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CHARACTERIZATION OF DRUG RESISTANCE PATTERNS OF *E. COLI* ISOLATED FROM MILK COLLECTED FROM SMALL SCALE DAIRY FARMS REARED IN HOLETA AND BURAYU, AND MEAT FROM ADDIS ABABA ABATTOIRS ENTERPRISE AND ALEMA FARM SLAUGHTER SLAB

MSc Thesis

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June, 2016

College of Veterinary Medicine and Agriculture, Bishoftu

CHARACTERIZATION OF DRUG RESISTANCE PATTERNS OF *E. COLI* ISOLATED FROM MILK COLLECTED FROM SMALL SCALE DAIRY FARMS REARED IN HOLETA AND BURAYU, AND MEAT FROM ADDIS ABABA ABATTOIRS ENTERPRISE AND ALEMA FARM SLAUGHTER SLAB

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Epidemiology

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June, 2016

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As members of the examining board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by Yohannes Equar Messele entitled as "**Characterization of drug resistance patterns of *E. coli* isolated from milk collected from small scale dairy farms reared in Holeta and Burayu, and meat from Addis Ababa abattoirs enterprise and Alema farm slaughter slab**" and recommend that it be accepted as fulfilling the thesis requirement for the Degree of Masters of Science in Veterinary Epidemiology

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STATEMENT OF AUTHOR

First, I declare that this thesis is my bonafide work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

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ABBREVATIONS

aac (3)-IV	Acetyltransferases- Gentamicin resistance gene
AACA	Addis Ababa City Administration
aadA1	Adenlytransferases-streptomycin resistance gene
ABC	ATP-Binding Cassette
aph	Adenylphosphotransferases- Aminoglycoside resistance gene
AMR	Antimicrobial Resistance
ATP	Adenosine Triphospahate
Bla ($_{CMY}$, $_{SHV}$, ampC, oxa, ctx-M, tem)	Beta-lactam resistance genes
bp	Base pair
cAMP	Cyclic Adenine Monophosphate
catA1	Chloramphenicol resistance gene
CDC	Centers for Disease Control and Prevention
cGMP	Cyclic Guanine Monophosphate
CI	Confidence Interval
CLIS	Clinical and Laboratory Standard Institute
cmlA	Chloramphenicol resistance gene
CMT	California Mastitis Test
dfrA	Dihydrofolate reductase - Trimethoprim resistant gene
DNA	Deoxyribonucleic acid
DHFR	Dihydrofolate reductase
dNTP	Deoxy Nucleotide Tri phosphate
EAggEC	Enteroaggressive E.coli
EDEC	Edema-disease E. coli
EHEC	Enterohaemorrhagic Escherichia coli
EIEC	Enteroinvasive E.coli
ELISA	Enzyme Linked Immunosorbant Assay
EMB	Eosin methylene blue agar

EPEC	Enteropathogenic E.coli
ETEC	Enterotoxigenic E.coli
ere (A)	Erythromycin resistance gene
FAO	Food and Agricultural Organisation
*gyr*A/B	Gyrase enzyme - Quinolones resistance gene
HUS	Hemolytic Uremic Syndrome
IFA	Immuno Fluorescent Assay
ISO	International Organization for Standardization
LIA	Lysine Iron Agar
LPS	Lipopolysacharide
LT	Heat labile enterotoxin
MDR	Multi Drug Resistant
mM	Milli Molar
MR	Methyl Red
MUDHCo	Ministry of Urban Development Housing and Construction
NCCLS	National Committee for Clinical Laboratory Standard
nm	Nano meter
NSF	Non Sorbitol Fermenting
OR	Odds ratio
PABA	P-Amino Benzoic Acid
*par*C /E	Topoisomerase IV- Quinolones resistance gene
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
SPSS	Statistical Package for the Social Sciences
STa	Heat stable enterotoxin
strA/B	Streptomycin resistance gene
STEC	Shiga Toxin-producing *Escherichia Coli*
Stx1	Shigatoxins 1
Stx2	Shigatoxins 2
sulI, II&III	Sulfonamide resistance genes
tet (A-E, M,O,P,Q,S)	Tetracycline resistance genes

TSI	Triple Sugar Iron
VTEC	Verotoxigenic E.coli
µg	Microgram

ABSTRACT

A total of 516 samples were collected from December 2015 to April 2016 from dairy cattle farms and slaughterhouses to investigate the prevalence and antimicrobial resistance trait of *E. coli* isolated from cow milk, beef, mutton, chevon and chicken meat. The overall prevalence of *E. coli* were found to be 79 (15.3%) from both milk and meat. Of these positive cases, the isolation of *E. coli* was the highest in chicken samples 27 (37.0%), followed by 17 (23.3%) in mutton, 15 (20.6%) in chevon, 16 (7.1%) from cow milk and 4 (5.5 %) in beef. Conventional and molecular method were used to identify *E. coli* antimicrobial susceptibility trait. The overall result by disk diffusion showed that significantly high resistance to ampicillin (70.9%) and tetracycline (40.5%). On the other hand, the antibiotic sensitivity test showed that highest number of *E. coli* isolates were sensitive to gentamicin (81%) and chloramphenicol (67.1%). In this study the overall multiple drug resistance was 73.4% and only 7 (8.9 %) of the isolates were sensitive to all antimicrobials tested. Identification of the genes associated with antimicrobial resistance was also done using PCR. The prevalence of *E. coli* isolates carrying antimicrobial resistance gene for tetracycline (*tet(A)),* beta lactams *(blaCMY)* and sulphanamide (*sulI)* genes found in milk, beef, mutton, chevon and chicken meat were significantly high. Fifty six out of the 79 (71%) meat and milk *E. coli* isolates have got multidrug resistance gene to two or more classes of drugs. The associations of antimicrobial resistance phenotypes and resistance genes was also determined. The most common drugs to which isolates demonstrated resistance were consistent with the most common resistance genes detected. Tetracycline, beta lactams and sulphamethoxazole were the top three drugs identified as being the most common for resistance measured either phenotypically or genotypically. So that the rising levels of resistance to multiple antimicrobials dictate the urgent need for frequent and close monitoring of resistance in bacterial pathogens.

Key words: *Antibiotic resistance*, *Dairy farm, Escherichia coli, Meat, Milk, Sloughter house*

1. INTRODUCTION

A number of *E.coli* strains are recognized as important pathogens of colibacillosis in food animals (poultry and ruminants) and some of them can cause severe human diseases such as hemorrhagic colitis and hemolytic uremic syndrome (Ferens and Hovde, 2011). The treatment of illnesses caused by this bacterium often requires antimicrobial therapy. The repeated and unsuitable use of antibiotics has led to an increasing rate of antimicrobial resistance (Mooljuntee *et al*., 2010). There is worldwide concern about the appearance and rise of bacterial resistance to commonly used antibiotics. The evolution, increasing prevalence and dissemination of pathogenic bacteria resistant to multiple antimicrobial agents is currently recognized as one of the most important problems in global public health (Bush, 2010). The rapid spread of antibiotic resistance genes, facilitated by mobile genetic elements such as plasmids and transposons, has led to the emergence of multidrug resistant (MDR) strains of many clinically important species that now frequently leave clinicians out of therapeutic options (Hawkey and Jones, 2009).

In this regard, programs for monitoring resistance have been implemented in many countries for the purpose of protecting the health of humans as well as animals (Li *et al*., 2010). These programs usually monitor indicator bacteria such as *Escherichia coli,* which is commonly found in human and animal intestinal tracts and, as a result of faecal contamination or contamination during food animal milking and slaughtering. Milk and meat are an excellent medium for the growth of numerous microbes which produce consequential spoilage of the milk and meat or infections in consumers (Oliver *et al.*, 2005).

Antibiotic usage selects for resistance not only in pathogenic bacteria but also in the endogenous flora of exposed individuals or populations. Therefore, the antibiotic selection pressure for resistance in bacteria in food animals is high and consequently, their faecal flora contains a relatively high proportion of resistant bacteria (Piddock, 1996). Resistance genes are encoded by plasmids and these are frequently transferred among species, being common in *Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, Enterobacter* spp., *Citrobacter freundii, Salmonella* spp., and *Serratia* spp., and their dissemination is apparently inevitable (De Champs *et al*., 1991).

High prevalence 0157:H7 (Shiga toxin producing) and enterotoxigenic, and high rates of multiple drug resistance in a given area indicates alarming situation that requires prompt prevention and control methods. In Ethiopia, scarce studies have shown that antibiotic resistance development among the bacteria poses a problem of concern. In this regard, a study on *E.coli* from all food samples of bovine origin in some woredas of Tigray has reported high resistance rates (greater than 80%) to cephalothin, chloramphenicol, tetracycline and (greater than 60%) to gentamicin and could be a potential public health threat (Abebe *et al*., 2014). However, there is no any documented information on the molecular pattern of antimicrobial resistance genes of *E.coli* in Ethiopia; therefore, this study was designed with the following objectives:

OBJECTIVES

- To determine the prevalence of *E. coli* from meat of cattle, sheep, goat and chicken and cow milk
- To assess the associations between antimicrobial resistance phenotypes and resistance genes in *E. coli* isolated from meat and milk

2. LITERATURE REVIEW

2.1. The organism and its characteristics

Escherichia coli (*E. coli*) were first described in 1885 by Theodor Escherich. Escherichia, a Bavarian pediatrician, had performed studies on the intestinal flora of infants and had discovered a normal microbial inhabitant in healthy individuals, which he named Bacterium coli commune. In 1919, the bacterium was renamed in his honor to *Escherichia coli*. *E.coli* commonly abbreviated *as E. coli;* is a Gram negative rod-shaped motile or nonmotile, facultative anaerobic, non-spore forming member of the Enterobacteriaceae family found in the gastrointestinal tract of warm-blooded animals and humans (Frydendahl, 2002).

Escherichia coli bacteria normally live in the intestines of healthy people and animals. In 1892, *Escherichia coli* was suggested as an indicator microorganism to monitor quality of water and foods (Purohit & Kapley, 2002). *E. coli* is transmitted by food and water, directly from one person to another, and occasionally through occupational exposure. Most food borne outbreaks have been traced to foods derived from cattle, especially ground beef and raw milk (Gyles, 2007).

2.2. Pathogenesis and virulence factor

There are several highly adapted *E. coli* clones that have acquired specific virulence attributes, which confers an increased ability to adapt to new niches and allows them to cause a broad spectrum of disease. Most varieties of *E. coli* are harmless or cause relatively brief diarrhea. But virulent strains can cause gastroenteritis, urinary tract infections, and neonatal meningitis. It can also be characterized by severe abdominal cramps, diarrhea that typically turns bloody within 24 hours, and sometimes fever. In rarer cases, virulent strains are also responsible for bowel necrosis (tissue death) and perforation without progressing to hemolytic-uremic syndrome, peritonitis, mastitis, septicemia, and Gram-negative pneumonia (Todar, 2007).

The pathogenic group of *E .coli* are divided into six groups on the basis of their virulence properties such as enterotoxigenic (ETEC, causative agent of diarrhea in humans, pigs, sheeps, goats, cattle, dogs and horses), enteropathogenic (EPEC, causative agent of diarrhoea in humans, rabbits, dogs, cats and horses), enteroinvasive (EIEC, found only in humans), verotoxigenic (VTEC, found in pigs, cattle, dogs and cats), enterohaemorrhagic (EHEC, found in human, cattle, and goats) and enteroaggregative *E. coli* (EAggEC) which found only in human (Biswas *et al*., 2006; Xia *et al.*, 2010). In terms of the zoonoses, the most important category is the enterohemorrhagic, which is also the most severe (Acha and Szyfres, 2001).

Pathogenic *E. coli* strains use a multi-step scheme of pathogenesis that is similar to that used by other mucosal pathogens, which consists of colonization of a mucosal site, evasion of host defences, multiplication and host damage. Most of the pathogenic *E. coli* strains remain extracellular, but EIEC is a true intracellular pathogen that is capable of invading and replicating within epithelial cells and macrophages. Other *E. coli* strains might be internalized by epithelial cells at low levels, but do not seem to replicate intracellularly (Nataro and Kaper, 1998).

Virulence factor in *E. coli* include the ability to resist phagocytosis, utilization of highly efficient iron acquisition systems, resistance to killing by serum, production of colicins and adhere, colonize, and invade the hosts' cells. Further to these are the secretion systems, production of cell surface molecules, transport and siderophore formation. The pathogenicity of STEC O157:H7 is associated with a number of virulence factors, including Shiga toxin 1 (encoded by the *stx1* gene), Shiga toxin 2(encoded by the *stx2* gene), intimin (encoded by the *eae A* gene) and enterohaemolysin (encoded by the *Ehly* gene) (Kang *et al*., 2004).

Two of the more prominent virulence factors identified for ETEC strains are expression of fimbrial (pili) antigens that enables the bacteria to adhere to and to elaboration of one or more enterotoxins that influence intestinal secretion of fluids (Holland, 1990) through increase cellular concentrations of cyclic AMP (cAMP) or cGMP. Although, the association with serotypes or serogroups does not confer virulence, results of several studies have shown that ETEC strains are limited to a few serotypes or serogroups (Soderlind *et al*., 1988). The most common observed fimbriae on ETEC from calves with diarrhea are *F5*, also named *K99* and *F41*, but strains with

F165 fimbriae have also been isolated (Contrepois *et al*., 1989). *K99* antigen is a fimbrial adhesion distinct from the capsular polysaccharide *K* antigens (Orskov *et al*., 1975). Two biological classes of enterotoxins are produced by ETEC: heat labile (LT) and heat stable (*STa* and *STb*) (Scotland *et al*., 1985), and most bovine ETEC produce *STa* enterotoxin and *K99* fimbriae (Kaeckenbeeck, 1981). The classification of shiga toxin producing *E. coli* is summarized in Table 1.

Table 1: Classification of shiga-toxin producing *Escherichia coli* (STEC) found in animals

Type	STEC subsets	Common serotypes/ serogroups	Geographical Distribution	Animal reservoir	Site of isolation in animals & derived products
Zoonotic	O157 EHEC	O157:H7	Worldwide, more common in industrialized countries	Cattle, sheep, goats, pigs	Intestine, faeces, meat, milk, cheese
	Non-O157 EHEC	O26,O111, O103,O113, O145	World wide	Cattle, sheep, goats, pigs, chickens	Intestine, faeces, meat, milk, cheese
Potentially zoonotic	None	O17, O56, O87, O108,O109,O130, O136, O149	World wide	Cattle, sheep, goats, pigs	Intestine, faeces, meat
Animal pathogenic	EDEC	O138, O139, O141	World wide	Pigs	Intestine

Source: Adopted from (Gyles, 2007).

