T.R.

KAHRAMANMARAŞ SÜTÇÜ IMAM UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOCHEMICAL IDENTIFICATION OF *ENTEROBACTERIACEAE* ISOLATES OBTAINED FROM LARGE INTESTINE OF CATTLE AND SHEEP AT KAHRAMANMARAS ABATTOIR AND DETERMINATION OF THEIR ANTIMICROBIAL SENSITIVITY PATTERNS

KARWAN HUSSEIN MAHMOOD

MASTER'S THESIS

DEPARTMENT OF BIOENGINEERING AND SCIENCES

KAHRAMANMARAŞ 2014

T.R.
KAHRAMANMARAŞ SÜTÇÜ IMAM UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOCHEMICAL IDENTIFICATION OF *ENTEROBACTERIACEAE* ISOLATES OBTAINED FROM LARGE INTESTINE OF CATTLE AND SHEEP AT KAHRAMANMARAS ABATTOIR AND DETERMINATION OF THEIR ANTIMICROBIAL SENSITIVITY PATTERNS

KARWAN HUSSEIN MAHMOOD

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
in
Bioengineering and Sciences

KAHRAMANMARAŞ 2014

M.Sc. thesis entitled "BIOCHEMICAL IDENTIFICATION OF *ENTEROBACTERIACEAE* ISOLATES OBTAINED FROM LARGE INTESTINE OF CATTLE AND SHEEP AT KAHRAMANMARAS ABATTOIR AND DETERMINATION OF THEIR ANTIMICROBIAL SENSITIVITY PATTERNS" and prepared by KARWAN HUSSIEN MAHMOOD, who is a student at Department of Bioengineering and Sciences, Graduate School of Natural and Applied Sciences, Kahramanmaraş Sütçü Imam University, was certified by all the/majority jury members, whose signatures are given below.

Doç. Dr. E. Banu BUYUKUNAL BAL (Supervisor) ……………………………..

Department of Biology

Kahramanmaraş Sütçü İmam University

Doç. Dr. Dr. Sevil TOROGLU (Member) ……………………………

Department of Biology

Kahramanmaraş Sütçü İmam University

Doç. Dr. Mehmet Ali BAL (Member) ……..……………………….

Department of Animal Science

Kahramanmaraş Sütçü İmam University

I approve that the above signatures related to the members.

Prof. Dr. M. Hakkı ALMA ……………………………….

Director of Graduate School

Kahramanmaraş Sütçü İmam University

DECLARATION

I hereby declare that all information in the thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

KARWAN HUSSEIN MAHMOOD

This study supported by: Kahramanmaraş Sütçü Imam University.
Project Number: 2013/6-17 YLS.
Note: the original and other sources used in this thesis, the declaration, tables, figures and photographs showing the use of resources, subject to the provisions of Law No. 5846 on Intellectual and Artistic Works.

KAHRAMANMARAŞ MEZBAHASINDAKİ SIĞIR VE KOYUNLARIN KALIN BAĞIRSAKLARINDAN ELDE EDİLEN *ENTEROBACTERİACEAE* İZOLATLARININ BİYOKİMYASAL İDENTİFİKASYONU VE ANTİBİYOTİK DUYARLILIK PROFİLLERİNİN BELİRLENMESİ

(YÜKSEK LİSANS TEZİ)

KARWAN HUSSEİN MAHMOOD

ÖZ

Enterobacteriaceae üyeleri gıda kaynaklı sağlıklı hayvanların sindirim sisteminin doğal elemanları olmakla beraber bazı durumlarda, patojen hale gelebilmektedir. Son on yılda *Enterobacteriaceae* üyelerinde ilaç direnci dünyada ciddi bir artış göstermiştir. Bu durum özellikle Genişlemiş Spektrumlu β-Laktamaz (GSBL) üreticilerinin prevalansındaki artıştan ve bu artışın bir sonucu olarak karbapenemlerin kullanımındaki yükselişten kaynaklanmaktadır. Bu çalışmanın amacı Kahramanmaraş mezbahasındaki sağlıklı hayvanlardan elde edilen *Enterobacteriaceae* izolatlarının identifikasyonu ve antibiyotik dirençlerinin belirlenmesidir. Toplam 40 adet (sığır=20, koyun=20) kolon içeriğinden 54 izolat elde edilmiştir. Bu izolatların identifikasyonu standart biyokimyasal testler ile yapılmış ve chromogenic besiyeri ile identifikasyon sonuçları doğrulanmıştır. *Enterobacteriaceae* izolatlarının 49 tanesi (%90.74) *E. coli* ve 1 tanesi (%1.85) ise diğer *Enterobacteriaceae* olarak identifiye edilmiştir. İzolatların antimikrobiyal duyarlılıkları Kirby-Bauer disk difüzyon duyarlılık testi kullanılarak 13 antibiyotik için belirlenmiştir. GSBL üreticilerinin saptanması amacıyla çift disk sinerji testi yapılmıştır. Sonuç olarak non-*E. coli* olarak tanımlanan izolatın tüm antibiyotiklere karşı duyarlı olduğu saptanmıştır. Ayrıca tüm *E. coli* izolatlarının çoğu antibiyotiğe karşı duyarlı olduğu bulunmuştur. Test edilen antibiyotiklere duyarlılık düşük olmakla beraber, 49 *E. coli* izolatının 6 tanesinin (%12.24) en az bir antibiyotiğe dirençli olduğu bulunmuştur. Tüm *E. coli* izolatlarında en yüksek direnç trimethoprim-sulfamethoxazole (n=3; %6.12) için tespit edilmiştir. Tüm *E. coli* izolatlarının 2 tanesinde (%4.08) carbapenemlere (imipenem) direnç görülmüştür. Bu nedenle, bu çalışma Türkiyedeki hayvan kaynaklı carbapenem-dirençli *E. coli* izolatları için ilk raporu sunmaktadır. Ayrıca, çalışma sonucunda GSBL pozitif bir izolat tespit edilmemiştir. Kümes hayvanlarında antibiyotik dirençli bakterilerin sürekli ve stratejik olarak taranması dirençli bakterilerin geçişinin önlenebilmesi için önem taşımaktadır.

Anahtar kelimeler: *Enterobacteriaceae, E. coli,* sığır, koyun, mezbaha, biyokimyasal testler, antibiyotik duyarlılık.

Kahramanmaraş Sütçü İmam Üniversitesi
Fen Bilimleri Enstitüsü
Biyomühendislik ve Bilimleri Anabilim Dalı, Temmuz, 2014

Danışman: Doç. Dr. E. Banu BUYUKUNAL BAL

Sayfa Sayısı: 104

BIOCHEMICAL IDENTIFICATION OF *ENTEROBACTERIACEAE* ISOLATES OBTAINED FROM LARGE INTESTINE OF CATTLE AND SHEEP AT KAHRAMANMARAS ABATTOIR AND DETERMINATION OF THEIR ANTIMICROBIAL SENSITIVITY PATTERNS

(M.Sc. THESIS)

KARWAN HUSSEIN MAHMOOD

ABSTRACT

Enterobacteriaceae members are normal inhabitants of the intestinal tract of healthy food animals. However, under some circumstances, several of them become pathogens, causing diseases. During the last decade, drug resistance in *Enterobacteriaceae* has increased dramatically worldwide, resulted from increased prevalence of Extended-Spectrum β-Lactamase (ESBL) producers and then led to increase of using the last-resort antimicrobial drugs which are carbapenems. The aim of this study was to identify the *Enterobacteriaceae* isolates and determine the occurrence of antibiotic resistance among them which obtained from healthy food animals at Kahramanmaras abattoir. A total of 40 samples of colon contents from 20 cattle and 20 sheep carcasses were taken just after evisceration, and overall 54 isolates were recovered. Identification was performed based on standard biochemical tests and verified by using chromogenic media. As many as (n=49; 90.74%) and (n=1; 1.85%) were identified as *E. coli* and other *Enterobacteriaceae*, respectively. Kirby-Bauer Disk Diffusion Susceptibility Test was used to determine their antimicrobial sensitivities against 13 antibiotic agents and Double Disk Synergy test was carried out to detect the ESBL producers among them. As a result, no resistance found for an isolate which was identified as non-*E. coli*. Additionally, all the *E. coli* isolates were susceptible to most of the antibiotics, but the resistance to tested antibiotics was low, out of 49 *E. coli* isolates (n=6; 12.24%) were resistant to at least one antibiotic agent. Among all the *E. coli* isolates, the highest resistance level (n=3; 6.12%) was detected for trimethoprim-sulfamethoxazole. Of 49 *E. coli* isolates (n=2; 4.08%) were resistant to carbapenems (imipenem), this resistance is of concern. This is the first report about the carbapenems resistant *E. coli* of animal origin in Turkey. Furthermore, no ESBL positive isolate was detected at all. Continuous and strategic surveillance of antimicrobial resistant bacteria in livestock is essential to suppress further dissemination of these bacteria into Turkish society at large.

Key words: *Enterobacteriaceae, E. coli,* cattle, sheep, abattoir, biochemical tests, antibiotic susceptibility.

Kahramanmaraş Sutcu Imam University
Graduate School of Natural and Applied Sciences
Department of Bioengineering and sciences, July, 2014

Supervisor: Doç. Dr. E. Banu BUYUKUNAL BAL
Page number: 104

ACKNOWLEDGEMENT

I hereby thank everyone that has helped me throughout the execution of this work. Specifically, I thank to Assoc. Prof. Dr. E. Banu BUYUKUNAL BAL, my supervisor. I am very grateful for all the knowledge, advices and guidance that she offered me during this study. It was an honour to work with her. I also thank to my parents, for their constant support and for believing in me.

TABLE OF CONTENTS

Page No

ÖZ i

ABSTRACT.. ii

ACKNOWLEDGEMENT.. iii

TABLE OF CONTENTS iv

LIST OF FIGURES vii

LIST OF TABLES viii

SYMBOLS AND ABBREVIATIONS ix

1. INTRODUCTION 1

2. LITERATURE REVIEW 4

2.1. Brief History of *Enterobacteriaceae* Family 4
2.2. Taxonomy and Classification of *Enterobacteriaceae* 4
2.3. Intestinal Habitat of *Enterobacteriaceae* 5
2.4. General Characteristics of *Enterobacteriaceae* 5
2.5. Identification of *Enterobacteriaceae* Members 6
2.6. Cell Wall of *Enterobacteriaceae* 7
2.7. Some Important *Enterobacteriaceae* Members 8
2.7.1. *Salmonella* serotypes 8
2.7.2. *Shigella* species 8
2.7.3. *Klebsiella* species 9
2.7.4. *Enterobacter* species 9
2.7.5. *Proteus* species 9
2.7.6. *Yersinia* species 10
2.8. *Escherichia* species 10
2.8.1. Morphology and characteristics of *E. coli* 11
2.8.2. Genomes of *E. coli* 11
2.8.3. Biochemical properties of *E. coli* 12
2.8.4. Unusual biochemical properties of *E. coli* 13
2.8.5. Serological characteristics of *E. coli* 13
2.8.6. *E. coli* virulence factors and virulence genes 14
2.8.7. Strain classification of *E. coli* 15

Page No

2.8.8. Some *E. coli* infections in cattle and sheep 15
2.8.9. Transmission of *E. coli* from food animals to human 16
2.9. Antibiotics 17
2.9.1. Classification of antibacterial agents 17
2.9.2. Types of resistance to antibiotics 18
2.9.3. Mechanisms of antibiotic resistance 18
2.9.4. β-Lactam antibiotics 20
2.9.5. β-Lactamases 21
2.9.6. Classification of β-lactamases 21
2.9.7. Extended-spectrum β-lactamases 22
2.9.8. Carbapenems resistance in *Enterobacteriaceae* 22
2.9.9. β-lactamase inhibitors 23
2.9.10. Transfer of antimicrobial resistance from animals to humans 23
2.9.11. Antibiotic susceptibility testing methods 24
3. MATERIALS AND METHODS 26
3.1. Sample Collection 26
3.2. Isolation of Single Bacterial Colonies 27
3.2.1. Culture media and reagents 27
3.2.2. Processing of the samples 29
3.3. Stock Culture Making of the Bacterial Isolates 30
3.4. Biochemical Characterization and Identification of the Bacterial Isolates 30
3.4.1. Sub-culture of the bacterial isolates 32
3.4.2. Oxidase test 32
3.4.3. Triple sugar iron test 33
3.4.4. Lysine decarboxylase test 35
3.4.5. Indole production 35
3.4.6. Methyl Red and Voges-Proskauer test 37
3.4.7. Citrate utilization test 39
3.5. Verification of Biochemical Identification of the Bacterial Isolates 41
3.5.1. CHROMagar ECC composition 41
3.5.2. CHROMagar ECC medium preparation 41
3.5.3. Inoculation of CHROMagar ECC agar plates 42
3.5.4. Interpretation of the results on CHROMagar ECC plates 42
3.6. Antibiotic Susceptibility Testing 42

Page No

3.6.1. Antibiotic susceptibility testing medium 43
3.6.2. Inoculum preparation 44
3.6.3. Performing of the antibiotic susceptibility testing 44
3.6.4. ESBL production detection 46

4. RESULTS AND DISCUSSION 47

4.1. Isolation of Single Bacterial Colonies 47
4.1.1. Culture of samples on EMB agar 47
4.1.2. Culture of isolates in TSB medium 47
4.2. Biochemical Characteristics of the Isolates 49
4.2.1. Isolates reactions on oxidase test strips 49
4.2.2. Isolates reactions in triple sugar iron agar 49
4.2.3. Isolates reactions in lysine decarboxylase broth 50
4.2.4. Isolates reactions in IMViC tests 51
4.3. Identification of the Isolates 56
4.3.1. Identification of *E. coli* 56
4.3.2. Identification of other *Enterobacteriaceae* members 59
4.3.3. Identification of Non-*Enterobacteriaceae* isolates 61
4.4. Verification of the Biochemically Identified Bacterial Isolates 63
4.5. Antibiotic Susceptibility Patterns of Isolates 66
4.5.1. Antibiotic susceptibility patterns of *E. coli* isolates 66
4.5.2. Antibiotic susceptibilities of other *Enterobacteriaceae* member 82
4.5.3. ESBL producer detection among the *Enterobacteriaceae* isolates 84

5. CONCLUSIONS 88

REFERENCES 90

CURRICULUM VITAE 104

LIST OF FIGURES

Page No

Figure 2.1. Schematic illustration of the components of gram-negative bacterial cell wall 7

Figure 3.1. Sample collection method 27

Figure 4.1. Reaction of isolates on oxidase test strips 49

Figure 4.2. Reactions in TSI agar 50

Figure 4.3. Reactions in lysine decarboxylase broth 51

Figure 4.4. Reactions in Indole test 51

Figure 4.5. Reactions in MR test 52

Figure 4.6. Reactions in VP test. 52

Figure 4.7. Reactions in simmon's citrate test 53

Figure 4.8. Percentages of total numbers of identified isolates from cattle and sheep samples 62

Figure 4.9. Colony colors of identified bacterial isolates 64

Figure 4.10. Antibiotic susceptibility and ESBL producer detection testing 73

Figure 4.11. Antibiotic sensitivity patterns of *E. coli* isolates from cattle samples in percentage 74

Figure 4.12. Antibiotic sensitivity patterns of *E. coli* isolates from sheep samples in percentage 75

Figure 4.13 Antibiotic sensitivity patterns of total no. of *E. coli* isolates in percentage 76

LIST OF TABLES

Page No

Table 3.1. Biochemical reactions of *Enterobacteriaceae* members which are of veterinary importance. 31

Table 3.2. Antimicrobial agents and their disc contents that used for this study 43

Table 3.3. Zone diameter interpretive criteria used for the tested antibiotics 45

Table 4.1. All bacterial isolates from cattle and sheep samples 48

Table 4.2. Biochemical tests results of all the isolates 54

Table 4.3. Biochemical characteristics of *E. coli* isolates 56

Table 4.4. Biochemical characteristics of other *Enterobacteriaceae* member 60

Table 4.5. Biochemical characteristics of Non-*Enterobacteriaceae* isolates 61

Table 4.6. Antibiotic inhibition zone diameters of *E. coli* isolates 69

Table 4.7. Interpretation of antibiotic inhibition zone diameters of *E. coli* isolates 71

Table 4.8. Antibiotic sensitivity patterns of *E. coli* isolates from cattle samples in percentage 73

Table 4.9. Antibiotic sensitivity patterns of *E. coli* isolates from sheep samples in percentage 74

Table 4.10. Antibiotic sensitivity patterns of total no. of *E. coli* isolates from cattle and sheep samples in percentage 75

Table 4.11. Antibiotic susceptibility patterns of other *Enterobacteriaceae* isolate 82

SYMBOLS AND ABBREVIATIONS

ATCC : American Type Culture Collection

CFU : Colony Forming Unit

CLSI : Clinical and Laboratory Standards Institute

DDS : Double Disk Synergy

EMB : Eosin Methylene Blue

ESBL : Extended Spectrum β-Lactamase

H_2S : Hydrogen Sulfide

IMViC : Indole, Methyl Red, Voges-Proskauer and Citrate Utilization

MHA : Mueller – Hinton Agar

MR : Methyl Red

PBPs : Penicillin Binding Proteins

PBS : Phosphate Buffered Saline

spp. : Species

TSA : Tryptic Soy Agar

TSB : Tryptic Soy Broth

TSI : Triple Sugar Iron

VP : Voges-Proskauer

1. INTRODUCTION

Enterobacteriaceae is a large family of organisms belonging to the Gram-negative bacteria, classified within the domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria and order Enterobacteriales. *Enterobacteriaceae* include many hundreds of species, but only a limited number is of clinical relevance. The most studied bacteria of clinical importance in this family are: *Escherichia coli*, *Klebsiella* spp., *Salmonella* serotypes, *Shigella* spp., *Proteus spp.*, *Enterobacter* spp. and *Yersinia* spp. *Enterobacteriaceae* are rod-shaped, 1 to 5 µm in length, non-sporulating, facultative anaerobic bacteria. They ferment sugars (e.g. glucose and lactose) to a variety of end products and reduce nitrate to nitrite, produce catalase and are oxidase negative. All these criteria help detecting them in the clinical microbiological laboratories. As the name implies they are common colonizers of the gastrointestinal tract and are highly important for the well being of human and most animals as part of the natural bacterial flora. *Enterobacteriaeae* are also widespread in the environment. However, they can also be pathogenic causing infections, such as, in central nervous system, lower respiratory tract, gastrointestinal and urinary tract. They possess many different characteristics that contribute to their survival, such as: endotoxin, capsule, antigenic phase variation, sequestration of growth factors (Madigan *et al.*, 2003; Murray *et al.*, 2009).

The term “enterics” is often used to describe this family, because the natural habitat of most of them is the lower gastrointestinal tract of animals and humans (Farmer *et al.*, 2008). *Escherichia* is the type genus of the family *Enterobacteriaceae*, and *Escherichia coli* is the type species of the genus *Escherichia* (Nataro *et al.*, 2007). Many members of this family can cause problems in the animal husbandry. For instance, Salmonellosis in poultry and eggs is a worldwide problem, both for poultry farmers and as a vehicle for human disease (Mishu *et al.*, 1994; Guard-Petter, 2001). Enterotoxigenic *E. coli* strains are primarily responsible for infections in lambs, pigs and calves (Janke *et al.*, 1989), while *Klebsiella pneumoniae, E. coli* and *Enterobacter aerogenes* are causes of bovine mastitis (Bannerman *et al.*, 2003). Furthermore, many species of *Enterobacteriaceae* can contaminate animal food products such as: milk and dairy products, raw fresh meat and poultry and processed meat products, by a variety of sources (Gustavsson and Borch, 1993; Castano *et al.*, 2002; Iversen and Forsythe, 2004; Hudson *et al.*, 2008).

Good agricultural practices, good manufacturing practices and hygiene control during handling and processing of food products help to reduce and avoid contamination with pathogenic *Enterobacteriaceae* members. Some species of this family are used as indicator organisms; the presence of these bacteria in food indicates poor hygiene of food handlers, inadequate processing or contamination after processing. *E. coli* and the coliform group are used as the index of fecal contamination. *E. coli* is considered as a better indicator of a possible fecal contamination (Gras *et al.*, 1994).

Antimicrobial agents, which include antibiotics, rank among the greatest discoveries in the history of a human kind. They are essential in the treatment of infectious diseases as well as in disease prophylaxis in human and animals. Antimicrobial resistance emergence, dissemination, propagation, and maintenance among bacteria have become a worldwide health concern, especially in human and veterinary medicine (Anderson, 1999; Levy and Marshall, 2004). The introduction of antimicrobials in food-producing animals started in the 1940s to treat diseases and for growth purposes (Hammerum and Heuer, 2009).

Resistance is defined as the relative insusceptibility of a microorganism to a specific treatment under a particular set of circumstances (Kummerer, 2004). Some researchers believe that resistance is an ecological phenomenon stemming from the response of bacteria to the widespread use of antibiotics and their presence in the environment (Levy, 2001; Esiobu *et al.*, 2002). Others believe that there is a "concerted attack" on the use of antibiotics, and that antibiotic resistance is as ancient as bacterial organisms since bacteria are able to secrete products (antimicrobials) to prevent an attack from other competitive organisms (Phillips *et al.*, 2004).

Moreover, the misuse and intensive use of therapeutic forms of antimicrobial agents, prophylactic use, and sub therapeutic use for growth promotion in food animals have substantially increased selective pressures on bacteria, favoring the propagation and maintenance of antimicrobial resistant bacteria. Antimicrobial resistance can develop in commensal (normal microflora) and pathogenic bacteria both in human and animal gastrointestinal tracts as well as in the environment (Levy, 2002). Furthermore, morbidity and mortality have increased in human medicine associated with resistant pathogens and thus failure therapeutic (Davies and Davies, 2010).

The need for empirical identification and quantification of antimicrobial resistant genes in animal populations, in addition to tracking the spread of antimicrobial resistant

bacteria to human and animal populations, is imperative (Singer *et al*., 2003). The impact of antimicrobial use and possible misuse in animals as a public health issue is focused on food-producing animals mainly. It is necessary to control and monitor the antimicrobial use in animals in order to avoid the increase of antimicrobial resistance. Additionally, the use of the critically important antimicrobials should be minimised in animals to maintain/preserve the efficacy of these for treating human's infections (SvHKS, 2013).

The author of the present thesis was focused on *E. coli* among the *Enterobacteriaceae* members during practical part and literature part in the current study.

Aims of the study

1. To isolate and identify the *Enterobacteriaceae* members in colon segment of the large intestine of healthy adult cattle and sheep at slaughter in Kahramanmaras abattoir.
2. To find out the percentage of the different members of the *Enterobacteriaceae* isolates.
3. To determine the antibiotic susceptibility patterns of the *Enterobacteriaceae* isolates.
4. To screen for the occurrence of Extended Spectrum β-Lactamase (ESBL) and carbapenems resistance among the *Enterobacteriaceae* isolates.

2. LITERATURE REVIEW

2.1. Brief History of *Enterobacteriaceae* Family

The name "*Enterobacteriaceae*" was first proposed in 1937 by Rahn to enclose the genus *Enterobacter* and other bacteria that shared the ability to ferment glucose with the production of gas (Rahn, 1937). At that time the name *Enterobacteriaceae* was referred to as bacteria which inhabit the "enteron" or intestinal tract of animals. Then, Sojka (1965) summarized the description provided by the 1958 *Enterobacteriaceae* Subcommittee, as follows: "The family *Enterobacteriaceae* is composed of Gram-negative, rod-shaped bacteria which are motile with peritricous flagella, or non motile. They ferment glucose rapidly with or without gas production, reduce nitrates to nitrites and grow on ordinary media". Based on biochemical and serological characteristics, members of the *Enterobacteriaceae* family are subdivided in groups, subgroups, serotypes, and biotypes (Sojka, 1965).

Nowadays several molecular techniques based on fragment analysis, techniques using Polymerase Chain Reaction (PCR) and sequencing of 16S rRNA genes emerged for the identification, characterisation and determination of relationships between bacterial groups (Christensen *et al.*, 1998; Sproer *et al.*, 1999).

2.2. Taxonomy and Classification of *Enterobacteriaceae*

Extensive research by Kauffmann (1966) and Edwards and Ewing (1972) contributed to the current general agreement both on the definition of the family *Enterobacteriaceae* and the main lines upon which it should be subdivided. The genera and species of *Enterobacteriaceae* should be distinguished by their biochemical characters, each of which may be further subdivided by DNA hybridization, serological, biochemical and other methods (Edwards and Ewing, 1972; Brenner, 1986; Jones, 1988). Currently, the *Enterobacteriaceae* make up a large family of Gram-negative rods that falls within the domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales (Donnenberg, 2000).

There are about 44 recognized genera and 176 named species of bacteria belonging to the family of *Enterobacteriaceae* and undoubtedly in the future the unnamed published

and unpublished genomospecies and presently undescribed groups will be included as new genera and species (Garrity *et al.*, 2005).

2.3. Intestinal Habitat of *Enterobacteriaceae*

In the large intestine there is a complex and dynamic interaction with high densities of living bacteria, which achieve concentrations of up to 10^{11} or 10^{12} cells/g in the luminal contents. *Enterobacteriaceae* represent a minority in faecal microbiota, the greatest number and the greatest species variation of bacteria are present in the colon. The total number of obligate anaerobes like *Bacteroides* and *Clostridium* is enormous (10^{10} - 10^{11} cells/gram of intestinal content), while facultative anaerobes like *Enterobacteriaceae* constitute generally less than 10^{7} cells/gram of intestinal content (Guarner and Malagelada, 2003). *Escherichia coli* is commonly abbreviated *E. coli,* it is present in the intestinal tract of domesticated animals, bird and humans. It is estimated that at least 10^{7} (Colony Forming Unit) CFU/g *E. coli* are present in human feces. Under normal circumstances, this population is considered as commensals (Vidotto *et al.*, 1990; Bettelheim, 1991).

2.4. General Characteristics of *Enterobacteriaceae*

Enterobacteriaceae are gram-negative, straight rods, of 0.3-1.0 x 1.0-6.0 µm in size, non-spore forming and facultatively anaerobic. They can be motile by peritrichous flagella or nonmotile. Most of them grow well at 22–35°C. However, optimal growth and maximal biochemical capacity of a number of genera (*Yersinia, Hafnia, Xenorhabdus, Photorhabdus*, and many *Erwiniae*) occurs at 25-28°C (Farmer, 1995; Garrity *et al.*, 2005).

They are chemoorganotrophic, having both a respiratory and a fermentative metabolism. Acids and visible gas are often produced during fermentation of glucose and other sugars. Most species are catalase-positive and oxidase-negative (Garrity *et al.*, 2005) and can reduce nitrate to nitrite. All ferment glucose, but those which ferment lactose are grouped together as coliform bacteria (e.g. *Citrobacter*, *Escherichia*, *Enterobacter* and *Klebsiella*) genera (Farmer *et al.*, 1985; Farmer, 1995; Farmer, 1999).

2.5. Identification of *Enterobacteriaceae* Members

Genera and species of the *Enterobacteriaceae* family have traditionally been differentiated based on biochemical tests. Biochemical reactions for *Enterobacteriaceae* include: The IMViC (Indole production, Methyl red, Voges-Proskauer, Citrate utilization) tests are a group of biochemical reactions, hydrogen sulfide production by plating on Triple Sugar Iron (TSI) agar, growth in Potassium Cyanide (KCN) broth, utilization of malonate, tartrate utilization by plating on Jordan's tartrate agar and acetate utilization (Farmer *et al.*, 1985; Farmer, 1995; Farmer, 1999). During fermentation, the production of gas (CO2) is a tool to differentiate between *Escherichia coli* and pathogens like *Shigella* spp. and *Salmonella* spp., which do not produce gas (Sawers, 2005). Similarly, because they possess formic hydrogenlyase, members of *Enterobacter* spp. are vigorous gas-producers but paradoxically *Serratia* spp. does not produce it (Kurokawa and Tanisho, 2005).

Enzymatic tests are also commonly used: hydrolysis of urea, gelatin or esculin, phenylalanine deaminase, lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, tartrate, lipase, DNase, oxidation of nitrate to nitrite, Kovacs oxidase test, ONPG (β-galactosidase) test, yellow pigment production, acid and gas production from D-glucose (Farmer *et al.*, 1985; Farmer, 1999; Farmer, 2003).

The lysine decarboxylase production test is used to distinguish *Proteus* species from *Salmonella* species as these organisms have similar reactions in TSI agar. *Proteus* species are negative in the test, whereas *Salmonella* species invariably produce the enzyme. Urease production can also distinguish *Proteus* species from *Salmonella* species, *Proteus* species produce urease whereas *Salmonella* species do not. Tests for motility allow differentiation of *Klebsiella* species (non-motile) from *Enterobacter* species (motile). Both species produce similar mucoid colonies which are difficult to distinguish visually (Quinn *et al*, 2011). Many genera and species of *Enterobacteriaceae* also have typical patterns of resistance and susceptibility to antibiotics; thus, the antibiogram of an isolate can also be used as an aid of identification (Farmer *et al.*, 1985; Farmer, 1999).

2.6. Cell Wall of *Enterobacteriaceae*

The cell wall of *Enterobacteriaceae* generally consists of three layers, the cytoplasmic membrane and the outer membrane, separated by a peptidoglycan layer (Figure 2.1). The outer cell membrane contains phospholipids, membrane proteins and lipopolysaccharide (LPS). LPS comprises the lipid-A, the lipopolysaccharide core and repeated polysaccharide units called O-antigens. Lipid-A is the lipophilic, inner part of LPS. The toxic effects of LPS also known as endotoxin, are caused by lipid-A. Here, the terms LPS and endotoxin are used synonymously. On the outer surface, bacteria may have fimbrias which protrude from the cell wall. The surface may be covered with a thick polysaccharide layer called a capsule (Cullor, 1996).

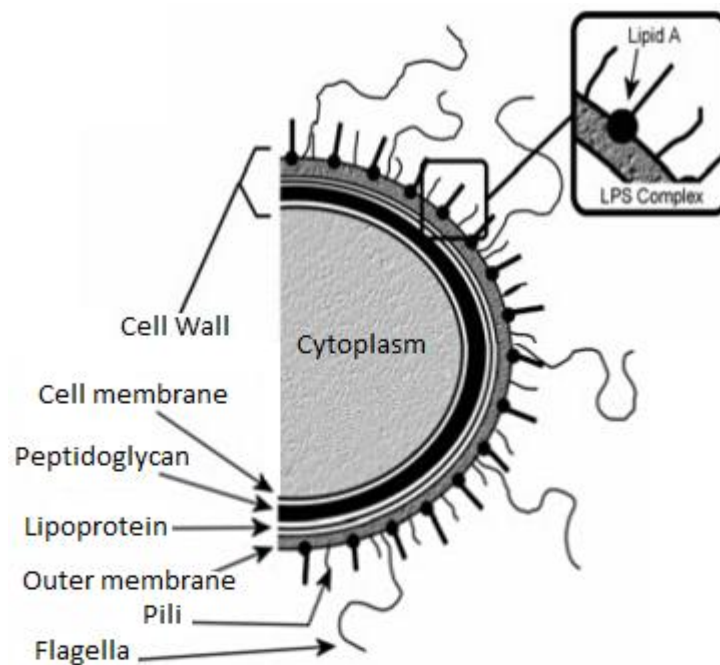


Figure 2.1. Schematic illustration of the components of gram-negative bacterial cell wall (Redrawn from Cullor, 1996).

