ANTIBIOGRAM PROFILING OF SORBITOL NON FERMENTING *E. coli* ISOLATED FROM RAW MEAT

A

Project work report submitted to

Department of Microbiology

Central Campus of Technology, Tribhuvan University

In Partial Fulfillment for the Award of the Degree of

Bachelor of Science in Microbiology

Submitted by

Santoshi Ghimire

Roll no: 80019

Department of Microbiology, CCT, Tribhuvan University

Hattisar, Dharan

July 2017

RECOMMENDATION

This is to certify that **Ms. Santoshi Ghimire** has completed project work entitled **"Antibiogram Profiling of Sorbitol non Fermenting *E. Coli* Isolated from Raw Meat"** for the partial fulfillment of the requirements of Bachelor's degree in microbiology under our supervision. To our knowledge this work has not been submitted to any other degree.

____

Mr. Prince Subba

Lecturer

Department of Microbiology

Central Campus of Technology

Tribhuwan University

Hattisar, Dharan, Sunsari

Date:

CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Prince Subba** this project work of **Ms. Santoshi Ghimire** entitled **“Antibiogram Profiling of Sorbitol Non Fermenting *E. Coli* from Raw Meat”** has been approved for examination and is submitted to Tribhuvan University in partial fulfillment of the requirements for Bachelor's degree in Microbiology.

____

Asst. Prof Shiv Nandan Shah
Head of Department
Department of Microbiology
Central Campus of Technology
Tribhuvan University
Hattisar, Dharan, Sunsari

Date:

BOARD OF EXAMINERS

Recommended by:

____

Mr. Prince Subba
Supervisor

____

Asst. Prof. Shiv Nandan Sah
Co –supervisor

Approved by:

____

Asst. Prof. Shiv Nandan Sah
Head, Department of Microbiology

Examined by:

____

(Internal Examiner)

____

(External Examiner)

Date:

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deep sense of gratitude to my respected supervisor, **Mr. Prince Subba**, Lecturer, Central Campus of Technology, Department of Microbiology for his continuous guidance, valuable suggestions and support during my research.

I would like to express my gratefulness to my co-supervisor, **Mr. Shiv Nandan Sah,** Head, Department of Microbiology for his support that helped for the timely completion of the project.

I acknowledge Department of Microbiology, Central Campus of Technology, for allowing me to perform the research work and for the laboratory materials to carry out the study.

I want to express my thankfulness to my classmates especially Subhekshya Rijal, Srijana Bastola, Nisha Koirala, Rojina Subedi, Yasodha Rai, and Chunsa Limbu for their cooperation during the practical and documentation phases of the project. I'm grateful to Kamana Bantawa, Aindra Karki and Saru Ghale, staffs of Microbiology Department for their good company and help in laboratory work.

Finally, I am thankful to my family members who have brought me up to this position with continuous support, blessings, and encouragement.

____

Santoshi Ghimire

Date:

ABSTRACT

The present study was carried out for microbiological analysis of raw meat marketed in Dharan Sub metropolitan city. Antibiotic resistance and its dissemination in food is a serious public health issue. The objective of study was to explore the occurrence and distribution of *E. coli* in meat and their susceptibility to antibiotics. Total of 24 samples (6 chicken, 6 buffalo,6 pork and 6 goats) were randomly collected when examined for stated microbiological parameters showed 41.66% Samples viz. 10 samples (3 Chicken, 3 Buffalo, 2 Pork, 2 Goat meat samples),were sorbitol non-fermenting *E. coli*. The highest Percentage of Sorbitol Non-Fermenting *E. coli* was found in Chicken and Buffalo meat with 50%, where's as Pig and goat meat had the lowest prevalence with 33.33% occurrence.

All the isolated *E. coli* were subjected to the antibiotic susceptibility test using 17 different antibiotics namely Amoxicillin, Ampicillin, Ceftazidime, Cefixime, Amikacin, Ciprofloxacin, Chloramphenicol, Nalidixic acid, Erythromycin, Nitrofurantoin, Teicoplanin, Impenin, Cotrimoxzole, Gentamycin, Ceftriaxone, Cefotaxime and Tetracycline. All *E. coli* showed resistance (100%) against Ampicillin, Amoxicillin, Ceftazidime, and Teicoplanin. Out of 6 chicken C_2 was found as the most pathogenic one because it showed resistance with greater number of drugs. The raw meat samples were found highly contaminated with sorbitol non-fermenting *E. coli*. And the people of Dharan are found at higher risk to be victim of such pathogenic *E. coli*. So, Strict hygienic practices, regular checks, sanitation, training to meat handlers, implementation of slaughterhouse and meat act and raising public awareness should be promoted to ensure quality of raw meat.

Key words: Antibiotic resistance, Antibiotic susceptibility test, Sorbitol non-fermenting *E. coli*

TABLE OF CONTENTS

CONTENTS	Page No

RECOMMENDATION ii
CERTIFICATE OF APPROVAL iii
BOARD OF EXAMINERS iv
ACKNOWLEDGEMENTS v
ABSTRACT vi
TABLE OF CONTENTS vii
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF APPENDICES xi
LIST OF PHOTOGRAPHS xii
ABBREVIATIONS xiii
CHAPTER I: INTRODUCTION 1
1.1 Background 1
1.2 Statement of the problem 2
1.3 Rationale of study 3
1.4 Objectives of the study 4
1.4.1 General objective 4
1.4.2 Specific objectives 4
CHAPTERII: LITERATURE REVIEW 5
2.1 Background 5
2.2 Biochemical Composition of Meat 5
2.3 Microbiology of Meat 5
2.4 Meat Marketing System in Dharan 6
2.5 Enterobacteriaceae 7
2.5.1 General overview of *E. coli* as pathogen 7
2.5.2 Taxonomic classification of *E. coli* 8
2.5.3 Cell structure and physiology of *E. coli* 9
2.5.4 Growth characteristics of *E. coli* 10
2.5.5 Sorbitol non-fermenting *E. coli* (*E. coli* O157) 11
2.6 Antibiotic Sensitivity Test 14
2.6.1 Principle 14
2.6.2 Antimicrobial Resistance in *E. coli* 14
CHAPTER III: MATERIALS AND METHODS 16
3.1 MATERIALS 16
3.2 METHODOLOGY 16

3.2.1 Study duration 16
3.2.2 Laboratory set up 16
3.2.3 Area of Study 16
3.2.4 Sampling Methods and Sample size 16
3.2.5 Sample Collection and Transport 16
3.3 Processing of Meat Sample 17
3.3.1 Homogenization 17
3.4 Laboratory Analysis 17
3.4.1 Bacterial Isolation 17
3.4.2 Identification 17
3.5 Antibiotic Susceptibility Test of Isolates 18
3.6 Quality Control 18
CHAPTER IV: RESULT 20
4.1 Bacterial Isolation 20
4.2 Antibiotic Susceptibility Pattern of *E. coli* 21
CHAPTER V: DISCUSSION 23
CHAPTER VI: CONCLUSION AND RECOMMENDATION 25
6.1 CONCLUSION 25
6.2 RECOMMENDATION 25
REFERENCES 26

LIST OF TABLES

Table 2.1 Gastrointestinal disease caused by different strains of *E. coli.*

Table 4.1 Occurrence of Sorbitol Non-Fermenting *E. coli*

Table 4.2 Colonial characteristics of Sorbitol Non-Fermenting *E. coli*

Table 4.3 Biochemical reaction of Sorbitol Non-Fermenting *E. coli*

Table 4.4 Antibiotic Susceptibility Pattern of Sorbitol Non-Fermenting *E. coli*

Table A1 Biochemical Characterization of Sorbitol Non-Fermenting *E. coli*

Table A2 Zone size interpretation chart of antibiotic susceptibility test of *E. coli*

Table A3 Antibiotic Susceptibility Pattern of sorbitol non-fermenting *E. coli*

LIST OF FIGURES

Figure 3.1 Flow chart of Isolation and Identification of Sorbitol Non-Fermenting *E. coli*

LIST OF APPENDICES

Appendix I	List of materials
Appendix II	Composition and Preparation of different culture media
Appendix III	Methodology of Biochemical Test used for the identification of bacteria
Appendix IV	Tables

LIST OF PHOTOGRAPHS

Photograph 1 Homogenate in EC reduced Bile Salt Broth
Photograph 2 *E coli* in Sorbitol MacConkey Agar
Photograph 3 *E. coli* in Eosin Methylene Blue
Photograph 4 Sub culture in Nutrient Agar
Photograph 5 *E. coli* in Sorbitol Broth
Photograph 6 Biochemical tests of Sorbitol Non-Fermenting *E. coli*
Photograph 7 Antibiotic susceptibility test of Sorbitol Non-Fermenting *E. coli*
Photograph 8 Antibiotic susceptibility test of Sorbitol Non-Fermenting *E. coli*
Photograph 9 Antibiotic susceptibility test of Sorbitol Non-Fermenting *E. coli*
Photograph 10 Antibiotic susceptibility test of Sorbitol Non-Fermenting *E. coli*

LIST OF ABBREVIATIONS

AST	Antibiotic Susceptibility Testing
CLSI	Clinical and Laboratory Standards Institute
EMBA	Eosin Methylene Blue Agar
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
ISO	International Standards Organization
SMCA	Sorbitol MacConkey Agar
MHA	Muller Hinton Agar
SIM	Sulfide Indole Motility
TSI	Triple Sugar Iron
WHO	World Health Organization
MDR	Multiple Drug Resistance
EHEC	Enterohaemorrhagic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EPEC	Enteropathogenic *E. coli*
EAEC	Enteroaggregative *E. coli*
EIEC	Enteroinvasive *E. coli*
DAEC	Diffusely Adherent *E. coli*

CHAPTER I: INTRODUCTION

1.1 Background

Food safety is one of the leading issues for the agricultural industry including livestock production sector (Barry and Richard, 2011). Some data from both developing and developed countries indicate that at least 10% of the population may experience a food borne disease (Cohen et al., 2006). This indicates that microbial food-borne illness still remains a global concern despite the extensive scientific progress and technological developments achieved in recent years especially in developed countries (Pal, 2012). Such problems are usually occurred because of the prevailing poor food handling and sanitation practices, inadequate food safety laws, weak regulatory system, lack of financial resources to invest in safer equipment and lack of education for food-handlers (Haileselassie et al., 2013).

