanic acid, Piperacillin + Tazobactum, Cefoperazone + Sulbactum, Cefotaxime + Sulbactum, Ceftriaxome + Sulbactum and monotherapy of amikacin showed higher sensitivity to Pseudomonas infections; however, the maximum sensitivity was shown by the Carbapenems.

Amani *et al*,(2015) used multiplex PCR to detect stx1 and stx2 genes from enterobacteria strains. The molecular analysis in this study, where stx1 and stx2 genes containing samples showed affirmation of shiga toxin residue in those *E. coli* isolates. In multiplex PCR, genes such as *lt, stx1, stx2, bfp, eae, aaiC, aat, ipaH,* and *ial*are examined. However, in the multiplex PCR all the other pathogenic genes were absent, therefore it can be concluded that the strains were STEC and are responsible for enterotoxigenic effect of *E.coli* isolates. These findings are supported by the experiment of Maktabi*et al*, (2016) who found that all their isolates of *E. coli O157:H7* (which is pathogenic) had more than one virulent genes, including stx1 and stx2. The stx1 gene can be found in the range of 255-350 bp in gel electrophoresis, Hence, sample that were analyzed in the laboratory of BRAC university was positive for stx1 gene also. This indicates the molecular characteristics of bacteria like *E coli* are constantly changing and they are becoming harmful compared to their nonpathogenic types. The most harmful type of *E. coli* is *E. coli 0157:H7*, which are responsible for outbreaks recently, the study plan on detecting suspecting *E. coli 0157:H7* based on their molecular characteristics (Deisingh and Thompson, 2003).

The present study further aims to analyze molecular characteristics for all *E. coli* isolates and other bacterial isolates, such as, *Pseudomonas spp.* are rapidly becoming antibiotic resistant and as a consequence only a few antibiotics currently effective against them (Lee *et al*, 2007). There are possibilities to determine which proteins are responsible for the outbreaks of diarrhea, as they showed the same symptoms while causing disease. By analyzing their molecular characteristics, there will be more potential to develop vaccines and antibiotics which will effectively work against them. This study also indicates the necessity of proper cooking, storage and eating habits of individuals, otherwise, these organisms will continue to cause disease and evolve.

5. REFERENCES

- Acharya A., Poudel A., Sah AK., Maharjan D., Tibrewal S. and Mandal PK., (2016). Isolation and Identification of Bacteria from Meat Processing Units of Kathmandu Valley. International Journal of Microbiology and Allied Sciences. 2(3):33-37.
- Adams M. R., Moss M. O. Food microbiology. Thomas Graham house, Service Park, Cambridge, UK: The Royal Society of Chemistry; 1999, p 192-202.
- Adu-Gyamfi A., Torgby-Tetteh W. and Appiah. V., (2012). Microbiological Quality of Chicken Sold in Accra and Determination of D10-Value of *E. coli*. Food and Nutrition Sciences. 3 (5), 2012, 693-698.
- Amani J., Ahmadpour A., Fooladi A. A. I., Nazarian S., (2015). "Detection of *E. coli* O157:H7 and Shigella Dysenteriae Toxins In Clinical Samples By PCR-ELISA". The Brazilian Journal of Infectious Diseases 19.3 (2015): 278-284. Web. 14 June 2017.
- Brahmbhatt M. N., Anjaria JM., (1991). Isolation of bacteria from market goat meat and their in vitro antibiotic sensitivity pattern. Indian J Animal Sci 1991; 63: 522-523.
- Bhandare S. G., Sherikarv A. T., Paturkar A. M., Waskar V. S., Zende R. J., (2007). A comparison of microbial contamination on sheep/goat carcasses in a modern Indian abattoir and traditional meat shops. Food Control 2007; 18: 854-868.
- Bauer A. W, Kirby W. M. M., Sherris J. C., Turck M., (1997). Antibiotic susceptibility testing by a standardised single disk method. Am J Clin Pathol 1997; 45:493-496.
- Bolton F. J, Crozier L., Williamson I. K., (1996). Isolation of *E.coli* 0157 from raw meat products. Let Appll Microbiol 1996; 23: 317-321.
- Deal, E. N., Micek, S. T., Ritchie, D. J., Reichley, R. M., Dunne, W. M. and Kollef, M. H., (2007), Predictors of In-Hospital Mortality for Bloodstream Infections Caused by *Enterobacter* Species or *Citrobacter freundii*. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 27: 191–199. doi:10.1592/phco.27.2.191