2.7. Some Important *Enterobacteriaceae* Members

2.7.1. *Salmonella* serotypes

Non-typhoidal *salmonellae*, which include > 2500 serotypes (or serovars) are widely distributed in nature, including the gastrointestinal tract of mammals, reptiles, birds, and insects (Riemann, 2006). Most *salmonellae* are motile, however, the serotypes *gallinarum* and *pullorum* are non-motile (Boone *et al.*, 2001).

Salmonella have been isolated from the feces of healthy cattle, where it may exist as a normal member of the gastrointestinal flora, or as a transient member of the gastrointestinal microbial population. Illness from Salmonellosis in the bovine is seen predominantly in young calves, although it is sometimes seen in adult cattle (Callaway *et al.*, 2005). Host-specific serotypes of *Salmonella* (such as *S. dublin*) can cause diseases such as abortion or severe gastroenteritis. However, ubiquitous serotypes can cause sub-acute septicemia and acute enteritis. In the subclinical form of the disease, animals may either have a latent infection or become a temporary or persistent carrier (Foley and Lynne, 2008). Many human illnesses can be linked to the consumption of bacterially contaminated ground beef, milk, or other dairy products (Callaway *et al.*, 2005). Direct contact with infected animals can also serve as a source for *Salmonella* infection (Foley and Lynne, 2008).

2.7.2. *Shigella* species

The genus *Shigella* consists of four species: *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei* which can be differentiated from each other by the fermentation of sugars or sugar alcohols, production of indole, and the synthesis of ornithine decarboxylase or arginine dehydrolase (Lampel *et al.*, 2000; Garrity *et al.*, 2005). *Shigella* produces pyruvate from the fermentation of glucose and other sugars and converts it primarily by mixed acid fermentation to formic acid, acetic acid, and ethanol. In contrast to *E. coli* strains, *Shigellae* do not produce gas from the fermentation of sugars, because they lack the formate hydrogen lyase system that splits formic acid into CO_2 and H_2 (Lampel *et al.*, 2000). In addition, members of the genus *Shigella* do not produce detectable amounts of acetoin or 2, 3-butanediol (Voges-Proskauer negative) (Lampel *et al.*, 2000; Garrity *et al.*, 2005).

2.7.3. *Klebsiella* species

Klebsiella species are facultative anaerobic, non motile, encapsulated, straight rods that are arranged singly, in pairs or in short chains. *Klebsiella* species have an optimum growth temperature of 37°C. This organism can be found in animal and human feces, soil, water, sewage, grains, fruits and vegetables. *Klebsiella* species include *ornithinolytica, oxytoca, planticola, pneumoniae, pneumoniae* subsp. *pneumoniae, pneumoniae* subsp. *ozaenae, pneumoniae* subsp. *rhinoscleromatis,* and *terrigena*. *Klebsiella pneumoniae* and *oxytoca* and sometimes other species are opportunistic pathogens which can cause bacteremia, pneumonia, urinary tract infections and other human infections. The ability of *Klebsiella* to cause gastroenteritis is unclear (Holt *et al.*, 1994; Janda and Abbott, 1998).

2.7.4. *Enterobacter* species

Enterobacter species are facultative anaerobic, straight rods that are usually motile by peritrichous flagella (except: *asburiae*) and have an optimum growth temperature of 30 to 37°C. This organism is widely distributed in nature and can be found in animal and human feces, soil, water, sewage, plants and vegetables. *Enterobacter* species include *agglomerans*, *aerogenes*, *amnigenus*, *asburiae*, *cancerogenes*, *cloacae*, *dissolvens*, *gergoviae*, *hormaechei*, *intermedius*, *kobei*, *nimipressuralis*, *pyrinus* and *sakazakii*. This organism may also be isolated from farm animals, primates, fish and insects. *Enterobacter cloacae*, *sakazakii*, *aerogenes*, *agglomerans*, and *gergoviae* are opportunistic pathogens that cause burn, wound, and urinary tract infections and sporadically cause septicemia and meningitis (Holt *et al.*, 1994; Janda and Abbott, 1998). They are biochemically similar to *Klebsiella*, but unlike *Klebsiella*, they are able to decarboxylate ornithine, in other words, ornithine positive. The *Enterobacter* strains are fimbriate and slime-forming. *E. sakazakii* may be confused with *E. cloacae*, but can be distinguished by yellow pigment production (Eisenstein and Zalenik, 2000).

2.7.5. *Proteus* species

The two species most representative of the genus *Proteus* are: *P. mirabilis* and *P. vulgaris* which were both described in 1885 by Hauser. He noted the swarming colonies nature and classified them according to their ability to liquefy gelatine (O'Hara *et al.*, 2000). This swarming ability is related to the flagella which translocate very fast along the plates, and this characteristic allows them to differentiate from other *Enterobacteriaceae* members. The genus *Proteus* are lactose negative and motile (Donnenberg, 2010). Bacteria

of this genus are a part of the commensal flora of the intestinal tract from humans and animals (O'Hara *et al.*, 2000; Giammanco *et al.*, 2011).

2.7.6. *Yersinia* species

Yersiniae are non-spore forming, straight rods or coccobacilli, responsible for self-limiting gastroenteritis and Yersiniosis, restricted to the intestinal tract and the intestinal lymphoid system. The genus *Yersinia* is composed of 14 species, of which only *Y. enterocolitica, Y. pseudotuberculosis* and *Y. pestis* are known pathogens for humans and animals. *Y. enterocolitica* and *Y. pseudotuberculosis* are enteropathogenic organisms that share common modes of transmission mainly through food and water. A special characteristic is that different serotypes can be positive to the Voges-Proskauer reaction when cultivated between 22°C to 25°C and negative between 35°C to 37°C (Kapperud and Bergan, 1984).

Bacteria currently classified as *Y. enterocolitica* do not constitute a homogeneous group. Within this species, there is a wide spectrum of biochemical variants; such variations form the basis for dividing *Y. enterocolitica* into biovars (Bercovier *et al.*, 1980; Swaminathan *et al.*, 1982). Infection with *Y. enterocolitica* includes the proliferation of cells in the lumen and in the lymphoid tissue of the intestine. Adherence and penetration into the epithelial cells of the intestinal mucosa are essential factors in the pathogenesis (Miller *et al.*, 1988; Bliska and Falkow, 1994).

2.8. *Escherichia* species

The genus *Escherichia* consists of five species; *Escherichia blattae*, *Escherichia coli, Escherichia fergusonii, Escherichia hermanii,* and *Escherichia vulneris* (Bruckner *et al.*, 1999). *Escherichia coli* is commonly abbreviated as *E. coli*. It is the most numerous commensal inhabitant of the large intestine in humans. *E. coli* can be commonly found in animal faeces and the lower intestines of mammals, and they possess adhesive fimbriae that promote binding to intestinal epithelial cells. *E. coli* can also be found in environments at a higher temperature, such as on the edge of hot springs. They prefer to live at a high temperature rather than low. *E. coli* is probably the most famous member of the *Enterobacteriaceae* family, since it is a model organism and lots of our knowledge of biochemical processes and genetics derived from this species. *E. coli* is also commonly

used as an indicator of faecal contamination. *E. coli* can be distinguished from most other coliforms by its ability to ferment lactose at 44°C (Janda and Abbott, 1998).

E. coli colonizes the gastro intestinal tract within hours after birth and there is a mutual relationship (symbiotic) between host and bacteria. However if the immune system or if the gastrointestinal barriers are compromised some strains can cause disease (Nataro and Kaper, 1998). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and by preventing the establishment of pathogenic bacteria within the intestine (Reid *et al.*, 2001).

2.8.1. Morphology and characteristics of *E. coli*

E. coli is a Gram-negative, non-spore forming, facultative anaerobe that ferments a wide range of sugars, including lactose, producing acid and gas (Feng, 2001). Its morphology is typically a straight, round-ended rod, approximately 0.5-1.0 x 1-6 µm in size. They usually occur singly or in pairs and most strains are motile and commonly have fimbriae and/or pili. Its cells may have a polysaccharide capsule or microcapsule and a typical Gram-negative-type cell wall. It forms colonies on nutrient agar (37°C, 24 h) which are usually 1-3 mm in diameter and may be smooth, low-convex and grayish-translucent. Mucoid and slime-producing strains may also occur (Singleton and Sainsbury, 1999).

On MacConkey agar *E. coli* colonies are bright pink surrounded by a precipitate, and on Eosin-methylene blue agar *E. coli* colonies have a dark green-black metallic sheen. In contrast to the frequent hemolysis observed on blood agar by mammalian pathogenic *E. coli*, this is not common of avian pathogenic *E. coli* (Barnes *et al.*, 2003).

2.8.2. Genomes of *E. coli*

The first complete DNA sequence of an *E. coli* genome was published in 1997. It was found to be a circular DNA molecule 4.6 million base pairs in length, containing 4288 annotated protein-coding genes, seven ribosomal RNA (rRNA) operons, and 86 transfer RNA (tRNA) genes. Also, it has been the subject of intensive genetic analysis for approximately 40 years, a large number of these genes were previously unknown. The coding density was found to be very high, with a mean distance between genes of only 118 base pairs. The genome was observed to contain a significant number of transposable genetic elements, repeat elements, cryptic prophages, and bacteriophage remnants (Blattner *et al.* 1997).

Today, over 60 complete genomic sequences of *Escherichia* species are available. Comparison of these sequences shows a remarkable amount of diversity; only about 20% of each genome represents sequences that are present in every one of the isolates, while approximately 80% of each genome can vary among isolates. The total number of different genes among all of the sequenced *E. coli* strains (the pan-genome) exceeds 16,000. This very large variety of component genes has been interpreted to mean that two-thirds of the *E. coli* pan-genome originated in other species and arrived through the process of horizontal gene transfer (Zhaxybayeva *et al.* 2011).

2.8.3. Biochemical properties of *E. coli*

E. coli strains are lactose fermenters, but strains of *E. coli* which do not ferment lactose are occasionally isolated; these non-fermenter strains should be differentiated from *Salmonella* (Barnes *et al.*, 2003). In most cases, *E. coli* are able to ferment a number of sugars including arabinose, glucose, galactose, fructose, saccharose, and maltose (Sojka, 1965). The ability to ferment glucose with the production of acid and gas is a basic characteristic of *E. coli*, these bacteria are methyl red positive, which means that they reduce the pH enough for methyl red to change in colour. *E. coli* also produce a mixture of almost equal amounts of hydrogen and carbon dioxide (Bettelheim, 1994) as well as indole (Sojka, 1965). The reduction of nitrate to nitrite is characteristic of *E. coli*, which allows them to grow anaerobically on nitrate and utilize the ammonia formed by its anaerobic reduction as a nitrogen source (Bettelheim, 1994).

E. coli do not grow in the presence of potassium cyanide, do not hydrolyze urea. The majority of strains will be inactivated at temperatures between 60°C for 30 min and 70°C for 2 min; however, they survive freezing temperatures for extended periods of time. A pH below 4.5 or above 9 will inhibit although it will not kill the replication of most *E. coli* strains. Organic acids are more effective at inhibiting bacterial growth than inorganic acids (Barnes *et al.*, 2003).

2.8.4. Unusual biochemical properties of *E. coli*

E. coli strains isolated from diseased conditions showing atypical biochemical behavior have been reported (Sutariya, 1993). Often these characters were found transmissible along with drug resistance. Hence, it is possible to use this unusual biochemical behaviour as an epidemiological tool in characterizing the isolates from disease outbreaks. The existence of the unusual biochemical characters of *E. coli* have been recognized by many researchers.

For example: H_2S production reported by Sutariya (1993) who found 4 out of 20 poultry *E. coli* isolates that produced H_2S on TSI. And Mishra *et al.* (2002) isolated few atypical poultry *E. coli* strains which produced H_2S. Urease production observed by Dubey and Sharda (2001) they observed that 4.3 % *E. coli* isolated from diarrhoeic goats produced urease. Ability to utilize citrate noticed by Dubey and Sharda (2001) they observed that *E. coli* strains isolated from diarrhoeic goats failed to utilize citrate in Simon's citrate medium but 48.9% were positive for using citrate as a sole carbon source when tested in Christensen's citrate agar. Adonitol and Raffinose fermentation of *E. coli* isolated from diseased broilers found by Cloud *et al.* (1985) as they reported that 30 out of 197 isolates fermented adonitol while 177 out of 197 fermented raffinose.

2.8.5. Serological characteristics of *E. coli*

In 1947, Kauffmann was the first to successfully use serological methods to classify *E. coli* (Ørskov *et al.*, 1977). Currently, 167 "O", 74 "K", 53 "H", and 17 "F" *E. coli* antigens are recognized (Barnes *et al.*, 2003).

2.8.5.1. Somatic antigens

The O antigens (lipopolysaccharide (LPS) antigens) are thermostable surface antigens found in all smooth or S forms of *Enterobacteriaceae* (Ørskov *et al.*, 1977). They are composed of phospholipid polysaccharide complexes with a heat resistant protein fraction (Lior, 1994). These antigens retain their immunogenic, agglutinating and binding capacity even after boiling (Ørskov *et al.*, 1977). The differences among O antigens are defined by their terminal group, mainly by its nature and by the order of the repeating units within the chain (Lior, 1994). However, due to common LPS and outer membrane proteins in Gram-negative bacteria, cross-reactivity among the O antigens of *E. coli*, *Salmonella* and *Shigella* is common (Ørskov *et al.*, 1977).

Moreover, K antigens (acidic polysaccharide factors) are polymeric acids on the cell surface, they contain 2% reducing sugars and are associated with virulence (Barnes *et al*., 2003). Lior (1994) hypothesized that the inability to agglutinate might have been caused by flagella, fimbriae or other surface structures.

2.8.5.2. Protein antigens

Flagellar (H) antigens are thermolabile determinants contained on flagellin, the constituent protein of flagella in motile organisms (Figure 2.1.). H antigens may be associated with any of the O antigens, so in human medicine the O:H combination, i.e., the serotype, should be determined in cases of diarrhoeal and extraintestinal disease caused by pathogenic *E. coli*. Fimbriae or pili (F) antigens are thread-like structures that project from the surface of many Gram-negative bacteria (Figure 2.1.). They play an important role in bacterial adherence to epithelial cell surfaces, especially in the intestinal and urinary tracts (Lior, 1994).

Curli are thin, coiled filaments present on the surface of *E. coli* and *Salmonella* spp. Curli mediate bacterial binding to the extracellular matrix and to serum proteins such as fibronectin, laminin, plasminogen and plasminogen activator protein, thus contributing to bacterial adherence and colonization (Olsen *et al*., 1989).

2.8.6. *E. coli* virulence factors and virulence genes

A compilation of multiple individual *E. coli* genome sequences revealed that there are approximately 2,200 genes in the core genome and approximately 13,000 genes in the pan-genome. The *E. coli* pathogenic genome is commonly encoded on more than 5,000 genes. Less than half of these are included in the core genome (Rasko *et al*., 2008). This makes *E. coli* a very diverse microorganism with a substantial genetic diversity and plasticity. Of all the pathogenic genes known of this organism, only 567 *E. coli* virulence genes have been identified. These have been grouped into 78 different types of virulence factors (Chen *et al*., 2012). Some of the most common virulence factors include: adhesins, toxins, capsules and secretion systems.

2.8.7. Strain classification of *E. coli*

In medicine, a broad classification of *E. coli* based on their genetics and clinical importance divides them in commensal strains, intestinal pathogenic, enteric or diarrheagenic, and extraintestinal pathogenic strains. Commensal strains of *E. coli* are the main component of the fecal flora in most healthy humans and animals. They lack the virulence factors carried by pathogenic intestinal and extraintestinal strains, thus they do not normally cause disease within the intestinal tract or outside it. Intestinal pathogenic *E. coli* strains are not present in the fecal flora of healthy hosts, their presence is associated with gastroenteritis or colitis (Russo and Johnson, 2000).

The intestinal pathogenic *E. coli* strains have been grouped in six pathotypes: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). The different pathotypes of *E. coli* are composed by clonal groups sharing O and H antigens (Nataro and Kaper, 1998). To cause disease in animals, EPEC, EHEC, and ETEC use the same virulence factors found in human strains but have specific colonization factors for animal species not found in human strains (Kaper *et al.*, 2004). An additional animal pathotype known as Avian Pathogenic *E. coli* (APEC) causes extraintestinal infections in poultry species (Barnes *et al.*, 2003).

2.8.8. Some *E. coli* infections in cattle and sheep

E. coli produce intestinal and systemic disease in domestic animals. Early exposure to pathogenic *E. coli* along with low levels of circulating immunoglobulins make neonatal calves and lambs susceptible to colisepticaemia, an acute fatal infection of dairy calves under 1 week of age (Gay and Besser, 1994) and neonatal lambs within 2 days of birth (Hodgson, 1994). Acute or chronic neonatal diarrhoea commonly affects calves during the first 4 week after birth. Calves affected by enteric colibacillosis produce large amounts of foul-smelling pasty to watery faeces, with colours varying from pale yellow to white, which produce characteristic soiling of the buttocks known as “white scours” (Butler and Clarke, 1994).

Cattle are a reservoir for EHEC O157:H7 but this serotype is not associated with disease in cattle (Butler and Clarke, 1994). In 1997, Blanco *et al.* characterized EHEC strains recovered from more than 350 healthy animals from 19 different farms in Spain; they concluded that 95% of the farms examined were positive for EHEC strains not only in

adult cows but also in calves (Blanco *et al.*, 1997). In cases of mastitis caused by *E. coli* clinical signs may vary from fatal per acute mastitis, through acute, chronic and subclinical mastitis (Hill, 1994).

2.8.9. Transmission of *E. coli* from food animals to human

As *E. coli* is commonly found in the normal intestinal microbiota of animals, it is hypothesized that food animals could be a reservoir for extraintestinal pathogenic *E. coli* (ExPEC). In previous studies, direct transmission of *E. coli* between animals (beef, pig and chicken) and humans (e.g., farmers, slaughterers and caretakers) in close contact with food animals have been documented (Ojeniyi, 1989; Marshall *et al.*, 1990). It has also been demonstrated that the human gut can be colonized by *E. coli* strains coming from food animals (Hummel *et al.*, 1986). As a normal constituent of the animal gut microbiota, *E. coli* from feces can readily contaminate carcasses and meat as well as the abattoir and the packing station environment (Shooter *et al.*, 1974). As a result, meat products are often contaminated during the slaughtering process, and dissemination between carcasses can occur (Howe *et al.*, 1976). *E. coli* is typically killed during cooking. However, meat handling can still lead to bacterial transmission via kitchen cross-contamination and by the consumption of inadequately cooked meat or poor hand and surface hygiene (Cogan *et al.*, 2002).

2.9. Antibiotics

The term antibiotic is used for substances or antimicrobial agents from natural or synthetic sources, that kill or inhibit the growth of microbes by specific interactions with bacterial targets, without harming the host harboring the infecting bacteria (Walsh, 2000; Davies and Davies, 2010). An antimicrobial agent must have the potency (enter into the bacterium cell) and access (should be able to reach the target) in order to exert its antimicrobial action (Fisher *et al.*, 2005).

2.9.1. Classification of antibacterial agents

Antibacterial agents can be classified according to their mechanism of action and by spectrum of activity. The five main target sites of antimicrobial agents are: (i) cell wall synthesis, (ii) protein synthesis, (iii) nucleic acid synthesis, (iv) metabolic pathways, and (v) cell membrane functions (Tenover, 2006; Maddison, 2009). Antibacterial drugs that act by inactivating the wall synthesis include the penicillins, cephalosporins and vancomycin. Aminoglycosides and tetracycline inhibit protein synthesis by binding to the ribosomal subunit 30S, while macrolides and chloramphenicol bind to the 50S ribosomal subunit to inhibit protein synthesis. Fluoroquinolones antimicrobial agents disrupt the DNA synthesis whereas sulphonamides and trimethoprims block the pathway for folic acid synthesis (Tenover, 2006).

Bacteriostatic agents inhibit bacterial growth as long as the drug concentration is above the minimal inhibitory concentration (MIC). Tetracycline, chloramphenicol and sulphonamides are examples of bacteriostatic agents. Bactericidal agents e.g. aminoglycosides, cephalosporins, fluoroquinolones, metronidazole, penicillins and potentiated sulphonamides kill the bacteria and are preferred in infections in which the host cannot control or eradicate the infection due to location of the infection itself or due to the compromised immune status of the host (Maddison, 2009).

2.9.2. Types of resistance to antibiotics

A. Intrinsic resistance: The resistance of all members of a bacterial species without any genetic extra-modifications. This type of resistance is due to either the lack of the target for the action of drug or inability of drug to enter the bacterial cell (Normark and Normark, 2002; Greenwood *et al.*, 2006).

B. Acquired resistance: This type of resistance includes:

1. Mutational resistance: Occur either by point mutation, deletion, inversion or insertion in the bacterial genome resulting in a very few individuals, among the huge bacterial populations, exhibiting spontaneous resistance. These resistant mutants proliferate under the action of antibiotic selective pressure to constitute the majority or even the whole population (Normark and Normark, 2002; Greenwood *et al.*, 2006).

2. Transferable resistance: in which a resistance gene (or genes) transfer from resistant to susceptible bacterial cell. Among the different DNA elements those transfer antibiotic resistances are plasmids, phages, transposons and integrons (Normark and Normark, 2002; Greenwood *et al.*, 2006).

2.9.3. Mechanisms of antibiotic resistance

Bacteria employ several basic strategies for evading the effects of antibiotics, such as followings.

2.9.3.1. Altered target sites

Alterations of antibiotic target sites are usually caused by point mutations in the regions of a specific gene necessary for antibiotic activity, resulting in lowered binding affinity between the antibiotic and its target. Examples that characterize this resistance mechanism are listed below:

Modification of penicillin binding proteins (PBPs) enzyme, the targets of β-lactams; this can be achieved by one of three mechanisms: i) amino acid substitutions, ii) the acquisition of new PBPs-encoding genes, iii) the recombination of genes coding for PBPs, resulting in mosaic genes encoding proteins with decreased affinity to β-lactams (Georgopapadakou and Liu, 1980). Point mutations in the *gyrA* and *gyrB* genes, coding for DNA topoisomerase II (also called DNA gyrase) or in topoisomerase IV, which also acts

as target proteins for the action of antibiotics (Hooper, 1995; Tankovic *et al.*, 1996), can alter the binding efficiency of fluoroquinolone antibiotics, thereby reducing their efficacy. In addition, not only single mutations, but multiple point mutations may also occur, eventually leading to higher levels of resistance, as observed for the quinolone resistance determining region (QRDR) of DNA topoisomerase II (*gyrA*, *gyrB*) and IV (*parC* and *parE*) genes. Although, resistance to quinolones can also be mediated by Qnr proteins, that protect the complex of DNA and DNA gyrase or topoisomerase IV enzymes from the inhibitory effect of those antibiotics (Strahilevitz *et al.*, 2009).

2.9.3.2. Decreased uptake

Antibiotic resistance may occur through a decrease in the permeability of the outer membrane of Gram-negative bacteria (Poole, 2002). The flow of molecules into the cell is ensured by complex membrane proteins, known as OMPs (Outer Membrane Proteins), which provide channels for the entry of molecules into the cell membrane (including antibiotics) based on charge, shape, and size. Loss of function of one of these porins due to a mutation event may possibly lead to antibiotic resistance (Delcour, 2009).

2.9.3.3. Active efflux system

The efflux of antibiotics provides a residual level of resistance; i.e., it is usually not enough for the expression of clinical resistance. However, together with other mechanisms, it may lead to treatment failures. Resistance is frequently caused by increased synthesis of protein machinery, due to, for example, mutations that occur in the transcriptional repressors of these proteins, eventually leading to increased efficiency of transport of antibiotics to the outside of the cell (Poole, 2004).

2.9.3.4. Enzymatic inactivation or modification

Enzymatic inactivation or modification of antibiotic agents is a common mechanism of resistance that reduces or eliminates antibiotic activity (Morar and Wright, 2010). The primary resistance mechanism to β-lactams, is enzymatic degradation. In β-lactams, hydrolysis of the β-lactam ring is mediated by β-lactamases. Enzymatic modification is the most common type of aminoglycoside resistance. One classical example of such modification involves aminoglycoside acetyltransferase, that acetylates and inactivates aminoglycosides such as gentamicin (Wright, 2005).

2.9.4. β-Lactam antibiotics

The β-lactam antibiotics, including penicillins, cephalosporins, monobactams and carbapenems are one of the most widely used group of antimicrobials due do their safety, low cost, ease of delivery, minimal side effects and high efficacy. β-lactam antibiotics are grouped together based upon a shared structural feature. β-lactam is a generic name for all β-lactam antibiotics that contain a β-lactam ring, a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom (Wilke *et al.*, 2005).

This group represents 60% of all antimicrobial used by weight and is used to treat infections caused by gram-negative bacteria and gram-positive, in human medicine (Livermore and Woodford, 2006). In veterinary medicine, different substances from the penicillin family, first to fourth generation cephalosporins and β-lactamase inhibitors are recommended for the treatment according to the animal species involved and the underlying disease (Smet *et al.*, 2010).

2.9.4.1. Mechanism of action of β-lactam antibiotics

β-lactam antibiotics inhibit the growth of bacteria by inactivating enzymes called penicillin binding proteins (PBPs), located in the bacterial cell wall which are involved in the third stage of cell wall synthesis (Poole, 2004). β-lactam antibiotics normally interfere with this process by reacting covalently with the active site serine to form a stable acyl-enzyme preventing the peptidoglycan synthesis (Hujer *et al.*, 2005). In contrast to gram-positive bacteria, in gram negative the peptidoglycan it is a thin layer between the cell wall and the cytoplasmic membrane. The antibiotic β-lactam must diffuse across the outer membrane of the gram negative cell, using pores formed by porin proteins, and then cross the periplasm before reaching its PBPs targets, which lie on the outer surface of the cytoplasmic membrane (Livermore and Woodford, 2006).

2.9.4.2. Resistance to β-lactam antibiotics

β-lactam resistance in gram-negative bacteria can occur by the below mechanisms; alterations of porin proteins in the cell membrane causing reduced permeability and blocking entry of the drug, the use of an efflux mechanism to pump out the antibiotic as it crosses the membrane, alterations of the target penicillin binding proteins (PBPs) to prevent β-lactam binding and the production of enzymes that inactivate the antimicrobial

agent (Black, 2002), one such enzyme, is β-lactamase. It is the main cause of clinically significant resistance to β-lactams in *Enterobacteriaceae* members.

2.9.5. β-Lactamases

β-lactamases are bacterial enzymes that catalyze the hydrolysis of the β-lactam ring to yield inactive products. In gram-negative bacteria β-lactamase enzymes remain in the periplasmic space of the cell wall, where they attack the β-lactam before it can reach the penicillin binding proteins (Bradford, 2001). Genes encoding β-lactamase enzymes are found on the chromosome and on plasmids. Plasmid-mediated β-lactamase genes are the most common ones as they can be transferred to other gram-negative bacteria by conjugation (Livermore, 1995).

In *Enterobacteriaceae*, the major mechanism of β-lactam resistance is the production of β-lactamases (Majiduddin *et al.*, 2002). This resistance mechanism is often associated with mobile genetic elements such as insertion elements, transposons and integrons, and is highly selected by the selective pressure caused by the use of β-lactam antibiotics (Massova and Mobashery, 1998).

2.9.6. Classification of β-lactamases

Since early 1970s different classifications based on phenotype, gene or amino acid protein sequences and function have been described (Bush *et al.*, 1995). Classification of β-lactamases can be done according to the functional properties (Bush-Jacoby-Medeiros scheme) defined by the substrate and inhibitor profile. There are four main groups and multiple subgroups in this system. This classification scheme is of much more immediate relevance to the physician or microbiologist in a diagnostic laboratory because it considers β-lactamase inhibitors and β-lactam substrates that are clinically relevant (Paterson and Bonomo, 2005). Group 1 includes cephalosporinases that are not well inhibited by clavulanic acid; group 2 refers to penicillinases, cephalosporinases, and broad spectrum β-lactamases that are generally inhibited by active site-directed β-lactamase inhibitors, the last group, group 3 includes the metallo β-lactamases that hydrolyse penicillins, cephalosporins, and carbapenems and that are poorly inhibited by almost all β-lactams (Bush *et al.*, 1995).

In contrast, the Ambler classification scheme divides the β-lactamases into 4 classes (A, B, C and D) upon their amino acid sequence and not phenotypic characteristics.

Class A, C, and D, all have a serine at their active site, while class B are metallo enzymes that require at least one active site zinc ion to facilitate β-lactam hydrolysis (Hall and Barlow, 2005; Bush and Jacoby, 2010).

2.9.7. Extended-spectrum β-lactamases

Resistance to β-lactams in *Enterobacteriaceae* is mainly due to the production of β-lactamases which may be encoded either chromosomally or on plasmids (Bradford, 2001). Resistance to extended-spectrum β-lactams has been associated with the production of broad-spectrum β-lactamases such as extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases and metallo-β-lactamases (MBLs) (Batchelor *et al.*, 2005). ESBLs confer resistance to most β-lactam antibiotics, but are not active against cephamycins and carbapenems and are inactivated by β-lactamase inhibitors such as clavulanic acid. This is in contrast to AmpC β-lactamases, which are not inhibited by clavulanic acid and usually confer resistance to all β-lactams, with the exception of dipolar ionic methoxy-imino-cephalosporins, such as cefepime and the carbapenems (Bradford, 2001). MBLs can hydrolize all clinical β-lactam substrates, with the exception of aztreonam.

The emergence and spread of extended-spectrum beta-lactamases (ESBLs) among members of *Enterobacteriaceae* family originating from food-producing animals is a major public health issue worldwide. Regarding ESBL variants, CTX-M-type enzymes have been widely identified in *E. coli* and *Salmonella* spp. strains from both healthy and diseased animals (Carattoli, 2008).

2.9.8. Carbapenems resistance in *Enterobacteriaceae*

Carbapenems (e.g. imipenem, meropenem, doripenem, ertapenem) cover a broad antibacterial spectrum with activity against both gram-positive and gram-negative aerobic and anaerobic bacteria. *Enterobacteriaceae* are generally carbapenem-susceptible, including ESBL- and AmpC-producing isolates. Unfortunately, in recent years emerging resistance to carbapenems has been observed. Most often, multiple mechanisms are responsible for the final resistance phenotype. It is likely that the interplay between efflux pumps, β-lactamases and altered membrane permeability through porin loss is what mediates the resistance. β-lactamases that are able to hydrolyze carbapenems are categorized as class A carbapenemases consisting of plasmid-encoded KPC, class D carbapenemases also known as OXA-type (mainly in *Acinetobacter* spp.), and class B

metallo β-lactamases MBLs, including IMP, VIM (Yoshiro and Paterson, 2010; Retamar, 2010).

2.9.9. β-lactamase inhibitors

The β-lactamase inhibition can be classified as either reversible or irreversible. Reversible inhibitors bind to an enzyme but activity may be restored after their removal. Irreversible inhibitors may be more effective than reversible inhibitors because the final outcome is the destruction of enzymatic activity. Specific irreversible inhibitors of β-lactamases include the clavulanic acid, the penicillanic acid, sulfones, sulbactam and aztreonam (Bush, 1988).

The compounds such as clavulanic acid and sulbactam exhibit low bactericidal activity when used alone but remain effective against β-lactamases enhancing the activity of β-lactam antibiotics. The combination of amoxicillin with clavulanate was selected due to the similarity in pharmacokinetics between the two compounds (Moosdeen *et al.*, 1988). These substances are used in the treatment of serious *Enterobacteriaceae* and penicillin resistant *staphylococcal* infections (Drawz and Bonomo, 2010).

