CHARACTERIZATION OF EXTENDED-SPECTRUM BETA-LACTAMASE (ESBL) - PRODUCING *ESCHERICHIA COLI* ISOLATED FROM RAW DAIRY CATTLE MILK IN PERI-URBAN FARMS IN NAIROBI, 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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…….. 30th November, 2021.

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DEDICATION

To my late mother, Agnes Mutei Mue, for all the support throughout my academic journey

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LIST OF ABBREVIATIONS

WHO	World Health Organization
ESBL	Extended spectrum beta lactamases
EMB	Eosin Methylene Blue
3GCs	Third-generation cephalosporins
AST	Antimicrobial susceptibility testing
BLAST	Basic Local Alignment Search Tool
CFU	Colony-forming units
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
HGT	Horizontal gene transfer
ICU	Intensive care unit
MDR	Multidrug-resistant
MGE	Mobile genetic element
NCBI	National Center for Biotechnology Information
PBP	Penicillin binding protein
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism

ABSTRACT

Antimicrobial resistance has become a global health concern as many pathogens are becoming resistant to more than one antibiotic, and new, last-resort antibiotics are expensive and often out of reach for those who need them from low income countries. Beta-lactams (penicillins and cephalosporins) are one of the most commonly used classes of antibiotics in the treatment of infections caused by multidrug-resistant gram-negative bacteria particularly *Escherichia coli* and *Klebsiella species* both in human and animals. Emergence of extended spectrum beta lactamase (ESBL) producing strains of bacteria represent one of the biggest threats to global health. There have been reports worldwide on food animals and foods of animal origin being potential sources of ESBL-producing strains raising serious food safety questions regarding the prevalence of these strains in foods of animal origin.

This study was carried out to characterize extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* isolated from raw dairy cattle milk in peri-urban farms in Nairobi Kenya.

The study was carried on 351 raw dairy milk samples collected between November 2016 to October 2017 from five dairy farms, namely: - University of Nairobi Veterinary Farm at Kanyariri (n=103), Department of Veterinary services farm at Ngong (n=52), Dominic Farm (n=106), Kabogo Farm (n=50) and Karuga Farm (n=40) all around Nairobi. Pooled milk samples of a volume of 10 ml from four teats of an individual cow were collected directly from the udder by hand milking into a sterile bijou bottle. Samples of dairy milk were then placed in cool boxes and transported to University of Nairobi, department of public health, pharmacology and toxicology laboratories. Isolation of *Escherichia coli* was by employing enrichment in buffered peptone water followed by inoculation in tryptone soy agar and then cultured in EMB Agar selective medium. Identification of *Escherichia coli* was done through morphological characteristics, biochemical reactions and final confirmation by polymerase

chain reaction (PCR) using primers specific to *gad*A gene of *Escherichia coli*. Antimicrobial susceptibility testing (AST) was performed as described by Kirby-Bauer disc diffusion method employing Clinical and Laboratory Standards Institute (CLSI) guidelines. A total of 12 commonly used antimicrobial agents were tested: Ampicillin (10µg), Amoxicillin/Clavulanic acid (20/10µg), Cefazolin (30µg), Cefuroxime (30µg), Cefoxitin (30µg), Cefotaxime (30µg), Ceftazidime (30µg), Cefepime (30µg), Imipenem (30µg), Gentamycin (30µg), Ciprofloxacin (30µg) and Tetracycline (30µg). CLSI zone diameter interpretive break points were employed. For each isolate, AST was done in triplicates and the mean zone diameter of inhibition was calculated. The mean diameter was then compared to the interpretive standard break points for *Escherichia coli* for each tested antibiotic. *Escherichia coli* ATCC 25922 was used as the quality control organism.

Presumptive ESBL producers were screened for extended spectrum beta lactamase production using standard disc-diffusion method on Mueller Hinton Agar. The selection of these isolates was based on zone diameters for ceftazidime 30µg and cefotaxime 30µg (Ceftazidime zone ≤ 22 mm or Cefotaxime zone ≤ 27 mm). ESBL test was performed using disc-diffusion method on Mueller Hinton agar employing the following sets of antimicrobial disks: Ceftazidime 30µg/Ceftazidime-clavulanate 30µg/10µg and Cefotaxime 30µg/Cefotaxime-clavulanate 30µg/10µg. The observations were considered positive if ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanate versus the zone diameter of the agent when tested alone was observed, else, the result was considered negative and in all these tests *Escherichia. coli* ATCC *25922* was still used as reference bacteria.

DNA of the phenotypically identified ESBL producers were extracted and thereafter specific PCR assays were conducted to screen for the presence of ESBL genes in the *Escherichia coli* isolates. The PCR amplicons were electrophoresed on 1.3 % agarose gel in Tris-acetate-

EDTA buffer supplemented with 0.5µg/ml of ethidium bromide and calibrated using 100 bp DNA ladder. The gels were visually inspected by UV-transilluminator.

The study confirmed by PCR assay 91 *Escherichia coli* isolates (25.9%) from the 351 samples collected. Seventeen isolates (18.7%) were susceptible to all antimicrobial agents. Seventy-four isolates (81.3%) showed resistance to at least one antimicrobial agent and twenty-six (28.6%) isolates were resistant to more than one antimicrobial agent. Five isolates (5.5%) were resistant to at least one agent in three or more antimicrobial categories indicating that they were MDR strains. *E. coli* showed resistance profiles to: Ampicillin 54 isolates (59.3%), tetracycline 19 isolates (20.9%), amoxiclav 10 isolates (11.0%), cefazolin 4 isolates (4.4%) and one isolate each (1.1%) for cefoxitin, ceftazidime and cefotaxime. None of the isolates was resistant to cefuroxime, cefepime, imipenem, ciprofloxacin and gentamycin.

Thirty-five phenotypically identified ESBL- producing *E. coli* isolates were assayed by PCR for presence of three ESBL genes: *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$. Thirty-three (36 %) *Escherichia coli* isolates were found to have at least one ESBL gene. Sixteen (17.6%) isolates harboured two ESBL genes. Nine (9.9%) isolates had all the three ESBL genes -*bla*$_{\text{TEM}}$, *bla*$_{SHV}$ and *bla*$_{\text{CTX-M}}$. *bla*$_{\text{CTX-M}}$ was the most predominant gene and was detected in 31 isolates (34.1%) followed by *bla*$_{\text{TEM}}$ which was observed in 24 isolates (26.4%). *bla*$_{\text{SHV}}$ was detected in 12 isolates (13.2%).

BLAST analysis of the sequenced PCR products revealed that all the resistance genes had 97 - 100 % nucleotide identity to sequences in the NCBI GenBank database.

This study shows that raw dairy milk harbours *Escherichia coli* resistant to more than one antimicrobial agent. This study further shows that *Escherichia coli* isolates obtained from cattle milk were susceptible to cefuroxime, cefepime, imipenem, ciprofloxacin and

gentamycin suggesting that these antimicrobials can still be used effectively. *Escherichia coli* isolates harboured bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$.

ESBL genes were similar to other strains previously found in several countries worldwide in isolates obtained from food producing animals as well as from human clinical samples. This study suggests that raw cow milk is a potential source of ESBL-producing strains and could therefore pose a public health risk. From the findings of this study, it is recommended that dairy milk should be pasteurized prior to consumption to avoid transmission of multidrug resistant ESBL strains to consumers.

1. CHAPTER ONE: INTRODUCTION

1.1 Background

Antimicrobial resistance is a huge concern to the livelihood of humankind as several microorganisms are becoming resistant to more than one antibiotic, and the new, last-resort antibiotics are expensive. It represents one of the biggest global health threats: increasing hospital stays, medical costs, and mortality. Increased misuse of antibiotics in agriculture and human and veterinary medicine primarily aggravates the problem of antimicrobial resistance (WHO, 2015; Barriere, 2015).

Beta-lactams (penicillins, cephalosporins and carbapenems) are one of the major classes of antibiotics in both human and veterinary medicine. In the early 1980s, a huge therapeutic advancement in medicine was marked by the introduction of third-generation cephalosporins (3GCs) to manage diseases caused by multidrug-resistant (MDR) gram-negative bacteria *Escherichia coli* and *Klebsiella species*. These two pathogens cause a wide range of infections (Gundogan and Avci, 2013). However, shortly after introducing 3GCs, transferable plasmid-mediated resistance was first reported in Germany with the identification of a mutant beta-lactamase SHV-2 (Sulfhydryl variable**)** and later in France with a similar mutant beta-lactamase TEM-3 being identified. Following the first reports of SHV- and TEM- derived ESBLs in Germany and France, these genes, together with a different type of ESBL gene (*bla*$_{\text{CTX-M}}$), have been common in other countries worldwide (Ewers *et al.,* 2012).

The worldwide prevalence of ESBL-producing *E. coli* and other Enterobacteriaceae is growing fast in both hospitals and communities. Significant similarities have been found in ESBL- producing *Escherichia coli* from food animals and humans based on resistant genes and other mobile resistance elements (Kluytmans *et al.,* 2013). Food animals are therefore recognized as a major carrier for ESBL-producing pathogens raising grave food safety concerns. The gastrointestinal tract harbours kilograms of bacteria, and these organisms serve

as an ideal reservoir for antibiotic resistance genes. With the increased use of antibiotics, resistant strains are accumulated following selective advantage by the immune system, increasing the possibility of transmission of resistant genes to other microorganisms (Brolund, 2014). Several ESBL dissemination methods have been identified between food animals, humans and the environment. The major pathways include contact with pastures contaminated with faecal matter and through the food chain from contaminated foods of animal origin (van Hall *et al*., 2011 and Collis *et al*., 2019).

Earlier studies show that ESBL genes, mobile genetic elements and *Escherichia coli* originating from food animals are spread to consumers via food chain (van Hall *et al*., 2011). Possible faecal contamination of foods originating from animals might occur during milking, slaughtering, milking, food processing and storage. Therefore, these foods act as carriers of ESBL *Escherichia coli* strains to human beings with poor hygienic practices.

A study conducted in Turkey reported ESBL producing strains at a prevalence of 44.4 %, among which *Escherichia coli* was the most dominant species isolated from raw meat, raw milk, white cheese and ice cream (Gundogan and Avci, 2013). The *E. coli* isolates were resistant to ampicillin and other antimicrobial agents, but none was resistant to imipenem, ertapenem, cefepime and piperacillin/tazobactam. In Japan, a study to evaluate the diversity of ESBL genes in rectal samples of food-producing animals established ESBL-carrying *Escherichia coli* in 60 % of broiler samples, 5.9 % of layers, 12.5 % of beef cattle and 3 % of pigs (Hiroi *et al*., 2012). Several other studies across the world have also reported food animals as reservoirs of ESBL-producing strains of *Escherichia coli* (Nadine *et al*., 2012; Njage *et al*., 2012; Reist *et al*., 2013; Schmid *et al*., 2013 and Karuppasamy *et al*., 2015).

Several surveys of ESBLs producing *E. coli* in food animals have also been conducted in Africa, with reports of the CTX-M-1 group being dominant in most surveys for both humans and animals (Gisele *et al*., 2011; Abdallah *et al*., 2015; and Falgenhauer *et al*., 2019). Certain

bla$_{\text{CTX-M-1}}$ -harbouring clones (ST131/B2 or ST405/D) which are majorly known to be found in humans, have also been reported in animals from Nigeria, Tunisia and Tanzania (Alonso, 2017).

In Kenya, some investigations have also been done on antibiotic resistance in ESBL producing pathogens in humans. A study from a hospital in Kenya reported 78 % of isolates of *Escherichia coli* and *Klebsiella species* displayed multiple resistances to various classes of antimicrobial agents except for carbapenems (1 %). Nitrofurantoin was also noted to retain good activity, with 23 % of the isolates resistant to nitrofurantoin (Maina *et al*., 2013). In another study conducted at County Hospital on malnourished and nourished children, the prevalence of ESBL phenotype was reported to be higher in severely malnourished children (39%) as compared to nourished children (7%) (Njoroge *et al*., 2014).

Despite several studies on antibiotic resistance in ESBL producing *Escherichia coli* in humans, little is known about the burden of ESBL pathogens in food-producing animals and foods of animal origin in Kenya. The occurrence of microbial contaminants with zoonotic potential in food-producing animals and foods of animal origin has greatly aggravated the situation. It is, therefore, difficult to recommend any possible interventions to combat the problem of antimicrobial resistance. Therefore, the current study was undertaken to establish the presence of ESBL *Escherichia coli* strains, their antimicrobial susceptibility, and molecular characterization of ESBL producing *Escherichia coli*, including sequencing of resistant strains in dairy milk from the peri-urban Nairobi region.

1.2 Hypothesis

Escherichia coli are present in raw cattle milk, and some isolates may have ESBL genes that confer resistance to beta-lactam antibiotics.

1.3 Overall objective

To characterize Extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* isolated from raw dairy cattle milk in peri-urban farms in Nairobi, Kenya.

1.4 Specific objectives

1. To determine the prevalence of *Escherichia coli* from raw dairy cattle milk in peri-urban farms in Nairobi, Kenya.

2. To determine the antimicrobial susceptibility profile of *Escherichia coli* isolated from raw dairy cattle milk in peri-urban farms in Nairobi, Kenya.

3. To determine the presence of ESBL genes among the *Escherichia coli* isolates from raw dairy cattle milk in peri-urban farms in Nairobi, Kenya.

4. To determine the relationship between ESBL genes in the *Escherichia coli* isolates and those in the gene bank.

2. CHAPTER TWO: LITERATURE REVIEW

2.1 Ecology of *Escherichia coli*

The ecology of *Escherichia coli* species varies from human to vertebrate animals and environmental sources (Berthe, 2013). Its natural habitat is intestinal tracts of animals and is released to the environment via faecal matter. Intrinsic (genetic adaptation) and extrinsic factors help *Escherichia coli* species in surviving in adverse environments such as soil, manure and water (Sadowsky, 2008). Earlier studies show certain strains of *Escherichia coli* having the ability to persist in adverse extra intestinal environments for longer time and potentially reproducing which poses a challenge in detecting and monitoring food and water safety for purposes of public health surveillance (Chekabab, 2013; van Elsas, 2011; Jang, 2017).

2.2 *Escherichia coli* infections

Most strains of *Escherichia coli* are non-pathogenic. However, some stains can cause severe enteric infections which are usually transmitted through consumption of contaminated water or food, such as undercooked meat products and raw milk. There are various intestinal pathogenic types of *Escherichia coli* including: enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC), and enteroadherent *E. coli* (EAEC). There is also an extraintestinal pathogenic *E. coli* (Jafari, 2012).

Other *Escherichia coli* infections include acute bacterial meningitis, pneumonia, intra-abdominal infections and urinary tract infections (Makvana, 2015).

2.2.1 Acute bacterial meningitis

Escherichia coli is the most common Enterobacteriaceae that causes meningitis. *E. coli* meningitis is still causing mortality and morbidity globally. *E coli* and *group B streptococcal* infections (28.5% and 34.1% overall, respectively) causes most of neonatal meningitis cases (Ishida, 2016). Expectant mothers are at a higher risk of infection with the K1 capsular antigen strain of *E coli* (Kim, 2016).

2.2.2 Pneumonia

Escherichia coli pneumonia mostly affects aged people and persons with predisposing factors such as immunosuppression, alcoholism or diabetes. *Escherichia coli* pneumonia is normally hospital acquired (Cunha, 1982). The pathogen reaches the respiratory tract either through aspiration of oropharyngeal secretions or by hematogenous transmission from a primary source (Palmer, 1984). In some cases, *Escherichia coli* pneumonia is community-acquired where persons have underlying disease conditions such as diabetes mellitus, chronic obstructive pulmonary disease, alcoholism and *E. coli* UTI (Lerner, 1980).

2.2.3 Intra-abdominal infections

Intra-abdominal infections can be caused by facultative and obligate anaerobic organisms, gram-negative facultative organism (Enterobacteriaceae with *E. coli* at the first place), other gram-negative bacilli and Enterococci (Sartelli, 2010).

E. coli intra-abdominal infections often result from a perforated viscus (e.g. appendix, diverticulum) or may be associated with intra-abdominal abscess, cholecystitis, and ascending cholangitis. Patients with diabetes mellitus are also at high risk of developing pylephlebitis of the portal vein and liver abscesses.

Intra-abdominal abscesses are usually polymicrobial and *E. coli* is one of the more common gram-negative bacilli observed together with anaerobes. Abscesses can be caused by spontaneous or traumatic gastrointestinal tract perforation or after anastomotic disruption with spillage of colon contents and subsequent peritonitis (Sartelli, 2010).

2.2.4 Enteric infections

As a cause of enteric infections, six different mechanisms of action of six different varieties of *Escherichia coli* have been reported. Enterotoxigenic *E. coli* (ETEC) causes traveller's diarrhoea. Enteropathogenic *E. coli* (EPEC) is a cause of childhood diarrhoea. Enteroinvasive *E. coli* (EIEC) causes a Shigella -like dysentery. Enterohemorrhagic *E. coli* (EHEC) is a cause of haemorrhagic colitis or haemolytic-uremic syndrome (HUS). Enteroaggregative *E. coli* (EAggEC) is primarily associated with persistent diarrhoea in children in developing countries, and enteroadherent *E. coli* (EAEC) is a cause of childhood diarrhoea and traveller's diarrhoea in Mexico and North Africa (Jafari, 2012).

ETEC, EPEC, EAggEC, and EAEC colonize the small bowel, and EIEC and EHEC preferentially colonize the large bowel prior to causing diarrhoea. Shiga toxin–producing *E. coli* (STEC) is among the most common causes of foodborne diseases. This organism is responsible for several GI illnesses, including non-bloody and bloody diarrhoea. Patients with these diseases, especially children, may be affected by neurologic and renal complications, including HUS. Strains of STEC serotype O157-H7 have caused numerous outbreaks and sporadic cases of bloody diarrhoea and HUS (Makvana, 2015).

2.2.5 Urinary tract infections

Urinary tract infections (UTIs) are among the most common types of bacterial infections occurring both in the community and hospital settings. The majority of UTIs are caused by *E. coli* bacteria, followed by *Proteus* spp., *Staphylococcus saprophyticus*, *Klebsiella* spp. and other *Enterobacteriaceae*. However, among bacteria causing UTIS, *E. coli* is considered as the most predominant cause of both community and nosocomial UTIs (Alanazi, 2018).

E. coli causes a wide range of UTIs, including uncomplicated urethritis/cystitis, symptomatic cystitis, pyelonephritis, acute prostatitis, prostatic abscess, and urosepsis. Uncomplicated cystitis occurs primarily in females who are sexually active and are colonized by a uropathogenic strain of *E. coli* (Marrs *et al*., 2005). Subsequently, the periurethral region is

colonized from contamination of the colon, and the organism reaches the bladder during sexual intercourse. Complicated UTI and pyelonephritis are observed in elderly patients with structural abnormalities or obstruction such as prostatic hypertrophy or neurogenic bladders or in patients with urinary catheters (Terlizzi, 2017).

2.2.6 *E coli* infections in cattle

Escherichia coli causes a wide range of infections in cattle including mastitis, cystitis, calf scours and neonatal mortality. Bovine mastitis caused by *Escherichia coli* can range from being a subclinical infection of the mammary gland to a severe systemic disease. Dairy cow-dependent factors such as lactation stage and age affect the severity of coliform mastitis. The only antimicrobials for which there is some scientific evidence of beneficial effects in the treatment for *E. coli* mastitis are fluoroquinolones and cephalosporins (Suojala, 2013).