Meat is animal flesh that is eaten as food. It is an important part of balance diet for most people and most widely used food items having both macro and micro nutrient. Its nutritive value is reflected by its chief constituents namely, 75% water, 20% protein, 5% fat, carbohydrate and assorted proteins (science of meat, 2015). Goat, buffalo, poultry, pig and sheep are the major meat animals in Nepal. The annual production of meat in Nepal is 181.867 million tones whereas present demand of meat is 189.700 million tons (Joshi et al., 2015). The healthy inner flesh of meat has been reported to contain less or nil microorganisms, although they have been found in lymph nodes, bone marrow and even flesh (Frazier et al., 2014). The contamination comes from external sources during bleeding, handling and processing. Knives, tools, cloths, hands and air serve as the sources of contaminants. The microbial contamination in meat has important consequences in public health, storage life and spoilage of meat (Gracey and Collins, 1994). Food borne pathogens are the leading causes of illness and death in less developed countries killing approximately 1.8 million people annually (Shah, 2012). The prevalence of pathogens in meat and its level depend upon the number of factors including origin of animal, sanitation and hygiene practices employed during handling and processing. High numbers of microorganism are found in intestinal content and some of them reach the surface of carcasses during dressing operations via the hands of workers, tools, clothing, water etc.

The most important food borne bacterial pathogens associated with meat are Salmonella species, *Staphylococcus aureus, Escherichia coli, Campylobacter*

jejuni, Listeria monocytogenes, Clostridium perfringes, Yersinia enterocoliticaand Aeromonashydrophila (Koutsoumanis and Sofos, 2004). Among them, Salmonella species, *Campylobacter jejuni, Listeria monocytogenes* and verocytotoxin producing *E. coli* O157 are major public health problem (Korsak et al., 1998).

According to Schroeder (2002), *E. coli* are facultative anaerobes and component of the usual intestinal flora in humans and animals, belongs to the Enterobacteriaceae family and is ubiquitously found in faeces of healthy humans, pig, goat, buffalo, chicken, and other domestic, wild mammals and birds. Among pathogenic strains identified, the commonest is the Enterohaemorrhagic *E. coli* (EHEC), others being Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely Adherent *E. coli* (DAEC) (Bolton, 2011).

E. coli O157:H7, termed as an EHEC is one of the most significant food-borne pathogens, differs from most others strains of *E. coli* in being unable to ferment sorbitol. Typical illness as a result of an *E. coli* O157:H7 infection can be life threatening, and susceptible individuals show a range of symptoms including haemolytic colitis, hemolytic uremic syndrome, and thrombocytopenia purpura (Sima et al., 2000; Chileshe and Ateba, 2013)

1.2 Statement of Problem

An evaluation of quality before consuming is the most for any food. Microbiological quality is important from public health point of view. Both consumers and sellers of Dharan are not very sensitive towards meat quality. Apparently the hygienic, condition of meat sold in Dharan market is very poor. Due to the deficiency of slaughterhouse, use of traditional methods of slaughtering, absence of monitoring and proceeding and reprisals, professional meat trade cannot be safe in Dharan. Lack of meat inspector, infrastructure, well-trained workers, use of unhygienic water and selling of meat in open places and poor sanitation of meat shop and meat animals play additive role to increase the rate of contamination in meat and increase the risk of acquiring the food borne illness via the consumption of meat. Food poisoning incidences due to consumption of poor quality of meat has not been recorded systematically to date. Hence, an investigation is essential to find out the prevalence of sorbitol non-fermenting *E. coli* in raw meat, to assess the

microbiological meat safety and to prevent the food poisoning incidence due to consumption of meat in Dharan.

1.3 Rationale of the Study

In order to improve livestock production, antibiotics are often used. This study provides much needed information on antibiotic Resistance pattern of Sorbitol Non-Fermenting *E. coli.* Despite the widely presence of the problems, there is still lack of compressive survey of such food borne pathogen (Sorbitol non-fermenting *E. coli*) at abattoirs. An evaluation of quality before consuming is the most for any food. Nepal is the member of World Health Organization (WHO). So, safe and wholesome meat is must in order to be able to compare in market. Since Nepal does not have any developed standards for microbial quality of meat, this research can help to establish suitable guideline for microbial safety in raw meat. The information that obtained can be useful to Dharan Sub-Metropolitan city and nation for the management of Sorbitol non-fermenting i.e. pathogenic *E. coli* in raw meat. This finding is able to evaluate the current status of hazards associated with meat consumption.

1.4 Objectives of the study

1.4.1 General objective

The general objective of the study is Antibiogram profiling of Sorbitol Non Fermenting *E. coli* from raw meat.

1.4.2 Specific objectives:

The specific objectives are as follows;

a. To isolate and identify Sorbitol Non-Fermenting *E. coli* from different meat samples.

b. To differentiate sorbitol fermenting and Sorbitol Non-Fermenting *E. coli.*

c. To perform antibiotic susceptibility test.

CHAPTER II: LITERATURE REVIEW

2.1 Background

Meat is the muscle tissue of an animal, eaten as food. The word ‘meat’ comes from old English word ‘mete’, Danish word ‘mad’, Swedish word ‘mat’ and Icelandic word ‘matur’ which means generally food. Since humans are omnivores, they used to hunt and kill animals for meat in ancient times (John, 2013). The civilization allowed the domestication of animals such as chickens, sheep, pigs and cattle and eventually their use in production in industrial scale. The paleontological evidence shows that meat was the diet of even earliest humans. Domestication and breeding of animals allowed the systematic production meat (Lawrie, 2006). The principal animal sources of meat are sheep, beef, pigs, horses, dogs, cats, guinea pig, buffalo, poultry, whales and dolphins.

2.2 Biochemical composition of meat

Biochemical composition of meat varies according to species, breed, sex, age, plane of nutrition, training and exercises of the animal as well as anatomical location (Lawrie, 2006). Adult mammalian flesh of meat contains roughly 75 percent water, 19 percent protein, 2.5 percent intramuscular fat, 1.2 percent carbohydrate and 2.3 percent other soluble non-protein substances. These include nitrogenous compounds such as amino acids and inorganic substances such as minerals (Lawrie, 2006). Muscle proteins are either soluble in water (about 11.5 % of total mass) or in concentrated salt solution (about 5.5 % of total mass). Water soluble proteins are called sarcoplasmic proteins and salt soluble proteins are called myofibrillar proteins. There are several sarcoplasmic proteins and are glycolytic enzymes. Myosin and actin are major myofibrillar proteins, responsible for muscles overall structure. The remaining protein mass contains connective tissue (collagen and elastin) as well as organelle tissue (Lawrie, 2006). Fat in meat can be either adipose tissue or intramuscular fat containing considerable quantities of phospholipids and of unsaponifiable components such as cholesterol (Lawrie, 2006).

2.3 Microbiology of meat

Being an ideal growth medium for many microorganisms, meat is highly susceptible to spoilage as well as frequently implicated in the spread of food

borne illness. Contaminated raw meat and retail meat shops are potential vehicles for transmitting food borne illness (Bhandari et al., 2007). During slaughtering and processing, potentially edible tissues are subjected to contamination from a variety of sources within and outside animal. The living animal surfaces in contact with the environment, harbors a variety of microorganisms. The contaminating organisms mainly derive from the hide of animal and from feces. The processed meat foods are prone to contamination with pathogenic microorganisms during various stages of processing. Bacterial genera commonly infecting meat while it is being processed, cut, packaged, transported, sold and handled include Salmonella species, Shigella species, *Escherchia coli, Vibrio cholerae, Staphylococcus epidermidis, S. aureus, Clostridium wellchii, Clostridium botulinam, Bacillus cereus* and fecal Streptococci. The molds commonly commonly infecting meat are Penicillium, Mucor, Cladosporium, Alternaria, Sporotrichum, and Thamnidium (Lawrie, 2006). When these organisms colonize a piece of meat, they begin to break it down leaving behind toxins that can cause enteritis or food poisoning. They do not survive a thorough cooking but several toxins and spores can survive (Lawrie, 2006). Microorganisms on the substrates will bring physical and chemical alterations when they grow resulting on the unwanted colors, odors and tastes. Visible colonies will appear if contaminated with molds. Among the bacteria, viruses, yeasts, molds and parasites, bacteria are the most frequent contaminant capable of causing disease or producing toxins that may be dangerous to consumer meat and poultry dishes have been major vehicle of food borne intoxications (Wilson et. al., 1981). The microbial populations growing in raw meat and meat products are the effects of the prevailing environmental conditions, type of microorganisms initially present in the raw meat and materials as well as cross contamination or processing. Both intrinsic and extrinsic factors affect the microbial growth on meat (Mossel and Ingram, 1955). Predominant intrinsic factors are concentration of nutrients, pH, redox potential, buffering capacity, availability of water and structure of meat and meat products. The main important extrinsic factors are storage and processing conditions.