Any new β-lactamase inhibitors should have the following characteristics: the molecules must be capable of preventing hydrolysis of well tolerated broad-spectrum β-lactam antibiotic, preferably inexpensive penicillin; the pharmacokinetics of the two molecules should be similar; side effects should be minimal and mild; the molecules should not be good inducers of cephalosporinase activity. Ideally, one would like to have molecules with oral activity (Bush, 1988).

2.9.10. Transfer of antimicrobial resistance from animals to humans

Animals are exposed to antimicrobials that are analogues to those used for therapy in humans, with the exception of ionophores, and bambermycins (Turnidge, 2004). This creates an issue where these antimicrobials may promote antimicrobial resistance in microbiota to antimicrobials used for therapy in humans (Phillips *et al.*, 2004). Bacteria resistance genes to antimicrobials could be spread through the environment by livestock through food products or via contamination of water or crops with animal excreta (Wegener, 2003). Bacterial resistance determinants to antibiotics and disinfectants have been detected in the environment in waste water, surface water, ground water, sediments and soils (Kummerer, 2004).

Furthermore, bacterial spread from livestock to food products has occurred with *Salmonella*, *Campylobacter*, and *E. coli* (Wegener, 2003). Because resistance genes are generally mobile, these resistance determinants within the bacterial flora could be transferred to more pathogenic bacteria. This could occur either in the livestock, or the human gastrointestinal tract (Winokur *et al.*, 2001). Series of studies on the resistance of *E. coli* which were isolated from animals and humans have strongly suggested that those bacteria which are resistant to antimicrobials used in animals would also be resistant to antimicrobials used in humans (Miles *et al.,* 2006; Umolu *et al.,* 2006). This phenomenon will cause major health problems by limiting antimicrobial treatment options for infectious diseases in humans.

There are two main pathways by which there could be a negative impact on human health. In one pathway, zoonotic pathogens in livestock may develop cross-resistance or co-resistance to antimicrobials used in people after the livestock are treated with antimicrobials (Angulo *et al.*, 2004; Schwarz *et al.*, 2006). The zoonotic pathogen could then infect people, primarily through the food supply but occasionally through direct contact of at risk groups, and cause disease that is difficult to treat due to antimicrobial resistance (Angulo *et al.*, 2004). The other pathway is through commensal organisms, carrying and spreading resistance determinants through the food, or possibly via the environment, to humans (Angulo *et al.*, 2004; Hurd *et al.*, 2004). Commensal organisms can transiently colonize humans and disseminate resistance determinants, through horizontal transfer, to the normal flora of the host that may persist if there is no fitness cost of resistance to the pathogen (Cohen and Murray, 2004; Foxman, 2007). Other intermediate steps may also occur, such as improper meat handling and cross contamination (Hurd *et al.*, 2004).

2.9.11. Antibiotic susceptibility testing methods

The primary methods used for susceptibility testing are agar disc diffusion, broth microdilution, agar dilution, broth macrodilution, and E-test. Since agar diffusion and broth microdilution are the two principal methodologies used in veterinary medicine (Brooks *et al.*, 2003).

Agar disc diffusion is based on diffusion of an antimicrobial agent from a commercially prepared disc placed on an agar surface inoculated with a standardized growth medium that has been used for a pure culture (Prescott, 2000). At the same time

that the inoculum is growing, the antimicrobial agent is diffusing from the disc. If the organism is susceptible to the antimicrobial, a zone of growth inhibition is created around the disc. The larger the zone of inhibition, the more susceptible the organism is to the antimicrobial. Agar disc diffusion techniques provide qualitative data, are flexible and low cost. However, the results of disc diffusion will vary unless the inoculum density, the agar thickness and the incubation are carefully controlled (Potz *et al.*, 2004).

3. MATERIALS AND METHODS

The present study was conducted in the laboratory of Biology Department, Faculty of Science and Literature, Kahramanmaras Sutcu Imam University (KSU). It was mainly aimed to focus on isolation, biochemical identification of *E. coli* in addition to some other members of *Enterobacteriaceae* which are of veterinary importance among the isolates from cattle and sheep samples of large intestine. So, different biochemical tests and selective plating methods have been applied.

3.1. Sample Collection

Samples were taken at Kahramanmaras city abattoir, where only healthy cattle and sheep were slaughtered daily, viscera of slaughtered animals were separated in a special place right away after evisceration. There, a total of 40 samples were taken from contents of colon which is one of the large intestine segments, these included 20 samples from cattle and 20 samples from sheep, i.e., one sample per animal. They were collected aseptically and immediately after evisceration, before cleaning process of the viscera by workers, in April 2013. The aseptic sampling process was done by making a small incision in colon wall with a sterile blade and insertion of a sterile cotton swab through the colon wall to get intestinal contents (Figure 3.1.), then replacing the swab at once in the sterile disposable transport tube (FIRATMED, Turkey) containing 1 ml of sterile 1X Phosphate Buffered Saline (PBS) in order to protect the osmotic pressure of the bacteria until processing them for microbiological examinations because PBS is isotonic and non-toxic to most cells. 1X PBS (1L) contains 800 ml of distilled water, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4. pH of solution was adjusted to 7.4 with HCl.

Every sample tube was labeled by writing first letter of animal name and number of sample accordingly. Turkish names for animals were used, as upper-case letter S was abbreviated for Sığır= Cattle, whereas upper-case letter K was abbreviated for Koyun = Sheep. Each letter was used with number 1 to 20, i.e., S1 to S20 and K1 to K20. Both cattle and sheep intestinal samples were randomly selected and collected. So the sex, age and locations of the farm of origin of the animals were not known because the ear tag numbers were not available and only viscera were available in the place that the intestinal samples were taken. Eventually, intestinal samples were transported to the laboratory within few hours of sample collection in a foam box containing ice packs to maintain a

fridge temperature and then they were subjected to streaking on Eosin Methylene Blue agar at the laboratory at the same day.

Figure 3.1. Sample collection method

3.2. Isolation of Single Bacterial Colonies

3.2.1. Culture media and reagents

3.2.1.1. Eosin Methylene Blue Agar, Levine (Acumedia, USA)

Eosin Methylene Blue Agar, Levine (EMB) agar, is selective medium for the isolation and differentiation of gram-negative enteric bacilli. Colonies of lactose fermenters are blue-black with or without a green metallic sheen. *E. coli* colonies typically are dark centered and usually have a green metallic sheen. Colonies of non-lactose fermenting bacteria are colorless and translucent (Acumedia, 2011).

3.2.1.1.1. Eosin Methylene Blue Agar, Levine (Acumedia, USA) Composition (g/liter)

Enzymatic Digest of Gelatin 10.0 g; Lactose 10.0 g; Dipotassium Phosphate 2.0g; Eosin Y 0.4 g; Methylene Blue 0.065 g; Agar 15.0 g.

Enzymatic Digest of Gelatin is the nitrogen source in EMB Agar, Levine. Lactose is the carbohydrate as energy source and Dipotassium Phosphate is the buffer. The dyes Eosin Y and Methylene Blue are the pH indicators. The Methylene Blue is also a selective

agent (inhibit the growth of most gram positive bacteria). During strong acidic conditions, the dyes impart a metallic sheen to certain lactose fermenters, such as *E. coli* (Acumedia, 2011).

3.2.1.1.2. Eosin Methylene Blue Agar, Levine (Acumedia, USA) medium preparation

The medium was prepared according to manufacturer's instructions, 37.5 grams of medium powder were added to 1000 ml of distilled water in a flask and the medium pH 7.1±0.2 was checked by using electronic pH meter at room temperature, then the liquid was heated with frequent agitation until boiling to dissolve the medium completely by using electric hot plate magnetic mixer. The medium was then sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was put into a water bath to decrease its temperature. After solidification of the medium in sterile Petri dishes, the medium color was dark red. Then they were enclosed by parafilm and stored in a refrigerator for future use.

3.2.1.2. Tryptic Soy Broth

Tryptic Soy Broth medium is a nutritious liquid medium that supports the growth of a wide variety of microorganisms, especially common aerobic and facultatively anaerobic bacteria. It is also a general purpose medium and is commonly abbreviated as TSB. In clinical microbiology, it may be used for the suspension, enrichment and cultivation of strains isolated on other media. Growth of bacteria in TSB media is indicated by the presence of turbidity, specks, or flocculation in the medium while an uninoculated control remains clear and without turbidity after incubation. Growth obtained in this medium must be subcultured onto appropriate solid media to obtain pure cultures which afterwards can be identified with methods appropriate for the isolates.

3.2.1.2.1. Tryptic Soy Broth (Merck, Germany) Composition (g/liter)

Peptone from casein 17.0g; Peptone from soymeal 3.0g; D(+)Glucose 2.5g; Sodium chloride 5.0g; Di-potassium hydrogen phosphate 2.5g.

3.2.1.2.2. Tryptic Soy Broth (Merck, Germany) medium preparation

The medium was prepared according to manufacturer's instructions, 30 grams of medium powder were added to 1000 ml of distilled water in a flask and the pH 7.3±0.2 of the liquid was checked using electronic pH meter at room temperature, then it was heated

until boiling to dissolve the medium completely by using an electric hot plate magnetic mixer. The medium was then sterilized by autoclaving at 121 °C for 15 minutes. After autoclaving, the medium was put into water bath of 45°C to decrease its temperature, then stored in a refrigerator for future use.

3.2.2. Processing of the samples

3.2.2.1. Inoculation of samples on Eosin Methylene Blue Agar, Levine

The collected samples were immediately processed upon arrival in the laboratory. Adequate numbers of the previously prepared EMB agar plates were labeled according to the collected sample numbers. All samples were inoculated on EMB agar by using Quadrant streak plate method for obtaining single isolated bacterial colonies, first the swab rotated against the side of the transport tube (above the fluid level) using firm pressure, to remove excess fluid. Then, streaking the small area of agar surface with the swab to get a sufficient amount of bacteria on the agar plate. Next, the sterile wooden sticks were used to streak the entire surface of the medium. Afterward, inoculated plates were incubated for 18-24 hours at 35°C. After incubation, the plates were examined for bacterial isolation process (Acumedia, 2011; Lal and Cheeptham, 2007).

3.2.2.2. Inoculation of bacterial single colonies in Tryptic Soy broth

Few individual colonies, 1 or 2 or 3 but up to 3 colonies from each grown bacterial sample on EMB agar plates were selected including well isolated single colonies. Then with the help of a sterile wooden stick, a selected colony was transferred to sterile culture glass tube containing 5 ml of previously prepared sterile TSB medium. The inoculated TSB tubes which containing isolated individual colonies were coded and labeled, this was recorded according to the original sample numbers. For instance, isolate number 1 of sample number 1 from cattle was coded as S11 and isolate number 2 of the same sample was coded as S12. While some isolates were coded similar to the original sample number because only one colony was taken from cultured EMB plate, for example: Isolate number 1 of sample number 1 from sheep was coded as K1. Then, all the inoculated TSB tubes were incubated in the water bath shaker for 18-24 hours at 35°C.

3.3. Stock Culture Making of the Bacterial Isolates

Two sets of sterile Eppendorf tubes which have 1.5 ml capacity were labeled as same as the number of each bacterial isolate and 300 micro liter of sterile 20% glycerol broth were added to each tube. Then, the overnight grown bacteria in TSB tubes which prepared previously for growth of bacterial colonies were mixed by vortex mixer, then 1 ml of this culture was added to each Eppendorf tube containing 20% glycerol broth, then both of the different types of liquid were mixed by a vortex mixer. Furthermore, one set of the culture in Eppendorf tubes was kept at -80˚C of freezing, this storage condition allowed long-term preservation of bacteria for archiving. While the other set of tubes was kept at -20˚C, this storage condition allowed in-use preservation of bacteria (daily use).

3.4. Biochemical Characterization and Identification of the Bacterial Isolates

Different biochemical tests were performed for biochemical characterization and identification of the isolates; they were selected according to the Veterinary Microbiology and Microbial Disease book (Quinn et al., 2011) as mentioned in Table 3.1. The biochemical tests were: Oxidase test, Triple sugar iron (TSI) agar, Lysine decarboxylase production, the IMViC tests (indole production test, methyl red test, Voges-Proskauer test, citrate utilization test).

Table 3.1. Biochemical reactions of *Enterobacteriaceae* members which are of veterinary importance. Adapted in part from (Quinn et al., 2011)*

Biochemical tests	*E. coli*	*Enterobacter aerogenes*	*Klebsiella pneumoniae*	*Salmonella* serotypes	*Yersinia* species	*Proteus* species
TSI, slant	Yellow	Yellow	Yellow	Red	Yellow or Red[b]	Red or Yellow[c]
TSI, butt	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
TSI, H_2S production	−	−	−	+[a]	−	+
Lysine decarboxylase production	+	+	+	+	−	−
IMViC, Indole production test	+	−	−	−	V	±[d]
IMViC, MR test	+	−	−	+	+	+
IMViC, VP test	−	+	+	−	−	V
IMViC, Citrate utilization test	−	+	+	+	−	V

*+, positive reaction.
−, negative reaction.
Yello, acid; Red, alkaline.
V, reaction varies with individual species.
a, exceptions include *S. choleraesuis* (H_2S −).
b, *Y. enterocolitica* (yellow); *Y. pseudotuberculosis* and *Y. pestis* (red).
c, *P. mirabilis* (red); *P. vulgaris* (yellow).
d, *P. vulgaris* +; *P. mirabilis*−.

3.4.1. Sub-culture of the bacterial isolates

3.4.1.1. Tryptic soy agar

Tryptic Soy Agar (TSA) is a general-purpose medium, providing enough nutrients to allow for a wide variety of microorganisms to grow. It is used for a wide range of applications, including enumeration (counting), isolation of pure cultures, or simply general culture.

3.4.1.2. Tryptic Soy Agar medium preparation

30 grams of Tryptic soy broth medium powder (Merck, Germany) were added to 1000 ml of distilled water in an Erlenmeyer flask, then 15 grams of Agar-agar (Merck, Germany) were added to the flask. The liquid was mixed and heated until boiling to dissolve the medium completely by using a hot plate magnetic mixer. The medium was then sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was put into water bath at 60°C to decrease its temperature. After solidification of the medium in the petri dishes, they were enclosed by parafilm and stored in a refrigerator for future use.

3.4.1.3. Inoculation of TSA plates

Enough numbers of the previously prepared TSA plates were labeled according to the preserved isolates numbers of -20°C stock culture. An inoculum from each isolate stock Eppendorf tube was sub-cultured on TSA plates with sterile wooden sticks and using Quadrant streaking method for obtaining isolated bacterial colonies on agar media. Then inoculated plates were incubated for 18-24 hours at 35°C.

3.4.2. Oxidase test

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, the enzyme sometimes called indophenol oxidase. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colorless reagent becomes an oxidized colored product.

The final stage of bacterial respiration involves a series of membrane-embedded components collectively known as the electron transport chain. The final step in the chain may involve the use of the enzyme cytochrome oxidase, which catalyzes the oxidation of cytochrome *c* while reducing oxygen to form water. The oxidase test often uses a reagent,

tetra-methyl-*p*-phenylenediamine dihydrochloride, as an artificial electron donor for cytochrome *c*. When the reagent is oxidized by cytochrome *c*, it changes from colorless to a dark blue or purple, indophenol blue (Shields and Cathcart, 2010).

3.4.2.1. Performing of the test

Oxidase test strips (Merck, Germany) were used for the present study and have been prepared by the manufacturer as test strips and are ready for use, no manual preparation is needed. A drop of overnight grown isolates in Tryptic Soy Broth medium was added onto the strip, within 20-30 seconds the appearance of purple color or a black color is an indicator of positive reaction, while yellowish to orange color is an indicator of negative reaction (Merck, Germany).

3.4.3. Triple sugar iron test (TSI)

Triple sugar iron agar is a tubed differential medium used in determining carbohydrate fermentation and H_2S production. Gas from carbohydrate metabolism can also be detected. Bacteria can metabolize carbohydrates aerobically (with oxygen) or fementatively (without oxygen). TSI differentiates bacteria based on their fermentation of lactose, glucose and sucrose and on the production of hydrogen sulfide. TSI is most frequently used in the identification of the *Enterobacteriaceae*, although it is useful for other gram-negative bacteria (Lehman, 2005).

Results interpretation after incubation: Three kinds of data may be obtained from the reactions.

- Sugar fermentations

  Alkaline slant over acid butt (red slant over yellow butt): glucose has been fermented but not sucrose or lactose.

  Acid slant over acid butt (yellow slant over yellow butt): lactose and/or sucrose has been fermented as well as the glucose.

  Alkaline slant over alkaline butt (red slant over red butt): neither glucose, lactose, nor sucrose has been fermented (Lehman, 2005).

- Gas production

  Indicated by bubbles in the butt. With large amounts of gas, the agar may be broken or pushed upward (Lehman, 2005).

- Hydrogen sulfide production

  Hydrogen sulfide production from thiosulfate is indicated by a blackening of the butt as a result of the reaction of H_2S with the ferrous ammonium sulfate to form black ferrous sulfide (Lehman, 2005).

3.4.3.1. Triple sugar iron agar (Merck, Germany) composition (g/liter)

Peptone from casein 10.0g; Peptone from meat 10.0g; Meat extract 3.0g; Yeast extract 3.0g; Sodium chloride 5.0g; Lactose 10.0g; Sucrose 10.0g; D(+)Glucose 1.0g; Ammonium iron(III) citrate 0.5g; Sodium thiosulfate 0.5g; Phenol red 0.024g; Agar-agar 12.0g.

3.4.3.2. Triple sugar iron agar (Merck, Germany) medium preparation

The medium was prepared according to manufacturer's instructions, 65 grams of a dehydrated medium was added to 1000 ml of distilled water in an Erlenmeyer flask and the pH was adjusted to 7.4 at room temperature, then the liquid was heated with frequent agitation and boiled for one minute to completely dissolve the medium by electric hot plate magnetic mixer.

After that, 7 ml of the above solution were dispensed into each culture glass tube and sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was allowed to solidify in a slanted position. Finally, after solidification of the medium, the uninoculated medium was reddish-orange (the original color) of the medium and then stored in a refrigerator for future use.

3.4.3.3. Performing of the TSI test

The stored TSI agar tubes were labeled according to the bacterial isolates numbers. A sterile straight inoculating wire was used to pick up a single isolated colony grown on the previously cultured TSA plates. Then the tube agar was inoculated by first stabbing the butt down to the bottom, on withdrawal the wire, the slant surface was inoculated. After inoculation, a loosely fitting closure of the tubes was done to permit access of air and incubated at 37°C for 18 to 24 hours (Lehman, 2005).

3.4.4. Lysine decarboxylase test (Taylor's modification)

Lysine Decarboxylase Broth is a diagnostic medium which distinguishes *Salmonellae* and some other *Enterobacteriaceae* by a distinct biochemical reaction to detect lysine decarboxylase production by the bacteria. During the initial stages of incubation, fermentation of glucose by the organism, with acid production results in a colour change to yellow. On further incubation, if lysine is decarboxylated to cadaverine, there will be an alkaline reaction. The colour then changes to purple (positive). If the colour remains yellow, the reaction is negative (Oxoid, UK).

3.4.4.1. Lysine decarboxylase broth (Oxoid, UK) tablets composition (gram/liter)

Yeast extract 3.0g; Glucose 1.0g; L-lysine 5.0g; Bromocresol purple 0.016g.

3.4.4.2. Lysine decarboxylase broth (Oxoid, UK) medium preparation

The medium was prepared according to the manufacturer's directions, 1 tablet was added to 5 ml of distilled water in a culture glass tube and shacked well to complete dissolving of the tablet. Then the solution pH 6.1±0.2 was checked by electronic pH meter at room temperature and sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was allowed to cool down and stored in a refrigerator until use, the prepared medium color was blue/ grey solution.

3.4.4.3. Performing of the Lysine decarboxylase test

Every sterile culture tube containing 5 ml of the medium broth were labeled according to the isolates numbers. A light inoculum from an isolated colony grown on previously cultured TSA plates, was transferred to the tube by using a sterile wooden stick and incubated at 35°C for 24 hours by using water bath shaker. After incubation, the purple color of the culture was indicated positive reaction, while yellow color was indicated negative reaction (Oxoid, UK).

3.4.5. Indole production

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of the IMViC procedures, a battery of tests designed to distinguish among members of the family *Enterobacteriaceae.* Tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria that express tryptophanase enzyme.

Tryptophan + water ⟶ indole + pyruvic acid + ammonia

The chief requirement for culturing an organism prior to performing the indole test is that the medium contains a sufficient quantity of tryptophan. The presence of indole when a microbe is grown in a medium rich in tryptophan demonstrates that an organism has the capacity to degrade tryptophan. Detection of indole, a by-product of tryptophan metabolism, relies upon the chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) under acidic conditions to produce the red dye (MacWilliams, 2009a).

3.4.5.1. Tryptone water (Merck, Germany) composition (g/liter)

Peptone from casein 10; Sodium chloride 5.

3.4.5.2. Tryptone water (Merck, Germany) medium preparation

The medium was prepared according to manufacturer's instructions, 15 grams of the dehydrated medium were added to 1000 ml of distilled water in a conical flask and dissolved the medium completely by using electric magnetic mixer, the pH was adjusted to 7.3 by using an electronic pH meter at room temperature. Then, 4 ml of the solution was dispensed into each culture glass tube and sterilized by autoclaving at 121 °C for 15 minutes. After autoclaving, the medium was allowed to cool down. The medium broth was clear with yellowish color and stored in a refrigerator until use.

3.4.5.3. Kovacs indole reagent (Merck, Germany)

Kovacs' reagent is composed of n-butanol; hydrochloric acid; 4-dimethylaminobenzaldehyde.

3.4.5.4. Performing of the indole test

The sterile culture tubes containing 4 ml of tryptone water were labeled according to the isolates numbers. A light inoculum from an isolated colony grown on previously cultured TSA plates, was transferred to the tube by using a sterile wooden stick and incubated at 35°C for 24 to 48 hours by using water bath shaker. After incubation, all cultured tubes were mixed by using Vortex mixer, then 5 drops of Kovács reagent were added directly to the tube. A positive indole test was indicated by the formation of a pink to red color ("cherry-red ring") in the layer on top of the medium within seconds of adding

the reagent. While for indole negative, the layer was remained yellow or slightly cloudy (MacWilliams, 2009a).

3.4.6. Methyl Red and Voges-Proskauer test (MR-VP)

Both the methyl red and Voges-Proskauer tests are commonly used in conjunction with the indole and citrate tests, to form a group of tests known as IMViC which aid in the differentiation of *Enterobacteriaceae*. Originally the paired MR-VP tests were used to distinguish between members of the family *Enterobacteriaceae*, but now they are used to characterize other groups of bacteria including *Actinobacteria* (McDevitt, 2009).

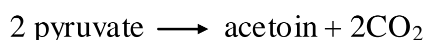
3.4.6.1. Methyl red test (MR)

E. coli and other members of the low-ratio organisms described by Clark and Lubs ferment sugars by the mixed acid pathway resulting in a low ratio of CO_2 to H_2 gas produced by fermentation. The mixed acid pathway gives 4mol of acidic products (mainly lactic and acetic acid), 1 mol of neutral fermentation product (ethanol), 1 mol of CO_2, and 1 mol of H_2 per mol of glucose fermented. The large quantity of acids produced causes a significant decrease in the pH of the culture medium. In contrast, *Enterobacter aerogenes* and other members of the high-ratio organisms (those that produce a high ratio of CO_2 to H_2 from the fermentation of glucose) ferment sugars via the butanediol fermentation pathway, producing only 1mol of acid per mol of glucose. This pathway results in a lower degree of acidification of the culture medium. The pH indicator methyl red (p-dimethylaminoaeobenzene-O-carboxylic acid) has been found to be suitable to measure the concentration of hydrogen ions between pH 4.4 (red) and 6.0 (yellow).

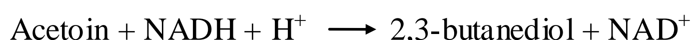
When the culture medium turns red after addition of methyl red, because of a pH at or below 4.4 from the fermentation of glucose, the culture has a positive result for the MR test. A negative MR test is indicated by a yellow color in the culture medium, which occurs when less acid is produced (pH is higher) from the fermentation of glucose (McDevitt, 2009).

3.4.6.2. Voges-proskauer test (VP)

Bacteria fermenting sugars via the butanediol pathway produce acetoin (i.e., acetyl methyl carbinolor 3-hydroxybutanone) as an intermediate which can be further reduced to 2,3-butanediol.



$$2\ \text{pyruvate} \longrightarrow \text{acetoin} + 2CO_2$$



$$\text{Acetoin} + \text{NADH} + H^+ \longrightarrow \text{2,3-butanediol} + NAD^+$$

In the presence of KOH, the intermediate acetoin is oxidized to diacetyl, a reaction which is catalyzed by α-naphthol. Diacetyl reacts with the guanidine group associated with molecules contributed by peptone in the medium, to form a pinkish-red-colored product. The α-naphthol in the Barritt's modification of the VP test serves as a color intensifier (McDevitt, 2009).

3.4.6.3. MR-VP broth (Merck, Germany) composition (g/liter)

Peptone from meat 7g; D(+)Glucose 5g; Phosphate buffer 5g.

3.4.6.4. MR-VP broth (Merck, Germany) medium preparation

The medium was prepared according to manufacturer's instructions, 17 grams of the dehydrated medium were added to 1000 ml of distilled water in a conical flask and dissolved the medium completely by using an electric magnetic mixer, the pH was adjusted to 6.9 by using electronic pH meter at room temperature. Then, 5 ml of the solution was dispensed into each culture glass tube and sterilized by autoclaving at 121 °C for 15 minutes. After autoclaving, the medium was allowed to cool down and stored in a refrigerator until use.

3.4.6.5. Methyl red solution

0.1 g of methyl red (Merck, Germany) were completely dissolved in 300 ml of ethanol (95%). Then 200 ml of deionized water were added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol. The prepared methyl red solution was stored at 4°C of refrigerator until use (McDevitt, 2009).

3.4.6.6. Voges-proskauer reagents

VP-1 (Barritt's reagent A): 5% (wt/vol) a-naphthol was prepared in absolute ethanol.

VP-2 (Barritt's reagent B): 40% (wt/vol) KOH was prepared in distilled water (McDevitt, 2009).

3.4.6.7. Performing of the MR-VP test

The sterile culture tubes containing 5 ml of MR-VP broth were labeled according to the isolates numbers. A light inoculum from an isolated colony grown on TSA plates, was transferred to the tube by using a sterile wooden stick and incubated at 35°C for 48 hours by using water bath shaker. After incubation, all the tubes were mixed by Vortex mixer (McDevitt, 2009).

3.4.6.7.1. MR test

2.5 ml of culture were transferred into a new sterile culture tube and labeled. 5 drops of the methyl red solution were added to each tube. The MR-positive showed a red coloration as a result of high acid production and a decrease in the pH of the culture medium to 4.4, while the MR-negative culture had a yellow color indicating a less acidic medium (McDevitt, 2009).

3.4.6.7.2. VP test

0.6 ml (or 12 drops) of VP-1 were added to the remaining 2.5 ml of culture grown in MR-VP broth. Then 0.2 ml (or 4 drops) were added to the same culture broth. The tubes were carefully shacked for 30 seconds to 1 minute to expose the medium to atmospheric oxygen (necessary for oxidation of acetoin to obtain a color reaction). Then the tubes were allowed to stand for at least 30 minutes. After that, VP-positive showed red coloration on top of the culture tube, whereas VP-negative had a yellowish color of the entire cultured medium (McDevitt, 2009).

3.4.7. Citrate utilization test

The citrate test is commonly employed as part of a group of tests, the IMViC tests, that distinguish between members of the *Enterobacteriaceae* family based on their metabolic by-products. In the most common formulation, citrate is the sole source of carbon in the Simmon's citrate medium while inorganic ammonium salt ($NH_4H_2PO_4$) is the sole fixed nitrogen source. When an organic acid such as citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates ultimately are produced. The visible presence of growth on the medium and the change in pH indicator color due to the increased pH are the signs that an organism can import citrate and use it as a sole carbon and energy source; such organisms are considered to be citrate positive (Prussian blue color) (MacWilliams, 2009b).

3.4.7.1. Simmon's citrate agar (Merck, Germany) composition (g/liter)

Ammonium dihydrogen phosphate 1.0g; di-potassium hydrogen phosphate 1.0g; Sodium chloride 5.0g; Sodium citrate 2.0g; Magnesium sulfate 0.2g; Bromothymol blue 0.08g; Agar-agar 13.0g.

3.4.7.2. Simmon's citrate agar (Merck, Germany) medium preparation

The medium was prepared according to manufacturer's instructions, 22.5 grams of dehydrated medium was added to 1000 ml of distilled water in an Erlenmeyer flask and the pH was adjusted to 6.6, then the liquid was heated with frequent agitation and boiled for one minute to completely dissolve the medium by electric hot plate magnetic mixer.

After that, 7 ml of the above solution were dispensed into each culture glass tube and sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was allowed to solidify in a slanted position. Finally, after solidification of the medium, the medium had a deep forest green color and then stored in a refrigerator until use.

3.4.7.3. Performing of the Simmon's citrate test

The stored Simmon's citrate agar tubes were allowed to warm up to room temperature prior to inoculation and were labeled according to the bacterial isolates numbers. A sterile straight inoculating wire was used to pick up a single isolated colony grown on the previously cultured TSA plates. Then the medium agar was inoculated by first stabbing the butt down to the bottom, on withdrawal the wire, the slant surface was inoculated. A loosely fitting closure of the tubes was done to permit access of air and incubated at 35°C for 18 to 48 hours.

After incubation, citrate positive: the medium was an intense Prussian blue. While citrate negative: no color change occurred; the medium remained as same as the deep forest green color of the uninoculated agar (MacWilliams, 2009b).

3.5. Verification of Biochemical Identification of the Bacterial Isolates by Using CHROMagar ECC

All the isolates were further investigated to verify the biochemical identification as a final step of isolates identification methods, so one of the commercial chromogenic media which is CHROMagar ECC (France) was selected to confirm the identification and differentiation between *E. coli* isolates and other isolates at the same time. CHROMagar is a new generation of selective microbiological testing media (chromogenic) permitting in a single step, the isolation and differentiation of micro-organisms by colony colors. They are powerful and convenient, as they allow a nice visualization of colored colonies even when the colonies are very close to each other which happen often from real samples containing a commensal microbial flora. The chromogenic media are very useful to pick colonies by color, thus providing an easy, rapid, sensitive and specific methodology for direct differentiation of species.

Furthermore, CHROMagar ECC is a chromogenic medium which can be used for the detection and enumeration of β-glucuronidase positive *E. coli* in processed food, raw materials, water, milk and environment samples. It can play a role as a selective culture medium showing directly *E. coli* colonies in blue color, thus making the detection and the enumeration of this important bacterium as simple as possible. In addition, it detects the other coliforms (other *Enterobacteriaceae* members) as mauve colonies, as well as non-*Enterobacteriaceae* bacteria as inhibited or colorless colonies (CHROMagar, 2013).

3.5.1. CHROMagar ECC (France) composition (g/liter)

Agar 15.0g; Peptone and yeast extract 8.0g; Sodium chloride (N_aCl) 5.0g; Chromogenic mix 4.8g.

3.5.2. CHROMagar ECC medium preparation

The medium was prepared according to manufacturer's instructions, 32.8 grams of dehydrated medium powder were added to 1000 ml of distilled water in an Erlenmeyer flask and the medium pH 7.2±0.2 was checked by electronic pH meter at room temperature, then the liquid was heated until boil (100^oC) with swirling in order to complete fusion of the agar grains has taken place (large bubbles replacing foam) by electric hot plate magnetic mixer.

