# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

The discovery of antibiotics is one of the most significant medical achievements of the 20th century. The introduction of antibiotics about a century ago controlled many life threatening diseases, reduced the tolls of death and illness and increased the life expectancy of individuals (Schlessinger, 1993). β- Lactam antimicrobial agents are the most common treatments for bacterial infections and account for over 50% of global antibiotic use (Kotra *et al*., 2002). Bacterial resistance to such antibiotics has significantly increased in recent years becoming a serious threat to global health (Livermore, 2004).

Antibiotic resistance is a growing health problem worldwide, with major impact on the length of hospital stay, morbidity, mortality and increasing cost of care (Carlet *et al.,* 2012). When antibiotics first came into use, resistance was not a problem. From 1945 to the late 1980s, new antibiotic agents were developed faster than the bacteria developed resistance (SMAC, 1998). While the 1950s and the 1960s saw the discovery of many new classes of antibiotic agents, the 1980s and 1990s saw only improvements within these classes. No new classes of antibiotic agents have been developed in the past 15-20 years while the bacterial resistance to antibiotics has been increasing at an alarming rate. The alarming increase in the rate of antibiotic resistance has been reported to be at a level that places future patients in real danger (Carlet *et al*., 2012)

The development of antibiotic resistance resulting from selective pressure posed by the use of antibiotics in human medicine, veterinary medicine and agriculture is of a serious concern. Reports show that multidrug resistance has been long discovered (Wantabe, 1963). The widespread use of antibiotics has led to the emergence of multidrug resistant organisms causing numerous outbreaks of infections (Shukla *et al.,* 2004). Lack of regular monitoring of antibiotics as well as frequent assessments of antimicrobial activities of commonly used antibiotics especially in developing countries like Nigeria has led to an increase in antibiotic resistance (Lucet *et al.,* 1999; Soge *et al.,* 2006). Reduced general hospital hygiene measures which has reduced

control of antibiotic resistant isolates has also led to an increase in outbreaks caused by these organisms as a result of drug resistant problems (Paterson *et al*., 2000).

β- Lactam antibiotics include penicillins, cephalosporins, carbapenems & monobactams. Increased use of antibiotics, particularly the third generation of cephalosporins, has been associated with the emergence of β-lactamases mediated bacterial resistance, which subsequently led to the development of extended-spectrum beta-lactamase (ESBL) producing bacteria. Extended-spectrum β-lactamases are enzymes which confer antibiotic resistance on certain bacteria in the family *Enterobacteriaceae (*Yan *et al.,* 2000). Extended spectrum β-lactamases are enzymes capable of hydrolyzing a wide range of extended-spectrum β-lactams, including oxyiminocephalosporins and azetronam, but are inactive against cephamycins and carbapenems (Pitout and Laupland, 2008).

The first plasmid mediated β-lactamase in Gram-negative bacteria, TEM-1, was described in the early 1960s (Datta and Kontomichalou, 1965). Afterwards it was detected in *Klebsiella* in Europe 1980, in Germany 1983, and in France 1985 (Perez *et al.,* 2007). Genetic control of β-lactamase production resides either on plasmids or on the chromosome, while expression is either constitutive or inducible (Chiang and Liaw 2005). Currently, over 150 plasmid borne natural ESBL variants frequently detected in enterobacteria are known. They represent a worldwide problem in hospitalized patients (Spanu *et al*., 2002; Turner, 2005). They were identified after the introduction of extended-spectrum β-lactams (Bradford, 2001). Recent studies indicate that these enzymes play an important role in the dissemination of antibiotic resistant bacterial isolates and may condition future choices for antibiotic regimens for treating life threatening infections due to these organisms (Karas *et al.,* 1996; Soge, 2007).

*Klebsiella pneumoniae and Escherichia coli* are the most common ESBL positive species but all enterobacteria can harbor plasmid mediated ESBL genes (Bradford, 2001). These β-lactamases have been found globally in many different genera of *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Friedman *et al.,* 2008). The extended spectrum cephalosporins have greater Gram-negative antimicrobial properties (Chambers *et al*., 1998) and this can be correlated with the ability of all

enterobaceteria to harbor plasmid mediated ESBL genes as a result of the extensive use of these cephalosporins. ESBLs are inhibited *in-vitro* by β-lactamase inhibitors such as clavulanic acid and tazobactam. Some ESBLs are derived from earlier, broad-spectrum β-lactamases (e.g., the TEM, SHV and OXA enzyme families) and differ from the parent enzyme by a few point mutations, which confer an extended spectrum of activity (Hawkey, 2008).

In enterobacteria, classical ESBLs evolved from the TEM and SHV families (Bradford, 2001). In recent years, several new ESBLs of non-TEM, non-SHV types have emerged such as enzymes of the CTX-M, PER, VEB and GES lineages (Jacoby *et al*., 2005). The CTX-M types are becoming increasingly common compared with the other types (Hawkey, 2008). Extended-spectrum beta-lactamase producing strains can increase the morbidity and mortality rates, in part as a result of associated resistance to other antibiotic families, which limits the treatment options, and raise healthcare costs (Paterson and Bonomo, 2005). Over the last 15 years, numerous outbreaks of infection with organisms producing extended-spectrum β-lactamases (ESBLs) have been observed worldwide (Palucha *et al.,* 1999). Many reports of ESBL-positive bacteria are available from American, European and Asian countries (Valverde *et al.,* 2004; Livermore and Hawkey, 2005; Bagattini *et al.,* 2006) with the prevalence of ESBLs being very high in Africa and other Mediterranean countries (Jacoby and Munoz-Price, 2005; Soge *et al.,* 2006). Reports show that the occurrence of a wide variety of β-lactamases in South Africa was believed to reflect overuse of the newer extended spectrum cephalosporins in medical practice (Pitout *et al.,* 1998). The prevalence of ESBLs among clinical isolates varies from country to country and from hospital to hospital because of different approaches to prevention and control procedures of infections they cause (Bradford, 2001). Extended-spectrum beta-lactamases have been reported from all parts of the world. However, prevalence varies widely even in closely related regions. The true incidence is difficult to determine because of the difficulty in detecting ESBL production and due to inconsistencies in testing & reporting (Yusha'u *et al.,* 2010). The collapse of primary healthcare system coupled with the unavailability of drugs in hospitals in developing countries like Nigeria has resulted into most people purchasing drugs illicitly exposing them to the danger of acquiring ESBL producing organisms (Yusha'u *et al.,* 2007).

Whereas much is known about the occurrence of different β-lactamases from bacteria causing infection in humans, there is lack of knowledge about the occurrence of such enzymes in bacteria from food processing animals. Concern about the development of antibiotic resistance due to the use of antibiotics in animals was first raised in the 1960's. Long term exposure to low doses of antibiotics can lead to selection of resistant forms of microorganisms to antibiotics in treated animals, as a natural and unavoidable phenomenon and also an inherent risk associated with the use of antibiotics in animals. The use of antibiotics in animal husbandry has contributed to the selection on antibiotic resistant animal enterobacterial pathogens. The major difference between the uses of antibiotics in animals and humans is that about 90% of all antibiotics used in food animals are used in sub-therapeutic doses and not for treatment of sick animals (Walker, 1994).

Animals, especially food-producing animals, have been assumed to represent a reservoir for ESBL-producing bacteria (Carattoli, 2008), and this observation might suggest a transmission from the animal reservoir to the community. There are many descriptions of fecal carriage of such *E.coli* among food producing animals especially poultry (broilers) and pigs (Costa *et al*., 2009; Bortolaia *et al*., 2010; Cortes *et al*., 2010; Fortini *et al.,* 2011). The reports concerning livestock cattle are much less abundant (Horton *et al*., 2011). A recent study reported high rates of resistance to quinolones in association with β-lactams among *E.coli* isolates from healthy animals in Nigeria (Fortini *et al.,* 2011). Confirmatory reports are yet to be obtained on the persistence and outbreaks of ESBL producers among bovine enterobacterial pathogens. In animals in the UK, figures indicate that the quantities of cephalosporins used are relatively low in comparison with certain other antimicrobial classes (Veterinary Medicines Directorate, 2004).

The first ESBL to be reported in UK livestock was detected in *E.coli* recovered from calves on a diary farm in Wales in 2004 (Teale *et al.,* 2005). Resistance to 3$^{rd}$ generation cephalosporins in veterinary bacteria in the UK mediated by ESBLs or plasmid-borne AmpC enzymes has been reported to be currently rare or infrequent (Teale *et al*., 2005). However, because of the importance of cephalosporins in human medicine and also in the treatment of some animal infections, surveillance for resistance to these compounds is essential. The particular resistance of current concern in veterinary bacteria has been reported to be AmpC or ESBL resistance in

*Salmonella* and ESBL resistance in *E. coli* (Teale *et al.,* 2005). The first pathway of resistance transfer is the direct transfer of a pathogen from animals to humans. This is the case for zoonotic agents such as *Salmonella*, where resistance to ß-lactams, including extended-spectrum cephalosporins, has been demonstrated (Bertrand *et al.*, 2006; Cloeckaert *et al.*, 2007). Animals may also harbour resistant genes in their residing commensal flora. Commensal *E. coli* isolates have been implicated in the transmission of genetic resistance traits (Kruse and Sorum, 1994) because the genes responsible for resistance may jump from one bacterium to another, mainly by means of mobile genetic elements such as transposons and plasmids.

It has been reported that ESBL producing *E. coli* strains are present in the chicken population in Nigeria (Chah and Oboegbulem, 2007; Fortini *et al.,* 2011) and they may therefore serve as reservoir of ESBL producing *E. coli* strains which could be transferred to humans and other animals. In Nigeria, these agents have been reported not to be in wide use in veterinary practice (Chah and Oboegbulem, 2007). Thus the presence of ESBL producing *E. coli* strains reported in poultry in the country may not be related to the overuse of these agents (Chah and Oboegbulem, 2007). However ampicillin is widely used in poultry production in the country (Chah and Nweze, 2001) and this agent may provide a selective pressure favoring the emergence of *Escherichia coli* strains that produce ESBL enzymes. Other reports also show that *E. coli* strains resistant to extended spectrum cephalosporins have been isolated from calves (Bradford *et al.*, 1999) and dogs (Warren *et al.*, 2001). Apart from ESBL enzymes, resistance to expanded spectrum cephalosporins in enterobacteriaceae has also been found to be mediated by Amp C β-lactamases (Papanicolaou *et al.*, 1990). These enzymes are resistant to cephalosporins/clavulanate combination.

Genes conferring plasmid mediated quinolone resistance (PMQRs) have been reported to be associated with ESBLs or other β-lactamases (Robicsek *et al.,* 2006). The first PMQR gene, *qnr*A, has been described in 1998 (Martínez-Martínez *et al.*, 1998). Qnr proteins protect the DNA-gyrase-complex, the target of quinolones and fluoroquinolones, and thus mediate resistance to quinolones and decreased susceptibility to fluoroquinolones. More qnr genes, *qnr*B, *qnr*C, *qnr*D and *qnr*S, and subtypes thereof have been identified (Hata *et al*., 2005; Jacoby *et al.,* 2006; Cavaco *et al.,* 2009; Wang *et al.,* 2009). Besides qnr genes the gene *aac(6')-Ib-cr*, coding for

an aminoglycoside acetyltransferase, has been detected, which confers resistance to kanamycin and decreased susceptibility to ciprofloxacin and norfloxacin by acetylating their piperazinyl substituent (Robicsek *et al.,* 2006). Two plasmid encoded efflux pumps, QepA1 and QepA2 (Yamane *et al.,* 2007; Cattoir *et al.,* 2008), have also been reported.

Many reports have described and characterized ESBLs in *Klebsiella sp.* and *E. coli* (Bradford, 2001; Paterson and Bonomo, 2005) including reports from African countries (Blomberg *et al.,* 2005; Gangoue-Pieboji *et al.,* 2005). However, few reports on the prevalence of ESBL resistant determinants and PMQR determinants in both human and bovine isolates in Nigeria have been documented. ESBL genes and PMQR genes have been described to be co-located either on the same plasmid or on different plasmids within the same isolate (Dionisi *et al.,* 2009; Woodford *et al.,* 2009; Richter *et al.,* 2010; Dolejska *et al*., 2011; Kirchner *et al.,* 2011; Müller *et al*., 2011; Yao *et al.,* 2011).

Just as physicians need new antibiotics to treat human diseases, veterinarians see needs for the use of new antibiotics in their practice. It is possible to have a drug closely related to one used in animals to be developed for human use and in that case resistance to the animal drug if transferred to bacteria that infect humans might be cross resistant to the human drug and reduce its efficacy. Whatever the reason for the use of antibiotics in animals, treatment of animals can result in contamination of meat by antibiotic resistant bacteria which may result in three things (OTA, 1995): The first is that antibiotic resistant pathogenic bacteria might be transferred to humans. Secondly, antibiotic resistant genes although present in non-pathogenic bacteria in the animal may be transferred to pathogenic organisms in humans. Thirdly, antibiotic resistant bacteria that don't normally affect humans will be ingested by people on antibiotic therapy which would have altered the human flora and that alteration may favor the growth of bacteria that pose a risk to human health (OTA, 1995).

The advent of these ESBL producers has posed a great threat to the use of many classes of antibiotics particularly the cephalosporins which are useful therapeutic agents. These organisms also cause difficulties in antimicrobial susceptibility testing

and increase morbidity and mortality rates. The misuse of antimicrobials in Nigeria calls for the need to detect the occurrence of ESBL producing organisms since selective pressure on the use and overuse of antibiotics in treatments has resulted in the emergence of new variants of β-lactamases (Cosgroove *et al*., 2002). This form of resistance is not only against expanded spectrum cephalosporins but even fluoroquinolones, aminoglycosides, tetracycline and chloramphenicol that were developed to counter the emergence of resistance to ordinary β-lacatmases (Paterson and Bonomo, 2005; Soge, 2007).

Preserving the effectiveness of antibiotics and other antimicrobials will require changes in all major areas of use: human medicine, veterinary medicine and agriculture.

### 1.2 Justification for this study

With reports on the prevalence and characterization of ESBL enzyme expression and plasmid mediated quinolone resistance (PMQR) among members of the family *Enterobacteriaceae* and paucity of information in Nigeria, this current study is aimed at determining the molecular characteristics of ESBL and PMQR determinants in *E. coli* clinical isolates from human and bovine in Oyo state, Nigeria This study will also determine the possible dissemination of the resistance genes. Little knowledge about the occurrence of ESBLs and PMQRs in bacteria causing infections in humans' exisit but there is paucity of information about these resistance mechanisms in bacteria from food-producing animals. This study will contribute to reducing the breadth of the problem and to define appropriate therapeutic options.

Characterization of the isolates from both the human and bovine sources in respect of their antibiotic resistant properties and demonstrating the transferability of the resistant genes may also contribute to the prudent use of antibiotics in both human and veterinary medicine and also increase the chances of having successful outcomes in therapeutic options.

This study will also allow an understanding on how isolates from Nigeria as a developing country compare with similar isolates from other parts of the world especially developed countries. This study therefore would demonstrate the need for

local surveillance and characterization of bacteria not only from human sources but also from bovine sources especially in developing countries which have limited resources and where antibiotics are indiscriminately used both in human and animals.

Detailed and reliable knowledge on the resistance of enterobacterial pathogens in both human and animals to therapeutic options available for infections they cause would be obtained from this study as an important requirement for safe and effective use of these drugs in both human and veterinary medicine.

## 1.3 Aim and Objectives

### General Objective

To determine the molecular characteristics of ESBL and PMQR determinants in *E. coli* clinical isolates from human and bovine in Oyo State, Nigeria, and to determine the possible dissemination of the resistance genes.

### Specific Objectives

(1) To isolate and characterise *Escherichia coli* from both human and bovine sources.
(2) To determine the antibiotic susceptibility patterns of *Escherichia coli* isolates to selected β-lactams, flouroquinolones, quinolones, aminoglycosides and trimethoprim by the disc- diffusion and Epsilometer test methods respectively.
(3) To determine the genetic basis for antibiotic resistance through phenotypic detection of ESBLs in the *Escherichia coli* isolates by the Double-disc synergy test.
(4) To genotypically characterise ESBL and PMQR genes using molecular methods
(5) To determine the genetic locations (plasmid and integron) of the ESBL and PMQR genes and determine the genetic transfer ability of the resistance genes by conjugation.
(6) To compare the molecular characterisation of extended-spectrum β-lactamase resistance and plasmid mediated quinolone resistance in bovine and human isolates of *Escherichia coli*.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 *Escherichia coli* as a pathogen

*Escherichia coli* was discovered by German pediatrician and bacteriologist Theodor Escherich in 1885 (Feng *et al* 2002) and is now classified as part of the Enterobacteriaceae family. It is a Gram-negative bacterium, facultatively anaerobic and non-sporulating.

*Escherichia coli* cells are typically rod-shaped and are about 2 micrometres (μm) long and 0.5 μm in diameter, with a cell volume of 0.6 - 0.7 $\mu m^3$ (Kubitschek, 1990). It can live on a wide variety of substrates. *Escherichia coli* use mixed-acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate and carbon-dioxide. Strains that possess flagella are motile. *Escherichia coli* and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population.

*Escherichia coli* normally colonize an infant's gastrointestinal tract within 40 hours of birth, arriving with food or water or with the individuals handling the child. In the bowel, it adheres to the mucus of the large intestine. The bowel is a rich environment for genetic exchange between commensal Enterobacteriaceae. Most *E. coli* strains are harmless, but some, such as serotype 0157:H7, can cause serious food poisoning in humans (Vogt and Dippold, 2005). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin $K_2$ (Bentley and Meganathan, 1982) or by preventing the establishment of pathogenic bacteria within the intestine (Reid *et al.,* 2001). It is the primary facultative organism of the human gastrointestinal tract (Todar, 2007). As long as these bacteria do not acquire genetic elements encoding for virulence factors, they remain benign commensals (Evans *et al.,* 2007). Faecal carriage of CTX-M producing bacteria has been described (Gazouli *et al.,* 1998; Ma *et al*., 1998).

On MacConkey agar, deep red colonies are produced as the organism is lactose-positive, and fermentation of this sugar medium will cause the medium's pH to drop,

leading to darkening of the medium. Growth on Levine EMB agar produces black colonies with greenish-black metallic sheen. This is diagnostic of *E. coli*. The organism is also lysine positive, and grows on TSI slant with a (A/A/g+/$H_2S$-) profile. Also, IMViC is ++-- for *E. coli*; as its indole positive (red ring) and methyl red positive (bright red), but VP negative (no change-colorless) and citrate negative (no change-green color).

*Escherichia coli* is not always confined to the intestine, and its ability to survive for brief periods outside the body makes it an ideal indicator organism to test environmental samples for fecal contamination (Thompson, 2007). *Escherichia coli* are the most abundant facultative anaerobe of the human intestinal microflora. Commensal *E. coli* strains rarely cause disease in humans, except in immunocompromised patients or when the normal gastrointestinal barriers are breached (Rolhion and Darfeuille-Michaud, 2007). A limited number of pathogenic *E. coli* clones have gained specific virulence attibutes (adherence factors and other pathogenic surface structures) which enable them to cause urinary tract infections, septicaemia, menigitis and diarrheal disease not only in immunocompromised patients but also in healthy individuals (Kaper *et al.,* 2004). Pathogenic *E. coli* strains are characterised by shared O (lipopolysaccharide) and H (flagellar) antigens that define serotypes or serogroups. *Escherichia coli* can be sub-divided further into four main phylogenetic groups; A, B1, D and B2. Commensal strains of *E. coli* belong mainly to the A and B1 phylogenetic groups (Picard *et al.,* 1999). Pathogenic phylogenetic lineages involved in extra-intestinal infections are mainly derived from the B2 and D groups.

*Escherichia coli* can also be grown easily and are easily-manipulated or duplicated through a process of metagenics, making it one of the best-studied prokaryotic model organisms, and an important species in biotechnology and microbiology. As a Gram-negative organism, *E. coli* is resistant to many antibiotics that are effective against Gram-positive organisms. Antibiotics which may be used to treat *E. coli* infection include amoxicillin as well as other semi-synthetic penicillins, many cephalosporins, carbapenems, azetronam, trimethoprim-sulphamethoxazole, ciprofloxacin, nitrofurantoin and the aminoglycosides

Antibiotic-resistant *E. coli* may also pass on the genes responsible for antibiotic resistance to other species of bacteria, such as *Staphylococcus aureus*. *Escherichia coli* often carry multidrug resistant plasmids and under stress readily transfer those plasmids to other species. Thus, *E. coli* and the other enterobacteria are important reservoirs of transferable antibiotic resistance (Salyers *et al.,* 2004).

## 2.2 Antibiotics

Following earlier experiments that had demonstrated interesting anti-bacterial effects from various bacterial secretions, the German scientist E. de Freudenreich in 1888 isolated a bacterial secretion and noted its antibacterial properties. Pyocyanase, secreted by *Bacillus pyocyaneus*, retarded the growth of other bacteria in situ and was toxic to many disease-causing bacteria. Unfortunately, pyocyanase's toxicity and unstable character within the human body prevented its use as an effective, safe antibiotic (Russell, 2006). The first effective antibiotic discovered was penicillin (Nester *et al.,* 1998; Tipton, 2002).

Originally, the term antibiotic referred only to organic compounds produced by bacteria or moulds that are toxic to other microorganisms but it’s now applied also to synthetic antimicrobials such as the sulphonamides. Antibiotics are substances produced by microorganisms and chemical synthesis but which at low concentrations will kill or inhibit the growth of infectious organisms particularly bacteria and fungi (Charles *et al*., 1977; Ngan, 2005).

Antibiotics are one class of "antimicrobials", a larger group which also includes anti-viral, anti-fungal, and anti-parasitic drugs. Antibiotics are not effective in viral, fungal and other nonbacterial infections, and individual antibiotics vary widely in their effectiveness on various types of bacteria. Some specific antibiotics (called "narrow-spectrum antibiotics") target both gram-negative or gram-positive bacteria, and others are more “broad-spectrum” antibiotics. The effectiveness of individual antibiotics varies with the location of the infection and the ability of the antibiotic to reach this site. Such antibiotic must display selectivity of action against the causative organism; as such selection of such antibiotic depends on proper and accurate identification of such organism. Major antibiotic producers are fungi such as *Penicillium spp* and *Cephalosporium spp* which produce penicillins and cephalosporins respectively and

also *Bacillus* and *Streptomyces spp* which produce bacitracin and tetracycline respectively. Antibiotics have been used in managing various human and animal bacterial infections. An ideal antibiotic or antibacterial agent, while displaying selectivity of action against the causative organism of a bacterial infection, must show minimal toxicity against the host tissue (Frobisher *et al.,* 1974)

**2.3 Classification of antibiotics**

Although there are several classification schemes for antibiotics, based on bacterial spectrum (broad/ narrow) or route of administration (injectable/ oral / topical), or type of activity (bactericidal/ bacteriostatic), the most useful is based on chemical structures or by mechanism of action. Antibiotics within a structural class will generally have similar patterns of effectiveness, toxicity, and allergic potential (Schwartz and Al-Mutairi 2010). Most commonly used types of antibiotics are: aminoglycosides, penicillins, fluoroquinolones, cephalosporins, macrolides, and tetracyclines. While each class is composed of multiple drugs, each drug is unique in some way. The important mechanism of action of most antimicrobial agents used in treating bacterial infections includes:

1. Inhibition of bacterial cell wall synthesis. Bacteria have murein in their cell walls, not found in the host, and murein (peptidoglycan) is essential to the viability of the bacterium (eg β-lactams).

2. Interference with protein synthesis (eg the tetracyclines)

3. Interference with nucleic acid synthesis (RNA and DNA), which exploits differences between RNA polymerases and DNA replication strategies in bacteria and eukaryotes (eg the flouroquinolones).
4. Inhibition of an essential metabolic pathway that exists in the bacterium but not in the host (eg trimethoprim-sulphamethoxazole). (Lamikanra, 1999; Army, 2004)
5. Membrane inhibition or disruption by antimicrobial peptides. The outer membrane of Gram-negative bacteria is a reasonable point of attack (eg the polymyxins) (Todar, 2008).

The figure and table below illustrate various antibiotic targets in the bacterial cell and the more important mechanisms of action of various classes of antibiotics on the bacterial cell.

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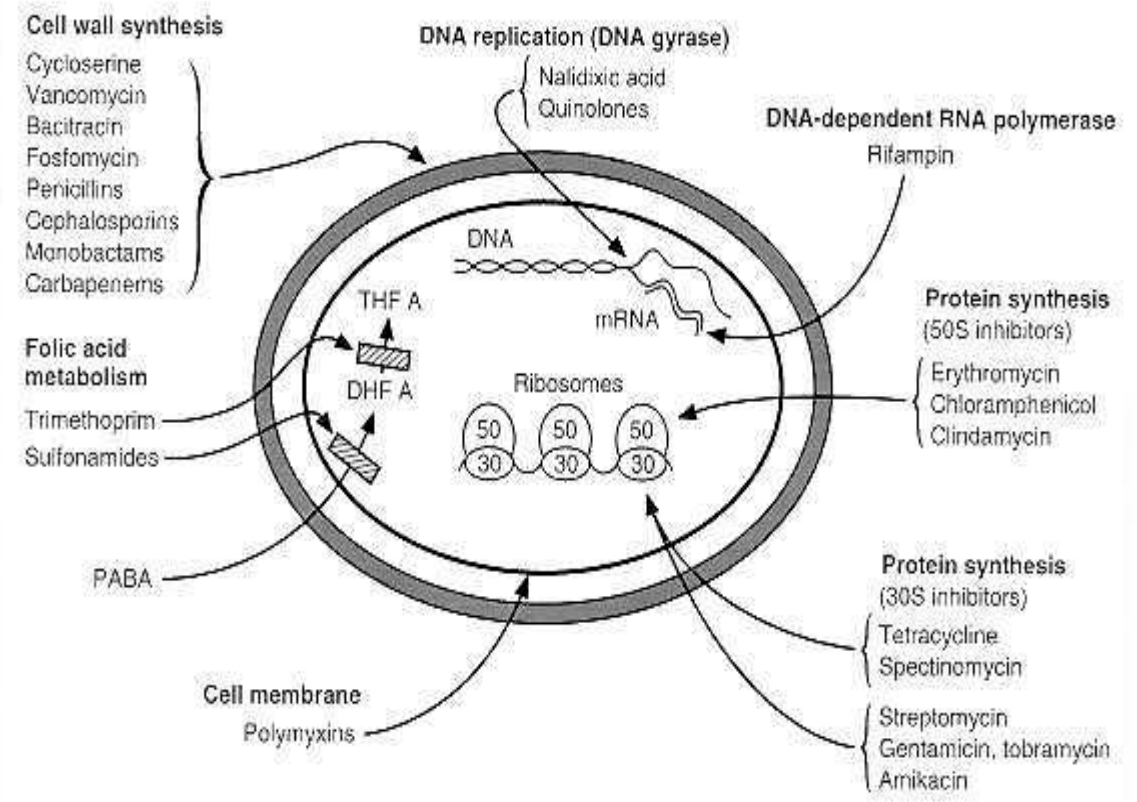



**Fig 2.1 Sites of action of different antibiotics in the bacterial cell.** PABA, paraminobenzoic acid; DHFA, dihydrofolic acid; THFA, tetrahydrofolic acid. **(Neu and Gootz 1996)**

## Table 2.1 Mechanisms of action of antibiotics (Neu and Gootz, 1996)

| Mechanism of Action | Antibiotics |
|---|---|
| 1 **Inhibition of Bacterial cell wall synthesis** | |
| -Drugs that inhibit biosynthetic enzymes | Fosfomycin, cycloserine |
| - Drugs that combine with carrier molecules | Bacitracin |
| -Drugs that combine with cell wall substrates | Vancomycin |
| -Drugs that inhibit polymerization and attachment of new peptidoglycan to cell wall | Penicillins, Cephalosporins, Carbapenems, Monobactams |
| 2 **Inhibition of Cytoplasmic membranes** | |
| -Drugs that disorganize the cytoplasmic membranes | Tyrocidins, Polymyxins |
| -Drugs that produce pores in membranes | Gramicidins, Polyenes (Amphotericin) |
| -Drugs that alter the structure of fungi | Imidazoles (Ketoconazole, fluconazole) |
| 3 **Interference with Nucleic acid synthesis** | |
| -Inhibitors of nucleotide metabolism | Flucytosine(Fungi) |
| - Inhibitors of DNA replication | Quinolones, Nitroimidazoles |
| - Inhibitors of RNA polymerase | Rifampicin |
| 4 **Inhibition of Protein Synthesis** | |
| -Drugs that bind to the 30S ribosomal unit | Streptomycin, Kanamycin, Gentamicin, Amikacin, Spectinomycin , Tetracyclines |
| -Drugs that bind to the 50S ribosomal unit | Clindamycin, Chloramphenicol, Erythromycin, Fusidic acid |
| 5 **Inhibition of a metabolic pathway (Folate metabolism)** | |
| -Inhibitors of pteroic acid synthetase | Sulfonamides |
| -Inhibitor of dihydrofolate reductase | Trimethoprim |

## 2.4 Human and veterinary uses of antibiotics

Antibiotics are used for treating a wide range of bacterial infections in both human and veterinary medicine. They are also used for prophylaxis of bacterial; infections, diagnosis of malignancies and also growth promotion in animals (McEwen and Fedorka-Cray *et al.*, 2002). Common forms of antibiotic misuse in humans include taking an inappropriate antibiotic, in particular the use of antibacterials for viral infections like the common cold, and failure to take the entire prescribed course of the antibiotic, usually because the patient feels better before the infecting organism is completely eradicated. In addition to treatment failure, these practices can result in antibiotic resistance. While some uses of antibiotics in animals are a matter of animal health, other uses have an economic motive referred to as sub-therapeutic uses of antibiotics (Abioye, 2002). Unlike in human medicine, antibiotics in food-producing animals are used for two different purposes: prevention and control of bacterial infections and growth promotion (Schwarz and Chaslus-Dancla, 2001). The major difference in antibiotic use in humans' and animals is that about 90% of all the antibiotics used in food animals are used in subtherapeutic doses and not for the treatment of sick animals. Intestinal commensal *Enterobacteriaceae* of animals reared in high-population flocks are usually under different selective pressures by the use of antibiotics for treating infections, for metaphylaxis and for prophylaxis (Schwarz and Chaslus-Dancla, 2001).

Several antimicrobial agents used in veterinary and human medicine belong to the same antibiotic families and hence different selective pressures exercised in distinct environments might contribute to the selection and dissemination of similar resistance genes (WHO, 1997; Aarestrup, 1999). The control and prevention of bacterial infections is achieved by either therapeutic, metaphylactic or prophylactic application of antimicrobials. For this, substances of mainly the same classes as used in human medicine are available for the treatment of food-producing animals (Schwarz and Chaslus-Dancla, 2001). Treatment of animals can result in contamination of meat by antibiotic-resistant bacteria which can result in antibiotic resistant bacteria been transferred to humans or antibiotic resistant genes transferring to pathogenic organisms in human even though present in non-pathogenic bacteria in the animal. Antibiotic resistant bacteria that do not normally affect humans may also as a result of

treatment of the animals be ingested by people on antibiotic therapy. The therapy will have altered the human flora, and the alteration will favor the growth of bacteria that pose a risk to human health. While no one denies that the unwise use of antibiotics in human medicine is a source of serious resistance problems, this view has prevented recognition of some of the most attractive opportunities to cut back on the use of those drugs in sub-therapeutic applications (Aarestrup *et al.,* 2008).

The use of antibiotics for growth promotion is specific to food producing animals. The most commonly used antibiotic agents in animals reared for food are from five major classes; β-lactams, tetracyclines, aminoglycosides, macrolides and sulphonamides (Johnston, 1998). Long term exposure to low doses of antibiotics can lead to selection of resistant forms of microorganisms to antibiotics in the treated animal which is a natural and unavoidable phenomenon. It's also an inherent risk associated with the use of antibiotics in the animals. These resistant forms of microorganisms can be passed on to caretakers of the animals and more broadly to people who prepare and consume undercooked meat (Abioye, 2002). The development of resistance can be minimized provided that a number of measures are observed to prolong the useful life of all antibiotics in both human and veterinary medicine especially in developing countries (Okeke *et al*, 2005a). More strict veterinary antibiotic policies are needed in order to prevent emergence and dissemination of these strains among animals and humans, limiting future problems of therapy failure.

Antibiotic uses should be limited to situations where they are needed and the selection of antibiotic both in human and veterinary medicine should take a number of factors into consideration. The use of certain drugs that have important uses in humans like flouroquinolones and third generation cephalosporins should be prohibited in animals (Federation of Veterinarians of Europe, 2006). Antibiotic misuse should be avoided both in human and veterinary medicine especially in most developing countries where socioeconomic factors contribute to the spread of resistant bacteria as a result of antibiotic misuse (Okeke *et al.,* 2005b).

## 2.5 Antibiotic resistance

Resistance may be defined as the ability of a microorganism to resist the action of antimicrobial agents at concentrations achievable in the body after normal dosage meaning that such microorganism survives antimicrobial therapy (Mims *et al.,* 1993; Brooks *et al.,* 2004). Most of the resistances in microbes which are now difficult to treat are of genetic origin and transferable between species and genera of bacteria (Rahman *et al*. 2004). The inappropriate use of antimicrobial agents does not achieve the desired therapeutic outcomes and is associated with the emergence of resistance. Under *in-vivo* conditions, a strain may either be resistant or sensitive to treatment depending on its location, the dosage and mode of drug administration, tissue distribution of the drug, and the state of the immune system of the individual under treatment (Aarestrup, 2006). In microbiology, a bacterial strain is described as being resistant to a specific antimicrobial agent if it is not inhibited by specific concentrations that inhibit majority of strains in the bacterial species. Such a strain can also be described as resistant because it grows in higher concentrations of a specific antimicrobial agent compared with phylogenetically related strains (Aarestrup, 2006).

Resistance to antibiotics has evolved due to misuse of antibiotics in clinical treatment (Essack, 2004) and resistance itself represents a serious threat to effective treatment. Lack of access, poor adherence and sub-standard antimicrobials may also play as important a role in antibiotic resistance (WHO, 2001). All antimicrobial agents have the potential to select drug- resistant sub-populations of microorganisms. With the widespread use of antimicrobials, the prevalence of resistance to each new drug has increased. The prevalence of resistance varies between geographical regions and over time, but sooner or later resistance will emerge to all antimicrobial agents (WHO, 2001).

### 2.5.1 Types of antibiotic resistance

Bacterial strains with antibiotic resistance are selected through the use of antibiotics and such antibiotic resistance could either be natural or acquired antibiotic resistance (Today, 1995). The three main mechanisms by which Gram-negative organisms exhibit resistance to antimicrobials are decreased permeability of the drug into the cell, hydrolysis of the drug by enzymes and decreased affinity of the antibiotic target (Piddock *et al.*, 1997 and Cheesebrough, 2000). The major mechanism of resistance in Gram-negative organisms which causes clinically significant infection is the expression of β-lactamases (Piddock *et al.*, 1997) which are enzymes capable of hydrolyzing the β-lactam ring of penicillins, cephalosporins and related antimicrobial drugs, rendering them inactive. Majority of drug resistant microorganisms have emerged as a result of genetic changes acquired through mutation or transfer of genetic material during the life of the microorganism and subsequent selection processes. All *Enterobacteriaceae* except *Salmonella spp* produce intrinsic chromosomal encoded beta-lactamases which are responsible for intrinsic resistance of individual species to some antibiotics (Susic, 2004).

#### 2.5.1.1 Natural resistance

Bacteria may be inherently resistant to an antibiotic which is due to a structural or functional trait allowing tolerance of a particular antibiotic or antimicrobial class by members of a bacterial group. More accurately, this should be referred to as insensitivity or reduced sensitivity (Aarestrup, 2006). Reduced sensitivity can be due to low affinity of the drug for the bacterial target (for example low affinity of nalidixic acid for enterococcal gyrase), inaccessibility of the drug into the bacterial cell (for example a Gram-negative bacterium with an outer membrane that establishes a permeability barrier against antibiotics like glycopeptides) (Today, 1995; David *et al.*, 2000), extrusion of the drug by chromosomally encoded active exporters (resistance to tetracyclines, chloramphenicol and quinolones in *Pseudomonas aeruginosa*), or innate production of enzymes inactivating the drug (for example AmpC beta-lactamase in some members of the family *Enterobacteriaceae*). The characteristic feature responsible for a bacterium that has natural resistance is inherent

or integral which has arisen through the process of evolution (Schweizer, 1998; Aarestrup, 2006; Soge, 2007).

Natural resistance is also found in fungi, protozoa and viruses which are resistant to most antibacterial agents. Natural resistance represents a clinical problem in dealing with bacterial species that are insensitive to a large number of antimicrobial classes, for example *Mycobacterium tuberculosis* or *Pseudomonas aeruginosa*, since it limits the range of drugs available for treatment and consequently increases the risk associated with emergence of acquired resistance. *Pseudomonas aeruginosa* is generally insensitive to most antibiotics because of cell membrane impermeability and natural resistance of the organism (Poole *et al*., 1993). Generally some organisms are intrinsically resistant to certain antibiotics for example Gram-positive bacteria have been reported to be much less susceptible to polymyxins than Gram-negative bacteria (David *et al.*, 2000).

#### 2.5.1.2 Acquired resistance: mutational and transferable resistance

Acquired resistance is a major threat to animal and human health because it causes the emergence and spread of resistance in susceptible bacterial populations and consequently may lead to therapeutic failure. Unlike natural resistance, it is a trait associated with strains of a particular bacterial genus or species. Many bacteria may acquire resistance to one or more of the antibiotics to which they were formerly susceptible. Acquisition is due to a genetic change in the bacterial genome, which can be the consequence of a mutation (endogenous resistance) or of horizontal acquisition of foreign genetic information (exogenous resistance) (Today, 1995; Soge, 2007). Resistance can also result from a combination of mutational and gene transfer events, like in the case of mutations that expand the spectrum of beta-lactamases or confer on them resistance to beta-lactamase inhibitors. Endogenous resistance plays an essential role in bacteria that are not known to acquire foreign DNA under natural conditions (for example *Mycobacterium spp*.). For all bacteria, it represents the main mode of acquiring resistance when high level of resistance is not conferred by mobile genetic elements (for example fluoroquinolone resistance).

Exogenous resistance can be secondary due to acquisition of free DNA by transformation, bacteriophages by transduction (Todar, 2008), and cell-to-cell transfer

by conjugation (Synder and Champness, 1997). Conjugation plays a more important role in the spread of antimicrobial resistance since resistance genes are often located on conjugative genetic elements such as plasmids or transposons which allow easy spread of resistance from one bacterium to another even from a specie of bacterium to another (Ricki, 1995).

Mutational resistance develops as a result of spontaneous mutation in a locus on the bacterial chromosome that controls susceptibility to a given antibiotic. The presence of the drug serves as a selecting mechanism to suppress susceptible microorganisms and promote the growth of resistant mutants (Today, 1995). Some examples include resistant genes acquired by some organisms leading to the synthesis of cephalosporinases and penicillinases which provide protection against β-lactam antibiotics. Mutations occur spontaneously in any gene of the bacterial genome but the frequency of mutation may differ among genes. Consequently, the frequencies of mutations leading to antimicrobial resistance may vary depending on the specific antimicrobial agent. A single mutation can determine a 1,000- fold increase in the level of resistance to a drug (e.g., streptomycin). In contrast, for other drugs (e.g., flouroquinolones) the acquisition of resistance by mutation is a gradual, stepwise process in which different mutations are involved. The frequencies of mutations conferring drug resistance also differ among species as well as strains of the same species (Aarestrup, 2006). Some bacteria synthesize 'pumps' in their plasma membrane through which they remove antibiotics like tetracycline from the interior of the cell wall, some methylate their ribosomes obscuring the target of the antibiotics for example erythromycin (Teneover, 2006) and some produce enzymes that destroy the antibacterial drug as a result of the acquisition of resistance genes in a bacterium etc.

Resistance can be mediated by genes located either on the chromosomes or on mobile genetic elements of extraneous origins such as resistant plasmids, including those associated with transposons and integrons (Normark and Normark, 2002). The Figure 2.2 and Table 2.2 show major mechanisms and methods of antibiotic resistance in bacteria.

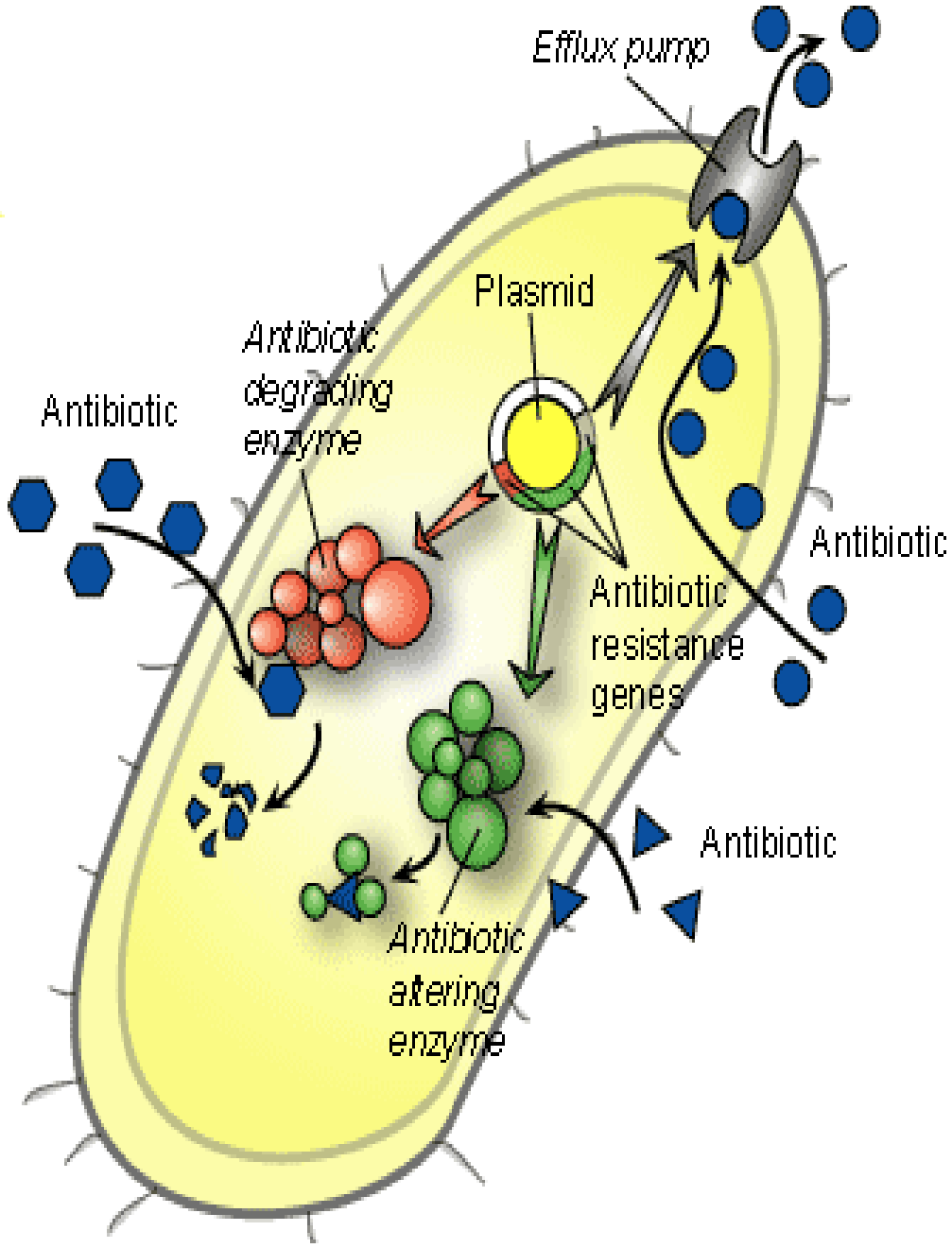



**Fig 2.2 Mechanisms of antibiotic resistance in bacteria cell (Todar, 2008)**

**Table 2.2 Methods of Antibiotic resistance (Todar, 2008)**

| Antibiotic | Method of Resistance |
|---|---|
| Chloramphenicol | Reduced uptake into the cell |
| Tetracycline | Active efflux from the cell |
| B-lactams, Erythromycin, Lincomycin | Eliminates or reduces binding of antibiotic to cell target |
| B-lactams, Aminoglycosides, Chloramphenicol | Enzymatic cleavage or modification to inactivate antibiotic molecule |
| Sulfonamides, Trimethoprim | Metabolic bypass of inhibited reaction |
| Sulfonamides, Trimethoprim | Overproduction of antibiotic target (titration) |

### 2.5.2 Elements involved in horizontal transfer of resistance genes

The rapid spread of antimicrobial resistant genes between bacteria of the same and of different species and genera is mainly the result of horizontal transfer events of mobile genetic elements carrying one or more resistance genes. Plasmids, transposons, integrons and gene cassettes play a major role in horizontal transfer of antimicrobial resistance genes. These four types of elements are composed of double-stranded DNA, but differ distinctly in their structures, sizes, biological properties, and ways of spreading (Schwarz and Chaslus-Dancla, 2001; Schwarz *et al.,* 2006). Plasmids which are small extrachromosomal DNA molecules and transposons and integrons which are short DNA sequences provide effective mechanism for rapid horizontal dissemination of antibiotic resistant determinants among bacteria specie and can also code for multiresistance (Ricki, 1995; Rice, 2002; Ferber, 2003). An alarming number of human pathogens have acquired genes to combat all presently used antibiotics and multidrug resistant strains arising are particularly common where antibiotic use is heavy and where patients often have weakened immune system especially in the hospital.