2.4 Meat marketing system in Dharan

Meat animals are usually purchased in livestock markets from brokers and traders who obtain the animals from villages of Nepal as well as from India. The meat shops are dispersed all over the city centering main market area. It is usual for butchers to slaughter in early morning in open field. Animals are

mistreated using cruel methods. The water used for cleaning is often heavily polluted. The condition of shops where meat is sold does not comply with minimum expected requirements for hygiene and quality. Animals do not undergo any health inspection. The transportation of buffalo, pig and goat meat from slaughtering site to meat shops have been used with public vehicles like auto-tempos, rickshaws, cars and hand carts all are used and eat is carried unwrapped exposing to flies and dust. Shops do not have cold chain facility. (Adhikari et. al., 2012)

2.5 Enterobacteriaceae

Members of this family are found primarily in the guts of humans and warm blooded animals. They are Gram-negative, rod-shaped, and non-sporulating facultative anaerobes. They also ferment different carbohydrates using them as the carbon source. They may grow as mucoid colonies when grown on agar plates but only Klebsiellaspp are truly encapsulated. The most infections caused by the Enterobacteriaceae are the urinary tract infections. Others include wound infections, pneumonia, septicaemia and infections involving the nervous system. Clinically important members of the family include Escherichia, Salmonella, Citrobacter, Enterobacter, klebsiella, Proteus, Providencia, Serratia, Shigella, Yersinia, Enterobacter, etc. Some genera in this family are known to cause intestinal infections such as enteritis and diarrhea. (Tärnberg, 2012)

2.5.1 General overview of *E. coli* as pathogen

E. coli are Gram-negative, facultative anaerobic, rod-shaped and highly motile bacteria. They are often classified under enterobacteriacea known to be normal inhabitants of the gastrointestinal tract of both animals and human beings but only some strains of *E. coli* have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases (Kubitschek, 1990). *E. coli* were first isolated by a German paediatrician, Theodore Esherich, in 1884 from faeces of human neonates (Khan and Steiner, 2002). The first confirmed isolation of *E. coli* O157:H7 in the United States of America was in 1975 from a Californian woman with bloody diarrhoea, while the first reported isolation of the pathogen from cattle was in Argentina in 1977 (Fernandez, 2008). It was since 1982 that EHEC have been recognized as an important aetiological agent of diarrhoeal diseases in man and animals. *E coli* O157 were described as a rare serotype (Karmali et al., 1983). Studies conducted between 1983 and

1985 in the United States and Canada, have linked EHEC infection to hemorrhagic colitis (HC) and it had a close relation with the classical form of haemolyticuraemic syndrome (HUS) (Karmali et al., 1985). As a result of these and other studies, Orskov et al. (1987) re-examined isolates of *E. coli* belonging to the O157 serogroup that had been submitted to the International Escherichia and Klebsiella Centre. Three isolates were found that had the H7 antigen (Orskov et al., 1987). These three isolates were from the faeces of one animal out of a batch of 39 calves with colibacillosis in Argentina. Orskov and colleagues (1987) also speculated that cattle might be the reservoir for these organisms.

2.5.2 Taxonomic classification of *E. coli*

The comparative analysis of 5S and 16S ribosomal RNA sequences suggest that Escherichia and Salmonella diverged from a common ancestor between 120 and 160 million years ago, which coincides with the origin of mammals. Escherichia and Shigella have been historically separated into different genera within the Enterobacteriaceae family. DNA sequence analysis of their genomes reveals a high degree of sequence similarity and suggests that they should be considered a single species (Ochman & Wilson, 1987). There are several types of *E. coli* strains but these strain types can be divided into six groups or pathotypes based on the mechanism they cause disease: Enteropathogenic *E. coli* (EPEC), Attaching and effacing *E. coli* (A/EEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), EHEC and Enteroaggregative *E. coli* (EAEC). *E. coli* strains that produce the Stx toxins have been referred to as Vero Toxin-producing *E. coli* (VTEC), Shiga-toxigenic *E. coli* (STEC) and Enterohaemorrhagic *E. coli* (EHEC) (Karmali, 1989; Nataro & Kaper 1998).

Scientific classification of *E. coli* is under:

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Family: Enterobacteriaceae
Genus: *Escherichia*
Species: *E.coli*
Source: Nataro & Kaper (1998)

EPEC organisms are a significant cause of infant diarrhea in developing nations. Enteropathogenic *E. coli* (EPEC) were historically recognized on the basis of serotypes such as O55:H6 and O127:H6. EPEC are an established etiological agent of human infantile diarrhea. In developing countries, Enteropathogenic *E. coli* (EPEC) is one of the most common pathogens (Fagundes & Scaletsky, 2000).

EIEC organisms often cause a broad spectrum of human's diseases. They are biochemically, genetically and pathogenetically closely related to Shigella species. Both characteristically cause an invasive inflammatory colitis, but either may also elicit a watery diarrhea syndrome indistinguishable from that caused by other *E. coli* pathogens. (Nataro & Kaper, 1998)

ETEC strains are a major cause of secretory diarrhea in both humans and animals (Bern et al., 1992). ETEC produce toxins which are heat-labile (LT) and/or heat-stable (STa and STb) that are also causing diarrhea. It is known to be a frequent cause of diarrhea in both humans and animals.

EAEC strains are defined by their distinctive adherence pattern on HEp-2 cells in culture (Nataro & Kaper, 1998). The essential element of the aggregative phenotype is the stacked brick pattern by lying side-by-side with an appreciable distinction of where one bacterium begins and another ends. (Nataro & Kaper, 1998).

Enterohaemorrhagic *E. coli* (EHEC) strains are implicated in food-borne diseases principally due to ingestion of uncooked minced meat and raw milk. These strains produce shiga like toxin 1 (Stx1), shiga-like toxin 2 (Stx2). Serotype EHEC O157:H7 is the prototype of increasing importance and is associated with hemorrhagic colitis, bloody diarrhea and the hemolytic uremic syndrome (Pickering et al., 1994; Cornick et al., 2000).

2.5.3 Cell structure and physiology of *E. coli*

E. coli is the head of the large bacterial family, Enterobacteriaceae, the enteric bacteria, which are facultative anaerobic and non spore forming bacilli having about 2μm long and 0.5μm in diameter with a cell volume of 0.6 to 0.7μm3 (Kubitschek, 1990). They are approximately 0.5 μm in diameter and 1.0–3.0 μm in length. Within the periplasm is a single layer of peptidoglycan. The peptidoglycan has a typical subunit structure where the N-acetylmuramic acid is linked by an amide bond to a peptide consisting of L-alanine, D-glutamic acid, meso-diaminopimelic acid and D-alanine. *E. coli* are commonly motile in liquid by means of peritrichous flagella. *E. coli* are commonly fimbriated.

The type I pili are the most common and are expressed in a phase switch on or off manner that leads to piliated and nonpiliated states (Eisenstein, 1987). One of the traits commonly encoded on the larger genetic islands of the different pathotypes of *E. coli* are additional pili (chaperone-usher and type IV pili families and non-pili adhesions (Schreiber & Donnenberg, 2002).

Among *E. coli* isolates, there is considerable variation and many combinations of somatic (O and K) and flagellar (H) antigens. *E. coli* are routinely characterized by serological identification of somatic O, flagellar H and capsular K antigens. However, while some serotypes correlate closely with certain clinical syndromes, differentiation of pathogenic strains from the normal flora depends on the identification of virulence characteristics. Among pathogenic strains, there are few patterns of these antigens and few phylogenetic groupings. For *E. coli*, there are over 150 antigenically unique O-antigens (Whitfield & Valvano, 1993). K type capsular material occurs in two or four forms on the basis of physical, biochemical and genetic criteria (Whitfield & Roberts, 1999).

Over 80 serologically and chemically distinct capsular polysaccharides have been recognized. In addition, a slime layer, colonic acid extracellular polysaccharide, is common to many *E. coli* isolates and can be co-expressed with some K-type capsules. There are 53 H-antigen specificities among E. coli (Schreiber & Donnenberg, 2002).

2.5.4 Growth characteristic of *E. coli*

E. coli is a facultative anaerobe. Though most *E. coli* strains are capable of growing over a wide range in temperature (approximately 15–48°C), the growth rate is maximal in the narrow range of 37–42°C. Escherichia coli can grow within a pH range of approximately 5.5–8.0 with best growth occurring at neutrality. Some diarrheagenic *E. coli* strains have the ability to tolerate exposure to pH 2.0. Such an acid shock mimics transit through the stomach and induces expression of sets of genes involved in survival and pathogenesis (Fotadar et al., 2005). It is capable of reducing nitrates to nitrites. When growing fermentatively on glucose or other carbohydrates, it produces acid and gas (mainly H_2 and CO_2). By traditional clinical laboratory biochemical tests, *E. coli* is positive for indole production and the methyl red test. Most strains are oxidase, citrate, urease and hydrogen sulfide negative. The classic differential test to primarily separate *E. coli* from Shigella and Salmonella is

the ability of *E. coli* to ferment lactose, which the latter two genera fail to do (Fotadar et al., 2005).

Aside from lactose, most *E. coli* strains can also ferment D-mannitol, D-sorbitol, and Larabinose, maltose, D-xylose, trehalose and Dmannose. There are limited instances where pathogenic strains differ from the commensals in their metabolic abilities. For example, commensal *E. coli* strains generally use sorbitol, but *E. coli* O157:H7 does not. Most diarrheagenic strains cannot utilize D-serine as a carbon and nitrogen source, but uropathogenic and commensal fecal strains can use this enantiomer of serine (Roesch et al., 2003).

2.5.5 Sorbitol non-fermenting *E. coli* (*E. coli* O157:H7)

E. coli O157: H7 differs from most other strains of *E. coli* in being unable to ferment sorbitol EHEC O157:H7 infections occur worldwide; infections have been reported on every continent except Antarctica. Other EHEC are probably also widely distributed. Species affected are Ruminants, especially cattle and sheep, are the major reservoirs for EHEC O157:H7 (Griffin, 1995). This organism can sometimes be found in other mammals including pigs, rabbits, horses, dogs, and other domestic and wild birds. In some instances, it is not known whether a species normally serves as a reservoir host or if it is only a temporary carrier. For example, rabbits shedding EHEC O157:H7 have caused outbreaks in humans, but most infected rabbits have been found near farms with infected cattle (Alam and Zurek, 2006).

EHEC O157:H7 are transmitted by the fecal–oral route. They can be spread between animals by direct contact or via water troughs, shared feed, contaminated pastures or other environmental sources. Birds and flies are potential vectors. The organism was thought to have become aerosolized during high pressure washing of pens, but normal feeding and rooting behavior may have also contributed (Griffin and Tauxe, 1991 Leomil, 2005).