Next, the solution was allowed to cool down in a water bath to 48°C. Then, the medium was poured into the sterile Petri dishes and allowed to solidify. After solidification, the agar plates were enclosed by parafilm and stored in a refrigerator in the dark and protected from light by enclosing them with aluminum foil.

3.5.3. Inoculation of CHROMagar ECC agar plates

The medium was inoculated according to the manufacturer's instructions, enough numbers of the previously prepared medium plates were labeled according to the bacterial isolates and allowed to warm to room temperature before inoculation. A sterile wooden stick was used to pick up a single isolated colony grown on the previously cultured TSA plates and inoculated on CHROMagar plate by using Quadrant streak method for obtaining isolated bacterial colonies. Then the plates were incubated at 37°C for 24 hours.

3.5.4. Interpretation of the results on CHROMagar ECC plates

After incubation, 3 kinds of bacterial colonies can appear according to the manufacturer's directions: Blue color colonies indicate *E. coli*; mauve color indicates other coliforms (other *Enterobacteriaceae* members); and inhibited or colorless colonies indicate other types of Gram-negative bacteria different than coliforms including non-*Enterobacteriaceae* members.

3.6. Antibiotic Susceptibility Testing

Susceptibilities of the isolates to different antimicrobial agents were measured *in vitro* by employing Kirby-Bauer Disk Diffusion Susceptibility Test method (Hudzicki, 2009). This method allows for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc. Furthermore, commercially available antimicrobial discs (Oxoid, U.K) were used for the test, 13 antibiotics were tested against all the *Enterobacteriaceae* isolates (Table 3.2).

Table 3.2. Antimicrobial agents and their disc contents that used for this study

Antibiotic susceptibility disks		Abbreviation	Disk content (μg)
Antibiotic group	Antibiotic agent		
β-Lactam/ β-Lactamase inhibitor combinations	Amoxicillin-Clavulanic acid	AMC	30 (20/10)
	Piperacillin-tazobactam	TZP	110 (100/10)
β-lactams: Cephalosporins	Ceftazidime	CAZ	30
	Cefotaxime	CTX	30
	Cefpodoxime	CPD	10
	Ceftriaxone	CRO	30
β-lactams: Carbapenems	Ertapenem	ETP	10
	Imipenem	IPM	10
	Meropenem	MEM	10
β-lactams: Monobactams	Aztreonam	ATM	30
Flouroquinolones	Ciprofloxacin	CIP	5
Aminoglycosides	Amikacin	AK	30
Folate Pathway Inhibitors	Trimethoprim-Sulphamethoxazole	SXT	25 (1.25/23.75)

3.6.1. Antibiotic susceptibility testing medium

The Mueller Hinton agar (Merck, Germany) was used as the antibiotic susceptibility testing medium and it's ingredients (g/liter) are: Infusion from meat 2.0g; Casein hydrolysate 17.5g; Starch 1.5g; Agar-agar 13.0g.

The medium was prepared according to the manufacturer's directions, 34 grams of the dehydrated medium were added to 1000 ml of distilled water in an Erlenmeyer flask and the medium pH 7.4±0.2 was checked by electronic pH meter at room temperature, then the liquid was heated with frequent agitation and boiled for 1 minute to completely dissolve the components by using an electric hot plate magnetic mixer. The solution was sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was poured into sterile Petri dishes and allowed to solidify at room temperature. Then the agar plates were enclosed by parafilm and stored in refrigerator until use.

3.6.2. Inoculum preparation

Adequate numbers of sterile glass tubes were labeled according to the isolates which were sub-cultured on TSA medium plates. 4 or 5 morphologically similar isolated colonies from each TSA plate were transferred into the glass tube containing 5 ml of sterile TSB medium by using a sterile wooden stick. Then the culture tubes were incubated at 35°C of water bath shaker until the visible growth turbidity was equal to the 0.5 McFarland standard (Hudzicki, 2009).

3.6.3. Performing of the antibiotic susceptibility testing

The procedure for antibiotic susceptibility testing was carried out according to Kirby-Bauer Disk Diffusion Susceptibility Test method (Hudzicki, 2009). Sufficient number of previously prepared Mueller Hinton agar plates were labeled according to the isolates numbers and allowed them to warm to room temperature in laminar flow hood. A sterile cotton swab was dipped into the inoculum tube fluid and the swab rotated against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid. Then the agar plate was inoculated by streaking the swab three times over the entire agar surface and the plate was allowed to sit at room temperature by leaving the plate lid slightly ajar at least 3 to 5 minutes, but no more than 15 minutes, for the surface of the agar plate to dry before proceeding to the next step. Afterward, antibiotic discs were placed aseptically on the surface of the inoculated plates at an appropriate arrangement of center to center distance with the help of a sterile forceps, 6 disks placed in one plate and other 7 disks placed in another plate. The plates were then inverted and incubated at 35°C for 16 to 18 hours.

After incubation, the plates were examined and the diameters of the zone of inhibition were measured in mm by using a ruler. Then the zone diameters for individual antimicrobial agents were translated into susceptible, intermediate and resistant categories by referring to a zone diameter interpretation standard table which obtained from Clinical and Laboratory Standards Institute (CLSI, 2013) as mentioned in Table 3.3. In addition, *E. coli* ATCC 25922 was used as the reference strain in antibiotics susceptibility testing.

Table 3.3. Zone diameter interpretive criteria used for the tested antibiotics. Adapted in part from CLSI, Table 2A in M100-S23 document (CLSI, 2013)

Antibiotic agent	Zone diameter breakpoints (mm)		
	Susceptible	Intermediate	Resistant
Amoxicillin-clavulanic acid	≥18	14-17	≤13
Piperacillin-tazobactam	≥21	18-20	≤17
Ceftazidime	≥21	18-20	≤17
Cefotaxime	≥26	23-25	≤22
Cefpodoxime	≥21	18-20	≤17
Ceftriaxone	≥23	20-22	≤19
Ertapenem	≥22	19-21	≤18
Imipenem	≥23	20-22	≤19
Meropenem	≥23	20-22	≤19
Aztreonam	≥21	18-20	≤17
Ciprofloxacin	≥21	16-20	≤15
Amikacin	≥17	15-16	≤14
Trimethoprim-Sulphamethoxazole	≥16	11-15	≤10

3.6.4. ESBL production detection

Double Disk Synergy (DDS) test was used for the phenotypic detection of ESBL producing *Enterobacteriaceae* isolates, this was done at the same time when antibiotic susceptibility testing of the isolates performed and in the same Muller-Hinton agar plate. The DDS test was carried out by swabbing the organism onto a Muller-Hinton agar plate then a disk containing amoxicillin-clavulanate (20µg/10µg) was placed in the center of the plate and the disks containing 30 µg of ceftazidime, ceftriaxone, cefotaxime, and aztreonam and the disc containing 10 µg of cefpodoxime were placed at a distance of 25 to 30 mm (center to center) far from amoxicillin-clavulanate disk in the same plate in order to observe the synergistic effect.

A clear extension or protrusion (synergistic effect) of the edge of the inhibition zone of any of the antibiotics toward the disk containing clavulanic acid was interpreted as positive for ESBL production. Cefpodoxime was used as the oxyimino-cephalosporin, as it gives test sensitivity up to 97% and test specificity up to 100% (Drieux *et al.*, 2008).

4. RESULTS AND DISCUSSION

4.1. Isolation of Single Bacterial Colonies

4.1.1. Culture of samples on EMB agar

The collected samples (intestinal contents) were numbered and inoculated onto EMB agar plates soon upon arrival in a laboratory as described in materials and methods chapter of the present study. After overnight incubation, out of 40 samples, 34 were grown on EMB agar plates with different colony colors, such as: greenish-black colonies with metallic sheen and brown dark-centered mucoid colonies, indicating lactose fermentation and acid production. While the samples were shown no growth on the EMB agar plates, are: 1 of cattle sample (S12) and 5 sheep samples (K2, K3, K9, K10 and K13), these were not investigated anymore.

4.1.2. Culture of isolates in TSB medium

The single isolated colonies on EMB agar were inoculated in TSB medium, after overnight incubation, some of them were not grown in TSB medium, so they were not recorded and were discarded. As a result, a total of 54 isolates were grown and recorded, these included 31 isolates (57.4%) from cattle samples and 23 isolates (42.59%) from sheep samples, and then they were processed for making stock culture. All the isolates are shown in Table 4.1.

Table 4.1. All bacterial isolates from cattle and sheep samples

Cattle sample no.	Isolate no.
S1	S11 S12
S2	S22 S23
S3	S32
S4	S41 S42
S5	S52
S6	S61 S62
S7	S71
S8	S81 S82
S9	S91 S92 S93
S10	S101 S102 S103
S11	S111 S112
S13	S132
S14	S141
S15	S151 S152
S16	S161
S17	S171 S172
S18	S181
S19	S191
S20	S201
Total no. 19	Total no. 31

Sheep sample no.	Isolate no.
K1	K1
K4	K4
K5	K51 K52
K6	K61
K7	K71
K8	K81 K82
K11	K111 K112
K12	K122
K14	K141 K142
K15	K151
K16	K161 K162 K163
K17	K171 K172
K18	K181
K19	K191
K20	K201 K202
Total no. 15	Total no. 23

4.2. Biochemical Characteristics of the Isolates

Biochemical characterization of all the isolates were performed by first subculture on TSA agar plates to get pure single isolated colonies and then processing the isolates into series of biochemical tests for determination of their biochemical characteristics as described in materials and methods chapter.

4.2.1. Isolates reactions on oxidase test strips

All the isolates were showed yellowish to orange colors on test strips after 20 to 30 seconds (Figure 4.1); these indicated oxidase negative (Table 4.2) according to the manufacturer's instructions for interpretation the results (Merck, Germany).

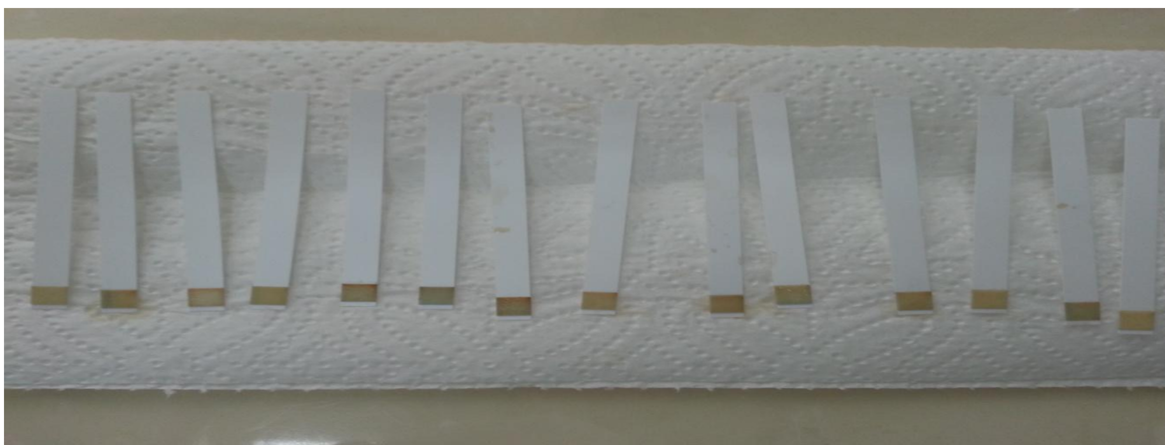


Figure 4.1. Reaction of isolates on oxidase test strips

4.2.2. Isolates reactions in triple sugar iron (TSI) agar

Various types of results were observed from the isolates reactions in TSI agar tubes, they are:

Sugar fermentations

Out of 54 isolates, 50 were shown yellow slant over yellow butt (acid slant over acid butt) in TSI agar tubes, these included 29 isolates of cattle samples and 21 isolates of sheep samples, this kind of reaction indicates lactose and/or sucrose has been fermented as well as glucose by the isolates (Figure 4.2; Table 4.2). While a total of 4 isolates were shown red slant over red butt (alkaline slant over alkaline butt), these included 2 isolates of cattle samples (S62 and S91) and 2 isolates of sheep samples (K71 and K161), this kind of

reaction indicates neither glucose, lactose, nor sucrose has been fermented by the isolates (Figure 4.2; Table 4.2).

Gas production

A total of 50 isolates were produced gas with reactions in TSI agar (acid slant over acid butt) these included 29 isolates of cattle samples and 21 isolates of sheep samples (Figure 4.2; Table 4.2), while 4 isolates (2 of cattle samples and 2 of sheep samples) were not produced gas with reactions in TSI agar (alkaline slant over alkaline butt) (Figure 4.2; Table 4.2).

Hydrogen sulfide production

No isolate was shown this kind of reaction.

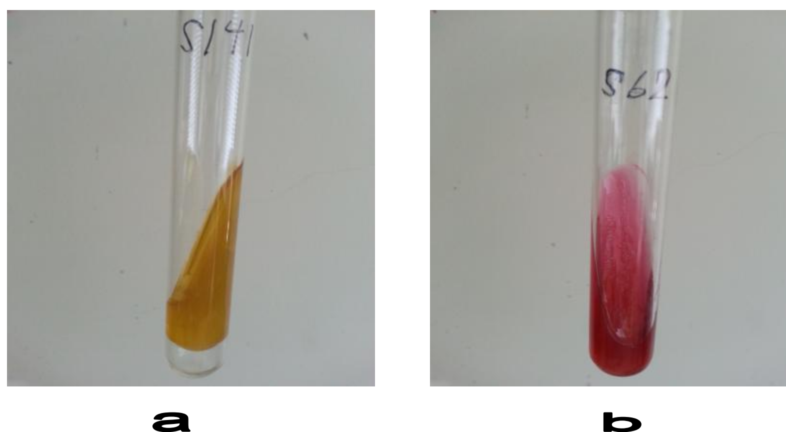


Figure 4.2. Reactions in TSI agar. a) acid slant over acid butt with gas production. b) alkaline slant over alkaline butt without gas production

4.2.3. Isolates reactions in lysine decarboxylase broth

All of the isolates were shown purple color, this indicated that all the isolates were produced positive reactions in the lysine decarboxylase broth (Figure 4.3; Table 4.2).

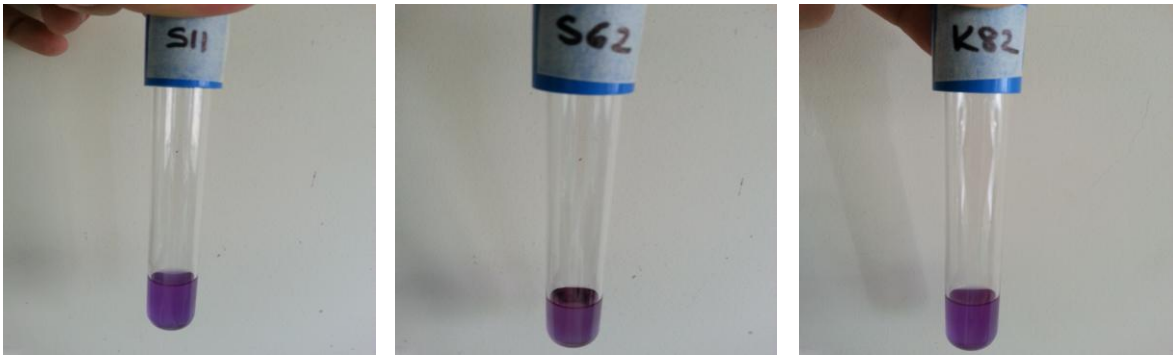


Figure 4.3. Reactions in lysine decarboxylase broth, all of them produced positive reactions

4.2.4. Isolates reactions in IMViC tests

4.2.4.1. Indole production

Out of 54 isolates, 49 were shown cherry-red ring on top of the medium, this was revealed the indole positive, including 28 isolates of cattle samples and 21 isolates of sheep samples (Figure 4.4; Table 4.2). Whereas only 5 isolates were shown slightly cloudy medium with darker ring on the top, this was indicated indole negative, including 3 isolates of cattle samples (S62, S71 and S91) and 2 isolates of sheep samples (K71 and K161), (Figure 4.4, Table 4.2).

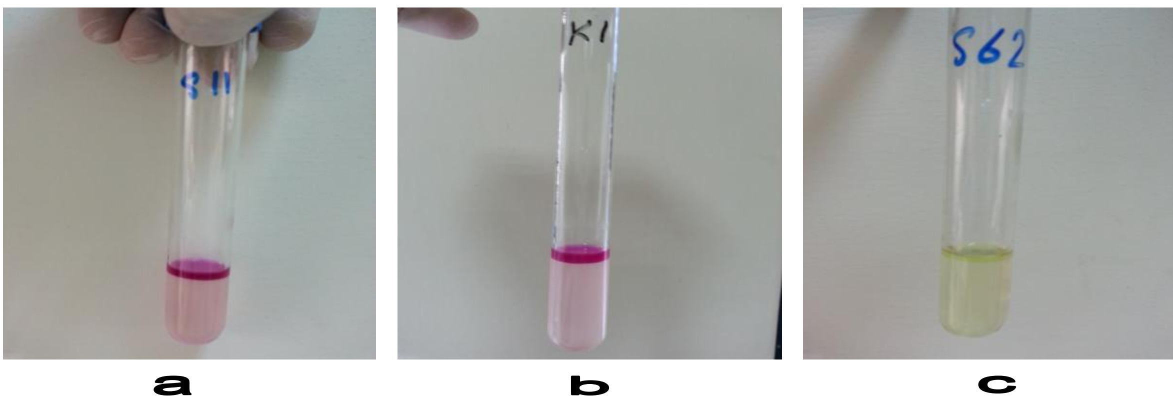


Figure 4.4. Reactions in Indole test. a, b) Indole positive. c) Indole negative

4.2.4.2. MR test

Out of 54 isolates, 49 were shown red color of the cultured medium, this was indicated MR positive, including 28 isolates of cattle samples and 21 isolates of sheep samples (Figure 4.5; Table 4.2). Whereas only 5 isolates were shown yellow color of the medium, this was indicated MR negative, including 3 isolates of cattle samples (S62, S71 and S91) and 2 isolates of sheep samples (K71 and K161) (Figure 4.5; Table 4.2).

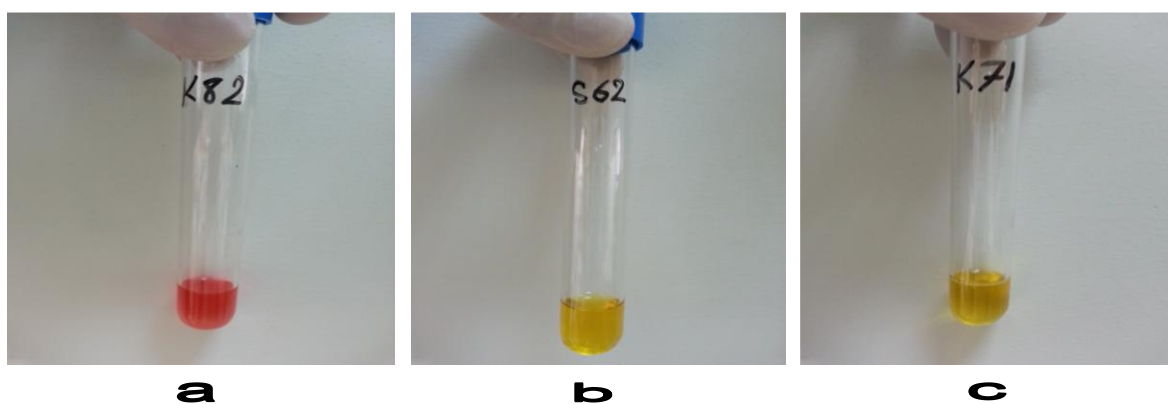


Figure 4.5. Reactions in MR test. a) MR positive. b, c) MR negative

4.2.4.3. VP test

Only 1 isolate of cattle sample among all of the isolates, was shown red coloration on top of the culture tube indicating VP positive which is S71 (Figure 4.6; Table 4.2). While other 53 isolates were shown cloudy yellow color of the entire culture medium indicating VP negative (Figure 4.6; Table 4.2).

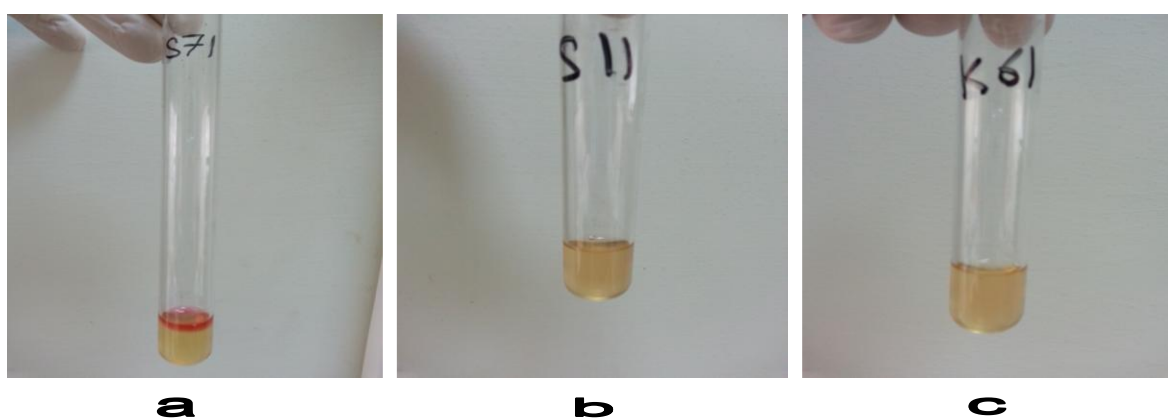


Figure 4.6. Reactions in VP test. a) VP positive. b, c) VP negative

4.2.4.4. Simmon’s citrate test

5 isolates among all of the isolates were shown intense prussian blue color indicating positive reaction (Figure 4.7; Table 4.2), these included 3 isolates of cattle samples (S62, S71 and S91) and 2 isolates of sheep samples (K71 and K161). While other 49 isolates were shown deep forest green color indicating negative reaction (Figure 4.7; Table 4.2).

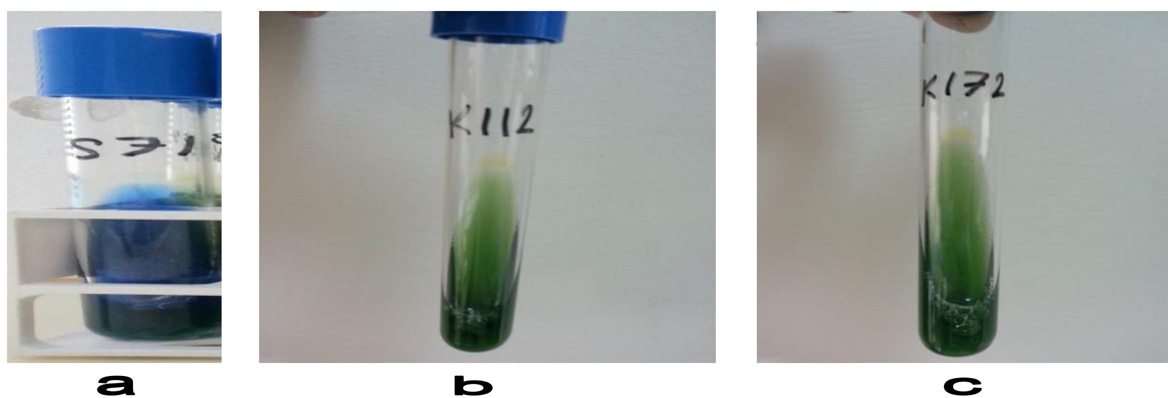


Figure 4.7. Reactions in simmon’s citrate test. a) citrate positive. b, c) citrate negative

Table 4.2 Biochemical tests results of all the isolates*

Isolate no.	TSI				Lysine decarboxylase	Indole	MR	VP	Simmon's citrate
	Slant	Butt	Gas	H_2S					
S11	Yellow	Yellow	+	−	+	+	+	−	−
S12	Yellow	Yellow	+	−	+	+	+	−	−
S22	Yellow	Yellow	+	−	+	+	+	−	−
S23	Yellow	Yellow	+	−	+	+	+	−	−
S32	Yellow	Yellow	+	−	+	+	+	−	−
S41	Yellow	Yellow	+	−	+	+	+	−	−
S42	Yellow	Yellow	+	−	+	+	+	−	−
S52	Yellow	Yellow	+	−	+	+	+	−	−
S61	Yellow	Yellow	+	−	+	+	+	−	−
S62	Red	Red	−	−	+	−	−	−	−
S71	Yellow	Yellow	+	−	+	−	−	+	+
S81	Yellow	Yellow	+	−	+	+	+	−	−
S82	Yellow	Yellow	+	−	+	+	+	−	−
S91	Red	Red	−	−	+	−	−	−	+
S92	Yellow	Yellow	+	−	+	+	+	−	−
S93	Yellow	Yellow	+	−	+	+	+	−	−
S101	Yellow	Yellow	+	−	+	+	+	−	−
S102	Yellow	Yellow	+	−	+	+	+	−	−
S103	Yellow	Yellow	+	−	+	+	+	−	−
S111	Yellow	Yellow	+	−	+	+	+	−	−
S112	Yellow	Yellow	+	−	+	+	+	−	−
S132	Yellow	Yellow	+	−	+	+	+	−	−
S141	Yellow	Yellow	+	−	+	+	+	−	−
S151	Yellow	Yellow	+	−	+	+	+	−	−
S152	Yellow	Yellow	+	−	+	+	+	−	−
S161	Yellow	Yellow	+	−	+	+	+	−	−
S171	Yellow	Yellow	+	−	+	+	+	−	−
S172	Yellow	Yellow	+	−	+	+	+	−	−

* Yellow, acid; Red, alkaline. +, positive reaction. −, negative reaction.

Table 4.2. (Continued) Biochemical tests results of all the isolates*

Isolate no.	TSI				Lysine decarboxylase	Indole	MR	VP	Simmon's citrate
	Slant	Butt	Gas	H_2S					
S181	Yellow	Yellow	+	−	+	+	+	−	−
S191	Yellow	Yellow	+	−	+	+	+	−	−
S201	Yellow	Yellow	+	−	+	+	+	−	−
K1	Yellow	Yellow	+	−	+	+	+	−	−
K4	Yellow	Yellow	+	−	+	+	+	−	−
K51	Yellow	Yellow	+	−	+	+	+	−	−
K52	Yellow	Yellow	+	−	+	+	+	−	−
K61	Yellow	Yellow	+	−	+	+	+	−	−
K71	Red	Red	−	−	+	−	−	−	+
K81	Yellow	Yellow	+	−	+	+	+	−	−
K82	Yellow	Yellow	+	−	+	+	+	−	−
K111	Yellow	Yellow	+	−	+	+	+	−	−
K112	Yellow	Yellow	+	−	+	+	+	−	−
K122	Yellow	Yellow	+	−	+	+	+	−	−
K141	Yellow	Yellow	+	−	+	+	+	−	−
K142	Yellow	Yellow	+	−	+	+	+	−	−
K151	Yellow	Yellow	+	−	+	+	+	−	−
K161	Red	Red	−	−	+	−	−	−	+
K162	Yellow	Yellow	+	−	+	+	+	−	−
K163	Yellow	Yellow	+	−	+	+	+	−	−
K171	Yellow	Yellow	+	−	+	+	+	−	−
K172	Yellow	Yellow	+	−	+	+	+	−	−
K181	Yellow	Yellow	+	−	+	+	+	−	−
K191	Yellow	Yellow	+	−	+	+	+	−	−
K201	Yellow	Yellow	+	−	+	+	+	−	−
K202	Yellow	Yellow	+	−	+	+	+	−	−

*Yellow, acid; Red, alkaline. +, positive reaction. −, negative reaction.

4.3. Identification of the Isolates

All the isolates were identified based on their biochemical characteristics which described previously and according to the Veterinary Microbiology and Microbial Disease book (Quinn *et al.*, 2011) as mentioned in Table 3.1 of the materials and methods chapter.

4.3.1. Identification of *E. coli*

When considering all the isolates (n=54) from cattle and sheep samples, (n=49; 90.74%) of total isolates were identified as *E. coli*, these included (n=28; 51.85%) and (n=21; 38.88%) of total isolates from cattle and sheep samples, respectively (Figure 4.8). All the isolates which are mentioned in Table 4.2, were identified as *E. coli*, except 5 isolates which are S62, S71, S91, K71, K161, they were not *E.coli*. Whereas, when considering all the *E. coli* isolates (n=49), these included (n=28; 57.14%) and (n=21; 42.85%) of total *E. coli* isolates from cattle and sheep samples, respectively.

All *E. coli* isolates were shown oxidase negative and they were shown acid slant over acid butt with gas production and H_2S negative in TSI agar. Also they produced positive reaction in lysine decarboxylase broth and they were IMViC +/ +/−/− as illustrated in Table 4.3.

Table 4.3. Biochemical characteristics of *E. coli* isolates*

Oxidase test	Reactions in TSI agar				Lysine decarboxylase	IMViC tests			
	Slant	Butt	H_2S	Gas production		Indole	MR	VP	Citrate
−	Yellow	Yellow	−	+	+	+	+	−	−

*Yellow, acid; +, positive reaction. −, negative reaction.

In the present study, one of the biochemical characters of the *E. coli* isolates is oxidase negative. It means that these isolates contain no cytochrome oxidase enzyme which catalyzes the oxidation of cytochrome *c* while reducing oxygen to form water (Shields and Cathcart, 2010). While *E. coli* isolates reactions in the TSI agar revealed that glucose, lactose and/or sucrose have been metabolized. During the incubation period, bacteria quickly metabolized the glucose, resulted in producing an acid slant and an acid butt in a few hours. The Emben-Meyerhof-Parnas pathway was used both aerobically (on the slant) and anaerobically (in the butt) to produce ATP and pyruvate. On the slant, the pyruvate was further metabolized to CO_2, H_2O, and energy. After further incubation (18

hours) the glucose was consumed, and then the bacteria utilized lactose and/or sucrose, maintaining an acid slant. The results are recorded as acid over acid (yellow/yellow). In the anaerobic butt, the bacteria convert pyruvate into stable acid end products, thus the butt remains acidic (yellow) (Lehman, 2005). *E. coli* is a facultative anaerobe that ferments a wide range of sugars, including lactose, producing acid and gas (Feng, 2001). During sugar fermentation, the production of gas is a tool to differentiate between *Escherichia coli* and pathogens like *Shigella* spp. and *Salmonella* spp., which do not produce gas (Sawers, 2005).

Furthermore, the positive reaction in lysine decarboxylase broth by all *E. coli* isolates indicated that glucose was fermented and acid produced by the bacteria during the initial stages of incubation, this was resulted in change of normal color of the medium to yellow. On further incubation the lysine was decarboxylated to cadaverine and due to an alkaline reaction, the color then changed to purple (positive reaction) (Oxoid, UK). However, the production of indole by all *E. coli* isolates illustrated that these isolates had the capacity to deamination and hydrolysis the amino acid tryptophan by tryptophanase enzyme under acidic conditions to produce the red dye of the ring on top of the medium (positive reaction) (MacWilliams, 2009).