2.3. Bacterial isolation

Bacteriological method for detecting pathogens typically involves culturing the organism in selective media and identifying isolates according to their morphological, biochemical, and/or immunological characteristics. This method is sensitive and permits the specific detection of

microorganism of interest in complex environments such as foods and certain clinical samples. However, this method is time consuming and usually requires 5-11 days (Riyaz *et al.,* 2004).

E. coli can be differentiated from other members of the *Enterobacteriaceae* on the basis of a number of sugar-fermentation and other biochemical tests. Classically an important group of tests used for this purpose are known by the acronym IMViC (Table 2). These tested for the ability to produce: indole from tryptophan (I); sufficient acid to reduce the medium pH below 4.4, the break point of the indicator methyl red (M); acetoin (acetyl methyl carbinol) (V); and the ability to utilize citrate (C) (Adams and Moss, 2008). Despite *E. coli* can be identified with a variety of biochemical reactions, the indole test remains the most useful method to differentiate *E. coli* from other members of the *Enterobacteriaceae* (Xia, 2010).

Table 2: The IMViC tests

Bacteria	Indole	Methyl Red	Voges proskauer	Citrate
Escherichia coli	+	+	-	-
Salmonella Typhimurium	-	+	-	+
Shigella	+/-	+	-	-
Klebsiella pneumonia	-	-	+	+
Enterobacter aerogenes	-	-	+	+

Source: Adams and Moss, 2008.

2.4. Serological method

Immunological detection methods, such as ELISA, immunofluorescent antibody (IFA) techniques, and radioimmunoassay, are based on the specific binding of antibodies to antigens.

Compared with the conventional methods, the immunological methods are faster, taking only 10-28 h to have the results. Immunoassays to identify O and H antigens and VT may be used to confirm the identity of the organisms once isolated from clinical, food or environmental samples, while others, including dip stick and membrane technologies, microplate assays and colony immune blotting, are used as rapid methods for detecting the presence of potential pathogens in samples prior to isolation, thus shortening the time for a presumptive diagnosis. Most assays for somatic and flagellar antigens are designed to detect the O157 LPS and H7 flagellar antigen. Enzyme immunoassays for O157 and VT, visual immunoassays for O157 and agglutination tests for O157, H7 and VT are available commercially as kits (Strockbine *et al., 1998)*.

Cho (2012), described that recently a commercial antigen capturing ELISA kit in form of a dipstick (Bovine Enterichek, Biovet) was made available to bovine practicioners and procedures for the rapid detection of *E.coli* K99+ in feces from diarrheic calves at acute stage of clinical disease with diagnostic sensitivity and specificity of 71.4 and 100% respectively in comparison to multiplex real time PCR.

The Plasmatic *E. coli* test kit is a latex agglutination test for the rapid identification of *E. coli* sero group O157. The test is best used in conjunction with Sorbitol MacConkey Agar. *E. coli O157* strains cannot ferment sorbitol and will therefore give colorless colonies. The majority of other *E.coli* strains is capable of fermenting sorbitol and therefore, gives characteristic pink colonies. The non-sorbitol fermenting (NSF) colonies can further tested by the latex agglutination method. A positive result is indicated by agglutination with the test reagent, whilst the control reagent should appear milky and smooth (Shelton *et al*., 2004).

The disadvantages include: (i) because they are based on the antigen and antibody reaction, false positive results may occur; (ii) they are not able to differentiate the dead and living cells since the antigens are present on both dead and living cells; and (iii) there are no isolates available for further studies. Due to these disadvantages, the immunological methods are usually used as a screening method. Immunological techniques can also be used in sample preparation for further bacterial identification, such as immunomagnetic separation (Parham *et al.,* 2003).

2.5. Antibiotic use in food animals

Antimicrobial agents are substances produced by various species of microorganisms (bacteria, fungi, actinomycetes) that suppress the growth of other microorganisms and may eventually destroy them. However, common usage often extends the term “antibiotics” to include synthetic or semisynthetic antibacterial agents, such as sulfonamides and metronidazole, which are not produced by microbes (Chambers and Sande, 1996).

The history of humankind can be regarded from a medical point of view as a struggle against infectious diseases. Infections were the leading cause of death worldwide at the beginning of the 20th century. Since the discovery of penicillin by Alexander Fleming in 1929 and the first introduction of the sulpha drugs by Domagk in 1932, the number of new antimicrobials available has increased tremendously between 1940 and 1960. ‘The era of antibiotics’ led to optimism till the early 1970s that infectious diseases can be controlled and prevented and mankind felt confident that modern medicine would prevail (Yoneyama and Katsumata, 2006).

The discovery of antibacterial agents radically changed the outcome of common human and animal diseases. Antimicrobials, such as antibiotics, are essential in human and veterinary medicine to treat infections caused by bacteria. Antibiotics are used for many purposes, including the therapeutic treatment of clinically sick animals, for disease prophylaxis during periods of high risk of infection, and for promotion of growth and feed efficiency (McEwen and Fedorka, 2002).

Many illnesses that were deadly before antibiotics became available are now readily treatable. However, infections are still the second-leading cause of death worldwide, causing over 13 million deaths each year. This fact is the result of the emergence of new diseases, the re-emergence of diseases once controlled and more specifically of the development of antimicrobial resistance. There is also considerable debate in veterinary medicine regarding use of antibiotics in animals raised for human consumption (food animals). The potential threat to human health resulting from inappropriate antibiotic use in food animals is significant, as pathogenic-resistant

organisms propagated in these livestock are poised to enter the food supply and could be widely disseminated in food products (Garofalo *et al.,* 2007).

The different antibiotics/antibacterial drugs have various targets on the bacteria including 1) cell wall and cell membranes, 2) ribosomes, 3) nucleic acids, 4) bacterial cellular metabolism and 5) bacterial cellular enzymes. There are many different mechanisms by which these agents inhibits the multiplication and growth, and the destruction of bacteria. Among these include 1) Inhibition of cell wall synthesis such as beta lactams, 2) Disruption of cell-membrane function, 3) Inhibition of protein synthesis (both 50S and 30S) 4) Inhibition of nucleic acid synthesis both the DNA synthesis and RNA synthesis and 5) action as antimetabolites. The difference in bacteria & mammalian cells especially the structural and metabolic differences enables the antibiotics or antibacterial agents to cause selective toxicity to the bacterial organisms without causing any damage to the host cells (Brunton *et al*, 2013). Currently there are a number of classes of antibiotics/antibacterial agents that are commonly used to treat bacterial infections (Table 3).

Table 3. Antibiotic targets and mode of action

Antimicrobial	Mode of Action	Resistance Mechanisms	Genes responsible
β-lactams (penicillins, cephalosporins, carbapenums)	Inhibition of enzymes required for peptidoglycan synthesis, alterations in penicillin binding	Production of β-lactamases, changes causing reduced uptake of the drug and active efflux	*bla*, *ampC*, *tem*, *shv*, cmy, *oxa, ctx-M*
Tetracycline	Prevents protein synthesis by binding reversibly to 30 S ribosomal subunit	Altered permeability, active efflux or ribosome protection	*tet (A-E), tet (M,O,P,Q,S)*
Sulfonamides and Trimethoprim	Inhibit conversion of p-amino benzoic acid (PABA) into	Decreased permeability and overproduction of PABA	*sul I, II&III*

	dihydrofolate or inhibit dihydrofolate reductase (DHFR) respectively		
Chloramphenicol	Prevents peptide elongation by binding to 50S ribosomal subunit	Inactivation of the drug by chloramphenicol acetyl transferases	*cat*, *cml*
Aminoglycosides (gentamicin, amikacin, tobramycin, kanamycin,strepto mycin, spectinomycin	Inhibit protein synthesis by binding irreversibly to 30 S ribosomal subunit	Decreased uptake of drug or enzymes modification	*aac*, *aad*, *strA/B, aph,*
Quinolones (nalidixic acid, ciprofloxacin, levofloxacin)	Inhibit DNA synthesis by acting on DNA gyrase and topoisomerase IV	Mutations in genes encoding gyrase or topoisomerase, decreased permeability and active efflux	*gyr*A, *gyr*B, *par*C and *par*E

Source: (Quintiliani *et al*., 1999, Aarts *et al.,* 2006)

2.6. Bacterial resistance strategies

Bacteria have a remarkable ability to adapt to adverse environmental conditions, which is an example of the ancient law of nature of ‘survival of the fittest. Microbial resistance is a natural biological response of microbes to a selective pressure, such as weather conditions, food, oxygen or water availability, or the presence of an antimicrobial drug. When a new class of antibiotic is introduced, it is effective at first, but will eventually select for survival of the small fraction of bacterial populations that have an intrinsic or acquired resistance mechanism (Walsh, 2003).

The ability of bacteria to evolve mechanisms to resist attack by antimicrobials was recognized soon after the widespread deployment of the first antibiotics (Angulo *et al.,* 2004). Increasing prevalence of resistance has been reported in many pathogens over the years in different regions of the world including developing countries (Byarugaba, 2005). In recent years, scientists have begun to understand at the molecular level the sophisticated mechanisms that enable bacteria to fend off or neutralize antibiotics. Further exacerbating the problem, pharmaceutical companies are developing fewer new antibiotics to replace those that are no longer effective (Silbergeld *et al*., 2008). Call for new antibiotic therapies have been issues, but there is continuing decline in the number of approved drugs (Cassir *et al.*, 2014).

Due to normal genetic variation in bacterial populations, individual organisms may carry mutations that render antibiotics ineffective, conveying a survival advantage to the mutated strain. In the presence of antibiotics, advantageous mutations can also be transferred via plasmid exchange within the bacterial colony, resulting in proliferation of the resistance trait (Courvalin, 2008). The emergence of drug resistance has been observed following the introduction of each new class of antibiotics, and the threat is compounded by a slow drug development pipeline and limited investment in the discovery and development of new antibiotic agents (Spellberg *et al*., 2004). Antimicrobial resistance can be classified into 3 groups: intrinsic, mutational and acquired resistance.

Intrinsic resistance refers to an inherent resistance to an antibiotic that is a naturally occurring feature of the microorganism. For example, certain oral bacteria such as many streptococci lack the nitro reductases necessary to convert metronidazole to its active metabolites and therefore are not affected by the drug (Walker, 1996).

Mutational resistance occurs due to a spontaneous chromosomal mutation that produces a genetically-altered bacterial population that is resistant to the drug. Mutations resulting from the change of a single nucleotide base can result in resistance, as has been well documented for aminoglycosides and for rifampin (Walker, 1996).

Acquired resistance refers to the horizontal acquisition from another microorganism of a genetic element that encodes antibiotic resistance. This process can occur by transduction, transformation or conjugation. Transduction is a process by which exogenous DNA is transferred from one bacterium to another by the intervention of a bacteriophage, while transformation is the process by which bacteria acquire segments of DNA that are free in the environment. In conjugation the passage of genetic material occurs by direct cell-to-cell contact, through a sex pilus or bridge. This is the most common mechanism of transferring antibiotic resistance genes (Furuya and Lowy, 2006).

Resistance can be caused by a variety of mechanisms: The presence of an enzyme that inactivates the antimicrobial agent, the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent, mutation in the antimicrobial agent's target which reduces the binding of the antimicrobial agent, posttranscriptional or posttranslational modification of the antimicrobial agent's target which reduces binding of the antimicrobial agent, reduced uptake of the antimicrobial agent, active efflux of the antimicrobial agent and overproduction of the target of the antimicrobial agent. In addition, resistance may be caused by a previously unrecognized mechanism (Maarten, 2001).

E. coli and related bacteria possess the ability to transfer DNA via bacterial conjugation or transduction, which allows genetic material to spread horizontally through an existing population (Figure 1). The process of transduction, which uses the bacterial virus called a bacteriophage, is where the spread of the gene encoding for the Shiga toxin from the *Shigella* bacteria to *E. coli* helped produce *E. coli* O157:H7, the Shiga toxin producing strain of *E. coli* (Brussow *et al.,* 2004).

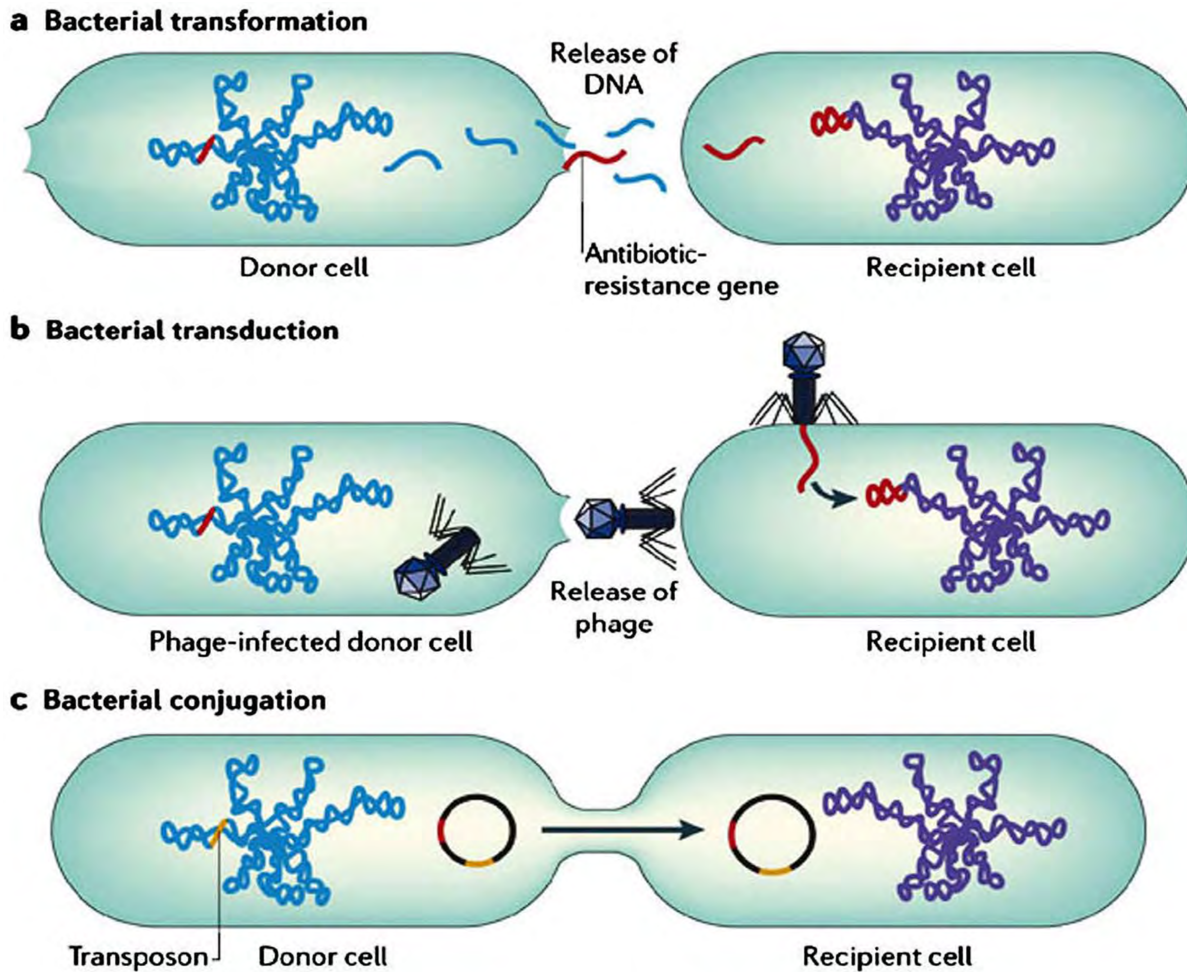

Figure 1. Antimicrobial-resistant bacteria in the community setting (Adopted from Furuya and Lowy, 2006).

