CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Extended-spectrum beta-lactamases (ESBLs) have been increasingly reported in the world since their first description in 1983, shortly after a variety of extended-spectrum cephalosporins were introduced in the 1980s. Due to indiscriminate use of these antibiotics, tremendous increase in antibiotics resistance among pathogenic organisms that were previously susceptible to these commonly used antibiotics in clinics/hospitals has resulted. Beta-lactam antimicrobial agents represent the most common treatment for bacterial infections. The persistent exposure of bacterial strains to a multitude of β-lactams has induced continuous production and mutation of β-lactamases in these bacteria thus, expanding their activity even against the newly developed β-lactam antibiotics (Livermore, 2012; Paterson and Bonomo, 2005; Pitout and Laupland, 2008). Extended spectrum beta lactamase (ESBLs) are enzymes produced by Enterobactericeae family (mainly *Escherichia coli* and *Klebsiella pneumoniae*) that can confer resistance to first, second and third generation cephalosporins. They are on the rise in hospital and community settings across the globe. During the 1990s, they were described mainly as member of the TEM- and SHV-beta-lactamase families in *Klebsiella pneumoniae* causing nosocomial outbreaks (Coque *et al.*, 2008).

Nowadays, they are mostly found in *Escherichia coli* that cause community-acquired infections and with increasing frequency contain CTX-M enzymes. Beta-lactam antibiotics account for approximately 50 % of global antibiotic consumption and this heavy usage has exerted considerable selection for resistance (Livermore, 2007). *Klebsiella pneumoniae* and *Escherichia coli* remain the major ESBL-producing organisms isolated worldwide (Jacoby and Munoz-Price,

2005) which are recommended to be routinely tested for and reported by the Clinical and Laboratory Standards Institute (CLSI, 2012).

Production of beta-lactamases is the most common cause of resistance to beta-lactam antibacterial agents among enterobacteriaceae or Gram-negative bacteria (Pitout *et al.,* 2004). These enzymes undermined the utilization of ampicillin, first and second-generation cephalosporins in the chemotherapy of infection caused by Gram-negative bacteria. To overcome the problems posed by the beta-lactamase enzymes, third and fourth generation extended-spectrum cephalosporins were developed. Unfortunately, members of the family of Enterobacteriaceae have developed resistance to these extended-spectrum cephalosporins via production of extended-spectrum beta-lactamase (ESBL) (Canton *et al.,* 2008; Behrooozi *et al*., 2010). Apart from the ESBL enzymes, resistance to extended spectrum cephalosporins in enterobacteria has also been found to be mediated by AMP-C beta-lactamase (Philippon *et al.*, 2002).

Extended-spectrum β-lactamases (ESBLs) as noted by some authors are heterogeneous group of plasmid-mediated bacterial enzymes that confer significant resistance to oxyimino-cephalosporin and monobactam antimicrobials (Canton *et al.,* 2008; Bradford, 2001) or they are enzymes that mediate resistance to beta-lactam antibiotics including third generation cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) and monobactams (aztreonam) but do not affect cephamycins (cefoxitin and cefotetan) or carbapenems (meropenem or imipenem) (Philippon *et al.,* 2002). A broader definition would include all β-lactamases capable of hydrolysing 3GCs (third generation cephalosporins). ESBLs are β-lactamase enzymes, generally acquired rather than inherent to a bacterial species, that confers resistance to oxyimino-cephalosporins (but not carbapenems) (Giske *et al.,* 2009). Some are mutant derivatives of well-established plasmid-

mediated β-lactamases (TEM and SHV) which, in their un-mutated form, are not able to degrade oxyimino-cephalosporins. Others (such as CTX-M types) have been mobilised on plasmids from chromosomal genes in environmental/opportunistic bacteria. Extended spectrum beta-lactamases (ESBLs) are on the rise in hospital settings across the globe (Livermore *et al.,* 2007; Stürenburg and Mack, 2003; Perez *et al.,* 2007; Romero *et al.,* 2007; Kuo *et al.,* 2007; Messai *et al.,* 2008).

Throughout the world, increasing attention is being focused on the growing involvement of ESBL-producing strains of *E. coli* in serious infections of hospitalized and non-hospitalized patients (Oteo *et al.,* 2010; Kalantar and Mansouri 2010; Peirano and Pitout 2010). This trend is largely due to the emergence of CTX-M type ESBLs, a rapidly expanding group of enzymes encountered with increasing frequency, especially in *E. coli* (Giske *et al.,* 2009). CTX-M type ESBLs have been spreading rapidly throughout Europe, America, Africa and Asia since early 21^{Ist}century and ESBL producing *E. coli* and *Klebsiella* species represents a major problem in human and veterinary medicine, particularly in nosocomial infections. Thus, ESBL-producing bacteria, mainly *E. coli* and *Klebsiella,* are rapidly increasing among human isolates and today more than 100 different ESBL types have been described, with the most widespread type being the CTX-M β-lactamases encoded by the $bla_{\text{CTX-M}}$ gene (Poirel *et al.,* 2002). It has also spread to other microorganisms such as *Salmonella*, *Shigella* and *Vibrio cholerae* (Livermore *et al.*, 2007). Human clinical strains of *Escherichia coli* producing ESBLs have been described from different parts of the world (Messai *et al.,* 2006). In Nigeria, these enzymes have been reported in *Enterobacter* species from human patients in Lagos (Aibinu *et al.,* 2003).

The first organisms producing β-lactamases of this type were identified as the single epidemic clinical isolates in the early 1990s (Bauernfeind *et al.,* 1996). Based on their amino-acid sequence diversity, the vast number (more than 50) of CTX-M variants identified thus far have

been classified into the following 5 major phylogenetic groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 (Livermore and Hawkey 2005; Nevine *et al.,* 2010). In contrast to TEM- and SHV-type ESBLs, most of the CTX-M enzymes are much more active against cefotaxime and ceftriaxone than against ceftazidime. Thus, most of the CTX-M producers display levels of resistance to cefotaxime significantly higher than those to ceftazidime. The gene coding for ESBLs are usually carried by plasmid, which facilitates their transfer among other genera of Gram-negative bacteria (Iroha *et al.,* 2008b). Strains with the ESBL phenotype are multi-drug resistant because the relevant plasmids may also carry other genes responsible for resistance to aminoglycosides and trimethoprim-sulfamethoxazole. Therefore, the rapid spread of ESBLs causes a significant threat to the therapy of infections in hospitals (Pitout *et al.,* 2005).

Staphylococcus aureus is a recognized human pathogen responsible for a great variety of pyogenic infection in man and animals, infecting about one-third of the world's population (Chahine *et al*., 2011). The pathogen is also capable of living a benign lifestyle in the nasal passage and skin (Okunye *et al*., 2011). It is the causative agent of many suppuration processes ranging from localized abscess which can occur in any part of the body, to fatal septicaemia and pneumonia. However, among Gram-positive cocci, the only β-lactamase of major clinical significance is Staphylococcal β-lactamase, which rapidly hydrolyses benzylpenicillin, ampicillin, cephalosporins, and related antimicrobials (Haque *et al*., 2011). This study was undertaken to assess extended spectrum beta-lactamase production and presence of resistance genes among *E.coli*, *Klebsiella pneumoniae and Staphylococcus aureus* isolated from soft tissue infections.

1.2 Statement of Problem

Massive quantities of beta-lactam antibiotics are being prepared and used each day in the hospitals for the treatment of bacterial infection. As a result of this, an increasing number of diseases are resisting treatment due to the spread of drug resistance, and much of the difficulty arises from drug misuse and over usage (Harrison and Bratcher 2008; Pitout and Laupland, 2008). This drug resistance is due to production of ESBL enzyme by certain organism and this makes the organism to manoeuvre different classes of beta-lactam drugs including cephalosporins, thereby making infections caused by such organism very difficult to treat. Therefore, patients with *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* infections are inherently resistant to many of the known cephalosporin classes of drugs and this may cause various kinds of infections which may lead to life-threatening diseases and sometimes death (Haque *et al*., 2011; Coque *et al.*, 2008). Thus, this study is greared towards investigating the presence of extended spectrum beta-lactamase production among *E.coli, Klebsiella pneumoniae and Staphylococcus aureus* isolated from patients suspected of wound, high vaginal and urinary tract infection.

1.3 Aim of the Study

The main aim of this study is to phenotypically and molecularly characterize ESBL-producing organisms isolated from patients attending Federal Teaching Hospital Abakaliki in Ebonyi State, Nigeria.

1.4 Objectives of the Study

1. To determine the presence of selected clinical isolates (*Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus*) in patient's specimen.

2. To determine the distribution of the isolates among patients with respect to age, sex, educational level and occupation.
3. To determine the resistance profiles of the clinical isolates to commonly used beta-lactam antibiotics.
4. To determine beta-lactamase production from the isolates.
5. To carry out phenotypic confirmation of extended spectrum beta-lactamase production.
6. To determine the types of ESBL gene haboured by the isolates.
7. To determine the location (plasmid-borne or chromosomal) of the resistance determinants in the ESBL positive bacteria isolates.
8. To determine the identity of the isolates through molecular analysis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Enterobacteriaceae

Enterobacteriaceae are a large group of heterogeneous Gram negative rods whose natural habitat is the intestinal tract of humans and animals (Brooks *et al.,* 2004). Most bacterial species under the Enterobacteriaceae grow well at 37^{o}C, and they are facultatively anaerobic, oxidase negative and catalase positive (except for *Shigella dysenteriae* type 1), and they are distributed worldwide and can also be found in the soil and water (Madigan *et al.,* 2009). Members of Enterobacteriaceae are Gram negative straight rods, some of which are sugar fermenters and motile in nature. They are also called enteric bacilli or bacteria, and they are made up of about 32 different genera and 130 different species (Madigan *et al.,* 2009). All members of bacteria in the Enterobacteriaceae are similar in morphology and cultural characteristics, and they can be differentiated biochemically using sugars of different types, and by their antigenic structure. The Enterobacteriaceae which are also known as Gram Negative Bacilli (GNB) have over the years continue to play an important role as a cause of healthcare- associated infections, and they are a common cause of sepsis, urinary tract infections, bacteremia and post surgical infections in patients in acute care hospitals or in the ICU's (Mumtaz *et al.,* 2008). Common genera of the family Enterobacteriaceae include: *Escherichia* species, *Klebsiella* species, *Citrobacter* species, *Enterobacter* species, *Salmonella* species, *Shigella* species, *Serratia* species, and *Proteus* species. The Enterobacteriaceae are non-spore forming bacteria, and majority of the organisms in this family of microorganisms produce a variety of toxins and other virulence factors which in most of the time forms the basis for their characterization (Zhang *et al.,* 2010). They are major pathogens in both humans and animals, normal commensal organisms in the colon of both man

and animals, and can also be referred to as coliforms or coliform bacteria. Enterobacteriaceae are indicators of water pollution owing to the fact that they are mostly found in the colon or gastrointestinal tract (GIT) of humans, and they are responsible for most cases of nosocomial infections in humans (Madigan *et al.,* 2009).