These are strains of *E. coli* that produce one or more types of cytotoxins known as Shigatoxin (Stx) or Verocytotoxin (VT). STEC was discovered in North America in 1982 in stool isolates of Escherichia coli from sporadic cases of haemolyticuraemic syndrome. Since then, they have been implicated in major foodborne illnesses reported in both developed countries and developing countries (Karmali et al., 1983; Gyles, 2007). Shiga toxin producing *E. coli* can also be referred to as Verocytotoxin-producing *E. coli* (VTEC) for its toxigenic effect to Vero cells (Xia et al., 2010). Largely, STEC

is frequently used in America, whereas VTEC is mostly used in Europe (Bolton, 2011). Various diseases caused by STEC include watery diarrhoea, bloody diarrhoea, haemorrhagic colitis (HC), and haemolyticuraemic syndrome (HUS). Hemolytic uremic syndrome is a rare disorder characterized by microangiopathic hemolytic anaemia, microthrombi, and multiorgan injury. It is one of the major causes of acute kidney failure in children globally (Tarr et al., 2005). Occurrences of illnesses caused by STEC have been epidemiologically associated with contact with animals and consumption of meat and fresh products (Kaspar et al., 2010). The STEC causing HC and HUS are also called Enterohemorrhagic *E. coli* (EHEC) (Gyles, 2007). Effective procedures are available for detection of O157, but the same methods cannot be applied to the non-O157 serogroups due to the complexity and diversity of these pathogens, which has prohibited the development of a standardized isolation and culturing method (Conrad et al., 2014).

Table: 2.1 Gastrointestinal disease caused by different strains of *E. coli.*

Serotypes	Diseases
Enterotoxigenic *E. coli* (ETEC) O6, O8, O25, O63, O78, O148, O159, O167 etc.	Traveler's diarrhea, infant diarrhea in developing countries, watery diarrhea, nausea, vomiting, cramps, low grade fever.
Enteropathogenic *E. coli* (EPEC) O26, O55, O86, O111, O114, O126, O128, O142 etc.	Infant diarrhea with fever, vomiting, nausea, and non-bloody stools.
Enterohaemorrhagic *E. coli* (EHEC or VTEC) O157	Haemorrhagic colitis with severe abdominal cramps, initial watery stool, followed by grossly bloody diarrhea.
Enteroinvasive *E. coli* (EIEC) O28 ac, O112 ac, O124, O144, O152, O164 etc.	Fever, cramping, watery diarrhea followed by development of dysentery with scanty bloody stools.
Enteroaggressive *E. coli* (EAggEC)	Infant diarrhea in developing countries persistent watery diarrhea with vomiting dehydration fever.

Source: P. Chakraborty

2.6 Antibiotic Sensitivity Test

Antibiotic Sensitivity describes the susceptibility of bacteria to various antibiotics. Antibiotic Sensitivity test is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection in vivo, because susceptibility can vary within a species. AST is often done by the Kirby-Bauer method. In this method small antibiotic discs are placed onto a plate upon which bacteria are growing. If the bacteria are sensitive to the antibiotic, a clear zone of inhibition is seen around the disc. Ideal theory is based on determination of the etiological agent and its relevant antibiotic sensitivity. The effectiveness of individual antibiotics varies with the location of the infection, the ability of antibiotic to reach the site of infection, and the ability of bacteria (bactericidal), whereas others prevent the bacteria from multiplying (bacteriostatic). Muller-Hinton agar is most frequently used in this susceptibility test.

2.6.1 Principle

In Kirby-Bauer method, the antibiotic impregnated discs are placed on the Muller Hinton Agar plates on which the bacterial culture is spread. As the antibiotic impregnated discs come in contact with the moist agar surface, water is absorbed in the disc paper diffusing antibiotic out in the surrounding medium. As the distance from the disc increases, there is a logarithmic reduction in the agar medium surrounding each disc. Though the diffusion of drug occurs, the bacteria that are inoculated on the agar surface are not inhibited by the concentration of antimicrobial agents but continue to multiply until the growth is visible. No growth occurs in the area where the concentration of drug is inhibitory thus forming a zone of inhibition. Thus when an organism is sensitive to any antibiotics, a clear zone appears around that specific disc where the growth has been inhibited (zone of inhibition) whereas if an organism is resistant, no clear zone of inhibition appears.

2.6.2 Antimicrobial resistance in *E. coli*

Antimicrobial resistance in *E. coli* has been reported worldwide and increasing rates of resistance among *E. coli* is a growing concern in both developed and developing countries. A rise in bacterial resistance to antibiotics complicates treatment of infections (Erb et al., 2007). In Ethiopia, a number of studies have been done on the prevalence and antimicrobial resistance patterns of *E. coli* from various clinical sources (Gebre-Sellassie, 2007). The recent research

findings of Taye et al. (2013); Hiko et al. (2008); Bekele et al. (2014) and Mohammed et al. (2013) confirmed that *E. coli* O157:H7 have developed already different degrees of resistant against various commonly used antibiotics including erythromycin Amoxycillin-Clavulanic acid, Sulfonamides, Ampicillin and Tetracycline, some strains also developed Multi drug resistant. From the recent research done by Dulo (2014) from carcass swabs taken from goats slaughtered at Dire Dawa municipal slaughter house showed the presence 100% and 83.3% resistant *E. coli* O157:H7 against Erythromycin and Ampicillin respectively. Such types of drug resistance are believed to be as a result of multiple factors like wide use of antibacterial drugs especially in food animals and transfer of drug resistance carrying plasmid gene among *E. coli* species.

The four main Mechanisms by which microorganism's resistance to Antimicrobials are: 1. Drug Inactivation or Modification, 2. Alteration of Target, 3. Reduced Accumulation

CHAPTER III: MATERIALS AND METHODS

3.1 Materials

The materials, equipment, media and reagents used in this study are listed in Appendix I

3.2 Methodology

3.2.1 Study duration

The study was conducted from December 2016 to February 2017.

3.2.2 Laboratory set up

Laboratory setting was done in Microbiology laboratory, Central Campus of Technology, Dharan.

3.2.3 Area of study

Dharan Sub metropolitan city has 2112-hectare area and located in the eastern Terai of Nepal stretching from the edge of northern Mahabharat hill range up to the Charkoshe Jhadi in south separating from the southern Terai. The meat consumption by people of Dharan is high i.e. the per capita meat consumption of Dharan is 13 kg whereas national per capita meat consumption is 9kg. The samples were collected from retail shops of different locations representing 20 wards of Dharan Sub metropolitan city.

3.2.4 Sampling method and sample size

Simple random sampling was done for the collection of sample and total of 24 meat samples, 6 chicken, 6 buffalo, 6 pork, and 6 goat meats from different places of Dharan.

3.2.5 Sample collection and Transport

Sample collection and processing was carried out according to U.S. FDA guideline (U.S. FDA, 2015). A sample size of 25g from each place was collected in sterile plastic bags and analyzed within 2 hrs. of collection. The samples were kept inside the sterile polythene bags without touching by the collector and transported to the laboratory maintaining cold chain. Generally,

the samples were collected in the morning time of at 8-9AM. Samples were processed immediately as soon as possible otherwise preserved at $4^{O}C$.

3.3 Processing of Meat Sample

3.3.1 Homogenization

In this study, 25 gram of meat sample was aseptically transferred into disinfected (treating with 70% ethanol followed by sterile water) meat mincer. Then the homogenate was poured in to the conical flash containing 225ml of *E. coli* reduced salt broth and incubated in incubator at $37^{O}C$ for 24hrs.

3.4 Laboratory Analysis

3.4.1 Bacterial Isolation

For the isolation of Sorbitol non Fermenting *E. coli*, at first, the meat was homogenate. *E. coli* Reduced Salt Broth, Sorbitol MacConkey Agar (SmcA) and Eosin Methylene Blue (EMB) Agar plates were prepared.

The homogenate was subjected to the conical flask containing 225ml of EC Reduced Salt Broth and incubated in incubator at $37^{O}C$ for 24 hours. After incubation, loopful of sample was streak on SmcA. Then the inoculated plates were incubated at $37^{0}C$ for 24 hrs. After incubation the plates were observed. Non-Sorbitol Fermenting colonies were selected and streaked on Eosin Methylene Blue (EMB) and plates were incubated at $37^{O}C$ overnight. After 24 hours of incubation, colonies giving green metallic sheen were sub culture on Nutrient Agar and incubated at $37^{O}C$ for 24hrs. Further, the pure culture was inoculated on the test tube containing Sorbitol Broth for reconfirmation of Sorbitol Non-Fermenting *E. coli* and test tubes were incubated at $37^{O}C$ for 16-18 hrs. After incubation, the test tubes which color remain same as the Sorbitol Broth (red) i.e. Sorbitol Non-Fermenting organisms were selected and streak again on Nutrient Agar (NA).

3.4.2 Identification

After obtaining the pure culture, the organisms were identified by using Standard Microbiological Techniques as described in Bergey's Manual of Systematic Bacteriology-1986. For this, colonies from both NA plates were taken and Gram Stained. Different biochemical tests were performed to confirm *E. coli* using Indole, Methyl red, Voges-Proskauer test, Nitra, Simon

Citrate Agar, Urease Production, and SIM. Each biochemical media was inoculated with *E. coli* taken from NA and incubated at $37^{O}C$ for 24 hours.