In general, bacteria use 3 main strategies to become resistant to different antibiotics: (a) inactivating the antibiotic (Robicsek *et al*., 2006), (b) preventing the drug from reaching its target (Nikaido, 2009), and (c) altering the target (Ince and Hooper, 2003),

2.6.1. Inactivating the antibiotic

It appears that the emergence of antimicrobial resistant bacteria is inevitable to most every new drug and it is recognized as a major problem in the treatment of microbial infections. Some bacteria produce modifying enzymes that reside within or near the cell surface, which selectively target and inactivate the drug. A key feature of the target sites for antimicrobial agents is their vital role in microbial growth and survival (Lambert, 2005). Enzymatic inactivation either by hydrolysis or by modification (group transfer and redox mechanisms) is a major mechanism of resistance to natural antibiotics in pathogenic bacteria. The resistant isolates in most cases inherit the antibiotic-resistance genes on resistance plasmids. These resistance determinants are most probably acquired by pathogenic bacteria from a pool of resistance genes in other microbial genera, including antibiotic producing organisms (Yoneyama and Katsumata, 2006).

2.6.2. Preventing the drug from reaching its target

Increasing the efflux plays a role, especially with hydrophobic compounds that presumably enter the cell *via* diffusion (Silver, 2003). At the same speed where these antimicrobials are entering the cell, efflux mechanisms are pumping them out again, before they reach their target. Members of the ATP-binding cassette (ABC) superfamily are primary transporters using energy liberated by ATP hydrolysis. A mutation resulting in overexpression of a multidrug efflux pump leads to resistance to a wide variety of structurally unrelated antimicrobials. Multidrug resistance proteins (MDRs) or multidrug efflux pumps are widespread in bacteria (Langton, 2005).

2.6.3. Altering the target

The presence of genes affording resistance to self-produced antibiotics, the outer membrane of Gram-negative bacteria, absence of an uptake transport system for the antimicrobial or general absence of the target or reaction hit by the antimicrobial (Wright, 2005).

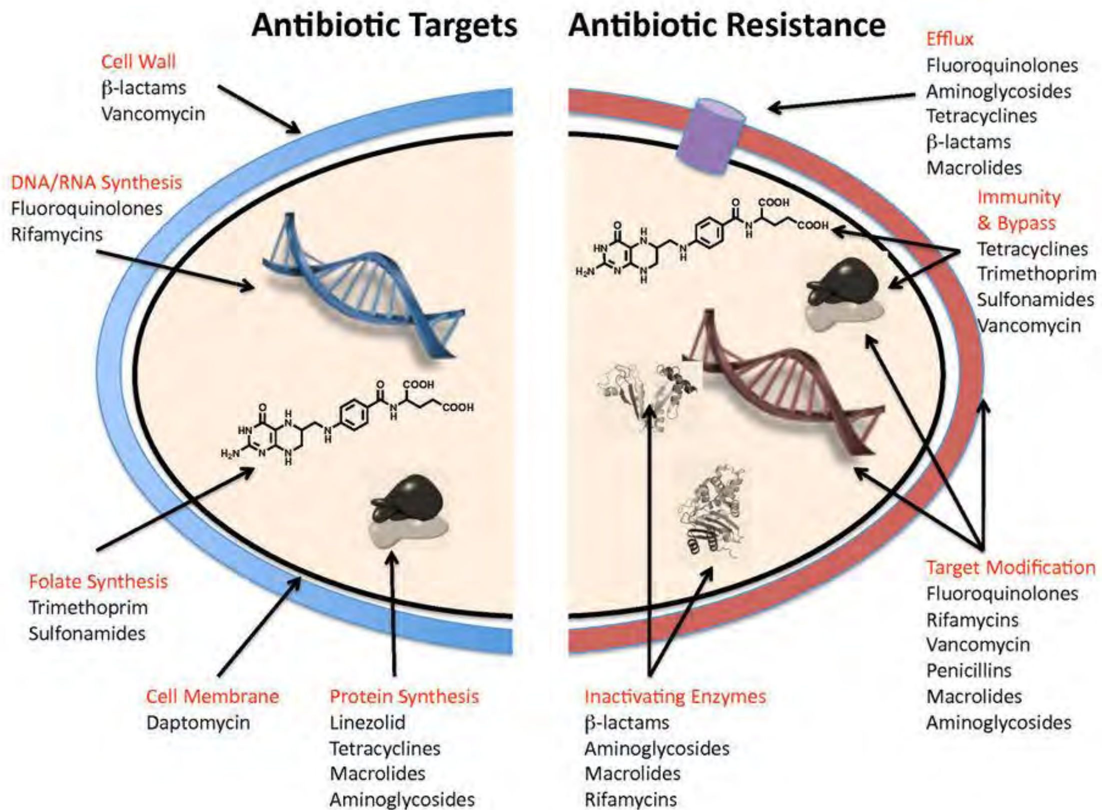

Figure 2. Mechanisms used by common antibiotics to deal with bacteria and ways by which bacteria become resistant to them (Gerard, 2010).

2.7. Detection of antimicrobial resistance

2.7.1. Conventional antimicrobial susceptibility test

Conventional antimicrobial susceptibility testing methods require that pathogens are first isolated from specimens by culture methods. In separate assays, isolated microorganisms are then exposed to various concentrations of antimicrobial agents under specified growth conditions, and the ability of these antimicrobs to inhibit growth is determined. Methods that are frequently used for testing cultivated bacteria and yeasts include disk diffusion, broth dilution, agar dilution, and gradient diffusion (Epsilometer test). Isolated bacteria can also be screened for antimicrobic-

modifying enzymes. The susceptibility of the bacterial isolates to each antimicrobial agent is measured and the result is interpreted in accordance with criteria provided by (CLIS, 2012).

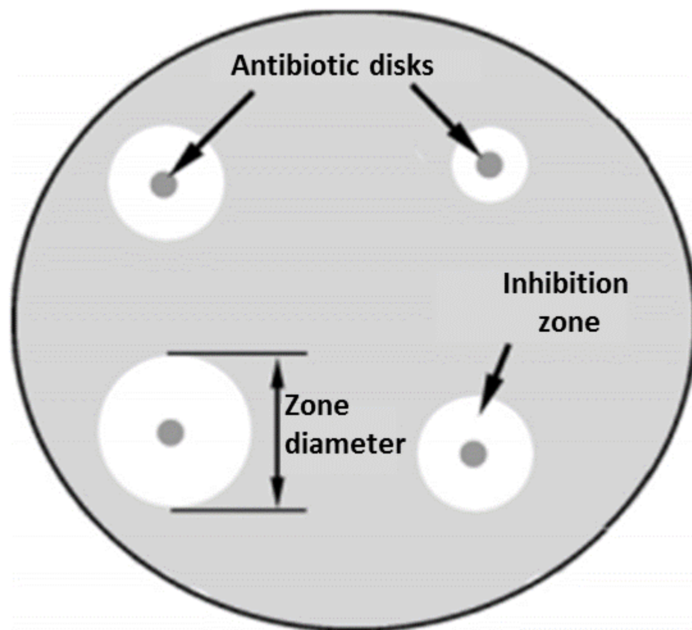

Figure 3. Kirby - Bauer antibiotic sensitivity tests
Source: CLIS, 2012

2.7.2. Molecular methods

Molecular techniques have been under development for the last 30 years, but progress throughout the last decade has been particularly rapid. A comprehensive pheno- and genotyping of resistance and virulence features was performed recently on a selected *E. coli* strain collection of poultry, pig, and bovine origin, representing diverse sample sources from healthy and sick animals (Szmolka *et al*. 2012). It was found, that regard- less of the host source, resistance genes were abundantly present to confer simultaneous resistance to three or more antimicrobial compounds/classes. Results indicated the persistence of a common"multiresistance pattern, "represented by associations between several important antimicrobial classes (and corresponding genes) including aminoglycosides (*aadA1*-likeand *strA/B*), β-lactams (*bla*TEM), sulfonamides (linked mainly to class1 integrons– *intI1*), and tetracyclines [*tet (A)* and *tet (B)]* is especially distributed and maintained in animal husbandry (Szmolka *et al*., 2012).

The molecular characterization of underlying resistance determinants reveal that common co-resistant phenotypes of animal *E.coli* are based on certain genes: ampicillin (*bla*TEM−1-like), streptomycin/spectinomycin (*aadA1*-like and *strA/B*), tetracycline [*tet*(A) and *tet*(B)], sulfamethoxazole (*sul1*),and trimethoprim (*dfrA1* like). Beside these major genes listed, several additional genes encoding the same resistance phenotype were identified: *bla*OXA−1-like (ampicillin), *sul2* (sulfamethoxazole), *dfrA14* and *dfrA17* (trimethoprim) (Guerra *et al.,* 2003; Bonnet *et al*., 2009).

The observation that a high number of same or closely related resistance genes are constantly circulating between bacteria of different species or even genera lead to the recognition, that the horizontal gene transfer represents the most effective tool in the acquisition and widespread dissemination of multiresistance pheno- and genotypes (Ochman *et al*., 2000). Clustering several resistance genes on mobile genetic elements (e.g., plasmids, integrons) ensure not only the co-transfer of these resistance traits, but also contributes to the persistence of resistance in the lack of antimicrobial pressure (Sandvang and Aarestrup, 2000).

Standard PCRs with amplicon sizing by gel electrophoresis are especially useful for identifying genes which encode antimicrobial resistance. These assays are highly specific, especially if there are no other nucleic sequences harbored by the organism which share significant homology with the target genetic material and large quantities of target nucleic acid are amplified. The latter condition exists when organisms are first propagated by culture and then isolated colonies are used for the PCR (Weller *et al.,* 1997).

The advent of DNA amplification by the PCR method and its application has significantly improved the specificity, sensitivity and the time necessary for detection of microbial pathogens in the environment. These molecular techniques are power full enough to detect the pattern of antibiotic resistance genes in bacterial chromosomes and /or plasmids, thus enabling to differentiate horizontal and/or vertical transfer of antimicrobial resistance genes in bacteria. PCR has also become a valuable tool for investigating food-borne outbreaks and identifying pathogens (Riyaz *et al*., 2004).

2.8. Prevention and control of *E. coli*

The evolution of bacterial antibiotic resistances, and its spread and emergence, represent one of the most threatening health care problems with worldwide consequences (Hawkey, 2008). The rise of multi-drug resistance urgently requires a better understanding of the factors and hot spots involved in its diffusion and development. Horizontal gene transfer events are responsible for the acquisition of resistance mechanisms among species and from antibiotic producers to commensal and pathogenic bacteria. Hospitals, human community, farms, aquacultures, and agriculture are reactors where the usage of antibiotics selects for resistant bacteria and promotes gene exchange (Lupo *et al*., 2012).

An effective control program to substantially reduce *E.coli* infections will require the implementation of intervention strategies throughout the food continuum, from farm to table. Promising intervention measures at the farm include competitive exclusion bacteria, bacteriophage, and targeted animal management practices addressing common points of contamination. Consumers also have a role in implementing intervention controls in food handling and preparation. Unfortunately, many consumers eat high-risk foods, improperly handle and store foods, and ignore warnings regarding foods known to be unsafe (Sanchez *et al*., 2002).

Ground beef should be cooked until it is no longer pink. Meat from cattle, like that of other mammalian and avian species, can be contaminated by feces during slaughter and processing. Thus, all precautions should be taken to minimize this risk, and foods of animal origin should be well cooked before they are eaten. Personal hygiene, particularly hand washing after relieving oneself, is also important (Pal, 2007).

In an effort to improve quantity and quality of food, FAO is promoting good management practices in the dairy and beef sector, often in collaboration with the private sector. Including the preparation of manuals such as the FAO Guide to Good Dairy Farming Practice or the development of training material and capacity building interventions in relation to hygienic milk handling and processing but also testing and quality control (Sargeant and Smith, 2003).

3. MATERIALS AND METHODS

3.1. Study area

The research was done from milk collected from small scale dairy farms reared in Holeta and Burayu and meat from Addis Ababa abattoir enterprise and Bishoftu Alema farm slaughter slub with the goal to determine the prevalence and antimicrobial resistance patterns of *E. coli*. Addis Ababa is situated at a latitude of 9°3'North and 38°43'east. It lies in the central highlands of Ethiopia at an altitude of 2500 m.a.s.l. It has an average rainfall of 1800 mm per annum and the annual average maximum and minimum temperature is 26°C and 11°C, respectively; with an overall average of 18.7°C. Addis Ababa has a relative humidity varying from 70% to 80% during the rainy season and 40% to 50% during the dry season (AACA, 2004).