2.1.1 Biology of *Escherichia coli*

Escherichia coli are bacterium that is commonly found as a normal microflora in the intestinal tract of humans and animals (Brooks *et al*, 2004; Prescott *et al*, 2008). *E. coli* is an indicator organism in the feacal contamination of water and food materials. But some of these bacteria can cause a variety of serious infections in human beings including diarrhea, gastrointestinal infections, septicaemia, bacteraemia and UTIs (Brooks *et al*, 2004). Six species of *Escherichia* are known to exist, and four out of this number are known to cause disease in humans. *E. coli* are known to produce a number of enterotoxins and virulence factors including those that are associated with invasiveness, and some strains of *Escherichia coli* are known to be capsulated with "K" or capsule antigen (HPA, 2010). *E. coli* is a lactose fermenter, producing pink and yellow/blue colonies on MacConkey agar and Cystein Lactose Electrolyte Deficient (CLED) medium respectively. They can also be identified biochemically in the laboratory by a number of tests including: Indole test, Motility test, and other sugar based tests like Lactose, Mannitol, and Glucose tests (Cheesbrough, 2010). *E. coli* has five different strains including: Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC), and Enterotoxigenic *E. coli* (ETEC). These strains of *E. coli* are responsible for causing a range of infections in humans including diarrhea in infants, traveler's diarrhea, hemorrhagic colitis, and acute and chronic diarrhea in adults (Brooks *et al.,* 2004).

Beta-lactam antibiotics are often used to treat infections caused by *E. coli*. However, some strains of *E. coli* can build up resistance against antibiotics. The resistance spreads further to other strains of *E. coli* and other bacteria through frequent use of antibiotics. Resistance to β-lactam antibiotics in *E. coli* and other intestinal strains is usually caused by enzymes (in particular β-lactamases) that are produced by the bacteria themselves (Prescott *et al*., 2008). These enzymes hydrolyze antibiotics *in vivo*, and render them inefficacious in the treatment of bacterial related infections. Various β-lactamases (extended spectrum beta-lactamases) that have an effect on different antibiotics including 3rd generation cephalosporins such as ceftazidime, cefotaxime, and ceftriaxone are now being produced by *E. coli* and other Gram negative bacteria (Bush and Jacoby 2010).

2.1.2 Biology of *Klebsiella pneumoniae*

The genus *Klebsiella* belongs to the tribe *Klebsiellae*, a member of the family Enterobacteriaceae. The organism is named after Edwin Klebs (1834-1913), a 19th century German microbiologist. *Klebsiella pneumoniae* is non-motile, rod-shaped, Gram-negative bacteria with parallel or bulging side and slightly pointed or rounded end. They have a prominent polysaccharide based capsule and appear mucoid in colonies (Ryan and Ray, 2004). *Klebsiella* species have no specific growth requirements and grow well on standard laboratory media such as MacConkey agar, CLED and chocolate agar between temperature of 35^{0}C-37^{0}C and a pH of 7.2. The species are facultative anaerobes, and most strains can survive with citrate and glucose as their carbon source and ammonia as their sole nitrogen source. *Klebsiella* species are routinely found in the human nose, mouth and gastrointestinal tract as normal flora. However, they can also occur as opportunistic human pathogens. It can lead to wide range of disease state notably pneumonia, urinary tract infections, septicemia, meningitis, diarrhea and soft tissue infection.

The antimicrobial resistance associated with *Klebsiella pneumoniae* is thought to be attributed mainly to multi-drug efflux pump and plasmid transfer (Adesiyun and Downes, 2012).

2.1.3 Biology of *Staphylococcus aureus*

Staphylococci were first described by the Scottish surgeon Sir Alexander Ogston as the cause of a number of pyogenic (pus forming) infection on human in 1882, he gave them the name *Staphylococcus*, which is coined from two Greek words Staphyle, meaning bunch of grapes; and coccus, meaning grain or berry. *Staphylococci* are Gram positive spherical cells, usually arranged in grape-like irregular clusters and measured 1 micrometer in diameter. Some of these organisms are members of the normal flora of the skin and mucous membrane of human; others cause suppuration, abscess formation, a variety of pyogenic infections and even fatal septicaemia (Jawetz *et al.,* 2007). The three main species of clinical importance are *Staphylococcus aureus, Staphylococcus epidemidis* and *Staphylococcus sapophyticus.* This organism produces alpha, beta, gamma and delta toxins. The colonies on solid media are round smooth, raised. *Staphylococcus aureus* are catalase positive which differentiate them from other species, oxidase, indole, and voges proskauer negative, facultative anaerobes. It has the ability to ferment glucose. They grow most rapid at 37 ^{0}C but form pigment best at room temperature of 20-25 ^{0}C. They usually form grey to deep golden yellow colonies. Mannitol salt agar can be used as a selective media (Prescott *et al.,* 2008). *Staphylococcus aureus* has been implicated in antimicrobial resistance in both hospitals and community environment, they harbor resistant genes which cause resistance to the antibiotics. However, among gram-positive cocci, the only β-lactamase of major clinical significance is *Staphylococcal* β-lactamase, which rapidly hydrolyses benzylpenicillin, ampicillin, cephalosporins, and related antimicrobials (Haque *et al*., 2011).

2.2 Beta-lactams

Beta lactams are group of antibiotics that contain a four member heterocyclic beta lactam ring. They are the most important groups of antibiotics both historically and medically (Madigan *et al.,* 2009). Beta lactams are cell wall inhibitors, thus they inhibit the synthesis of peptidoglycan, an important component of bacterial cell wall, leading to the death of the organism (Prescott *et al.,* 2008). They are among the most frequently prescribed antibiotics used worldwide in many hospitals, and they are by far the largest group of antibiotics used in clinical medicine today (Duerden, 1987). They include the first true antibiotic (penicillin) to be discovered and brought into clinical practice, and the entire natural and semi-synthetic derivatives with their wide range of properties and applications in clinical medicine. The beta lactam antibiotics remain a foundation of antibiotic therapy for the range of most systemic infections (Madigan *et al.,* 2009). The antibacterial activity of the beta lactams is derived from the four membered carbon-nitrogen (C-N) beta-lactam rings that all the antibiotics in this group possess (Prescott *et al.,* 2008). Inactivation of the beta-lactam ring of the beta-lactams by beta lactamase enzymes produced by pathogenic bacteria will lead to the production of a compound which is devoid of any antibacterial activity. For example, the inactivation of Penicillin by beta lactamase enzyme will eventually result to the formation of penicilloic acid, which is devoid of any antibacterial activity. Thus, the beta lactam ring of the beta-lactam antibiotics is the active site and most important part of this class of antibiotics, and it is the point of target by beta lactamase enzymes including ESBLs (Madigan *et al.*, 2009).

2.2.1 Types of beta–lactams

There are several different types of beta lactam antibiotics that are invaluable and potentially important for the treatment of bacterial related infections. Beta lactam antibiotics can be divided

into four main groups according to the molecular structures that surround and supports the active site (beta-lactam ring) of this class/group of antibiotics and they include: penicillins, cephalosporins, carbapenems and monobactams, and another group known as the beta lactamase inhibitors like clavulanic acid, sulbactam and tazobactam that do not necessarily have intrinsic antibacterial activity, but work in synergy with other beta lactam drugs to inhibit the growth of a bacterium *in vitro* (Prescott *et al.,* 2008). The structures of beta-lactam drugs are shown in Appendix 1.

Penicillins: Penicillin was discovered in 1929 by the British scientist, Alexander Fleming (Duerden, 1987). It is an antibacterial product of the fungus, *Penicillium chrysogenum*, but today penicillin is now produced by semi-synthetic and synthetic methods. Penicillins have a nucleus or chemical structure known as 6-aminopenicillanic acid. Benzyl penicillin (penicillin G) was the early and original member of the group of antibiotics called penicillins, and they remain the most active antibacterial agent against sensitive bacteria. Other members of the Penicillins include: Phenoxymethyl penicillin (penicillin V), ampicillin, amoxycillin, piperacillin, oxacillin, cloxacillin and ticarcillin amongst others. The penicillins are broad spectrum antibiotics showing activity on Gram positive and Gram negative bacteria (Prescott *et al.*, 2008).

Cephalosporins: Cephalosporins like the penicillins are another group of clinically important beta lactam antibiotics. Produced as a by-product by the fungus, *Cephalosporium acremonium*, the cephalosporins have antibacterial activity predominantly amongst Gram negative bacteria, showing variable amount of activity against Gram positive bacteria. Unlike the penicillins, the cephalosporins have a chemical structure called 7-aminocephalosporanic acid. The cephalosporins are divided into four (4) generations of antibiotics and they are: first generation cephalosporins (cephalexin), second generation cephalosporins (cefuroxime), third generation

cephalosporins (ceftazidime) and fourth generation cephalosporins (cefepime) (Duerden, 1987; Prescott *et al.,* 2008).

Carbapenems: The carbapenems comprises of a group of fused beta lactam antibiotics. Examples include imipenem, meropenem and ertapenem. Carbapenems are known to have good antibacterial activity against Gram negative bacteria including: *P. aeruginosa, Bacteriodes* and *Staphylococci*. They are used in conjunction with an enzyme inhibitor because of their easy breakdown by liver enzymes upon ingestion into the body (Duerden, 1987). Carbapenems are effective on multi-drug resistant bacteria including those that produce extended spectrum beta lactamases (ESBLs) (Walsh *et al.,* 2005).

Monobactams: Monobactams are monocyclic beta lactam antibiotics that are produced by a variety of bacteria strains. The monobactams are unique in that their beta-lactam ring is not linked to a second ring like the other beta lactam antibiotics. They have good antibacterial activity against many Gram negative bacteria including *P. aeruginosa,* but they show no activity against most Gram positive organisms and anaerobes. Typical example of a monobactam is aztreonam (Duerden, 1987; Prescott *et al.,* 2008).

2.2.2 Beta-lactamases synthesis and mode of transfer

The synthesis of β lactamases is either chromosomal (constitutive), as in *Pseudomonas aeruginosa,* or plasmid mediated (inducible), as in *Aeromonas hydrophila, E. coli* and *Staphylococcus aureus*. Plasmids are a major cause of bacterial resistance spreading, as they can be transferred between Gram negative bacteria by conjugation and between Gram positive bacteria by bacterial viruses called transducing phages. This transferability is responsible for many outbreaks of resistance, especially when appropriate infection control measures are breached in hospital settings. However, in the Gram positive bacteria β lactamases are located or

secreted to the outside membrane environment as exoenzymes. In the Gram negative bacteria they remain in the periplasmic space, where they attack the antibiotic before it can reach its receptor site (Brooks *et al.*, 2004).