- Ercolini. D, Russo F., Torrieri E., Masi P. and Villani F., (2006). Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. Applied and Environmental Microbiology.72 (7), 2006, 4663-4671.
- Forward, K. R., Matheson, K. M., Hiltz M. H., Musgrave and Poppe C., (2004). Recovery of cephalosporin-resistant Escherichia coli and Salmonella from pork, beef and chicken in Nova Scotia. Canadian Journal of Infectious Diseases and Medical Microbiology, 15(4), 2004, 226-230.
- Gebeyehu A, Yousuf M, Sebsibe A., (2013) Evaluation of Microbial Load of Beef of Arsi Cattle in Adama Town, Oromia, Ethiopia. J Food Process Technol 4:234. doi:10.4172/2157-7110.1000234.
- Adeyanju G. T., Ishola O., Springerplus., (2014); 3: 139. Published online 2014 Mar 12. doi: 10.1186/2193-1801-3-139, PMCID: PMC4320193.
- Gill CO, McGinnis JC, Bryant J., (2007). Microbiological contamination of meat during the skinning of beef carcass hindquarters at three slaughtering plants. Int J Food Microbiol 1998; 42: 175-184.
- Laura F, Mauro S., (2007). Characterisation of *Pseudomonas spp.* isolated from foods. Annals of Microbiol 2007; 57 (1): 39-47.
- Gill G. O, Harris L. M., (1982). Survival and growth of Campylobacter fetus subsp. Jejuni on meat and in cooked foods. Appl Environ Microbiol 1982; 44:259-263.
- Gould S. and Friedman L. F., (2015). The countries where people eat the most meat. Sep. 26, 2015, 9:30 AM. Business insider, Technews.
- Haque M. A, Siddique M. P, Habib M. A, Sarkar V, Choudhury K. A., (2008). Evaluation of sanitary quality of goat meat obtained from slaughter yards and meat stalls at late market hours. Bangladesh J Vet Med 2008; 6(1): 87-92.
- Hegde A, Ballal M, Shenoy S., (2012). Detection of diarrheagenic *Escherichia coli* by multiplex PCR. Indian J Med Microbiol 2012;30:279-84.
- Ikeme I. A., (1990). Meat Science and Technology. A comprehensive approach. Onitsha, Nigeria: Africana – FEP publishers Ltd; 1990.

- Izat A. L., Tidwell N. M., Thomas R. A., Reiber M. A., Adams M. H., Colberg M., Waldroup P. W., (1990). Effect of a buffered propionic acid in diets on the performance of broiler chickens and on microflora of the intestine and carcass. Poult Sci 1990, 69: 818-826.
- Javiya, V. A., Ghatak, S. B., Patel, K. R., & Patel, J. A., (2008, October). Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* at a tertiary care hospital in Gujarat, India. Retrieved June 13, 2017, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2792624/.
- Kibret, M., & Abera, B., (2011, August). Antimicrobial susceptibility patterns of *E. coli* from clinical sources in northeast Ethiopia. Retrieved June 13, 2017, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3220125/.
- Komba E. V. G., Komba, Mkupasi E. M., Mbyuzi A. O., Mshamu S., Luwumbra D., Busagwe Z. and Mzula A., (2012). Sanitary practices and occurrence of zoonotic conditions in cattle at slaughter in Morogoro Municipality, Tanzania: implications for public health. Tanzania Journal Health Research, 14 (2), 2012.
- Lee Y. C, Ahn B. J, Jin J. S, Kim J. U, Lee S. H, Song D. Y, Lee W. K, Lee J. C., (2007). Molecular characterization of Pseudomonas aeruginosa isolates resistant to all antimicrobial agents, but susceptible to colistin, in Daegu, Korea. 2007 Aug;45(4):358-63. NCBI; PubMed.
- J Microbiol. 2007 Aug;45(4):358-63. PMID: 17846591.
- Li, M., Wang, F., & Li, F. (2011, April). Identification and Molecular Characterization of Antimicrobial-Resistant Shiga Toxin–Producing *Escherichia coli* Isolated from Retail Meat Products. Retrieved June 13, 2017, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3123925/.
- Li M. Y., Zhou G. H., Xu X. L., C. B. Li and Zhu W. Y., (2006). Changes of bacterial diversity and main flora in chilled pork during storage using PCR- DGGE. Food Microbiology. 23 (7), 2006, 607-611.
- Maktabi, Siavash , Zarei M., and Mohammadpour H,. (2016). "Isolation And Molecular Characterization Of Non-Sorbitol Fermenting Escherichia Coli Isolated From Fresh Ground Beef". Jundishapur Journal of Health Sciences 8.1 (2016).

