ANTIMICROBIAL SUSCEPTIBILITY PROFILES AND MOLECULAR CHARACTERIZATION OF ARCHIVED ISOLATES OF *ESCHERICHIA COLI* FROM THE GUT OF HEALTHY FOOD ANIMALS AND ENVIRONMENTAL SOURCES IN SELECTED COUNTIES IN KENYA

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Antimicrobial Susceptibility Profiles and Molecular Characterization of Archived Isolates of *Escherichia coli* from the Gut of Healthy Food Animals and Environmental Sources in Selected Counties in Kenya

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DECLARATION

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

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DEDICATION

This thesis is dedicated to my children Danny, Dan and Don, their uncle Sennel and the Community of *BENE BERNADETTA* Sisters who have tirelessly supported me in hard moments, after the death of my wife and all the way since the beginning of my studies. Their prayers and moral support have been the driving force that has enabled me to achieve the goal in my studies.

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ABBREVIATIONS AND ACRONYMS

A/E	attaching and effacing
AA	aggregative adherence
AAF	aggregative adherence fimbria
ADP	adenosine diphosphate
AfaE	afimbrial adhesion E
AIEC	adherent Invasive *E.coli*
ATCC	American Type Culture Collection
BD	Becton and Dickinson Company
BFP	bundle-forming pili
cAMP	cyclic adenosine monophosphate
CF	colonizing factor
CFA	colonizing factor A
CLSI	clinical and laboratory standards institute
CS	coli surface antigen
CTX-M	Cefotaximase-munich
DA	diffusely adherence
DAEC	diffusely adherent *E. coli*
DNA	deoxyribonucleic acid
EAEC	enteroaggregative *E. coli*
EAF	EPEC adherence factor
EDTA	ethylene diamine tetra acetic acid
EHEC	enterohaemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E. coli*
ERC	ethical review committee
ESBL	extended spectrum beta-lactamase
ESBL-PE	extended spectrum beta lactamase producing *E. coli*
ETEC	enterotoxigenic *E. coli*
G	guanosine

GARP:	Global Antibiotic Resistance Partnership-Kenya Working Group.
GNB	Gram negative bacteria
GNP	Gram negative pathogen
GTP	guanosine triphosphate
HC	hemorrhagic colitis
HCL	hydrogen chloride
HUS	hemolytic uremic syndrome
InPEC	intestinal pathogenic *E. coli*
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenyan Medical Research Institute
LEE	locus of enterocyte effacement
LT	heat-labile toxin
MDa	Milli Dalton
MIC	minimal inhibitory concentration
NCCLS	National Committee for Clinical Laboratory Standard
NM	non motile
pAA	plasmid aggregative adherence
PAI	Pathogenicity Island
PCR	polymerase chain reaction
pINV	plasmid invasion
RFLP	restriction fragment length polymorphism
RSI	restriction site insertion
SERU	scientific and ethics review unit
SHV	sulfhydryl variable
SPSS	statistical package for social science
SSC	standard steering committee
ST	heat-stable toxin
STEC	shiga toxin-producing *E. coli*
TEM	Temoneira *E. coli* mutant
T3SS	type III secretion system
UK	United Kingdom
UPEC	uropathogenic *Escherichia coli*

USA	United States of America
UTI	urinary tract infections
UV	ultraviolet
VTEC	verotoxigenic *E. coli*
Vtx	verotoxin
WHO	world health organization

ABSTRACT

Escherichia coli is present in the intestinal tract of both humans and animals and is released into the environment through fecal material. *E. coli* is a reservoir for antibiotic resistance genes. Some strains of *E. coli* are pathogenic by acquisition of different virulence factors. The aim of this study was to determine antimicrobial susceptibility profiles, the pathotypes and the proportion of ESBL producers and ESBL genes among archived isolates of *E. coli* from the gut of healthy food animals and environmental sources in selected Counties in Kenya (Kiambu, Nairobi, Kisumu, Mombasa and Kwale). A laboratory based cross sectional study design was used. The sample size encompassed 375 archived isolates of *E. coli* (non-duplicate) at the Center for Microbiology Research, Kenya Medical Research Institute. Antimicrobial susceptibility profiles were determined by disk diffusion technique, *E. coli* pathotypes were determined by PCR. Phenotypic ESBL detection and ESBL encoding genes detection were done by Double Disk Synergy Test and PCR respectively. Overall, 78.4% archived isolates of *E. coli* were resistant to at least one of the 13 tested antibiotics among which 28.8% showed multidrug resistance whereas 21.6% were fully susceptible. Resistance to tetracycline was 55.2%, co-trimoxazole 44%, trimethoprim 43.7%, ampicillin 28.8%, nalidixic acid 18.7%, azithromycin 14.9%, ciprofloxacin 10.1%, chloramphenicol 8.8%, imipenem 0.8%, cefotaxime 5.9%, ceftazidime 3.7%, cefuroxime 2.4%, and gentamicin 1.1%. The proportion of Enteroaggregative *E. coli* (harbouring *agg*R gene) was 88.3% and other pathotypes were not found. The proportion of ESBL producing *E. coli* was 8.8% (33/375) and the antimicrobial resistance was significantly different between ESBL producers and non ESBL producers ($p \leq 0.05$). All ESBL producers harboured bla_{TEM}, 18.1% harboured $bla_{\text{CTX-M}}$ and none had bla_{SHV}. Archived isolates of *E. coli* were found to be reservoirs for resistance determinants and virulence factor genes. Stringent regulations governing the usage of antimicrobials in animal agriculture should be applied to curb the development antibiotic resistance. Homestead and barns should be kept clean to prevent the spread of resistant pathogens in the environment by arthropods.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Escherichia coli (*E. coli*) is present in the intestinal tracts of both humans and animals and is released into the environment through fecal material and is also a reservoir for antibiotic resistance genes (Sahoo *et al.*, 2012). *E. coli* is an extremely versatile species with a high adaptation capacity to new and variable niches (Gordo *et al.*, 2014). Although most *E. coli* strains are non-pathogenic, some of them are highly pathogenic (Barbosa *et al.,* 2014). *E. coli* pathogenicity is a complex multi-factorial mechanism involving a large number of virulence factors (Kuhnert *et al.*, 2000). Some strains of *E. coli* have acquired virulence factors that enable them to cause important intestinal and extra intestinal diseases (Mora *et al.,* 2011).

Most bacterial pathogens involved in human enteric diseases originate from animals and can be conveyed directly from animals to humans or indirectly through food of animal origin or contaminated water (Newell *et al.*, 2010; Schlundt *et al.*, 2004). *E. coli* strains involved in diarrheal diseases are one of the most important of the various etiological agents of diarrhea where strains have evolved by the acquisition, through horizontal gene transfer, of a particular set of characteristics that have successfully persisted in the host (Gomes *et al.*, 2016).

Intestinal pathogenic *E. coli* (InPEC) strains can cause different types of gastroenteritis and can be divided into six pathotypes including enterohaemorrhagic *E. coli*; enteropathogenic *E. coli*; enteroaggregative *E. coli*; enterotoxigenic *E. coli*; enteroinvasive *E. coli* and diffusely adherent *E. coli* (Filho *et al.,* 2015; Lamprecht *et al.,* 2014)**.** Diarrheagenic *E. coli* is an important agent of endemic and epidemic diarrhea worldwide particularly in developing countries (Nataro & Kaper, 1998). The widespread use of agricultural antimicrobials contributes to increased clinical resistance to antimicrobials (Brower, *et al.*, 2017). The identification of

diarrheagenic *E. coli* pathotypes is through the use of molecular methods for the detection of the genes responsible for mediating the substances responsible for pathogenicity (Onanuga *et al.*, 2014).

E. coli has shown increased resistance to one or more antibiotics, a fact that has generated concern for public health (Lima *et al.*, 2017). *E. coli* from livestock is exposed to a great selective pressure because in some countries, more than a half of the antimicrobial agents are used in food producing animals (Al Haj *et al.*, 2007). There are two main ways of acquiring antibiotic resistance and they include mutation in different chromosomal loci and horizontal gene transfer (Dzidic *et al*, 2008). One of the current most relevant resistance mechanisms in *Enterobacteriaceae* like *E. coli* is the production of enzymes that lead to higher generation cephalosporins and even carbapenems resistance (Zurfluh *et al.,* 2013). Resistance in *E. coli* develops either through mutations (fluoroquinolone resistance) or by acquisition of mobile genetic elements, which has been the case for broad spectrum penicillin and resistance to third-generation cephalosporins conferred by enzymes known as extended spectrum β-lactamases (W H O, 2014). Humans and animals are probably main reservoirs of antimicrobial resistant *E. coli* (Hoang *et al*., 2017).

Food animals and their production environments are reservoirs of both resistant bacteria and resistance genes (Adelowo *et al.,* 2014). In the natural environment the resistant bacteria and resistance genes from animal or environmental origin might be transferred to humans (Sahoo *et al.,* 2012). Several reports indicate that these resistance genes are spread through the food chain and via direct contact with humans and animals (Oppegaard *et al.*, 2001; Winokur *et al.*, 2001).

The prevalence of bacteria producing ESBL varies globally across the world including Africa, United States of America, Europe, and Asian countries (Sah &Hemalatha, 2015)**.** In general, the spread of infection due to ESBL producers has been greater in countries with lower economic resources (Villegas *et al.*, 2008).

Antimicrobial resistance conferred by ESBLs severely limit treatment possibilities due to the multidrug resistance nature of ESBL producing bacteria resulting in increased prevalence of *Enterobacteriaceae* resistant to last resort antibiotics such as carbapenems (Blaak *et al.*, 2014). Livestock and animal-derived foods are regarded as relevant sources for colonization of humans with ESBL producing *E. coli* (Hille *et al.*, 2014; Sharp *et al.*, 2014). Recent studies have shown frequent colonization of poultry, cattle, pigs and other animal species with ESBL producing bacteria (Valentin *et al.*, 2014). A report in Germany showed a high prevalence of ESBL producing *E. coli* in livestock (88.2 % in pigs, 54.5 % cattle and 50 % in chickens) and provided the risk of transfer between livestock and farm workers (Dahms *et al.*, 2015).

An increasing number of ESBL producing *Enterobacteriaceae* has been detected in humans as well as in a wide range of animal species ranging from companion animals to food animals and in food (Schmid *et al.*, 2013). These enzymes are predominantly found in *E. coli* and *Klebsiella* although present also in other members of the *Enterobacteriaceae* (Saedii *et al.*, 2017). A worrisome aspect is the spread of ESBLs and carbapenemase-producers into the environment (Abgottspon, 2014). Extended spectrum beta-lactamase-producing strains have also emerged in healthy human carriers (Zurfluh *et al.*, 2013), in healthy food-producing animals and household pets as well as on food products like meat, fish and raw milk (Geser *et al.,* 2011).

Almost every class of anti-microbial is used in agriculture, including many closely related to clinically relevant antimicrobials, such as penicillins, cephalosporins, fluoroquinolones, tetracyclines, sulfonamides, and amino-glycosides (Brower *et al.*, 2017). Averagely, antimicrobial use is higher in the animal industry than in human medicine (Hu *et al.*, 2017). The increasing number of multiple-antibiotic resistant pathogens has become a serious threat to human health (Kappell *et al.,* 2015). Since antimicrobials are routinely added to animal feeds, bacterial populations are repeatedly exposed to subtherapeutic doses ideal for the emergence and spread of antimicrobial resistance (Brower *et al.*, 2017). The continued exposure of bacterial

strains to a multitude of beta-lactams has induced dynamic and continuous production and mutation of beta-lactamases, resulting in synthesis of enzymes known as ESBLs in these bacteria (Shaikh *et al.,* 2015).

Extended spectrum beta-lactamase producing *Enterobacteriaceae* are increasing in prevalence worldwide (Cantón *et al.*, 2008). The incidence of ESBL-producing organisms is difficult to resolve due to various reasons, i.e. difficulty in detecting ESBL production and inconsistencies in reporting (Shaikh *et al.,* 2015). The general objective of this study was to determine the susceptibility profiles and molecular characteristics of archived isolates of *E. coli* from the gut of healthy food animals and associated environmental sources as an important and frequent cause of diarrhea in humans. Food animals included cattle, pigs and chicken whereas environmental resources included effluents from food animal shed and food animal droppings. Selected counties include Nairobi, Kiambu, Mombasa, Kisumu and Kwale.

1.2 Statement of the problem

E. coli is an important human and animal pathogen worldwide (Wang *et al.*, 2013). Diarrheagenic *E. coli* is a major cause of gastroenteritis in children in the developing world and is associated increased resistance to antibiotics (Ochoa *et al.*, 2009). Data from Kenya demonstrated a high prevalence (86.5%) of diarrheagenic *E. coli* among hospitalized diarrheic children (Shah *et al.*, 2016). ESBLs have emerged as a major source of antimicrobial resistance among Gram negative pathogens including *E. coli* (Goyal *et al.*, 2008; Thenmozhi *et al.,* 2014). The prevalence of ESBL-producing *Enterobacteriaceae* such as *E. coli* was 37.4 % in hospital and community samples in Kenya (Storberg, 2014). Drug-resistant pathogens such as *E. coli* are difficult to identify by routine diagnostic techniques and lack of new antibiotics for their management (Sah & Hemalatha, 2015). Therefore, it is important to know the occurrence of *E. coli* pathotypes, the proportion ESBL producers and the antimicrobial susceptibility patterns among archived *E. coli* isolates from healthy food animals and their immediate environments in Kenya and this will help to

institute measures to curb the spread of the pathogens and preserve good health among farmers as well as the whole population.

1.3 Justification

Systematic studies on diarrheagenic *E. coli* and ESBL-producing *E. coli* from food animals are not routinely done in Kenya and indeed East African region. The extent and impact associated with drug resistant foodborne pathogens such as *E. coli* is not well understood. Yet these data are essential for performing risk assessment and management. There is an indiscriminate and increasing use of antimicrobials in livestock worldwide; contributing to the development of multidrug resistance. This is coupled by a weak legislation on dispensing and/or inadequate enforcement to regulate dispensing and distribution of antimicrobial agents. A good understanding of the proportion of antimicrobial resistance, resistance determinants and the associated *E. coli* pathotypes will contribute greatly towards on-going efforts to develop control measures of the main routes of transmission of these foodborne pathogens. Any success in the control of antibiotic resistant *E. coli* would be worth because it is likely to lead to a similar decline in several other types of antimicrobial resistant foodborne pathogens. Findings from this study will be essential for decision makers to develop stringent measures to regulate the use of antimicrobial agents in food animals in Kenya. The data from this study will help the decision makers to develop awareness programs towards farmers on the proper management and disposal of droppings and effluents from food animals. Finding from this study will be beneficial to whoever will read it in Kenya and elsewhere.

1.4 Research questions

1) What are the antimicrobial susceptibility patterns of archived isolates of *E. coli* from the gut of healthy food animals and environmental sources in selected counties in Kenya?

2) Which are the pathotypes of *E. coli* among archived isolates of *E. coli* from the gut of healthy food animals and environmental sources in selected counties in Kenya?

3) What is the proportion of ESBL-producers among archived isolates of *E. coli* from the gut of healthy food animals and environmental sources in selected counties in Kenya?

1.5 Objectives

1.5.1 Broad objective

To determine antimicrobial susceptibility patterns, the pathotypes and the proportion of ESBL producers and their phenotypes among archived isolates of *E. coli* from the gut of healthy food animals and environmental sources in selected counties including Nairobi, Kiambu, Mombasa, Kisumu and Kwale in Kenya.

1.5.2 Specific objectives

1) To determine antimicrobial susceptibility patterns of archived isolates of *E. coli* from the gut of healthy food animals and environmental sources in selected counties in Kenya to commonly used antimicrobials.
2) To determine the *E. coli* pathotypes among archived isolates of *E. coli* from the gut of healthy food animals and environmental sources in selected counties in Kenya.
3) To determine the proportion of ESBL-producers and their genotypes among archived isolates of *E. coli* from the gut of healthy food animals and environmental sources in selected counties in Kenya.

1.6 Limitations of the study

The present study used conventional methods and was limited to detection of the main types of ESBL genes (bla_{TEM}, bla_{SHV}, and $bla_{\mathrm{CTX\text{-}M}}$), and detected only key virulence factors or genes for each *E. coli* pathotype.

CHAPTER TWO

LITERATURE REVIEW

2.1 Genus *Escherichia*

Escherichia are straight, cylindrical, Gram-negative rods with rounded ends that are 1.1–1.5 µm in diameter and 2.0–6.0 µm in length. They occur singly or in pairs and can be motile by peritrichous flagella or non-motile (Scheutz & Strockbine, 2005).

2.1.1 History

E. coli, originally called "*Bacterium coli commune*," was first isolated from the feces of a child in 1885 by the Austrian pediatrician Theodor Escherich (Manasa, 2016; Welch, 2006). *E. coli* got its first name, *Escherichia*, from Theodor Escherich, who discovered the bacteria (Paiva de Sousa, 2006). Its second name, *coli*, means "from the colon", which is the organism's natural habitat (Ingerson-Mahar & Reid, 2011). *E. coli* are currently the best studied bacteria (Paiva de Sousa, 2006).

2.1.2 *Escherichia* species

Several species have been identified and all are known to cause human disease (Public Health England, 2015). Other *Escherichia* species include *E. adecarboxylata*, *E. blattae*, *E. fergusonii*, *E. hermannii*, and *E. vulneris* have been isolated from a variety of clinical and environmental sources (Rice *et al.,* 1991; Rice *et al.,* 1993). The sources of the clinical isolates were predominantly infections of peripheral limbs secondary to trauma; these species are not routinely isolated from stool specimens and are not considered as fecal contamination indicator (Rice *et al.,* 1993). *E. coli* occur naturally in the lower part of the intestine of warm-blooded animals, *E. blattae* in the hind-gut of cockroaches, and *E. fergusonii*, *E. hermannii*, and *E. vulneris* are found in the intestine, as well as extraintestinal sites of warm blooded animals (Scheutz & Strockbine, 2005).

2.1.3 *Escherichia coli* habitat

E. coli are ubiquitous bacterial species commensal of humans and warm blooded animals (Kabiru *et al.*, 2015). They can occasionally be isolated in association with the intestinal tract of non-mammalian animals and insects (Welch, 2006). Many *E. coli* strains are harmless and are commonly found in the intestinal tract of warm-blooded organisms (Djurdjevic-Milosevic *et al.*, 2011).

2.2 *Escherichia coli* pathotypes

2.2.1 Introduction

Although most *E. coli* strains are non-pathogenic, some of them are highly pathogenic (Barbosa *et al.,* 2014). *E. coli* pathogenicity is a complex multi-factorial mechanism involving many virulence factors which vary according to the pathotypes; and include attachment functions, host cell surface modifying factors, invasins, and many different toxins as well as secretion systems which export toxins and other virulence factors to the target host cells (Kuhnert *et al.*, 2000).

Some strains of *E. coli* have acquired virulence factors that enable them to cause important intestinal and extraintestinal diseases, such as diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), urinary tract infections (UTI), septicemia, and neonatal meningitis (Mora *et al.,* 2011). InPEC strains can cause different types of gastroenteritis and can be divided into six pathogenic groups: enterohaemorrhagic (EHEC); enteropathogenic (EPEC); enteroaggregative (EAEC); enterotoxigenic (ETEC); enteroinvasive (EIEC) and diffusely adherent (DAEC) *E. coli*. Each of the InPEC types has different infection mechanisms and symptoms (Filho *et al.,* 2015; Lamprecht *et al.,* 2014). Two further pathotypes have recently emerged; Adherent Invasive *E. coli* (AIEC) which is thought to be associated with Crohn disease but does not cause diarrheagenic infection and the Shiga Toxin (Stx) producing Enteroaggregative *E. coli* (STEAEC) responsible for the 2011 Germany *E. coli* outbreak (Clements *et al.,* 2012). Many amongst the enteric infections caused by *E. coli* are transmitted by inter-human contacts such as those caused by EIEC, EPEC or

EAEC while those ascribed to ETEC or Shiga toxin-producing *E. coli* (STEC) are primarily transmitted to humans through the consumption of contaminated water or food (Kabiru *et al.,* 2015).

2.2 2 Enterotoxigenic *Escherichia coli* (ETEC)

ETEC is a water and food-borne pathogen that infects the small intestine of the human gut and causes diarrhea (Kim *et al.,* 2014; Gonzales-Siles & Sjöling, 2016). ETEC is one of the most common causes of acute watery diarrhea among children and adults in the developing world and is the main cause of travelers' diarrhea (Hussain, 2015; Sahla *et al*., 2015). The diarrhea is mediated by 1 or 2 plasmid-encoded enterotoxins; the heat-stable toxin (ST) and/or the heat-labile toxin (LT) (Nicklasson *et al.,*2010). The ST includes two heat stable enterotoxin STa called STh and STp encoded by estA gene (Bolin *et al*., 2006). The LT enterotoxin has an enzymatic activity that is identical to that of the cholera toxin and binds to the same intestinal receptors (ganglioside GM1) that are recognized by the cholera toxin (Hölmgren *et al*., 1975; Jacques de Muinck, 2013). LT is an 84-kDa multimeric protein which catalyses the activation of adenylate cyclase, production of excessive amounts of cAMP, disruption of electrolyte transport across the intestinal lumen, and diarrhea (Tauschek *et al*., 2002).

ETEC causes disease by colonizing the small intestine, utilizing antigenically distinct fimbriae (CFA/I, CFA/II and CFA/IV) and fibrillar or afimbrial colonization factors (CFs), production of either LT and/or ST enterotoxins leads to diarrhea (Tomar *et al*., 2015). The most prevalent CFs are CFA/I and coli surface antigens 1–6 (CS1-CS6), although CS7, CS14 and CS17 are also common (Gonzales-Siles & Sjöling, 2016). ETEC strains are antigenically diverse, exhibiting many different O: H serotypes (78 different O serotypes and 34 different H serotypes), multiple fimbrial colonization factors and different toxin phenotypes (Sizemore *et al*., 2004).

2.2.3 Enteropathogenic *Escherichia coli* (EPEC)

EPEC was the first responsible for many cases of infantile diarrhea in several developing countries in the 1940s and 1950s (Vieira *et al.*, 2001; Huiwen & Gad, 2005). EPEC continues to be a significant cause of infantile diarrhea in developing nations, contributing to high morbidity and mortality (Brendan *et al.*, 1997). EPEC employs multiple adhesins to colonize the small intestine and produces characteristic 'attaching and effacing' (A/E) lesions on small intestinal enterocytes (Bueris *et al.,* 2007). EPEC adhesins that have been associated with A/E adhesion and intestinal colonization include bundle-forming pili (BFP), EspA filaments and intimin (Cleary *et al.,* 2004). EPEC possesses type 3 secretion system (T3SS), some of the effectors from EPEC and EHEC including Tir, intimin, Esp $F_{U,}$ EspB and EspL2 affect the morphology of actin networks in infected host cell and change the shape of the cell (Hamda *et al.,* 2010). EPEC has been sub-divided into typical and atypical EPEC depending on their adhesion mechanisms in human epithelial cells (Caine *et al.,*2014). Typical EPEC carries the EPEC adherence factor (EAF)-plasmid encoding bundle forming pili (BFP) and atypical EPEC that do not carry the EAF-plasmid and do not express BFP (Kozub-Witkowski *et al.*, 2008). The presence of locus of enterocyte effacement has been used to identify atypical EPEC and this locus codes for the gene of attaching and effacing, *eae* gene which enables the bacteria to adhere and destroy human intestinal enterocytes (Campellone, 2010).

2.2.4 Enteroaggregative *Escherichia coli* (EAEC)

The definition EAEC is the ability of the micro-organism to adhere to epithelial cells such as HEp-2 in a very characteristic 'stacked-brick' pattern (Weintraub, 2007). EAEC is a subgroup of DEC that during the past decade has received increasing attention as a cause of watery diarrhea, which is often persistent (Wallace-Gadsden *et al.*, 2007; Mostafa *et al.,*2014). EAEC is transmitted by the fecal-oral route where contamination of food and water play a central role in transmission (Cennimo *et al*, 2007).

EAEC strains cause persistent (≥14 days) diarrhea in children (Scaletsky *et al.*, 2002). They are associated with acute diarrhea and may be associated with nosocomial and community outbreaks worldwide and participation as a causative agent of diarrheal diseases in HIV infected adults in developed world has been reported (Villaseca *et al.,* 2005; Scavia *et al.*, 2008). EAEC is also commonly associated with traveler's diarrhea in developing countries (Pérez *et al,* 2010). Putative virulence factors like adhesins, toxins and secreted proteins, majority of them being plasmid borne, none of them is found in all EAEC strains (Gomes *et al*, 2016). They include a 60 MDa plasmid encoded proteins, aggregative adherence (AA) factor (I, II, III, IV), ST-like enterotoxin (EAST), an anti-aggregation protein transporter (CVD432), dispersin secretory protein (aap; aspU) and transcriptional activator *aggR* (Ahangarzadeh-Rezaee *et al*., 2015). The most studied adhesins are the aggregative adherence fimbria (AAF/I-AAF/V) family, which includes five types that mediate the aggregative adherence (AA) pattern and biofilm formation (Gomes *et al*, 2016). Laboratory studies on prototype strains revealed that the aggregative adherence (AA) phenotype was encoded on 55-65 MDa plasmids, collectively called pAA (Harrington *et al.,* 2006).

2.2.5. Enterohemorrhagic *Escherichia coli* (EHEC)

EHEC strains are a highly pathogenic subgroup of Shiga toxin–producing *E. coli* (STEC) that causes severe human diseases, including bloody diarrhea and hemolytic uremic syndrome (HUS) (Mellmann *et al.,* 2008; Mellies & Lorenzen, 2014). HUS is defined by a triad of features: acute renal failure, thrombocytopenia, and microangiopathic haemolytic anemia occurs in 2-15% of cases (Welinder-Olsson & Kaijser, 2005). EHEC pathogenicity is usually linked to a Shiga toxin and virulence factors, including adhesins, toxins, invasins, protein secretion systems, iron uptake systems, and several unidentified functions (Gerrish *et al*., 2007). EHEC is the main group of verotoxigenic strains which has emerged as the leading cause of haemorrhagic colitis (HC) and (HUS) in humans, shigatoxins are categorized into two main groups, *stxI* and *stxII* (Marejkova *et al*, 2013) and the majority of *Stx* genes

are bacteriophage-borne, which may be important for the spread of shiga toxin-producing *E. coli* (STEC) (Bonyadian *et al.,* 2010).

EHEC combine the virulence features of 2 other *E. coli* pathotypes that cause diarrheal disease in human; with STEC they share bacteriophage-encoded Shiga toxins (Mellies & Lorenzen, 2014); and with EPEC, they share a chromosomally located pathogenicity island (PAI), the locus of enterocyte effacement (LEE) (Caprioli *et al.*, 2005). The LEE encodes proteins necessary to produce an "attaching and effacing effect," whereby the bacteria attach to intestinal epithelial cells and induce loss of the microvilli on the apical surface (Page & Liles, 2013). EHEC like EAEC produce one or more toxins that are variously known as verotoxins, verocytotoxins or shiga toxins which act on vero cells (Joris *et al*., 2012). There are two major families of verotoxins, Vtx1 and Vtx2, each of which can be further divided into subtypes (Vtx1a, 1c and 1d; Vtx2a to Vtx2e) (Kaper,1998); Vtx2, and especially Vtx2a, seems to be more common than Vtx1 in people with the most severe disease complications (Caprioli *et al.*, 2005; Iowa State University, 2016).

A number of serotypes are known to contain EHEC, some well-known organisms involved in human disease include *E. coli* O157:H7, *E. coli* O157: H- (also known as *E. coli* O157: NM, for "nonmotile"), and members of serogroups O26, O55, O91, O103, O111, O121 and O145 (Iowa State University, 2016). Characteristics of *E. coli* serotype O157: H7 (EHEC) infection includes abdominal cramps and bloody diarrhea, as well as the life-threatening complication HUS (Nguyen & Sperandio, 2012). The majority of clinical cases have been caused by strains belonging to the O157:H7 serotype (Joris *et al*., 2012). The source of *E. coli* O157:H7 infection was generally found to be foods of bovine origin or other foods cross-contaminated by beef products or cow manure (Pierard *et al*.,1997). Transmission of EHEC to humans occurs by many ways such as through consumption of undercooked meat, vegetables, and water contaminated by feces of carriers, person-to-person and contaminated environment contact (Garbaj *et al.,* 2016). Ruminants, especially cattle, sheep, and possibly goats, are the major reservoirs for EHEC 0157:H7 (Rice *et al*., 2016).

2.2.6 Enteroinvasive *Escherichia coli* (EIEC)

Enteroinvasive *Escherichia coli* (EIEC) bacteria are human enteric pathogens associated with bacillary dysentery responsible for a substantial proportion of acute diarrheal diseases worldwide (Hoseinzadeh *et al*., 2016). EIEC has been found to be endemic to developing countries, particularly where sanitation is poor, and causes illness in both adults and children (Constantiniu *et al*., 2001; Newitt *et al*.,2016). Majority of EIEC strains have a virulence encoding plasmid of 120 - 140 mDa which is identical of those of *Shigella* group (Constantiniu *et al*., 2001). The invasion associated pathogen antigen or *ipaH* gene is a key virulence factor for EIEC, and thus dysentery is characterized by fever, painful abdominal cramps and diarrhea, sometimes vomiting and the stool contains blood and leukocytes (Hoseinzadeh *et al*., 2016). Some target genes are located on the invasion plasmid (pINV) or, like the *ipa*H gene, on both IP and the chromosomes of these pathogens (Pal *et al.,* 1997). EIEC represent a group of diarrheagenic *E. coli* biochemically, genetically, and pathogenetically related to *Shigella spp* (Health Louisiana Office of Public, 2016; EU Reference Laboratory for *E.coli*, 2013).

2.2.7 Diffusely Adherent *Escherichia coli* (DAEC)

DAEC strains are defined by a pattern of diffuse adherence (DA), in which the bacteria uniformly cover the entire cell surface (Scaletsky *et al*., 2002). DAEC possess no typical markers for other categories of diarrheagenic *E. coli* and uniformly adhere to the entire surface of HEp-2 cells or HeLa cells (Meraz *et al*., 2007). In many cases, the DA pattern of DAEC isolates is due to the production of adhesins such as fimbrial F1845, Dr and afimbrial AfaE-I, and AfaE-III encoded by a family of afa/dra/daa related operons (Le Bouguenec & Servin, 2006; Patzi-Vargas *et al.*, 2013). DAEC is considered a heterogeneous group of isolates of which supposed diarrheagenic *E. coli* strains would be included (Pereira & Giugliano, 2013).

DAEC has been associated with the watery diarrhea that can become persistent in young children in both developing and developed countries as well as recurring urinary tract infections (Jafari *et al*., 2012). The invasion of epithelial cell lines by

the adherent, invasive *E coli* (AIEC) in vitro varies between cell lines and is a property found in other *E coli* including diffusely adherent *E. coli* (DAEC) and uropathogenic *E coli* (Prorok-Hamon *et al*., 2013). However, the implication of DAEC strains in diarrhea remains controversial, since some studies have reported that these strains are found similarly in children with and without diarrhea (Dedeić-Ljubović *et al*., 2009).

2.2.8 Uropathogenic *Escherichia coli* (UPEC)

Uropathogenic *Escherichia coli* (UPEC) are the most important group of microorganisms responsible for urinary tract infections (UTI) and produce specific virulence factors and pathogenic islands which carries genes for virulence factors which enable the bacteria to establish UTIs (Slavchev *et al*., 2009). UPEC virulence factors such as adhesins, siderophores and toxins; some of which encoded in chromosomal DNA segments known as PAI which are mobile genetic elements (Calhau, 2014).

2.3 Phylotypes

The species *E. coli* is composed of four main phylogenetic groups including A, B1, B2 and D (Wassenaar, 2018). These groups can be divided into six phylogenetic subgroups (A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2) according to the combination of the three genetic markers *chu*A, *yja*A and DNA fragment TspE. C2 (Hemati *et al*., 2014). Phylogenetic group distribution may be related to antimicrobial resistance prevalence in *E. coli* isolates. Commensal *E. coli* strains frequently belong to phylotype A but are found also in group B1 (Hussain, 2015; Johnson *et al*, 2008). The extraintestinal pathogenic strains normally belong to groups B2 and D (Hussain, 2015). Not all *E. coli* pathotypes group together, ETEC was found in group A and B1 while EAEC was found in group A, B1, B2 and D (Croxen *et al*., 2013).

2.4 *Escherichia coli* serotypes

E. coli strains are normally identified by serological typing of their surface antigens such as O (polysaccharides), H (flagellar) and K (capsular) (Sharma *et al*., 2016).

Serotyping of the antigens is a useful, but complex (56 H-antigens, 80 K-antigens, 173 O-antigens) criterion for identifying pathogenic *E. coli* strains in food, environmental and clinical samples, and for epidemiological purposes (Hussain, 2015). The O, H, K antigens can be found in nature in many of the possible combinations, the final number of *E. coli* serotypes range between 50,000 to 100,000 or more (Orskov & Orskov, 1992).

2.5 *Escherichia coli* and antimicrobial drug resistance

2.5.1 Introduction

E. coli is a reservoir for antibiotic resistance genes (Sahoo *et al.,* 2012). The development and spread of resistance in *E. coli* as in other bacteria is a complex process that is influenced by selective pressure, pre-existence of resistance genes and use of infection control measures (Rupp & Fey, 2003). In the natural environment the resistant bacteria such as *E. coli* and resistance genes from animal or environmental origin might transfer to humans; the use of antibiotics is one of the factors contributing to resistance (Sahoo *et al.*, 2012). Food animals and their production environments are reservoirs of both resistant bacteria including *E. coli* and resistance genes that could be transferred to humans either by direct contact between animals and humans or indirectly via the food production chain (Adelowo *et al.,* 2014). Studies have demonstrated that pathogenic *E coli* (PEC) have developed resistance to a wide range of antibiotics (Kipkorir *et al*, 2016). The increased resistance to commonly used antibiotics in Kenya raises the need of regular antimicrobial resistance surveillance and strengthening antimicrobial stewardship programme (Wangai *et al.*, 2019)

2.5.2 Antimicrobial resistance epidemiology

Antimicrobial resistance (AMR) poses a serious global threat of growing concern to human, animal, and environment health (Aslam *et al*., 2018). Research shows that a continued rise in AMR could lead to10 million deaths every year and a 3% reduction in gross domestic product by the year 2050 (CDC-Kenya, 2017). Yet the magnitude

of AMR is still not well understood in many parts of the world (Tadesse *et al.*, 2017). Causes of the global AMR are overpopulation, enhanced global migration, increased use of antibiotics in clinics and animal production, selective pressure, poor sanitation, wildlife spread, and poor sewerage disposal system (Marshall & Levy, 2011; Singer *et al.*, 2016). WHO report on antibiotic resistance reveals high antimicrobial resistance levels in both high and low income countries, 8% to 65% of *E. coli* associated with urinary tract infections showing resistance to ciprofloxacin (WHO, 2018).

In some WHO European region, the resistance of some pathogens now reaches over 50%; the situation is worrying for Gram-negative bacteria, such as *Klebsiella pneumoniae* and *Escherichia coli* (WHO, 2019). Each year, in the in the US at least 2 million people get an antibiotic-resistant infection, and at least 23,000 people die (CDC, 2018). Although circumstances may vary by region or country, it is clear that some Asian countries are epicenters of resistance having seen rapid increases in the prevalence of antimicrobial resistance of major bacterial pathogens, Multidrug resistance (MDR) was observed in 59.3% of the isolates from Asian countries (Kang & Song, 2013).