The location of resistant genes determines how rapid they are disseminated. Reports show that resistant genes encoded on genetic elements (plasmids, integrons and transposons) can be transferred horizontally by conjugation as well as vertically to the progeny (Summers, 2006; Soge, 2007). Horizontal transfer of resistant genes accounts for a much faster rate at which antibiotic resistant genes on mobile genetic elements are disseminated when compared with chromosomal genes which are only transferred vertically.

Reports show that bacteria can become resistant either through mutation or horizontal transfer of resistant genes by transformation, transduction and conjugation. The most common method of transfer of antibiotic resistance however is conjugation. Plasmids and transposons carrying antibiotic resistant genes can readily move from one cell to another (Teneover, 2006).

## Plasmids

Plasmids are self-replicating circular DNA, smaller and separate from the bacterial genome that can be transferred (some are transmissible) into another bacterial strain or species (Baker, 1999). They have been detected in virtually all bacterial Genera of medical and veterinary importance, bacteria which constitute the physiological flora of the skin and the various mucosal surfaces in humans and animals (Schwarz and Chaslus-Dancla, 2001). They vary in size between < 2 and > 100kb. They encode multiple resistance phenotypes and carry genetic information that may provide selective advantage to the bacteria (Mims *et al.*, 1993). They can code for resistance to antimicrobial agents, disinfectants, heavy metal cations, anions, nucleic acid-binding substances, or bacteriocins, but also for metabolic or virulence properties, and fertility functions (Stanisich, 1988). Bacterial plasmids that encode proteins responsible for antibiotic resistance are referred to as resistant (R) plasmids (Hindler *et al.,* 1994) and at times, they carry resistance genes in addition to genes for other traits.

Due to the presence of plasmids and their exchange among bacteria, resistance genes have been widely spread and are also subject to mutation (Lee *et al*., 2001). The ability of plasmids to transfer genes from one cell to another was first discovered in the 1950's by Joshua Lederberg and Edward Tatum (Atlas *et al.,* 1998). This plays a major role in the dissemination of resistance genes and can be transferred among organisms of same and different species of bacteria (Butaye *et al.,* 2003).

Plasmids have systems which guarantee their autonomous replication but also have mechanisms controlling their copy number and ensuring stable inheritance during cell division (Carattolli, 2009). Hedges and Datta proposed a plasmid classification scheme based on the stability of plasmids during conjugation, a phenomenon called plasmid incompatibility (Datta and Hedges, 1971). Incompatibilty is a manifestation of the relatedness of plasmids that share common replication controls. Incompatibility was defined as the inability of two related plasmids to be propagated stably in the same cell line; thus, only compatible plasmids can be rescued in transconjugants. Plasmids with the same replication control are "incompatible", whereas plasmids with

different replication controls are “compatible” (Carattolli *et al*., 2005). The knowledge that some plasmid types are prevalent in resistant bacterial populations could be useful to explore the possibility of identifying drugs targeting these plasmid families as a medicinal strategy for the treatment of drug-resistant bacteria.

The PCR-based replicon typing scheme targets the replicons of the major plasmid families occurring in Enterobacteriaceae and is useful in tracking the spread of plasmids conferring drug resistance. This method could be applied to monitor the circulation of plasmids within strains from different environments or to follow the horizontal transmission of antimicrobial resistance genes among the enterobacteriaceae. Plasmid classification gives relevant information about the potential host range of a resitance gene located on a specific plasmid type. Acquisiton of replicons on a plasmid by a bacterium enables the bacteria to acquire the ability to replicate more.

Although plasmids are capable of autonomous replication due to their replication systems, it is noteworthy that not every plasmid can replicate in every host bacterium (Schwarz *et al.,* 2006). Therefore, when transferred into a new host cell, plasmids may stably replicate; form cointegrates with other plasmids; or integrate, either in part or completely, into the chromosomal DNA. Plasmids usually act as vectors for transposons and integrons/gene cassettes (Bennett, 1995).

**Transposons**

Transposons do not possess replication systems and therefore must integrate, for their stable maintenance, into replication-proficient vector molecules such as chromosomal DNA or plasmids in the cell. Transposons also vary in size (<1kbp to 60kbp) and structure.

The smallest transposons, also known as insertion sequences, solely carry the gene for a transposase which is responsible for the movement of the element (Schwarz and Chaslus-Dancla, 2001). Transposons are segments of DNA that can move around to different positions in the genome of a single cell and in the process may cause mutations or increase/decrease the amount of DNA in the genome. These mobile segments of DNA are sometimes called ‘jumping genes’. The larger transposons are

known to usually carry one or more additional genes, most of which code for antibiotic resistance properties.

There are 3 distinct types of transposons- Class II transposons consisting only of DNA that moves from one location to another, Class III transposons also called miniature inverted repeats transposable elements and Class I transposons known as retrotransposons that first transcribe DNA to RNA and use reverse transcriptase to make a DNA copy of the RNA so as to insert it in a new location (Heritage *et al.,* 1999). The widely found members of the Tn 21 transposon family have arisen from repeated insertions of novel transposable elements and carry diverse antibiotic resistance genes. Members of the Tn 21 family are among the most widely found transposable elements in facultative Gram-negative bacteria (Brown *et al*., 1996). Some transposons integrate site specifically, whereas others can insert at various positions in the chromosomal or plasmid DNA. Similar to the situation among plasmids, there are also non-conjugative and conjugative transposons (Bennett, 1995; Salyers *et al.,* 1995).

**Integrons**

The increased frequency of antibiotic resistance is known to be associated with the dissemination of integrons in the Enterobacteriaceae. In recent years, the incidence of multidrug resistance in the Enterobacteriaceae is on the increase in hospital settings (Bush, 2010). The resistance in these isolates has been linked with the carriage of integrons, genetic elements which allow the integration of antimicrobial drug resistance genes through site-specific recombination events. Integrons are capable of recognizing, capturing and expressing multiple resistance genes in cassette structures, and hence, are assumed to play important roles in the dissemination of antimicrobial resistance (White *et al*., 2001). High prevalence of integrons among clinical isolates of Enterobacteriaceae particularly *Escherichia coli* and *Klebsiella* spp. has been reported worldwide (Martinez-Freijo *et al.,* 1998; Chang *et al.,* 2000; Schmitz *et al*., 2001; Rao *et al*., 2006; Su *et al*., 2006; Yao *et al*., 2007; Bhattacharjee *et al*., 2010). Multidrug resistance in the Enterobacteriaceae has been linked with the carriage of integrons, in particular, aminoglycoside and anti-folate resistances are significantly associated with integron carriage in the Enterobacteriaceae (White *et al*., 2001).

Integrons are highly efficient recombination and expression systems, which are capable of capturing DNA sequences known as gene cassettes by site-specific recombination (Courvalin, 2008). Integrons can move in and out of the genome and in this way remodel it, but they are not able to move among bacteria. They have an integrase gene (intI), a nearby recombination site (attI), and a promoter which allows expression of genes resulting from the integration of several genes into the variable region (Mazel, 2006). Several different integron classes have been reported according to the homology of their integrase genes (Machado *et al.,* 2005). Class 1 integron, followed by class 2 integrons is most commonly found in nosocomial and community settings. Gene cassettes represent small mobile elements of less than 2kbp that are present in Gram-negative and Gram-positive bacteria (Reechia and Hall, 1995; Nandi *et al.,* 2004). They commonly consist of only a specific recombination site and a single gene which most often is an antimicrobial resistance gene. They don't have replication systems or transposition systems, but move by site- specific recombination. They are usually present at specific sites within an integron (Recchia and Hall, 1995; Hall and Collis, 1998).

Classes 1 and 2 integrons represent intact or defective transposons and commonly consist of a 5'- and a 3'-conserved region. Class 1 integrons have been reported to be extensively studied with a variable region bordered by 5' and 3' conserved regions. The 5' region is made up of the integrase (intI) gene whose product (IntI) catalyzes the site-specific insertion of the gene cassettes, a receptor site (attI) and the promoter which drives transcription of genes within the variable region i.e allow expression of the cassette- borne genes. The 3' region consists of an ethidium bromide resistance locus (qacEDI), a semi- functional derivative of the quaternary ammonium compounds resistance gene (qacE) (Paulsen *et al.,* 1996), a sulfonamide resistance gene (sulI) in class 1 integrons and transposition genes in class 2 integrons (Reechia and Hall, 1995; Carattoli, 2001) and an open reading frame containing a gene of unknown function (Hall *et al.,* 1996).

Integrons can carry several different gene cassettes and therefore play an important role in the dissemination of multiple antimicrobial resistance genes. Antibiotic resistance genes that integrons capture are located on gene cassettes (Waites, 2000). Integrons play an important role in the antimicrobial resistance of clinical *E. coli*

strains since they capture, integrate and express gene cassettes encoding proteins associated with antimicrobial resistance. Their role in the capture and spread of antibiotic resistance is particularly important when they are associated with conjugative plasmids and transposons (Fluit and Schmitz, 1999; Huaxi *et al.,* 2009).

Integrons of enterobacteria such as *E. coli* and the *Klebsiella* spp. are capable of encoding every class of beta-lactamase including AmpC-type cephalosporinases, metallo-beta lactamases and extended-spectrum beta-lactamases (Bush, 2010). Integrons allow bacteria to acquire additional resistance genes that can be disseminated to other bacteria when integrons are mobilized by plasmids. Integrons can't self transfer however when associated with conjugative plasmids the potential horizontal transfer ability of integrons is considered a risk with regard to the dissemination of multiresistance (Martinez- Freijo *et al*., 1998).

The impact of horizontally transmitted genetic determinants in the evolution of resistance is particularly evident when resistant genes are physically associated in clusters and transferred en bloc to the recipient cell (Grape *et al.,* 2005). Chromosomally encoded resistance is either inherited or arises from mutation or by integration of a transposable element. Multiple antibiotic resistance may also be attributed to chromosomally encoded systems in some bacteria especially the Enterobacteriaceae (Soge, 2007).

**Insertion Sequences**

Genetic elements such as the insertion sequences (IS) IS*Ecp1* have been found to be involved in the mobility of *bla* CTX-M genes (Poirel *et al.,* 2008). IS*Ecp1*-like elements belong to the IS*1380* family of insertion sequences and have been identified in association with genes belonging to the *bla*CTX-M-1, *bla*CTX-M-2, *bla*CTX-M-25 and *bla*CTX-M-9 ESBL gene clusters (Navon-Venezia *et al.,* 2008; Shen *et al.,* 2008).Extensive analysis has shown that IS*Ecp1* is responsible for the mobility of a transposition unit including itself and a *bla* CTX-M gene. This insertion sequence element is located upstream of a *bla*CTX-M gene (Canton and Coque, 2006). IS*Ecp1* element which contains a transposase gene *tnpA* enables various β-lactamase genes to be transposed to other DNA target sites. Moreover IS*Ecp1* element provides promoter activity for expression of a downstream CTX-M type β-lactamase gene.

### 2.5.3 Efflux mediated resistance

Efflux pumps play a major role in antibiotic resistance and also serve other functions in bacteria such as the uptake of essential nutrients and ions, excretion of metabolic end products and deleterious substances as well as the communication between cells and environment (Li and Nikaido, 2004). Different bacteria are able to pump out antibiotics from the cell; and in doing so the organism can prevent it from reaching the concentration necessary for effective action. This can cause resistance not only to the prescribed antibiotic but also to multiple other antibiotics (Waterer and Wunderink, 2001). Active efflux is an energy-dependent mechanism used by bacteria and transmembrane proteins, known as efflux pumps or active transporters mediate active efflux. Such proteins generally have broad substrate specificity, and only some of them confer resistance to antimicrobial agents (Guardabassi *et al.*, 2006). Resistance is determined by reduction in the concentration of drug in the cytoplasm, thus preventing or limiting access of the drug to its target.

Some pumps act on specific drugs (specific-drug-resistance [SDR] pumps), whereas others are active on multiple drugs (multiple-drug-resistance [MDR] pumps). SDR efflux pumps are the most important mechanism of resistance to tetracyclines, especially in Gram-negative bacteria. They confer resistance to Macrolides-Lincosamides-Streptogramin and phenicols. These pumps generally confer high-level resistance and are associated with mobile genetic elements (Butaye *et al.*, 2003). Most of the genes coding for specific efflux systems are inducibly expressed in the presence of specific antibiotics. The substrate of MDR pumps may include various antibacterial agents of medical importance. These efflux pumps generally confer low-level resistance and are frequently encoded by the chromosome. MDR efflux pumps are divided into two main groups depending on the source of energy used for active efflux: ATP-binding cassette (ABC) transporters and secondary drug transporters (Putman *et al.*, 2000). ABC transporters utilize ATP hydrolysis as a source of energy, have broad physiological functions and usually mediate the export of specific antimicrobial classes such as Macrolides-Lincosamides-Streptogramin. Secondary drug transporters utilize the transmembrane electrochemical gradient of proton or sodium ions to extrude drugs from the cell which accounts for most pumps mediating resistance to multiple antimicrobial agents (Guardabassi and Agerso, 2006).

### 2.5.4 Modes of acquisition of resistance genes

Plasmids, transposons and gene cassettes/integrons are spread vertically during the division of the host cell, but can also be transferred horizontally among bacteria of the same or different species or genera via transduction, conjugation/mobilization and transformation which could result in acquisition of new genetic material by antimicrobial-susceptible bacteria from resistant strains of bacteria (Fig 2.3) (Bennett, 1995; Schwarz and Noble, 1999; Todar, 2008). Resistance genes can be spread far by horizontal gene transfer mechanisms like conjugation, transformation and transduction. Such gene transfer mechanisms allow mobilization of specific DNA fragments from one region to another, from plasmids to plasmids, from chromosome to chromosome and between plasmids and chromosomes. Genetic sequences capable of coding for resistance can migrate from a plasmid to a chromosome and then back to the plasmid. These sequences are transpositional and are known as transposons. A number of transposons responsible for the transfer of resistance factor also have been isolated, characterized, and identified.

**Transduction**

Transduction describes a bacteriophage-mediated transfer process (Bennett, 1995; Todar, 2008). Bacteriophages inject their DNA into host cells, where it can direct the production of new phage particles. This includes expression of phage-borne genes, replication of the phage DNA, and packaging of this DNA into new phage particles, which are released from the bacterial cell (lytic cycle). However phage DNA may also integrate as a “prophage” into the host cell chromosome and remain there for long periods in an inactive state (lysogenic cycle). External factors, such as UV irradiation, can activate the prophage and initiate a lytic cycle. The spread of resistant genes via transduction is limited by the amount of DNA that can be packaged into a phage head and the requirement of specific receptors for phage attachment on the surface of the new host cell. Transduction is mainly observed among bacteria of the same species. Transducing phages have been detected in a wide variety of bacteria (Kokjohn, 1989).

## Conjugation

Conjugation describes the self-transfer of a conjugative plasmid or transposon from a donor cell to a recipient cell (Bennett, 1995; Schwarz *et al.,* 2006). Close contact between donor and recipient is one of the major requirements for efficient conjugation. Conjugation occurs when there is direct cell-cell contact between two bacteria (which need not be closely related) and transfer of small pieces of DNA called plasmids takes place. This is thought to be the main mechanism of horizontal gene transfer (Todar, 2008). In conjugation, the DNA passes from the donor cell to the recipient via a bridge formed during direct cell-to-cell contact.

This is the most sophisticated form of transmission because, for transfer to occur at all, the donor must have the necessary surface appendage (sex pilus) to form the bridge. This pilus is coded for by a resistance transfer factor on the plasmid and is called a conjugative sequence. General facets of conjugation make it an important process for gene transfer under natural conditions. Many types of bacteria can act as recipients, and resistance can pass freely from organisms normally saprophytic in the gut of animals to pathogenic bacteria (Boothe, 2012). In general, transfer occurs more frequently among Gram-negative bacteria and only rarely among Gram-positive organisms. Conjugation allows the passage of a number of distinct genes at one time. Thus, resistance to several antibiotics, all mediated by different biochemical means, may be acquired in a single step. The great efficiency of the conjugation process makes the probability of gene transfer to a super-infecting pathogen high.

Non-conjugative plasmids are not self-transmissible and their genetic transfer can only occur by mobilization. Small nonconjugative plasmids that co-reside in the same host cell may use the transfer apparatus provided by the conjugative element, as long as they have an origin of transfer (oriT region) and mobilization proteins are present to start a strand separation at the oriT in a process known as mobilization (Schwarz *et al.,* 2006). Conjugation and mobilization are believed to be of major importance for the spread of resistance genes under in vivo conditions (Schwarz *et al.,* 2006).

## Transformation

In transformation, naked DNA passes from a donor to a recipient through the growth medium. This process appears to be confined to a limited range of bacteria. It is a process where parts of DNA are taken up by bacteria from the external environment where DNA is normally present due to the death and lysis of another bacterium. Transformation is the major way of introducing plasmids into new host bacteria under *in-vitro* conditions. Under *in-vivo* conditions, transformation is considered to play only a limited role in transfer of resistance genes (Bennett, 1995). Only a few bacteria, such as *Streptococcus pneumoniae* or *Bacillus spp.* exhibit a natural ability to take up DNA from their environment (Schwarz and Chaslus-Dancla, 2001). Figure 2.3 shows mechanisms of horizontal gene transfer in bacteria.

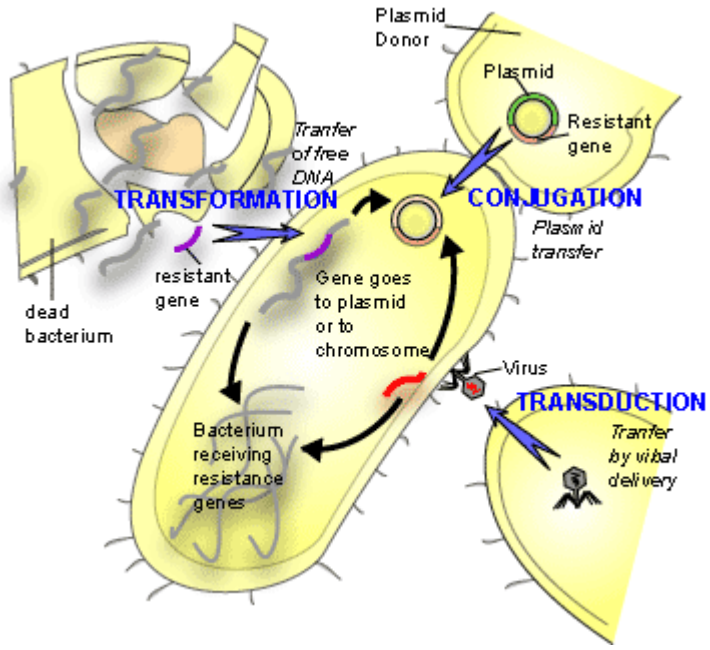



**Fig 2.3 Mechanisms of horizontal gene transfer in bacteria (Todar, 2008)**

## 2.6 Beta-Lactam Antibiotics and Extended Spectrum Beta Lactamases

### 2.6.1 Beta-Lactam Antibiotics

β-Lactam antibiotics are a broad class of antibiotics that include penicillin derivatives, cephalosporins, monobactams and carbapenems that is, any antibiotic agent that contains a β-lactam nucleus in its molecular structure (Holten and Onusko, 2000). Different groups within the family are distinguished by the structure of the ring and the side chain attached to the β-lactam nucleus. They all have a beta lactam ring (Fig 2.4a), which can be hydrolyzed by beta lactamases. The groups differ from each other by additional rings (Fig 2.4b-e) e.g. Thiazolidine ring for penicillin, Cephem nucleus for cephalosporin, none for monobactam, Double ring structure for carbapenem (Levinson, 2010). ESBLs are able to hydrolyze the oxyimino group (Fig 2.4f), thus conferring resistance to most β-lactam antibiotics. β-Lactam antibiotics act on bacteria by two mechanisms: at first, they incorporate in bacterial cell wall and inhibit the action of transpeptidase, responsible for completion of cell wall. Secondly, they attach to the penicillin binding proteins (PBPs) that normally suppress cell wall hydrolases, thus freeing these hydrolases, which in turn act to lyse the bacterial cell wall. To bypass these antimicrobial mechanisms of action, bacteria resist by producing beta lactam inactivating enzymes (beta-lactamases) (Samaha-Kfoury and Araj, 2003). Other mechanisms are decreased permeability or active transportation via efflux pumps (Chambers, 2005).

β-Lactams are the most widely used group of antibiotics because of their low toxicity and strong bactericidal activity (Matabane, 2005). β-Lactam antibiotics are indicated for the prophylaxis and treatment of bacterial infections caused by susceptible organisms. At first, β-lactam antibiotics were mainly active only against Gram-positive bacteria, yet the recent development of broad-spectrum β-lactam antibiotics active against various Gram-negative organisms has increased their usefulness. As a group, these drugs are active against many gram-positive, gram-negative and anaerobic organisms (Holten and Onusko, 2000).

### 2.6.2 Mode of action of β-lactam antibiotics

β-Lactam antibiotics are bactericidal, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins (PBPs) which are the primary targets for β-lactam antibiotics (Wright, 1999).

β-Lactam antibiotics are analogues of D-alanyl-D-alanine - the terminal amino acid residues on the precursor peptide subunits of the nascent peptidoglycan layer. The structural similarity between β-lactam antibiotics and D-alanyl-D-alanine facilitates their binding to the active site of penicillin-binding proteins (PBPs) hence the enzyme catalyzing the transpeptidation reaction is inhibited (Wright, 1999). This irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis (Wright, 1999).

The antibacterial effect of all β-lactam antibiotics depends on the capacity of the antibiotic to diffuse through the cell membrane of the bacteria cell, it’s affinity for target proteins of the bacterium and stability of the antibiotic against the bacterium degradation complex system (Pitout *et al.*, 1997).

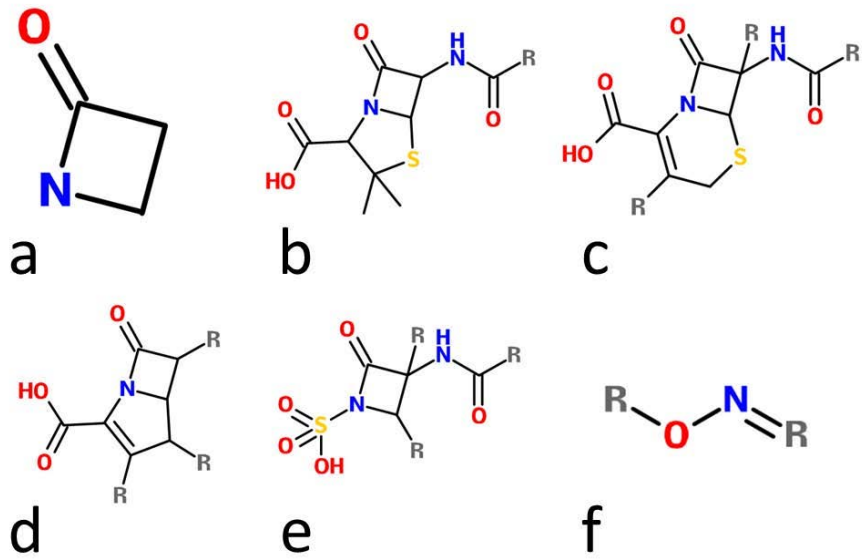



**Fig 2.4 Chemical structures of a) the beta-lactam ring; the core structure of b) penicillins, c) cephalosporins, d)carbapenems and e) monobactam; and f) an oxyimino group. (Levinson, 2010)**

### 2.6.3 Mechanisms of Bacterial resistance to β-Lactam antibiotics

Resistance to beta-lactam antibiotics has become a particular problem in recent decades, as strains of bacteria that produce extended-spectrum beta-lactamases have become more common (Paterson and Bonomo, 2005). These beta-lactamase enzymes make many, if not all, of the penicillins and cephalosporins ineffective as therapy. By definition, all β-lactam antibiotics have a β-lactam ring in their structure. The effectiveness of these antibiotics relies on their ability to reach the penicillin-binding protein (PBP) intact and their ability to bind to the PBP. Resistance to β-lactam antibiotics is mainly due to inactivation by β-lactamases (Livermore, 1995) and decreased ability to bind to penicillin-binding proteins (Georgeopapadakou, 1993) in both Gram-positive and Gram-negative bacteria, but may also be based on decreased uptake of β-lactams due to permeability barriers or increased efflux via multidrug transporter systems (Paulsen *et al*., 1996; Quintiliani *et al*., 1999). Inactivation via β-lactamases is most commonly seen, with a wide range of β-lactamases involved.

#### 2.6.3.1 Enzymatic inactivation

This is based on the cleavage of the amino bond in the β-lactam ring by β-lactamases (Livermore, 1995; Wiegand and Al-Agamy, 2003). The initial classification scheme based on the similarities in the amino acid sequences subdivided the β-lactamases into four classes, A to D (Ambler, 1980). The currently used classification of β-lactamases is done on the basis of their substrate spectra and their susceptibility to β-lactamase inhibitors such as clavulanic acid subdividing the β-lactamases into four classes (1 to 4) (Bush *et al*., 1995). Class 1 β-lactamases, such as AmpC, are widespread among Gram-negative bacteria. The AmpC genes are commonly located on the chromosome, but may also be found on plasmids. Some of these genes are expressed inducibly, others constitutively (Wiedemann *et al*., 1998). The substrate spectrum includes all β-lactams except carbapenems. These enzymes are not inhibited by Clavulanic acid.

Most enzymes in Class 2 β-lactamases are sensitive to inhibition by clavulanic acid. Subclass 2a includes enzymes such as *bla*Z from *Staphylococci* that can inactivate only penicillins. Subclass 2b includes broad spectrum β-lactamases that can hydrolyze penicillins and broad spectrum cephalosporins such as TEM-1, SHV-1 etc. Subclass 2be represent extended spectrum β-lactamases (ESBLs) that can also inactivate

oxyimino cephalosporins and monobactams and due to their wide spectrum of activity represent a serious cause of concern (Bradford, 2001). Extended spectrum β-lactamases include TEM-3 to TEM-20, SHV-2 etc. Most ESBLs currently known belong to the TEM, SHV, CTX-M or OXA families of β-lactamases. The enzymes of subclass 2br which are also broad spectrum β-lactamases includes enzymes such as TEM-30 to TEM-40 which however cannot be inhibited by clavulanic acid. Subclass 2c includes the inhibitor-sensitive carbenicillinases whereas the β-lactamases of subclass 2d exihibit relative insensitivity to inhibitors and can hydrolyze oxacillin and cloxacillin.

The β-lactamases of subclass 2e and 2f represent cephalosporinases or serine carbapenemases both of which are inhibited by clavulanic acid. While the β-lactamases of classes 1 and 2 have a serine residue in the catalytic center, the β-lactamases of class 3 hydrolyze β-lactams by divalent cations ($Zn^{2+}$) and are referred to as metallo-β-lactamases. These enzymes can inactivate all β-lactams except monobactams and are insensitive to clavulanic acid (Schwarz *et al.,* 2006). Class 4 enzymes comprise all so far nonsequenced β-lactamases that cannot be assigned to any of the other groups. The location of many of the β-lactamase genes on either plasmids, transposons, or gene cassettes favours their dissemination (Bonnet, 2004; Weldenhagen, 2004). Analysis of β-lactamases especially those of the TEM or SHV types, revealed the presence of mutations that either extended the substrate spectrum or affected the enzyme stability (Livermore, 1995; Petrosino *et al.,* 1998). Extended-spectrum beta-lactamase–producing *E. coli* is highly resistant to an array of antibiotics and infections by these strains are difficult to treat (Kumar *et al.,* 2006; Jalalpour, 2012). Susceptibility testing should guide treatment in all infections in which the organism can be isolated for culture

### 2.6.3.2 Altered PBPs

These are often associated with resistance due to decreased binding of β-lactam antibiotics (Georgeopapadakou, 1993). PBPs are transpeptidases that play an important role in cell wall synthesis and are present in most cell wall-containing bacteria, but they vary from species to species in number, size, amounts, and affinity for β-lactam antibiotics (Georgeopapadakou, 1993). PBPs with low affinity for β-lactams have been detected in microorganisms such as *Staphylococcus aureus,*

*Streptococcus* and *Enterococcus spp*. It is noteworthy that alterations in PBPs do not necessarily result in complete resistance to all β-lactams, but can also lead to elevated MICs of selected β-lactam antibiotics. Several different PBPs which show decreased binding of a more or less extended spectrum of β-lactams have been identified in Gram-negative bacteria (Georgeopapadakou, 1993).

### 2.6.3.3 Reduced β-lactam uptake

This is usually due to decreased outer membrane permeability and/or the lack of certain outer membrane proteins, which serve as entry for β-lactams to the bacterial cell. It has been described in *Enterobacteriaceae*, *Pseudomonas spp* and other bacteria (Hopkins and Towner, 1990; Charrel *et al.,* 1996). In *Escherichia coli* and *Klebsiella pneumoniae*, β-lactam resistance can be based on the decreased expression or the structural alteration of the porins OmpF (Simonet *et al.,* 2000) and OmpK38 (Martinez-Marzinez *et al*., 1996), by which β-lactams cross the outer membrane. In *Pseudomonas aeruginosa,* resistance to imipenem has been shown to be based on the loss of the porin OprD (Wolter *et al.,* 2004).

### 2.6.3.4 Multidrug transporters

Several multidrug transporters such as the MexAB-OprM and the MexCD-OprJ systems in *Pseudomonas aeruginosa* and the AcrAb-To1C system in *Salmonella* and *Escherichia coli* (Putman *et al*., 2000; Poole, 2002) are known to mediate the export of β-lactam antibiotics.

### 2.6 4 Classification of extended spectrum beta-lactamases

Since the late 1960's, many attempts have been made to categorize and classify β-lactamases as a result of the diversity of enzymatic characteristics of the many β-lacatamases discovered. The Ambler molecular classification and the Bush-Jacoby-Medeiros functional classification are the two most commonly used classification systems for β-lactamases (Ambler *et al.,* 1991; Bush *et al.,* 1995). Ambler scheme divides β-lactamases into four major classes (A to D). The basis of this classification scheme rests upon protein homology (amino acid similarity) and not phenotypic

characteristics. In the Ambler classification scheme, β-lactamases of classes A, C and D are serine β-lactamases. In contrast, the class B enzymes are metallo-β-lactamases.

With the exception of OXA-type enzymes (which are class D enzymes), the ESBLs are of molecular class A and may be defined as plasmid-mediated enzymes that hydrolyze oxyimino-cephalosporins and monobactams but not cephamycins and carbapenems (Bradford, 2001). They are inhibited in-vitro by clavulanate (Paterson and Bonomo, 2005). There are various types of ESBLs. Of these the most common are the SHV, TEM and CTX-M types (Rupp and Paul, 2003). Other clinically important types include VEB, PER, BEL-1, BES-1, SFO-1, TLA, and IBC (Jacoby *et al.*, 2005).

The Bush-Jacoby-Medeiros classification scheme groups β-lactamases according to functional similarities (substrate and inhibitor profile). There are four main groups and multiple subgroups in this system. This classification scheme is of much more immediate relevance to the physician or microbiologist in a diagnostic laboratory because it considers β-lactamase and β-lactam substrates that are clinically relevant. In this classification, ESBLs belong to group 2be or group 2d (OXA-type), the latter sharing most of the fundamental properties of group 2be enzymes though differing in being inhibitor resistant (Bush *et al.*, 1995). The 2be designation shows that these enzymes are derived from group 2b β-lactamases (for example, TEM-1, TEM-2 and SHV-1); the 'e' of 2be denotes that the β-lactamases have an extended spectrum. The ESBLs derived from TEM-1, TEM-2 or SHV-1 differs from their progenitors by as few as one amino acid. This results in a profound change in the enzymatic activity of the ESBLs, so that they can now hydrolyze the third-generation cephalosporins or aztreonam (hence the extension of spectrum compared to the parent enzymes).

Inhibition by β-lactamase inhibitors such as clavulanic acid and inability to hydrolyze cephamycins differentiates the ESBLs from the AmpC-type β-lactamases, which have third-generation cephalosporins as their substrates but which are not inhibited by clavulanic acid. Selection of stably de-repressed mutants which hyperproduce the AmpC-type β-lactamases has been associated with clinical failure when third-generation cephalosporins are used to treat serious infections with organisms producing these enzymes (Kaye *et al.*, 2001, Cosgrove *et al.*, 2002). In general, the

fourth-generation cephalosporin, cefepime, is clinically useful against organisms producing AmpC-type β-lactamases (Sanders *et al.,* 1996) but may be less useful in treating ESBL-producing organisms (Yaun *et al*., 1998). Additionally, the metalloenzymes produced by organisms such as *Stenotrophomonas maltophilia* can hydrolyze third-generation cephalosporins (and carbapenems) but are inhibited by ethylenediaminetetraacetic acid (EDTA),a heavy-metal chelator but not clavulanic acid (Wachino *et al.,* 2004). Table 2.3 and Figure 2.5 show the different classification of β-lactamases.

**Table 2.3 Classification of β-lactamases. Modified from (Bush *et al.*, 1995)**

| Bush-Jacoby-Medeiros Group | Molecular class (Ambler) | Preferred substrates | Representative enzymes | Resistance or susceptibility to β-lactamase inhibitors |
|---|---|---|---|---|
| 1 | C | Cephalosporins | AmpC | Resistant |
| 2a | A | Penicillins | Z | Susceptible |
| 2a | A | Penicillins | TEM, SHV | Susceptible |
| 2b | A | Penicillins, Cephalosporins | TEM, SHV | Susceptible |
| 2be | A | Penicillins, extended-spectrum cephalosporins,monobactam | TEM, SHV | Susceptible |
| 2br | A | Penicillins, extended-spectrum cephalosporins,monobactam | TEM, SHV | Resistant/ Susceptible |
| 2c | A | Penicillin, cloxacillin | TEM, SHV | Susceptible |
| 2d | A/D | Penicillin, cloxacillin | OXA | Resistant |
| 2e | A | Cephalosporins | Inducible cephalosporinases from *Proteus vulgaris* | Susceptible |
| 2f | A | Penicillins, Cephalosporins, Carbapenems | NMC-A from *Enterobacter cloacae* | Resistant |
| 3 | B | Most β-lactams including carbapenems | L1 from *Stenotrophomonas maltophilia* | Resistant |
| 4 | - | Penicillins | - | Resistant |

CTX-M was not classified in the above original scheme but fulfils the above criteria for group 2be enzymes.

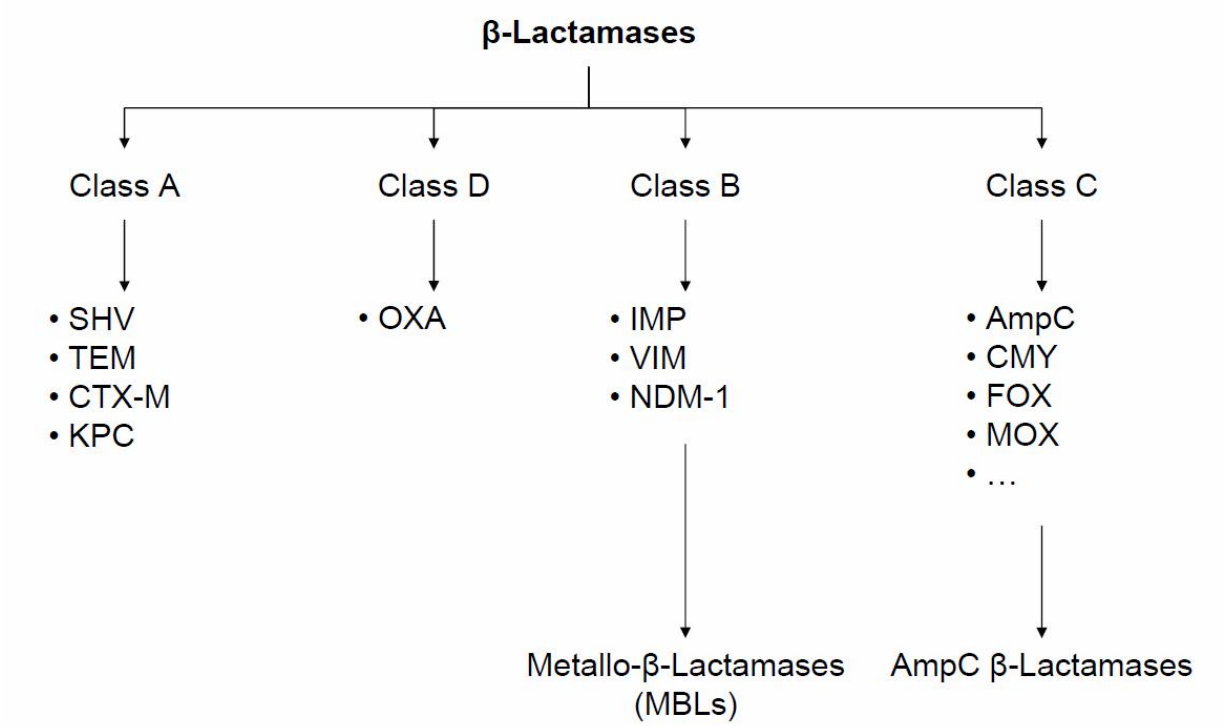



**Fig 2.5 Molecular classification of β-lactamases. (Yasmin, 2012)**

### 2.6.5 Extended Spectrum β-lactamases and their Clinical significance

Beta- lactamases are enzymes that catalyze the hydrolysis of beta-lactam. The genes encoding these enzymes were originally located on the bacterial chromosome (Hanson *et al*.1999; Yusha'u *et al*. 2010). The first β-lactamase was identified in *Escherichia coli* prior to the release of penicillin for use in medical practice (Abraham and Chain1940). Many Genera of Gram-negative bacteria possess a naturally occurring, chromosomally mediated β-lactamase. These enzymes are thought to have evolved from penicillin-binding proteins, with which they show some sequence homology. Presently there are more than 500 different beta-lactamases that have been found in nature (CLSI, 2010). These versatile enzymes are present in both Gram-positive and Gram-negative bacteria (Holten and Onusko, 2000). Beta-Lactamase producing-Gram-positive bacteria release the enzyme into the surrounding medium but Gram-negative bacteria release the enzyme into the periplasmic space (Samaha Kfoury and Araj, 2003). Furthermore, these enzymes are inducible and constitutively expressed in low quantities. In 1965, the first report of a plasmid-encoded beta-lactamase in a Gram-negative bacterium appeared from Greece (Datta and Kontomichalou, 1965).

Over the last 20 years, many new β-lactam antibiotics have been developed that were specifically designed to be resistant to the hydrolytic action of β-lactamases. However, with each new class that has been used to treat patients, new β-lactamases emerged that caused resistance to that class of drug. β-lactamase producing bacteria are increasing in number and causing more severe infections (Shobha *et al.* 2007; Andrews 2009). Presumably, the selective pressure of the use and overuse of new antibiotics in the treatment of patients with bacterial infections has selected for new variants of β-lactamase. One of these new classes was the oxyimino-cephalosporins, which became widely used for the treatment of serious infections due to gram-negative bacteria in the 1980s.

Not surprisingly, resistance to these expanded-spectrum β-lactam antibiotics due to β-lactamases emerged quickly. The first of these enzymes capable of hydrolyzing the newer β-lactams, SHV-2, was found in a single strain of *Klebsiella ozaenae* isolated in Germany (Kliebe *et al.*, 1985). Because of their increased spectrum of activity,

especially against the oxyimino-cephalosporins, these enzymes were called extended-spectrum β-lactamases (ESBLs). The most common cause of resistance to expanded-spectrum cephalosporins in *Escherichia coli* is the production of extended-spectrum β-lactamases (ESBLs) (Paterson, 2006). Currently, more than 600 ESBL variants are known. There are now >150 TEM-type β-lactamases and >90 SHV-type β-lactamases (http://www.lahey.org/studies/webt.htm). Over 100 CTX-M enzymes reported have been grouped into five main subgroups (Bonnet, 2004). These β-lactamases have been found worldwide in many different Genera of *Enterobacteriaceae* and *P. aeruginosa*. The distribution of ESBL genotypes has been found to vary according to the antimicrobial agents used in each hospital or local community (Bassetti *et al.,* 2006; Ko *et al.,* 2007).

In recent years a new family of plasmid-mediated ESBLs, called CTX-M, that preferentially hydrolyze cefotaxime has arisen. They have mainly been found in strains of *Salmonella enterica* serovar Typhimurium and *E. coli*, but have also been described in other species of *Enterobacteriaceae* (Bradford, 2001). There has been an increase in the number of organisms reported in literature that produce ESBLs including CTX-M β-lactamases both in nosocomial and community settings globally (Bonnet, 2004). Since the end of the 1990s, the CTX-M β- lactamases, has spread among continents, becoming the most prevalent in the world (Canton and Coque, 2006).