- Marks, M. J. (n.d.). (2017). Diarrhea Symptoms, Causes & Natural and Home Remedies. Retrieved June 13, 2017, from http://www.medicinenet.com/diarrhea/article.htm.
- Nørrung B., Andersen J. K. and Buncic S. (2009). Main Concerns of Pathogenic Microorganisms in Meat Safety of Meat and Processed Meat. F. Toldrá, ed. Food Microbiology and Food Safety. (Springer New York), 2009, pp. 3-29.
- Papadopoulos D., Schneider D., Meier-Eiss J., Arber W., Lenski R. E., and Blot M., (1999). Genomic evolution during a 10,000-generation experiment with bacteria. 1999 Mar 30; 96(7): 3807–3812. NCBI; PMC.
- Robert H., (2014). Bangladesh anthrax cases up to 170 in different areas of the country. (2014, August 29). Retrieved June 13, 2017, from http://outbreaknewstoday.com/bangladesh-anthrax-cases-up-to-170-in-different-areas-of-the-country-93331/.
- Roger S., Nicole B., Claudio Z., Miguel B., Jesús E. B. (2004). First isolation and further characterization of enteropatho genic *Escherichia coli* (EPEC) O157:H45 strains from cattle. *BMC Microbiol* 2004; 4: 10.
- Sinha B. K, Mandal L. N., (1977). Studies on bacteriological quality of market goat meat and its public health importance. Indian J Animal Sci 1977; 47:478-481.
- Viljoen B. C, Geornaras I, Lamprecht A, Von Holy A. (1988). Yeast population associated with processed poultry. J Appl Microbiol 1998; 15: 113-117.
- Skippington, E., & Ragan, M. A. (2012, September 01). Phylogeny rather than ecology or lifestyle biases the construction of Escherichia coli–Shigella genetic exchange communities. Retrieved June 13, 2017, from http://rsob.royalsocietypublishing.org/content/2/9/120112.
- Thanigaivel G., Anandhan A. S., (2015); Isolation and Characterization of Microorganisms from Raw Meat Obtained from Different Market Places in and Around Chennai. J Pharm Chem Biol Sci 2015; 3(2):295-301.
- Elizabeth S., Mark A. R., (2012). Phylogeny rather than ecology or lifestyle biases the construction of *Escherichia coli–Shigella* genetic exchange communities, Published 12 September 2012.DOI: 10.1098/rsob.120112.
- Theresa J. O., Carmen A. C. (2011). Enteropathogenic *E. coli* (EPEC) infection in children. PMC, NCBI. PMCID: PMC3277943. NIHMSID: NIHMS348474

Appendix I

List of Medias and Broths

EC Broth	
Components	Gms/ Litre
Casein enzymicHydrolysate	20.00
Lactose	5.00
Bile salt mixture	1.50
Dipotassium phosphate	4.00
Monopotassium phosphate	1.50
Sodium chloride	5.00
Final pH should be 6.9±0.2 (at 25◦C)	

Nutrient Agar (NA) per 1000 ml	
Ingredients	**Measurements (grams)**
Nutrient Agar (NA)	28
**** Boiled to dissolve and autoclaved for sterilization.**	

Mannitol Salt Agar (MSA) per 1000ml	
Ingredients	**Measurements (grams)**
Mannitol Salt Agar (MSA)	111.02
****Boiled to dissolve and autoclaved for sterilization.**	

Simmons Citrate Agar per 1000ml	
Ingredients	**Measurements (grams)**
Citrate Agar	23
** **Boiled to dissolve and autoclaved for sterilization.**	

MRVP broth per 1000ml	
Ingredients	**Measurements (grams)**
Peptone	7
Dextrose	5
Potassium phosphate	5
**** Autoclaved for sterilization.**	

MIU agar per 950 ml	
Ingredients	**Measurements (grams)**
MIU agar	18g
**** Autoclaved for sterilization.**	

TSI agar per 1000 ml	
Ingredients	**Measurements (grams)**
TSI agar	64.42
**** Autoclaved for sterilization.**	

Glucose Fermentation broth per 100ml	
Ingredients	**Measurements (grams)**
Casesin Enzyme Hydrosylate	1
Glucose	0.5
NaCl	0.5
Phenol red	0.0189
**** Autoclaved for sterilization.**	

Nitrate broth per 1000ml	
Ingredients	**Measurements (grams)**
Peptone	5
Beef extract	3
Potassium nitrate	5
**** Autoclaved for sterilization.**	

Mueller-Hinton Agar (MHA) per 1000ml	
Ingredients	**Measurements (grams)**
Mueller-Hinton Agar (MHA)	38
****Boiled to dissolve and autoclaved for sterilization.**	

Cetrimide agar	
Ingredients	**Measurements (grams)**
Enzymatic Digest of Gelatin	20
Magnesium Chloride	1.4
Potassium Chloride	10
Cetrimide (Cetyltrimethylammonium Bromide)	0.3
Glycerol	10
Agar	13.6
****Boiled to dissolve and autoclaved for sterilization.**	