Escherichia coli causes two common diseases of new born calves: coli-septicemia in which the bacteria invade the systemic circulation and internal organs and enteric colibacillosis in which the bacteria are localized to the lumen and mucosal surface of the small intestine (Stephen, 1985). The *E. coli* that causes septicaemia survives and multiply in the blood and internal organs of calves. Conversely, those that causes diarrhoea are equipped to survive locally in the gastrointestinal tract. Other infectious agents for calf diarrheic diseases include S*almonella, Clostridium perfringens* and *Campylobacter species* (Muktar, 2015).

2.2.7 Treatment of *Escherichia coli* infections

Several antimicrobial agents effectively inhibit the growth of *Escherichia coli* and are regimens for treatment of community and hospital acquired *E. coli* infections. The antimicrobial agents include: β-lactams, fluoroquinolones, aminoglycosides, trimethoprim-sulfamethoxazole and nitrofurantoin for uncomplicated UTIs (Pitout, 2014 and Morrill, 2015). The β-lactam antibiotics, especially the cephalosporins and β-lactams/β-

lactamases inhibitor combinations, are major drug classes used in the treatment of infections caused by extended spectrum beta lactamase producing pathogens. The carbapenems are widely regarded as the drugs of choice for the treatment of severe infections caused by ESBL-producing Enterobacteriaceae (Morrill, 2015).

2.3 Beta- Lactams

Beta-lactams are a group of antibiotics most widely used both in human and animal production.

Chemistry

All β-lactam antibiotics have β-lactam ring which is a highly reactive amide. There are five β-lactam ring structures including the penam, cephem, penem, carbapenem and monobactam (Fernandes, 2013).

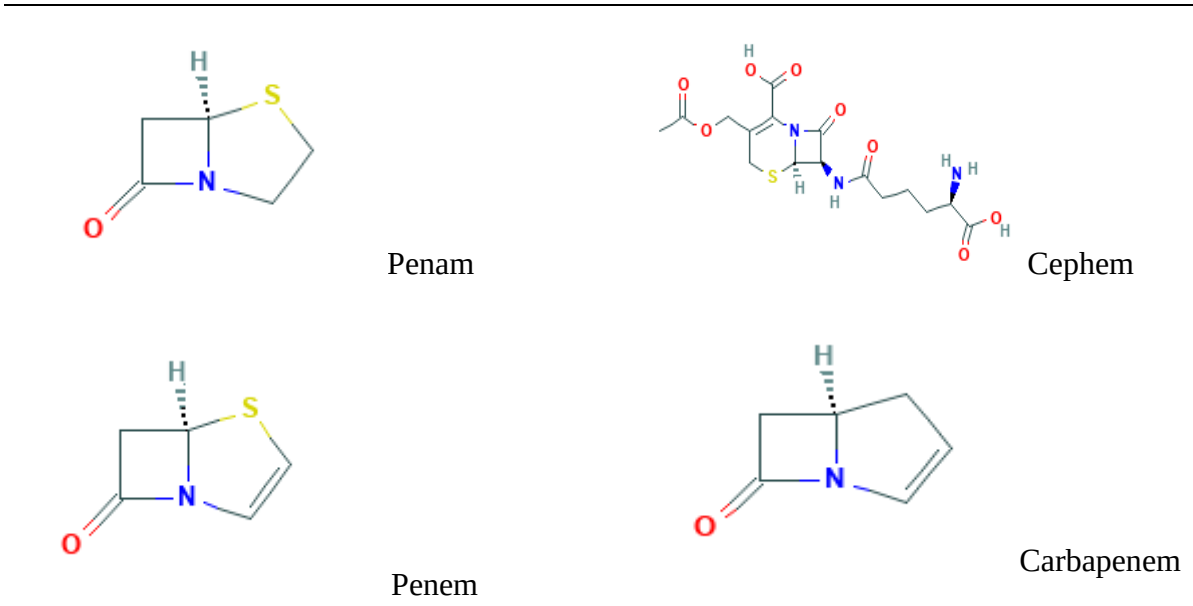

Fig 2.1 Four common β-lactam ring structures

Mechanism of action

Beta-lactams act by impeding enzymes that are responsible for the formation of peptidoglycan layer thus interfering with bacterial cell wall synthesis. The targets for the actions of beta-lactam antibiotics are known as penicillin-binding proteins (PBPs). This binding, in turn, interrupts the terminal transpeptidation process and induces loss of viability and lysis, also through autolytic processes within the bacterial cell (Pandey and Cascella, 2020).

Pharmacokinetics

Beta-lactam antibiotics are generally available for parenteral application, but some are absorbed from the gastrointestinal tract. The serum half-life of most beta-lactams is 1-2 hours. Penicillins and cephalosporins are eliminated by glomerular filtration and varying degrees of active transport across the epithelial cells of the renal tubuli and hepatobiliary system (Bergan, 1984).

Toxicity of Beta-lactams

The use of β-lactam antibiotics has been linked to triggering allergic reactions like urticaria, bronchoconstriction, also severe conditions like immune-mediated haemolytic anaemia and intravascular haemolysis. It is known that some β-lactam antibiotics are neurotoxic, some are nephrotoxic, some are genotoxic, and some are toxic to urogenital system (Bozcal and Dagdeviren, 2017).

2.3.1 Penams: Penicillins (e.g. ampicillin, amoxicillin, cloxacillin)

Penicillin was first discovered by Alexander Fleming in 1929 from samples of mold *Penicillium notatum*. Structurally, they consist of β-lactam ring fused to a thiazolidine ring together referred to as penam structure. Since the discovery of penicillin, multiple synthetic analogues have been developed and are classified in various subgroups.

2.3.1.1 Natural Penicillins

They are directly purified from cultures of penicillium mold. Despite bacterial resistance, they are still used in treatment of infections caused by gram-positive organisms including *Treponema pallidum*, the causative agent for syphilis. Examples of natural penicillins include penicillin G and Penicillin V (Fig. 2.2).

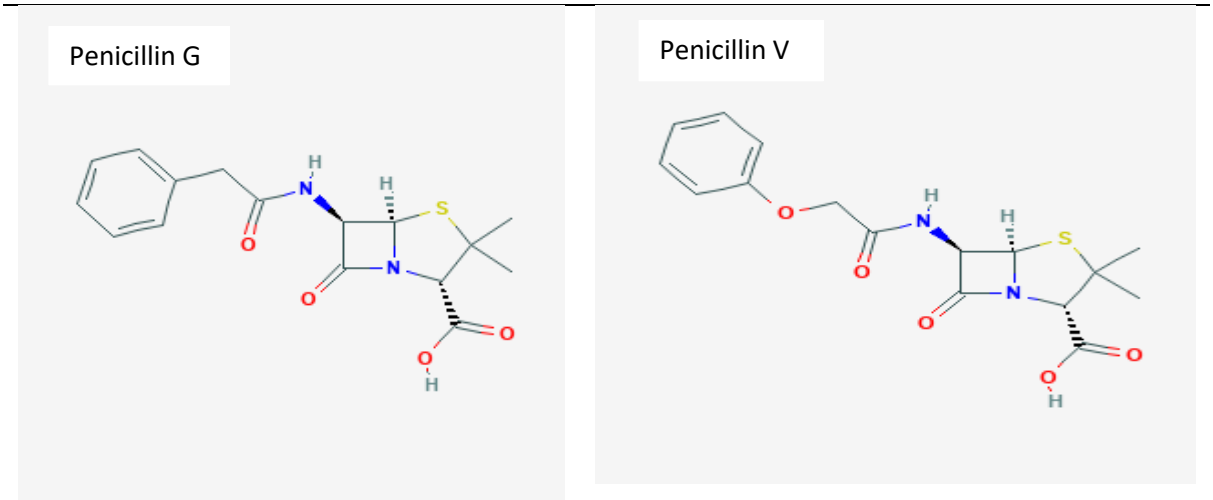

Figure 2.2 Structures of natural peniciilins

2.3.1.2 Antistaphylococcal penicillins

They are resistant to inactivation by penicillinases and are useful in treatment of infections caused by sensitive *Staphylococcus aureus* and *Staphylococcus epidermidis*. However, they are not effective against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE). The agents are also not effective against enterococcal and streptococcal infections. Examples of these agents include nafcillin, oxacillin, methicillin and dicloxacillin (Fig. 2.3).

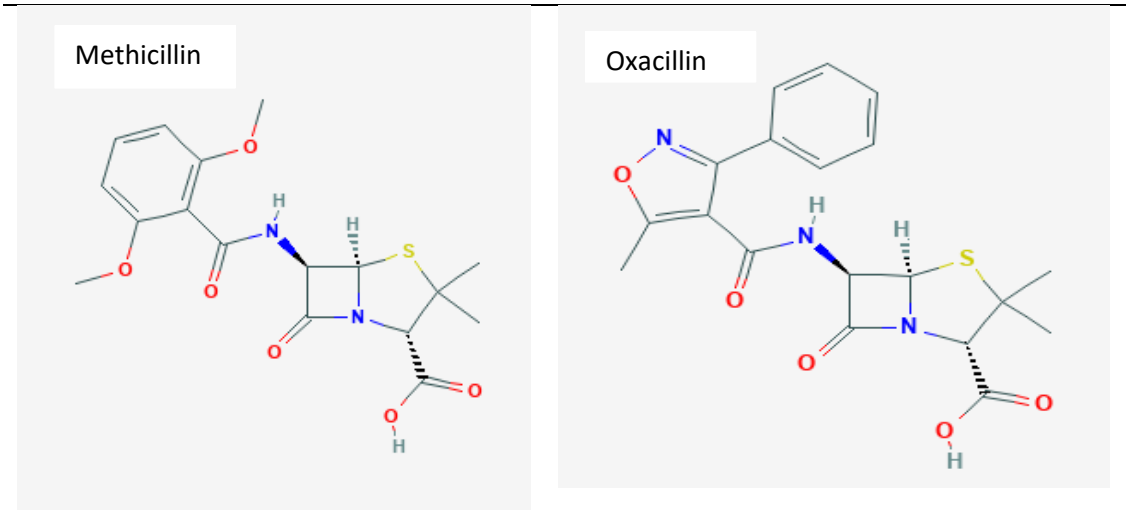

Figure 2.3 Structures of antistaphylococcal penicillins

2.3.1.3 Aminopenicillins

Examples include ampicillin and amoxicillin (Fig. 2.4). They are prone to cleavage by beta-lactamases like natural penicillins hence are commonly combined with beta-lactamase inhibitor. The agents are active against some gram-negative bacteria for instance *Escherichia coli, Proteus mirabilis and Shigella species.*

These penicillins are primarily used in treatment of *Listeria meningitis* and enterococcal infections.

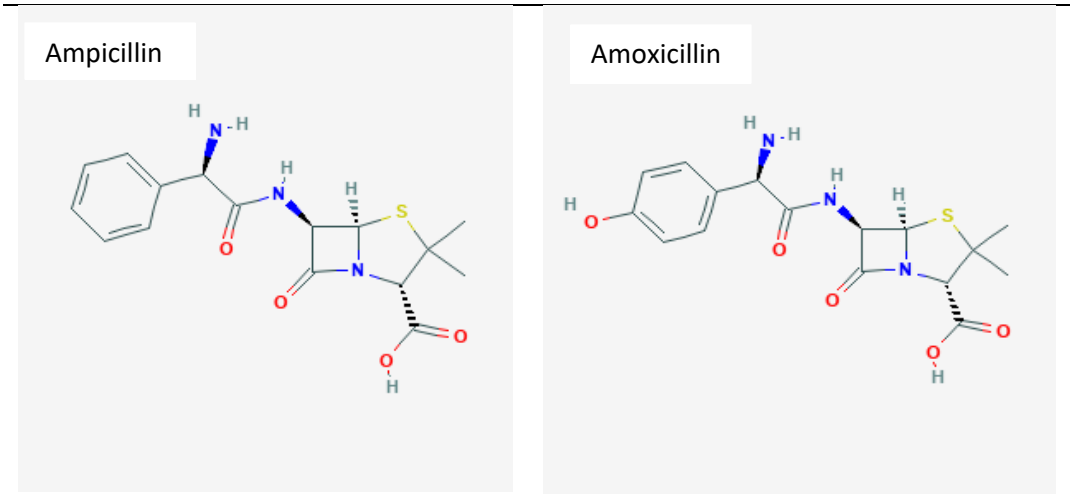

Figure 2.4 Structures of aminopenicillins

2.3.1.4 Extended-spectrum (antipseudomonal) penicillins

These penicillins are generally more resistant to beta lactamases compared to aminopenicillins. They have good activity against *Pseudomonas aeruginosa*. These drugs achieve their best antimicrobial activity when in combination with beta-lactamase inhibitors and have good activity on many aerobic gram-positive, gram-negative organisms and all anaerobic organisms except *Clostridium deficile* and examples of these agents include piperacillin and ticarcillin (Fig. 2.5).

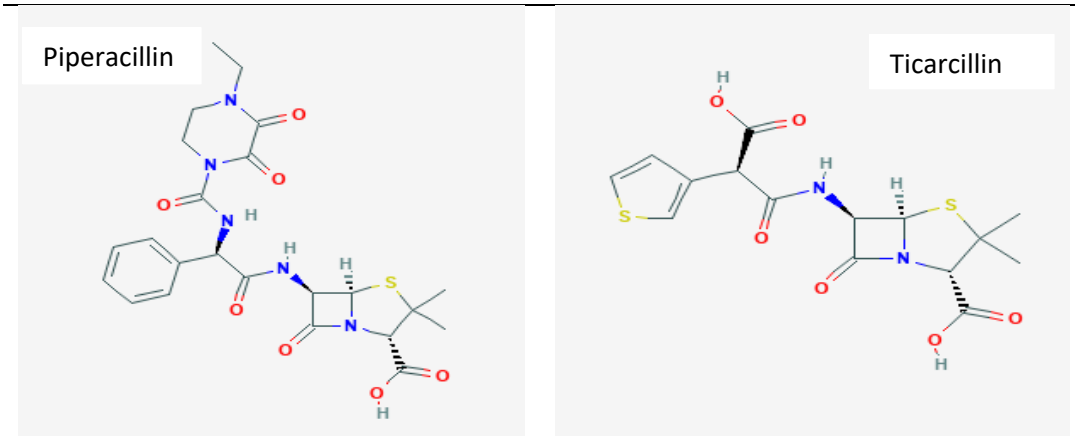

Figure 2.5 Structures of extended-spectrum (antipseudomonal) penicillins

2.3.2 Cephems: Cephalosporins

2.3.2.1 First generation cephalosporins

First generation cephalosporins are very active against gram positive cocci except enterococci and methicillin-resistant staphylococci, and moderately active against some gram-negative bacteria primarily *Escherichia coli, Proteus* and *Klebsiella species*. They have moderate or poor activity against anaerobes. Examples of First generation cephalosporins include cefazolin, cephalexin, cephradine, cefadroxil, cephalothin and cephapirin (Fig. 2.6). These cephalosporins are primarily used in treatment of urinary tract infections and respiratory tract infections.

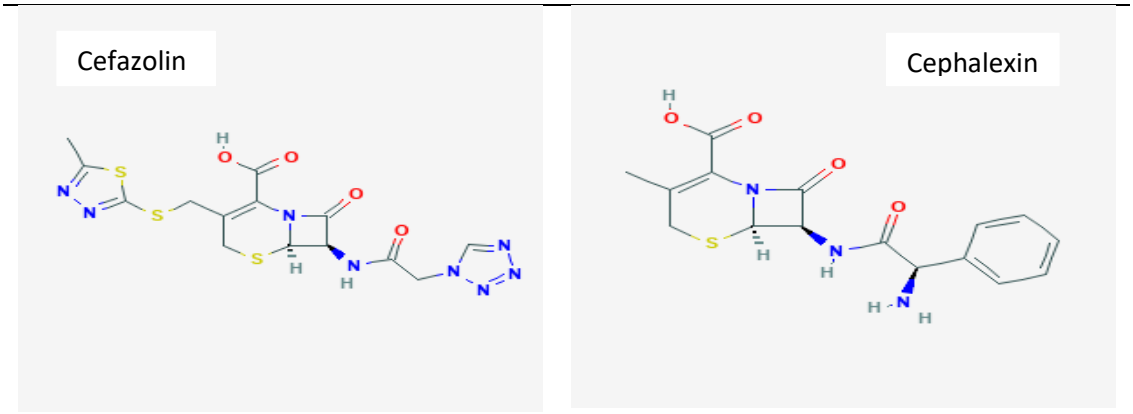

Figure 2.6 Structures of first generation cephalosporins

2.3.2.2 Second generation cephalosporins

These cephalosporins are active against organisms sensitive to first generation cephalosporins but they have increased activity against gram-negative rods including *Escherichia coli*, *Proteus* and *Klebsiella species*. However, these drugs have no activity against *Pseudomonas aeruginosa*. Some oral second generation cephalosporins have activity against *Hemophilus influenzae*, including beta-lactamase producing strains. Examples include cefaclor, cefoxitin and cefotetan (Fig.2.7).

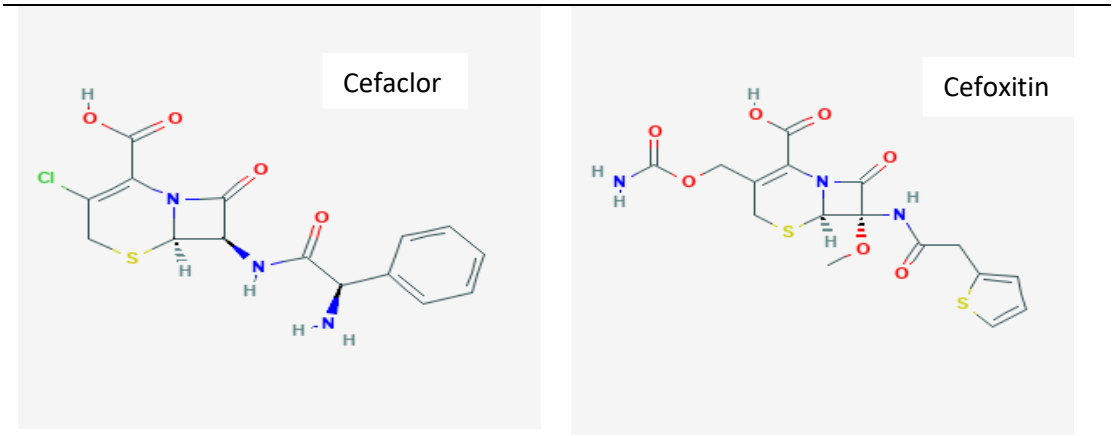

Figure 2.7 Structures of second generation cephalosporins

2.3.2.3 Third generation cephalosporins

They have decreased activity against gram-positive cocci. However, most third generation cephalosporins are active against *Staphylococci* but ceftazidime is weakly active. Some are active against *Pseudomonas aeruginosa*. These drugs are majorly useful in treatment of

hospital acquired gram-negative infections. A distinguishing feature of third generation cephalosporins is their ability to reach central nervous system and therefore useful in management of meningitis caused gram-negative bacteria. Examples include cefotaxime, ceftazidime, ceftiofur, ceftriaxone and cefixime (Fig.2.8).