Despite limited laboratory capacity to monitor AMR; available data suggest that the African region shares the worldwide trend of increasing drug resistance (Ndihokubwayo *et al.*, 2013). Resistance to the trimethoprim (33.9%–100%), ampicillin (7.9%–100%) and penicillin (0%–75%) was reported to be high in all regions, *E. coli* was the most common reported organism but AMR data was not available for 42.6% of the countries in the African continent (Tadesse *et al.,* 2017). A study done in Eastern Cape, South Africa on *E. coli* strains isolated from treated waste water effluent showed varying (1.7–70.6%) degrees of resistance to 15 of the test antibiotics and multidrug resistance was exhibited by 32.7% of the isolates (Adefisoye & Okoh, 2015). According to a study done in Lagos, Nigeria on prevalence and antimicrobial resistance in *E. coli* from food animals where 22 antimicrobials were tested, *E. coli* exhibited the highest resistance to tetracycline (58.8%), trimethoprim/sulfamethoxazole (39.8%), and ampicillin (34.1%)

(Adenipekun *et al*., 2015). In East Africa, high levels of AMR to commonly-used antibiotics were reported, including 50%−100% resistance to ampicillin and co-trimoxazole, 20%–47% to gentamicin, relatively high resistance to ceftriaxone (46%-69%) among Gram-negative infections, much resistance was found to be in *Klebsiella* species and *Escherichia coli* (Ampaire *et al*., 2016).

In Kenya, data from Kenyatta National Hospital where 624 isolates were analyzed reported AMR rates higher than most recent and international reports; 88% of isolates tested were multi-drug resistant whereas 25% were extensively drug resistant (Wangai *et al.,* 2019). According to this report *E. coli* and *K. pneumoniae* showed high resistance to penicillins (8-48%), cephalosporins (16-43%), fluoroquinolones (22-44%), monobactams (17-29%) (Wangai *et al.,* 2019). A report on epidemiology of antimicrobial resistance among *Escherichia coli* strains in Trans-Nzoia county showed that 42.2% of *E. coli* isolates were multidrug resistant to sulfamethoxazole, co-trimoxazole, ampicillin, chloramphenicol, tetracycline, kanamycin and streptomycin (Kipkorir *et al*., 2016).

2.5.3 Antimicrobial resistance evolution

Over the past nearly seventy-five years, the intensity and character of concerns about antibiotic resistance have depended on factors including the distribution of resistant organisms, diagnostic capacity, models of antibiotic resistance and their impact on socio-economic development, the usage of antibiotics in human and animal medicine, and the practice of agribusiness (Podolsky, 2018). The golden era of antibiotics ranged from the 1930s to 1960s which gave rise to many antibiotics (Nathan & Cars, 2014). Since the introduction in 1937 of the first effective antimicrobials (sulfonamides), the development of specific mechanisms of resistance plagued their therapeutic use (Davies & Davies, 2010).

During the late 1950s and early 1960s (Fig. 2.1), antibiotic resistance to multiple antimicrobial agents was detected, for the very first time, among enteric bacteria including *Salmonella*, *Shigella,* and *Escherichia coli* (Aslam *et al*., 2018). Increased

use of broad spectrum antibiotics such as third generation cephalosporins has been correlated with the development of beta-lactamases mediated bacterial resistance, which subsequently led to the emergence of ESBLs producing organisms (Raut *et al.*, 2015). Similar to the resistance to the third generation cephalosporins, there were reports of fluoroquinolone resistance in *E. coli* exceeding 50% in five of the WHO regions (WHO, 2014).

The evolving response to antibiotic resistance can be traced through five eras: 1945−1963, a period during which the pharmaceutical industry appeared to be more capable to control antibiotic resistance; 1963−1981, characterized by increasing concern in the wake of the discovery of the transmission of antibiotic resistance via extrachromosomal plasmids; 1981-1992, antibiotic resistance was recognized as a shared global problem ; 1992−2013 where antibiotic resistance became linked to larger concerns regarding emerging infections; and 2013 to date with antibiotic resistance still regarded as a concern over global emerging infections, social justice, and development goals, attracting interest for investigators (Podolsky, 2018).

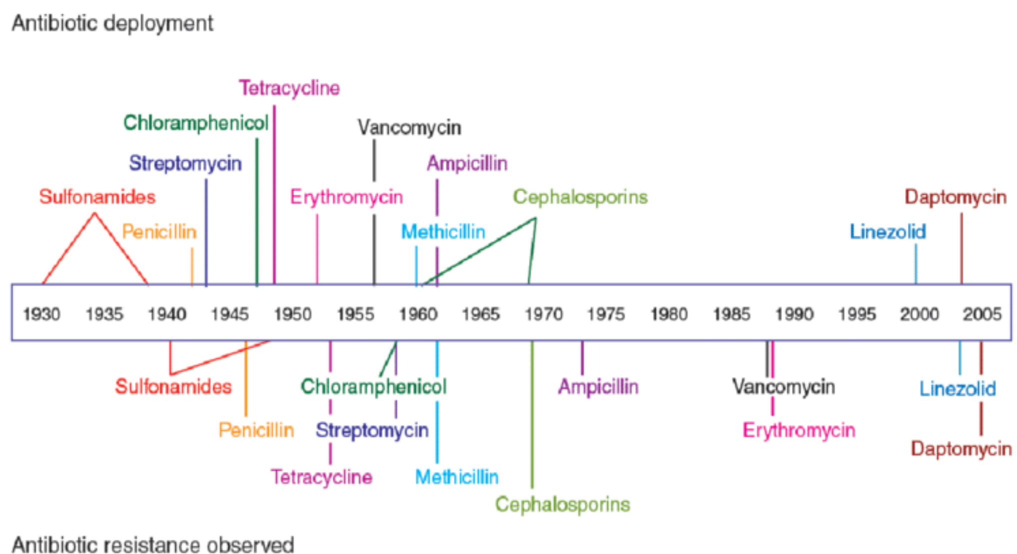


Figure 2.1 Timeline representing antibiotic deployment and resistance

This timeline represents both antibiotic deployment (top of timeline) and almost parallel antibiotic resistance (bottom of timeline).Reproduced from Clatworthy *et al.* (2007)

2.5.4 Mechanism of antimicrobial drug resistance and their transmission mode

In *E. coli* as in other bacteria, antimicrobial resistance can be an intrinsic property of the bacteria themselves or it can be acquired (Yanling *et al.*, 2013). Two ways of acquiring antibiotic resistance include chromosomal mutations and horizontal gene transfer (Munita & Arias, 2016; Yanling *et al.*, 2013). Mutations occur randomly as replication errors or an incorrect repair of a damaged DNA in actively dividing cells (spontaneous mutations), during a prolonged non-lethal antibiotic selective pressure whereby a small bacterial population enters a transient state of a high mutation rate (hypermutators) and adaptive mutations arising in non-dividing or slowly dividing cells during the presence of a non-lethal selective pressure that favours them (Yanling *et al.*, 2013).

Horizontal genes transfer of genetic material among bacteria including *E. coli* is the most commonly used way to spread antibiotic resistance (Partridge, 2015; Yanling *et al.*, 2013). The exchange of genetic material is achieved through several processes such as transduction (via bacteriophages), conjugation (via plasmids and conjugative transposons), and transformation (via incorporation into the chromosome of chromosomal DNA, plasmids, and other DNAs) (Levy & Marshall., 2004, Munita & Arias, 2016; Sultan *et al.*, 2018).

Plasmid mediated antibiotic resistance involves different classes of antibiotics in including aminoglycosides, cephalosporins and fluoroquinolones (Fig. 2.2) (Nordmann & Poirel, 2002) Transposons spread quicker than genes in chromosomes and are transferred by conjugation, transformation, or transduction (Alekshun & Levy, 2007; Giedraitiene *et al*., 2011). Integrons acquire and exchange exogenous DNA, known as gene cassettes, by a site-specific recombination mechanism (Nordmann & Poirel, 2002).

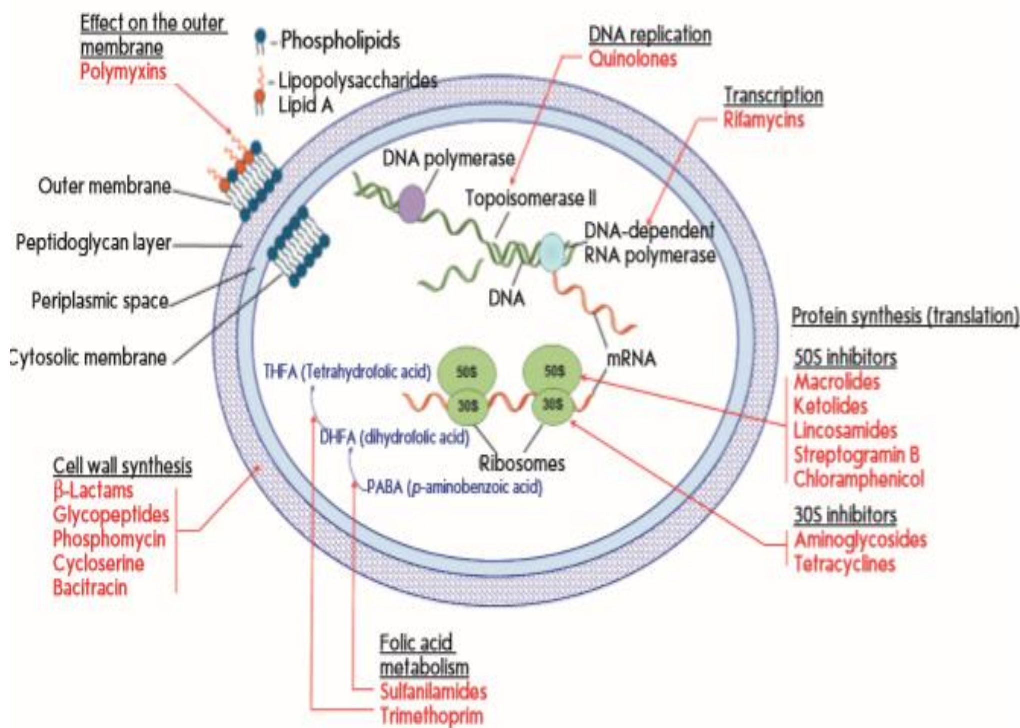


Figure 2.2: Main classes of antimicrobial drugs and their targets

The figure indicates the main classes of antimicrobial drugs, their targets, and their effect on the main processes of vital activity of a bacterial cell (Egorov *et al.*, 2018)

Biochemical mechanisms of resistance in bacteria such as *E. coli* include antibiotic inactivation by enzymatic modification of the drug, alteration of the sensitivity to the antibiotic by modification of the target; efflux pumps and outer membrane permeability, disruption of the nucleic acid machinery, interference with metabolic pathways and disruption of bacterial membrane structure (Fig 2.3) (Golkar *et al.* , 2018**;** Kumar & Varela, 2013; Sultan *et al.*, 2018; Yanling *et al.*, 2013).

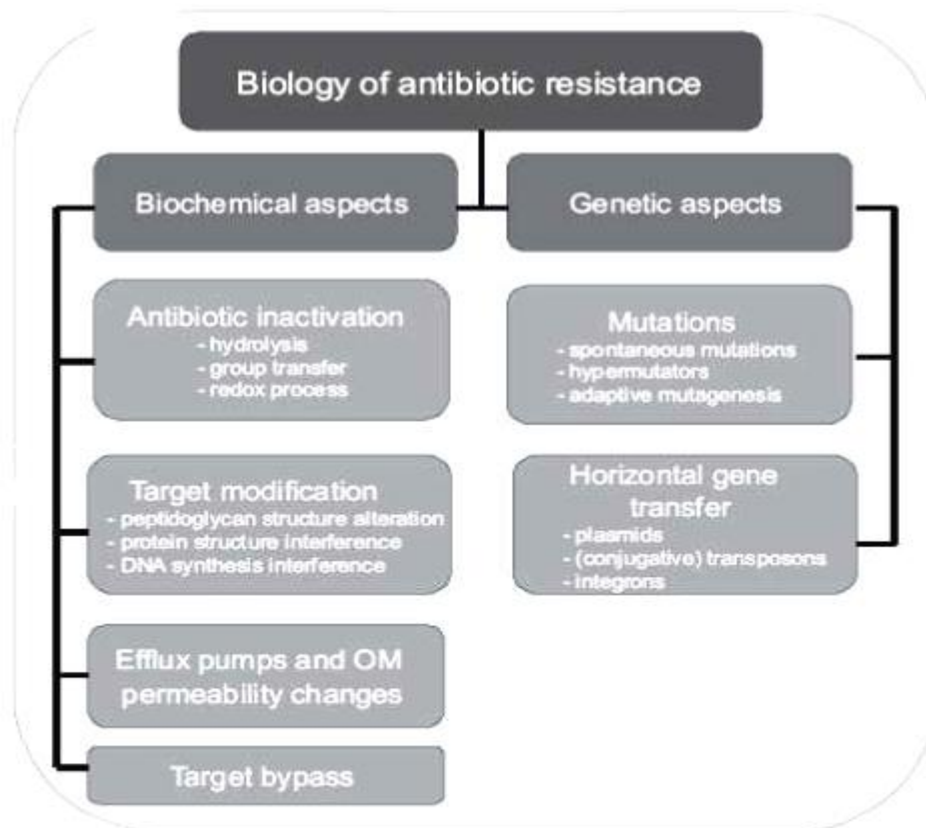


Figure 2.3: Biology of antibiotic resistance

The figure represents the biology of antimicrobial resistance as stated by Yanling *et al* (2018)

Inactivating enzymes (Fig. 2.4) include beta-lactamases that degrade antibiotics and others that perform chemical transformations (Yanling *et al.*, 2013). The beta-lactamases are more common and important weapons for Gram-negative bacteria such as *E. coli* (Jacoby & Munoz-Price, 2005, Rahman *et al.*, 2018). Many types of modifying enzymes have been described, the biochemical reactions they catalyze include acetylation (aminoglycosides, chloramphenicol, streptogramins), phosphorylation (aminoglycosides, chloramphenicol), and adenylation (aminoglycosides, lincosamides) (Munita & Arias, 2016)

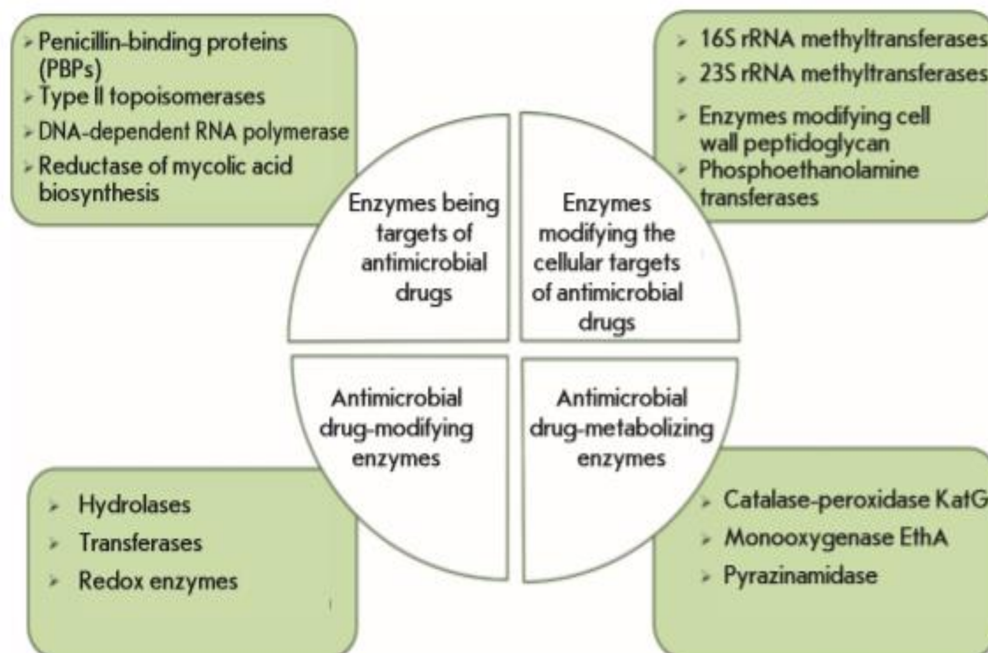


Figure 2.4: Enzymes involved in antimicrobial resistance mechanisms

Classes of enzymes involved in enzyme mediated resistance mechanisms (Fig. 2.5) are subdivided into hydrolases, transferases, and oxidoreductases (Egorov *et al.*, 2018):

i) Hydrolases: beta-lactamases and macrolide esterases destroy beta-lactams and macrolides, respectively and are the most common enzymes catalyzing antibiotic hydrolysis (Sultan *et al.*, 2018; Wright, 2005). Beta-lactamases hydrolyze the amide bond in the beta-lactam ring, the common structural element of all beta-lactam antibiotics including penicillins, cephalosporins, carbapenems, and monobactams (Bonomo, 2017, Deshpande *et al.*, 2004). Macrolide esterases hydrolyse the lactone ring of the 14- and 15-membered macrolides such as erythromycin, azithromycin (Fyfe *et al.*, 2016; Garneau-Tsodikova & Labby, 2016).

ii) Transferases: they modify molecules by covalently binding various chemical groups and represent a large superfamily of enzymes (Morar & Wright, 2010; Wright, 2005). They include aminoglycoside-modifying enzymes (N-acetyltransferases (AAC), O-phosphotransferases (APH), O-adenyltransferases (ANT)) which catalyze enzymatic modification of aminoglycoside antibiotics (Hon

et al., 1997; Khosravi *et al.*, 2017; Krause *et al.*, 2016; Ngo & Garneau-Tsodikova, 2016; Sultan *et al.*, 2018). Chloramphenicol acetyltransferases (CATs) catalyze the addition of the acetyl group of acetyl-CoA to the 3-hydroxyl group of chloramphenicol or its synthetic analogues (thiamphenicol, azidamphenicol) and prevent the binding of the antibiotic molecule to ribosomes (Sultan *et al.*, 2018; Tharian *et. al.*, 2013; Wright, 2005). Macrolide phosphotransferases (MPHs) modify the structure of macrolides by adding a phosphate group to the 2'-OH group (Leclercq, 2002**;** Wright, 2005) and Macrolide glycosyltransferases are enzymes that inactivate macrolides by glycosylating the 2'-OH group of the macrolide ring (Dinos, 2017; Leclercq, 2002; Morar & Wright, 2010). Streptogramin acetyltransferases inactivate streptogramins A by acetylation of an unbound hydroxyl group (Leclercq, 2002; Wright, 2005). Phosphomycin-modifying enzymes such FosA, FosB, and FosX epoxidases, as well as FomA and FomB kinases, are metalloenzymes that inactivate phosphomycin (Nikolaidis *et al.*, 2014; Silver, 2017). Rifamycin-modifying enzymes inactivate rifamycins by modifying the hydroxyl group involved in the binding of an antibiotic molecule to the β-subunit of RNA polymerase (Egorov *et al.*, 2018)

iii) Monooxygenases: the flavin-dependent monooxygenase TetX confers resistance to all tetracyclines, including the broad-spectrum antibiotic tigecycline (Wright, 2005). TetX catalyzes monohydroxylation of tetracyclines in the presence of NADPH, O2, and Mg2+, leading to intramolecular cyclization and decomposition of the molecule (Egorov *et al.*, 2018; Linkevicius *et al.*, 2016). Flavin-dependent monooxygenases Rox inactivate rifamycins by oxidating the naphthyl group at position 2, leading to ring opening and linearization of the antibiotic molecule (Koteva *et al.*, 2018).

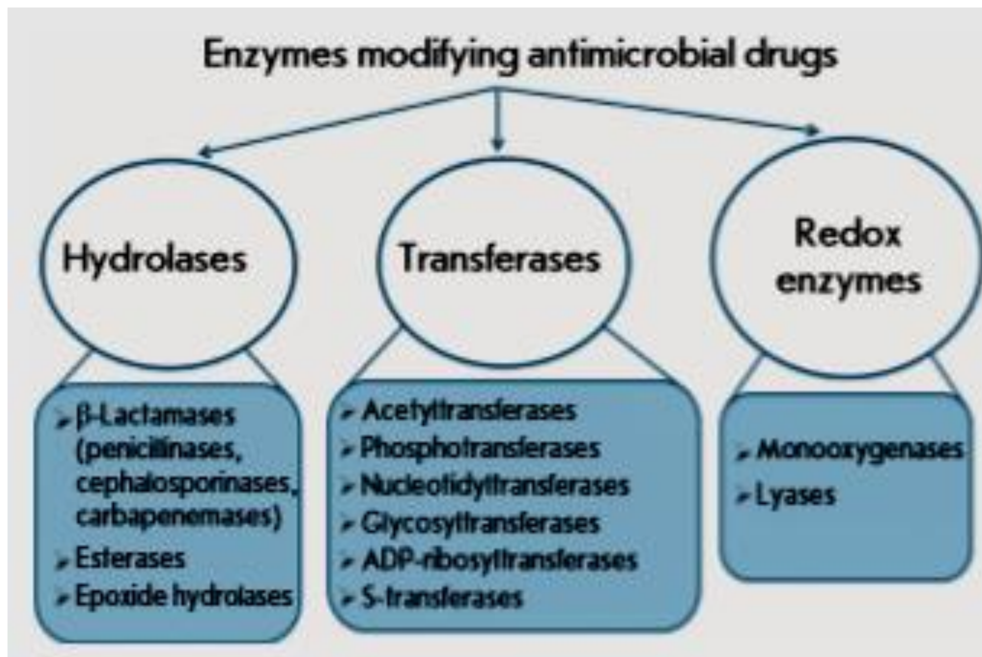


Figure 2.5: Different classes of enzymes modifying antimicrobial drugs

The chart shows different classes of enzymes involved in the modification of antibiotic (Egorov *et al.*, 2018)

2.6 Extended spectrum beta-lactamases (ESBLs)

2.6.1 Definition and mechanism of action

ESBLs are enzymes whose rate of hydrolysis of the extended-spectrum beta-lactam antibiotics such as ceftazidime, cefotaxime, or aztreonam is > 10% that of benzylpenicillin (Sridhar, 2015). ESBLs are defined as beta-lactamases that have the following characteristics: they are transferable; they can hydrolyze penicillins, first, second, and third generation cephalosporins, and aztreonam (Raut *et al.*, 2015; Sridhar, 2015). Carbapenems and cephomycins are effective against ESBL producing strains (Ghafourian *et al*., 2015).

ESBLs can be blocked *in vitro* by beta-lactamase inhibitors such as clavulanic acid. Beta-lactamases hydrolyse the beta-lactam ring of antibiotics (Fig. 2.6) and confer resistance to antibiotics (Miyagi *et al.,* 2012).

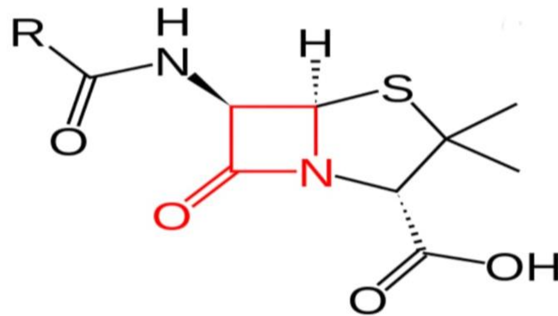


Figure 2.6: Diagram representing the beta-lactam ring

The diagram represents in red the beta-lactam ring and was reproduced from (Khan Academy, 2019)

Beta-lactam antimicrobials are characterized by having a four-membered cyclic amide (Fig. 2.6) as part of their chemical structure (Walkty *et al*., 2016). This broad group includes the penicillins, cephalosporins, carbapenems, and monobactam (Walkty *et al*., 2016). ESBLs are a subset of beta-lactamases that confer resistance to penicillins, cephalosporins, and monobactams and are less efficiently antagonized by beta-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam (Rawat & Nair, 2010). ESBL-producing GNB like *E. coli* remain susceptible to the carbapenems and cephamycins (Malloy & Campos, 2011). ESBLs are usually plasmid-mediated beta-lactamases, most commonly found in *Klebsiella pneumoniae*, *E. coli* and other Gram-negative bacilli (Goyal *et al*., 2008). *E. coli* and *K. pneumoniae* are the most common ESBL producing bacterial species (Juma *et al*., 2016).

ESBL-producing enterics such as *E. coli* have 3 various resistance mechanisms where the most widespread is production of beta-lactamases which hydrolyze the beta-lactam ring in penicillins and cephalosporins, the second is mutation which decreases beta-lactam uptake and existence of efflux pumps, which exports antibiotics outside of the cell (Adelowo *et al*., 2014). The first plasmid-borne beta-lactamase gene was detected in GNB in Greece in the 1960s and was designated bla_{TEM} after the name of the patient (Temoneira) who carried the pathogen (Kaur &

Aggarwal, 2013). TEM-1 is the most common beta-lactamase in GNB, and it can hydrolyze penicillins (ampicillin) (Bruton *et al*., 1986). The beta-lactamases also quickly spread to other bacteria and compared to the TEMs, the sulfhydryl-variable (SHV) beta-lactamases are similar in biochemical structure but are more common in *Klebsiella spp* (Kaur & Aggarwal, 2013). The third-generation cephalosporins were stable against hydrolysis by the original TEMs and SHVs (Tham, 2012).

2.6.2 ESBL evolution

In 1983, Knothe found a single nucleotide mutation in a sulfhydryl variable (SHV) that represented the first plasmid-encoded beta-lactamase that could hydrolyze the extended-spectrum cephalosporins in an isolate of *K. ozaenae* (Knothe *et al.,*1983). Outbreaks of primarily *Klebsiella spp* with mutated TEM and SHV enzyme derivates were reported from French hospitals at the end of the 1980s, and, to distinguish these enzymes from broad-spectrum beta-lactamases (mainly TEM-1, TEM-2, and SHV-1), the term ESBL was coined by Philippon *et al.,* 1989 (Sirot *et al*., 1987).

Members of the CTX-M group are now the most common ESBLs worldwide (Bonnet, 2004). The first CTX-M was described in Japan in 1985 in a laboratory dog used for pharmacokinetic studies of beta-lactam antibiotics (Matsumoto *et al*., 1988). In Germany in 1989, an *E. coli* strain resistant to cefotaxime and producing a non-TEM–non-SHV enzyme was isolated, was named CTX-M-1 due to its elevated cefotaximase activity (Bauernfeind *et al*., 1990). The CTX-M enzymes are naturally produced by *Kluyvera spp*., are found in the chromosomes of those bacteria and have also been transferred to a plasmid that carries these enzymes (Decousser *et al*., 2001).

A class of beta-lactamase capable to hydrolyse expanded spectrum beta-lactam antibiotic was described first in a *K. pneumoniae* strain in1985s (Kliebe *et al*., 1985). Ten years later, a wide range of organisms producing these enzymes could be found in health care settings worldwide (Correa-Martinez *et al*, 2019). Less than 20 years after their identification, these organisms were one of the most important groups of

healthcare associated pathogens (Gniadkowski, 2001). It has been reported that ESBL-producing bacteria play an important role beyond the healthcare settings (Coque *et al.*, 2008). Today, ESBLs are the most common beta-lactam resistance mechanism of Gram negative bacteria such as *E. coli* (Al-Bayssari *et al.*, 2015). ESBLs have become a concern for public health with increasing infections and colonization rates worldwide (McDaniel *et al.*, 2017).

2.6.3 ESBL classification

ESBLs are grouped into major and minor types depending on the general prevalence (Sridhar, 2015). *E. coli* and *K. pneumoniae* remain the major ESBL- producing organisms isolated worldwide but these enzymes have been identified in several other *Enterobacteriaceae* members (Thenmozhi *et al.*, 2014). The total number of ESBLs now characterized exceeds 200 (Raut *et al*, 2015). The most common ESBL isolated from clinical specimen are the CTX-M, SHV and TEM (Hackman *et al.*, 2014). Beta-lactamases are commonly classified according to two general schemes, the Ambler molecular classification scheme (Hall & Barlow, 2005) and the Bush-Jacoby-Medeiros functional classification (Paterson & Bonomo, 2005).

The molecular classification is based on the amino-acid sequence and divides beta-lactamases into class A, C, D enzymes which utilize serine for beta-lactam hydrolysis and class B metallo-enzymes which requires divalent zinc ions for substrate hydrolysis (Bush & Jacoby, 2010; Subashini & Kannabiran, 2013). The functional groups use the characteristics of the enzymes, including their hydrolytic substrate profile, and response to inhibitors (Bush & Jacoby, 2010). The most famous scheme was developed by Bush, Jacoby and Medeiros (Ghafourian *et al.*, 2015). It takes into account substrate and inhibitor profiles in attempt to group enzymes in a way that can correlate with their phenotypes in clinical isolates (Bush & Jacoby, 2010; Tärnberg, 2012).

Group 1 includes cephalosporinases that are not inhibited by clavulanic acid belonging to the molecular class C (Samaha-Kfoury & Araj, 2003). These enzymes

can show some resistance to carbapenems when they present in high levels, can act on cephamycins; the subgroup 1e (extended spectrum AmpC beta-lactamases) has an activity against ceftazidime and oxyimino-beta-lactams (Chandra, 2013). The enzymes in this group are found in *Enterobacteriaceae* as well as in *Pseudomonas* and they are inducible (Shah *et al.*, 2004). Group 1 enzymes are not inhibited by clavulanic acid (Urumova, 2015).

Group 2 (Ambler class A and D) broad-spectrum, inhibitor-resistant, ESBLs and serine carbapenemases (Rahman *et al*, 2018): these enzymes are carried by plasmid and beta-lactamase inhibitors such clavulanate, sulbactam and tazobactam inhibit the original group 2 enzymes (Cormican *et al.*, 1996).

Group 3 metallo-beta-lactamases (Ambler class B) are mostly broad spectrum and hydrolyse a variety of penicillins and cephalosporins but not aztreonam (Fakhuruddin *et al.*, 2014, Pari *et al.*, 2018). Group 3 gathers zinc containing molecular class B, can hydrolyse also carbapenem and are not inhibited by clavulanic acid (Urumova, 2015), they are frequently encountered in *P. aeruginosa*, *Bacteroides fragilis* and *Stenotrophomonas maltophila* (Ghafourian *et al.*, 2015).

Group 4 consists of penicillinases resistant to clavulanic acid that could not be referred any molecular class (Samaha-Kfoury & Araj, 2003; Urumova, 2015).

2.6.4 ESBL genes

More than 350 different natural ESBL encoding genes are known (Bajpai e*t al.*, 2017). Among the many ESBLs genes described in a variety of pathogens, $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} are most common across various epidemiological niches (Rahman *et al.*, 2018).

bla_{TEM} (Temoneira *E. coli* mutant) encodes for ESBL whose substrates and inhibition profiles are similar to those mediated by $bla_{SHV\text{-}1}$ (Shaikh *et al.*, 2015). The first bla_{TEM} variant with increased activity against extended spectrum cephalosporins was

bla$_{TEM-3}$ (Ullah *et al*., 2017). *bla*$_{TM-3}$ was the first *bla*$_{TEM}$ gene encoding for a beta-lactamase that displayed the ESBL phenotype (Bradford , 2001). *bla*$_{TM-3}$ may not have been the first ESBL encoding *bla*TEM, *Klebsiella oxytoca* harboring a plasmid borne gene encoding ceftazidime resistance; the responsible beta-lactamase gene was what is now called *bla*$_{TEM-12}$ (Paterson & Bonomo, 2005).

bla$_{SHV}$ (Sulfhydryl variable) family of beta-lactamase gene appears to be derived from *Klebsiella spp* (Shaikh *et al*, 2015). *bla*$_{SHV-1}$ encoded beta lactamase was found in *Klebsiella pneumoniae* and *E. coli*; it confers resistance to broad-spectrum penicillins such as ampicillin and piperacillin but not to the oxyimino substituted cephalosporins (Livermore, 1995; Turner, 2005). *bla*$_{SHV-2}$ encoded beta-lactamase was the first beta-lactamase to hydrolyse expanded spectrum beta-lactam antibiotics and was described in 1985 for a *Klebsiella pneumoniae* isolated in Germany (Turner, 2005)

bla$_{CTX-M}$ (Cefotaximase-munich) constitutes a distinct lineage encoding molecular class A beta-lactamase (Mininder & Aruna, 2013). *bla*$_{CTX-M}$ encoded beta-lactamases inhibit better with beta-lactamase inhibitor tazobactam than sulbactam and clavulanate (Bethel *et al*, 2011). Unlike *bla*$_{TEM}$ and *bla*$_{SHV}$ encoded enzymes, there is no point mutation in CTX-M encoding gene and it is believed that *bla* $_{CTX-M}$ was first identified from the chromosome *Kluyvera spp* (Kocsis & Szabó, 2013) following its transforming to plasmid (Cantón *et al.*, 2012). *bla*$_{CTX-M-15}$ encoded beta-lactamase is the most dominant and belongs to *bla*$_{CTX-M-1}$ encoded beta-lactamases sub-group (Kocsis & Szabó, 2013). They are found in different *Enterobacteriaceae* (Pallecchi *et al*., 2004). Various reports suggest that the *bla*$_{CTX-M}$ encoded ESBLs may now be the most frequent ESBL type worldwide, endemic in most countries of Europe, Asia and South America, with high rates of CTX-M-type ESBLs among *E. coli* (30 to 90 %) and *K. pneumonia* (Bonnedahl *et al.*, 2009; Ouedraogo *et al*., 2016; Rawat & Nair, 2010).

OXA (oxacillinase) gene: *bla*$_{OXA}$ encoded beta-lactamases were among the earliest beta-lactamases detected (Evans & Amyes, 2014). The *bla*$_{OXA}$ encoded ESBLs were

originally discovered in *P.aeruginosa* isolates from a single hospital in Ankara (Naas *et al.*, 2008).

Minor ESBLs encoding genes include bla_{BES-1} (Brazil ESBL-1) bla_{SFO} (Serratia fonticola), bla_{BEL} (Belgium ESBL), bla_{TLA} (Tlahuicas), bla_{GES} (Guiana ESBL-1), bla_{PER} (*Pseudomonas* extended resistance) and bla_{VEB-1} (Vietenamese ESBL) (Bajpai e*t al.*, 2017; Naas *et al.*, 2008). Some of these ESBL genes are infrequently found or restricted ; others are becoming locally prevalent or progressively isolated globally (Amirkamali *et al.*, 2017; Bajpai e*t al.*, 2017; Naas *et al.*, 2006; Rahman *et al.*, 2018; Villegas *et al*., 2008).

2.6.5 ESBL epidemiology

Certain levels to consider for ESBL epidemiology include the wider geographical area, the country, the hospital, the community, the host, the bacteria (*E. coli* is more endemic, *K. pneumoniae* is more epidemic) and their mobile genetic elements, various reservoirs including the environment, wild animals, farm animals, and pets and the transmission mechanisms (Shaikh *et al*, 2015; Tham, 2012). Some of the risks have been positively associated with colonization or infection by ESBL producers and include African /Asian country of birth, long hospital stay (>7days), nursing home residence, transfer from another healthcare facility and antibiotics use (Demirdag & Hosoglu, 2010).

The increasing prevalence of ESBL has been associated with an epidemiological shift to increasing rates of ESBL in community acquired infections. The highest beta-lactamase rates were consistently observed in Asia and South America, lower in North America and the data are particularly variable in Africa (Moxon & Paulus, 2016). In general, the spread of infection due to ESBL producers has been greater in countries with lower economic resources (Villegas *et al.*, 2008).