2.2.3 Beta-lactamases

Beta lactamases are enzymes that hydrolyze or break down beta lactam antibiotics such as penicillin and ampicillin. They are produced by both Gram positive and Gram negative bacteria, and they have the ability to destroy beta lactam drugs and make them ineffective for treatment of bacterial related diseases (Prescott *et al*., 2008 and Brooks *et al*., 2004). All beta-lactam drugs have a common element known as beta-lactam ring in their structures. This beta-lactam ring is the basis for the antibacterial activity of all beta-lactam drugs, and the target of beta lactamase enzymes as well. Beta-lactamase enzyme breaks the beta-lactam ring by opening the carbon-nitrogen bond that binds the ring together, and this deactivates the antibacterial properties of the drug (Prescott *et al*., 2008). The earlier beta-lactamases include TEM-1, TEM-2 and SHV-1 enzymes which used to be active against previous beta-lactam antibiotics such as penicillins. But the introduction of newer and very potent antibiotics into clinical practice has led to an upsurge in a variety of beta-lactamase enzymes with more power to inactivate many potent antibiotics including 3rd generation cephalosporins amongst others. Normally, the earlier beta-lactamases (TEM-1, TEM-2 and SHV-1 enzymes) which are commonly found in bacteria of the *Enterobacteriaceae* family, confer high level resistance to early beta-lactams such as penicillins and low level resistance to the first generation cephalosporins (Bush, 2001, Paterson *et al*., 2006, Jacoby and Munoz-Price, 2005).

However, the wide spread use of the 3rd generation cephalosporins and aztreonam is believed to be the major cause of mutations in these enzymes that has led to the emergence of newer enzymes like TEM 3 with extended substrate profiles (Livermore, 2004; Jacoby and Munoz-Price, 2005). These enzymes are called extended spectrum beta-lactamases (ESBLs) because of their greatly enhanced substrate range, which is their exceptional ability to hydrolyze and render ineffective the newer beta lactam drugs such as cephalosporins. ESBLs are plasmid-mediated beta-lactamase enzymes and the large plasmids carrying genes for ESBL production in pathogenic bacteria may also harbour genes that confer resistance to several other non-beta lactam antibiotics including aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and sulphamethoxazole-trimethoprim, thus limiting the available treatment options for bacterial related diseases (Bradford, 2001; Bush *et al*., 1995). Though ESBL hydrolyze and destroy cephalosporins and other non-beta lactam antibiotics, they are still inhibited by suicide inhibitors such as clavulanic acids, and this phenomenon is exploited in the laboratory detection of pathogenic bacteria that produce these all important enzymes. Though ESBL-producing organisms may appear susceptible to some extended-spectrum cephalosporins, treatment with such antibiotics has been associated with high failure rates. However, carbapenems such as imipenem and meropenem are the treatment of choice for serious infections due to ESBL-producing bacterial pathogens (Lye *et al*., 2008).

2.2.4 Classification of beta-lactamase enzymes

Beta-lactamase enzymes have several classifications but the Ambler classification and Bush classification are often the most versatile and most comprehensive methods of classifying beta-lactamase enzymes. Ambler classification is solely based on the amino acid sequence and

molecular structure of the enzyme while Bush's classification is based on the substrate that the enzyme breaks down (the particular antibiotic it hydrolyzes) and on the ability of other compounds such as clavulanic acid and EDTA to inhibit the enzyme production (Jacoby and Munoz-Price, 2005; Bush *et al*., 1995). In Ambler classification, beta-lactamases are classified into four groups labeled A-D, and they include all beta-lactamase enzymes that are inhibited by a chelating agent such as ethylene diamine-tetra-acetic acid (EDTA). The Bush classification arranged beta-lactamase enzymes into four groups as follows:

Group 1: Group one beta-lactamase enzymes includes all cephalosporinase enzymes (enzymes that hydrolyze third-generation cephalosporins) but are not inhibited by clavulanic acid, a beta-lactamase inhibitor. They also include beta-lactamase enzymes in molecular class C of Ambler classification of beta-lactamases.

Group 2: Group two beta-lactamase enzymes include all penicillinases (enzymes that hydrolyze penicillins), cephalosporinases and enzymes that are inhibited by a beta lactamase inhibitor such as clavulanic acid. They are the earlier beta-lactamase enzymes such as TEM-1 and SHV-1 enzymes. Group 2 beta-lactamase enzymes correspond to the molecular classes A and D of Ambler classification of beta-lactamase enzymes. Beta-lactamase enzymes in this group are broad and are further classified into sub-groups that include extended spectrum beta-lactamase enzymes, carbenicillinases, cloxacilinases, oxicilinases, and carbapenemases which hydrolyze drugs that are used for the treatment of infections caused by ESBL producing bacterial pathogens.

Group 3: Group three beta-lactamase enzymes include all beta-lactamase enzymes that hydrolyze antibiotics such as cephalosporins and carbapenems. Beta-lactamase enzymes in this group are

majorly known as metallo beta lactamase enzymes because they are zinc-based enzymes that are inhibited by chelating agents such as EDTA. They correspond to beta-lactamase enzymes in the molecular class B of Ambler classification. Metallo beta-lactamase enzymes are able to hydrolyze very potent drugs such as imipenem and meropenem which are both used to treat serious bacterial infections caused by ESBLs.

Group 4: Group four beta-lactamase enzymes do not belong to any molecular class of enzymes. They include beta-lactamase enzymes such as penicillinases which are not well inhibited by clavulanic acid. No molecular class has been assigned to this group of beta-lactamase enzymes (Bush *et al.*, 1995; Jacoby and Munoz-Price, 2005).

2.3 Beta-lactam antibiotics modes of resistance

All β-lactam agents have a four-membered β-lactam ring in their structure. A beta-lactam ring is a cyclic amide with a heteroatomic ring structure consisting of three carbon atoms and one nitrogen atom. The effectiveness of these antibiotics relies on their ability to reach the Penicillin Binding Protein intact and their ability to bind to the PBP. These antimicrobial agents prevent the bacterial cell wall from forming by interfering with the final stage of peptidoglycan synthesis through acting on penicillin binding proteins (PBPS). The number of PBPS varies between bacterial species and these PBPS are found as both membrane-bound and cytoplasmatic proteins. The peptidoglycan layer maintains the cell shape and protects the bacterium against osmotic forces. In Gram-positive bacteria, peptidoglycan forms a thick layer on the cytoplasmic membrane, whereas in Gram-negative bacteria and mycobacteria, peptidoglycan is a thin layer sandwiched between the outer membrane and the cytoplasmic membrane. Hence, there are two

main modes of bacterial resistance to β-lactams (Drawz and Bonomo, 2010; Giguère *et al*., 2006; Poole, 2004).

2.3.1 Enzymatic hydrolysis of the β-lactam ring

If the bacterium produces the enzyme beta-lactamase or the enzyme penicillinase, the enzyme will hydrolyse the β-lactam ring of the antibiotic, rendering the antibiotic ineffective. The genes encoding these enzymes may be inherently present on the bacterial chromosome or may be acquired through plasmid transfer 'plasmid mediated resistance' and β-lactamase gene expression may be induced by to much exposure to β-lactams (Drawz and Bonomo, 2010). The production of a β-lactamase by a bacterium does not necessarily rule out all treatment options with β-lactam antibiotics. In some instances, β-lactam antibiotics may be co-administered with a β-lactamase inhibitor. For example, Augmentin is made of amoxicillin, a β-lactam antibiotic, and clavulanic acid, a β-lactamase inhibitor. The clavulanic acid is designed to overwhelm all β-lactamase enzymes, bind irreversibly to them, and effectively serve as an antagonist so that the amoxicillin is not affected by the β-lactamase enzymes. However, in all cases where infection with β-lactamase-producing bacteria is suspected, the choice of a suitable β-lactam antibiotic should be carefully considered prior to treatment. In particular, choosing appropriate β-lactam antibiotic therapy is of utmost importance against organisms with inducible β-lactamase expression. If β-lactamase production is inducible, then failure to use the most appropriate β-lactam antibiotic therapy at the onset of treatment will result in induction of β-lactamase production, thereby making further efforts with other β-lactam antibiotics more difficult (Drawz and Bonomo, 2010).

2.3.2 Possession of altered penicillin binding proteins

As a response to increased efficacy of β-lactams, some bacteria have changed the proteins to which β-lactam antibiotics bind. Beta-lactams cannot bind as effectively to these altered penecillin binding protines, and, as a result, the β-lactams are less effective at disrupting cell wall synthesis. Notable examples of this mode of resistance include methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant *Streptococcus pneumoniae*. Altered PBPs do not necessarily rule out all treatment options with β-lactam antibiotics (Drawz and Bonomo, 2010).

2.4 Bata-lactamase inhibitor (Clavulanic acid)

Clavulanic acid is not an antibiotic. It is a beta-lactamase inhibitor sometimes combined with semi-synthetic beta-lactam antibiotics to overcome resistance in bacteria that produce beta-lactamase enzyme, which otherwise inactivate the antibiotic. Most commonly, it is combined with amoxicillin as argumentin (trade name). Clavulanic acid has negligible intrinsic antimicrobial activity, despite sharing the beta-lactam ring that is characteristic of beta-lactam antibiotics. However, the similarity in chemical structure allows the molecule to interact with the enzyme beta-lactamase secreted by certain bacteria to confer resistance to beta-lactam antibiotics. Clavulanic acid is a suicide inhibitor, covalently bonding to a serine residue in the active site of the beta-lactamase. This restructures the clavulanic acid molecule, creating a much more reactive species that is attacked by another amino acid in the active site, permanently inactivating it, and thus inactivating the enzyme. This inhibition restores the antimicrobial activity of beta-lactam antibiotics against lactamase secreting resistant bacteria. Despite this,

some bacterial strains that are resistant even to such combinations have emerged (Stratton, 2000).

2.5 Description and definition of ESBL

ESBL is an 'acronym' which stands for extended spectrum beta-lactamases. Though there is no consensus on the definition of ESBL, extended spectrum beta-lactam (ESBLs) are Class A β-lactamases and may be defined as plasmid-mediated enzymes that hydrolyse oxyimino-cephalosporins, and monobactams but not cephamycins or carbapenems. They are inhibited in vitro by clavulanate (Paterson and Bonomo, 2005). Extended spectrum beta-lactamases (ESBL) are beta-lactamase enzymes that are produced by both Gram positive and Gram negative bacteria, and which hydrolyze or breakdown 3rd-generation cephalosporins such as ceftriaxone, cefotaxime and ceftazidime and makes them less efficacious in the treatment of bacterial related diseases (Bradford, 2001; Bush and Jacoby, 2010). Extended-spectrum β-lactamases (ESBLs) are enzymes that compromise the efficacy of all β-lactams, except cephamycins and carbapenems, by hydrolysis of the β-lactam ring and are inhibited by β-lactamase inhibitors such as clavulanic acid. There are more than 400 ESBLs described so far, most derived from the groups TEM, SHV and CTX-M, with 183, 134 and 103 variants, respectively (Coque *et al.*, 2008). ESBL production is by far one of the most important mechanisms that accounts for the less antibacterial activity of some available antibiotics against pathogenic bacteria due to their ability to degrade 3rd-generation cephalosporins, adhoc antibiotics in clinical medicine. The efficacy of some potent drugs (ceftazidime, ceftriaxone, and cefotaxime) has been badly compromised due to the emergence and spread of extended spectrum beta-lactamases (in particular CTX-M type ESBLs) in both the community and hospital environment (Padmini *et al*, 2008).

