PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF PATHOTYPES, AND ANTIMICROBIAL RESISTANCE PROFILES OF *Escherichia coli* FROM FOOD, WATER AND LIVESTOCK IN SELECTED INFORMAL SETTLEMENTS IN NAIROBI.

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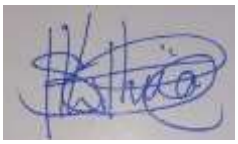
A thesis submitted in partial fulfilment for the degree of Master of Veterinary Public Health (MVPH) of the University of Nairobi.

Department of Public Health, Pharmacology and Toxicology

DECLARATION

This thesis is my original work and has not been submitted for a degree award in any other University.



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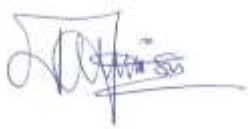

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DEDICATION

To my beautiful mothers Mukami and Kavinya, and my father, Mengo. To my loving husband William and daughters Princess Annie, Princess Lisa and Princess Olivia.

To Dr Margaret Ruinge.

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ABBREVIATIONS AND ACRONYMS

AIDS - Acquired Immunodeficiency Syndrome

AMC - amoxycillin- clavulanic acid

AMP - ampicillin

AMR - antimicrobial resistance

APHRC - Population & Health Research Centre

AST - Antimicrobial SusceptibilityTesting

Bla TEM - β-lactam

Bp - base pairs

C - chloramphenicol

CAZ - ceftazidime

CDC - Centres for Disease Control and Prevention

CIP - ciprofloxacin

CLSI - Clinical and Laboratory Standards Institute

CN - gentamicin

CRO - cefuroxime

CXM - ceftriaxone

DAEC - diffusely adhering *E.coli*

DNA - Deoxyribonucleic Acid

dNTPS - Deoxynucleotide Triphosphates

E.coli - *Escherichia coli*

EAEC - entero-aggregative *E.coli*

EAF - EPEC adherence factor

EHEC - entero-haemorrhagic *E.coli*

EIEC - enteroinvasive *E.coli*

EPEC - entero-pathogenic *E.coli*

ESBL - Extended Spectrum B-lactams

ESEI - Environmental and Social Ecology of Human Infectious Diseases Initiative

ETEC - eltA entero-toxigenic *E.coli*

ETEC - estA entero-toxigenic *E.coli*

FAO - Food Agriculture Organization

HIV - Human Immunodeficiency Virus

HUS - Haemolytic uremic syndrome

IERC - International Ethical Review Committee

ILRI - International Livestock Research Institute

KEMRI - Kenya Medical Research Institute

LT - heat-labile

MDR - Multiple drug resistance

MDRS - Multi-drug resistant

MHA - Mûeller Hinton Agar

MIC - Minimum Inhibitory Concentration

MRSA - methicillin-resistant *Staphylococcus aureus*

NA - nalidixic acid

O and H - antigens

OIE - World Organisation for Animal Health

PCR - Polymerase Chain Reaction

RNA - Ribonucleic Acid

rRNA - Ribosomal Ribonucleic Acid

S - streptomycin

ST - heat-stable

STEC - Shiga toxin-producing *E.coli*

SXT - trimethoprim-sulfamethoxazole

TAE - Tris-Acetate EDTA

TE - tetracycline

TSA - Tryptone Soy Agar

USA - United States of America

UTI - Urinary tract infections

WHO - World Health Organization

WTO - World Trade Organization

ABSTRACT

Escherichia coli (E.coli) are globally recognised as important causes of diarrhoeagenic infections as well as other extra-intestinal infections in both animals and man. In developing countries, *E.coli* is the most important agent causing diarrhoeal diseases which results in significant economic losses. In veterinary medicine, domestic animals are indicated as an essential harbour of multidrug-resistant *E.coli* which can be transferred food animals to man. The global rise in antibiotic resistance and particularly multi-drug resistance (MDR) has made treatment of infections challenging. The objective of this research study was to determine the pathotypes, and antimicrobial resistance profiles of *E.coli* isolated from foods, water and livestock in selected informal settlements in Nairobi.

The study was carried out in two informal settlements in Nairobi, namely Viwandani and Korogocho. It entailed reviving of previously isolated and biochemically confirmed *E.coli* isolates. These isolates were streaked onto MacConkey agar plates and then incubated overnight at 37ºC. Distinct pink colonies were then picked from MacConkey agar, streaked on Tryptone Soy Agar (TSA) and incubated for 24hrs at 37ºC to obtain pure colonies. The Kirby-Bauer disk diffusion method was employed to determine the phenotypic antimicrobial susceptibilityof the isolates in accordance with the Clinical and Laboratory Standards Institute(CLSI) guidelines (CLSI 2014). Inoculated plates with Mueller Hinton Agar (MHA) were seeded with antimicrobial disks and the zone of inhibition measured after incubation. Each isolate was screened for antimicrobial susceptibility with twelve different antimicrobial agents of veterinary and human health importance.The reference organism used was *Escherichia coli* ATCC 25922.

The highest resistance was shown to ampicillin where 79(45.4%) isolates showed resistance to the antimicrobial agents, while the least resistance (0%) was demonstrated to ceftriaxone where all the isolates were susceptible. For livestock isolates, 74.5% of the isolates were susceptiple to antimicrobial agents tested, 8% were intermediate, and 17.5 were resistant.The highest resistance in livestock isolates was shown to tetracycline at 53% and sulfamethoxazole/ trimethoprim at 53% while amoxicillin/clavulanate and ciprofloxacin showed the least resistance at 1%. Antibiotic resistance genes detected in food isolates included *blaTEM* (91%) and tetA31% while in livestock, the antimicrobial resistance profiles included *blaTEM* (35%), and tetA (15%).The most prominent pathotype in food isolates was enteropathogenic enteropathogenic *E. coli* ETEC estA with 2% of the isolates testing positive while enterohaemorhagic *E.coli* (EHEC) was the most prominent pathotype in livestock with 4%.

This study also showed that *Escherichia coli* from Viwandani and Korogocho are pathogenic and resistant to antibiotics. Since most antibiotics are shared between animals and human, the detection of resistance *E.coli* strains points at a risk of spread of resistant strains to man. This calls for further research and collaboration of public health department in a one health approach.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Escherichia coli (E.coli) are Gram-negative bacteria first described by Theodore Escherich in 1885. They belong to the Enterobacteriaceae family and form part of the healthy gut flora of both animals and humans. However, some strains of these lactose forming bacteria are pathogenic and cause many diseases which include urinary tract infections, wound infections, abscesses, meningitis, pneumonia and septicaemia, among others. These extra-intestinal infections can override illnesses caused by other micro-organisms (Diekema *et al*., 2000). In *E.coli* has become the most important pathogen causing infections and colonisation of food production animals (Aidara-Kane *et al*., 2013; Lahlaoui *et al*., 2014). One of the significant causes of deaths in neonatal foals is diarrhoea which leads to substantial socio-economic losses. *Escherichia coli,* coupled with other infectious agents, management practices, nutrition status and environmental conditions, plays a vital role in this diarrhoea (Frederick *et al*., 2009).

In veterinary practice, colibacillosis is one of the challenging diarrhoeal diseases which results in economic losses. *Escherichia coli* has been confirmed to be the number one organism causing diarrhoea, particularly in developing countries. In poultry farming, *E.coli* has been shown to cause numerous losses due to reduced production of affected birds, high morbidity, mortality as well as slaughter condemnation. Colibacillosis in birds is extra-intestinal, affecting the upper respiratory tract. The air sacs are infected, and the sacculitis can spread to other organs causing multiple lesions including cellulitis, salpingitis, polyserositis, peritonitis, hepatitis, septicaemia and eventual death (Dho-Moulin and Fairbrother, 1999; Awad and Arafat, 2016). In developing

countries, *E.coli* is an essential agent causing diarrhoeal diseases (Prats *et al*., 2003). In humans, Kyaw *et al*.(2003) and Kariuki *et al*. (1999) indicated that *E.coli* are of great importance in gastroenteritis in children and immune-compromised individuals. Infections caused by diarrhoeagenic *E.coli* are a vital public health concern in developing countries because they are closely linked to high morbidity and mortality in young children 0- 5 years old (Gomes *et al*., 2016).

Pathogenic *E.coli* are classified according to their mechanism of virulence. These classes include entero-adherent *E.coli* (EAEC), enterotoxigenic *E.coli* (ETEC), entero-pathogenic *E.coli* (EPEC), entero-haemorrhagic (Shiga toxin-producing) *E.coli* (EHEC/STEC), enteroinvasive *E.coli* (EIEC) and diffusely adhering *E.coli* (DAEC) (Paterson, 2006; Luz *et al*., 2015; Prats *et al*., 2003). .

The treatment regime assumed to manage *E.coli* infections is antimicrobial therapy to eliminate the pathogen as well as symptomatic management of the diarrhoea. Most antibiotics used in the treatment of *E.coli* infections are shared between veterinary practice and human medicine.The use of antimicrobials in production animals has been demonstrated to cause the rise in resistant bacteria along the food chain (Casey *et al*., 2013; Price et al., 2012). *Escherichia coli* have been shown to develop resistance to many antimicrobials commonly used in therapeutic treatment. In veterinary practice, the state of antimicrobial resistance is alarming and has been investigated with emphasis on food production animals (Mitema *et al*., 2001; Philips et al., 2004).

1.2 Problem Statement

Infections caused by pathogenic antibiotic resistant bacteria usually result in grave and costly grave and costly public health problems in both animal and human health (Aarestrup *et al*., 2006). Such infections lead to illnesses resulting in high morbidity and mortality unless they are combated in time with alternative antimicrobial agents. Domesticated animals have been indicated as an essential reservoir of MDR *E.coli* responsible for the transfer of bacterial genetic material to man (Awadallah *et al*., 2016). Rising antimicrobial resistance rates in clinical isolates result in high morbidity and mortality, long periods of treatment and therefore increased economic costs. Antimicrobial resistance of pathogenic *E.coli* in animals threaten the health of not only animals but that of humans as well. There is heightened scientific and public concern in the application of antibiotics in animals due to the rise and spread of MDR bacterial pathogens which are of zoonotic importance (Bebora *et al*., 1994; Mitema *et al*., 2001).

According to a recent review of research and investigations of antimicrobial resistance in East Africa, there is a significant gap in the level of studies in this field. The report showed that only 24% of the AMR research studies were conducted in animals compared to 76% of studies in humans. This indicates an urgent need for a surveillance network for the region aimed at controlling antimicrobial use and curbing resistance (Omulo *et al*., 2015). In Kenya, several studies have been carried out on the significance of *E.coli* in childhood diarrhoea (Senerwa *et al*., 1989a; Kariuki et al., 2006; Sang and Schnabel, 2012). Although previous studies have been undertaken in Kenya investigating *E.coli* and antimicrobial resistance in animals and their products (Mitema *et al*., 2001; Mapenay *et al*., 2007; Kariuki 2002; Kariuki 2013), studies in

informal settlements are limited. The current study investigated the pathotypes of *E.coli* present in food, water and livestock and described their antimicrobial resistance profile in respect to twelve commonly used antimicrobials.

1.3 Justification

Characterization of pathogenic *E.coli* isolated from foods, water and livestock described in this study, provides knowledge of the virulence strains of *E.coli* circulating in informal settlements. This knowledge is useful to the public health department to control and ensure food safety. The finding and description of virulent strains in this study can also advise policy makers in formulating policies for food hygiene from farm to fork. In addition, understanding the phenotypic and characterizing the genotypic antimicrobial resistance profile of pathogenic *E.coli* from foods, water and livestock will provide ideas on the emergence of resistance in informal settlements in livestock and food value chains. This knowledge can also be used to develop a guideline for therapeutic interventions in animals and humans as well. Consequently,the findings of this study can also form a baseline for further research on the virulence and AMR characteristics of pathogenic *E.coli* in informal settlements in Nairobi.

1.4 Null Hypothesis

Food, water and livestock faecal material obtained from informal settlements in Nairobi are not contaminated with pathogenic and antimicrobial resistant *E.coli*.

1.5 General Objective

The general objective of this research study was to determine the pathotypes and characterise the antimicrobial resistance trends of *Escherichia coli* isolated from livestock, food and water in Viwandani and Korogocho peri-urban areas of Nairobi.

1.5.1 Specific objectives

The specific objectives were:-

1. To determine the pathotypes of *Escherichia coli* isolated from foods, water and livestock in Viwandani and Korogocho informal settlements of Nairobi.
2. To determine the phenotypic antimicrobial susceptibility of *Escherichia coli* isolated from foods and water and livestock in Viwandani and Korogocho informal settlements of Nairobi.
3. To characterise genetic determinants responsible for the antimicrobial resistance phenotypes of the *Escherichia coli* isolates mentioned above.

CHAPTER TWO: LITERATURE REVIEW

2.1 Characteristics of *Escherichia coli*

Escherichia coli are gram-negative and facultative anaerobic bacteria of the family Enterobacteriaceae and the genus *Escherichia.* The motile, rod-shaped bacteria have peritrichous flagella and are normally found in the colons of mammals, where they are part of the healthy gut flora. They play a pivotal role in preventing pathogenic bacteria from colonising the intestines and are also responsible for the production of vitamin K (Kaper *et al*., 2004; Gomes et al., 2016). This non-spore-forming bacteria is a chemoheterotroph, and its growth medium must include a source of energy and carbon. The most commonly used media is MacConkey agar and after incubation at 37degrees Celsius under aerobic conditions, yields lactose fermenting pink colonies (Cavalieri *et al*.). It is catalase positive, oxidase negative and indole positive. Although most *E.coli* strains are not harmful, several serotypes are highly pathogenic and are the leading cause of many infections of both man and animals (Kaper *et al*., 2004). They are introduced in the environment through the faeces of the host organism. Subsequently, the pathogenic *E.coli* strains cause disease through the faecal contamination of food and water.. *Escherichiacoli* is easily cultured in the laboratory and therefore, has been widely used in biotechnology and microbiology fields.

2.2 Pathotypes of *Escherichia coli*

Escherichia coli are subdivided into three categories, namely intestinal non-pathogenic isolates (commensal), intestinal pathogenic isolates and extra-intestinal pathogenic isolates (Pitout, 2012). The mechanism which enables pathogenic bacteria to cause infections is its virulence factor. The

genes which code for factors which contribute to the virulence in pathogens can be identified using genomics (Dobrindt, 2005). The currently known pathotypes of diarrhoeagenic *E.coli* are classified depending on their virulence factors/mechanisms, preferential host colonisation sites and the resultant clinical symptoms. Each pathotype contains a group of clones sharing specific virulence determinants (Kaper *et al*., 2004; Cabrera-Sosa and Ochoa, 2020; Nataro and Kaper, 1998). This is summarised in Table 2.1.These classes are enterotoxigenic *E.coli* (ETEC) which produce toxins resulting in traveller's diarrhoea, entero-haemorrhagic (Shiga toxin-producing) *E.coli* (EHEC/STEC) which produces haemolysin resulting in diarrhoea and enteroinvasive *E.coli* (EIEC) which perforates the epithelial cells that line intestinal mucosa resulting in diarrhoea.

Other classes are entero-pathogenic *E.coli* (EPEC) which is considered a causative agent in infant diarrhoea, entero-aggregative *E.coli* (EAEC) responsible for prolonged diarrhoea and entero-adherent *E.coli* (EAEC) or diffusely adhering *E.coli* (DAEC) which adheres to epithelial cells (Paterson, 2006; Luz *et al*., 2015; Prats *et al*., 2003). However, with the discovery of many new strains like *E.coli* 0157:H7, there are proposals of reclassification (Brenner, 2005; Farmer *et al*., 2004; Levin *et al*.,2000; Paterson, 2006). Several studies have been conducted to better understand the mechanism of pathogenicity of *E.coli* strains.