Data from a review published in 2005 showed that 10% of *Enterobacteriaceae* isolates expressed ESBLs in Australia, Sweden, Japan, Korea and Singapore while

rates were higher than 30% in Portugal, Italy, Turkey and most Latin American countries (Paterson & Bonomo, 2005; Rupp & Fey, 2003). Rates of ESBL-producing *E. coli* were over 50% in China, India and Thailand (Hawser *et al*., 2009).
According to data from the European Antimicrobial Resistance Surveillance System (EARSS), the proportion of ESBL producing *E. coli* from blood cultures was 28% in Bulgaria, 16% in Cyprus and Romania (EARSS, 2005). First reports of ESBLs in the USA in the late 1980s were reported with TEM-type and the major enzymes appeared to be the TEM and SHV types, with a minimum countenance of CTX-M types (Sah & Hemalatha, 2015). In Gemany, a prevalence of 12.9% of ESBL-producing *E. coli* in flies was reported (Wetzker *et al*, 2019). A study investigating livestock and farm workers reported high level of ESBL production (70.6%) in *E. coli* from farms in Germany (Dahms *et al*., 2015). The incidence of ESBL producing *E. coli* and ESBL-*Klebsiella* infections in the United States has increased with slightly higher rates of ESBL-*Klebsiella* infections according to a literature review of US studies published between 2000 and 2015 (McDanel *et al*., 2017).

The ESBL prevalence varies across Africa, being highest in North Africa (16.4–77.8%), and least in South Africa (8.8–13.1%) (Ampaire *et al*., 2017). Globally, few investigations have been conducted in sub-Saharan Africa, and they have provided very little data (Ghafourian *et al*., 2015). Twenty-six studies from 13 African countries showed that in total the proportion of ESBL-producing isolates was 15% in 16 out of 26 studies (Tansarli *et al*., 2014).

In East Africa, studies reported a prevalence of ESBL ranging from 37.4 to 62.8% (Ampaire *et al*., 2017). A high prevalence of ESBL-production in *Enterobacteriaceae* (*E. coli., K. pneumonia* and *K. oxytoca*) (78.57%), was reported among Ethiopian children (Legese *et al*., 2017). The ocurrence of ESBL production in clinical isolates was reported to be 38.3 % (36/94) in inpatients (13.8% of *E. coli*) and 5.9% (6/102) in outpatients (1.9% of *E. coli*) in Rwanda (Muvunyi *et al*., 2013). Reports from Uganda showed high levels of ESBl production in (56%-89%) in clinical isolates including *E coli* and *Klebsiella spp* and high occurrence of ESBL production was found in *E. coli* (Ampaire *et al*., 2017; Baguma *et al*., 2017; Kasango

et al., 2018). In Tanzania, 24.3% ESBL-producing *Enterobacteriaceae* (16 *E. coli* and 1 *K. pneumoniae*) in 70 latrine samples were reported and five ESBL *E. coli* strains were detected on door handles (Erb *et al.*, 2018).

A study done in Kenya on *E. coli* from community-acquired urinary tract infections resistant fluoroquinolones and extended-spectrum beta-lactams showed that twelve of the 17 *E. coli* (70.6%) were resistant to multiple drugs, including ampicillin, co-amoxyclav, cefotaxime, ceftriaxone, ceftazidime and gentamicin and nalidixic acid and produced plasmid-mediated CTX-M-15 type ESBLs and CMY-2 AmpC type enzymes (Kariuki *et al.*, 2007). The prevalence of ESBL phenotypes from faecal *E. coli* from severely malnourished and nourished children attending Mbagathi district hospital was 39% (Njoroge *et al.*, 2014).

A study to assess the prevalence of ESBL-producing *E. coli* and *K pneumonia* from patients presenting with diarrhea in Machakos district hospital showed that out of the 200 *K. pneumonia* and 100 *E. coli* tested, (18) 6% were positive for ESBL production phenotypically. Eight (4%) and 2 (1%) of the 200 *K. pneumonia* isolates had bla_{TEM} and bla_{SHV}, respectively (Juma *et al.*, 2016). A study investigating the carriage and acquisition of extended-spectrum beta-lactamase-producing enterobacterales among neonates admitted to hospital in Kilifi, showed that 10 % of neonates carried ESBL producing enterics at admission; among the admitted non carriers, 55% acquired ESBL producing enterics during their hospital stay. The incindence of ESBL-producing *Enterobacteriaceae* acquisition was 21.4%, *K. pneumoniae* and *E. coli* were the predominant ESBL producers (Kagia *et al.*, 2019).

These epidemiological data show that there is a wide variation in the geographical distribution of ESBL genes across the world coupled with an increasing incidence of ESBL-producing *Enterobacteriaceae*. *E. coli and K. pneumoniae* are the most important ESBL producers in different settings. However few studies on occurrence of ESBL producing *Enterobacteriaceae* have been done in food animals and their immediate environment, in Kenya. Therefore, more investigations are of a paramount

importance in this particular area since resistant organisms present in food animals and environment might transfer to humans through food chain.

2.7 Laboratory detection of ESBLS

Phenotypic tests for ESBL detection involve screening and confirmatory phenotypic methods that use non-molecular techniques, which detect the ability of the ESBL enzymes to hydrolyse different cephalosporins (Pitout & Laupland, 2008). Genotypic methods use molecular techniques to detect the gene responsible for the production of the ESBL (Pitout & Laupland, 2008).

2.7.1 Screening methods

These methods involve detection of resistance to any of the 3rd generation cephalosporins such as cefotaxime, ceftazidime, ceftriaxone, cefpodoxime aztreonam by disk diffusion method and MIC estimation (Goel *et al*, 2014; Taneja & Sharma, 2008).

2.7.1.1 Disk diffusion methods

The clinical and laboratory standards institute (CLSI) proposed the disk diffusion method for screening for ESBL by *K. pneumoniae, K. oxytoca, E. coli* and *Proteus mirabilis* (CLSI, 2009) Laboratories using disk diffusion methods for antimicrobial susceptibility testing can screen for ESBL production by noting specific zone diameters which indicate a high level of suspicion for ESBL production. Since the affinity of ESBLs for different substrates is variable, using more than one antibiotic for screening improves the sensitivity of detection; cefpodoxime, ceftazidime, aztreonam, cefotaxime or ceftriaxone disks are used (CLSI, 2009). It is advisable to use cefotaxime which detect CTX-M and ceftazidime which detect TEM; if only one drug can be used, the best indicator is cefpodoxime though it can lead to a high number of false positive resulting from mechanisms other than ESBL production (Jarlier *et al.,* 1988; Livermore & Paterson, 2006; Steward *et al.*, 2001). If isolates

show reduced sensitivity to any of the five antimicrobials, it indicates suspicion for ESBL production (Rawat & Nair, 2010)

2. 7.1.2 Screening by dilution antimicrobial susceptibility test

The CLSI has proposed dilution method for screening for ESBL production by *K. pneumoniae, K. oxytoca, Escherichia coli* and *Proteus mirabilis*. (CLSI, 2009) Ceftazidime, aztreonam, cefotaxime or ceftriaxone can be used at screening concentration of 1 µg /ml or cefpodoxime at a concentration of 1 µg /ml for *Proteus mirabilis* or 4 µg /ml for the others. Growth at or above this screening antibiotic concentration is suspicious of ESBL production and is an indication for the organism to be tested by a phenotypic confirmatory test. (CLSI, 2009)

2.7.2 Detection of ESBL by phenotypic confirmatory methods

The methods used rely on inactivation of chromogenic substrate by rapid test, inactivation of antibiotic by the extract of the cells by three dimensional tests and inhibition of ESBL by clavulanate (Sridhar, 2015). Automated methods such as Vitek 2 system are also used.

2.7.2.1 Rapid tests

Nordmann Dortet Poirel ESBL test (NDP test) is based on a technique designed to identify the hydrolysis of the beta-lactam ring of a cephalosporin which generates a carboxyl group by acidifying a culture medium; the acidity is identified by the color change generated using a pH indicator and inhibition of ESBL activity is evidenced by adding tazobactam in a complementary well (Nordmann *et al.*, 2012).

2.7.2.2 Three dimension test

Muller Hinton Agar plates are seeded with the inoculum of a standard sensitive strain (*E. coli* ATCC 25922) McFarland 0.5 standard. Four wells (4 mm in diameter), are made on inoculated agar plates. A 30 µl suspension of test organism in sterile distilled water 5.0 McFarland standards is poured into each well. Discs of

ceftazidime, ceftriaxone, cefotaxime and aztreonam are placed about 2 mm away towards the center of the plate from the wells. The plate is then incubated at 37 °C for 16-18 hours. Heart shaped distortion of zone of inhibition around the antibiotic disc indicates ESBL production (Biswas *et al.*, 2013).

2.7.2.3 Double disc synergy test

A plate is inoculated with the test organism as for a routine susceptibility test; discs containing cefotaxime and ceftazidime 30 µg (or cefpodoxime 10 µg) are applied on either side of one with co-amoxiclav 20±10 µg; and are placed 20mm away (center to center) from it; this distance is optimal for cephalosporin 30 µg discs; ESBL production is confirmed when the zone of inhibition of antimicrobials (one or more) is extended towards amoxiclav disk (Paul *et al.*, 2014; Public Health England, 2016).

2.7.2.4 Inhibitor potentiated disk diffusion

This test is based on a modification of the standard disk diffusion method as described by NCCLS. Mueller Hinton agar (MHA) supplemented with 4 mg/l of clavulanate is used. The inoculum is prepared (0.5 McFarland standard). Antibiotic discs containing ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) and aztreonam (30 µg) are placed on 85 mm clavulanate containing and clavulanate-free MH agar. After overnight incubation at 37°C, a difference of ≥10 mm in inhibition zone diameters of beta-lactam discs on clavulanate-free medium against those in clavulanate-containing medium is considered positive for ESBL production (Ho *et al.*, 1998)

2.7.2.5 CLSI Phenotypic confirmatory test

Also known as combined Disk Method, this test is recommended by CLSI for confirmation of ESBL producing isolates of *E. coli*, *K pneumonia*, *K. oxytoca* and *P. mirabilis*; the test can be performed either as disk diffusion method or MIC estimation (Sridhar, 2015). A Mueller Hinton agar plate is inoculated with the test organism; then cefotaxime (30 µg) and ceftazidime (30 µg) are used alone and with clavulanic acid (10 µg); a ≥ 5mm increase in the zone diameter for either

antimicrobial agents tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL production(Goel *et al*, 2014).

2.7.2.6 E-Test

E-test strips that contain either a combination of ceftazidime and ceftazidime/clavulanic acid or cefotaxime and cefotaxime/ clavulanic acid; both strips have a decreasing gradient of ceftazidime or cefotaxime alone on one end and a decreasing gradient of ceftazidime or cefotaxime plus a fixed gradient of clavulanic acid on the other end; the presence of ESBL is confirmed by the appearance of a phantom zone or when the minimum inhibitory concentration of the antibiotic mixture side is reduced by ≥ 8 times in the presence of beta-lactamases inhibitor (Rupp & Fey, 2003; Paul *et al*, 2014).

2.7.2.7 Automated system

Automated ESBL detection tests such as Vitex Legacy, Vitek 2 (BioMerieux), Microscan (Siemens Medical Solution Diagnostic), Sensititre (TREK Diagnostic System) and Phoenix (BD Diagnostic System) detect ESBL on the principle of inhibition by clavulanic acid (Sridhar, 2015). The Vitek 2 ESBL test (BioMérieux) is a common tool for rapid detection of ESBL production which is based on simultaneous assessment of the inhibitory effects of cefepime, cefotaxime, and ceftazidime, alone and in the presence of clavulanic acid (Spanu *et al.*, 2006).

2.7.3 Molecular methods in the detection of ESBL genes

Detection and identification of beta-lactamase (*bla*) genes became accurate when nucleic acid sequencing was introduced. Earlier the detection of *bla* genes was undertaken by DNA hybridization assay using labeled probes but with the advent of Polymerase Chain Reaction (PCR) technology, detection and identification of *bla* genes have been convenient (Sridhar, 2015). Molecular techniques for the detection of resistance genes are more reliable as compared to the conventional phenotypic methods (Malik *et al.*, 2018).

2.7.3.1 Nucleic Acid based molecular techniques

By designing suitable primers, entire *bla* gene can be amplified by PCR technique. By detecting specific parts of *bla* genes, beta lactamase genes can be identified to a certain extent but for complete identification, the entire *bla* gene must be amplified and sequenced by use of conventional PCR that can be run either as uniplex or multiplex. Currently RT PCR has been developed and can also be used (Sridhar, 2015).

2.7.3.2 Non-nucleic acid based molecular techniques

Matrix Assisted Laser Desorption/ Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) is based on the cellular proteome, which reflects gene products and metabolic products of the organisms (Vrioni *et al.*, 2018). The method is based on the analysis of highly abundant, mainly ribosomal, proteins of microorganisms in the mass range of 2,000 to 20,000 Daltons (Vrioni *et al.*, 2018). These proteins are ionized into charged molecules by addition or loss of one or more than one protons, in order to measure the mass to charge (m/z) ratio (Vrioni *et al.*, 2018). An energy absorbent solution called "matrix" is mixed with the sample for analysis (Vrioni *et al.*, 2018). When the matrix crystallizes on drying, the sample entrapped within it also co-crystallizes (Vrioni *et al.*, 2018). A laser beam then ionizes the sample, generating singly protonated ions (Vrioni *et al.*, 2018). These ions are then accelerated at a fixed potential and they separate from each other on the basis of their m/z ratio, which is measured by determining the time required for each ion to travel the length of the flight tube (TOF). Based on the TOF information, a characteristic mass spectrum called "Peptide Mass Fingerprint" (PMF) is generated (Vrioni *et al.*, 2018). This PMF, with peaks specific to genera and species unique to individual types of microorganisms, is then compared to a database (Vrioni *et al.*, 2018). The PMF of unknown microbial isolates is compared with those of known microbial isolates contained in the database and thus the unknown organism is identified at the family, genus, and species level (Vrioni *et al.*, 2018).

2.7.4 Controls for ESBL tests

Quality control when performing screening and confirmatory involves performing simultaneous testing with non ESBL producer (*E. coli* ATCC 25922) and an ESBL producer (*K. pneumoniae* ATCC 700603) (CLSI, 2009).

CHAPTER THREE

MATERIAL AND METHOD

3.1 Study sites

This study was carried out at Centre for Microbiology Research, Kenya Medical Research Institute (CMR KEMRI). The work made use of an existing archived culture collection of *E. coli* isolates. The isolates were obtained from rectal swabs of food animals (pigs, cattle, chickens) and environmental sources (whereby food animals droppings and effluents from food animal sheds were collected aseptically in sterile vials) in an on-going approved study, 'Ecology and epidemiology of antimicrobial-resistant foodborne pathogens (*E. coli, Salmonella spp* and *Campylobacter spp*) in selected sites in five counties (Kiambu, Nairobi, Mombasa, Kisumu and Kwale) in Kenya. The study investigated farmer knowledge and attitudes towards antimicrobial use and resistance as well as public health significance (SERU/CMR/P0036/3205).

The five counties were chosen based on certain socio-economic characteristics and represented the entire country. Nairobi and Mombasa represented urban and high income counties; Kwale represented rural and low income counties. Kiambu represented poultry farming counties, Kisumu represented pig farming counties. A Kenya map is shown in Fig. 3.1.

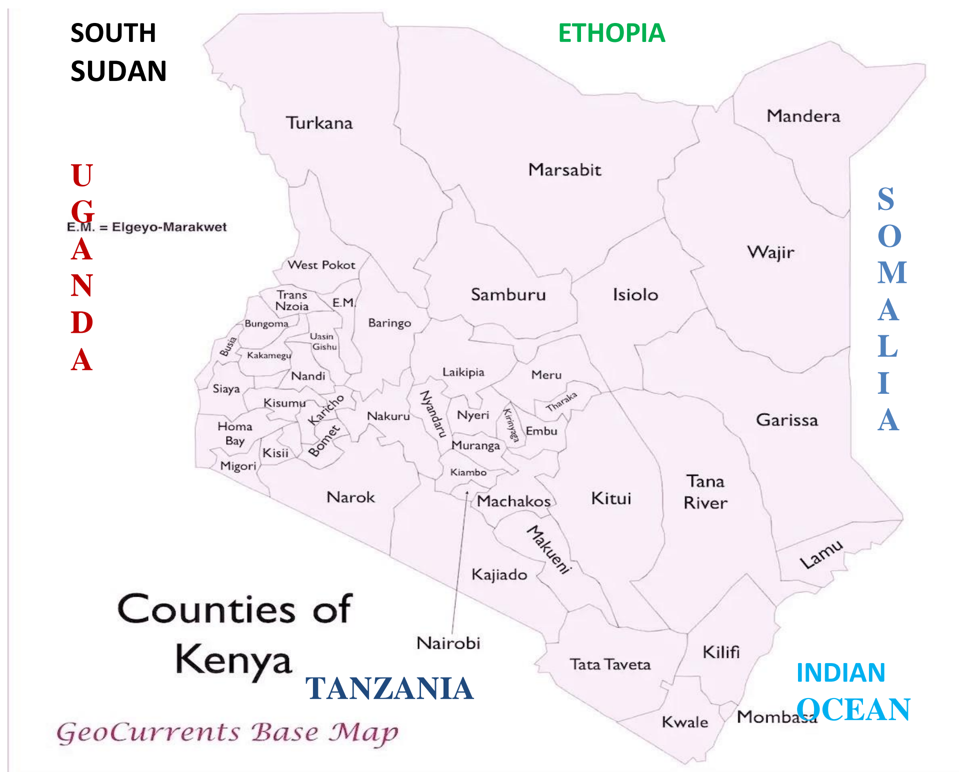


Figure 3.1: Map of Kenya which shows different counties

The samples were collected in five selected counties including Kiambu, Nairobi, Mombasa, Kwale and Kisumu (Kwach, 2018)

3.2 Study design

The study was a laboratory based retrospective, cross-sectional study

3.3 Study population

The study involved 1849 archived (-80 °C freezer) isolates of *E. coli* at the enteric bacteriology laboratories, Center for microbiology research, KEMRI. *E. coli* isolates have been archived since May 2016 to September 2017. Of the 1849 archived isolates of *E. coli*, 1627 archived isolates of *E. coli* were from food animals whereas 222 were collected from environmental sources.

3.4 Inclusion and exclusion criteria

3.4.1 Inclusion criteria

E. coli isolates from archived isolates obtained from healthy food animals and environmental sources and correctly identified as *E. coli* were used.

3.4.2 Exclusion criteria

E. coli isolates from unknown sources and from clinical material as well as cultures identified as contaminated were excluded.

3.5 Sampling

3.5.1 Sample Size determination

Sample size was determined according to Cochran equation (1963) or Fisher *et al* (1998) to calculate sample size for prevalence or proportions as follow:

$$n = \frac{Z^2_{(1-\alpha)}pq}{e^2}$$

Z^2 is the abscissa of the normal curve that cuts off an area α at the tails, (1 – α) equals the desired confidence level, e.g., 95%); e is the desired level of precision; p is the estimated proportion of an attribute that is present in the population from previous or pilot study when not available use 50%, q is 1-p. The value for Z is found in statistical tables which contain the area under the normal curve. e. g. Z = 1.96 for 95 % level of confidence. The prevalence of *E. coli* resistance to antibiotics used in this study comes from the study done in Kenya on epidemiology of antimicrobial resistance among *Escherichia coli* strains in Trans-Nzoia County where 42.2% of *E. coli* isolates were multidrug resistant to sulfamethoxazole, co-trimoxazole, ampicillin, chloramphenicol, tetracycline, kanamycin and streptomycin; all isolates that were resistant to ampicillin harbored bla_{TEM} gene (Kipkorir *et al.*, 2016).

For the prevalence of 42.2%, $n = (1.96)^2 0.422(1- 0.422)/ (0.05)^2 =$ **375**

Of the 375 archived isolates of *E. coli*, 208 (55.5%) were from Kiambu, 85 (22.7%) from Nairobi, 34 (9%) from Kisumu, 31 (8.3%) from Mombasa and 17 (4.5 %) from Kwale.

3.5.2 Sampling procedures

Simple random sampling was used. Whereby the isolates in the population were allocated numbers, they were sampled by a random process using a random number table (random table number, appendix I), to select study units that meet the inclusion criteria.

3.6 Laboratory procedures

The following procedures were carried out for resuscitation, identification of archived *E. coli* isolates, antimicrobial susceptibility testing, determination of *E. coli* pathotypes, detection of ESBL and ESBL genes. The laboratory tests were non-duplicate

3.6.1 Resuscitation of archived *E. coli* isolates

MacConkey agar was prepared according to the instructions of the manufacturer (media preparation shown appendix II). Archived *E. coli* isolates at the Center for Microbiology Research, Kenya Medical Research Institute were cultured onto the MacConkey agar, without salt (Oxoid, United Kingdom) and incubated at 37 °C for 18-24 h by streak plate method for revival (Zinnah *et al*., 2007; Zhang *et al*., 2017).

3.6.2 Confirmation of resuscitated *E. coli* isolates identity

Discreet pink colonies (lactose fermenters) and colourless colonies (non-lactose fermenters or late lactose fermenters) were identified as *E. coli* based on Gram stain and biochemical tests including Triple Sugar Iron, Citrate utilization, Motility Indole and Ornithine and Urease Test (Coico, 2005; Zinnah, *et al*., 2007; Hassan *et al.*, 2016; Panchalingam *et al*, 2012; Zhang *et al.*, 2017). The tests were non duplicate

but were done once (Gram stain procedures and biochemical tests results interpretation are shown in appendices III and IV respectively).

3.6.2.1 Gram Staining

Gram staining tests were carried for all the isolates and the smear was examined on microscope with immersion oil (Coico, 2005; Zinnah, *et al.*, 2007, Zhang *et al.*, 2017). Positive and negative controls were respectively *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 (Bhat *et al.*, 2017).

Biochemical test media were prepared according to the instructions of the manufacturer (Oxoid, United Kingdom), and inoculated as following:

3.6.2.2 Triple sugar iron (TSI)

Three characteristics such as gas production, hydrogen sulfide production and carbohydrates fermentation (dextrose, lactose, sucrose) were studied on this media (Zinnah *et al.*, 2007; Hassan *et al.*, 2016). To inoculate the butt and the slant of TSI agar (Oxoid, United Kingdom), a pure colony was picked on MacConkey with a sterile straight wire loop and the tube was stabbed all the way to the bottom for the butt and streaked in zigzag pattern on the surface of the slant (Zinnah *et al.*, 2007; Hassan *et al.*, 2016). The tube was incubated at 37 °C overnight and color change of the media (acid (yellow color) if carbohydrates were fermented, alkaline (red color) when carbohydrates were not fermented), gas (cracking and splitting of the media) and hydrogen sulfide (black precipitate) production in the butt and the slant were noted (Zinnah *et al.*, 2007; Hassan *et al.*, 2016).

3.6.2.3 Citrate utilization

The same straight wire loop used on TSI agar (without flaming in between) was streaked in zigzag pattern on the surface of the slant of Simmons' Citrate agar (Oxoid, United Kingdom) (Zhang *et al.*, 2017). The tube was incubated aerobically overnight at 37 °C, the change of the media from green to blue noted (Zhang *et al.*, 2017).

3.6.2.3 Motility indole ornithine decarboxylase test

Three characteristics such as motility, indole and ornithine decarboxylase production were studied. The MIO agar (Becton, Dickson; Netherlands) was inoculated with the same straight wire loop used for Simmons' citrate agar without flaming up to the half way; the tube was incubated overnight, aerobically at 37 °C and different characteristics noted (Panchalingam *et al*, 2012); motility (diffusion of the inoculation line) and ornithine decarboxylase (dark purple) were noted; indole production (red ring at the interface) was observed after addition of a drop of Kovac's reagent.

3.6.2.4 Urease Test

Urease agar (Oxoid, United Kingdom) was stabbed four times with the same straight wire loop used for the previous tests, without flaming. The test tube was incubated aerobically overnight at 37 °C, urease production indicated by the color change of the media from orange to pink (Hassan *et al.*, 2016; Zhang *et al.*, 2017). The biochemical characteristics of archived isolates of *E. coli* are shown in Table 3.1. Confirmed *E. coli* isolates were suspended in tryptone soy broth (Oxoid, United Kingdom) added with 15 % of glycerol and were kept at -80 °C for further use (Villegas *et al.*, 2013) (stocking media preparation shown in appendix V).

Table 3.1: Biochemical characteristics of archived isolates of *E. coli*

TEST	INTERPRETATION
TSI	Acid butt (yellow) Acid slant (yellow) Gas: Positive (cracking of the media) H_2S :Negative (no black precipitate)
Citrate utilization	Negative (green)
MIO	Motility: variable Indole: variable (red ring at the interface if positive) Ornithine decarboxylase: variable (dark purple or colorless)
Urease Test	Negative (orange)

H_2S: Hydrogen sulphide

3.6.3 Antimicrobial susceptibility testing

Antibiotic susceptibility testing of the identified *E. coli* isolates for this study was performed using the disk diffusion technique (Bauer, *et al.*, 1966) for commonly used antimicrobial discs including; ampicillin (AMP 10 µg), tetracycline (TE 30 µg), trimethoprim (TMP 5 µg), co-trimoxazole (SXT 1.25/23.75 µg), chloramphenicol (C 30 µg), azithromycin (AZM 15 µg), gentamicin (CN 10 µg), cefuroxime (CXM 30 µg) , cefotaxime (CTX 30 µg), ceftazidime CAZ 30 µg), imipenem (IMP 10 µg), ciprofloxacin (CIP 5 µg) and nalidixic acid (NA 30 µg). The tests were performed on Mueller Hinton agar (Oxoid, United Kingdom). Quality control for the microbial growth and the antimicrobial discs potency was performed using *E. coli* ATCC 25922 (Zhang *et al.*, 2017). To prepare the inoculum, a suspension of pure colonies from an overnight growth of *E. coli* ATCC 25922 at 37 °C grown on Tryptone Soy Agar (Oxoid, United Kingdom) was done in sterile normal saline and the turbidity was compared to barium chloride 0.5 McFarland standard (approximately 10^8 CFU/ml) for equivalence (normal saline preparation shown in appendix VI). A sterile cotton swab was dipped in the inoculum and pressed on the inner wall of the tube to remove excess of the inoculum from the swab; the plate was streaked uniformly over the entire surface of the Mueller Hinton Agar and the plate rotated 3 times; the streaking was completed by passing the swab on the rim of the plate. The antibiotics discs were dispensed using a disc dispenser and a sterile forceps. The plate was incubated upside down, aerobically at 37 °C for 16 to 18 h. After incubation, the inhibition zones diameters were measured using a ruler and the results were interpreted according to the breakpoints provided by the CLSI, 2017 (Table 3.2). CLSI 2017 breakpoints for *E. coli* are shown in appendix VII. The *E. coli* isolates were treated the same way as *E. coli* ATCC 25922, the antibiograms generated were used to cluster the isolates into various susceptibility profiles ranging from sensitive to resistant. The antimicrobial susceptibility tests were not duplicated.

Table 3.2: Antimicrobial susceptibility testing interpretation

Class	Antibiotics	S	I	R
Penicillins	AMP (10 µg)	≥ 17	14-16	≤ 13
	AUG (30 µg)	≥ 18	14-17	≤ 13
Cephalosporins	CXT (30 µg)	≥ 26	23-25	≤ 22
	CAZ (30 µg)	≥ 21	18-20	≤ 17
	CXM (30 µg)	≥ 18	15-17	≤ 14
	CRO (30 µg)	≥ 23	20-22	≤ 19
Carbapenem	IMP (10 µg)	≥ 23	20-22	≤ 19
Aminoglycoside	CN (10 µg)	≥ 15	13-14	≤ 12
Macrolides	AZM (15 µg)	≥ 13	-	≤ 12
Tetracyclines	TET (30 µg)	≥ 15	12-14	≤ 11
Quinolones & Fluoroquinolones	CIP (5 µg)	≥ 21	16-20	≤ 15
	NA (30 µg)	≥ 19	14-18	≤13
Sulphonamides	SXT (25 µg)	≥ 16	11-15	≤ 10
Trimethoprim	TMP (5 µg)	≥ 16	11-15	≤ 10
Phenicols	C (30 µg)	≥ 18	13-17	≤12

S: sensitive I: intermediate R: resistant

3.6.4 Determination of *E. coli* pathotypes by PCR

3.6.4.1 DNA extraction

DNA extraction was done using the boiling method (Dashti *et al.*, 2009). Pure colonies were scraped off an overnight culture on Mueller Hinton agar plate with a sterile swab and were suspended in an Eppendorf tube containing 1ml of sterile distilled water (DNase /RNase free) and boiled at 95 °C for 10 min on a heating block (Dashti *et al.*, 2009). After boiling, the tubes were centrifuged at 14,000 revolutions per minutes (rpm) for 5 min (Dashti *et al.*, 2009). The supernatant containing the released DNA was transferred to new Eppendorf tubes and stored at -20 °C for further PCR use.

3.6.4.2 DNA amplification of virulence genes for intestinal *E. coli* pathotypes

PCR amplification reactions (van der Bij *et al*, 2011) were performed in a total volume of 25 µl containing 12.5 µl of One Taq Master Mix with standard buffer (20 mM Tris-HCL, 22mM KCl, 22 mM NH_4 Cl 0.5 µmol, 1.8 mM $MgCl_2$, 5% glycerol, 0.05% Tween®20, 0.06% IGEPAL® CA-630, 0.2 mM dNTPs, 25 units/ml One Taq® DNA polymerase, pH 8.9), 0.5 µl of each primer, 1.5 µl of DNA template and 10 µl of nuclease free water (Biolabs, UK). The amplification was performed using a GeneAmp® PCR System 9700 Thermocycler (Applied Biosystems) (van der Bij *et al*, 2011). PCR amplification cycling conditions consisted of an initial denaturation of 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 seconds, an annealing step whose temperature is primer specific for min, extension step at 72 °C and whose extension time is fragment-size dependent and final extension at 72 °C for 7 min; the annealing temperature for the used primers and extension time are depicted in Table 3.3 The primers designs were obtained from previously published papers (Pérez *et al.,* 2010; Fujioka *et al*., 2009; Gomez-Duarte *et al*., 2010; Le Bougénec *et al.*, 1992). The amplifications reactions were non-duplicate

Table 3. 3: Primers used for identification of *E. coli* virulence factors

Target genes		Primer sequence (5'-3')	ET	EF size	AT (°C)
***bfp*A**					
	Forward	AATGGTGCTTGCGCTTGCTGC	30s	324 bp	62
	Reverse	GCCGCTTTATCCAACCTGGTA			
stx1					
	Forward	AGTTAATGTGGTGGCGAA	45s	817 bp	58
	Reverse	GACTCTTCCATCTGCCG			
***agg*R**					
	Forward	GTATACACAAAAGAAGGAAGC	30s	254 bp	58
	Reverse	ACAGAATCGTCAGCATCAGC			
LT					
	Forward	GCACACGGAGCTCCTCAGTC	30s	218 bp	60
	Reverse	TCCTTCATCCTTTCAATGGCTTT			
sth					
	Forward	CCCTCAGGATGCTAAACCAG	30s	166 bp	56
	Reverse	TTAATAGCACCCGGTACAAGC			
***st*p**					
	Forward	TCTGTATTATCTTTCCCCTC	30s	186 bp	65
	Reverse	ATAACATCCAGCACAGGC			
inv					
	Forward	ATATCTCTATTTCCAATCGCGT’	35s	382 bp	58
	Reverse	GATGGCGAGAAATTATATCCCG			
afa					
	Forward	GCTGGGCAGCAAACTGATAACTCT	40s	750 bp	66
	Reverse	CATCAAGCTGTTTGTTCGTCCGCCG			

AT=annealing temperature ET: extension time EF size: expected fragment size s: seconds

3.6.4.3 Gel electrophoresis of the amplicons

Electrophoresis was carried out in 1.5 % agarose (1.5 g/100ml) in Tris-borate EDTA (TBE) (Alzahrani *et al.*, 2016). A volume of 10 µl EZ-vision® In-Gel solution 10,000X was added to the agarose gel solution and poured in the rack with combs to form reaction wells; the agarose gel was put in the electrophoresis chamber and flooded by a solution of 1 X TBE. 3 µl of molecular ladder (Hyper Ladder 1kb;

Bioline) was loaded in the first lane of the gel wells to aid in the estimation of the PCR product sizes and 10 µl of the PCR product samples and control was added to the wells for electrophoresis. The amplicons are already stained in green by the master mix dye. Electrophoresis was performed at 80 V for 40 minutes. After electrophoresis, the gel was placed in the gel casting tray and observed under UV light transilluminator (Gelmax® UV imager) and image captured using a UV light software program.

3.6.5 Phenotypic ESBL detection

The ESBL detection was based on Double Disk Synergy test (Drieux *et al.*, 2008). 33 *E. coli* isolates which showed resistance to third generation cephalosporins such as ceftazidime and cefotaxime were tested for the production of ESBL. In this test, disks of third-generation cephalosporins including ceftazidime (CAZ 30 µg), cefotaxime (CTX 30 µg), and ceftriaxone (CRO 30 µg) were placed on either side of augmentin (AUG 30 µg); 30 mm apart, center to center, on inoculated Mueller-Hinton Agar test (Drieux *et al.*, 2008). The test was considered positive for ESBL production when a decreased susceptibility to any of the third generation cephalosporins used was associated to a clear-cut increase of the clear zone of the third generation cephalosporin in front of augmentin showing a characteristic shape-zone referred to as “champagne-cork”, “keyhole” (Drieux *et al.*, 2008). *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive controls respectively (Mathur *et al.*, 2002). The double disk synergy tests were non-duplicate

3.6.6 Detection of ESBL-PE genes

3.6.6.1 DNA Extraction

DNA extraction was done using the boiling method (Dashti *et al.*, 2009). Pure colonies were scraped off an overnight culture on Mueller Hinton agar plate with a sterile swab; they were suspended in an Eppendorf tube containing 1ml of sterile distilled water (DNase /RNase free) and boiled at 95 °C for 10 min on a heating

block. After boiling, the tubes were centrifuged at 14,000 revolutions per minutes (rpm) for 5 min. The supernatant containing the released DNA was transferred to new Eppendorf tubes and stored at -20 °C for further use.

3.6.6.2 DNA amplification of ESBL genes

PCR amplification reactions (van der Bij *et al*, 2011) were performed in a total volume of 25 µl containing 12.5 µl of One Taq Master Mix with standard buffer (20 mM Tris-HCL, 22mM KCl, 22 mM NH_4 Cl 0.5 µmol, 1.8 mM $MgCl_2$, 5% glycerol, 0.05% Tween®20, 0.06% IGEPAL® CA-630, 0.2 mM dNTPs, 25 units/ml One Taq® DNA polymerase, pH 8.9), 0.5 µl of each primer, 1.5 µl of DNA template and 10µl of nuclease free water (Biolabs, UK). The amplification was done using a GeneAmp® PCR System 9700 Thermocycler (Applied Biosystems) (van der Bij *et al*, 2011). The cycling conditions consisted of an initial denaturation of 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 seconds, an annealing step whose temperature is primer specific for 1min, extension step at 72 °C whose time is fragment size dependent and final extension at 72 °C for 7 min. The targeted genes, primer specific annealing temperatures, extension time are depicted in Table 3.4. The primer designs were obtained from previously published papers (Olsen *et al.*, 2004; Monstein *et al.*, 2007). The amplification reactions were non-duplicated.