Burayu, Holeta and Bishoftu cities are located in Oromia National Regional State at a distance of 15, 40 and 47 km respectively from Addis Ababa. Burayu is located in 9º02'30''North latitude and 38º03'30''- 38º41'30'' East longitude. Its altitude is 2450–2560 m.a.s.l, it has mean annual temperature of 14ºC and mean annual rainfall of 1188 mm. Bishoftu is located in 8º 43'- 8º 45' North latitude and 38º 56'- 39º 01' East longitude. Its altitude is 1900 -1995 m.a.s.l., mean annual temperature is 27ºC and mean annual rainfall is 746.6 mm. Holeta town is located in 9º30' North latitude and 38º30' East longitude. It has mean annual temperature of 22.2º and mean annual rainfall of 1037mm. Its altitude is 2214–2498 m.a.s.l (MUDHCo, 2013).

3.2. Sample collection

A cross-sectional study was conducted from December 2015 to April 2016 and sampling was held by systematic random sampling technique.

3.2.1. Meat sampling

Sample size from meat was determined using the formula by Thrusfield (2005), based on expected prevalence of *E. coli* O157 in sheep and goat meat, which was estimated at 5% (Mersha

et al., 2009). The confidence level was 95% and the precision was 5%. Thus, the required sample size was 73 for each meat type.

Sample size was determined

$$n = \frac{1.96^2 \, Pexp\,(1\text{-}Pexp)}{d^2}$$

Where, n = required sample size.

Pexp = expected prevalence of *E. coli O157:H7* in goat meat, which was estimated at 5% following Mersha *et al*. (2009).

d = desired absolute precision of 0.05.

A total of 292 meat sample including beef meat (n= 73), sheep meat (n=73), goat meat (n=73) were collected from Addis Ababa municipal abattoir and chicken meat (n=73) from Alema farm slaughter slub located in Bishoftu city. Sample were collected by swabbing from carcass gluteal muscle and inserted in to 10 ml peptone water containing test tube. The Sample were taken following the guidelines of the International Organization for Standardization (ISO, 2013). Each sampling area covered 100 cm^2 by placing a sterile template (10 cm x 10 cm) on the carcass. For each sampling area, a sterile cotton-tipped swab (2 X 3 cm), fitted with shaft, was first moistened in 10 ml of peptone water and then rubbed first horizontally and then vertically several times across the carcass surface. On completion of the rubbing process, the shaft was broken by pressing it against the inner wall of the test tube and disposed leaving the cotton swab in the test tube. The sample were transported to Holeta National Agricultural Biotechnology Research Center in an ice box (+4^{0}C) for further processing and microbiological analysis.

3.2.2. Milk sampling

Milk sample were collected from a total of 14 small scale dairy farms registered at Holeta and Burayu cities. The number of lactating animals ranged 1 to 15 on the visited farms. All cows of the farms were milked by hand and a total of 224 lactating cows with California Mastitis Test positive were included in the study. During sampling of raw milk directly from teats, the udder and teats were cleaned and dried before sampling; each teat end was scrubbed gently with cotton

swabs moistened with 70% ethyl alcohol. The first 3–4 streams of milk were discarded, and approximately 10ml of milk was collected by sterile screw capped bottle. And the sample were transported to Holeta National Agricultural Biotechnology Research Center in an ice box (+4^{0}C) for further processing and microbiological analysis. Isolation and identification of bacteria from milk samples were carried out on the basis of colony morphology in different media, staining characteristics and biochemical properties.

3.3. Isolation of bacteria

The respective specimens from milk and meat were incubated firstly on nutrient agar at 37◦C for 24h and then subcultured on MacConkey agar for 18 to 24 h at 37 °C. *E.coli* isolates revealed characteristics colony morphology such as smooth, circular, white to grayish white colony in nutrient agar. Colonies with the typical color and appearance of *E. coli* were picked and streaked again on blood agar plates and re-streaked on Eosin Methylene Blue (EMB) agar. Green metallic sheen isolates were considered to be *E. coli* and the presumptive colonies were biochemically tested for growth on triple sugar iron agar (TSI) and lysine iron agar (LIA), and for oxidative/fermentative degradation of glucose, citrate utilization, urease production, indole test (tryptophan degradation), glucose degradation (methyl red test) and motility. The *E. coli* isolates revealed a complete fermentation of 5 basic sugars by producing both acid and gas. The isolates also revealed positive reaction in MR test and Indole test but negative reaction in VP test. The *E. coli* isolates were stored in nutrient broth with 15% glycerol at –20 °C (Mooljuntee *et al.,* 2010).

3.4. Antimicrobial susceptibility test

Antimicrobial susceptibility test of the isolates were performed using the Kirby–Bauer disc diffusion method and Mueller–Hinton agar (Merck, Germany) according to Clinical and Laboratory Standards Institute guidelines (CLIS, 2012). Bacterial suspension was prepared by adding 2-4 colonies to a 5 ml tube containing 0.9% normal saline (NaCl), to achieve absorbance of 0.17 – 0.18 at wavelength of 600 nm (equivalent to 0.5 McFarland standards (Walker, 2000)).

The suspension was spread onto Mueller-Hinton agar media using a sterile cotton swab, and the antibiotic disc were laid on the top of the agar plate. Inoculated plates were incubated aerobically at 37°C for 18–24 h, after which antimicrobial susceptibility in the *E.coli* isolates were tested and interpreted according the standard described on Table 4. All *E.coli* isolates were tested for susceptibility and their corresponding concentrations were as follows: tetracycline (30 µg/disk); streptomycin (10 µg/disk); chloramphenicol (30 µg/disk), sulphonametaxazole-trimethoprim (25µg/disk), gentamycin (10 µg/disk); ampicillin, (10µg/disk); erythromycin (15µg/disk); by the Kirby-Bauer disk diffusion method (MAST House, Merseyside, United Kingdom) (Bauer *et al.,* 1966).

Table 4. Inhibition zone diameter interpretation chart for enterobacteriacea

Antibiotics	Resistance (mm)	Intermediate (mm)	Susceptible (mm)
Gentamycin (10 µg)	≤ 12	(12-15)	≥ 15
Erythromycin (15 µg)	≤ 13	(13-18)	≥ 18
Streptomycin (10 µg)	≤ 11	(11-15)	≥ 15
Chloramphenicol (30 µg)	≤ 12	(12-18)	≥ 18
Sulphamethazole/Trimethoprim (25 µg)	≤ 10	(10-16)	≥ 16
Tetracycline (30 µg)	≤ 14	(14-19)	≥ 19
Ampicillin (10 µg)	≤ 13	(13-17)	≥ 17

Source: adapted from Clinical Laboratory Institute Standards (CLIS, 2005)

3.5. Detection of antibiotic resistance genes

3.5.1. DNA extraction

Bacterial strains were grown overnight in nutrient agar (Himedia, India) at 37°C. Bacterial DNA was extracted by boiling a bacterial suspension in water. A loop full of the colonies was added to 100 µl of sterile water. After boiling the suspension for 13 minutes, the suspension were frozen for 5 minutes in ice and centrifuged at 14,000 rpm for 15 min to pellet the cell debris (Reischl *et al*., 2002). The supernatant from the centrifuged tubes was transferred to new 1.5 ml clean plastic tube and used as a template for PCR amplification. The purified DNA were detected by electrophoresis in 1.5 % agarose gel and then stored at -20°C for further use.

3.5.2. Detection of antibiotic resistance genes by PCR

Molecular identification of drug resistance gene from *E. coli* isolates were performed as described by Fode *et al.* (2003). Primers for antibiotics drug resistance genes such as streptomycin (*aadA1*), tetracycline [*tet* (A)], gentamicin [*aac(3)-IV*], sulfonamides (*sul1*), beta-lactams (*bla*SHV, *bla*CMY), erythromycin [*ere*(A)] and chloramphenicol (*catA1, cmlA*) were used from published article by H. Momtaz *et al*., (2012). The specific primer sequences and the predicted size of the amplified products for the different pathogenic gene coding regions are presented in (Annex 1).

Each PCR assay were carried out with a 50 µL mixture containing 10X PCR buffer (5 µL), 2.5 mM $MgCl_2$ (5 µL), 2.5 mM dNTP (1 µL), 5.0 U of Taq DNA polymerase (1 µL) (Himedia, India), 0.5 mM of each primer set (1 µL) and 1 µL of the DNA template. PCR assay were done on a thermocycler using the following cycle: after an initial denaturation cycle of three minutes at 95°C, the reaction mixes will be subjected to 35 amplification cycles of 1 minute at 94°C and 1 minute at 55°C and one minutes at 72°C, and final extension of ten minutes at 72°C. The PCR products were then separated by electrophoresis on a 1.5 % agarose gel, stained with gel red

Huang *et al*., 2010) and visualized using UV illumination. A 100 bp DNA molecular marker were used to determine the size of the amplicons.

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3.6. Statistical analysis

The coded data was entered in MS Excel and then analyzed using the SPSS (statistical package for the social sciences) version 20 software (SPSS Inc., Chicago, IL, USA). The prevalence of *E.coli* in milk and meat samples were determined by dividing the number of positive samples by the total number of samples examined multiplied by 100. P-values were calculated using Chi-square and Odds ratio (OR) analysis to determine significant relationships between various criteria and distribution of antibiotic resistance properties of *E. coli* strain isolated from chicken, mutton, beef, goat meat and cow milk. A P-value less than 0.05 were considered statistically significant.

4. RESULTS

4.1. Prevalence of *E. coli* in milk and meat

From December 2015 to April 2016, a total of 516 samples were collected from dairy cattle farm and slaughterhouses for isolation and identification of bacteria and antimicrobial susceptibility testing. Isolates of *E. coli* were detected in seventy nine samples (15.3%) in both meat and milk samples. Of these positive cases, the isolation of *E.coli* was the highest in chicken samples 27 (37.0%), followed by 17 (23.3%) in mutton, 15 (20.6%) in goat meat, 16 (7.1) from cow milk and 4 (5.5 %) in beef. The test statistics among the types of raw samples indicated that there was significance difference in prevalence (Table 5).

Table 5. Prevalence of *Escherichia coli* isolated from milk and meat

Type of Sample	Total Sample	Number of positives	Apparent prevalence (%)	95 % CI for apparent prevalence	OR	P-value
Cow milk	224	16	7.1	0.9 – 39.5	0.13	
Beef	73	4	5.5	2.1 -13.7	0.10	
Mutton	73	17	23.3	3.4 – 72.3	0.51	< 0.001
Chevon	73	15	20.6	2.9 – 69.3	0.44	
Chicken meat	73	27	37.0	6.6 – 83.1	1.00	
Total	516	79	15.3	12.5 – 18.7		

4.2. Phenotypic antibiotic susceptibility test in *E. coli*

All isolated *E. coli* were confirmed by microbiological examination and further, antibiogram studies were also conducted for the isolates by disk diffusion method using 7 antibiotics which were used frequently for the treatment of animal disease. The overall susceptibility patterns of *E. coli* isolated from various samples is displayed in Table 6. Significantly high resistance were detected to ampicillin (70.9%) followed by tetracycline (40.5%) and Sulphamethazole-trimethoprim (38). On the other hand, the antibiotic sensitivity test showed that highest number of *E. coli* isolates were sensitive to gentamicin (81%) and chloramphenicol (67.1%).

Table 6. Overall antimicrobial susceptibility patterns of *E. coli* isolates

Antimicrobials	Total number of isolates tested	Resistant N (%)	Intermediate N (%)	Sensitive N (%)
Erythromycin	79	29 (36.7)	29 (36.7)	21 (26.6)
Ampicillin	79	56 (70.9)	8 (10.1)	15 (19.0)
Gentamicin	79	3 (3.8)	12 (15.2)	64 (81.0)
Streptomycin	79	27 (34.2)	17 (21.5)	35 (44.3)
Tetracycline	79	32 (40.5)	8 (10.1)	39 (49.4)
Sulphamethazole -trimethoprim	79	30 (38.0)	8 (10.1)	41 (51.9)
Chloramphenicol	79	15 ((19.0)	11 (13.9)	53 (67.1)

Resistance to ampicillin were found 68.7, 50, 64.7, 86.7 and 70.4 % for *E. coli* isolated from cow milk, beef, mutton, chevon and chicken meat respectively. The distribution of antimicrobial resistance within the *E. coli* isolated from meat and milk samples is shown in Table 7. When analyzed by source, *E. coli* isolated from chicken meat were more resistant than those isolated from cow milk, beef, mutton and chevon meat. For individual antimicrobial agents, *E. coli* isolated from chicken meat exhibited a high level of antimicrobial resistance with more than 40% of isolates resistant to each antimicrobial except for gentamycin.

Table 7. Antimicrobial drug resistance phenotypes of *Escherichia coli* isolates from different sources

Antimicrobials	*E.coli* positive sample, n (%)														
	Cow milk (n=16)			Beef meat (=4)			Mutton (=17)			Chevon (n=15)			Chicken meat (n=27)		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Erythromycin	1 (6.3)	2 (12.5)	13 (81.3)	0	2 (50)	2 (50)	9 (53)	7 (41.2)	1 (5.9)	7 (46.7)	8 (53.3)	0	11 (40.7)	10 (37.0)	6 (22.2)
Ampicillin	11 (68.7)	0	5 (31.2)	2 (50)	1 (25)	1 (25)	11 (64.7)	3 (17.6)	3 (17.6)	13 (86.7)	0	2 (13.3)	19 (70.4)	3 (11.1)	5 (18.5)
Gentamicin	0	3 (18.7)	13 (81.2)	0	0	4 (100)	2 (11.8)	1 (5.9)	14 (82.3)	1 (6.7)	1 (6.7)	13 (86.7)	0	7 (26.0)	20 (74)
Streptomycin	4 (25)	2 (12.5)	10 (62.5)	2 (50)	1 (25)	1 (25)	7 (41.2)	3 (17.6)	7 (41.2)	1 (6.7)	5 (33.3)	9 (60)	13 (48.1)	6 (22.2)	8 (29.6)
Tetracycline	2 (12.5)	0	14 (87.5	2 (50)	0	2 (50)	4 (23.5)	2 (11.8)	11 (64.7)	3 (20)	2 (13.3)	10 (66.7)	21 (77.8)	4 (14.8)	2 (7.4)
Sulphamethazole-trimethoprim	8 (50)	0	8 (50)	1 (25)	0	3 (75)	3 (17.6)	3 (17.6)	11 (64.7)	1 (6.7)	2 (13.3)	12 (80)	17 (63)	3 (11.0)	7 (26)
Chloramphenicol	0	2 (12.5)	14 (87.5	1 (25)	1 (25)	2 (50)	2 (11.8)	3 (17.6)	12 (70.6)	1 (6.7)	0	14 (93.3)	11 (40.7)	5 (18.5)	11 (40.7)
$\chi 2$ (P-value)	36.0(< 0. 001)	8.0 (0. 24)	19.7(< 0. 001)	5.9(0. 429)	5.8(0. 441)	6.9(0. 331)	21.0(0. 002)	8.1(0. 232)	36.6 <0. 001	44.3(< 0. 001)	24.3(< 0. 001)	49.0(< 0. 001	43.4(< 0. 001)	8.7(0. 191)	34.8(< 0. 001)

S = Sensitive, I = Intermediate, R = Resistant.