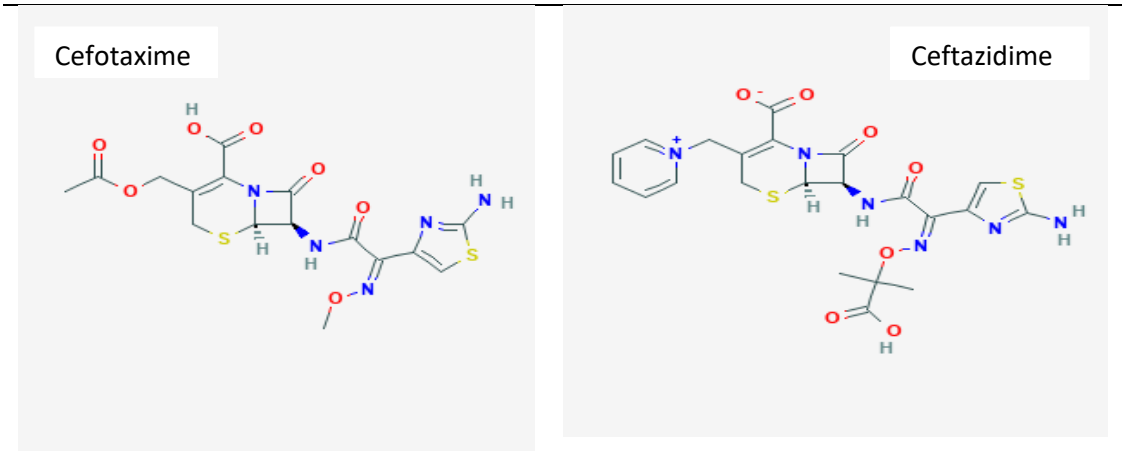

Figure 2.8 Structures of third generation cephalosporins

2.3.2.4 Fourth generation cephalosporins

They have enhanced activity against Enterobacter and Citrobacter which are resistant against third generation cephalosporins. They are also active against *Pseudomonas aeruginosa*. Only cefepime and cefpirome are currently available in the market (Fig. 2.9).

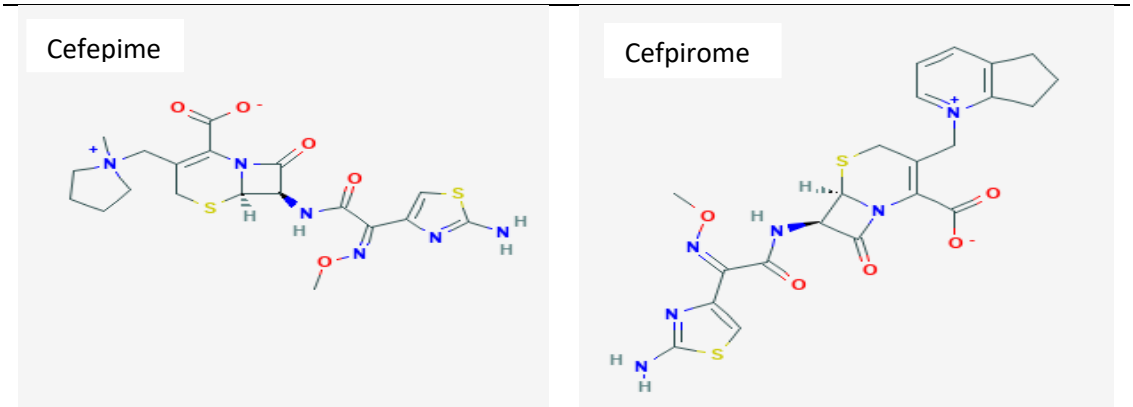

Figure 2.9 Structures of fourth generation cephalosporins

2.3.2.5 Fifth generation cephalosporins

These are active against gram-positive bacteria and retains activity of other generation of cephalosporins with broad spectrum activity against gram-negative bacteria. An example is ceftaroline newly approved by FDA as Ceftaroline fosamil (Fig.2.10).

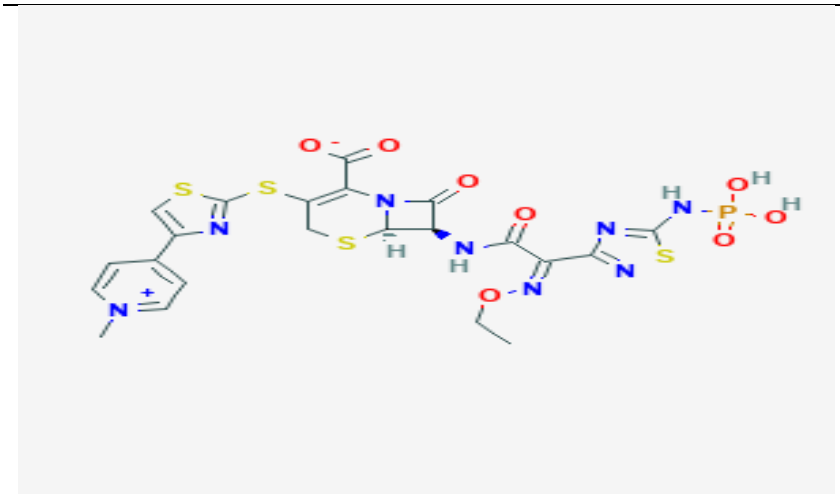

Figure 2.10 Structure of ceftaroline, a fifth generation cephalosporin

2.3.3 Carbapenems and Penems

They have the broadest spectrum of all beta-lactams, with good activity against gram negative organisms and anaerobes such B. fragilis and narrower spectrum against gram positives. Carbapenems are highly resistant to cleavage by most β-lactamases including ESBLs. They are currently reserved for treatment of infections caused by multi-drug resistant organisms which are not sensitive to available penicillins, cephalosporins and b-lactam/b-lactamase inhibitor combinations. Carbapenems are effective against infections caused by ESBL producing strains of Enterobacteriaceae and *Pseudomonas aeruginosa* (Papp-Wallace, 2011). Examples include imipenem and ertapenem (Fig.2.11). Other examples are meropenem and doripenem.

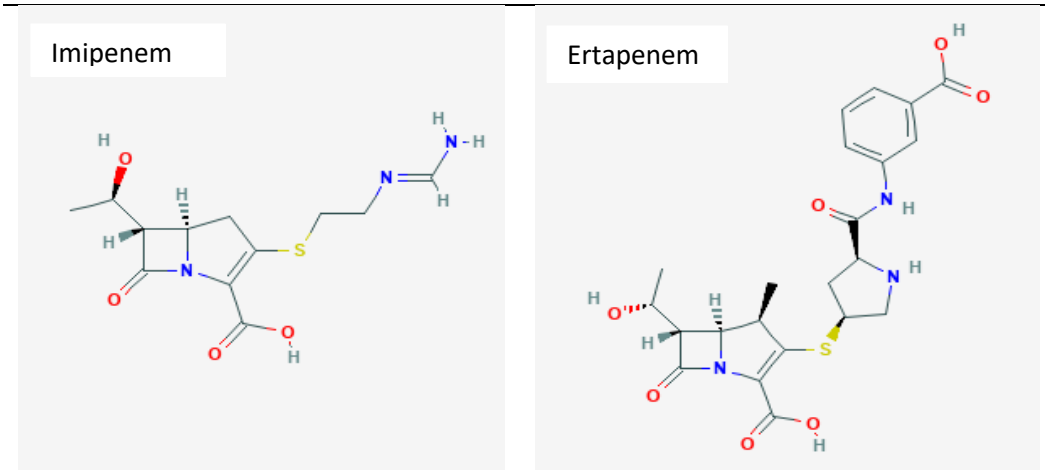

Figure 2.11 Structures of carbapenems

2.3.4 Monobactams

They have monocyclic beta-lactam ring. They are active against gram negative rods including *Pseudomonas aeruginosa* but not against gram positive bacteria and anaerobes. Monobactams are resistant to some beta-lactamases but are inactivated by ESBLs (Bonner, 1985). Monobactams are poorly absorbed after oral administration, so are given parenterally. Excretion is via renal route and the usual half-life (one to two hours) is increased in renal failure.

Example include aztreonam which is the only commercially available monobactam (Fig.2.12).

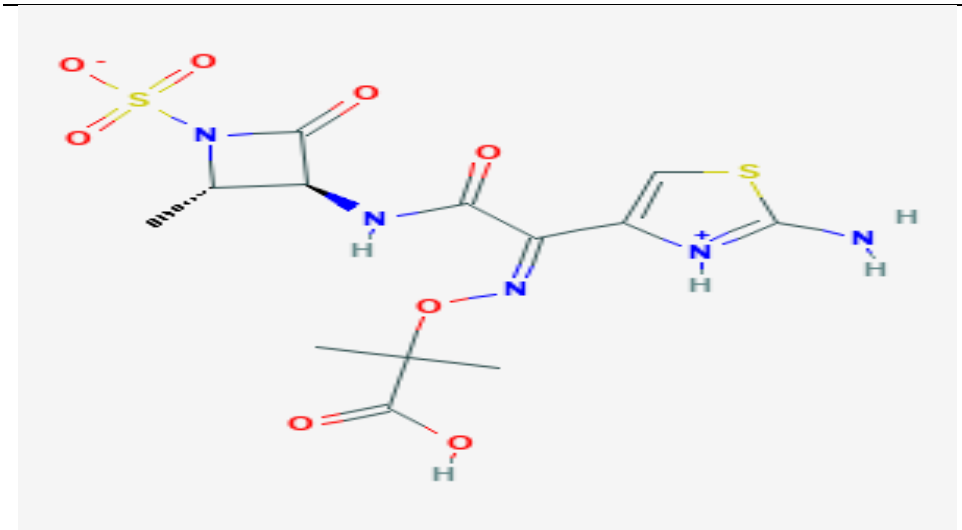

Figure 2.12 Structure of aztreonam

All structures of beta lactams presented above are referred from *pubchem.ncbi.nlm.nih.gov*. Table 2.1 below illustrates the mechanisms of action of beta-lactam antibiotics and other antimicrobial agents.

Table 2.1: Mechanisms of action of various classes of antimicrobial agents

Antimicrobial Class	**Effect on Bacteria**	**Mode of Action**
B-lactams	Bactericidal	Inhibition of cell wall synthesis
Polypeptide antibiotics	Bactericidal	Inhibition of cell wall synthesis
Quinolones	Bactericidal	Inhibition of DNA synthesis
Metronidazole	Bactericidal	Inhibition of DNA synthesis
Rifamycins	Bactericidal	Inhibits RNA transcription
Lincosamides	Bactericidal	Inhibits protein synthesis
Aminoglycosides	Bactericidal	Inhibits protein synthesis
Macrolides	Bacteriostatic	Inhibits protein synthesis
Tetracycline	Bacteriostatic	Inhibits protein synthesis
Chloramphenicol	Bacteriostatic	Inhibits protein synthesis
Sulphonamides	Bacteriostatic	Competitive Inhibition

2.4 Antimicrobial resistance

Antibiotic resistance is the reduction in effectiveness of an antimicrobial agent in curing a disease or condition. European Centre for Disease Control (ECDC) defines Multidrug resistant (MDR) as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories (ECDC, 2011). Treatment of bacterial infections is compromised by the emergence of organisms that are resistant to multiple antibiotics.

2.4.1 Basic mechanisms of antibiotic resistance

Mechanisms of antimicrobial resistance are classified as either innate or acquired mechanisms. Innate resistance mechanisms are further classified into intrinsic or induced. Intrinsic resistance mechanisms are those specified by naturally occurring genes in the host's chromosome. It is a trait shared universally within a bacterial species and is independent of

previous antibiotic exposure. The common mechanisms that are involved in intrinsic resistance include: reduced permeability of the outer cell wall limiting uptake of the drug, drug inactivation and natural activity of efflux pumps. For induced mechanisms, genes are naturally occurring in bacteria but are expressed to resistance levels only after exposure to a specific antibiotic. The common mechanism of induced resistance is multidrug-efflux pumps activity, target modification and drug inactivation (Reygaert, 2016).

Acquired/Secondary resistance mechanisms on the other hand involve mutations in genes targeted by the antibiotics or acquisition of genetic material through horizontal gene transfer as shown in Table 2.2. The transfer of resistance determinants is through plasmids, bacteriophages, transposons and other mobile genetic material. This exchange of resistance genes is accomplished through the processes of transduction via bacteriophages, conjugation via plasmids and conjugate transposons and transformation via incorporation into chromosomal DNA or plasmids. Plasmid-mediated transmission of resistance genes is the most commonly seen route for the acquisition of outside genetic material. Once established resistant clone persists and spread worldwide, causing clinical failures in the treatment of infections (Alekshun and Levy, 2007; Reygaert, 2016).

Table 2.2: Types of acquired resistance mechanisms for various antimicrobial classes and their common encoding genes.

Antimicrobial Class	**Target within bacteria**	**Type of acquired resistance mechanism**	**Specific resistance mechanism**	**Common encoding genes**
Beta-lactams	Penicillin-binding protein (PBP)	Target modification	PBP with reduced affinity	*mec*A
		Inactivating enzymes	β-lactamase enzymes including	bla_{TEM}, bla_{SHV}, bla_{CMY}, bla_{Z}
			AmpC enzymes	
			Extended-spectrum β-lactamases	bla_{TEM}, bla_{SHV} bla_{CTX-M}, bla_{OXA}
Quinolones	DNA topoisomerase enzymes	Target modification	Altered QRDR of topoisomerase enzymes	Chromosomal mutations of *gyr*A and *par*C
		Target protection	Pentapeptide molecule (protection factor)	*qnr*A, *qnr*B, *qnr*S
		Inactivating Enzymes	Acetyltransferase enzymes	*ACC*(6′)-lb-cr
		Antimicrobial efflux	Efflux pumps	*qep*A
Tetracyclines	30S ribosomal subunit	Target protection	Ribosomal protection factor	*tet*(M)
		Antimicrobial efflux	Efflux pumps	*tet*(A), *tet*(B), *tet*(C)

Aminoglycosides	30S ribosomal subunit	Inactivating enzymes	Adenylyltransferase/ Acetyltransferase enzymes	*aad*A1, *aad*A2, *aad*A4, *aac*(3)-l, *aac*(6´)-lb
		Target modification	Methylase enzymes	*arm*A, *rtm*B
Macrolides & Lincosamides	50S ribosomal subunit	Target modification	Altered ribosomal binding site	*erm*A, *erm*B
		Antimicrobial efflux	Efflux pumps	*mef*(A), *msr*(D)
		Inactivating enzymes	Esterase, acetyltransferase enzymes	*mph*(C)
			acetyltransferase enzyme	*Cat*
Chloramphenicol	50S subunit	Inactivating enzymes	Esterase, acetyltransferase enzymes	*mph*(C), *cat*
Sulfonamides	Dihydropteroic acid synthase	Target modification	Alternative dihydropteroic acid synthase	*sul*1, *sul*2
Trimethoprim	Dihydrofolate reductase	Target modification	Alternative dihydrofolate reductase	*dfr*A1
Glycopeptides	Cell wall peptidoglycans	Target modification	Alternative peptidoglycan precursors	

2.4.2 Mechanisms of antimicrobial resistance by *Escherichia coli*

There are four main mechanisms of antimicrobial resistance that bacteria use: limiting the uptake of a drug, modifying a drug target, inactivating a drug, and actively effluxing a drug. Gram negative bacteria including *Escherichia coli* species can utilize all four mechanisms to resist antibiotics (Reygaert, 2016).

2.4.2.1 Limiting drug uptake

In the *Escherichia coli* species, the structure and functions of the lipopolysaccharide (LPS) cell wall layer provide a barrier to certain molecules conferring innate resistance to certain antimicrobial agents. Drug uptake is limited by changes in porins: either a decrease in the number of porins or changes in the selectivity of the porin channel, usually through mutations which affect porin channel structure or charge (Reygaert, 2016).

2.4.2.2 Modification of drug targets

The bacterial cell contains many components that may be potential targets for antimicrobial agents. The bacteria are capable of modifying any or all of these targets to enable resistance to those drugs. Drugs that target nucleic acid synthesis, such as the fluoroquinolones, are resisted via modifications in DNA gyrase (gram negative bacteria – e.g. *gyr*A) or topoisomerase IV (gram positive bacteria – e.g. *grl*A). The mutations in those genes cause changes in the structure of gyrase or topoisomerase. Those changes decrease or eliminate the ability of the drug to bind to these components. There are specific types of drugs that target bacterial metabolic pathways. Resistance to these drugs is via mutations in specific enzymes (DHPS – dihydropteroate synthase, DHFR – dihydrofolate reductase) that are involved in the folate biosynthesis pathway and/or overproduction of resistant DHPS and DHFR enzymes. The sulfonamides (target - DHPS) and trimethoprim (target - DHFR) are able to bind to their respective enzymes because they are structural analogs of the natural substrates.

Resistance to the β-lactam drugs is commonly achieved via alterations in the structure and/or number of penicillin-binding proteins (PBPs), which are transpeptidases involved in the

construction of peptidoglycan in the cell wall (a mechanism used almost exclusively by gram positive bacteria). A change in PBP structure may decrease the ability of the drug to bind, or stop the ability of a drug to bind (Reygaert, 2016).

2.4.2.3 Active efflux of drugs

Most bacteria have genes for efflux pumps that are chromosomally encoded. Some of the genes for these pumps are expressed constitutively while other pumps are only induced (or sometimes overexpressed) by certain environmental stimuli or presence of a suitable substrate. High level drug resistance by efflux pumps usually occurs via a mutation that modifies the inside of the transport channel of the pump. The main function of efflux pumps is to protect the bacteria from toxic substances. Many of these pumps are capable of transporting a wide variety of compounds and are known as multi-drug (MDR) efflux pumps (Reygaert, 2016).

E. coli possesses several efflux pumps for various classes of drugs including tetracycline and fluoroquinolones (Xian-Zhi Li, 2015).

The up regulation of efflux pumps and plasmid-mediated resistance mechanisms (e.g. *qnr* determinants) can reduce fluoroquinolone susceptibilities in *E. coli*, however high level resistance to the fluoroquinolones typically requires 1-2 point mutations within the quinolone resistance determining regions of *gyrA* and *parC***,** the chromosomal genes encoding for DNA gyrase and topoisomerase IV respectively (Johnson, 2013).

2.4.2.4 Inactivation of drugs

Bacteria inactivate drugs by using two main mechanisms: by actual degradation of the drug, or by transfer of a chemical group to the drug. One very large group of drug hydrolysing enzymes is the β-lactamases. The process by which the β-lactamases inactivate a drug is though hydrolyzation of a site in the ring structure. This action breaks the ring and inactivates the drug (Reygaert, 2016).

Production of b-lactamases by Escherichia coli species remains the most important resistance mechanism to b-lactam antibiotics. b-lactamases are bacterial enzymes that inactivate b-lactam antibiotics by hydrolysis, which results in ineffective compounds. However, Enterobacteriaceae that produce carbapenemases, which are enzymes that deactivate carbapenems and most other ß-lactam antibiotics, have emerged and are increasingly being reported worldwide (Morrill, 2015).

Tetracycline is another drug that can be inactivated by hydrolyzation, via the *tet*X gene.

2.4.3 Extended-spectrum beta-lactamases in *Escherichia coli*

The most important resistance mechanism by *Escherichia coli* to beta lactam antibiotics is production of Extended-spectrum beta-lactamases (ESBL). Extended-spectrum β-lactamases (ESBLs) are generally acquired by horizontal gene transfer, some being mutant derivatives of established plasmid-mediated β-lactamases (e.g. bla_{TEM}/bla_{SHV}) or mobilized from environmental bacteria (e.g. $bla_{\text{CTX-M}}$) (Hawkey, 2009). These enzymes reduce the efficacy of penicillins, modern expanded-spectrum cephalosporins (except cephamycins and carbapenems) and monobactams by hydrolyzing their beta lactam ring. However, these enzymes are inhibited by β-lactamase inhibitors such as clavulanic acid, salbactam and tazobactam, a feature used as a criterion for classification of β-lactamases and for diagnostic ESBL detection purposes (Paterson and Bonomo, 2005).