2.2.1 Enterotoxigenic Escherichia coli

Enterotoxigenic *E.coli* (ETEC) strains are characterised by the production of adhesions or colonisation factors (CFs), which aid the bacteria attach itself to the host's intestinal epithelial

cells and confer host specificity. The pathogenicity of ETEC is determined by the present enterotoxins. These toxins are divided into two structurally and functionally different classes; the heat-labile (LT) and the heat-stable (ST) toxins (Tabaran *et al*., 2016). The toxins mediate membrane ion channels resulting in a massive loss of water and ions which clinically presents as watery diarrhoea (Fleckenstein *et al*., 2010; Gomes *et al*., 2016). In developing countries, ETEC is considered a significant cause of infant diarrhoea and also traveller's diarrhoea. In veterinary medicine, this strain is responsible for many diarrhoeal outbreaks in broiler production, pigs and cattle production as well. Infections with ETEC strains have been shown to result in massive deaths and numerous diarrhoea episodes. Enterotoxigenic *E.coli* diagnosis relies on the identification of LT and /or ST toxins. Also, PCR assays to detect virulence genes like clay, *tia, east-1* and *eatA* are more specific and can be employed (Fleckenstein *et al*., 2010; von Mentzer *et al*., 2014; Sahl and Rasko, 2012; Kotloff *et al*., 2013).

2.2.2 Enterohaemorrhagic (Shiga toxin-producing) Escherichia coli

The enterohaemorrhagic (Shiga toxin-producing) *E.coli* (EHEC/STEC) have a worldwide distribution and are known to produce of the Shiga toxin (Stx) type cytotoxins. The EHEC/STEC can adhere to intestinal epithelial cells resulting in infections ranging from bloody and acute diarrhoea to grave conditions like haemolytic uremic syndrome (HUS). Infections with EHEC/STEC affect mainly infants and children and is associated with acute renal failure in children (Stevens and Frankel, 2014). *Escherichia coli* 0157:H7 is considered to be the prototype for EHEC/STEC and has been linked to numerous outbreaks globally as well as haemorrhagic colitis and HUS cases. The cytotoxin Stx causes pro-inflammatory and pro-apoptosis responses by acting on cell signal transduction and immune modulation. Through PCR, specific primers

targeting *Stx* gene which is the major virulence factor are used to accurately diagnose EHEC/STEC infections(Gomes *et al*., 2016; Krüger and Lucchesi, 2015). An outbreak of EHEC 0104:H4 strain occurred in Germany and other countries in the region in May 2011. The infections were thought to arise from contaminated fenugreek seeds from Egypt. The infected persons developed HUS. The transmission pathways include food-borne, faecal-oral, between persons and environmental transmission. (Kintz *et al*., 2016).

2.2.3 Enteroinvasive Escherichia coli

Enteroinvasive *E.coli* (EIEC) is the leading cause of dysentery in third world countries and results in an infection akin to the one caused by *Shigella* species. EIEC causes diarrhoea through penetration and invasion of bacteria, thereby destroying the enterocytes (Gomes *et al*., 2016; Cabrera-Sosa and Ochoa, 2020; Orskov *et al*., 1991). Virulence genes present include *ipaABCD, icsA, icsB, virF and virB.* Milk and milk by-products, as well as beef, have been incriminated as a source of EIEC infections in outbreaks (Matsushita *et al*., 1993). In Italy, an outbreak of EIEC was reported whereby 109 cases were investigated in 2012 while the United Kingdom reported 50 cases of EIEC in an outbreak in 2014 (Newitt *et al*., 2016; Michelacci *et al*., 2016). Investigation of the *ipaH* gene by PCR is recommended in the identification of EIEC.

2.2.4 Enteropathogenic Escherichia coli

Enteropathogenic *E.coli* (EPEC) is extensively described by Kaper *et al*., (2004). A significant feature of EPEC is the localised adherence to intestinal wall, mediated and aided by the EPEC adherence factor (EAF) and plasmid-encoded bundle-forming pilus (BFP) gene. The type IV fimbriae BFP contributes to the auto-aggregation, antigenicity and biofilm formation of this

strain. Typical EPEC are therefore *eae* positive, do not produce Shiga toxins and posses *EAF* plasmid. Enteropathogenic *E.coli* strains cause diarrhoea resulting in histopathology of the intestinal epithelium called the attaching and effacing (EA) lesion. Diarrhoea due to EPEC is rarely reported in adults except in those who are immune-compromised and global studies have shown EPEC to be strongly linked to diarrhoea in children under the age of one year. According to Kotloff *et al*., (2013), significant association of EPEC with severe to moderate diarrhoea in children under the age of two years in Kenya was described. Enteropathogenic *E.coli* can be isolated in the laboratory by DNA probes or PCR assays using specific primers targeting the *stx* and *eae* genes (Nataro and Kaper, 1998; Cabrera-Sosa and Ochoa, 2020; Gomes *et al*., 2016)

2.2.5 Enteroaggregative Escherichia coli

The Enteroaggregative *E.coli* (EAEC) causes acute diarrhoea which is self- limiting although it may be protracted in some patients. The diarrhoea is often watery with mucus, sometimes bloody, and patients experience abdominal pain, vomiting and low-grade fever. They have also been shown to cause urinary tract infections. This pathotype displays aggregative (AA) pattern on epithelial cells in culture (Gomes *et al*., 2016). The virulence factors of EAEC include adhesins which mediate biofilm formation, enterotoxins as well as excreted proteins which are mainly plasmid-borne. The presence of an *aggR* gene that encodes virulence gene regulator for EAEC splits the pathotype into two: typical EAEC and atypical EAEC (Hebbelstrup *et al*., 2014; Cabrera-Sosa and Ochoa, 2020; Navarro-Garcia and Elias, 2011). The typical EAEC has more pathogenic potential through *AggR* virulence factor. Enterotoxins include EAEC heat-stable enterotoxin 1 (EAST-1). There was a foodborne outbreak due to EAEC reported in Europe involving 4000 people in 2011 Rasko *et al*., 2011). The PCR assays target the virulence genes

are, *aap* and *aatA* which encode the *AggR* regulator are useful in the characterisation of this pathotype (Gomes *et al*., 2016; Luz *et al*., 2015).

2.2.6 Diffusely adhering Escherichia coli (DAEC)

Diffusely adhering *E.coli (*DAEC) is linked to UTIs in adults and with infant diarrhoea. The diarrhoea is often watery and persistent, especially in young children below 5 years of age. It has also been implicated in pregnancy complications. (Meza-Segura and Estrada-Garcia, 2016; Cabrera-Sosa and Ochoa, 2020). This pathogen adheres to the whole epithelial cell surface, causing diarrhoea, subsequent epithelial damage and sometimes other intestinal diseases (Giron *et al*., 1991; Cabrera-Sosa and Ochoa, 2020). The diffuse adherence pattern of DAEC in Hela or Hep-2 cells is a unique description of this pathotype. This pattern is aided by fimbria (Dr) and afimbrial (Afa) which are referred to as *Afa/Dr adhesins* (Giron *et al*., 1991; Campos *et al*., 1999). Some atypical DAEC strains lack the Afa/Dr adhesins but instead have diffuse adhering adhesins called *AIDA-1*. Molecular characterization of DAEC involves detection of genes of *Afa, dra* and *daa* operons which encode the *Afa, Dr* and F1845 adhesins respectively. The virulence genes investigated in this study were VT of EHEC, *daaE* of DAEC, *eae* of EPEC *AggR* of EAEC, *eltA* and *estA* of ETEC (Cabrera-Sosa and Ochoa, 2020).

Figure 2. 1: Clinical Description and the Virulence Determinants of Diarrhoeagenic *E.coli* (Cabrera-Sosa and Ochoa, 2020).

Pathotype	**Diarrhoea's Characteristics**	**Complications or Associated Conditions**	Virulence Genes*
EPEC	Watery; acute or persistent	Malnutrition in children	***eae, bfp***
STEC	Bloody; acute	HUS	***stx1, stx2, eae,*** *ehx, fliC*
EAEC	Watery; acute or persistent	Malnutrition in children; traveler's diarrhoea; HIV infection	***aggR, astA, aatA,*** *aaiC, aap, set1A*
ETEC	Watery; acute	Dehydration; traveller's diarrhoea	**LT, ST,** and CF genes
EIEC	Bloody; acute		***ipaH, ial***
DAEC	Watery; acute or persistent	UTI	Afa/Dr adhesin genes: ***daaC, daaD, daaE***
AIEC	Watery; acute or persistent	Crohn's disease	No specific gene reported

*In bold, main genes used for diagnosis of each pathotype.

AIEC, Adherent invasive *E.coli; CF,* colonization factors; *DAEC,* diffusely adherent *E.coli; EAEC,* enteroaggregative *E.coli; EIEC,* enteroinvasive *E.coli; EPEC,* enteropathogenic *E.coli; ETEC,* enterotoxigenic *E.coli; HUS,* hemolytic uremic syndrome; *LT,* heat-labile enterotoxin; *ST,* heat-stable enterotoxin; *STEC,* Shiga toxin-producing *E.coli; UTI,* urinary tract infections.

2.3 *Escherichia coli* Infections in Animals

In animals, *E.coli* causes disease syndromes which include systemic colibacillosis due to invasive *E.coli* strains, mastitis in adult cattle and enteric colibacillosis which may be colibacillary toxaemia or colibacillary diarrhoea (Wray and Morris, 1985). Colibacillary diarrhoea which is often acute occurs in calves, piglets and lambs in the first three days after birth and is termed neonatal colibacillary diarrhoea. Piglets may, however, develop the disease during weaning. In porcine, colibacillary toxaemia is expressed in three distinct clinical syndromes namely; haemorrhagic enteritis which is characterised by haemorrhagic lesions in the gut mucosa and the sudden death of the pigs, shock in weaners described by sudden death, and a third syndrome termed oedema disease common in young pigs and characterised by ataxia and convulsions in infected animals. Systemic colibacillosis, which is often fatal occurs in lambs, poultry and calves (Wray and Morris, 1985).

Shiga-toxin producing *E.coli* (STEC) exist as normal flora in ruminants and do not produce disease symptoms in animals but can cause severe infections in man (Kintz *et al*., 2016). According to Herrera-Luna *et al*., (2009) the occurrence of diarrhoea in livestock is significant because of the subsequent economic losses but more importantly because numerous infectious agents causing diarrhoea have an impact on zoonoses and are closely linked with foodborne diseases.

2.4 *Escherichia coli* Infections in Man

Escherichia coli has been demonstrated to be prolific due to faecal contamination and persists for weeks in the environment under ideal conditions. While most strains of *E.coli* are considered

relatively harmless, several others have been shown to produce toxins which cause diarrhoeal diseases and other extra-intestinal infections as well. The primary cause of diarrhoea, meningitis in neonates, UTI's, septicaemia, and nosocomial infections in man are intestinal strains of *E.coli.* Several studies have demonstrated a high risk of infection in persons who are immune-compromised than in healthy individualsThere have been outbreaks of diarrhoea due to EPEC in daycare centres and hospital nurseries, but incidences are rare in adults. The role of *E.coli* in diarrhoea has been described in Kenya and several studies have been conducted (Senerwa *et al*., 1989b; Kariuki *et al*., 1999; Sang and Schnabel, 2012; Kiiru *et al*., 2012).

Acute bacterial meningitis caused by colonisation with a strain of *E coli*; K1 capsular antigen has 8% mortality, and 28.5% of neonatal meningitis is caused by *E coli*. They have also been shown to cause nosocomial pneumonia which manifests as bronchopneumonia of the lower lobes and as intra-abdominal infections. *Escherichia coli* causes more than 90% of uncomplicated UTI's which include pyelonephritis in elderly patients, prostatic abscess, urethritis and symptomatic cystitis. Other diseases caused by *E.coli* include suppurative thyroiditis, sinusitis, septic arthritis, skin infections and osteomyelitis. The risk factors in man include close contact with livestock, consumption of raw or undercooked meat, use of unpasteurized milk and dairy products and impure water.

2.5 Treatment of *Escherichia coli* Infections

The treatment regime assumed to manage *E.coli* infections is antimicrobial therapy to eliminate the pathogen as well as symptomatic management of the diarrhoea. The presence of antimicrobial agents in the gut may kill the bacterial organism, or it may induce the development of

antimicrobial resistance. Application of antibiotics in animals has been demonstrated to cause the development of resistant bacteria along the meat and dairyfood chains (Mitema *et al*., 2001; Bebora *et al*., 1994). These studies have shown *E.coli* as the leading cause of acquired bacteraemia in immune-compromised persons and therefore, a primary target for bombardment with antimicrobial agents. The antimicrobial agents employed in the treatment of *E.coli* infections are *β*-lactams, cephalosporins, fluoroquinolones, tetracyclines.and aminoglycosides.

2.5.1 β-lactams

β-lactam antimicrobials include penicillins, carbapenems, cephalosporins as well as glycoproteins kill microbes through cell wall synthesis inhibition, resulting in activation of bacterial autolytic pathways. β lactamases are microbial enzymes which render β lactam antimicrobials inactive and subsequently ineffective, through hydrolysis process. (Livermore and Woodford, 2006). A transcriptional attenuator regulates the cephalosporinase gene (*ampC)* of *E.coli* in addition to a weak promoter. The enzyme extended-spectrum β-lactamase is in class A and includes *blaTEM* and *blaSHV*. Its spectrum of resistance extends to cephalosporins, penicillins and monobactams.

The class C CMY is a plasmid-mediated *AmpC* βeta lactamase and can hydrolyse monobactams, penicillins, cephalosporins, and cephamycins. The resistance of *E.coli* to β-lactams is by intrinsic and acquired βeta lactamases (Jacoby, 2005; Livermore and Woodford, 2006; Pitout, 2012; Woo *et al*., 2003). Most non-*β*-lactam antimicrobials are not effective against Extended Spectrum β-lactams (ESBL) producing pathogens. This results from either the occurence of genes encoding resistance to other drugs in the plasmid carrying the ESBL gene (Paterson, 2006), or the close association with resistance to other antimicrobials. The development of AMR to the penicillin-

based AMC (amoxicillin-clavulanic acid) is caused by mutation of TEM-1 or TEM-2 β-lactamases resulting in the development of Inhibitor Resistant TEM (IRT) enzymes. These IRT enzymes have lower affinity for the carboxy-, amino- and ureido-penicillins (Ri`os *et al*., 2015). The current study investigated three β-lactamase genes, namely; *bla-SHV*, *bla-TEM* and *bla-CMY*.

2.5.2 Fluoroquinolones

Quinolones such as nalidixic acid act through the inhibition of the enzymes topoisomerase IV and DNA gyrase, which are vital for bacterial DNA synthesis. The bacterial enzyme DNA gyrase introduces negative superhelical twists during bacterial DNA synthesis before the replication fork. Therefore, resistance to fluoroquinolones results from chromosomal mutations in the DNA target enzymes of topoisomerase IV and DNA *gyrase* (Lambert, 2005; Woo *et al*., 2003).

2.5.3 Aminoglycosides

Aminoglycosides like gentamicin and streptomycin inhibit bacterial growth when they bind to the 30S subunit of the microbial ribosome, thereby causing a mismatch between codons and anticodons. Subsequently, the biosythesised proteins will contain incorrect amino acids. The antimicrobial resistance may result from several mechanisms namely inactivation and enzymatic modification which is the most prevalent, mutations of target site through methylation of ribosomal RNA and porin loss from diffusion through the outer membrane subsequent to porin loss (Fritsche *et al*., 2008; Woo *et al*., 2003).

The aminoglycoside modifying enzymes are grouped into three: phosphotransferases (APH) Acetyltransferases *(aac)* and nucleotidyltransferases*(ant).* After modification, the aminoglycoside is not able to bind to the bacterial ribosome. The current study investigated the acetyltransferases *aac(3*), which has been described as a bifunctional aminoglycoside modifying enzyme (Dubois *et al*., 2002).

2.6 Antimicrobial Resistance (AMR)

According to Fortman and Mukhopadhyay (2016), antimicrobial resistance is on the rise, but there is a paucity in the generation of new antimicrobials. The effectiveness of antibiotics is decreasing because of the rising antimicrobial resistance to drugs currently available and a diminishing pipeline for new drugs. Antimicrobial stewardship should, therefore, focus not only on the control of resistance but the effectiveness of currently available antimicrobial agents (Fishman, 2006). The World Organisation for Animal Health (OIE), World Health Organization (WHO) and the Food and Agriculture Organization (FAO) recognise the evolution of multiple antimicrobial-resistant pathogenic bacteria as a severe global danger to both animal and human health. Implementation of antimicrobial stewardship programs and a global force of research should be employed to combat this problem (WHO, 2014). This evolution is alarming because of the high rate of emerging resistant phenotypes in many bacterial pathogens and commensal organisms as well.