The overall rate of multiple drug resistance was 73.4 % and only 7 (8.9 %) of the isolates were sensitive to seven antimicrobials tested (Table 8).

Table 8. Determination of antimicrobial resistance pattern of *E. coli* isolates using disc diffusion test

Drug type	Number of isolates	Prevalence (%)
Susceptible to all drugs	7	8.9
Resistance to one drug	14	17.7
Resistance to two drugs	26	32.9
AMP*C	1	1.3
AMP*S	3	3.8
AMP*Sxt	5	6.3
AMP*TE	6	7.6
E*AMP	6	7.6
E*S	1	1.3
E*TE	3	3.8
TE*Sxt	1	1.3
Resistance to three drugs	14	17.7
AMP*S*Sxt	2	2.5
AMP*Sxt*C	1	1.3
AMP*TE*C	1	1.3
AMP*TE*Sxt	3	3.8
E*AMP*S	1	1.3
E*AMP*Sxt	2	2.5
S*Sxt*C	1	1.3
S*TE*Sxt	3	3.8
Resistance to four drugs	9	11.4
AMP*S*TE*C	1	1.3
AMP*S*TE*Sxt	1	1.3
E*AMP*GN*S	3	3.8
E*AMP*S*TE	2	2.5
E*AMP*Sxt*C	1	1.3
E*AMP*TE*C	1	1.3
Resistance to five drugs	7	8.9
AMP*S*TE*Sxt*C	2	2.5
E*AMP*S*TE*Sxt	2	2.5
E*S*TE*Sxt*C	3	3.8
Resistance to six drugs	2	2.5
E*AMP*S*TE*Sxt*C	2	2.5
Grand Total	79	100.0

E (erythromycin), AMP (ampicillin), GN (gentamicin), S (streptomycin), TE (tetracycline), SXT (sulphamethazole-trimethoprim), C (chloramphenicol)

The maximum number of antimicrobials to which an isolate demonstrated resistance was 6. Resistance to at least 2 antimicrobials was observed in 73.4% (58/79) of the isolates. The most common pattern found in the multiresistant isolates was ampicillin, tetracycline, sulphamethoxazole, streptomycin, and erythromycin. Of the isolates with resistance to at least two antimicrobials, 53.2% (42/79) had a pattern including ampicillin.

4.3. Genotypic detection of drug resistance in *E. coli*

In the current research DNA templates were prepared from pure cultures of *E.coli* by thermal extraction and the presence of DNA was confirmed by running the extracted DNA samples on gel electrophoresis as shown on Figure 4.

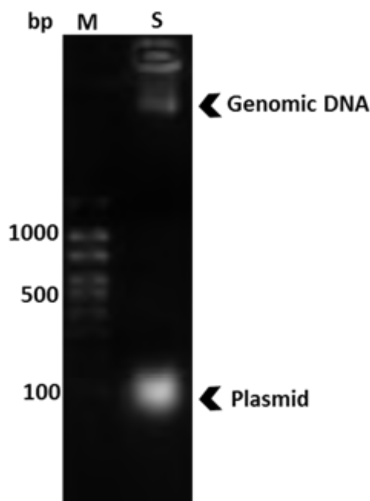


Figure 4. Determination of extracted DNA by gel electrophoresis; bp (base pair), M (marker) and S (sample).

E. coli isolates recovered from milk and meat samples (n=79) were used for PCR analysis of antibiotic resistance pattern. PCR was carried out to reconfirm the phenotypically characterized drug resistance *E. coli* by using gene specific primers. And permit sufficient sensitivity and specificity for the direct detection of *E.coli* in food samples. This study was developed to examine the presence of the following antibiotic resistance gene, such as *blaSHV* and *blaCMY* (extended β-lactam resistance conferring broad resistance to penicillin and cephalosporin), aadA1 (streptomycin resistance), aac (3)-IV (gentamicin resistance), *sul1* (sulfonamide resistance), *ere (A)* (erythromycin resistance), *catA1* and *cml (A)* (chloramphenicol resistance)

and *tetA* (tetracycline resistance) in *E. coli*. The prevalence of different resistance genes varied from 50% for both *cmlA and tet (A)* to 12. 5% for the *aad1*, *sul1*, bla_{SHV}, bla_{CMY}, *ere (A)* and *catA1* gene in *E.coli* isolated from dairy cattle milk. The overall prevalence of resistance gene of *E.coli* isolated from different sample type varies from 62% for *tet (A)* to 1.3 % for *ere (A)* gene. The bla_{SHV}, β-lactamase gene and bla_{CMY} gene encoding cephalosporin resistance was identified in (6.3%) and 54.4 % of the isolates respectively. The gene bla_{CMY} is an important cephalosporinases encoded on the chromosome of many Enterobacteriaceae. The *aac (3)-IV* gene which codes resistance against gentamycin was found also in 19% of isolates. Those most commonly detected genes include *sulI* (sulphonamide resistance), *tetA* (tetracycline resistance) and *blaCMY* (cephalosporin resistance). The resistance genes for erythromycin (*ère(A)*) and streptomycin (*aadA1*) were not detected in *E.coli* isolates from various type of meat. The results obtained from analysis of the antimicrobial resistance genes showed that all the 9 resistance genes were identified in *E. coli* strains isolated from dairy cattle milk (Figure 5 A). However, only 7 resistance genes were confirmed from *E. coli* isolated from meat sample (Figure 5 B). The distribution of resistance genes among *E. coli* isolates is summarized in Table 9.

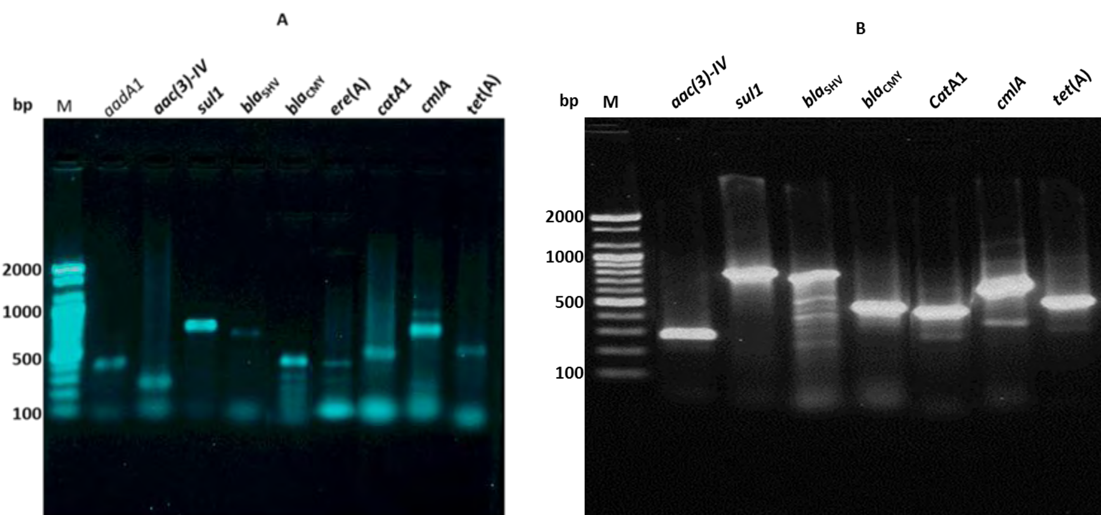


Figure 5. Determination of antimicrobial resistance genes by gel electrophoresis. (A) *E.coli* isolated from cow milk and (B) isolated from meat. The name of specific antibiotic resistance gene are indicated on top. bp= base pair, M= molecular weight standard, *aad1* (447bp) = Streptomycin, *aac (3)-IV* (286 bp) = Gentamicin, *sul1* (822 bp) = Sulfonamide, bla_{SHV} (768 bp) and bla_{CMY} (462 bp) = Beta lactams, *ere(A)* (419 bp) =Erythromycin, *catA1* (547 bp) and *cmlA* (698 bp) = Chloramphenicol and *tet(A)* (577 bp) for Tetracycline

Table 9. Distribution of antimicrobial resistance genes in *E. coli* isolated from different source

Total *E. coli* Positive Samples (n)	Resistance Gene, n (%)								
	aadA1	*aac(3)-IV*	*sul1*	bla_{SHV}	bla_{CMY}	*ere*(A)	*catA1*	*cmlA*	*tet*(A)
Cow milk (16)	2 (12.5)	6 (37.5)	2 (12.5)	2 (12.5)	2 (12.5)	1 (6.3)	2 (12.5)	8 (50)	8 (50.0)
Beef (4)	0	0	1 (25)	0	0	0	1 (25.0)	0	0
Mutton (17)	0	0	5 (29.4)	0	11 (64.7)	0	1 (5.9)	0	11 (64.7)
Chevon (15)	0	1 (6.7)	9 (60.0)	0	14 (93.3)	0	0	0	5 (33.3)
Chicken meat (27)	0	8 (29.6)	19 (70.4)	3 (11.1)	16 (59.2)	0	3 (11.1)	7 (26.0)	25 (92.6)
Total	2 (2.5)	15 (19.0)	36 (45.6)	5 (6.3)	43 (54.4)	1 (1.3)	7 (8.9)	15 (19.0)	49 (62.0)
$\chi 2$	77.1								
P-value	< 0. 001								

aadA1 (streptomycin), *aac (3)-IV* (gentamicin), *sul1* (sulfonamide), bla_{SHV} and bla_{CMY} (beta lactams), *ere (A)* (erythromycin), *catA1* and *cmlA* (chloramphenicol), *tet (A)* (tetracycline)

All the 79 isolates were tested to determine the presence of any antimicrobial resistance gene. Isolates that showed `susceptibility and resistance pattern are shown in Table 10. Overall, 56/79 (71%) isolates from all sample sources have got resistance gene responsible to at least two drug. The most commonly detected genes were tetracycline resistance *tetA,* sulphonamide resistance sulI, bla_{CMY} a gene for cephalosporin resistance and *cmlA* a gene responsible for chloramphenicol resistance. The most common pattern detected was bla_{CMY} and *tetA* together (n=29, 36.7%) followed by *sulI* and *tet (A)* (n=27, 34.2%) and bla_{CMY} and *sulI* (n=27, 34.2%). Of the isolates which have got resistance genes to at least two antimicrobials, 59.5% (47/79) had a pattern including tetracycline *tet (A)*.

Table 10: Molecular determination of antimicrobial resistance gene pattern in *E. coli* isolates

Type of resistance gene	Number of isolates	Prevalence (%)
No resistance gene	11	14.0
Resistance gene to one drug	12	15.2
Resistance gene to two drugs	25	31.6
aac(3)-IV * *bla*$_{CMY}$	1	1.3
aac(3)-IV * *tet*(A)	4	5.1
cmlA * *tet*(A)	3	3.8
bla$_{CMY}$ * *tet*(A)	7	8.9
*sul1** *bla*$_{CMY}$	5	6.3
*sul1** *catA1*	1	1.3
*sul1** *tet*(A)	4	5.1
Resistance to three drugs	17	21.5
aac(3)-IV * *cmlA* * *tet*(A)	2	2.5
*sul1** *bla*$_{CMY}$ * *tet*(A)	10	13.0
*sul1** *cmlA* * *tet*(A)	2	2.5
aac(3)-IV * *sul1** *tet*(A)	1	1.3
bla$_{CMY}$ * *cmlA* * *tet*(A)	1	1.3
bla$_{CMY}$ * *catA1** *tet*(A)	1	1.3
Resistance to four drugs	10	12.6
*aadA1** *sul1** *bla*$_{SHV}$* *cmlA*	2	2.5
bla$_{CMY}$ * *catA1** *cmlA** *tet*(A)	2	2.5
*sul1** *bla*$_{CMY}$ * *catA1** *tet*(A)	2	2.5
aac(3)-IV * *sul1** *bla*$_{CMY}$ * *tet*(A)	2	2.5
*sul1** *bla*$_{CMY}$ * *cmlA* * *tet*(A)	1	1.3
sul1 * *bla*$_{SHV}$ * *bla*$_{CMY}$ * *tet*(A)	1	1.3
Resistance to five drugs	4	5.1
aac(3)-IV * *sul1** *bla*$_{CMY}$ * *cmlA* * *tet*(A)	2	2.5
aac(3)-IV * *sul1** *bla*$_{SHV}$ **bla*$_{CMY}$* *tet*(A)	2	2.5
Grand Total	79	100.0

aad1 (streptomycin), *aac (3)-IV* (gentamicin), *sul1* (sulfonamide), *bla*$_{SHV}$ and *bla*$_{CMY}$ (beta lactams), *ere (A)* (erythromycin), *catA1* and *cml A* (chloramphenicol), *tet (A)* (tetracycline)

4.4. Phenotypic antimicrobial susceptibility and associated resistance genes

The current research showed that fifteen isolates from all sample carried resistance genes to gentamycin, but only three isolates were found resistance phenotypically. Alternatively there were also 28 isolates from all *E. coli* isolates that were classified as resistant to erythromycin based on disk diffusion method but with only one resistance genes identified by molecular method.

Phenotypic and genotypic characterization of the isolates demonstrated comparable patterns. Additionally, the most common drugs to which isolates demonstrated resistance were consistent with the most common resistance genes detected. Tetracycline, beta lactams and sulphamethoxazole were the top three drugs identified as being the most common for resistance measured either phenotypically or genotypically.

Generally an association was detected between certain phenotypes and genotypes. Examples of such isolates from this sample size would include tetracycline and *tet (A)*, Ampicillin and bla_{CMY}, sulphamethazole-trimethoprim and *sulI,* chloramphenicol and *cmlA*. On the other hand, there was a lack of association between erythromycin resistance and *ere (A),* ampicillin and bla_{SHV}, gentamycin and *aac (3)-IV,* streptomycin and *aadA1,* chloramphenicol and *catA1*resistance gene (Table 11).