Beta-Lactamases are classified according to two general schemes: the Ambler molecular classification scheme and the Bush-Jacoby-Medieros functional classification system as shown in Table 2.3. The Ambler scheme divides beta-lactamases into four major classes; A, B, C and D. The basis of this classification scheme rests upon protein homology (amino acid similarity), and not phenotypic characteristics. Classes A, C, and D are serine beta-lactamases while the class B enzymes are metallo-β-lactamases. The Bush-Jacoby-Medeiros classification scheme groups beta-lactamases according to functional similarities (substrate

and inhibitor profile) (Paterson & Bonomo, 2005). Using this scheme, ESBLs are defined as β-lactamases capable of hydrolyzing oximino-cephalosporins and are inhibited by clavulanic acid and are placed into functional group 2be.

Table 2.3: ESBLs classification, distinctive substrate and representative enzymes

Bush-Jacoby-Medeiros	Ambler Classification	Distinctive substrate	Inhibitor	Representative Enzyme
1	C	Cephalosporins	None	AmpC
2b	A	Penicillins, Narrow-spectrum cephalosporins	B-lactamase inhibitors	TEM-1, TEM-2, TEM-13, SHV-1
2be	A	Extended-spectrum cephalosporins and Aztreonam	B-lactamase inhibitors	TEM-3, SHV-2, PER, VEB, CTX-M-15
2d	D	Cloxacillin	B-lactamase inhibitors	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	B-lactamase inhibitors	OXA-11, 0XA-15
2df	D	Carbapenems	B-lactamase inhibitors	OXA-23, OXA-48
2f	A	Carbapenems	B-lactamase inhibitors	KPC, IMI, SME, NMC
3a	B	Carbapenems	EDTA	MBL

2.4.4 Extended-spectrum beta-lactamase genes in *Escherichia coli*

Extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* strains are widely distributed among humans and animals (Schmid et al., 2013). The most common ESBLs are SHV, TEM and CTX-M families. SHVs are prevalent in Europe; TEMs are dominantly present in USA while CTX-Ms are being increasingly detected worldwide. Three other families of ESBLs have also been documented.

SHV Type (Sulfhydryl variable): The progenitor of the SHV class of enzymes, SHV-1 is primarily found in *Klebsiella pneumoniae*. The gene encoding SHV-1 beta-lactamase resides within the bacterial chromosome in *Klebsiella spp*. However, gene evolution and subsequent incorporation into a plasmid has led to spread of this gene to other enterobacteria. SHV-1 confers resistance to broad spectrum penicillins such as ampicillin and piperacillin but not to oxyimino substituted cephalosporins. An example of SHV variant with increased activity against extended spectrum cephalosporins is SHV-2, formed by mutation of SHV-1 through replacement of glycine by serine at the 238 position (Shaikh *et al*., 2015).

TEM Type (Patient's name: Temoneira): TEM-1 is capable of hydrolyzing penicillins and first generation cephalosporins but is unable to attack oxyimino cephalosporins. TEM variants with increased activity against extended spectrum cephalosorins include TEM-3 and TEM-12. The emergence of these TEM variants was as a result of the selective pressure induced by extended spectrum cephalosporins (Shaikh *et al*., 2015).

CTX Type (cefotaximase-munich): They have a potent hydrolytic activity against cefotaxime. They are thought to have originated from chromosomal ESBL genes found in *Kluyvera species*; an opportunistic pathogen of the Enterobacteriaceae found in the environment. The gene sequences encoding CTX-M enzymes show a high similarity to those of beta-lactamases of *Kluyvera species*. More than 100 CTX-M proteins of have been sequenced to date. These enzymes are comprised in five sub-groups as CTX-M-1, -2, -8, -9 and -25. The most commonly found enzyme of these ESBLs is CTX-M-15 which belongs to CTX-M-1 sub-group (Shaikh *et al*., 2015). Unlike TEM- and SHV- ESBLS which were generated by amino acid substitutions of their parent enzymes, CTX-M ESBLs dissemination is through horizontal gene transfer mediated by mobile elements such as conjugative plasmids or transposons. There has been evidence that the proliferation of CTX-M-producing *E. coli* is due to the growth of indigenous CTX-M-producing strains and the possible

emergence of strains that acquired CTX-M genes by horizontal transfer (Hiroi *et al*., 2012). CTX-M is currently the most predominant ESBL worldwide (Fig.2.13).

OXA Type: They are so named because of their oxacillin-hydrolyzing abilities. The OXA type beta-lactamases are characterized by hydrolysis rates for cloxacillin and oxacillin greater than 50% as that of benzyl penicillin. They are mostly found in Pseudomonas aeruginosa but have also been detected in other Gram-negative bacteria such as *E. coli*. An example of OXA type ESBL is OXA-19 believed to have evolved from narrow spectrum OXA-13 beta-lactamase (Shaikh *et al*., 2015).

PER Type (Pseudomonas extended resistance): The PER-type ESBLs share only around 25 to 27% homology with known TEM- and SHV-type ESBLs. PER-1 beta-lactamase efficiently hydrolyses penicillins and cephalosporins and is susceptible to clavulanic acid inhibition. PER-1 was first detected in Pseudomonas aeruginosa, and later in *Salmonella enterica serovar Typhimurium* and *Acinetobacter* (Paterson and Bonomo, 2005).

VEB Type (Vietnam extended-spectrum beta-lactamase): VEB-1 has greatest homology with PER-1 and PER-2. It confers high-level resistance to ceftazidime, cefotaxime, and aztreonam, which is reversed by clavulanic acid. The gene encoding VEB-1 was found to be plasmid mediated; such plasmids also confer resistance to non-beta-lactam antibiotics. These beta-lactamases have properties resembling those of Class A ESBLs. (Paterson & Bonomo, 2005).

The minor ESBLs include GES, BES, TLA, SFO and BEL as they are rarely identified and are geographically localized.

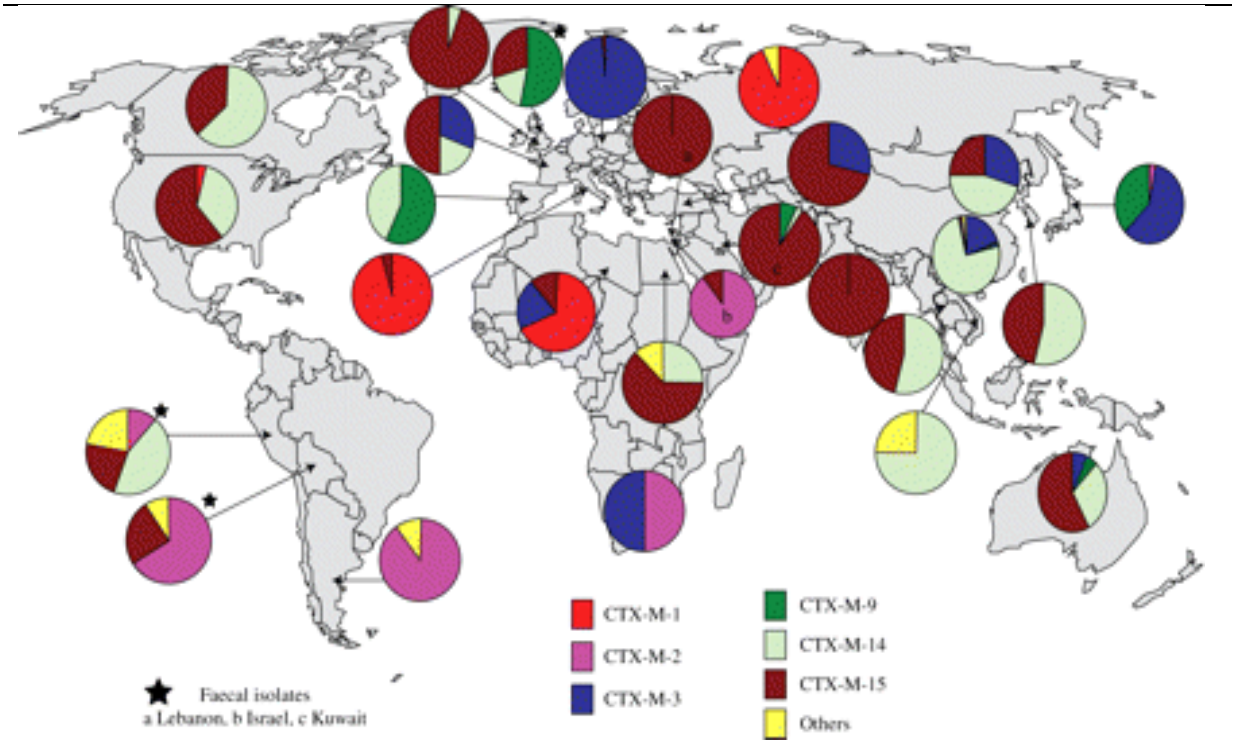

Figure 2.13 Global distribution of CTX-M genotypes

2.4.5 ESBLs producing *Escherichia coli* in food producing animals

The worldwide prevalence of ESBL-producing *Escherichia coli* and other Enterobacteriaceae is increasing rapidly in both hospitals and community. Significant similarities have been found among ESBL- producing *Escherichia coli* isolates from food animals and humans according to mobile resistance elements, virulence genes and genomic backbone (Kluytmans, 2013). Food animals are therefore increasingly recognized as a reservoir for ESBL-producing strains raising serious food safety questions. Several ESBL transmission pathways between dairy cattle, the environment, and humans have been proposed, including contact with manure-contaminated pastures, direct contact, or through the food chain from contaminated animal-derived products (van Hall *et al*., 2011; Collis *et al*., 2019).

A study in Turkey indicated a prevalence of 44.4% of ESBL producing strains among Escherichia coli isolated from raw meat, raw milk, white cheese and ice cream. The isolates

were found to be resistant to ampicillins and other antimicrobial agents but none was resistant to imipenem, ertapenem, cefepime and piperacillin/tazobactam (Gundogan and Avci, 2013).

A study on ESBL producing *E coli* from diseased food-producing animals in Germany conducted by Michael, *et al*., (2017) identified 11.2 % as ESBL producers among *Escherichia coli* isolates collected from cattle, 4.8% from pigs and 0.8% from poultry. ESBL genes detected were: *bla*$_{\text{CTX-M-1}}$ (69.9%), *bla*$_{\text{CTX-M-15}}$ (13.6%), *bla*$_{\text{CTX-M-14}}$ (11.7%), *bla*$_{\text{TEM-52}}$ (1.9%), *bla*$_{\text{SHV-12}}$ (1.4%), *bla*$_{\text{CTX-M-3}}$ (1.0%), and *bla*$_{\text{CTX-M-2}}$ (0.5%). Most of the ESBL-producing isolates were from animals suffering from gastrointestinal infections. In 95.2% of the isolates, additional resistance to non-β-lactam antibiotics was seen, which may facilitate the co-selection of ESBL genes, when located on the same mobile genetic element as the others resistance genes and may compromise the therapeutic options (Michael, *et al*., 2017). Analysis of extended-spectrum-β-lactamase-producing *Escherichia coli* isolates collected in the GERM-Vet indicated that the *bla*$_{\text{CTX-M-1}}$ gene is the predominant ESBL gene among *E. coli* isolates from diseased animals in Germany and a considerable structural heterogeneity was found in the regions flanking the *bla*$_{\text{CTX-M-1}}$ gene. The *bla*$_{\text{CTX-M-1}}$ genes were carried on IncN (n=16), IncF (n=3), IncI1 (n=2) or multireplicon (n=1) plasmids. A *bla*$_{\text{CTX-M-3}}$ gene was located on an IncN plasmid and a *bla*$_{\text{CTX-M-15}}$ gene was located on an IncF plasmid. A multireplicon plasmid and an IncHI1 plasmid harboured *bla*$_{\text{CTX-M-2}}$. A *bla*$_{\text{TEM-52c}}$ gene was identified within Tn2 on an IncI1 plasmid. The *bla*$_{\text{CTX-M}}$ genes located within the same or related genetic contexts showed differences due to the integration of insertion sequences. Various MLST types were detected, with ST10 (n=7), ST167 (n=4) and ST100 (n=3) being the most common (Schink, 2013). In another study, *Escherichia coli* the isolates which harboured *bla*$_{\text{CTX-M-1}}$ gene belonged to the novel multilocus sequence typing (MLST) types ST410, ST1576 and ST1153. Detailed sequence analysis showed that the integration of

insertion sequences, as well as interplasmid recombination events, accounted for the structural variability in the *bla*$_{\text{CTX-M}}$ gene regions (Schink, 2013).

Several surveys of ESBLs producing *E. coli* in food producing animals have been conducted in Africa; the *bla*$_{\text{CTX-M-1}}$ group being reported as dominant in most the surveys for both animals and humans. Certain *bla*$_{\text{CTX-M-1}}$ -harbouring clones (ST131/B2 or ST405/D) are mainly identified in humans, but they have also been reported in livestock species from Tanzania, Nigeria and Tunisia. Moreover, several reports suggested an inter-host circulation of specific plasmids (e.g. *bla*$_{\text{CTX-M-1}}$ -carrying IncI1/ST3 in Tunisia, IncY- and Inc-untypeable replicons co-harbouring *qnr*S1 and *bla*$_{\text{CTX-M-15}}$ in Tanzania and the worldwide distributed *bla*$_{\text{CTX-M-15}}$ -carrying IncF-type plasmids) (Alonso, 2017).

Use of third- and fourth-generation cephalosporins in animals is associated with high prevalence of ESBL/AmpC producing pathogens (Gonggrijp, 2016).

Hiroi et al., (2012) evaluated the diversity of ESBL genes in rectal samples of food producing animal and ESBL-carrying Escherichia coli were isolated from 60% of broiler rectal samples, 5.9% of layers, 12.5% of beef cattle and 3% of pigs.

Several other studies across the world have also reported food animals to be reservoirs of ESBL-producing strains of *Escherichia coli* (Nadine *et al*., 2012; Reist *et al*., 2013; Karuppasamy *et al*., 2015; Njage *et al*., 2012; Schmid *et al*., 2013; Heuvelink, 2019).

2.4.6 Transmission of ESBL genes

Transmission of genes encoding ESBL enzymes can occur either by emerging bacterial clones or by horizontal gene transfer (HGT).

Horizontal gene transfer is one of the most drivers of bacterial evolution and is frequently responsible for development of antimicrobial resistance. Three main strategies are involved in HGT: transformation, transduction and conjugation. Transformation is the uptake by bacteria

and subsequent integration, and functional expression of naked fragments of extracellular DNA. Transduction involves bacteriophages in transfer of ARGs to their microbial hosts, in turn promoting their own survival and dissemination. The transferable DNA sequences range from chromosomal DNA to MGEs such as plasmids, transposons and genomic islands. Of the thre strategies of horizontal gene transfer, conjugation is thought to have the greatest influence on the dissemination of antimicrobial resistance genes. It involves cell-to-cell contact and occurs at a high rate compared to other mechanisms. Conjugation uses mobile genetic elements (MGEs) as vehicles of transferring resistance genetic information from one bacterium to another of the same and/or different species. However, transfer of genetic information from chromosome to chromosome has been documented. The most important MGEs are plasmids and transposons (Munita, 2016; Von Wintersdorff, 2016; Sun, 2018).

Another common mechanism of accumulating antimicrobial resistance genes is through integrons. These are site-specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes (Munita, 2016). HGT has caused antibiotic resistance to spread from commensal and environmental species to pathogenic ones,

Multireplicon FII plasmids are shown to carry the most widely distributed $bla_{\text{CTX-M-15}}$ across continents, paving the way for $bla_{\text{CTX-M-15}}$ into different genetic lineages of *Escherichia coli* (Naseer, 2011).

The gut flora is an ideal reservoir for antibiotic resistance genes, where kilograms of bacteria of different species can interact, most often without causing disease and hence to much interference with the immune system. When antibiotics are used, resistant strains gain a selective advantage and are accumulated. This increases the probability for genes important for survival to be further disseminated (Brolund, 2014). Two different studies in Netherlands revealed genetic similarities between ESBL genes, plasmids and *Escherichia coli* strains isolated from Dutch patients and those isolated from retail chicken meat and poultry. The

findings were suggestive for transmission of ESBL genes, plasmids and Escherichia coli isolates from poultry to humans, most likely through food chain (van Hall *et al*., 2011).

3 CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Site

The study was conducted in farms within the peri-urban region of Nairobi City, Kenya. Nairobi City is located at approximately 1° 9'S, 1° 28'S and 36° 4'E, 37° 10'E. It occupies an area of about 696 km^2 the altitude varies between 1,600 and 1,850 meters above sea level. The climate is of temperate tropical type with two rainy seasons in March and April and another in November and December.

Five dairy farms were included in this study: University of Nairobi (UON) Veterinary Farm, Department of Veterinary services (DVS) farm at Ngong, Dominic Farm at Githunguri, Kabogo Farm at Githunguri and Karuga Farm at Githunguri. The selected farms were based on owners' consent to take part in this study. UON and DVS farms were institutional farms whereas the other three were private farms.

3.2 Study Design

This was a cross-sectional study involving three phases, where the first phase was sampling of fresh milk at source by hand milking from identified farms and isolation of *Escherichia coli* in the laboratory. The second phase involved antibiotic susceptibility testing (AST) and the third phase involved molecular characterization of phenotypically resistant *Escherichia coli* and sequencing.

3.3 Sampling

A total of 351 milk samples were collected from all cows in the 5 dairy farms: The Faculty of Veterinary Medicine farm (n=103), Department of Veterinary Services farm (n=52) and three private farms at Githunguri: Dominic Farm (n=106), Kabogo Farm (n=50) and Karuga Farm (n=40). The samples were collected in the morning between November 2016 and October 2017. Pooled milk samples of a volume of 10 ml from four teats of an individual cow were collected directly from the udder by hand milking into a sterile bijou bottle. Proper

disinfection of cow's udder and hands was done before milking. The first strip from each teat was cast-off and sample collected from the second strip.

The samples were transported in cool boxes to University of Nairobi, Department of Pharmacology and toxicology bacteriology laboratories for further analysis. Processing of the samples was done within six hours of samples collection.

3.4 Bacteriological analysis

The samples were enriched in buffered peptone water (Oxoid, Hampshire, England) and incubated at 37ºC for 24 hours. After 24 hours incubation, a loopful of the culture was inoculated onto the Tryptone Soya Agar (HiMedia Laboratories Pvt. Ltd, Mumbai, India) and incubated at 37°C for 24 hours (Zinnah, et al., 2007). Single colonies were then further cultured on the selective medium EMB agar (HiMedia Laboratories Pvt. Ltd, Mumbai, India) at 37ºC for 24 hours. The organisms showing characteristic colony morphology of *Escherichia coli* were further sub-cultured onto EMB agar to obtain pure culture with homogenous colonies.

The presumptive cultures of *Escherichia coli* obtained were further identified through four biochemical reactions: Indole test, Methyl red test, Citrate test and Voges-proskauer reaction. *Escherichia coli* ATCC® 25922 was used as the quality control strain during this microbiological analysis. After identification, the pure *Escherichia coli* isolates were stored in skimmed milk (Oxoid, Hampshire, England) and kept under refrigerated conditions.