Although there is a highly infectious disease burden in third world countries, the widespread application of newer and often more expensive drugs is not possible due to cost constraints (Laxminarayan *et al*., 2013). According to Okeke (2009) antimicrobial resistance has reached

highly alarming levels in the majority of pathogens of importance in developing countries. Consequently, there is a need to evaluate the role of agriculture in selective pressure. Suboptimum doses of antimicrobial agents aid in selection of resistance. With the rise and rapid spread of carbapenem-resistant Enterobacteriaceae pathogens, the world is at the dawn of a post-antibiotic era where all the previous achievements due to antibiotics could be lost (Centers for Disease Control and Prevention (CDC), 2013).

According to Shapiro *et al*. (2001), emerging AMR to commonly used agents coupled with inadequate surveillance to monitor the AMR trends renders empirical therapy ineffective. Excessive oral administration of antimicrobial agents could create pressure on the target bacteria predisposing it to gain resistance (Mitema *et al*., 2001). In addition, over the counter drugs and self-medication contribute markedly to the rise in antimicrobial resistance (Morgan *et al*., 2011). Investigations have demonstrated that antibiotics in Kenya are sold without a prescription for the management of infections for both man and animals (Kariuki *et al*., 1999). The indiscriminate utilisation of antimicrobials is a key factor in the rise and dissemination of AMR (Fishman, 2006; Omulo *et al*., 2015). According to Dancer (2004), there is an urgent need for appropriate prescribing by doctors and all stakeholders to address this global problem. In the United States of America (USA), the estimated costs associated with antimicrobial resistance lie at US$400-US$18.6 billion (Hensher, 1999). In 2013 alone, the CDC approximated 23000 deaths caused by infections with resistant bacteria. Approximately 25000 people die from AMR bacteria each year in Europe. (CDC), 2013)

2.6.1 Antimicrobial Resistance Transfer

Most drivers of AMR arise from inappropriate antimicrobial use in humans and animal health care, agriculture or environmental contamination (Holmes *et al*., 2016). Pathogen mobility and transmission in an ecosystem is vital for predicting the potential of disease occurrence in wildlife. It is also useful in the development of disease control strategies. The proximity of wildlife, livestock and man in many African countries coupled with the high prevalenceas well as diversity of pathogens necessitates its understanding (Cleaveland *et al*., 2001).

Nevertheless, there is insufficient data regarding pathogen mobility in densely populated urban areas and AMR transfer pathways from animals to man and across species. Two AMR transfer pathways from animals to humans have been described. One is a direct transference of the resistant bacteria from animal to man and occurs in zoonotic diseases. This is explained in *Salmonella* resistance to β-lactam (Bertrand *et al*., 2006; Cleaveland *et al*., 2001; Cloeckaert *et al*., 2007). The second pathway occurs when animals harbour AMR genes which are transferred from animals to man using transposons or plasmids, and it has been shown to be the case in *Escherichia coli* (Aarestrup, 2006; Philips *et al*., 2004; Kruse and Sørum, 1994). According to Nataro and Kaper (1998) plasmid transmission is vital in AMR dissemination.

Escherichia coli can transfer bacterial DNA through conjugation or transduction, allowing the horizontal spread of genetic material in a population. The Shiga toxin-producing *E.coli* 0157:H7 was produced through a transduction process which uses bacteriophage. An analysis of antimicrobial resistance genes, transposons and plasmids using molecular tools has shown that elements present in animals and man are identical. Therefore, the use of antimicrobials in

animals selects for resistant pathogenic strains (Teuber, 2001). Studies in China demonstrated 80.8% horizontal transfer of antimicrobial resistance through conjugation (Yin *et al*., 2013). While investigating antimicrobial resistance of commensal bacteria from cattle and cattle attendants, *E.coli* resistant to tetracycline was detected in 40.8% and 33.1% in human and cattle respectively suggesting frequent gene exchange between the two hosts (Madoshi *et al*., 2016).

In developing countries and especially sub Saharan Africa, there is an increased demand for use of antimicrobials for prophylaxis and for treatment as well as. This demand is due to the endemic nature of diarrhoeal diseases majority of which is due to *E coli*, tuberculosis, HIV/ AIDS, malaria, acute respiratory and helminth infections (Shears, 2001; Okeke, 2009). While investigating *E.coli* isolates of wild rabbits, Silva *et al*. (2010) detected isolates resistant to antimicrobials commonly used on man. The resistance in that study though lower than what has been reported from production animals shows that wild animals are significant in the spread of AMR in the environment.

Investigations by Oscar Madzingira (2016) indicated that sheep are reservoirs of STEC strains including 0157:H7 which could spread to man and thus food safety measures should be put in place. Studies in Turkey suggest that raw chicken retail meat markets have a high level of ESBL producing *E.coli* contamination, thus posing a risk to human health and require regular monitoring (Önen *et al*., 2015). The study also recommended the controlled use of carbapenems in veterinary practice. Studies have shown the possibility of the chicken being possible sources of trimethoprim-sulfamethoxazole resistance gene for humans, and vice versa (Bebora *et al*., 1994). Research has established the importance of poultry meat in AMR transmission, especially

multidrug-resistant bacterial strains in general human populations as well as the environment. This has subsequently resulted in heightened food safety concerns and general environment health (Aidara-Kane *et al*., 2013).

Several studies (Fey *et al*., 2000; Bertrand *et al*., 2006; Cloeckaert *et al*., 2007) have demonstrated AMR transfer from animals to man. A study by Awadallah *et al*. (2016) established the livestock to be key players in the transmission of zoonotic bacteria to man via the consumption of contaminated milk. It has been established that the leading and most significant factor in the spread and increase of AMR is uncontrolled antimicrobial use in animals, plants and man (Fishman, 2006; Mitema *et al*., 2001). Adeyanju and Ishola (2014) caution against the indiscriminate use of antibiotics which could result in lost effectiveness against microbes. According to Sheikh *et al*. (2012), multiple resistance genes can in some instances occur on the same genetic element, and more than one gene can present as similar phenotypic appearance. Therefore the application of one antibiotic may give rise to *E.coli* strains resistant to other unrelated antimicrobials (Sheikh *et al*., 2012). There is a doubtless global rise in antimicrobial resistance (Adeyanju and Ishola, 2014) and an opposing decline in the formulation of new antibiotics.One study in China on the potential health impact of bio-aerosols on both humans and poultry, observed tetracycline resistance genes in the air of feeding operations in China (Gao *et al*., 2016).

2.7 Need for Global Action on Antimicrobial Resistance

The world bank has estimated that about 28 million people in developing countries could be plunged into poverty due to increased human deaths and treatment failure linked to AMR. The

global rise in antimicrobial resistance is strongly associated with financial and health burdens which strain health care budgets as well as resources (OIE,2014). The WHO has emphasised the public health risks of AMR, terming it one of the most significant threats to human health as a result of AMR. The risk has been attributed to resistance against critical antimicrobial agents including fluoroquinolones, carbapenems and cephalosporins (WHO, 2014). In 2011 on world health day, WHO issued a policy package for governments and their partners to halt the dissemination of AMR.

Rapid action is needed to contain the use of antimicrobial agents. Long-term political commitment by all nations is necessary, a coordinated, multisectoral effort by all stakeholders for national control plans for use and dissemination of drugs (OIE, 2014). Antimicrobial resistance is a dangerous situation, but there is a potential to arrest this through emerging technologies including vaccines against pathogenic bacteria, and use of antibodies coupled with antibiotic stewardship (Fortman and Mukhopadhyay, 2016; Lu and Koeris, 2011).

Studies by Delgado-Valverde *et al*. (2013) indicate a need for rapid susceptibility testing and accurate diagnosis before antimicrobial therapy. Animal husbandry and management of manure in both urban and peri-urban farming should be addressed to lower the risk of transmission of enteropathogens between livestock and man. A study to determine the faecal transmission between man and livestock detected ampicillin and tetracycline-resistant *E.coli* in both man and cattle living in close contact (Lupindu *et al*., 2015). Efforts to develop *E.coli* vaccine are currently focused on ETEC, but there are many challenges because of the diverse nature of this pathogen. A study by Shaheen *et al*.(2003) detected and described 164 strains of ETEC.

The contamination of foods and water with faecal matter is the primary mode of transmission of *E.coli* to man, and subsequently, prevention methods include improved sanitation/hygiene, hand washing and access to clean water. Large-scale dissemination of multi-resistant pathogenic organisms in hospitals is an emerging public health threat. Therefore, mitigation strategies should be taken focusing on medical, agricultural and environmental changes to arrest this trend (Wellington *et al*., 2013). Antimicrobial resistance is paramount because it is not restricted to bacteria only but extends to viral, fungal and parasitic diseases. New, accurate diagnostics like PCR before prescription and therapeutics are required to achieve rapid identification and selection of organism and the use of effective therapies (Levy, 2005). Routine surveillance of antimicrobial resistance trends will generate useful data and promote stewardship. The research studies should have an ecology approach to address all factors of emergence and spread (Diekema and Pfaller, 2013; Kerremans *et al*., 2008).

2.8 Global Studies in Antimicrobial Resistance

In an attempt to improve diagnostics not to rely on culture and growth of pathogens, which takes time, there is a need for strain-level identification for the accurate and effective choice of agent. Diagnostics development is listed in the USA as one of the White House National Action Plan for Combating Antimicrobial Resistance (Fortman and Mukhopadhyay, 2016). Bacterial isolates from surface waters of Lake Taihu in China showed multiple antimicrobial resistance (62.0%) indicating the urgency of this menace and the health risks to animals and people dependent on the lake for water consumption (Yin *et al*., 2013). Kumarasam *et al*. (2010) while investigating β-lactam resistant *E.coli* in India, recommended coordinated international surveillance to arrest the global public health problem. In Malaysia, Aliyu *et al*. (2016) observed *E.coli* resistance to ESBL

to be 48.8% while investigating poultry meat in retail markets. High resistance rates to β-lactams: ampicillin 100%, cefotaxime 100%, ceftazidime 100% were reported by Chishimba *et al*. (2016).

A research study in Argentina demonstrated chicken and their products as significant sources of atypical EPEC strains of *E.coli,* and thus hygiene should improve in handling and slaughter (Alonso *et al*., 2016).In Australia, a study aimed at defining the frequency of AMR in *E.coli* sampled from production animals established the presence of antimicrobial resistance to amoxicillin-clavulanic acid (4.14%), ampicillin (39%), streptomycin (28%) (Abraham *et al*., 2015). Globally, studies on AMR of *E.coli* and other pathogens have been conducted with a number focusing on foods of animal origin (Kluytmans *et al*., 2013; Adeyanju and Ishola, 2014; Abraham *et al*., 2015; Önen *et al*., 2015; Tew *et al*., 2016; Kikuvi *et al*., 2006).

2.9 Antimicrobial Resistance Studies in Africa

In East Africa, Uganda, studies showed resistance of *E.coli* obtained from hospital patients' urine to sulfamethoxazole /trimethoprim 70%, ceftriaxone 3%, AMC 36%, ciprofloxacin 11%, chloramphenicol 20% and gentamicin 11%, (Najjuka *et al*., 2016). Another study in Uganda (Byarugaba, 2004) attributed the rise of AMR in developing countries to poverty and related factors that will require coordinated interventions and a global approach to combat it. In Tanzania, Madoshi *et al*. (2016) investigated *E.coli* strains in cattle and their handlers and found 16 ERIC-PCR genotypes which were shared between the two hosts, an indicator of transfer of strains between two hosts. This study demonstrated that human *E.coli* isolates are more resistant than those isolated from cattle.

There was 100% AMR to amoxicillin, and AMC in *E.coli* samples obtained from frozen poultry meat in Nigeria, indicating an alarming rise in AMR (Adeyanju and Ishola, 2014). Studies conducted in Morocco investigating ESBL in *E. coli* strains causing UTI's, attributed the rise in the prevalence of β-lactam resistance to poor hygiene and living conditions and uncontrolled antimicrobial consumption in the community (Barguigua *et al*., 2013). The rise of AMR is a complex interplay of man, animals, environmental and pathogen-related factors (Shears, 2001; Okeke, 2009). The selection of AMR has been driven by antimicrobial exposure in agriculture, environment and health care (Holmes *et al*., 2016, Eltayb et al, 2012). In Zambia, 20% of the analysed isolates from poultry contained ESBL producing *E.coli* (Chishimba *et al*., 2016). The research findings demonstrated that frequent antimicrobial administration in poultry might contribute to the emergence of resistant strains. Poultry is an important reservoir for AMR genes which could spread to man. A study investigating antimicrobial residues in South Africa by Ntuli *et al*. (2016) detected 70% antimicrobial-resistant *E.coli* isolates.

2.10 Antimicrobial Resistance Studies in Kenya

Gakuya *et al*. (2001) demonstrated that wild rats carry antimicrobial resistant bacteria which could spread to man and other animals and thus are a threat to general public health. Rats could act as reservoirs of genetic pools and foci of multiplying resistant bacteria with resulting transmission to other animals as well as man. A total of 20% of samples showed resistance to one or more of the antibiotics investigated in that study. Kariuki *et al*. (1999) investigated *E. coli* in children and from chickens living in close contact. Studies by Albrechtova *et al*. (2012) in Northern Kenya found ESBLs in companion animals of pastoralists. According to Kiiru *et al*. (2012), there is an alarmingly high rates of βeta-lactam resistance gene *bla*, in strains of *E. coli*

causing infections in Kenyan patients. A study investigating the occurrence of pathogenic *E.coli* from hotel food workers in Kenya expressed concern over strains resistant to ampicillin 53%, tetracycline 56% and SXT 53.8% with 61.5% being MDRS in that study. Similar studies by Onono *et al*. (2010) in Nairobi investigated AMR profiles of *E.coli* isolates from cattle and milk detected resistance to mpicillin, tetracycline, nalidixic acid and sulfamethoxazole.

According to Kuria *et al*. (2002), two-thirds of veterinary drugs are dispensed by unqualified personnel. This fact confounds the finding of antimicrobial agents residues in foods from animal sources. A rounded approach is thus necessary in order to arrest antimicrobial resistance locally, regionally and internationally (Ombui, 1994; Kang'ethe *et al*., 2005, Kang'ethe *et al*., 2007). Several other studies in Kenya have focused on AMR of *E. coli* in humans (Senerwa *et al*., 1989b; Bebora *et al*., 1994; Sang and Schnabel, 2012; Kariuki *et al*., 1993; Kiiru *et al*., 2012; Kariuki *et al*., 2005) and from food-producing animals and animal products (Bebora *et al*., 1994; Ombui, 1994; Mitema *et al*., 2001; Mapenay *et al*., 2007; Kariuki 2002; Kariuki 2013).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Area and Design

The research study was carried out in Korogocho and Viwandani informal settlements of Nairobi. The two informal settlements are densely populated locations of Kasarani and Makadara sub-counties of Nairobi respectively. The sample framework was provided by the African Population & Health Research Centre (APHRC). This was a cross-sectional study where data were collected from the target population and analyzed to answer research questions.

3.2 Urban Zoo

This research study was supported by the Urban Zoo project, directed by the International Livestock Research Institute (ILRI) and funded by the Natural Environment Research Council, The Medical Research Council, the Economic and Social Research Council as well as the Biotechnology and Biosciences Research Council through the Environmental and Social Ecology of Human Infectious Diseases Initiative (ESEI). The project collaborated with other partners including the University of Nairobi, The African Population and Health Research Centre (APHRC), The Food Agriculture Organization (FAO), The Royal Veterinary College, University of London, The Centre for Immunity, Infection and Evolution at the University of Edinburgh, The International Livestock Research Institute (ILRI), The Wellcome Trust Sanger Centre, The Roslin Institute and The Kenya Medical Research Institute (KEMRI). Urban Zoo has a focus on socioeconomics, ecology and epidemiology of zoonotic disease rise in urban areas. The primary objective of Urban Zoo is to comprehend the mechanisms that result in the introduction and subsequent spread of pathogens into urban populations through production animal value chains.

3.3 Clearance to undertake the research

Approval of the research was granted by the University of Nairobi's Faculty of Veterinary Medicine's Biosecurity, Animal Use and Ethics Research Committee, the Institutional Research Ethics Committee at ILRI and the International Ethical Review Committee (IERC) of the African Medical Research Foundation (AMREF). Animal owners gave their consent before sample collection from their livestock.