Moreover, the positive results of methyl red test of all *E. coli* isolates demonstrated that these isolates fermented sugars by mixed acid pathway resulting in producing a low ratio of CO_2 to H_2 gas. This pathway were given large quantity of acids produced and caused the pH of the medium at or below 4.4 from the fermentation of glucose, then the culture medium turned red as positive reaction with help of pH indicator methyl red (Bettelheim, 1994; McDevitt, 2009). On the other hand, the negative results of VP test of all *E. coli* isolates indicated that the isolates were not fermented sugars via the butanediol pathway and acetoin was not produced, thus the medium was cloudy yellow (negative reaction) (McDevitt, 2009).

Additionally, the citrate negative results of all *E. coli* isolates revealed that they were unable to utilize citrate in simmon’s citrate medium as their carbon and energy source and the alkaline by-products of citrate metabolism were not produced, thus the pH of the medium was not increased and its color was remained deep forest green at neutral pH (MacWilliams, 2009).

In the current study, all the tested samples have been taken from contents of the colon segment of the large intestine of cattle and sheep digestive tracts because there is no

report about the prevalence of *E. coli* in colon contents of healthy food producing animals at slaughter in Turkey. On the other hand, the studies on the prevalence of *E. coli* in rectum contents of slaughtered animals have been documented more frequently around the world. Furthermore, the study in England by Laven *et al.* (2003) they reported that the population density of *E. coli* in the colon significantly exceeds that in the rumen but there was no significant difference between the population density of *E. coli* in colon and faeces (or caudal rectal contents) of slaughtered healthy cattle. Despite the fact that the rumen volume of cattle is around 10 times larger than that of the colon (Van Soest, 1994).

There are several reports about the prevalence of *E. coli* in large intestine of healthy food producing animals at slaughter worldwide. For example: a study in Portugal by Ramos *et al.* (2013) detected 192 *E. coli* isolates from rectal samples (fecal) of healthy food animals at slaughter, these included pigs (n = 66; 34.37%), sheep (n = 73; 38.02%) and cattle (n = 53; 27.60%) of total *E. coli* isolates from that animals, in part of their study. Whereas in the present study, *E. coli* isolates from cattle were (n=28; 57.14%) and from sheep were (n=21; 42.85%) of total *E. coli* isolates. These data reveal that occurrence of *E. coli* in cattle and sheep in our findings was higher than the findings of Ramos *et al.* (2013). As well, the study in Jordan by Tarawneh *et al.* (2009) they found 207 *E. coli* isolates from 233 fecal samples taken from colon contents of healthy sheep at slaughter, in part of their study. These *E. coli* isolates were identified by phenotypic characterization and conventional biochemical activities using an IMViC test (a positive identification of *E. coli* was positive for the indole and methyl red tests, negative for the Voges-Proskauer and citrate utilization tests). While, in the present study, 21 *E. coli* isolates were recovered from 20 fecal samples from colon contents of slaughtered sheep. These data indicate that our findings were higher than the findings by Tarawneh *et al.* (2009). However, the *E. coli* isolates of sheep samples in the current study were shown same biochemical characters with IMViC tests of the sheep *E. coli* isolates in their study.

E. coli is one of the bacteria that exist in the normal micro flora of the intestinal tract of humans and warm blooded animals. Most strains of *E. coli* are nonpathogenic (Stender *et al.*, 2001). However some strains differ from commensals in that they express virulence factors molecules directly involved in pathogenesis thereby causing disease (Schroeder *et al.*, 2004). Shiga-toxin producing *E. coli* strains (STEC) causing human infections belong to a large number of O, H serotypes are also named as verotoxin producing *E. coli* (VTEC). STEC strain of *E. coli* O157:H7 is a food borne pathogen being

the most common enterohaemorrhagic (EHEC) serotype and has been associated with a variety of diseases in humans including diarrhoea, haemorrhagic colitis and the haemolytic uraemic syndrome (Griffin and Tauxe, 1991; Tarr *et al.*, 1999). In the present study, *E. coli* isolates were not tested for determination of serotypes and virulence genes, so further studies might focus on pathogenic properties of the *E. coli* isolates of the current study.

Besides, cattle have been regarded as a natural reservoir of VTEC organisms for infections (Mead and Griffin, 1998; Meng and Doyle, 1998). The organism is carried in the gastrointestinal tract and is shed in the feces (Bach, 2004). *E. coli* O157 was detected more frequently in the colon than in the rumen of slaughtered healthy cattle (Laven *et al.*, 2003). *E. coli* O157:H7 can be transmitted to carcass surfaces when gut contents or fecal materials get in contact with meat surfaces during the processes of skinning and evisceration (Dickson and Anderson, 1992). In Turkey, few reports have been addressed regarding prevalence of *E. coli* O157:H7 in fecal samples of healthy live cattle at farms and fecal samples and carcass swabs of healthy slaughtered cattle at abattoirs (Inat and Siriken, 2010; Kuyucuoglu *et al.*, 2011). Most human infections with *E. coli* O157:H7 have been primarily associated with the consumption of contaminated and improperly cooked ground beef (Armstrong *et al.*, 1996). In Turkey, some studies have been published regarding *E. coli* O157:H7 in minced beef, soudjouk, hamburger, cooked meat, raw meat samples and beef doner kebabs (Akkus, 1996; Gun *et al.*, 2000; Ulukanli *et al.*, 2010). So, the addressed remarks recommend adequate slaughter procedures and meat inspection must be carried out in slaughterhouses to ensure not contamination of the carcasses by pathogenic *E. coli* strains. Also, continuous monitoring and surveillance are valuable for risk management of carcass contamination at abattoirs in Turkey.

4.3.2. Identification of other *Enterobacteriaceae* members

Out of 54 isolates, only 1 isolate from cattle samples which is S71 was identified as other *Enterobacteriaceae* member (non-*E. coli*) based on its biochemical characters and according to the Veterinary Microbiology and Microbial Disease book (Quinn *et al.*, 2011). It's percentage among total numbers of isolates was 1.85% (Figure 4.8). Biochemical characters of isolate S71 are mentioned in Table 4.4.

Table 4.4. Biochemical characteristics of other *Enterobacteriaceae* member (isolate S71)*

Oxidase test	Reactions in TSI agar				Lysine decarboxylase	IMViC tests			
	Slant	Butt	H_2S	Gas production		Indole	MR	VP	Citrate
−	Yellow	Yellow	−	+	+	−	−	+	+

*Yellow, acid; +, positive reaction. −, negative reaction.

The isolate S71 was not fully identified because the biochemical tests used for identification of this isolate, are same to those biochemical tests which were selected for identification of *E. coli* isolates as mentioned in Table 3.1 of materials and methods chapter. According to Quinn et al. (2011) and based of the biochemical characters mentioned in Table 4.4, the isolate S71 was not *E. coli* and was identified as other member of *Enterobacteriaceae* (*Enterobacter aerogenes* or *Klebsiella pneumoniae*) and further biochemical tests need to be used for differentiation between both of these bacteria. In addition, tests for motility allow differentiation of *Klebsiella* species (non-motile) from *Enterobacter* species (motile), as both species produce similar mucoid colonies which are difficult to distinguish visually (Quinn et al., 2011).

The biochemical characters of isolate S71 were similar to those of *E. coli* isolates except of IMViC tests. The indole negative result of isolate S71 indicated that this isolate was unable to degrade the amino acid tryptophan because it was not expressed tryptophanase enzyme, so less acidity of the medium shown by slightly cloudy of the fluid (negative reaction) (MacWilliams, 2009).

Also, a negative result of methyl red test of isolate S71 demonstrated that the isolate fermented sugars via butanediol fermentation pathway resulting in producing a high ratio of CO_2 to H_2 gas. This pathway were produced less quantity of acids and caused the pH of the medium at 6 or higher, thus the culture medium turned yellow as negative reaction with help of pH indicator methyl red (McDevitt, 2009). While the VP positive result of isolate S71 revealed that the isolate was able to ferment sugars via the butanediol pathway to produce acetoin. In the presence of KOH in VP-2 reagent the acetoin was oxidized to diacetyl, a reaction which is catalyzed by a-naphthol in VP-1 reagent. Diacetyl reacted with the guanidine group associated with molecules contributed by peptone in the medium, to form a pinkish-red-colored product (McDevitt, 2009).

Furthermore, the positive result of simmon's citrate test of isolate S71 illustrated that the isolate was able to utilize citrate in simmons citrate medium as sole carbon and energy source, then alkaline carbonates and bicarbonates ultimately were produced. Thus the pH of the medium was increased and its color was Prussian blue (positive reaction) (MacWilliams, 2009). Additionally, the isolate S71 was produced gas in TSI agar, this result is compatible with the description by Lehman (2005) who has mentioned bacteria that commonly producing an acid over acid reaction with gas (CO_2 and O_2) include: *Enterobacter aerogenes, E. cloacae, Escherichia coli, Klebsiella oxytoca,* and *Klebsiella pneumonia*, however, some strains do not produce gas.

4.3.3. Identification of Non-*Enterobacteriaceae* isolates

Only 4 isolates among all of the isolates were shown different biochemical reactions than *Enterobacteriaceae* biochemical reactions which are mentioned in the Veterinary Microbiology and Microbial Disease book (Quinn et al., 2011), they were 2 isolates of cattle samples which are S62 and S91 and 2 isolates of sheep samples which are K71 and K161. Thus, they were identified as non-*Enterobacteriaceae* members. Their percentage among total numbers of isolates was 7.4% (Figure 4.8). Their biochemical characters mentioned in Table 4.5.

Table 4.5. Biochemical characteristics of Non-*Enterobacteriaceae* isolates*

Oxidase test	Reactions in TSI agar				Lysine decarboxylase	IMViC tests			
	Slant	Butt	H_2S	Gas production		Indole	MR	VP	Citrate
−	Red	Red	−	−	+	−	−	−	+

*Red, alkaline; +, positive reaction. −, negative reaction.

The non-*Enterobacteriaceae* isolates were not further investigated to distinguish their genus and species, because this job was not part of the specified objectives of the current study, as all of the objectives were related only to *Enterobacteriaceae* family members. The reactions mechanisms of non-*Enterobacteriaceae* isolates in oxidase test, lysine decarboxylase broth and VP test were similar to those of *E. coli* isolates. While their reactions mechanisms in indole test, MR test and simmon's citrate test are same to those of isolate S71 (other *Enterobacteriaceae* member).

On the other hand, their reactions in TSI agar indicated that all three sugars (glucose, lactose and sucrose) have not been metabolized. These isolates were unable to

ferment sugars and they can be called as non glucose fermenter isolates, so the bacteria derived energy from peptones either aerobically or anaerobically. Utilization of peptones caused the release of ammonia (NH_3) resulting in the pH indicator, phenol red, turning from pink to red and the entire TSI agar was red in color (Lehman, 2005). Also, they were not produced H_2S and gas in the TSI medium.

Also, based on these isolates reactions in TSI agar mentioned above, they were definitely identified as non-*Enterobacteriaceae* members and this compatible to Lehman (2005) who described that bacteria producing alkaline over alkaline (red over red) include non-*Enterobacteriaceae*. Furthermore, the EMB agar was used as the selective medium for getting only gram negative bacteria from culture of collected samples in the present study, thus the non-*Enterobacteriaceae* isolates were definitely gram negative and this is compatible with Lal and Cheeptham (2007) as they described that EMB agar is selective for gram-negative bacteria, the dye methylene blue in the medium inhibits the growth of gram-positive bacteria, small amounts of this dye effectively inhibit the growth of most gram-positive bacteria.

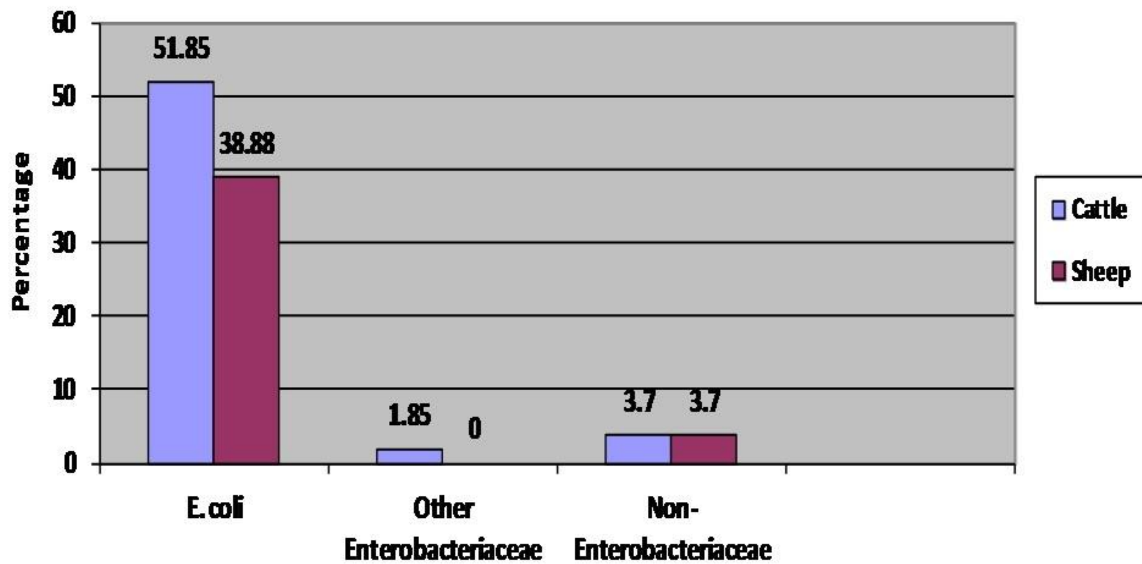


Figure 4.8. Percentages of total numbers of identified isolates from cattle and sheep samples

4.4. Verification of the Biochemically Identified Bacterial Isolates by Using CHROMagar ECC

Culture on chromogenic agar media (CHROMagar ECC) was applied to all the identified isolates in order to verify phenotypical identification by biochemical tests as described in the materials and methods chapter. After incubation of inoculated CHROMagar ECC plates, the results were exactly compatible with the biochemical tests results and no distinction was found, i.e., the biochemical identification (phenotypic) of the *Enterobacteriaceae* and non-*Enterobacteriaceae* isolates were confirmed according to the manufacturer's instructions for interpretation of the results in CHROMagar ECC media.

Thus, every *E. coli* isolates which were biochemically identified previously, produced blue colonies on CHROMagar ECC plates (Figure 4.9), other *Enterobacteriaceae* including isolate S71 was produced mauve colonies (Figure 4.9) and non-*Enterobacteriaceae* isolates were not grown (inhibited) on the medium agar plates (Figure 4.9). These results were well-matched to the CHROMagar directions for interpretation of the results.

Furthermore, Li *et al.* (2014) monitored the blue colonies of *E. coli* and red colonies of other *Enterobacteriaceae* including 4 genera (*Citrobacter*, *Klebsiella*, *Enterobacter* and *Raoultella)* isolates on CHROMagar from river water samples; as they reported that based on the sequencing results, 46 among 47 tested blue colonies selected on the medium were identical to the cultured *E. coli*, so the rate of false identifications was as low as 2% which proved that it is reliable to distinguish *E. coli* from other coliform bacteria using CHROMagar ECC medium.

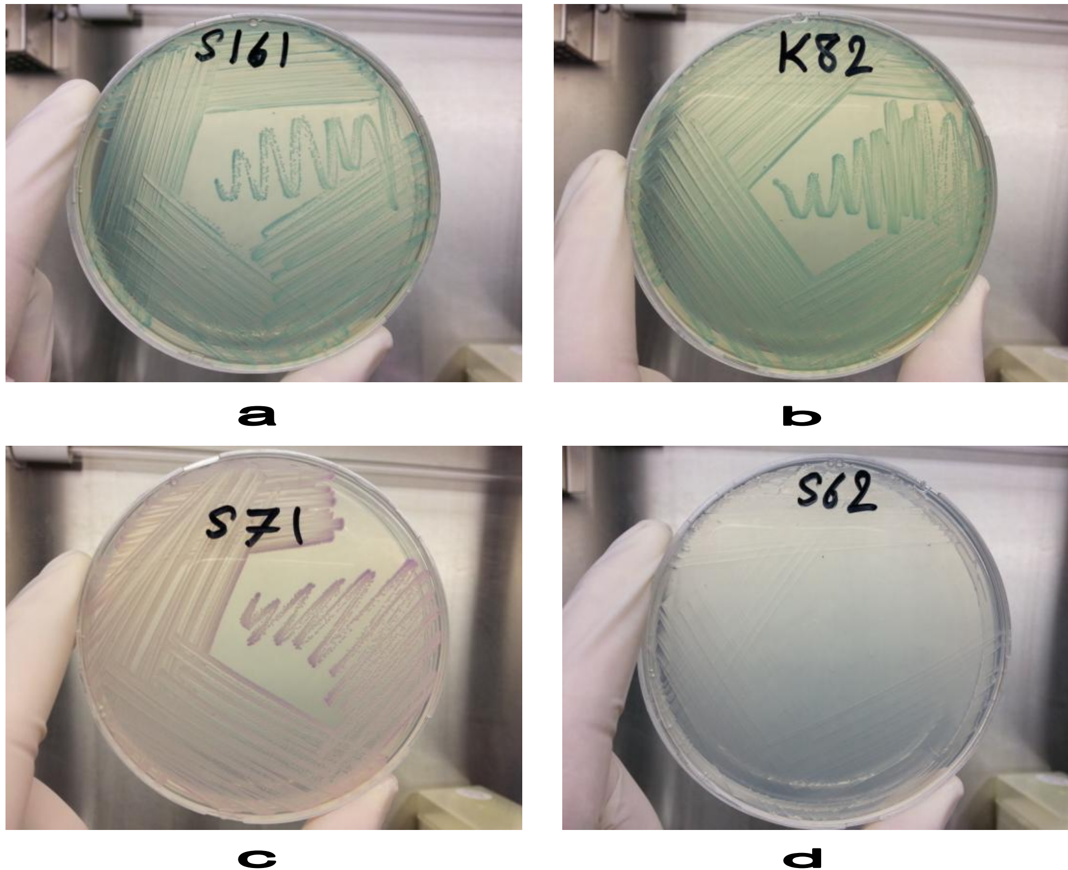


Figure 4.9. Colony colors of identified bacterial isolates. a, b) *E. coli*. c) other *Enterobacteriaceae*. d) non-*Enterobacteriaceae*

CHROMagar ECC is a differential agar, can be used for the rapid identification of *E. coli* and other organisms. One of the medium ingredients is chromogenic mixture which contains chromogenic agents (chromogenic substrates). One of chromogenic substrates is cleaved by the enzyme β-glucuronidase which is specific for *E. coli* and produced by approximately 97% of *E. coli* strains, this results in producing blue colonies on the agar plate. However, rare β-glucuronidase negative *E. coli* strains are false negative on this medium (typically 0157 *E. coli*). Therefore, CHROMagar manufacture recommend to use CHROMagar O157 product when focusing on pathogenic strains such as *E. coli* O157 (CHROMagar, 2013). But the present study was not focused on the pathogenic strains of *E. coli* isolates. The other chromogenic substrate is cleaved by β-galactosidase, an enzyme produced by the majority of coliforms (other *Enterobacteriaceae* members). This results in producing mauve colonies on the agar plate.

Since today, there is no report about using CHROMagar ECC for identifying bacteria of animal origin in Turkey; to our knowledge, the current study is the sole report

related to that purpose has been conducted in Turkey. Whereas, there are several reports on using CHROMagar ECC for samples of animal origin worldwide. For example: Liebana *et al.* (2006) and Hasan and Tinni (2011) observed blue colonies of *E. coli* isolates on CHROMagar ECC from animal sources in their studies. As well, Gosling *et al.* (2012) reported blue colonies of *E. coli* isolated from turkeys in Great Britain. Also, other researchers used CHROMagar ECC medium for different bacterial sources than animal sources. Such as: Giri *et al.* (2005) counted blue and red (or could be mauve) colonies for fecal and non fecal coliform bacteria, respectively in sludge, since majority of fecal coliform bacteria consist of *E. coli*, blue colonies were taken as *E. coli* counts in their study, by using pour-plate technique for inoculation of the plates in part of their study. Whereas, in the present study, a streak plate technique was used for inoculation of the plates, but in both studies the results of the colony colors are parallel to each other.

Additionally, Rincon and Pulgarin (2003) used CHROMagar ECC for detection and simultaneous enumeration of *E. coli* (blue colonies), fecal coliform including non-*E. coli* member of *Enterobacteriaceae* (violet colonies) and other different types of coliform (white colonies) for the experiment made on waste-water samples in their study. While in the present study, the non-*Enterobacteriaceae* isolates were not grown on CHROMagar plates. Yet, both inhibited growth and colorless (white) colonies are well-suited to the manufacturer's directions for interpretation of the results in CHROMagar ECC.

4.5. Antibiotic Susceptibility Patterns of Isolates

Out of 54 identified isolates, 50 *Enterobacteriaceae* isolates including 49 *E. coli* isolates and 1 other *Enterobacteriaceae* isolate (S71) were tested to determine their antimicrobial susceptibility profiles for 13 antibiotic agents including 10 β-lactams and 3 non-β-lactams, as described in materials and methods chapter. After incubation, the plates were examined and the diameters of the zone of inhibition were measured in mm by using a ruler (Figure 4.10). Then those values were interpreted and isolates were sorted as susceptible, intermediate and resistant by using the criteria of CLSI, 2013.

4.5.1. Antibiotic susceptibility patterns of *E. coli* isolates

The antimicrobial susceptibility profiles of *E. coli* isolates for 13 antibiotic agents were determined including the diameter of inhibition zones are shown in Table 4.6 and interpretive criteria (susceptible, intermediate and resistant) are mentioned in Table 4.7.

When considering all *E. coli* isolates, susceptibility rates to tested antibiotics were more higher than intermediate and resistance rates and more variable among all the *E. coli* isolates (Table 4.10; Figure 4.13). All the *E. coli* isolates were susceptible to most of the antibiotics, but the more active antibiotics were found among β-lactam group including penicillins (amoxicillin-clavulanic acid) and cephalosporins (ceftriaxone), i.e., all *E. coli* isolates 100% from cattle and sheep samples were susceptible to both of antibiotic agents. Following to that, the second highest susceptibility 97.95% of total *E. coli* isolates was detected in carbapenems group including ertapenem and imipenem antibiotics, while the lowest susceptibility level 57.14% of total *E. coli* isolates was only seen for cephalosporins (cefotaxime) which was the less active antibiotic agent (Table 4.10; Figure 4.13).

On the other hand, the susceptibility rates were higher in *E. coli* isolates of sheep samples than cattle samples. Among the sheep isolates, out of 13 antibiotics, 6 were more active against them, i.e., all *E. coli* isolates of sheep samples were 100% susceptible to these antibiotic agents which were penicillins (amoxicillin-clavulanate), cephalosporins (cefttriaxone), two of carbapenems (ertapenem and meropenem), monobactams (aztreonam) and non-β-lactam antibiotic (trimethoprim-sulfamethoxazole). Whereas, all *E. coli* isolates of cattle samples were 100% susceptible to only two β-lactams including amoxicillin-clavulanate and ceftriaxone. While, the lowest susceptibility for cephalosporins (cefotaxime) was found in *E. coli* isolates of cattle and sheep samples with 46.42% and 71.42% respectively (Table 4.8; 4.9; Figure 4.11; 4.12).

Furthermore, when considering all the *E. coli* isolates intermediate level of antibiotic susceptibilities were more variable. The highest intermediate level 42.85% of total *E. coli* isolates was observed for cephalosporins (cefotaxime) and the lowest intermediate level 2.04% of total *E. coli* isolates was seen for each one of two carbapenems (ertapenem and meropenem) and fluoroquinolones (ciprofloxacin) (Table 4.10; Figure 4.13). However, the highest intermediate level 53.57% of cattle isolates and 28.57% of sheep isolates were determined for cefotaxime, but the lowest intermediate level was seen in cattle isolates more than sheep isolates, as 3.57% of cattle isolates was found for each monobactams (ertapenem and meropenem), penicillins (piperacillin-tazobactam) and fluoroquinolones (ciprofloxacin) and 4.76% of sheep isolates was determined for piperacillin-tazobactam (Table 4.8; 4.9; Figure 4.11; 4.12).

Additionally, when considering all the *E. coli* isolates, resistance to tested antibiotics was low, out of 49 *E. coli* isolates from cattle and sheep samples, 6 (12.24%) were resistant to at least one antibiotic agent. Also, the highest resistance level (n=3; 6.12%) of total *E. coli* isolates was detected for non β-lactam (trimethoprim-sulfamethoxazole) and the lowest resistance level 2.04% of total *E. coli* isolates was observed for cephalosporins (cefpodoxime) (Table 4.10; Figure 4.13). Moreover, the resistance rate in *E. coli* isolates of cattle samples were higher than sheep samples. As, of 28 *E. coli* isolates of cattle samples, 4 (14.28%) were resistant to at least one antibiotic agent and of 21 *E. coli* isolates of sheep samples, 2 (9.52%) were resistant to at least one antibiotic agent. Also, the highest resistance level 10.71% of cattle isolates was found for trimethoprim-sulfamethoxazole and highest resistance level 9.52% of sheep isolates was seen for ciprofloxacin (Table 4.8; 4.9; Figure 4.11; 4.12).

Also, out of 49 *E. coli* isolates from cattle and sheep samples, 2 (4.08%) were found as multiple drug resistant (resistance to two antibiotics). Multiple resistant isolates (S11 and S12) were only isolated from cattle samples, and they were resistant to both of the carbapenems (imipenem) and non β-lactam (trimethoprim-sulfamethoxazole) (Table 4.7). Along with those isolates, two sheep isolates (K112 and K151) were resistant to one antibiotic agent (ciprofloxacin). Whilst, there were resistance to more than one antibiotic agents among the *E. coli* isolates from cattle samples, resistance to β-lactam (cefpodoxime, imipenem) and non β-lactam (trimethoprim-sulfamethoxazole) were observed. Among those *E. coli* isolates, only one cattle *E. coli* isolate (S23) was shown resistance to β-lactam antibiotic (cefpodoxime). In addition, out of 28 *E. coli* isolates of cattle samples, 4

(14.28%) were susceptible to all tested antibiotics, while of 21 *E. coli* isolates of sheep samples, 10 (47.61%) were susceptible to all tested antibiotics (Table 4.7).

Table 4.6. Antibiotic inhibition zone diameters of *E. coli* isolates

Isolates	AMC	TZP	CAZ	CTX	CPD	CRO	ETP	IPM	MEM	ATM	CIP	AK	SXT
S11	18	21	21	24	21	25	24	18	23	21	21	20	0
S12	19	23	25	25	23	27	25	19	23	26	26	20	0
S22	21	23	20	26	21	26	26	23	28	25	30	19	18
S23	20	22	21	27	17	25	22	25	26	20	26	20	18
S32	21	20	24	26	20	27	24	25	24	27	30	20	19
S41	20	23	24	27	18	28	26	27	26	22	27	18	28
S42	19	21	20	26	22	25	20	23	27	22	26	16	24
S52	20	23	20	26	21	26	24	20	20	25	23	18	25
S61	22	22	23	25	23	25	25	21	27	26	25	18	22
S81	21	21	22	25	21	26	24	23	25	24	22	20	22
S82	18	21	21	24	19	23	23	25	26	21	22	19	25
S92	20	21	25	26	23	30	25	25	24	26	26	19	25
S93	18	22	21	25	21	25	26	25	23	23	26	17	24
S101	22	25	25	27	22	29	31	28	30	27	34	22	26
S102	21	23	24	25	21	27	28	27	29	25	24	22	25
S103	19	21	24	26	22	27	28	25	28	26	25	19	24
S111	20	27	21	25	20	26	28	26	26	25	26	21	22
S112	19	22	21	23	19	23	26	23	27	18	29	19	26
S132	21	25	20	25	21	27	26	28	28	25	27	18	22
S141	21	23	21	25	20	28	24	22	26	26	28	20	28
S151	20	21	24	25	20	26	26	23	27	24	26	19	25
S152	22	22	24	27	19	27	24	26	25	24	26	18	25
S161	20	24	25	27	21	29	32	29	31	28	28	19	27
S171	19	22	21	24	22	26	30	27	28	23	16	20	0

Table 4.6. (Continued) Antibiotic inhibition zone diameters of *E. coli* isolates

Isolates	AMC	TZP	CAZ	CTX	CPD	CRO	ETP	IPM	MEM	ATM	CIP	AK	SXT
S172	20	23	22	26	20	25	25	24	26	22	28	19	24
S181	20	25	24	25	20	28	26	26	28	23	31	22	29
S191	22	20	22	27	21	26	22	28	25	20	25	16	28
S201	20	23	22	24	20	26	27	21	26	26	28	20	25
K1	19	24	21	25	20	27	26	22	28	26	25	17	26
K4	21	21	26	28	23	28	25	25	26	28	30	20	24
K51	22	26	27	28	24	30	27	27	30	27	30	20	20
K52	21	23	24	26	21	28	24	22	28	25	25	15	26
K61	22	23	24	27	23	31	28	30	27	27	29	18	24
K81	19	21	21	25	22	27	24	24	25	26	26	18	23
K82	20	22	25	26	23	28	23	24	23	26	26	18	24
K111	26	27	24	27	22	32	33	23	30	27	30	18	28
K112	21	26	22	26	22	28	30	28	30	28	15	18	20
K122	20	23	24	27	23	29	26	28	28	26	28	22	18
K141	23	24	27	26	23	28	30	31	30	26	28	19	26
K142	20	23	26	25	21	24	23	26	26	25	28	18	24
K151	20	22	20	27	22	28	28	26	26	27	13	18	18
K162	20	23	18	24	21	30	22	28	29	23	26	20	21
K163	19	24	22	25	20	27	29	28	28	24	26	16	18
K171	19	23	21	25	19	25	22	29	28	26	23	18	19
K172	21	21	20	27	18	28	27	28	28	26	26	19	19
K181	21	20	26	28	22	28	26	26	28	26	25	17	26
K191	21	23	22	27	22	26	29	26	28	27	25	17	24
K201	21	22	25	28	23	32	26	27	25	26	28	19	18
K202	20	22	26	28	23	29	25	26	26	29	28	20	17

Table 4.7 Interpretation of antibiotic inhibition zone diameters of *E. coli* isolates*

Isolates	AMC	TZP	CAZ	CTX	CPD	CRO	ETP	IPM	MEM	ATM	CIP	AK	SXT
S11	S	S	S	I	S	S	S	R	S	S	S	S	R
S12	S	S	S	I	S	S	S	R	S	S	S	S	R
S22	S	S	I	S	S	S	S	S	S	S	S	S	S
S23	S	S	S	S	R	S	S	S	S	I	S	S	S
S32	S	S	S	S	I	S	S	S	S	S	S	S	S
S41	S	S	S	S	I	S	S	S	S	S	S	S	S
S42	S	S	I	S	S	S	I	S	S	S	S	I	S
S52	S	S	I	S	S	S	S	I	I	S	S	S	S
S61	S	S	S	I	S	S	S	I	S	S	S	S	S
S81	S	S	S	I	S	S	S	S	S	S	S	S	S
S82	S	S	S	I	I	S	S	S	S	S	S	S	S
S92	S	S	S	S	S	S	S	S	S	S	S	S	S
S93	S	S	S	I	S	S	S	S	S	S	S	S	S
S101	S	S	S	S	S	S	S	S	S	S	S	S	S
S102	S	S	S	I	S	S	S	S	S	S	S	S	S
S103	S	S	S	S	S	S	S	S	S	S	S	S	S
S111	S	S	S	I	I	S	S	S	S	S	S	S	S
S112	S	S	S	I	I	S	S	S	S	I	S	S	S
S132	S	S	I	I	S	S	S	S	S	S	S	S	S
S141	S	S	S	I	I	S	S	I	S	S	S	S	S
S151	S	S	S	I	I	S	S	S	S	S	S	S	S
S152	S	S	S	S	I	S	S	S	S	S	S	S	S
S161	S	S	S	S	S	S	S	S	S	S	S	S	S
S171	S	S	S	I	S	S	S	S	S	S	I	S	R

**S, susceptible; I, intermediate; R, resistant.

Table 4.7. (Continued) Interpretation of antibiotic inhibition zone diameters of *E. coli* isolates*

Isolates	AMC	TZP	CAZ	CTX	CPD	CRO	ETP	IPM	MEM	ATM	CIP	AK	SXT
S172	S	S	S	S	I	S	S	S	S	S	S	S	S
S181	S	S	S	I	I	S	S	S	S	S	S	S	S
S191	S	I	S	S	S	S	S	S	S	I	S	I	S
S201	S	S	S	I	I	S	S	I	S	S	S	S	S
K1	S	S	S	I	I	S	S	I	S	S	S	S	S
K4	S	S	S	S	S	S	S	S	S	S	S	S	S
K51	S	S	S	S	S	S	S	S	S	S	S	S	S
K52	S	S	S	S	S	S	S	I	S	S	S	I	S
K61	S	S	S	S	S	S	S	S	S	S	S	S	S
K81	S	S	S	I	S	S	S	S	S	S	S	S	S
K82	S	S	S	S	S	S	S	S	S	S	S	S	S
K111	S	S	S	S	S	S	S	S	S	S	S	S	S
K112	S	S	S	S	S	S	S	S	S	S	R	S	S
K122	S	S	S	S	S	S	S	S	S	S	S	S	S
K141	S	S	S	S	S	S	S	S	S	S	S	S	S
K142	S	S	S	I	S	S	S	S	S	S	S	S	S
K151	S	S	I	S	S	S	S	S	S	S	R	S	S
K162	S	S	I	I	S	S	S	S	S	S	S	S	S
K163	S	S	S	I	I	S	S	S	S	S	S	I	S
K171	S	S	S	I	I	S	S	S	S	S	S	S	S
K172	S	S	I	S	I	S	S	S	S	S	S	S	S
K181	S	I	S	S	S	S	S	S	S	S	S	S	S
K191	S	S	S	S	S	S	S	S	S	S	S	S	S
K201	S	S	S	S	S	S	S	S	S	S	S	S	S
K202	S	S	S	S	S	S	S	S	S	S	S	S	S

*S, susceptible; I, intermediate; R, resistant.