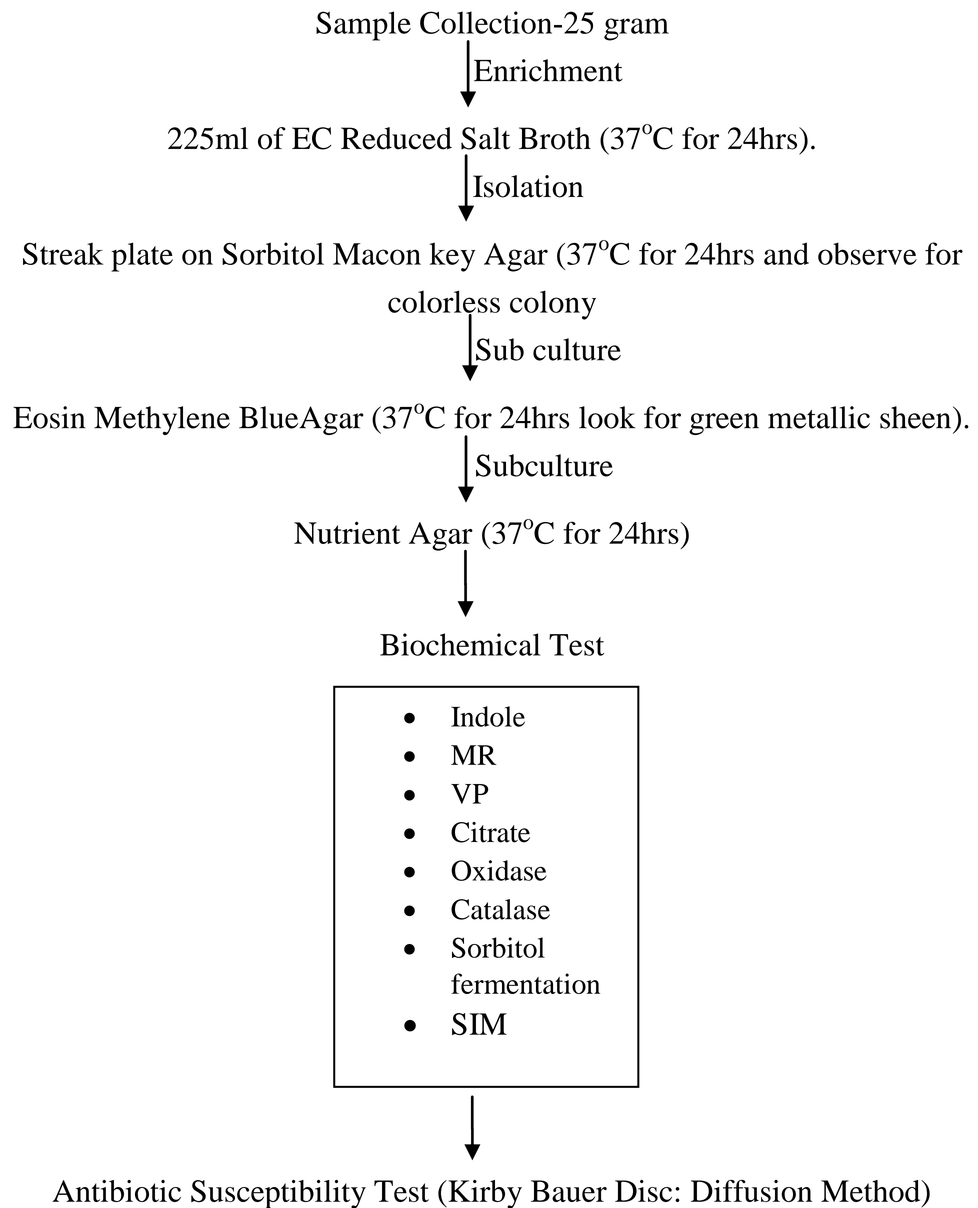
3.5 Antibiotic Susceptibility Test of isolates

Antimicrobial Susceptibility Tests of isolated pathogens were done by Modified Kirby Bauer Disk Diffusion method following CLSI guidelines (CLSI, 2011). Fresh colonies were selected and transferred into the Nutrient Broth (NB) to obtain turbidity equivalent to 0.5 McFarland barium sulfate standards (1.5x108CFU/ml). MHA plates were inoculated with sterile cotton swabs then antibiotic discs were placed with sterile forceps and allowed to stand at room temperature for 15 minutes for pre-diffusion then incubated at $37^{O}C$ for 16-18 hours. The zones of inhibition were interpreted as susceptible, intermediate and resistant according to CLSI "Diffusion Supplemental Table" (2013). A wide range of antibiotics namely Amoxicillin, Ampicillin, Cefotaxime, Cefixime, Amikacin, Tetracycline, Ceftriaxone, Ceftazidime, Ciprofloxacin, Chloramphenicol, Nalidixic acid, Erythromycin, Nitrofurantine, Teicoplanin, Impenem, Cotrimoxzole, Gentamycin were used for Antibiotic Susceptibility Test.

3.6 Quality Control

Antibiotic sensitivity of Sorbitol non-fermenting *E. coli* was compared with ATCC 25922 *E coli* on Muller Hinton Agar.

Figure 3.1: Flow chart of Isolation and Identification of *E. coli*



CHAPTER IV: RESULT

4.1 Bacterial Isolation

In this study, different 24 meat samples (6 Chicken, 6 Buffalo, 6 Pig, 6 Goat) from different location analyzed microbiologically. Out of 24 samples Sorbitol Non-Fermenting *E. coli* were found in 41.66% Samples viz. 10 samples (3 Chicken, 3 Buffalo, 2 Pork, 2 Goat meat samples). The highest Percentage of Sorbitol Non-Fermenting *E. coli* was found in Chicken meat and Buffalo meat with 50% where's as Pig meat and goat had the lowest prevalence of sorbitol Non-Fermenting *E. coli* with 33.33% occurrence. (Table 4.1)

The isolated organisms were identified on the basis of biochemical characteristics. The Colonial Characteristics and Biochemical Reactions are given in Table no. 4.2 and Table no. 4.3 respectively.

Table 4.1: Occurrence of sorbitol non-fermenting *E. coli*

Source sample	*E. coli*	
	Growth	Percentage
Chicken(n=6)	3	50
Buffalo(n=6)	3	50
Pig(n=6)	2	33.33
Goat(n=6)	2	33.33
Total(n=24)	10	41.66

Table 4.2: Colonial characteristics of sorbitol non-fermenting *E. coli*

Organisms	Colonial characteristics			
E. coli	Configuration	Margin	Elevation	Color
	Round	Smooth colonies with entire edge	Raised	colorless and yellowish white

Table 4.3: Biochemical reactions of sorbitol non-fermenting *E. coli*

Organism	Biochemical Tests							
E. coli	Catalase	Oxidase	Indole	MR	VP	Citrate Utilization	SIM	Gram Stain
	+	_	+	+	_	_	+	_

4.2 Antibiotic Susceptibility Pattern of *E. coli*

All isolated *E. coli* were subjected to Antibiotic Susceptibility Test (AST) by using Kirby-Bauer Disc Diffusion Method. The Antibiotic used were Amoxicillin (AMX), Ampicillin (AMP), Cefotaxime (CTX), Cefixime (CFM), Amikacin (AK), Nalidixic acid (NA), Chloramphenicol (C), Gentamycin (GEN), Erythromycin (E), Cefrtiaxone (CTR), Cotrimazole (COT), Impenem (IPM), Teicoplanin (TEI), Tetracycline (TE), Ceftazidime (CAZ), Ciprofloxacin (CIP), Nitrofurantoin (NIT). All *E. coli* showed resistance (100%) against Amoxicillin, Ampicillin, Teicoplanin, and Ceftazidime. 80% resistance to cefotaxime. 60% resistance to Nalidixic acid, 50% resistance to Erythromycin, 40% resistance to ciprofloxacin, 30% resistance to Nitrofurantoin, Imipenem, Tetracycline and Cotrimazole. 10% resistance to Ceftriaxone, Cefixime, Amikacin, and Chloramphenicol. And 90% were susceptible to Gentamycin, 80% susceptible to Ceftriaxone, Amikacin, and Chloramphenicol. 70% susceptible to Tetracycline. 60% susceptible to Cotrimazole. 50% susceptible to Cefixime, 40% susceptible to Nalidixic acid and 30% susceptible to Nitrofurantoin and Imipenem.

Table 4.4: Antibiotic Susceptibility pattern of sorbitol non-fermenting *E. coli*

Antibiotic class	Antibiotic used	Samples										R (%)	I (%)	S (%)
		C_1	C_2	C_3	B_1	B_3	B_{3a}	P_2	P_3	M_1	M_5			
Ampicillins	AMX	R	R	R	R	R	R	R	R	R	R	100	0	0
	AMP	R	R	R	R	R	R	R	R	R	R	100	0	0
Cephalosporin	CAZ	R	R	R	R	R	R	R	R	R	R	100	0	0
	CTX	R	R	R	I	I	R	R	R	R	R	80	20	0
	CTR	S	R	S	S	S	S	S	S	I	S	10	10	80
	CFM	I	R	S	S	S	I	S	S	I	I	10	40	50
Sulpha groups	COT	S	R	S	S	I	R	S	S	R	S	30	10	60
Aminoglycosides	AK	S	S	R	S	S	S	S	S	I	S	10	10	80
	GEN	S	S	I	S	S	S	S	S	S	S	0	10	90
	TE	S	S	R	S	S	R	S	S	R	S	30	0	70
Quinolone	NA	S	R	R	R	R	R	S	S	R	S	60	0	40
Fluoroquinolone	CIP	I	I	R	I	R	R	I	I	R	I	40	60	0
Phenolic	C	S	S	S	S	S	R	S	S	I	S	10	10	80
Carbapenems	IPM	R	S	R	I	R	S	S	I	I	I	30	40	30
Macrolids	E	R	I	R	R	I	R	I	I	R	I	50	50	0
Glycopeptides	TEI	R	R	R	R	R	R	R	R	R	R	100	0	0
Nitroflorentine	NIT	I	R	R	S	I	I	S	S	R	I	30	40	30