Extended spectrum beta-lactamases contain a number of mutations that allow them to hydrolyze expanded-spectrum β-lactam antibiotics. Different ESBL types vary in their ability to hydrolyze cephalosporins and aztreonam. The CTX-M β-lactamases have been reported to hydrolyze cefotaxime and ceftriaxone better than they hydrolyze ceftazidime (Bonnet, 2004; Paterson and Bonomo, 2005; Perez *et al*., 2007), while ceftazidime is usually the best substrate for SHV ESBLs (Babini *et al.,* 2000a; Bonnet, 2004). While TEM- and SHV-type ESBLs retain their ability to hydrolyze penicillins, they are not catalytically as efficient as the parent enzymes (Bush and Singer, 1989). In addition, the expansion of the active site that allows the increased activity against expanded-spectrum cephalosporins may also result in the increased susceptibility of ESBLs to β-lactamase inhibitors (Jacoby and Medeiros, 1991). ESBLs are not active against cephamycins, and most strains expressing ESBLs are

susceptible to cefoxitin and cefotetan. However, it has been reported that ESBL-producing strains can become resistant to cephamycins due to the loss of an outer membrane porin protein (Vatopoulos *et al*., 1990; Martinez-Marzinez *et al*., 1996). As a matter of growing concern, resistance caused by ESBLs is often associated with resistance to other classes of antibiotics like fluoroquinolones, aminoglycosides and trimethoprim-sulfmethoxazole thereby contributing to the selection and persistence of multidrug-resistant ESBL strains and plasmids in both clinical and community settings (Canton and Coque, 2006; Canton *et al.,* 2008; Gniadkowski, 2001; Morosini *et al*., 2006). The OXA-type enzymes are another growing family of ESBLs. The OXA-type β-lactamase confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid (Bush *et al.,* 1995).

While most ESBLs have been found in *E. coli, K. pneumoniae,* and other *Enterobacteriaceae,* the OXA-type ESBLs have been found mainly in *P. aeruginosa* (Bradford, 2001). While the majority of ESBLs are derived from TEM or SHV β-lactamases and others can be categorized with one of the newer families of ESBLs, a few ESBLs have been reported that are not closely related to any of the established families of β-lactamases (Bradford, 2001). The PER-1, PER-2, VEB-1, CME-1, and TLA-1 β-lactamases are related but show only 40 to 50% homology (Bradford, 2001). These enzymes all confer resistance to oxyimino-cephalosporins, especially ceftazidime, and aztreonam. They also show some homology to the chromosomal cephalosporinases in *Bacteroides* spp. and may have originated from this Genus (Rossolini *et al.,* 1999). GES-1 is another uncommon ESBL enzyme that is not closely related to any other plasmid-mediated β-lactamase but does show 36% homology to a carbenicillinase from *Proteus mirabilis* (Poirel *et al.,* 2000).

A distinction must be made between other β-lactamases like AmpC ß-Lactamases and ESBL enzymes. The AmpC ß-Lactamases are encoded by genes located on chromosomes, are often inducible, and are commonly found in *Enterobacter* sp, *Citrobacter freundii, Morganella morganii, Serratia marcescens,* and *Pseudomonas aeruginosa (*Bush *et al.,* 1995). Since genes encoding these enzymes are located on chromosomes, they are not easily transferable to other bacterial species. Resistance patterns associated with AmpC ß-Lactamase enzymes are strikingly similar to those

of ESBLs; however, AmpC ß-Lactamases are only weakly inhibited by β-Lactamase inhibitors and usually confer resistance to cephamycins (Nathisuwan *et al.*, 2001). In contrast, ESBLs are generally well inhibited by β-Lactamase inhibitors and usually retain sensitivity to the cephamycins (Jacoby and Han, 1996). Also, the carbapenemases have a broader range activity but are not as widely distributed as the CTX-M-type ESBLs (Walsh *et al.*, 2005). The carbapenemases although are of great concern because they have fast eroded the effectiveness of carbapnems which have for many years been the drugs of choice for treating infections caused by multi-drug resistant organisms. The ESBLs are encoded by genes located on plasmids, resulting in easy transfer to other bacterial species (Nathisuwan *et al*., 2001). A brief summary of different β-lactamases produced by Gram-negative bacteria and their characteristics is described below (Table 2.4) (Jacoby *et al.*, 2005).

**Table 2.4 Selected β-Lactamases of Gram-negative bacteria (Jacoby *et al.*, 2005)**

| B-Lactamase | Examples | Substrates | Inhibition by Clavulanic acid | Molecular class |
|---|---|---|---|---|
| Broad-spectrum | TEM-1, TEM-2, SHV-1 | Benzylpenicillin (penicillin G), aminopenicillins (amoxicillin and ampicillin), carboxypenicillins (carbenicillin and ticarcillin), ureidopenicillin (piperacillin), narrow-spectrum cephalosporins (cefazolin, cephalothin, cefamandole, cefuroxime, and others) | +++ | A |
| | OXA family | Substrates of the broad-spectrum group plus cloxacillin, methicillin, and oxacillin | + | D |
| Expanded-spectrum | TEM family and SHV family | Substrates of the broad-spectrum group plus oxyimino-cephalosporins (cefotaxime, cefpodoxime, ceftazidime, and ceftriaxone) and monobactam (aztreonam) | ++++ | A |
| | Others (BES-1, GES/IBC family, PER-1, PER-2, SFO-1, TLA-1, VEB-1, and VEB-2) | Same as for TEM family and SHV family | ++++ | A |
| | CTX-M family | Substrates of the expanded-spectrum group plus, for some enzymes, cefepime | ++++ | A |
| | OXA family | Same as for CTX-M family | + | D |
| AmpC | ACC-1, ACT-1, CFE-1, CMY family, DHA-1, DHA-2, FOX family, LAT family, MIR-1, MOX-1, and MOX-2 | Substrates of expanded-spectrum group plus cephamycins (cefotetan, cefoxitin, and others) | - | C |
| Carbapenemase | IMP family, VIM family, GIM-1, and SPM-1 | Substrates of the expanded-spectrum group plus cephamycins and carbapenems (ertapenem, imipenem, and meropenem | - | B |
| | KPC-1, KPC-2, and KPC-3 | Same as for IMP family, VIM family, GIM-1, and SPM-1 | +++ | A |
| | OXA-23, OXA-24, OXA25, OXA-26, OXA-27, OXA-40, and OXA-48 | Same as for IMP family, VIM family, GIM-1, and SPM-1 | + | D |

+ denotes relative sensitivity to inhibition

The presence of ESBLs in some *K. pneumoniae* and *E. coli* strains poses an important challenge in clinical practice, since these organisms are common causes of serious infections. Extended-spectrum beta-lactamases (ESBLs) are an increasingly important cause of multi-drug resistant infections throughout the world (Livermore *et al.,* 2007).

Bacteria carrying such enzymes have long been recognized as a cause of healthcare-associated infection (Paterson and Bonomo, 2005). They are associated with severe infections such as bacteraemia, intra-abdominal infection, respiratory tract infections and urinary tract infections (particularly in the community setting) (Pitout *et al.,* 2008). However, of concern, the incidence of such organisms also appears to be increasing in the community, typically as a cause of urinary tract infection (Pitout *et al.,* 2005; Livermore *et al*., 2007). Infections due to ESBL-producing organisms can pose a major threat to life; are often difficult and expensive to treat; and can delay discharge from hospital (Kola, 2007; Melzer and Peterson, 2007). ESBL genes are often located within mobile genetic elements such as plasmids, transposons and integrons which also contain other resistance genes, conferring resistance to antimicrobials that could be extensively used among animals and humans (eg trimethoprim, sulfamethoxazole, streptomycin) and this could play an important role in the co-selection of these ESBL genes. Plasmids responsible for ESBL production may also carry genes encoding resistance to other drug classes, for example, aminoglycosides, trimethoprim, and fluoroquinolones (Turner, 2005; Girlich *et al.,* 2007). This explains why ESBL bacteria are resistant to other antibiotics such as cotrimoxazole, aminoglycosides and fluoroquinolones (Paterson and Bonomo, 2005; Pitout *et al.,* 2005), which implies a prognostic impact and therapeutic challenge becomes greater.

Previous reports have demonstrated that ESBL genes can spread not only by epidemic strains but also by plasmid dissemination among unrelated strains (Ben-Hamouda *et al.,* 2004). Therefore, antibacterial drug options in the treatment of patients with ESBL-producing organisms may be very limited. High mortality rates have been reported when patients infected by ESBL-producing organisms were treated with cephalosporins (Colodner, 2005). Of all available β-Lactams, carbapenems are the most effective and reliable antimicrobial agents against ESBL isolates. Carbapenems are highly resistant to the hydrolytic activity of all ESBL enzymes, due to the trans-6-

hydroxyethyl group although they are very expensive (Livermore, 1998). Although carbapenems are the most reliable agents against ESBL infection, an increase in carbapenem use also poses a significant problem as it is very disturbing that there are now reports of plasmid-mediated carbapenemases (Walsh *et al*., 2005; Paterson, 2006). Polymixin B and colistin are now being used to treat infections caused by carbapnemases producing organisms (Li *et al.,* 2005).

The proportion of ESBL-producing isolates resistant to fluoroquinolones has increased over time, initially in *K. pneumoniae* and later also in *E. coli* (Lautenbach *et al*., 2001; Canton *et al.,* 2008; Garcia-Fernandez *et al.,* 2008). This increase has apparently occurred in parallel to the increase in plasmid-mediated resistance mechanisms including *Qnr* proteins (*qnrA*, *qnrB* or *qnrS*), acetylases that can affect the action of certain fluroquinolones (*aac(6')-Ib-cr*) or systems pumping fluoroquinolones out of the bacteria (qepA) (Nordmann and Poirel, 2005; Cattoir *et al.,* 2008). These determinants increase the minimum inhibitory concentrations (MICs) of quinolones by 8- to 32-fold, preventing the inhibition of DNA gyrase (Tran *et al.,* 2002).

ESBL genes and PMQRs have been described to be co-exist either on the same plasmid or on different plasmids within the same isolate (Dionisi *et al.,* 2009; Woodford *et al*., 2009; Richter *et al.,* 2010; Dolejska *et al*., 2011; Kirchner *et al*., 2011; Müller *et al*., 2011; Yao *et al*., 2011).

### 2.6.6 Types of ESBLs

In the early years the most common beta lactamases were the *bla*Z, TEM and SHV varieties (Florijn *et al.* 2002; Shobha *et al.* 2007; Pitout, 2010). TEM-2 and SHV-2 ESBL are derived from parental TEM-1 and SHV-1 by point mutation. All CTX-M enzymes are ESBL (Al-Agamy *et al.,* 2009). Now, CTX-M enzymes are being discovered throughout the world and becoming the most prevalent beta lactamase (Xu *et al.,* 2005).

### 2.6.6.1 TEM and SHV

The first plasmid-mediated β-lactamase in Gram-negative bacteria TEM 1 was described in the early 1960s (Datta *et al*., 1965; Bradford, 2001). It was designated as TEM as it was originally found in a single strain of *E. coli* isolated from a blood culture of a patient named Temoniera in Greece (Medeiros, 1984). Being plasmid and transposon mediated, TEM-1 enzymes spread worldwide and are now found in many different species of the family Enterobacteriaceae, *Pseudomonas aeruginosa, Hemophilus influenzae and Neissiria gonorrhea* (Fonze *et al.,* 1995). TEM-type ESBLs have also been found in non-Enterobacteriaceae Gram-negative bacteria.The TEM-42 β-lactamase was found in a strain of *P. aeruginosa* (Bradford, 2001). Up to90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. SHV-1 (for sulphydryl variable type 1) (Turner, 2005) is another β-lactamase commonly found in *Klebsiella pneumoniae* and *Escherichia coli* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in *Klebsiella pneumoniae*. The SHV-1 β-lactamase is chromosomally encoded in the majority of isolates of *K. pneumoniae* but is usually plasmid mediated in *E. coli*.

Over the years, the use of newer β-lactam antibiotics has enabled selection of new variants of β-lactamases. In the early 1980s, the third-generation, or oxy-imino, cephalosporins were introduced into clinical practice in response to the increasing prevalence and spread of the β-lactamases. Resistance to these extended-spectrum cephalosporins emerged quickly, and the first report of an SHV-2 enzyme which was capable of hydrolyzing these antibiotics was published as early as 1983 in Germany. More than 60 SHV varieties are known. SHV-5 and SHV-12 are the most common (Farkosh, 2007). SHV variants are important worldwide (Rahman *et al*., 2004). The majorityof SHV ESBLs are characterized by the substitution of a serine for glycine at position 238. These enzymes were called extended-spectrum β-lactamases because of their increased spectrum of activity, especially against the oxyimino cephalosporins.The largest groups are the mutants of TEM and SHV β-lactamases, with over 150 members. Based upon different combinations of changes, currently 195 TEM-type enzymes have been described (Yasmin, 2012). The first variant described

is TEM-2. It differed from TEM-1 through the substituition of a lysine for a glutamine at position 39 (Rupp and Paul, 2003).

TEM-3, the first TEM-type β-lactamases to exhibit the ESBL phenotype was first reported in 1989 (Bradford, 2001). The mutations which affect a small number of critical amino acids enlarge the enzyme's active site and enable it to deflect the oxyimino substitutes, which normally shield the β-lactam ring. As a result, whereas the classical TEM and SHV enzymes are unable to significantly hydrolyze the oxyimino cephalosporins, the mutants can do so, conferring resistance to their host strains (Livermore and Woodford, 2006). TEM and SHV are transferred by both plasmid and chromosome (Sharma *et al.,* 2010).

### 2.6.6.2 CTX-M-type

A CTX-M-type ESBL, related to the chromosomal β-lactamase of *Kluyvera ascorbata* (Humeniuk *et al.,* 2002), was reported in 1989 and characterized by a better hydrolysation of cefuroxime, cefotaxime and cefepime than that of ceftazidime (Bernard *et al.,* 1992). CTX-M β-lacatamases are the second largest group of ESBLs. CTX-M β-lactamases (i.e. 'active on Cefotaxime, first isolated in Munich) were first reported from Japan in 1986 (the enzyme was initially named TOHO-1 and was later changed to CTX-M) (Matsumoto *et al.,* 1988). Currently, more than 100 variants have been sequenced. During the general dissemination and occasional nosocomial outbreak in the 1990s, most of CTX-M-2-producing Enterobacteriaceae, were reported from South America (especially Argentina) (Peirano and Pitout, 2010). However, since 2000, *E. coli* producing CTX-M β-lactamases have emerged worldwide as an important cause of community-onset urinary tract infections (UTIs) (Radice *et al.,* 2001). These enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates (e.g., ceftazidime, ceftriaxone, or cefepime) (Pitout and Laupland, 2008). Rather than arising by mutation, they represent examples of plasmid acquisition of beta-lactamase genes normally found on the chromosome of *Kluyvera* species, a group of rarely pathogenic commensal organisms (Coque *et al.,* 2008). These enzymes are not very closely related to TEM or SHV beta-lactamases in that they show only approximately 40% identity with these two commonly isolated beta-lactamases. The change at position 102 mainly enhances resistance to Ceftazidime, while the change at position 236 predominantly augments

resistance to Cefotaxime, with a slight effect for Ceftazidime (Rahman *et al.,* 2004). It has been suggested that the serine residue at position 237 which is present in all of the CTX-M enzymes, plays an important role in the extended spectrum activity of the CTX-M-type β-lactamases (Tzouvelekis *et al.,* 2000).

CTX-M enzymes are currently devided into 5 clusters on the basis of amino acid sequence: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Al-Agamy *et al.,* 2009; Smet *et al.,* 2010) and each cluster is named after the enzyme first discovered for each lineage (Pagani *et al.,* 2003). Despite their characteristics, a few are more active on ceftazidime than cefotaxime. The flanking sequences of the β-lactamases can be very different (Peirano and Pitout, 2010). Another unique feature of these enzymes is that they are inhibited better by the β-lactamase inhibitor tazobactam than by sulbactam or clavulanate (Bradford *et al.,* 1998 and Bradford, 2001)

The initial observation of infections caused by bacteria harboring ESBLs in hospitals would suggest that CTX-M arose in the nosocomial setting and spread to the community (Perez *et al.,* 2007).The epidemiology of organisms producing CTX-M enzymes is very different from those that produce TEM-derived and SHV-derived ESBLs (Pitout and Laupland, 2008). Epidemiological reports demonstrate that some enzymes are more frequently reported than others, that predominant enzyme type varies with country and that diverse CTX-M types often exist within a single country (Ensor *et al.,* 2006). CTX-M-15-producing *E. coli* are emerging worldwide, especially since 2003, as an important pathogen causing community-onset and hospital-acquired infections (Peirano and Pitout, 2010). CTX-M-15 was first identified in an isolate from India in 1999 (Karim *et al.,* 2001) and then became prevalent worldwide (Boyd *et al.,* 2004; Livermore *et al.,* 2007; Carattoli *et al.,* 2008). Reports from India indicate that *E. coli* producing CTX-M-15 is very common in the community as well as hospital settings (Ensor *et al.,* 2006). India represents a significant reservoir and source of *E. coli* producing CTX-M-15 β-lactamases (Peirano and Pitout, 2010). CTX-M-15 has been reported from most countries in Europe, Asia, Africa, North America, South America and Australia (Peirano and Pitout, 2010). Group 9 (CTX-M-9 and 14) enzyme is dominant in Spain and Group 1 enzymes (particularly CTX-M 3 and CTX-M-15) is everywhere (Livermore, 2007).

### 2.6.6.3 OXA β-lacatamases

The OXA-type β-lactamases which are also plasmid-mediated β-lactamases are so named because of their oxacillin-hydrolyzing abilities (oxacillin and related anti-staphylococcal penicillins). They predominantly occur in *Pseudomonas aeruginosa* (Weldhagen, 2004) but have been detected in many other gram-negative bacteria (Livermore, 1995) The OXA-type ESBLs were originally discovered in *Pseudomonas aeruginosa* isolates from Turkey. The evolution of ESBL OXA-type β-lactamases from parent enzymes with narrower spectra has many parallels with the evolution of SHV- and TEM-type ESBLs. The OXA-type beta-lactamases confer resistance to ampicillin and cephalothin and are characterised by their high hydrolytic activity against oxacillin and cloxacillin.

They are poorly inhibited by clavulanic acid. OXA-10 hydrolyzes (weakly) cefotaxime, ceftriaxone and aztreonam, giving most organisms reduced susceptibility to these antibiotics; but OXA-11, -14, -16, -17, -19, -15, -18, -28, -31, -32, -35 and -45 confer frank resistance to cefotaxime and sometimes ceftazidime and aztreonam (Daniel *et al.,* 1998; Toleman *et al.,* 2003) The simultaneous production of a carbapenem-hydrolyzing metalloenzyme and an aztreonam-hydrolyzing OXA enzyme can readily lead to resistance to all β-lactam antibiotics (Toleman *et al.,* 2003).

A variety of other β-lactamases (PER, VEB, GES, BES, TLA, SFO, IBC groups) which are plasmid-mediated or integron-associated class A enzymes have been discovered (Bauernfeind *et al.,* 1996; Matsumoto and Inowe, 1999; Poirel *et al.,* 1999*;* Bonnet *et al.,* 2000; Giakkoupi *et al.,* 2000; Poirel *et al.,* 2000*;* Silva *et al.,* 2000 ) They are not simple point-mutant derivatives of any known β-lactamases and have been found in a wide range of geographic locations. Novel chromosomally encoded ESBLs have also been described (Bellais *et al.,* 2001). Chromosomal ESBL/ESBL-like genes are commonly found in clinically encountered Enterobacteriaceae such as *K. oxytoca* (K1/K /OXY), *Proteus vulgaris* (CumA) and *Proteus penneri* (HUGA). They are also found in a variety of more uncommon species, occasionally involved in infections of particularly the immunocompromised (e.g. *Citrobacter* spp. (CdiA, CKO, and SED-1), *Kluyvera* spp. (KLUA, KLUC, and

KLUG), *Serratia fonticola* (FONA) or *Rahnella aquatilis* (RAHN-1). (Bradford, 2001; Bonnet, 2004; Naas *et al.*, 2008)

### 2.6.7 Prevalence of ESBLs

So far, the presence of ESBLs among commensal Enterobacteriaceae has been found to range from 0.2 to 40.7%. Some ESBLs seem to be confined to specific individual countries, such as TEM-106 in Belgium, CTX-M-8 and SHV-5 in Tunisia and several CTX-M enzymes in China (Duan *et al.*, 2006; Jouini *et al.*, 2007; Smet *et al.*, 2008; Tian *et al.*, 2009). Other ESBLs have been found to be more widely distributed. With the emergence of the CTX-Ms, there has been a marked shift in the epidemiology of ESBLs (Bonnet, 2004; Canton and Coque, 2006). Currently, the CTX-Ms are the most prevalent ESBL enzymes, and *E. coli* is the main ESBL producer.The predominance of CTX-Ms has not only been observed in hospitals but also in the community, from nursing homes and long-term facilities. CTX-M-15 is the most commonly reported ESBL-enzyme in Europe and globally (Livermore, 2007; Canton *et al.*, 2008). It was earlier descibed in the UK (Woodford *et al.*, 2004; Livermore and Hawkey, 2005). CTX-M-15 is derived from CTX-M-3 by a single amino acid substitution at position 240 (Asp →Gly). This substitution confers an increased catalytic activity against ceftazidime (Poirel *et al.*, 2002). The prevalence of ESBLs in Europe is higher than in the USA but lower than in Asia and South America (Girlich *et al.*, 2004).

A study performed in Turkey showed a prevalence of 21% ESBL producers among *E. coli* causing community acquired urinary tract infection (UTI) during 2004 and 2005 (Coque *et al.*, 2008). In Norway, a prospective survey of clinical *E. coli* isolates with reduced susceptibility to oxyimino-cephalosporins demonstrated the dominance of CTX-M-15 (46%) and CTX-M-9-like (30%) enzymes among ESBL-positive *E. coli* and of SHV-5 (47.4%) and SHV-2 (21.0%) among ESBL-positive *K. pneumoniae* isolates (Coque *et al.*, 2008). In Italy, the prevalence of ESBL producers among clinical isolates has also increased over the past ten years. The most prevalent ESBL-positive species are *E. coli* among hospitalised patients and *Proteus mirabilis* among outpatients (Luzzaro *et al.*, 2006).

The prevalence of ESBLs is over 10% in Hungary, Poland, Romania, Russia and Turkey. *K. pneumoniae* is the most frequent ESBL-producing species in Hungary and Russia, and an increase in the percentage of ESBL producers among *K. pneumoniae* isolates has been reported from Poland, Turkey, Bulgaria, and Romania (Edelstein *et al*., 2003). Asia probably has a long history of the occurrence of extended-spectrum β-lactamase (ESBL)-producing bacteria (Kim *et al*., 2007). There were, a number of sporadic reports of ESBLs, notably of the SHV-2 type, from China in 1988 (Rupp *et al*., 2003), and the TOHO-1 ESBL produced by *Escherichia coli* from Japan in 1993 (Ishii *et al.,* 1995). ESBLs mediated resistance in *Klebsiella spp.* ranged from 20-40% throughout Southeast Asia, China and Japan (Rupp and Paul, 2003). CTX-M-15 has probably been present in India for some considerable time, and is present in both *E. coli and Klebsiella spp*. at a high frequency and it is assumed that spreads of CTX-M-15 from India to other countries is more likely. The pattern of ESBL genotypes in Japan is quite different from that seen in surrounding countries, although universally successful types e.g CTX-M-14, has recently become more common (Hirakata *et al*., 2005). Both India and Pakistan have reported high rates of ESBLs since the 1990s (Mathai *et al*., 2002; Grover *et al*., 2006). With the populations of India and China, these two countries surely represent the largest reservoirs of CTX-M ESBL genes in the world. Increasing travel and trade will contribute to the worldwide spread of locally evolved CTX-M genotypes (Hawkey, 2008).

ESBL producing organisms have also been isolated in Africa. South Africa and Kenya have reported several outbreaks of infections due to ESBL producing *Klebsiella spp* (Shipton *et al.,* 2001). Studies have reported that 14.7% of all *E. coli* and 20.8% of all *K. pneumoniae* strains in Nigeria are ESBL producers (Aibinu *et al*., 2003). The success of the CTX-Ms over the classical ESBL-enzymes SHVs and TEMs is linked to the way by which CTX-M enzymes are spread. Through mobile genetic elements, resistance genes disseminate within the same species and also among bacteria of different species (Canton and Coque, 2006; Courvalin, 2008). Mobile elements involved in the dissemination of $bla_{\text{CTX-M}}$ genes have been described in recent reviews (Canton and Coque, 2006; Canton *et al.,* 2008).

Horizontal dissemination of genes encoding ESBLs occurs by conjugative plasmids and transposons. The motility and multidrug-resistance of the CTX-Ms is sometimes associated with integrons (Bonnet, 2004; Machado *et al.,* 2005). The gene cassettes, which harbour genes encoding CTX-Ms, usually carry one or several other genes encoding antibiotic resistance. One of the major factors involved in the current prevalence of ESBL-producing Enterobacteriaceae is clonal spread. As a consequence of horizontal gene transfer by transposon-plasmid vectors, most ESBL-producing *E.coli* were clonally unrelated until a few years ago (Diaz *et al.,* 2010). Recently, CTX-M-15 was identified in an international clone of *E. coli,* which has been detected in both in-patients and out-patients (Coque *et al.,* 2008; Lau *et al.,* 2008). This clone belongs to the phylogenetic group B2, MLST-type 131 and exhibits a specific lipopolysaccharide-type (O25b). This O25b-ST131 clone has not only a considerable ability to disseminate, it is also equipped with a high virulence potential, causing significant morbidity and mortality. This is partly explained by its capacity to produce biofilm, which might contribute to their long-term persistence in various environments and to their exhibited resistance to antimicrobial agents and disinfectants (Clermont *et al.,* 2008). It is also possible that the production of biofilm leads to an increased resistance to host immune defences. Dissemination in conjunction with this clone results in resistance to many low-cost and easily available antimicrobials commonly used to treat *E. coli* infections.

Majority of reports on the clonal spread of ST131 *E.coli* producing CTX-M β-lactamases emanate from the developed world. Little is known about the distribution of ST131 in many parts of the developing world, areas postulated as reservoirs of the pathogen (Pitout, 2010). Multilocus sequence typing (MLST) is well suited to characterizing the genetic relationships between the organisms of bacterial species including *E. coli* (Tartof *et al.,* 2005). The successful dispersion of CTX-M-15 has been associated with specific clones, such as ST131 and ST405, which belong to virulent phylogenetic groups B2 and D, respectively (Coque *et al.,* 2008; Rodgers *et al.,* 2011).

Antibiotic overuse in humans and animals, hospital cross infection, the food chain, trade and human migration seem to have contributed to the recent dissemination of ESBLs outside hospitals, although the role of these factors is variable and linked to

particular epidemiological situations. The epidemiology of ESBL genes is changing rapidly and shows marked geographic differences in distribution of genotypes of $bla_{CTX-M}$ β-lactamases (Hawkey and Jones, 2009). The epidemiology of ESBL-producing enterbacteriaceae is a challenge to clinical microbiology laboratories as it can be characterized by a wide diversity of clones and mobile genetic elements.

Infections caused by enterobacteria producing ESBLs are associated with increased morbidity, mortality, and health care-associated costs (Du *et al.,* 2002; Lautenbach *et al.,* 2001). Extended-spectrum beta-lactamase producing strains can increase the frequency and severity of infections with such organisms especially as a result of associated resistance to other antibiotics, limit treatment options and raise healthcare costs. They also cause difficulties in antimicrobial susceptibility testing (Pitout and Laupland, 2008). The emergence of ESBL-producers along with multiple resistant isolates poses a serious problem in the hospital setting. Prevalence of ESBLs vary from country to country, hospital to hospital even very closely related regions.

### 2.6.8 Risk factors associated with ESBL-producing organisms

The widespread uses of antibiotics coupled with the transmissibility of resistance determinants mediated by plasmids, transposons, and gene cassettes in integrons are factors that contribute to the increase in antibiotic resistance in bacterial pathogens (Kang *et al.,* 2005). The use of antibiotics, particularly oxyimino-cephalosporins, and hospital transfer are well-defined risk factors for the acquisition of ESBL-producing bacteria. Many research groups have investigated the risk factors associated with the acquisition of infections with ESBL-producing *Enterobacteriaceae* (Tumbarello *et al*., 2006; Skippen *et al*., 2006). Patients at high risk for developing colonization or infection with ESBL producing organisms are often seriously ill patients with prolonged hospital stays (Denton 2007; McGowan 2008) and in whom medical invasive devices are present (urinary catheters, nasogastric feeding tubes, endotracheal tubes) for a prolonged duration (Paterson and Bonomo, 2005; Pfaller and Sagreti, 2006; Silva *et al.,* 2006). One important risk factor is prior exposure to antibiotics, predominantly third generation cephalosporins and fluoroquinolones (Kang *et al.,* 2005; Mendelson *et al.,* 2005; Paterson and Bonomo, 2005). Other risk factors are the presence of severe underlying disease, recent surgery, poor nutritional status (Mangeney *et al*., 2000), delay in appropriate treatment, presence of ulcers (Paterson *et al.,* 2005; Pfaller and Sagreti, 2006) and recent hospitalization (Lytsy,

2010). Intensive care units are typically “risk units” due to their high selective pressure in combination with susceptible patients (Menashe *et al*., 2001; Mendelson *et al.,* 2005). Risk factors for colonization or infection with CTX-M producing organisms include history of recent hospitalization (Menashe *et al.*, 2001), age 65 years or higher, dementia and diabetes etc (Paterson and Bonomo, 2005).
Although there is no conclusive evidence, one potential source of colonization with the ESBL producers in the community may be the use of veterinary oxyimino cephalosporins like ceftiofur in livestock (Livermore and Woodford, 2006).

### 2.6.9 Extended spectrum ß-lactamases among *Enterobacteriaceae* from animals

Several antimicrobial agents used in veterinary and human medicine belong to the same antibiotic families and hence different selective pressures exercised in distinct environments might contribute to the selection and dissemination of similar resistance genes (WHO, 1997; Aarestrup, 1999). Gut colonizers of both humans and animals may play an important role in the dissemination of resistance (Caratolli, 2008). Resistance in bacteria of animals and its impact on human health has drawn much attention worldwide (Phillips *et al.*, 2004; Aarestrup, 2006). More recently, several studies have reported the dissemination of ESBL producing *Enterobacteriaceae* to healthy food producing animals in several countries in Europe and Asia (Duan *et al*., 2006; Meunier *et al*., 2006; Tian *et al*., 2009 Cortes *et al.,* 2010; Goncalves *et al.,* 2010) and food products like meat, fish and raw milk (Jensen *et al*., 2006; Jouini *et al*., 2007; Hammad *et al*., 2008). The first pathway of resistance transfer is the direct transfer of a pathogen from animals to humans.

Third-generation cephalosporins are rarely used in poultry and only under very limited conditions for treatment of valuable poultry stocks (Guardibassi *et al.*, 2008). However, ceftiofur, licensed for veterinary use in the USA since 1988, has been given to one-day-old chicks to prevent early mortality in the USA (Batchelor *et al.,* 2005). In Europe, cephalosporins are not allowed for use in poultry (Schwarz & Chaslus-Dancla, 2001; Smet *et al*., 2008), although extra-label use may occur. The first detection of an ESBL in an animal was reported in Japan in 1988, from a laboratory dog infected by an FEC-1-producing *E. coli* strain (Matsumoto *et al.,* 1988).

Broad-spectrum SHV-1-, TEM-1- and OXAtype β-lactamases have been frequently described in *E. coli* and *Salmonella spp*. from animals and food of animal origin in Spain, Germany, the USA and the UK. TEM-1 was the most common variant among these isolates (Miriagou *et al*., 2004; Batchelor *et al*., 2005), but it is only in the last few years that some ESBLs known to be relevant to human medicine have been described in isolates from animals. In animals, the spread of resistance genes could be due to promiscuity in animal breeding and the genes selected by the use of antimicrobial agents. It is interesting to note that despite the use of different cephalosporins in veterinary medicine, there is no specific β-lactamase associated with animals and that only a few *E. coli* producers of the CTX-M-15 variant, which is one of the most prevalent in humans have been identified in animals (Poirel *et al*., 2002; Muenier *et al.,* 2006). It is important to emphasize that often, some ESBL genes are located within mobile genetic elements, associated with other resistance genes, conferring resistance to antimicrobials that could be extensively used among animals and humans (e.g., trimethoprim, sulfamethoxazole, streptomycin) which could play an important role in the co-selection of these ESBL genes. Animals, especially food-producing animals, have been assumed to represent a reservoir for ESBL-producing bacteria (Carattoli, 2008), and this observation might suggest a transmission from the animal reservoir to the community. Although the transmission of ESBL-producing bacteria through the food chain or direct contact between humans and animals has seldom been proven (Riaño *et al.*, 2006; Cavaco *et al.,* 2008; Bertrand *et al.,* 2006), animals should be considered as an important reservoir of ESBL-strains and highly transmissible plasmids.

Although the use of of cephalosporins and flouroquinolones for prophylaxis and treatment of bovine animals is rare in Nigeria, their resistant determinants could be selected by other drugs frequently used in food animals. Chah and Oboegbulem, 2007 reported the wide use of ampicillin in poultry production in Nigeria which may provide a selective pressure favouring the emergence of *E. coli* strains that produce ESBL enzymes. These other food animals may serve as reservoir of ESBL producing *E. coli* strains which could be transferred to humans and other animals

The number of publications reporting commensal broad-spectrum cephalosporin resistant *Enterobacteriaceae* isolated from food-producing animals has increased dramatically especially from developed countries. The diversity among the ESBL encoding genes in Enterobacteriaceae from food-producing animals is by far larger than what is seen for the AmpC encoding genes.

So far, TEM-52- and SHV-12 producing *Enterobacteriaceae,* isolated especially from poultry, have only been described on the European continent (Cloeckaert *et al.*, 2007; Chiaretto *et al.*, 2008; Machado *et al.*, 2008; Smet *et al.*, 2008; Costa *et al.*, 2009). ESBLs such as CTX-M-1, CTX-M-2 and CTX-M-14 have been found in many European countries, being associated with *E. coli* mainly from poultry (Jouini *et al.*, 2007; Machado *et al.*, 2008; Smet *et al.*, 2008; Costa *et al.*, 2009). The CTX-M-15 enzyme, the most widely diffused enzyme among human *Enterobacteriaceae,* was only recently detected among *E. coli* from poultry and pigs (Smet *et al.*, 2008; Tian *et al.*, 2009). A recent report characterized PMQR determinants and β-lactamase (CTX-M-15) among *E. coli* from healthy chickens and pigs at slaughter in Ibadan, Nigeria, which showed a high prevalence of these resistant determinants (Fortini *et al.*, 2011).

### 2.6.10 Detection of ESBLs

The methods for detection of ESBLs can be broadly divided into two groups: phenotypic methods that use non-molecular techniques, which detect the ability of the ESBL enzymes to hydrolyse different cephalosporins; and genotypic methods, which use molecular techniques to detect the gene responsible for the production of the ESBL.

Clinical diagnostic laboratories use mostly phenotypic methods because these tests are easy to perform and are also cost effective. ESBL testing using phenotypic methods involves two important steps. The first is a screening test with an indicator cephalosporin which looks for resistance or diminished susceptibility, thus identifying isolates likely to be harboring ESBLs. The second one tests for synergy between an oxyimino cephalosporin and clavulanate, distinguishing isolates with ESBLs from those that are resistant for other reasons. Failure to detect ESBL production by routine disk-diffusion tests has been well documented (Tenover *et al.*, 1999; Paterson and Yu, 1999). The current CLSI recommendations (2010) for detection of ESBL's in

*Klebsiella spp*. and *E. coli* includes an initial screening test with any two of the following beta- lactam antibiotics: cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone. Isolates exhibiting a MIC > 1µg/ml should be confirmed phenotypically using ceftazidime plus ceftazidime/clavulanic acid and cefotaxime plus cefotaxime/clavulanic acid.

### 2.6.10.1 Screening test for ESBLs

**2.6.10.1.1 Disc diffusion method** Clinical and Laboratory Standards Institute (CLSI) has proposed disc-diffusion methods for screening for ESBL production by *Klebsiellae pneumoniae, K. oxytoca, Escherichia coli and Proteus mirabilis*. Laboratories using disc-diffusion methods for antibiotic susceptibility testing can screen for ESBL production by noting specific zone diameters which indicate a high level of suspicion for ESBL production. Cefpodoxime, ceftazidime, aztreonam, cefotaxime or ceftriaxone discs are used. Since the affinity of ESBLs for different substrates is variable, the use of more than one of these agents for screening improves the sensitivity of detection (CLSI, 2009). If an organism is resistant to any of the cephalosporins, a phenotypic confirmatory test is performed to ascertain the diagnosis. However, it is adequate to use cefotaxime, which is consistently susceptible to CTX-M; and ceftazidime, which is a consistently good substrate for TEM and SHV variants. If only one drug can be used, then the single best indicator has been found to be cefpodoxime (Jarlier *et al.*, 1988; Steward *et al*., 2001). Recently, chromogenic media designed specifically for screening and identification of ESBLs producing Enterobacteriaceae, have become comercially available (Black *et al.*, 2005). The CLSI has proposed dilution methods for screening for ESBL production by *Klebsiella pneumoniae* and *K oxytoca, Escherichia coli and Proteus mirabilis*. Ceftazidime, aztreonam, cefotaxime or ceftriaxone can be used at a screening concentration of 1 µg/mL or cefpodoxime at a concentration of 1 µg/mL for *Proteus mirabilis*; or 4 µg/mL, for the others. Growth at or above this screening antibiotic concentration is suspicious of ESBL production and is an indication for the organism to be tested by a phenotypic confirmatory test (CLSI, 2009).

### 2.6.10.2 Confirmatory tests for ESBLs

The disc synergy test (DDST) is the oldest method for phenotypic confirmation of ESBLs producing organisms, first proposed in 1980 (Jarlier *et al*. 1988). In this, test discs of third-generation cephalosporins and augmentin are kept 30 mm apart, center to center, on inoculated Mueller-Hinton agar (MHA) (Jarlier *et al.,* 1988). A clear extension of the edge of the inhibition zone of cephalosporin towards augmentin disc is interpreted as positive for ESBL production. The sensitivity and specificity of this method are 94.1% and 81.4% respectively for all species (Drieux *et al.,* 2008). Sensitivity of the method may be reduced when ESBL activity is very low. Using cephalosporins/clavulanate combination discs, the CLSI advocates use of cefotaxime (30 μg) or ceftazidime (30 μg) discs with or without clavulanate (10 μg) for phenotypic confirmation of the presence of ESBLs in *Klebsiella* and *Escherichia coli, P. mirabilis and Salmonella species*.

The CLSI recommends that the disk tests be performed with confluent growth on Mueller-Hinton agar. A difference of ≥5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/ clavulanate discs is taken to be phenotypic confirmation of ESBL production (CLSI, 2009). This method has a sensitivity and specificity of 92.9% and 96.6% respectively for all species (Drieux *et al.,* 2008). Phenotypic confirmatory testing can also be performed by broth microdilution assays using ceftazidime (0.25-128 μg/mL), ceftazidime plus clavulanic acid (0.25/4 - 128/4 μg/mL), cefotaxime (0.25-64 μg/mL), or cefotaxime plus clavulanic acid (0.25/4 - 64/4 μg/mL) (Quenaan *et al.,* 2004). Broth microdilution is performed using standard methods.

According to CLSI guidelines, isolates which have a positive phenotypic confirmatory test should be reported as resistant to all cephalosporins (except the cephamycins, cefoxitin and cefotetan) and aztreonam, regardless of the MIC of that particular cephalosporin. Several other tests have been developed to confirm the presence of ESBLs

#### 2.6.10.2.1 Three-dimensional test

The three-dimensional test gives phenotypic evidence of ESBL-induced inactivation of extended-spectrum cephalosporins or aztreonam without relying on demonstration of inactivation of the β-lactamases by a β-lactamase inhibitor (Paterson and Bonomo, 2005)

In this test, the surface of the susceptibility plate is inoculated by standard methods for disk-diffusion testing, but additionally a circular slit is cut in the agar concentric with the margin of the plate. A heavy inoculum of the test organism ($10^9$ to $10^{10}$ CFU of cells) is pipetted into the slit. β-lactam–impregnated disks are then placed on the surface of the agar 3 mm outside of the inoculated circular slit. β-lactamase–induced inactivation of each test antibiotic is detected by inspection of the margin of the zone of inhibition in the vicinity of its intersection with the circular three-dimensional inoculation. The presence of β-lactamase–induced drug inactivation is visualized as a distortion or discontinuity in the usually circular inhibition zone or as the production of discrete colonies in the vicinity of the inoculated slit (Paterson and Bonomo, 2005).

#### 2.6.10.2.2 Agar supplemented with clavulanate

Antibiotic disks containing ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg) and aztreonam (30 μg) are placed on the clavulanate-containing agar plates and regular clavulanate-free Mueller-Hinton agar plates (Ho *et al.,* 1998) A difference in β-lactam zone width of ≥10 mm in the two media was considered positive for ESBL production. A major drawback of the method is the need to freshly prepare clavulanate-containing plates. The potency of clavulanic acid begins to decrease after 72 hours (Paterson and Bonomo, 2005).

#### 2.6.10.2.3 Cephalosporin/clavulanate combination disks on iso-sensitest agar

The British Society for Antimicrobial Chemotherapy has recommended the disk-diffusion method for phenotypic confirmation of ESBL presence using ceftazidime-clavulanate and cefotaxime-clavulanate combination disks, with semiconfluent growth on Iso-Sensitest agar (rather than confluent growth on Mueller-Hinton agar). A ratio of cephalosporin/clavulanate zone size to cephalosporin zone size of 1.5 or greater was taken to signify the presence of ESBL activity. Using this method, the

sensitivity of the test for detecting ESBLs was 93% using both ceftazidime and cefotaxime. The test did not detect ESBL production by strains producing SHV-6 (M'zali *et al.,* 2000).

### 2.6.10.3 Commercial available methods for ESBL Detection

#### 2.6.10.3.1 Vitek ESBL cards

The Vitek ESBL test utilize cefotaxime and ceftazidime, alone (at 0.5 µg/ml), and in combination with clavulanic acid (0.4 µg/ml). Inoculation of the cards is identical to that performed for regular Vitek cards. After inoculation, cards are introduced into the VITEK machine, and for each antibiotic tested, turbidity is measured at regular intervals. Analysis of all wells is performed automatically once the growth control well reached a set threshold (4 to 15 hr of incubation).

A predetermined reduction in the growth of the cefotaxime or ceftazidime wells containing clavulinic acid, compared with the level of growth in the well with the cephalosporin alone, indicates a positive result (Drieux *et al.,* 2008). Sensitivity and specificity of the method exceed 90%.

#### 2.6.10.3.2 BD Phoenix Automated Microbiology System

This is a short-incubation system for bacterial identification and susceptibility testing (Sturenberg *et al*., 2003; Sanguinetti *et al.,* 2003). The Phoenix ESBL test uses growth response to cefpodoxime, ceftazidime, ceftriaxone and cefotaxime, with or without clavulinic acid, to detect the production of ESBLS. The test algorithm has been identified by Sanguinetti *et al.,* (2003). Results are usually available within 6 hours. The method has a sensitivity of 98.8% and specificity of 52.2% for all species (Drieux *et al.,* 2008).