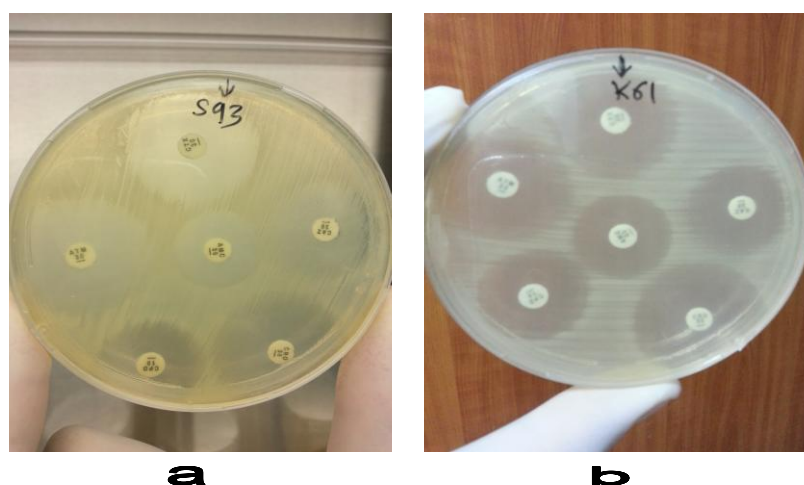


Figure 4.10. Antibiotic susceptibility and ESBL producer detection testing. a) *E. coli* isolate of cattle sample. b) *E. coli* isolate of sheep sample

Table 4.8. Antibiotic sensitivity patterns of *E. coli* isolates from cattle samples in percentage

Antibiotic agent	Susceptible		Intermediate		Resistant	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
AMC	28	100	0	0	0	0
TZP	27	96.42	1	3.57	0	0
CAZ	24	85.71	4	14.28	0	0
CTX	13	46.42	15	53.57	0	0
CPD	16	57.14	11	39.28	1	3.57
CRO	28	100	0	0	0	0
ETP	27	96.42	1	3.57	0	0
IPM	22	78.57	4	14.28	2	7.14
MEM	27	96.42	1	3.57	0	0
ATM	25	89.28	3	10.71	0	0
CIP	27	96.42	1	3.57	0	0
AK	26	92.85	2	7.14	0	0
SXT	25	89.28	0	0	3	10.71

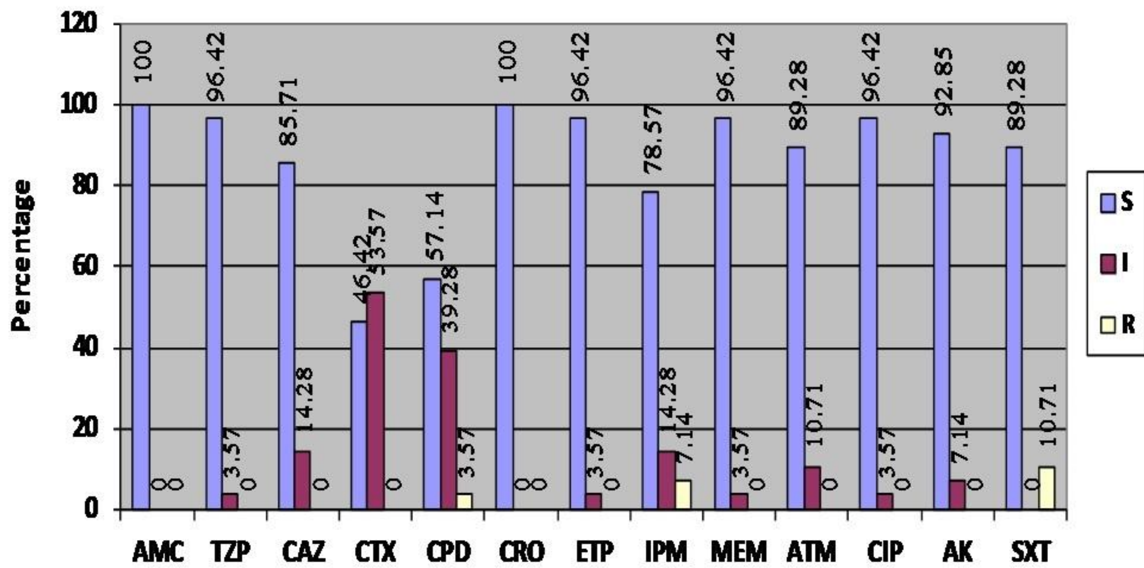


Figure 4.11. Antibiotic sensitivity patterns of *E. coli* isolates from cattle samples in percentage

Table 4.9. Antibiotic sensitivity patterns of *E. coli* isolates from sheep samples in percentage

Antibiotic agent	Susceptible		Intermediate		Resistant	
	No. of islates	%	No. of isolates	%	No. of isolates	%
AMC	21	100	0	0	0	0
TZP	20	95.23	1	4.76	0	0
CAZ	18	85.71	3	14.28	0	0
CTX	15	71.42	6	28.57	0	0
CPD	17	80.95	4	19.04	0	0
CRO	21	100	0	0	0	0
ETP	21	100	0	0	0	0
IPM	19	90.47	2	9.52	0	0
MEM	21	100	0	0	0	0
ATM	21	100	0	0	0	0
CIP	19	90.47	0	0	2	9.52
AK	19	90.47	2	9.52	0	0
SXT	21	100	0	0	0	0

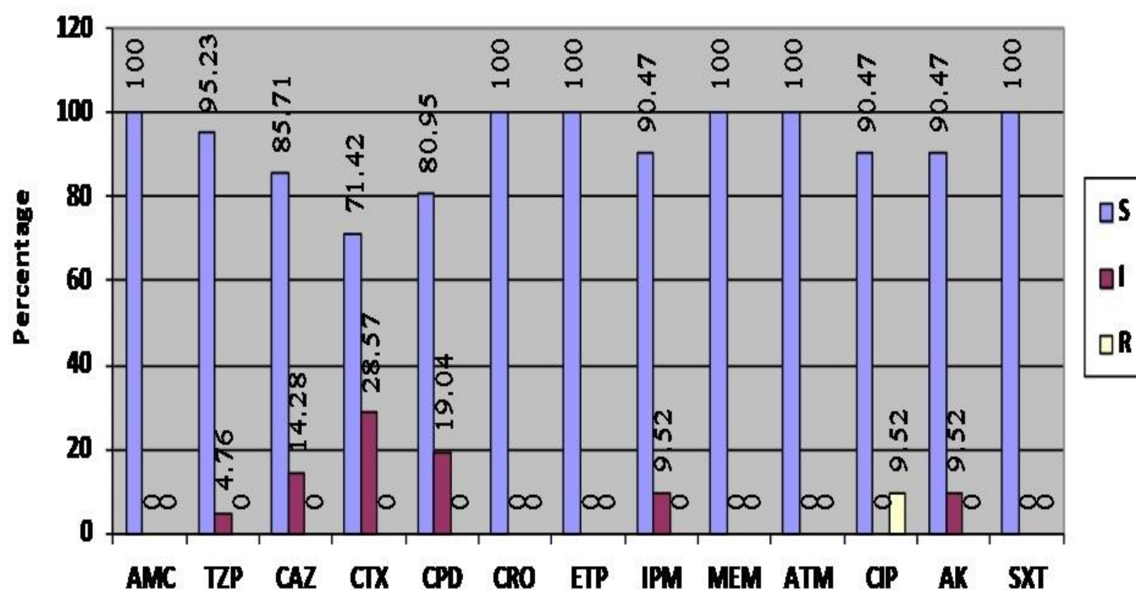


Figure 4.12. Antibiotic sensitivity patterns of *E. coli* isolates from sheep samples in percentage

Table 4.10. Antibiotic sensitivity patterns of total no. of *E. coli* isolates from cattle and sheep samples in percentage

Antibiotic agent	Susceptible: total no. and (%)			Intermediate: total no. and (%)			Resistant: total no. and (%)		
	Cattle	Sheep	Total	Cattle	Sheep	Total	Cattle	Sheep	Total
AMC	28	21	49 (100)	0	0	0	0	0	0
TZP	27	20	47 (95.91)	1	1	2 (4.08)	0	0	0
CAZ	24	18	42 (85.71)	4	3	7 (14.28)	0	0	0
CTX	13	15	28 (57.14)	15	6	21 (42.85)	0	0	0
CPD	16	17	33 (67.34)	11	4	15 (30.61)	1	0	1 (2.04)
CRO	28	21	49 (100)	0	0	0	0	0	0
ETP	27	21	48 (97.95)	1	0	1 (2.04)	0	0	0
IPM	22	19	41 (83.67)	4	2	6 (12.24)	2	0	2 (4.08)
MEM	27	21	48 (97.95)	1	0	1 (2.04)	0	0	0
ATM	25	21	46 (93.87)	3	0	3 (6.12)	0	0	0
CIP	27	19	46 (93.87)	1	0	1 (2.04)	0	2	2 (4.08)
AK	26	19	45 (91.83)	2	2	4 (8.16)	0	0	0
SXT	25	21	46 (93.87)	0	0	0	3	0	3 (6.12)

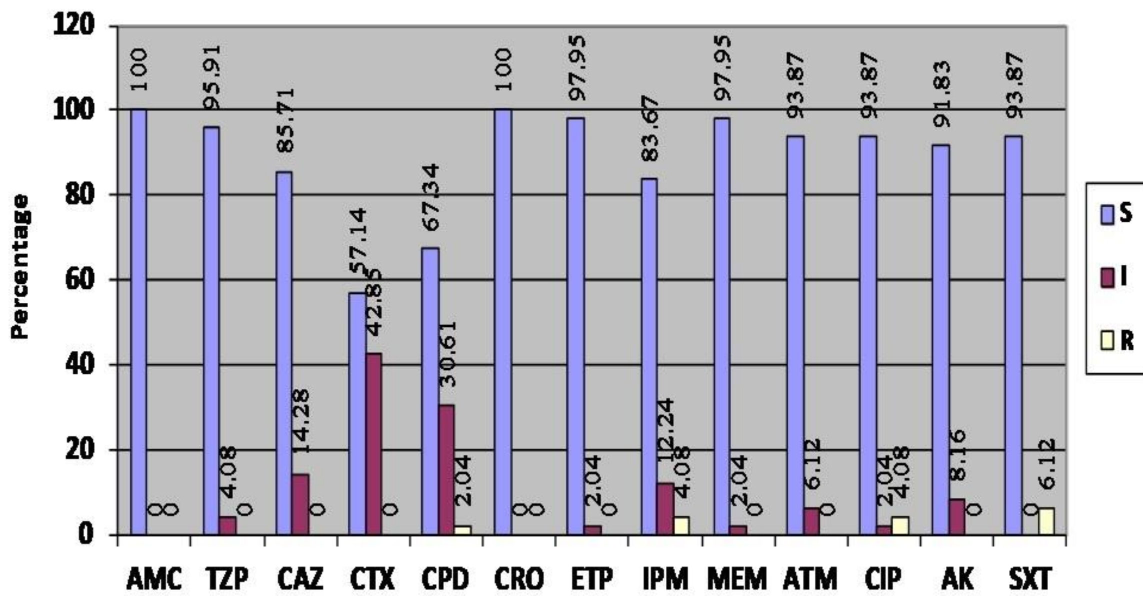


Figure 4.13 Antibiotic sensitivity patterns of total no. of *E. coli* isolates in percentage

Enterobacteriaceae are the significant causes of serious infection, and many of the most important members of this family are becoming increasingly resistant to currently available antimicrobials. Two organisms of concern are *E. coli* and *Klebsiella pneumoniae*, an opportunistic pathogens of humans and animals responsible for a wide range of infections (Slama *et al.*, 2010). Fecal contamination of foods of animal origin might occur during animal slaughtering, milking, and/or processing, and the growth of the contaminating bacteria may occur during the product transport and storage phases. Consequently, without good hygienic practices, foods may act as a vehicle of transfer of β-lactam-resistant bacteria to the gastrointestinal tract of consumers (Overdevest *et al.*, 2011). It is well known that β-lactam use is the major selective factor influencing β-lactamase production by pathogens (Livermore, 1995). Evidence obtained from laboratory and epidemiological studies indicated that the persistence of resistant bacteria was related to the persistence of antimicrobial drug use, i.e., if an antimicrobial drug is used, continuously, the persistence of resistant organisms will go on (Andersson, 2003).

Since today, there is no specific data or reports about the antibiotic sensitivity patterns, especially the prevalence of β-lactam resistance of commensal *Enterobacteriaceae* members in healthy adult cattle and sheep at slaughter in Turkey. On the other hand, only 1 report was addressed about ESBLs producing *Enterobacteriaceae* isolated from food producing animals in Turkey and it was by Kucukbasmaci *et al.* (2008)

in the northwest of Turkey. To our knowledge, the current study is the first report about the occurrence of β-lactam resistance of intestinal commensal *Enterobacteriaceae* has been carried out for healthy adult cattle and sheep at slaughter in south of Turkey. Kucukbasmaci *et al.* (2008) reported that out of 349 samples from 239 cattle, 38 calves and 72 sheep from three different slaughterhouses and two farms, they found (n=5; 2.1%) ESBL producing *Enterobacteriaceae* prevalance from healthy cattle fecal samples at slaughter including 3 *E. coli* and 2 *Citrobacter* species. In their published article, they only focused on those 5 isolates and no data about the β-lactam resistant patterns of all the isolates were given. In their findings, all the ESBL producing isolates were susceptible to imipenem and ciprofloxacin but four of the five isolates were resistant to trimethoprim-sulphamethaxazole, along with other data about other types of antibiotic agents which were not used in our study. While in the present study, out of 28 *E. coli* isolates from cattle samples, n=22; 78.57% and n=27; 96.42% were susceptible to imipenem and ciprofloxacin respectively, but n=3; 10.71% were resistant to trimethoprim-sulphamethaxazole.

Raw materials and foods of animal origin may be not only a source of pathogenic bacteria causing alimentary tract infections but also a source of bacteria with a dangerous extent of resistance to antibiotics, potentially entering the human food chain (Bardon *et al.*, 2013). Because of the intensive use of antimicrobial agents in food animal production, meat is frequently contaminated with antimicrobial-resistant *E. coli.* Thus, humans can be colonized with *E. coli* of animal origin, and because of resistance to commonly used antimicrobial agents, these bacteria may cause infections for which limited therapeutic options are available. Bacteria from the animal reservoir that carry resistance to antimicrobial agents that are regarded as highly or critically important in human therapy (e.g., aminoglycosides, fluoroquinolones and third- and fourth-generation cephalosporins) are of especially great concern (Hammerum and Heuer, 2009).

In addition, there are several reports addressed to happening the antibiotic resistance of *Enterobacteriaceae* members in foods of animal origin in Turkey. For example, the most recent study by Gundogan and Avci (2013), they isolated 63 *Klebsiella* spp. and 45 *E. coli* from 75 samples of raw calf meat (minced), chicken meat, raw milk, white cheese and ice cream collected from various supermarkets, dairy plants and pastry shops in Ankara, Turkey. In their study, antibiotic susceptibility testing was carried out using disk diffusion method and CLSI. They observed that *E. coli* isolates have been found resistant to cefotaxime 33.3%, ciprofloxacin 31.1%, aztroenam 28.9%, ceftazidime 8.9%,

ceftriaxone 8.9%, amoxcillin/clavulanic acid 6.7%, and amikacin 4.4%. All *E. coli* isolates which were studied have been found sensitive to imipenem, ertapenem and piperacillin/tazobactam. In their study, they used some other antibiotic agents which were not used in our study. Whereas, in the present study, out of 49 *E. coli* isolates, 1(2.04%), 2(4.08%), 2(4.08%) and 3(6.12%) resistant to cefpodoxime, imipenem, ciprofloxacin and trimethoprim-sulfamethoxazole respectively. Besides, all *E. coli* isolates were susceptible to amoxicillin-clavulanate and ceftriaxone.

The above data elucidate that resistances of *E. coli* isolates to β-lactam were higher in findings by Gundogan and Avci (2013) than our findings. The most effective β-lactam antibiotics were imipenem, ertapenem and piperacillin/tazobactam in their findings, but it was imipenem in retail meat *E. coli* isolates in Bolu, Turkey (Arslan and Eyi, 2011). While in our study, amoxicillin-clavulanate and ceftriaxone were most active. Cephalosporins are an important class of antibacterial agents in use for both humans and animals. The use of cephalosporins in food-producing animals could be selective factor for the appearance of ESBL producing and multiple antimicrobial resistant bacteria in such animals (Cavaco *et al.*, 2008). On the other hand, increasing resistance to third generation cephalosporins (for example, cefotaxime, ceftazidime, ceftriaxone) has become a cause for concern about *Enterobacteriaceae* (Okesola and Makanjuola, 2009). In our study, the resistance to third generation cephalosporins was very low and it was only 2.04% for cefpodoxime. Also, the susceptibility to ceftazidime (85.71%) in the present study was higher than susceptibility (73.6%) in the study by Arslan and Eyi (2011). In the present study and study by Arslan and Eyi (2011) among the combinations of β-lactam/β-lactamase inhibitors, the most effective one was amoxicillin/clavulanic acid, but in findings by Gundogan and Avci (2013) it was piperacillin/tazobactam. Aminoglycosides are active against clinically important gram-negative bacilli (Ramirez *et al.*, 2010). The susceptibility to amikacin in our study higher than the findings by Arslan and Eyi (2011). Ciprofloxacin is a broad spectrum fluoroquinolone antimicrobial agent that is highly effective for the treatment of a variety of infections in humans and animals (Periti *et al.*, 1998). The observed resistances of *E. coli* isolates to ciprofloxacin in Gundogan and Avci (2013) and Arslan and Eyi (2011) studies were 31.9% and 31.1% respectively but in our study it was much less (4.08%). Van den Bogaard *et al.* (2001) speculated that high percentages of fluoroquinolone resistance of the animal isolates were probably due to the therapeutic use of such antibiotic in animals and/or widespread addition of it to the animal feed. As

resistance to ciprofloxacin emerged, resistance to β-lactam antibiotics became prominent. This resistance was largely a result of ESBLs, which mediate resistance to newer β-lactam agents, such as ceftazidime, ceftriaxone, cefotaxime, and aztreonam, that have an oxyamino group (Gundogan *et al.*, 2011). Furthermore, in our study, *E. coli* resistance to trimethoprim-sulphamethoxazole was 6.12% but higher resistance 45.8% was noticed in retail meat in Bolu, Turkey (Arslan and Eyi, 2011).

During the past decade, drug resistance in *Enterobacteriaceae* has increased dramatically worldwide. This increase is mainly the result of an increased prevalence of ESBL-producing *Enterobacteriaceae* and then led to the increase of using the last-resort antimicrobial drugs which are the carbapenems (Kuzucu *et al.*, 2011). Since, ESBLs are plasmid-encoded β-lactamases capable of hydrolyzing penicillins, cephalosporins and monobactams but not cephamycins and carbapenems (Bradford, 2001). Therefore, the current worldwide emergence of resistance to the powerful antibiotic carbapenem in *Enterobacteriaceae* constitutes an important growing public health threat (Nordmann *et al.*, 2012). Thus, there remains a need for continued surveillance and prudent use of the β-lactam (carbapenems) as a recent report in Turkey has documented the occurrence of carbapenems resistant in two *E. coli*, one *K. pneumoniae* and one *K. oxytoca* isolates from clinical specimens of hospitalized patients and outpatients between January 2007-February 2008 (Kuzucu *et al.*, 2011). Similarly, in our study, we detected the carbapenems (imipenem) resistant *E. coli* isolates which were (n=2; 4.08%) of total *E. coli* isolates. Therefore, prevalence of carbapenems resistant isolates from human and animals in Turkey should be monitored routinely for risk assessment of those isolates. Since, food animals are increasingly recognized as a reservoir for ESBL-producing strains, these strains can be transmitted via the food chain (Overdevest *et al.*, 2011). Eventually, with carbapenems resistant pathogens the treatment options in both diseased humans and animals might be more restricted and limited. So, the results in the current study, suggest that further investigations at molecular level are needed to detect the similarity between resistant genes in both human and animals, along with the increase of the testing samples numbers in other abattoirs throughout Turkey to screen the prevalence of carbapenems resistant *Enterobacteriaceae* of animal origin. To our knowledge, our study is the first report about the occurrence of carbapenems resistant *E. coli* in healthy food animals at slaughter in Turkey.

Furthermore, there are more studies about the occurrence of antibiotic sensitivity patterns of bacterial isolates of an animal origin and reports about the prevalence of β-lactam resistant *Enterobacteriaceae* members in healthy ruminant domesticated animals at slaughter in several countries around the world. For example: the recent study in Portugal by Ramos *et al.* (2013) reported that among 192 *E. coli* isolates from faecal samples of slaughtered animals including 66 from pigs, 73 from sheep and 53 from cattle, the resistance of *E. coli* isolates to β-lactam/β-lactamase combination (amoxicillin-clavulanic acid) was 31 (42.5%) and 2 (3.8%) of sheep and cattle isolates respectively, in contrast to that study, in the current study, all (100%) of *E. coli* isolates from cattle and sheep samples were susceptible to that antibiotic agent. As well, no resistances were detected to cefotaxime and aztreonam in our study, but their resistances were (n=1; 1.9%) of cattle isolates to each of both of the agents in their study. In our study, the resistance to imipenem was (n=2; 7.14%) of cattle *E. coli* isolates, whereas such resistance was not observed in their study. However, in their study, the resistance of *E. coli* isolates to amikacin was (n=2; 2.7%) and (n=1; 1.9%) of sheep and cattle isolates respectively, but such resistance was not recorded in our study. On the other hand, the resistance to trimethoprim-sulfamethoxazole was (n=18; 24.6%) and (n=1; 1.9%) of sheep and cattle isolates respectively in their study, but it was (n=3; 10.71%) of cattle isolates in our study. In addition, the resistance to ciprofloxacin was (n=1; 1.4%) and (n=3; 5.7%) of sheep and cattle isolates respectively in their study, but it was (n=2; 9.52%) of sheep samples in our study. In their study, some other antibiotics have been used but they were not used in our study. These data elucidate that overall resistances to the above mentioned β-lactams (except carbapenems) and aminoglycosides (amikacin) in sheep and cattle samples were not observed in the present study, but they were found in the study by Ramos *et al.* (2013). Whereas carbapenems (imipenem) resistant *E. coli* isolates were detected in the current study, but it was not found in their study. Additionally, resistances to trimethoprim-sulfamethoxazole and flouroquinolones (ciprofloxacin) were higher in their findings than our findings.

However, the resistant *E. coli* isolates from cattle and sheep samples were much less than the susceptible ones in the present study. Nevertheless, the obtained results were consistent to previous studies around the world. For example: the most recent study in Poland by Wasyl *et al.* (2013) reported that most (79.9%) of cattle *E. coli* isolates showed no resistance in their study, since those isolates originated mostly from adult cattle

(medium age at slaughter: 46 months, max. 214 months; data not shown) it might be presumed that some animals were slaughtered due to insufficient milk productivity and had not been treated with antimicrobials due to restriction on milk during withdrawal period. On the opposite, poultry might be treated until few days before slaughter and thus high resistance levels were found: only 5.1% of broiler and 11.3% of turkey isolates showed no resistance in their study. Besides, the less resistance of *E. coli* isolates from cattle and sheep samples were noticed when comparing to pig *E. coli* isolates in previous studies worldwide. For example: In Great Britain, Enne *et al.* (2008) stated that out of 836 *E. coli* isolates from cattle tested, only 5.7% were resistant to one or more antimicrobials, while only 3.0% of 836 isolates from sheep were resistant to one or more agents. However, 92.1% of 2480 isolates from pigs were resistant to at least one antimicrobial. Such variation may reflect the differences in the amounts of therapeutic antimicrobials used in the husbandry of the three animal species studied. Similar findings were documented in Korea by Lim *et al.* (2007) detected the prevalence of resistance in *E. coli* isolates of pigs was much higher than that in cattle, with 98.3% of pig isolates and 37.1% of cattle isolates showing resistance to one or more of the antimicrobial agents tested.

Although, there is only one Turkish study which focused on fuoroquinolones (ciprofloxacin) resistant *E. coli* isolated from animals (Mustak *et al.*, 2012). They reported that according to antimicrobial susceptibility tests, 47 (50.0%) of 94 chicken, 3 (4.5%) of 66 sheep, 5 (9.6%) of 52 cattle and 3 (6.4%) of 47 dog *E. coli* strains were found to be resistant to ciprofloxacin. In contrast to that study, there has been resistance to ciprofloxacin only in sheep *E. coli* isolates and it was 2 (9.52%) of sheep isolates.

Series of studies on the resistance of *E. coli* which were isolated from animals and humans have strongly suggested that those bacteria which are resistant to antimicrobials used in animals would also be resistant to antimicrobials used in humans (VSPA, 2006; Miles *et al.,* 2006; Umolu *et al.,* 2006). A most important side effect of the use of antibiotics is the emergence and dissemination of resistant bacteria. Most retrospective and prospective studies show that after the introduction of an antibiotic not only the level of resistance of pathogenic bacteria, but also of commensal bacteria increases. Commensal bacteria constitute a reservior of resistance genes for pathogenic bacteria. Resistant commensal bacteria of food animals might contaminate, like zoonotic bacteria, meat (products) and so reach the intestinal tract of humans. To safeguard public health, the selection and dissemination of resistant bacteria from animals should be controlled. This

can only be achieved by reducing the amounts of antibiotics used in animals (Van den Bogaard and Stobberingh, 2000). Prudent use of β-lactams, especially the extended-spectrum cephalosporins, would be a critical factor in containing β-lactamase producing bacteria, which include minimizing both enrichment and dissemination of resistant bacteria. In this regard, the transfer of resistant clones between animal and human communities warrants further investigation (Fey *et al.*, 2000).

4.5.2. Antibiotic susceptibilities of other *Enterobacteriaceae* member (isolate S71)

The antimicrobial susceptibility profile of other *Enterobacteriaceae* isolate, i.e., non-*E. coli* which is isolate no. S71 for 13 antibiotic agents were determined including the diameter of inhibition zones and interpretive criteria (susceptible, intermediate and resistant) (Table 4.11).

Isolate S71 was susceptible to most of the tested β-lactam antibiotic agents including penicillins (amoxicillin-clavulanate), three of cephalosporins (ceftazidime, cefpodoxime and ceftriaxone), two of carbapenems (ertapenem amd meropenem) and monobactams (aztreonam). As well, it was susceptible to all the non β-lactam antibiotic agents including fluoroquinolones (ciprofloxacin), aminoglycosides (amikacin) and folate pathway inhibitors (trimethoprim-sulfamethoxazole). The other-*Enterobacteriaceae* isolate was neither susceptible nor resistant (intermediate) to three β-lactam antibiotic agents amongst all tested agents, that are penicillins (piperacillin-tazobactam), cephalosporins (cefotaxime) and carbapenems (imipenem). However, no resistance was noticed for isolate S71 among all antibiotic agents, while resistances to few agents were found among *E. coli* isolates, this elucidated that almost all the tested antibiotic agents were active against other-*Enterobacteriaceae* isolate (S71) but more active ones were non β-lactam agents (Table 4.7; 4.11).

Table 4.11. Antibiotic susceptibility patterns of other *Enterobacteriaceae* isolate (S71)*

AMC	TZP	CAZ	CTX	CPD	CRO	ETP	IPM	MEM	ATM	CIP	AK	SXT
18	20	21	25	22	24	24	21	23	23	24	19	21
S	I	S	I	S	S	S	I	S	S	S	S	S

*S, susceptible; I, intermediate; R, resistant.

Up to date, there is no report about the antibiotic susceptibility patterns of non-*E. coli* members among *Enterobacteriaceae* family in healthy adult cattle and sheep at slaughter in Turkey. Whereas, there are some reports addressed to happening the antibiotic resistance of non-*E. coli* members of *Enterobacteriaceae* in foods of animal origin in Turkey. For example, the more recent study, Gundogan and Avci (2013) isolated 63 *Klebsiella* spp. and 45 *E. coli* from 75 samples of raw calf meat (minced), chicken meat, raw milk, white cheese and ice cream collected from various supermarkets, dairy plants and pastry shops in Ankara, Turkey. In their study, antibiotic susceptibility testing was carried out using disk diffusion method and interpreted according to CLSI. They observed that *Klebsiella* spp. isolates have been found resistant to aztroenam (42.9%), ciprofloxacin (23.8%), cefotaxime (17.5%), ceftriaxone (17.5%), ceftazidime (11.1%), amikacin (4.8%) and amoxcillin/clavulanic acid (3.1%). Besides, all *Klebsiella* spp. isolates were susceptible to imipenem, ertapenem and piperacillin/tazobactam. In their study, they used some other antibiotic agents which were not used in our study. As well, Gundogan *et al.* (2011) reported that of 45 *Klebsiella* isolates from 60 calf and chicken meat samples purchased from various supermarkets in Ankara, Turkey, 13 (29%) were produced ESBL. They noticed that all isolates were resistant to two or more antimicrobial agents using disk diffusion method to test their susceptibility for 14 antibiotic agents (data not shown). And all ESBL producing *Klebsiella* isolates were highly resistant to cephalosporins and monobactams. In contrast to our finding, the non-*E. coli* isolate (S71) was not resistant to any of the tested antibiotics, as we knew that S71 isolate might presume to be *Klebsiella* or *Enterobacter* spp. based on biochemical identification results.