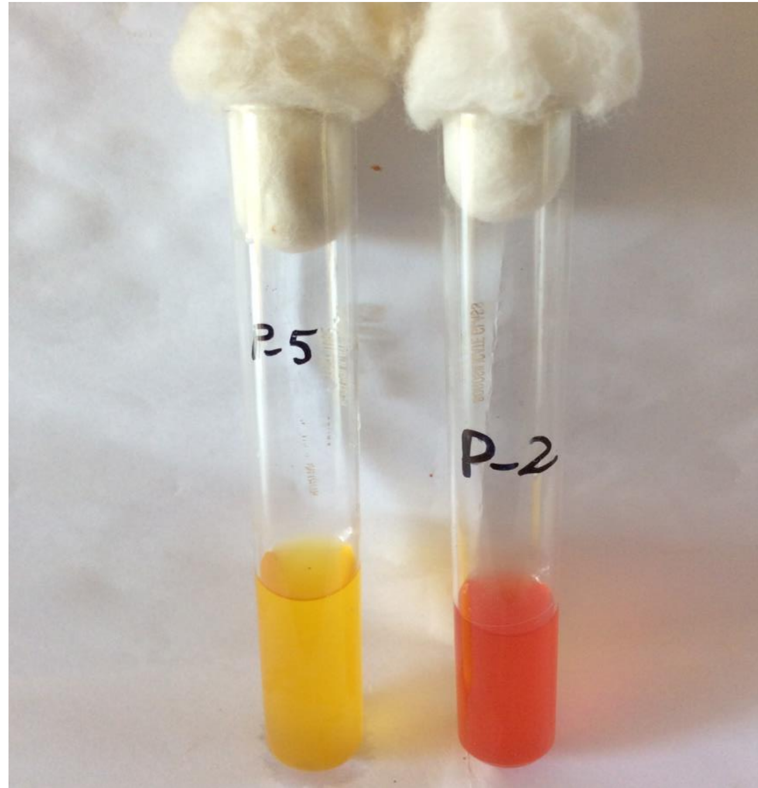
PHOTOGTAPHS

Photograph 1: Homogenate in EC Reduced Bile Salt Broth

Photograph 2: *E coli* in sorbitol MacConkey Agar

Photograph 3: ***E. coli*** **in Eosin Methylene Blue**

Photograph 4: Sub Culture in Nutrient Agar



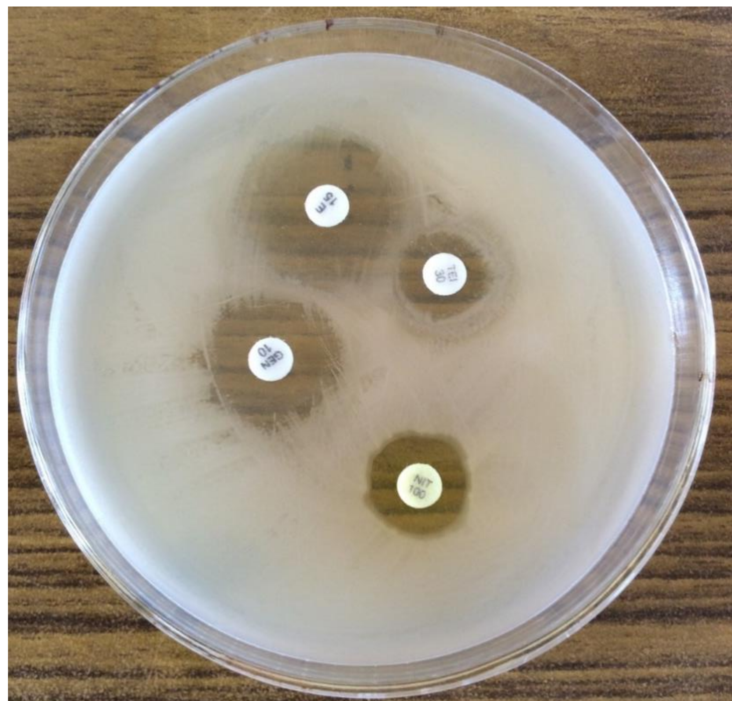
Photograph 5: *E. coli* in Sorbitol broth (P5- sorbitol fermenting, P2 sorbitol non- fermenting)

Photograph 6: Biochemical test of sorbitol non fermenting *E. coli*

1. Indole 2. SIM 3. VP 4. MR 5. Citrate 6. Urease

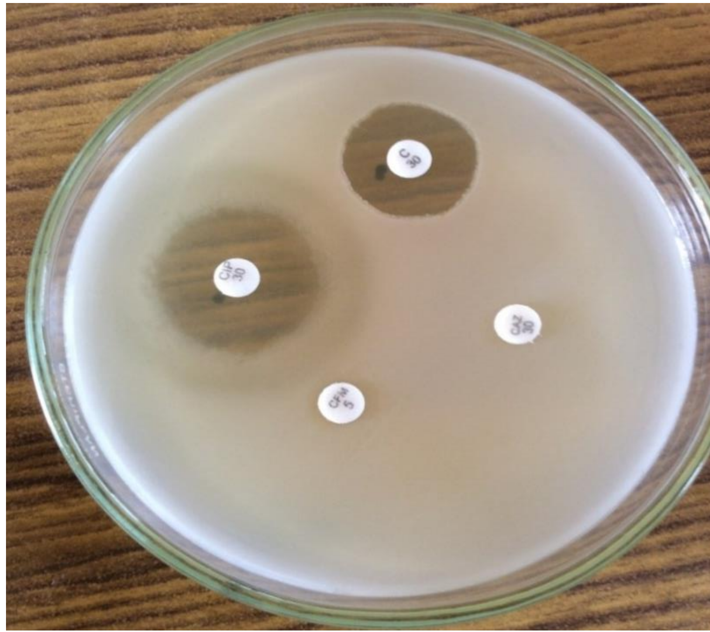
Photograph 7: Antibiotic Susceptibility test of sorbital non-fermenting *E .coli*

Antibiotics used are: Nalidixic acid (NA), Cotrimazole (COT), Tetracycline (TE), Ceftriaxone (CTR), Cefotaxime (CTX)



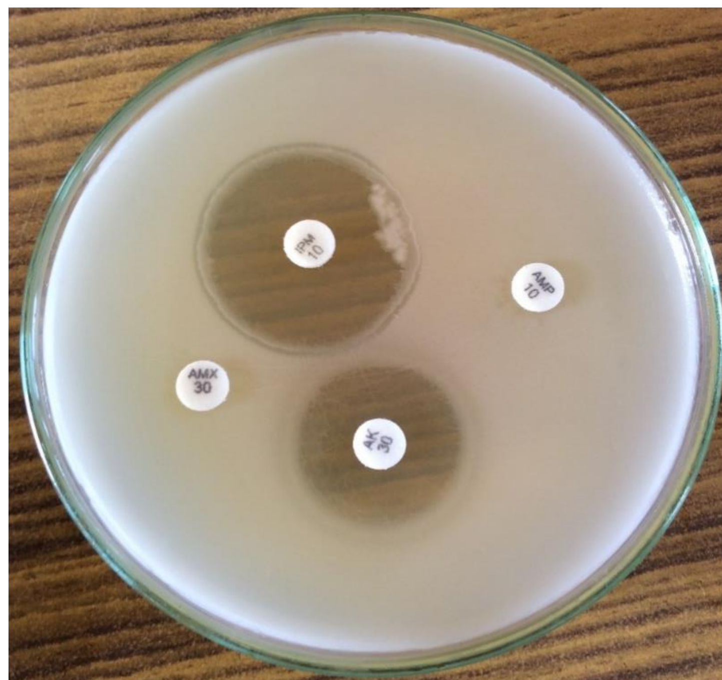
Photograph 8: Antibiotic Susceptibility test of sorbitol non-fermenting *E. coli*

Antibiotics used are: Erythromycin (E), Gentamycin (GEN), Nitroflurentoin (NIT), Teicoplanin (TEI)



Photograph 9: Antibiotic Susceptibility test of sorbital non-fermenting *E. coli*

Antibiotic used are: Chloramphenicol (C), Ciprofloxacin (CIP), Cefexime (CFM), Ceftazidime(CAZ)



Photograph 10: Antibiotic Susceptibility test of sorbital non fermenting *E. coli*

Antibiotic used are: Imipenem (IPM), Amoxicillin (AMX), Amikacin (AK), Ampicillin (AMP)

CHAPTER V: DISCUSSION

Meat and meat products are rapidly contaminated and favoured by the organisms. The increasing population, urbanization, modernization and industrialization of Dharan are also responsible for the pollution. The impact of pollution is also on various food borne diseases due to contamination by various pathogenic bacteria. Microorganisms set into the meat and meat products by water, unclean utensils, knives, unscientific slaughtering practices and cruel handling methods, besides, environmental contamination and handling of meat in its preparation and sales. Due to lack of scientific methods of storage and due to lack of knowledge of microorganisms, many types of microorganisms gain access to meat. Once microorganisms are introduced into the meat, they multiply rapidly and reach levels sufficient to produce infections or intoxications depending upon the types of invasion. The number of microbes in the meat and meat products at any given time depends on its handling, storage condition, storage temperature and length of time it has been kept. The contaminating organisms may include those responsible for food borne illness. But the number or dose of organisms necessary to infect or to produce sufficient toxin to cause symptoms not only varies with the species and kind of organisms but also varies with the resistance of the person who consumed the meat and its products. Even though the microbial population in the meat does not necessarily cause food borne disease, certain microbial contamination is an indicator of poor sanitary practice in the processing and storage of meat.

Numerous epidemiological reports have implicated food of animal origin as the major vehicles associated with illness caused by food borne pathogens, such as *E. coli*, Salmonella, Shigella and Campylobacter for human infection and this problem is highly aggravated in the developing world (Zhao et al., 2001; Humphrey and Jorgensen, 2006; Pal, 2007). Meat is generally checked for the presence of indicator organisms such as *E. coli* and coli forms to indicate the possible contamination with viscera or fecal material (Brown and Baird-Parker, 1982). The present finding is an objective revelation of very poor microbiological quality of meat traded in Dharan. Although FAO, 1992 has reported to minimize contamination of meat during slaughtering, dressing, and subsequent handling of meat by enteropathogenic organism none of the market sample were found in such uncontaminated condition by these organisms. Meat of Dharan is found to be very high contamination with

sorbitol non-fermenting *E. coli* which indicates poor hygienic condition during its processing.