EMB Agar	
Ingredients	**Measurements (grams)**
Peptic digest of animal tissue	10
Dipotassium phosphate	2
Lactose	5
Sucrose	5
Eosin - Y	0.4
Methylene blue	0.065
Agar	13.5

XLD agar	
Ingredients	**Measurements (grams)**
Lactose	7.5
Yeast Extract	3
Sodium Deoxycholate	2.5
Ferric Ammonium Citrate	0.8
Phenol Red	0.08
****Boiled to dissolve and autoclaved for sterilization.**	

Blood agar	
Ingredients	**Measurements (grams)**
Beef heart infusion from (beef extract)	500
Tryptose	10
Sodium chloride	5
Agar	15

MACconkey Agar	
Ingredients	**Measurements (grams)**
Peptic digest of animal tissue	1.5
Casein enzymichydrolysate	1.5
Pancreatic digest of gelatin	17
Lactose	10
Bile salts	1.5
Crystal violate	0.001
Neutral red	0.03
Agar	15

Appendix II

List of reagents

Methyl Red reagent per 100ml	
Ingredients	**Measurements**
Methyl red	0.02 g
Ethyl alcohol	40ml
**** The 100ml mark is reached by addition of 60 ml distilled water.**	

Barritt's reagent A	
Ingredients	**Measurements**
Alpha-Napthol, 5%	50g
Absolute Ethanol	1000ml

Barritt's reagent B per 1000ml	
Ingredients	**Measurements**
Potassium Hydroxide (KOH)	40g

40% Urea Solution	
Ingredients	**Measurements**
Urea crystals	40g
Distilled water	100ml
**** Filter sterilized within laminar air flow cabinets as urea breaks down upon heating.**	

Kovac's reagent	
Ingredients	**Measurements**
Concentrated HCl	25ml
Amyl alcohol	75ml
Paradimethylamino-benzaldehye	5g

Nitrate Reduction Reagent A (sulfanilic acid)	
Ingredients	**Measurements**
Sulfanilic acid	8g
Acetic acid	1000ml
** Acetic acid must be diluted to 5N to 100% glacial acetic acid.	

Nitrate Reduction Reagent B (α-napthylamine)	
Ingredients	**Measurements**
α-napthylamine	5g
Acetic acid	1000ml
** Acetic acid must be diluted to 5N to 100% glacial acetic acid.	

3% hydrogen peroxide solution	
Ingredients	**Measurements**
35% Hydrogen peroxide	2.57ml
Distilled water	27.43ml

Crystal Violet	
Ingredients	**Measurements**
Crystal violet	2g
Ammonium oxalate monohydrate	0.8g
95% ethyl alcohol	20ml
Distilled water	80ml

Gram's Iodine	
Ingredients	**Measurements**
Iodine	1g
Potassium iodide	2g
Distilled water	300ml

95% ethanol	
Ingredients	**Measurements**
Absolute ethanol	95ml
Distilled water	5ml

Safranin	
Ingredients	**Measurements**
Safranin	2.5g
95% ethyl alcohol	10ml
Distilled water	100ml

0.85% salt solution	
Ingredients	**Measurements**
NaCl	0.85grms
Distilled water	100 ml
The solution should be done Autoclaved	

TE Buffer	
Ingredients	**Measurements**
10mM Tris with HCL	1ml
1mM EDTA	0.2ml
Distilled water	100 ml
The solution should be done Autoclaved and pH should be 8	

TBE Buffer	
Ingredients	**Measurements**
Tris Base	54grms
Boric acid	27.5grms
0.5M EDTA	20ml
Distilled water	1000ml
The solution should be done Autoclaved at pH should be 8.3	

Appendix III

List of gadgets that were used during the study

Instrument	Manufacturer
Weighing Machine	Adam equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS-20R	Daihan Scientific Companies, Korea
Spectrophotometer, UV mini - 1240	Shimadzu Corporation, Australia
NanoDrop 2000 Spectrophotometer	Thermo Scientific, USA
Microscope	A. Krüssoptronic, Germany
UV Transilluminator, Model: MD-20	Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
Vortex Machine	VWR International
Microwave Oven, Model:MH6548SR	LG, China
pH Meter: pHep Tester	Hanna Instruments, Romania
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland

List of abbreviations

ml - milliliter

µl - microliter

TBE – Tris borate EDTA

TE – Tris EDTA

PCR – Polymerase chain Reaction

DNA – Deoxyribonucleic acid

TSI – Triple Sugar Iron test

EMB – Eosin Methylene Blue

XLD - Xylose lysine deoxycholate