#### 2.6.10.3.3 The E test method (Epsilometer test)

These are plastic drug-impregnated strips, one end of which generates a stable concentration gradient of cephalosporin (i.e., ceftazidime 0.5–32 µg/ml, cefotaxime and cefepime 0.25–16 µg/ml) and the remaining end of which generates a gradient of cephalosporin (i.e., ceftazidime and cefepime 0.064–4 µg/ml, cefotaxime 0.016–1 µg/ml) plus a constant concentration of clavulanate (4 µg/ml). ESBL production is inferred if the MIC ratio for cephalosporin alone/cephalosporin plus clavulanate MIC is equal to 8 (Health Protection Agency, 2005). These strips are useful for both

screening and phenotypic confirmation of ESBL production. Florijn *et al.*, 2002 found that E- test was more sensitive than the disc diffusion test.

The phenotypic detection of ESBLs in bacteria other than *E coli, Klebsiella spp,* and *Proteus spp* remains a problematic and controversial issue. The reason for this is that the clavulanate effect noticed with these ESBL-producing species is not always present in species such as *Enterobacter* and *Citrobacter* (Pitout and Laupland, 2008).

**2.6.10.4 Genotypic detection**

Phenotypic methods are not able to distinguish between the specific enzymes responsible for ESBL production (SHV, TEM, and CTX-M types). Several research or reference laboratories use genotypic methods for the identification of the specific gene responsible for the production of the ESBL, which have the additional ability to detect low-level resistance (i.e, can be missed by phenotypic methods). Furthermore, molecular assays also have the potential to be done directly on clinical specimens without culturing the bacteria, with subsequent reduction of detection time.

The determination of whether a specific ESBL present in a clinical isolate is related to TEM and SHV enzymes is a complicated process because point mutations around the active sites of the TEM and SHV sequences have led to amino acid changes that increase the spectrum of activity of the parent enzymes, such as in TEM-1, TEM-2, and SHV-1 (Farkosh, 2007). The molecular method commonly used is the PCR amplification of the TEM and SHV genes with oligonucleotide primers, followed by sequencing. Sequencing is essential to discriminate between the non-ESBL parent enzymes (eg, TEM-1, TEM-2, or SHV-1) and different variants of TEM or SHV ESBLs (eg, TEM-3, SHV-2, etc). Molecular methods that do not use sequencing have been developed to characterize ESBLs. These include PCR with Restriction fragment length Polymorphism (RFLPs), DNA probes method, Oligotyping method, PCR with single-strand conformational polymorphism, ligase chain reaction, restriction site insertion PCR, and real-time PCR. Polymerase chain reaction amplification followed by nucleotide sequencing remains the gold standard for the identification of specific point mutation of TEM or SHV ESBL genes. Sequencing is the only method for identifying CTX-M genes, which is labour intensive, time-consuming and expensive. Xu *et al.,* (2005) reported that the development of a rapid

and accurate multiplex PCR assay for simultaneous amplification of all CTX-M genes and differentiation of the five clusters.

Newer technologies such as the molecular techniques above in tandem with mass spectrometry (matrix assisted light desorption ionization-time-of-flight; MALDI-TOF) are being mooted as quicker alternatives to conventional laboratory diagnosis (Rishi and Clark, 2012). However, these technologies are still relatively new in development and are not for use in most clinical institutions.

### 2.6.11 Treatment of infections due to ESBL producing organisms

Infections due to ESBL-producing organisms present a major therapeutic dilemma as the choice of antibiotics is extremely limited (Pitout *et al.,* 2005). The presence of ESBLs complicates the selection of antibiotics, particularly in patients with serious infections such as bacteraemia. The reason for this is that ESBL-producing bacteria are often multiresistant to various antibiotics, and CTX-M-producing isolates are co-resistant to the fluoroquinolones. The factors which determine the choice of antibiotics and other management options include a) site of infection; b) severity of infection; c) presence of a prosthetic device or implant; d) metabolic parameters — liver and renal function; e) patient-related factors such as age, pregnancy, lactation (Bhattacharya, 2006). The therapeutic options for ESBL-producing organisms are very limited. ESBLs confer on them the ability to be resistant to most β-lactam antibiotics except cephamycins and carbapenems.

In addition, the plasmids bearing genes-encoding ESBLs frequently also carry genes encoding resistance to other antimicrobial agents, such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (Paterson, 2000; Livermore and Woodford, 2006).

There has also been increasing reports of plasmid-encoded decrease in susceptibility to quinolones, frequently in association with plasmid-mediated cephalosporin resistance (Wang *et al.,* 2003; Mammeri *et al.,* 2005). There appears to be a strong association between quinolone resistance and ESBL production even in the absence of plasmid-encoded decrease in quinolone susceptibility although the reason for this association is not well understood (Babini *et al.,* 2000b; Brisse *et al.,* 2000). Fluoroquinolones may be used for the treatment of uncomplicated urinary tract

infections (UTIs) when found to be susceptible, although increasing *in-vitro* resistance of ESBL producers to quinolones will limit the role of these antibiotics in the future. Studies have found carbapenems to be superior to quinolones for treatment of serious infections caused by ESBL-producing organisms (Endimiani *et al.,* 2004; Paterson *et al*., 2004). Some infections due to organisms testing resistant to ceftazidime but susceptible to cefotaxime or ceftriaxone have responded to treatment with these alternate cephalosporins. However, MICs of these agents rise dramatically as the inoculum is increased (Chaudhary and Aggarwal, 2004) .Thus isolates giving a positive synergy test are inferred to have ESBLs, and all cephalosporins should be avoided as therapy, irrespective of susceptibility results.

Although ESBL activity is inhibited by clavulanic acid, β-lactam/ β-lactamase inhibitor combinations are not considered optimal therapy for serious infections due to ESBL producers as their clinical effectiveness against serious infections due to ESBL-producing organisms is controversial (Paterson *et al.,* 2005). There is also concern that misuse of carbapenems in uncomplicated cases will result in carbapenem resistance. Thus the therapeutic options are limited to carbapenems, colistin, polymyxin, temocillin, tigecycline for serious infections. However uncomplicated infections like non-bacteremic urinary tract infections can be managed with a variety of antibiotics, depending on their susceptibility. These include oral antibiotics like trimethoprim, nitrofurantoin, fosfomycin, co-amoxiclav, mecillinam; or intravenous agents like aminoglycoside (gentamicin, amikacin) and inhibitor combinations (Bhattacharya, 2006; Rawat *et al.,* 2009). Among these carbapenems are the drugs of choice for serious infections with ESBL producers. Imipenem and meropenem are preferred in nosocomial infections, while etrapenam is preferred in community-acquired infections (Shah *et al.*, 2003)

Although *in-vitro* studies have demonstrated no synergy, additivity or antagonism in combination therapy (carbapenem + aminoglycoside), the bactericidal activity of imipenem in combination with amikacin was found to be greater than that of imipenem alone. This was due to the faster killing rates of amikacin (Bhattacharya, 2006) Thus carbapenems may be combined with a second agent (amikacin) for the first few days in the treatment of life-threatening infections like septicemia, hospital-

acquired pneumonia, intra-visceral abscesses (Bhattacharya, 2006). Tigecycline, temocillin, colistin and polymyxin are reserved for patients resistant to all of the other antibiotics, including the carbapenems.

**2.7 Flouroquinolones**

The first quinolone, nalidixic acid, was derived from the antimalarial drug, chloroquine (Andriole, 2005).Subsequent agents were derived through side chain and nuclear manipulation (Ball, 2003).The development of the fluoroquinolone class may be described in generational terms, with each generation sharing similar features or antimicrobial spectra (Table 2.5) (Ball, 2003; Andriole, 2005). First-generation agents possess activity against aerobic Gram-negative bacteria, but little activity against aerobic Gram-positive bacteria or anaerobes. Second-generation agents are the original fluoroquinolones, so named because of the addition of a fluorine atom at position C-6 (Fig. 1).

These agents offer improved coverage against gram-negative bacteria and moderately improved gram-positive coverage. Third-generation agents achieve greater potency against gram-positive bacteria, particularly pneumococci, in combination with good activity against anaerobes. Fourth-generation fluoroquinolones have superior coverage against pneumococci and anaerobes.

**Table 2.5 Evolution of the flouroquinolone class of antimicrobials (Andriole, 2005)**

| Generation | Agent |
|---|---|
| First Generation | Nalidixic acid |
| | Cinoxacin |
| Second Generation | Norfloxacin |
| | Ciprofloxacin |
| | Lomefloxacin |
| | Ofloxacin |
| | Levofloxacin |
| Third Generation | Sparfloxacin |
| | Gatifloxacin |
| | Grepafloxacin |
| Fourth generation | Trovafloxacin |
| | Moxifloxacin |
| | Gemifloxacin |
| | Garenoxacin |

The fluoroquinolone class of antimicrobial agents has had broad acceptance in hospitalized and community patients, and usage appears to be increasing (Chen *et al.*, 1999; Hooper, 2000). Although some members of the class (temafloxacin, grepafloxacin, and trovafloxacin) have been withdrawn or restricted because of adverse events, new members continue to be developed and approved (gatifloxacin and moxifloxacin). The recently released fluoroquinolones eg Moxifloxacin are for treating patients with respiratory tract infections, the single most common group of infections (Low and Scheld, 1998). The important use and convenience of fluoroquinolones (once or twice a day oral dosing), suggests that their use will increase (Hooper, 2000). Resistance has already emerged in some species of bacteria and some clinical settings as a result of flouroquinolone use.

### 2.7.1 Mechanism of action of flouroquinolones

Fluoroquinolones interfere with bacterial cell replication, transcription, and DNA repair by disabling two bacterial enzymes crucial to these processes, DNA gyrase (formerly topoisomerase II) and topoisomerase IV. These enzymes are necessary for bacteria to manage the topological challenge of containing their genetic material. Fluoroquinolones (and earlier quinolones) are novel among antimicrobial agents in clinical use because they directly inhibit DNA synthesis (Hooper, 2001). Inhibition appears to occur by interaction of the drug with complexes composed of DNA and either of the two target enzymes, DNA gyrase and topoisomerase IV. These enzymes are structurally related to each other, both being tetrameric with pairs of two different subunits.

The gyrA and gyrB subunits of DNA gyrase are respectively homologous with the parC and parE subunits of topoisomerase IV. Both enzymes are type 2 topoisomerases, which act by breaking both strands of a segment of DNA, passing another segment through the break, and then resealing the break. For DNA gyrase, this topoisomerization reaction results in introduction (or removal) of DNA supercoils, thus affecting the negative supercoiling of DNA necessary to initiate DNA replication and remove positive supercoils that accumulate before an advancing replication fork. For topoisomerase IV, the topoisomerization reaction results in separation of the interlocking of daughter DNA strands that develops during

replication which facilitates the segregation of daughter DNA molecules into daughter cells. In both cases, fluoroquinolones appear to trap the enzyme on DNA during the topoisomerization reaction, forming a physical barrier to the movement of the replication fork (Hiasa *et al.*, 1996), RNA polymerase (Willmott *et al*., 1994), and DNA helicase (Shea and Hiasa, 1999). The collision of the replication fork with these trapped complexes triggers other poorly defined events within the cell that ultimately result in cell death.

### 2.7.2 Mechanisms of resistance to flouroquinolones

In recent years, use of fluoroquinolones has increased in many countries and emergence of resistance of bacterial isolates to fluoroquinolones has been observed. Consistent step-wise increase in *E. coli* resistance to ciprofloxacin was observed from 1995 (0.7%) to 2001 (2.5%) by Bolon *et al*., (2004). Ciprofloxacin resistance in Portugal was 25.8% and Italy 24.3% while in Germany and Netherlands it was 15.2% and 6.8% respectively (Oteo *et al*., 2005). In previous years, *E. coli* was 100% susceptible to the fluoroquinolones. In 1996, Egri-Okwaji reported 100% susceptibility of *E. coli* isolates to ofloxacin in Nigeria. In another study carried out by Kesah *et al*. (1999), resistance of *E. coli* to fluroquinolone was 2%.

Resistance to fluoroquinolones was traditionally believed to be caused by one of two possible mechanisms: mutation of the target enzymes or reduction of intracellular drug concentrations by way of efflux pumps or alterations in porin channels. Quinolone resistance has multiple mechanisms and significant clinical impact. Mutations may occur rapidly during fluoroquinolone therapy and may be the most significant factor limiting the use of these antimicrobials. The discovery of transferable resistance due to plasmids has uncovered additional mechanisms (Jacoby, 2005). Overuse of a single agent will ultimately result in resistance to the entire class (Hooper, 2000).

#### 2.7.2.1 Target Mutations

Mutations in DNA gyrase or topoisomerase IV are due to amino acid substitutions in the corresponding genes (gyrA or gyrB for DNA gyrase and parC or parE for topoisomerase IV) at a site known as the quinolone resistance determining region (QRDR). This location corresponds to a region on the DNA-binding surface of the

enzyme and influences drug affinity at the DNA–enzyme complex (Jacoby, 2005). Resistance to fluoroquinolones occurs in a stepwise fashion, with accumulation of additional mutations resulting in a greater degree of resistance. The primary target enzyme for an organism is generally the first affected by mutation. Thus, gyrA mutations are the first to occur in *E. coli* because DNA gyrase is the primary target of fluoroquinolones in gram-negative organisms (Oram and Fisher, 1991; Hawkey, 2003).

Additional mutations in gyrA and parC lead to higher levels of resistance in gram-negative organisms (Khodursky *et al.*, 1995). The fact that relatively more mutations are required for high-level resistance in *E. coli* may account for the superior activity of fluoroquinolones in *E. coli* compared to that of other gram-negatives with intrinsic resistance to fluoroquinolones (Vila *et al.*, 1996; Vila *et al.*, 1997). Topoisomerase IV is believed to be the primary target of fluoroquinolones in the gram-positive organisms. Typically, parC mutations are the first to occur in *Staphylococcus aureus* or *Streptococcus pneumoniae* and are associated with low-level resistance. Progressive resistance in gram-positives also occurs in a stepwise fashion with accumulation of subsequent mutations in gyrA leading to higher levels of fluoroquinolone resistance (Ferrero *et al.*, 1995; Pan *et al.*, 1996). One continuing challenge for clinicians is the failure of standard susceptibility testing methods to identify isolates with low-level resistance caused by single-step mutations. These isolates may develop high-level resistance upon exposure to fluoroquinolone therapy.

#### 2.7.2.2 Efflux Pumps

More recently, resistance caused by reduced accumulation has been shown to require the presence and enhanced expression of endogenous efflux systems that actively pump drug from the cytoplasm. In gram-negative bacteria, these systems typically have three components: the efflux pump located in the cytoplasmic membrane, an outer membrane protein, and a membrane fusion protein thought to link the two (Hooper, 2001). Drug is actively extruded from the cytoplasm or cytoplasmic membrane across the periplasm and outer membrane to the cell exterior. The energy for this process is derived from the proton gradient across the membranes. Pumps of this type also exist in gram-positive bacteria including *S. aureus* (Kaatz *et al.*, 1993). They appear to be present in many if not all bacteria.

Efflux pumps are intrinsic components of the bacterial cell membrane that expel waste and other harmful substances from cells. In general, efflux pumps are responsible for lower levels of resistance to fluoroquinolones than target enzyme mutations (Kaatz *et al.,* 1993). However, by allowing short-term survival of the organism in the presence of the drug, efflux pumps encourage the development of mutations in the QRDR (Dalhoff and Schmitz, 2003). The efflux transport mechanism is seen in wild-type *E. coli* (Cohen *et al.,* 1988) and may explain the intrinsic fluoroquinolone resistance among *P. aeruginosa* (Li *et al*., 1994). Agents such as moxifloxacin are less susceptible to efflux pumps (Scheld, 2003). Efflux pumps may expel multiple antimicrobial agents and an over expression the pumps may contribute to the selection of multidrug-resistant organisms.

### 2.7.3 Plasmid-mediated Resistance Mechanisms

The resistance mechanisms of Enterobacteriaceae to fluoroquinolones were restricted to mutations in the chromosomal DNA gyrase (topoisomerase II) and topoisomerase IV genes and to changes in the efflux pumps or porins that decrease intracellular drug concentration (Hooper, 1999). However, the plasmid-mediated fluoroquinolone-resistance protein, Qnr, was detected in a clinical isolate of *Klebsiella pneumoniae* from the United States in 1994, confirming that horizontal transfer of fluoroquinolone-resistance is achievable (Martinez-Martinez *et al.,* 1998). Since the initial discovery of plasmid-mediated fluoroquinolone resistance, several additional plasmid-mediated resistance determinants have been described. Qnr-type determinants, which are now thought to produce proteins that protect DNA gyrase and topoisomerase IV from fluoroquinolone inhibition, are geographically widespread and have been identified in many species of *Enterobacteriaceae.* Qnr proteins have been identified worldwide with a frequent association with clavulanate inhibited expanded spectrum β-lactamases and plasmid-mediated cephalosporinases. Qnr proteins protect DNA from the inhibitory activity of quinolones such as nalidixic acid. The first transferable plasmid-encoded quinolone resistance gene (qnrA, qnrB and qnrS) was isolated from a clinical isolate of ciprofloxacin- resistant *Klebsiella pneumoniae* in 1998 (Martinez-Martinez *et al.,* 1998). The isolated qnrA, qnrB and qnrS gene products protect DNA gyrase from inhibition by ciprofloxacin (Tran and Jacoby, 2002; Wu *et al.,* 2007; Jacoby *et al.,* 2008.) QnrB determinants are associated with

the ESBL SHV-12 in several isolates which may explain in part the frequent association between fluoroquinolone and expanded-spectrum cephalosporin resistance in *Enterobacteriaceae* (Wu *et al.,* 2007; Jiang *et al.,* 2008).

Two other plasmid-mediated quinolone resistance mechanisms have been described: *aac (6 )-Ib-cr*, was first detected in *E. coli* isolates from China in 2003 and was reported to be disseminating (Wang *et al*., 2003). The *aac(6')-Ib-cr* determinant, a variant of aminoglycoside acetyltransferase, which acts in *Enterobacteriaceae* by acetylating the piperazinyl substituent of ciprofloxacin and norfloxacin and reducing its activity. It is widely prevalent and seems to be associated to qnr genes (Karisik *et al.,* 2006; Robicsek *et al.,* 2006; Pitout and Laupland, 2008). It is frequently found in association with extended-spectrum β-lactamases (Yamane *et al*., 2007; Poirel *et al.,* 2008). Very recent studies indicate that the *aac(6')-Ib-cr* gene seems to be confined to *E. coli* ST131 and thus has mainly been linked to CTX-M-15 isolates in different surveys, whereas *qnr* genes are mostly associated with enzymes from the CTX-M-9 or CTX-M-1 groups, which reflects the fact that genes coding for resistance to beta-lactams and quinolones are located on the same plasmid and thus passed on together among different enterobacterial species (Nordmann *et al.,* 2005; Jones *et al.,* 2008). Recent studies have demonstrated the co-transfer of *qnr,* encoding reduced susceptibility to the quinolones, with ESBLs on a plasmid (Paterson, 2000).

Another plasmid-mediated quinolone resistance mechanism is the quinolone efflux pump gene, qepA, has been identified in *E. coli* isolates (Yamane *et al*., 2007; Poirel *et al.,* 2008). The QepA was discovered in Belgium and Japan (Perichon *et al.,* 2007; Yamane *et al.,* 2007). This protein confers resistance to hydrophilic quinolones i.e norfloxacin, ciprofloxacin and enrofloxacin by the efflux pump system (Yamane *et al.,* 2007). It is mediated by a probable transposable element flanked by two copies of IS26 (Yamane *et al.,* 2007; Cattoir *et al.,* 2008). Usually, PMQR determinants have been reported to be associated with extended-spectrum β-lactamases (ESBLs) or other β-lactamases (Robicsek *et al.,* 2006). The emergence of plasmid-mediated quinolone resistance determinants in *Enterobacteriaceae* may compromise further the efficacy of quinolones that are, together with ß-lactams and aminoglycosides, the most commonly prescribed antibiotics for treating human infections.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Equipment, Media, Chemicals and other materials

**3.1.1. Equipment**: The equipment used in this study includes

i) Autoclave (Autester) – It was used for the sterilization of all relevant culture media at 121$^{o}$C for 15 minutes.

ii) Hot air oven (Thermo Scientific Heraeus$^{R}$) - It was used for the sterilization of all items at 160 $^{o}$C for 1 hour.

iii) Incubator (Binder) – It was used for incubating culture media at 37 $^{o}$C.

iv) Microscope – It was used in the characterization of the bacteria

v) Micro centrifuge (Thermo Scientific) – It was used in the isolation of plasmid DNA.

vi) Weighing balance (Mettler PC 400, England) - It was used for the purpose of weighing appropriate antibiotic powder samples and media.

vii) Glassware (Pyrex, England) – Conical flask, measuring cylinder, petri dishes, glass beakers, pipettes and test tubes were the items of glassware used.

viii) Vortex – It was used to mix small vials of liquid during the experiments.

ix) Micropipettes, Eppendorf tubes and tips (Sigma Aldrich)- They were used in the Polymerase chain reactions

x) Agarose gel casting trays and combs- It was used in determining the molecular weight of DNA amplicons from the polymerase chain reactions

xi) Gel chambers - It was used in determining the molecular weight of DNA amplicons from the polymerase chain reactions

xii) Electrophoresis tanks - It was used in determining the molecular weight of DNA amplicons from the polymerase chain reactions.

xiii) UV transilluminator (Sigma Aldrich) – It was used in visualizing DNA amplicons on the agarose gels before recording by photography.

xiv) Polaroid camera (Sigma Aldrich) – It was used to obtain gel pictures of DNA amplicons after electrophoresis.

xv) Water bath (Electrothermal, England) – It was used for the melting of aqeous suspended agar powder prior sterilization and for general moist heating purpose.

xvi) Refrigerator (Snijders Scientific)- It was used for storing the bacteria on culture media at -80 $^{o}$C, the antibiotics at – 20 $^{o}$C and other relevant materials used in the study at 4$^{o}$C

### 3.1.2. Media

The culture media used in this study are listed below

i) Luria Bertani (LB) Agar
ii) Mueller Hinton Agar
iii) MacConkey Agar
iv) Luria Bertani (LB) Broth
v) Eosin Methylene Blue Agar
vi) Nutient Agar

The different compositions and preparations of the different media are given in Appendix I

### 3.1.3 Chemicals and Other materials

Chemicals used in this study include

i) Gram's Iodine Solution
ii) Safranin solution
iii) Crystal violet solution
iv) Kovac's Indole reagents
v) Koser's citrate medium
vi) Plasmid extraction solutions
vii) Sodium Dodecyl sulphate (SDS) (Merck, Germany)
viii) Agarose (Fisher Scientific, USA)
ix) Bromophenol blue (Promega, Madison, WI, USA)

x) Chloroform (Merck KGaA, Germany)

xi) EDTA (Merck KGaA, Germany)

xii) Acetic acid (Merck, Darmstadt, F.R. Germany)

xiii) Ethidium bromide

xiv) Ethanol (Merck KGaA, Germany)

xv) HCl (Baker Analyzed Reagent, Holand)

xvi) Lysozyme(SIGMA, Inc. St. Louis, MO, USA)

xvii) Potassium acetate

Enzymes include

i) Restriction enzymes(New England Biolabs, Ipswich USA)

ii) RNase

The compositions of the chemicals are given in Appendix I

Antibiotic discs and powders used include

| Antimicrobial Agent | | Disc Potency (µg) |
|---|---|---|
| i) | Ceftazidime | 30 |
| ii) | Cefoxitin | 30 |
| iii) | Cefotaxime | 30 |
| iv) | Amoxicillin-Clavulanic acid | 20/10 |
| v) | Imipenem | 10 |
| vi) | Azetronam | 30 |
| vii) | Cefepime | 30 |
| viii) | Ciprofloxacin | 5 |
| ix) | Nalidixic acid | 30 |
| x) | Amoxicillin | 25 |
| xi) | Trimethoprim | 5 |
| xii) | Gentamicin | 10 |

### 3.2 Bacterial isolates

From August 2010-August 2011 Fifty seven *Escherichia coli* isolated from urine, high vaginal swabs, stool, sputum, endocervical swab and catheter tip samples (Table 3.1) of different patients were collected from four hospitals located in Ibadan and Ogbomoso in Oyo state Nigeria respectively. The hospitals comprised of two Tertiary hospitals (University College Hospital Ibadan and Bowen University Teaching Hospital Ogbomoso) and two Secondary Hospitals (Oluyoro Catholic Hospital Ibadan and General Hospital Adeoyo Ibadan) (Table 3.2). Similarly, Fifty seven *Escherichia col*i isolated during the same period from 100 fecal samples of healthy bovine animals from five farms from three different locations (Ibadan, Ogbomoso and Iseyin) (Table 3.2) in Oyo State, Nigeria were used in the study. Sixty percent (n=34) of *E. coli* isolates were recovered from urine samples, while 18% (n=10) were from high vaginal swabs, 16% (n=9) were from stool, 5% (n=3) were from sputum, and 2% (n=1) was from an endocervical swab and a catheter tip sample. All the bovine *E.coli* isolates were isolated from faecal samples.

*Escherichia coli* J53 (Sodium azide resistant) was used as a recipient strain for conjugation mating assay. All isolates both Human and Bovine were identified using standard diagnostic methods (Cheesebrough, 2000).

### 3.3 Linearized DNA markers

Linearized DNA ladders used in this study are listed in Table 3.3

### 3.4 Polymerase chain reaction (PCR) and sequencing primers

Specific primers (NZYTech, Lda, Lisboa, Portugal) used for the detection of the various resistance genes investigated in this study and for the sequencing of the genes are listed in Table 3.4. Primers used for the amplication of the various plasmid replicon types are also listed in Table 3.5.

**Table 3.1 Clinical Sources of the human *Escherichia coli* isolates**

| ISOLATE NUMBER | CLINICAL SOURCE | HOSPITAL INVOLVED |
|---|---|---|
| H1 | Urine | UCH |
| 2 | High Vaginal swab | UCH |
| 3 | Urine | OCH |
| 4 | Stool | OCH |
| 5 | Sputum | OCH |
| 6 | Urine | UCH |
| 7 | Stool | OCH |
| 8 | Stool | GHA |
| 9 | Stool | GHA |
| 10 | Urine | UCH |
| 11 | Urine | UCH |
| 12 | High vaginal Swab | UCH |
| 13 | High Vaginal Swab | UCH |
| 14 | Urine | UCH |
| 15 | High vaginal Swab | GHA |
| 16 | Endocervical Swab | UCH |
| 17 | Stool | GHA |
| 18 | Urine | UCH |
| 19 | Urine | UCH |
| 20 | Sputum | UCH |
| 21 | Urine | UCH |
| 22 | Urine | UCH |
| 23 | High Vaginal Swab | UCH |
| 24 | Catheter tip | UCH |
| 25 | Urine | UCH |
| 26 | Urine | UCH |
| 27 | Urine | UCH |
| 28 | Stool | UCH |
| 29 | Stool | UCH |
| 30 | Urine | BUTH |
| 31 | Urine | BUTH |
| 32 | Urine | BUTH |
| 33 | Urine | BUTH |
| 34 | Stool | BUTH |
| 35 | Stool | BUTH |
| 36 | High Vaginal Swab | BUTH |
| 37 | High Vaginal Swab | BUTH |
| 38 | Urine | BUTH |
| 39 | Urine | BUTH |
| 40 | Urine | BUTH |
| 41 | Urine | BUTH |
| 42 | Urine | BUTH |

| ISOLATE NUMBER | CLINICAL SOURCE | HOSPITAL INVOLVED |
|---|---|---|
| 44 | Sputum | UCH |
| 45 | Urine | UCH |
| 46 | Urine | UCH |
| 47 | Urine | UCH |
| 48 | Urine | BUTH |
| 49 | High Vaginal Swab | UCH |
| 50 | Urine | BUTH |
| 51 | Urine | UCH |
| 52 | Urine | BUTH |
| 53 | Urine | UCH |
| 54 | Urine | UCH |
| 55 | Urine | OCH |
| 56 | Urine | OCH |
| 57 | Urine | UCH |
| 58 | High Vaginal Swab | UCH |

**Legend**-UCH-University College Hospital, Ibadan, OCH-Oluyoro Catholic Hospital, Ibadan, BUTH-Bowen University Teaching Hospital, Ogbomoso, GHA-General Hospital Adeoyo Ibadan

**Table 3.2 Distribution of the Human and Bovine *E.coli* isolates among the hospitals and farms**

| **Location sample** | **Number of *E.coli* Isolates per** |
|---|---|
| University College Hospital Ibadan | 31-Urine (19), High vaginal swab (6), Sputum (2), Endocervical swab (1), Stool (2), Catheter tip (1) |
| Oluyoro Catholic Hospital Ibadan (1) | 6-Urine (3), Stool (2), Sputum |
| General Hospital Adeoyo Ibadan | 4-High vaginal swab (1), Stool (3) |
| Bowen University Teaching Hospital | 16-Urine (12), High vaginal swab (2), Stool (2) |
| Farms at Ibadan | 42 |
| Farm at Iseyin | 7 |
| Farm at Ogbomoso | 8 |

**Table 3.3 Linearized DNA ladders (markers) used in this study**

| Linearized DNA ladders | Description |
|---|---|
| 50bp ladder (Biorion, Just Fine Molecular Biology, Singapore) | It has 10 fragments consisting 50 -400bp by 50; 500 and 700 bp |
| 100bp ladder (Promega, Promega Coorporation, Madison, USA) | It consists of 40 blunt-ended DNA fragments ranging from 100bp-4,000bp in 100bp increaments. |
| 1 kb ladder (Thermo Scientific Gene ruler) | It has 14 fragments consisting 250-10,000bp. |

**TABLE 3.4 PRIMERS USED FOR PCR AMPLIFICATION OF RESISTANCE GENES**

| GENE | PRIMER | PRIMER SEQUENCE(5'-3') | PCR PRODUCT SIZE(bp) | REFERENCES |
|---|---|---|---|---|
| *bla$_{TEM}$* | P1 | TACGATACGGGAGGGCTTAC | 716 | Belanouaj *et al*., 1984 |
| | P2 | TTCCTGTTTTTGCTCACCCA | | |
| | FIN | ATTCTTGAAGACGAAAGGGC | 1091 | Canica *et al*., 1997 |
| | DEB | ATGAGTAAACTTGGTCTGAC | | |
| *bla$_{CTXM}$* | CTXf | TTTGCGATGTGCAGTACCAGTAA | 543 | Eckert *et al*., 2004 |
| | CTXr | CGATATCGTTGGTGGTGCCATA | | |
| *bla$_{SHV}$* | SHVf1 | TCAGCGAAAAACACCTTG | 471 | M'zali *et al*., 1996 |
| | SHVf2 | TCCCGCAGATAAATCACCA | | |
| *ampC* | ampCAB1 | GATCGTTCTGCCGCTGTG | 271 | Caroff *et al*., 2000 |
| | ampC2 | GGGCAGCAAATGTGGAGCAA | | |
| *QnrA* | QnrAm-f | ATT TCT CAC GGA TGG ACT TG | 515 | Cattoir *et al*., 2007 |
| | QnrAm-r | GATCGGCAAAGGTCAGGTCA | | |
| *QnrB* | QnrBm-f | GATCGTGAAACCAGGAAAGG | 468 | Cattoir *et al*., 2007 |
| | QnrBm-r | ACGATGCCTGGGTATTGTCC | | |
| *QnrS* | QnrSm-f | ACGACATTCGTCAACTGCAA | 416 | Cattoir *et a*l., 2007 |
| | QnrSm-r | TAAATTGGCAGTAGGC | | |
| *QepA* | QepA-f | CTTCCTGCCCGAGTATCGTG | 391 | Ma *et al*., 2009 |
| | QepA-r | GAACCGATGACGAAGCACAG | | |
| *Aac(6)-lb* | aac-F | TTGCGATGCTCTATGAGTGGCTA | 482 | Park *et a*l., 2006 |
| | aac-R | CTCGAATGCCTGGCGTGT TT | | |
| *IntI1* | HS463a | CTGGATTTCGATCACGGCACG | | Levesque *et al*., |
| | HS464 | ACATGCGTGTAAATCATCGTCG | Variable | 1995 |
| Class 1 | 5Cs | GGCATCCAAGCAGCAAG | | Barlow *et al.,* 2004 |
| integrons | 3Cs | AAGCAGACTTGACCTGA | Variable | |
| *Isecp1* | Isecp1 | AAAAATGATTGAAAGGTGGT | Variable | Saladin *et al*., 2002 |
| *IS26* | IS26 | AGCGGTAAATCGTGGAGTGA | Variable | Mendonca *et al*., 2007 |
| *IS903* | IS903 | CGGTTGTAATCTGTTGTCCA | Variable | Mendonca *et al*., 2007 |

**Table 3.5- Primers used for the Amplification of the various Replicon types (Caratolli *et al.,* 2005)**

| Replicon Name | DNA sequence 5'-3' | Amplicon size(bp) |
|---|---|---|
| HII FW | ggagcgatggattacttcagtac | 471 |
| HIIRV | tgccgtttcacctcgtgagta | |
| HI2FW | tttctcctgagtcacctgttaacac | 644 |
| HI2RV | ggctcactaccgttgtcatcct | |
| II FW | cgaaagccggacggcagaa | 139 |
| IIRV | tcgtcgttccgccaagttcgt | |
| X FW | aaccttagaggctattaagttgctgat | 376 |
| X RV | tgagagtcaattttatctcatgttttagc | |
| L/M FW | ggatgaaaactatcagcatctgaag | 785 |
| L/M RV | ctgcaggggcgattcctttagg | |
| N FW | ctctaacgagcttaccgaag | 559 |
| NRV | gtttcaactctgccaagttc | |
| FIA FW | ccatgctggttctagagaaggtg | 462 |
| FIA RV | gtatatccttactggcttccgcag | |
| FIB FW | ggagttctgacacacgattttctg | 702 |
| FIB RV | ctcccgtcgcttcagggcatt | |
| W FW | cctaagaacaacaaagcccccg | 242 |
| W RV | ggtgcgcggcatagaaccgt | |
| Y FW | aattcaaacaacactgtgcagcctg | 765 |
| Y RV | gcgagaatggacgattacaaaacttt | |
| P FW | ctatggccctgcaaacgcgccagaaa | 534 |
| P RV | tcacgcgccagggcgcagcc | |
| FIC FW | gtgaactggcagatgaggaagg | 262 |
| FIC RV | ttctcctcgtcgccaaactagat | |
| A/C FW | gagaaccaaagacaaagacctgga | 465 |
| A/C RV | acgacaaacctgaattgcctcctt | |
| T FW | ttggcctgtttgtgcctaaaccat | 750 |
| T RV | cgttgattacacttagctttggac | |
| $FII_S$ FW | ctgtcgtaagctgatggc | 270 |
| $FII_S$ RV | ctctgccacaaacttcagc | |
| FrepB FW | tgatcgtttaaggaattttg | 270 |
| FrepB RV | gaagatcagtcacaccatcc | |
| K/B FW | gcggtccggaaagccagaaaac | 160 |
| K RV | tctttcacgagcccgccaaa | |
| B/O RV | tctgcgttccgccaagttcga | 159 |

**Table 3.6 List of primers used in the rep-PCR genomic fingerprinting method and MLST (Wirth *et al.*, 2006; Mohapatara *et al.*, 2007)**

| Primer | DNA sequence 5'-3' | Annealing temperature (ºC) |
|---|---|---|
| BOX AIR | CTA CGG CAA GGC GAC GCT GAC | 53 |
| adkF1 | TCATCATCTGCACTTTCCGC | 56 |
| adkR1 | CCAGATCAGCGCGAACTTCA | |
| fumCF | TCACAGGTCGCCAGCGCTTC | 62 |
| fumCR1 | TCCCGGCAGATAAGCTGTGG | |
| gyrBF | TCGGCGACACGGATGACGGC | 62 |
| gyrBR1 | GTCCATGTAGGCGTTCAGGG | |
| icdF' | ATGGAAAGTAAAGTAGTTGTTCCGGCACA | 56 |
| icdR 5' | GGACGCAGCAGGATCTGTT | |
| mdhF1 | AGCGCGTTCTGTTCAAATGC | 56 |
| mdhR1 | CAGGTTCAGAACTCTCTCTGT | |
| purAF1 | TCGGTAACGGTGTTGTGCTG | 60 |
| purAR | CATACGGTAAGCCACGCAGA | |
| recAR1 | AGCGTGAAGGTAAAACCTGTG | 56 |
| recAF1 | ACCTTTGTAGCTGTACCACG | |

### 3.5 Biochemical identification of isolates

The methods adopted for the identification of the bacterial isolates were as described by Cowan (1974); Harrigan and McCance (1976); Benson (1978), Cruickshan *et al* (1982); Ogbulie *et al* (1998); Cheesebrough (2000).

#### 3.5.1 Gram Staining

Gram staining classifies bacterial isolates into Gram-positive and Gram-negative on the basis of differential interactions of Gram reagents with the varying cell wall components of these two groups of bacteria. A thin smear of each isolate from a 24 - hr old culture was prepared on clean grease-free slide and heat fixed by flaming. The smear was then flooded with crystal violet as primary stain for 30 – 60 seconds, rinsed with distilled water and flooded with Gram's iodine, which was left for 60 seconds before rinsing with distilled water. The smear was decolorized by flooding the smear with 95% ethanol until no more violet coloration was observed, followed by counterstaining with dilute safranin for 30 seconds before rinsing off with distilled water and air-drying. The stained smear was examined under the X100 immersion oil objective of a light microscope and the Gram reaction was noted. Gram-positive organisms stained blue to purple while Gram-negative organisms were pink to red.

#### 3.5.2 Citrate utilization test

This test works on the ability of an organism to utilize sodium citrate as the sole carbon source for growth and metabolism which results in the alkalinization of the medium. The Koser's citrate medium used contains sodium citrate as the only carbon source and bromo-thymol blue as indicator. The utilization of citrate for bacterial growth results in the production of alkaline by-products which raises the pH of the medium and eventually causes a colour change from green to blue. A 24 hr-old culture of the test isolate was inoculated into a 3 mL aliquot of the medium. This was incubated at 37 $^{o}$C for 4 days and observed daily for degradation of citrate leading to alkalinisation of the medium, which is indicated by the pH indicator bromothymol blue changing colour from green to deep blue along with the growth of the organism, to indicate a positive result. A negative test reaction was shown by no-change in colour, without any growth of the isolate.

### 3.5.3 Indole test

The indole peptone water medium was inoculated with the test isolate and incubated at 44 $^{o}$C±0.25 $^{o}$C in a water bath for 48 hrs. Kovac's reagent was then added in 0.5 mL volume to the culture and shaken gently. A red colour in the surface (the alchol layer) of the medium implied a positive reaction. No-colour change indicates a negative reaction.

### 3.5.4 Lactose fermentation test

All isolates belonging to enterobacteriaceae were inoculated on Mac-conkey agar plates and incubated for 18 hours at 37 $^{o}$C. The plates were then observed for growth coloration, a pinkish coloration of colonies as a positive reaction.

### 3.5.5 Methyl Red (MR) test

This is a qualitative test of the acidity produced by bacteria as a result of the fermentation of the carbohydrates present in the growth medium. The test organism was inoculated into a tube containing the medium followed by incubation at 37 $^{o}$C for 24 hours after which a few drops of methyl red indicator was added and the colour change was observed. A positive result was indicated by the immediate development of a red coloration

### 3.5.6 Voges- Proskauer (VP) Reaction

Bacteria positive to this test produce acetylmethylcarbinol or acetoin, which reacts with the reagents to produce a red colour. This test involves the production of acetoin through the fermentation of sugar with the resultant condensation of pyruvates. The isolates were each cultured in buffered glucose broth cultures at 37 $^{o}$C for 48 hours. After two days of incubation, 1 mL of alpha-naphthol solution and of 10% sodium hydroxide were added successfully to the broth cultures. An immediate appearance of a red coloration indicated a positive result. The medium was left for up to an hour to check for slow reaction in cases of negative result.

### 3.6 Antimicrobial susceptibility tests

#### 3.6.1 Disc Diffusion

The Antibiotic susceptibility testing of both the human and bovine strains was determined by the Kirby Bauer method (Bauer *et al.,* 1966) and results were interpreted using CLSI criteria. Bacterial suspension of each isolate was prepared to match 0.5 MacFarland standards for antibiotic susceptibility as described by CLSI (CLSI, 2012). A sterile swab was dipped into the bacterial suspension and firmly rotated several times against the wall of the tubes to expel excess fluid. The swab was used to inoculate the entire surface of the plates ensuring to obtain uniform inoculation. The antibiotic discs (cetfazidime 30 µg, cefotaxime 30 µg, cefoxitin 30 µg, amoxicillin/clavulanic acid 20/10 µg, imipenem 10 µg, azetronam 30 µg, cefepime 30 µg, ciprofloxacin 5 µg, nalidixic acid 30 µg, amoxicillin 25 µg, trimethoprim 5µg and gentamicin 10 µg) were aseptically applied onto the surface of the inoculated agar plates. The antibiotic susceptibility of each isolate was carried out on Mueller Hinton agar (Oxoid, England) and inoculated plates were incubated overnight in an incubator at 37 $^{o}$C. Antimicrobial susceptibilities of the transconjugants were also determined by the disc diffusion on Mueller-Hinton agar (Oxoid, England) plates and results were also interpreted using CLSI criteria (CLSI, 2012).

#### 3.6.2 Detection of ESBLs in *E. coli* isolates

The double disc synergy test was used to test for the production of ESBLs in both human and bovine isolates. Discs containing cefotaxime, ceftazidime, aztreonam and cefepime and in the centre the amoxicillin-clavulanic acid (20:10 µg) were utilized (Bradford, 2001). Bacterial suspension of each isolate was prepared to match 0.5 MacFarland standards for antibiotic susceptibility as described by CLSI (CLSI, 2012). A sterile swab was dipped into the bacterial suspension and firmly rotated several times against the wall of the tubes to expel excess fluid. The swab was used to inoculate the entire surface of the Mueller-Hinton agar plates ensuring to obtain uniform inoculation. The antibiotic discs were aseptically applied onto the surface of the inoculated agar plates. The plates were incubated overnight in an incubator at 37 $^{o}$C. Extended spectrum beta lactamase was detected after incubation by the enhanced zone of inhibition between amoxycillin/clavulanic acid, cefotaxime and ceftazidime.

### 3.6.3 Determination of MIC by Epsilometer-test

The E-test was used to determine the MIC of five selected antibiotics (ampicillin, cefotaxime, ceftazidime, levofloxacin and gentamicin) by the human ESBL *E. coli* isolates. Bacterial suspension of each isolate was prepared to match 0.5 MacFarland standards for antibiotic susceptibility as described by CLSI (CLSI, 2012). A sterile swab was dipped into the bacterial suspension and firmly rotated several times against the wall of the tubes to expel excess fluid. The swab was used to inoculate the entire surface of the Mueller-Hinton agar plates ensuring to obtain uniform inoculation. The surface of the agar plates were allowed to dry for about 15 minutes on the bench before the E-test strips were applied to the agar plates aseptically with the MIC scale of the E-test strip facing the opening of the plate. Inoculated plates were incubated overnight in an incubator at 37 $^{o}$C. The MIC was interpreted as the point of intersection of the inhibition ellipse with the E-test strip edge.