These ESBLs enzymes are plasmid borne and have evolved from point mutations altering the configuration of the active site of the original and long known β lactamases designated TEM-1, TEM-2, and SHV-1. The activity of these enzymes is limited to ampicillin, penicillin, and carbenicillin. The original TEM was first discovered 1960s in *E. coli* in a patient named Temoniera in Greece, but it spread rapidly to other bacteria. Although TEM-type β lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found in other genera of Enterobacteriacae and in other penicillin or ampicillin resistant Gram negative bacteria such as *Haemophilus influenzae* and *Neisseria gonorrhoeae*. The SHV enzymes, named after the "sulfhydryl variable" active site, are commonly associated with *K. pneumoniae*. At first these bacteria contained a single ESBL gene, but now multiple ESBL genes are commonly present in a single strain, further complicating the process of detecting them and identifying an appropriate treatment regimen (Bradford *et al.,* 1994). Nowadays, more than 90 TEM-type and more than 25 SHV-type β lactamases have been identified. Other recently recognized genes with similar activity include PER-1 β lactamases, first discovered in *Pseudomonas aeruginosa* in Turkey. Infections with ESBL producing bacteria can result in avoidable failure of treatment and increased cost in patients who have received inappropriate antibiotic treatment. Nosocomial outbreaks of this form of resistance are most often associated with intensive care units and oncology, burns, and neonatal wards. They can result in prolongation of hospital stay, as well as devastating or even fatal consequences (Ejikeugwu *et al.,* 2013).

2.5.1 The origin and history of ESBLs

ESBLs was first isolated in 1983 from a *Klebsiella* isolate in Germany and in the United States of America in 1989, and has since then proliferated to other enteric bacteria and non-enteric

bacteria such as *Pseudomonas* and *Acinetobacter* species. The production of extended spectrum beta-lactamase (ESBL) enzymes by pathogenic bacteria especially members of the Enterobacteriaceae family has been extended following the introduction of cephalosporins into clinical practice in the early 1980's. The introduction of the first, second, third and fourth generation cephalosporins into clinical medicine for the treatment of difficult bacterial related infection was welcomed due to their activity against the earlier beta-lactamase enzymes which rendered the earlier beta-lactams such as penicillins ineffective in treating some bacterial infections. ESBL production by pathogenic bacteria has been reported from across the world, and their prevalence is steadily on the increase (Jacoby and Munoz-Price, 2005; Livermore and Woodford, 2004). Mutations in the active site of the earlier beta-lactamase enzymes such as TEM-1 and SHV-1 have allowed ESBLs to be widespread in both the community and hospital environment. This makes pathogenic bacteria that produce ESBLs to be resistant to a good portion of available antibiotics.

Since the early 2000s, CTX-M-type ESBLs have been increasingly reported, and these enzymes have now replaced TEM and SHV as the most common type of ESBL. The genes encoding ESBLs are usually located on plasmids that are highly mobile and can harbor resistance genes to several other unrelated classes of antimicrobials (Baudry *et al.*, 2009), such as the plasmid-mediated quinolone-resistance (PMQR) genes and aminoglycoside-resistance genes (Crémet *et al.*, 2011; Rodríguez-Baño *et al.*, 2010). A shift in the distribution of different ESBLs plasmid mediated, TEM and SHV derived enzyme has recently occurred in Africa and Europe, with a dramatic increase of CTX-M enzyme over TEM and SHV variants. Other non-TEM and non-SHV enzymes, such as PER and OXA types have also been found in some countries (Livermore *et al.*, 2007). ESBLs plasmid mediated, TEM, SHV, and CTX-M derived enzymes are most

common in *Klebsiella spp*, followed by *E. coli*, these enzymes are capable of hydrolyzing broad spectrum cephalosporins and monobactams but inactive against cephamycins and imipenem. CTX-M enzymes, particularly CTX-M-15, have been involved in various epidemiological situations and have disseminated throughout all continents as a result of epidemic plasmids and/or particular epidemic strains (Ruppé, 2010). CTX-M-producing *Escherichia coli* and *Klebsiella pneumoniae* are becoming increasingly involved in urinary tract infections, especially among outpatients. Furthermore, these bacteria seem to have been imported from the community into the hospital setting (Arpin *et al.*, 2009).

Some studies have shown a high prevalence of ESBL-producing Enterobacteriaceae in hospitals in African countries including Morocco, South Africa and Nigeria (Iabadene *et al.*, 2008; Sekhsokh *et al.*, 2008; Pitout *et al.*, 2008; Aibinu *et al.*, 2003 and Iroha *et al*., 2008). However, little information is available regarding their prevalence within the community. Arpin *et al.*, 2009; detected a variety of β-lactamases among the isolates of *E. coli* and *K. pneumoniae*, namely SHV-, CTX-M-, OXA- and TEM-type enzymes. The CTX-M type was the most common ESBL in our setting. The predominance of CTX-M-15 indicates that this allele is now common in Morocco, as in other countries as a result of worldwide dissemination. These higher rates of CTX-M among total ESBL enzymes are most probably associated with high mobilization of the encoding genes. Barlow *et al.* (2008) found that $bla_{\text{CTX-M}}$ genes are mobilized to plasmids almost ten times more frequently than other class A β-lactamases. However, resistance to cephalosporin drugs has been reported frequently in Europe and America in the past years (Canton *et al.*, 2008). After a variety of extended spectrum cephalosporin were introduced in the 1980s, bacteria strain producing extended spectrum beta-lactermases (ESBLs) such as TEM and SHV- derived ESBLs, emerged in Europe and since then, their variant have been

proliferating around the world. The first ESBLs isolates were discovered in Western Europe in mid 1980s and subsequently in USA in late 1980s. In Nigeria, ESBLs enzymes have been reported in *Enterobacter spp* from clinical samples in human patient in Lagos (Aibinu *et al.,* 2003). Chau and Obegbunam (2007) reported the presence of high resistant extended spectrum beta-lactamase producing *Escherchia coli* in Enugu State.

2.6 Types of ESBLs

Extended spectrum beta-lactamase enzymes produce varying effects *in vivo* depending on the type of available substrate (i.e. antibiotic) they are acting on. Over 150 ESBLs gene have been currently recognized and characterized worldwide (Livermore *et al.,* 2007; Jacoby and Munoz-Price, 2005; Bush and Jacoby, 2010). ESBL types may include TEM type ESBLs, SHV type ESBLs, OXA type ESBLs, CTX-M ESBLs and other classes of ESBLs which are not normally found worldwide, but are only restricted to some parts of the world.

2.6.1 OXA type of ESBLs

OXA types ESBLs are groups of beta-lactamase enzymes that could hydrolyze oxacillin and other related anti-*staphylococcal* penicillins (Bush *et al*., 1995). Oxacillin is a beta-lactam drug that is used to treat bacterial infections when penicillin resistance arises, but the OXA types ESBLs now exists and they are able to hydrolyze and render this agent inefficacious. They exhibit high hydrolytic ability against oxacillin and cloxacillin, and may also extend their resistance to ampicillin and cephalothin antibiotics (Jacoby and Munoz-Price, 2005). OXA types ESBLs differ from the TEM and SHV enzymes in that they belong to molecular class D of Ambler classification of beta-lactamase enzymes. Beta-lactamase inhibitors such as clavulanic acid have little or no effect on these types of ESBLs. OXA types ESBLs usually arises in the

hospital environment and amongst bacterial pathogens following an amino acid substitutions in the active sites of the OXA enzymes. OXA-type ESBLs have been found mainly in *Pseudomonas aeruginosa* isolates from Turkey and France. They rarely exist in members of bacteria in the Enterobacteriaceae family, and have been reported as one of the most prevalent ESBLs in the community (Bradford, 2001).

2.6.2 TEM type of ESBLs

TEM is an acronym which stands for 'Temoneira' the name of patient from which TEM-1 beta-lactamase enzyme was first isolated from in 1965 from *E. coli* isolate in Athens, Greece (Paterson *et al.,* 2005). TEM type ESBLs are usually the most commonly-encountered type of ESBLs produced by Gram-negative bacteria including *Escherichia coli, K. pneumoniae, K. oxytoca*, and other enteric bacteria (Jacoby and Munoz-Price, 2005). They are largely responsible for bacterial resistance to penicillins, ampicillin and even the cephalosporins. TEM type ESBLs arises following amino acid substitution or point mutation that occurs at the active site of TEM-1 (the earlier beta-lactamase) enzymes (Bush *et al*., 1995). This gives pathogenic bacteria producing this particular class of beta lactamase enzymes the exceptional ability to hydrolyze a wider spectrum of expanded spectrum drugs. Opening the active site to beta-lactam substrates also typically enhances the susceptibility of the enzyme to beta-lactamase inhibitors, such as clavulanic acid. TEM type ESBLs have been reported from across the world even here in Nigeria (Iroha *et al*., 2010).

2.6.3 SHV type of ESBLs

The acronym SHV stands for sulfhydryl variants and they represent a special group of beta-lactamase enzymes that share 68 percent of its amino acids with TEM-1 enzymes (Bush and Jacoby, 2012; Jacoby and Munoz-Price, 2005). SHV type ESBLs share similar homology or structure with TEM type ESBLs. They are commonly found in *K. pneumoniae* isolates and are responsible for up to 20% of the plasmid-mediated ampicillin resistance experienced in bacterial related infections (Livermore, 1995). More than 60 SHV variant of ESBLs are known, and ESBLs in this family also have amino acid changes around the active site, most commonly at positions 238 or 238 and 240. Mutation in codon 238 of *K. pneumoniae* expressing SHV-1 beta lactamase give rise to a high level of resistance to fast penetrating cephalosporins (SHV-2), and when a second mutation occurs in codon 240 (SHV-5), a ceftazidime resistance is raised to untreatable levels in the host or patient (SCIEH, 2004). SHV type ESBLs are usually the predominant type of ESBLs in Europe and the United States of America. However, SHV type ESBLs are found worldwide, with SHV-5 and SHV-12 being reported among the most common enzymes amongst pathogenic bacteria strains.