Table3 4: Primers used to amplify ESBL genes

Target gene	Primer sequence (5'-3')	ET	EF size	AT °C
***bla*$_{TEM}$**				
Forward	ACCAATGCTTAATCAGTGAG	55s	963bp	52
Reverse	GCGGAACCCCTATTTG			
***bla*$_{SHV}$**				
Forward	TTAGCGTTGCCAGTGTTC	50s	851bp	58
Reverse	TTCGCCTGTGTATTATCTCCCTG			
***bla* $_{CTX-M}$**				
Forward	ATGTGCAGYACCAGTAARGTKATGGC	35s	593bp	65
Reverse	TGGGTRAARTARGTSACCAGAAYCAGCG			

AT: Annealing temperature ET: Extension time EF size: Expected fragment size s: seconds

3.6.6.3 Gel electrophoresis

Electrophoresis was carried out in 1.5 % agarose (1.5g/100 ml) in TBE (Alzahrani *et al.*, 2016). A volume of 10 µl EZ-vision® In-Gel solution 10,000X was added to the agarose gel solution and poured in the rack with combs to form reaction wells; the agarose gel was put in the electrophoresis chamber and flooded by a solution of 1 X TBE. 3 µl of molecular ladder (Hyper Ladder 1kb; Bioline) was loaded in the first lane of the gel wells to aid in the estimation of the PCR product sizes and 10 µl of the PCR product samples and control was added to the wells for electrophoresis. The amplicons are already stained in green by the master mix dye. Electrophoresis was performed at 80 V for 40 minutes. After electrophoresis, the gel was placed in a gel casting tray and was observed under UV light transilluminator (Gelmax® UV imager) and image captured using a UV light software program. Control strains for *E. coli* virulence factors and *bla* genes are shown in Table 3.5 (Canizalez-Roman *et al.*, 2016; Chen *et al.*, 2014; Cho *et al.*, 2018; Mathur *et al.*, 2002, Nguyen *et al.*, 2005; Rahmouni *et al.*, 2018)

Table 3.5: Control strains of diarrheagenic *E. coli* and ESBL genes

Strains		target genes
EHEC	ATCC 43890	*stx1*
EPEC	ATCC 43887	*bfpA*
ETEC	ATCC 35401	LT, STp, STh
EAEC	ATCC 29552	*agg*R
EIEC	ATCC 43893	*InvE*
DAEC	DAEC (*afa*A30)	*afa*
KPN	ATCC 700603	bla_{TEM}, bla_{CTXM}, bla_{SHV} (positive control)
E. coli	ATCC 25922	Negative control

EHEC=Enterohemorrhagic *E. coli*, EPEC=Enteropathogenic *E. coli*, ETEC=Enterotoxigenic *E. coli*, EAEC=Enteroaggregative *E. coli*, EIEC=Enteroinvasive *E. coli*, DAEC= Diffusely adherent *E. coli*, KPN ATCC =*Klebsiella pneumoniae* American type culture collection.

3.7 Data Management and analysis

The laboratory tests were not duplicated but were done once. Data were entered in Microsoft Excel database coded and analyzed with SPSS, version 21.0. The antimicrobial susceptibility data, the *E. coli* pathotypes findings and ESBL production data were expressed in percentage. A chi square test (Fisher's exact) was used to assess the difference between *E. coli* isolates from food animals and environmental sources as well as between ESBL producers and non ESBL producers with regard to antimicrobial resistance. The difference in ESBL production in *E. coli* isolates from food animals and environmental sources was also assessed using a Chi-square test (Fisher's exact) ($P \leq 0.05$ was regarded as statistically significant). The data were presented in graphs and charts. Data on the proportion of ESBL producing pathogens will be essential for performing risk assessment and risk management by developing measures to control the transmission routes of ESBL producing food borne pathogens and therefore contributing to reduce the morbidity and mortality due to ESBL producing foodborne pathogens and thus to overcome the massive threat posed by the pathogens.

3.8 Ethical considerations

Institutional approval was sought from the School of Biomedical Sciences (SOBMS) at Jomo Kenyatta University of Agriculture and Technology (JKUAT). Ethical clearance to carry out the study was sought from KEMRI Scientific and Ethics Review Unit (Appendix VIII). Scientific and Ethical approval for the use of archived bacterial isolates in Kenya was granted by KEMRI Scientific and Ethics Review Unit (Appendix IX) for an approved on-going study, 'Ecology and epidemiology of antimicrobial-resistant foodborne pathogens (E. *coli, Salmonella spp* and *Campylobacter spp*) in selected sites in five Counties in Kenya: Investigating farmer knowledge and attitudes towards antimicrobial use and resistance and public health significance (SERU/CMR/P0036/3205).

CHAPTER FOUR

RESULTS

4.1 Confirmation of archived *E. coli* isolates identity

All 375 archived isolates of *E. coli* were Gram negative bacilli. On MacConkey, *E. coli* isolates were lactose fermenters (Fig. 4.1). Colonies were circular (shape), medium sized (1-3mm approximately), entire (margin), smooth (surface), slightly elevated (elevation) and opaque (Fig. 4.1). Archived isolates of *E. coli* fermented carbohydrates (acid butt and acid slant), produced gas and were H_2S negative on TSI (Fig. 4.2). All archived isolates of *E. coli* were citrate negative (no colour change of the media); 94.5% (353) isolates were motile (diffused zone of growth out from the inoculation line), 90.03% (341) were indole positive (red colour ring at the interface), 48.53 % (182) isolates produced ornithine decarboxylase (dark purple colour of the media) (Fig. 4.2). *E. coli* isolates were urease negative as shown in Fig. 4.2.

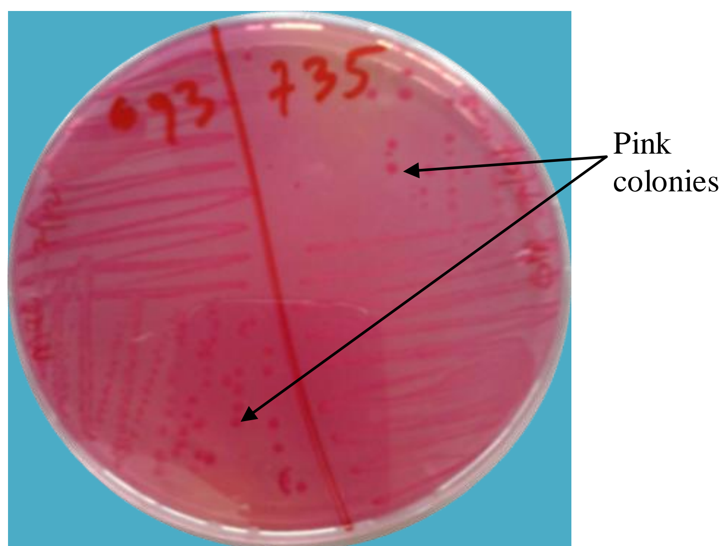


Figure 4.1 Pink colonies of *E. coli* isolates on MacConkey

The figure shows colonies of *E. coli* isolates on MacConkey after incubation at 37 °C for 18-24h

Figure 4.2: ***E. coli*** **on TSI, Simmons citrate, MIO and urease test media**

On TSI, after an overnight incubation at 37 °C, carbohydrates were fermented with gas production and absence of H_2S. Simmons citrate test was negative; the *E. coli* isolate was motile, indole positive produced ornithine decarboxylase. Urease test was negative.

4.2 Antimicrobial resistance patterns of archived *E. coli* isolates

4.2.1 Occurrence of antimicrobial resistance among archived *E. coli* isolates

Antimicrobial susceptibility profiles to 13 commonly used antibiotics were performed for all the 375 selected archived isolates of *E. coli*. In total, 21.6% (81) of the isolates were sensitive to all antibiotics tested, 78.4% (274) isolates were resistant to at least one of the tested drugs among which 28.8% (108) were multidrug resistant (resistant to ≥ 3 classes of antibiotics) (Fig. 4.3).

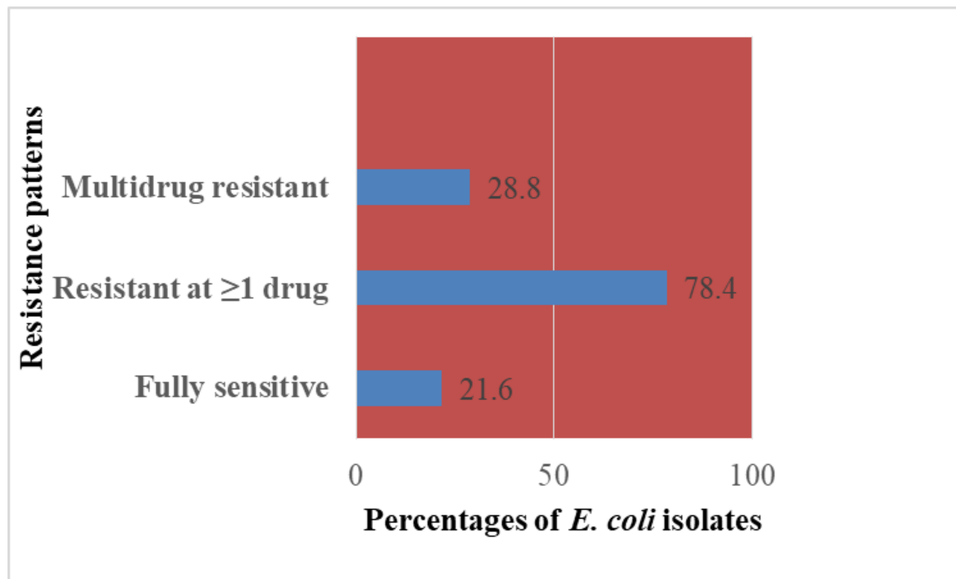


Figure 4.3: Occurrence of antimicrobial resistance among *E. coli* isolates

4.2.2 Antimicrobial resistance profiles of archived isolates of *E. coli*

Resistance to tetracycline was 55.2% (207), co-trimoxazole 44% (165), trimethoprim 43.7% (164), ampicillin 28.8% (108), nalidixic acid 18.7% (70), azithromycin 14.9% (56), ciprofloxacin 10.1% (38), chloramphenicol 8.8% (33), cefotaxime 5.9% (22), ceftazidime 3.7% (14), cefuroxime 2.4% (9), gentamicin 1.1% (4) and imipenem 0.8% (3). The highest resistance rate was recorded for tetracycline whereas the lowest resistance rate was observed to imipenem (0.8 %). The archived isolates of *E. coli* were more susceptible to cefuroxime, imipenem and gentamicin (Table 4.1).

Table 4.1: Antimicrobial susceptibility profiles for *E. coli* isolates

Sensitive isolates		Intermediate isolates		Resistant isolates		
Ni	**%**	**Ni**	**%**	**Ni**	**%**	**Antibiotic**
226	60.3	41	10.9	108	28.8	Ampicillin (10µg)
365	97.3	1	0.3	9	2.4	Cefuroxime (30µg)
338	90.1	23	6.1	14	3.7	Ceftazidime (30µg)
318	84.8	35	9.3	22	5.9	Cefotaxime (30µg)
365	97.3	7	1.9	3	0.8	Imipenem (10µg)
346	92.3	25	6.7	4	1.1	Gentamicin (10µg)
319	85.1	-	-	56	14.9	Azithromycin (15µg)
161	42.9	7	1.9	207	55.2	Tetracyclines (30µg)
271	72.3	34	9.1	70	18.7	Nalidixic Acid (30µg)
305	81.3	32	8.5	38	10.1	Ciprofloxacin (5µg)
199	53.1	11	2.9	165	44	Co-trimoxazole (25µg)
208	55.5	3	0.8	164	43.7	Trimethoprim (5µg)
335	89.3	7	1.9	33	8.8	Chloramphenicol (30µg)

Ni: number of isolates %: percentage

4.2.3 Antimicrobial resistance between *E. coli* isolates from different sources

The difference with regard to antimicrobial resistance between *E. coli* isolates from food animals and isolates from environmental sources was determined by Chi-square test (two tailed Fisher's exact ,where applicable), $p \leq 0.05$. The difference was statistically significant for ampicillin, cefuroxime, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, co-trimoxazole and trimethoprim (Table 4.2). Of the 108 multidrug resistant *E. coli* isolates, 90.7% (98) were from food animals and 9.3% (10) were from environmental sources. The multidrug resistance was significantly high in *E. coli* isolates recovered from healthy food animals ($p=0.05$).

Table 4.2: Resistance profiles in *E. coli* isolates from different sources

	HFA (n=330)		**E S (n=45)**		
	Resistance		**Resistance**		
Antibiotics	Ni	%	Ni	%	p value
Ampicillin (10 µg)	96	88.9	12	11.1	**0.03**
Cefuroxime (30 µg)	8	88.9	1	11.1	**0.02**
Cefotaxime (30 µg)	20	90.9	2	9.1	**0.02**
Ceftazidime (30 µg)	12	85.7	2	14.3	**0.01**
Imipenem (30 µg)	2	66.7	1	33.3	**0.06**
Gentamicin (10 µg)	4	100	0	0	**0.05**
Azithromycin (15 µg)	52	92.9	4	7.1	**0.2**
Tetracyclines (30µg)	184	88.9	23	11.1	**0.07**
Nalidixic acid (10 µg)	64	91.4	6	8.6	**0.06**
Ciprofloxacin (5 µg)	33	88.6	5	13.2	**0.01**
Co-trimoxazole (25 µg)	154	87.9	20	12.1	**0.03**
Trimethoprim (5 µg)	142	86.6	22	13.4	**0.04**
Chloramphenicol (30 µg)	29	87.9	4	12.1	**0.07**

HFA: healthy food animals ES: environmental sources Ni: number of isolates %: Percentage

4.3 Carriage of virulence genes for *E. coli* pathotypes

4.3.1 Proportion of *E. coli* pathotypes

Overall, 120 multidrug resistant *E. coli* isolates and/or screened phenotypic ESBL positive were tested for the carriage of virulence genes. 106 out of 120 (88.3%) archived isolates of *E. coli* were found to harbour *aggR*, a virulence gene for Enteroaggregative *E. coli*. Other virulence genes such as stx1 for Enterohemorrhagic *E. coli*, *bfp*A for Enteropathogenic *E. coli*, LT, STh and STp for Enterotoxigenic *E. coli*, *invE* gene specific for Enteroinvasive *E. coli* and *afa* gene for Diffusely adherent *E. coli* were not found (Fig. 4.4). Of the 106 *E. coli* isolates positive for EAEC pathotypes, 96 (92.5%) originated from food animals whereas 10 (7.5%) of EAEC were from environmental sources. The gel electrophoresis image for *agg*R gene characteristic of EAEC products is shown in Fig. 4.5.

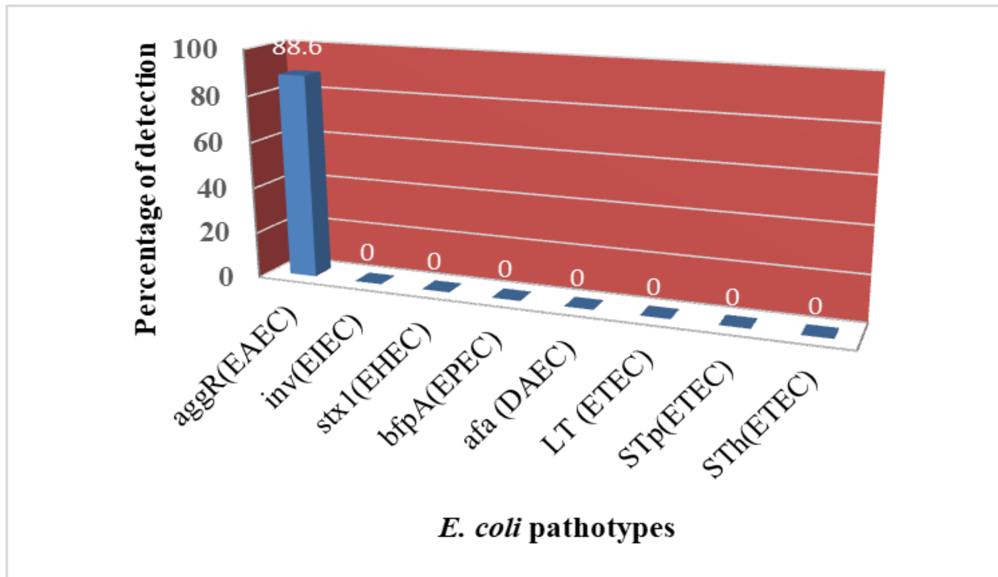


Figure 4.4: Shows the level of detection of different virulence genes

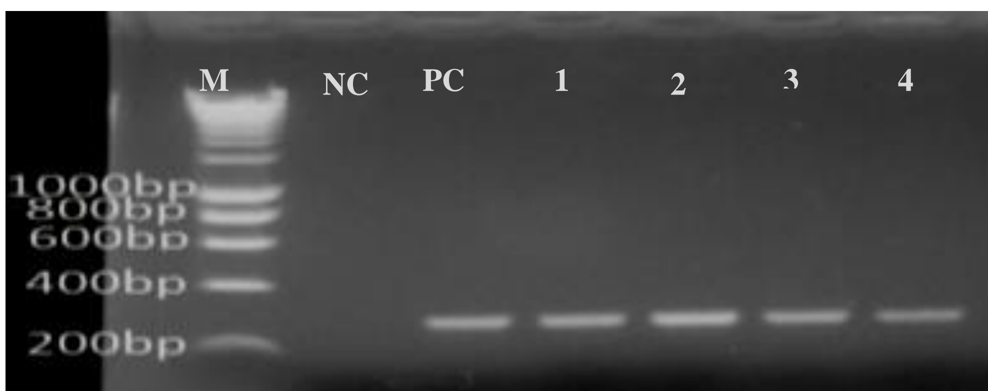


Figure 4.5 Gel image of *aggR* for Enteroaggregative *E. coli* (254 bp)

Isolate numbers: 1-4. NC *E. coli* ATCC 25922 and *E. coli* ATCC 29552 are negative and positive controls respectively while M is 1kb molecular ladder.

4.3.2 Distribution of EAEC (*aggR*) in different counties

Enteroaggregative *E. coli* virulence determinant (*aggR*) was detected in 88.3% (106/120) of the isolates tested. A high proportion of EAEC (*aggR*) was found in Kiambu 60.4% (64/106) whereas a low rate of EAEC (*agg*R) was detected in Kwale 6.6% (7/106) (Fig. 4.6).

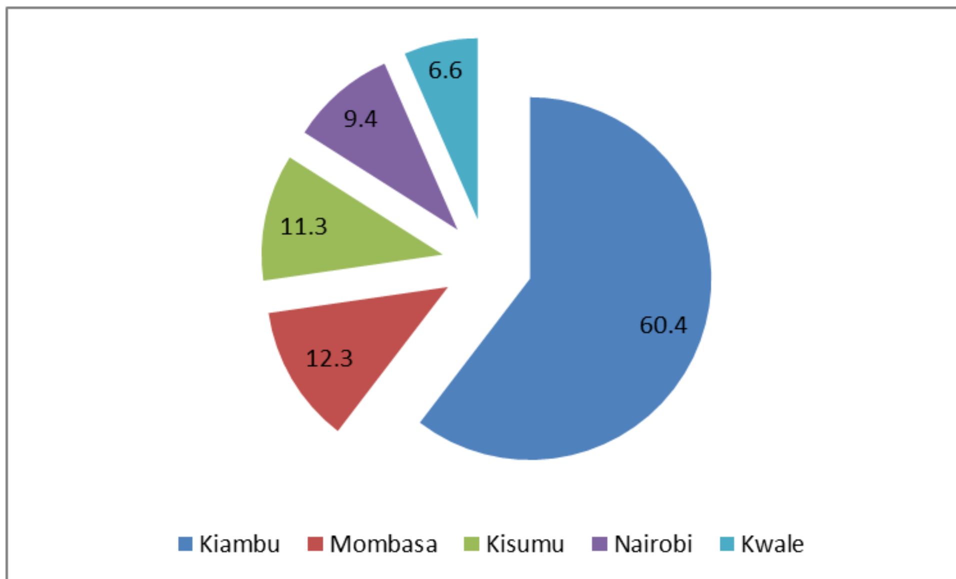


Figure 4.6: Distribution of EAEC (*aggR*) across different counties

A high level of *aggR* was found in *E. coli* isolates from Kiambu while a lower rate was found in *E. coli* isolates from Kwale (Fig. 4.6). 12.3% (13/106) of EAEC were found in Mombasa, 11.3% (12/106) in Kisumu and 9.4% (10/106) in Nairobi.

4.4 Proportion ESBL producers among archived isolates of *E. coli*

4.4.1 Proportion of ESBL producing *E. coli* phenotypes

All archived isolates of *E. coli* screened positive for ESBL production were confirmed to be ESBL producers by the double disk synergy test. Out of 375 archived isolates of *E. coli,* 33 were ESBL-producers translating to 8.80 % while non ESBL producers were 91.20% (342). Fig. 4.7 demonstrates a double disk synergy test positive for ESBL production among archived *E. coli* isolates; this is exhibited by a synergy zone between augmentin and cefotaxime or a phantom zone between augmentin and ceftriaxone.

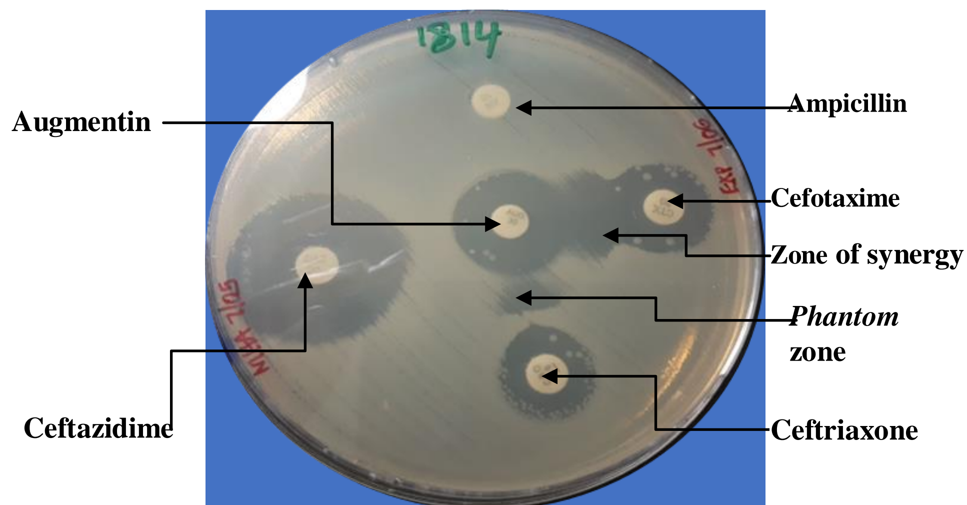


Figure 4.7: Double disk synergy test positive for ESBL producer

The figure shows a double disk synergy test positive for ESBL production in archived isolates *E. coli*

The difference with respect to antimicrobial resistance between ESBL and non ESBL producing *E coli* isolates was analyzed using chi-square test (two tailed Fisher's exact test). A $p \leq 0.05$ was considered as statistically significant. The difference in resistance levels between the two groups of archived isolates of *E. coli* was significant for cefuroxime, ceftazidime, cefotaxime, gentamicin, nalidixic acid and ciprofloxacin (Table 4.3). The difference in antimicrobial resistance was not significant high for ampicillin, imipenem, tetracycline, azithromycin, co-trimoxazole, trimethoprim and chloramphenicol (Table 4.3).

The multidrug resistance was statistically significant in ESBL producers ($p=0.001$) since 63.6% (21/33) of ESBL-producing *E coli* isolates were multidrug resistant against 25.4% (87/342) of non ESBL producers. The difference in ESBL production among *E. coli* isolates from food animals and environmental sources was statistically significant ($p=0.02$). Of the 33 ESBL-producing *E. coli* isolates, 90.9 % (30) isolates were from food animals whereas 9.1% (3) isolates were from environmental sources.

Table 4.3: Resistance profiles in ESBL producers vs non-ESBL producers

	ESBL producers n=33 (8.8%)		Non ESBL producers n=342 (91.2 %)		
	Resistance		Resistance		
Antibiotics	**Ni**	**%**	**Ni**	**%**	**p value**
Ampicillin (10µg)	15	45.5	93	27.2	**0.06**
Cefotaxime (30µg)	22	66.7	0	0	**0.001**
Ceftazidime (30µ)	13	39.4	1	0.3	**0.001**
Imipenem (10µg)	0	0	3	0.9	**0.1**
Gentamicin (10µg)	1	3	3	0.9	**0.001**
Azithromycin (15µg)	8	24.2	48	14	**0.1**
Tetracyclines (30µg)	20	60.6	187	54.7	**0.6**
Ciprofloxacin (5µg)	7	21.2	31	9.1	**0.006**
Co-trimoxazole (25µg)	10	30.3	155	45.3	**0.1**
Trimethoprim (5µg)	10	30.3	154	45	**0.2**
Chloramphenicol (30µg)	5	15.2	28	8.2	**0.3**
Nalidixic Acid (30µg)	15	45.5	55	16.1	**0.001**
Cefuroxime (30µg)	6	18.2	3	0.9	**0.001**

Ni: number of isolates %: Percentage

4.4.2 Proportion of ESBL genes among ESBL producing *E. coli*

All 33 positive ESBL producing *E. coli* phenotypes isolates were found to carry *bla* genes (Fig 4.8); bla_{TEM} was detected in all the phenotypic ESBL producers (100%) whereas $bla_{CTX\text{-}M}$ was found in 6 isolates (18.1%) as shown in figure Fig. 4.8. None of the ESBL producers had bla_{SHV}. Gel electrophoresis images for bla_{TEM} and $bla_{CTX\text{-}M}$ genes are shown in Fig. 4.9 and Fig. 4.10 respectively.

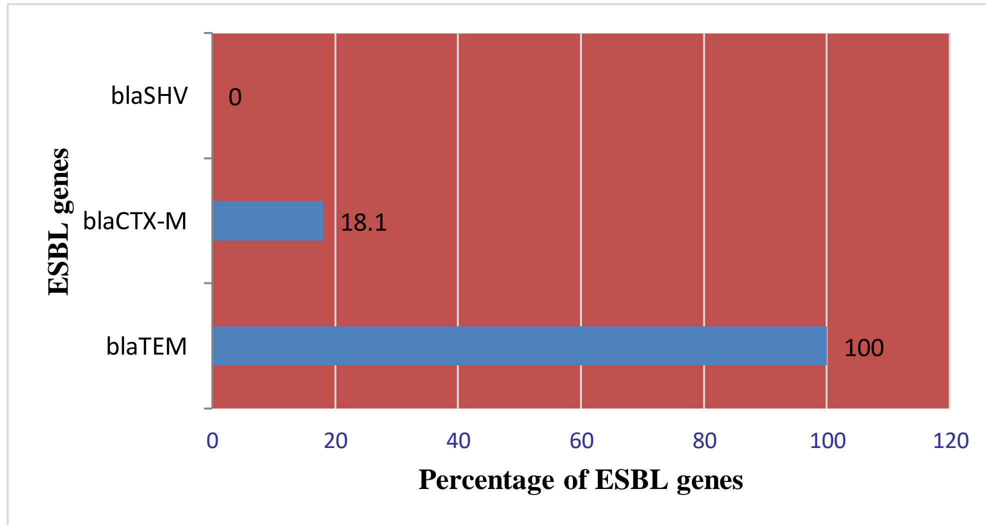


Figure 4.8: carriage of *bla* genes such as *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{SHV}$

The figure shows different level of carriage of *bla* genes such as *bla*$_{TEM}$, *bla*$_{CTX-M}$ with absence of *bla*$_{SHV}$ among archived *E. coli* isolates

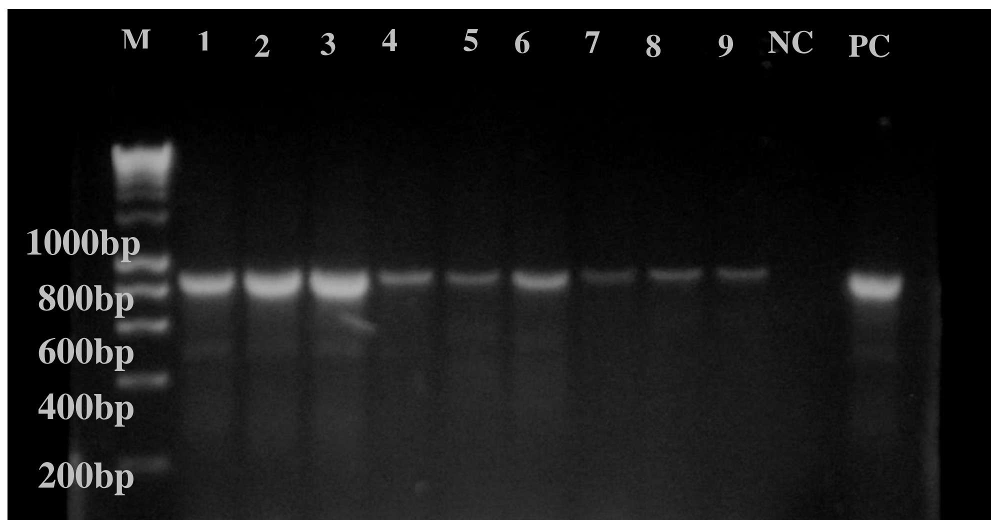


Figure 4.9: Shows gel image of *bla*$_{TEM}$ with a size of 963bp

Isolate numbers: 1-9, NC (ATCC 25922) and PC (ATCC 700603) are negative and positive controls respectively while M is 1kb molecular ladder

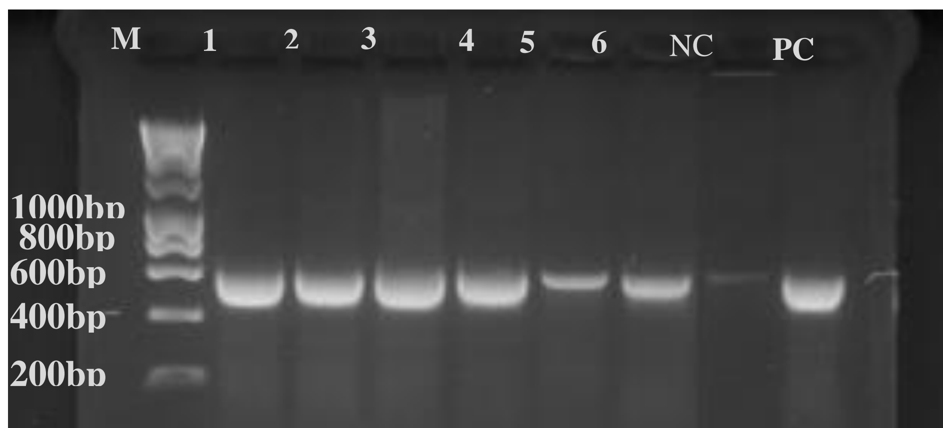


Figure 4.10: Shows gel image of *bla*$_{CTX-M}$ gene with a size of 593bp

Isolates numbers: 1-6, NC (ATCC 25922) and PC (ATCC 700603) are negative and positive controls respectively while M is 1kb molecular ladder

4.4.3 Distribution of *bla*$_{TEM}$ across counties

Of the 33 *E. coli* isolates harboring *bla*$_{TEM}$, 23 (69.7%) were recovered in Kiambu, 4 (12.1%) in Mombasa county. Three isolates (9.1%) containing *bla*$_{TEM}$ were found in *E. coli* isolates from Nairobi while 2 (6.1%) and 1(3%) *bla*$_{TEM}$ containing isolates were detected in *E. coli* isolates respectively in Kisumu and Kwale (Fig. 4.11).

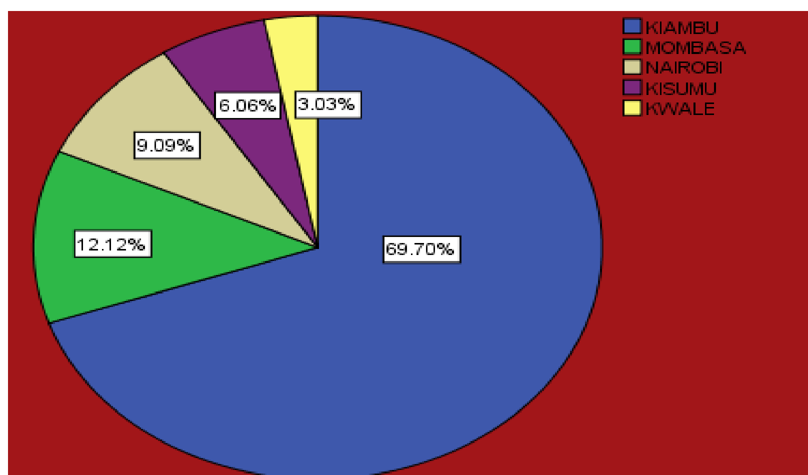


Figure 4.11: Distribution of *bla*$_{TEM}$ in five selected counties.

The figure shows the distribution of *bla*$_{TEM}$ in selected counties including Kiambu, Mombasa, Nairobi, Kisumu and Kwale. A high proportion of *bla*$_{TEM,}$ 69.7% (23/33) was found in *E. coli* isolates from Kiambu.

4.4.4 Distribution of *bla*$_{CTX-M}$ in five selected counties

Overall *bla* $_{CTX-M}$ gene was detected in 18.1% (6/33) of ESBL producers. Of these *bla*$_{CTX-M}$ genes, 88.3% (5/6) were found in *E. coli* isolates from Kiambu County while 16.7 % (1/6) were detected in isolates from Mombasa County. The *bla*$_{CTX-M}$ gene was not detected in *E. coli* isolates from other counties as shown in Fig. 4.12.

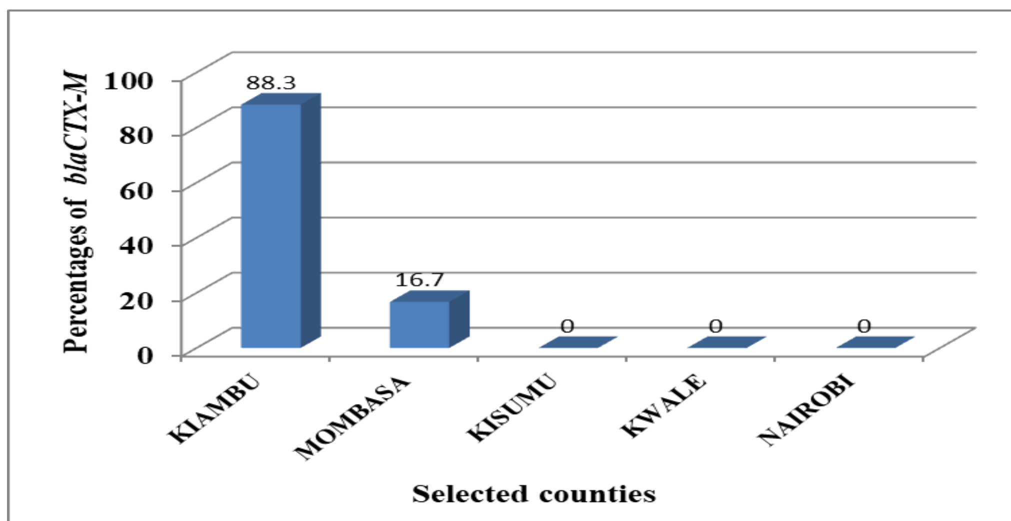


Figure 4.12 Distribution of *bla*$_{CTX-M}$ in five selected counties

A high rate of *bla*$_{CTX-M}$ gene was found in *E. coli* isolates from Kiambu County. This gene was not detected in *E. coli* isolates from Nairobi, Kisumu and Kwale.