Table 11. Antimicrobial resistance phenotype and genotype prevalence (n=79)

Antimicrobial	Number of resistance isolates (%)	Antimicrobial	Resistance gene	Number of resistance genes (%)
Erythromycin	28 (35.4)	Erythromycin	*ere(A)*	1 (1.3)
Ampicillin	46 (58.2)	Beta lactams (Ampicillin)	bla_{SHV}	5 (6.3)
			bla_{CMY}	43 (54.4)
Gentamicin	3 (3.8)	Gentamicin	*aac(3)-IV*	15 (19.0)
Streptomycin	27 (34.2)	Streptomycin	*aadA1*	2 (2.5)
Tetracycline	32 (40.5)	Tetracycline	*tet(A)*	49 (62.0)
Sulphamethazole-trimethoprim	30 (38)	Sulphamethazole -trimethoprim	*sul1*	36 (45.6)
Chloramphenicol	15 (19)	Chloramphenicol	*catA1*	7 (8.9)
			cmlA	15 (19.0)

5. DISCUSSION

To the best of our knowledge this study provides some of the first available information describing antimicrobial resistance both phenotypically and genotypically in *E. coli* isolated from milk and meat in Ethiopia. Additionally, it took a novel approach to investigate associations between AMR phenotype and genotype.

E. coli outbreaks have been associated with meat, dairy products, and mayonnaise (Arun, 2006). Transmission to humans occurs through consumption of undercooked meat, unpasteurized dairy products, and water contaminated by feces of carrier animals (Songer and post, 2005). Animals are asymptomatic reservoirs for Shiga toxin-producing *Escherichia coli* (STEC), which are major foodborne pathogens (Karmali *et al*, 2010). The organisms reside in the hindgut of animals and are shed in feces, which can serve as a source of contamination of meat, milk, and water for infections in humans. Human illness from an STEC infection can result in clinical manifestations ranging from mild to bloody diarrhea to potentially life-threatening complications, such as hemolytic uremic syndrome primarily in children, but also in other individuals (Ferens and Hovde, 2011).

Raw meat and milk in value chain is commonly distributed locally to consumers with no controlled measures to maintain the safety and quality before it reaches to consumers in Ethiopia. It has been reported that contamination of *E. coli* occurs in meat and milk chain because of poor hygienic practices (Vahedi *et al*., 2013). So that the purpose of this study is to investigate the prevalence and antimicrobial resistance pattern of *E. coli* isolated from cow milk and different type of meat. This approach may provide insights into the emergence of novel antimicrobial resistance (AMR) genes into the food chain.

5.1. Prevalence of *E. coli* in different types of meat and cow milk

Out of 516 specimens collected, 79 (15.3%) *E. coli* isolates were found from both milk and meat samples. This result is in line with 15.89% prevalence reported from meat samples from Dire Dawa ELFORA and Municipal abattoir (Ousman *et al.,* 2014). The overall prevalence of *E coli* isolate from meat were 63 isolates (21.6%) and these results are in the agreement with previous

studies in which *E coli* species were identified as the most common bacterial enteric pathogen. The prevalence of *E. coli* in the present study was comparable with reports by Haimanot *et al*. (2010) and Bitew *et al*. (2010) with an isolation rate of 26.6 % and 20.3 % respectively. However, significantly higher prevalence of carcass contamination were also reported 40% and 30.97 % by Mekonen *et al.* (2012) and Taye *et al*. (2013) respectively. In this study, the prevalence of *E. coli* in cow milk was also found 7.1% and this is lower than 11.6 % and 26.57% previously reported by Ayano *et al.* (2012) and Sori *et al*. (2005) respectively.

Observed variation in prevalence among studies could be attributed to difference in sampling and isolation procedures, variability in sampled populations, diverse geographical origins of animals, numbers of animals, study design, season, sanitation and treatment with antimicrobial substances during the process. Estimates of the prevalence of *E.coli* among populations of animal vary considerably (NCCLS, 1997).

5.2. Phenotypic antimicrobial susceptibility in *E. coli*

There is a worldwide concern about the appearance and rise of *E. coli* resistance to commonly used antibiotics. In this regard, programs for monitoring resistance have been implemented in many countries for the purpose of protecting the health of humans as well as amals (Aarestrup 2004; Li *et al*. 2010). The overall result showed that significantly high resistance rate to ampicillin (70.9%), tetracycline (40.5%) and sulphamethazole-trimethoprim (38.0%). As expected, the most common resistance were found to older drugs such as ampicillin (Introduced in 1961), tetracycline (Introduced in 1948) followed by sulphamethazole (Introduced in 1936) (Daniel *et al*., 2012).

Wu *et al*. (2010) demonstrated that streptomycin and ampicillin are the two most frequently co-transferred resistance phenotypes among sulfonamide resistant *E. coli* isolates recovered. Daniel *et al.*, (2012) also reported that resistance to sulfonamide was one of the most common resistance profiles identified and 80% and 74% of sulfonamide-resistant *E. coli* isolates were also resistant to tetracycline and streptomycin, respectively.

Similarly, tests conducted on stool samples collected from diarrheic patients in Korem, Ethiopia showed that 53% of *E. coli* strains were found to be resistant to ampicillin, 47% to Chloramphenicol, and 67% to Tetracycline (Meng *et al*., 1998). Although there is significant variation between this study and the present study, there were resistant nature of *E.coli* species to ampicillin and tetracycline. The high resistance to streptomycin in this study is in agreement with Hiko *et al*. (2008) who reported antimicrobial resistance to *E. coli* O157:H7 isolates from raw meat samples to some of above mentioned antimicrobials especially to streptomycin.

The significantly high level of resistance to these antimicrobials was probably an indication of their extensive usage in the veterinary sector for therapeutic and prophylactic purpose both for *E. coli* and other infections. On the other hand, the antibiotic sensitivity test showed that highest number of *E. coli* isolates were sensitive to gentamicin (81%) and chloramphenicol (67.1%). Similar study by Tesfaye *et al.,* (2009) also showed that *E. coli* isolates were sensitive to gentamicin, nitrofurantoin, ciprofloxacin and chloramphenicol.

Recently, multidrug resistant (MDR) phenotypes have been spread widely among Gram negative bacteria. There is concern about the loss of efficacious treatment options as a result of AMR. However, the bigger issue facing veterinarians and the livestock industry is the public health aspect of the issue because of evidence that agricultural use of antimicrobials contributes to increasing AMR in the human population. In this study the overall rate of multiple drug resistance was 73.4% and only 8.9 % of the isolates were sensitive to all antimicrobials tested. This is in agreement with the findings by other researchers, who reported multidrug resistance among *E. coli* O157:H7 isolates (Hiko, 2008). Several studies have investigated the potential link between antimicrobial use and AMR in animals and the development of resistance in humans (Winokur *et al*., 2001 Swartz, 2002). Other studies of commensal and pathogenic resistant bacteria have been conducted in poultry, feedlot, and dairy operations in order to more fully understand the type and level of resistance that is present in livestock (Khachatryan *et al*., 2004, Hershberger *et al*., 2005).

The widespread use of antibiotics in animal husbandry and agriculture for treating or preventing infection and promoting growth, can play an important role in the development and spread of antibiotic resistance. Antimicrobial resistance may arise either spontaneously by selective

pressure or due to antimicrobial misuse by humans or overuse in feeding or treatment of animals by farmers. Resistance development also might be related to exchange of resistance factors between related bacteria (Tenover, 2006). Little information is available regarding the fate of antibiotics in the environment and their link to the emergence of the resistant genotypes. The transfer among microorganisms has long been recognized as a serious threat, contributing to the evolution and emergence of antibiotic resistant bacteria, thereby reducing the therapeutic potential against pathogens (Hawkey and Jones, 2009).

5.3. Genotypic detection of drug resistance in *E. coli*

Identification of genes associated with antimicrobial resistance was accomplished using PCR analysis. The overall prevalence of resistance gene of *E. coli* isolated from different sample type varies from 62% for *tet (A)* to 1.3 % for *ere (A)* gene. The *aac (3)-IV* gene which codes resistance against gentamycin was found in 19% of isolates. Although most isolates were susceptible to gentamicin, it should also be noted that gentamicin use in cattle is discouraged because of prolonged tissue residues (Prescott, 2000).

Similar research in Iran by H. Momtaz *et al*, (2012) found that resistance to tetracycline was the most common finding (91.2%), followed by resistance to sulfamethoxazole (45.6%), chloramphenicol and trimethoprim (29.8%). The prevalence of different resistance genes from pediatric patient *E.coli* isolate were also reported 85.06%, 60.38%, 57.79%, 90.25%, 40.25% and 54.54% positive for *tetA* (tetracycline), *cmlA* (chloramphenicol), *SHV β-lactamase* (cephalithin), *CITM* (ampicillin), *sul1* (sulfonamide) and *aac(3)-IV* (gentamycin) resistance genes respectively by Maryam *et al* (2014).

Gram negative bacteria and related organisms often harbor different plasmids, which confer to them multiple antibiotic resistance to many unrelated antibiotics, contributing to survival in microbial hostile environments. Horizontal transfer of antibiotic resistance gene through conjugative plasmids often occurs among coliforms and Gram negative bacteria, making them important bacteria in the development of multiple antibiotic resistances to several antibiotics (Shakibaie *et al*., 2009).

5.4. Phenotypic antimicrobial susceptibility and associated resistance genes

The relationship between isolates resistant to specific antimicrobials and related resistance genes was expected, since a strong correlation between the phenotypic resistance pattern of a strain and the presence of resistance genes has previously been observed (Shaw *et al*., 1991). In situations where a very strong association was detected between certain phenotypes and genotypes, the phenotypic expression of resistance to a particular antimicrobial may be a good indicator of the underlying resistance gene for that antimicrobial. In this research tetracycline and *tet(A)*, ampicillin and *blaCMY*, sulphamethazole-trimethoprim (*sulI),* chloramphenicol (*cmlA)* were strongly associated. On the other hand, there was a lack of association between erythromycin resistance and *ere (A),* ampicillin and *blaSHV,* gentamycin and *aac (3)-IV,* streptomycin and *aadA1,* chloramphenicol and *catA1*resistance gene.

There are many different genetic determinants of antimicrobial resistance (AMR) and each determinant may present a different distribution among bacterial populations (Lanz *et al*., 2003). Typically, AMR is reported based only on the expressed phenotype derived from susceptibility testing of the organism. However, resistance phenotypes alone do not always represent all of the underlying resistance genes. Alternatively, the presence or absence of a resistance gene does not imply that the particular isolate is resistant or susceptible to an antimicrobial (Aarts *et al*., 2006). Evaluating both phenotype and genotype together provides a more complete understanding of the epidemiology of AMR.

Resistant phenotypes can emerge from many different genetic determinants and each determinant may present unique epidemiological features (Lanz *et al*., 2003). The divergence between genotype and phenotype could simply be explained by not testing for all possible resistance genes or by genes not being turned on within certain isolates. One other explanation for the difference in phenotypic resistance and the presence of resistance genes could be that the breakpoint may be misplaced resulting in misclassification of isolates as susceptible and resistance. Finally, some resistance phenotypes may be caused by point mutations rather than gene acquisition; therefore, no associated resistance gene would be expected. The genotypic and phenotypic polymorphism in this study was also described by Blake *et al*. (2003).

For the phenotypes and resistance genes investigated this study did demonstrate that a phenotype does not necessarily reflect the underlying genotype and that a resistance gene does not always have an expressed phenotype. The associations between phenotype and underlying resistance genes were numerous and complex illustrating the likelihood of molecular linkage of resistance genes in this population. This is important since often only phenotype is reported.

6. CONCLUSION AND RECOMMENDATION

The overall result of *E.coli* isolates showed that significantly high resistance to ampicillin, tetracycline and sulphamethazole-trimethoprim. A strong correlation between the phenotypic resistance pattern of an isolates and the presence of resistance genes has been observed. Multiple antimicrobial resistances were high among the isolated bacteria. However, the results from this study contribute to our understanding regarding the occurrence, distribution, and fate of antibiotic resistance in livestock operations, while adding a new and important perspective to aid in the advancement of this critical problem. Overall observation of this study clearly indicated that the indiscriminate use of different kinds of antibiotics for the treatment of animal disease certainly initiated the emergence of multidrug resistant *E. coli* organisms which are definitely potential threat for animal as well as human health of this region. Without this measure of surveillance, the management of this problem on a gradual basis could result in a further decline of the effectiveness of antimicrobials and additionally lead to a reduction in the number of available antibiotics used to treat animal or human infections. Hence implementation of *E. coli* prevention and control strategies from farm production to consumption of meat and milk products are crucial. This study illustrates the distribution patterns of antimicrobial resistance genes in different sectors; however, further studies are required.

Based on the above findings obtained, the following recommendations are forwarded:

- A comprehensive educational programme about the potential hazards associated with *E.coli* infection and antibiotic resistance in foods of animal origin should be in place.
- Monitoring the use of antibiotics in animal husbandry and human therapy is necessary to minimize the development of antibiotic resistance and to keep these valuable drugs functional.
- Additional studies should be done to elaborate the PCR detection of resistance genes and virulence genes (associated with pathogenicity).
- Plasmid sequencing must be carried out in order to provide a more comprehensive understanding of the plasmids associated with antibiotic resistance gene.

7. REFERENCES

Aarestrup, F.M., Wegener, H.C. and Collignon, P. (2008): Resistance in Bacteria of the food chain epidemiology and control strategies. *Expert Rev. Anti. Infect. Ther.* **6**(5): 733-750.

Aarts, H., Guerra, B. and Malorny, B. (2006): Molecular methods for detection of antimicrobial resistance p.37-48 in Antimicrobial Resistance in Bacteria of Animal Origin F. M. Aarestrup (*ed*.) ASM press, Washington, D.C.

Abebe, M., Hailelule, A., Abrha, B., Nigus, A., Birhanu, M., Adane, H., Genene, T., Daniel, H., Getachew, G., Merga, G. and Haftay A. (2014): Antibiogram of *Escherichia coli* strains isolated from food of bovine origin in selected Woredas of Tigray, Ethiopia. *J. Bac. Res*. **6**(3): 17-22.

Acha, P. N. and Szyfres, B. (2001): Colibacillosis. In: Zoonoses and Communicable Diseases Common to Man and Animals: Bacterioses and Mycoses. Pan American Health Organization, Washington, D.C. 3rd *ed*. 90-97.