In addition, common sources of *E. coli* and *Klebsiella* are feces (of animal and human origin), personnel, water and containers (Slama *et al.*, 2010). Antibiotics used for growth promotion or preventing/ treating bacterial infections in food-animals have executed an immense pressure for the selection of antibiotic resistant commensal and/or pathogenic bacteria, including species from genera *Salmonella*, *Escherichia*, *Campylobacter* and *Enterococcus*. These bacteria may also colonize humans and/or become sources of antimicrobial resistance genes for human endogenous microbiota (Trobos *et al.*, 2009).

Although, meat and its products represent potential hazardous sources of multidrug-resistant and virulent *Klebsiella* species (Gundogan *et al.*, 2011). The above both studies conducted in Ankara, Turkey, reveal that high antibiotic resistance of *Klebsiella* species in

foods of animal origin should be take in consideration in order to help to make known the probability of transmission the resistant *Enterobacteriaceae* members of animal origin at slaughter to human via food chain, in view of the fact that the contamination of foods with resistant *Enterobacteriaceae* may occur in a variety of ways including slaughtering, meat production and processing, distribution, retail marketing and handling. Therefore, no detection of resistance to antibiotics of non-*E. coli* isolate of *Enterobacteriaceae* (isolate S71) in our study, do not necessarily mean that those resistant organisms were absent in the slaughtered animals at Kahramanmaras abattoir because of the limited number of the overall findings including non-*E. coli* isolate of *Enterobacteriaceae* members which was only (n=1; 1.85%) of total isolates in the current study.

4.5.3. ESBL producer detection among the *Enterobacteriaceae* isolates

Double disk synergy (DDS) was used for the phenotypic detection of ESBL producing *Enterobacteriaceae* isolates (Figure 4.10). Eventually, no positive ESBL was detected among the *Enterobacteriaceae* isolates in the current study. This result was appeared to be concordance with Carattoli (2008) who reviewed that ESBLs are widely detected in various human medical institutions but they are not so frequently reported in the bacterial population circulating in animals, this could indicate that these enzymes are less prevalent in animals than in humans, but also that they have not been extensively sought.

Extended spectrum β-lactamases are plasmid-encoded β-lactamases capable of hydrolyzing penicillins, cephalosporins and monobactams (but not cephamycins and carbapenems) and they are usually inhibited by β-lactamase inhibitors (Bradford, 2001). Gram-negative bacteria expressing ESBLs have emerged globally and this has limited the treatment strategies available for bacterial infections, as it is also well known that plasmids carrying genes encoding ESBLs may also carry genes encoding resistance to non β-lactam antibiotics such as aminoglycosides, chloramphenicol and trimethoprim-sulphamethoxazole. Besides, most ESBL producing bacteria are also resistant to fluoroquinolones which even further restricting the treatment options (Paterson *et al.* 2000; Tolun *et al.* 2004; Jensen *et al.*, 2006). Recent reports show that the bacteria producing ESBLs are increased among food producing animals in several countries. While, true prevalence can be underestimated because of the fact that the studies on beta-lactamases including ESBLs in food and companion animals are still limited. Fortunately, these studies can still help us to elucidate what extent the use of antimicrobials in veterinary

medicine contribute the antimicrobial resistance in humans (Carratoli, 2008; Li *et al.*, 2007). The factors leading to the emergence of ESBLs among bacteria of animal origin are not fully clear. The use of ceftiofur which is a third generation cephalosporin in veterinary medicine may have contributed to selection and maintenance of ESBL producing bacteria. In Turkey ceftiofur can easily be purchased even without a veterinary prescription and unfortunately reliable data are lacking on the antibiotic consumption in veterinary medicine in Turkey (Kucukbasmaci *et al.*, 2008). As well, according to a study in Japan, CTX-M-2 type of ESBLproducing *E. coli* might have originated from cattle through the use of cephalosporins such as ceftiofur and cattle could be a reservoir and subsequently been transmitted to the food chain by contamination of cattle carcasses (Shiraki et al., 2004). A study by Kluytmans *et al.* (2012) comparing ESBL-producing *E. coli* isolates in chicken meat and humans found significant similarities between animal and human strains at a molecular genetics level. Therefore, livestock should be considered a potential source of these bacteria in humans.

Since today, only one report was addressed about ESBL producing *Enterobacteriaceae* isolated from healthy food producing animals (Kucukbasmaci *et al.*, 2008) in Turkey. Up to date, our study is the second trial for detection of ESBL positive *Enterobacteriaceae* in healthy food animals at slaughter has been carried out in Turkey. Kucukbasmaci *et al.* (2008) reported that out of 349 samples from 239 cattle, 38 calves and 72 sheep from three different slaughterhouses and two farms, they found (n=5; 2.1%) ESBL producing *Enterobacteriaceae* from healthy cattle fecal samples at slaughter including 3 *E. coli* and 2 *Citrobacter* species by using ESBL phenotypic detection method according to CLSI and different than DDS method, then PCR and sequencing were used to determine the underlying genetic determinants responsible for the ESBL phenotypes. All of the five isolates harboured TEM-1 and SHV (four isolates SHV-5 and one isolate SHV-12) type β−lactamases and none of the isolates harboured CTX-M type ESBLs. Also, they found no ESBL producers in calve and sheep samples. Approximately similar prevalence by Duan *et al.* (2006) noticed a 3.1% prevalance of ESBL producers among *E. coli* isolates from fecal samples of healthy cattle in Hong Kong. Whereas, higher prevalence by Geser *et al.* (2011) found 17.1% prevalence of ESBL producing *Enterobacteriaceae* including 11 *E. coli* and 1 *Citrobacter youngae* from fecal matter of cattle at slaughterhouse after cutting the large intestine with sterile scissors after evisceration, in Switzerland.

Furthermore, ESBL production is particularly high in some *Enterobacteriaceae* species (e.g. *K. pneumoniae, E. coli*), but it has also been spread to other species like *Enterobacter* spp., *Citrobacter* spp., *Salmonella* spp. (Kucukbasmaci *et al.*, 2008). Although, detection of no ESBL producers in our study might be related to the number of bacterial isolates obtained from this study and might not be enough for confirming whether the ESBLs producing *Enterobacteriaceae* of animal origin in Kahramanmaras city is existing or not, so processing the higher numbers of samples is recommended in further studies. This result is consistent with those obtained in previous studies, since there is other reports worldwide about trial to find ESBL production in cattle and sheep slaughtered at abattoir and then without getting positive results. For example: Wasyl *et al.* (2012) reported that the prevalence of *E. coli* with putative extended-spectrum cephalosporin resistance was assessed in cattle, pigs, broilers, layers, and turkey slaughtered in Poland, but no cephalosporin-resistant *E. coli* was found in cattle, i.e., no ESBL producing *E. coli* was detected in cattle samples.

Additionally, during the past decade, extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* have become a matter of great concern in human medicine. ESBL-producing strains are found in the community, not just in hospital-associated patients, which raises a question about possible reservoirs. Recent studies describe the occurrence of ESBL-producing *Enterobacteriaceae* in meat, fish, and raw milk; therefore, the impact of food animals as reservoirs for and disseminators of such strains into the food production chain must be assessed (Geser *et al.* 2011). ESBLs producing *Enterobacteriaceae* have been found in foods of animal origin in Turkey. Such as, Gundogan and Avci (2013) observed ESBL positive *Enterobacteriaceae* from food of animal origin in Ankara, Turkey using DDS test, of 20 ESBL-producing *E. coli,* 5 (25%) were from raw calf meat (minced) and of the 13 ESBL-producing *K. oxytoca* isolates, 4 (30.8%) were from minced meat and of the 5 ESBL-producing *K. pneumonia,* 1 (20%) were from minced meat along with other types of ESBL producing bacteria of other types of animal food products in their study. Similar findings of ESBL positive *Klebsiella* spp. detected by Gundogan *et al.* (2011) in Ankara, Turkey using DDS test. Whereas, lower occurrence of ESBL positive *E. coli* reported in Bolu, Turkey using DDS test (Arslan and Eyi, 2011).

Moreover, there are several reports about the prevalence of ESBL producing *Enterobacteriaceae* on carcasses of slaughtered animal at an abattoir worldwide. For

example, Shiraki *et al.*, (2004) found (n=2; 0.7%) of 270 surface swabs of cattle carcasses at abattoir in Japan and these *E. coli* strains were CTX-M-2 type ESBL producers. As well, Bardon *et al.* (2013) noticed (n=5; 3.57%) of 140 surface swabs of healthy cattle carcasses at an abattoir in Czech Republic and the types of ESBLs were CTM-M-1 in three isolates but both CTX-M-1 and TEM production were found in other two isolates.

Briefly, due to the intensive use of antimicrobial agents for animal food production, the food products like meat and milk are frequently seen with antimicrobial resistant bacteria. Multidrug resistant and ESBL-producing *E. coli* and *Klebsiella* species can be transmitted by different foods, including meat, milk and their products. The increasing prevalence of resistance in the isolates from animal origin may have important therapeutic implications. Thus, monitoring of ESBL-producing *Enterobacteriaceae* should be continued at various levels (animals, human, and environment), while investigating the factors that contribute to their selection and dissemination (Gundogan and Avci, 2013).

5. CONCLUSIONS

This study has led to the first investigation of distribution and diversity of *Enterobacteriaceae* members in large intestine of healthy cattle and sheep at slaughter in Kahramanmaras city located at south of Turkey. In the current study, one of the aims was to identify the *Enterobacteriaceae* members which are normal inhabitant in colon segment of digestive tract of healthy food producing animals at slaughter. Many of the previous studies around the world revealed that commensal *Enterobacteriaceae* in the gut of healthy animals could be pathogenic to animals themselves under some circumstances such as immune system inadequacy of host, or animals could be natural reservoir of those pathogenic strains, such as *E. coli* O157:H7 in colon and rectum of cattle and recently found in sheep and goat, these could be transmitted to human via fecal contamination of food chain and causing a variety of health problems. As well, in Turkey, few reports showed the occurrence of those pathogenic strains of bacteria in animals at slaughter, on carcasses and in foods of animal origin in other regions different than Kahramanmaras city. In the present study, most of the bacterial isolates were *E. coli* and few ones were not *E. coli*, but they were not tested for determination of serotypes and virulence genes, so further study at molecular level might focus on pathogenic properties of the isolates of the present study in order to elucidate the scope of commensal *E. coli* could be changed to pathogenic strains, their spread and possible threats for human and animal health in the current city. Furthermore, the pointed remarks recommend adequate slaughter procedures and meat inspection must be carried out in slaughterhouses to ensure not fecal contamination of the carcasses by pathogenic bacteria.

Additionally, another aims of the current study were to screen the occurrence of antibiotic resistance along with ESBL producer and carbapenem resistant *Enterobacteriaceae* isolates in healthy cattle and sheep at slaughter in Kahramanmaras abattoir. It is known that commensal bacteria of food-producing animals are considered as an important reservoir of antibiotic resistance and lead to health problems in both human and veterinary medicine. Extended spectrum β-lactamases (ESBLs) production is one of the currently most important resistance mechanisms in *Enterobacteriaceae*, which reduces the efficacy even of modern broad-spectrum cephalosporins and monobactams. As a matter of growing concern, resistance caused by ESBLs is often associated with resistance to other classes of antibiotics like fluoroquinolones, aminoglycosides and trimethoprim-sulfmethoxazole. Moreover, the emergence and worldwide spread of carbapenemase

producing (carbapenems resistant) *Enterobacteriaceae* is of great concern to public health services and a major threat to the efficacy of carbapenem antibiotics such as imipenem, ertapenem or meropenem, which are drugs of choice for the treatment of infections due to ESBL producing strains. Since ESBL producing organisms are potential risk for human and animals due to limitation of treatment options in diseased individuals.

As expected from the high level of susceptibilities of isolates to β-lactams in the present study, none of the isolates was ESBL positive. Also, overall results pointed out that the occurrence of antibiotic resistant *Enterobacteriaceae* from food animals at Kahramanmaras abattoir was low and less than 15% of the total isolates. This result was valuable for public health when considering the transmission of β-lactamase resistant bacteria from animal to human. ESBL negative results in the current study, however, do not necessarily mean that ESBL producing organisms are absent in the Kahramanmaras animal herds because of the limited size of the overall taken samples at abattoir. Whereas, the resistance to carbapenems was detected at less than 5% of the isolates but this resistance is of concern and support the data concerning about the increase of resistance to carbapenems and possible transmission from animals to human through food chain. As well, a recent report in Turkey has documented the occurrence of carbapenems resistant *Enterobacteriaceae* in few clinical specimens of hospitalized patients and outpatients. To our knowledge, our study is a first report about the occurrence of carbapenems resistant bacteria of animal origin at slaughter in Turkey.

In fact, due to the intensive use of antimicrobial agents for animal food production, antimicrobial resistant *Enterobacteriaceae* became increased worldwide and could be transmitted to human by different foods, including meat, milk and their products. In addition, the development of resistance to commonly used antimicrobials agents has been associated with limitation in the treatment options. Thus, prudent use of β-lactams, especially the extended-spectrum cephalosporins in animals, would be a critical factor in reducing of β-lactamase producing *Enterobacteriaceae*. Also, monitoring of antibiotic resistance should be continued at various levels (animals, human, and environment), while investigating the factors that contribute to their selection and dissemination.

REFERENCES

Acumedia, 2011. Eosin Methylene Blue Agar, Levine (7103). Document PI 7103, Rev 4, February 2011. Acumedia, 620 Lesher Place, Lansing, MI 48912 USA. Retrieved from http://www.neogen.com/Acumedia/pdf/ProdInfo/7103_PI.pdf

Akkus, F., 1996. Isolation of Verocytotoxin Producing *Escherichia coli* O157:H7 in Minced Beef. Doctorate Thesis. Ankara University Graduate School of Health Sciences. Ankara.

Anderson, R.M., 1999. The Pandemic of Antibiotic Resistance. *Nat. Med*, 5: 147-149.

Andersson, D.I., 2003. Persistence of Antibiotic Resistant Bacteria. *Curr. Opin. Microbiol,* 6(5): 452-456.

Angulo, F.J., Nunnery, J.A., Bair, H.D., 2004. Antimicrobial Resistance in Zoonotic Enteric Pathogens. *Rev Sci Tech Off Int Epiz,* 23(2): 485-496.

Armstrong, G.L., Hollingsworth, J., Morris, J.G., 1996. Emerging Foodborne Pathogens: *Escherichia coli* O157:H7 as a Model of Entry of a New Pathogen Into the Food Supply of the Developed World. *Epidemiol. Rev,* 18: 29-51.

Arslan, S., Eyi, A., 2011. Antimicrobial Resistance and ESBL Prevalence In *Escherichia Coli* From Retail Meats. *Journal of Food Safety*, 31(2): 262-267.

Bach, S. J., McCallister, T. A., Mears, G. J., Schwartzkopf-Genswein, K. S., 2004. Long-haul Transport and Lack of Preconditioning Increases Fecal Shedding of *Escherichia coli* and *Escherichia coli* O157:H7. *Journal of Food Protection*, 67: 672–678.

Bannerman, D.D., Paape, M.J., Hare, W.R., Sohn, E.J., 2003. Increased Levels of LPS-Binding Protein in Bovine Blood and Milk Following Bacterial Lipopolysaccharide Challenge. *Journal of Dairy Science*, 86(10): 3128–3137.

Bardoň, J., Husičková, V., Chromá, M., Kolář, M., 2013. Prevalence and Characteristics of *Escherichia coli* Strains Producing Extended-Spectrum b-Lactamases in Slaughtered Animals in the Czech Republic. *Journal of Food Protection*, 76(10): 1773-1777.

Barnes, J.H., Vaillancourt, J.P., Gross, W.B., 2003. Diseases of Poultry. In: Colibacillosis (Editors: Saif, Y.M., Barnes, H.J., Fadly, A.M.). Blackwell Publishing, Ames, Iowa, USA. pp. 631-652.

Batchelor, M., Threlfall, E.J., Liebana, E., 2005. Cephalosporin Resistance Among Animal-Associated Enterobacteria: A Current Perspective. *Expert Rev Anti Infect Ther,* 3: 403-417.

Bercovier, H., Brenner, D.J., Ursing, J., Steigerwalt , A.G., Fanning, G.R., Alonso, J.M., Carter, G.P., Mollaret, H.H., 1980. Characterization of *Yersinia enterocolitica* sensustricto. *Current Microbiology*, 4: 201–206.

Bettelheim, K.A., 1991. The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. In: The Genus *Escherichia*. Springer-Verslag, New York, N.Y. pp. 2696-2736.

Bettelheim, K.A., 1994. *Escherichia coli* in Domestic Animals and Humans. In: Biochemical Characteristics of *Escherichia coli* (Editor: Gyles, C.L.). CAB International, Wallingford, UK. pp. 3-30.

Black, J.G., 2002. Antimicrobial Therapy: the Resistance of Microorganisms. In: Microbiology Principles and Explorations (Editors: Roesch, B., Swain, E.). (5th Edition). J Wiley and Sons, New York. pp. 342-343.

Blanco, M., Blanco, J.E., Blanco, J., Mora, A., Prado, C., Alonso, M.P., Mouriño, M., Madrid, C., Balsalobre, C., Juárez, A., 1997. Distribution and Characterization of Faecal Verotoxin-Producing *Escherichia coli* (VTEC) Isolated from Healthy Cattle. *Vet. Microbiol*, 54: 309-319.

Blattner, F.R., Plunkett, G., Bloch, C.A., Riley, M., Collado-vides, J., Glasner, J.D., 1997. The Complete Genome Sequence of *Escherichia coli* K-12, *Science*, 277(53): 1453-1462.

Bliska, J.B., Falkow, S., 1994. Interplay between Determinants of Cellular Entry and Cellular Disruption in the Enteropathogenic *Yersinia*. Current Opinion in Infectious Diseases, 7(3): 323–328.

Boone, D.R., Castenholz, R.W., Garrity, G.M., 2001. Bergey's Manual of Systematic Bacteriology (Editor: Garrity, G.M.). (2nd Edition). Springer, New York.

Bradford, P.A., 2001. Extended-Spectrum β-lactamases in the 21st Century: Characterization, Epidemiology, and Detection of this Important Resistance Threat. *Clinical microbiology reviews*, 14(4): 933-951.

Brenner, D.J., 1986. Bergey's Manual of Systematic Bacteriology. In: Facultatively Anaerobic Gram-negative Rods, (Editors: Krieg, N.R.). Williams and Wilkins, Baltimore. pp. 409-516.

Brooks, M.B., Morley, P.S., Dargatz, D.A., Hyatt, D.R., Salman, M.D., 2003. Antimicrobial Susceptibility Testing Practices of Veterinary Diagnostic Laboratories in the U.S. *J Am Vet Med Assoc*, 222: 168-173.

Bruckner, D.A., Colonna, P., Bearson, B.L., 1999. Nomenclature for Aerobic and Facultative Bacteria. *Clin Infect Dis,* 29: 713-723.

Bush, K., 1988. β-lactamase Inhibitors from Laboratory to Clinic. *Clinical Microbiology Reviews*, 1(1): 109–23.

Bush, K., Jacoby, G.A., 2010. Updated Functional Classification of β-lactamases. *Antimicrobial Agents and Chemotherapy*, 54(3): 969–976.

Bush, K., Jacoby, G.A., Medeiros, A.A., 1995. A Functional Classification Scheme for β-Lactamases and Its Correlation with Molecular Structure. *Antimicrobial Agents and Chemotherapy*, 39(6): 1211–1233.

Butler, D.G., Clarke, R.C., 1994. *Escherichia coli* in Domestic Animals and Humans. In: Diarrhoea and Dysentery in Calves (Editor: Gyles, C.L.). CAB International, Wallingford, UK. pp. 91-116.

Callaway, T. R., Keen, J.E., Edrington, T.S., Baumgard, L.H., Spicer, L., Fonda, E.S., Griswold, K.E., Overton, T.R., Van Amburgh, M.E., Anderson, R.C., Genovese, K.J., Poole, T.L., Harvey, R.B., Nisbet, D.J., 2005. Fecal Prevalence and Diversity of *Salmonella* Species in Lactating Dairy Cattle in Four States. *J Dairy Sci*, 88: 3603-3608.

Carattoli, A., 2008. Animal Reservoirs for Extended Spectrum β-lactamase Producers. *Clin Microbiol Infect*., 14 Suppl 1: 117-123.

Castano, A., Garcia Fontan, M.C., Fresno, J.M., Tornadijo, M.E., Carballo, J., 2002. Survival of *Enterobacteriaceae* During Processing of Chorizo de cebolla, a Spanish Fermented Sausage. *Food Control*, 13: 107–115.

Cavaco, L.M., Abitith, E., Aarestrup, F.M., Guardabassi, L., 2008. Selection and Persistence of CTX-M-Producing *Escherichia coli* in the Intestinal Flora of Pigs Treated with Amoxicillin, Ceftiofur, or Cefquinome. *Antimicrob. Ag. Chem*, 52: 3612-3616.

Chen, L., Xiong, Z., Sun, L., Yang, J., Jin, Q., VFDB 2012 update: toward the genetic diversity and molecular evolution of bacterial virulence factors. *Nucleic acids research* 2012, 40 (Database issue), D641-5.

Christensen, H., Nordentoft , S., Olson, J.E., 1998. Phylogenetic Relationship of *Salmonella* Based on rRNA Sequences. *International Journal of Systematic Bacteriology*, 48: 605–610.

CHROMagar, 2013. The Chromogenic Media Pioneer: CHROMagar ECC. Instructions for Use; English Version. Document NT-EXT-016, Version 6, 21-Oct-2013. CHROMagar 4 place du 18 juin 1940, 75006 Paris, France. pp. 1-2.

Cloud, S.S., Rosenberger, J.K., Fries, P.A., Wilson, R.A., Odor, E.M., 1985. *In vitro* and *In vivo* Characterization of Avian *Escherichia coli*. Serotypes, Metabolic Activity and Antibiotic Activity and Antibiotic sensitivity. *Avian Dis*, 29: 1084-1093.

CLSI, 2013. Clinical and Laboratory Standards Institute: Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. CLSI Document M100-S23, Table 2A., *Enterobacteriaceae*. Wayne, PA 19087, USA. 33(1): 44-50

Cogan, T. A., Slader, J., Bloomfield, S.F., Humphrey, T.J., 2002. Achieving Hygiene in the Domestic Kitchen: the Effectiveness of Commonly Used Cleaning Procedures. *J Appl. Microbiol*, 92: 885-892.

Cohen, T., Murray, M., 2004. Modeling Epidemics of Multidrug-Resistant *M. tuberculosis* of Heterogeneous Fitness. *Nat Med,* 10: 1117-1121.

Cullor, J., 1996. Endotoxin and Disease in Food animals. *Comp. Cont. Ed. Food Animal*, 18: 31-38.

Davies, J., Davies, D., 2010. Origins and Evolution of Antibiotic Resistance. *Microbiology Mollecular Biology Reviews*, *74*(3): 417-33. doi: 10.1128/MMBR.00016-10

Delcour, A.H., 2009. Outer Membrane Permeability and Antibiotic Resistance. *Biochim Biophys Acta*, 1794: 808-816.

Dickson, J. S., Anderson, M. E., 1992. Microbiological Decontamination of Food Animal Carcasses by Washing and Sanitizing Systems: A Review. *Journal of Food Protection*, 55: 133–140.

Donnenberg, M., 2010. Principles and Practice of Infectious Diseases. In: *Enterobacteriaceae* (Editors: Mandel, G.L., Bennett, J.E., Dolin, R.). Elsevier, Churchill, Livingstone, London. pp. 2567–2586.

Donnenberg, M.S., 2000. Principles and Practice of Infectious Diseases. In: *Enterobacteriaceae*, (Editors: Mandell, G.L., Benett, J.E., Dolin, R.). Elsevier Churchill Livingstone, New York, NY. pp. 2567-2578.

Drawz, S.M., Bonomo, R.A., 2010. Three Decades of β-Lactamase Inhibitors. *Clinical Microbiology Reviews*, 23(1): 160–201.

Drieux, L., Brossier, F., Sougakoff, W., Jarlier, V., 2008. Phenotypic Detection of Extended-Spectrum β-lactamase Production in *Enterobacteriaceae*: Review and Bench Guide. *Clin. Microbiol. Infect*, 14 Suppl. 1: 90-103.

Duan, R. S., Sit, T. H., Wong, S. S., Wong, R. C., Chow, K. H., Mak, G. C., Yam, W.C., Ng, N.G., Yuen, K.Y., Ho, P. L., 2006. *Escherichia coli* Producing CTX-M β-lactamases in Food Animals in Hong Kong. *Microbial Drug Resistance*, 12(2): 145-148.

Dubey, S., Sharda, R., 2001. Prevalence, Serotypes and Pathogenecity of *E. coli* Associated with Diarrhoea in Goats. *Indian J. Anim. Sc*, 71: 92-94.

Edwards, P.R., Ewingwh, 1972. Identification of *Enterobacteriaceae*. 3rd ed. Burgess: Minneapolis. pp. 1-336.

Eisenstein, B.I., Zalenik, D.F., 2000. Principles and Practice of Infectious Diseases. In: *Enterobacteriaceae* (Editors: Mandell, G.L., Bennett, J.E., Dolin, R.). Philadelphia, Pennsylvania, Churchill Livingstone, USA. pp. 2294-2310.

Enne, V. I., Cassar, C., Sprigings, K., Woodward, M. J., Bennett, P. M., 2008. A High Prevalence of Antimicrobial Resistant *Escherichia coli* Isolated from Pigs and a Low Prevalence of Antimicrobial Resistant *E. coli* from Cattle and Sheep in Great Britain at Slaughter. *FEMS microbiology letters*, 278(2): 193-199.

Esiobu, N., Armenta, L., Ike, J., 2002. Antibiotic Resistance in Soil and Water Environments. *Int J Environ Health Res,* 12: 133-44.

Farmer, J., Boatwright, K.D., Janda, J., 2008. Manual of Clinical Microbiology. In: *Enterobacteriaceae*: Introduction and Identification (Editors: Murray, P.R., Baron, E.J., Jorgensen, J.H., Landry, M.L., Pfaller, M.). ASM Press, Washington DC. pp. 649-669.

Farmer, J.J.III., 1995. Manual of Clinical Microbiology. In: *Enterobacteriaceae*: Introduction and Identification (Editors: Murray, Baron, Pfaller, Tenover, Yolken). American Society for Microbiology, Washington, D.C. pp. 438–449.

Farmer, J.J.III., 1999. Manual of Clinical Microbiology. In: *Enterobacteriaceae*: Introduction and Identification (Editors: Murray, Baron, Pfaller, Tenover, Yolken). (7th Edition). American Society for Microbiology, Washington D.C. pp. 442–458.

Farmer, J.J.III., 2003. Manual of Clinical Microbiology. In: *Enterobacteriaceae*: Introduction and Identification (Editors: Murray, Baron, Pfaller, Jorgensen, Yolken). (8th Edition). American Society for Microbiology, Washington, D.C., pp. 636–653.

Farmer, J.J.III., Davis, B.R., Hickman-Brenner, F.W., McWhorter, A., Hunt ley-Carter, J.P., Asbury, M.A., Riddle, C., Wathen-Grady, H.G., Elias, C., Fanning, G.R., 1985. Biochemical Identification of New Species and Biogroups of *Enterobacteriaceae* Isolated from Clinical Specimens. *Journal of Clinical Microbiology*, 21(1): 46–76.

Feng, P., 2001. Guide to Foodborne Pathogens. In: *Escherichia coli* (Editors: Labbe, G.G., Garcia, S.). John Wiley and Sons, Inc.

Fey, P.D., Safranek, T. J., Rupp, M. E., Dunne, E. F., Ribot, E., Iwen, P. C., Bradford P.A., Angulo F.J., Hinrichs, S. H., 2000. Ceftriaxone-Resistant *Salmonella* Infection Acquired by a Child from Cattle. *New England Journal of Medicine*, 342(17): 1242-1249.

Fisher, J.F., Meroueh, S.O., Mobashery, S., 2005. Bacterial Resistance to β-lactam Antibiotics: Compelling Opportunism, Compelling Opportunity. *Chemical Reviews*, 105(2): 395-424.

Foley, S.L., Lynne, A.M., 2008. Food Animal Associated *Salmonella* Challenges: Pathogenicity and Antimicrobial Resistance. *J Anim Sci,* 86: 173-187.

Foxman, B., 2007. Contributions of Molecular Epidemiology to the Understanding of Infectious Disease Transmission, Pathogenesis, and Evolution. *Ann Epidemiol,* 17: 148-156.

Garrity, G.M., Brenner, D.J., Krieg, N.R., Staley, J.R. (Editors), 2005. Bergey's Manual of Systematic Bacteriology. In: The Proteobacteria. Springer–Verlag. pp. 587–607.

Gay, C.C., Besser, T.E., 1994. *Escherichia coli* in Domestic Animals and Humans. In: *Escherichia coli* Septicaemia in Calves (Editor: Gyles, C.L.). CAB International, Wallingford, UK pp. 75-116.

Georgopapadakou, N.H., Liu, F.Y., 1980. Binding of β-lactam Antibiotics to Penicillin-Binding Proteins of *Staphylococcus aureus* and *Streptococcus faecalis*: Relation to Antibacterial Activity. *Antimicrob Agents Chemother,* 18: 834-836.

Geser, N., Stephan, R., Kuhnert, P., Zbinden, R., Kaeppeli, U., Cernela, N., Haechler, H., 2011. Fecal Carriage of Extended-Spectrum β-lactamase–Producing *Enterobacteriaceae* in Swine and Cattle at Slaughter in Switzerland. *Journal of Food Protection*, 74(3): 446-449.

Giammanco, G., Grimont, P. A. D., Grimont, F., Giammanco, G. M., 2011. Genotyping of the Genus *Proteus* by rpoB Sequence Analysis. *Italian Journal of Public Health*, *61*(7): 1638–1644.

Giri, R. R., Takeuchi, J., Ozaki, H., 2005. Influence of Night Soil Contamination on Activated Sludge Microbial Communities in Bangkok, Thailand. *Ecological Engineering,* 25(4): 395-404.

Gosling, R. J., Clouting, C. S., Randall, L. P., Horton, R. A., Davies, R. H., 2012. Ciprofloxacin Resistance in *E. coli* isolated from Turkeys in Great Britain. *Avian Pathology*, 41(1): 83-89.

Gras, M.H., Druet-Michaud, C., Cerf, O., 1994. La Flore Bacterienne Des Feuilles De Salade Fraiche. *Sciences des Aliments*, 14(2): 173–188.

Greenwood, D., Finch, R., Davey, P., Wilcox, M., 2006. Antimicrobial Chemotherapy. (5th Edition). Oxford University Press Inc, New York.

Griffin, P.M., Tauxe, R.V., 1991. The Epidemiology of Infections Caused by *Escherichia coli* O157:H7, Other Enterohaemorrhagic *E. coli*, and the Associated Hemolytic Syndrome. *Epidemiol. Rev*, 30: 60-98.

Guard-Petter, J., 2001. The Chicken, the Egg and *Salmonella enteritidis*. *Environmental Microbiology*, 3(7): 421–430.