CHAPTER FIVE

DISCUSSION

5.1 Prevalence of antimicrobial resistance among archived isolates of *E. coli*

The level of antimicrobial resistance in *E. coli* is a useful indicator of resistance dissemination in bacterial populations, and of selective pressure imposed by antimicrobials used in food animals (Zhao *et al.*, 2012). Antimicrobial usage in food animals is common and runoff from animal waste may contain non metabolized antibiotics, antimicrobial resistant bacteria and antibiotic resistance genes (Ng & Gin, 2019).

In this study, the overall proportion of antibiotic resistance among archived isolates of *E. coli* was 78.4% of which 28.8% showed multidrug resistance. The multidrug resistance observed in the current findings is significantly higher in *E. coli* isolates from food animals (90.7%) than in environmental sources (9.3%). This could be due to the direct exposure undergone by *E. coli* present in intestinal tract of food animals to drugs used for growth promotion, disease prevention and therapy. Antimicrobial resistance in enterics such as *E. coli* from animals and humans is increasing as it is reported from a review done from 1974 to 2013 of enteric antimicrobial resistance in East African countries including Kenya, Uganda, Tanzania, Rwanda, Burundi and Ethiopia (Omulo *et al.*, 2015). The multidrug resistance observed in the current study is nearly the same as the findings from a study conducted in Kenya where *E. coli* isolates from healthy food animals (swine, poultry and cattle) exhibited a multidrug resistance rate of 26% (Mapenay *et al.,* 2006). The prevalence of antibiotic resistance as well as the multidrug resistance observed in the present study is high and this constitutes a public health concern since farmers can readily acquire resistant organisms due to the permanent contact with animal or their droppings. Manure and effluent containing resistant organisms may be discharged to the environment jeopardizing people's lives. A report from Reunion showed a high prevalence of antimicrobial resistance among *E. coli* isolates recovered from poultry and swine,

70±16.7% and 53.3±18.2% respectively (Gay *et al.,* 2018). In a study conducted the Netherlands on poultry and farmers, *E. coli* isolates showed a resistance level of 32%, 23%, 22% in turkeys, broilers and farmers respectively (Van den Bogaard *et al.*, 2001). Since antimicrobials used in animal production and in human are similar and some are even the same (Nhung *et al.,* 2016; Teuber, 2001), dissemination of antimicrobial resistance determinant to humans could lead to limited therapeutic options and associated consequences including treatment failure, economic loss and high rate of morbidity and mortality.

From the current findings, it was demonstrated that *E. coli* isolates from healthy food animals and associated environmental sources are highly resistant to commonly used antibiotics with a high level of multidrug resistance. This is a risk factor for acquisition of antimicrobial resistant organisms particularly for farmers who are in permanent contact with food animals and for the population in general since resistant organism can spread into the environment as demonstrated in the current study.

5.2 Antimicrobial resistance profiles among archived isolates of *E. coli*

In the current study, the highest resistance rates were recorded for tetracycline (55.2%) and co-trimoxazole (44%) whereas the lowest resistance rates were observed for gentamicin (1.1%) and imipenem (0.8%). From these findings, the antimicrobial resistance levels were significantly higher in *E. coli* isolates from food animals than in environmental sources for ampicillin, cefuroxime, cefotaxime, ceftazidime, gentamicin, ciprofloxacin, co-trimoxazole and trimethoprim. These findings concur with the report from Kenya in *E. coli* isolates recovered from food animals including pigs, cattle where resistance rates were 20-50% to tetracycline, 9-30% to co-trimoxazole, 20-40 % to ampicillin, 0.5-20% to chloramphenicol, 0.5-14% to nalidixic acid, 0-5% to ciprofloxacin and 0-2% to gentamicin (Kariuki, 2016). However, higher resistance levels to commonly used antibiotics particularly tetracycline (75.9%), co-trimoxazole (72.4%) and ampicillin (39%) were also reported in *E. coli* from broiler chickens in Kenya (Adelaide *et al.*, 2008).

Different studies conducted in others countries including Tanzania, Nigeria, Belgium, *E. coli* from food animals including broilers, cattle, pigs, turkeys, goats and associated environments exhibited high resistance to commonly used antimicrobials such as ampicillin (30-95%), chloramphenicol (5-95%), tetracyclines (50-60%), co-trimoxazole (20-80%), nalidixic acid (10-80%), ciprofloxacin (20-80%), trimethoprim (20-60%), gentamicin (8-20%), cefotaxime (0-30%) and ceftazidime (0-10%) as demonstrated in the current study (Adelowo *et al*., 2014; Adenipekun *et al.*, 2015; Chantziaras *et al*., 2014; Hamisi *et al.*, 2014; Nsofor, C. & Iroegbu, 2012; Nsofor *et al.*, 2013; Ogunleye *et al*., 2013).

However low resistance rates to tetracycline (10.61%), ampicillin (6.02%) and cotrimoxazole (4.49%) were reported in *E. coli* from dairy cattle in Zambia (Mainda *et al.*, 2015). The antimicrobial resistance observed in the present study could be due to the use of antibiotics in food animals for various purposes including growth promotion, disease prevention and therapy. Antibiotics are used in animal husbandry for therapeutic and prophylactic purposes in infectious diseases but also as growth promoting agents at low and subtherapeutic doses (Alonso *et al*., 2017; Szmolka & Nagy, 2013), thereby contributing to the emergence of bacterial strains which could be highly resistant to those drugs. There is a widespread use of tetracycline and sulfonamides in livestock in Kenya (GARP, 2011; Mitema *et al*, 2001; NRL, 2017); this could explain the particularly high prevalence of resistance to tetracycline and co-trimoxazole demonstrated in the current study. Low resistance (less than 20%) to drugs including gentamicin, chloremphenicol and cephalosporines such as cefuroxime was observed in a similar study conducted in Nigeria and Rwanda (Adenipekun *et al.*, 2015, Manishimwe *et al.*, 2017).

In early 1952, antimicrobials were introduced to commercial feed for cattle, pigs and poultry (Mazurek *et al.*, 2015); this might have contributed to the overtime development of antimicrobial resistance observed in food producing animals due to the possible selective pressure exerted by those drugs on the normal flora. Data from surveys conducted in Nigeria, Zambia and South Africa indicated that tetracycline and beta-lactams were among the first four antibiotics used in food animals

(mammals and poultry), sulfonamides and macrolides, quinolones and cephalosporins are also used in livestock production (Alonso et *al* 2017). Those data corroborate the reason why *E. coli* isolates from this study are highly resistant to those antibiotics. The use of antimicrobial agents in the veterinary field influence the emergence, prevalence, and spread of antimicrobial resistance in bacteria isolated from food-producing animals and the resulting resistant bacteria reduce the efficacy of the antimicrobial agents in humans (Harada & Asai, 2010).

Farm animals may act as vectors to the dissemination of drug resistant genes due to intensive use of antibiotics in animal husbandry resulting in a wide range resistance to antibiotics including those used in human medicine (Caruso, 2018). When multidrug resistance is conveyed to pathogenic bacteria, it becomes a public health challenge since resistance will leads to treatment failure and implies resort to second line antibiotics for therapy which is very costly and contributing to the development of antimicrobial resistant strains (Chantziaras *et al*., 2014).

Therefore, multidrug resistant *E. coli* from food animals and their immediate environments represent a great risk particularly to farmers who are permanently exposed through direct contact with the animals and their waste and to the public in general given that effluents and animals feces containing resistant organisms can soil vegetables through watering with contaminated water, can soil meat during the slaughtering process exposing thus people through food chain.

5.3 Proportion of *E. coli* pathotypes

In the current study, 88 .3% Enteroaggregative *E. coli* were recovered from archived isolates of *E. coli* from healthy food animals and environmental sources. Among these EAEC, 92.5% were detected in healthy food animals whereas 7.5% were found in environmental sources. A high occurrence of EAEC was recorded in Kiambu (60.4%). The proportion of EAEC was 12.3%, 11.3%, 9.4% and 6.6 % in Mombasa, Kisumu, Nairobi and Kwale respectively. This discrepancy in the occurrence of EAEC across Counties could be due to the difference in the distribution of the

samples since 55.5% of archived isolates of *E. coli* were from Kiambu. All EAEC were multidrug resistant and/or were carrying extended spectrum beta lactamase genes. In the current study pathotypes such as EIEC, EHEC, ETEC, EPEC and DAEC were not found. A worrying level of multidrug resistance in EAEC strains was reported in several studies, ESBL productions as well as enhanced resistance to quinolones were reported (Amin *et al.*, 2018; Franz *et al.*, 2015; Jensen *et al.,* 2012).

Though few studies were done in Kenya on healthy food animals and environmental sources, the findings from this study concur with those from a recent study conducted in Kenya which demonstrated 80% of EAEC in fecal samples from healthy cows; EPEC, EHEC, EIEC were not detected in animal samples (Ochi *et al*, 2017). Different findings were reported in Kenya with a lower level (7%) of EAEC (*asp*U and *agg*R genes) in fecal sample from goats, pathotypes such as ETEC, EPEC, and STEC were found (Njoroge *et al.*, 2013)
. The *E coli* pathotypes showed relatively low resistance rates to tetracyclines (15%), chloramphenicol (4%) and co-trimoxazole (2%) (Njoroge *et al.*, 2013)

Different studies were conducted in other parts of the world and revealed different results. For instance, in Burkina Faso *E. coli* pathotypes were isolated from fecal samples from slaughtered food animals at varying rates, EAEC (32% in pigs, 6% in cattle and 7% in chickens), EIEC (1% in chickens), STEC (37% in cattle, 6% in Chicken, 30% in pigs), ETEC (4% in cattle, 5% in chicken and 18% in pigs) and EPEC (8% in cattle, 37% in chicken and 32% in pigs) were recovered (Kagambèga *et al*, 2012) while in China 9% EAEC were recovered from fecal samples from non-clinical settings including healthy animals (pigs and companion animals) and healthy humans (Zhang *et al*, 20 16). In a study conducted in Vietnam, 88.2% of EAEC exhibited a high multidrug resistance, 64.7 % were resistant to 3rd generation cephalosporins and 50% were ESBL positive (Trung *et al*, 2016). EAEC is more likely to be resistant to antibiotics than are other diarrheagenic pathotypes (Okhuysen & DuPont, 2010).
In several large-scale studies carried out in the United States, EAEC was reported to be the most common bacterial pathogen identified in diarrheal stool samples (Croxen

et al., 2013; shah *et al.*, 2018). This frequency of EAEC in clinical samples could explain the abundance of EAEC observed in the current findings suggesting that food animals might have been exposed to contaminated feeds and water. Some strains of EAEC possess additional virulence factors which have been linked with the ability to cause diarrhea and other symptoms which can be life-threatening for susceptible people (EFSA, 2015).

The current findings demonstrate that food animals and associated environments are potential reservoirs for EAEC. Given the frequent pathogenicity associated with EAEC in humans persistent as stated by Jafari *et al.*, 2012 (diarrhea, traveler's diarrhea, diarrhea in HIV/AIDS patients, acute diarrhea in both infants and adults, endemic and epidemic diarrhea worldwide), proper disposal of food animals wastes must be practiced to protect the food animals, farmers, the population and to obviate the spread of EAEC in the environment.

5.4 Proportion of phenotypic ESBL producers.

In the current study, the proportion of ESBL producers was 8.8 % (33 out of 375), 63.6% of ESBL producing *E. coli* were multidrug resistant. The level of production of ESBL was significantly higher in *E. coli* isolates from food animals than in environmental sources. This is alarming given that multidrug resistant bacteria are currently acknowledged as one of the most important public health problems and antimicrobial resistance as one of the greatest threats to human health worldwide (van Duin & Paterson, 2016). The multidrug resistance in ESBL producing *E coli* observed in the current study was statistically significant which concur with the statement that ESBL are world widely responsible of transferrable multidrug resistance in GNB (Bali *et al.*, 2010). The highest resistance rates among ESBL producers were observed for cefotaxime (66.7%), tetracyclines (60.6%), ampicillin and nalidixic acid (45.5%); the lowest resistance rates were exhibited to gentamicin (3%) whereas none of the ESBL producers from this study was resistant to imipenem.

In the current study, the difference in antibiotic resistance between ESBL producers and non ESBL producing organisms was statistically significant for ceftazidime, cefotaxime, cefuroxime , gentamicin, nalidixic acid and ciprofloxacin; this is due to the fact that ESBL producers commonly harbour resistance determinants to other classes of antibiotics including aminoglycosides, fluoroquinolones, piperacillin-tazobactam and cefepime (Turner, 2005). Studies conducted in other parts of the world showed different ESBL production rates in *E. coli* isolates from food animals samples such as 2 %, 7.2%, 20.1% and 88.8% in Zambia, India, China respectively (Bandyopadhyay *et al.*, 2014; Chishimba *et al.*, 2016; Li *et al.*,2016; Xu *et al.*, 2018). In accordance with the current study, ESBL producing *E. coli* exhibited high resistance rate to non-beta-lactam drug such tetracycline, nalidixic acid, trimethoprim, co-trimoxazole and ciprofloxacin and the multidrug resistance rate was significantly higher comparing to non ESBL producers (Wu *et al.*, 2018; Xu *et al.,* 2018).

The high susceptibility to imipenem in ESBL-producing *E coli* from this study may be explained not only by the fact that ESBLs do not hydrolyse carbapenems (Walkty *et al.*, 2016), but also by the lack of direct selection pressure since carbapenem are not approved to be used in food producing animals (Michael *et al.*, 2015). The resistance level observed in ESBL producing *E coli* isolates was statistically significant for beta lactam drugs such as ceftazidime, cefotaxime, cefuroxime but also for non-beta lactam antibiotics including ciprofloxacin, nalidixic acid and gentamicin suggesting that ESBL production as a cause of resistance transferred to beta lactams antibiotics was associated with other resistance mechanisms. The most negative aspect of *E. coli* as an ESBL-producing organism like other ESBL producing GNB such as *Proteus*, *Klebsiella*, *Enterobacter*, *and Pseudomonas* is the fact that they frequently carry genes encoding for resistance to other classes of antibiotics for example aminoglycosides, quinolones and sulfonamides (Thenmozhi *et al.*, 2014).

Cross-resistance (a single mechanism confers resistance to an entire class of antibiotics) in beta-lactam antibiotics (ampicillin, cefuroxime, ceftazidime,

cefotaxime) and co-resistance (presence of resistance to more than one class of antibiotics in the same bacterial strain) with other classes of antibiotic (ciprofloxacin, azithromycin, chloramphenicol, Nalidixic acid, co-trimoxazole, trimethoprim and tetracycline) was noted in the current study. This co-resistance shows that resistance mechanisms including plasmid mediated resistance mechanisms and chromosomal resistance mechanisms such plasmid mediated quinolones resistance (PMQR) mediating resistance to fluoroquinolones, plasmid mediated resistance to sulfonamides, plasmid mediated dihydrofolate reductase (DHFR) conferring resistance to trimethoprim, acquired resistance to macrolides, chromosomal resistance mechanisms to tetracyclines and to chloramphenicol among others may have been involved. It has been found out that in many cases resistance mechanisms act jointly (Li *et al.*, 2014; van Hoek *et al.*, 2011).

It is highly probable that food animals have become one of the most important sources for the spread of these resistance-gene-carrying bacteria to humans through horizontal transfer of genes included on mobile elements such plasmid, integrons and transposons (Sihem *et al.*, 2015; Wu *et al.*, 2018). *E. coli* can cause infections at almost any site of the body in humans (Hughes & Heritage, 2004). The carriage of resistance determinants in archived isotes of *E. coli* from healthy food animals and their immediate environment is a serious threat to public health especially because these bacteria together with *Salmonella*, *Campylobacter* and *Enterococci* are among bacteria whose resistance build up is most commonly associated with the use of growth promoters and are likely to be transmitted frequently from animals to humans (Hughes & Heritage, 2004).

The present findings show that archived isolates of *E. coli* are resistant to third generation cephalosporine which are often considered as last resort antibiotics in human therapy. This is a serious problem for public health especially for farmers since infections with ESBL producing *E. coli* could lead to high morbidity and mortality rates as well as increased health care costs. Given that the emergence of drug resistant organisms can result from the selective pressure due to the large scale use of antibiotics, the use of antibiotics in food animals should be restricted. The use

of critically important drug in human therapy such as cephalosporins and fluoroquinolones should be banned.

5.5 Proportion of resistance genes in ESBL producers

The resistance genes that were detected in ESBL producers from the current study were bla_{TEM} and $bla_{CTX-M.}$ All the ESBL producers namely 8.8% (33/375) archived *E. coli* isolates were found to harbour *bla* genes; bla_{TEM} was detected in all the ESBL producing *E. coli* (100%) while bla_{CTX-M} was detected in 18.1%. Of the 33 ESBL producing *E. coli,* 18.1% had two *bla* genes (bla_{TEM} and $bla_{CTX-M)}$; bla_{SHV} was not found in these isolates. The absence of bla_{SHV} in *E. coli* isolates from the current finding could be due to the fact that this *bla* gene is more common in *Klebsiella pneumoniae* than *E. coli* (Doi *et al*., 2017; Rahman *et al*., 2018). The occurrence of bla_{TEM} and *bla* $_{CTX-M}$ was higher in Kiambu (69.7% and 88.3% respectively) than in other Counties. This difference is likely the result of an uneven distribution of *E. coli* isolates in the five selected Counties.

To my knowlwdge, this is the first study which investigated the carriage of ESBL genes among archived isolates of *E. coli* from healthy food animals and their immediate environment in Kenya. Reports from Madagascar, Mayotte, Reunion, Tunisia and Japan exhibited high prevalence of bla_{CTX-M} (70%-100%) in *E. coli* isolated from poultry, pigs and cattle samples while bla_{TEM} and bla_{SHV} were either low or absent (Gay *et al*., 2018; Nahar *et al*., 2018). The findings from the current study demonstrated the presence of bla_{TEM} and bla_{CTX-M} with absence of bla_{SHV} among archived isolates of *E. coli* from healthy food animals and environmental sources which concur with the report from a study done in China which demonstrated high rates of these *bla* genes (bla_{CTX-M} 51.7%, *bla* $_{TEM}$ 79.3%) with absence of bla_{SHV} in *E. coli* isolates from chickens (Yuan *et al*., 2009). Other studies conducted in China and Spain demonstrated high level of carriage of bla_{TEM} (79.3%, 80% , 83.9%) while bla_{CTX-M} rates were 16.1% , 51.7%, 92.7% repectively in *E. coli* isolates from healthy food animals such as chickens; bla_{SHV} was not detected (Costa *et al*., 2009; Wu *et al*., 2018; Yuan *et al*., 2009). The presence of bla_{TEM} and bla_{CTX-M}

in one *E. coli* isolate was reported in *E. coli* isolates recovered from food animal including pigs and chickens in China and Portugal (Gonçalves *et al*., 2010, Li *et al*., 2014). The rise of ESBL genes in archived isolates of *E. coli* from the current study could be due to the the selective pressure resulting from the increased veterinary use of antibiotics such as penicillins and cephalosporins in Kenya as it was demonstrated by other researchers (van Boeckel *et al*., 2015; Yuan *et al.,*2009)**.**

From the current findings, it is demonstrated that healthy food animals and associated environmental sources are reservoirs of resistance genes and resistant Gram negative bacteria (*E. coli*). These resistant bacteria might transfer to humans through contaminated water and food. Preventive measures such as good hygiene of homesteads, consumption of thoroughly cooked food especially food of animal origin, drinking of clean water should be put in place to curb the spread of resistant organisms from food animals to farmers and to the general population.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

The archived *E. coli* isolates from healthy food animals and environmental sources in selected counties in Kenya were found to be resistant to commonly used antibiotics. A good proportion of *E. coli* isolates 28% (108/3375) revealed to be multidrug resistant, cross-resistance (presence of resistance to more than one class of antibiotics in the same bacterial strain) and co-resistance (single mechanism confers resistance to an entire class of antibiotics) were observed. Archived isolates of *E. coli* were highly resistant to tetracycline, ampicillin, co-trimoxazole and trimethoprim. Resistance to cephalosporins, quinolones and fluoroquinolones, macrolides and chloramphenicol was noted. The increased antimicrobial resistance observed in these archived *E. coli* isolates could be due to the intensified antimicrobial usage in food animal husbandry for various purposes including disease prevention, growth promotion and therapy; exerting thus a selective pressure on *E. coli* as a normal flora resulting in antimicrobial resistance.

From this study, *E. coli* isolates harbouring virulence genes characteristic of Enteroaggregative *E. coli* (88.3% of the isolates tested)were detected amongst archived isolates of *E. coli* from healthy food animals and environmental sources suggesting that healthy food animals can be regarded as potential reservoirs for pathogenic strains of *E. coli*. The *E. coli* pathotypes were resistant to antimicrobials indicating a combination of carriage of virulence factors and resistance determinants making the strain an important hazard for public health. Proportion of ESBL producing *E. coli* was 8.8% and all ESBL producers harboured extended spectrum beta-lactamase genes, bla_{TEM} and $bla_{\mathrm{CTX\text{-}M.}}$ ESBL inactivate 3rd generation cephalosporins which are often considered as last resort antibiotic in human therapy; *bla* genes are plasmid born and can be transferred from *E. coli* to other bacteria including pathogenic bacteria. This is risky for public health since infection by resistant pathogens could result in treatment failure ending in human lives loss.

Archived isolates of *E. coli* from healthy food animals and environmental sources were found to be reservoirs for resistance determinants and virulence genes.

6.2 Recommendations

On the basis of the findings from this study, the following recommendations are made:

1. Stringent regulations governing the commerce, distributions and usage of antimicrobial agents should be applied to curb the development of antibiotic resistant organisms in food animals
2. The veterinary use of antibiotics for prevention and growth promotion should be rigorously monitored and the usage of critically important drug in human therapy such as cephalosporins and fluoroquinolones banned.
3. Farm level monitoring, individual level monitoring and national level monitoring of antimicrobial resistance should be implemented within the framework of a better surveillance.
4. Awareness program to sensitize the population about the spread of antimicrobial resistance through animal wastes, the safe handling and proper disposal of animal waste should be carried out to protect the population, farmers and the environment.
5. Farmers should wear protective equipment when it is unavoidable to be in contact with their livestock
6. Homestead and barns should be kept clean to protect animals and to limit the propagation of resistant organisms by arthropods

These data are already published; the abstract of the publication is shown in appendix X

REFERENCES

Abgottspon, H. (2014). *Enterobacteriaceae* with extended-spectrum and pampc-types beta-lactamase encoding genes isolated from freshwater fish from two lakes in Switzerland. *Antimicrobial agents and chemotherapy*, 2482-2484

Adefisoye, M.A. & Okoh, A.I. (2015). Identification and antimicrobial resistance prevalence of pathogenic *Escherichia coli* strains from treated wastewater effluents in Eastern Cape, South Africa. *Microbiology open*, *5*(1), 143- 151

Adelaide, O. A., Bii, C. & Okemo, P. (2008). Antibiotic resistance and virulence factors in *Escherichia coli* from broiler chickens slaughtered at Tigoni processing plant in Limuru, Kenya. *East african medical journal*, *85*(11), 508-517

Adelowo, O. O., Fagade, O. E. & Agers, Y. (2014). Antibiotic resistance and resistance genes in *Escherichia coli* from poultry farms,southwest Nigeria. Journal of infection in developing countries, 8(9), 1103-1112

Adenipekun, E. O., Jackson, C. R., Oluwadun, A, Iwalokun, B. A., Frye, J. G., John, B., …& Woodley, T. A. (2015). Prevalence and antimicrobial resistance in *Escherichia coli* from food animals in Lagos, Nigeria. Microbial drug resistance, 21(3), 372

Ahangarzadeh-Rezaee, M., Shahbazi, G., Hasani, A., Asgharzadeh, M., RahimRhahimi, A. A. & Alebouyeh, M. (2015). Prevalence of enteroaggregative *Escherichia coli* among children with gastroenteritis in the northwest of Iran. *Journal of analytical research in clinical medicine*, *3*(3), 183-9

Al-Bayssari, C., Dabboussi, F., Hamze, M. & Rolain, J. M. (2015). Detection of expanded-spectrum beta-lactamases in Gram-negative bacteria in the 21st century. *Expert review of anti infective therapy*, *13*, 1139-1158

Alekshun, M. N & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell*, 128(6), 1037-1050

Al Haj, N., Mariana, N. S., Raha, A. R. & Ishak, Z. (2007). Phenotypic and genotypic characterization of antimicrobial resistance among diarrheagenic *Escherichia coli*. *Research journal of medical sciences*, *1*(5), 245-250

Alonso, C. A., Zarazaga, M., Ben Sallem, R., Jouini, A., Ben Slama, K. & Torres, C. (2017). Antibiotic resistance in *Escherichia coli* in husbandry animals: the African perspective. *Letters in applied microbiology*, *64*, 318-334

Alzahrani, K. A., Farag, M. M., Abbadi, H. S., Hassan, M. M., Gaber, A. & Abdel-Moneim, A. S. (2016). Antibiotic resistance profile and random amplification typing of beta-lactamase- producing *Enterobacteriaceae* from the local area of Al-Taif and nearby cities in Saudi Arabia. *Asian biomedicine*, *10*(3), 219-228

Amin, M., Sirous, M., Javaherizadeh, H., Motamedifar, M., Saki, M.,Veisi, J., …& Hashemzadeh, M. (2018). Antibiotic resistance pattern and molecular characterization of extended-spectrum beta-lactamase producing enteroaggregative *Escherichia coli* isolates in children from southwest Iran. *Infection and drug resistance*, *11*, 1097-1104

Amirkamali, S., Naserpour-Farivar, T., Azarhoosh, K. & Peymani, A. (2017). Distributon of bla_{OXA}, $bla_{\text{VEB-1}}$ and $bla_{\text{GES-1}}$ genes and resistance pattern of ESBL-producing *Pseudomonas aeruginosa* isolated from hospitals in Tehran and Qazvin, Iran. *Revisita da sociedade brasileira de medicina tropical*, *50*(3), 315-320

Ampaire, L., Muhindo, A. & Orikiriza, P. (2016). A review of antimicrobial resistance in East Africa. *African journal of laboratory medicine*, *5*(1), a432

Ampaire, L., Nduhura, E. & Wewedru, I. (2017). Phenotypic prevalence of extended spectrum beta-lactamases among *Enterobacteriaceae* isolated at Mulago National Referral Hospital: Uganda. Biomed central research notes, *10*, 448

Asai, T., Masani, K., Sato, C., Hiki, M., Usui, M., Baba, K., …& Sawada, T. (2011). Phylogenetic resistance group and cephalosporin resistance genes of *Escherichia. coli* from diseased food-producing animals in Japan. *Acta veterinaria scandinavica*, *53*

Aslam, B., Wang, W., Arshad, I.M., Khurshid, M., Muzammil, S., Rasool, H. M., …& Baloch, Z. (2018). Antibiotic resistance: a rundown of a global crisis. *Infection and drug resistance* , *11*

Bajpai, T., Pandey, M., Varma, M. & Bhatambare, G. S. (2017). Prevalence of TEM, SHV and CTX-M beta-lactamase genes in the urinary isolates of a tertiary care hospital. *Avicenna journal of medicine*, *7*(1), 12-16

Bali, E. B., Acik, L. & Sultan, N. (2010). Phenotypic and molecular characterization of SHV, TEM, CTX-M and extended-spectrum beta-lactamase produced by *Escherichia coli, Acinobacter baumannii* and *Klebsiella* isolates in a Turkish hospital. *African journal of microbiology research*, *4*(8), 650-654

Baguma, A., Kagirita, A. & Bazira, J. (2017). Prevalence of extended-spectrum Beta-lactamases-producing microorganisms in patients admitted at Kabale regional referral hospital, southwestern Uganda. *International journal of microbiology,* 1-6

Bandyopadhyay, S., Kar, D., Bhattacharyya, D., Mondal, B., Samanta, I., Mahanti, A., …& Brandyopadhay, S. (2014). Molecular characterization of extended spectrum beta-lactamase (ESBL) producing *E. coli* in diverse food producing animals from Eastern India. *Journal of veterinary science and techonology*, *5*(3)

Barbosa, M. M., Pinto, F., Ribeiro, L. F., Guriz, C. S. L., Ferraudo, A. S., Maluta, R.P., …& Amaral, L. A. (2014). Serology and patterns of antimicrobial susceptibility in *Escherichia coli* isolates from pay-to-fish ponds. *Food safety/scientific article*, *81*(1), 43-48

Bauer, A. W., Kirby, W. M. M., Sherris, J. C. & Turck, M (1966). Antibiotic susceptibility testing by a standardized single disk method. *American journal clininial pathology*, *45*, 493-6

Bauernfeind, A., Grimm, H. & Schweighart, S. (1990). A new plasmid cefotaximase in clinical isolate of *Escherichia coli*. *Infection*, 294-298

Bethel, C. R., Tarcila, M., Shyr, T., Thomson, J. D., Distler, A. M., Hujer, K. M., …& Bonomo, R. A. (2011). Exploring the inhibition of CTX-M-9 by beta-lactamase inhibiotors and carbapenems. *Antimicrobial agent and chemotherapy, 55*(7), 3465-3475

Bhat, V. G, Vira, H. J, Kelkar, R. S, Biswas, S. K. & Chavan, P. D. (2017). Gram negative bacterial sepsis in a cancer centre: bacteriological spectrum and antibiotic susceptibility profiles. *Journal of basic and clininical pharmacy*, *8*, 144-148

Biswas, S. M., Mia, M. R. A., Ara, N., Ibrahim, M., Nasir, T. A. &Yunus, S. (2013). Comparison of three dimensional test and double disc synergy test for detection of extended spectrum beta-lactamase (ESBL) producing gram negative bacteria. *Pulse*, 6 (1 & 2), 12-19

Blaak, H., Hamidjaja, R. A., van Hoek, A. H. A. M, de Heer, L., Husman, A. M. & Schets, F. M. (2014). Detection of extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* on flies at poultry farms. *Applied and environmental microbiology*, *80*(1), 239-246

Bolin, I., Wiklund, G., Qadri, F., Torres, O., Bourgeois, A. L., Savarino, S. & Svennerholm, A.M. (2006). Enterotoxigenic *Escherichia coli* with *STh* and

STp genotypes is associated with diarrhea both in children in area of endemicity and in travelers. *Journal of clinicinical Microbiology, 44*(11), 3872-3877

Bonnedahl, J., Drobni, M., Gauthier-Clerc, M., Hernandez, J., Granholm, S., Kayser, Y., …& Olsen, B. (2009). Dissemination of *Escherichia coli* with CTX-M type ESBL between humans and yellow-legged gulls in the South of France. *Plos one, 4*(6), 1-6

Bonnet, R. (2004). Growing group of extended spectrum beta-lactamase: the CTX-M enzymes. *Antimicrobial agents chemomotherapy*, *48*(1), 1-14

Bonomo, R. A. (2017). Beta-lactamases: a focus on current challenges. *Cold Spring Harbor Perspective in Medicine*, *7*(1), 1–16

Bonyadian, M., Momtaz, H., Rahimi, E., Habibian, R.,Yazdani, A. & M. Zamani, M. (2010). Identification and characterization of shiga toxin-producing *Escherichia coli* isolates from patients with diarrhoea in Iran. *Indian joural of medical research*, *132*, 328-331

Bradford, A. P. (2001). Extended-spectrum beta-lactamases in the 21st century: characterization,epidemiology, and detection of this important resistance threat. C*linical microbiology reviews, 14*(4), 933-951

Brendan, K., DeVinney, R.; Stein, M., Reinscheid, D. J, Frey, E. A. & Finlay, B. B. (1997). Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell*, *91*, 511-522

Brower, C. H., Mandal, S., Hayer, S., Sran, M., Zehra, A., Patel, S. J., ...& Laxminarayan, R. (2017). The prevalence of extended-spectrum beta-lactamase-producing multidrug-resistant E*scherichia coli* in poultry,chickens and variation according to farming practices in Punjab, India. *Environmental health perspectives,* 077015-10

Bruton, J., Clare, D. & Meier, D. (1986). Molecular epidemiology of antibiotic resistance plasmids of *Haemophilus* species and *Neisseria gonorrhoeae*. *Reviews of infectious disease*, *8*(5), 713-24

Bueris,V., Sircili, M. P., Taddei, C. R., Fernandes dos Santos, M., Franzolin, M. R., Martinez, M. B., …& Trabulsi, L. R. (2007). Detection of diarrheagenic *Escherichia coli* from children with and without diarrhea in Salvador, Bahia, Brazil. *Memórias do instituto oswaldo cruz, 102*(7), 839-844

Bush, K., & Jacoby, G. A. (2010). Updated functional classification of beta-lactamases. *Antimicrobial. agents and chemotherapy.*, 969-976.

Calhau, V. T. (2014). *Virulence factors associated with antimicrobial resistance determinants among Escherichia coli and Klebsiella spp.* Coimbra: Universidade de Coimbra

Caine, L. A., Nwodo, U. U., Okoh, A. I., Rolahnd N., Ndip, R. N. & Green, E. (2014). Occurrence of virulence genes associated with diarrheagenic *Escherichia coli* isolated from raw cow's milk from two commercial dairy farms in the Eastern Cape Province, South Africa. *International journal of environmental research and public health*, *11*, 11950-11963

Campellone, K. G. (2010). Cytoskeleton-modulating effectors of enteropathogenic and enterohemorrhagic *Escherichia coli*: Tir, EspF U and actin pedestal Assembly. *Federation of europian biochemical socieaty jounal*, *277*(11), 2390-2402

Canizalez-Roman, A., Flores-Villaseñor, H. M., Gonzalez-Nuñez, E., Valzquez, J.,Vdal, J. E., Muro-Amador, S., …& León-Scairos, N. (2016). Surveillance of diarrheagenic strains isolated from diarrhea cases from children, adults and eldery at Northwest of Mexico. *Frontiers in microbiology, 7*, 1924

Cantón, R., Novais, A., Valverde, A., Machado, E., Peixe, L., Baquero, L., & Coque, T.M. (2008). Prevalence and spread of extended-spectrum beta-lactamase

producing E*nterobacteriaceae* in Europe. *Clinical microbiology and infection*, *14*(suppl. 1), 144-153

Cantón, R., gonzalez-Alba, J. M. & Galan, J. C. (2012). CTX-M enzymes: origin and diffusion. *Frontiers in microbiology*, *3*, 110

Caprioli, A., Morabito, A. S., Brugere, H. & Oswald, E. (2005). Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Veterinary research*, *36*(3), 289-311

Caruso, G. (2018). Antibiotic resistance in *Escherichia coli* from farm livestock and related analytical methods: a review. *Journal of the association of official agricultural chemists international, 101(*4), 016-922

Cennimo, D. J., Koo, H. , Mohamed, J. A., Huang, D. B. & Chiang, T. (2007). Enteroaggregative *Escherichia coli:* a review of trends,diagnosis, and treatment. *Infection in medicine*, 100-110

Centers for Disease control and Prevention (2018). Antibiotic/Antimicrobial resistance. *About antimicrobial resistance*. Retrieved from: https:// www.cdc.gov

Centers for Disease control and Prevention-Kenya (2018). Annual report 2017. CDC-Kenya-Annual-report-2017-5-508.pdf. Retrieved from: www.cdc.gov/globalhealth/countries/kenya

Chandra, S. (2013). Extended-spectrum beta-lactamase infections. *Current emergency and hospital medicine reports*, *1,*145-148

Chantziaras, I., Dewulf, J., Boyen, F., Callens, B. & Butaye, P. (2014). Antimicrobial resistance prevalence of pathogenic and commensal *Escherichia coli* in food producing animals in Belgium. *Vlaams diergeneeskundig tijdschrift, 83,* 225-233

Chen, Q., Shi, X., Li, Y., Jiang, Y., Lin, Y., Qiu, Y., …& Hu, Q. (2014). Rapid genetic typing of diarrheagenic *Escherichia coli* using a two tube modified molecular beacon based multiplex real-time PCR assay and clinical application. *Annals of clinical microbiology and antimicrobials,* 13, 30

Chishimba, K., Hang'ombe, B. M., Muzandu, K., Mshana, S. E., Matee, M. I, Nakajima, C. & Suzuki, Y. (2016). Detection of extended-spectrum beta-lactamase-producing *Escherichia coli* in market-ready chickens in Zambia. *International journal of microbiology,* 2016, 1-5 http://dx.doi.org/10.1155/2016/5275724

Cho, S., Hiott, L. M., Barrett, J. B., McMillan, E. A., House, S. L., Humayoun, S. B., Adams, E. S., Jackson, C. R., & Frye, J. G. (2018). Prevalence and characterization of *Escherichia coli* isolated from the Upper Oconee Watershed in Northeast Georgia. *Plos one, 13*(5), e0197005

Clatworthy, A. E., Pierson, E. & Hung, T. D. (2007). Targeting virulence. *A new paradigm for antimicrobial therapy, 3*(9), 541-548

Cleary, J., Lai, L. , Shaw, R. K., Straatman-Iwanowska, A., Donnenberg, M. S., Frankel, G. & Knutton, S. (2004). Enteropathogenic *Escherichia coli* (EPEC) adhesion to intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. *Microbiology*, *150*, 527-538

Clements, A., Young, J. C., Constantinou, N. & Frankel, G. (2012). Infection strategies of enteric pathogenic *Escherichia coli. Gut microbes*, 3(2), 71-87.