3.4 Sampling and Sample Collection

Sampling and data collection was conducted by Urban Zoo. The proportional random sampling technique was employed and a simple random selection of households and livestock. The samples collected were faecal samples for cattle, goats, sheep, pigs and cloacal swabs for poultry (chicken and ducks) and rabbits.

The sample framework was provided by the African Population & Health Research Centre (APHRC), and the samples were computer-generated. The sample size was obtained using the formula by Dohoo *et al*. (2003).

$$n = \frac{Z^2(P)(q)}{L^2} \quad \text{Eq. 1}$$

Where:

n= required sample size,

Z= Z value (at 95% level of confidence)

P= the expected prevalence

q= 1-p

L= level of precision

$$n = \frac{1.96^2(0.5)(1-0.5)}{0.05^2}$$

n=384

A prevalence value of 50% was used, and a design effect of 2 was applied to cater for clustering. Samples were collected from livestock namely cattle, goats, sheep, pigs, chicken, ducks and rabbits; foods and water in Korogocho and Viwandani informal settlements, Nairobi. Conventional biochemical methods were used to identify and isolate *E.coli* which were then stored in skimmed milk at -80 degrees Celsius.

3.5 Reviving the *Escherichia coli* isolates

The *E.coli* isolates had previously been collected by Urban Zoo, isolated, biochemically tested and confirmed, and stored in skimmed milk at -80ºC. Samples were revived for pathotyping, antimicrobial susceptibility testing and genetic characterization of AMR. The isolates were taken out of the freezer and were left to stand at room temperature for two hours before streaking on McConkey agar (Oxoid) plates and incubating at 37 degrees Celsius for 24 hours to obtain pink colonies. Distinct colonies were then picked from MacConkey agar and streaked on Tryptone Soya Agar (Oxoid Hampshire, England) and incubated overnight at 37 degrees Celsius to obtain pure colonies (See Appendix1for media preparation).

3.6 Extraction of Genomic DNA

Bacterial colonies on Trypton Soy Agar were suspended in 2ml centrifuge tubes with sterile distilled water, then placed in a water bath previously set at 115^0 C for 30 minutes. This was followed by centrifuging at 12000 rpm for five minutes and decanting the supernatant into sterile Eppendorf tubes. This was stored at -20^0 C awaiting DNA amplification (Nazik *et al*., 2011).

3.7 Pathotyping of *Escherichia coli* Isolates

The extracted DNA for each isolate was taken through polymerase chain reaction (PCR) to amplify the gene for each pathotype using specific primers for respective target genes Table 3.1 below lists the primers that were used in the amplification of each gene encoding for virulence factors . These isolates were assayed for the presence of *VT* of EHEC, *eae* of EPEC, *AggR* of EAEC, *daaE* of DAEC, *eltA* and *estA* of ETEC.The PCR reactions were conducted in a total volume of 25 µl, including 2.5µl PCR Buffer Coraload, 0.5 µl dNTPs, 0.1µl primers, 0.125 µm Taq DNA polymerase, and 5µl of DNA. Amplification reactions were performed using a DNA thermo-cycler (Veriti® 96 well Thermo Cycler, Applied Biosystems) programmed as follows: Initial denaturation at 95ºC for 1 minute followed by 35 amplification cycles of denaturation at 95ºC for 15 seconds, primer annealing at 55ºC for 15 seconds, primer extension at 72ºC for 30 seconds and final primer extension at 72ºC for 7 minutes. The negative control used for each pathotype was DNAse free water.

The electrophoresis gel was prepared by adding 2% gel to Tris-Acetate Buffer (TAE) at 8.0 Ph and warming it until it dissolved completely. It was then cooled, and 0.5µg/ml of Ethidium Bromide added to 100ml of Tris-Acetate buffer. The gel was cast and set to dry. Ten microlitres

of the PCR products were loaded on the gel previously cast, and 6µl of a molecular weight marker with 100bp increments (100bp DNA ladder) from Qiagen GelPilot®, USA was used as a size standard. The amplified samples were analysed in a horizontal electrophoresis unit (Consort Electrophoresis EV245) at 250 Volts for 40 minutes. The results were visualised using UVP Gelmax Imager projected to a computer screen and saved.

3.8 Antimicrobial SusceptibilityTesting

Antimicrobial susceptibility to selected antimicrobial agents was assessed using the Disk Diffusion method of Kirby Bauer Hudzick (2013). Individual distinct colonies of each isolate were picked from TSA (Oxoid, Hampshire, England) and suspended in normal saline to obtain a 0.5 turbidity solution according to McFarland's principles. A visual comparison was prepared to a 0.5 McFarland standard tube for each inoculum according to WHO protocol "Global Salm-Surv, 2003"(See Appendix). The suspension obtained contained approximately 1 to 2×10^8 colony forming units/ml.

Using sterile swabs, each suspended isolate was swabbed onto plates with Müeller Hinton Agar (MHA) as described by the Clinical Laboratory Standard Institute (CLSI, 2014). The antibiotic disks were obtained from Oxoid Ltd, United Kingdom, England. Three plates of MHA were used for each isolate. Antimicrobial discs were placed on the swabbed MHA with proper spacing to prevent the overlapping of the diameter zone of inhibition. The disks were pressed down to allow total and complete contact with the agar surface. Each isolate was screened for antimicrobial susceptibility testing with twelve different antimicrobial drugs. The antimicrobial agents and their corresponding concentrations were as follows: - the first plate with Amoxycillin- clavulanic acid

(20/10ug), ampicillin (10ug), ceftazidime (30ug), cefuroxime (30ug) and ceftriaxone (30ug). The second plate contained nalidixic acid (30ug), tetracycline (30ug), ciprofloxacin (5ug), and trimethoprim-sulfamethoxazole (1.25/23.75ug) and the third plate with gentamicin (10ug), streptomycin (10ug) and chloramphenicol (30ug). *β*-lactam antimicrobial agents were placed next to the amoxicillin-clavulanate (AMC) disc at inter disc distances centre to centre of 20mm. The swabbed MHA plates were aerobically incubated at 37 degrees Celsius for 24 hours. Antimicrobial susceptibility and resistance of each isolate were obtained by measuring the diameter zone of inhibition using vernier callipers, and the measurements in millimetres were recorded. The interpretation was made in accordance with (CLSI) guidelines for each tested antibiotic (See Appendix). Each isolate was scored Susceptible, Intermediate or Resistant accordingly. *Escherichia coli* ATCC 25922 was the quality control organism used for susceptibility testing in this study (Table 3.2).

3.9 Genetic characterization of antimicrobial resistance

For isolates that displayed resistance to one or more of the above-listed antimicrobials, specific antibiotic-resistant genes were detected using multiplex polymerase chain reaction (PCR). Table 3.3 below shows the antibiotic type and the genetic determinant of resistance tested for each category. Table 3.4 shows the list of primers used in testing for genetic resistance of the different antimicrobial agents. The PCR reactions were conducted in a total volume of 25 µl, including 2.5µl PCR Buffer Coraload, 200 µm dNTPs, 1µm primers, 0.125 µm Taq DNA polymerase, and 5µl of DNA as a template. Amplification reactions were performed using a DNA thermo-cycler (Veriti® 96 well Thermo Cycler, Applied Biosystems) programmed as follows:Initial denaturation at 95ºC for 8 minutes followed by 35 amplification cycles of denaturation at 94ºC

for 2 minutes, primer annealing at 56ºC for 70 seconds, primer extension at 72ºC for 2 minutes and final primer extension at 72ºC for 8 minutes.

The electrophoresis gel was prepared by adding 1.5% gel to Tris Acetate-EDTA (TAE) buffer at 8.0 pH and warming it until it dissolved. It was then cooled, and 0.5µg/ml of Ethidium Bromide added to 100ml of TAE. The gel was cast and set to dry. Ten µl of the PCR products were loaded on the gel previously cast. Six µl of a molecular weight marker with 100bp increments (100bp DNA ladder) from Qiagen GelPilot®, USA was used as a size standard. The amplified samples were analysed in a horizontal electrophoresis unit (Consort Electrophoresis EV245) at 250 Volts and 250 Amps. The results were read using UVP Gelmax Imager, projected to a screen and saved.

3.10 Data Entry, Cleaning and Analysis

After completion of laboratory work, the data was keyed into the computer using Microsoft Excel® software database. Data cleaning and coding were done. Microsoft Excel 2010 was used for descriptive analysis including frequencies, proportions, charts and graphs). Statistical analysis was prepared using SPSS 14.0. The rates of resistance were compared by Chi square test. A P value of <0.05 were considered to be statistically significant. and inferential analysis. Chi-square tests were used to carry out comparisons between the various proportions, including pathotypes and antibiotic resistance profiles.

Table 3. 1: Primers used in Pathotyping (Macharia, 2019)

Oligo Name	Sequence 5' – 3'	Target Gene	Product Size (bp)
STXF	GAGCGAAATAATTTATATGTG	VT of EHEC	518
STXR	TGATGATGGCAATTCAGTAT		
AEAF	CTGAACGGCGATTACGCGAA	eae of EPEC	918
AEAR	CGAGACGATACGATCCAG		
AGGF	GTATACACAAAAGAAGGAAGC	AggR of EAEC	254
AGGR	ACAGAATCGTCAGCATCAGC		
LTF	GCACACGGAGCTCCTCAGTC	eltA of ETEC	218
LTR	TCCTTCATCCTTTCAATGGCTTT		
STF	GCTAAACCAGTAGAGSTCTTCAAAA	estA of ETEC	147
	CCCGGTACAGRGCAGGATTACAACA		
DAAEF	GAACGTTGGTTAATGTGGGGTAA	daaE of DAEC	750
DAAER	TATTCACCGGTCGGTTATCAGT		

Table 3. 2: Quality control ranges for *Escherichia coli* ATCC 25922 for the selected antimicrobial agents

Antimicrobial Agent	**Antibiotic concentration (micrograms)**	***Cut off Diameter zone of Inhibition for Escherichia coli* ATCC 25922**
Ampicillin	10µg	16-22 mm
Amoxicillin-clavulanic acid	20/10 µg	18-24 mm
Ceftriaxone	30 µg	29-35 mm
Cefuroxime	30 µg	20-26 mm
Gentamicin	10 µg	19-26 mm
Streptomycin	10 µg	12-20 mm
Tetracycline	30 µg	18-25 mm
Ciprofloxacin	Five µg	30-40 mm
Nalidixic acid	30 µg	22-28 mm
Chloramphenicol	30 µg	21-27 mm
Trimethoprim-sulfamethoxazole	1.25/23.75 µg	23-29 mm
Ceftazidime	30 µg	25-32 mm

Table 3. 3: Antibiotic Type and genetic determinant of Resistance Tested

ANTIBIOTIC TYPE	RESISTANCE GENES
β-lactam	*bla SHV, bla CMY, bla TEM*
Tetracycline	*tet(A), tet(B)*
Aminoglycosides	*aac(3) IV, aadA1*
Sulphonamides	*sul1, sul2*

Table 3. 4: Primers used in genetic characterization of antimicrobial resistance

Antimicrobial Agent	Target Gene	Sequence	Amplicon size(bp)	Reference
Tetracycline	Tet(A)	F-GGTTCACTCGAACGACGTCA R-CTGTCCGACAAGTTGCATGA	577	Randall *et al*. 2004
Tetracycline	Tet(B)	F-CCTCAGCTTCTCAACGCGTG R-GCACCTTGCTGATGACTCTT	634	(Randall *et al*., 2004)
Beta-lactam	blaSHV	F-CGCCTGTGTATTATCTCCC R-CGCAGATAAATCACCACAATG	787	(Van *et al*., 2008)
	blaCMY	F-TGGCCAGAACTGACAGGCAAA R-TTTCTCCTGAACGTGGCTGGC	462	(Van *et al*., 2008)
	blaTEM	F-GAGTATTCAACATTTTCGT R-ACCAATGCTTAATCAGTGA	284	(Van *et al*., 2008)
Aminoglycoside	aadA1	F-TATCCAGCTAAGCGCGAACT R-ATTTGCCGACTACCTTGGTC	490	(Van *et al*., 2008)
	aac(3)- IV	F-CTTCAGGATGGCAAGTTGGT R-TCATCTCGTTCTCCGCTCAT	286	(Van *et al*., 2008)
Sulfonamide	sull	F-TTCGGCATTCTGAATCTCAC R-ATGATCTAACCCTCGGTCTC	822	(Van *et al*., 2008)
	sul2	F-GCGCTCAAGGCAGATGGCATT RGCGTTTGATACCGGCACCCGT	285	(Van *et al*., 2008)
Chloramphenicol	catA1	F-AGTTGCTCAATGTACCTATAACC R-TTGTAATTCATTAAGCATTCTGCC	547	(Randall *et al*., 2004)
	cmlA-	F-CCGCCACGGTGTTGTTGTTATC R-CACCTTGCCTGCCCATCATTAG	698	(Mammeri *et al*., 2005)

CHAPTER FOUR: RESULTS

4.1 *Escherichia coli* Revived from Skimmed Milk

A total number of 174, 38, and 1168 viable *E. coli* isolates were revived successfully as evidenced by growth on Mac-Conkey agar. The E.coli were isolated from food, water and livestock samples. Respectively.

4.2 Pathotyping of *Escherichia coli* Isolates

Each of the revived *E.coli* isolates was tested for pathotype with specific primers using multiplex Polymerase Chain Reaction Technique. Table 4.1 shows the obtained results for food isolates with six pathotypes. The most prominent pathotype among food isolates was ETEC *estA* with 2% of the isolates testing positive while ETEC *eltA* was the least detected with only one isolate 0.5%. The EHEC, EPEC and EAEC and DAEC pathotypes were identified in 1% of the isolates. Figure 4.1 and 4.2 show amplicons obtained with primers specific to Enterohaemorrhagic *E.coli* isolated from food Multiplex PCR was used to test each isolate from water for genetic determinants of virulence. A total of 38 isolates were tested. Three isolates (8%) of the water isolates were positive for ETEC *estA* while .1% contained the virulence gene for ETEC *eltA*. None of the tested isolates contained EHEC or EPEC. Table 4. 1 shows the obtained results for water isolates. Each revived *E.coli* isolates from livestock was investigated for virulence gene with six specific primers using multiplex PCR. Among these isolates,the most prominent pathotype was EHEC with 4% of the isolates testing positive followed by DAEC and EPEC at 2% each; while ETEC *estA* was the least detected at 0.1%. The virulence determinant *AggR* of EAEC and the heat-labile virulence gene *eltA* of ETEC were detected in 0.3% and 0.4% isolates respectively. Table 4.3

shows the obtained results with six virulence determinants. See Appendix for PCR amplicons for different pathotypes.