3.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed as described in Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (2017) using Mueller-Hinton Agar (HiMedia Laboratories Pvt. Ltd, Mumbai, India). A single pure bacterial colony was suspended in normal saline to give an opacity equivalent to that of 0.5 McFarland standards and a uniformly thin lawn of the suspension was carefully spread on

Mueller-Hinton agar plates. Incubation was done at 37ºC, ambient air, for 16-18 hours. *Escherichia. coli* ATCC *25922* was used as the standard reference organism for quality control (CLSI, 2017).

A panel of 12 commonly used antimicrobial agents were tested using antimicrobial disks purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Antibiogram panel included: Ampicillin (10µg), amoxicillin/clavulanic acid (20/10µg), cefazolin (30µg), cefuroxime (30µg), cefoxitin (30µg), cefotaxime (30µg), ceftazidime (30µg), cefepime (30µg), imipenem (10µg), gentamycin (10µg), ciprofloxacin (5µg) and tetracycline (30µg). *E. coli* isolates were categorized as susceptible, intermediate, and resistant based on interpretive criteria developed by Clinical and Laboratory Standards Institute (CLSI, 2017). CLSI zone diameter interpretive break points for these antimicrobial agents were presented in the order; sensitive, intermediate and resistant: ampicillin (≥17, 14-16, ≤13), amoxicillin/clavulanic acid (≥18, 14-17, ≤13), cefazolin (≥15, ≤14), cefuroxime (≥18, 15-17, ≤14), cefoxitin (≥18, 15-17, ≤14), cefotaxime (≥26, 23-25, ≤22), ceftazidime (≥21, 18-20, ≤17), cefepime (≥25, 19-24, ≤18), imipenem (≥23, 20-22, ≤19), gentamycin (≥15, 13-14, ≤12), ciprofloxacin (≥21, 16-20, ≤15), and tetracycline (≥15, 12-14, ≤11).

3.6 Antimicrobial susceptibility testing (AST)

3.6.1 Reviving of *Escherichia coli* isolates

The samples were removed from storage medium and cultured onto selective medium EMB agar (HiMedia Laboratories Pvt. Ltd, Mumbai, India) at 37ºC for 24 hours. Single colonies were then further cultured on the nonselective medium Tryptone Soya agar plate (Oxoid, Hampshire, England) and incubated at 37ºC for 24 hours.

3.6.2 Inoculum preparation

The direct colony suspension method was used for inoculum preparation. The inoculum was prepared by making a direct saline suspension of isolated colonies selected from a 24-hour

Tryptone Soya agar plate (Oxoid, Hampshire, England), a nonselective medium, incubated at 37^{o}C. The suspension was adjusted to achieve a turbidity equivalent to 0.5 McFarland standard. The resultant suspension contained approximately 1 to 2×10^{8} colony-forming units (CFU)/ml. To perform this step accurately, a visual comparison was made for each inoculum prepared to a 0.5 McFarland standard tube.

3.6.3 Inoculation of test plates

A sterile cotton swab was dipped into the adjusted suspension within 15 minutes after the preparation of the inoculum suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This was to remove excess fluid from the swab. The dried surface of the Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid of the plate was left open for three to five minutes, to allow for any excess surface moisture to be absorbed before application of the drug-impregnated disks.

3.6.4 Application of disks to inoculated agar plates

The antimicrobial disks were dispensed onto the surface of the inoculated agar plate. Each disk was pressed down to ensure complete contact with the agar surface. The plates were inverted and incubated at 37°C for 18 hours.

3.6.5 Determination of diameters of zones of inhibition

The zone diameters of complete inhibition, including that of the disks, were measured to the nearest whole millimetre using a ruler. For each isolate, the antimicrobial susceptibility testing was done in triplicates and the mean zone diameters of inhibition calculated. For purposes of interpretation, these mean diameter zones of inhibition were compared with standard break points for *Escherichia coli* spp. for each tested antibiotic using CLSI

guidelines (CLSI, 2017). The antimicrobial susceptibility was scored as susceptible, intermediate or resistant. The overall results were tabulated on an antibiogram (Appendix 1).

3.6.6 Quality control of AST

Each batch of AST agar plates were tested alongside standard quality control organism to validate the results obtained for the batch (Fig. 3.1). *Escherichia. coli* ATCC *25922* was used as quality control organism according to CLSI guidelines (CLSI, 2017). Uninoculated agar plate was also incubated alongside each batch of tests run to verify sterility of the medium.

Figure 3.1 Antibiotic susceptibility testing in progress

3.7 Phenotypic screening for Extended Spectrum β-Lactamases (ESBLs) producers

Presumptive ESBL producers were screened using standard disc-diffusion method on Mueller Hinton agar incubation conditions of 35 ± 2°C, ambient air for 16 -18 hours. Selection of isolates for screening for ESBL production was based on zone diameters for ceftazidime 30µg and cefotaxime 30µg. The isolates were categorized as ESBL producers if either ceftazidime zone ≤ 22 mm or cefotaxime zone ≤ 27 mm were observed (CLSI 2017). ESBL test was performed using standard disc-diffusion method on Mueller Hinton agar, incubation conditions of 35 ± 2°C, ambient air for 16-18 hours. The following antimicrobial disk sets

were used for determination of ESBL production: ceftazidime 30µg and ceftazidime-clavulanate 30µg/10µg and cefotaxime 30µg & cefotaxime-clavulanate 30µg/10µg. The results were considered positive if ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanate vs the zone diameter of the agent when tested alone was observed, else, the result was considered negative. *Escherichia. coli* ATCC *25922* was used for quality control. (CLSI 2017).

3.8 PCR detection of ESBL genes

Phenotypically identified ESBL- producing *E. coli* isolates were assayed by PCR for presence of three ESBL genes: bla_{TEM}, $bla_{SHV}$and $bla_{CTX\text{-}M}$ using PCR. The primers used were described by Hakki and Ozpinar (2016) and are shown in Table 4.

3.8.1. DNA extraction

Extraction of DNA was performed as described by Díaz, (2012). Two or three colonies were obtained from 18 – 48 hours cultures inoculated on tryptic soy agar (4.1 %) and suspended in 400 µl of sterile distilled water. The bacterial suspension was boiled at 95°C for 7 minutes and then centrifuged at 15,000 *g* for 1 min and the supernatant was collected. The DNA supernatant extracts were stored at -20°C until used as a template for the PCR reactions.

3.8.2 Validation of isolates

Amplification of *gad*A gene of all strains were performed at first to confirm that they were *Escherichia coli.* This was performed in a protocol adapted from Grant *et al*. (2001). PCR reaction was done in a total volume of 20 µl containing 5 µl of DNA template, 10 µl of mastermix, 1.28 µl each of primers *gad*A-F and *gad*A-R and 2.44 µl of distilled water . Thermal cycling reactions consisted of an initial denaturation at 94°C for 10 min; followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 65°C for 15 s, extension at 72°C for 1 min; and a final elongation at 72°C for 5 min. Amplification products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. Gels were visualised

under U.V light. Amplification of the 715bp PCR product confirmed that the strains were *Escherichia coli.*

3.8.3 Detection of ESBL genes

For detection of each ESBL gene, a volume of 5 µL of the DNA was subjected to singleplex PCR in a 25 µL reaction mixture containing 1x PCR buffer (10mM Tris-Hcl, pH 8.3/ 50mM Kcl/ 1.5 mM $MgCl_2$), 200µM concentration of each deoxynucleotide triphosphate, a variable concentration of specific group primers and IU of Taq polymerase (Sigma Aldrich, St. Quentin fallarien, France).

The amplification was done in an MJ minicycler (MJ Research Inc., USA) under various conditions as shown in Table 3.1. The amplicons were electrophoresed on 1.3 % agarose gels in Tris-acetate-EDTA buffer supplemented with 0.5 µg/ml of ethidium bromide and calibrated using 100bp DNA ladder (100) (GelPilot, QIAGEN, USA). The gels were visually inspected by UV-transilluminator (TF-35M Vilber Lourmat illuminator, France). Gel image was captured using a camera.

Table 3.1: Primers used for amplifying and sequencing resistant genes

Primer (F/R)	**Sequence**	**Amplicon size (bp)**	**Thermocycler condition (TC)**
bla TEM-F	5-GCTCACCCAGAAACGCTGGT-3	686	1 cycle for 15 min at 95◦C, followed by 35 cycles for 1 min at 95◦C, 1 min at 63◦C, and 1 min at 72◦C, and finally 10 min at 72◦C.
bla TEM-R	5-CCATCTGGCC CCAGTGCTGC-3		
bla SHV-F	5-CCCGCAGCCGCTTGAGCAAA-3	733	1 cycle for 15 min at 95◦C, followed by 30 cycles for 30 s at 94◦C, 30 s at 58.5◦C, and 45 s at 72◦C, and finally 10 min at 72◦C
bla SHV-R	5-CATGCTCGCCGGCGTATCCC-3		
bla CTX-M-F	5-SCSATGTGCAGYACCAGTAA-3	585	1 cycle for 15 min at 95◦C, followed by 30 cycles for 30 s at 94◦C, 30 s at 55◦C, and 45 s at 72◦C, and a final elongation at 72◦C for 10 min
bla CTX-M-R	5-ACCAGAAYVAGCGGBGC-3		

3.9 Sequencing of resistant genes

The amplicons obtained using gene-specific primers were sent to Macrogen Europe, 1105

AZ, Amsterdam, Netherlands for purification and sequencing.

4 CHAPTER FOUR: RESULTS

4.1 Isolation and identification of *Escherichia coli*

Out of 351 samples collected from 5 dairy farms and analysed for presence of *Escherichia coli* using selective medium EMB Agar, 139 presumptive *Escherichia coli* isolates were detected after their morphology showed green metallic sheen colonies. One hundred and one isolates (n=101) were identified as *Escherichia coli* through biochemical reactions. The distribution of positive cases was as follows: 30 isolates from University of Nairobi Veterinary Farm samples, 18 isolates from Department of Veterinary services farm at Ngong, 41 isolates from Dominic Farm at Githunguri, 7 isolates from Kabogo Farm at Githunguri and 5 isolates from Karuga Farm at Githunguri. Ninety-one isolates (n=91) were confirmed to be *Escherichia coli* by PCR assay.

4.2 Antimicrobial susceptibility profiles

Antimicrobial susceptibility testing was done on 91 isolates of which 17 isolates (18.7%) were susceptible to all the antimicrobial agents. Seventy-four isolates (81.3%) showed resistance to at least one antimicrobial agent and twenty-six (28.6%) isolates were resistant to more than one antimicrobial agent. Five isolates (5.5%) were resistant to at least one agent in three or more antimicrobial categories indicating that they were MDR strains. The phenotypic resistance profiles were: - ampicillin 54 (59.3%) isolates followed by tetracycline in 19 (20.9%) isolates and amoxiclav 10 (11.0%) isolates. Four isolates (4.4%) were observed to be resistant to cefazolin, one isolate (1.1%) each found resistant to cefoxitin, ceftazidime and cefotaxime. None of the isolates was resistant to cefuroxime, cefepime, imipenem, ciprofloxacin and gentamycin. Intermediate susceptibility to some of the antibiotics tested were- ampicillin 12 (13.2%), amoxiclav 33 (36.3%), cefotaxime 5 (5.5%), cefepime 4 (4.4%), tetracycline 3 (3.3%) and gentamycin 1 (1.1%). The resistance profile to the antimicrobial agents is shown in Table 4.1

4.3 Phenotypic characterization of ESBL producing isolates

Forty-five isolates (49.5%) were categorized as presumptive ESBL producers based on their ceftazidime and cefotaxime zone diameters from the AST data in table 5, (Ceftazidime zone ≤ 22 mm or Cefotaxime zone ≤ 27 mm).

Thirty-five (38.5%) isolates were phenotypically identified as ESBL producing strains and the remaining 10 isolates tested negative for ESBL production. The characteristics of 45 *Escherichia coli* strains tested are shown in appendix 2.

4.4 Detection of ESBL genes by PCR

Thirty-five phenotypically identified ESBL- producing *E. coli* isolates were assayed for presence of three ESBL genes: bla_{TEM}, bla_{SHV} and bla_{CTX-M} using PCR (Fig 4.1, 4.2 and 4.3). Primers used in this assay are shown in Table 3.1.

Thirty-three out of 35 *Escherichia coli* isolates were found to have at least one ESBL gene while two isolates though phenotypically identified as ESBL strains did not harbour any ESBL gene. Sixteen (17.6%) isolates harboured two ESBL genes. Nine (9.9%) isolates had all three ESBL genes screened; bla_{TEM}, bla_{SHV} and bla_{CTX-M}. bla_{CTX-M} was the most predominant gene detected in 31 isolates (34.1%) followed by bla_{TEM} which was observed in 24 isolates (26.4%). bla_{SHV} was detected in 12 isolates (13.2%). The ESBL genes detected in the 33 *Escherichia coli* isolates are shown in Table 4.2.

Table 4.1: Antimicrobial resistance profile of 91 *Escherichia coli* isolates to selected antimicrobial agents

Antimicrobial Agent	**Resistance Profile (No. of isolates & percentage)**		
	Susceptible	**Intermediate**	**Resistant**
Ampicillin	25 (27.5%)	12 (13.2%)	54 (59.3%)
Amoxiclav	8 (8.8%)	33 (36.3%)	10 (11.0%)
Cefazolin	87 (95.6%)	00 (0.0%)	04 (4.4%)
Cefuroxime	91 (100.0%)	00 (0.0%)	00 (0.0%)
Cefoxitin	90 (98.9%)	00 (0.0%)	01 (1.1%)
Ceftazidime	90 (98.9%)	00 (0.0%)	01 (1.1%)
Cefotaxime	85 (93.4%)	05 (5.5%)	01 (1.1%)
Cefepime	87 (95.6%)	04 (4.4%)	00 (0.0%)
Imipenem	91 (100.0%)	00 (0.0%)	00 (0.0%)
Ciprofloxacin	91 (100.0%)	00 (0.0%)	00 (0.0%)
Tetracycline	69 (75.8%)	03 (3.3%)	19 (20.9%)
Gentamycin	90 (98.9%)	01 (1.1%)	00 (0.0%}

Table 4.2: ESBL genes in 35 *Escherichia coli* isolates phenotypically detected for ESBL production.

S/No.	**Sample Lab No.**	**Phenotype**	***bla*TEM**	***bla*SHV**	***bla*CTX-M**
1	1	ESBL	+VE	+VE	+VE
2	2	ESBL	+VE	+VE	+VE
3	4	ESBL	+VE	–VE	+VE
4	6	ESBL	+VE	+VE	+VE
5	10	ESBL	–VE	–VE	–VE
6	11	ESBL	+VE	–VE	+VE
7	13	ESBL	+VE	–VE	+VE
8	14	ESBL	+VE	+VE	+VE
9	16	ESBL	+VE	+VE	+VE
10	22	ESBL	+VE	+VE	+VE
11	23	ESBL	+VE	–VE	+VE
12	30	ESBL	+VE	–VE	+VE
13	31	ESBL	+VE	–VE	+VE

14	32	ESBL	+VE	–VE	+VE
15	33	ESBL	+VE	–VE	+VE
16	34	ESBL	–VE	–VE	–VE
17	48	ESBL	–VE	–VE	+VE
18	49	ESBL	–VE	+VE	–VE
19	50	ESBL	–VE	–VE	+VE
20	53	ESBL	+VE	–VE	+VE
21	54	ESBL	+VE	–VE	+VE
22	58	ESBL	–VE	–VE	+VE
23	61	ESBL	+VE	+VE	–VE
24	67	ESBL	+VE	+VE	+VE
25	69	ESBL	+VE	+VE	+VE
26	70	ESBL	+VE	+VE	+VE
27	71	ESBL	–VE	–VE	+VE
28	72	ESBL	–VE	+VE	+VE
29	76	ESBL	–VE	–VE	+VE
30	77	ESBL	+VE	–VE	+VE
31	80	ESBL	+VE	–VE	+VE
32	81	ESBL	–VE	–VE	+VE
33	83	ESBL	+VE	–VE	+VE
34	85	ESBL	–VE	–VE	+VE
35	87	ESBL	+VE	–VE	+VE
TOTAL		**Positive**	**24**	**12**	**31**
		Negative	**11**	**23**	**04**

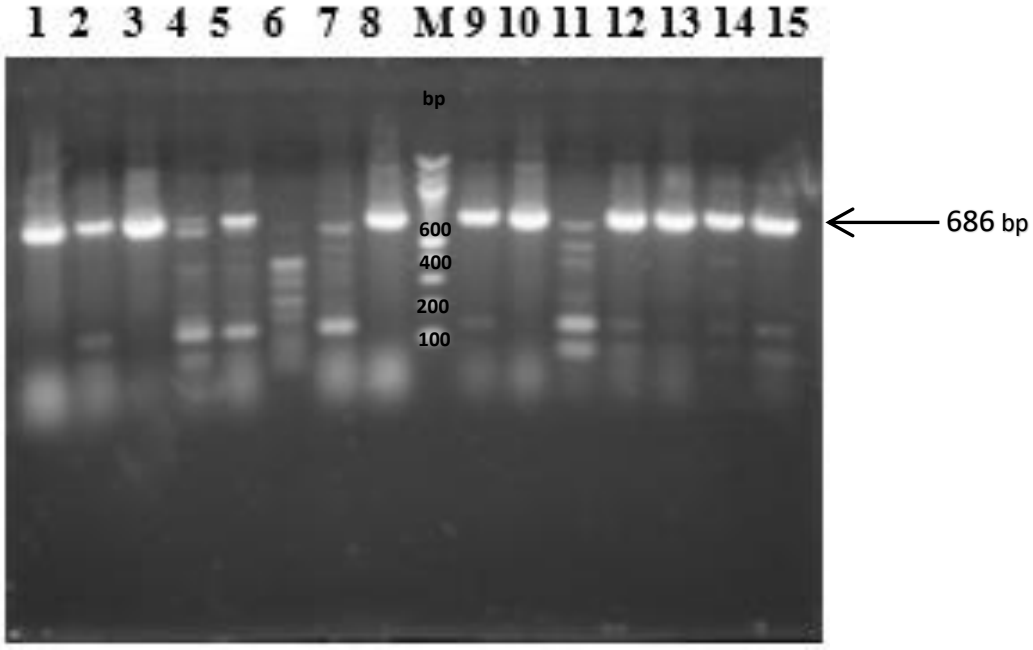

Figure 4.1: Electrophoretic analysis of the *bla*TEM genes' polymerase chain reaction (PCR) screening results from representative *Escherichia coli* isolates obtained from milk samples. The amplification of the gene is seen by presence of a specific band approximately 686 bp. M is 100 bp DNA ladder marker.