Adam, M. and Moss, M. (2008): Food Microbiology. 3rd Edition. Royal Society of Chemistry, Cambridge. 216-224.

Addis Ababa City Administration (AACA) (2004): Proclamation for establishment of "Kifle Ketema" and "Kebele" in Addis Ababa city, Addis Ababa, Ethiopia

Angulo, F., Nargund, V. and Chiller, T. (2004): Evidence of an Association between Use of Anti-microbial Agents in Food Animals and Anti-microbial Resistance among Bacteria Isolated from Humans and the Human Health Consequences of Such Resistance. *J.Vet.Med.B.Infect.Dis.Vet.Public Health*. 51: 374-37

Arun, K. (2008): Foodborne Microbial Pathogens: Mechanisms and Pathogenesis. Food science text series. Purdue University West Lafayette, IN USA, 184.

Ayano, A., Hirko, F., Simayalew, A. and Yohannes, A. (2013): Prevalence of subclinical mastitis in lactating cows in selected commercial dairy farms of Holeta district. *J Vet. Med. Anim. Health.* **5**(3): 67-72.

Bauer, A., Kirby, W., Sherris, J. and Turck, M. (1966): Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol.* **45**(4): 493–6.

Bitew, M., Tafere, A. and Tolosa, T. (2010). Study on bovine mastitis in dairy farms of Bahir Dar town and its environment. *J. Anim. Vet. Adv*. 9: 2912- 2917.

Biswas, P., Uddin, G., Barua, H., Roy, K., Biswas, D., Ahad, A. and Debnath, N. (2006): Causes of loss of Sonali chickens on small holder households in Bangladesh. *Prev. Vet. Med.* **76**: 185-195.

Blake, D., Humphry, R., Scott, K., Hillman, K., Fenlon, D. and Low, J. (2003): Influence of tetracycline exposure on tetracycline resistance and the carriage of tetracycline resistance genes within commensal *Escherichia coli* populations. *J. App. Microbiol*. **94**: 1087-1097.

Brussow, H., Canchaya, C. and Hardt, W. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol and Molecu Bio. Rev*. **68** (3): 560–602.

Bush, K. (2010): Alarming β-lactamase-mediated resistance in multidrug-resistant enterobacteriaceae. *Curr Opin Microbiol.* **13**: 558–564.

Bonnet, C., Diarrassouba, F., Brousseau, R., Masson, L., Topp, E., and Diarra, M. (2009): Pathotype and antibiotic resistance gene distributions of *Escherichia coli* isolates from broiler chickens raised on antimicrobial- supplemented diets. *Appl.Env. Microbiol.* **75**: 6955–6962.

Brunton, L.L., Chabner, B.A. and Knollmann, B.C. (2013): Chemotherapy of microbial diseases: Goodman & gilman's the pharmacological basis of therapeutics, 12*ed*. McGraw-Hill Global Education Holdings, LLC, Section VII.

Byarugaba, D. (2005). Antimicrobial resistance and its containment in developing countries. In Antibiotic Policies: Theory and Practice, *ed*. I. Gould and V. Meer, 617–646.

Cassir, N., Rolain J. and Brouqui P. (2014): A new strategy to fight antimicrobial resistance: the revival of old antibiotics. *Front in microbiol.* **5**: 551.

Chambers, H. and Sande, M. (1996): Antimicrobial agents. General considerations. In: Gilman AG, Rall TW, Nies AS, Taylor P. The pharmacological basis of therapeutics. New York: *Pergamon Press*: 1026-9.

Cho Yong-il (2012): Ecology of calf diarrhea in cow-calf operations. Iowa State University Graduate theses and dissertation paper. 126-142.

Clinical and Laboratory Standards Institute (2012): Performance standards for antimicrobial susceptibility testing; twenty-second informational supplement. Wayne, Pa: M100-S21, CLS.

Clinical Laboratory Institute Standards (2005): Performance standards for antimicrobial susceptibility testing; fifteenth information supplement CLSI document M100-S15. **26** (3).

Contrepois, M., Fairbrother, J., Kaura, Y. and Girardeau, J. (1989): Prevalence of CS31 A and F165 surface antigens in Escherichia coli isolates from animals in France, Canada and India. *FEMS Microbiol. Lett*. **59**: 319-323.

Courvalin, P. (2008): Predictable and unpredictable evolution of antibiotic resistance. *J Intern Med*. 264: 4-16.

Daniel, A., Shaohua, Z., Emily, T., Sherry, A., Aparna, S., Mary, J. and Patrick, F. (2012): Antimicrobial drug resistance in *Escherichia coli* from Humans and Food Animals, United States, 1950–2002. *Emer. Infect. Dis.* **18** (5): 741-749.

De Champs, C., Sirot, C., Chanal, C., Poupart, M., Dumas, M. and Dumas J. (1991): Concomitant dissemination of three extended-spectrum β-lactamase among different Enterobacteriaceae isolated in a French hospital. *J. Antimicrob Chemother*. **27**: 441-457.

Ferens, W. and Hovde, C. (2011): Escherichia coli O157:H7: animal reservoir and sources of human infection. *Foodborne Patho and Dis*. **8:** 465–485.

Fode-Vaughan, K., Maki, J., Benson, J. and Collins, M. (2003): Direct PCR detection of Escherichia coli O157:H7. *Lett. Appl. Microbiol.* **37**: 239–243.

Frydendahl, K. (2002): Prevalence of serogroups and virulence genes in Escherichia coli associated with postweaning diarrhea and edema disease in pigs and a comparison of diagnostic approaches. *Vet. Microbiol*. 85:169-182.

Furuya, E. and Lowy, F. (2006) Antimicrobial-resistant bacteria in the community setting. *N. Rev. Microbiol*. **4**: 36-45.

Garofalo C, Vignaroli C, Zandri G, Aquilanti L, Bordoni D, Osimani A, (2007). Direct detection of antibiotic resistance genes in specimens of chicken and pork meat. *Int J Food Microbiol*. **113**:75-83.

Gerard, D. (2010): Antibiotic targets and mechanisms of resistance. See text for details. Wright BMC Biology. **8**: 123.

Guerra, B., Junker, E., Schroeder, A., Malorny, B., Lehmann, S. and Helmuth, R. (2003): Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichiacoli* isolates from cattle, swine and poultry. *J. Antimi- crob.Chemother.* **52**: 489–492.

Gyles, C. (2007): Shiga toxin-producing *Escherichia coli*: An overview. *J. Anim. Sci.* **85**: E45-E62.

H. Momtaz, E. Rahimi, and S. Moshkelani (2012): Molecular detection of antimicrobial resistance genes in *E.coli* isolated from slaughtered commercial chickens in Iran. *Vet. Med.* ***57***(4): 193–197.

Haimanot, T., Alemseged, A., Getnet, B. and Solomon G. (2010): Microbial Flora and Foodborne Pathogens on Minced Meat and Their Susceptibility to Antimicrobial Agents, Ethiop. *J. Health Sci.* **20**(3): 137-143.

Hawkey, P.M. 2008. Molecular epidemiology of clinically significant antibiotic resistance genes. *Br. J. Pharmacol*. **153**: S407-S413.

Hawkey, P. and Jones, A. (2009): The changing epidemiology of resistance. *J Antimicrobial Chemother Suppl*. **1**: 3–10.

Hershberger, E., Oprea, S., Donabedian, S., Perri, M., Bozigar, P., Bartlett, P. and Zervos, M. (2005): Epidemiology of antimicrobial resistance in enterococci of animal origin. *J Antimicrob Chemother*. **55**: 127-130

Hiko, A., Daniel, A. and Girma, Z. (2008): Occurrence of Escherichia coli O157:H7 in retail raw meat products in Ethiopia. *Infect. Dev. Ctries*. **2**(5): 389-393.

Holland, R. (1990): Some infectious causes of diarrhea in young farm animals. *Clin. Microbiol. Rev*. **3**:345-375.

Huang, Q., Baum, L. and Fu W. (2010): Simple and practical staining of DNA with GelRed in agarose gel electrophoresis. **56**:149-52.

Ince D and Hooper DC. (2003): Quinolone resistance due to reduced target enzyme expression. *J Bacterial*. **185**:6883-92.

International Organization for Standardization (2013): Microbiology of food and animal feeding stuffs carcass sampling for microbiological analysis. Geneva, Switzerland.

James, B., James, P. and Harry, L. (2004): Pathogenic Escherichia coli; *N. Rev.Microbiol*. **2**, 123-140.

Kaeckenbeeck, A. (1981): Resistance and pathogenic plasmids. CEC Seminar, NIVR, Brussels. 275.

Karmali, M., Gannon, V., Sargeant, J. (2010): Verocytotoxin-producing *Escherichia coli* (VTEC). *Vet Microbiol.* **140**: 360–370.

Kang, S., Ryu, S., Chae, J., Woo, G. and Lee, J. (2004): Occurrence and characteristics of enterohemorrhagic *Escherichia coli* O157 in calves associated with diarrhea. *Vet. Microbiol.* **98**: 323-328.

Khachatryan, A., Hancock, D., Besser, T. and Call, D. (2004): Role of calf adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. *Appl Environ Microbiol*. **70**: 752-757.

Lambert, P. (2005): Bacterial resistance to antibiotics: modified target sites. *Adv. Drug Deliv. Rev*. **57**: 1471-1485.

Langton, K., Henderson, P. and Herbert, R. (2005): Antibiotic resistance: multidrug efflux proteins, a common transport mechanism. *Nat. Prod. Rep*. **22**: 439-451.

Lanz, R., Kuhnert, P. and Boerlin, P. (2003): Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland. *Vet Microbiol*. **91**: 74-83.

Li, D., Liu, B., Chen, M., Guo, D., Guo, X., Liu, F., Feng, L. and Wang, L. (2010): A multiplex PCR method to detect 14 *Escherichia coli* serogroups associated with urinary tract infections. *J. Microbiol Met.* **82**:71–77.

Lupo, A., Coyne, S. and Berendonk, T. (2012): Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Front. Microbiol*. **3**: 1-13.

McEwen, S. and P.J. Fedorka-Cray, (2002): Antimicrobial use and resistance in animals. *Clin Infect Dis*,*Suppl.* **3**: S93-S106.

Maarten, R., Fluit, C. and Franz-Josef Schmitz, (2001): Molecular Detection of Antimicrobial Resistance. *Clin. Microbiol Rev*. **14**: 836–871.

Maryam, H., Hassan, M. and Mahboobeh M. (2014): Characterization of Diarrheagenic Antimicrobial Resistant *Escherichia coli* Isolated From Pediatric Patients in Tehran, Iran. *Red Crescent Med J*. **16**(4): e12329.

Mekonnen, H., Habtamu, T., Kelali, A. and Shewit K. (2012): Food safety knowledge and practices of abattoir and butchery shops and the microbial profile of meat in Mekelle city, Ethiopia. *Asian Pac.J. Trop. Biomed*. 952-957.

Meng, J., Zhao, S., Doyle, M. and Joseph, S. (1998): Antibiotic resistance of *Escherichia coli* O157:H7 and O157: NM isolated from animals, food, and humans. *J Food Prot.* **61**: 1511-1514

Mersha, G., Asrat, D., Zewde, B. and Kyule, M. (2009): Occurrence of *Escherichia coli* O157:H7 in faeces, skin and carcasses from sheep and goats in Ethiopia. *Lett Appl Microbiol*. **50**(1): 71–6.

Ministry of Urban Development Housing and Construction (MUDHCo) (2013): The Construction Industry Registration Draft Proclamation, Addis Ababa, Ethiopia.

Mooljuntee, S., Chansiripornchai, P. and Chansiripornchai, N. (2010): Prevalence of the cellular and molecular antimicrobial resistance against *E.coli* isolated from Thai broilers. *Thai J. Vet. Med.* **40**: 311–315.

Nataro, J. and Kaper, J. (1998): Diarrheagenic *Escherichia coli.* A comprehensive review of the pathogenesis, epidemiology, diagnosis and clinical aspects of diarrhoeagenic *E.coli*. *Clin. Microbiol.* **11**: 142–201.

National Committee for Clinical Laboratory Standard (1997): Performance Standard for Antimicrobial Disk Standard Susceptibility Test. Lippincott and Williams Wilkon, USA.

Nikaido, H. (2009). Multidrug resistance in bacteria. *Annu Rev Biochem*. **78**: 119-46.

Ochman, H., Lawrence, J. and Groisman, E. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature.* **405**: 299–304.

Oliver, S., Jayarao, B. and Almeida, R. (2005): Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Food borne Pathogenic Dis.* **2**(2): 115-129.

Orskov, I., Orskov, F., Smith, H. and Sojka W. (1975): The establishment of K99, a thermostable, transmissible *Escherichia coli* K antigen, previously called "Kco'', possessed by calf and lamb enteropathogenic strains. *Acta, Pathol. Microbiol*. **83**: 31-36.

Ousman, M., Dawit, S., Petros, A. and Teka, F. (2014): Prevalence and Antimicrobial Susceptibility Pattern of *E.coli* Isolates from Raw Meat Samples Obtained from Abattoirs in Dire Dawa City, Eastern Ethiopia. *Intern. J. Microbiol Res***. 5** (1): 35-39.

Pal, M. (2007): Zoonoses. 2nd *Edition*. Satyam Publishers, Jaipur, India. 100-134.

Parham, N., Spencer, D., Taylor, H., Ternent, G., Innocent, D., Mellor, M., and Williams A. (2003): An adapted Immuno Magnetic cell separation method for use in quantification of Escherichia coli O157:H7 from bovine faeces. *J Microbiol Met.* **53**:1-9.

Piddock L (1996): Does the use of antimicrobial agents in veterinary medicine and animal husbandry select antibiotic-resistant bacteria that infect man and compromise antimicrobial chemotherapy. *J. Antimicrobial Chemother.* **38**: 1–3.

Purohit, H. and Kapley, A. (2002): PCR as an emerging option in the microbial quality control of drinking water. *Trends Biotechnol*. **20**, 325.

Quintiliani, R., Sahm, D. F JR., and Courvalin, P. (1999): Mechanisms of Resistance to Antimicrobial Agents. *Manual of clinical microbiology*, 7th *ed* American Society for Microbiology, Washington, DC.

Reischl, U., Youssef, M., Kilwinski, J., Lehn, N., Zhang, W., Karch, H. and Strockbine, N. (2002): Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. *J Clin Microbiol*. **40**:2555–2565.