Table 4. 2: Pathotype frequency, food isolates

	EPEC	DAEC	EHEC	EAEC	ETEC *eltA*	ETEC *estA*
POSITVE	2(1%)	2(1%)	2(1%)	… 2(1%)	1(0.5%)	4(2%)
NEGATIVE	172(99%)	172(99%)	172(99%)	.. 172(99%)	173(99.5%)	170(98%)
n=174						

Key: EPEC-Enteropathogenic *E. coli*, DAEC - Diffusely Adhering *E. coli*, EHEC - Enterohaemorrhagic *E. coli*, ETEC *eltA* - Enterotoxigenic *E. coli* (heat-labile toxin) ETEC *estA* - Enterotoxigenic *E. coli* (heat-stable toxin)

Table 4. 3: Pathotype frequency water isolates

	EPEC	DAEC	EHEC	EAEC	ETEC *eltA*	ETEC *estA*
POSITVE	0(0%)	1(3%)	0(0%)	2(5%)	1(3%)	3(8%)
NEGATIVE	38(100%)	37(97%)	38(100%)	36(95%)	37(97%)	35(92%)

n=38

Key: EPEC-Enteropathogenic *E. coli*, DAEC - Diffusely Adhering *E. coli*, EHEC - Enterohaemorrhagic *E. coli*, ETEC *eltA* - Enterotoxigenic *E. coli* (heat-labile toxin) ETEC *estA* - Enterotoxigenic *E. coli* (heat-stable toxin)

Table 4. 4: Frequency of pathotypes detected among livestock isolates

	EPEC	DAEC	EHEC	EAEC	ETEC eltA	ETEC estA
POSITVE	17(2%)	24(2%)	46(4%)	4(0.3%)	5(0.4%)	1(0.1%)
NEGATIVE	1148(98%)	1147(98%)	1122(96%)	1164(99.7%)	1163(99.6%)	1167(99.9%)
n=1168						

Key: EPEC-Enteropathogenic *E. coli*, DAEC - Diffusely Adhering *E. coli*, EHEC - Enterohaemorrhagic *E. coli*, ETEC ***eltA*** - Enterotoxigenic *E. coli* (heat-labile toxin) ETEC ***estA*** - Enterotoxigenic *E. coli* (heat-stable toxin)

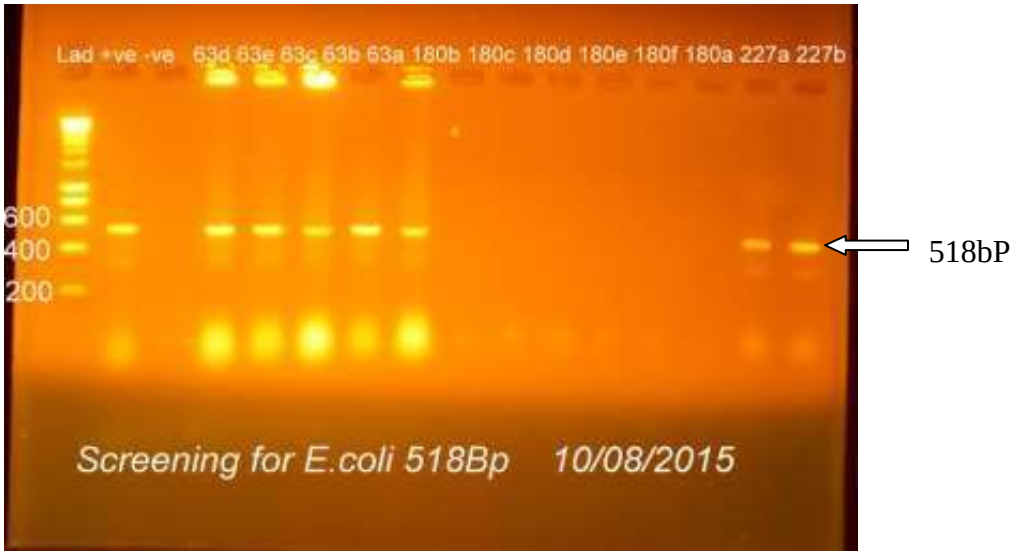

Figure 4. 1: PCR amplicons obtained by primer specific to EHEC (518bp).

818 bP - Lane 1 is 200 bp DNA Ladder. Lane 2 is a positive control, and lane 3 is a negative control. Lane 4-8, 15, 16 are positive results for 518 bp and lanes 9-14 are negative for 25bp.

Figure 4. 2*: PCR amplicons obtained by primer specific to EHEC 518bp.*

EHEC - Lane 1 is 200bp DNA Ladder. Lane 2-8,13,14 are positive results for 518bp and lanes 19-12,15 and16 are negative for EHEC.

Figure 4. 3**:**Analysis of *E.coli* pathotype genes by PCR.

Lane 1 is 50 bp DNA ladder. Lane 2 is a positive control, and lane 3 is a negative control. Lane 4,5,6,7,8,9,10 and 12 show negative results for ETEClt pathotype (218 base pairs). Lanes 13,14,15 and 16 show positive result after PCR amplification.

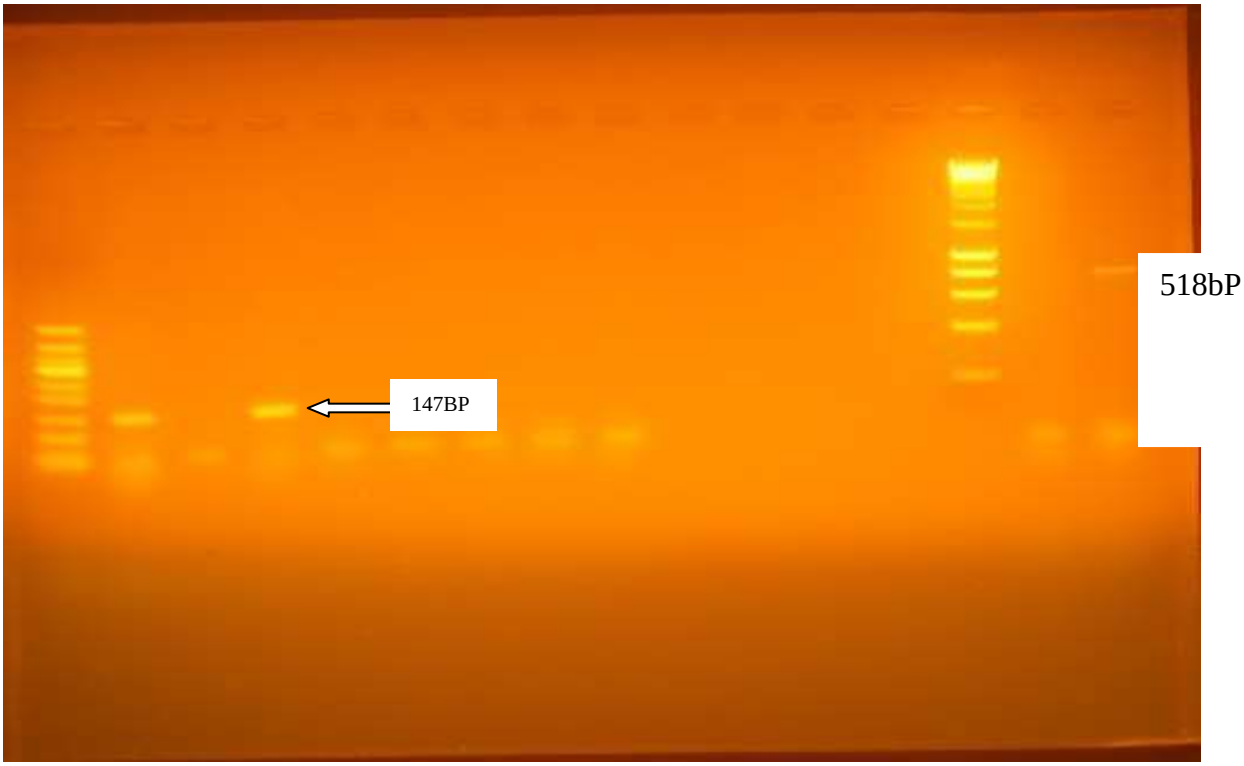

Figure 4. 4**:**Analysis of *E.coli* pathotype genes by PCR.

Lane 1 is 50 bp DNA ladder. Lane 2 is a positive control, and lane 3 is a negative control. Lane 4,5,6,7,8,9,10 and 12 show negative results for EAEC pathotype (147 base pairs). Lanes 13,14,15 and 16 show positive result after PCR amplification.

4.3 Phenotypic Antimicrobial Susceptibility Testing

4.3.1 Antimicrobial Susceptibility of Escherichia coli From Food Isolates

A total number of 174 isolates from food were tested for antimicrobial susceptibility using the disk diffusion method with twelve antimicrobial agents. Figure 4.2 shows one of the isolates streaked on TSA with five antimicrobial disks. The disks surrounded by clear zones indicate a lack of *E.coli* growth confirming susceptibility of the isolates to the antibiotic. On the other hand, discs without clear zones around them confirmed that the bacterium was resistant since it was able to grow around the disc.

Out of the food isolates tested, 8.9% were shown to be resistant to antimicrobial agents, 15.1% of isolates were intermediate, and 76% were found to be susceptible to antimicrobial agents tested. Table 4.4 shows the results obtained with twelve antimicrobial agents. The highest resistance was shown to ampicillin (45.4%), followed by sulfamethoxazole/trimethoprim (41.2%) and tetracycline (29.3%). Resistance to streptomycin was detected in 39 (22.5%) isolates while 22 (12.6%) isolates were resistant to amoxicillin-clavulanic acid. Among the food isolates, the least resistance (0%) was demonstrated to cefuroxime where all the isolates were susceptible. Three isolates (1.8%) were resistant to ciprofloxacin, while only one (0.6%) of the isolates was resistant to gentamicin. Twelve (6.9%) and 18 (10.4%) *E. coli* isolates were found to be resistant to and chloramphenicol and gentamicin respectively.

4.3.1 Antimicrobial Susceptibility of Escherichia coli From Water Isolates

A total number of 38 isolates from water were tested for antimicrobial susceptibility using the disk diffusion method with twelve antimicrobial agents. Table 4.5 shows the results obtained with twelve antimicrobial agents. The highest resistance was shown to ampicillin (73.6%) and to tetracycline (63.1%). The least resistance (0%) was demonstrated to cefuroxime and ceftriaxone where all the isolates were susceptible. Four (10.5%) isolates were shown to be resistant to chloramphenicol.

4.3.2 Antimicrobial Susceptibility of Escherichia coli From Livestock Isolates

A total number of 1168 isolates from livestock were tested for antimicrobial susceptibility using the Disk diffusion method. Out of these, 74.5 % were shown to be susceptible to antimicrobial agents, 8% isolates were intermediate, and 17.5% were resistant to antimicrobial agents tested. The highest resistance was shown to tetracycline at 56% and Sulfamethoxazole/ trimethoprim at 53%, followed by streptomycin with 45% of the isolates showing resistance. The least resistance was shown to amoxicillin/clavulanate and ciprofloxacin at 1% each. Resistance to cefuroxime and ceftriaxone was seen in 2% and 3% of the isolates respectively. Table 4.6 shows the antimicrobial resistance profile of the isolates with twelve antimicrobial agents.

Ciprofloxacin and amoxicillin/clavulanic acid were the most effective antimicrobial agents, with 99% of the isolates showing susceptibility to the two antibiotics followed by ceftriaxone with 98% susceptible isolates. Resistance to tetracycline was the most common finding in livestock isolates (56%) followed by sulfamethoxazole-trimethoprim (53%), streptomycin (45%) and ampicillin (32%).

Table 4. 5: Frequency of AMR for food isolates

	AMC	AMP	CAZ	CRO	CXM	NA
Resistant	22(12.6%)	79(45.4%)	3(1.7%)	0	2(1.1%)	18(10.4%)
Intermediate	16(9.2%)	37(21.3%)	13(7.5%)	19(11%)	11(6.4%)	15(8.6%)
Susceptible	136(78.2%)	58(33.3%)	158(90.8%)	155(89%)	161(92.5%)	141(81%)

	TE	CIP	SXT	CN	S	C
Resistant	51(29.3%)	3(1.8%)	68(41.2%)	1(0.6%)	39(22.5%)	12(6.9%)
Intermediate	11(6.3%)	11(5.7%)	10(3.3%)	12(6.9%)	37(21.4%)	12(6.9%)
Susceptible	112(64.4%)	161(92.5%)	96(55.5%)	161(92.5%)	97(56.1%)	150(86.2%)

n=174

Table 4. 6: Frequency of AMR for water isolates

	AMC	AMP	CAZ	CRO	CXM	NA
Resistant	10(26.3%)	28(73.6%)	0	0	1(2.6%)	8(21.1%)
Intermediate	6(15.8%)	5(13.2%)	13(34.2%)	7(18.4%)	13(34.2%)	7(18.4%)
Susceptible	22(57.9%)	5(13.2%)	25(65.8%)	21(81.6%)	14(63.2%)	23(60.5%)

	TE	CIP	SXT	CN	S	C
Resistant	24(63.1%)	2(5.3%)	17(44.7%)	1(2.6%)	11(28.85%)	4(10.5%)
Intermediate	6(15.8%)	12(31.6%)	10(26.3%)	27(71.1%)	13(34.2%)	8(21.1%)
Susceptible	8 (21.1%)	24(63.1%)	11(29%)	10(26.3%)	14(36.8%)	26(68.4%)

n=38

Table 4. 7: AMR profile with 12 antimicrobial agents for livestock isolates

	AMC	AMP	CAZ	CRO	CXM	NA
Resistant	9(1%)	367(32%)	36(3%)	37(3%)	25(2%)	88(8%)
Intermediate	91(8%)	121(10%)	62(5%)	42(4%)	79(7%)	71(6%)
Susceptible	1068(91%)	680(58%)	1070(92%)	1089(93%)	1064(91%)	1009(86%)
	TE	**CIP**	**SXT**	**CN**	**S**	**C**
Resistant	648(56%)	16(1%)	618(53%)	30(3%)	524(45%)	49(4%)
Intermediate	3(0.3%)	32(3%)	70(.6%)	93(8%)	498(43%)	21(2%)
Susceptible	517(43.7%)	1120(96%)	543(46.4%)	1045(89%)	146(12%)	1098(94%)
n=1168						

Key: AMC- Amoxicillin-Clavulanic acid, AMP - Ampicillin, CAZ - Ceftazidime, CRO - Ceftriaxone, CXM - Cefuroxime, NA - Nalidixic Acid, TE - Tetracycline, CIP - Ciprofloxacin, SXT - Trimethoprim-Sulfamethoxazole, CN - Gentamicin, S - Streptomycin, C - Chloramphenicol

4.4 Genetic Determinants of Resistance

4.4.1 Genetic Determinants of Resistance for Food Isolates

The isolates which were phenotypically resistant to antimicrobial agents were screened for resistance genes using PCR obtaining results in Table 4.7. *β*-lactam TEM gene was the most prominent and was detected in 91% of the resistant *E.coli* isolates. This was followed by *bla* CMY resistance gene at 51%. The *β*-lactam gene *bla* SHV was the least detected in this category at 7.8%. The most prominent tetracycline resistance gene was *tet* A at 31% and *tet* B at 29% while *aac3* (V) was detected in 17% of the test isolates. In sulphonamides, the most frequent resistant gene was *sul2* (18%) while *sul1* was seen in 10% of the *E.coli* isolates. Chloramphenicol resistance genes *cml* and *cat*1 were detected in 8% and 16% respectively.

4.4.1 Genetic Determinants of Resistance for Water Isolates

The isolates which were phenotypically resistant to antimicrobial agents were screened for resistance genes using PCR obtaining results in Table 4.8. *β*-lactam *TEM* gene was the most prominent and was present in 25% of the resistant *E.coli* isolates. This was followed by *bla SHV* resistance gene at 21.4%. The *β*-lactam gene *bla CMY* was the least detected in this category with only 3 isolates (10.7%). The most prominent tetracycline resistance gene was *tet* A at 41.7% and *tet* B at 41.7% while *aac3* (V) was detected in 25% of the tested isolates. In sulphonamides, the most frequent resistant gene was *sul2* (63.6%) while *sul1* was detected in 36.4% of the isolates.

4.4.2 Genetic Determinants of Resistance for Livestock Isolates

Among the sulphonamide resistance genes investigated, *sul1* was detected in 19% of the *E.coli* isolates while *sul2* was detected in 26% of isolates. The trimethoprim resistance gene investigated *aadA1*was detected in 80 (17%) isolates from livestock. The extended spectrum *β*-lactamase gene bla *TEM* and *bla CMY* were detected in 35% and 20% respectively, while *bla SHV* were only detected in 1.5% of the isolates. The tetracycline resistance genes most frequently detected were *tetA* (15%) and *tetB* (24%) of isolates (Table 4.9). Chloramphenicol resistance genes *cat1* and *cml* were detected in 18% and 14% of the test samples, respectively.

4.5 Antimicrobial Resistance of *Escherichia coli* Isolates from Viwandani and Korogocho

4.5.1 From food isolates

The antimicrobial resistance profile demonstrated a higher percentage of resistant strains in Korogocho than in Viwandani in all food samples (Table 4.10). Eighty-nine percent of the isolates resistant to amoxicillin/clavulanate were from Korogocho and 11% from Viwandani respectively. Korogocho area harbours 71% of the isolates resistant to Ampicillin and 78% of those resistant to ceftazidime.

4.5.2 From livestock isolates

Animal species from Korogocho area contributed a higher percentage of resistance isolates than those from Viwandani for all antimicrobial agents except for ciprofloxacin, as shown in Table 4.10. Four hundred and seventy-eight (74%) of isolates resistant to tetracycline were from Korogocho while Viwandani contributed 26% resistant isolates to the same antimicrobial, while

86% and 14% of those resistant to chloramphenicol were from Korogocho and Viwandani respectively. In ciprofloxacin, 38% and 62% of the resistant isolates were from Korogocho and Viwandani, respectively.