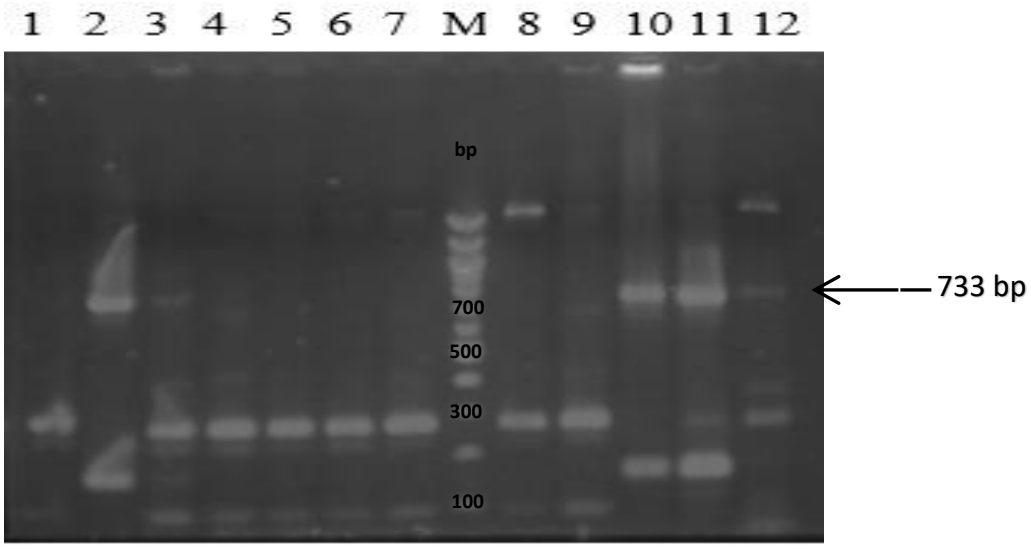

Figure 4.2: Electrophoretic analysis of the *bla*SHV genes' polymerase chain reaction (PCR) screening results from representative *Escherichia coli* isolates obtained from milk samples. The amplification of the gene is seen by presence of a specific band approximately 733 bp. M is 100 bp DNA ladder marker.

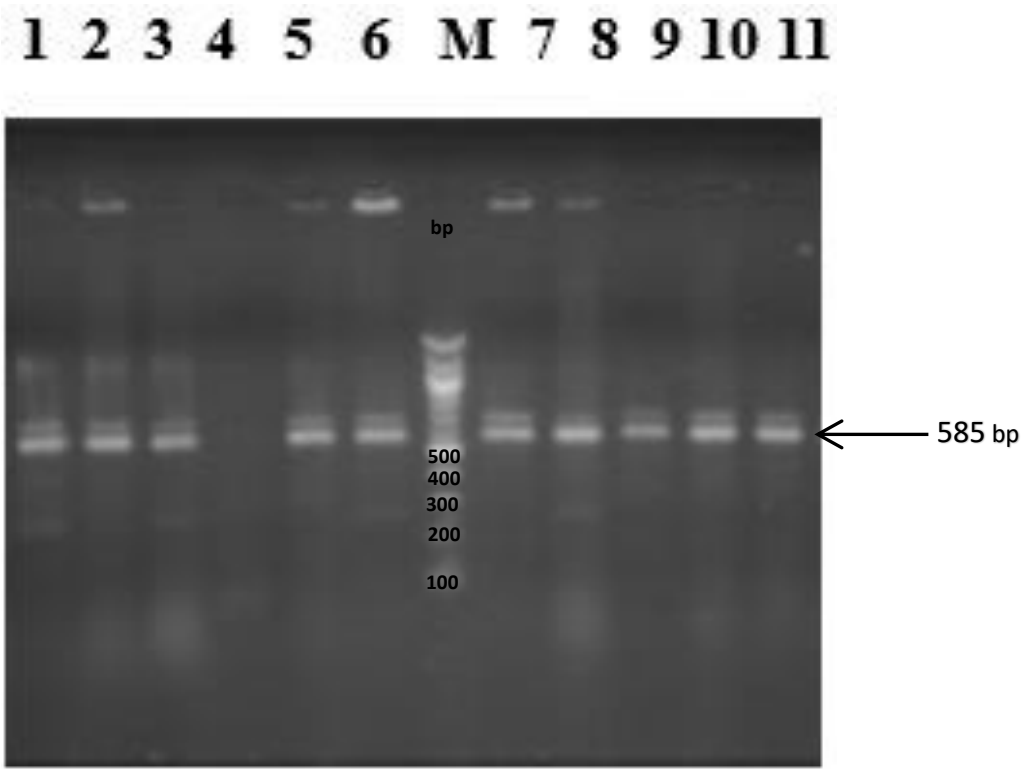

Figure 4.3: Electrophoretic analysis of the *bla*CTX-M genes' polymerase chain reaction (PCR) screening results from representative *Escherichia coli* isolates obtained from milk samples. The amplification of the gene is seen by presence of a specific band approximately 585 bp. M is 100 bp DNA ladder marker.

4.5 BLAST analysis of DNA sequences

Sequence analysis revealed by *Escherichia coli* strains harboured all resistant genes. All resistance genes revealed 97 - 100 % nucleotide identity to sequences in the NCBI database (Table 4.3).

Table 4.3: Resistant gene nucleotide homologues and their percentage identities

Isolate Id	Homologue	% Identity	Accession Number
UEC 688/16	*Escherichia coli* strain 3EC TEM family beta-lactamase (*bla*TEM) gene	98.18	MN158355.1
UEC 783/16	*Escherichia coli* strain ESBL 15 chromosome	99.62	CP041678.1
UEC 755/16	*Escherichia coli* strain ESBL 15 chromosome	99.23	CP041678.1
UEC 714/16	*Escherichia coli* 5971R *bla*TEM gene for class A extended-spectrum beta-lactamase TEM-207	99.08	NG_050239.1
NEC K40/17	*Escherichia coli* strain ESBL 15 chromosome	99.81	CP041678.1
NEC 3005/17	*Escherichia coli* strain A130 plasmid pA130-TEM	99.09	MN816372.1
NEC 3017/17	*Escherichia coli* strain ESBL 15 chromosome	99.25	CP041678.1
GEC 86/17	*Escherichia coli* strain ESBL 15 chromosome	99.04	CP041678.1
GEC104/17	*Escherichia coli* strain ESBL 15 chromosome	99.81	CP041678.1
GEC110/17	*Escherichia coli* strain A130 plasmid pA130-TEM	97.84	MN816372.1
GEC155/17	*Escherichia coli* strain A130 plasmid pA130-TEM	98.31	MN816372.1

5 CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATION

5.1 DISCUSSION

The study identified multidrug-resistant *Escherichia coli* isolates in milk sampled from five dairy farms in Kenya. This finding has important implications on healthcare sector as presence of resistant strains of *E. coli* complicates the management of associated diseases given that a narrow range of antibiotics can be used effectively for treatment. Besides, new, last-resort antibiotics used in treatment of these infections are expensive and not affordable to many. This represents one of the biggest threats to global health today; increasing hospital stays, medical costs and mortality rate.

Out of 351 milk samples collected in the present study, *Escherichia coli* was isolated from 91 (25.6%) samples based on the morphological and cultural characteristics, biochemical reactions and PCR assay. A study by Palaha *et al*., (2012) showed that *E. coli* isolates were present in the udder of dairy cows with 17.3% being reported from samples collected directly from cows' udder. The observed prevalence in the current study was higher than 13.8 % reported in milk samples collected recently from Northern Kenya (Ngaywa, 2019). The prevalence was however lower than 34 % reported in Ethiopia (Disassa, 2017), 38% in India (Thaker, 2012), 42% in South Africa (Ntuli, 2016) and 65% in Malaysia (Chye, 2004). The disparity in prevalence could be attributed to milking technique and culture method. Poor hygiene in dairies may result in the contamination at the time of milking can be a possible cause for the presence of *E. coli* in milk.

The pattern of multidrug resistance observed in this study is consistent with other studies in Taiwan (Yaochi *et al*., 2016) and Ethiopia (Messele *et al*., 2019) which reported multidrug resistance patterns in 70% and 68.7% of *Escherichia coli* isolates respectively.

The resistance patterns were consistent with findings in a study from Turkey (Gundogan and Avci, 2013) where all isolates of *Escherichia coli* and *Klebsiella spp* from foods of animal origin showed resistance to ampicillin but none exhibited resistance to imipenem, cefepime and piperacillin/tazobactam. In the same study, some *Escherichia coli* and *Klebsiella spp*. isolates were resistant to cefotaxime, ceftazidime, ceftriaxone, aztroenam, tetracycline and ciprofloxacin but all isolates were resistant to two or more antimicrobial agents. Ampicillin resistance is common in other studies which have reported highest frequencies of non-susceptibility among respective isolates of *E. coli* to ampicillin (Amanda *et al*., 2018 and Simona, 2014).

In this study, none of the isolates was found resistant to cefuroxime, cefepime, imipenem, ciprofloxacin and gentamycin. This observation is similar to that of Rafael et al., (2014) where all *Escherichia coli* isolates from fish were susceptible to ciprofloxacin, gentamycim and imipenem. However, this observation differs from a study by George *et al*., (2012) which reported resistance of *Escherichia coli* isolates to ciprofloxacin and gentamycin. The observation also differs from a study by Omololu-Aso *et al*., (2017) who observed a high proportion of *Escherichia coli* isolates (92.86%) being resistant to ciprofloxacin. For both studies, investigators collected samples from human patients in hospitals in Turkey and Nigeria respectively and this may explain the disparity. The prevalence of ESBL- producing *E. coli* is similar to that reported by Schmid *et al*., (2013). In their study a total of 598 samples yielded 196 (32.8%) samples that contained ESBL-producing *E. coli.*

In the present study, presence of ESBL genes in *Escherichia coli* isolates is similar to other studies within or outside of Kenya (Maina *et.al*, 2012; Gundogan and Avci, 2013; Kluytmans *et al*., 2013 and Abdallah *et al*., 2015). Presence of all three ESBL genes - *bla*TEM, *bla*SHV and *bla*CTX-M observed in this study was similar to a study by Michael *et al*., (2017) where

the three genes were detected in samples collected from food animals including cattle, pigs and poultry.

In a study by Tekinar and Ozpinar (2016) the frequency rates of *bla*$_{TEM}$, *bla*$_{CTX-M}$, and *bla*$_{SHV}$ were 96.4%, 53.7%, and 34.5%, respectively. This is different from observations in this study where *bla*$_{CTX-M}$ is the predominant gene observed. They further reported co-existence of *bla* genes was observed in 82 % of extended spectrum beta-lactamases producers with a distribution of *bla*$_{TEM}$ and *bla*$_{CTX-M}$ (52.7%), *bla*$_{TEM}$ and *bla*$_{SHV}$ (20%), *bla*$_{TEM}$ and *bla*$_{CTX-M}$ & *bla*$_{SHV}$ (12.7%), and *bla*$_{SHV}$ & *bla*$_{CTX-M}$ (1.8%) which is similar to the current study.

A study by Chakraborty *et al*., (2015) on ESBL producing *Escherichia coli* isolates from extra-intestinal infections in humans indicated that *bla*$_{CTX-M}$ was the most common gene with 62 % of the isolates being positive for this gene, 14 % being positive for b*la*$_{TEM}$ and only 4% having *bla*$_{SHV}$ gene. Further, their study reported b*la*$_{TEM}$ and *bla*$_{CTX-M}$ being 7 % of the isolates compared to only 2 % of b*la*$_{SHV}$ and bla$_{CTX-M}$ combination which is in agreement with the present study. Schmid *et al*., (2013) also reported *bla*$_{CTX-M}$ as the predominant ESBLgenes from cattle in Germany where 93.4% of ESBL-producing *E. coli* strains harbored CTX-M genes.

Several surveys of ESBLs producing *E. coli* in food producing animals in Africa have also reported CTX-M as the dominant gene in both animals and humans (Gisele, *et al*., 2011; Abdallah, *et al*. 2015 and Falgenhauer, *et al*., 2019). Certain *bla*$_{CTX-M-1}$ -harbouring clones (ST131/B2 or ST405/D) are mainly identified in humans, but they have also been reported in livestock species from Tanzania, Nigeria and Tunisia (Alonso, 2017). Moreover, several reports suggested an inter-host circulation of specific plasmids (for example *bla*$_{CTX-M-1}$ -carrying IncI1/ST3 in Tunisia, IncY- and Inc-untypeable replicons co-harbouring *qnr*S1 and *bla*$_{CTX-M-15}$ in Tanzania and the worldwide distributed *bla*$_{CTX-M-15}$ -carrying IncF-type

plasmids (Alonso, 2017). Other studies across the world have also reported food animals to be reservoirs of ESBL-producing strains of *Escherichia coli* (Nadine *et al*., 2012; Reist *et al*., 2013; Karuppasamy *et al*., 2015; Njage *et al*., 2012; Schmid *et al*., 2013; Heuvelink, 2019).

Use of third- and fourth-generation cephalosporins in animals is associated with high prevalence of ESBL/AmpC producing pathogens (Gonggrijp, 2016).

Two different studies in Netherlands revealed genetic similarities between ESBL genes, plasmids and *Escherichia coli* strains isolated from Dutch patients and those isolated from retail chicken meat and poultry (van Hall *et al*. 2011 and Stefan et al. 2016). van Hall *et al*. (2011) reported $bla_{\text{CTX-M}}$ and bla_{TEM} genes as being the most predominant genes in chicken. In their study, 86 % of ESBL genes in Dutch patients, retail chicken meat and poultry were $bla_{\text{CTX-M}}$ and bla_{TEM}. They further reported that, of the retail meat samples, 94 % contained ESBL-producing isolates of which 39 % belonged to *E. coli* genotypes also present in human samples. Although they found no evidence of clonal spread of ESBL/pAmpC-producing *E. coli* from farm animals or foods to humans, ESBL/pAmpC-producing *E. coli* with identical genes and plasmids were present in farm animals, foods, and humans suggesting possible transmission of resistant genes from food animals to humans.

Kluytmans *et al*., (2013) also reported significant genetic similarities among ESBL-producing strains of *Escherichia coli* from chicken meat and humans according to mobile resistance elements, virulence genes, and genomic backbone. These findings are suggestive of transmission of ESBL genes, plasmids and *E. coli* isolates from food producing animals to humans through the food chain

There has been evidence that the proliferation of CTX-M-producing *E. coli* is due to the growth of indigenous CTX-M-producing strains and the possible emergence of strains that acquired CTX-M genes by horizontal transfer (Hiroi, *et al*., 2012).

Analysis of the sequenced resistant determinants showed that the resistant genes were harboured by *Escherichia coli* strains and that the resistant determinants are geographically widespread across various regions of the globe and having previously been reported from other countries in isolates obtained from food producing animals as well as from human clinical samples.

5.2 CONCLUSIONS

1. *Escherichia coli* is present is raw dairy cattle milk when good hygienic practices are not observed.
2. *Escherichia coli* isolates phenotypically depicted multidrug resistance patterns with several isolates being resistant to more than one antimicrobial agent. *Escherichia coli* isolates obtained from cattle milk were not resistant to cefuroxime, cefepime, imipenem, ciprofloxacin and gentamycin suggesting that these antimicrobials can still be used effectively.
3. Resistance of *Escherichia coli* isolates to beta-lactams was attributed to the presence of ESBL genes- bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M.}}$
4. ESBL genes bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ detected in *Escherichia coli* isolates from raw cattle milk have been previously found in several countries worldwide in isolates obtained from food producing animals as well as from human clinical samples. This suggests that food animals and foods of animal origin are potential sources of ESBL-producing strains and could therefore pose a public health risk.

5.3 RECOMMENDATION

From the findings of this study, it is recommended that dairy milk should be pasteurized prior to consumption to avoid transmission of multidrug resistant ESBL strains to consumers.

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7 APPENDICES

Appendix 1: Disk diffusion antimicrobial susceptibility testing with zone diameter readings in mm

Sample No.	Sample Code	Replicate	Ampicillin AMP 10 µg	Amoxiclav AMC 20/10 µg	Cefazolin KZ 30 µg	Cefuroxime CXM 30 µg	Cefoxitin FOX 30 µg	Ceftazidime CAZ 30 µg	Cefotaxime CTX 30 µg	Cefepime CPM 30 µg	Imipenem IMI 10 µg	Ciprofloxacin CIP 5 µg	Tetracycline TE 30 µg	Gentamycin CN 10 µg
	ATCC 25922	R1	18	19	23	23	24	25	29	33	27	32	22	20
		R2	17	18	23	20	23	27	29	32	28	32	19	20
		R3	18	19	23	22	24	25	30	33	27	33	20	20
		Av	**18**	**19**	**23**	**22**	**24**	**26**	**29**	**33**	**27**	**32**	**20**	**20**
	ATCC 25922	R1	17	20	23	20	23	26	30	33	28	34	19	21
		R2	17	20	23	23	24	26	29	32	28	33	22	21
		R3	17	19	23	22	24	26	31	32	28	34	21	20
		Av	**17**	**20**	**23**	**22**	**24**	**26**	**30**	**32**	**28**	**34**	**21**	**21**
	ATCC 25922	R1	16	20	25	27	27	28	32	32	28	30	20	21
		R2	17	22	24	25	26	27	30	32	29	33	19	21
		R3	16	20	24	25	27	26	30	32	27	33	19	21
		Av	**16**	**21**	**24**	**26**	**27**	**27**	**31**	**32**	**28**	**32**	**19**	**21**
1	UEC 862/16	R1	0	0	14	17	20	21	25	23	23	27	19	18
		R2	0	0	13	17	20	21	26	23	25	29	20	17
		R3	0	0	14	18	21	21	26	23	25	28	18	15
		Av	**0**	**0**	**14**	**17**	**20**	**21**	**26**	**23**	**24**	**28**	**19**	**17**
2	UEC 688/16	R1	11	16	17	18	15	22	25	23	23	26	18	16
		R2	11	16	17	18	15	22	25	24	23	26	18	16
		R3	11	16	16	18	15	21	25	23	23	26	18	16
		Av	**11**	**16**	**17**	**18**	**15**	**22**	**25**	**23**	**23**	**26**	**18**	**16**
3	UEC 653/16	R1	0	17	21	20	22	26	31	30	23	30	24	16

		R2	0	17	20	20	21	22	26	28	22	29	23	16
		R3	0	18	19	20	21	24	28	29	24	29	22	16
		Av	**0**	**17**	**20**	**20**	**21**	**24**	**28**	**29**	**23**	**29**	**23**	**16**
4	UEC 783/16	R1	0	-	15	19	24	20	27	24	24	31	20	17
		R2	0	14	13	19	22	19	25	24	23	28	19	18
		R3	0	14	14	17	21	20	25	24	23	28	18	14
		Av	**0**	**14**	**14**	**18**	**22**	**20**	**26**	**24**	**23**	**29**	**19**	**16**
5	UEC 740/16	R1	11	15	18	17	22	20	25	29	23	29	20	18
		R2	12	18	19	18	24	20	25	28	24	28	20	16
		R3	13	18	19	19	21	21	28	27	22	28	20	17
		Av	**12**	**17**	**19**	**18**	**22**	**20**	**26**	**28**	**23**	**28**	**20**	**17**
6	UEC 790/16	R1	11	13	20	18	21	21	24	28	25	33	21	20
		R2	11	18	18	18	21	21	25	26	23	30	20	18
		R3	11	18	19	18	22	20	24	27	24	32	20	18
		Av	**11**	**16**	**19**	**18**	**21**	**21**	**24**	**27**	**24**	**32**	**20**	**19**
7	UEC 679/16	R1	-	17	18	24	25	25	30	29	25	30	25	18
		R2	8	18	19	21	25	24	29	28	22	28	23	17
		R3	8	18	18	21	25	24	29	27	23	26	22	16
		Av	**8**	**18**	**18**	**22**	**25**	**24**	**29**	**28**	**23**	**28**	**23**	**17**
8	UEC 837/16	R1	12	13	14	19	21	25	28	29	25	30	21	15
		R2	13	14	13	19	22	24	28	28	24	26	21	16
		R3	13	14	14	19	22	23	26	28	26	26	21	17
		Av	**13**	**14**	**14**	**19**	**22**	**24**	**27**	**28**	**25**	**27**	**21**	**16**
9	UEC 772/16	R1	10	16	18	18	24	17	17	25	24	26	18	16
		R2	14	20	20	20	25	22	28	30	24	26	23	18
		R3	13	20	20	19	24	22	26	30	24	28	20	17
		Av	**12**	**19**	**19**	**19**	**24**	**20**	**24**	**28**	**24**	**27**	**20**	**17**
10	UEC 697/16	R1	15	17	20	19	23	21	28	28	25	28	19	16