2.6.4 CTX-M type of ESBLs

CTX-M is the short form of the 3rd-generation cephalosporin, cefotaxime, and it is used to denote bacterial pathogens that hydrolyze this class of antibiotics. Thus, bacterial pathogens that produce beta-lactamase enzymes that hydrolyze cefotaxime are said to be positive for CTX-M ESBL production. CTX-M type ESBLs were named for their greater activity against cefotaxime than other oxyimino 3rd-generation cephalosporins such as ceftazidime, ceftriaxone, or cefepime (Jacoby and Munoz-Price, 2005). This group of ESBLs does not arise by mutation or amino acid

substitution as is the case for other types of ESBLs such as TEM and SHV ESBLs that are known to arise by mutation. Instead, CTX-M type ESBLs represents examples of plasmid acquisition of beta-lactamase genes normally found on the chromosome of *Kluyvera* species, a group of rarely pathogenic commensal organisms that occur commonly in the community or non-hospital environments. CTX-M type ESBLs are not very closely related to the TEM- and SHV beta-lactamases. CTX-M ESBLs have mainly been found in strains of *Escherichia coli, Salmonella enterica* serovar *Typhimurium*, but have also been described in other species of Enterobacteriaceae from the community (Bush and Jacoby, 2010). CTX-M ESBLs are now widely distributed and they are responsible for a variety of hospital and community-acquired bacterial infections (Livermore and Hawkey, 2005). More than 50 CTX-M enzymes have been so far isolated from across the world and this includes CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-15, CTX-M-9, and CTX-M-25 (Paterson *et al*., 2005). These CTX-M enzymes appear worldwide and have been reported in enteric bacteria from France, Italy, Portugal, Spain, USA, Germany, and in some parts of Africa too with varying prevalence (Jacoby and Munoz-Price, 2010; Iroha *et al*., 2010).

2.6.5 PER type of ESBLs

The PER-type ESBL is a difficult to understand type of extended spectrum lactamase enzyme. It was first discovered in bacterial strains of *Pseudomonas aeruginosa* from specimens of patients in Turkey (Bradford, 2001). PER type of ESBLs are clinically important beta lactamase enzymes with very strong ESBL activity which can efficiently breakdown penicillins and cephalosporins but is susceptible to inhibition by clavulanic acid. The PER type of ESBL genes (^{bla}PER-1) are also found in organisms like *Acinetobacter* species, *Salmonella* species, *Proteus* species and *Alcaligenes faecalis*. Though the PER type of ESBLs was detected first in Turkey, it has also

been detected and reported in Korea, France, Belgium and Italy. Apart from the ^{bla}PER-1 genes, there is also another class of enzymes called PER-2 enzyme which has only been found exclusively in South America, and it shares about 86% amino acid homology with the PER-1 enzymes (Al-Jasser, 2006).

2.6.6 Other ESBL types

There are varieties of other ESBLs which are plasmid mediated or integron associated enzymes that are characterized by their geographic diversity (Bush and Jacoby, 2010). It is noteworthy that the majority of ESBLs are derived from TEM or SHV beta lactamases, but there are a few other ESBLs that are not closely related to either the TEM or SHV beta lactamases, and they include the PER type of ESBLs (already expanded above), VEB-1, GES-1 and so on. These other ESBLs do not arise by point mutations like the TEM and SHV-ESBLs but are remarkable for their geographic diversity (Paterson *et al*., 2005; Al-Jasser, 2006). They have been found to confer a high level of resistance to the third generation cephalosporins including cefotaxime and ceftazidime, and aztreonam, but they are also inhibited by the action of clavulanic acid, a beta lactamases inhibitor. The VEB-1 gene was first described in a Vietnamese infant hospitalized in France from whom an *E. coli* isolate that produced the VEB-1 enzyme was recovered from. Also, an identical beta lactamase has also been found in isolates of *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *Enterobacter* species from Thailand, Kuwait and China. The other ESBLs including PER-1, VEB-1, and GES are all non-SHV, non-TEM ESBLs that have been found in a wide range of geographic locations, and did not arose by point mutations, despite the fact that they hydrolyze a given range of third – generation cephalosporins and are yet inhibited by clavulanic acid (Bush and Jacoby, 2010).

2.7 Genetics of ESBLs

Mutations at the active sites of the earlier beta lactamases (TEM and SHV- enzymes) lead to the extension of antibiotic spectrums that this beta lactamases can resist (Bush and Jacoby, 2010). ESBLs are plasmid mediated borne organisms, though some are chromosomally mediated. Many ESBL- producing bacteria now have relaxed active sites with about 1-7 amino acid substitutions, which allows the enlargement of their active sites; thus allowing attack on oxyimino – cephalosporins (Jacoby and Munoz-Price, 2005). These changes together with other factors gave impetus to the resistance of ESBL- producing bacteria (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) to virtually all beta lactam antibiotics (the most widely used class of antibiotics in clinical medicine) (Bush and Jacoby, 2010). Plasmids are responsible for the spread of ESBL-producing bacteria, despite the fact that the genes that encodes these enzymes can also be found on the bacterial chromosome, and it is through plasmids that they transfer their resistance genes to non ESBL- producing bacteria. Genes that encode some ESBLs can also be found on bacterial transposons and integrons. The genes encoding the TEM-1 and TEM-2 ESBLs are also carried by transposons while the genes encoding SHV-1 is found on the chromosome of most strains of *K. pneumoniae*. SHV genes also occur on transmissible plasmids. The genes encoding the CTX-M- type ESBLs are found on the chromosome of a rarely pathogenic genus of *Kluyvera* species, a commensal organism (Jacoby and Munoz-Price, 2005).

The selective pressure that drives ESBL evolution has usually been attributed to the strong use of the third generation cephalosporins (especially irrationally both within and outside the hospital) (Bush and Jacoby, 2010). This intense selective pressure imposed on ESBL- producing bacteria by these antibiotics over time operates not only on the ESBL gene coding regions, but it also impacts or operates on their promoter regions, copy number and other genes. These genetic

changes may significantly affect the beta lactam resistance of bacterial strains by increasing its level, expanding it for non ESBL substrates, and compromising the activity of beta lactamase inhibitors. Genetic changes promote up mutations and insertion of transposable elements close to the promoter region or multiplication of the ESBL gene copy number can make a bacterial strain to hyper-produce ESBLs (Ginadkowski, 2001). The genes responsible for the extension of ESBLs in pathogenic bacteria are often located on bacterial plasmids, and these plasmids also carry genes that mediate resistance to other classes of antibiotics including chloramphenicol, fluoroquinolones, aminoglycosides, and antimetabolites such as sulphonamides and trimethoprim (Pitout *et al.,* 2005).

2.8 Risk factors for ESBL acquisition

The extensive use of antibiotics including 3rd-generation cephalosporins whether rationally or irrationally creates a wide reservoir of resistant pathogenic bacteria in both the community and hospital environment, and this could also result in the emergence and spread of resistance genes, thus opposing the good qualities of these agents in the treatment of bacterial related infections. The use of non-beta lactam drugs such as quinolones, trimethoprim, sulphamethoxazole and aminoglycosides has also been found to be responsible for the acquisition of ESBLs. Several risk factors are associated with the possible acquisition of ESBL-producing bacterial pathogen from either the community or hospital environment, but the single usage of extended spectrum cephalosporins enhances the chances of a person to acquire the infection (Bradford, 2001; Jacoby and Munoz-Price, 2005). In addition to antibiotic usage (which is a key factor in the acquisition of ESBLs), patients who are also at increased risk of ESBL infection include those who are hospitalized for longer periods and who also may have a serious underlying disease (Mohamudha *et al.*, 2010; Bush and Jacoby, 2010). Hospitalized individuals who use invasive

medical devices such as urine catheters, nasogastric tubes, jejunostomy tubes, and endotracheal tubes for a very long period are also at a very high risk of acquiring ESBL related infection (Jacoby and Munoz-Price, 2005).

2.9 Laboratory detection of ESBLs

Detection of isolates expressing ESBLs in the clinical microbiology laboratory is not a trivial undertaking. Although a particular ESBL will typically confer resistance to at least one particular expanded-spectrum cephalosporin or aztreonam, the minimum inhibitory concentration (MIC) may not be high enough for the strain to be called resistant under current interpretations of the National Committee for Clinical Laboratory Standards, now Clinical Laboratory Standards Institute (CLSI, 2012). Because of the clinical significance of ESBLs, specific guidelines for the detection of ESBL-expressing organisms were proposed in 1999 by the NCCLS. The presence of an ESBL is suggested if bacterial growth is observed despite a concentration of 1g/ml of at least one of three expanded-spectrum cephalosporins (ceftazidime, ceftriaxone or cefotaxime) or aztreonam, or growth occurs despite a concentration of 4g/ml of cefpodoxime. The use of more than one antibacterial agent for screening improves the sensitivity of detection of ESBLs. Phenotypic confirmatory tests include the addition of clavulanic acid to both ceftazidime and cefotaxime. A serial dilution concentration decrease in a MIC for either antibacterial agent tested in combination with clavulanic acid versus its MIC when tested alone constitutes a positive phenotypic test for an ESBL. If disk diffusion is used by the laboratory, a 5mm increase in zone diameter for either cefotaxime or ceftazidime tested with clavulanic acid versus its zone size when tested alone is considered a positive phenotypic ESBL test. It is important to note that the NCCLS confirmatory tests are only intended for the detection of ESBLs found in *E. coli, K. pneumoniae*, and *K. oxytoca*. Chromosomally encoded AmpC β -

lactamases found in Enterobacteriaceae, such as *Enterobacter cloacae* can interfere with clavulanic acid inhibition of an ESBL. Because of the fact that *E. cloacae* are important nosocomial pathogens, specific NCCLS methodology guidelines are needed to detect ESBLs in species that produce chromosomally encoded inducible AmpC β-lactamases (Tzelepi *et al.*, 2000). Although the methods described in the previous paragraph are those published by the NCCLS as ESBL confirmatory tests, there are other methods that investigators have used to phenotypically detect ESBLs in the clinical microbiology laboratory such as Three-Dimensional Test and E-Test.

2.10 Screening for ESBL production

The increase in the frequency of Gram negative bacilli producing extended spectrum beta-lactamases (ESBLs) generate a need for the accurate screening of clinical isolates (especially *E. coli*, *K. pneumoniae, P. aeruginosa* and *K. oxytoca*) in order to identify potential ESBL producers that warrants either a phenotypic confirmation (using corresponding cephalosporins) or genotypic confirmation (using PCR) that will authenticate the presence of ESBLs in the test bacteria (Bradford, 2001). According to the Health Protection Agency (HPA), the basic procedure or strategy to detect ESBL producers are to use an indicator cephalosporin to screen for likely producers of this all important enzymes, and then test the possible synergistic effect of a combination of the cephalosporin with a beta lactamase inhibitor, clavulanic acid (HPA, 2008). The ideal indicator cephalosporins that should be used to screen for ESBL production in clinical isolates is one to which all ESBLs are known to confer antibiotic resistance to. The CLSI (formerly NCCLS) recommends the use of ceftazidime, cefotaxime, cefpodoxime, aztreonam and ceftriaxone to screen for ESBL production (CLSI, 2012). The choice of the indicator cephalosporin to use for screening is often based on the substrate that these enzymes are known

to hydrolyze. For example, TEM and SHV- type ESBLs hydrolyze and confer resistance to ceftazidime while CTX- type ESBLs hydrolyze and confer resistance to cefotaxime. But all ESBLs are known to confer obvious resistance to cefpodoxime, which is why most researchers prefer to use cefpodoxime to screen for potential ESBLs than the other cephalosporins; though false positive results have been reported by some researchers on the use of cefpodoxime to screen for ESBL production in clinical isolates (HPA, 2008). The CLSI has proposed both a disk diffusion method and a dilution method for the proper screening of clinical isolates for ESBL production. In each of these methods, specific inhibition zone diameters, IZD (for disk diffusion methods) and minimum inhibitory concentrations, MICs (for dilution methods) are noted (SCIEH, 2004). These specific IZDs and MICs raise a very high level of suspicion for the possible production of ESBLs by the test bacteria. For the disk diffusion methods, the NCCLS recommends some established breakpoints for ESBL screening, and MICs for which the screening of clinical isolates for the production of ESBLs shall be based. ESBL zone of inhibition breakpoints are as given in the Appendix 1 (CLSI, 2012). According to the CLSI, ceftazidime, ceftriaxone, aztreonam and cefotaxime can be used at a screening concentration of 1 µg/ml, and bacterial growth at this particular antibiotic screening concentration (MIC of the test cephalosporin of ≥2 µg/ml) is indicative of a potential ESBL production in the test organism. For cefpodoxime, an MIC ≥8 µg/ml is taken as the screening concentration when screening clinical isolates for ESBL production (Paterson *et al*., 2005).