### 3.7 Strain typing of the ESBL *E. coli* isolates

Repititive-PCR genomic fingerprinting method (Box PCR) was carried out on the ESBL producing human strains to assess the relatedness of the different isolates as determined by the similarity of the patterns produced. A specific primer was used for this PCR (Table 3.6).

Biometra T1 Thermocycler PCR machine was used for the PCR. A volume of 1 µL of DNA templates was added to the reaction mixture (DyNAzyme $^{TM}$ II, PCR master mix-10 µL each, Primer (5')- 1 µL) and sterile distilled water was added to each tube to make a final volume of 20µL. DyNAzyme $^{TM}$II PCR master mix (1.25 mL) contains 0.04 U/µL DyNAzyme $^{TM}$ II DNA polymerase, 20 mM Tris-HCl (pH 8.8 at 25 $^{o}$C), 3 mM $MgCl_2$, 100 mM KCl, Stabilizers and 400 µM of dNTPs.

The reactions for the BOX PCR were run under the following conditions: An initial DNA denaturation at 95 $^{o}$C for 1 minute followed by 30 cycles of DNA denaturation at 94 $^{o}$C for 1minute. At 53 $^{o}$C for 1 minute annealing of specific primers occured from which synthesis of DNA started. Extension of the DNA occurred at 65 $^{o}$C for 8 minutes and a final extension of the DNA follows at 65 $^{o}$C for 16 minutes (Mohapatra *et al.,* 2007).

Polymerase chain reaction products were analyzed on a 2% agarose gel. Polymerase chain reaction products were run for 100-120 volts for 24 hours. The agarose gel was then photographed under UV illumination and the picture was retrieved on the computer. The genetic relatedness of the ESBL *E. coli* isolates were determined by the similarity of the DNA band patterns obtained.

### 3.8 PCR amplification for ESBLS and DNA sequencing

The ESBL positive human *E. coli* isolates were subjected to PCR amplification to screen for the $bla_{TEM}$, $bla_{CTXM}$, $bla_{SHV}$ genes. Specific primers were used for the PCR and sequencing (Table 3.4). A volume of 1 µL of DNA templates was added to the reaction mixture (DyNAzyme $^{TM}$ II, PCR master mix-10 µL each, Primers (Forward)-0.5 µL, Primers (Reverse)-0.5 µL) and sterile distilled water was added to each tube to make a final volume of 20 µL. The reactions for this PCR were run under the following conditions: An initial DNA denaturation at 95 $^{o}$C for 5 minutes followed by 26 cycles of DNA denaturation at 95 $^{o}$C for 1 minute. At 56 $^{o}$C (for $bla_{CTXM}$ amplification) and 58 $^{o}$C (for other amplification of β-lactamase genes) for 1 minute annealing of specific primers occured from which synthesis of DNA started. Elongation (or extension) of the DNA occured at 72 $^{o}$C for 1 minute and a final extension followed at 72 $^{o}$C for 10 minutes (Mendonca *et al.*, 2007). PCR products were analyzed on a 1% agarose gel. A successful PCR reaction yielded visible DNA fragments of the expected size. Linearized DNA ladders were used as size reference standards for the agarose gel electrophoresis of the PCR products.

Positive controls *E .coli* FFC39 ($bla_{TEM}$) and *E. coli* FFC144 ($bla_{CTX-M}$) and *Klebsiella pneumoniae* FFC339 ($bla_{SHV}$), were included for the PCRs. Polymerase chain reaction products were purified with Exosap IT (Affymetrix, Santa Clara, USA) and sequenced in both strands (Macrogen, Seoul Korea). Nucleotide sequences were analyzed with BioEdit software and database searches were performed using the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

### 3.9 Detection of Plasmid mediated quinolone resistance genes

Twenty-nine human and two bovine *E. coli* isolates with reduced susceptibility/resistance to quinolones used in this study were screened by multiplex PCR for qnr genes (*qnrA, qnrB, qnrS*) (Cattoir *et al.,* 2007). Two simplex PCR were carried out to detect *aac(6,)-lb* and *qepA* genes (Park *et al.,* 2006; Ma *et al*., 2009). Specific primers were used for the PCR (Table 3.4). A volume of 1 µL of DNA templates was added to the reaction mixture (DyNAzyme $^{TM}$ II, PCR master mix-10 µL each, Primers (Forward)-0.5 µL, Primers (Reverse)-0.5 µL) and sterile distilled water was added to each tube to make a final volume of 20 µL. The reactions for this PCR were run under the following conditions: An initial DNA denaturation at 95 $^{o}$C for 5 minutes followed by 26 cycles of DNA denaturation at 95 $^{o}$C for 1 minute. At 56 $^{o}$C for 1 minute, annealing of specific primers occurred from which synthesis of DNA started. Elongation or extension of the DNA occurred at 72 $^{o}$C for 1 minute and a final extension followed at 72 $^{o}$C for 10 minutes.

The *aac (6')-lb* gene in six human ESBL *E. coli* isolates were further characterized as the *aac (6')-lb-cr* variant by digestion with *Bts*CI restriction endonucleases (New England Biolabs, Ipswich, USA) to identify *the aac (6')-lb-cr* , which lacks the *Bts*CI restriction site present in the wild-type gene. A reaction mixture of 10 µL of the PCR amplicons of the *aac (6')-lb* gene, 2.5 µL of NE buffer 4 (New England Biolabs, Ipswich, USA), 1.5 µL of the *Bts*CI enzyme (New England Biolabs, Ipswich, USA) and 11 µL of water was made for each of the isolates positive for *aac(6')-lb* gene. The reaction mixture was kept at 50 $^{o}$C for two hours before analyzing by electrophoresis. PCR products were analyzed on a 1% agarose gel for the simplex PCR and 2% agarose gel for the multiplex PCR.

### 3.10 Plasmid Extraction and Analysis

Plasmid extraction and analysis were carried out on the only bovine strain with plasmid mediated quinolone resistance determinant and the eight human ESBL isolates. The extraction of plasmids was carried out by the Brinboim and Doly alkaline lysis method (Brinboim and Doly, 1979). From a fresh culture tube, inoculated and incubated for 18 hr at 37 $^{o}$C with shaking, 1.5 mL of the culture was removed into an eppendorf tube and centrifuged at 18,000 g for 3 minutes. Removing the supernatant, the pellets were resuspended in a 100 µL of solution 1 (1 mL of 500 mM glucose, 1 mL of 100 mM EDTA, 2.5 mL 100 mM Tris HCl pH 8, 40 mg Sigma lysozyme, made up to the volume of 10 mL sterile water) at 4 $^{\circ}$C. After staying 10 minutes at room temperature, the suspension became viscous, the tubes were then placed on ice and 200 µL of solution 2 (2N NaOH 0.6 mL, 0.6 mL 10% SDS and 4.8 mL of distilled water), at room temperature, was added. After shaking by inversion 1-2 times, the tubes were left on ice for 5 minutes, before adding 150 µL of solution 3 (6 mL 5M Potassium acetate pH 4.8, 1.5 mL of glacial acetic acid and 2.5 mL of distilled water) at 4 °C, stirring gently by inversion. All solutions were freshly prepared. After staying 45 minutes on ice, the suspension was centrifuged at 18000 g. The mixture of 200 µL saturated phenol and 200 µL of chloroform was prepared in another set of tubes.

After centrifuging, 400 µL of supernatant was introduced into new tubes containing chloroform and phenol and stirred by gentle inversion 30 times before centrifuging at 18,000 g for 5 minutes. 900 µL absolute ethanol at -20 $^{o}$C was introduced into a new set of tubes to which was added 350 to 400 µL of supernatant from the phenol-chloroform extraction. The tubes were mixed by inversion and placed at -20 $^{o}$C for 1.5 hours after which the suspension was centrifuged at 18,000 g for 30 minutes.The supernatant from the tubes were eliminated carefully, taking into account the possible detachment of the pellet. 400 µL of 70% ethanol at -20 $^{o}$C was added to the tubes to wash the pellets by inverting 2-3 times and centrifuged at 18000 g for 15 minutes. The supernatant was carefully removed and the washing of the pellets was repeated. After drying the pellet in an oven at 37 °C, the pellets were resuspended in 10 µL of sterile water, to which were added 4 µL 1 mg/mL RNase. The suspension was left for 15 minutes, at room temperature, before verifying on an agarose gel.

Gel extraction of the plasmid DNAs was carried out using a QIAquick gel extraction kit. The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane. Special buffers provided with each kit were optimized for efficient recovery of DNA and removal of contaminants in each specific application. DNA adsorbs to the silica membrane in the presence of high concentrations of salt while contaminants pass through the column. Impurities were efficiently washed away, and the pure DNA was eluted with Tris buffer or water.

DNA fragment from the agarose gel was excised with a clean sharp scalpel. The gel size was weighed in a colorless tube and 3 volumes of Buffer QG was added to 1 volume of the gel. The gel was incubated at 50 $^{o}$C for 10 minutes, vortexing the tube every 2-3 minutes to ensure the gel slice completely dissolved. 1 gel volume of isopropanol was then added to the sample and mixed. The QIAquick spin column was then placed in a 2 mL collection tube and the sample was applied to the column to bind the DNA and centrifuged for 1 minute. Flow-through was discarded and the QIAquick column was placed back in the same collection tube. 0.5 mL of Buffer QG was added to the column and centrifuged for 1 minute. O.75 mL of Buffer PE was then added to the column to wash and centrifuged for 1 minute. The flow-through was discarded again and column was centrifuged for an additional 1 minute at 10,000 g. The QIAquick column was then placed in a clean 1.5 mL microcentrifuge tube, and to elute DNA, 30 µL of Buffer EB (10 mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane and allowed to stand for 1 minute before centrifuging for 1 minute.

Purified DNA was then used for detection of CTX-M β-lactamases and plasmid mediated quinolone resistance determinants by PCR.

### 3.11 Plasmid replicon typing

The PCR method based on replicons (inc/rep PCR) of the major plasmid incompatibility groups among *Enterobacteriaceae* is used for tracing plasmids conferring drug resistance. Plasmids were identified in the only one bovine strain found with plasmid mediated quinolone resistance determinant and all the eight human ESBL isolates and assigned to different incompatibility groups by this method. The PCR-based inc/rep typing method consists of five different multiplex-PCRs recognizing three different replicon types and three simplex PCRs for K, F and B/O replicon types. The DNA templates obtained from the strains were used in the PCR based replicon typing to identify plasmids. Specific primers were used for the amplification of the various replicon types (Table 3.5).

A volume of 1 µl of DNA templates was added to the reaction mixture (DyNAzyme $^{TM}$II, DNA polymerase PCR Mastermix-10 µL each, Primers (Forward)-0.5 µL, (Reverse)-0.5 µL, DMSO (Used in multiplex reactions)-1 µL) and sterile distilled water was added to each tube to make a final volume of 20 µL. The reactions for this PCR were run under the following conditions: An initial DNA denaturation at 94 $^{o}$C for 5 minutes, followed by 30 cycles of DNA denaturation at 94 $^{o}$C for 1 minute, annealing at 60 $^{o}$C for 30 seconds, and extension of the DNA at 72 $^{o}$C for 1 minute. A final extension followed at 72 $^{o}$C for 10 minutes. The F simplex PCR was performed with same amplification programme but an annealing temperature of 52 $^{o}$C. PCR products were analyzed on 2% agarose gels. Positive controls were included for the PCR.

### 3.12 Detection of integrase genes and characterization of integrons

The eight human ESBL isolates and a bovine strain with a plasmid quinolone resistance determinant were screened by PCR for the presence of integrase genes *intI*1 using specific primers designed to amplify conserved regions of the gene (Table 3.4). The primers were used to detect intI1 positive *E.coli* isolates. A volume of 1 µL of DNA templates was added to the reaction mixture (DyNAzyme $^{TM}$ II, PCR master mix-10 µL each, Primers (Forward)-0.5 µL, Primers (Reverse)-0.5 µL) and sterile distilled water was added to each tube to make a final volume of 20 µL. The reactions for this PCR were run under the following conditions: An initial DNA denaturation at

95 ºC for 5 minutes followed by 26 cycles of DNA denaturation at 95 ºC for 1 minute. At 58 ºC for 1 minute, annealing of specific primers occured from which synthesis of DNA started. Elongation or extension of the DNA occurred at 72ºC for 1 minute and a final extension of the DNA followed at 72 ºC for 10 minutes. PCR products were analyzed on a 1% agarose gel.

Six ESBL isolates positive for integrase genes *intI*1 were screened with primers able to amplify the inserted gene cassette regions of class 1 integrons. The primers were used to determine the sizes of the inserted cassette region. A volume of 1 µL of DNA templates was added to the reaction mixture (DyNAzyme $^{TM}$ II, PCR master mix-10 µL each, Primers (Forward)-0.5 µL, Primers (Reverse)-0.5 µL) and sterile distilled water was added to each tube to make a final volume of 20 µL. The reactions were run under the following conditions: An initial DNA denaturation at 94 ºC for 5 minutes followed by 35 cycles of DNA denaturation at 94 ºC for 1 minute. At 55 ºC for 1 minute, annealing of specific primers occurred from which synthesis of DNA started. Elongation or extension of the DNA occurred at 72 ºC for 1 minute and a final extension of the DNA followed at 72 ºC for 10 minutes. PCR products were analyzed on a 1% agarose gel. PCR products were purified with Exosap IT (Affymetrix, Santa Clara, USA) and sequenced in both strands. Nucleotide sequences were analyzed with BioEdit software and database searches were performed using the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

### 3.13 Exploration of the Upstream Sequence of the *bla*$_{CTX-M}$ genes

The presence of *ISEcp1* was investigated in the eight human ESBL isolates by a PCR amplification using primers *ISEcp1* (5' AAAAATGATTGAAAGGTGGT-3) and CTX-M-R (Table 3.4) as described previously (Saladin *et al*., 2002; Mendonca *et al*., 2007). This sequence ensures the transfer of the resistance genes from the bacterial chromosome to the plasmids and is involved in the spread of such ESBLs.
*Is 26* and *Is 903* elements were also screened for by PCR in the eight human ESBL positive isolates by PCR using specific primers (Table 3.4). PCR mixture and cycling conditions were as for *bla*$_{CTX-M}$ gene amplification.

### 3.14 Conjugation experiments

Broth mating assays were carried out with the eight human ESBL clinical strains as donors and *E. coli* J53 (Sodium Azide resistant) as recipient at a bacterial cell ratio of 1:10 to determine the transfer of ESBL determinants. The mating mixture was centrifuged at 10,000 g for 10 minutes after 24 hours of incubation and the supernatant removed. The cells obtained were resuspended in 50 µL of phosphate buffer solution and 20 µL was plated in duplicates on Lauria-Bertani agar containing sodium azide (100 mg/L) supplemented with cefotaxime (1 mg/L). Selected transconjugants were reconfirmed by growing the strains in Lauria-Bertani broth containing an equivalent concentration of Sodium azide and cefotaxime. Transconjugants were identified and confirmed as *E. coli* and their antibiotic susceptibility profile determined. PCR amplification of the ESBL determinants, plasmid quinolone resistance determinants and plasmid replicon typing were also carried out in the transconjugants to determine the resistance determinants transferred and plasmid replicon type involved in the transfer of resistance.

### 3.15 Multilocus sequence typing of *E. coli*

Internal fragments of the following seven housekeeping genes adk (adenylate kinase), fumC (fumarate hydratase), icd (isocitrate/ isopropylmalate dehydrogenase), purA (adenylosuccinate dehydrogenase), gyrB (DNA gyrase), recA (ATP/GTPbinding motif), and mdh (malate dehydrogenase) (Wirth *et al*., 2006) were amplified in the two isolates successful for transfer of

resistance determinants by conjugation using specific primers (Table 3.6) with reaction conditions essentially as described at the MLST website www.mlst.ucc.ie having little modifications. A volume of 1 µL of DNA templates was added to the reaction mixture (DyNAzyme $^{TM}$ II, PCR master mix-10 µL each, primers (Forward)-0.5 µL, primers (Reverse)-0.5 µL.) and sterile distilled water was added to each tube to make a final volume of 20 µL. The reactions for the MLST were run under the following conditions: An initial DNA denaturation at 95 $^{o}$C for 5 minutes followed by 26 cycles of DNA denaturation at 95 $^{o}$C for 1 minute. At 56 $^{o}$C (ADK, ICD, MDH, RECA gene amplification), 60 $^{o}$C for (PURA gene amplification) and 62 $^{o}$C for (FUMC and GYRB gene amplification) for 1 minute, annealing of specific primers occurred from which synthesis of DNA started. Elongation or extension of the DNA occurred at 72 $^{o}$C for 1 minute and a final extension followed at 72 $^{o}$C for 10 minutes.

Polymerase chain reaction products were analyzed on a 1% agarose gel. Polymerase chain reaction products were purified for sequencing with Exosap IT (Affymetrix, Santa Clara USA). The forward and reverse DNA strands were sequenced with the same PCR primer sets. Raw sequences were reviewed by visual inspection using the BioEdit software. Sequences of each allele were trimmed and compared to all alleles in the database. New alleles had to be submitted to the curator of the MLST *E. coli* submission page for assignment of new allele and Sequence Type (ST) number.

# CHAPTER FOUR

# RESULTS

## 4.1 Identification of isolates

The cultural and biochemical characteristics of the bacterial isolates produced results that agreed with their identities. The human isolates were previously identified as *E.coli* at the Clinical Microbiology Departments of the Hospitals where they were collected. Both the human and animal isolates were further identified by biochemical tests at the Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria. Tables 4.1 and 4.2 show the characteristics obtained.

The Majority of the human isolates of *Escherichia coli* 31(54%) were from the University College Hospital, Ibadan, Nigeria (Fig 4.1). Most of the samples (n=42, 74%) of the bovine animals were obtained from farms in Ibadan, the capital city of Oyo state, Nigeria (Fig 4.2).

**Table 4.1 Biochemical Characteristics of Human *E. coli* isolates**

| Isolates | G.S | MOR | L.F.T | I.T | M.R.T | V.P.T | C.U.T |
|---|---|---|---|---|---|---|---|
| H1 | Gram-neg | Small rods | + | + | + | - | - |
| 2 | Gram-neg | Small rods | + | + | + | - | - |
| 3 | Gram-neg | Small rods | + | + | + | - | - |
| 4 | Gram-neg | Small rods | + | + | + | - | - |
| 5 | Gram-neg | Small rods | + | + | + | - | - |
| 6 | Gram-neg | Small rods | + | + | + | - | - |
| 7 | Gram-neg | Small rods | + | + | + | - | - |
| 8 | Gram-neg | Small rods | + | + | + | - | - |
| 9 | Gram-neg | Small rods | + | + | + | - | - |
| 10 | Gram-neg | Small rods | + | + | + | - | - |
| 11 | Gram-neg | Small rods | + | + | + | - | - |
| 12 | Gram-neg | Small rods | + | + | + | - | - |
| 13 | Gram-neg | Small rods | + | + | + | - | - |
| 14 | Gram-neg | Small rods | + | + | + | - | - |
| 15 | Gram-neg | Small rods | + | + | + | - | - |
| 16 | Gram-neg | Small rods | + | + | + | - | - |
| 17 | Gram-neg | Small rods | + | + | + | - | - |
| 18 | Gram-neg | Small rods | + | + | + | - | - |
| 19 | Gram-neg | Small rods | + | + | + | - | - |
| 20 | Gram-neg | Small rods | + | + | + | - | - |
| 21 | Gram-neg | Small rods | + | + | + | - | - |
| 22 | Gram-neg | Small rods | + | + | + | - | - |
| 23 | Gram-neg | Small rods | + | + | + | - | - |
| 24 | Gram-neg | Small rods | + | + | + | - | - |
| 25 | Gram-neg | Small rods | + | + | + | - | - |
| 26 | Gram-neg | Small rods | + | + | + | - | - |
| 27 | Gram-neg | Small rods | + | + | + | - | - |
| 28 | Gram-neg | Small rods | + | + | + | - | - |
| 29 | Gram-neg | Small rods | + | + | + | - | - |
| 30 | Gram-neg | Small rods | + | + | + | - | - |

| Isolates | G.S | MOR | L.F.T | I.T | M.R.T | V.P.T | C.U.T |
|---|---|---|---|---|---|---|---|
| 31 | Gram-neg | Small rods | + | + | + | - | - |
| 33 | Gram-neg | Small rods | + | + | + | - | - |
| 34 | Gram-neg | Small rods | + | + | + | - | - |
| 35 | Gram-neg | Small rods | + | + | + | - | - |
| 36 | Gram-neg | Small rods | + | + | + | - | - |
| 37 | Gram-neg | Small rods | + | + | + | - | - |
| 38 | Gram-neg | Small rods | + | + | + | - | - |
| 39 | Gram-neg | Small rods | + | + | + | - | - |
| 40 | Gram-neg | Small rods | + | + | + | - | - |
| 41 | Gram-neg | Small rods | + | + | + | - | - |
| 42 | Gram-neg | Small rods | + | + | + | - | - |
| 43 | Gram-neg | Small rods | + | + | + | - | - |
| 44 | Gram-neg | Small rods | + | + | + | - | - |
| 45 | Gram-neg | Small rods | + | + | + | - | - |
| 46 | Gram-neg | Small rods | + | + | + | - | - |
| 47 | Gram-neg | Small rods | + | + | + | - | - |
| 48 | Gram-neg | Small rods | + | + | + | - | - |
| 49 | Gram-neg | Small rods | + | + | + | - | - |
| 50 | Gram-neg | Small rods | + | + | + | - | - |
| 51 | Gram-neg | Small rods | + | + | + | - | - |
| 52 | Gram-neg | Small rods | + | + | + | - | - |
| 53 | Gram-neg | Small rods | + | + | + | - | - |
| **54** | Gram-neg | Small rods | + | + | + | - | - |
| **55** | Gram-neg | Small rods | + | + | + | - | - |
| **56** | Gram-neg | Small rods | + | + | + | - | - |
| **57** | Gram-neg | Small rods | + | + | + | - | - |
| **58** | Gram-neg | Small rods | + | + | + | - | - |

Legend: GS-Gram-stain, MOR-Morphology, LFT- Lactose fermenting test, IT- Indole test, MRT-Methyl red test, VPT- Voges Proskauer test, CUT- Citrate Utilisation test, Gram-neg- Gram-negative, H-Human isolates

+- Positive, - -Negative

**Table 4.2 Biochemical Characteristics of Animal *E. coli* isolates**

| Isolates | G.S | MOR | L.F.T | I.T | M.R.T | V.P.T | C.U.T |
|---|---|---|---|---|---|---|---|
| A1 | Gram-neg | Small rods | + | + | + | - | - |
| 2 | Gram-neg | Small rods | + | + | + | - | - |
| 3 | Gram-neg | Small rods | + | + | + | - | - |
| 4 | Gram-neg | Small rods | + | + | + | - | - |
| 5 | Gram-neg | Small rods | + | + | + | - | - |
| 6 | Gram-neg | Small rods | + | + | + | - | - |
| 7 | Gram-neg | Small rods | + | + | + | - | - |
| 8 | Gram-neg | Small rods | + | + | + | - | - |
| 9 | Gram-neg | Small rods | + | + | + | - | - |
| 10 | Gram-neg | Small rods | + | + | + | - | - |
| 11 | Gram-neg | Small rods | + | + | + | - | - |
| 12 | Gram-neg | Small rods | + | + | + | - | - |
| 13 | Gram-neg | Small rods | + | + | + | - | - |
| 14 | Gram-neg | Small rods | + | + | + | - | - |
| 15 | Gram-neg | Small rods | + | + | + | - | - |
| 16 | Gram-neg | Small rods | + | + | + | - | - |
| 17 | Gram-neg | Small rods | + | + | + | - | - |
| 18 | Gram-neg | Small rods | + | + | + | - | - |
| 19 | Gram-neg | Small rods | + | + | + | - | - |
| 20 | Gram-neg | Small rods | + | + | + | - | - |
| 21 | Gram-neg | Small rods | + | + | + | - | - |
| 22 | Gram-neg | Small rods | + | + | + | - | - |
| 23 | Gram-neg | Small rods | + | + | + | - | - |
| 24 | Gram-neg | Small rods | + | + | + | - | - |
| 25 | Gram-neg | Small rods | + | + | + | - | - |
| 26 | Gram-neg | Small rods | + | + | + | - | - |
| 27 | Gram-neg | Small rods | + | + | + | - | - |
| 28 | Gram-neg | Small rods | + | + | + | - | - |
| 29 | Gram-neg | Small rods | + | + | + | - | - |
| 30 | Gram-neg | Small rods | + | + | + | - | - |

| Isolates | G.S | MOR | L.F.T | I.T | M.R.T | V.P.T | C.U.T |
|---|---|---|---|---|---|---|---|
| 31 | Gram-neg | Small rods | + | + | + | - | - |
| 33 | Gram-neg | Small rods | + | + | + | - | - |
| 34 | Gram-neg | Small rods | + | + | + | - | - |
| 35 | Gram-neg | Small rods | + | + | + | - | - |
| 36 | Gram-neg | Small rods | + | + | + | - | - |
| 37 | Gram-neg | Small rods | + | + | + | - | - |
| 38 | Gram-neg | Small rods | + | + | + | - | - |
| 39 | Gram-neg | Small rods | + | + | + | - | - |
| 40 | Gram-neg | Small rods | + | + | + | - | - |
| 41 | Gram-neg | Small rods | + | + | + | - | - |
| 42 | Gram-neg | Small rods | + | + | + | - | - |
| 43 | Gram-neg | Small rods | + | + | + | - | - |
| 44 | Gram-neg | Small rods | + | + | + | - | - |
| 45 | Gram-neg | Small rods | + | + | + | - | - |
| 46 | Gram-neg | Small rods | + | + | + | - | - |
| 47 | Gram-neg | Small rods | + | + | + | - | - |
| 48 | Gram-neg | Small rods | + | + | + | - | - |
| 49 | Gram-neg | Small rods | + | + | + | - | - |
| 50 | Gram-neg | Small rods | + | + | + | - | - |
| 51 | Gram-neg | Small rods | + | + | + | - | - |
| 52 | Gram-neg | Small rods | + | + | + | - | - |
| 53 | Gram-neg | Small rods | + | + | + | - | - |
| **54** | Gram-neg | Small rods | + | + | + | - | - |
| **55** | Gram-neg | Small rods | + | + | + | - | - |
| **56** | Gram-neg | Small rods | + | + | + | - | - |
| **57** | Gram-neg | Small rods | + | + | + | - | - |
| **58** | Gram-neg | Small rods | + | + | + | - | - |

Legend: GS-Gram-stain, MOR-Morphology, LFT- Lactose fermenting test, IT- Indole test, MRT-Methyl red test, VPT- Voges Proskauer test, CUT- Citrate Utilisation test, Gram-neg- Gram-negative, H-Human isolates

+- Positive, - -Negative

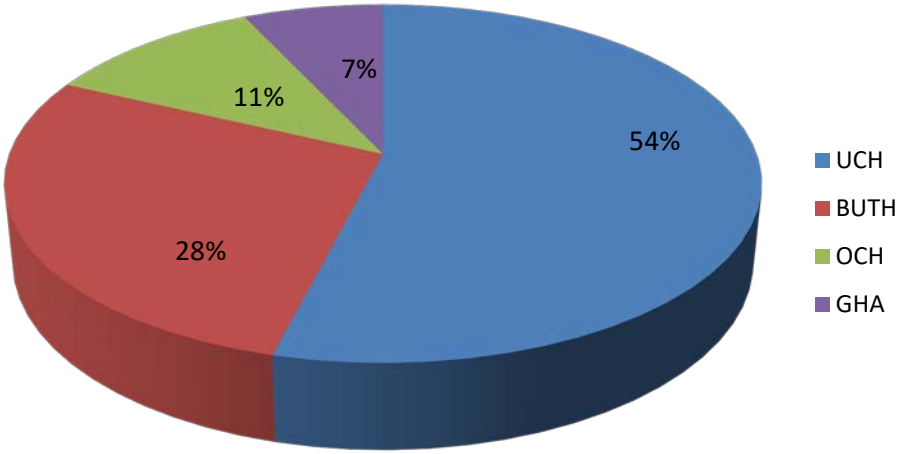



**Fig 4.1 Distribution of human *E.coli* isolates by Hospitals**

UCH, University College Hospital; BUTH, Bowen University Teaching Hospital; GHA, General Hospital Adeoyo; OCH, Oluyoro Catholic Hospital;

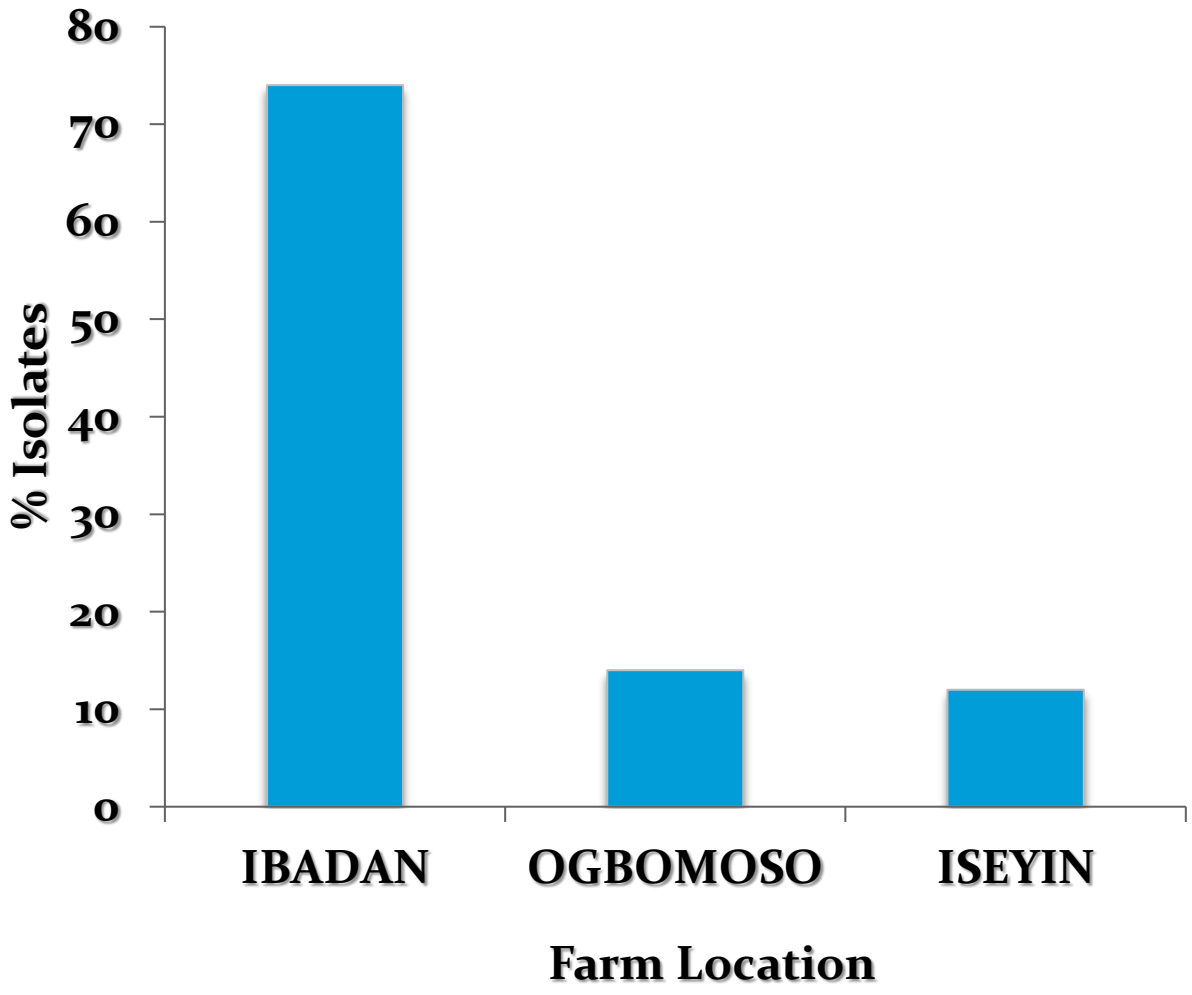


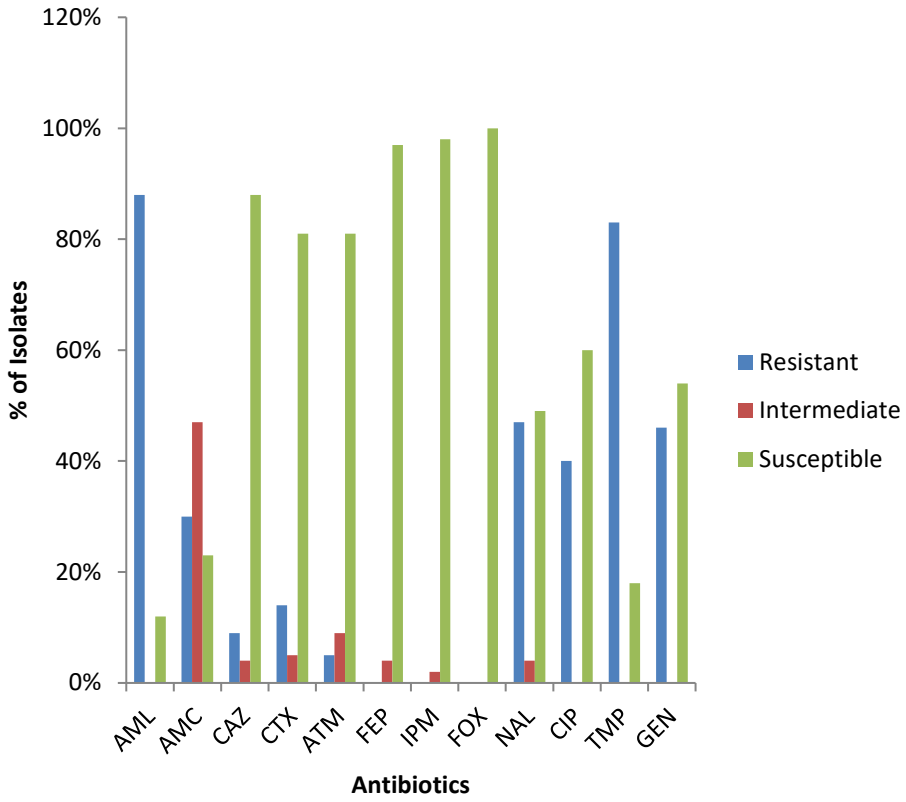

**Fig 4.2 Distribution of bovine isolates of *E.coli* by farm location**

## 4.2 Antimicrobial Susceptibility

### 4.2.1 Disc diffusion Test

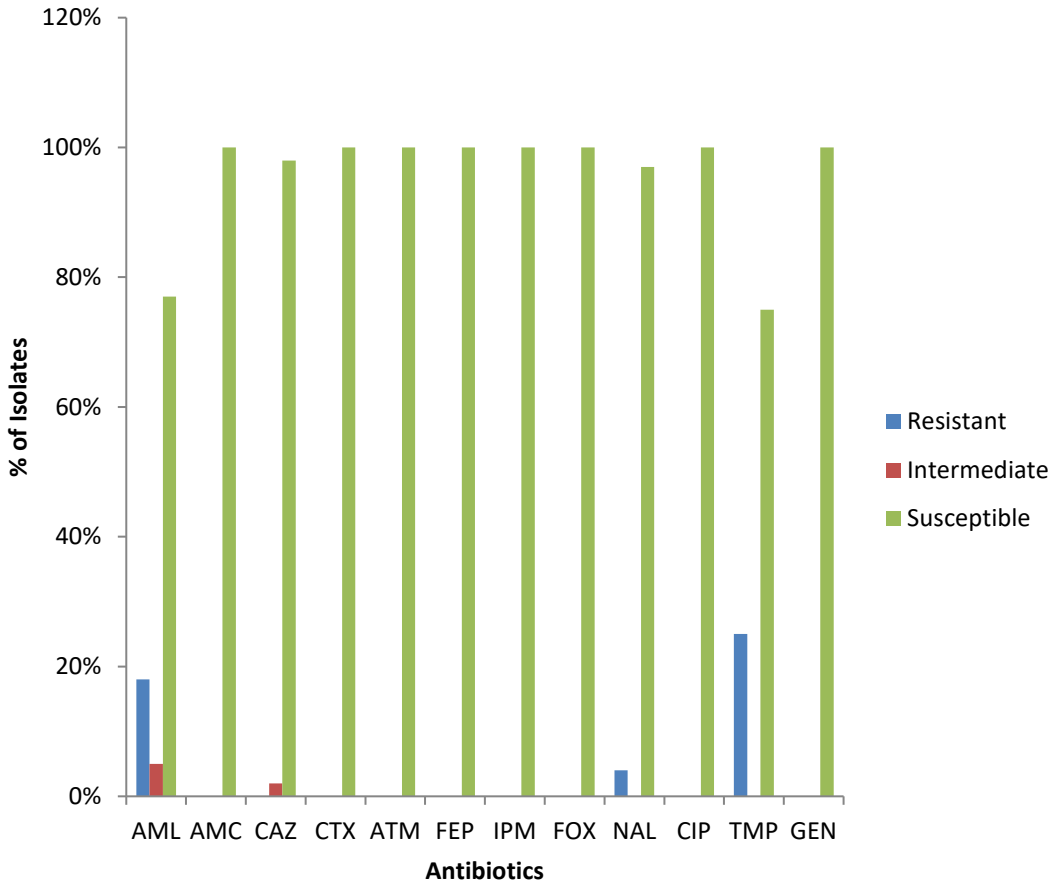
The antimicrobial susceptibilities of the human and bovine *E. coli* isolates are shown in Appendix II-III. Antibiotic susceptibility pattern of the human *E. coli* isolates showed 100% susceptibility to cefoxitine while the bovine *E. coli* isolates had 100% susceptibility to amoxicillin plus clavulanic acid, gentamicin, cefoxitine, ciprofloxacin, imipenem, cefepime, cefotaxime and azetronam (Fig 4.3- 4.4). The human isolates showed a varying level of resistance to most of the antibiotics in the antibiotic susceptibility testing but high prevalence of resistance to amoxicillin (87.7%), trimethoprim (82.5%) and nalidixic acid (47.7%) were remarkable (Fig 4.3). Generally the bovine isolates were more susceptible to the antibiotics (Fig 4.4).

Tables 4.3 and 4.4 show the resistance pattern of the human and bovine *E. coli* isolates. The human *E. coli* isolates had nineteen distinct resistance patterns. The most prevalent resistance pattern by the human *E. coli* isolates was to trimethoprim and amoxicillin followed by ciprofloxacin, nalidixic acid, trimethoprim, gentamicin and amoxicillin. Five resistance patterns were shown by the bovine *E. coli* isolates. The most prevalent resistance pattern by the bovine *E. coli* isolates was to trimethoprim only as well as trimethoprim and amoxicillin. Multi-resistant isolates were high among the human isolates (n=30, 53%) compared with the bovine isolates (n=9, 16%) (Fig 4.5). There was no relationship in the antibiotic susceptibility pattern of the bovine and human *E. coli* isolates (Table 4.5- 4.9, Appendix IV)




Legend- AML- Amoxicillin, AMC-Amoxicillin-clavulanic acid, CAZ-Ceftazidime, CTX-Cefotaxime, ATM-Azetronam, FEP-Cefepime, IPM-Imipenem, FOX-Cefoxitine, NAL-Nalidixic acid, CIP-Ciprofloxacin, TMP-Trimethoprim, GEN-Gentamicin

**Fig 4.3 Distribution of human *E. coli* isolates by antibiotic susceptibility pattern**




Legend- AML- Amoxicillin, AMC-Amoxicillin-clavulanic acid, CAZ-Ceftazidime, CTX-Cefotaxime, ATM-Azetronam, FEP-Cefepime, IPM-Imipenem, FOX-Cefoxitine, NAL-Nalidixic acid, CIP-Ciprofloxacin, TMP-Trimethoprim, GEN-Gentamicin

**Fig 4.4 Distribution of bovine *E. coli* isolates by antibiotic susceptibility pattern**

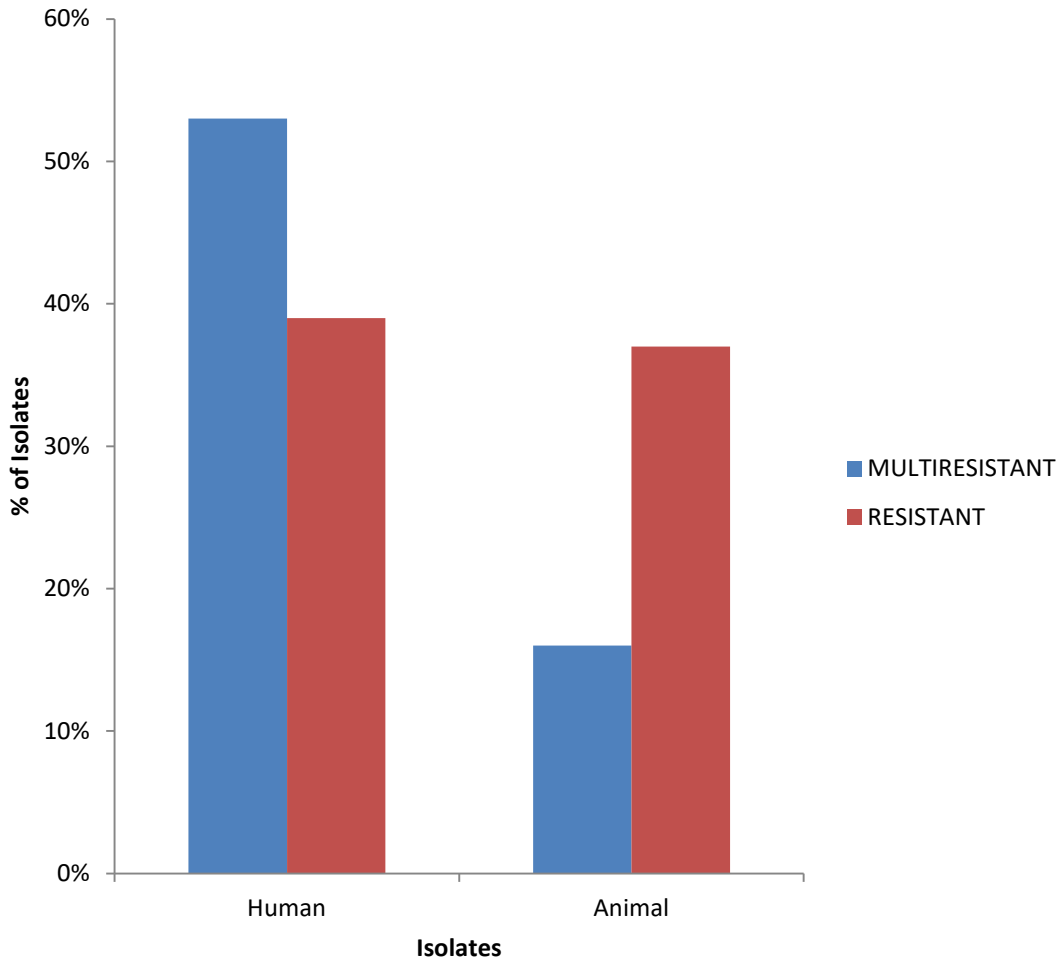



**Fig 4.5 Distribution of human and bovine *E. coli* isolates by antimicrobial resistance pattern**

**Table 4.3 Resistance pattern of the Human *E. coli* isolates**

| ISOLATE | RESISTANCE PATTERN |
|---|---|
| H1 | CAZ-CTX-ATM-TMP-GEN-AML |
| 2 | - |
| 3 | CIP-NAL-TMP-GEN-AML |
| 4 | CIP-NAL-TMP |
| 5 | - |
| 6 | NAL-TMP-AML |
| 7 | CIP-NAL-TMP-GEN-AML |
| 8 | CIP-NAL-TMP-GEN-AML |
| 9 | CIP-NAL-TMP-GEN-AML |
| 10 | AML |
| 11 | TMP-AML |
| 12 | CAZ-CTX-ATM- CIP-NAL-TMP-GEN-AML |
| 13 | CAZ-CTX- CIP-NAL-TMP-GEN-AML |
| 14 | AML |
| 15 | CAZ-CTX-AMC-ATM- CIP-NAL-TMP-GEN-AML |
| 16 | - |
| 17 | TMP-AML |
| 18 | - |
| 19 | TMP-AML |
| 20 | AMC-CIP-NAL-TMP-AML |
| 21 | AMC-AML |
| 22 | CAZ-CTX-AMC- CIP-NAL-TMP-GEN-AML |
| 23 | AMC- CIP-NAL-TMP-GEN-AML |
| 24 | AMC- CIP-NAL-TMP-GEN-AML |
| 25 | AMC- CIP-NAL-TMP-GEN-AML |
| 26 | CIP-NAL-TMP-GEN-AML |
| 27 | AMC- CIP-NAL-TMP-GEN-AML |
| 28 | AMC- CIP-NAL-TMP-GEN-AML |
| 29 | AMC- CIP-NAL-TMP-GEN-AML |
| 30 | TMP-AML |
| 31 | TMP-AML |
| 32 | CIP-NAL-TMP-GEN-AML |
| 33 | CIP-NAL-TMP-GEN-AML |
| 34 | CIP-NAL-TMP-GEN-AML |
| 35 | CIP-NAL-TMP-GEN-AML |
| 36 | AMC-NAL-TMP-GEN-AML |

| ISOLATE | RESISTANCE PATTERN |
|---|---|
| H37 | AMC-NAL-TMP-GEN-AML |
| 38 | TMP-AML |
| 39 | TMP-AML |
| 40 | AMC-TMP-AML |
| 41 | AMC-TMP-AML |
| 42 | AML |
| 44 | TMP-AML |
| 45 | CTX-TMP-GEN-AML |
| 46 | TMP-AML |
| 47 | CTX-AMC-TMP-GEN-AML |
| 48 | TMP-AML |
| 49 | - |
| 50 | TMP-AML |
| 51 | CIP-NAL-TMP-GEN-AML |
| 52 | CIP-NAL-TMP-GEN-AML |
| 53 | TMP |
| 54 | AMC-TMP-AML |
| 55 | AMC-NAL-TMP-AML |
| 56 | AML |
| 57 | TMP-AML |
| 58 | TMP-AML |

Legend- AML- Amoxicillin, AMC-Amoxicillin-clavulanic acid, CAZ-Ceftazidime, CTX-Cefotaxime, ATM-Azetronam, FEP-Cefepime, IPM-Imipenem, FOX-Cefoxitine, NAL-Nalidixic acid, CIP-Ciprofloxacin, TMP-Trimethoprim, GEN-Gentamicin, H-Human, - stands for No resistance in isolate.