As an indicator of hygiene and sanitary quality, presence of *E. coli* suggests that consumers are at risk of being food poisoned and presence of other pathogenic flora. Table 4.1 revealed that among 25 samples Sorbitol non-fermenting *E. coli* were found in 40% samples viz. 10 samples (3 Chicken, 3 Buffalo, 2 Pig and 2 Goat meats). The highest percentage of sorbitol non-fermenting *E. coli* was found in chicken meat and Buffalo meat with 50% whereas pig meat had the lowest prevalence of sorbitol non-fermenting *E. coli* with 33.33%. The prevalence of *E coli* is also higher than previous studies in USA, Greece and Egypt. E.g. Zhao et al (2001) reported 38.7% Escherichia coli in chicken, 19% in beef, 16.3% in pork and 11.9% in turkey in retail meat samples in USA. In Greece, out of 428 samples, 153 *E. coli* were isolated (Gousia et al, 2010). Khalafalla et al, (2015) isolated 89.97% *E. coli* from raw poultry in Egypt.

Antimicrobial Resistance emerges from the use of antimicrobials in animals and human, and the subsequent transfer of resistance genes and bacteria among animals, humans, animal products and the environment (scott et al., 2002). With regard to the Antibiogram of *E. coli* in the current study, 17 different commercially available Antimicrobials discs were used and all the 10 *E. coli* isolates subjected to Antimicrobial Sensitivity test were found to be 100% resistance against Amoxicillin, Ampicillin, which is agreement with the result of Aly et al. (2012). 80% resistance to Cefotaxime, 60% resistance to Nalidixic acid, 50% resistance to Erythromycin, 40% resistance to ciprofloxacin, 30% resistance to Nitrofurantoin, Imipenem, Tetracycline and Cotrimazole, 10% resistance to Ceftriaxone, Cefixime, Amikacin, and Chloramphenicol. And 90% susceptible to Gentamycin, 80% susceptible to Ceftriaxone, Amikacin, and Chloramphenicol, 70% susceptible to Tetracycline, 60% susceptible to Cotrimazole, 50% susceptible to Cefixime, 40% susceptible to Nalidixic acid and 30% susceptible to Nitrofurantoin and Imipenem.

Multidrug Resistance is defined as resistance of an isolated to more than two Antimicrobials tested (Dominic et al. 2005). Multiple drug Resistance was also seen. All *E. coli* resist against more than two drugs. This finding was supported by Bekel et al. (2014); who reported the existence of multidrug resistance of *E. coli*.

CHAPTER VI: CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The result of present study of raw meat was found contaminated with Sorbitol non-fermenting *E. coli*. Resistance to a wide range of antibiotics was observed. MDR cases of sorbitol non fermenting *E. coli* was found. Resistance to Amoxicillin, Ampicillin, Teicoplanin, and Ceftazidime are higher. This study revealed that unhygienic processing and poor sanitation of meat shops. Meat retailers are unaware of basic requirements of basic guidelines of meat.

6.2 RECOMMENDATIONS

Based on the above concluding remarks, the following Recommendations are forwarded.

1. Intensive training should be given to those personnel working in municipal, slaughter houses to ensure the hygienic practices during slaughtering of animals.
2. Strict hygienic practices should be maintained to ensure contamination free meat while handling and processing.
3. Effective and adequate sanitation facility (wash basins, soap/ detergent, toilets, sanitized towels etc.) should be available on the meat shops and premises.
4. Regular antimicrobial susceptibility is essential.

REFRENCES

Adhikari BM, Subedi RP and Subba D (2012). A Study on Standard of Buffallo meat Hygiene in Dharan, J. Food Sciences and Tech. Nepal, Vol-7, 98-101

Alam MJ and Zurek L (2006). Seasonal prevalence of Escherichia coli O157:H7 in beef cattle feces. J Food Prot. 2006; 69(12):3018-20.

Barry B and Richard B (2011).The veterinarian's role in food safety. Vet. Bull. Agri 1: 6-10

Bekele T, Zewde, G, Tefera, G, Feleke, A. and Zerom, Z (2014). Escherichia coliO157:H7 in Raw Meat in Addis Ababa, Ethiopia. Prevalence at an Abattoir and Retailers and Antimicrobial Susceptibility.Int J. of Food Contamination, 1:4

Brown MH and Baird Parker AC (1982). The Microbiological Examination of Meat Microbiology, Applied Science Publishers LTD, England

Bolton DJ (2011).Verocytotoxigenic (Shiga toxin-producing) Escherichia coli: virulence factors and pathogenicity in the farm to fork paradigm. Foodborne Pathogens and Disease, 8, 357–365

Brown MH (1982). The microbiological examination of meat in meat microbiology. In: "Meat Microbiology" (Baird Parker), Applied Science Publ. Ltd, England.

Cohen N, Ennaji H, Hass M, and Karib H (2006): The bacterial quality of red meat and offal in Csablanca (Morocco). Mol. Nut.Food Res. 50:557.

Dulo F (2014). Prevalence and antimicrobial resistance profile of *E. coli* O157:H7 in goat slaughtered in Dire Dawa municipal abattoir as well as food safety knowledge, attitude and hygiene practice assessment among slaughter staff, Ethiopia. MSc thesis submitted to the College of Veterinary Medicine and Agriculture, Addis Ababa University, p.36.

Erb A, Stürmer T, Marre R and Brenner H (2007): Prevalence of antibiotic resistance in Escherichia coli: overview of geographical, temporal, and methodological variations. Eur J Clin Microbial Infect Dis. 2007; 26: 83–90.

FAO (1992) Guidelines for slaughtering, meat cutting and further processing. FAO Animal Production and Health Paper 91, Rome.

Frazier WC and Dennis CW (2014). Food Microbiology, McGraw Hill Education Private Limited New Delhi, India, pp 202-203.

Fotadar U, Zaveloff P and Terracio L (2005).Journal of basic microbiol.45 (5), 403.

Gebre-Sellassie,S (2007).Antimicrobial resistance patterns of clinical bacterial isolates in southern Ethiopia. Ethiop Med J. 2007; 45(4): 363-370.

Gousia P, Economou V, Sakkas H, Leveidiotou S and Papadopoulou C (2010).AntimicrobialResistance of Major Food Borne Pathogens from Major Meat Products, Food Borne Pathogens and Disease, Vol-8, DOI:10.1089/fpd.2010.0577

Gracey JF and Collins DS (1994). Meat Hygiene, Edition, BlliereTindall, London.Haileselassie, M, Taddele, H, Adhana, K. and Kalayou, S. (2012). Study on food safety knowledge and practices of abattoir and butchery shops and the microbial profile of meat in Mekelle City, Ethiopia. Asian Pacific Journal of Tropical Biomedicine.6

Griffin PM and Tauxe RV (1991). The epidemiology of infections caused by Escherichia coli O157:H7, other enterohemorrhagic E. coli, and the associated hemolytic uremic syndrome. Epidemiology Review, 13:60-97.

Khalafalla FA, Abdel NS, Abdel-Wanis A and Hanafy AS (2005).Food Poisoning Microorganisms in Chicken Broiler Meat. Global Vetereneria, 14(2):211-218, DOI:10.5829/idosi.gv2015.14.02.9319.

Kaper JB, NataroJP, & Mobley HL (2004).Pathogenic Escherichia coli. Nature Reviews. Microbiology, 2, 123–140.

Kubitschehk E (1990). Cell volume increase in Escherichia coli after shifts to richer media. Journal of Bacteriology 172: 94-101.

Karmali MA, Petric M, Lim C, Fleming PC, Arbus GS and Lior H (1985). The association between ideopathichaemolyticuraemic syndrome and infection byverotoxin-producing Escherichia coli. J Dis Imm 151: 775-782.

Nataro J and Kaper J (1998). Diarrheagenic E. coli.Clin.Microbiol. Rev. 11: p216.

O'brien A and Laveck G (1983). Purification and characterization of a Shigelladysenteriae type 1-like toxin produced by Escherichia coli. Infect. Immun. 40:675–683.

Karmali MA (1989). Infection by verotoxin producing Escherichia coli.Clin.Microbiol. Rev., 2:15–38.

Pal M (2012). Raw meat poses public health risks. The Ethiopian Herald, May 15, 2014. Pp. 2-3.

RoeschPL, Redford P and Batchelet S (2003).Uropathogenic Escherichia coli used serine deaminase to modulate infection of the murine urinary tract. Molecular Microbiology, 49: 55–67.

Science of Meat: What is Meat?
https://www.exploratium,edu/cooking/meat/INT-what is meat.html

Schroeder C, Zhao C, DebRoy C, Torcolini J, Zhao S, White G, Wagner D, McDermott F, Walker D and Meng J (2002). Antimicrobial Resistance of Escherichia coli O157:H7 Isolated from Humans, Cattle, Swine, and Food. J Appl. Environ. Microbial.68: 576–581.

Shah M Faraque (2012). Food Borne Pathogens: Epidemiology, Evolution and Molecular Biology, Caster Academic Press, pp330.

Tärnberg M (2012). Extended-spectrum beta-lactamase producing Enterobacteriaceae : aspects on detection, epidemiology and multi-drug resistance. Linkoping University, 153.

Taye, M., Tamiru, B., Yenehiwot, B., Firao, T. and Dechassa, T. (2013): Study on Carcass Contaminating Escherichia coli in Apparently Healthy Slaughtered Cattle in Haramaya University Slaughter House with Special Emphasis on Escherichia coli O157:H7, Ethiopia. J VeterinarSciTechnolo., 4:1.

U.S. Food and Drug Administration, (2015). "Microbiological Methods and Bacteriological Analytical Manual(BAM)Online.https://www.fsis.usda.gov/wps/portal/fsis/topics/science /laboratoriesandprocedures/guidebooks-and-methods/microbiology laboratoryguidebook/microbiology-laboratory-guidebook

Whitfield C and Valvano MA (1993). Biosynthesis and expression of cell-surface polysaccharides in Gram-negative bacteria. Advanced in Microbial Physiology, 35:135-246.