4.6 Antimicrobial Resistance of *Escherichia coli* From the Various Sources of sample

4.6.1 Antimicrobial Resistance of Escherichia coli From the Various Livestock Species

Overall, in comparison with cattle, pigs and rabbits, chicken isolates demonstrated the highest resistance prevalence (Table 4.11) for most antimicrobial agents. The differences between species were statistically significant ($P < 0.05$) for seven out of twelve antimicrobial agents evaluated in this study. Two hundred and seventy-three (74%) of all isolates that were resistant to ampicillin and 89% of isolates resistant to amoxicillin-clavulanic acid were from chicken. Chicken isolates demonstrated a remarkably high frequency of resistance to trimethoprim-sulfamethoxazole (80%) and gentamicin (77%). Goats also contributed markedly to resistant isolates with 24% and 14% of isolates resistant to ceftriaxone and ceftazidime being from this species. Seven (44%) of isolates that showed resistance to ciprofloxacin were from pigs and chicken simultaneously.

4.6.2 Antimicrobial Resistance of Escherichia coli From the Various Foods samples

The highest resistance among food isolates was recorded for tea with milk which was resistant to nine of the antimicrobials investigated. The highest resistance (54.5%) was recoreded for sulfamethoxazole/trimethoprim.

Table 4. 8: Genetic determinants of resistance for food isolates

	βla TEM	βla SHV	βla CMY	tet A	tet B	aaC3(V)
Positive	17(91%)	1(7.8%)	4(51%)	11(31%)	10(29%)	6(17%)
Negative	11(9%)	27 (92.2%)	24(49%)	24(69%)	25(71%)	30(83%)
Total	28	28	28	35	35	36
	sul 1	**sul 2**	**aadA1**	**cml**	**cat 1**	
Positive	4(10%)	7(18%)	3(8%)	1(14%)	2(16%)	
Negative	35(90%)	32(82%)	36(92%)	11(92%)	9(84%)	
Total	39	39	39	12	12	

Key *βla TEM* - resistance gene of β-lactam, *βla SHV* - resistance gene of β-lactam, *βla CMY* - resistance gene of *β*-lactam, *tet* A - resistance gene of tetracycline, *tet* B - resistance gene of tetracycline, *aaC3(V)* - resistance gene of aminoglycosides, *sul 1* -resistance gene of sulphonamides, *sul 2 - resistance gene of sulphonamides, aadA1 - resistance gene of aminoglycosides, cml - resistance gene of chloramphenicol, cat1* - resistance gene of chloramphenicol

Table 4. 9: Genetic determinants of resistance for water isolates

	βla TEM	βla SHV	βla CMY	tet A	tet B	aaC3(V)
Positive	7(25%)	6(21.4%)	3(10.7%)	11(45.8%)	10(41.7%)	6(25%)
Negative	21(75%)	22(78.6%)	25(89.3%)	13(54.2%)	14(58.3%)	18(75%)
Total	28	28	28	24	24	24
	sul 1	**sul 2**	**aadA1**	**cml**	**cat 1**	
Positive	4(36.4%)	7(63.6%)	3(27.3%)	1(25%)	2(50%)	
Negative	7(63.6%)	4(36.4%)	8(72.7%)	3(75%)	2(50%)	
Total	11	11	11	4	4	

Key: *βla TEM* - resistance gene of β-lactam, *βla SHV* - resistance gene of β-lactam, *βla CMY* - resistance gene of *β*-lactam, *tet* A - resistance gene of tetracycline, *tet* B - resistance gene of tetracycline, *aaC3(V)* - resistance gene of aminoglycosides, *sul 1* -resistance gene of sulphonamides, *sul 2 - resistance gene of sulphonamides, aadA1 - resistance gene of aminoglycosides, cml - resistance gene of chloramphenicol, cat1* - resistance gene of chloramphenicol

Table 4. 10: Genetic determinants of resistance for livestock Isolates

Bla TEM	bla SHV	bla CMY	TET A	TET B	aaC3(V)	
Positive	217(35%)	9(1.5%)	119(20%)	91(15%)	143(24%)	100(16%)
Negative	391(65%)	603(98.5%)	490(80%)	518(85%)	466(76%)	544(84%)
Total	609	609	609	609	609	644

	SUL 1	SUL 2	aadA1	cml	cat 1
Positive	92(19%)	126(26%)	80(17%)	7(14%)	9(18%)
Negative	388(81%)	354(74%)	400(83)	42(86%)	40(82%)
Total	480	480	480	49	49

Key: *βla TEM* - resistance gene of *β*-lactam, *βla SHV* - resistance gene of *β*-lactam, *βla CMY* - resistance gene of *β*-lactam, *tet A* - resistance gene of tetracycline, *tet B* - resistance gene of tetracycline, *aaC3(V)* - resistance gene of aminoglycosides, *sul 1* -resistance gene of sulphonamides, *sul 2* - resistance gene of sulphonamides, *aadA1* - resistance gene of aminoglycosides, *cml* - resistance gene of chloramphenicol, *cat1* - resistance gene of chloramphenicol

Table 4. 11: Antimicrobial Resistance from Korogocho and Viwandani

Sample from	Site	Antimicrobial type											
		AMC	AMP	CAZ	CRO	CXM	NA	TE	CIP	SXT	CN	S	C
Food	Korogocho (n=)	13(81 %)	28(76%)	9(69%)	0	10(83 %)	6(40%)	10(91 %)	9(82 %)	10(91 %)	11(92 %)	24(65 %)	6(50 %)
	Viwandani (n=)	3(19%)	9(24%)	4(31%)	0	2(17%)	9(60%)	1(9%)	2(18 %)	1(9%)	1(8%)	13(35 %)	6(50 %)
Livestock	Korogpcho (n=)	8(89%)	259(71%)	28(78 %)	28(76 %)	17(68 %)	62(71%)	478 (74)	6 (62)	450 (73)	23 (77)	391 (75)	48 (86)
	Viwandani (n=)	1(11 %)	108(29 %)	8(22 %)	9(24 %)	8(32 %)	26(29 %)	170(26)	10 (62)	168 (27)	7 (23)	133 (25)	7 (14)

Key: **AMC- Amoxicillin-Clavulanic acid, AMP - Ampicillin, CAZ - Ceftazidime, CRO - Ceftriaxone, CXM - Cefuroxime, NA - Nalidixic Acid, TE - Tetracycline, CIP - Ciprofloxacin, SXT - Trimethoprim-Sulfamethoxazole, CN - Gentamicin, S - Streptomycin, C – Chloramphenicol**

Table 4. 12: Antimicrobial Resistance of *Escherichia coli* From Various Livestock Species

Source of Isolate	ANTIMICROBIAL TYPE											
	AMC	AMP	CAZ	CRO	CXM	NA	TE	CIP	SXT	CN	S	C
Cattle	1(11%)	14(4%)	2(5.5%)	-	-	3(3.5%)	17(2.9%)	1(6%)	15(2.4%)	2(7%)	25(5%)	2(4%)
Chicken	8(89%)	273(74%)	25(69%)	29(79%)	16(64%)	66(75%)	522(81%)	7(44%)	495(80%)	23(77%)	366(70%)	31(63%)
Doves	-	8(2%)	1(3%)	3(8%)	-	4(4.5%)	16(2%)	-	15(2.4%)	2(7%)	16(3%)	-
Duck	-	8(2%)	1(3%)	-	1(4%)	2(2%)	5(0.8%)	-	10(1.6%)	-	7(1%)	-
Goat	-	29(8%)	5(14%)	3(8%)	6(24%)	5(6%)	38(6%)	-	43(7%)	2(7%)	57(11%)	11(22%)
Pig	-	33(9%)	-	-	1(4%)	6(7%)	36(5.6%)	7(44%)	31(5%)	1(3%)	42(8%)	2(4%)
Rabbit	-	2(1%)	2(5.5%)	2(5%)	1(4%)	2(2%)	11(1.7%)	1(6%)	9(1.6%)	-	11(2%)	3(6%)
TOTAL	9	367	36	37	25	88	648	16	618	30	524	49

Key: AMC- Amoxicillin-Clavulanic acid, AMP - Ampicillin, CAZ - Ceftazidime, CRO - Ceftriaxone, CXM - Cefuroxime, NA - Nalidixic Acid, TE - Tetracycline, CIP - Ciprofloxacin, SXT - Trimethoprim-Sulfamethoxazole, CN - Gentamicin, S - Streptomycin, C - Chloramphenicol

Table 4. 13: Antimicrobial Resistance of *Escherichia coli* From the Various Types of Foods samples

Source of Isolate	ANTIMICROBIAL TYPE											
	AMC	AMP	CAZ	**CRO	CXM	NA	TE	CIP	SXT	CN	S	C
Black Tea	-	-	-	-	-	6(33.3%)	-	-	-	-	4(0.6%)	-
Food	-	-	-	0			-	-	-	-	3(0.6%)	3(25%)
Githeri	-	-	-	-	-	-	-	-	-	-	-	-
Mashed potato/rice	-	-	-	-	-	-	-	-	-	-	3(0.6%)	-
Mashed potato beans	-		1(7.7%)	0	-	-	-	-	-	-	-	-
Milk	-			0	-	-	-	-	-	-	-	-
Milk Tea	7(31.2%)	19(27%)	1(46.1%)	0	1(45.5%)		20(45.5%)	3(50%)	26(54.5%)		10(4%)	5(41.7%)
Porridge	-	12(8.1%)	-	-	-	2(6.7%)	-	-	9(%0%0)	-	4(2.9%)	-
Potato stew	-	-	-	0		1(6.7%)	7(0.6%)	-	-	-	-	-
Rice	2(6.3%)	9(2.8%)	-	-	-	1(6.7%)	-	-	7(…%)	-	4(8.3%)	-
Swab	-	-	-	-	-	-	-	-	9(9.1%)	-	-	-
Ugali sukuma	2(6.3%)	11(8.1%)	1(7.7%)	0	-	-	-	-	-	-	-	-
TOTAL	11	51	3	0	1	10	27	3	51	0	28	8

Key: AMC- Amoxicillin-Clavulanic acid, AMP - Ampicillin, CAZ - Ceftazidime, CRO - Ceftriaxone, CXM - Cefuroxime, NA - Nalidixic Acid, TE - Tetracycline, CIP - Ciprofloxacin, SXT - Trimethoprim-Sulfamethoxazole, CN - Gentamicin, S - Streptomycin, C – Chloramphenicol

CHAPTER FIVE: DISCUSSION

The virulence determinant *estA* of enterotoxigenic *E.coli (*ETEC) was the most prominent pathotype in food isolates and was detected in 4(2%). Three of the four isolates were from water, and the other one was isolated from milk tea. Two isolates (1%) showed virulence genes for EPEC, DAEC, EHEC and EAEC. Three out of the eight were isolated from milk tea; two were from water, two from porridge and one from ugali. The findings of the current study are lower than those found in Japan foods by Wang et al. (2017) who found 56% EPEC in food samples. The virulence gene *Stx* of EHEC was the most prevalent and was detected in 4% of all livestock isolates. Two percent of livestock isolates were positive for EPEC and DAEC, and the least prominent pathotype was ETEC *estA.* While investigating poultry, Stromberg *et al*. (2017) found 24% pathogenic *E.coli* from chickens. Awad *et al* (2020) found 2.7% EPEC from cattle and 7.3% EAEC pathotypes. The present study detected 1% EHEC in food and 4% in livestock respectively. Majority of the EAEC isolated harboured the *aggR* gene. The *aagR* gene controls the expression of adherence factors, a dispersin protein, and a large cluster of genes encoded on the EAEC. The ETEC *ltA* virulence determinant was detected in 0.4% of food and 0.5% of livestock isolates. ETEC *estA*, which is associated with travellers' diarrhoea, was prevalent in 2% and 0.1% of food and livestock isolates respectively.

While investigating *E. coli* from water samples in Northern Mexico, Canizalez-Roman A et al (2019) found 31% EPEC, results which are higher than the current study which did not record any EPEC strains in water. He also found 27% DAEC, values which are higher than those found in the current study. Harada *et al.*(2018) while investigating stored water in a slum in Bangladesh, found 7.8% ETEC in drinking water. The same study also found 6.3% EPEC in waste water. These findings are higher than those of the current study.

In this study, the most effective antibiotic among β-lactams was cefuroxime with 100% of the food isolates susceptible to this agent, followed by gentamicin and ceftriaxone with 99.4% and 98.9% of the isolates being susceptible respectively. In Canada Sheikh *et al*. (2012), investigations into antimicrobial resistance of *E.coli* isolated from retail meats detected higher resistance to amoxicillin-clavulanic acid (16.8%) and ceftriaxone (12.65%). These values are higher than those of the current study which was detected as 12.6% and 1.1% respectively.

Among aminoglycosides, 22.5% and 45% were resistant to streptomycin in food and livestock isolates, respectively. Resistance to streptomycin detected by Musgrove (2006) was, however, lower (6.2%) than the findings of this study. While investigating *E.coli* from hen eggshells (Burgos *et al*., 2016) reported a higher (37.3%) prevalence of streptomycin resistance. In food isolates, the highest resistance was detected in ampicillin (45%) followed by sulfamethoxazole-trimethoprim (38%), tetracycline (29.3%) and streptomycin (22.5%). The findings in this study are lower than those detected by Tew *et al*. (2016) in Malaysia when he showed antimicrobial resistance in Enterobacteriaceae bacteria isolates from retail sausages as 100% to amoxicillin/clavulanic acid, 71.4% to cefotaxime and 83.3% to ampicillin. While investigating *E.coli* from commercial eggshells, Musgrove (2006) found 29.9% resistance to tetracycline results which are similar to the current study. Saenz *et al*. (2001) however, reported a higher prevalence of tetracycline (75%). Msolo *et al*. (2016) also detected higher resistance for tetracycline (81%) while investigating the potential public health risk of AMR posed by dairy farms in South Africa. The resistance to tetracycline reported in this study is a significant finding because previous studies have shown tetracycline to be the most commonly used antibiotics in food producing animals (Mitema *et al*., 2001). In this study, the resistance of food isolates to nalidixic acid was 8.6%, and that of ampicillin was 45.4%. Saenz *et al*. 2001 detected higher

frequencies for nalidixic acid (88%) as well as ampicillin 58%. He also recorded higher resistance to sulfamethoxazole-trimethoprim (65%) and ciprofloxacin 38% than the current study which detected 41.2% and 1.8% respectively. Resistance to chloramphenicol was 12% in the current study. This is in agreement with Saenz *et al*. (2001) who found similar results in chloramphenicol (12%). In Spain, *E.coli* isolates from patients with UTI showed 40% resistance to both amoxicillin-clavulanic acid and ciprofloxacin (Ri`os *et al*., 2015). The resistance patterns in this study are similar to other studies which stated that *E.coli* isolates are MDR especially to tetracycline, trimethoprim-sulfamethoxazole, ampicillin and streptomycin.

Among the *β*-lactam resistance genes investigated, *bla TEM* was the most prevalent in 91% of food isolates and 35% of livestock isolates. The resistance gene *bla CMY* was detected in 51% and 20% of food and livestock isolates, respectively. Studies showing *E.coli* as an essential foodborne ESBL carrier in Turkey revealed *bla TEM* was the most frequent gene (Önen *et al*., 2015). The critical health risk to Turkish consumers of food of animal origin established potential reservoirs for diverse ESBL producing *E.coli* and their encoding genes and resistance. (Tekiner and Özpınar, 2016). In his research, Tew *et al*. (2016) reported a higher prevalence of *blaSHV* (22.2%) in retail sausages. The resistance gene *bla SHV* was detected in 7.8% of the food isolates in this study. Antimicrobial resistance genes determinants *tetA* and *tetB* were detected in 31% and 29% respectively in food isolates results which are similar to findings *by* Burgos *et al*. (2016) who reported *tet B* 29.63% and *sul1* (14.81%). In the present study, however, sulfamethoxazole resistance gene *sul1* which is closely associated with type 1 integrons (Poeta *et al*., 2010) was detected in 10% of the isolates, a proportion less than that detected by Burgos *et al*. (2016). In his study, Tew *et al*. (2016) identified an almost similar prevalence of sulphonamide resistance gene *sul1*(11.1%). The other sulphonamide resistance

gene *aac3* was detected in 17% and 16% food and livestock isolates respectively. The gene encoding *aac (3*)-IV which is associated with gentamicin resistance was identified in 6 (17%), and 100 (16%) of food and livestock *E.coli* isolates respectively. Chloramphenicol resistance gene determinants *cml* and *cat1* were detected in 16.7% and 33.3% respectively among food isolates; 14% and 18% among livestock isolates. The resistance gene *aadA1* were identified in 8% and 17% food and livestock isolates respectively.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. Livestock, food and water from Viwandani and Korogocho are contaminated with *E.coli* isolates, which habour various virulence genes indicating that they can be potentially pathogenic.
2. *Escherichia coli* from foods, water and livestock from Viwandani and Korogocho are resistant to antimicrobials and carry antimicrobial resistant genes.
3. More resistant strains of *E.coli* were detected in Korogocho and less in Viwandani settlement areas.