2.11 Confirmation for ESBL production

In as much as it is very important to screen Enterobacteriaceae for the production of ESBLs, it is also necessary to confirm the production of these enzymes in these isolates so that it can be established whether or not they truly produce ESBLs. Any bacterial isolate that is resistant (or

that shows reduced antibiotic susceptibility) to any of the indicator cephalosporins (ceftazidime, cefotaxime, ceftriaxone or cefpodoxime) warrants a confirmatory test to investigate the production of ESBLs (Bradford, 2001). Confirmation of ESBL production depends on demonstrating synergy between clavulanic acid and any of the indicator cephalosporin (s) to which the isolate was initially found resistant. Clavulanate (amoxicillin - clavulanic acid) is a beta – lactamase inhibitor, and in combination with a cephalosporin, clavulanate helps to reduce the level of resistance to the cephalosporin. According to the NCCLS, a ≥5 mm increase in inhibition zone diameter (IZD) for either of the cephalosporin(s) tested in combination with clavulanic acid versus its zone when tested alone, is indicative of or confirms ESBL production phenotypically (NCCLS, 2002). Several methods have been described for the detection of ESBLs in clinical isolates, and these methods can be two – folds depending on the one the researcher decides to use. ESBL confirmation tests can either be by phenotypic methods or genotypic methods depending on the level of resources available to the researcher. Phenotypic confirmatory testes available for ESBL detection include; double disk synergy test (DDST), Epilometer (E) test strip and VITEK ESBL test (Kader *et al.,* 2004; Okesola and Adeniji, 2010; Pfaller *et al.*, 2006).

2.12 Phenotypic ESBL detection

The phenotypic methods of ESBL detection are based upon the reduced antibiotic susceptibility that ESBL- producing bacteria confer to oxyimino-cephalosporins and the ability of clavulanic acid (a beta lactamase inhibitor) to block this resistance posed by the enzymes (Bradford, 2001). There are several methods for the phenotypic detection of ESBL production including double disk synergy test (DDST), Epsilometer (E) test method amongst others. In any of these methods, the test organism is swabbed onto an agar plate (preferably Mueller-Hinton agar), and an

antibiotic disk containing amoxicillin-clavulanic acid is placed aseptically in the center of the agar plate (Iroha *et al.,* 2008a). Antibiotic disks containing any of the indicator cephalosporin (s) are placed on either side of the central disk (amoxicillin-clavulanic acid) at a distance of about 15mm. Enhancement of the IZD of the indicator cephalosporins caused by the synergy of the central disk is indicative of ESBL production phenotypically (Kader and Kumar, 2004). And when compared with the indicator cephalosporin (s), a 5 mm difference in IZD confirms ESBL production phenotypically (Bradford, 2001). The phenotypic method of ESBL detection is a reliable method of detecting the production of ESBLs in Enterobacteriaceae, and it has been employed by many researchers in the detection of ESBLs in other non enteric bacteria around the world (Okesola and Adeniji, 2010).

2.12.1 Double disk synergy test (DDST) method

The double disk synergy test (DDST) method is a phenotypic method of confirming ESBL production in Enterobacteriaceae (especially *E. coli*, *K. pneumoniae* and *K. oxytoca*) by testing synergy between amoxicillin-clavulanic acid and an indicator cephalosporin like cefotaxime, ceftazidime or cefpodoxime (Iroha *et al.,* 2008a). DDST have been employed by researchers elsewhere in the phenotypic confirmation of ESBL production in test isolates, and it appears to be one of the most versatile phenotypic methods of detecting ESBLs worldwide. DDST is a disk diffusion test which examines for the expansion of the indicator cephalosporin(s) IZD adjacent to an amoxicillin-clavulanic acid disk that is placed at the center of an already inoculated Mueller Hinton (MH) agar plate at a distance of about 15mm away from the indicator cephalosporin. The inhibition zone diameter (IZD) around the combination disc (containing amoxicillin-clavulanic acid and an indicator cephalosporin) is compared to the IZD around the indicator cephalosporin tested alone. According to the CLSI, 2012 an increase or difference of IZD ≥5mm between the

IZD of the amoxicillin-clavulanic acid and the indicator cephalosporin is taken as the phenotypic confirmation of ESBL production in the test isolates by the DDST method; NCCL, 2002). The DDST method of confirming ESBL production phenotypically was the first detection test for ESBL production in the 1980s, and it has shown sensitivities and specificities ranging from 79 % to 97 % and 94 % and 100 % respectively (Al-Jasser, 2006).

2.12.2 Epsilometer (E) test strip method

The epsilometer (E) test strip method of detecting ESBLs from clinical isolates is based on minimum inhibitory concentration (MIC) (Livermore and Woodford, 2004). In this method, nylon strips that have a linear gradient of the indicator cephalosporin lyophilized on one side of the strip are used. And on the other side of the strip are a series of lines and figures that denotes the MIC values of the indicator cephalosporin. The E- test ESBL strip is a phenotypic ESBL detection method like the DDST, except that it is much more expensive than the DDST method and the E- test strips are not readily available in most developing parts of the world such as Nigeria. E- test strips improves the availability of detecting the different types of ESBLs in clinical isolates, and the reported sensitivity of the ESBL E- test strip method is 87 % - 100 %, and 95 % - 100 % specificity when used for the phenotypic detection of ESBLs (Paterson and Bonomo, 2005). ESBL productions in the test isolates using the E- test ESBL strip method is inferred if the MIC ratio for cephalosporin alone: cephalosporin + clavulanate MIC is ≥8 (Livermore and Woodford, 2004).

2.12.3 VITEK ESBL test method

The VITEK ESBL test is an automated confirmatory test for the detection of ESBLs in *E. coli*, *K. pneumoniae*, and *K. oxytoca* using an automated VITEK 2 system. It measures MICs and

compares the growth of bacteria in the presence of an indicator cephalosporin (e.g. cefotaxime) versus cefotaxime-clavulanic acid (Pfaller and Segreti, 2006). The VITEK ESBL test method contains specific antimicrobial concentrations in cards known as VITEK cards, and the antibiotics these cards contain includes: cefepime (1.0 µg/ml), cefotaxime (0.5 µg/ml), ceftazidime (0.5 µg/ml), cefepime/clavulanic acid (1.0/10 µg/ml), cefotaxime/clavulanic acid (0.5/4.0 µg/ml), and ceftazidime/clavulanic acid (0.5/4.0 µg/ml). VITEK ESBL test makes use of an indicator cephalosporin alone and in combination with a beta lactamase inhibitor (clavulanic acid). The use of VITEK ESBL cards risk incorrectly reporting ESBL- producing organisms as being susceptible to cephalosporins when MICs are ≤8 µg/ml (Paterson and Bonomo, 2005).

2.12.4 Three dimensional tests

The three-dimensional test method is used to obtain phenotypic confirmation of ESBL production in Enterobacteriaceae (especially *E. coli*, *K. pneumoniae, K. oxytoca*). It does not rely on the inactivation of the beta lactamase enzymes produced by the test bacterium by a beta lactamase inhibitor, but rather it depends on the phenotypic evidence of ESBL induced inactivation of extended spectrum cephalosporins or aztreonam. In performing the three dimensional test for ESBL detection, 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 is pipetted into the slit, and beta lactam impregnated disks are then placed on the surface of the agar 3mm outside of the inoculated circular slit. The beta lactamase induced inactivation of each test antibiotic is detected by inspection of the margin of the zones of inhibition in the vicinity of its intersection with the circular three-dimensional inoculation. The presence of beta lactamase

induced drug inactivation is visualized as a distortion or discontinuity in the usually circular inhibition zone (Paterson and Bonomo, 2005).

2.13 Causes of false negative and false positive results in ESBL confirmation tests

The phenotypic methods of confirming ESBL production in Gram negative bacteria are based mainly on *in vitro* tests that works on the principle of inhibition of ESBL production by amoxicillin-clavulanic acid (20/10 µg) in combination with some selected corresponding third generation cephalosporins (cefotaxime 30 µg, ceftazidime 30 µg, ceftriaxone 30 µg). These tests according to the Clinical Laboratory Standard Institute (CLSI) were personalized to detect ESBL production in *E. coli, K. pneumoniae*, *K. oxytoca*, but they can be equally applied with some modifications to detect the production of these enzymes in other Enterobacteriaceae and even some non Enterobacteriaceae like *P. aeruginosa* and *Acinetobacter baumanii* (SCIEH, 2004; CAT, 2011). On the other hand, a false negative result or false positive result can also be obtained in the *in vitro* phenotypic detection of ESBLs; and this could be mainly as a result of the co-production of other related enzymes in the test bacteria other than ESBLs. False negative results in ESBL detection phenotypically can be obtained in: Bacterial strains that co-produce an inducible chromosomal AmpC beta-lactamase, Bacterial strains that co-produce plasmid-mediated AmpC beta-lactamase, *P. aeruginosa*, having not only an inducible AmpC enzyme but also a much higher degree of impermeability than Enterobacteriaceae and efflux-mediated resistance mechanisms, and Carbapenemase co-producing organisms (CAT 2011).

It is noteworthy that AmpC enzymes may be induced by clavulanic acid (which inhibits them poorly) and then attack the cephalosporins, thus masking synergy arising from inhibitions of the ESBLs (Paterson and Bonomo, 2005; Rupp and Fey, 2003). Also, bacteria strains that co-

produce ESBLs and carbapenemases can inherently hydrolyze cephalosporins but are not or poorly inhibited by clavulanic acid. False positive can also ensue from a phenotypic ESBL detection method, and this can be obtained in: *K. pneumoniae* isolates that hyper-produce the chromosomal beta – lactamase KL, and SHV-1 hyper-producers or KPC producers. It is in cognizance of this prevailing fact that it is very important for further evaluation of the phenotypic ESBL detection methods by a more specific procedure such as PCR and DNA sequencing amongst others so that ESBL- producing bacteria can be properly characterized and established.