**Table 4.4 Resistance pattern of the Bovine *E. coli* isolates**

| ISOLATE | RESISTANCE PATTERN |
|---|---|
| A1 | - |
| 2 | - |
| 3 | - |
| 4 | - |
| 5 | AML |
| 6 | AML |
| 7 | - |
| 8 | - |
| 9 | - |
| 10 | - |
| 11 | - |
| 12 | - |
| 13 | - |
| 14 | - |
| 15 | TMP |
| 17 | - |
| 18 | TMP |
| 19 | TMP |
| 20 | TMP |
| 21 | - |
| 22 | AML |
| 23 | - |
| 24 | - |
| 25 | - |
| 26 | - |
| 27 | - |
| 28 | NAL-TMP |
| 29 | - |
| 30 | - |
| 31 | TMP-AML |
| 32 | - |
| 33 | - |
| 34 | - |
| 35 | - |
| 36 | TMP-AML |
| 37 | - |

| ISOLATE | RESISTANCE PATTERN |
|---|---|
| A38 | - |
| 39 | TMP-AML |
| 40 | TMP-AML |
| 41 | TMP-AML |
| 42 | NAL-TMP-AML |
| 43 | - |
| 44 | - |
| 45 | - |
| 46 | TMP |
| 47 | - |
| 48 | - |
| 49 | - |
| 50 | - |
| 51 | - |
| 52 | - |
| 53 | - |
| 54 | TMP-AML |
| 55 | TMP |
| 56 | - |
| 57 | - |
| 58 | - |

Legend- AML- Amoxicillin, AMC-Amoxicillin-clavulanic acid, CAZ-Ceftazidime, CTX-Cefotaxime, ATM-Azetronam, FEP-Cefepime, IPM-Imipenem, FOX-Cefoxitine, NAL-Nalidixic acid, CIP-Ciprofloxacin, TMP-Trimethoprim, GEN-Gentamicin, A-Animal, - stands for No resistance in isolate.

**Table 4.5: Correlation coefficient between the susceptibility pattern of the bovine and human *E. coli* isolates to Gentamicin**

| Isolate Number | X (Zone of inhibition for bovine isolates) | Y (Zone of inhibition for human isolates) | $X^2$ | $Y^2$ | XY |
|---|---|---|---|---|---|
| 1 | 29 | 0 | 841 | 0 | 0 |
| 2 | 27 | 26 | 729 | 676 | 702 |
| 3 | 26 | 0 | 676 | 0 | 0 |
| 4 | 26 | 23 | 676 | 529 | 598 |
| 5 | 27 | 26 | 729 | 676 | 702 |
| 6 | 24 | 15 | 576 | 225 | 360 |
| 7 | 28 | 0 | 784 | 0 | 0 |
| 8 | 27 | 0 | 729 | 0 | 0 |
| 9 | 26 | 0 | 676 | 0 | 0 |
| 10 | 26 | 24 | 676 | 576 | 624 |
| 11 | 26 | 29 | 676 | 841 | 754 |
| 12 | 26 | 0 | 676 | 0 | 0 |
| 13 | 27 | 0 | 729 | 0 | 0 |
| 14 | 26 | 29 | 676 | 841 | 754 |
| 15 | 28 | 0 | 784 | 0 | 0 |
| 16 | 29 | 25 | 841 | 625 | 725 |
| 17 | 27 | 25 | 729 | 625 | 675 |
| 18 | 22 | 28 | 484 | 784 | 616 |
| 19 | 25 | 21 | 625 | 441 | 525 |
| 20 | 24 | 27 | 576 | 729 | 648 |
| 21 | 27 | 27 | 729 | 729 | 729 |
| 22 | 26 | 0 | 676 | 0 | 0 |
| 23 | 26 | 0 | 676 | 0 | 0 |
| 24 | 28 | 0 | 784 | 0 | 0 |
| 25 | 25 | 0 | 625 | 0 | 0 |
| 26 | 30 | 0 | 900 | 0 | 0 |
| 27 | 28 | 0 | 784 | 0 | 0 |

| Isolate Number | X (Zone of inhibition for | Y (Zone of inhibition for | $X^2$ | $Y^2$ | XY |
|---|---|---|---|---|---|

| | bovine isolates) | human isolates) | | | |
|---|---|---|---|---|---|
| 28 | 25 | 0 | 625 | 0 | 0 |
| 29 | 26 | 0 | 676 | 0 | 0 |
| 30 | 26 | 26 | 676 | 676 | 676 |
| 31 | 26 | 27 | 676 | 729 | 702 |
| 32 | 29 | 0 | 841 | 0 | 0 |
| 33 | 20 | 0 | 400 | 0 | 0 |
| 34 | 22 | 0 | 484 | 0 | 0 |
| 35 | 26 | 0 | 676 | 0 | 0 |
| 36 | 29 | 0 | 841 | 0 | 0 |
| 37 | 23 | 0 | 529 | 0 | 0 |
| 38 | 28 | 27 | 784 | 729 | 756 |
| 39 | 27 | 23 | 729 | 529 | 621 |
| 40 | 29 | 24 | 841 | 576 | 696 |
| 41 | 28 | 29 | 784 | 841 | 812 |
| 42 | 25 | 27 | 625 | 729 | 675 |
| 43 | 25 | 27 | 625 | 729 | 675 |
| 44 | 27 | 0 | 729 | 0 | 0 |
| 45 | 27 | 26 | 729 | 676 | 702 |
| 46 | 25 | 0 | 625 | 0 | 0 |
| 47 | 25 | 27 | 625 | 729 | 675 |
| 48 | 27 | 25 | 729 | 625 | 675 |
| 49 | 26 | 25 | 676 | 625 | 650 |
| 50 | 26 | 0 | 676 | 0 | 0 |
| 51 | 25 | 0 | 625 | 0 | 0 |
| 52 | 22 | 28 | 484 | 784 | 616 |
| 53 | 27 | 23 | 729 | 529 | 621 |
| 54 | 26 | 22 | 676 | 484 | 572 |
| 55 | 25 | 25 | 625 | 625 | 625 |
| 56 | 23 | 21 | 529 | 441 | 483 |
| 57 | 26 | 22 | 676 | 484 | 572 |
| **n = 57** | **1487 ∑X** | **779 ∑Y** | **39007 $\sum X^2$** | **19837 $\sum Y^2$** | **20216 ∑XY** |

$$r = \frac{n(\Sigma XY)-(\Sigma X)(\Sigma Y)}{\sqrt{\{ n (\Sigma X^2) - (\Sigma X)^2\} \{n (\Sigma Y^2)-(\Sigma Y)^2\}}}$$

Coefficient of correlation, r = -0.075721659

**Table 4.6: Correlation coefficient between the susceptibility pattern of the bovine and human *E. coli* isolates to Ciprofloxacin**

| Isolate Number | X (Zone of inhibition for bovine isolates) | Y (Zone of inhibition for human isolates) | $X^2$ | $Y^2$ | XY |
|---|---|---|---|---|---|
| 1 | 32 | 22 | 1024 | 484 | 704 |
| 2 | 34 | 30 | 1156 | 900 | 1020 |
| 3 | 31 | 0 | 961 | 0 | 0 |
| 4 | 32 | 0 | 1024 | 0 | 0 |
| 5 | 30 | 31 | 900 | 961 | 930 |
| 6 | 31 | 26 | 961 | 676 | 806 |
| 7 | 32 | 0 | 1024 | 0 | 0 |
| 8 | 30 | 0 | 900 | 0 | 0 |
| 9 | 34 | 0 | 1156 | 0 | 0 |
| 10 | 32 | 32 | 1024 | 1024 | 1024 |
| 11 | 32 | 34 | 1024 | 1156 | 1088 |
| 12 | 34 | 0 | 1156 | 0 | 0 |
| 13 | 30 | 0 | 900 | 0 | 0 |
| 14 | 32 | 30 | 1024 | 900 | 960 |
| 15 | 34 | 0 | 1156 | 0 | 0 |
| 16 | 29 | 33 | 841 | 1089 | 957 |
| 17 | 36 | 34 | 1296 | 1156 | 1224 |
| 18 | 29 | 35 | 841 | 1225 | 1015 |
| 19 | 32 | 32 | 1024 | 1024 | 1024 |
| 20 | 30 | 0 | 900 | 0 | 0 |
| 21 | 30 | 35 | 900 | 1225 | 1050 |
| 22 | 29 | 0 | 841 | 0 | 0 |
| 23 | 34 | 0 | 1156 | 0 | 0 |
| 24 | 30 | 0 | 900 | 0 | 0 |
| 25 | 31 | 0 | 961 | 0 | 0 |
| 26 | 33 | 0 | 1089 | 0 | 0 |
| 27 | 29 | 0 | 841 | 0 | 0 |
| 28 | 32 | 0 | 1024 | 0 | 0 |
| 29 | 31 | 0 | 961 | 0 | 0 |
| 30 | 31 | 29 | 961 | 841 | 899 |
| 31 | 32 | 31 | 1024 | 961 | 992 |

| | X (Zone of inhibition for bovine isolates) | Y (Zone of inhibition for human isolates) | $X^2$ | $Y^2$ | XY |
|---|---|---|---|---|---|
| 32 | 34 | 0 | 1156 | 0 | 0 |
| 33 | 28 | 0 | 784 | 0 | 0 |
| 34 | 31 | 0 | 961 | 0 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| 35 | 27 | 0 | 729 | 0 | 0 |
| 36 | 34 | 29 | 1156 | 841 | 986 |
| 37 | 33 | 24 | 1089 | 576 | 792 |
| 38 | 30 | 30 | 900 | 900 | 900 |
| 39 | 32 | 30 | 1024 | 900 | 960 |
| 40 | 29 | 29 | 841 | 841 | 841 |
| 41 | 33 | 33 | 1089 | 1089 | 1089 |
| 42 | 30 | 32 | 900 | 1024 | 960 |
| 43 | 30 | 30 | 900 | 900 | 900 |
| 44 | 35 | 24 | 1225 | 576 | 840 |
| 45 | 34 | 34 | 1156 | 1156 | 1156 |
| 46 | 30 | 25 | 900 | 625 | 750 |
| 47 | 30 | 35 | 900 | 1225 | 1050 |
| 48 | 31 | 33 | 961 | 1089 | 1023 |
| 49 | 32 | 33 | 1024 | 1089 | 1056 |
| 50 | 35 | 0 | 1225 | 0 | 0 |
| 51 | 33 | 0 | 1089 | 0 | 0 |
| 52 | 33 | 33 | 1089 | 1089 | 1089 |
| 53 | 34 | 30 | 1156 | 900 | 1020 |
| 54 | 33 | 22 | 1089 | 484 | 726 |
| 55 | 30 | 30 | 900 | 900 | 900 |
| 56 | 31 | 34 | 961 | 1156 | 1054 |
| 57 | 35 | 32 | 1225 | 1024 | 1120 |
| | **1805** | **1036** | **57379** | **32006** | **32905** |
| **n = 57** | **∑X** | **∑Y** | **$\sum X^2$** | **$\sum Y^2$** | **∑XY** |

$$r = \frac{n(\Sigma XY)-(\Sigma X)(\Sigma Y)}{\sqrt{\{n(\Sigma X^2)-(\Sigma X)^2\}\{n(\Sigma Y^2)-(\Sigma Y)^2\}}}$$

Coefficient of correlation, r = 0.057668221

**Table 4.7: Correlation coefficient between the susceptibility pattern of the bovine and human *E. coli* isolates to Amoxicillin**

| Isolate Number | X (Zone of inhibition for bovine isolates) | Y (Zone of inhibition for human isolates) | $X^2$ | $Y^2$ | XY |
|---|---|---|---|---|---|
| 1 | 21 | 0 | 441 | 0 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| 2 | 23 | 24 | 529 | 576 | 552 |
| 3 | 22 | 0 | 484 | 0 | 0 |
| 4 | 20 | 20 | 400 | 400 | 400 |
| 5 | 0 | 26 | 0 | 676 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 |
| 7 | 22 | 0 | 484 | 0 | 0 |
| 8 | 24 | 0 | 576 | 0 | 0 |
| 9 | 22 | 0 | 484 | 0 | 0 |
| 10 | 23 | 0 | 529 | 0 | 0 |
| 11 | 25 | 0 | 625 | 0 | 0 |
| 12 | 28 | 0 | 784 | 0 | 0 |
| 13 | 27 | 0 | 729 | 0 | 0 |
| 14 | 25 | 0 | 625 | 0 | 0 |
| 15 | 25 | 0 | 625 | 0 | 0 |
| 16 | 24 | 24 | 576 | 576 | 576 |
| 17 | 27 | 0 | 729 | 0 | 0 |
| 18 | 16 | 29 | 256 | 841 | 464 |
| 19 | 14 | 0 | 196 | 0 | 0 |
| 20 | 23 | 0 | 529 | 0 | 0 |
| 21 | 11 | 0 | 121 | 0 | 0 |
| 22 | 23 | 0 | 529 | 0 | 0 |
| 23 | 25 | 0 | 625 | 0 | 0 |
| 24 | 25 | 0 | 625 | 0 | 0 |
| 25 | 24 | 0 | 576 | 0 | 0 |
| 26 | 27 | 0 | 729 | 0 | 0 |
| 27 | 29 | 0 | 841 | 0 | 0 |
| 28 | 25 | 0 | 625 | 0 | 0 |
| 29 | 25 | 0 | 625 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 0 |
| 31 | 23 | 0 | 529 | 0 | 0 |
| 32 | 22 | 0 | 484 | 0 | 0 |

| **Isolate Number** | **X (Zone of inhibition for bovine isolates)** | **Y (Zone of inhibition for human isolates)** | **$X^2$** | **$Y^2$** | **XY** |
|---|---|---|---|---|---|
| 33 | 26 | 0 | 676 | 0 | 0 |
| 34 | 25 | 0 | 625 | 0 | 0 |
| 35 | 0 | 0 | 0 | 0 | 0 |
| 36 | 26 | 0 | 676 | 0 | 0 |
| 37 | 22 | 0 | 484 | 0 | 0 |
| 38 | 0 | 0 | 0 | 0 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| 39 | 11 | 0 | 121 | 0 | 0 |
| 40 | 0 | 0 | 0 | 0 | 0 |
| 41 | 0 | 0 | 0 | 0 | 0 |
| 42 | 24 | 0 | 576 | 0 | 0 |
| 43 | 21 | 0 | 441 | 0 | 0 |
| 44 | 28 | 0 | 784 | 0 | 0 |
| 45 | 26 | 0 | 676 | 0 | 0 |
| 46 | 25 | 0 | 625 | 0 | 0 |
| 47 | 24 | 0 | 576 | 0 | 0 |
| 48 | 25 | 24 | 625 | 576 | 600 |
| 49 | 24 | 0 | 576 | 0 | 0 |
| 50 | 21 | 0 | 441 | 0 | 0 |
| 51 | 24 | 0 | 576 | 0 | 0 |
| 52 | 14 | 23 | 196 | 529 | 322 |
| 53 | 0 | 0 | 0 | 0 | 0 |
| 54 | 29 | 0 | 841 | 0 | 0 |
| 55 | 25 | 0 | 625 | 0 | 0 |
| 56 | 25 | 0 | 625 | 0 | 0 |
| 57 | 27 | 0 | 729 | 0 | 0 |
| | **1142** | **170** | **27404** | **4174** | **2914** |
| **n = 57** | **∑X** | **∑Y** | **$\sum X^2$** | **$\sum Y^2$** | **∑XY** |

$$r = \frac{n(\Sigma XY)-(\Sigma X)(\Sigma Y)}{\sqrt{\{n(\Sigma X^2)-(\Sigma X)^2\}\{n(\Sigma Y^2)-(\Sigma Y)^2\}}}$$

Coefficient of correlation, r = -0.120787442

**Table 4.8: Correlation coefficient between the susceptibility pattern of the bovine and human *E. coli* isolates to Ceftazidime**

| Isolate Number | X (Zone of inhibition for bovine isolates) | Y (Zone of inhibition for human isolates) | $X^2$ | $Y^2$ | XY |
|---|---|---|---|---|---|
| 1 | 27 | 14 | 729 | 196 | 378 |
| 2 | 31 | 27 | 961 | 729 | 837 |
| 3 | 30 | 27 | 900 | 729 | 810 |

| | | | | | |
|---|---|---|---|---|---|
| 4 | 30 | 24 | 900 | 576 | 720 |
| 5 | 29 | 25 | 841 | 625 | 725 |
| 6 | 28 | 18 | 784 | 324 | 504 |
| 7 | 28 | 27 | 784 | 729 | 756 |
| 8 | 30 | 25 | 900 | 625 | 750 |
| 9 | 26 | 21 | 676 | 441 | 546 |
| 10 | 32 | 25 | 1024 | 625 | 800 |
| 11 | 30 | 26 | 900 | 676 | 780 |
| 12 | 30 | 12 | 900 | 144 | 360 |
| 13 | 25 | 16 | 625 | 256 | 400 |
| 14 | 30 | 21 | 900 | 441 | 630 |
| 15 | 28 | 0 | 784 | 0 | 0 |
| 16 | 30 | 26 | 900 | 676 | 780 |
| 17 | 29 | 26 | 841 | 676 | 754 |
| 18 | 31 | 25 | 961 | 625 | 775 |
| 19 | 29 | 27 | 841 | 729 | 783 |
| 20 | 27 | 24 | 729 | 576 | 648 |
| 21 | 30 | 22 | 900 | 484 | 660 |
| 22 | 30 | 14 | 900 | 196 | 420 |
| 23 | 30 | 25 | 900 | 625 | 750 |
| 24 | 30 | 25 | 900 | 625 | 750 |
| 25 | 27 | 26 | 729 | 676 | 702 |
| 26 | 31 | 24 | 961 | 576 | 744 |
| 27 | 33 | 2 | 1089 | 4 | 66 |
| 28 | 32 | 26 | 1024 | 676 | 832 |
| 29 | 33 | 27 | 1089 | 729 | 891 |
| 30 | 29 | 25 | 841 | 625 | 725 |
| 31 | 26 | 26 | 676 | 676 | 676 |
| 32 | 25 | 22 | 625 | 484 | 550 |

| **Isolate Number** | **X (Zone of inhibition for bovine isolates)** | **Y (Zone of inhibition for human isolates)** | **$X^2$** | **$Y^2$** | **XY** |
|---|---|---|---|---|---|
| 33 | 30 | 25 | 900 | 625 | 750 |
| 34 | 33 | 24 | 1089 | 576 | 792 |
| 35 | 29 | 26 | 841 | 676 | 754 |
| 36 | 29 | 28 | 841 | 784 | 812 |
| 37 | 31 | 26 | 961 | 676 | 806 |

| | | | | | |
|---|---|---|---|---|---|
| 38 | 30 | 25 | 900 | 625 | 750 |
| 39 | 29 | 26 | 841 | 676 | 754 |
| 40 | 29 | 28 | 841 | 784 | 812 |
| 41 | 26 | 28 | 676 | 784 | 728 |
| 42 | 29 | 26 | 841 | 676 | 754 |
| 43 | 29 | 28 | 841 | 784 | 812 |
| 44 | 27 | 21 | 729 | 441 | 567 |
| 45 | 27 | 30 | 729 | 900 | 810 |
| 46 | 30 | 20 | 900 | 400 | 600 |
| 47 | 31 | 25 | 961 | 625 | 775 |
| 48 | 34 | 25 | 1156 | 625 | 850 |
| 49 | 28 | 30 | 784 | 900 | 840 |
| 50 | 30 | 29 | 900 | 841 | 870 |
| 51 | 31 | 27 | 961 | 729 | 837 |
| 52 | 30 | 28 | 900 | 784 | 840 |
| 53 | 29 | 27 | 841 | 729 | 783 |
| 54 | 30 | 30 | 900 | 900 | 900 |
| 55 | 30 | 27 | 900 | 729 | 810 |
| 56 | 30 | 30 | 900 | 900 | 900 |
| 57 | 29 | 29 | 841 | 841 | 841 |
| | **1676** | **1368** | **49488** | **34784** | **40249** |
| **n = 57** | **∑X** | **∑Y** | **$\sum X^2$** | **$\sum Y^2$** | **∑XY** |

$$r = \frac{n(\Sigma XY)-(\Sigma X)(\Sigma Y)}{\sqrt{\{n(\Sigma X^2)-(\Sigma X)^2\}\{n(\Sigma Y^2)-(\Sigma Y)^2\}}}$$

Coefficient of correlation, r = 0.039261029

**Table 4.9: Correlation coefficient between the susceptibility pattern of the bovine and human *E. coli* isolates to Cefotaxime**

| Isolate Number | X (Zone of inhibition for bovine isolates) | Y (Zone of inhibition for human isolates) | $X^2$ | $Y^2$ | XY |
|---|---|---|---|---|---|
| 1 | 31 | 0 | 961 | 0 | 0 |
| 2 | 33 | 30 | 1089 | 900 | 990 |

| | | | | | |
|---|---|---|---|---|---|
| 3 | 35 | 29 | 1225 | 841 | 1015 |
| 4 | 30 | 26 | 900 | 676 | 780 |
| 5 | 34 | 30 | 1156 | 900 | 1020 |
| 6 | 30 | 10 | 900 | 100 | 300 |
| 7 | 32 | 28 | 1024 | 784 | 896 |
| 8 | 34 | 30 | 1156 | 900 | 1020 |
| 9 | 29 | 25 | 841 | 625 | 725 |
| 10 | 35 | 30 | 1225 | 900 | 1050 |
| 11 | 33 | 28 | 1089 | 784 | 924 |
| 12 | 33 | 11 | 1089 | 121 | 363 |
| 13 | 30 | 15 | 900 | 225 | 450 |
| 14 | 32 | 28 | 1024 | 784 | 896 |
| 15 | 31 | 0 | 961 | 0 | 0 |
| 16 | 32 | 28 | 1024 | 784 | 896 |
| 17 | 34 | 30 | 1156 | 900 | 1020 |
| 18 | 32 | 31 | 1024 | 961 | 992 |
| 19 | 32 | 30 | 1024 | 900 | 960 |
| 20 | 30 | 29 | 900 | 841 | 870 |
| 21 | 32 | 26 | 1024 | 676 | 832 |
| 22 | 33 | 13 | 1089 | 169 | 429 |
| 23 | 33 | 25 | 1089 | 625 | 825 |
| 24 | 30 | 29 | 900 | 841 | 870 |
| 25 | 32 | 26 | 1024 | 676 | 832 |
| 26 | 33 | 29 | 1089 | 841 | 957 |
| 27 | 32 | 27 | 1024 | 729 | 864 |
| 28 | 31 | 26 | 961 | 676 | 806 |
| 29 | 35 | 28 | 1225 | 784 | 980 |
| 30 | 30 | 30 | 900 | 900 | 900 |
| 31 | 27 | 28 | 729 | 784 | 756 |
| 32 | 25 | 26 | 625 | 676 | 650 |

| **Isolate Number** | **X (Zone of inhibition for bovine isolates)** | **Y (Zone of inhibition for human isolates)** | **$X^2$** | **$Y^2$** | **XY** |
|---|---|---|---|---|---|
| 33 | 32 | 27 | 1024 | 729 | 864 |
| 34 | 32 | 25 | 1024 | 625 | 800 |
| 35 | 32 | 30 | 1024 | 900 | 960 |
| 36 | 30 | 30 | 900 | 900 | 900 |
| 37 | 33 | 29 | 1089 | 841 | 957 |
| 38 | 33 | 28 | 1089 | 784 | 924 |

| | | | | | |
|---|---|---|---|---|---|
| 39 | 29 | 30 | 841 | 900 | 870 |
| 40 | 30 | 30 | 900 | 900 | 900 |
| 41 | 27 | 29 | 729 | 841 | 783 |
| 42 | 29 | 29 | 841 | 841 | 841 |
| 43 | 30 | 30 | 900 | 900 | 900 |
| 44 | 29 | 0 | 841 | 0 | 0 |
| 45 | 30 | 33 | 900 | 1089 | 990 |
| 46 | 32 | 0 | 1024 | 0 | 0 |
| 47 | 30 | 29 | 900 | 841 | 870 |
| 48 | 32 | 28 | 1024 | 784 | 896 |
| 49 | 32 | 29 | 1024 | 841 | 928 |
| 50 | 33 | 28 | 1089 | 784 | 924 |
| 51 | 33 | 26 | 1089 | 676 | 858 |
| 52 | 32 | 29 | 1024 | 841 | 928 |
| 53 | 33 | 30 | 1089 | 900 | 990 |
| 54 | 31 | 30 | 961 | 900 | 930 |
| 55 | 31 | 29 | 961 | 841 | 899 |
| 56 | 34 | 30 | 1156 | 900 | 1020 |
| 57 | 32 | 30 | 1024 | 900 | 960 |
| | **1796** | **1449** | **56814** | **40761** | **45760** |
| **n = 57** | **∑X** | **∑Y** | **∑X²** | **∑Y²** | **∑XY** |

$$r = \frac{n(\Sigma XY)-(\Sigma X)(\Sigma Y)}{\sqrt{\{n(\Sigma X^2)-(\Sigma X)^2\}\{n(\Sigma Y^2)-(\Sigma Y)^2\}}}$$

Coefficient of correlation, r = 0.110617033

### 4.2.2 Phenotypic detection of ESBLs

ESBLs were detected only in the human clinical *E. coli* isolates. Eight out of the fifty seven human isolates were positive for the double disc synergy test by the enhancement of inhibition zones of either or both of the cephalosporins, cefotaxime and ceftazidime towards the amoxicillin-clavulanic acid disc (Appendix III, Table 4.11). The increase occurred because the clavulanic acid present in the amoxicillin/clavulanic acid disc inactivates the ESBL produced by the test organism. Fourteen percent of the clinical *E. coli* isolates showed resistance to cefotaxime with reduced susceptibility to ceftazidime in some of the isolates. The ESBL producing isolates were predominantly isolated from

urine samples followed by high vaginal swabs. ESBL positive strains were also resistant to gentamicin (87.5%), nalidixic acid (62.5%) and ciprofloxacin (50%) but susceptible to cefoxitin and imipenem (100%).

### 4.2.3 Minimum Inhibitory Concentrations of Human ESBL producing *E. coli* isolates

MIC range for the eight human isolates to the β-lactams used was 6->256 µg/ml and 0.047->256 µg/ml for the bovine isolates. The MICs of the human isolates to Levofloxacin ranged from 0.125->32 and 0.012-0.064 µg/ml for the bovine isolates. The human isolates showed a MIC range of 12-32 µg/ml for gentamicin compared to the bovine isolates that had a MIC range of 0.125-0.25 µg/ml (Table 4.10). MIC results confirmed data from antibiotic susceptibility tests showing the high resistance rates among the human isolates especially the ESBL producing isolates.

**Table 4.10 MIC Results by Human ESBL producing *E. coli* isolates**

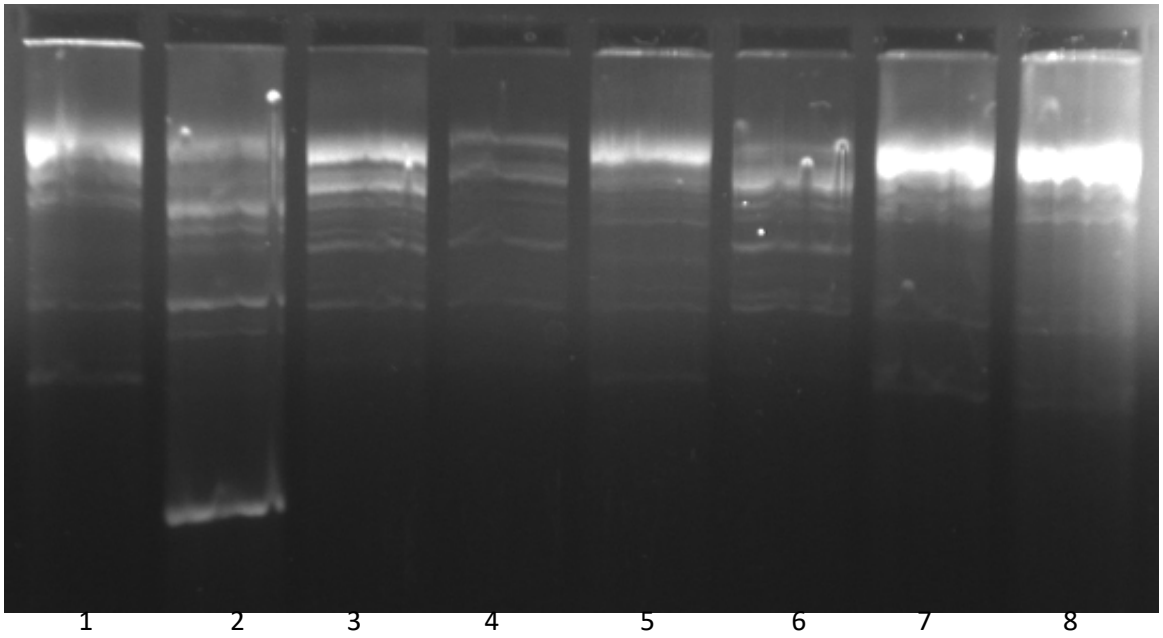
| Human ESBL *E. coli* Isolates | Ampicillin (µg/ml) | Cefotaxime (µg/ml) | Ceftazidime (µg/ml) | Levofloxacin (µg/ml) | Gentamicin (µg/ml) |
|---|---|---|---|---|---|
| **H1** | >256 | 128 | 12 | 0.125 | 12 |
| **H6** | >256 | 64 | 6 | 0.125 | 12 |
| **H12** | >256 | 48 | 24 | 8 | 16 |
| **H13** | >256 | 32 | 24 | 8 | 24 |
| **H15** | >256 | 64 | 24 | >32 | 32 |
| **H22** | >256 | 96 | 24 | 6 | 12 |
| **H45** | >256 | 128 | 6 | 8 | 16 |
| **H47** | >256 | 64 | 6 | 0.125 | 32 |

## 4.3 Typing of *E.coli* ESBL producers

BOX-PCR identified three DNA fingerprint patterns among the ESBL positive isolates. The Human *E. coli* isolate H1, 12, 15, 45 and 47 had a unique DNA fingerprint pattern as well as Human *E. coli* isolate H13 and 22. The Human *E. coli* isolate H6 also had a distinct DNA fingerprint pattern (Fig 4.6). The ESBL producing isolates were from two different hospitals in Oyo state with UCH having the highest number of isolates (n=7). Most of the isolates shared a common fingerprinting pattern.

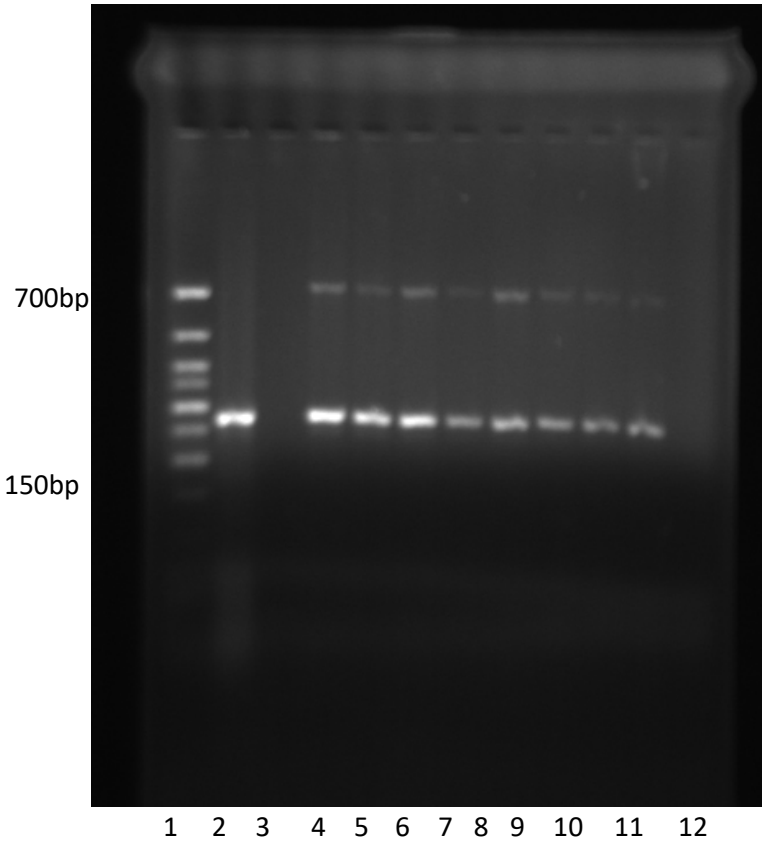
## 4.4 Beta-lactamase and PMQR gene characterization

*Bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes were detected in all the ESBL positive strains (Fig 4.7 and 4.8). *bla*$_{SHV}$ was not detected. CTX-M15 was identified in all the ESBL positive strains by DNA sequencing. Two (3.5%) and six (10.5%) *E. coli* ESBL producers had the *qnrB* gene and *aac(6')-lb* gene respectively (Fig 4.9). The *aac(6')-lb* gene was further characterized as the *aac(6')-lb-cr* variant (Fig 4.10). Two isolates carried both the *qnrB* gene and the *aac(6')-lb-cr* gene. A *qnrS* gene was identified in a bovine strain (Fig 4.9). None of the isolates carried the *qepA* gene.