6.2 Recommendations

1. Further investigations on the contamination of water with potentially pathogenic and antimicrobial resistant *E.coli* should be conducted as the number of isolates in this study were limited.
2. Whole genome sequencing should be done in order to understand the genomic diversity of *E.coli* strains from foods, water and livestock in informal settlements and source of contamination.
3. The role of other virulent factors of *E.coli* which were not considered in this study should be investigated for each strain of *E.coli*.

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LIST OF APPENDICES

Appendix 1: Preparation of media

MacConkey Agar

Fifty grams of the powder was suspended in 1000ml of distilled water. This was heated to boiling in order to dissolve the medium entirely. This was sterilized by autoclaving at 121ºC for 15 minutes. The molten agar was cooled to 45-50 ºC. An approximate 20ml of the molten agar was poured into Petri dishes which were 90mm in diameter on a level horizontal surface to give uniform depth. They were allowed to cool at room temperature.

Müller Hinton Agar

Thirty-eight grams of the powder was suspended in 1000ml of distilled water. This was heated with frequent agitation and boiled for one minute to ensure the medium dissolves completely. Sterilization was done by autoclaving at 121ºC for 15 minutes. An approximate 20ml of the molten agar was poured into Petri dishes which were 90mm in diameter on a level horizontal surface to give uniform depth. They were allowed to cool at room temperature

Tryptone Soy Agar

Forty grams of the powder was suspended in 1000ml of distilled water. This was heated with frequent agitation and boiled for one minute until complete dissolution of the medium. Sterilization was done by autoclaving at 121C for 15 minutes. An approximate 20ml of the molten agar was poured into Petri dishes on a level, horizontal surface to give uniform depth. These were allowed to stand and cool at room temperature.

Appendix 2: WHO Global Foodborne Infections Network (formerly WHO Global Salm-Surv) Susceptibility testing of Enterobacteriaceae using disk diffusion

WHO Global Foodborne Infections Network

(formerly WHO Global Salm-Surv)

"A WHO network building capacity to detect, control and prevent foodborne and other enteric infections from farm to table"

Laboratory Protocol:

“Susceptibility testing of Enterobacteriaceae using disk diffusion”

Issued

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With acknowledgments for significant technical and editorial contributions to the: WHO Global Foodborne Infections Network Laboratory Sub-Committee

LABORATORY SOP

Title: Susceptibility testing of Enterobacteriaceae using disk diffusion	
Protocol Number: 2010GFNLAB002	**Effective Date:** Feb 2010 **Revision Number:**

REVISION HISTORY

HISTORY OF CHANGES				
Rev. Level	**Sections Changed**	**Description of Change (From—To) Date A**	**Proval**	
0				
1				

I. PURPOSE

A standardised method for determining the antimicrobial susceptibility profile of Enterobacteriaceae, e.g. *Salmonella* and *Shigella*.

II. TEST PRINCIPLES

Disk diffusion testing is one of several phenotypic assays which can be utilised to determine the antimicrobial resistance profile (antibiogramme) of an organism. Disk diffusion tests estimate *in vitro* susceptibility.

The principle of agar diffusion is simple: Agar plates are inoculated with a standardised inoculum of the bacteria and an antimicrobial disk is placed on the inoculated agar plate. The disks used for a disk diffusion assay contain a standardised known amount of an antimicrobial agent, which diffuses into the agar when in contact with the agar surface. The plate is incubated under standardised conditions following Clinical and Laboratory Standards Institute (CLSI) guidelines. During incubation, the antimicrobial agent diffuses into the agar and inhibits growth of the bacteria, producing a “zone of inhibition”

around the disk. Following incubation, the diameter of this zone is measured and the results are interpreted as resistant, intermediate, or susceptible using standard guidelines (e.g. CLSI M100).

The size of the diameter zone of inhibition indicates the degree of resistance, and might also give important information about the resistance mechanism and the resistance genes involved. In addition, the disk diffusion method can be used for determination of MIC values provided the necessary reference curves for conversion of diameter zone of inhibition into MIC values are available.

Highly standardised methods are essential for all types of susceptibility testing. These assays are highly sensitive to variations in: inoculum density, media formulation, media pH, and incubation conditions. In addition, agar diffusion methods are strongly influenced by agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria. To insure the accuracy and reproducibility of antimicrobial susceptibility test results, internal quality control testing must be regularly performed as recommended by CLSI (CLSI M2).

Diffusion tests are low-cost compared to most MIC determination methods, but MIC determination performed as agar dilution is regarded as the gold standard for susceptibility testing. It should be noted that the WHO-GFN does not recommend any specific method for the susceptibility testing of microorganisms.

The disk diffusion method described in this protocol is in accordance with the international recommendations given by CLSI (M2). The CLSI sets international guidelines for interpretation of the results (e.g. M7, M31, and M100). The most recent update of the guidelines should be used as reference.

III. RESPONSIBILITIES

A. Staff Responsibilities

Refer to applicable manuals within the facility/location for complete set of responsibilities to properly conduct this procedure. Specific Safety Requirements and Responsibilities

Carry out all procedures in accordance with the local codes of safe practice.

IV. SAMPLE PREPARATION

The test material must be a pure 18- to 24-hour culture of the Enterobacteriaceae grown on non-selective agar. Single, isolated colonies should be present.

V. MATERIALS/SUPPLIES

Media

- Sterile saline solution (0.85%) 3-4 mL each tube
- Mueller-Hinton agar plates (with a uniform agar depth of approximately 4 mm)
- Antimicrobial Disks
- Nutrient agar plates or other non-selective agar

Bacterial strains

• Enterobacteriaceae strains on non-selective agar (colonies from selective isolation plates should not be used because they could be mixed cultures and could influence on the susceptibility test result)

• Strain for quality control: *Escherichia coli* ATCC 25922

VI. EQUIPMENT

• McFarland standard 0.5 (the tube containing the McFarland standard must be the same type/material and diameter as the tubes used for the test suspension)

• Nephelometer or white paper with black lines

• Vortex

• Scissors

• Disk dispenser (alternatively, forceps can be used)

• Forceps

• Loops (1 µl and 10 µl)

• Bunsen burner (or other to secure sterilisation of forceps and loops)

• Small sterile cotton swabs or drigalski (hockeystick)

• Ruler or calliper

• CLSI M100: Performance Standards for Antimicrobial Susceptibility Testing

• CLSI M2: Performance Standards for Antimicrobial Disk Susceptibility

• QUALITY ASSURANCE

Each lot of media utilized in this SOP will be quality control tested prior to use. The results of QC testing (performance characteristics and sterility) will be recorded. Only media which has passed QC will be used for testing.

Quality assurance of the susceptibility testing of Enterobacteriaceae includes testing of *E.coli* ATCC 25922. The test results from this microorganism must be within the QC ranges set by the CLSI. The test thereby serves as quality assurance that the method was carried out with all variable factors standardized according to the CLSI guidelines. If the test results of the *E.coli* ATCC 25922 QC strain are out of range, do not report results from test strains and proceed with the troubleshooting guidelines according to CLSI (M100, Table 3C).

Reference strains should be stored in a suitable stabilizer such as 50% fetal calf serum in broth, 10-15% glycerol in tryptic soy broth, defibrinated sheep blood or skim milk. Store at
-20°C to -80°C (preferably -70°C to -80°C).

Before using rejuvenated strains for QC, subculture to check for purity and viability. Set up on agar slants with appropriate medium, store at 4-8°C and subculture weekly. Replace the working strain with a stock culture at least

monthly. If a change in the organisms inherent susceptibility occurs, obtain a fresh stock culture or a new strain from a reference culture collection e.g. ATCC.

Antimicrobial discs should be stored in a freezer (-10°C to -20°C) until needed. A small working supply of discs can be kept in a refrigerator, in containers with a desiccant. To prevent condensation, the jars and disc dispensers should be allowed to warm to room temperature before being opened. The unused portion of the discs should be put back into the refrigerator as soon as possible to minimize the exposure to room temperature and humidity. Only those discs that have not reached the manufacturer's expiration date stated on the label may be used. Discs should be discarded on the expiration date.

VII. PROCEDURE

Day 1

Standardisation of inoculum:

Prior to preparing the inoculum, visually examine the agar plates containing the test organism and control strain. If culture appears mixed, a fresh sub-culture will be prepared.

With a loop or sterile swab, touch the top of at least 4 to 5 well isolated colonies. Transfer the growth to the tube of saline. Emulsify the inoculum on the inside of the tube to avoid clumping of the cells.

Picking cells from more than one colony ensures the selection of sufficient bacterial numbers and minimizes the risk of selecting bacteria that have lost their resistance.

Adjust the inoculum standard to a 0.5 McFarland:

Compare turbidity to that in the 0.5 McFarland standard using a nephelometer or paper with black lines. Adjust turbidity of inoculum to match that standard.

McFarland 0.5 equals approximately 10^8 CFU/mL. Use of a standardised inoculum is essential for the accurate performance of the assay. If the suspension is too light,

confluent growth will not be obtained; if the suspension is too heavy, zone sizes will be artificially small (CLSI/Kirby-Bauer).

Inoculation of Mueller-Hinton plate:

Visually examine the Mueller Hinton agar plates prior to use, insure plates are:

- free of visible contamination
- poured to a uniform depth of approximately 4mm
- not excessively wet
- not cracked or dry

Within 15 minutes of preparing the adjusted inoculum, dip a sterile cotton swab into the inoculum. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.

Streak the swab over the entire surface of the Mueller Hinton agar plate. Rotate the plate approximately 60° then repeat streaking motion. Rotate 60° again and repeat streaking for the third time. Complete inoculation by running the swab around the rim of the agar.

Homogeneous plating is important to yield reliable results. An alternative method for obtaining a confluent lawn is to inoculate each 10 cm plate with 50 µL and use a drigalski (hockeystick) to create the uniform lawn. This method is a practical, and by most laboratories' experience, an acceptable deviation from the recommended standard.

Allow any excess moisture on the agar surface to be absorbed prior to applying the antimicrobial disks. The lid of the plate may be left ajar for 3-5 minutes (no more than 15 minutes) to allow any excess moisture to be absorbed before applying disks.

Dispensing Antimicrobial Disks:

Dispense disks to the agar surface with a disk dispenser or sterile forceps (forceps can be sterilized by flaming with alcohol. Avoid using over-heated forceps). Do not relocate any disks after contact with the agar. After application, insure that the disk has made complete contact with the agar surface by touching the top of the disk with forceps.

The disks cannot be moved after being placed onto the agar surface. Diffusion of the drug begins immediately when the disk contacts the agar. Moving the disks after contact with the agar will produce distorted zones and result in unreliable results.

Note: Selection of disks should be guided by plate size and the intended use of the results (clinical or epidemiological). Ordinarily, no more than 5 disks should be placed on a 10 cm agar plate and no more than 12

disks should be placed on a 15 cm agar plate. In a clinical setting, only ampicillin, a quinolone and/or fluoroquinolone, and trimethoprim-sulphamethoxazole should be reported for faecal isolates of Salmonella and Shigella. Chloramphenicol and a third-generation cephalosporin should also be tested and reported for extra-intestinal isolates of Salmonella. Other agents and drug classes (e.g. aminoglycosides) may provide valuable epidemiologic data; however in- vitro susceptibility of Salmonella to these agents may not correlate with in-vivo efficacy. The reader is advised to consult the current CLSI interpretative standards (M100) for further guidance.

Verifying Purity of Inoculum:

To verify the purity of the inoculum, the inoculum is plated to a nutrient agar plate (or other non-selective media).

Use a sterile 10 µL loop, to collect inoculum from the tube and plate to a nutrient agar plate (or other non-selective media).

Incubate plates inverted at 36±1°C for 16 to 18 hours in ambient air.

Day 2

Reading Results:

Check the purity of the purity control plate. If the growth appears mixed, attempt to obtain a pure culture.

Check that the growth is a confluent lawn. Individual colonies of resistant organisms may be observed around some antimicrobial disks; however, if individual colonies are dispersed across the plate, the inoculum was too light and the sample must be retested.

An overly dense inoculum will yield artificially small zones (this becomes apparent when comparing the results of the control strain, E.coli ATCC 25922, to the QC-ranges given in the CLSI guidelines M100, Table 3).

Check that zones are round; not oval, deformed or have jagged edges.

Sometimes when disks are placed closely together, interaction between antimicrobials may produce distortion of the diameter zone of inhibition (i.e. antagonism, synergism, inhibition and/or induction). Such valuable additional information should not be considered in the reading of the diameter zone of inhibition but provides important information about the putative mechanism of resistance, bacterial ID, etc.

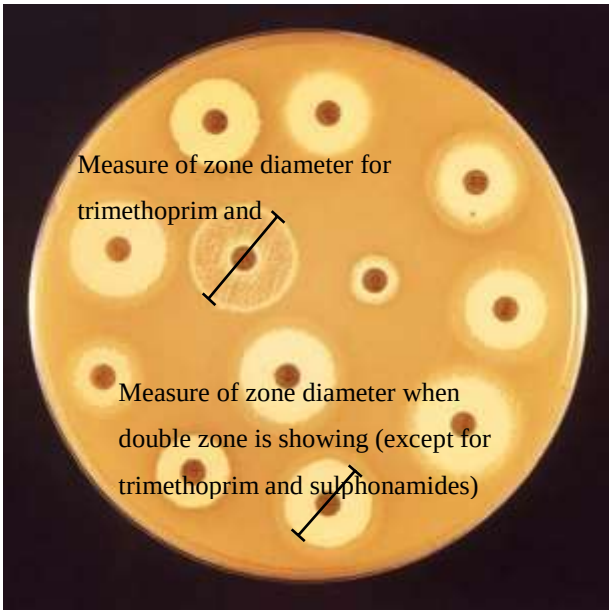


Photo: Disk diffusion on a 150mm Mueller Hinton agar plate. From CDC Public Health Image Library (PHIL) www.phil.cdc.gov

Measure the diameter of inhibition zones. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye (a haze should be disregarded). The antibiotic trimethoprim and the sulphonamides allow growth of the bacteria for some generations before inhibition occurs, therefore for these antimicrobials, disregard slight growth (20% or less of the lawn of growth), and measure the more obvious margin to determine the zone diameter. If no inhibition is present (confluent growth is present up to the border of the disk), the diameter of the disk should be recorded (6mm).

VIII. INTERPRETATION OF RESULTS

CLSI Guideline M100, Table 2A ('Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae') is the interpretation guideline for the categorization of the test strain as susceptible, intermediate or resistant.

Interpretation of the diameter zone of inhibition is based on a confluent lawn of growth and on regression lines found by testing and comparing a large population of isolates.

IX. LIMITATIONS OF PROCEDURES

Disk diffusion testing, like other antimicrobial susceptibility testing assays, is an *in vitro* determination of antimicrobial susceptibility. These *in vitro* results may not always correlate with *in vivo* efficacy. Additionally, antimicrobial agents with limited clinical efficacy (aminoglycosides against *Salmonella* and

Shigella) or antimicrobials which are subject to legal restrictions (chloramphenicol in food animals) often provide valuable markers for specific resistance phenotypes are still included in many susceptibility panels.

Susceptibility testing microorganisms by disk diffusion should be performed on a pure culture. An indication (non-reportable zone size) of the results can, however, be obtained even though the culture is not pure. To obtain a correct result, subculture the test strain and re-test.