Clinical and Laboratory Standard Institute (2009). *Perfomance standards for antimicrobial suscepibility testing, 20th informational supplement*. CLSI document M100-S 19. CLSI, Wayne, PA: Clinical and laboratory standard institute

Coico, R. (2005). Gram staining. *Current protocols in microbiology*, doi.org/101002/9780471729259.mca03cs00

Constantiniu, S., Buzdugan, I., Romaniuc, A., Cozma, L., Filimon, R., Dumbrava, M., …& Danis, G. (2001). Setotypes of *Escherichia coli* enteroinvasive in North-Eastern districts of Romania. *The journal o f preventive medicine*, *9*(3), 67-73

Coque, T., Baquero, F., & Canton, R. (2008). Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. *Eurosurveillance*, *13*, 19044.

Correa-Martínez, L. C, Idelevich, A. E., Sparbier, K, Kostrzewa, M & Becker, K. (2019). Rapid detection of extended-spectrum beta-lactamases (ESBL) and ampc beta-lactamases in enterobacterales: development of a screening panel using the MALDI-TOF MS-based direct-on-target microdroplet growth assay. Frontiers in microbiology, *10*, 13

Cormican, M. G., Marshall, S. A. & Jones, R. N. (1996). Detection of extended-spectrum beta-lactamase (ESBL)-producing strains by the E-test ESBL screen. *Journal of clinical microbiology*, *34*(8), 1880-1884.

Costa, D., Vinué, L., Poeta, P., Coelho, A. C., Matos, M., Saenz,Y., …& Torres, C. (2009). Prevalence of extended spectrum beta-lactamase-producing isolates in faecal of samples of broilers. *Veterinary microbiology*, 138(3-4), 339

Croxen, A. M, Law, J. R., Scholz, R., Keeny, M. K, Wlodarska, M. & Finlay, B. B. (2013). Recent advances in understanding enteric pathogenic *Escherichia coli*. *clinical microbiology review*, *26*(4), 822-880

Dadie, A., Kouassi, N., Dako, E., Dje, M. & Dosso, M. (2014). Virulence, serotype and phylogenetic groups of diarrhoeagenic *Escherichia coli* isolated during digestive infections in Abidjan, Côte d'Ivoire. *African journal of biotechnology*, 13(9), 999-1008

Dahms, C., Hübner, N., Kossow, A., mellmann, A., Dittmann, K. & Kramer, A. (2015). Occurrence of ESBL producing *Escherichia coli* in livestock and farm workers in Mecklenburg-Western Pomerania, Germany. *Plos one*, 1-13

Dashti, A. A., Jadaon, M. M., Abdulsalamad, A. M. & Dashti, H. M. (2009). Heat treatment of bacteria: a simple method of DNA extraction for molecular techniques. *Kuwait Medical Journal, 41*(2), 117-122

Davies, J & Davies, D. (2010). Origin and evolution of antibiotic resistance. *Microbiology and molecular biology reviews,* 417-433

Decousser, J. W., Poirel, L. & Nordmann, P (2001). Characterization of chromosomally encoded extended spectrum classA beta lactamase from *Klyuvera cryocrescens. Antimicrobial Agent and Chemotherapy*, *45*(12), 3595-3598

Dedeić-Ljubović, A., Hukić, M., Bekić, D. & Zvizdić, A. (2009). Frequency and distribution of diarrhoeagenic *Escherichia coli* strains isolated from pediatric patient with diarrhoea in Bosnia and Herzegovina . *Bosnian journal of basic medical sciences*, *9*(2), 148-155

Demirdag, K., & Hosoglu, S. (2010). Epidemiology and risk factors for ESBL-producing *Klebsiella pneumoniae*: a case control study. *Journal of infection in developing countries,* 4(11), 717-722

Deshpande, A. D., Baheti, K. G. & Chatterjee, N. R. (2004) Degradation of beta-lactam antibiotics. *Current science*, *87*(12), 25

Dinos, P. G. (2017). The macrolide antibiotic renaissance. *Pharmacology British journal of pharmacology*, *174*, 2967–2983

Djurdjevic-Milosevic, D. M., Solaja, M. M.,Topalic-Trivunovic, L. J. N., Stijepic, M. J & Glusac, J. R. (2011). The survival of *Escherichia coli* upon exposure to irradiation with non-coherent polychromatic polarized light. *Veterinarni Medicina*, *56*(10), 520-527

Doi, Y., Park, Y. S., Rivera, J. I., Adams-Haduch, J. M., Hingwe, A., Sordillo, E. M. (2012). Community-associated extended-spectrum beta-lactamase-producing

Escherichia coli infection in the United States. *Clinical infectious diseases, 56*, 641-648

Doi,Y., Iovleva, A. & Bonomo, A. R. (2017). The ecology of extended-spectrum beta-lactamases (ESBLs) in the developed world. *International society of travel medicine*, *24*(suppl 1), S44-S51

Drieux, L., Brossier, F., Sougakoff, W. & Jarlier, V. (2008). Phenotypic detection of extended-spectrum beta-lactamase production in *Enterobacteriaceae:* review and bench guide. *Clinical microbiology and infectious diseases, 14*(Suppl. 1), 90-103

Dzidic, S., Suskovic J. & Kos, B., (2008). Antibiotic resistance mechanisms in bacteria:biochemical and genetic aspects. *Food technology and biotechnolology*, 4*6*(1), 11-21

Egorov, A. M., Ulyashova, M. M. & Rubtsova, M. Y. (2018). Bacterial enzymes and antibiotic resistance. *Acta naturae*, *10*(4), 1-39

Erb, S., D'Mello-Guyett, L., Malebo, M. H., Njee, M. R., Matwewe, F., Ensink, J., …& Frei, R. (2018). High prevalence of ESBL-Producing *E. coli* in private and shared latrines in an informal urban settlement in Dar Es Salaam, Tanzania. *Antimicrobial resistance and infection control,* 7, 3

Europian Food Security Authority BIOHAZ Panel (EFSA Panel on Biological Hazards) (2015). Scientific opinion onpublic health risks associated with Enteroaggregative *Escherichia coli* (EAEC) as a food-borne pathogen. *Europian food security authority journal, 13*(12), 4330-87

EU Reference Laboratory for *E.coli.* (2013). Detection of enteroinvasive *Escherichia coli* in food by real time PCR amplification of the *ipa*h gene. *European-Reference Laboratory for Verocyototoxin producing E. coli.* Retrieved from http://ec.europa.eu

European Antimicrobial Resistance Surveillance System, EARSS (2006). Annual report *2006*. Retrieved from https://www.rivm.nl

Evans, A.B. & Amyes, G.B.S. (2014). OXA beta-lactamases. *Clinical microbiology review*, *27*(2), 241-263

Fakhuruddin, Durrani, A. M., Ahmed, R., Kumar, M. & Bakar, I. (2014) Frequency of class B carbapenemases (MβL) in *Enterobacteriaceae*. *Journal of the pakistan medical association*, *64*(5), 519-523

Filho, K. H., Carvalho, D., Grassotti, T. T. , Soares, B. D., Rossato, J. M. , Cunha, A.C., …& Brito, B. G. (2015). Avian pathogenic *Escherichia coli* - methods for improved diagnosis. *World's poultry science journal*, 71, 249-258

Fisher, A. A., Laing, J. E, Stoeckel, J. E & Townsend, J. W. (1998). *Family planning operations research design*. New York: Population council

Fleming A (1999). *Penicillin*.World Scientific Publishing Company, Singapore

Franz, E., Veenman, C., van Hoek, M. A. H. A., Husman, R. R. & BlaaK, H. (2015). Pathogenic *Escherichia coli* producing extended-spectrum beta-lactamases isolated from surface water and wastewater. Scientific reports, *5*, 14372, doi: 10.1038/srep14372

Fujioka, M., Kasai, K., Miura, T., Sato, T., & Otomo, Y. (2009). Rapid diagnostic method for the detection of diarrheagenic *Escherichia coli* by multiplex PCR. *Japan journal of infectious disease*, *62*, 476-480

Fyfe. C., Grossman, T. H., Kerstein, K. & Sutcliffe, J. (2016). Resistance to macrolide antibiotics in public health system. *Cold Spring Harbor. Perspectives in. Medicine*, *6*(10), 1-38

Garbaj, A. M., Awad, E. M, Azwai, S., Abolghait, S. K., Naas, H. T., Moawad, A. A., …& Eldaghayes, I. M. (2016). Enterohemorrhagic *Escherichia coli* O157

in milk and dairy products from Libya: isolation and molecular identification by partial sequencing of 16s rDNA. *Veterinary world*, *9*(11), 1184-1189

Garneau-Tsodikova, S. & Labby, K. J. (2016). Mechanisms of resistance to aminoglycosides antibiotics: overview and perspectives. *MedChemComm*, *7*(1), 11-27

Gay, N., Leclaire, A., Laval, M., Miltgen, G., Jégo, M., Stéphane, R., Jaubert, J., …& Cardinale, E. (2018). Factors of extended spectrum beta-lactamase producing *Enterobacteriaceae* occurrence in farms in Reunion, Madagascar and Mayotte Islands, 2016-2017. *Veterinary sciences*, 5(22), 1-14

Gerrish, S. R., Lee , J. E., Reed, J., Williams, J., Farrell, L. D., Spiege, K. M., Sheridan, P. P., …& Shields, M. S. (2007). PCR versus hybridization for detecting virulence genes of enterohemorrhagic *Escherichia coli. Emerging infectious diseases*, *13*(8), 1253-1255

Geser, N., Stephan, R., Kuhnert, P., Zbinden, R, Kaeppeli, U., Cernela, N. & Haecler, H. (2011). Fecal carriage of extended-spectrum beta lactamse producing *Enterobacteriaceae* in swine and cattle at slaughter in switzerland. *Journal of food protection*, *73*(3), 446-9

Ghafourian, S., Sadeghifard, N., Soheili, S. & Sekawi, Z. (2015). Extended spectrum beta-lactamases: definition, classification and epidemiology. *Current issues in molecular biolology*, *17*, 11-22

Global Antibiotic Resistance Partnership-Kenya Working Group (2011). Situation Analysis and Recommendations. *Antibiotic Use and Resistance in Kenya*. Retrieved from https://cddep.org

Giedraitiene, A, Vitkauskiene, A. & Naginiene, R. (2011). Antibiotic resistance mechanisms of clinically important bacteria. *Medicina*, 47(3), 137-146

Gniadkowski, M. (2001). Evolution and epidemiology of extended-spectrum beta-lactamases (ESBLs) and ESBL-producing microorganisms. *Clinical microbiology and infection*, *7*, 597-608

Goel, N. I., Sachin, S., Kumar, P. C., Nikhil, P. & Suneet, Y. (2014). Different phenotypic methods for detection of ESBL production in bacteria: a review. *Journal of pharmaceutical and scientific innovation*, *3*(3), 197-198

Golkar, T., Zieliński, M. & Berghuis, M. A. (2018). Look and look on enzyme mediated macrolide resistance. *Frontiers in microbiology*, *9*(1942), 1-15

Gomes, T. A., Elias, W. P., Scaletsky, I. C. A., Gutha, B. E. C., Rodrigues, J. F., Piazza, R. M. F., …& Martinez, M. B. (2016). Diarrheagenic *Escherichia coli. Brazilian journal of medical microbiology*, *475*, 3-10

Gomez-Duarte , O. G., Arzuza, O., Urbina, D., Bai., J., Guerra, J., Montes,O., …& Castro, Y. J. (2010). Detection of *Escherichia coli* enteropathogens by multiplexpolymerase chain reaction from children's diarrheal stools in two caribbean colombian cities. *Foodborne pathogens and disease, 7*(2), 199-206

Gonçalves, A., Torres, C., Silva, N., Carneiro,C., Radhouani, H., Coelho, C., …& Igrejas, G. (2010). Genetic characterization of extended-spectrum beta-lactamases in *Escherichia coli* isolates of pigs from a portuguese intensive swine farm. *Foodborne pathogens and disease*, *7*(12), 1569-1573

Gonzales-Siles, L. & Sjöling, Å. (2016). The different ecological niches of enterotoxigenic *Escherichia coli*. *Environmental microbiology*, *18*(3), 741-751

Gordo, I., Demengeot, J. & Xavier, K. (2014). *Escherichia coli* adaptation to the gut environment: a constant fight for survival. *Future Microbiology*, *9*(11), 1235-1237

Goyal, A., Prasad, K. N., Prasad, A., Gupta, S., Ghoshal, U. & Ayyagari, A. (2008). Extended spectrum beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* and associated risk factors. *Indian journal of medical research*, 695-700

Hall, B. G., & Barlow, M. (2005). Revised Ambler classification of beta-Lactamases. *Journal of antimicrobial chemotherapy, 55*(6), 1050-1051

Hamda, D. (2010). Cytoskeleton modulating effectors of enteropathogenic and Enterohemorrhagic *Escherichia coli*. *Federation of european biochemical societies journal*, 277(11), 2389

Harrington, S. M., Dudley, E. G. & Nataro, J. P. (2006). Pathogenesis of Enteroaggregative *Escherichia coli* infection. *Federation of european microbiological societies*, 254(1), 12-18

Health Louisiana Office of Public (2016*). Escherichia coli (E. coli)* infections. *Infectious disease epidemiology section.* Retrieved from https://www.infectiousdisease.dhh.louisiana.gov

Hackman, H. K., Brown, C. A. & Twum-Danso, K. (2014). Antibiotic resistance profile of CTX-M-type extended-spectrum beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in Accra , Ghana. *Journal of natural sciences research,* 4(12)

Hamisi, Z., Tuntufye, H. & Shahada, F. (2014). Antimicrobial resistance phenotypes of *Escherichia coli* isolated from tropical free range chickens. *International journal of science and research*, *3*(9), 34-37

Harada, K. & Asai, T. (2010). Role of antimicrobial selective pressure and secondary Factors on antimicrobial resistance prevalence in *Escherichia coli* from food-producing animals in japan. *Journal of biomedicine and biotechnology*, 2012, 1-12

Hassan, M. D., Ibrahim, M. G. & Ibrahim, G. D. (2016). A novel single tube method for biochemical identification of *Escherichia coli*. *Life science journal*, *13* (8), 8-12

Hawser, S. P., Bouchillon, S. K., Hoban, D. J., Badal, R. E., Hsueh, P. R. & Paterson, D. L. (2009). Emergence of high levels of extended spectrum beta-lactamase producing Gram negative bacilli in the Asian- Pacific region: data from the Study for Monitoring Antimicrobial resistance Trends (SMART) program. *Antimicrobial. agents and chemother*apy, *53*, 3280-3284

Hemati, Z., Ghanibarpour, R. & Alizade, H. (2014). The distribution of beta-lactamase genes in *Escherichia coli* phylotypes isolated from diarrhea and UTI cases in Northwest Iran. *Advances in clinical and experimental Medicine*, *23*(4), 523-529

Hille, K., Fischer, J., Falgenhauer, L., Sharp, H., Michael, Brenner, G., …& Kreienbrock, L. (2014). On occurrence of extended spectrum and AmpC beta-lactamase producing *Escherichia coli* in livestock: results of selected European studies. *Berliner und münchener tierärztliche wochenschrif,* 127

Ho, P. L., Chow, K. H., Yuen, K. Y., Ng, W. S. & Chaua, P. Y. (1998). Comparison of a novel, inhibitor-potentiated disc-diffusion test with other methods for the detection of extended-spectrum beta-lactamases in *Escherichia coli* and Klebsiella pneumoniae. *Journal of antimicrobial chemotherapy* , *42*, 49–54

Hoang, P. H., Awasthi, S. P., Nguyen, P. D. O , Nguyen, N. L. H., Nguyen, D. T. A., Le, N. H.,…& Yamasaki, S. (2017). Antimicrobial resistance profiles and molecular characterization of *Escherichia coli* strains isolated from healthy adults in Ho Chi Minh City, Vietnam. *Journal of veterinary medical science*, *79*(3), 479–485

Hölmgren, J., Lonnroth, I., Månsson, J & Svennerholm, L. (1975). Interaction of cholera toxin and membrane GM1 ganglioside of small intestine.

Proceedings of the national academy of sciences of the United states of America, *72*(7), 2520-2524

Hon, W., Mckay, A. G., Thompson, R. P., Sweet, M. R., Yang, C. S. D.,Wright, D. G. & Berghuis, M. A. (1997). Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. *Cell*, *89*, 887-895

Hoseinzadeh, T., Ghanbarpour, R. & Rokhbakhsh-Zamin, F. (2016). Phylogenetic background of enterotoxigenic and enteroinvasive *Escherichia coli* from patients with diarrhea in Sirjan, Iran. *Iran journal of microbiology*, 8(3), 187-192

Hughes, P. & Heritage, J. (2004). Antibiotic growth promoters in food animals. *Food and agriculture organization animal production and health*, 160

Hu, Y. S., Shin, S., Park, Y. H. & Park, K. T. (2017). Prevalence and mechanism of fluoroquinolone resistance in *Escherichia coli* isolated from swine feces in Korea. *Journal of food protection, 80*(7), 1145-1151

Hussain, T. (2015). An introduction to the serotypes, pathotypes and phylotypes of *Escherichia coli. International journal of microbiology and allied sciences*, *2*(16), 9-16

Huiwen , D. C., & Gad, F. (2005). Enteropathogenic *Escherichia coli*: unravelling pathogenesis. *Federation of european microbiology societies microbiology reviews*, 83-98

Ingerson-Mahar , M., & Reid, A. (2011). Faq: *E coli*: good ,bad and deadly. *The american academy of microbiology,* 1-16

Iowa State University. (2016). Enterohemorrhagic *Escherichia coli* and other *E. coli* causing hemolytic uremic syndrome. *Enterohemorrhagic Escherichia. coli infections*. Retrieved from https://lib.dr.iastate.edu

Jacoby, G. A. & Munoz-Price, L. S. (2005) The new beta-lactamases. *The new England journal of medecine, 352*(4), 380-391

Jacques de Munick, E. (2013). *Deep characterization of Escherichia coli in a cohort of mothers and their infants*. Oslo: Akademika publishing

Jafari, A., Aslani, M. & Bouzari, S. (2012). *Escherichia coli*: a brief review of diarrheagenic pathotypes and their role in diarrheal diseases in Iran. *Iran journal of microbiology*, *4*(3), 102-117

Jarlier, V., Nicolas, M. H., Fournier, G. & Fillipon, A. (1988). Extended spectrum beta-lactamase conferring transferable resistance to newer lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility partens, *review of infectious diseases*, *10*, 867-878

Jensen, B. H., Olsen, K. E. P., Struve, C., Krogfelt, K. A. & Petersen, A. M. (2014). Epidemiology and clinical manifestations of enteroaggregative *Escherichia coli. Clinical microbiology reviews, 27*(3), 614-630

Johnson, J. R., Johnston, B., Kuskowski, M. A., Nougayrede, J. P. & Oswald, E. (2008). Molecular epidemiology and phylogenetic distribution of Escherichia coli pks genomic island. *Jounal of clinical microbiology*, *46*(12), 3906-11

Joris, A. M., Vanrompay, D. K., Verstraete, K., De Reu, K. & De Zutter, L., (2012). Enterohemorrhagic *Escherichia coli* with particular attention to the german outbreak strain O104:H4. *Vlaams diergeneeskundig tijdschrift*, 81(3), 3-8

Juma, B.W., Kariuki, S., Waiyaki, P. G., Mutugi, M. M. & Bulimo, W. D. (2016). The prevalence of TEM and SHV genes among extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli. African journal of pharmacology and therapeutics, 5*(1), 1-7

Kabiru, L. M., Bello, M., Kabir, J., Grande, L. & Morabito, S. (2015). Detection of pathogenic *Escherichia coli* in samples collected at an abattoir in Zaria,

Nigeria and at different points in the surrounding environment. *International journal of environmental research and public health*, *12*(1), 680-691

Kagia, N., Kosgei, P., Ooko, M., Wafula, L., Mturi, N., Anampiu, K., …& Morpeth, C. S. (2019). Carriage and acquisition of extended-spectrum beta-lactamase-producing enterobacterales among neonates admitted to hospital in Kilifi, Kenya. Clinical infectious diseases, 1-9. Doi: 10.1093/cid/ciy976

Kagambèga, A., Martikainen, O., Siitonen, A., Traore, A. S., Barro, N., Haukka, K. (2012). Prevalence of diarrheagenic *Escherichia coli* virulence genes in the feces of slaughtered cattle, chickens, and pigs in Burkina Faso. *Microbiology open*, *1*(3), 276-284

Kang, C. & Song, J. (2013). Antimicrobial resistance in Asia: current epidemiology and clinical implications. *Infection and chemotherapy* , *45*(1), 22-3

Kaper, J. B. (1998). Enterohemorrhagic *Escherichia coli*. *Current opinion in microbiology*, *1*,103-108

Kappell, A. D., DeNies, M. S., Ahuja, N. H., Ledeboer, N. A, Newton, R. J. & Hristova, K. R (2015). Detection of multi-drug resistant *Escherichia coli* in the urban waterways of Milwaukee, WI. *Frontiers in microbiology*, *6*(336), 1-5

Kariuki, S., Revathi, G., Corkill, J., Kiiru, Mwituria, J. J., Mirza, N. & Hart, C. A (2007). *Escherichia coli* from community-acquired urinary tract infections resistant to fluoroquinolones and extended-spectrum beta-lactams. The *Journal of infection in developing countries, 1*(3), 257-262

Kariuki, S. (2016). *Antimicrobial resistance in key foodborne bacterial pathogens in Kenya.* Kenya medical Research Institute, Wellcome trust, Sanger institute.

Kasango, D. S., Lutoti, S., Wewedru, Aboce, E. & Angol, C. D. (2018). Prevalence and antimicrobial susceptibility pattern of extended spectrum beta-lactamase

producers in Gram-negative urine isolates at MBN clinical laboratories, Kampala Uganda. *Archives of microbiology & immunology*, *2*(3), 042-052

Kaur, M., & Aggarwal, A. (2013). *Enterobacteriaceae* in a tertiary care hospital of North India. *Journal of clinical diagnostic research*, 642-645

Khan Academy (2019). The chemical structure consequences of beta-lactams. Retrieved from Khan Academy website: https:// www.khanacademy.org

Khosravi, D. A., Jenabi, A. & Montazeri, A. E. (2017). Distribution of genes encoding resistance to aminoglycoside modifying enzymes in methicillin-resistant Staphylococcus aureus (MRSA) strains. *Kaohsiung journal of medical sciences*, 33, 587-593

Kliebe, C., Nies, B. A., Meyer, J. F., Tolxdorff-Neutzling, R. M., & Wiedemann, B. (1985). Evolution of plasmid-coded resistance to broadspectrum cephalosporins. *Antimicrobial agents chemotheray,* 28, 302-307

Kim, J. S., Kim, J., Kim, S., Jeon, S., Oh, K.H., Cho, S., …& Chung, G.T. (2014). Characterization of CTX-M-type extended-spectrum beta-lactamase-producing diarrheagenic *Escherichia coli* isolates in the Republic of Korea during 2008-2011. *Journal of microbiology and biotechnolology, 24*(3), 421-426

Kipkorir, K. C., Bett, P. K., Onyango, P. O., Onyango, D. M., Ayieko, C. & Angienda, P. (2016). Epidemiology of antimicrobial resistance among *Escherichia coli* strains in Trans-Nzoia County, Kenya. *Journal of microbiology and infectious diseases*, *6* (3), 107- 112

Knothe, H., Shah, P., Krcmery, V., Antal, M. & Mitsuhashi, S. (1983). Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens* . *Infection*, 11(6), 315-7

Kocsis, B., & Szabó, D. (2013). Antibiotic resistance mechanisms in *Enterobacteriaceae*. *Formatex*, 251-257

Koteva, K., Cox, G., Kelso, J. K., Surette, M. D., Zubyk, H. L., Ejim, L., …& Wright, G. D. (2018). Rox, a rifamycin resistance enzyme with an unprecedented acion. *Cell chemical. Biology*, *25*(4), 403-412

Kozub-Witkowski, E., Krause, G., Franke, G., Kramer, D., Appel, B. & Beutin, L. (2008). Serotypes and virutypes of enteropathogenic and enterohaemorrhagic *Escherichia coli* strains from stool samples of children with diarrhoea in Germany. *Journal of applied microbiology*, 403-410

Krause, M. K, Serio, W. A., Kane, R. T. & Connolly, E. L. (2016). Aminoglycosides: an overview. *Cold spring harbor perspective in medicine*, *6*(6), 1-18

Kuhnert, P., Boerlin, P. & Frey, J. (2000). Target genes for virulence assessment of *Escherichia coli* isolates from water, food and the environment. *Federation of european microbiological societies microbial review*, *24*(1), 107-117

Kumar, S., & Varela, M.F. (2013). Molecular mechanisms of bacterial resistance to antimicrobial agents. *Microbial pathogens and strategies for combating them: science, technology and education*, 522-534

Kwach, J. (2018). Nairobi sub-county explained. Retrieved from:https://www.tuko.co.ke/261967-nairobi-sub-counties-explained.html

Lamprecht, C., Romanis, M., Huisamen, N., Carinus, A., Schoeman, N., Sigge, G.O. & Britz, T.J. (2014). *Escherichia coli* with virulence factors and multidrug resistance in the Plankenburg river. *E. coli virulence factors and multidrug resistance*, *110*(9/10), 1-6

Le Bouguenec, C., Archambaud, M. & Labigne, A. (1992). Rapid and specific detection of *pap*, *afa*, and *sfa* adhesn-encoding operons in uropathogenic

Escherichia coli strains by polymerase cham reaction. *Journal of clinical microbiology, 30,* 1189-93

Le Bouguénec, C. & Servin, A. L. (2006). Diffusely adherent *Escherichia coli* strains expressing Afa/Dr adhesins (Afa/DrDAEC): hitherto unrecognized pathogens. *Federation of europian microbiology society*, 185-194

Leclercq, R. (2002). Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implicationsclinical. *Infectious diseases*, *34*, 482-92

Legese, H. M., Weldearegay, M. G. & Asrat, D. (2017). Extnded spectrum and carbapenemase-producing *Enterobacteriacea* among Ethiopian children. *Infection and drug resistance*, *10*, 27-34

Levy, S. B. & Marshall, B (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature medicine*; *10*(12 Suppl), S122-129

Li, L., Wang, B., Feng, S., Li, J., Wu, C., Wang, Y., …& Zeng, M. (2014). Prevalence and characteristics of extended-spectrum beta-lactamase and plasmid-mediated fluoroquinolone resistance genes in *Escherichia coli* isolated from chickens in Anhui Province, China. *Plos one*, 9(8), e104356. doi:10.1371/journal.pone.0104356

Li., S., Zhao, M., Liu, J., Zhou,Y. & Miao, Z. (2016). Prevalence and antibiotic resistance profile of extended-spectrum β-lactamase producing *Escherichia coli* isolated from healthy broilers in Shandong province , China. *Journal of food protection*, 79(7), 1169-1173

Lima, C. M., Souza, I. E. G. L., Alves, T. S., Leite, C. C., Evangelista-Barreto, N. S. & Almeida, R. C. C (2017). Antimicrobial resistance in diarrheagenic *Escherichia coli* from ready-to-eat foods. *Journal of food science and technology*, *54*(11), 3612-3619

Linkevicius, M., Sandegren, L. & Andersson, I. D. (2016). Potential of tetracycline resistance proteins to evolve tigecycline resistance. *Antimicrobial agents and chemotherapy*, *60*(2), 789-796

Livermore, D. M. (1995). Beta-lactamases in laboratory and clinical resistant. *Clinical microbiology review,* 8(4), 557-584.

Lvermore, D. M & Paterson, D. L. (2006). *Pocket guide to extended spectrum beta-lactamases in resistance*. New Delhi: Current medicine group

Mainda, G., Bessell, P. R., Muma, J. B., McAteer, S. P., Chase-Topping, M. E., Gibbons, J., …& Bronsvoort, B. M. (2015). Prevalence and patterns of antimicrobial resistance among *Escherichia coli* isolated from Zambian dairy cattle across different production system. *International journal of scientific reports*, *5*, 14162

Malloy, A. M., & Campos, J. M. (2011). Extended-spectrum beta-lactamases. *The pediatric infectious disease journal*, *30*(12), 1092-1093

Manasa, D. P. (2016). Pathology, ecology and infection of *E. coli*. *Research and reviews journal of medical and health sciences*, 5(3)

Marejkova, M., Blahova, K., Janda, J., Fruth, A. & Petras, P. (2013). Enterohemorrhagic *Escherichia coli* as causes of hemolytic uremic syndrome in the Czech Republic. *Plos one*, *8*(9), e73927

Marshall, B. M. & Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clinical microbiology review*, *24*(4), 718-733.

Mazurek, J., Bok, E., Stosik, M. & Baldy-Chudzik, K. (2015). Antimicrobial resistance in commensal *Escherichia coli* from pigs during metaphylactic trimethoprim and sulfamethoxazole treatment and in the post-exposure period. *International jounal of environmental research and public health, 12*(2), 2150-63

Manishimwe, R., Buhire, M., Uyisunze, A., Turikumwenayo, J. B. & Tukei, M. (2017). Characterization of antibiotic resistant *Escherichia coli* in different poultry farming systems in the Eastern Province and Kigali City of Rwanda. *Revue d, élevage et de. Médecine veterinaire des pays tropicaux, 70*(1), 13-19

Mapenay, I. M., Kikuvi, G. M., Mitema, E. S. & Ombui, J. N. (2006). Antibiotic resistance of *Escherichia coli* isolated from healthy food animals in Kenya. *The Kenya veterinarian*, *30*(1), 23-27

Mathur, P., Kapil, A., Das, B. & Dhawan, B. (2002). Prevalence of exttended spectrum beta-lactamase producing gram negative bacteria in a tertiary care hospital. *Indian journal of medical research*, 115, 153-7

Matsumoto, Y., Ikeda, F., Kamimura, T., Yokota, Y. & Mine,Y. (1988). Novel plasmid mediated beta lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. *Antimicrobial agents and chemotheapy*, *32*(8), 1243-6

McDanel, J., Schweizer, M., Crabb, V. & Nelson, R. (2017). Incidence of extended Spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella* infections in the United States: a systematic literature review. *Infection control and hospital epidemiology*, *38*(10), 1209-1215

Mellies, L. J., & Lorenzen, E. (2014). Enterohemorrhagic *Escherichia coli* virulence gene regulation. *Microbiol Spectrum*, *2*(4), 1-17.

Mellmann, A., Bielaszewska, M., Köck, R., Friedrich, A. W., Fruth, A., Middendorf, B., …& Karch, H. (2008). Analysis of collection of hemolytic uremic syndrome–associated enterohemorrhagic *Escherichia coli*. *Emerging infectious diseases*, *14*(8), 1287-1290

Meraz, I. M., Arikawa, K., Nakamura, H., Ogasawara, J., Hase, A. & Nishikawa, Y. (2007). Association of IL-8-inducing strains of diffusely adherent

Escherichia coli with sporadic diarrheal patients with less than 5 years of age. *The brazilian journal of infectious diseases*, *11*(1), 44-49

Michael, G. B., Freitag, C., Wendlandt, S., Eidam, C., Feßler, A. T., Lopes, G. V., Kadlec, K. & Schwarz, S. (2015). Emerging issues in antimicrobial resistance of bacteria from food-producing animals. *Future Microbiology*, 10(3), 427-443

Mininder, K., & Aruna, A. (2013). Occurrence of the CTX-M, SHV and the TEM genes among the extended spectrum beta- lactamase producing isolates of *Enterobacteriaceae* in tertiary care hospital of North India. *Journal of clinical and diagnostic research, 7*(4), 642-645.

Mitema, E. S., Kikuvi, G. M., Wegener, H. C. & Stohr, K. (2001). An assessment of antimicrobial consumption in food producing animals in Kenya. *Journal of veterinary pharmacolology and therapeutics*, *24*(6), 385-90

Miyagi, H., Okada, T., Honda, S., Minato, M. & Taketomi, A. (2012). Appendical perforation by infection with extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*: case report. *Surgical Science*, *3*, 53-55

Morar, M. & Wright., G. D. (2010). Genetic enzymology of antibiotic resistance. *The annual review of genetics*, *44*(1), 25-51

Monstein, H. J., Östhelm-Balkhed, A., Nilsson., M. V., Nilsson, M., Dornbusch & Nilsson, L. E. (2007). Multiplex PCR amplification assay for the detection of bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M}$ genes in *Enterobacteriaceae*. *Acta pathologica microbiologica et immunologica scandinavica journal*, 115, 1400-8

Malik, T., Naim, A. & Saeed, A. (2018). Molecular detection of TEM, SHV and CTX-M genes amomg Gram negative *Klebsiella* isolates. *Current drug delivery*, *15*(3), 417-423

Mora, A., Herrera, A., López, C., Dahbi, G., Mamani, R., Pita, J. M., …& Jorge Blanco, J. (2011). Characteristics of the Shiga-toxin-producing enteroaggregative *Escherichia coli* O104: H4 German outbreak strain and of STEC strains isolated in Spain. *International microbiology* , 14, 121-141.

Mostafa, M. M., Ahmed, S. F., Klena, D. J., Mohamed, Z. K., Moussa, T. A. & Ghenghesh, K. S. (2014). Enteroaggregative *Escherichia coli* in diarrheic children in Egypt: molecular characterization and antimicrobial susceptibility. The *Journal of infectionin in develpimg countries*, *8*(5), 589-596.