2.14 Genotypic ESBL detection

Because some ESBL expressing bacterial isolates may be missed by phenotypic detection methods, the use of phenotypic method alone is not sufficient enough to confirm ESBL production, thus the need for genotypic ESBL detection methods (which are DNA - based) unlike the phenotypic detection methods (which are antibiotic - based). Molecular detection of ESBL production in suspected isolates is necessary to validate the phenotypic ESBL detection methods and to clear all doubts that an ESBL- producing bacteria was missed or wrongly detected (Al-Jasser, 2006). The phenotypic methods of detecting the production of ESBLs in clinical isolates only identify the presence of an ESBL presumptively (SCIEH, 2004). These methods do not characterize the different types of ESBLs present in the test isolates, or detects the genes responsible for the production of these enzymes even though they can give us a clue as to the type of gene present due to the substrate (antibiotic) they utilize (Jacoby and Munoz-Price, 2005; Bradford, 2001).

The genotypic methods of ESBL detection in clinical isolates are much more complicated and expensive than the phenotypic ESBL detection methods. A number of methods are available for

the molecular characterization of ESBL genes in clinical isolates, including isoelectric focusing (IF), polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), PCR-single - stranded conformation polymorphism (SSCP), ligase - chain reaction (LCR) and nucleotide sequencing amongst others (Al-Jasser, 2006). Genotypic or molecular ESBL detection methods unlike the phenotypic detection methods are usually used in reference laboratories or for research purposes. They are used to differentiate or discriminate between the specific types of ESBLs, and they have the ability to detect low levels of resistance in clinical isolates. With the genotypic ESBL detection methods, specific point mutations in bacterial genes that confer ESBL activity can be detected, and these methods are less – time consuming and do not require culture like the phenotypic detection methods (Rubtsova *et al.,* 2010). According to the CLSI (formerly NCCLS), the genotypic or molecular methods of detecting ESBL production in clinical isolates are the "Gold Standards" by which these enzymes (ESBLs) can be substantially detected in clinical isolates; and though they can be lapses in its use, the genotypic methods of detecting ESBLs are more sensitive and specific than the phenotypic methods (Pitout and Laupland, 2008). The determination of whether a specific ESBL present in a clinical isolate is related to TEM- or SHV- enzymes is a very complex 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 (the earlier beta - lactamases) such as TEM-1, TEM-2, and SHV-1. The genotypic or molecular method of ESBL detection makes use of PCR to amplify the ESBL genes with specific oligonucleotide primers (Rubtsova *et al.,* 2010). This is usually followed by sequencing of the genes – which is essential to discriminate between the non- ESBL parent enzymes (TEM- 1, TEM- 2, or SHV- 1) and different variants of TEM or SHV ESBLs (SHV- 2, TEM- 3) (Rubtsova *et al.*, 2010; Rupp and Fey, 2003).

2.14.1 PCR amplification

Polymerase chain reaction (PCR) amplification of ESBL genes in clinical isolates is one of the easiest genotypic detection methods for ESBL- producing bacteria. It is specific for ESBL gene family (TEM or SHV) but cannot distinguish between ESBLs and non- ESBLs and it cannot also distinguish between the different variants of TEM- or SHV- ESBLs (Bradford, 2001). PCR amplification is performed by heating a suspension of colonies from the test isolate (s) in 50 µl of water to 95 ^{o}C for 10 minutes in order to obtain the DNA (Messai *et al*., 2006). The DNA-containing supernatant after heating is used as a template in specific PCR for the detection of specific ESBL genes (*bla*TEM and *bla*SHV) (Rubtsova *et al.,* 2010). PCR amplification is performed by using specific primers (forward and reverse primers) that are complementary to the *bla*TEM, *bla*CTX or *bla*SHV ESBL genes (Bali *et al.,* 2010). Cycling conditions of the thermo cycler is performed at 94 ^{o}C for 5mins followed by 30 cycles of denaturation at 94 ^{o}C for 30 seconds and annealing at a temperature ranging between 50 ^{o}C to 60 ^{o}C. The final elongation or extension stage of the PCR amplification of the ESBL genes is extended to 10mins at 72 ^{o}C, and the PCR products were separated on 1.5 % agarose gel in order to separate the fragments according to their sizes in an agarose gel electrophoresis (Bali *et al.,* 2010; Messai *et al.,* 2006). The different bands after electrophoresis is visualized under ultraviolet (UV) light after being stained with ethidium bromide and photographed. Because of the demerits of PCR in detecting ESBLs, other genetic techniques based especially on identification of specific point mutations that extend the substrate specificity is paramount in the genotypic ESBL detection methods.

2.15 Medical significance of ESBL detection

Extended spectrum beta - lactamases (ESBLs) are clinically important enzymes produced by pathogenic bacteria including *E. coli*, *K. pneumoniae* and *P. aeruginosa* to mention only but a

few. They represent an important mechanism of antibiotic resistance in Enterobacteriaceae and even non- Enterobacteriaceae such as *Pseudomonas aeruginosa*, reducing the therapeutic efficacy of available drugs especially the beta-lactams. ESBLs hydrolyze and inactivate penicillins, cephalosporins and some non-beta lactams including quinolones, sulphamethoxazole-trimethoprim and aminoglycosides (Uzunovic-Kamberovic *et al.,* 2006). Gram negative bacteria producing ESBLs are a clinical threat and have been associated with increased morbidity and mortality in patients with severe infections (SCIEH, 2004). The detection of ESBLs in clinical isolates is associated with many health problems such as destruction of oxyimino - cephalosporins (workhorse hospital antibiotics), delayed recognition of the infection, inappropriate treatment of ESBL infections and they are multidrug resistant (MDR) (Livermore and Woodford, 2004). The clinical microbiology laboratories in Nigeria do not screen for ESBL producing bacteria in their routine laboratory work, and because of this, they should be up and doing in the detection of ESBLs in clinical isolates according to the CLSI (formally NCCLS) recommended standards (CCLS, 2012) since these enzymes gradually erode the efficacy of one of the most widely used and efficacious regimens in clinical medicine (the cephalosporins and other beta - lactams).

2.16 Epidemiology of ESBLs

The prevalence of organisms producing extended spectrum beta-lactamase (ESBL) enzymes vary greatly from one hospital to another and from one country to another; and this is due in part to the genetic diversity of the organisms producing these all important enzymes coupled to the usage of antibiotic regimens in the affecting regions. ESBL production have been reported from across the world (Nigeria inclusive), and they have been implicated in a variety of stubborn bacterial-related infections such as bacteraemia, pneumonia, wound infections and so on

(Ejikeugwu *et al*., 2013; Iroha *et al*., 2010; Bush and Jacoby, 2010; Jacoby and Munoz-Price, 2005; Bradford, 2001; Bush *et al*., 1995). ESBL production have been widely reported amongst Gram positive and Gram negative bacterial strains but the majority of bacterial organisms that ESBL have been recorded amongst members of the genus Enterobacteriaceae. In particular, *Klebsiella pneumoniae, Klebsiella oxytoca* and *Escherichia coli* are most notorious in the production of ESBL enzymes (Jacoby and Munoz-Price, 2005). Though an emerging and increasing problem in clinical medicine, ESBL production have rendered many antibiotics ineffective for the treatment of bacteria related infections. For the past twenty years, the frequency of ESBL production according to available data has progressively increased, and this is due to the uncontrolled usage of antibiotics, which is largely responsible for exerting the selective pressure that drives the emergence and spread of antibiotic resistant genes including ESBLs (Harrison and Bratcher, 2008; Emery *et al*., 1997). *K. pneumoniae* and *E. coli* are the bacterial species incriminated mostly, but outbreaks have been observed due to *Enterobacter* spp., *Pseudomonas* species, *Citrobacter* species, and *Salmonella* species. The potential diversity and promiscuity of microbes was documented in one hospital outbreak in Poland in which seven different species of Enterobacteriaceae were involved. Reservoirs and vectors for infection have included thermometers, oxygen probes, liquid soap, cockroaches, ultrasound gel and healthcare workers (Macrae *et al.*, 2001).

Non-enteric bacteria including *Pseudomonas* and *Acinetobacter* now produce ESBLs. Bacterial strains that produce ESBLs have been reported in from around the world even in some countries such as USA, UK, and China, where the usage of antibiotics is strictly controlled by federal laws. However, the increasing frequency of ESBL producing bacteria have made them to be regarded as emerging pathogens since they can now be found all over the world. Most outbreaks have

occurred in debilitated, hospitalized patients located in Intensive care units (ICUs). However, outbreaks have been described in out-of hospital locations such as nursing homes, geriatric centre's and rehabilitation units (Hollander *et al.,* 2001) Both adult and pediatric patients have been involved. Other specific patient populations have included solid organ transplant recipients, oncology patients, burn patients and neonates. Organisms producing ESBLs are also responsible for infections in the intensive care units (ICUs) of hospitals, and they also causes a variety of community-associated and nosocomial infections as well (Iroha *et al*., 2011; Yusha'u *et al.,* 2011; Jacoby and Munoz-Price, 2005).

2.17 Prevention and control of the spread of ESBL- producing bacteria

Over the last two decades, ESBL- producing Enterobacteriaceae (especially *E. coli* and *K. pneumoniae*) have evolved, and they cause great harm to both patients and our therapeutic armamentarium (especially the expanded - oxyimino cephalosporins), limiting treatment options for some bacterial related infections caused by these organisms (Jacoby and Munoz-Price, 2005; Paterson and Bonomo, 2005; Bush and Jacoby, 2010). According to a researcher, Amyes S.G in the Scottish Center for Infection and Environmental Health Weekly Report (SCIEH, 2004), it is too late to try and stop the development of ESBL- producing bacteria because the mutated genes (responsible for the production of ESBLs in these organisms) now exist and have spread amongst clinical microbial populations through plasmids. There is therefore need to review our hospital infection control measures and antibiotic (drug) usage policies so as to try and keep ESBL-producers at bay, both in the hospital and community settings. ESBL- producing bacteria may be endemic in most of our hospitals unknowingly, thus it is vital that measures to detect and control their spread be put in place, and such infection control mechanisms should be adhered to by both hospital and non-hospital staff visiting the hospital (SCIEH, 2004).

To prevent the spread of ESBL- producing bacteria, the use of standard hospital precautions at all times for all patients should be observed and strict hand hygiene is very essential using liquid soap, warm water, alcohol hand gel and disposable paper towels (NYYPCT, 2008). The use of separate hand gloves for different patients is also important since ESBL- producing bacteria can easily spread within a given hospital environment, and the main focus of any infection control measure should be to stop the spread of ESBL- producers from patient to patient. Since ESBL-producing organisms are multidrug resistant (MDR), a multidisciplinary approach is necessary in its prevention and control. Gram negative bacilli (especially the Enterobacteriaceae and *Pseudomonads*) producing ESBLs have been described on almost every continent of the world (except for some Scandinavian countries where antibiotic resistant pathogens are at a minimal level) and they are increasing in prevalence as well. Because ESBL production or colonization is spurred by an initial or prior exposure to antibiotics (especially the cephalosporins), there is need for a proper restriction on the use of extended spectrum cephalosporins (Rupp and Fey, 2003). The selective pressure imposed on Enterobacteriaceae as a result of the overuse of beta lactam drugs should be avoided through the prudent use of antibiotics. Apart from the beta lactams and cephalosporins which are known to spur ESBL production in pathogens, other drug classes (such as the quinolones, aminoglycosides, fluoroquinolones, sulphamethoxazole) which might contribute should be used wisely and third generation cephalosporins should be used in rational and limiting amounts since this measure have shown to be effective in alleviating ESBL-producing bacteria (Mohamudha *et al.*, 2010).