Riyaz-Ul-Hassan, S., Verma, V., and Qazi, G. (2004): Rapid detection of Salmonella by polymerase chain reaction. *Mol. Cell. Probes*. **18**: 333.

Robicsek, A., Strahilevitz, J., Jacoby, G., Macielag, M., Abbanat, D. and Park C. (2006): Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat Med*. **12**: 83-8.

Sanchez, S., Lee, M., Harmon, B., Maurer, J. and Doyle, M. (2002): Zoonosis Update: Animal issues associated with *Escherichia coli* O157:H7. *Veterinary Medicine Today*. **221**: 1122-1126.

Sandvang, D. and Aarestrup, F. (2000): Characterization of aminoglycoside resistance genes and class1 integrons in porcine and bovine gentamicin-resistant *Escherichia coli*. *Microb.Drug Resist.* **6**: 19–27.

Sargeant, J. and Smith D. (2003): The Epidemiology of *Escherichia coli* O157:H7. Microbial Food Safety in Animal. 131-174.

Scotland, S., Gross, R. and Rowe, B. (1985): Laboratory tests for enterotoxin production, enteroinvasive and adhesion in diarrhoegenic *Escherichia coli.* The virulence of *Escherichia coli*: *reviews and methods*. 395-405.

Silbergeld, E., Price, L. and Graham, J. (2008): Antimicrobial Resistance and Human Health. Retrieved from Pew Commission on Industrial Farm Animal Production website

Shakibaie, M., Jalilzadeh, K. and Yamakanamardi, S. (2009): Horizontal transfer of antibiotic resistance genes among Gram negative bacteria in sewage and lake water and influence

of some physic-chemical parameters of water on conjugation process. J. *Environ. Biol.* **30**: 45-49.

Shaw, K., Hare, R., Sabatelli, F., Rizzo, M., Cramer, C., Naples, L., Kocsi, S., Munayyer, H., Mann, P. and Miller, G. (1991): Correlation between aminoglycoside resistance profiles and DNA hybridization of clinical isolates. *Antimicrob Agents Chemother*. **35**: 2253-2261.

Shelton, R., Higgins, J., Kessel, Y., Pachepsky, K. and Karns, S. (2004): Estimation of viable *Escherichia coli* O157 in surface waters using enrichment in conjunction with immunological detection. *J. Microbiol. Met.*, **58**:223-231.

Silver, L. (2003): Novel inhibitors of bacterial cell wall synthesis, *Curr. Opin. Microbiol.* **6**: 431-438.

Soderlind, O., Thafvelin, B. and Mollby, R. (1988): Virulence factors in *Escherichia coli* strains isolated from Swedish piglets with diarrhea. *J. Clin. Microbiol*. **26**: 879-884.

Songer, J., and Post (2005): Veterinary Microbiology: Bacterial and Fungal Agents of Animal Diseaes; *Elsever Health*, New York, USA.

Sori, H., Zerihun, A and, Abdicho, S. (2005). Dairy cattle mastitis in and around Sebeta, Ethiopia. *Int. J. Appl. Res. Vet. Med*. **3**: 332-338.

Spellberg, B., Powers, J., Brass, E., Miller, L. and Edwards, J. (2004): Trends in antimicrobial drug development: implications for the future. *Clin Infect Dis*. **38**: 1279-86.

Strockbine, N., Wells, J., Bopp, C. and Barrett, T. (1998): Overview of Detection and Subtyping Methods. *Escherichia coli* O157:H7 and Other Shiga Toxin-Producing *E.coli* Strains. ASM Press, Washington, D. C., USA. 331-356.

Swartz, J. (2002): Human diseases caused by foodborne pathogens of animal origin. *Clin Infect Dis*. **34**: S111-S122

Szmolka, A., Anjum, M., Ragione, R., Kaszanyitzky, J. and Nagy, B. (2012).Microarray based comparative genotyping of gentamicin resistant *Escherichia coli* strains from food animals and humans. *Vet.Microbiol.* **156**: 110– 118.

Taye, M., Berhanu, T., Berhanu, Y., Tamiru, F. and Terefe, D. (2013): Study on Carcass Contaminating *Escherichia coli* in Apparently Healthy Slaughtered Cattle in Haramaya University Slaughter House with Special Emphasis on *Escherichia coli* O157:H7, Ethiopia. *J Veterinary Sci Technol*. **4**: 132.

Tenover, F. (2006): Mechanisms of antimicrobial resistance in bacteria. *Amer. J. Med,* **119**(6A): S3–S10.

Tesfaye, G., Asrat, D., Woldeamanuel Y. and Gizaw M. (2009): Microbiology of discharging ears in Ethiopia. *Asian Pac J Trop Med*. **2**(91): 60-67.

Thrusfield, M. (2005): Veterinary Epidemiology, 3rd *ed*. Blackwell Science Ltd, UK. 233-250.

Todar, K. (2007): Pathogenic *E.coli*. *Online Textbook of Bacteriology*. University of Wisconsin Madison Department of Bacteriology.

Vahedi, M., Nasrolahei, M., Sharif, M. and Mirabi, A. (2013): Bacteriological Study of Raw and Unexpired Pasteurized Cow's Milk Collected at the dairy Farms and Super Markets in Sari City in 2011. *J. Prev. Med and Hyg.* **54**: 120-123.

Walker CB (1996). The acquisition of antimicrobial resistance in the periodontal microflora. Periodontal 2000. 1**0**:79-88.

Walker RD (2000): Antimicrobial susceptibility testing and interpretation of results. Antimicrobial Therapy in Veterinary Medicine, RD Walker Iowa State Press, **2**:12-26.

Walsh C. (2003): Where will new antibiotics come from? *Nat Rev Microbiol*. **1**: 65-70.

Weller, T., Crook, D., Crow, M., Ibrahim, W., Pennington, T. and Selkon J. (1997): Methicillin susceptibility testing of *staphylococci* by E-test and comparison with agar dilution and *mecA* detection. *J Antimicrob Chemother*. **39**: 251–253.

Winokur, P., Vonstein, D., Hoffman, L., Uhlenhopp, E. and Doern, G. (2001): Evidence for transfer of CMY-2 AmpC β-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. *Antimicrob Agents Chemother*. **45**: 2716-2722.

Wright, G. (2005): Bacterial resistance to antibiotics: enzymatic degradation and modification. *Adv. Drug Deliv. Rev*. **57**: 1451-1470.

Wu, S., Dalsgaard, A., Hammerum, A., Porsbo, L. and Jensen, L. (2010): Prevalence and characterization of plasmids carrying sulfonamide resistance genes among *Escherichia coli* from pigs, pig carcasses and human. *Acta Vet Scand.* **52**:47.

Xia, X., Meng, J., McDermott, P. F., Ayers, S., Blickenstaff, K., Tran, T., Abbott, J., Zheng, J. and Zhao, S. (2010): Presence and characterization of shiga toxin-producing *Escherichia*

coli and other potentially diarrheagenic *E.coli* strains in retail meats. *App and Environ Microbiol*. **76**: 1709-1717.

Yoneyama, H. and Katsumata, R. (2006): Antibiotic resistance in bacteria and its future for novel antibiotic development. *Biosci. Biotechnol. Biochem*. **70**: 1060-1075.

8. APPENDICES

Annex 1**:** *Escherichia coli* antimicrobial resistant genes and primer sequences used for PCR identification

Anti-microbial agent)	Resistance gene	primer	Sequence 5'-3'	Sequence Size (bp	Source
Streptomycin	*aadA1*	*aadA1*F	TATCCAGCTAAGCGCGAACT	447	Van et al. 2008
		*aadA1*R	ATTTGCCGACTACCTTGGTC		
Gentamicin	*aac(3)-IV*	*aac(3)-IV*F	CTTCAGGATGGCAAGTTGGT	286	
		*aac(3)-IV*R	TCATCTCGTTCTCCGCTCAT		
Sulfonamide	*sul1*	*sul1*F	TTCGGCATTCTGAATCTCAC	822	
		sul1R	ATGATCTAACCCTCGGTCTC		
Beta-lactams	bla_{SHV}	bla_{SHV}F	TCGCCTGTGTATTATCTCCC	768	
		bla_{SHVR}	CGCAGATAAATCACCACAATG		
	bla_{CMY}	bla_{CMY}F	TGGCCAGAACTGACAGGCAAA	462	
		bla_{CMY}R	TTTCTCCTGAACGTGGCTGGC		
Erythromycin	*ere*(A)	*ere*(A)F	GCCGGTGCTCATGAACTTGAG	419	
		ere(A)R	CGACTCTATTCGATCAGAGGC		
Chloramphenicol	*catA1*	*catA1*F	AGTTGCTCAATGTACCTATAACC	547	
		*catA1*R	TTGTAATTCATTAAGCATTCTGCC		

	cmlA	*cmlA*F	CCGCCACGGTGTTGTTGTTATC	698	
		*cmlA*R	CACCTTGCCTGCCCATCATTAG		
Tetracycline	*tet*(A)	*tet*(A)F	GGTTCACTCGAACGACGTCA	577	Randall et al. 2004
		tet(A)R	CTGTCCGACAAGTTGCATGA		

Annexes 2: Sample collection and laboratory activities work sheet for the isolation of *E.coli*

No	Date	Spp	Sample type	Sample ID	Milk sample CMT result	Enrichment	Colony Characteristics	Growth on Selective media	Biochemical test	Antibiotic susceptibility test	Molecular Identification

Annexes 3: Media and buffer used for isolation and identification of *E.coli*

- Peptone water (Sisco Research Laboratories pvt.Ltd)
- Nutrient agar(Himedia Laboratories pvt.Ltd, India)
- MacConkey agar (Himedia Laboratories pvt.Ltd, India).
- Simmons Citrate Agar (Laboratories Conda S.A, Spain)
- Muller-Hinton agar (Himedia Laboratories pvt.Ltd, India)
- EMB agar (Himedia Laboratories pvt.Ltd, India)
- MR/VP test (Himedia Laboratories pvt.Ltd, India)
- 50x TAE buffer

Composition:

Tris base ……………… ……………..242.0 g

Glacial acetic acid …………………… 57.1 ml

EDTA …………………………………18.6 g

Distilled water ………………………. 1 Litter

Final pH; 8.0

- DNA loading buffer

Composition:

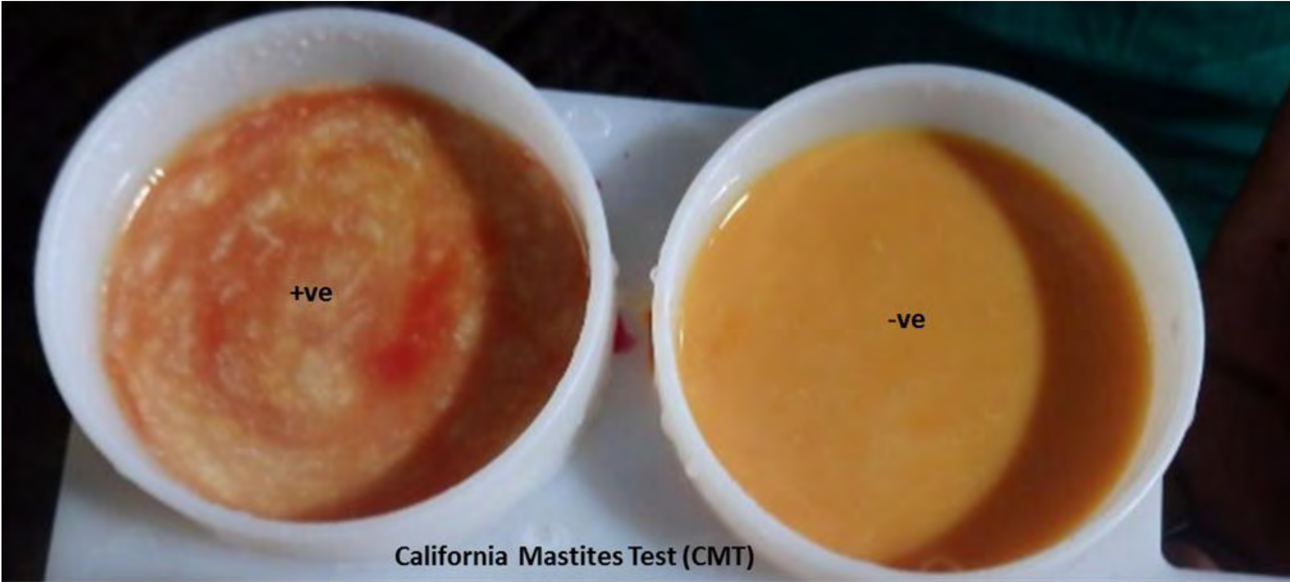
Bromophenol blue………………….. 0.25 g

Xylene cyanol ….................................0.25 g

Glycerine …………………………..30.0 ml

Distilled water …………………….70.0 ml

Annex 4: Sample collection and identification of *E.coli* by Eosin Methylene Blue Agar



California Mastites Test (CMT)

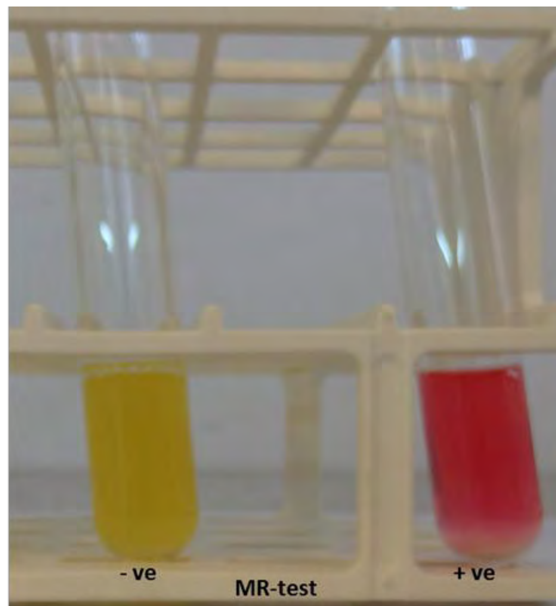
Green metallic sheen on EMB Agar (E.coli)

Annexes 5: identification and isolation of *E.coli* by biochemical test

Triple sugar iron agar (TSI)

Simmon citrate Agar (SCA)

Indole test



MR-test

Annex 6. Antimicrobial sensitivity test and gel electrophoresis