Moxon, A. C. & Paulus, S. (2016). Beta lactamases in *Enterobacteriaceae* infection in children. *Journal of infection*, *72*, S41-S49

Munita, M. J. & Arias, A. C. (2016). Mechanisms of antibiotic resistance. *Microbiology spectrum*, *4*(2), 1-37

Muvunyi, M. C., Masaisa, F., Bayingana, C., Mutesa, L & Musemakweri, A. (2013). Antimicrobial resistance and extended-spectrum beta-lactamase (ESBL) producing clinical isolates from urinary tract infection at two teaching hospitals in Rwanda. *Rwanda Journal*, *1*(1), 1-14

Naas, T., Coignard, B., Carbonne, A., Blanckaert, K., Bajolet, O., Bernet, C., …& Nordmann, P. (2006). VEB-1 extended spectrum beta-lactamase-producing *Acinetobacter baumanii*, France. *Emerging infectious diseases*, *12*(8), 1214-1222

Naas, T., Poirel, L. & Nordmann, P. (2008). Minor extended-spectrum beta-lactamases. *Clinical microbiology infection, 14*(Suppl. 1), 42-52

Nahar, A., Awasthi, S. P., Hatanaka, N., Okuno, K., Hoang, P. H., Hassan, J., …& Yamasaki, S. (2018). Prevalence and characteristics of extended spectrum beta-lactamase-producing *Escherichia coli* in domestic and imported chicken meats in Japan. *The journal. of veterinary. medical. sciences*, *80*(3), 510-517

Nataro, J. P. & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbialology Review, 11*, 142-201

Nathan, C. & Cars, O. (2014). Antibiotic resistance-problems, progress, and prospects. *The new england journal of medicine*, *371*(19), 1761–1763

Naval Research Laboratory (2017). High prevalence of antibiotic resistance in Kenya. Retrieved from ScienceDaily website: https://www.sciencedaily.com

Ndihokubwayo, J. B., Yahaya, A. A., Desta, T. A., Ki-Zerbo, G., Odei, A. M., Bah, K., …& Nkhoma, W. (2013). Antimicrobial resistance in the African Region: issues, challenges and actions proposed. African health monitor, *16*, 27-30

Newell, D. G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., …& Kruse, H. (2010). Food borne diseases- the challenges of 20 years ago still persist while new ones continue to emerge. *International Journal of food Microbiology, 139*(Suppl.1), S3-S15

Newitt, S., MacGregor, V., Robbins, V., Bayliss, L., Chattaway, M. A., Dallman,T., …& Hawker, J. (2016). Two linked Enteroinvasive *Escherichia coli* outbreaks Nottingham, UK, June 2014. *Emerging infectious diseases*, *22*(7), 1178-1184.

Ng, C & Gin, K. Y. (2019). Monitoring antimicrobial resistance dissemination in aquatic systems. *Water*, *11*,71

Ngo, X. H. & Garneau-Tsodikova, S. (2016). Flipping the switch “on” for aminoglycosides resistance enzymes: the mechanism is finally revealed! *Cell*, structure *24*, 1011-1013

Nguyen, Y., & Sperandio, V. (2012). Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *cellular and infection microbiology*, 2-7

Nguyen, V. T., Van, L. P., Huy, L. C., Gia, N. K. & Weintraub, A. (2005). Detection and characterization of diarrheagenic escherichia coli from young children in Hanoi, Vietnam. *Journal of clinical microbiology*, *43*(2), 755–760

Nhung, N. T., Cuong, N. V., Thwaites, G. & Carrique-Mas, J. (2016). Antimicrobial usage and antimicrobial resistance in animal production in Southeast Asia: A Review. *Antibiotics, 5*(37), 1-24

Nicklasson, M., Klena, J., Rodas, C., Bourgeois, A. L., Torres, O., Svennerholm, A. & Sjöling, A. (2010). Enterotoxigenic *Escherichia coli* multilocus sequence types in Guatemala and Mexico. *Emerging infectious diseases*, *16*(1), 143-146.

Nikolaidis, I., Favini-Stabile, S. & Dessen, A. (2014). Resistance to antoibiotics targeted to bacterial cell wall. *Protein sciences*, *23*(3), 243-259

Njoroge, S., Muigai, A. W. T., Njiruh, P. N. & Kariuki, S. (2013). Molecular characterisation and antimicrobial resistance patterns of *Escherichia coli* isolates from goats slaughtered in parts of Kenya. *East African Medical Journal, 90*(3), 73-83

Njoroge, M. S., Kiiru, N. J. & Kikuvi, M. G. (2014). Broad spectrum β-lactam resistance in faecal *Escherichia coli* isolated from severely malnourished and nourished children attending Mbagathi district hospital, Nairobi: A case... *Chronicles of Young Scientists*, *5*(1), 39

Nordmann, P., Dortet, L. & Poirel, L. (2012). Rapid detection of extended spectrum beta-lactamase-producing *Enterobacteriaceae*. *Journal of clinical microbiology*, *50*(9), 3016-3022

Nordmann, P. & Poirel, L. (2002). Emerging carbapenemases in Gram-negative aerobes. *Clininical microbiology and infection*, *8*(6), 321-331.

Nsofor, C. A. & Iroegbu, C. U. (2012). Antibiotic resistance profile of *Escherichia coli*isolated from apparently healthy domestic livestock in South-East Nigeria. *Journal of cell and animal biology, 6*(8), 129-135

Nsofor, C. A. Iroegbu, C. U., Call, R. D. & Davies, A. M. (2013). The genetic relatedness of drug resistant *E.coli* isolates of human and animal origin in Nigeria. *International journal of genetics and molecular biology, 5*(3), 47-41

Ochoa, T. J., Ruiz, J., Molina, M., Del Valle, L. J., Vargas, M., Gil, A. I., …& Lanata, C. F. (2009). High frequency of antimicrobial drug resistance of diarrheagenic *Escherichia coli* in infants in Peru. *The american jounal of tropical medicine and hiegene*, *81*(2), 296-301

Ochi, S., Shah, M., Odoyo, E., Bundi, M., Miringu, G., Guyo, S., …& Ichinose., Y (2017). An outbreak of diarrhea in Mandera, Kenya, due to *Escherichia coli* sero-group non typable strain that had a coding gene for Enteroaggregative *E. coli* heat-stable enterotoxin 1. *The american journal of tropical medicine and hiegene*, *96*(2), 457-464

Ogunleye, A. O, Okunlade, A. O, Jeminlehin, F. O. & Ajuwape, A. T. P (2013). Antibiotic resistance in *Escherichia coli* isolated from healthy cattle at a major cattle market in Ibadan, Oyo State, South Western, Nigeria. *African journal of microbiology research, 7*(37), 4572-4575

Okhuysen, P. C. & DuPont, H. L. (2010). Enteroaggregative *Escherichia coli* (EAEC): a cause of acute and persistent diarrhea of worldwide importance. *The journal of infectious diseases, 202*(4), 503-505

Olsen, I., Hasman, H. & Aarestrup, F. M. (2004). Prevalence of beta-lactamases among ampicillin-resistant *Escherichia coli* and Salmonella isolated from food animals in Denmark. *Microbial drug resistance*, *10*(4), 334-342

Omulo, S., Thumbi, M. S., Njenga, K. M. & Call, I. D. (2015). A review of 40 years of enteric antimicrobial resistance research in Eastern Africa: what can be done better. *Antimicrobial resistance and infection control*, *4*, 1

Onanuga, A., Igbeneghu, O. & Lamikanra, A. (2014). A study of the prevalence of diarrheagenic *Escherichia coli* in children from Gwagwalada, federal captal territory, Nigeria. *The pan african medical journal*, *17*, 46

Oppegaard, H., Steinum, T. M. & Wasteson, Y. (2001). Horizontal transfer of a multi-drug resistance plasmid coliform bacteria of human and bovin origin in a farm environment. *Applied and environmental microbiology*, *67*, 3732-3734

Ouedraogo, A. S., Sanou, M., Kissou, A., Sanou, S., Solaré, H., Kaboré, F., …& Godreui, S. (2016). High prevelence of extended-spectrum beta-lactamases producing *Enterobacteriaceae* among clinical isolates in a Burkina Faso. *Infectious diseases*, *16*, 326.

Orskov, E. & Orskov, I. (1992). *Escherichia coli* serotyping and disease in man and animals. The *canada journal microbiology*, *38*(7), 699-704

Page, A. V., & Liles, C. W. (2013). Enterohemorrhagic *Escherichia coli* infections and the hemolytic-uremic syndrome. *Medical clinics of north america*, *97*, 681-695

Paiva de Sousa, C. (2006). *Escherichia coli* as a specialized bacterial pathogen. *Revista de biologia E ciências da terra*, *6*(2), 341-352

Pal, T., Al-Sweih, N. A., Herpay, M. & Chugh, T.D. (1997). Identification of Enteroinvasive *Escherichia coli* and *Shigella* strains in pediatric patients by an IpaC-specific enzyme-linked immunosorbent assay. *Journal of clinical microbiology, 35*(7), 1757-1760

Pallecchi, L., Malossi, M., Mantella , A, Gotuzzo, E., Trigoso, C., Bartoloni, A., …& Rossolini, G. M. (2004). Detection of CTX-M-Type beta-Lactamase genes in

fecal *Escherichia coli* isolates from healthy children in Bolivia and Peru. *Antimicrobial agents and chemotherapy*, 4556-4561.

Panchalingam, S., Antonio, M, Hossain, A., Mandomando, I, Ochieng, B., Oundo, J., …& Nataro, P. J. (2012). Diagnostic microbiologic methods in the GEMS-1 case-control study. *Clinical infectious diseases*, 55(S4), S294-302

Pari, Q. F. J., Rojas, I. O. J., Mucha, C. M. A., Ortega, C. L. M., Hinojosa, C. J. H., Otivo, M. R., …& Sagüez, S. D. L. F. (2018). Carbapenemase producing *Klebsiella pneuminiae* in Peru: a case report and antimicrobial resistance discussion. *Medwave*, *18*(2), e7191

Partridge, S. R (2015). Resistance mechanisms in *Enterobacteriaceae*. *Pathology*, *47*(3), 276-284

Paterson, D. L., & Bonomo, R .A. (2005). extended-Spectrum bata lactamases: a clinical update. *Clinical microbiology reviews*, 657-686.

Patzi-Vargas, S., Zaidi, M., Bernal-Reynaga, R., León-Cen, M. , Michel, A. & Estrada-Garcia, T. (2013). Persistent bloody diarrhoea without fever associated with diffusely adherent *Escherichia coli* in a young child. *Journal of medical microbiology*, *62*, 1907-1910

Paul, S., Jhora, T. S & Dey, P. P. (2014). Evaluation of phenotypic method to identify extended spectrum beta-lactamase (ESBL) producing gram negative bacteria. *Bangladesh journal of medical microbiology*, *08*(01), 21-24

Pereira, A. L., & Giugliano, L. G. (2013). Adhesion of diarrheagenic *Escherichia coli* and inhibition by glycocompounds engaged in the mucosal innate immunity. *Biology*, *2*, 810-831

Pérez, C., Gómez-Duarte , O. G. & Arias, M. L. (2010). Diarrheagenic *Escherichia coli* in children from Costa Rica. *The american journal of tropical medicine and hiegene*, *83*(3), 292-297

Pierard, D., Damme, L. V., Moriau, L., Stevens, D. & Lauwers, S. (1997). Virulence factors of verocytotoxin-producing *Escherichia coli* isolated from raw meats. *Applied and environmental microbiology*, *63*(11), 4585-4587

Pitout, J. D., & Laupland, K. B. (2008). Extended-spectrum beta-lactamase-producing *Enterobacteriaceae:* an emerging public-health concern. The *Lancet infectious diseases, 8, 159-66*

Prorok-Hamon, M., Friswell, M. K , Alswied, A., Roberts, C. L., Song, F, Flanagan, P.K., …& Campbell, B.J (2013). Colonic mucosa-associated diffusely adherent afaC+ *Escherichia coli* expressing lpfA and pks are increased in inflammatory bowel disease and colon cancer. *Gut microbiota*, *0,* 1-10.

Philippon, A., Labia, R. & Jacoby, G. (1989). Extended-spectrum beta-lactamases. *Antimicrobial agents and chemotherapy*, *33*(8), 1131-1136.

Podolsky, H. S. (2018) The evolving response to antibiotic resistance (1945-2018). *Algrave communications*, 4,124,

Public Health England. (2015*)*. United Kingdom for microbiology investigations *Identification of Enterobacteriaceae.* London. UK Standards for Microbiology Investigations. Retrieved from: www.apsi.it>ufiles>smi>i...

Public Health England (2016). United Kingdom standards for microbiology investigation. *Detection of Enterobacteriaceae producing extended spectrum Beta-lactamases*. *Bacteriology, B 59(4*.1), 1-31

Rahman, U. S., Ali, Pariq, Ali, I., Khan, N. A, Han, B. & Gao, J. (2018). The growing genetic and functional diversity of extended spectrum beta-lactamases. *Biomed research international, 14*

Rahmouni, O, Vignal, C., Titécat, M., Foligné, B., Pariente, B., Dubuquoy, L, Desreumaux, P. & Neut, C. (2018). High carriage of adherent invasive *E. coli* in wildlife and healthy individuals. *Gut pathogen, 10*(23), 1-13

Raut, S., Gokhale, S. & Adhikari, B. (2015). Prevalence of extended spectrum beta-lactamases among *Escherichia coli* and *Klebsiella spp* isolates in Manipal Teaching Hospital, Pokhara, Nepal. *Journal of microbiology and infectious diseases*, *5*(2), 69-75

Rawat, D., & Nair, D. (2010). Extended-Spectrum beta lactamases in Gram Negative Bacteria. *Journal global infectious diseases, 2*(3), 263-274.

Rice, E. W., Allen, M. J., Brenner, D. J., Stephen, C. & Edberg, S. C. (1991). Assay for beta-glucuronidase in species of the genus *Escherichia* and its applications for drinking-water analysis. *Applied and environmental microbiology*, *57*(2), 592-593.

Rice, E. W., Allen, M. J., Covert, T. C., Langewis, J. & Standridge, J. (1993). Identifying *Escherichia species* with biochemical tests kits and standard bacteriological tests. *American water works associations*, 74-76

Rice, T., Quinn, N., Sleator, R. D. & Lucey, B. (2016). Changing diagnostic methods and increased detection of verotoxigenic *Escherichia coli*, Ireland. *Emerging infectious diseases*, *22*(9), 1656-1657.

Rupp, M. E., & Fey, P. E. (2003). Extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*. *Drug*, *63*(4), 363-365.

Saedii, A. A. F., Abdelraheim, A. R., Abdel-Aziz, A. A. & Swelam, S. H. (2017). ESBL-Producing *E. coli* and *Klebsiella* among patients treated at Minia University Hospitals. *The journal of infectious diseases and preventive medicine, 5*(2)*,*10.4172/2329-8731.1000156.

Sah, S. K., & Hemalatha, S. (2015). Extended spectrum beta-lactamase (ESBL) mechanism of antibiotic resistance and epidemiology. *Ineternational.journal of pharm tech research, 7*(2), 303-309

Sahla, J. W., Sistrunk, J. R, Fraser, C. M., Hine, E., Baby, N., Begum, Y., …& Gilmore, M. S. (2015). Examination of Enterotoxigenic *Escherichia coli* population structure during human infection. *mBio*, 6(3), e00501-15

Sahoo , K. C., Tamhankar, A. J, Sahoo S., Sahu, P. S., Klintz, S. R. & Lundborg, C. S. (2012). Geographical variation in antibiotic-resistant *Escherichia coli* isolates from stool, cow-dung and drinking water. *International journal of environmental research and public health*, *9*, 747-759

Samaha-Kfoury, J. R. & Araj, F. G (2003). Recent development in β lactamases and extended spectrum beta-lactamases. *British medical journal*, h(22), 1209-13

Scalestsky, I. C. A., Fabbricotti, S. H., Carvalho, R. L. B., Nunes, C. R., Maranhao, H. S., Morais, M. B. & Fagundes-Neto, U. (2002). Diffusely Adherent *Escherichia coli* as a cause of acute diarrhea in young children in northeast Brazil: a Case-Control Study. *Journal of clinical microbiology*, *40*(2), 645–648.

Scaletsky, I. C., Fabbricotti, S. H., Silva, S. O. C., Morais, M. B. & Fagundes-Neto, U. (2002). HEp-2-Adherent *Escherichia coli* strains associated with acute infantile diarrhea São Paulo, Brazil. *Emerging infectious diseases*, *8*(8), 855-858.

Scavia, G., Stoffalani, M., Fisichella, S., Striano, G., Colletta, S., Ferri, G., …& Caprioli, A. (2008). Enteroaggregative *Escherichia coli* associated with a foodborne outbreak of gastroenteritis. *Journal of medical microbiology*, *57*, 1141-1146

Scheutz, F., & Strockbine, N. A. (2005). Genus I. *Escherichia. Family I. Enterobacteriaceae,* 608-624

Schlundt, J.,Toyofuku, H., Jansen, J. & Herbst, S. A (2004). Emerging food-borne zoonoses. *Revue scientifique et technique, 23*, 513-533

Schmid, A., Hörmansdorfer, S., Messelhäusser, U., Käsbohrer, A., Sauter-Louis, C. & Mansfeld, R. (2013). Prevalence of extended spectrum β-lactamase producing *Escherichia coli* on bavarian dairy and beef cattle farms. *Applied and environmental microbiology*, *79*(9), 3027-3032

Shah, A. A., Hassan, F., Ahmed, S. & Hameed, A. (2004). Extended spectrum beta-lactamases (ESBLs): characterization, epidemiology and detection. *Clinical reviews in microbiology*, *30*(1), 25-32

Shah, M., Kathiiko, C., Wada, A., Odoyo, E., Bundi, M., Miringu, G., …& Ichinose, Y. (2016). Prevalence, seasonal variation and antibiotic resistance pattern of enteric bacterial pathogens among hospitalized diarrheic children in suburban regions of cental Kenya. *Tropical medicine and health, 44*, 39

Shah, K. M., Aziz, A. S., Zakaria, Z., Lin, C. L. & Goni, D. M. (2018). A review on pathogenic *Escherichia coli* in Malaysia. *Advances in animal and veterinary sciences*, *6*(2), 95-107

Shaikh, S., Fatima, J., Shakil, S., Mohd, S., Rizvi, D. & Kamal, M. A. (2015). Antibiotic resistance and extended spectrum beta-lactamases: types, epidemiology and treatment. *Saudi journal biological science*, *22*(1), 90-101.

Sharma, J., Sharma, M. & Ray, P. (2010). Detection of TEM and SHV genes in *Escherichia coli* and *Klebsiella pneumoniae* isolates in a tertiary care hospital from India. *Indian journal of medical research*, *132*, 332-336

Sharma, S., Kaur, N., Malhotra, S., Madan, P., Ahmad, W. & Hans, C. (2016). Serotyping and antimicrobial susceptibilty pattern of *Escherichia coli* isolates from Urinary tract infections in pediatric population in a Tertiary Care Hospital. *Journal of pathogens*, *4*

Sharp, H., Valentin, L., Fisher, Y., Guerra, B., Appel, B. & Käsbohrer, A. (2014). Estimation of the transfer of ESBL producing *Escherichia coli* to humans in

Germany. *Berliner und Münchener Tierärztliche. Wschenschrift, 127*, 463-476

Sihem, M., Salah, H., Riadh, M. & Salah, A. M. (2015). Overview of ESBL-producing *Escherichia coli* of animal origin in Tunisia; in the way of global spread of CTX-M beta-lactamases. *Archives of clinical microbiology*, *6*(24)

Silver, L. L. (2007). Fosfomycin: mechanism and resistance. *Cold spring harbor perspectives in medicine, 7*(2), 1-12

Singer, A. C, Shaw, H, Rhodes, V. & Hart, A. (2016). Review of antimicrobial resistance in the environment and its relevance to environmental regulators. *Frontiers microbiology*, 7, 1728

Sirot, D., Sirot, J., Labia, R. & Cluzel, R. (1987). Transferable resistance to third generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-M1, a novel beta-lactamase. *The Journal of antimicrobial chemotherapy*, *20*(3), 323-334.

Sizemore, D., Roland, K. L. & Ryan, U. S. (2004). Enterotoxigenic *Escherichia coli* virulence factors and vaccine approaches. *Expert review of vaccines*, 3(5), 585-595.

Slavchev, G., Pisareva, E. & Markova, N. (2009). Virulence of uropathogenic *Escherichia coli*. *Journal of culture collections*, *6*, 3-9.

Spanu, T., Sanguinetti, M., Tumbarello, M., D'Inzeo, T., Fiori, B., Posteraro, B., …& Fadda, G. (2006). Evaluation of the new vitek 2 extended-spectrum beta-lactamase (ESBL) test for rapid detection of ESBL production in *Enterobacteriaceae* isolates. *Journal of clinical microbiology*, *44*(9), 3257-3262

Sridhar, R. P. (2015). Extended Spectrum beta-lactamase. *A comprehensive review*, 1-109

Steward, C. D., Rasheed, J. K., Hubert, S. K., Biddle, J.W., Raney, P. M. & Anderson, G. J. (2001). Characterization of clinical isolates from *Klebsiella pneumoniae* from 19 laboratories using the National committee for clinical laboratory standards etended spectrum beta-lactamase detection. *Journal of clinical microbiology*, *39*, 2864-2872

Storberg, V. (2014). ESBL producing *Enterobacteriaceae* in Africa-a non systematic literature review of research published 2008-2014. *Infection ecology and epidemiology, 4*, 20342

Subashini, J. & Kannabiran, K. (2013). Screening and identification of extended spectrum beta-lactamase (ESBL) pathogens in urine sample of UTI patient's. *Tropical Medicine and Surgery, 1*(3), 1-4

Sultan, I., Rahman, S., Jan, T. A. , Siddiqui, T. M., Mondal, H. A. & Haq, M. R. Q. (2018). Antibiotics, resistome and resistance mechanisms: a bacterial persepective. *Frontiers in microbiology*, *9*(2066), 1-16

Szmolka, A. & Nagy, B. (2013). Multidrugresistant commensal *Escherichia coli* in animals and its impact for public health. *Frontiers in microbiology, 4*(258), 1-13

Tadesse, T.B., Ashley, A. E., Ongarello, S., Havumaki, J., Wijegoonewardena, M., González, J. I & Dittrich, S. (2017). Antimicrobial resistance in Africa: a systematic review. *Biomed central infectious diseases, 17*, 616

Taneja, N. & Sharma, M. (2008). ESBLs detection in clinical microbiology: why & how ? *Indian Journal of Medical Research, 127*, 297-300.

Tansarli, G. S., Poulikakos, P., Kapaskelis, A. & Falagas, M. E. (2014). Proportion of extended-spectrum beta-lactamase (ESBL)-producing isolates among Enterobacteriaceae in Africa: evaluation of the evidence-systematic review. *Journal of antimicrobial chemotherapy, 69*, 1177-1184.

Tharian, A. J, Padmapriya, R. &Thirunalasundari, T. (2013). Chloramphenicol acetyl transferase producing bacteria. *Advances in applied science research*, *4*(4), 413-419

Tärnberg, M. (2012). *Extended-spectrum beta-lactamase producing Enterobacteriaceae: aspects on detection, epidemiology and multi-drug resistance*. Sweden: LiU-Tryck.

Tauschek, M., Gorrell, R.J, Strugnell, R. A & Robins-Browne, R. M. (2002). Identification of a protein secretory pathway for the secretion of heat-labile enterotoxin by an enterotoxigenic strain of *Escherichia coli*. *Proceedings of the national academyof sciences* , *99*(10), 7066-7071

Teuber, M. (2001). Veterinary use and antibiotic resistance. *Current opinion in microbiology, 4*(5), 493-9

Tham, J. (2012). *Extented-spectrum beta-lactamase producing Enterobacteriaceae: epidemiology,risk factors and duration of carriage*. Sweeden: Lund University.

Thenmozhi, S., Moorthy, K., Sureshkumar, B. T. & Suresh, M. (2014). Antibiotic resistance mechanism of ESBL producing *Enterobacteriaceae* in clinical field: Review. *International journal of pure and applied science*, *2*(3), 207-226

Tomar, R. S., Agarwal, M. & Jyoti, A. (2015). Determination of drug resistance and virulent gene signatures in potable water isolates of *Escherichia coli* in Gwalior city. *Journal of pharmaceutical sciences and research*, *7*(11), 967-971

Trung, N. V., Nhung, H. N., Carrique-Mas, J. J., Mai, H. H., Tuyen, H. T., Campbell, J., …& Hoa, N. T. (2016). Colonization of Enteroaggregative *Escherichia coli* and shiga toxin -producing *Escherichia coli* in chickens and humans in Southern Vietnam. *Biomed central* Microbiology, *16*, 208

Turner, J. P. (2005). Extended spectrum beta-lactamases. *Clinical infectious diseases, 41*, S273-5

Ullah, W., Qasim, M., Rahman, H., Bari, F., Khan, S., Dworeck, T. & Muhammad, N. (2017). Molecular identification of TEM-116 beta-lactamase gene in isolates of pathogenic *Pseudomonas aeruginosa:* a first report from Pakistan. *Tropical journal for pharmaceutical research, 16*(1), 149-154

Urumova, V. (2015). Extended spectrum beta-lactamase producing animal *Enterobacteriaceae* isolates as potential risk to public health-review. *Revue de médecine vétérinaire*, *166*(7-8), 192-207

Valentin, L., Sharp, H., Hille, K., Seibt, U., Fischer, J., Pfeifer, Y., …& Käsbohrer, A. (2014). Subgrouping of ESBL-producing *Escherichia coli* from animal and human sources: an approach to quantify the distribution of ESBL types between different reservoirs. *International journal of medical microbiology*. *304*, 805-816

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T.P, …& Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the national academyof sciences*, *112*(18), 5649-5654

van den Bogaard, A. E, London, N., Driessen, C. & Stobberingh, E. E. (2001). Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultryslaughterers. *Journal of antimicrobial chemotherapy*, *47*, 763–771

van der Bij, K. A., Pierano, G., Goessens, F. H. W., van der Vorm, E. R., van Westreenen, V. & Pitout, D. D. J. (2011). Clinical and molecular characteristics of extended spectrum beta-lactamase-producing *Escherichia coli* causing bacteremia in the Rotterdam area, Netherlands. *Antimicrobial agents and chemotherapy*, *55*(7), 3576-3578

van Duin, D. & Paterson, D. (2016). Multidrug Resistant Bacteria in the Community: Trends and Lessons Learned. *Infectious disease clinics*, *30*(2), 377-390

van Hoek, A. H. A. M., Mevius, D., Guerra, B., Mullany, B., Roberts, A. P. & Henk, J. M. (2011). Acquired antibiotic resistance gene: an overview. *Frontiers in microbiology*, *2*, 203

Vieira, M. A .M, Andrade, J. R. C., Trabulsi, L. R., Rosa, A. C. P., Angela, M. G., Dias, A. M. G.,…& Gomes, T. A. T. (2001). Phenotypic and genotypic characteristics of *Escherichia coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry eae and lack the EPEC adherence factor and shiga toxin DNA probe sequences. *The journal of infectious diseases*, *183*, 762-772.

Villaseca, J. M., Hernandez, U., Sainz-Espunes, T. R., Rosario, C. & Eslava, C. (2005). Enteroaggregative *Escherichia coli* an emergent pathogen with different virulence properties. *Rev latinoam microbiol*, *47*(3-4), 140-159

Villegas, A. N., Baronetti, J., Albesa, I., Polifroni, R., Parma, A., Etcheverría, B., …& Paraje, M. (2013). Relevance of biofilms in the pathogenesis of shiga-toxin producing *Escherichia coli* infection. *The scientific world journal*, 2013, 1-7

Villegas, M. V, Kattan, J. N., Quinteros, M. G. & Casellas, J. M. (2008). Prevalence of extended spectrum beta-lactamases in South America. *Clinical microbiology and infection*, *14*(suppl.1), 154-158.

Vrioni, G., Tsiamis, C., Oikonomidis, G., Theodoridou, K., Kapsimali, V. & Tsakris, A. (2018). Matrix-assited laser desorption/ionization-time of flight mass spectrometry technology for detecting biomarkers of antimicrobial resistance: current achievements and future perspectives. *Annals of translational medicine*, *6*(12), 240

Walkty, A., Lagacé-Wiens, A. & Karlowsky, J. (2016). Extended-spectrum beta-lactamase producing *Escherichia coli*: increasing incidence of a resistant pathogen. *micronotes*, 1-3.

Wallace-Gadsden, F., Johnson, J.R., Wain, J. & Okeke, N.I. (2007). Enteroaggregative *Escherichia coli* related to uropathogenic clonal group A. *Emerging Infectious Diseases*, *13*(5), 757-760.

Wang, X., Yan, Q., Xia, X., Zhang, Y., Li, D.,Wang, C., …& Houf, R. (2013). Serotypes, virulence factors, and antimicrobial susceptibilities of vaginal and fecal isolates of *Escherichia coli* from giant pandas. *Applied and environmental microbiology*, 5146-5150

Wangai, F. K., Masika, M. M., Lule, G. N, Karari, E. M., Maritim, M. C. & Jaoko, W. G. (2019). Bringing antimicrobial resistance knoweldge gaps: the East african perspective on a global problem. *PLos one*, *14*(2), e0212131

Wassenaar, M. T. (2018). *Escherichia coli* and colorectal cancer: a complex relationship that deseves a critical mindset. *Critical reviews in Microbiology*, 44, 5

Weintraub, A. (2007). Enteroaggregative *Escherichia coli*: epidemiology, virulence and detection. *Journal of Medical Microbiology*, *56*, 4-8.

Welch, R. A. (2006). The genus *Escherichia. Procaryotyes*, *6*, 60-71.

Welinder-Olsson, C. & Kaijser, B. (2005). Enterohemorrhagic *Escherichia coli* (EHEC). *Scandinavian journal of infectious diseases*, *37*, 405-416

Wetzker, W., Pfeifer, Y., Wolke, S., Haselbeck, A., Leistner, R., Kola, A., …& Salm, F. (2019). Extended-spectrum beta-lactamase (ESBL)-producing escherichia coli isolated from flies in the urban center of Berlin, Germany. *International journal of environmental research and public health, 16*, 1530

Winokur, P. L., Vonstein, D. L., Hoffman, L. J., Uhlenhopp, E. K. & Doern, G. V. (2001). Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. *Antimicrobial. Agents and chemotherapy.*, *45*, 2716-2722

World Health Organization (2014). Antimicrobial resistance: global Report on Surveillance. *World Health Organization*. Retrieved from: https://apps.who.int/iris/handle/10665/112642

World Health Organization (2018). Antimicrobial resistance in the WHO European Region. *World health organization*. Retrieved from: http://www.euro.who.int.

World Health Organization (2018). High levels of antibiotic resistance found worldwide, new data shows. *World Health Organization*. Retrieved from: Retrieved from: https://apps.who.int

Wu, C., Wang, Y., Shi, X., Wang, S., Ren, H., Shen, Z., …& Wang, S.(2018). Rapid rise of the ESBL and mcr-1 genes in *Escherichia coli* of chicken origin in China, 2008–2014. *Emerging Microbes and Infections*, 7(30), 1-10

Wright, G. D. (2005) Bacterial resistance to antibiotics: enzymatic degradation and modication. *Advanced drug delivery. Reviews*, *57*(10), 1451–1470.

Xu, Y., Sun, H., Bai, X., Fu, S., Fan, R. & Xiong, Y.(2018). Occurrence of multidrug-resistant and ESBL-producing atypical enteropathogenic *Escherichia coli* in China. *Gut Pathogens, 10*(8), 1-12

Yanling, J., Xin, L. & Zhiyuan, L. (2013). The antibacterial drug discovery. *The antibacterial drug discovery*, 290-307, http://dx.doi.org/10.5772/52510

Yuan, L., Liu, J., Hu, G., Pan, Y., Liu, Z., Mo, J. & Wei, Y. (2009). Molecular of extended-spectrum b-lactamase-producing *Escherichia coli* isolates from chickens in Henan Province, China. *Journal of Medical Microbiology*, *58*, 1449–1453

Zhang, H., Rehman, U. M., Li, K., Luo, H., Lan, Y., Nabi, F., & Li, J. (2017). Antimicrobial resistance of *Escherichia coli* isolated from Tibetan piglets suffering from white score diarrhea. *Paskan veterinary journal*, *37*(1), 43-46

Zhang, R., Gu, D., Huang, Y., Chan, E.W., Gong-Xiang Chen, G. & Chen, S. (2016). Comparative genetic characterization of Enteroaggregative *Escherichia coli* strains recovered from clinical and non-clinical settings. *Scientific Reports*, *6*, 24321.