Guarner, F., Malagelada, J. R., 2003. Gut Flora in Health and Disease. *Lancet*, *361*(9356), 512–9. doi:10.1016/S0140-6736(03)12489-0

Gun, A., Yilmaz, A., Turker, S., Tanlasi, A., Yilmaz, H., 2000. Contamination of Bovine Carcasses and Abattoir Environment by *Escherichia coli* O157:H7 in Istanbul. *Int. J. Food. Microbiol,* 2640: 1-6.

Gundogan, N., Avci, E., 2013. Prevalence and Antibiotic Resistance of Extended-Spectrum Beta-lactamase (ESBL) Producing *Escherichia coli* and *Klebsiella* Species Isolated from Foods of Animal Origin in Turkey. *African Journal of Microbiology Research*, 7(31): 4059-4064.

Gundogan, N., Citak, S., Yalcin, E. 2011. Virulence Properties of Extended Spectrum β-Lactamase–Producing Klebsiella Species in Meat Samples. *Journal of Food Protection*, 74(4): 559-564.

Gustavsson, P., Borch, E., 1993. Contamination of Beef Carcasses by Psychrotrophic Pseudomonas and *Enterobacteriaceae* at Different Stages Along the Processing Line. *International Journal of Food Microbiology*, 20: 67–83.

Hall, B.G., Barlow, M., 2005. Revised Ambler Classification of {beta}-lactamases. *Journal of Antimicrobial Chemotherapy*, 55(6): 1050–1051.

Hammerum, A.M., Heuer, O.E., 2009. Human Health Hazards From Antimicrobial Resistant *Escherichia coli* of Animal Origin. *Clinical Infectious Diseases: an official publication of the Infectious Diseases Society of America*, *48*(7): 916–921. doi:10.1086/597292

Hasan, M. M., Tinni, S. R., 2011. Detection of Integrons from *Escherichia coli* Isolates Obtained from Some Selected Animals in Republic of Korea. *Indian journal of microbiology*, 51(2): 144-146.

Hill, A.W., 1994. Escherichia coli in Domestic Animals and Humans. In: Escherichia coli Mastitis (Editor: Gyles, C.L.). CAB International, Willingford, UK. pp. 117-133.

Hodgson, J.C., 1994. *Escherichia coli* in Domestic Animals and Humans. In: Diseases Due to *Escherichia coli* in Sheep (Editor: Gyles, C.L.). CAB International, Willingford, UK. pp. 135-150.

Holt, J. G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T., (9th ed.). 1994. Bergey's Manual of Determinative Bacteriology. (9th Edition). Lippincott Williams and Wilkins, Baltimore, MD.

Hooper, D.C., 1995. Quinolone Mode of Action. *Drugs*, 49: 10-15.

Howe, K., Linton, A.H., Osborne, A.D., 1976. An Investigation of Calf Carcass Contamination by *Escherichia coli* from the Gut Contents at Slaughter. *J Appl. Bacteriol*, 41: 37-45.

Hudson, J.A., King, N.J., Cornelius, A.J., Bigwood, T., Thom, K., Monson, S., 2008. Detection, Isolation and Enumeration of *Yersinia enterocolitica* from Raw Pork. *International Journal of Food Microbiology*, 123: 25–31.

Hudzicki, J., 2009. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. *American Society for Microbiology*. URL (access date: 29.03.2014) http://www.microbelibrary.org/library/laboratory-test/3189-kirby-bauer-disk-diffusion-susceptibility-test-protocol

Hujer, A.M., Kania, M., Gerken, T., Anderson, V.E., Buynak, J.D., Ge, X., Caspers, P., Page, M.G.P., Rice, L., Bonomo, R.A., 2005. Structure-Activity Relationships of Different β -Lactam Antibiotics Against a Soluble Form of *Enterococcus faecium* PBP5, a Type II Bacterial Transpeptidase. *Antimicrobial Agents and Chemotherapy*, *49*(2): 612–618.

Hummel, R., Tschape, H., Witte, W., 1986. Spread of Plasmid-Mediated Nourseothricin Resistance Due to Antibiotic Use in Animal Husbandry. *J Basic Microbiol*, 26: 461-466.

Hurd, H.S., Doores, S., Hayes, D., Mathew, A., Maurer, J., Silley, P., Singer, R.S., Jones, R.N., 2004. Public Health Consequences of Macrolide Use in Food Animals: A Deterministic Risk Assessment. *J Food Prot,* 67: 980-992.

Inat, G., Siriken, B., 2010. Detection of *Escherichia coli* O157 and *Escherichia coli* O157: H7 by the Immunomagnetic Separation Technique and stx1 and stx2 genes by Multiplex PCR in Slaughtered Cattle in Samsun Province, Turkey. *Journal of veterinary science*, 11(4): 321-326.

Iversen, C., Forsythe, S., 2004. Isolation of *Enterobacter sakazakii* and Other *Enterobacteriaceae* from Powdered Infant Formula Milk and Related Products. *Food Microbiology*, 21: 771–777.

Janda, J. M., Abbott, S.L., 1998. The Enterobacteria. Lippincott Raven Publishers, Philadelphia, PA.

Janke, B.H., Francis, D.H., Collins, J.E., Libal, M.C., Zeman, D.H., Johnson, D.D., 1989. Attaching and Effacing *Escherichia coli* Infections in Calves, Pigs, Lambs, and Dogs. *Journal of Veterinary Diagnostic Investigation*, 1(1): 6–11.

Jensen, L. B., Hasman, H., Agersø, Y., Emborg, H. D., Aarestrup, F. M., 2006. First Description of An Oxyimino-Cephalosporin-Resistant, ESBL-Carrying *Escherichia coli* Isolated From Meat Sold in Denmark. *Journal of Antimicrobial Chemotherapy*, 57(4): 793-794.

Jones, D., 1988. Composition and Properties of the Family *Enterobacteriaceae*. *Soc Appl Bact Symp Ser*, 17: 1-19.

Kaper, J.B., Nataro, J.P., Mobley, H.L.T., 2004. Pathogenic *Escherichia coli. Nat. Rev. Microbiol.* 2: 123-141.

Kapperud, G., Bergan, T., 1984. Methods in Microbiology. In: Biochemical and Serological Characterization of *Yersinia enterocolitica* (Editors: Bergan, T., Norris, J.R.). (Vol. 15). Academic Press, London. pp. 295–344.

Kluytmans, J. A., Overdevest, I.T., Willemsen, I., Kluytmansvan den Bergh, M.F., Van der Zwaluw, K., Heck, M., Rijnsburger, M., Vandenbroucke-Grauls, C.M., Savelkoul, P.H., Johnston, B.D., Gordon, D., Johnson, J.R., 2012. Extended Spectrum b-Lactamase Producing *Escherichia coli* from Retail Chicken Meat and Humans: Comparison of Strains, Plasmids, Resistance Genes, and Virulence Factors. *Clin. Infect. Dis*., 56: 478–487.

Kucukbasmaci, O., Ciftcioglu, G., Midilli, K., Issa, G., 2008. Detection of Extended Spectrum β-lactamase Producing *Enterobacteriaceae* from Food Animals in Turkey. *Rev. Med. Vet*, 159: 586-592.

Kummerer, K., 2004. Resistance in the Environment. *J Antimicrob Chemother,* 54: 311-20.

Kurokawa, T., Tanisho, S., 2005. Effects of Formate on Fermentative Hydrogen Production by *Enterobacter aerogenes*. *Marine Biotechnology*, 7(2): 112-118.

Kuyucuoglu, Y., Şeker, E., Uguz, C., Sareyyupoglu, B., Konak, S., 2011. Virulence Genes of Shiga Toxin-Producing *Escherichia coli* O157:H7 Strains Isolated from Calves and Cattle. *Ankara Üniv Vet Fak Derg*, 58: 255-260.

Kuzucu, C., Yetkin, F., Görgeç, S., Ersoy, Y., 2011. Investigation of the Susceptibilities of Extended-Spectrum Beta-lactamase-Producing *Escherichia coli* and *Klebsiella spp*. Strains to Ertapenem and other Carbapenems. *Mikrobiyoloji bulteni*, 45(1): 28-35.

Lal, A., Cheeptham, N., 2007. Eosin-Methylene Blue Agar Plates Protocol. *American Society for Microbiology*. URL (access date: 05.04.2014) http://www.microbelibrary.org/library/laboratory-test/2869-eosin-methylene-blue-agar-plates-protocol

Lampel, K.A., Sandlin, R.C., Formal, S., 2000. Encyclopedia of Food Microbiology. In: *Shigella*: Introduction and Detection by Classical Culture Techniques (Editors: Robinson, R.K., Batt, C.A., Patel, P.D.). Academic Press, London. pp. 2015–2020.

Laven, R. A., Ashmore, A., Stewart, C. S., 2003. *Escherichia coli* in the Rumen and Colon of Slaughter Cattle, with Particular Reference to *E. coli* O157. *The Veterinary Journal*, 165(1): 78-83.

Lehman, D., 2005. Triple Sugar Iron Agar Protocols. *American Society for Microbiology*. URL (access date: 09.04.2014) http://www.microbelibrary.org/library/laboratory-test/2842-triple-sugar-iron-agar-protocols

Levy, S.B., 2001. Antibiotic Resistance: Consequences of Inaction. *Clin Infect Dis 33 Suppl*, 3: 124-129.

Levy, S.B., 2002. The Antibiotic Paradox. How the Misuse of Antibiotics Destroys Their Curative Powers, 2nd ed. Perseus Publishing Services, Cambridge, MA. pp. 149-180.

Levy, S.B., Marshall, B., 2004. Antibacterial Resistance Worldwide: Causes, Challenges and Responses. *Nat. Med*, 10: 122-129.

Li, J., Wang, H., Liu, Y., Lin, M., Liu, X., Hu, X., 2014. Distribution and Diversity of Coliform Bacteria in Estuary of Jiahe River, China. *International Journal of Environmental Research*, 8(2): 501-508.

Li, X. Z., Mehrotra, M., Ghimire, S., Adewoye, L., 2007. β-Lactam Resistance and β-lactamases in Bacteria of Animal Origin. *Veterinary microbiology*, 121(3): 197-214.

Liebana, E., Batchelor, M., Hopkins, K. L., Clifton-Hadley, F. A., Teale, C. J., Foster, A., Barker, L., Threlfall, E. J., Davies, R. H., 2006. Longitudinal Farm Study of Extended-Spectrum β-lactamase-Mediated Resistance. *Journal of clinical microbiology*, 44(5): 1630-1634.

Lim, S. K., Lee, H. S., Nam, H. M., Cho, Y. S., Kim, J. M., Song, S. W., Park, Y.H., Jung, S. C., 2007. Antimicrobial Resistance Observed in *Escherichia coli* Strains Isolated from Fecal Samples of Cattle and Pigs in Korea During 2003–2004. *International journal of food microbiology*, 116(2): 283-286.

Lior, H., 1994. *Escherichia coli* in Domestic Animals and Humans. In: Classification of *Escherichia coli* (Editor: Gyles, C.L.). CAB International, Wallingford, UK. pp. 31-72.

Livermore, D. M., 1995. Beta-Lactamases in Laboratory and Clinical Resistance. *Clinical microbiology reviews*, 8(4): 557-584.

Livermore, D.M., Woodford, N., 2006. The β-lactamase Threat in *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter*. Trends in Microbiology, 14(9): 413–420.

MacWilliams, M.P., 2009a. Indole Test Protocol. *American Society for Microbiology*. URL (access date: 10.04.2014) http://www.microbelibrary.org/library/laboratory-test/3202-indole-test-protocol

MacWilliams, M.P., 2009b. Citrate Test Protocol. *American Society for Microbiology*. URL (access date: 02.04.2014) http://www.microbelibrary.org/library/laboratory-test/3203-citrate-test-protocol

Maddison, J., 2009. Making Drug Choices: Rational Antibacterial Therapy. *Irish Veterinary Journal*, *62*(7): 469–475.

Madigan, M.T., Martinko, J.M., Parker, J., 2003. Brock Biology of Microorganisms. Upper Saddle River, NJ: Prentice Hall/Pearson Education.

Majiduddin, F.K., Materon, I.C., Palzkill, T.G., 2002. Molecular Analysis of Betalactamase Structure and Function. *International Journal of Medical Microbiology*, 292(2): 127–37.

Marshall, B., Petrowski, D., Levy, S.B., 1990. Inter- and Intra Species Spread of *Escherichia coli* in a Farm Environment in the Absence of Antibiotic Usage. *Proc. Natl. Acad. Sci,* 87: 6609-6613.

Massova, I., Mobashery, S., 1998. Kinship and Diversification of Bacterial Penicillin Binding Proteins and β-Lactamases. *Antimicrobial Agents and Chemotherapy*, 42(1): 1-17.

McDevitt, S., 2009. Methyl Red and Voges-Proskauer Test Protocols. *American Society for Microbiology*. URL (access date: 08.04.2014) http://www.microbelibrary.org/library/laboratory-test/3204-methyl-red-and-voges-proskauer-test-protocols

Mead, P.S., Griffin, P., 1998. *Escherichia coli* O157:H7. *Lancet*, 352: 1207-1212.

Meng, J., Doyle, M.P., 1998. Microbiology of Shiga Toxin Producing *Escherichia coli* in Foods. In: *Escherichia coli* O157:H7 and Other Shiga Toxin Producing *E. coli* Strains (Editors: Kasper, J.B., O'Brien, A.D.). *ASM*., Washington DC. pp. 92-108.

Miles, T.D., McLaughlin, W., Brown, P.D., 2006. Antimicrobial Resistance of *Escherichia coli* Isolates from Broiler Chickens and Humans. *BMC Vet. Res*, 2:7.

Miller, V.L., Finlay, B.B., Falkow, S., 1988. Factors Essential for the Penetration of Mammalian Cells by *Yersinia*. *Current Topics in Microbiology and Immunology*, 138: 15–39.

Mishra, A., Sharda, R., Chhabra, D., Moghe, M.N., 2002. *Escherichia coli* Isolates from Domestic Poultry. *Indian J. Anim. Sc*, 79: 727-729.

Mishu, B., Koehler, L.A., Rodrigue, L.D., Brenner, F.H., Blake, P., Tauxe, R.V., 1994. Outbreaks of *Salmonella enteritidis* Infections in the United States, 1985-1991. *Journal of Infectious Diseases*, 169: 547–552.

Moosdeen, F., Williams, J.D., Yamabe, S., 1988. Antibacterial Characteristics of YTR 830, a Sulfone β-Lactamase Inhibitor, Compared with Those of Clavulanic Acid and Sulbactam. *Antimicrobial Agents and Chemotherapy*, 32(6): 925–927.

Morar, M., Wright, G.D., 2010. The Genomic Enzymology of Antibiotic Resistance. *Annu Rev Genet*, 44: 25-51.

Murray, P.R., Rosenthal, K.S., Pfaller, M.A., 2009. Medical Microbiology. MOSBY Elsevier.

Mustak, H. K., Ica, T., Ciftci, A., Diker, K. S., 2012. Plasmid-Mediated Quinolone Resistance in *Escherichia coli* Strains Isolated from Animals in Turkey. *Vet J Ankara Univ*, 59: 255-258.

Nataro, J.P., Bopp, C.A., Fields, P.I., Kaper, J.B., Strockbine, N.A., 2007. Manual of Clinical Microbiology. In: *Escherichia, Shigella*, and *Salmonella* (Editors: Murray, P.R., Baron, E.J., Jorgensen, J.H., Landry, M.L., Pfaller, M.A.). ASM Press, Washington, D.C. pp. 670-687.

Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli. Clin Microbial Rev,* 11: 142-201.

Nordmann, P., Dortet, L., Poirel, L., 2012. Carbapenem Resistance in *Enterobacteriaceae*: Here is the Storm. *Trends in molecular medicine*, 18(5): 263-272.

Normark, B.H., Normark, S., 2002. Evolution and Spread of Antibiotic Resistance. *J. Intern. Med*, 252: 91-106.

O'Hara, C.M., Brenner, F.W., Miller, J.M., 2000. Classification, Identification, and Clinical Significance of Proteus, Providencia, and Morganella. *Clinical Microbiology Reviews*, *13*(4): 534–46.

Ojeniyi, A.A., 1989. Direct Transmission of *Escherichia coli* from Poultry to humans. *Epidemiol. Infect*, 103: 513-522.

Okesola, A. O., Makanjuola, O., 2009. Resistance to Third-Generation Cephalosporins and Other Antibiotics by *Enterobacteriaceae* in Western Nigeria. *American Journal of Infectious Diseases*, 5(1): 17.

Olsen, A., Jonsson, A., Normark, S., 1989. Fibronectin Binding Mediated by a Novel Class of Surface Organelles on *Escherichia coli. Nature*, 338: 652-655.

Ørskov, I., Ørskov, F., Jann, B., Jann, K., 1977. Serology, Chemistry, and Genetics of O and K Antigens of *Escherichia coli. Bacteriol. Rev*, 41: 667-710.

Overdevest, I., Willemsen, I., Rijnsburger, M., Eustace, A., Xu, L., Hawkey, P., Heck, M., Savelkoul, P., Vandenbroucke-Grauls, C., Zwaluw, K., Huijsdens, X., Kluytmans, J., 2011. Extended-Spectrum β-lactamase Genes of *Escherichia coli* in Chicken Meat and Humans, The Netherlands. *Emerg Infect Dis*, 17(7): 1216-22.

Paterson, D. L., Mulazimoglu, L., Casellas, J. M., Ko, W. C., Goossens, H., Von Gottberg, A., Mohapatra, S., Trenholme, G.M., Klugman, K.P., McCormack, J.G.,Victor, L. Y. 2000. Epidemiology of Ciprofloxacin Resistance and Its Relationship to Extended-Spectrum β-lactamase Production in *Klebsiella pneumoniae* Isolates Causing Bacteremia. *Clinical infectious diseases*, 30(3): 473-478.

Paterson, D.L., Bonomo, R.A., 2005. Extended-Spectrum β-Lactamases: a Clinical Update. *Clinical Microbiology Reviews*, 18(4): 657–686.

Periti, P., Mazzei, T., Elena Curti, M., 1998. Efficacy and Safety of High Dose Intravenous Ciprofloxacin in the Treatment of Bacterial Pneumonia. *International journal of antimicrobial agents*, 10(3): 215-222.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell., J., 2004. Does the Use of Antibiotics in Food Animals Pose a Risk to Human Health? A Critical Review of Published Data. *J Antimicrob Chemother*, 53: 28-52.

Poole, K., 2002. Outer Membranes and Efflux: the Path to Multidrug-Resistance in Gram-Negative Bacteria. *Curr Pharm Biotechnol,* 3: 77-98.

Poole, K., 2004. Efflux-Mediated Multiresistance in Gram-Negative Bacteria. *Clin Microbiol Infect*, 10: 12-26.

Potz, N.A.C., Mushtaq, S., Johnson, A.P., Henwood, C.J., Walker, R.A., Varey, E., Warner, M., James, D., Livermore, D.M., 2004. Reliability of Routine Disc Susceptibility Testing by the British Society for Antimicrobial Chemotherapy (BSAC) Method. *J Antimicrob Chemother,* 53: 729-738.

Prescott, J.F., 2000. Antimicrobial Therapy in Veterinary Medicine. In: Antimicrobial Drug Resistance and Its Epidemiology (Editors: Prescott, J.F., Baggot, J.D., Walker, R.D.). (3rd Edition). Iowa State Press, Ames, Iowa.

Quinn, P.J., Markey, B.K., Leonard, F.C., FitzPatrick, E.S., Fanning, S., Hartigan, P.J., 2011. Veterinary Microbiology and Microbial Disease. In: *Enterobacteriaceae*: Differentiation of the *Enterobacteriaceae.* (2nd Edition). ISBN: 978-1-4051-5823-7, Wiley-Blackwell Publishing, Chichester, West Sussex, UK. pp. 700-705.

Rahn, O., 1937. New Principles for the Classification of Bacteria. Zentralbl Bakteriol. Parasitenkd. Infekt ionskr. Hyg. Abt ., 96: 237–286.

Ramirez, M. S., Tolmasky, M. E., 2010. Aminoglycoside Modifying Enzymes. *Drug Resistance Updates*, 13(6): 151-171.

Ramos, S., Silva, N., Caniça, M., Capelo-Martinez, J. L., Brito, F., Igrejas, G., Poeta, P., 2013. High Prevalence of Antimicrobial-Resistant *Escherichia coli* from Animals at Slaughter: A Food Safety Risk. *Journal of the Science of Food and Agriculture*, 93(3): 517-526.

Rasko, D.A., Rosovitz, M.J., Myers, G.S.A., Mongodin, E.F., Fricke, W.F., Gajer, P., Crabtree, J., Sebaihia, M., Thomson, N.R., Chaudhuri, R., Henderson, I.R., Sperandio, V., Ravel, J., 2008. The Pangenome Structure of *Escherichia coli*: Comparative Genomic Analysis of *E. coli* Commensal and Pathogenic Isolates. *Journal of Bacteriology*, 190(20): 6881-6893.

Reid, G., Howard, J., Gan, B.S., 2001. Can Bacterial Interference Prevent Infection. *Trends Microbiol*, 9: 424.428.

Retamar, P., 2010. Ertapenem, in Kucers. The Use of Antibiotics. pp. 526-531.

Riemann, H., 2006. Foodborne Infections and Intoxications, 3rd ed. Elsevier, Boston, MA.

Rincon, A. G., Pulgarin, C., 2003. Photocatalytical Inactivation of *E. coli*: Effect of (continuous–intermittent) Light Intensity and of (suspended–fixed) TiO2 Concentration. *Applied Catalysis B: Environmental*, 44(3): 263-284.

Russo, T., Johnson, J.R., 2000. Proposal for a New Inclusive Designation for Extraintestinal Pathogenic Isolates of *Escherichia coli*: ExPEC. *J. Infect. Dis*, 181: 1753-1754.

Sawers, R.G., 2005. Formate and Its Role in Hydrogen Production in *Escherichia coli*. *Biochemical Society Transactions*, 33: 42–46.

Schroeder, C.M.,White, D.G., Meng, J., 2004. Retail Meat and Poultry as a Reservoir of Antimicrobial Resistant *E. coli*. *Food Microbiol*, 21: 249- 255.

Schwarz, S., Cloeckaert, A., Roberts, M.C., 2006. Antimicrobial Resistance in Bacteria of Animal Origin. In: Mechanisms and Spread of Bacterial Resistance to Antimicrobial Agents (Editor: Aarestrup, F.M.). Washington: ASM Press.

Shields, P., Cathcart, L., 2010. Oxidase Test Protocol. *American Society for Microbiology*. URL (access date: 07.04.2014) http://www.microbelibrary.org/library/laboratory-test/3229-oxidase-test-protocol

Shiraki, Y., Shibata, N., Doi, Y., Arakawa, Y., 2004. *Escherichia coli* Producing CTX-M-2 beta-lactamase in Cattle, Japan. *Emerging infectious diseases*, 10(1): 69-75.

Shooter, R.A., Cooke, E.M., O'Farrell, S., Bettelheim, K.A., Chandler, M.E., Bushrod, F.M., 1974. The Isolation of *Escherichia coli* from a Poultry Packing Station and an Abattoir. *J Hyg*, 73: 245-247.

Singer, R.S., Finch, R., Wegener, H.C., Bywater, R., Walters, J., Lipsitch, M., 2003, Antibiotic Resistance – the Interplay between Antibiotic Use in Animals and Human Beings. *Lancet Infect. Dis*, 3: 47-51.

Singleton, P., Sainsbury, D., 1999. Dictionary of Microbiology and Molecular Biology. 2nd Edition. John Wiley and Sons, Inc.

Slama, K.B., Jouini, A., Sallem, R. S., Somalo, S., Saenz, Y., Estepa, V., Boudabous, A., Torres, C., 2010. Prevalence of Broad-Spectrum Cephalosporin-Resistant *Escherichia coli* Isolates in Food Samples Tunisia, and Characterization of Integrons and Antimicrobial Resistance Mechanisms Implicated. *Int. J. Food Microbiol*, 137: 281-286.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Herman, L., Haesebrouck, F., Butaye , P., 2010. Broad-Spectrum β-lactamases Among *Enterobacteriaceae* of Animal Origin: Molecular Aspects, Mobility and Impact on Public Health. *FEMS Microbiolgy Review*, *34*(3): 295–316.

Sojka, W.J., 1965. *Escherichia coli* Infection in Domestic Animals and Poultry. The Common Wealth Agricultural Bureaux, Farnham Royal, Bucks, England.

Sproer, C., Mendrock, U., Swiderski, J., Lang, E., Stackebrandt , E., 1999. The Phylogenetic Position of *Serratia, Buttiauxella* and Some Other Genera of the Family *Enterobacteriacea*. *International Journal of Systematic Bacteriology*, 49: 1433–1438.

Stender, H., Oliveira, K., Rigby, S., Bargoot, F., Coull, J., 2001. Rapid Detection, Identification, Enumeration of *Escherichia coli* by Fluorescence in Situ Hybridization Using an Array Scanner. *J. Microbiol. Methods*., 45: 31-39.

Strahilevitz, J., Jacoby, G.A., Hooper, D.C., Robicsek, A., 2009. Plasmid-Mediated Quinolone Resistance: A Multifaceted Threat. *Clin Microbiol Rev,* 22: 664-689.

Sutariya, P.H., 1993. Studies on the Biochemical Characters, Drug Resistance, Colicinogeny and Virulence Associated Characters of *E. coli* Isolated from Clinical Samples. Master's Thesis. Gujarat Agricultural University, Anand Campus, Anand.

SvHKS, 2013. Danish Small Animal Veterinary Association: Antibiotic Use Guidelines for Companion Animal Practice. Emdrupvej 28A, 2100 København Ø, Denmark. ISBN 978-87-870703-0-0

Swaminathan, B., Harmon, M.C., Mehlman, I.J., 1982. A Review: *Yersinia enterocolitica. Journal of Applied Microbiology*, 52: 151–183.

Tankovic, J., Perichon, B., Duval, J., Courvalin, P., 1996. Contribution of Mutations in gyrA and parC Genes to Fluoroquinolone Resistance of Mutants of *Streptococcus pneumoniae* Obtained in vivo and in vitro. *Antimicrob Agents Chemother,* 40: 2505-2510.

Tarawneh, K. A., Al-Tawarah, N. M., Abdel-Ghani, A. H., Al-Majali, A. M., Khleifat, K. M., 2009. Characterization of Verotoxigenic *Escherichia coli* (VTEC) Isolates from Faeces of Small Ruminants and Environmental Samples in Southern Jordan. *Journal of basic microbiology*, 49(3): 310-317.

Tarr, P.I., Tran, N.T., Wilson, R., 1999. *Escherichia coli* O157:H7 in Retail Ground Beef in Seattle: Results of a One Year Prospective Study. *J. Food Prot,* 62: 133-139.

Tenover, F.C., 2006. Mechanisms of Antimicrobial Resistance in Bacteria. *American Journal of Medicine*, *119*(6 Suppl 1), S3–10; discussion S62–70. doi:10.1016/j.amjmed.2006.03.011

Tolun, V., Küçükbasmacı, Ö., Törümküney-Akbulut, D., Catal, C., Anğ-Küçüker, M., Anğ, Ö., 2004. Relationship Between Ciprofloxacin Resistance and Extended-Spectrum β-lactamase Production in *Escherichia coli* and Klebsiella *pneumoniae* strains. *Clinical microbiology and infection*, 10(1): 72-75.

Trobos, M., Lester, C.H., Olsen, J.E., Frimodt-Moller, N., Hammerum, A.M., 2009. Natural Transfer of Sulphonamide and Ampicillin Resistance between *Escherichia coli* Residing in the Human Intestine. *Journal of Antimicrobial Chemotherapy*, 63(1): 80–86

Turnidge, J., 2004. Antibiotic Use in Animals – Prejudices, Perceptions and Realities. *J. Antimicrob. Chemother,* 53: 26-27.

Ulukanli, Z., Çavli, P., Tuzcu, M., 2010. Detection of *Escherichia coli* O157: H7 from Beef Doner Kebabs Sold in Kars. *Gazi University Journal of Science*, 19(2): 99-104.

Umolu, P.I., Ohenhen, E.R., Okwu, I.G., Ogiehor, I.S., 2006. Multiple Antibiotic Resistant Index and Plasmid of *Escherichia coli* in Beef in Ekpoma. *Am. J. Sci*, 2(3): 1-4.

Van den Bogaard, A. E., London, N., Driessen, C., Stobberingh, E. E., 2001. Antibiotic Resistance of Faecal *Escherichia coli* in Poultry, Poultry Farmers and Poultry Slaughterers. *Journal of Antimicrobial Chemotherapy*, 47(6): 763-771.

Van den Bogaard, A. E., Stobberingh, E. E., 2000. Epidemiology of Resistance to Antibiotics: Links between Animals and Humans. *International journal of antimicrobial agents*, 14(4): 327-335.

Van Soest, P.J., 1994. Nutritional Ecology of the Ruminant. New York: Cornell University Press.

Vidotto, M.C., Muller, E.E., de Freitas, J.C., 1990. Virulence Factors of Avian *Escherichia coli*. *Avian Dis*, 34: 531-538.

VSPA (World Society for the Protection of Animals), 2006. Resistant Racteria in People and Farm Animals Around the World. http://wspafarmwelfare.org/hhantibiotics.html

Walsh, C., 2000. Molecular Mechanisms that Confer Antibacterial Drug Resistance. *Nature*, 406(6797): 775-781.

Wasyl, D., Hasman, H., Cavaco, L. M., Aarestrup, F. M., 2012. Prevalence and Characterization of Cephalosporin Resistance in Nonpathogenic *Escherichia coli* from Food-Producing Animals Slaughtered in Poland. *Microbial Drug Resistance*, 18(1): 79-82.

Wasyl, D., Hoszowski, A., Zając, M., Szulowski, K., 2013. Antimicrobial Resistance in Commensal *Escherichia coli* Isolated from Animals at Slaughter. Frontiers in Microbiology, 4. doi: 10.3389/fmicb.2013.00221

Wegener, H.C., 2003. Antibiotics in Animal Feed and Their Role in Resistance Development. *Curr. Opin. Microbiol*, 6: 439-445.

Wilke, M.S., Lovering, A.L., Strynadka, N.C.J., 2005. β-lactam Antibiotic Resistance: A Current Structural Perspective. *Current Opinion in Microbiology*, *8*(5): 525–533.

Winokur, P.L., Canton, R., Casellas, J.M., Legakis, N., 2001. Variations in the Prevalence of Strains Expressing an Extended-Spectrum β-Lactamases Phenotype and Characterization of Isolates from Europe, the Americas, and the Western Pacific Region. *Clin. Infect. Dis*, 32: 94-103.

Wright, G.D., 2005. Bacterial Resistance to Antibiotics: Enzymatic Degradation and Modification. *Adv Drug Deliver Rev,* 57: 1451-1470.

Yoshiro, H., Paterson, D.L., 2010. Imipenem, in Kucers. The Use of Antibiotics. pp. 471-479.

Zhaxybayeva, O., Doolittle, W.F., 2011. Lateral Gene Transfer. *Current Biology*, 21(7): 242-246.

CURRICULUM VITAE

Personal Identity

Name : Karwan Hussein Mahmood

Nationality : Iraqi

Date of birth : 01, January, 1986

Place of birth : Diyala, Iraq

Marital Status : Married

Telephone : (+964) 770 193 1986

e-mail : karwanhmahmood@gmail.com

Educational Background

Degree	Place of Education	Date of Graduation
Master Degree	KSU, Graduate School of Natural and Applied Sciences, Department of Bioengineering and Sciences	2014
Bachelor's Degree	Sulaimani University College of Veterinary Medicine	2009

Language Skills

Kurdish, English, Arabic