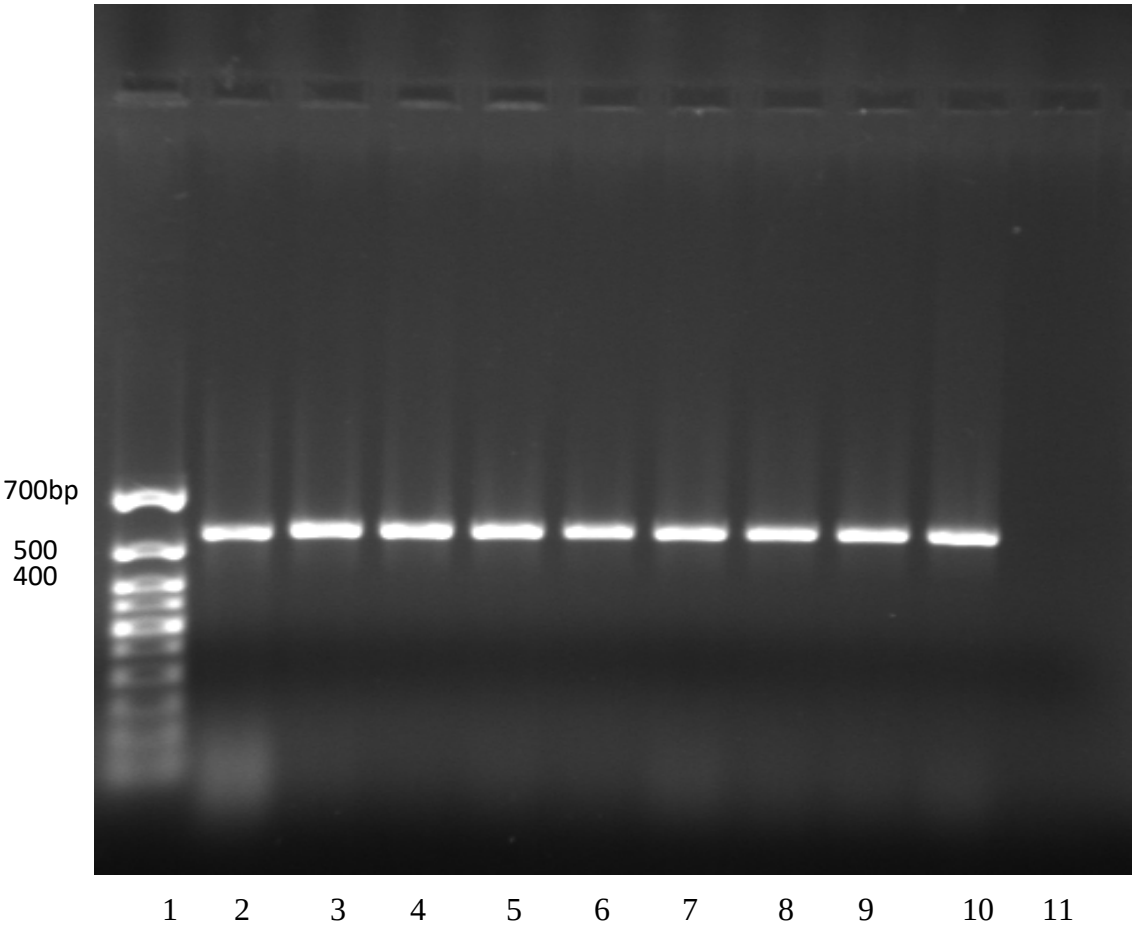

**Legend:** Lane 1- H1, Lane 2- H6, Lane 3- H12, Lane 4- H13, Lane 5- H15, Lane 6- H22, Lane 7- H45,Lane 8- H47

**Fig 4.6 Agarose gel electrophoresis of BOX-PCR for ESBL isolates showing their genetic relatedness as determined by the similarity of DNA band patterns**



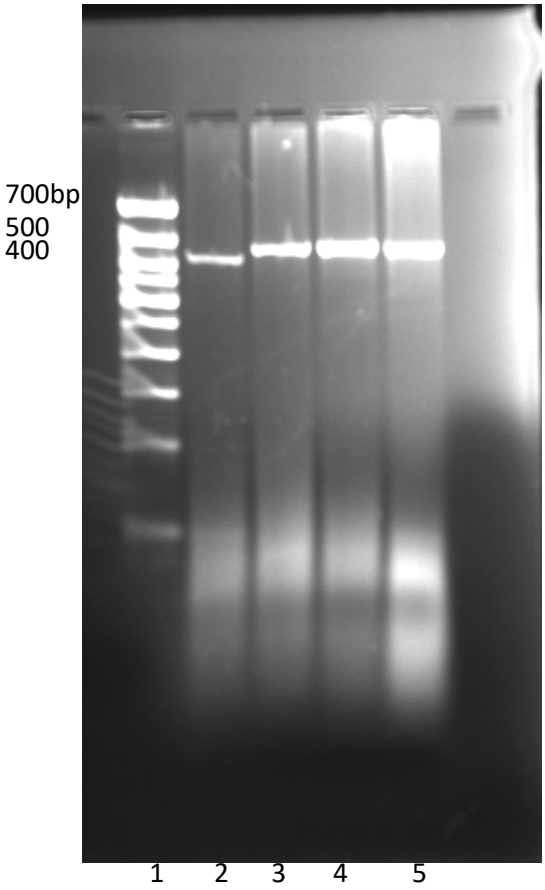

LEGEND-Lane 1-50bp DNA ladder (Biorion), Lane 2- Positive control for TEM enzymes E144, Lane 4-H1 ,Lane 5-H6, Lane 6-H12, Lane 7-H13, Lane 8-H15, Lane 9-H22, Lne10-H45, Lane 11-H47, Lane12-ve -Negative control for TEM enzymes

**Fig 4.7 Agarose gel electrophoresis for *bla*$_{\text{TEM}}$ in ESBL *E. coli* isolates**



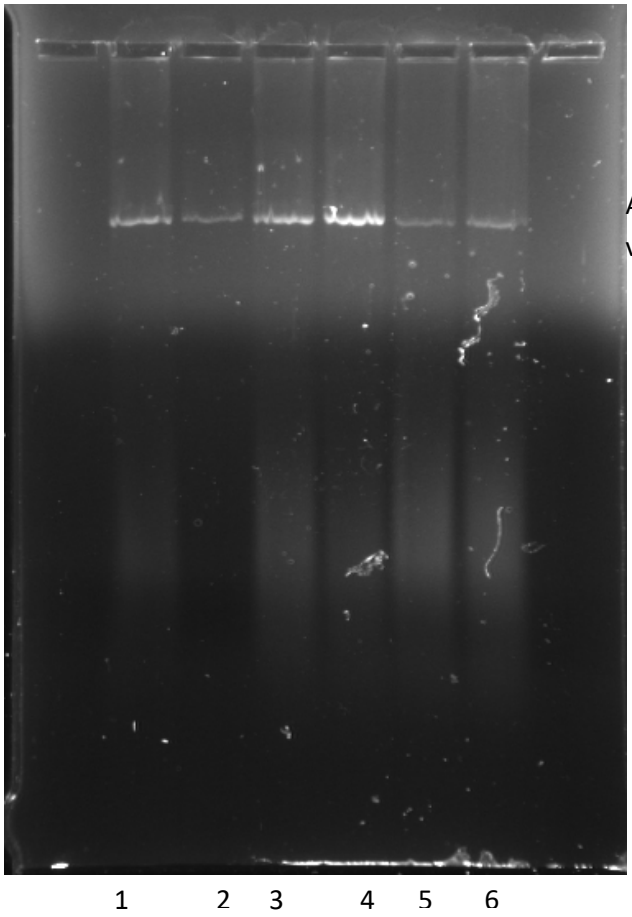

**Legend:** Lane 1- 50bp DNA ladder (Biorion), Lane 2- positive control for CTX-M enzymes FFC *E.coli* 144, Lane 3- H1, Lane 4- H6, Lane 5- H12, Lane 6- H13, Lane 7- H15, Lane 8- H22, Lane 9-H45, Lane 10- H47, Lane 11—ve- Negative control for CTX-M enzymes

**Fig 4.8 Agarose gel electrophoresis for *bla*$_{\text{CTX-M}}$ in ESBL *E. coli* isolates**




**Legend:** Lane 1- 50bp DNA ladder (Biorion), Lane 2- QnrS amplicon for bovine *E. coli* isolate, Lane 3- QnrB amplicon in H1 transconjugant *E. coli* isolate, Lane 4- QnrB amplicon in H1 human ESBL *E. coli* isolate, Lane 5- QnrB amplicon in H15 human ESBL *E. coli* isolate

**Fig 4.9 Agarose gel elctrophoresis for human ESBL *E. coli* isolates and bovine isolate with PMQRs (*Qnr* proteins)**

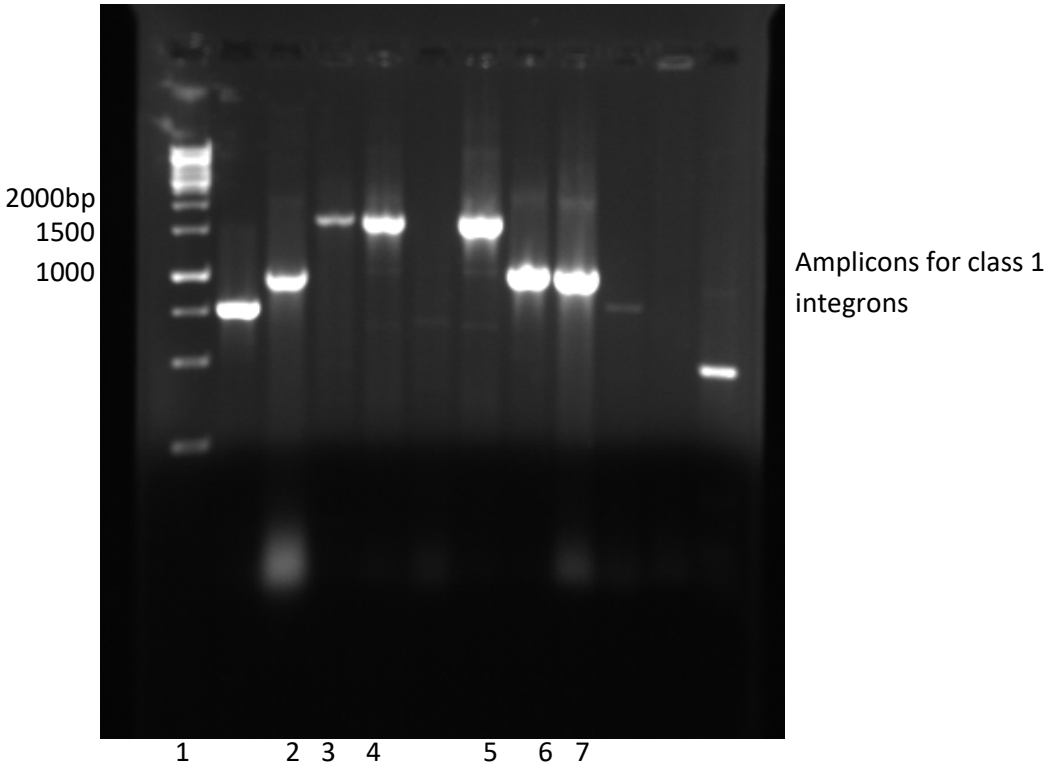



**Legend:** Lane 1- H1, Lane 2- H12, Lane 3- H15, Lane 4- H22, Lane 5-H45, Lane 6- H47

**Fig 4.10 Agarose gel electrophoresis for Human ESBL *E. coli* isolates with PMQRs (*aac(6')-lb-cr* variant)**

## 4.5 Integron carriage and antimicrobial resistance in ESBL producing *E. coli* isolates.

PCR detection of *IntI1* genes demonstrated the presence of an integrase gene in the bovine strain with the plasmid-mediated quinolone resistance determinant and (7) 87.5% of the human ESBL positive isolates. Integrons with inserted gene cassettes were found in only (6) 75% of class 1 integrase containing ESBL positive isolates (Fig 4.11). Two different gene cassette arrays with sizes ranging from 1000bp-1500bp were detected in the isolates. Sequence analysis of the gene fragments obtained was identical with those of known sequences: *aad*A1 and *dfr*A17-*aad*A5 encoding resistance to streptomycin and spectinomycin and streptomycin/spectinomycin and trimethoprim respectively. The *aad*A1 cassette carried by class 1 integrons which confers resistance to streptomycin and spectinomycin was detected in 50% of the integrons and the *dfr*A17-*aad*A5 gene arrays conferring resistance to streptomycin/spectinomycin and trimethoprim were also detected in 50% of the integrons. Integrons with inserted gene cassettes were absent in the bovine strain with the PMQR determinant and one of the positive ESBL human clinical isolates which were positive for the integrase genes. Isolates that had amplicons for the integrase genes but were negative for PCR detection of class 1 integrons indicated the possible presence of complex class 1 integrons but were not further investigated.




LEGEND-Lane 1- 1kb DNA ladder (Thermo Scientific Gene ruler), Lane 2- H1, Lane 3-H6, Lane 4-H12, Lane 5-H22, Lane 6-H45, Lane7-H47

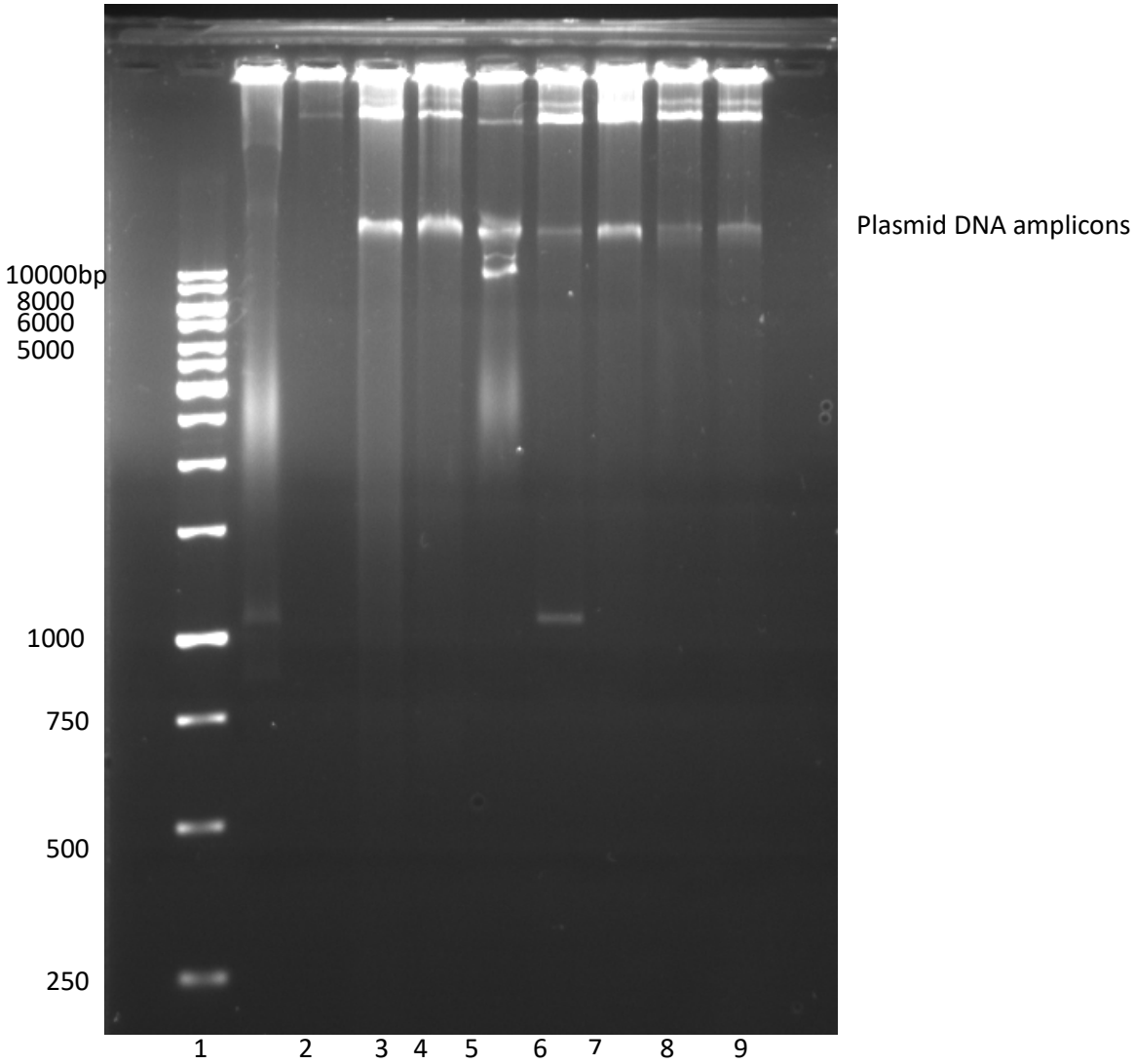
**Fig 4.11 Agarose gel electrophoresis for class 1 integrons in human ESBL producing *E. coli* isolates**

## 4.6 Exploration of the upstream sequence of the *bla*$_{\text{CTX-M}}$ genes.

In all the ESBL positive strains the IS*Ecp1* element was detected upstream of the *bla*$_{\text{CTX-M}}$ genes. The IS*26* element was located in three of the ESBL positive isolates and one isolate had both the IS*26* and IS*903* element.

## 4.7 Plasmid extraction and analysis

Most of the plasmids extracted were of large sizes >10kb (Fig 4.12). However one of the ESBL isolates H13 had a plasmid DNA of size 8kb and another H15 of size 1kb. PCR detected *bla*CTX-M and *aac(6')-lb-cr* genes on the plasmids (Fig 4.15).

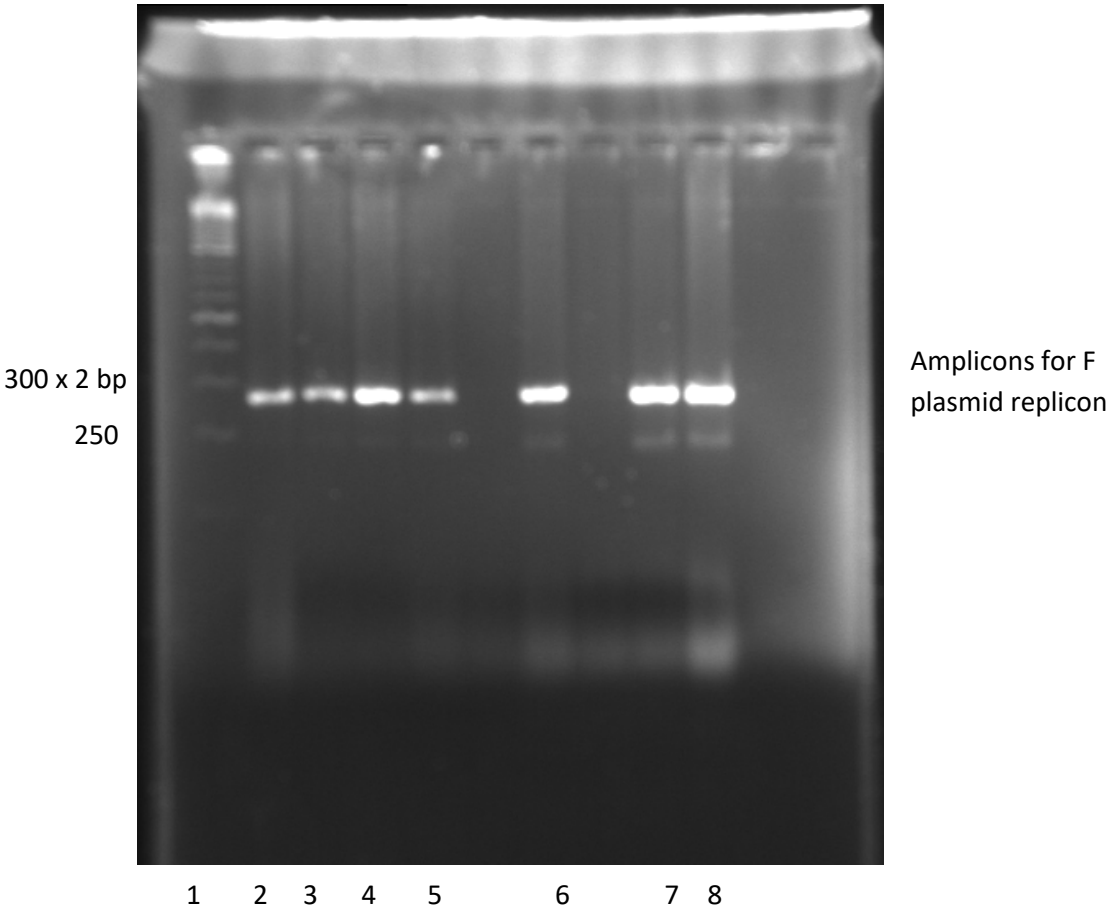



LEGEND-Lane 1- 1kb DNA ladder (Thermo Scientific Gene ruler), Lane 2- H1, Lane 3-H6, Lane 4-H12, Lane 5-H13, Lane 6-H15, Lane7-H22, Lane 8- H45, Lane 9-H47

**Fig 4.12 Agarose gel electrophoresis of Plasmids from Human ESBL *E. coli* isolates**

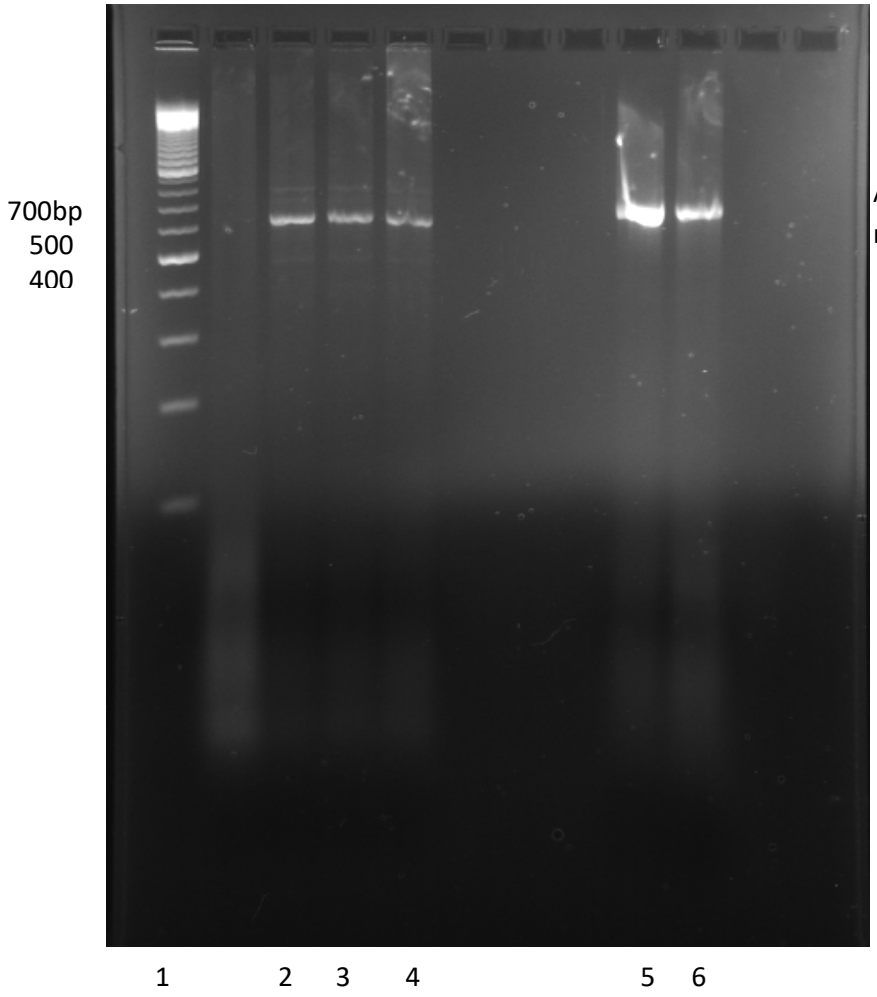
## 4.8 Association of plasmid replicon type with ESBLs and PMQR determinants.

Plasmids carrying the CTX-M 15 enzymes and the quinolone resistance determinants were assigned to the FIA, FIB, HI2, F and K replicon types (Fig 4.13 and 4.14). Plasmids associated with the quinolone resistance determinant in the bovine strain were assigned to the HII, FIB and Y replicon types.




**Legend:** Lane 1- 50bp DNA ladder (Biorion), Lane 2- positive control for F plasmid replicon, Lane 3- H1, Lane 4- H6, Lane 5- H12, Lane 6- H15, Lane 7- H45, Lane 8- H47

**Fig 4.13 Agarose gel electrophoresis for F plasmid replicon in human ESBL *E. coli* isolates**

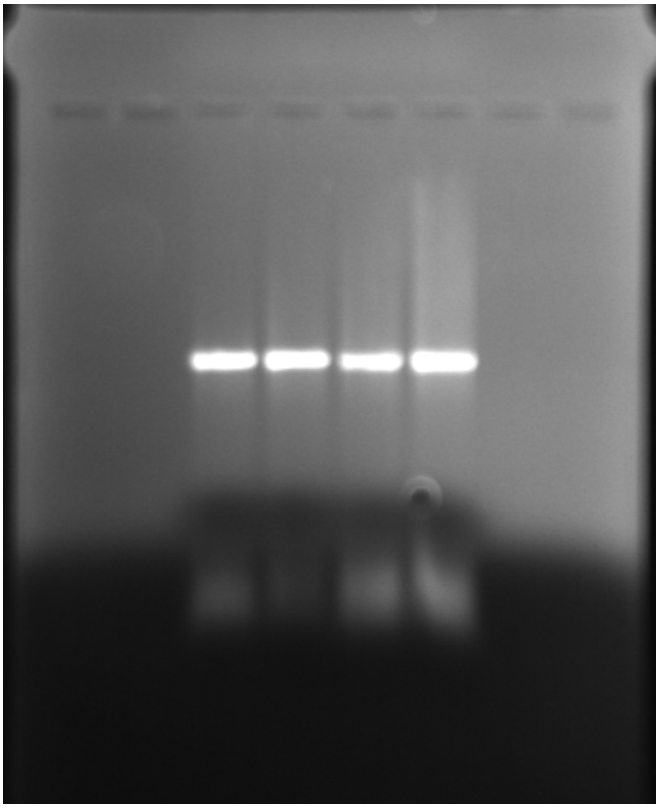



**Legend:** Lane 1- 50bp DNA ladder (Biorion), Lane 2- H1, Lane 3- H45, Lane 4- H47, Lane 5- H1 transconjugant *E. coli* isolate, Lane 6- Positive control for H12 plasmid replicon

**Fig 4.14 Agarose gel electrophoresis for H12 plasmid replicon in human ESBL *E. coli* isolates**

## 4.9 Transfer of Resistance Determinants.

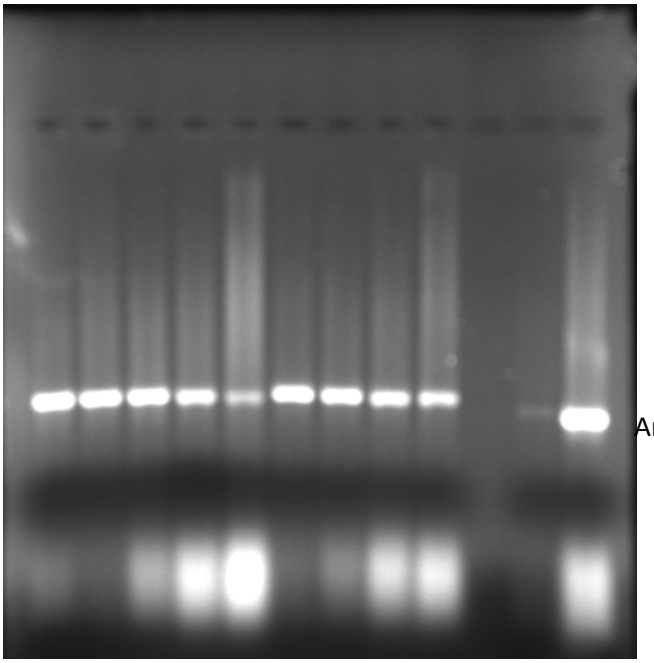
Transfer of the ESBLs and plasmid mediated quinolone resistance phenotype to *E. coli* J53 was successful for two of the ESBL strains by conjugation. Production of ESBLs was detected in the transconjugants by the double disc synergy test. Transconjugants obtained were confirmed for the $bla_{TEM}$ gene, $bla_{CTX\text{-}M}$ gene, *qnr*B gene and the *aac(6')-lb-cr* gene by PCR (Fig 4.9, 4.15 and 4.16). Plasmids carrying the resistance determinants in the transconjugants were assigned to the FIB and HI2 replicon types. The transconjugants had resistance profiles identical to those of their parental clinical strains.




Amplicons for tem enzymes in transconjugant *E. coli* isolates

**Legend:** Lane 1- H1, Lane 2- H15, Lane 3- H1 transconjugant *E. coli* isolate, Lane 4- H15 transconjugant *E. coli* isolate

**Fig 4.15 Agarose gel electrophoresis for TEM enzymes in transconjugant *E. coli* isolates**




Amplicons for CTX-M enzymes and *aac(6')-lb-cr* variant

**Legend:** Lane 1-4, 6-9- Amplicon of *bla*$_{\text{CTX-M}}$ on purified plasmid DNAs of Human ESBL *E. coli* isolate, Lane 5- Amplicon of *bla*$_{\text{CTX-M}}$ on *E. coli* transconjugant isolate, Lane 10 & 11- Amplicon of *aac(6')-lb-cr* variant on *E. coli* clinical and transconjugant isolate

**Fig 4.16 Agarose gel elctrophoresis for the resistant determinants on purified plasmid DNAs of human ESBL *E. coli* isolate and *E. coli* transconjugant.**

Table 4.11 show the antimicrobial resistance pattern and genetic characteristics of Human *E. coli* ESBL producers and Bovine *E. coli* isolate with a PMQR determinant.

### 4.10 Multilocus sequence typing

Sequence type ST 131 was identified in one of the human ESBL *E. coli* isolate screened. The other isolate was assigned to ST 2695, a new allele not previously described

**Table 4.11 Characteristics of Human ESBL producers and Bovine strain with PMQR determinant**

| Strain | Antimicrobial resistance pattern[a] | PMQR Gene | Beta-lactamase Gene | Transfered genes[b] | Inc Plasmid Group | Is elements | Gene Cassette(s) on Integrons | ST[c] |
|---|---|---|---|---|---|---|---|---|
| BV36 | TMP/AML | *Qnr*S | - | - | HII, Y, FIB | - | - | - |
| H1 | CAZ/CTX/ATM/W/GEN/AML | *Qnr*B, *aac(6')-lb-cr* | *bla*$_{TEM}$, *bla*$_{CTX-M\ 15}$ | *Qnr*B, *aac(6')-lb-cr*, *bla*$_{TEM}$, *bla*$_{CTX-M\ 15}$ | FIB, H12, K | *IsEcp1* | *aadA1* | 2695 |
| H6 | CTX/NAL/TMP/AML | - | *bla*$_{TEM}$, *bla*$_{CTX-M\ 15}$ | - | FIB | *IsEcp1, IS26* | *aadA5,dfrA17* | - |
| H12 | CAZ/CTX/ATM/CIP/NAL/TMP/GEN/AML | *aac(6')-lb-cr* | *bla*$_{TEM}$, *bla*$_{CTX-M\ 15}$ | - | FIA,FIB | *IsEcp1* | *aadA5, dfrA17* | - |
| H13 | CAZ/CTX/CIP/NAL/TMP/GEN/AML | - | *bla*$_{TEM}$, *bla*$_{CTX-M\ 15}$ | - | FIA,FIB | *IsEcp1, IS26* | - | - |
| H15 | CAZ/CTX/AMC/ATM/CIP/NAL/TMP/GEN/AML | *aac(6')-lb-cr* | *bla*$_{TEM}$, *bla*$_{CTX-M\ 15}$ | *aac(6')-lb-cr*, *bla*$_{TEM}$, *bla*$_{CTX-M\ 15}$ | FIB | *IsEcp1* | - | 131 |
| H22 | CAZ/CTX/AMC/CIP/NAL/TMP/GEN/AML | *aac(6')-lb-cr* | *bla*$_{TEM}$, *bla*$_{CTXM\ 15}$ | - | FIA,FIB | *IsEcp1* | *aadA5, dfrA17* | - |
| H45 | CTX/TMP/GEN/AML | *aac(6')-lb-cr* | *bla*$_{TEM}$, *bla*$_{CTXM\ 15}$ | - | H12, K, FREPB | *IsEcp1, IS26* | *aadA1* | - |
| H47 | CTX/AMC/TMP/GEN/AML | *Qnr*B, *aac(6')-lb-cr* | *bla*$_{TEM}$, *bla*$_{CTXM\ 15}$ | - | H12, FREPB | *IsEcp1, IS26, Is903* | *aadA1* | - |

[a]AML- Amoxicillin, AMC-Amoxicillin-clavulanic acid, CAZ-Ceftazidime, CTX-Cefotaxime, ATM-Azetronam, FEP-Cefepime, IPM-Imipenem, FOX-Cefoxitine, NAL-Nalidixic acid, CIP-Ciprofloxacin, TMP-Trimethoprim, GEN-Gentamicin

[b]- No transferred gene. Conjugation not successful

[c], Sequence type

# CHAPTER FIVE

# DISCUSSION AND CONCLUSION

.

*Escherichia coli* has been widely implicated in various clinical infections as hospital acquired and community infections: e.g. in urinary tract infections (Shah and Isaacs, 2003). The distribution of isolates with respect to clinical sources in this study agrees with this report as shown in the majority of the isolates (58%) from urine (Table 3.1).

Iroha *et al*., (2012) reported urine and vaginal swabs in a Nigerian study as the highest clinical sources from where human *E.coli* isolates were obtained which agrees with this study. Majority of the ESBL strains were also found in urine samples which supports data from previous studies (Kader and Kumar, 2005; Fang *et al.,* 2008; Ben Slama *et al.,* 2011; Iroha *et al.,* 2012). Most of the ESBL-producing strains were from samples obtained at the University College Hospital (UCH), Ibadan. This may be due to the fact that most patients in this tertiary care hospital are usually referred from other hospitals where they may have been started on antibiotic therapy. In Developing countries like Nigeria, resources are limited and antibiotics are indiscriminately used both in humans and animals which could result in outbreaks of ESBL producers. Persistence and outbreaks of ESBL producers have been convincingly correlated with the extensive use of cephalosporins (Sirot *et al.,* 1991).

The high resistance rates of all the 57 human isolates of *E. coli to* amoxicillin (88%) and trimethoprim (83%) find support in an earlier study by Aibinu *et al*., (2004), Umolu *et al*., (2006) and Olowe *et al*., (2008). Interestingly, all the human *E. coli* isolates had high susceptibility pattern to cefoxitin (100%), imipenem (98%) and cefepime (97%). Pathogenic *E. coli* isolates have relatively high potentials for developing resistance (Karlowsky *et al.,* 2004). The antibiotic resistance rates of the human *E. coli* isolates tested in this study were higher than those observed among the bovine *E. coli* isolates. This is contrary to a report in the United States that *E. coli* isolates from animals were more resistant than those of humans (Tadesse *et al.,* 2012), similar to the report in Nigeria of Ajayi *et al.,* (2011) of a high prevalence of bovine resistant isolates to antibiotics.

The bovine *E. coli* had 100% susceptibility to the antibiotics tested which included cefotaxime, azetronam, cefepime, amoxicillin-clavulanic acid, imipenem, cefoxitin, ciprofloxacin and gentamicin. The antibiotic susceptibility patterns of the bovine *E. coli* isolates correlate with data obtained from reports on bacteria isolated from meat tables in the Nigerian geographical location where the study was carried out (Olatoye, 2010; Adetunji *et al.,* 2011). In contrast with other reports (Chah and Oboegbulem, 2007; Akujobi *et al.,* 2008; Fortini *et al.,* 2011), the healthy animals in this study did not show considerable antibiotic resistance. Twenty five percent (25%) of the bovine *E. coli* isolates showed resistance to trimethoprim and 23% were also resistant to amoxicillin. Also, a recent study in Ibadan, Nigeria, characterized PMQR determinants and β-lactamases among *E. coli* from healthy chickens and pigs at slaughter in Ibadan, Nigeria, which showed a high prevalence of these resistance determinants compared to results obtained in this study from the bovine animals (Fortini *et al.,* 2011). Another study reported PMQR determinants in commensal *E. coli* from faeces of apparently healthy cattle in Nigeria (Ajayi *et al*., 2012) which contrast with results obtained from this study. Ajayi *et al.,* (2011) reported that generally, bovines are less medicated compared to other animals such as pigs which require more antibiotic doses to prevent infections or illness in them. The antibiotic susceptibility of the bovine isolates in this study may be attributed to less antibiotic dosing for the bovines in the geographical location where the study was carried out.

The percentage of nalidixic acid and ciprofloxacin resistance observed among the human isolates in this study was 47% and 40% respectively which is on the high side. Olowe *et al.,* 2012 also reported high resistance rate of human clinical *E. coli* isolates in Osogbo, Southwest, Nigeria to ciprofloxacin which correlate with results obtained from this study. The reason for the high resistance rate in this study may be due to an irrational increase in the use of flouroquinolones including ciprofloxacin, transmission of resistant isolates among people and consumption of food from animals that have received antibiotics. Self medication, non-compliance with medication and sales of substandard drugs may also account for the rise in antibiotic resistance observed among the human *E.coli* isolates in this study.

One of the most important resistance mechanisms in Enterobacteriaceae which reduces the efficacy of modern expanded spectrum cephalosporins (except cephamycins and carbapenems) and monobactams is based on plasmid-mediated

production of extended-spectrum β-lactamases (ESBLs). The human *E. coli* isolates that showed resistance to cefotaxime and ceftazidime, and also reduced susceptibility to ceftazidime in some of the isolates were phenotypically positive for ESBL detection by the double disc synergy test. Reports concerning *E. coli* carrying broad spectrum β-lactamases isolated from food producing animals and human have been published globally (Mesa *et al.*, 2006; Tadesse *et al.*, 2012).

Previous report show that the most prevalent ESBL-producer currently is *E. coli*, incidentally, also the leading cause of urinary tract infections and septicaemia, thereby rendering the organism as a significant problem not only in hospitals but also in the community (Lytsy, 2010). The ESBL human *E. coli* isolates in this study were also resistant to gentamicin (87.5%), nalidixic acid (62.5%) and ciprofloxacin (50%), but susceptible to cefoxitin and imipenem (100%). This correlate with previous reports that ESBL producing *E. coli* isolates were resistant to more antimicrobial agents than non ESBL producing isolates (Gniadkowski, 2001; Girlich *et al.*, 2007; Bourjilat *et al.*, 2011; Olowe *et al.*, 2012). Plasmids with $bla_{\text{CTX-M}}$ genes have been reported to often carry genes conferring resistance to quinolones, aminoglycosides and cotrimoxazole (Morosini *et al.*, 2006; Canton *et al.*, 2008), consistent with the results from this study. The MIC determination confirmed the antibiotic resistance rate among the human ESBL *E. coli* isolates using the selected antibiotics: cefotaxime, ceftazidime, ampicillin, levofloxacin and gentamicin.

The use of cephalosporins and fluoroquinolones for prophylaxis and treatment of bovine animals is rare in Nigeria which supports the high susceptibility rate of the bovine *E. coli* isolates. However, the resistance determinants could be selected against other drugs frequently used in food animals. Chah and Oboegbulem, (2007) reported the wide use of ampicillin in poultry production in Nigeria which may provide a selective pressure favouring the emergence of *E. coli* strains that carry plasmids with ESBL determinants. These other food animals may serve as reservoir of ESBL producing *E. coli* strains transferrable to humans and lower animals. However in this study, there was no association between *E. coli* from human and bovine with respect to their antibiotic susceptibility pattern. Wu *et al.*, (2013) also reported from a public health perspective that ESBL producing *E. coli* from animals may represent a reservoir of virulence and resistance genes rather than being the direct cause of human infections.

The CTX-M β-lactamase has been recognized globally as an important cause of resistance to oxyimino-cephalosporins in Gram-negative bacteria (Bonnet, 2004; Ho *et al.,* 2007; Mendonca *et al.,* 2007; Coque *et al.,* 2008; Rossolini *et al.,* 2008). Results from this study are consistent with these reports. Thus, it has been suggested that an increase in ESBL producers could be ascribed to clonal expansion of CTX-M producing *E. coli* (Coque *et al*., 2008; Fang *et al*., 2008). The emergence and dissemination of ESBL *E. coli* might have two possible explanations: dissemination of mobile genetic elements between non-clonally related strains, or clonal spread of the organism. The two models can occur simultaneously, thereby contributing to the rapid dissemination of ESBL *E. coli* (Blanco *et al.,* 2013). Evaluation of the genetic relatedness among the ESBL *E. coli* isolates in this study showed that a prevalent clone existed among the isolates.

The enzyme CTX-M-15 has been reported in different parts of the world (Boyd *et al*., 2004; Livermore *et al.,* 2007; Carattoli *et al*., 2008) including African countries (Blomberg *et al*., 2005; Gangoue-Pieboji *et al*., 2005). This enzyme was identified in this study in all the human ESBL *E. coli* isolates. In Nigeria, the enzyme has also been identified in previous studies in human clinical *Klebsiella spp* and *E. coli* isolates (Soge *et al*., 2006; Olowe *et al*., 2010, Iroha *et al*., 2012; Ogbolu *et al*., 2013). This shows the wide dissemination of the ESBL (CTX-M-15) which could also be attributed to the widespread and indiscriminate use of beta-lactam antibiotics. The resistance to nalidixic acid and ciprofloxacin shown by the human ESBL *E. coli* isolates could be linked partially to the simultaneous carriage of PMQRs. A recent report showed the high resistance rates to β-lactams and quinolones among Gram-negative isolates from different hospitals in Nigeria (Ogbolu *et al*., 2011), which may support findings in this study, at a molecular level. The *aac(6')-lb-cr* variant was shown in this study to be prevalent among the human ESBL *E. coli* isolates. The *qnr*B gene was also identified in this study in two ESBL *E. coli* isolates. This report supports previous findings suggesting a larger dissemination of *aac(6')-lb-cr* than *qnr* determinants, especially in multiple clones of *E. coli* carrying the $bla_{\text{CTX-M15}}$ gene (Fihman *et al.,* 2008; Warburg *et al*., 2009; Nazik *et al.,* 2011). The *aac(6')-lb-cr* gene has spread rapidly among Enterobacteriaceae, and although only conferring a low level of resistance, it may create an environment facilitating the selection of

highly resistant determinants especially in organisms harbouring topoisomerase mutation.

In this study, all strains with PMQR determinants were ESBL producers thereby supporting a strong association between ESBL production and quinolone resistance previously reported in Enterobacteriaceae (Nordmann and Poirel, 2005; Robicsek *et al.,* 2006). Nazik *et al.,* 2011 reported a high prevalence of *aac(6')-lb-cr* and CTX-M type β-lactamase in ESBL producing *E. coli* isolates from Hospitals in Turkey which correlate with results from this study. The acquisition and accumulation of resistance determinants have given rise to multi-drug resistant ESBL producers further limiting therapeutic options of infections caused by these organisms. They may also facilitate subsequent dissemination of these resistance genes to other members of Enterobacteriaceae through horizontal transfer because of their co-selection by various antimicrobials (Robicsek *et al.,* 2006; Cremet *et al.,* 2009).

Most of the plasmids extracted from the human ESBL *E. coli* isolates were of large sizes which is consistent with previous reports that ESBL production is coded by genes that are prevalently located on large conjugative plasmids of 80-160 Kb in size (Podshun and Ullmann, 1998; Mshana *et al*., 2009; Wang *et al*., 2013). ESBL genes of the TEM, SHV and CTX-M families can reside on large conjugative plasmids (Podschun and Ullmann, 1998; Bradford, 2001) and has been demonstrated for CTX-M 15 enzyme (Eckert *et al.,* 2004). This correlates with results obtained in this study. Genes normally present on the same plasmid carrying $bla_{\text{CTX-M-15}}$ like $bla_{\text{TEM}}$ and $bla_{\text{OXA}}$, as well as *aac-(6)-Ib-cr* and qnr determinants, explain the multiresistant phenotype of CTX-M 15-producing bacteria (Jacoby 1997; Nicolas-Chanoine *et al*., 2008; Rodgers *et al.,* 2011). Both the CTX-M 15 and TEM determinants as well as the *qnr*B and *aac(6')-lb-cr* variant among the human ESBL *E. coli* isolates could be transferred by conjugation (Table 4.11). This demonstrates the potential of co-dissemination by horizontal gene transfer of resistance to β-lactams and quinolones. Only two ESBL *E. coli* isolates were able to transfer their resistance determinants by conjugation and they belonged to distinct sequence types, as determined by multilocus sequence typing (MLST).

Lack of conjugative transfer from six (6) of the ESBL human isolate into the recipient strain *E. coli* J53 used in this study may indicate that the resistance determinants were

encoded on non-conjugative plasmids. The large plasmids isolated in this study carried multiple resistance genes which were transferred to the transconjugants. The transfer of the resistance determinants to the transconjugants creates the possibility that such multiresistant plasmids could be transferred between bacteria of different genera. Generally, the transconjugants had resistance profiles similar to their parental clinical strains.

Sequence type (ST) 131 was identified in a human *E. coli* isolate carrying *bla*$_{\text{CTX-M15}}$ and *aac (6')-lb-cr* by multilocus sequence typing (MLST). The clonal spread of ST131 *E. coli* producing CTX-M ESBLs has been reported in many countries (Woodford *et al*., 2004; Lau *et al*., 2008; Warren *et al.,* 2008). The first report of complex type clone *E. coli* ST 131 recently made in Nigeria found predominant among hospital isolates of CTX-M 15 producing *E. coli* which also harboured the *aac(6')-lb-cr* gene, was by Aibinu *et al*., (2012). Results from this study support the dissemination of *E. coli* ST 131 in Nigeria since the isolates were obtained in a different geographical location. Recent reports indicate that the worldwide dissemination of CTX-M 15 is mediated by clonally related *E. coli* strains which include specific clones of phylogroup B2, ST131 (Coque *et al.,* 2008). The other *E. coli* isolate harboring bla$_{\text{CTX-M 15}}$, *qnr*B and *aac (6')-lb-cr* genes was assigned ST 2695, a new allele not previously reported to be associated with CTX-M -15 enzyme.

The most frequently encountered plasmid replicon type analyzed in this study belonged to the IncF family which includes the F1A and F1B groups commonly reported to be associated with *bla*$_{\text{CTX-M}}$ and *aac(6')-lb-cr* resistance genes (Carattoli, 2009). IncF plasmids are low-copy number plasmids, often carrying more than one replicon (Carattolli, 2009). IncF replicons are widely distributed among *E. coli* strains and seem to be well adapted to this species (Boyd *et al.,* 1996). Reports show that IncF plasmids have become frequent in the UK since 2003 (Woodford *et al.,* 2004). Ogbolu *et al*., (2013) also reported the dissemination of IncF plasmids in ESBL producing isolates in Nigeria, which is consistent with reports from this study. *Bla*$_{\text{CTX-M}}$ resistance genes have also been found associated with the IncH12 and IncK group of plasmids (Caratolli, 2009). However, reports on association of the *qnrB* resistance

gene with the plasmid replicon types found in this study are limited. Also, limited reports of the *qnrS* resistance gene with the plasmid replicon types in this study exist.

Class 1 integrons coding for resistance to other antibiotic families were also associated with the CTX-M 15 human *E. coli* isolates though their genetic location was not investigated. A previous report showed the wide distribution of class 1 integrons in *E. coli* strains (White *et al.,* 2001). The genes included in the variable region of the class 1 integrons in the strains studied, encoded resistance to trimethoprim and aminoglycosides (streptomycin and spectinomycin). Mokracka *et al*., (2013) reported the genetic content of integrons among human extra-intestinal *E. coli* isolates from in-patients in Poznan hospitals comprised genes determining resistance to aminoglycosides, sulfonamides and trimethoprim which is consistent with results in this study. Another report in Madagascar revealed a predominance of *aadA* and *dfrA* genes in multi-drug resistant ESBL producing Enterobacteriaceae which confer resistance to aminoglycosides and trimethoprim respectively (Rakotonirina *et al*., 2013). These results correlate with studies in Africa of Enterobacteriaceae isolates (Frank *et al*., 2007; Dahmen *et al*., 2010) and are also consistent with results from this study. The gene cassette array *dfrA17-aadA5*, found in 50% of the ESBL isolates screened for class 1 integrons has been reported to be widely distributed among food animals and in the environment (Guerra *et al.,* 2003; Yu *et al.,* 2003; Ajiboye *et al.,* 2009). Chen *et al*., 2013 reported 69% of clinical ESBL producing isolates from in-patients in a Chinense Hospital with class 1 integrons indicating that these integrons are widely present in these isolates and may influence their level of antibiotic resistance. The presence of class 1 integrons found in the human CTX-M 15 producers shows the higher ability of the isolates to capture additional resistance genes.

In all the eight ESBL positive isolates the IS*Ecp1* element was detected upstream from the $bla_{\text{CTX-M}}$ genes which ensure the transfer of the resistance genes from the bacterial chromosome to the plasmids and is involved in the spread of ESBLs. The detection of the IS*Ecp1* element upstream of the $bla_{\text{CTX-M}}$ gene is consistent with the hypothesis that the IS*Ecp1* element is responsible for the wide distribution of the $bla_{\text{CTX-M}}$ gene (Poirel *et al.,* 2004). This insertion sequence has been reported to facilitate the mobilization of $bla_{\text{CTX-M}}$ genes (Canton and Coque, 2006) which is

consistent with findings from previous reports (Mshana *et al.,* 2009; Mokracka *et al.,* 2013; Rakotonirina *et al.,* 2013). The IS*Ecp1* element provides promoter activity for expression of a downstream CTX-M type β-lactamase gene.