Zhao, S., Blickenstaff, K., Bodeis-Jones, S., Gaines, S. A, Tong, E. & McDermott, P.F. (2012). A comparison of the prevalence and antimicrobial resistance of *Escherichia coli* from 2 different retail meats in the United States: 2002-2008. *Applied Environmental micobiology,* doi: 10.1128/AEM.07522-11

Zinnah, M. A., Bari, M. R, Islam, M. T., Hossain, M. T., Rahman, M. T., Haque, M.H., …& Islam, M. A. (2007). Characterization of *Escherichia coli* isolated from samples of different biological and environmental sources. *Bangladesh journal of veterinary medicine*, *5*(1 and 2), 25-32

Zurfluh, K., Hächler, H., Nüesch-Inderbinen, M. & Stephan, R. (2013). Characteristics of extended-spectrum beta-lactamase (ESBL)-and carbapenemase producing *Enterobacteriaceae* isolated from rivers and lakes in Switzerland. *Applied environmental microbiolology*. doi:10.1128/AEM.00054-13

APPENDICES

Appendix I: Random number table

TABLE A Random Digits

	00000 12345	00001 67890	11111 12345	11112 67890	22222 12345	22223 67890	33333 12345	33334 67890	44444 12345	44445 67890
01	85967	73152	14511	85285	36009	95892	36962	67835	63314	50162
02	07483	51453	11649	86348	76431	81594	95848	36738	25014	15460
03	96283	01898	61414	83525	04231	13604	75339	11730	85423	60698
04	49174	12074	98551	37895	93547	24769	09404	76548	05393	96770
05	97366	39941	21225	93629	19574	71565	33413	56087	40875	13351
06	90474	41469	16812	81542	81652	45554	27931	93994	22375	00953
07	28599	64109	09497	76235	41383	31555	12639	00619	22909	29563
08	25254	16210	89717	65997	82667	74624	36348	44018	64732	93589
09	28785	02760	24359	99410	77319	73408	58993	61098	04393	48245
10	84725	86576	86944	93296	10081	82454	76810	52975	10324	15457
11	41059	66456	47679	66810	15941	84602	14493	65515	19251	41642
12	67434	41045	82830	47617	36932	46728	71183	36345	41404	81110
13	72766	68816	37643	19959	57550	49620	98480	25640	67257	18671
14	92079	46784	66125	94932	64451	29275	57669	66658	30818	58353
15	29187	40350	62533	73603	34075	16451	42885	03448	37390	96328
16	74220	17612	65522	80607	19184	64164	66962	82310	18163	63495
17	03786	02407	06098	92917	40434	60602	82175	04470	78754	90775
18	75085	55558	15520	27038	25471	76107	90832	10819	56797	33751
19	09161	33015	19155	11715	00551	24909	31894	37774	37953	78837
20	75707	48992	64998	87080	39333	00767	45637	12538	67439	94914
21	21333	48660	31288	00086	79889	75532	28704	62844	92337	99695
22	65626	50061	42539	14812	48895	11196	34335	60492	70650	51108
23	84380	07389	87891	76255	89604	41372	10837	66992	93183	56920
24	46479	32072	80083	63868	70930	89654	05359	47196	12452	38234
25	59847	97197	55147	76639	76971	55928	36441	95141	42333	67483
26	31416	11231	27904	57383	31852	69137	96667	14315	01007	31929
27	82066	83436	67914	21465	99605	83114	97885	74440	99622	87912
28	01850	42782	39202	18582	46214	99228	79541	78298	75404	63648
29	32315	89276	89582	87138	16165	15984	21466	63830	30475	74729
30	59388	42703	55198	80380	67067	97155	34160	85019	03527	78140
31	58089	27632	50987	91373	07736	20436	96130	73483	85332	24384
32	61705	57285	30392	23660	75841	21931	04295	00875	09114	32101
33	18914	98982	60199	99275	41967	35208	30357	76772	92656	62318
34	11965	94089	34803	48941	69709	16784	44642	89761	66864	62803
35	85251	48111	80936	81781	93248	67877	16498	31924	51315	79921
36	66121	96986	84844	93873	46352	92183	51152	85878	30490	15974
37	53972	96642	24199	58080	35450	03482	66953	49521	63719	57615
38	14509	16594	78883	43222	23093	58645	60257	89250	63266	90858
39	37700	07688	65533	72126	23611	93993	01848	03910	38552	17472
40	85466	59392	72722	15473	73295	49759	56157	60477	83284	56367
41	52969	55863	42312	67842	05673	91878	82738	36563	79540	61935
42	42744	68315	17514	02878	97291	74851	42725	57894	81434	62041
43	26140	13336	67726	61876	29971	99294	96664	52817	90039	53211
44	95589	56319	14563	24071	06916	59555	18195	32280	79357	04224
45	39113	13217	59999	49952	83021	47709	53105	19295	88318	41626
46	41392	17622	18994	98283	07249	52289	24209	91139	30715	06604
47	54684	53645	79246	70183	87731	19185	08541	33519	07223	97413
48	89442	61001	36658	57444	95388	36682	38052	46719	09428	94012
49	36751	16778	54888	15357	68003	43564	90976	58904	40512	07725
50	98159	02564	21416	74944	53049	88749	02865	25772	89853	88714

Appendix II: Media Preparation

Material

- Calibrated analytical balance
- Spatula
- Small dish
- Autoclave
- Heating plate
- Water bath
- Petri dishes
- Fillers
- Pipettes
- Micropipettes
- Gas and burner
- Tins of powered media
- Measuring cylinder
- Glycerin (100%)
- Screwable tubes
- Cryovial of 1.5ml

MacConkey Agar (without salt)

- Suspend 47g in 1liter of distilled water
- Bring to the boil to dissolve completely
- Sterilize by autoclaving at 121^0C for 15minutes
- Well mix before pouring
- Burn to remove bubbles
- Dry surface of the gel before inoculation

Tryptone soy agar

- Suspend 40g in 1liter of distilled water
- Bring to the boil to dissolve completely
- Sterilize by autoclaving at 121^{0}C for 15minutes
- Well mix before pouring
- Burn to remove bubbles
- Dry surface of the gel before inoculation

Mueller Hinton agar

- Suspend 38g in 1liter of distilled water
- Bring to the boil to dissolve completely
- Sterilize by autoclaving at 121°C for 15minutes
- Well mix before pouring
- Burn to remove bubbles
- Dry surface of the gel before inoculation

Triple sugar iron

- Suspend 65g in 1 liter of distilled water
- Bring to the boil to dissolve completely
- Mix well and distribute into tubes
- Sterilize by autoclaving at 121^{0}C for 15 minutes
- Allow to set as slope with 2.5 cm butt

Simmons citrate

- Suspend 23g in 1liter of distilled water
- Bring to the boil to dissolve completely
- Sterilize by autoclaving at 121^{0}C for 15 minutes
- Allow to set as slant

Urea agar

- Suspend 2.4g in 95 ml of distilled water
- Bring to boil to dissolve completely
- Sterilize by autoclaving at 115^0C for 20 minutes
- Cool to 50 ^{0}C and aseptically add one ampoule of sterile urea solution
- Mix well , distribute 10ml amount into sterile containers and allow to set as a butt

Motility Indole Ornithine Medium

- Suspend 31g of the powder in liter of purified water. Mix thoroughly.
- Heat with frequent agitation and boil for 1minute to completely dissolve the powder
- Distribute 10 ml amount into sterile containers
- Autoclave at 121°C for 15 minutes
- Allow to set as butt

Appendix III: Gram staining procedures

Material

- Crystal violet
- Safranin solution
- Gram’s stain procedures
- Decolorizer solution
- Glass microscope slides
- Straight sterile wire loop
- Bunsen burner
- Lighter
- Gas
- Normal saline
- Hands gloves
- 70% ethanol and 5% bleach in spray
- Biohazard waste bin
- Staining rack
- Sink with tap water
- Microscope
- *Staphylococcus aureus 25923*
- *Escherichia coli ATCC 25922*

Procedures

- A drop of normal saline is put on a clean glass slide and a smear prepared by picking a pure colony using a sterile loop spreading the material evenly over a wide area and leaving to air dry
- The smear is heat-fix by passing over the flame, smear side up
- The slide is placed on a rack for cooling
- The slide is flooded with crystal violet for one minute and then washed for 5 seconds with tap water

- The slide is flooded with iodine solution for one minute and then rinsed for 5 seconds
- The ethanol (de-colorizer) is applied for 30 seconds and rinsed with running tap water
- The slide is flooded with Safranin (counter stain) for one minute and rinse with running tap water.

Gram positive bacteria appear blue violet whereas gram negative bacteria appear pink.

Appendix IV: Interpretation of biochemical tests

Triple Sugar Iron

Description and principle

TSI is used to determine whether a gram negative bacillus utilizes glucose and lactose or sucrose fermentatively and forms hydrogen sulfide (H_2S). TSI contains lactose (10 parts), sucrose (10 parts), glucose and peptone (1 part). Phenol red and ferrous sulfate serve as indicators of acidification and (H_2S) formation respectively. When glucose is utilized by a fermentative organism, the entire media becomes acidic (yellow) in 18 to 12 hours. The butt remains acidic after the recommended 18 to 24 hours incubation period because of the presence of organic acids resulting from the fermentation of glucose under anaerobic conditions in the butt of the tube. The slant however reverts to the alkaline (red) state because of oxidation of the fermentation products under aerobic conditions in the slant. This change is a result of the formation of carbon dioxide and water and the oxidation of peptones in the medium to alkaline amines. When in addition the glucose, lactose and/or sucrose are fermented, the large amount of fermentation products formed on the slant will neutralize the alkaline amines and render the slant acidic (yellow), provided the reaction is read in 18 to 24 hours. Reactions in TSI should not be read beyond 24 hours of incubation, because aerobic oxidation of the fermentation products from lactose and/or sucrose does not proceed and the slant will eventually revert to the alkaline state. The formation of carbon dioxide and water (hydrogen gas) is indicated by the presence of bubbles or cracks in the agar or by separation in the tube. The production of H_2S requires an acidic environment and is manifested by blackening of the butt of the medium.

Interpretation of results

Alkaline slant/no change in the butt (K/NC) = glucose, lactose, and sucrose non utilizer: this may also be recorded as K/K (alkaline slant /alkaline butt).

Alkaline slant/acid butt (K/A) = glucose fermentation only. Acid slant/acid butt (A/A) = glucose, sucrose, and /or lactose fermenter.
Note: A black precipitate in the butt indicates production of ferrous sulfide and H_2S gas (H_2S +). Bubbles or cracks in the tube indicate the production of carbon dioxide or hydrogen.

Quality control

A/A: *Escherichia coli*
K/A H2S+: *Salmonella typhi, Salmonella typhimurium*
K/NC: *Pseudomonas aeruginosa, S.typhimurium* produces H_2S while *Salmonella enteritidis* does not

Simmons citrate

Description

The test is used to determine the ability of an organism to utilize sodium citrate as its only carbon source and inorganic ammonium salts as its nitrogen source. Bacteria that can grow on this medium turn bromothymol blue indicator from green to blue.

Interpretation

Positive: Growth on the medium, with or without change in color of the indicator. The color change of the indicator is due to acid or alkali production by the test organism as it grows on the medium. Growth usually results in the bromothymol blue indicator, turning from green to blue.

Quality control

Known citrate positive; *Klebsiella pneumonia* and citrate negative; *Escherichia coli.* Both *Salmonella enteritidis* and *Salmonella typhimurium* do not utilize citrate as their source of carbon therefore they do not grow in this medium.

Urease Test

The urease test helps in the identification of certain species of *Enterobacteriaceae*. Urease enzyme hydrolyses substrate urea into ammonia, water, and carbon dioxide. The presence of the enzyme is determined by inoculating an organism to broth or agar that contains urea as the primary carbon source and detecting the production of ammonia. Ammonia increases the pH of the medium so its presence is readily detected using a common indicator of metabolic process and, because pH indicators change color which increases (alkalinity) or decrease (acidity) in the medium's pH. The test is used to determine the ability of an organism to produce the enzyme urease, which hydrolyses urea. Hydrolysis of urea produces ammonia and carbon dioxide. The formation of ammonia alkalinizes the medium, and the pH change is detected by the color change of phenol red indicator from light orange at pH 6.8 to magenta pH 8.1

Interpretation

Positive: Change in color of slant from light orange to magenta
Negative: No color change in media.

Quality control

Known urease positive, Proteus vulgaris and urease negative *Escherichia coli* should always be included when doing the test.
Both *Salmonella typhimurium* and *Salmonella enteritidis* are urease negative.

MIO

Motility, indole and ornithine medium (MIO) is used for identification of *Enterobacteriaceae* on the basis of motility, indole and ornithine decarboxylase activity in a single tube. Motility is shown by a diffused zone of growth out from the inoculation line. Indole test is done to determine the ability of an organism to break down tryptophan by a tryptophanase with production of indole. A positive reaction is indicated by a formation of a red ring at the interface after addition of Kovac's

reagent. If the organism has a specific decarboxylase, ornithine is decarboxylated to putrescine, an amine resulting in a subsequent rise in the pH of the medium changing the media to dark purple. Organisms that ferment dextrose will produce acids, thereby lowering the pH. A decreased pH results in the indicator, bromocresol purple turning from purple to yellow.

Appendix V Tryptone soy broth

- Dissolve 30g in 1 liter of distilled water
- Add 15% of glycerin and distribute into final containers
- Sterilize by autoclaving at 121^0C for 15 minutes

Appendix VI: Normal saline preparation

- Dissolve 0.85g in 100 ml of distilled water
- Distribute into Screwable tubes and cap
- Sterilize by autoclaving at 121^0C for15 minutes
- Set to cool

Appendix VII: Clinical Laboratory standard institute (CLSI, 2017)

Table 2A-1. Zone Diameter and Minimal Inhibitory Concentration Breakpoints for *Enterobacteriaceae*

Testing Conditions	Routine QC Recommendations (See Tables 4A and 5A for acceptable QC ranges.)
Medium: Disk diffusion: MHA Broth dilution: CAMHB Agar dilution: MHA **Inoculum:** Growth method or direct colony suspension, equivalent to a 0.5 McFarland standard **Incubation:** 35°C ± 2°C; ambient air Disk diffusion: 16–18 hours Dilution methods: 16–20 hours	*Escherichia coli* ATCC®* 25922 *Pseudomonas aeruginosa* ATCC® 27853 (for carbapenems) *Escherichia coli* ATCC® 35218 (for β-lactam/β-lactamase inhibitor combinations) When a commercial test system is used for susceptibility testing, refer to the manufacturer's instructions for QC test recommendations and QC ranges.

* ATCC® is a registered trademark of the American Type Culture Collection.

Refer to Tables 3A, 3B, 3C, and 3D for additional testing, reporting, and QC for *Enterobacteriaceae*.

General Comments

(1) For disk diffusion, test a maximum of 12 disks on a 150-mm plate and no more than 6 disks on a 100-mm plate; disks should be placed no less than 24 mm apart, center to center (see M02-A12, Subchapter 3.6). Each zone diameter should be clearly measurable; overlapping zones prevent accurate measurement. Measure the diameter of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk. Hold the Petri plate a few inches above a black background illuminated with reflected light. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth. Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus* spp., ignore the thin veil of swarming growth in an otherwise obvious zone of growth inhibition. With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth) and measure the more obvious margin to determine the zone diameter.

(2) When fecal isolates of *Salmonella* and *Shigella* spp. are tested, only ampicillin, a fluoroquinolone, and trimethoprim-sulfamethoxazole should be reported routinely. In addition, for extraintestinal isolates of *Salmonella* spp., a third-generation cephalosporin should be tested and reported, and chloramphenicol may be tested and reported if requested. Susceptibility testing is indicated for typhoidal *Salmonella* (*S.* Typhi and *Salmonella* Paratyphi A–C) isolated from extraintestinal and intestinal sources. Routine susceptibility testing is not indicated for nontyphoidal *Salmonella* spp. isolated from intestinal sources. In contrast, susceptibility testing is indicated for all *Shigella* isolates.

Table 2A-1. *Enterobacteriaceae* (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)				Interpretive Categories and MIC Breakpoints (µg/mL)				Comments
			S	SDD	I	R	S	SDD	I	R	
PENICILLINS											
A	Ampicillin	10 µg	≥17	–	14–16	≤13	≤8	–	16	≥32	(4) Results of ampicillin testing can be used to predict results for amoxicillin. See general comment (2).
O	Piperacillin	100 µg	≥21	–	18–20	≤17	≤16	–	32–64	≥128	
O	Mecillinam	10 µg	≥15	–	12–14	≤11	≤8	–	16	≥32	(5) For testing and reporting of *E. coli* urinary tract isolates only.
β-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS											
B	Amoxicillin-clavulanate	20/10 µg	≥18	–	14–17	≤13	≤8/4	–	16/8	≥32/16	
B	Ampicillin-sulbactam	10/10 µg	≥15	–	12–14	≤11	≤8/4	–	16/8	≥32/16	
B	Ceftolozane-tazobactam	–	–	–	–	–	≤2/4	–	4/4	≥8/4	**(6) Breakpoints are based on a dosage regimen of 1.5 g every 8 h.**
B	Piperacillin-tazobactam	100/10 µg	≥21	–	18–20	≤17	≤16/4	–	32/4–64/4	≥128/4	
O	Ticarcillin-clavulanate	75/10 µg	≥20	–	15–19	≤14	≤16/2	–	32/2–64/2	≥128/2	
CEPHEMS (PARENTERAL) (Including cephalosporins I, II, III, and IV. Please refer to Glossary I.) **(7) WARNING:** For *Salmonella* spp. and *Shigella* spp., first- and second-generation cephalosporins and cephamycins may appear active *in vitro*, but are not effective clinically and should not be reported as susceptible. (8) Following evaluation of PK-PD properties, limited clinical data, and MIC distributions, revised **breakpoints** for cephalosporins (cefazolin, cefotaxime, ceftazidime, ceftizoxime, and ceftriaxone) and aztreonam were first published in January 2010 (M100-S20) and are listed in this table. Cefuroxime (parenteral) was also evaluated; however, no change in **breakpoints** was necessary for the dosage indicated below. When using the current **breakpoints**, routine ESBL testing is no longer necessary before reporting results (ie, it is no longer necessary to edit results for cephalosporins, aztreonam, or penicillins from susceptible to resistant). However, ESBL testing may still be useful for epidemiological or infection control purposes. For laboratories that have not implemented the current **breakpoints**, ESBL testing should be performed as described in Table 3A. Note that **breakpoints** for drugs with limited availability in many countries (eg, moxalactam, cefonicid, cefamandole, and cefoperazone) were not evaluated. If considering use of these drugs for *E. coli*, *Klebsiella*, or *Proteus* spp., ESBL testing should be performed (see Table 3A). If isolates test ESBL positive, the results for moxalactam, cefonicid, cefamandole, and cefoperazone should be reported as resistant. (9) *Enterobacter*, *Citrobacter*, and *Serratia* may develop resistance during prolonged therapy with third-generation cephalosporins as a result of derepression of AmpC β-lactamase. Therefore, isolates that are initially susceptible may become resistant within 3 to 4 days after initiation of therapy. Testing of repeat isolates may be warranted.											
A	Cefazolin	30 µg	≥23	–	20–22	≤19	≤2	–	4	≥8	(10) **Breakpoints** when cefazolin is used for therapy of infections other than uncomplicated UTIs due to *E. coli*, *K. pneumoniae*, and *P. mirabilis*. **Breakpoints** are based on a dosage regimen of 2 g every 8 h. See comment (8).

Table 2A-1. *Enterobacteriaceae* (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm) S	SDD	I	R	Interpretive Categories and MIC Breakpoints (µg/mL) S	SDD	I	R	Comments
CEPHEMS (PARENTERAL) (including cephalosporins I, II, III, and IV. Please refer to Glossary I.) (Continued)											
U	Cefazolin	30 µg	≥15	–	–	≤14	≤16	–	–	≥32	**(11) Breakpoints** when cefazolin is used for therapy of uncomplicated UTIs due to *E. coli*, *K. pneumoniae*, and *P. mirabilis*. **Breakpoints** are based on a dosage regimen of 1 g every 12 h. See additional information below under CEPHEMS (ORAL).
C	Ceftaroline	30 µg	≥23	–	20–22	≤19	≤0.5	–	1	≥2	**(12) Breakpoints** are based on a dosage regimen of 600 mg every 12 h.
B	Cefepime	30 µg	≥25	19–24	–	≤18	≤2	4–8	–	≥16	(13) The **breakpoint** for susceptible is based on a dosage regimen of 1 g every 12 h. The **breakpoint** for SDD is based on dosing regimens that result in higher cefepime exposure, either higher doses or more frequent doses or both, up to approved maximum dosing regimens. See Appendix E for more information about **breakpoints** and dosing regimens. Also see the definition of SDD in the Instructions for Use of Tables section.
B B	Cefotaxime or ceftriaxone	30 µg 30 µg	≥26 ≥23	–	23–25 20–22	≤22 ≤19	≤1 ≤1	–	2 2	≥4 ≥4	(14) **Breakpoints** are based on a dosage regimen of 1 g every 24 h for ceftriaxone and 1 g every 8 h for cefotaxime. See comment (8).
B	Cefotetan	30 µg	≥16	–	13–15	≤12	≤16	–	32	≥64	
B	Cefoxitin	30 µg	≥18	–	15–17	≤14	≤8	–	16	≥32	**(15) Breakpoints** are based on a dosage regimen of at least 8 g per day (eg, 2 g every 6 h).
B	Cefuroxime (parenteral)	30 µg	≥18	–	15–17	≤14	≤8	–	16	≥32	**(16) Breakpoints** are based on a dosage regimen of 1.5 g every 8 h. See comment (8).
C	Ceftazidime	30 µg	≥21	–	18–20	≤17	≤4	–	8	≥16	**(17) Breakpoints** are based on a dosage regimen of 1 g every 8 h. See comment (8).

Table 2A-1. *Enterobacteriaceae* (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)				Interpretive Categories and MIC Breakpoints (µg/mL)				Comments
			S	SDD	I	R	S	SDD	I	R	
CEPHEMS (PARENTERAL) (including cephalosporins I, II, III, and IV. Please refer to Glossary I.) (Continued)											
O	Cefonicid	30 µg	≥ 18	–	15–17	≤ 14	≤ 8	–	16	≥ 32	See comment (8).
O	Cefoperazone	75 µg	≥ 21	–	16–20	≤ 15	≤ 16	–	32	≥ 64	See comment 8).
O	Ceftizoxime	30 µg	≥ 25	–	22–24	≤ 21	≤ 1	–	2	≥ 4	**(18) Breakpoints** are based on a dosage regimen of 1 g every 12 h. See comment (8).
O	Moxalactam	30 µg	≥ 23	–	15–22	≤ 14	≤ 8	–	16–32	≥ 64	See comment (8).
CEPHEMS (ORAL)											
B	Cefuroxime	30 µg	≥ 23		15–22	≤ 14	≤ 4		8–16	≥ 32	See comment (20).
U	Cefazolin (surrogate test for oral cephalosporins and uncomplicated UTI)	30 µg	≥ 15	–	–	≤ 14	≤ 16	–	–	≥ 32	**(20) Breakpoints** are for cefazolin when cefazolin results are used to predict results for the oral agents cefaclor, cefdinir, cefpodoxime, cefprozil, cefuroxime, cephalexin, and loracarbef when used for therapy of uncomplicated UTIs due to *E. coli*, *K. pneumoniae*, and *P. mirabilis*. Cefdinir, cefpodoxime, and cefuroxime may be tested individually because some isolates may be susceptible to these agents while testing resistant to cefazolin.
O	Loracarbef	30 µg	≥ 18	–	15–17	≤ 14	≤ 8	–	16	≥ 32	**(21)** Do not test *Citrobacter*, *Providencia*, or *Enterobacter* spp. with cefdinir or loracarbef by disk diffusion because false-susceptible results have been reported. See comment (20).
O	Cefaclor	30 µg	≥ 18	–	15–17	≤ 14	≤ 8	–	16	≥ 32	See comment (20).
O	Cefdinir	5 µg	≥ 20	–	17–19	≤ 16	≤ 1	–	2	≥ 4	See comments (20) and (21).
O	Cefixime	5 µg	≥ 19	–	16–18	≤ 15	≤ 1	–	2	≥ 4	**(22)** Do not test *Morganella* spp. with cefixime, cefpodoxime, or cefetamet by disk diffusion.
O	Cefpodoxime	10 µg	≥ 21	–	18–20	≤ 17	≤ 2	–	4	≥ 8	See comments (20) and (22).
O	Cefprozil	30 µg	≥ 18	–	15–17	≤ 14	≤ 8	–	16	≥ 32	**(23)** Do not test *Providencia* spp. with cefprozil by disk diffusion because false-susceptible results have been reported. See comment (20).
Inv.	Cefetamet	10 µg	≥ 18	–	15–17	≤ 14	≤ 4	–	8	≥ 16	See comment (22).
Inv.	Ceftibuten	30 µg	≥ 21	–	18–20	≤ 17	≤ 8	–	16	≥ 32	**(24)** For testing and reporting of urinary tract isolates only.

Table 2A-1. *Enterobacteriaceae* (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)				Interpretive Categories and MIC Breakpoints (µg/mL)				Comments
			S	SDD	I	R	S	SDD	I	R	
MONOBACTAMS											
C	Aztreonam	30 µg	≥ 21	–	18–20	≤ 17	≤4	–	8	≥ 16	(25) **Breakpoints** are based on a dosage regimen of 1 g every 8 h. See comment (8).
CARBAPENEMS (26) Following evaluation of PK-PD properties, limited clinical data, and MIC distributions that include recently described carbapenemase-producing strains, revised **breakpoints** for carbapenems were first published in June 2010 (M100-S20-U) and are listed below. Because of limited treatment options for infections caused by organisms with carbapenem MICs or zone diameters in the intermediate range, clinicians may wish to design carbapenem dosage regimens that use maximum recommended doses and possibly prolonged intravenous infusion regimens, as has been reported in the literature.[1-4] Consultation with an infectious diseases practitioner is recommended for isolates for which the carbapenem MICs or zone diameter results from disk diffusion testing are in the intermediate or resistant ranges. Laboratories using *Enterobacteriaceae* MIC **breakpoints** for carbapenems described in M100-S20 (January 2010) should perform the MHT, the Carba NP test, **mCIM**, and/or a molecular assay when isolates of *Enterobacteriaceae* are suspicious for carbapenemase production based on imipenem or meropenem MICs of 2–4 µg/mL or ertapenem MIC of 2 µg/mL (refer to Tables 3B, 3C, **and 3D**). After implementation of the current **breakpoints, these additional tests** do not need to be performed other than for epidemiological or infection control purposes (refer to Table 3B). The following information is provided as background on carbapenemases in *Enterobacteriaceae* that are largely responsible for MICs and zone diameters in the intermediate and resistant ranges, and thus the rationale for setting revised carbapenem breakpoints: • The clinical effectiveness of carbapenem treatment of infections produced by isolates for which the carbapenem MIC or disk diffusion test results are within the intermediate range is uncertain due to lack of controlled clinical studies. Imipenem MICs for *Proteus* spp., *Providencia* spp., and *Morganella morganii* tend to be higher (eg, MICs in the intermediate or resistant range) than meropenem or doripenem MICs. These isolates may have elevated imipenem MICs by mechanisms other than production of carbapenemases.											
B	Doripenem	10 µg	≥ 23	–	20–22	≤ 19	≤ 1	–	2	≥4	(27) **Breakpoints** are based on a dosage regimen of 500 mg every 8 h.
B	Ertapenem	10 µg	≥ 22	–	19–21	≤ 18	≤0.5	–	1	≥2	(28) **Breakpoints** are based on a dosage regimen of 1 g every 24 h.
B	Imipenem	10 µg	≥ 23	–	20–22	≤ 19	≤ 1	–	2	≥4	(29) **Breakpoints** are based on a dosage regimen of 500 mg every 6 h or 1 g every 8 h.
B	Meropenem	10 µg	≥ 23	–	20–22	≤ 19	≤ 1	–	2	≥4	(30) **Breakpoints** are based on a dosage regimen of 1 g every 8 h.
AMINOGLYCOSIDES (31) **WARNING:** For *Salmonella* spp. and *Shigella* spp., aminoglycosides may appear active *in vitro* but are not effective clinically and should not be reported as susceptible.											
A	Gentamicin	10 µg	≥ 15	–	13–14	≤ 12	≤4	–	8	≥ 16	
A	Tobramycin	10 µg	≥ 15	–	13–14	≤ 12	≤4	–	8	≥ 16	
B	Amikacin	30 µg	≥ 17	–	15–16	≤ 14	≤ 16	–	32	≥ 64	
O	Kanamycin	30 µg	≥ 18	–	14–17	≤ 13	≤ 16	–	32	≥ 64	

Table 2A-1. *Enterobacteriaceae* (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)				Interpretive Categories and MIC Breakpoints (µg/mL)				Comments
			S	SDD	I	R	S	SDD	I	R	
AMINOGLYCOSIDES (Continued)											
O	Netilmicin	30 µg	≥ 15	–	13–14	≤ 12	≤ 8	–	16	≥ 32	
O	Streptomycin	10 µg	≥ 15	–	12–14	≤ 11	–	–	–	–	(32) There are no MIC interpretive standards.
MACROLIDES											
Inv.	Azithromycin	15 µg	≥ 13	–	–	≤ 12	≤ 16	–	–	≥ 32	(33) *Salmonella* Typhi only: breakpoints are based on MIC distribution data and limited clinical data. For *Shigella flexneri* and *Shigella sonnei* see Table 2A-2.
TETRACYCLINES (34) Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline. However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline, minocycline, or both.											
C	Tetracycline	30 µg	≥ 15	–	12–14	≤ 11	≤ 4	–	8	≥ 16	
O	Doxycycline	30 µg	≥ 14	–	11–13	≤ 10	≤ 4	–	8	≥ 16	
O	Minocycline	30 µg	≥ 16	–	13–15	≤ 12	≤ 4	–	8	≥ 16	
QUINOLONES AND FLUOROQUINOLONES for *Enterobacteriaceae* except *Salmonella* spp. (Please refer to Glossary I.)											
B	Ciprofloxacin	5 µg	≥ 21	–	16–20	≤ 15	≤ 1	–	2	≥ 4	
B	Levofloxacin	5 µg	≥ 17		14–16	≤ 13	≤ 2	–	4	≥ 8	
O	Cinoxacin	100 µg	≥ 19	–	15–18	≤ 14	≤ 16	–	32	≥ 64	See comment (24).
O	Enoxacin	10 µg	≥ 18	–	15–17	≤ 14	≤ 2	–	4	≥ 8	See comment (24).
O	Gatifloxacin	5 µg	≥ 18	–	15–17	≤ 14	≤ 2	–	4	≥ 8	See comment (24).
O	Gemifloxacin	5 µg	≥ 20	–	16–19	≤ 15	≤ 0.25	–	0.5	≥ 1	(35) FDA-approved for *Klebsiella pneumoniae*.
O	Grepafloxacin	5 µg	≥ 18	–	15–17	≤ 14	≤ 1	–	2	≥ 4	
O	Lomefloxacin	10 µg	≥ 22	–	19–21	≤ 18	≤ 2	–	4	≥ 8	
O	Nalidixic acid	30 µg	≥ 19	–	14–18	≤ 13	≤ 16	–	–	≥ 32	See comment (24).
O	Norfloxacin	10 µg	≥ 17	–	13–16	≤ 12	≤ 4	–	8	≥ 16	See comment (24).
O	Ofloxacin	5 µg	≥ 16	–	13–15	≤ 12	≤ 2	–	4	≥ 8	
Inv.	Fleroxacin	5 µg	≥ 19	–	16–18	≤ 15	≤ 2	–	4	≥ 8	
QUINOLONES AND FLUOROQUINOLONES for *Salmonella* spp. (Please refer to Glossary I.)											

Table 2A-1. *Enterobacteriaceae* (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)				Interpretive Categories and MIC Breakpoints (µg/mL)				Comments
			S	SDD	I	R	S	SDD	I	R	
QUINOLONES AND FLUOROQUINOLONES for *Salmonella* spp. (Please refer to Glossary I.) (Continued)											
B B	Ciprofloxacin Levofloxacin	5 µg –	≥ 31 –	– –	21–30 –	≤ 20 –	≤0.06 ≤0.12	– –	0.12–0.5 0.25–1	≥ 1 ≥ 2	**(39)** Isolates of *Salmonella* spp. that test not susceptible to ciprofloxacin, levofloxacin, ofloxacin, or pefloxacin may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with salmonellosis.
O	Ofloxacin	–	–	–	–	–	≤0.12	–	0.25–1	≥ 2	
Inv.	Pefloxacin (surrogate test for ciprofloxacin)	5 µg	≥ 24	–	–	≤ 23	–	–	–	–	**(40) Report results as ciprofloxacin susceptible or resistant based on the pefloxacin test result. Pefloxacin will not detect resistance in *Salmonella* spp. due to *aac(6')-Ib-cr*. Pefloxacin disks are not available in the United States.** **See Comment (38)**
FOLATE PATHWAY INHIBITORS											
B	Trimethoprim-sulfamethoxazole	1.25/ 23.75 µg	≥ 16	–	11–15	≤ 10	≤2/38	–	–	≥4/76	**See general** comment (2).
U	Sulfonamides	250 or 300 µg	≥ 17	–	13–16	≤ 12	≤256	–	–	≥512	**(41)** Sulfisoxazole can be used to represent any of the currently available sulfonamide preparations.
U	Trimethoprim	5 µg	≥ 16	–	11–15	≤ 10	≤8	–	–	≥16	
PHENICOLS											
C	Chloramphenicol	30 µg	≥ 18	–	13–17	≤ 12	≤8	–	16	≥32	**(42)** Not routinely reported on isolates from the urinary tract.
FOSFOMYCINS											
U	Fosfomycin	200 µg	≥ 16	–	13–15	≤ 12	≤64	–	128	≥256	**(43)** For testing and reporting of *E. coli* urinary tract isolates only. **(44)** The 200-µg fosfomycin disk contains 50 µg of glucose-6-phosphate. **(46)** The only approved MIC method for testing is agar dilution using agar media supplemented with 25 µg/mL of glucose-6-phosphate. Broth dilution MIC testing should not be performed.

Table 2A-1. *Enterobacteriaceae* (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)				Interpretive Categories and MIC Breakpoints (µg/mL)				Comments
			S	SDD	I	R	S	SDD	I	R	
NITROFURANS											
U	Nitrofurantoin	300 µg	≥ 17	–	15–16	≤ 14	≤ 32	–	64	≥ 128	

Abbreviations: ATCC®, American Type Culture Collection; CAMHB, cation-adjusted Mueller-Hinton broth; ESBL, extended-spectrum β-lactamase; I, intermediate; mCIM, modified carbapenem inactivation method; MHA, Mueller-Hinton agar; MHT, modified Hodge test; MIC, minimal inhibitory concentration; PK-PD, pharmacokinetic-pharmacodynamic; QC, quality control; R, resistant; S, susceptible; SDD, susceptible-dose dependent; UTI, urinary tract infection.

Appendix VIII: Ethical approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **October 5, 2017**

TO: **IHORIMBERE THEOGENE,**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CMR,**
NAIROBI

Dear Sir,

RE: **KEMRI/SERU/CMR/00057/3521 (*RESUBMISSION 2 OF INITIAL SUBMISSION*): MOLECULAR CHARACTERISTICS OF ARCHIVED ISOLATES OF *ESCHERICHIA COLI* FROM THE GUT OF HEALTHY FOOD ANIMALS AND ENVIRONMENTAL SOURCES IN SELECTED COUNTIES IN KENYA.**

Reference is made to your letter dated 28th September 2017. KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study protocol on September 29, 2017.

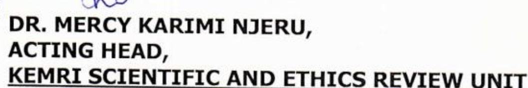
This is to inform you that the Committee notes that the issues raised during the 265th C meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **July 18, 2017**, have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day **5th October 2017** for a period of one year. Please note that authorization to conduct this study will automatically expire on **4th October 2018.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **23rd August 2018**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,



DR. MERCY KARIMI NJERU,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix IX: Permission for MSc student to use *E. coli* samples

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Microbiology Research, P.O. Box 19464 - 00202, NAIROBI - Kenya,
Tel: (254) (020) 2720794, 2720038, Nairobi. Website: www.kemri.org

24th August, 2017

AG. HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT SERU

Dear Madam,

RE: PERMISSION FOR MSC STUDENT TO USE E. COLI SAMPLES FROM SERU 3205 PROJECT

This is to acknowledge that permission has been granted to the following MSc student to use archived *E. Coli* samples from our 2-year project: 'Ecology and epidemiology of antimicrobial-resistant foodborne pathogens (*E. coli*, *Salmonella spp* and *Campylobacter* spp) in selected sites in five Counties in Kenya: *Investigating farmer knowledge and attitudes towards antimicrobial use and resistance and public health significance' **KEMRI/SERU/CMR/P0036/3205.*** The project received SERU approval on April 14, 2016.

Name: Ihorimbere Theogene
Institution: Jomo Kenyatta University of Agriculture and Technology (JKUAT)
Course: MSc. Medical Microbiology
Reg. No. TM302-0918/2016

The 2-year project started late due to delays in grant processing and SERU approval and we have just submitted application for continuation approval for the 2nd and final year and expect a response from SERU in a month's time. However, please note that the student will be using only samples that we have already archived in the 1st year of the project.

Yours Sincerely,

Prof. Samuel Kariuki
Principal Investigator & Director
Centre for Microbiology Research, KEMRI

In Search of Better Health

Appendix X: Abstract of the published data

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Molecular Characteristics of Archived Isolates of Escherichia coli from the Gut of Healthy Food Animals and Environmental Sources in Selected Counties in Kenya

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Abstract

E. coli is a reservoir of resistance genes encoding resistance enzymes including extended-spectrum beta-lactamases (ESBL) and carbapenemases. This study aims to determine the resistance pattern, the pathotypes, and the proportion of ESBL producers among archived *E. coli* isolates from healthy food animals and their immediate environments at the Center for Microbiology Research, Kenya Medical Research Institute. Of the 375 isolates, 78.4% isolates were resistant to at least one of the 13 tested antibiotics and 28.8% showed multidrug resistance. Resistance was higher to tetracycline (55.2%), co-trimoxazole (44%), trimethoprim (43.7%), and ampicillin (28.8%). The proportion of Enteroaggregative *E. coli* was 88.3% while other pathotypes were not found. The proportion of ESBL producers was 8.8% of which 100% harboured bla_{TEM} 18.1% harboured $bla_{CTX\text{-}M;}$ bla_{SHV} was not found. *E.coli* isolates from healthy food animals were multidrug resistant and harboured virulence genes and ESBL genes. Risk assessment and management is necessary to protect farmers and the public in general.

Keywords: Archived *E. coli* isolates, antimicrobial resistance, multidrug resistance, pathotypes, ESBLs.