To insure accurate and reproducible results, the agar used for the disk diffusion should follow international guidelines (CLSI M2):

- The pH of the Mueller-Hinton agar should be between 7.2 and 7.4 (at room temperature after gelling), otherwise some antimicrobials will appear to lose potency while other agents may appear to have excessive activity
- Excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulfonamides and trimethoprim, thus yielding smaller and less distinct zones, or even no zone at all, which may result in false-resistance reports
- Variations in the content in the Mueller-Hinton agar of Ca^{++} and Mg^{++} affect the zone sizes for tetracycline. The zones will be too small when the content of Ca^{++} and Mg^{++} is too high and too large when the content of Ca^{++} and Mg^{++} is too low

Testing the QC strain *E.coli* ATCC 25922 is the QC reference, and if problems with obtaining values within the CLSI acceptable zone diameters, proceed with the troubleshooting guidelines according to CLSI (M100, Table 3C).

X. REPORTING

Results are reported as S, I or R following interpretation according to the CLSI guidelines

XI. REFERENCES

1. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests, 9th ed. Approved standard. M2-A9. CLSI, Wayne, Pennsylvania, 2006

2. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 7th ed. Approved standard. M7-A7. CLSI, Wayne, Pennsylvania, 2006

3. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, 3rd ed. Approved standard. M31-A3. CLSI, Wayne, Pennsylvania, 2001

3. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 19th Informational Supplement.

M100-S19. CLSI, Wayne, Pennsylvania, 2009

XII. ATTACHMENTS

Attachment 1: Composition and preparation of culture media and reagents Attachment 2: Example of Record sheet

Attachment 1: Composition and preparation of culture media and reagents

If no reference is given, it is the procedure used at DTU Food, Copenhagen, Denmark.

The media and reagents are available from several companies including Oxoid, Merck and Difco.

Mueller Hinton Agar

Mueller-Hinton Agar should be prepared from a commercially available base. Only Mueller-Hinton agar formulations that have been tested according to, and meet the quality standards prescribed by CLSI document M6 *Protocols for Evaluating Dehydrated Mueller-Hinton Agar*, should be used.

The media should be prepared in accordance with the manufacturer's instructions. Specifications: Final pH 7.2-7.4. Agar depth 4 mm

Nutrient agar (according to ISO 6579:2002 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.)

Meat extract 3.0g Peptone 5.0g Agar 12g to 18g[1)] Water 1000 mL

[1)] Depending on the gel strength of the agar

Preparation: Dissolve the dehydrated medium in the water by heating. If necessary, adjust pH to ~7.0 after sterilisation, transfer into bottles and autoclave at 121°C for 20 min. Pour 15 mL of melted medium into each sterile 10 cm petri dish.

Saline solution Sodium chloride 8.5g Water 1000 Ml

Preparation: Dissolve the sodium chloride in the water by heating if necessary. Adjust pH to
7.0. Dispense the solution into tubes so 4 mL is obtained after autoclaving at 121°C for 20 min

Attachment 2: Example of Record sheet

Record sheet

Date:_____

Name:_____

Antimicrobial	Strain 1:#		Strain2:#	
	Zone diameter (mm)	Interpretation (R-I-S)	Zone diameter (mm)	Interpretation (R-I-S)
Ampicillin (10µg)				
Ceftriaxone (30µg)				
Chloramphenicol (30µg)				
Ciprofloxacin (5µg)				
Nalidixic acid (30µg)				
Trimethoprim-Sulphamethoxazole (1.25/23.75µg)				

Method used*	Strain Antimicrobial	*E.coli* ATCC 25922 Zone diameter (mm)	Within the QC range
Cotton swab 50µL 100µL 150µL 200µL	Ampicillin (10µg)	0	
	Ceftriaxone (30µg)		
	Chloramphenicol (30µg)		
	Ciprofloxacin (5µg)		
	Nalidixic acid (30µg)		
	Trimethoprim-Sulphamethoxazole (1.25/23.75µg)		

*Homogeneous plating is important to yield reliable results. An alternative method to the standard is to inoculate the plate with a certain volume of suspension (e.g. 50 µL pr. 10 cm agar plate) and use a drigalski (hockeystick) to create the uniform lawn. The method chosen as the routine method should ensure a confluent growth which yields zones for the reference strain *E.coli* ATCC 25922 within the QC-ranges given by the CLSI guidelines (in M100, Table 3). The obtained zone diameters for the *E.coli* reference strain should preferably be in the middle of the QC-ranges for as many antimicrobials as possible.

Appendix 3: Zone diameter interpretive standard breakpoints

Table 1: Zone diameter interpretive standard breakpoints to the nearest whole mm

Antimicrobial Agent	Disk Content	Susceptible	Intermediate	Resistant
Ampicillin	10µg	≥17	14-16	≤13
Amoxicillin-clavulanic acid	20/10 µg	≥18	14-17	≤13
Ceftriaxone	30µg	≥23	20-22	≤19
Cefuroxime	30µg	≥18	15-17	≤14
Gentamicin	10 µg	≥15	13-14	≤12
Streptomycin	10 µg	≥15	12-14	≤11
Tetracycline	30 µg	≥15	12-14	≤11
Ciprofloxacin	5 µg	≥21	16-20	≤15
Naladixic acid	30 µg	≥19	14-18	≤13
Chloramphenicol	30 µg	≥18	13-17	≤12
Trimethoprim-sulfamethoxazole	1.25/23.75 µg	≥16	11-15	≤10
Ceftazidime	30 µg	≥21	18-20	≤17

Appendix 4: Pathotype prevalence by species, Livestock

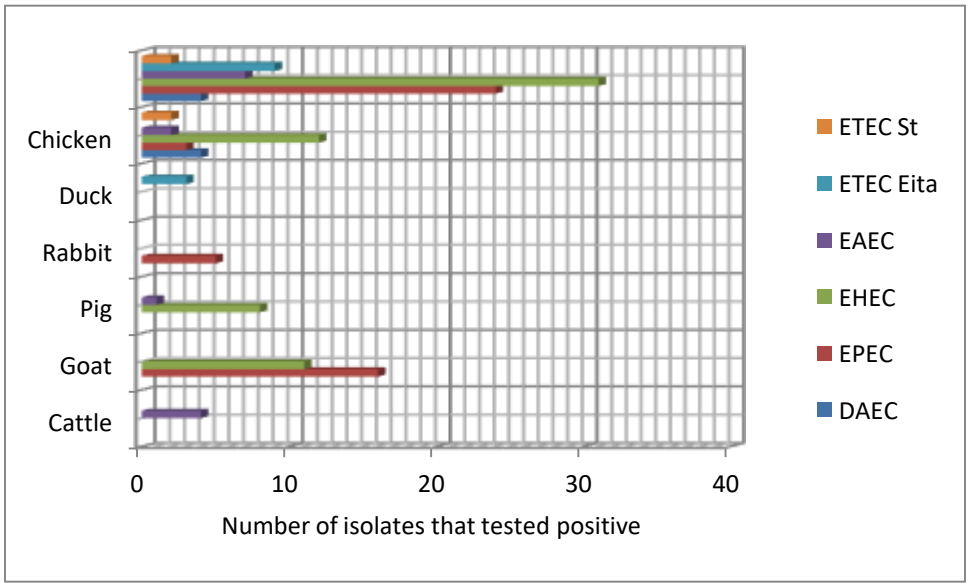


Figure 1. 1:Graph showing the distribution of pathotypes in the livestock samples

Appendix 5: Selected PCR amplicons for pathotypes and antimicrobial resistance genes ladder.

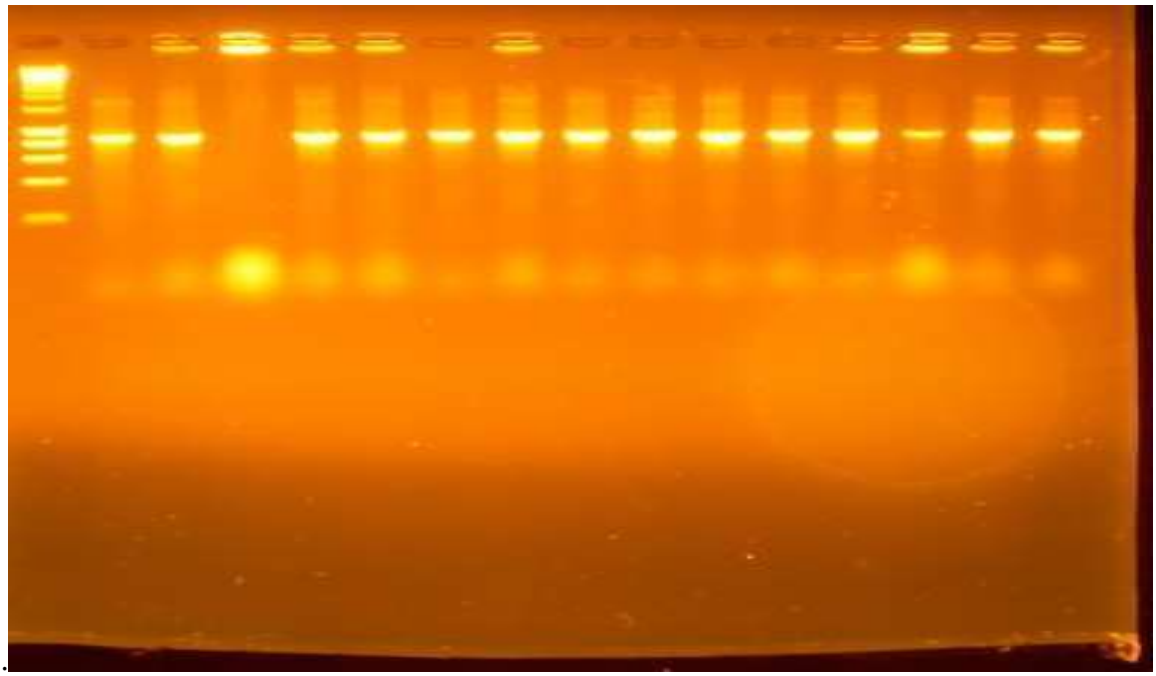


Figure 1: PCR amplicons obtained by primer specific to sulphonamide resistance gene sul1. Lane 1 is 200 bp DNA ladder. Lane 2,3, 5 16 shows bands of 822 bp of the respective *E.coli* isolates after PCR amplification. Lane 4 is a negative result.

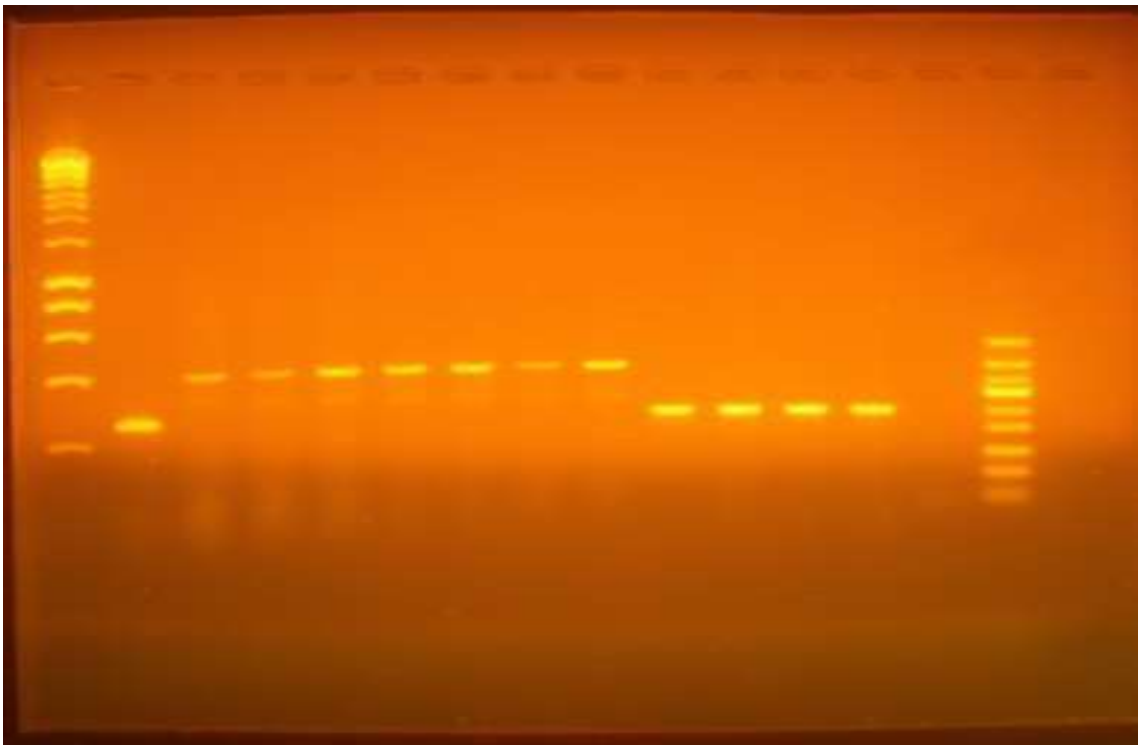
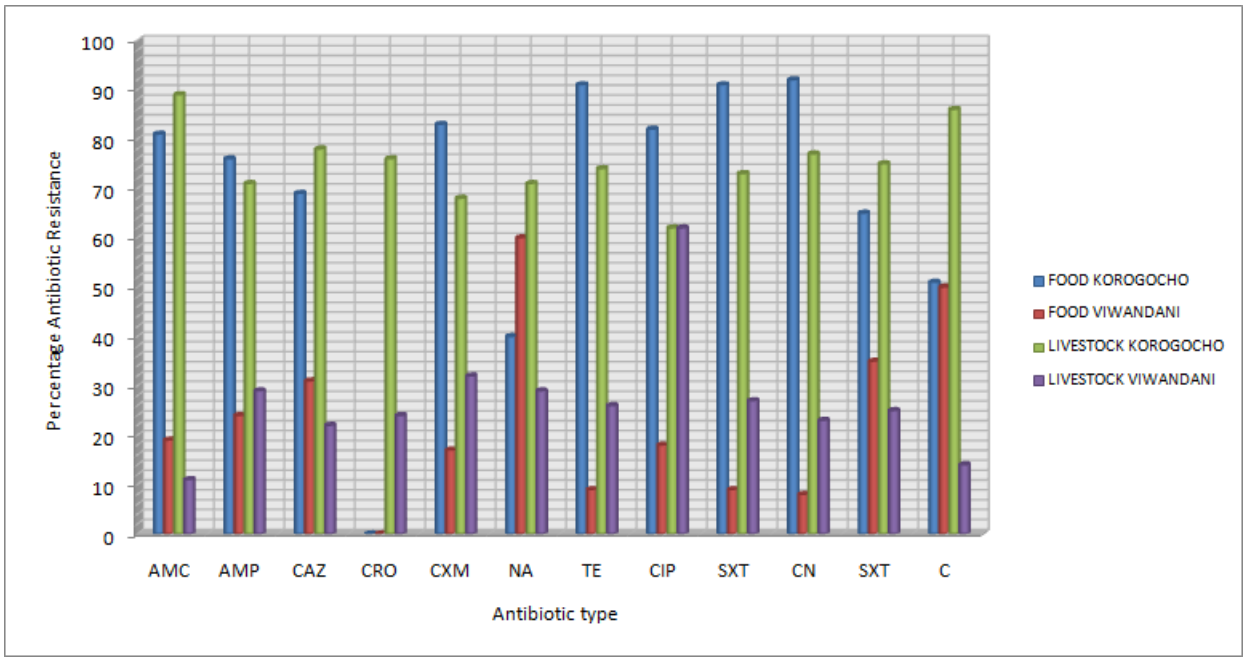


Figure 2: PCR amplicons obtained by primer specific to 254Bp.

254 BP - Lane 1 is 50Bp DNA Ladder. Lane 2 is a positive control, and lane 3 is a negative control. Lane 4-9,10 are negative results for 254Bp, and lanes 10-13 are positive for 25Bp.

Appendix 6: Graphical summary of the comparison of antimicrobial resistance (livestock and foods) by location of samples



Key: AMC- Amoxicillin-Clavulanic acid, AMP - Ampicillin, CAZ - Ceftazidime, CRO - Ceftriaxone, CXM - Cefuroxime, NA - Nalidixic Acid, TE - Tetracycline, CIP - Ciprofloxacin, SXT - Trimethoprim-Sulfamethoxazole, CN - Gentamicin, S - Streptomycin, C - Chloramphenicol