		R2	15	18	20	20	23	23	28	29	24	29	20	16
		R3	13	18	18	19	21	21	26	29	22	26	19	16
		Av	**14**	**18**	**19**	**19**	**22**	**22**	**27**	**29**	**24**	**28**	**19**	**16**
11	UEC 755/16	R1	12	15	19	19	22	21	26	25	23	32	8	15
		R2	13	18	19	19	23	23	28	27	24	32	8	17
		R3	12	18	20	19	23	22	28	26	22	30	8	18
		Av	**12**	**17**	**19**	**19**	**23**	**22**	**27**	**26**	**23**	**31**	**8**	**17**
12	UEC 716/16	R1	0	12	19	25	25	22	29	26	24	30	25	16
		R2	0	18	15	24	25	23	29	28	23	28	25	18
		R3	0	18	16	24	24	23	29	28	24	28	24	18
		Av	**0**	**16**	**17**	**24**	**25**	**23**	**29**	**27**	**24**	**29**	**25**	**17**
13	UEC 811/16	R1	0	-	14	17	23	21	25	25	23	28	20	15
		R2	0	16	14	18	22	21	26	24	23	24	19	15
		R3	0	16	14	18	23	21	27	24	23	26	20	14
		Av	**0**	**16**	**14**	**18**	**23**	**21**	**26**	**24**	**23**	**26**	**20**	**15**
14	UEC 792/16	R1	15	16	20	19	24	21	26	26	23	30	16	18
		R2	13	16	17	20	24	21	28	25	23	29	19	17
		R3	13	16	16	19	22	21	26	26	23	29	19	16
		Av	**14**	**16**	**18**	**19**	**23**	**21**	**27**	**26**	**23**	**29**	**18**	**17**
15	UEC 727/16	R1	17	22	20	23	23	27	31	28	28	29	20	16
		R2	16	21	19	25	24	25	30	29	27	30	20	17
		R3	18	23	20	28	24	26	32	30	26	32	23	18
		Av	**17**	**22**	**20**	**25**	**24**	**26**	**31**	**29**	**27**	**30**	**21**	**17**
16	UEC 714/16	R1	0	-	14	19	23	21	26	26	24	28	20	18
		R2	0	14	16	18	21	20	28	28	24	30	20	16
		R3	0	14	15	18	22	22	28	28	24	30	20	18
		Av	**0**	**14**	**15**	**18**	**22**	**21**	**27**	**27**	**24**	**29**	**20**	**17**
17	UEC 734/16	R1	0	18	17	19	0	24	30	26	25	27	22	14

		R2	0	18	18	18	0	24	30	26	24	30	20	17
		R3	0	15	18	18	0	24	28	25	25	26	20	16
		Av	**0**	**17**	**18**	**18**	**0**	**24**	**29**	**26**	**25**	**28**	**21**	**16**
18	UEC 852/16	R1	17	18	21	20	25	21	26	28	22	29	18	17
		R2	18	19	22	21	25	22	28	28	23	30	20	17
		R3	17	20	23	20	28	24	30	30	24	30	21	18
		Av	**17**	**19**	**22**	**20**	**26**	**22**	**28**	**29**	**23**	**30**	**20**	**17**
19	UEC 795/16	R1	17	19	18	21	25	20	25	24	26	34	19	17
		R2	17	18	20	19	24	21	27	25	25	33	19	16
		R3	17	20	21	20	24	21	28	29	25	34	21	17
		Av	**17**	**19**	**20**	**20**	**24**	**21**	**27**	**26**	**25**	**34**	**20**	**17**
20	UEC 765/16	R1	19	20	20	21	26	22	28	20	22	32	8	20
		R2	18	20	21	21	26	21	28	26	23	30	8	18
		R3	17	20	20	24	28	22	32	32	24	32	8	20
		Av	**18**	**20**	**20**	**22**	**27**	**22**	**29**	**26**	**23**	**31**	**8**	**19**
21	UEC 695/16	R1	17	19	18	20	24	22	26	28	24	30	20	16
		R2	16	19	21	20	24	24	26	26	24	30	20	16
		R3	17	20	22	22	24	23	26	20	22	32	20	16
		Av	**17**	**19**	**20**	**21**	**24**	**23**	**26**	**25**	**23**	**31**	**20**	**16**
22	NEC 2991/17	R1	0	18	19	20	26	13	16	28	24	28	19	16
		R2	0	21	20	16	24	8	19	28	22	28	21	17
		R3	0	21	17	20	22	11	18	28	24	28	21	18
		Av	**0**	**20**	**19**	**19**	**24**	**11**	**18**	**28**	**23**	**28**	**20**	**17**
23	NEC K40/17	R1	0	7	18	16	24	20	23	26	24	29	20	15
		R2	0	8	19	17	24	20	24	25	25	30	20	16
		R3	0	8	19	24	26	19	26	24	24	28	19	15
		Av	**0**	**8**	**19**	**19**	**25**	**20**	**24**	**25**	**24**	**29**	**20**	**15**
24	NEC 3090/17	R1	15	20	22	20	24	24	30	28	24	30	19	23

		R2	11	20	21	22	26	24	30	28	26	34	21	18
		R3	12	21	23	19	26	25	30	26	24	30	21	18
		Av	**13**	**20**	**22**	**20**	**25**	**24**	**30**	**27**	**25**	**31**	**20**	**20**
25	NEC 2996/17	R1	19	20	24	21	20	26	30	26	22	32	24	19
		R2	22	22	24	20	26	26	30	26	24	32	22	19
		R3	18	20	24	22	26	26	30	26	24	32	22	19
		Av	**20**	**21**	**24**	**21**	**24**	**26**	**30**	**26**	**23**	**32**	**23**	**19**
26	NEC 3040/17	R1	17	21	24	26	28	24	30	26	25	30	22	20
		R2	18	22	22	24	26	24	30	26	24	32	22	19
		R3	18	22	24	22	26	26	30	30	24	30	22	17
		Av	**18**	**22**	**23**	**24**	**27**	**25**	**30**	**27**	**24**	**31**	**22**	**19**
27	NEC 2938/17	R1	17	17	24	23	24	24	31	32	27	32	22	19
		R2	16	18	23	23	26	24	30	32	26	32	21	20
		R3	17	18	22	23	24	24	30	33	27	33	22	21
		Av	**17**	**18**	**23**	**23**	**25**	**24**	**30**	**32**	**27**	**32**	**22**	**20**
28	NEC 3058/17	R1	0	15	22	21	26	29	32	32	26	33	10	20
		R2	0	13	20	21	25	23	30	30	26	32	10	20
		R3	0	14	21	20	25	25	31	31	26	33	11	20
		Av	**0**	**14**	**21**	**21**	**25**	**26**	**31**	**31**	**26**	**33**	**10**	**20**
29	NEC K40/17	R1	15	18	24	21	28	24	32	34	27	32	22	20
		R2	15	17	23	21	26	24	30	32	27	32	21	20
		R3	15	18	24	20	27	24	31	33	26	32	21	20
		Av	**15**	**18**	**24**	**21**	**27**	**24**	**31**	**33**	**27**	**32**	**21**	**20**
30	NEC 2930/17	R1	0	9	20	19	24	23	29	27	24	32	21	18
		R2	0	9	19	18	24	19	28	26	24	32	21	18
		R3	0	8	20	21	26	25	30	28	24	32	20	18
		Av	**0**	**9**	**20**	**19**	**25**	**22**	**29**	**27**	**24**	**32**	**21**	**18**
31	NEC 3005/17	R1	0	8	19	17	24	20	26	28	24	30	19	17

		R2	0	9	20	18	26	21	27	24	24	30	18	17
		R3	0	8	20	17	28	22	26	-	23	33	19	17
		Av	**0**	**8**	**20**	**17**	**26**	**21**	**26**	**26**	**24**	**31**	**19**	**17**
32	NEC 3017/17	R1	0	9	23	19	26	22	30	26	24	28	22	14
		R2	0	10	21	19	24	22	29	24	23	27	21	12
		R3	0	14	21	19	24	22	30	26	25	30	22	15
		Av	**0**	**11**	**22**	**19**	**25**	**22**	**30**	**25**	**24**	**28**	**22**	**14**
33	NEC 3351/17	R1	0	7	18	24	26	20	25	32	23	30	15	16
		R2	0	7	19	25	25	19	25	31	23	31	20	16
		R3	0	7	20	25	26	22	26	34	23	32	20	16
		Av	**0**	**7**	**19**	**25**	**26**	**20**	**25**	**32**	**23**	**31**	**18**	**16**
34	NEC 3058/17	R1	0	19	20	22	26	25	30	30	26	34	22	20
		R2	0	17	22	23	28	24	30	30	25	34	23	20
		R3	0	18	22	24	28	24	30	30	25	34	22	20
		Av	**0**	**18**	**21**	**23**	**27**	**24**	**30**	**30**	**25**	**34**	**22**	**20**
35	NEC 3077/17	R1	0	15	18	23	23	24	29	28	23	30	21	15
		R2	0	15	19	24	25	23	28	26	23	30	24	16
		R3	0	15	18	24	24	24	30	29	23	30	24	17
		Av	**0**	**15**	**18**	**24**	**24**	**24**	**29**	**28**	**23**	**30**	**23**	**16**
36	NEC 3389/17	R1	0	15	19	23	23	25	30	30	24	30	23	18
		R2	0	16	17	23	24	24	29	32	25	30	23	18
		R3	0	15	17	25	23	23	30	30	23	29	25	18
		Av	**0**	**15**	**18**	**24**	**23**	**24**	**30**	**31**	**24**	**30**	**24**	**18**
37	NEC NK05/17	R1	0	14	18	25	24	24	30	33	24	30	26	19
		R2	0	14	18	26	26	25	29	32	24	31	24	20
		R3	0	16	21	27	27	25	32	32	26	31	25	19
		Av	**0**	**15**	**19**	**26**	**26**	**25**	**30**	**32**	**25**	**31**	**25**	**19**
38	NEC NK40/17	R1	0	16	22	25	26	25	30	30	25	32	26	19

		R2	0	16	20	25	25	25	30	32	26	32	26	19
		R3	0	16	21	25	26	27	30	31	26	31	25	19
		Av	**0**	**16**	**21**	**25**	**26**	**26**	**30**	**31**	**26**	**32**	**26**	**19**
39	NEC 2938/17	R1	15	21	24	24	25	25	31	31	29	33	22	19
		R2	14	21	21	24	22	25	31	32	28	34	22	20
		R3	13	20	20	24	24	25	30	30	28	33	20	18
		Av	**14**	**21**	**22**	**24**	**24**	**25**	**31**	**31**	**28**	**33**	**21**	**19**
40	GEC05/17	R1	16	22	21	24	22	25	30	31	25	32	25	20
		R2	15	20	21	22	22	25	32	32	24	31	23	18
		R3	16	24	24	26	25	28	32	32	26	33	26	22
		Av	**16**	**22**	**22**	**24**	**23**	**26**	**31**	**32**	**25**	**32**	**25**	**20**
41	GEC07/17	R1	19	20	21	22	25	23	29	32	26	31	22	20
		R2	19	20	20	23	25	24	30	32	29	36	24	18
		R3	20	22	22	25	26	27	32	31	29	36	25	20
		Av	**19**	**21**	**21**	**23**	**25**	**25**	**30**	**32**	**28**	**34**	**24**	**19**
42	GEC12/17	R1	15	20	21	23	27	27	30	31	28	32	22	20
		R2	16	22	25	25	24	25	31	32	28	36	24	21
		R3	18	23	24	25	25	26	30	30	28	32	23	21
		Av	**16**	**22**	**23**	**24**	**25**	**26**	**30**	**31**	**28**	**33**	**23**	**21**
43	GEC15/17	R1	15	22	24	25	26	27	30	34	29	32	24	20
		R2	15	22	24	25	25	27	32	33	29	31	24	20
		R3	16	22	23	23	25	27	32	33	28	32	25	20
		Av	**15**	**22**	**24**	**24**	**25**	**27**	**31**	**33**	**29**	**32**	**24**	**20**
44	GEC16/17	R1	12	21	21	24	22	25	30	31	28	30	24	20
		R2	11	20	22	21	25	24	30	31	28	32	24	18
		R3	12	20	21	21	24	25	30	32	29	31	24	17
		Av	**12**	**20**	**21**	**22**	**24**	**25**	**30**	**31**	**28**	**31**	**24**	**18**
45	GEC17/17	R1	0	10	20	21	24	25	30	28	26	32	8	17
		R2	0	10	18	24	24	25	30	28	27	30	7	19

		R3	0	10	20	24	26	25	30	32	26	30	8	19
		Av	**0**	**10**	**19**	**23**	**25**	**25**	**30**	**29**	**26**	**31**	**8**	**18**
46	GEC20/17	R1	19	22	23	22	25	22	28	32	26	30	22	21
		R2	16	24	20	20	25	23	30	29	24	30	25	21
		R3	18	23	22	21	25	22	29	31	25	30	23	21
		Av	**18**	**23**	**22**	**21**	**25**	**22**	**29**	**31**	**25**	**30**	**23**	**21**
47	GEC25/17	R1	20	20	26	24	26	25	31	34	28	33	22	24
		R2	21	22	24	26	26	25	30	35	31	34	25	24
		R3	21	21	25	25	26	25	30	34	30	32	23	24
		Av	**21**	**21**	**25**	**25**	**26**	**25**	**30**	**34**	**30**	**33**	**23**	**24**
48	GEC28/17	R1	12	20	20	20	21	21	26	27	21	28	19	18
		R2	14	20	20	20	21	21	25	27	25	30	20	18
		R3	11	20	19	20	23	20	26	28	24	28	20	17
		Av	**12**	**20**	**20**	**20**	**22**	**21**	**26**	**27**	**23**	**29**	**20**	**18**
49	GEC31/17	R1	8	15	19	21	21	21	25	28	23	26	20	18
		R2	8	16	20	21	23	21	26	28	23	27	20	19
		R3	8	16	21	22	21	23	28	28	23	26	21	18
		Av	**8**	**16**	**20**	**21**	**22**	**22**	**26**	**28**	**23**	**26**	**20**	**18**
50	GEC34/17	R1	12	19	20	21	21	22	26	27	22	26	20	16
		R2	13	19	20	21	24	22	27	30	22	30	21	18
		R3	11	19	19	20	23	21	25	30	25	28	20	19
		Av	**12**	**19**	**20**	**21**	**23**	**22**	**26**	**29**	**23**	**28**	**20**	**18**
51	GEC36/17	R1	8	23	28	28	26	25	31	34	28	33	8	20
		R2	8	22	28	28	26	26	32	34	28	33	7	20
		R3	7	23	27	28	26	26	31	35	28	34	7	20
		Av	**8**	**23**	**28**	**28**	**26**	**26**	**31**	**34**	**28**	**33**	**7**	**20**
52	GEC38/17	R1	14	21	23	23	25	22	30	31	24	30	9	18
		R2	12	23	23	23	26	24	30	30	24	30	9	19
		R3	11	22	23	23	25	23	30	30	24	31	9	18
		Av	**12**	**22**	**23**	**23**	**25**	**23**	**30**	**30**	**24**	**30**	**9**	**18**

53	GEC40/17	R1	14	19	24	24	23	20	27	34	25	30	20	20
		R2	14	20	23	24	25	22	29	33	25	33	21	19
		R3	14	20	24	24	24	22	28	33	25	32	21	20
		Av	**14**	**20**	**24**	**24**	**24**	**21**	**28**	**33**	**25**	**32**	**21**	**20**
54	GEC43/17	R1	9	18	23	23	26	23	29	33	25	32	23	19
		R2	10	19	23	22	26	22	29	34	25	31	21	19
		R3	9	18	23	23	26	22	29	34	25	32	22	19
		Av	**9**	**18**	**23**	**23**	**26**	**22**	**29**	**34**	**25**	**32**	**22**	**19**
55	GEC44/17	R1	19	20	21	23	25	22	27	30	23	30	22	21
		R2	19	21	22	22	24	22	27	30	24	30	22	19
		R3	23	22	21	28	23	23	32	31	22	32	26	20
		Av	**20**	**21**	**21**	**24**	**24**	**22**	**29**	**30**	**23**	**31**	**23**	**20**
56	GEC54/17	R1	24	21	20	28	20	24	32	32	26	33	25	22
		R2	25	19	15	30	12	25	33	34	25	35	25	24
		R3	23	22	21	28	23	23	32	31	26	32	26	20
		Av	**24**	**21**	**19**	**29**	**18**	**24**	**32**	**32**	**26**	**33**	**25**	**22**
57	GEC56/17	R1	21	26	25	24	27	25	30	32	28	32	0	21
		R2	23	27	24	25	25	25	31	32	28	34	0	20
		R3	22	26	25	24	26	25	30	32	28	33	0	20
		Av	**22**	**26**	**25**	**24**	**26**	**25**	**30**	**32**	**28**	**33**	**0**	**20**
58	GEC57/17	R1	13	20	20	19	22	18	24	28	28	29	20	17
		R2	17	21	17	19	23	18	25	27	27	30	19	15
		R3	17	19	19	19	22	19	24	28	28	29	20	18
		Av	**16**	**20**	**19**	**19**	**22**	**18**	**24**	**28**	**28**	**29**	**20**	**17**
59	GEC58/17	R1	0	17	19	22	25	23	27	28	27	29	11	23
		R2	0	17	21	22	24	23	28	31	28	30	13	24
		R3	0	17	21	22	25	23	28	29	27	30	12	24
		Av	**0**	**17**	**20**	**22**	**25**	**23**	**28**	**29**	**27**	**30**	**12**	**24**
60	GEC61/17	R1	0	18	22	23	26	24	29	32	25	32	21	21
		R2	0	20	21	23	26	24	26	32	25	32	20	21