Remarkably the IS*26* element characteristic of the epidemic strain from the United Kingdom (Woodford *et al.,* 2004) was detected in three of the ESBL positive isolates in this study. Cullik *et al.,* (2010) also reported the important role of IS 26 element in the spread of ESBL genes. The IS 903 element was also detected in one of the human ESBL *E. coli* isolates. This element has also been reported to be linked with $bla_{\text{CTX-M}}$ genes (Mendonca *et al.,* 2007; Cullik *et al.,* 2010). These insertion sequences could explain the dissemination of the $bla_{\text{CTX-M}}$ genes.

## CONCLUSION AND RECOMMENDATIONS

This study highlights the dissemination of *E. coli* CTX-M-15 producers in Oyo state, Nigeria, possibly as a result of prevalent clones of this organism in the hospital where most of the isolates were obtained and of the horizontal transfer of plasmids or mobile elements. This is the first report of *E. coli* ST2695 found in an hospital isolate of CTX-M 15 producing *E. coli* which harboured the *qnrB* gene, *aac(6')-lb-cr* gene and was also successful for its transfer of antibiotic resistance in this study. The result of this study furthermore suggests, that the association of CTX-M 15, *qnr*B and *aac(6')-lb-cr* resistance determinants in addition to mobile elements (ISE*cp1*, class 1 integrons) may facilitate the rapid dissemination of antimicrobial resistance into other Gram-negative bacteria in Nigeria limiting the choice of antibiotic therapy.

Many factors have been found to contribute to such high rates of resistance in developing countries. These include: poor drug quality or inadequate posology, the long-term treatments, misuse of antibiotics by health professionals, unskilled practitioners, auto medication (antibiotics can be purchased without prescription), unhygienic conditions accounting for the spread of resistant bacteria and inadequate surveillance programs (Tande *et al*., 2009; Hounsa and De Mol, 2009). In Nigeria the collapse of primary healthcare system coupled with the unavailability of drugs in hospitals in Nigeria has resulted in most people resorting to purchase of drugs over the counter and in some cases from roadside sellers which expose them to the danger of acquiring ESBL-producing organisms.

There are currently few treatment options available, which include the carbapenems (Gupta, 2007). Sader *et al*., (2003) also reported that the carbapenems are still the most effective agents against Enterobacteriaceae. This was confirmed in this study by the human and bovine isolates including the ESBL isolates showing 100% susceptibility to imipenem.

Unlike the situation in developed countries, the financial resources to provide alternative agents such as carbapenems are lacking in developing countries and the option to tailor therapy based on antimicrobial resistance testing is unavailable except in a few hospitals. To control the emergence and spread of ESBL-producing *E. coli*, it is essential for the public to practise good hygiene habits and comply with recommendations on the proper use of antibiotics. Proper infection-control practices and barriers are essential to prevent spreading and outbreaks of ESBL-producing bacteria. The reservoir for these bacteria seems to be the gastrointestinal tract of patients (Samaha-Kfoury and Araj, 2003). Alternative reservoirs could be the oropharynx, colonized wounds and urine. The contaminated hands and stethoscopes of healthcare providers are important factors in spreading infection between patients (Samaha-Kfoury and Araj 2003). Essential infection-control practices should include avoiding unnecessary use of invasive devices such as indwelling urinary catheters or IV lines, hand washing by hospital personnel, increased barrier precautions, and isolation of patients colonized or infected with ESBL producers. At an institutional level, practices that can minimize the spread of such organisms include clinical and bacteriological surveillance of patients admitted to intensive care units and antibiotic cycling; as well as policies of restriction, especially on the empirical use of broad-spectrum antimicrobial agents such as the third- and fourth-generation cephalosporins and quinolones (Bhattacharya, 2006)

The worrisome development of antibiotic resistance in ESBL producing *E. coli* in communities could be curtailed by enhanced capabilities for the detection of resistance mechanisms by private as well as public laboratories to implement appropriate infection control practices, and to prescribe appropriate chemotherapeutic agents. The use of broad-spectrum cephalosporins and fluoroquinolones in humans

and animals should be urgently limited to cases in which other therapeutic alternatives according to evidence-based guidelines are not possible. Limiting antimicrobial use may curtail the selection and persistence of predominant ESBL clones and the probable dissemination of conjugative plasmids among strains, thus decreasing not only the number of potential ESBL donors but also the accumulation of antibiotic resistance genes on common genetic elements.

Methods should be improved to efficiently detect and track those bacterial clones and plasmids that constitute the major vehicles for the spread of ESBL-mediated resistance. Ideally, such methods of detection should be accessible to medium-level diagnostic microbiology laboratories, to assure the possibility of performing interventions in real time. More studies are needed to make a more accurate risk assessment concerning the spread of antimicrobial resistance, as well as on the mechanisms of linkage and transferability of β-lactam resistance determinants in natural environments. Therefore, the evaluation of the possible impact of this resistance in animals for human health studies should not be limited to pathogenic bacteria, but must also include commensals, since they may be a major reservoir of resistance genes, as has already been shown to be the case in poultry (Smet *et al.*, 2008).

The importation of ESBL-producing bacterial strains through food animals and pets has the potential to cause the wide dissemination of antibiotic resistance among countries and their spread to humans. It highlights the need for national and supra-national public health efforts to implement surveillance, epidemiologic, environmental health, and policy-making components. The implementation of ecological surveillance of ESBL producing organisms, including environmental (particularly water environments, as sewage) and faecal colonisation surveillance studies in community-based individuals and animals is urgently needed to address the "colonisation pressure" outside hospitals, to detect circulation of highly epidemic clones and to monitor ESBL trends. These ecological studies could be useful as biosensors of modifications in the ESBL landscape. The clinical and commercial pressure to use β-lactams, as well as the global mobility of humans, animals and food products guarantee that the spread of ß-lactamase genes will continue. β-Lactam antibiotics may enter the environment, such as water sources, having been excreted in

the faeces and/or urine of treated animals. Water may therefore also be a potential source of selective pressure. An improvement is needed in the methods for detecting multidrug-resistant ESBL producers that express a low level of resistance to beta-lactams or might contain silenced antibiotic resistance genes not detectable by standard phenotype. Also strongly suggested is a standardisation of beta-lactam breakpoints recommended by the different agencies and committees.

The scientific and public health community should be aware that the potential interventions directed to control the world-wide spread of ESBL-producing organisms have a limited time-window for effective action. Once a number of thresholds are crossed (critical absolute number of ESBL-genes in the microbial world, critical associations of these genes with widespread genetic platforms, critical dissemination of ESBLs among different bacterial species and clones), the control will be simply impossible by applying the standard measures. Action should be taken now and preparation made for the future, by promoting innovative ways of controlling ESBL-producing organisms.

This study suggests the need for local surveillance and characterization of bacteria not only from human sources but also from bovine sources especially in developing countries with limited resources and where antibiotics are used indiscriminately in humans and animals. This study also highlights the need to adopt measures to monitor the dissemination of ESBLs in Nigeria. The detailed and reliable knowledge on the resistance of human and bovine pathogens is required for a controlled and safe use of antibiotics in clinical and community as well as veterinary medicine.

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## Appendix I

### Growth media, reagents, standard solutions, indicators and buffers

**Composition and preparation of media**

**Luria Bertani (LB) Agar**

Approximate formula per litre

| | |
|---|---|
| Tryptone | 10g |
| Yeast Extract | 5.0g |
| Sodium chloride | 5.0g |
| Agar | 15g |

Thirty-five grams of the powder was dissolved in 1L of purified water. It was then mixed and heated to ensure the complete dissolution of the powder. The thoroughly dissolved medium was sterilized by autoclaving at 121$^{o}$C for 15 minutes.

**Mueller Hinton Agar**

Approximate formula per litre

| | |
|---|---|
| Beef Infusion | 300g |
| Technical | 17.5g |
| Starch | 1.5g |
| Agar | 17.0g |

Thirty-eight grams of the powder was dissolved in 1L of purified water. It was then mixed and heated to ensure the complete dissolution of the powder. The thoroughly dissolved medium was sterilized by autoclaving at 121$^{o}$C for 15 minutes.

**MacConkey Agar**

Approximate Formula per litre

| | |
|---|---|
| Bacto Peptone | 17g |
| Bacto Proteose Peptone | 3g |
| Bacto Lactose | 10g |
| Bacto Bile Salts No.3 | 1.5g |
| Sodium Chloride | 5g |
| Bacto Agar | 13.5g |
| Neutral Red | 0.03g |
| Bacto Crystal Violet | 0.001g |

Fifty grams of the powder was dissolved in 1L of purified water. It was then mixed and heated to ensure the complete dissolution of the powder. The thoroughly dissolved medium was sterilized by autoclaving at 121$^{o}$C for 15 minutes.

**Luria Bertani (LB) Broth**

Approximate Formula per litre

| | |
|---|---|
| Pancreatic Casein Digest | 10g |
| Yeast Extract | 5.0g |
| Sodium Chloride | 5.0g |

Twenty grams of the powder was dissolved in 1L of purified water. The thoroughly dissolved medium was sterilized by autoclaving at 121$^{o}$C for 15minutes.

**Eosin Methylene Blue Agar**

Approximate Formula per litre

| | |
|---|---|
| Gelatin Peptone | 10g |
| Sucrose | 5.0g |
| Eosin Y | 0.4g |
| Agar | 13.5g |
| Lactose | 5.0g |
| Dipotassium Phosphate | 2.0g |
| Methylene Blue | 0.065g |
| pH | 7.2$\pm$ 0.2 |

Thirty six grams of the powder was dissolved in 1L of purified water. It was then mixed and heated to ensure the complete dissolution of the powder. The thoroughly dissolved medium was sterilized by autoclaving at 121$^{o}$C for 15 minutes.

**Nutrient Agar**

Approximate Formula per litre

| | |
|---|---|
| Peptone | 5.0g |
| Beef Extract | 3.0g |
| Sodium Chloride | 8.0g |
| Agar No.2 | 12.0g |
| pH | 7.3± 0.2 |

Twenty eight grams of the powder was dissolved in 1L of purified water. It was then mixed and heated to ensure the complete dissolution of the powder. The thoroughly dissolved medium was sterilized by autoclaving at 121$^{o}$C for 15 minutes

**Gram-stains and Reagents**

**Gram's Iodine Solution**

| | |
|---|---|
| Iodine | 1.0g |
| Potassium iodide | 2.0g |
| Distilled water | 300ml |

**Safranin solution**

| | |
|---|---|
| Safranin | 1.0g |
| Distilled water | 100ml |

**Crystal violet solution**

| | |
|---|---|
| Crystal violet | 500mg |
| Distilled water | 100ml |

**Kovac's Indole Reagents**

| | |
|---|---|
| Para-dimethyl aminobenzaldehyde | 10.0g |
| Pure amyl alcohol | 150ml |
| Conc. Pure hydrochloric acid | 50ml |

The aldehyde is dissolved in alcohol and the acid is slowly added before storing at 4$^{o}$C.

**Koser's citrate medium**

| | |
|---|---|
| Sodium ammonium hydrogen phosphate | 0.15g |
| Potassium dihydrogen phosphate | 0.10g |
| Magnesium sulphate | 0.20g |
| Sodium citrate | 0.20g |
| Bromothymol blue | 0.16mg |
| Distilled water | 100ml |

**Plasmid extraction solutions**

Solution 1

1ml of 500mM glucose

1ml of 100mM EDTA

2.5ml of 100mM Tris HCl pH 8

40mg Lysozyme

Made up to the volume 10ml sterile water

Solution 2

0.6ml of 2N NaOH

0.6ml of 10% SDS

4.8ml of sterile water

Solution 3

6ml of 5M Potassium acetate pH4.8

1.5ml glacial acetic acid

2.5ml sterile water

## Appendix II

## Antibiogram for the Human isolates

| ISOLATE | CAZ | FOX | CTX | AMC | IPM | ATM | FEP | CIP | NAL | TMP | GEN | AML | ESBL DETECTION |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H1 | 14(R)[a] | 28(S) | R | 14(I) | 29(S) | 17(R) | 19(S) | 22(S) | 17 | R | R | R | + |
| 2 | 27(S) | 27(S) | 30(S) | 17(I) | 28(S) | 29(S) | 30(S) | 30(S) | 23 | 25 | 26 | 24 | - |
| 3 | 27(S) | 25(S) | 29(S) | 16(I) | 27(S) | 29(S) | 30(S) | R | R | R | R | R | - |
| 4 | 24(S) | 23(S) | 26(S) | 20(S) | 27(S) | 30(S) | 29(S) | R | R | R | 23 | 20 | - |
| 5 | 25(S) | 27(S) | 30(S) | 19(S) | 27(S) | 29(S) | 29(S) | 31(S) | 25 | 26 | 26 | 26 | - |
| 6 | 18(I) | 26(S) | 10(R) | 16(I) | 28(S) | 20(I) | 22(S) | 26(S) | 10 | R | 15 | R | + |
| 7 | 27(S) | 27(S) | 28(S) | 16(I) | 26(S) | 30(S) | 30(S) | R | R | R | R | R | - |
| 8 | 25(S) | 20(S) | 30(S) | 17(I) | 26(S) | 27(S) | 29(S) | R | R | R | R | R | - |
| 9 | 21(S) | 20(S) | 25(I) | 17(I) | 26(S) | 25(S) | 26(S) | R | R | R | R | R | - |
| 10 | 25(S) | 25(S) | 30(S) | 17(I) | 26(S) | 29(S) | 29(S) | 32(S) | 25 | 25 | 24 | R | - |
| 11 | 26(S) | 27(S) | 28(S) | 19(S) | 27(S) | 32(S) | 30(S) | 34(S) | 26 | R | 29 | R | - |
| 12 | 12(R) | 23(S) | 11(R) | 16(I) | 30(S) | 16(R) | 20(S) | R | R | R | R | R | + |
| 13 | 16(R) | 26(S) | 15(R) | 19(S) | 30(S) | 20(I) | 20(S) | R | R | R | R | R | + |
| 14 | 21(S) | 20(S) | 28(S) | 19(S) | 24(S) | 28(S) | 25(S) | 30(S) | 24 | 25 | 29 | R | - |
| 15 | R | 24(S) | R | 11(R) | 30(S) | 16(R) | 16(I) | R | R | R | R | R | + |
| 16 | 26(S) | 21(S) | 28(S) | 20(S) | 26(S) | 28(S) | 30(S) | 33(S) | 24 | 23 | 25 | 24 | - |
| 17 | 26(S) | 27(S) | 30(S) | 15(I) | 26(S) | 28(S) | 29(S) | 34(S) | 26 | R | 25 | R | - |
| 18 | 25(S) | 25(S) | 31(S) | 20(S) | 30(S) | 30(S) | 29(S) | 35(S) | 26 | 27 | 28 | 29 | - |
| 19 | 27(S) | 25(S) | 30(S) | 15(I) | 26(S) | 28(S) | 29(S) | 32(S) | 25 | R | 21 | R | - |
| 20 | 24(S) | 22(S) | 29(S) | 10(R) | 27(S) | 26(S) | 27(S) | R | R | R | 27 | R | - |
| 21 | 22(S) | 22(S) | 26(S) | 13(R) | 22(I) | 25(S) | 25(S) | 35(S) | 25 | 24 | 27 | R | - |
| 22 | 14(R) | 25(S) | 13(R) | 12(R) | 27(S) | 18(I) | 22(S) | R | R | R | R | R | + |
| 23 | 25(S) | 26(S) | 25(I) | R | 28(S) | 29(S) | 25(S) | R | R | R | R | R | - |
| 24 | 25(S) | 27(S) | 29(S) | R | 26(S) | 26(S) | 25(S) | R | R | R | R | R | - |
| 25 | 26(S) | 25(S) | 26(S) | R | 27(S) | 30(S) | 26(S) | R | R | R | R | R | - |

| ISOLATE | CAZ | FOX | CTX | AMC | IPM | ATM | FEP | CIP | NAL | TMP | GEN | AML | ESBL DETECTION |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 26 | 24(S) | 27(S) | 29(S) | 15(I) | 27(S) | 26(S) | 27(S) | R | R | R | R | R | - |
| 27 | 27(S) | 25(S) | 27(S) | R | 27(S) | 30(S) | 25(S) | R | R | R | R | R | - |
| 28 | 26(S) | 27(S) | 26(S) | R | 28(S) | 27(S) | 26(S) | R | R | R | R | R | - |
| 29 | 27(S) | 29(S) | 28(S) | R | 26(S) | 28(S) | 24(S) | R | R | R | R | R | - |
| 30 | 25(S) | 26(S) | 30(S) | 19(S) | 27(S) | 29(S) | 29(S) | 29(S) | 25 | R | 26 | R | - |
| 31 | 26(S) | 25(S) | 28(S) | 19(S) | 26(S) | 30(S) | 27(S) | 31(S) | 25 | R | 27 | R | - |
| 32 | 22(S) | 24(S) | 26(S) | 16(I) | 26(S) | 27(S) | 28(S) | R | R | R | R | R | - |
| 33 | 25(S) | 24(S) | 27(S) | 16(I) | 25(S) | 26(S) | 29(S) | R | R | R | R | R | - |
| 34 | 24(S) | 21(S) | 25(I) | 15(I) | 25(S) | 26(S) | 24(S) | R | R | R | R | R | - |
| 35 | 26(S) | 25(S) | 30(S) | 15(I) | 25(S) | 26(S) | 28(S) | R | R | R | R | R | - |
| 36 | 28(S) | 28(S) | 30(S) | 12(R) | 28(S) | 30(S) | 30(S) | 29(S) | R | R | R | R | - |
| 37 | 26(S) | 27(S) | 29(S) | 13(R) | 25(S) | 27(S) | 29(S) | 24(S) | R | R | R | R | - |
| 38 | 25(S) | 25(S) | 28(S) | 17(I) | 26(S) | 27(S) | 30(S) | 30(S) | 24 | R | 27 | R | - |
| 39 | 26(S) | 25(S) | 30(S) | 16(I) | 25(S) | 27(S) | 26(S) | 30(S) | 20 | R | 23 | R | - |
| 40 | 28(S) | 28(S) | 30(S) | R | 28(S) | 28(S) | 30(S) | 29(S) | 23 | R | 24 | R | - |
| 41 | 28(S) | 26(S) | 29(S) | R | 28(S) | 30(S) | 27(S) | 33(S) | 27 | R | 29 | R | - |
| 42 | 26(S) | 23(S) | 29(S) | 17(I) | 27(S) | 30(S) | 30(S) | 32(S) | 26 | 25 | 27 | R | - |
| 44 | 28(S) | 22(S) | 30(S) | 14(I) | 28(S) | 29(S) | 27(S) | 30(S) | 25 | R | 27 | R | - |
| 45 | 21(S) | 26(S) | R | 15(I) | 30(S) | 18(I) | 19(S) | 24(S) | 20 | R | R | R | + |
| 46 | 30(S) | 29(S) | 33(S) | 20(S) | 30(S) | 31(S) | 30(S) | 34(S) | 26 | R | 26 | R | - |
| 47 | 20(I) | 26(S) | R | 13(R) | 26(S) | 20(I) | 17(I) | 25(S) | 18 | R | R | R | + |
| 48 | 25(S) | 23(S) | 29(S) | 14(I) | 25(S) | 27(S) | 30(S) | 35(S) | 27 | R | 27 | R | - |
| 49 | 25(S) | 24(S) | 28(S) | 20(S) | 25(S) | 28(S) | 30(S) | 33(S) | 26 | 25 | 25 | 24 | - |
| 50 | 30(S) | 27(S) | 29(S) | 15(I) | 28(S) | 28(S) | 29(S) | 33(S) | 26 | R | 25 | R | - |
| 51 | 29(S) | 24(S) | 28(S) | 14(I) | 28(S) | 30(S) | 30(S) | R | R | R | R | R | - |
| 52 | 27(S) | 24(S) | 26(S) | 14(I) | 26(S) | 26(S) | 27(S) | R | R | R | R | R | - |

| ISOLATE | CAZ | FOX | CTX | AMC | IPM | ATM | FEP | CIP | NAL | TMP | GEN | AML | ESBL DETECTION |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 53 | 28(S) | 23(S) | 29(S) | 18(S) | 25(S) | 30(S) | 29(S) | 33(S) | 27 | R | 28 | 23 | - |
| 54 | 27(S) | 24(S) | 30(S) | 13(R) | 28(S) | 30(S) | 27(S) | 30(S) | 28 | R | 23 | R | - |
| 55 | 30(S) | 24(S) | 30(S) | R | 27(S) | 34(S) | 26(S) | 22(S) | R | R | 22 | R | - |
| 56 | 27(S) | 25(S) | 29(S) | 18(S) | 30(S) | 30(S) | 27(S) | 30(S) | 26 | 29 | 25 | R | - |
| 57 | 30(S) | 25(S) | 30(S) | 18(S) | 30(S) | 29(S) | 28(S) | 34(S) | 27 | R | 21 | R | - |
| 58 | 29(S) | 24(S) | 30(S) | 17(I) | 27(S) | 30(S) | 30(S) | 32(S) | 28 | R | 22 | R | - |

CAZ-Ceftazidime 30µg, FOX-Cefoxitin 30µg, CTX-Cefotaxime 30µg, AMC-Amoxicillin-Clavulanic acid-20/10µg, IPM-Imipnem 10µg, ATM-Azetronam 30µg,FEP-Cefepime 30µg CIP-Ciprofloxacin 5µg, NAL-Nalidixic acid 30µg, TMP-Trimethoprim 5µg, GEN-Gentamicin-10µg, Amoxicillin- 25µg,+(Positive for ESBL Detection), a-Zone of inhibition in mm (-Negative for ESBL Detection), S- Sensitive, I-Intermediate, R- Resistant, H-Human

## Appendix III

## Antibiogram for the Bovine isolates

| ISOLATE | CAZ | FOX | CTX | AMC | IPM | ATM | FEP | CIP | NAL | TMP | GEN | AML | ESBL DETECTION |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A**1 | 27(S)[a] | 27(S) | 31(S) | 21(S) | 28(S) | 30(S) | 33(S) | 32(S) | 26 | 29 | 29 | 21 | - |
| 2 | 31(S) | 27(S) | 33(S) | 20(S) | 26(S) | 27(S) | 31(S) | 34(S) | 26 | 25 | 27 | 23 | - |
| 3 | 30(S) | 23(S) | 35(S) | 21(S) | 30(S) | 30(S) | 34(S) | 31(S) | 25 | 23 | 26 | 22 | - |
| 4 | 30(S) | 27(S) | 30(S) | 20(S) | 26(S) | 31(S) | 29(S) | 32(S) | 23 | 26 | 26 | 20 | - |
| 5 | 29(S) | 29(S) | 34(S) | 20(S) | 30(S) | 32(S) | 30(S) | 30(S) | 25 | 27 | 27 | R | - |
| 6 | 28(S) | 25(S) | 30(S) | 22(S) | 26(S) | 30(S) | 32(S) | 31(S) | 28 | 25 | 24 | R | - |
| 7 | 28(S) | 27(S) | 32(S) | 20(S) | 24(S) | 30(S) | 31(S) | 32(S) | 24 | 28 | 28 | 22 | - |
| 8 | 30(S) | 27(S) | 34(S) | 21(S) | 26(S) | 29(S) | 29(S) | 30(S) | 24 | 25 | 27 | 24 | - |
| 9 | 26(S) | 26(S) | 29(S) | 20(S) | 26(S) | 30(S) | 30(S) | 34(S) | 23 | 24 | 26 | 22 | - |
| 10 | 32(S) | 26(S) | 35(S) | 20(S) | 26(S) | 31(S) | 32(S) | 32(S) | 23 | 25 | 26 | 23 | - |
| 11 | 30(S) | 29(S) | 33(S) | 20(S) | 29(S) | 30(S) | 30(S) | 32(S) | 25 | 26 | 26 | 25 | - |
| 12 | 30(S) | 27(S) | 33(S) | 19(S) | 30(S) | 35(S) | 32(S) | 34(S) | 28 | 27 | 26 | 28 | - |
| 13 | 25(S) | 26(S) | 30(S) | 20(S) | 28(S) | 27(S) | 29(S) | 30(S) | 27 | 25 | 27 | 27 | - |
| 14 | 30(S) | 25(S) | 32(S) | 22(S) | 27(S) | 27(S) | 30(S) | 32(S) | 26 | 27 | 26 | 25 | - |
| 15 | 28(S) | 28(S) | 31(S) | 20(S) | 26(S) | 30(S) | 30(S) | 34(S) | 25 | R | 28 | 25 | - |
| 17 | 30(S) | 26(S) | 32(S) | 21(S) | 27(S) | 31(S) | 31(S) | 29(S) | 24 | 29 | 29 | 24 | - |
| 18 | 29(S) | 26(S) | 34(S) | 22(S) | 26(S) | 32(S) | 33(S) | 36(S) | 27 | R | 27 | 27 | - |
| 19 | 31(S) | 27(S) | 32(S) | 21(S) | 27(S) | 32(S) | 32(S) | 29(S) | 24 | R | 22 | 16 | - |
| 20 | 29(S) | 25(S) | 32(S) | 22(S) | 27(S) | 30(S) | 31(S) | 32(S) | 24 | R | 25 | 14 | - |
| 21 | 27(S) | 25(S) | 30(S) | 20(S) | 29(S) | 31(S) | 32(S) | 30(S) | 23 | 25 | 24 | 23 | - |
| 22 | 30(S) | 27(S) | 32(S) | 21(S) | 27(S) | 34(S) | 30(S) | 30(S) | 24 | 24 | 27 | 11 | - |
| 23 | 30(S) | 26(S) | 33(S) | 22(S) | 26(S) | 31(S) | 30(S) | 29(S) | 25 | 26 | 26 | 23 | - |
| 24 | 30(S) | 25(S) | 33(S) | 21(S) | 30(S) | 34(S) | 32(S) | 34(S) | 24 | 25 | 26 | 25 | - |

| ISOLATE | CAZ | FOX | CTX | AMC | IPM | ATM | FEP | CIP | NAL | TMP | GEN | AML | ESBL DETECTION |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 30(S) | 24(S) | 30(S) | 20(S) | 26(S) | 31(S) | 28(S) | 30(S) | 25 | 26 | 28 | 25 | - |
| 26 | 27(S) | 27(S) | 32(S) | 20(S) | 29(S) | 31(S) | 30(S) | 31(S) | 23 | 24 | 25 | 24 | - |
| 27 | 31(S) | 25(S) | 33(S) | 19(S) | 30(S) | 32(S) | 32(S) | 33(S) | 25 | 28 | 30 | 27 | - |
| 28 | 33(S) | 25(S) | 32(S) | 21(S) | 30(S) | 35(S) | 33(S) | 29(S) | R | R | 28 | 29 | - |
| 29 | 32(S) | 30(S) | 31(S) | 21(S) | 29(S) | 31(S) | 33(S) | 32(S) | 27 | 29 | 25 | 25 | - |
| 30 | 33(S) | 29(S) | 35(S) | 20(S) | 30(S) | 33(S) | 30(S) | 31(S) | 25 | 25 | 26 | 25 | - |
| 31 | 29(S) | 24(S) | 30(S) | 18(S) | 26(S) | 30(S) | 29(S) | 31(S) | 25 | R | 26 | R | - |
| 32 | 26(S) | 24(S) | 27(S) | 21(S) | 24(S) | 30(S) | 30(S) | 32(S) | 25 | 27 | 26 | 23 | - |
| 33 | 25(S) | 23(S) | 25(I) | 18(S) | 26(S) | 26(S) | 28(S) | 34(S) | 25 | 24 | 29 | 22 | - |
| 34 | 30(S) | 26(S) | 32(S) | 21(S) | 26(S) | 309S) | 31(S) | 28(S) | 21 | 25 | 20 | 26 | - |
| 35 | 33(S) | 27(S) | 32(S) | 19(S) | 28(S) | 30(S) | 29(S) | 31(S) | 24 | 27 | 22 | 25 | - |
| 36 | 29(S) | 27(S) | 32(S) | 19(S) | 28(S) | 32(S) | 31(S) | 27(S) | 21 | R | 26 | R | - |
| 37 | 29(S) | 26(S) | 30(S) | 22(S) | 30(S) | 31(S) | 30(S) | 34(S) | 25 | 30 | 29 | 26 | - |
| 38 | 31(S) | 26(S) | 33(S) | 21(S) | 30(S) | 35(S) | 31(S) | 33(S) | 21 | 25 | 23 | 22 | - |
| 39 | 30(S) | 26(S) | 33(S) | 22(S) | 29(S) | 32(S) | 32(S) | 30(S) | 25 | R | 28 | R | - |
| 40 | 29(S) | 24(S) | 29(S) | 20(S) | 27(S) | 30(S) | 30(S) | 32(S) | 22 | R | 27 | 11 | - |
| 41 | 29(S) | 24(S) | 30(S) | 19(S) | 30(S) | 32(S) | 32(S) | 29(S) | 23 | R | 29 | R | - |
| 42 | 26(S) | 25(S) | 27(S) | 19(S) | 26(S) | 28(S) | 28(S) | 33(S) | R | R | 28 | R | - |
| 43 | 29(S) | 26(S) | 29(S) | 21(S) | 27(S) | 30(S) | 31(S) | 30(S) | 24 | 25 | 25 | 24 | - |
| 44 | 29(S) | 24(S) | 30(S) | 22(S) | 28(S) | 32(S) | 289S) | 30(S) | 21 | 25 | 25 | 21 | - |
| 45 | 27(S) | 25(S) | 29(S) | 22(S) | 27(S) | 28(S) | 28(S) | 35(S) | 26 | 32 | 27 | 28 | - |
| 46 | 27(S) | 27(S) | 30(S) | 21(S) | 31(S) | 35(S) | 32(S) | 34(S) | 25 | R | 27 | 26 | - |
| 47 | 30(S) | 25(S) | 32(S) | 20(S) | 25(S) | 28(S) | 30(S) | 30(S) | 28 | 26 | 25 | 25 | - |
| 48 | 31(S) | 25(S) | 30(S) | 22(S) | 29(S) | 31(S) | 30(S) | 30(S) | 24 | 25 | 25 | 24 | - |
| 49 | 34(S) | 28(S) | 32(S) | 19(S) | 31(S) | 33(S) | 30(S) | 31(S) | 25 | 27 | 27 | 25 | - |

| ISOLATE | CAZ | FOX | CTX | AMC | IPM | ATM | FEP | CIP | NAL | TMP | GEN | AML | ESBL DETECTION |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 28(S) | 25(S) | 32(S) | 18(S) | 27(S) | 29(S) | 29(S) | 32(S) | 26 | 26 | 26 | 24 | - |
| 51 | 30(S) | 27(S) | 33(S) | 21(S) | 30(S) | 32(S) | 33(S) | 35(S) | 23 | 25 | 26 | 21 | - |
| 52 | 31(S) | 30(S) | 33(S) | 20(S) | 32(S) | 35(S) | 30(S) | 33(S) | 24 | 28 | 25 | 24 | - |
| 53 | 30(S) | 25(S) | 32(S) | 18(S) | 28(S) | 33(S) | 30(S) | 33(S) | 24 | 25 | 22 | 14 | - |
| 54 | 29(S) | 29(S) | 33(S) | 22(S) | 30(S) | 30(S) | 27(S) | 34(S) | 25 | R | 27 | R | - |
| 55 | 30(S) | 27(S) | 31(S) | 24(S) | 28(S) | 30(S) | 33(S) | 33(S) | 29 | R | 26 | 29 | - |
| 56 | 30(S) | 26(S) | 31(S) | 21(S) | 27(S) | 32(S) | 29(S) | 30(S) | 24 | 27 | 25 | 25 | - |
| 57 | 30(S) | 28(S) | 34(S) | 22(S) | 30(S) | 33(S) | 31(S) | 31(S) | 26 | 28 | 23 | 25 | - |
| 58 | 29(S) | 26(S) | 32(S) | 21(S) | 26(S) | 30(S) | 30(S) | 35(S) | 25 | 29 | 26 | 27 | - |

CAZ-Ceftazidime 30µg, FOX-Cefoxitin 30µg, CTX-Cefotaxime 30µg, AMC-Amoxicillin-Clavulanic acid-20/10µg, IPM-Imipnem 10µg, ATM-Azetronam 30µg, FEP-Cefepime 30µg CIP-Ciprofloxacin 5µg, NAL-Nalidixic acid 30µg, TMP-Trimethoprim 5µg, GEN-Gentamicin-10µg, Amoxicillin- 25µg, a- Zone of inhibition in mm
(-Negative FOR ESBL Detection), S- Sensitive, I-Intermediate, R-Resistant, A-Animal

## Appendix IV

## Statistical analysis

**COEFFICIENT OF CORRELATION = r**

$$r = \frac{n(\Sigma XY)-(\Sigma X)(\Sigma Y)}{\sqrt{\{n(\Sigma X^2)-(\Sigma X)^2\}\{n(\Sigma Y^2)-(\Sigma Y)^2\}}}$$

Where n = total number of paired observations.

$\Sigma XY$ = Sum of the total number of the multiplication of X and Y terms

$\Sigma X$ = Sum of X variables.

$\Sigma Y$ = Sum of Y variables.

$\Sigma X^2$ = Sum of squares of X variable.

$\Sigma Y^2$ = Sum of the square root of each of the Y term.

$(\Sigma X)^2$ =2 Square of the sum of the total number of X term.

$(\Sigma Y)^2$ = Square of the sum of the total number of Y term.

**COEFFICIENT OF DETERMINATION = $r^2$**

$r^2$ = Square root of coefficient of correlation

$$r^2 = \left[\frac{n\Sigma xy - \Sigma x\Sigma y}{\sqrt{\left[n\Sigma x^2 - (\Sigma x)^2\right]\left[n\Sigma y^2 - (\Sigma y)^2\right]}}\right]^2$$

Coefficient of variation = $r^2$ X 100%

T-test for correlation coefficient $= \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$

Where r = coefficient of correlation

n = Total number of population terms

$r^2$ = Coefficient of determination.

t-tabulated at 0.05 level of significance

t-tabulated = + or - t 0.05 (n-2)

Where n = Total number of population term.

**To test for correlation coefficient between the susceptibility pattern of the bovine and human *E.coli* isolates to Gentamicin**

$n\sum XY$ 1152312

| | |
|---|---|
| $\sum X \sum Y$ | 1158373 |
| $n\sum X^2$ | 2223399 |
| $(\sum X)^2$ | 2211169 |
| $n\sum Y^2$ | 1130709 |
| $(\sum Y)^2$ | 606841 |

| | |
|---|---|
| $n\sum X^2 - (\sum X)^2$ | 12230 |
| $n\sum Y^2 - (\sum Y)^2$ | 523868 |
| $[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]$ | 6406905640 |
| $\{[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]\}^{0.5}$ | 80043.14861 |

Coefficient of correlation, r = -0.075721659

From the formulae above

This indicates a weak negative correlation or relationship between the susceptibility pattern of the bovine and the human *E.coli* isolates to Gentamicin

: . Coefficient of determination = $r^2 = (-0.075721659)^2 = 0.00573377$

: . $r^2 = 0.6\%$

Therefore 0.6% of the variation in the susceptibility pattern of the bovine isolates is accounted for by the variation in the susceptibility pattern of the human isolates

t-test for correlation coefficient = t calculated (-0.60).

t-Tabulated = + or – t 0.05 (55)

t-Tabulated = 2.00

t -calculated (- 0.60) is less than the t tabulated (2.00).

Therefore we accept the Ho (null) hypothesis indicating no correlation and reject the alternative at 0.05 level of significant. This indicates enough evidence to say that there is no relationship in the susceptibility pattern of the bovine isolates and the human isolates to gentamicin

**To test for correlation coefficient between the susceptibility pattern of the bovine and human *E.coli* isolates to Ciprofloxacin**

n∑XY 1875585

| | |
|---|---|
| $\sum X \sum Y$ | 1869980 |
| $n\sum X^2$ | 3270603 |
| $(\sum X)^2$ | 3258025 |
| $n\sum Y^2$ | 1824342 |
| $(\sum Y)^2$ | 1073296 |

| | |
|---|---|
| $n\sum XY - \sum X \sum Y$ | 5605 |
| $n\sum X^2 - (\sum X)^2$ | 12578 |
| $n\sum Y^2 - (\sum Y)^2$ | 751046 |
| $[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]$ | 9446656588 |
| $\{[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]\}^{0.5}$ | 97193.9123 |

Coefficient of correlation, r = 0.057668221

From the formulae above

This indicates a weak positive correlation or relationship between the susceptibility pattern of the bovine and the human *E.coli* isolates to Ciprofloxacin

: . Coefficient of determination = $r^2 = (0.057668221)^2 = 0.003325624$

: . $r^2$ = 0.3%

Therefore 0.3% of the variation in the susceptibility pattern of the bovine isolates is accounted for by the variation in the susceptibility pattern of the human isolates

t-test for correlation coefficient = t calculated (0.45).

t-Tabulated = + or – t 0.05 (55)

t-Tabulated = 2.00

t -calculated (0.45) is less than the t tabulated (2.00).

Therefore we accept the Ho (null) hypothesis indicating no correlation and reject the alternative at 0.05 level of significant. This indicates enough evidence to say that there is no relationship in the susceptibility pattern of the bovine isolates and the human isolates to ciprofloxacin

**To test for correlation coefficient between the susceptibility pattern of the bovine and human *E.coli* isolates to Amoxicillin**

| | |
|---|---|
| $n\sum XY$ | 166098 |
| $\sum X\sum Y$ | 194140 |
| $n\sum X^2$ | 1562028 |
| $(\sum X)^2$ | 1304164 |
| $n\sum Y^2$ | 237918 |
| $(\sum Y)^2$ | 28900 |

| | |
|---|---|
| $n\sum XY - \sum X\sum Y$ | -28042 |
| $n\sum X^2 - (\sum X)^2$ | 257864 |
| $n\sum Y^2 - (\sum Y)^2$ | 209018 |
| $[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]$ | 53898217552 |
| $\{[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]\}^{0.5}$ | 232159.8965 |

Coefficient of correlation, r = -0.120787442

From the formulae above

This indicates a weak negative correlation or relationship between the susceptibility pattern of the bovine and the human *E.coli* isolates to Amoxicillin

: . Coefficient of determination = $r^2 = (-0.120787442)^2$

$= 0.014589606$

: . $r^2 = 1.5\%$

Therefore 1.5% of the variation in the susceptibility pattern of the bovine isolates is accounted for by the variation in the susceptibility pattern of the human isolates

t-test for correlation coefficient = t calculated (-0.9).

t-Tabulated = + or – t 0.05 (55)

t-Tabulated = 2.00

t -calculated (-0.9) is less than the t tabulated (2.00).

Therefore we accept the Ho (null) hypothesis indicating no correlation and reject the alternative at 0.05 level of significant. This indicates enough evidence to say that there is no relationship in the susceptibility pattern of the bovine isolates and the human isolates to Amoxicillin

**To test for correlation coefficient between the susceptibility pattern of the bovine and human *E.coli* isolates to Ceftazidime**

$n\sum XY$ 2294193

| | |
|---|---|
| $\sum X \sum Y$ | 2292768 |
| $n\sum X^2$ | 2820816 |
| $(\sum X)^2$ | 2808976 |
| $n\sum Y^2$ | 1982688 |
| $(\sum Y)^2$ | 1871424 |

$$n\sum XY - \sum X \sum Y \qquad 1425$$

$$n\sum X^2 - (\sum X)^2 \qquad 11840$$

$$n\sum Y^2 - (\sum Y)^2 \qquad 111264$$

$$[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2] \qquad 1317365760$$

$$\{[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]\}^{0.5} \qquad 36295.53361$$

Coefficient of correlation, r = 0.039261029

From the formulae above

This indicates a weak positive correlation or relationship between the susceptibility pattern of the bovine and the human *E.coli* isolates to Ceftazidime

: . Coefficient of determination = $r^2 = (0.039261029)^{2 =} 0.001541428$

: . $r^2 = 0.15\%$

Therefore 0.15% of the variation in the susceptibility pattern of the bovine isolates is accounted for by the variation in the susceptibility pattern of the human isolates

t-test for correlation coefficient = t calculated (0.3).

t-Tabulated = + or – t 0.05 (55)

t-Tabulated = 2.00

t -calculated (0.3) is less than the t tabulated (2.00).

Therefore we accept the Ho (null) hypothesis indicating no correlation and reject the alternative at 0.05 level of significant. This indicates enough evidence to say that there is no relationship in the susceptibility pattern of the bovine isolates and the human isolates to Ceftazidime

**To test for correlation coefficient between the susceptibility pattern of the bovine and human *E.coli* isolates to Cefotaxime**

| | |
|---|---|
| n∑XY | 2608320 |
| ∑X∑Y | 2602404 |

| | |
|---|---|
| $n\sum X^2$ | 3238398 |
| $(\sum X)^2$ | 3225616 |
| $n\sum Y^2$ | 2323377 |
| $(\sum Y)^2$ | 2099601 |

| | |
|---|---|
| $n\sum XY - \sum X\sum Y$ | 5916 |
| $n\sum X^2 - (\sum X)^2$ | 12782 |
| $n\sum Y^2 - (\sum Y)^2$ | 223776 |
| $[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]$ | 2860304832 |
| $\{[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]\}^{0.5}$ | 53481.81777 |

Coefficient of correlation, r = 0.110617033

From the formulae above

This indicates a weak positive correlation or relationship between the susceptibility pattern of the bovine and the human *E.coli* isolates to Cefotaxime

: . Coefficient of determination = $r^2 = (0.110617033)^{2 =} 0.012236128$

: . $r^2 = 1.2\%$

Therefore 1.2% of the variation in the susceptibility pattern of the bovine isolates is accounted for by the variation in the susceptibility pattern of the human isolates

t-test for correlation coefficient = t calculated (0.82).

t-Tabulated = + or – t 0.05 (55)

t-Tabulated = 2.00

t -calculated (0.82) is less than the t tabulated (2.00).

Therefore we accept the Ho (null) hypothesis indicating no correlation and reject the alternative at 0.05 level of significant. This indicates enough evidence to say that there is no relationship in the susceptibility pattern of the bovine isolates and the human isolates to Cefotaxime

**PUBLISHED PAPER AND ABSTRACT FROM THIS RESEARCH THESIS**

**Publication:**

**Inwezerua, C**., Mendonça, N., Calhau, V., Domingues, S., Adeleke, O.E., Da Silva G.J. 2014. Occurrence of extended-spectrum beta-lactamases in human and bovine *Escherichia coli* isolates from Oyo state, Nigeria. *Journal of Infection in developing countries* **8**(6): 774-779

**Abstract:**

Prevalence and characterization of extended-spectrum beta-lactamases in human and bovine isolates of *Escherichia coli* from Oyo state, Nigeria. Abstract of the 22nd European congress of Clinical Microbiology and Infectious Diseases held March 31st – April 3rd 2012, East London United Kingdom.