Zhao C, Ge B, Villena-De, J Yeh, E White, D Wanger, D and Meng J (2001). Prevalence of campylobacter spp., Escherichia coli, and Salmonella serovars in retail chicken, turkey, pork, and beef from the greater Washington DC area. Appl. Environ. Microbial 67: 5431-5436

APPENDICES

APPENDIX-I

LIST OF MATERIALS

1. EQUIPMENTS USED

1. Autoclave
2. Weighing balance
3. Hot air oven
4. Incubator
5. Microscope
6. Refrigerator
7. Water bath shaker
8. Micropipette

2. Microbiological media and Biochemical media

1. EC Reduced Salt Broth
2. Sorbitol MacConkey Agar
3. Eosin Methylene Blue Agar
4. Nutrient Agar
5. Nutrient Broth
6. Sorbitol Broth
7. Simmons Citrate Agar
8. Sulphide Indole Motility Media
9. Urease Agar
10. MR-VP Broth
11. Muller Hinton Agar

3. Chemicals and Reagents

1. Catalase reagent (3%H2O2)
2. Potassium hydroxide
3. Alpha-napthol
4. Crystal violet
5. Sulfuric acid
6. Gram's Iodine
7. Kovacs Reagent
8. Methyl Red
9. Oxidase reagent
10. Lysol
11. Ethanol
12. Safranin

4. Glasswares

1. Test tubes
2. Pipettes
3. Conical flask
4. Petriplates
5. Beakers
6. Glass rod and glass tubes
7. Reagent Bottle
8. Slides
9. Measuring Cylinder

5. Miscellaneous

1. Aluminum foils

2. Inoculating loop/ needles
3. Forceps
4. Cotton plugs
5. Cotton Swab
6. Sample collecting bottles
7. Labeling tape
8. Measuring scale
9. Blotting paper
10. Test tube holder
11. Detergent

APPENDIX-II

Composition and Preparation of Different Culture Media

1. **EC Broth (Reduced Bile Salts)**

Ingredient	Gm/liter
Tryptone	20.00
Lactose	5.00
Bile salts no.3	1.12
Di-Potassium Phosphate	4.00
Mono-Potassium Phosphate	1.50
Sodium Chloride	5.00
P^H	6.9 ± 0.2 at 25^0C

Direction: 36.62 gm of the medium was suspended in 1000ml distilled water and then boiled to dissolve completely. Then, the medium was sterilized by autoclaving at 121^0C (15 lbs. Pressure) for 15 minutes.

2. **Sorbitol MacConkey Agar**

Ingredient	Gm/liter
Peptone	20.00
Sorbitol	10.00
Bile Salt	1.50
Sodium Chloride	5.00
Neutral Red	0.03
Crystal Violet	0.001
Agar	15.00
P^H	7.1 ± 0.2 at 25^0C

Direction: 51.531gm of the medium was suspended in 100ml of distilled water and boiled to dissolve completely. Then sterilized by autoclaving at 121^0C for 15 minutes.

3. Eosin Methylene Blue

Ingredient	Gm/liter
Peptic digest of animal tissue	10.00
Dipotassium Phosphate	2.00
Lactose	5.00
Sucrose	5.00
Eosin	0.04
Methylene blue	0.065
Agar	15.00
P^H	7.2± 0.2 at 25^0C

Direction: 36.96 gm of the medium was suspended in 1000ml of distilled water and boiled to dissolve completely. Then sterilized by autoclaving at 121^0C for 15 minutes.

4. Phenol Red Sorbitol Broth

Ingredients	Gm/liter
Proteose peptone	10.00
Beef extract	1.00
Sodium Chloride	5.00
Sorbitol	5.00
Phenol Red	0.018
P^H	7.4±0.2

Directions: 21 gm of the medium was suspended in 1000ml of distilled water and boiled to dissolve completely. Then sterilized by autoclaving at 121^0C for 15 minutes.

5. Nutrient Agar

Ingredients	Gm/liter
Peptone	5.00
Sodium Chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Agar	15.00
P^H	7.20

Directions: 28 gm of the medium was suspended in 1000ml of distilled water and boiled to dissolve completely. Then sterilized by autoclaving at 121^0C for 15 minutes. For the preparation of Nutrient Broth Agar was not added.

6. Muller Hinton Agar

Ingredients	**Gm/liter**
Beef infusion	2.00
Casamino acid/ acid hydrolysate of casein	17.50
Starch	1.50
Agar	170
P^H	7.4±0.2

Direction: 38 gm of the medium was suspended in 1000ml of distilled water and boiled to dissolve completely. Then it was sterilized by autoclaving at 121^0C for 15 minutes.

APPENDIX-III

Methodology of Biochemical Test for the Identification of Bacteria

A. Catalase test

This test is performed to detect the presence of catalase, an enzyme that catalase's the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase splits hydrogen peroxide to water and oxygen. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being Streptococcus spp.

Procedure: A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop is used.

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-p-phenylenediaminedihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test. The test is used for screening *E. coli* which gives negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (What man's No. 1 filter paper impregnated with 1% tetramethyl-pphenylenediaminedihydrochloride). Then the colony of the test organism was smeared on the filter paper. The negative test is indicated without the appearance of blue-purple color.

C. Indole Production test

This test detects the ability of the organisms to produce an enzyme Tryptophanase. Tryptophan is oxidized by some bacteria by the enzyme Tryptophanase resulting in the formation of indole, pyruvic acid and ammonia.

Procedure: The bacterial colony was inoculated on tryptone broth and then incubated ad 37^0C for 24 hours of incubation, 1ml of Kovac's Reagent was added. Appearance of Red color(red ring) on the top of the medium indicates Positive Indole Test.

D. Methyl Red test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4- 6.0.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by thedevelopment of bright red color, indicating acidity.

E. Voges-Proskauer (VP) test

The principle of this test is to determine the ability of some organisms to produce an acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates. An organism of the Enterobacteriaceae group is usually either methyl red positive and Voges-Proskauer-negative or methyl red negative and Voges-Proskauer positive. The Voges-Proskauer test for acetoin is used primarily to separate E. coli from Klebsiella and Enterobacter species.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red color.

F. Citrate Utilization test

This test is performed to detect whether an organism utilizes citrate as the sole source of carbon. The utilization of citrate depends on the presence of an enzyme citrase produced by the organisms that breaks down the citrate to

oxaloacetic acid and acetic acid which later converted to pyruvic acid and carbondioxide. Simmons Citrate Agar is used for this test, where sodium citrate is the only source of carbon and energy. Once the CO_2 is generated, it combines with sodium and water to form Sodium Carbonate an alkaline product, which changes the color of the indicator (Bromothymol Blue) from green to blue.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. Bromothymol blue is green when acidic (P^H6.8 and below) and blue when alkaline (P^H 7.6 and higher).

G. Motility test

This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli; however, a few cocci forms are motile. Motile bacteria may contain single flagella. The motility media used formotilitytest is semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37ºC for 48 hours. Motile organisms migrate from the stablineand diffuse into the medium causing turbidity. Whereas, non-motile bacteria show the growth along the stab line only.

H. Urea Hydrolysis test:

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37°C for overnight. Positive organism shows pink red color due to the breakdown of urea to ammonia. With the release of ammonia, the medium becomes alkaline as shown by a change in color of the indicator to pink.

APPENDIX- IV

Table A1: Biochemical Characterization of *E. coli*

Biochemical test	Reaction
Catalase	+
Oxidase	–
Indole	+
Methyl Red	+
Voges-Proskauer	–
Citrate Utilization	–
Urease Activity	–
SIM	+

Table A2: Zone size interpretation chart of Antibiotic susceptibility testing of *E. coli*

Antibiotic Used	Symbol	Disc Content (mcg)	Diameter of zone Inhibition (mm)		
			Resistance	Intermediate	Susceptible
Amoxicillin	AMX	30	13	14-17	18
Ampicillin	AMP	10	13	14-16	17
Amikacin	AK	30	14	15-16	17
Imipenem	IPM	10	19	20-22	23
Cefotaxime	CTX	30	22	23-25	26
Cotrimazole	COT	25	10	11-15	16
Teicoplanin	TEI	30	-	-	-
Ceftriaxone	CTR	30	19	20-22	23
Tetracycline	TE	30	11	12-14	15
Nalidixic acid	NA	30	13	14-18	19
Cefixime	CFM	5	15	16-18	19
Ceftazidime	CAZ	30	21	18-20	17
Chloramphenicol	C	30	12	13-17	18
Ciprofloxacin	CIP	30	20	21-30	31
Gentamycin	GEN	10	12	13-14	15
Nitrofurantoin	NIT	100	14	15-16	17
Erythromycin	E	15	13	14-22	23

Source: CLSI document M100-S25(M02-A12): “Disc diffusion supplemental table

Table A3: Antibiotic Susceptibility pattern of Sorbitol Non-Fermenting *E. coli*

Antibiotic class	Antibiotic used	Samples										R (%)	I (%)	S (%)
		C_1	C_2	C_3	B_1	B_3	B_{3a}	P_2	P_3	M_1	M_5			
Ampicillins	AMX	0	0	0	0	0	0	0	0	0	0	100	0	0
	AMP	0	0	0	0	0	0	0	0	0	0	100	0	0
Cephalosporin	CAZ	14	0	10	13	10	14	14	14	12	12	100	0	0
	CTX	19	0	18	24	24	18	21	22	18	19	80	0	20
	CTR	24	0	24	26	26	24	24	25	21	23	10	10	80
	CFM	18	0	23	27	25	18	19	19	17	16	10	40	50
Sulpha groups	COT	21	0	20	19	12	0	21	21	0	20	70	10	20
Aminoglycosides	AK	18	20	14	21	17	17	19	19	16	19	80	10	10
	GEN	17	19	14	15	18	17	15	17	17	16	0	10	90
	TE	16	19	0	20	18	9	17	18	7	15	30	70	0
Quinolone	NA	19	13	12	0	0	12	19	21	7	20	40	0	60
Fluoroquinolone	CIP	29	21	17	21	20	20	30	30	17	26	0	60	40
Phenolic	C	24	19	19	28	2	11	26	26	16	24	90	10	0
Carbapenems	IPM	19	24	13	21	19	24	23	22	22	21	30	40	30
Macrolids	E	12	21	10	13	14	13	20	16	13	18	0	60	40
Glycopeptides	TEI	11	10	0	0	0	0	11	0	0	10	100	0	0
Nitroflorentine	NIT	15	14	8	20	15	15	17	18	11	15	30	40	30