		R3	0	19	22	24	26	24	28	32	25	32	22	21
		Av	**0**	**19**	**22**	**23**	**26**	**24**	**28**	**32**	**25**	**32**	**21**	**21**
61	GEC62/17	R1	9	16	18	19	21	19	26	25	25	28	18	19
		R2	16	20	20	20	24	20	28	30	25	32	20	20
		R3	15	18	19	19	22	20	28	28	25	30	19	20
		Av	**13**	**18**	**19**	**19**	**22**	**20**	**27**	**28**	**25**	**30**	**19**	**20**
62	GEC64/17	R1	0	18	24	23	26	25	29	33	26	31	11	22
		R2	0	20	23	24	26	26	30	32	26	34	12	23
		R3	0	19	24	24	26	25	30	32	26	32	12	22
		Av	**0**	**19**	**24**	**24**	**26**	**25**	**30**	**32**	**26**	**32**	**12**	**22**
63	GEC66/17	R1	0	13	22	21	25	24	31	31	25	32	10	21
		R2	0	13	20	21	24	22	31	31	25	32	10	20
		R3	0	13	21	21	25	24	31	32	25	33	10	20
		Av	**0**	**13**	**21**	**21**	**25**	**23**	**31**	**31**	**25**	**32**	**10**	**20**
64	GEC76/17	R1	0	15	21	21	26	24	31	32	25	32	10	20
		R2	0	14	19	21	25	23	30	31	24	31	10	20
		R3	0	14	20	21	25	25	31	31	25	31	10	20
		Av	**0**	**14**	**20**	**21**	**25**	**24**	**31**	**31**	**25**	**31**	**10**	**20**
65	GEC77/17	R1	15	16	25	24	26	24	32	32	30	34	23	23
		R2	15	16	23	24	27	24	30	30	30	33	23	22
		R3	15	16	24	24	27	24	31	30	30	33	22	22
		Av	**15**	**16**	**24**	**24**	**27**	**24**	**31**	**31**	**30**	**33**	**23**	**22**
66	GEC78/17	R1	0	16	23	24	28	25	30	32	27	34	10	22
		R2	0	13	20	23	27	23	31	32	28	31	11	22
		R3	0	15	22	24	28	24	30	32	28	33	10	22
		Av	**0**	**15**	**22**	**24**	**28**	**24**	**30**	**32**	**28**	**33**	**10**	**22**
67	GEC79/17	R1	11	15	18	20	20	17	24	29	21	23	17	14
		R2	14	18	21	22	24	20	28	30	25	26	17	20
		R3	13	17	20	21	21	20	27	29	23	25	17	19
		Av	**13**	**17**	**20**	**21**	**22**	**19**	**26**	**29**	**23**	**25**	**17**	**18**

68	GEC80/17	R1	17	18	21	20	24	21	28	29	25	30	19	19
		R2	18	18	21	19	24	22	27	30	25	30	19	19
		R3	17	18	21	20	24	23	28	30	25	30	19	19
		Av	**17**	**18**	**21**	**20**	**24**	**22**	**28**	**30**	**25**	**30**	**19**	**19**
69	GEC81/17	R1	15	19	21	21	23	20	27	28	24	29	21	19
		R2	16	20	20	20	23	21	28	28	23	28	23	19
		R3	16	20	20	21	23	21	28	28	24	29	22	19
		Av	**16**	**20**	**20**	**21**	**23**	**21**	**28**	**28**	**24**	**29**	**22**	**19**
70	GEC82/17	R1	18	20	20	20	24	20	27	30	23	33	22	20
		R2	19	20	22	21	25	20	29	30	23	31	22	22
		R3	19	20	21	20	24	20	28	30	23	32	22	21
		Av	**19**	**20**	**21**	**20**	**24**	**20**	**28**	**30**	**23**	**32**	**22**	**21**
71	GEC86/17	R1	19	22	17	18	20	21	26	26	22	29	23	14
		R2	18	21	20	20	20	20	26	25	25	29	21	18
		R3	19	22	20	19	20	21	26	25	23	29	22	16
		Av	**19**	**22**	**19**	**19**	**20**	**21**	**26**	**25**	**23**	**29**	**22**	**16**
72	GEC88/17	R1	15	22	23	21	25	21	28	30	23	31	21	19
		R2	16	20	22	23	25	20	25	30	23	30	18	19
		R3	16	21	21	22	24	21	27	30	23	31	19	19
		Av	**16**	**21**	**22**	**22**	**25**	**21**	**27**	**30**	**23**	**31**	**19**	**19**
73	GEC92/17	R1	0	16	24	22	28	26	32	34	25	33	21	20
		R2	0	14	22	20	27	25	29	31	23	32	20	19
		R3	0	15	23	21	28	24	29	32	24	32	21	20
		Av	**0**	**15**	**23**	**21**	**28**	**25**	**30**	**32**	**24**	**32**	**21**	**20**
74	GEC93/17	R1	0	14	24	22	29	25	32	33	26	31	11	21
		R2	0	12	20	21	25	24	30	30	25	30	11	21
		R3	0	13	22	22	28	25	31	31	25	30	11	21
		Av	**0**	**13**	**22**	**22**	**27**	**25**	**31**	**31**	**25**	**30**	**11**	**21**
75	GEC96/17	R1	14	20	18	25	28	24	30	34	27	32	23	20
		R2	12	20	25	25	27	21	29	32	26	30	21	19

		R3	12	20	22	25	28	22	29	33	27	31	21	20
		Av	**13**	**20**	**22**	**25**	**28**	**22**	**29**	**33**	**27**	**31**	**22**	**20**
76	GEC97/17	R1	16	19	21	20	23	20	28	30	25	29	21	19
		R2	16	20	20	21	23	21	28	29	25	29	23	19
		R3	16	19	20	20	23	21	27	28	25	29	21	20
		Av	**16**	**19**	**20**	**20**	**23**	**21**	**28**	**29**	**25**	**29**	**22**	**19**
77	GEC104/17	R1	18	17	21	21	24	22	28	30	26	30	0	20
		R2	16	18	20	20	25	22	27	30	24	30	0	20
		R3	17	17	20	20	24	22	28	30	25	30	0	20
		Av	**17**	**17**	**20**	**20**	**24**	**22**	**28**	**30**	**25**	**30**	**0**	**20**
78	GEC105/17	R1	17	18	20	20	25	23	28	30	25	30	0	19
		R2	18	18	22	21	26	22	29	30	25	30	0	20
		R3	18	17	21	21	26	22	28	30	26	30	0	19
		Av	**18**	**18**	**21**	**21**	**26**	**22**	**28**	**30**	**25**	**30**	**0**	**19**
79	GEC106/17	R1	17	18	19	20	23	20	28	28	25	29	0	18
		R2	17	18	20	23	24	22	27	30	25	30	0	19
		R3	17	17	20	21	23	21	28	29	25	30	0	18
		Av	**17**	**18**	**20**	**21**	**23**	**21**	**28**	**29**	**25**	**30**	**0**	**18**
80	GEC110/17	R1	0	15	18	21	24	21	27	30	26	30	10	21
		R2	0	15	18	22	24	23	29	30	26	30	10	21
		R3	0	15	18	21	24	22	28	30	26	30	10	22
		Av	**0**	**15**	**18**	**21**	**24**	**22**	**28**	**30**	**26**	**30**	**10**	**21**
81	GEC121/17	R1	18	18	20	20	24	21	27	30	27	29	18	20
		R2	17	18	21	22	25	21	29	30	28	30	19	20
		R3	17	16	21	21	24	21	28	30	27	29	19	20
		Av	**17**	**17**	**21**	**21**	**24**	**21**	**28**	**30**	**27**	**29**	**19**	**20**
82	GEC122/17	R1	18	18	21	22	24	22	30	29	26	28	20	19
		R2	16	18	21	22	25	22	28	30	28	28	20	20
		R3	17	17	21	22	25	22	28	30	27	28	20	20
		Av	**17**	**18**	**21**	**22**	**25**	**22**	**29**	**30**	**27**	**28**	**20**	**20**

83	GEC124/17	R1	0	15	18	20	24	21	26	28	24	28	10	20
		R2	0	15	19	21	26	21	28	28	25	29	9	20
		R3	0	15	19	20	25	21	28	28	25	30	10	20
		Av	**0**	**15**	**19**	**20**	**25**	**21**	**27**	**28**	**25**	**29**	**10**	**20**
84	GEC136/17	R1	0	15	18	20	24	21	26	28	24	30	10	20
		R2	0	16	19	21	26	21	27	29	25	32	10	20
		R3	0	16	19	20	25	21	26	28	25	30	10	20
		Av	**0**	**16**	**19**	**20**	**25**	**21**	**26**	**28**	**25**	**31**	**10**	**20**
85	GEC142/17	R1	20	18	20	20	22	21	26	30	23	32	22	20
		R2	19	18	20	21	22	22	28	29	25	32	20	20
		R3	20	18	20	20	22	21	27	30	24	32	21	20
		Av	**20**	**18**	**20**	**20**	**22**	**21**	**27**	**30**	**24**	**32**	**21**	**20**
86	GEC143/17	R1	20	19	18	19	22	23	29	29	26	35	22	19
		R2	16	18	23	21	24	21	27	30	27	31	20	22
		R3	18	19	20	20	23	22	28	30	26	32	20	20
		Av	**18**	**19**	**20**	**20**	**23**	**22**	**28**	**30**	**26**	**33**	**21**	**20**
87	GEC155/17	R1	0	16	17	20	25	21	23	26	22	30	10	17
		R2	0	19	20	21	26	22	29	29	25	31	12	20
		R3	0	19	19	21	25	22	28	28	24	30	12	18
		Av	**0**	**18**	**19**	**21**	**25**	**22**	**27**	**28**	**24**	**30**	**11**	**18**
88	GEC164/17	R1	0	10	19	20	26	24	27	28	25	32	20	18
		R2	0	9	20	20	25	23	28	28	25	30	20	17
		R3	0	12	20	20	24	22	30	28	25	-	21	18
		Av	**0**	**10**	**20**	**20**	**25**	**23**	**28**	**28**	**25**	**31**	**20**	**18**
89	GEC185/17	R1	0	17	19	22	25	23	28	28	27	29	11	23
		R2	0	17	21	23	24	24	28	31	28	30	13	24
		R3	0	17	21	22	25	23	28	29	27	30	12	24
		Av	**0**	**17**	**20**	**22**	**25**	**23**	**28**	**29**	**27**	**30**	**12**	**24**
90	GEC186/17	R1	0	17	20	22	28	25	32	33	25	34	19	20
		R2	0	18	22	22	28	24	31	32	25	33	19	20

		R3	0	18	21	22	28	24	31	32	24	33	19	19
		Av	**0**	**18**	**21**	**22**	**28**	**24**	**31**	**32**	**25**	**33**	**19**	**20**
91	GEC194/17	R1	0	17	19	20	25	21	26	30	25	31	10	21
		R2	0	16	20	22	26	21	27	30	26	30	11	22
		R3	0	17	20	22	26	21	27	30	26	31	11	23
		Av	**0**	**17**	**20**	**21**	**26**	**21**	**27**	**30**	**26**	**31**	**11**	**22**

TOTAL	SENSITIVE	25	48	87	91	90	90	85	87	91	91	69	90
	INTERMEDIATE	12	33	0	0	0	0	5	4	0	0	3	1
	RESISTANT	54	10	4	0	1	1	1	0	0	0	19	0

Appendix 2: Difference between zone diameter readings for cefotaxime & ceftazidime and their clavulanate combinations, the basis of phenotypic detection of ESBL production

Sample No.	Duplicate	Cefotaxime	Cefotaxime/ Clavulanate	Difference	Ceftazidime	Ceftazidime/ Clavulanate	Difference	Phenotype
		CTX	CTL	D	CAZ	CAL	D	
1	D1	29	34	5	20	26	6	
	D2	29	33	4	21	25	4	
	Av	29	34	5	21	26	5	ESBL
2	D1	32	36	4	23	30	7	
	D2	32	36	4	23	30	7	
		32	36	4	23	30	7	ESBL
4	D1	30	34	4	21	28	7	
	D2	31	34	3	22	28	6	
		30.5	34	3.5	21.5	28	6.5	ESBL
5	D1	30	34	4	21	26	5	
	D2	30	34	4	22	26	4	
		30	34	4	21.5	26	4.5	NON-ESBL
6	D1	30	34	4	21	28	7	
	D2	31	34	3	20	27	7	
		30.5	34	3.5	20.5	27.5	7	ESBL
10	D1	31	35	4	23	30	7	
	D2	32	35	3	23	30	7	
		31.5	35	3.5	23	30	7	ESBL
11	D1	29	31	2	20	26	6	
	D2	29	31	2	21	27	6	
		29	31	2	20.5	26.5	6	ESBL
13	D1	30	34	4	19	26	7	
	D2	30	34	4	21	27	6	
		30	34	4	20	26.5	6.5	ESBL
14	D1	30	34	4	21	28	7	
	D2	31	34	3	22	28	6	
		30.5	34	3.5	21.5	28	6.5	ESBL
16	D1	32	35	3	20	26	6	
	D2	31	35	4	22	27	5	
		31.5	35	3.5	21	26.5	5.5	ESBL
19	D1	29	31	2	21	24	3	
	D2	30	32	2	20	24	4	
		29.5	31.5	2	20.5	24	3.5	NON-ESBL
20	D1	29	31	2	21	26	5	
	D2	28	31	3	22	26	4	
		28.5	31	2.5	21.5	26	4.5	NON-ESBL

22	D1	29	31	2	20	25	5	
	D2	29	31	2	21	26	5	
		29	31	2	20.5	25.5	5	ESBL
23	D1	30	32	2	20	26	6	
	D2	30	33	3	21	27	6	
		30	32.5	2.5	20.5	26.5	6	ESBL
30	D1	30	33	3	21	29	8	
	D2	31	33	2	21	29	8	
		30.5	33	2.5	21	29	8	ESBL
31	D1	28	31	3	20	25	5	
	D2	29	32	3	20	26	6	
		28.5	31.5	3	20	25.5	5.5	ESBL
32	D1	30	32	2	21	26	5	
	D2	30	32	2	20	26	6	
		30	32	2	20.5	26	5.5	ESBL
33	D1	29	32	3	20	27	7	
	D2	29	32	3	20	26	6	
		29	32	3	20	26.5	6.5	ESBL
34	D1	28	31	3	22	27	5	
	D2	28	32	4	20	27	7	
		28	31.5	3.5	21	27	6	ESBL
48	D1	31	33	2	20	26	6	
	D2	30	33	3	22	27	5	
		30.5	33	2.5	21	26.5	5.5	ESBL
49	D1	29	32	3	20	25	5	
	D2	29	33	4	21	26	5	
		29	32.5	3.5	20.5	25.5	5	ESBL
50	D1	29	33	4	20	27	7	
	D2	30	33	3	21	27	6	
		29.5	33	3.5	20.5	27	6.5	ESBL
53	D1	29	32	3	20	26	6	
	D2	28	32	4	21	26	5	
		28.5	32	3.5	20.5	26	5.5	ESBL
54	D1	28	31	3	20	26	6	
	D2	28	31	3	19	26	7	
		28	31	3	19.5	26	6.5	ESBL
58	D1	29	33	4	21	27	6	
	D2	29	32	3	20	27	7	
		29	32.5	3.5	20.5	27	6.5	ESBL
61	D1	30	32	2	20	25	5	
	D2	29	32	3	18	25	7	
		29.5	32	2.5	19	25	6	ESBL
67	D1	29	32	3	20	26	6	
	D2	29	32	3	18	25	7	
		29	32	3	19	25.5	6.5	ESBL
68	D1	28	31	3	20	24	4	

	D2	29	32	3	20	24	4	
		28.5	31.5	3	20	24	4	NON-ESBL
69	D1	28	31	3	19	27	8	
	D2	27	31	4	20	28	8	
		27.5	31	3.5	19.5	27.5	8	ESBL
70	D1	28	32	4	19	29	10	
	D2	28	31	3	20	30	10	
		28	31.5	3.5	19.5	29.5	10	ESBL
71	D1	28	31	3	20	26	6	
	D2	29	32	3	22	27	5	
		28.5	31.5	3	21	26.5	5.5	ESBL
72	D1	29	31	2	19	27	8	
	D2	29	31	2	20	27	7	
		29	31	2	19.5	27	7.5	ESBL
76	D1	28	30	2	20	27	7	
	D2	28	30	2	21	28	7	
		28	30	2	20.5	27.5	7	ESBL
77	D1	27	29	2	18	27	9	
	D2	28	30	2	19	28	9	
		27.5	29.5	2	18.5	27.5	9	ESBL
78	D1	28	31	3	20	23	3	
	D2	28	30	2	21	22	1	
		28	30.5	2.5	20.5	22.5	2	NON-ESBL
79	D1	28	32	4	21	25	4	
	D2	28	32	4	21	25	4	
		28	32	4	21	25	4	NON-ESBL
80	D1	29	35	6	21	26	5	
	D2	28	34	6	20	26	6	
		28.5	34.5	6	20.5	26	5.5	ESBL
81	D1	29	34	5	21	27	6	
	D2	29	35	6	21	27	6	
		29	34.5	5.5	21	27	6	ESBL
82	D1	29	31	2	20	24	4	
	D2	29	31	2	20	24	4	
		29	31	2	20	24	4	NON-ESBL
83	D1	27	31	4	20	25	5	
	D2	28	31	3	20	26	6	
		27.5	31	3.5	20	25.5	5.5	ESBL
84	D1	27	31	4	20	24	4	
	D2	27	31	4	20	24	4	
		27	31	4	20	24	4	NON-ESBL
85	D1	29	34	5	21	26	5	
	D2	28	34	6	21	27	6	

		28.5	34	5.5	21	26.5	5.5	ESBL
86	D1	27	30	3	20	24	4	
	D2	28	31	3	21	25	4	
		27.5	30.5	3	20.5	24.5	4	NON-ESBL
87	D1	28	33	5	20	25	5	
	D2	28	33	5	20	25	5	
		28	33	5	20	25	5	ESBL
91	D1	26	30	4	20	23	3	
	D2	27	31	4	20	24	4	
		26.5	30.5	4	20	23.5	3.5	NON-ESBL
ATCC 25922	D1	31	33	2	28	30	2	NON-ESBL
	D2	31	33	2	28	30	2	

Appendix 3: ESBL genes detected in 35 Escherichia coli isolates phenotypically detected for ESBL production.

S/No.	Sample Lab No.	Phenotype	blaTEM	blaSHV	blaCTX-M
1	1	ESBL	+VE	+VE	+VE
2	2	ESBL	+VE	+VE	+VE
3	4	ESBL	+VE	–VE	+VE
4	6	ESBL	+VE	+VE	+VE
5	10	ESBL	–VE	–VE	–VE
6	11	ESBL	+VE	–VE	+VE
7	13	ESBL	+VE	–VE	+VE
8	14	ESBL	+VE	+VE	+VE
9	16	ESBL	+VE	+VE	+VE
10	22	ESBL	+VE	+VE	+VE
11	23	ESBL	+VE	–VE	+VE
12	30	ESBL	+VE	–VE	+VE
13	31	ESBL	+VE	–VE	+VE
14	32	ESBL	+VE	–VE	+VE
15	33	ESBL	+VE	–VE	+VE
16	34	ESBL	–VE	–VE	–VE
17	48	ESBL	–VE	–VE	+VE
18	49	ESBL	–VE	+VE	–VE
19	50	ESBL	–VE	–VE	+VE
20	53	ESBL	+VE	–VE	+VE
21	54	ESBL	+VE	–VE	+VE
22	58	ESBL	–VE	–VE	+VE
23	61	ESBL	+VE	+VE	–VE
24	67	ESBL	+VE	+VE	+VE
25	69	ESBL	+VE	+VE	+VE
26	70	ESBL	+VE	+VE	+VE
27	71	ESBL	–VE	–VE	+VE
28	72	ESBL	–VE	+VE	+VE
29	76	ESBL	–VE	–VE	+VE
30	77	ESBL	+VE	–VE	+VE
31	80	ESBL	+VE	–VE	+VE
32	81	ESBL	–VE	–VE	+VE
33	83	ESBL	+VE	–VE	+VE
34	85	ESBL	–VE	–VE	+VE
35	87	ESBL	+VE	–VE	+VE
TOTAL		**Positive**	**24**	**12**	**31**
		Negative	**11**	**23**	**04**