As stated above, tough hospital infection control measures like intermittent hand washing, patient isolation when an ESBL infection is suspected and strict adherence to infection control measures together with the prudent use of high grade antibiotics (in and outside the hospital) are

important to preventing the spread of ESBL infection. In addition, antibiotics (especially the extended spectrum cephalosporins) should be prescribed to patients only when necessary and in the right dose and duration so as to avert any emergence of ESBL- producing organism. Patients should also stick to their therapeutic regimens and ensure that they complete their therapy even after getting well before the treatment has been exhausted. Of utmost importance is a continuous education program in both our health institutions and communities so as to tackle ESBL problems head on, and government should support research studies which are geared towards discovering new antimicrobials and deciphering the presence of these enzymes in both the hospitals and non hospital environment so that appropriate control and preventive blue print can be drawn from such studies and implemented. This will help in preserving our available antibiotics and making the world a better place for all, free of antibiotic resistant organisms especially at this time when the discovery of new drugs is slow compared to the fast pace of emergence and spread of drug resistant pathogens (NYYPCT, 2008).

2.18 Treatment of ESBL infection

Bacterial resistance to beta-lactamase and even extended spectrum beta-lactamases (ESBLs) is on the increase, and this has been largely reported amongst bacteria in the Enterobacteriaceae family from across the world including here in Nigeria (Dhillon and Clark, 2011; Iroha *et al*., 2010; Chau and Oboegbunam, 2007; Aibinu *et al*., 2003; Pitout and Laupland, 2008). Clinically, patients infected with ESBL-producing bacteria tend to have less satisfactory treatment outcomes than those individuals who are apparently infected by bacterial pathogens that do not produce ESBLs. The reason for this development or phenomenon is that bacterial pathogens that express ESBLs are often multidrug resistant, and can continue to remain active even in the face of potent antimicrobial onslaught. This has further compounded the treatment options available for treating

bacterial related infections caused by ESBL-producing pathogens since this group of microbes have the ability to render a significant number of antibiotics used in clinical medicine today inactive. Infections caused by ESBL pathogens can render all penicillins, cephalosporins and even the monobactams ineffective for treatment (Jacoby and Munoz-Price, 2005). The best choice of antibiotics for the effective treatment of ESBL infections is therefore very challenging due to the limited number of drugs that can be used to assuage the menace caused by the infection. Beta-lactamase inhibitors such as clavulanic acid show very remarkable antibacterial activity against ESBL producing bacteria *in vitro*, but the clinical effectiveness of these agents in managing bacterial infections caused by ESBLs has not been fully established since there exist some bacterial strains that hyper-produce beta-lactamase enzymes (AmpC enzymes) which makes beta-lactamase inhibitors to be ineffective. Cephamycins such as cefoxitin can be effective in treating ESBL infections since the pathogen is sensitive to the drug. However, the drugs of choice for the effective treatment of bacterial infections caused by ESBLs still remain the carbapenems. Carbapenems are very potent antibiotics that include meropenem, imipenem and ertapenem, and they still remain the drug of choice for serious infections caused by ESBL-producing bacteria. In addition to the carbapenems, non-beta lactam drugs such as fluoroquinolones, aminoglycosides and sulphamethoxazole-trimethoprim are other viable alternatives for the treatment of ESBL infections (Bradford, 2001). Nonetheless, some level of resistance to these agents by ESBL producing bacterial strains have been noted in some quarters, thus ESBL infections must be handled and treated with caution especially with the use of a good antimicrobial susceptibility result which will help to guide therapy and choose the line of treatment to take (Jacoby and Munoz-Price, 2005; Dhillon and Clark, 2011).

2.19 History of antibiotic resistance

Antibiotic resistant bacteria were first discovered soon after the medicinal use of penicillin began. The first signs of antibiotic resistance were actually observed in 1940, five years before penicillin became commercially available to the public. In that year, the first observed bacterial enzyme (beta-lactamase) that destroyed penicillin was described (Rodriguez-Bano *et al*., 2010; Stephan and William, 2006). This was the first observed evidence of bacterial resistance to an antibiotic action. Therefore, the history of antibiotic resistance coincided with the history of antibiotics themselves. The number of antibiotics belonging to various families, their varied mode of action and the number of bacteria in which antibiotic resistance has been documented suggests that, in principle, any microbe could develop resistance to any antibiotic. Antibiotic resistance is one of the biggest challenges that bedevil our health sector worldwide. Resistance of microbes to antibiotics has been documented not only against antibiotics of natural and semi – synthetic origin, but also against purely synthetic compounds (such as the fluoroquinolones) or those which do not even enter the cells (such as vancomycin). And unfortunately, the discovery and development of newer antibiotics have not kept pace with the emergence and rate at which bacteria develops and mount resistance to antibiotics. Thus, the rate at which microbes are developing resistance to antibiotics is much faster than the rate at which the drugs are developed to curb the problem (Al-Zarouni *et al*., 2008).

2.20 Disseminating antibiotic resistance

There are two routes for acquired resistance, vertical evolution via mutation and selection or horizontal evolution via exchange of genes between similar and different species. Vertical evolution is determined by natural selection whereby a spontaneous mutation in the bacterial chromosome bestows resistance to a bacterium and its progeny within the population. Horizontal

evolution (or lateral gene transfer) generally occurs via three routes; transformation (uptake of naked DNA), conjugation (direct contact transfer of mobile plasmids) or transduction (bacteriophage). Lateral gene transfer is believed to be the major route for widespread global dissemination of antibiotic resistance and is responsible for transfers of plasmids carrying antibiotic resistance genes (R plasmids) in 60-90 % of gram-negative bacteria (Levy *et al*., 1988). Some other researchers deduced that 17.6 % of *E. coli* genes have been acquired by lateral gene transfer. Regardless of their physical location, chromosome, plasmid or integrons within transposons, antibiotic resistance genes can undergo lateral gene transfer. Transposons are the most conducive means of transferring antibiotic resistance genes amongst bacterial populations. They typically carry a selectable phenotype (antibiotic resistance) bordered by two insertion sequences, and are unique in their ability to 'jump' from one genetic locus into another, irrespective of taxanomic class. Transposons often contain integrons, genetic elements which harbor a range of antibiotic resistance genes, a promoter site, a recombination site downstream of the resistant genes and an integrase coding gene. They are transferred between bacteria, integrating into bacterial genomes and/or plasmids. Multi-resistance is achieved when several antibiotic resistance cassettes are inserted into the integron. There are five major classes of integrons (Mazel *et al.,* 2006). Class 1 integrons are derived from transposon Tn402 that can insert into the large Tn21 transposon; Class 2 is exclusively derived from the Tn7 transposon which is highly adept at integrating into the chromosome of *E. coli* and other *Proteobacteria* thus disseminating its resistance genes throughout a large community of bacteria; Class 3 is probably transposon associated; and Classes 4 and 5 are linked to trimethoprim resistance in *Vibrio* species. Integrons are thought to play a major role in the spread of bacterial antibiotic resistance. Some resistance genes reside within highly efficient transfer elements. For example

dihydrofolatereductase (*dfr1),* the most common trimethoprim resistance gene, is located on both bacteria isolated from humans and animals (Mazel *et al.,* 2006).

2.21 Types of antibiotic resistance

Bacteria have evolved to survive in diverse environments. They survive exposure to harsh chemicals including antibiotics, and they also survive difficult growth conditions. They have learned to "detoxify" harmful substances e.g. antibiotics. Antibiotic resistance can either be intrinsic or acquired.

2.21.1 Intrinsic (Innate) resistance

Some bacteria are said to possess innate/intrinsic resistance against antibacterial action put forward by antibiotics. They mount a great ingenuity in devising means or ways of neutralizing the killing or inhibiting action of antibiotics directed towards them. This innate form of antibiotic resistance in bacteria shows the different variations in the structure of the cell envelope of the organism, which allows them to mount resistance against drugs. It is a vertical means by which bacteria acquire resistance. Intrinsic or innate form of antibiotic resistance can occur by any one of the following route:

- Spontaneous mutation in the chromosomal DNA of bacteria.
- Accumulation of several point mutations in bacteria.
- An evolutionary process occurring only under selective pressure e.g. prior exposure of bacteria to antibiotics (Prescott *et al.,* 2008).

2.21.2 Acquired (Phenotypic) resistance

This type of antibiotic resistance is acquired by bacteria from the environment or other microorganisms by one of the means of genetic transfer (conjugation, transformation, and transduction). In acquired/phenotypic resistance, the bacteria acquire reduced susceptibility to

antibiotics through adaptation to growth within a specific environment. Acquired resistance is a horizontal means by which bacteria become resistant to antibacterial properties of antibiotics. This form of antibiotic resistance can be achieved in bacteria by one of the following route:

- Resistance can be maintained on horizontal mobile elements like plasmids, integrons and transposons.
- Resistant genes can be transferred among bacteria through means of genetic transfer.
- Resistance genes can be integrated into the bacterial chromosome or can be maintained in an extra chromosomal state (plasmids) (Prescott *et al.*, 2008; Mazel *et al.*, 2006).

2.22 Plasmids

Plasmids are genetic elements that replicate independently of the host chromosomes (Madigan *et al*., 2009). Like chromosomes, most plasmids are double- stranded DNA molecules that have an origin of replication and therefore can be replicated by the cell before it divides. Both circular and linear plasmids have been documented, but most known plasmids are circular. Linear plasmids possess special structures or sequences at their ends to prevent their degradation and to permit their replication. Plasmids have relatively few genes, generally less than 30. Their genetic information is not essential to the host, and cells that lack them usually function normally. However many plasmids carry genes that confer a selective advantage to their hosts in certain environments. Plasmids play many important roles in the lives of the organisms that have them. They also have proved invaluable to microbiologists and molecular geneticists in constructing and transferring new genetic combinations and in cloning genes. Plasmids are able to replicate autonomously. Single-copy plasmids produce only one copy per host cell. Multiply plasmids

may be present at concentrations of 40 or more per cell. Some plasmids are able to integrate into the chromosome and are thus replicated with the chromosome. Such plasmids are called episomes (Prescott *et al*., 2008).

Plasmids are inherited stably during cell division, but they are not always equally apportioned into daughter cells and sometimes are lost. The loss of a plasmid is called curing. It can occur spontaneously or be induced by treatments that inhibit plasmid replication but not host cell reproduction. Some commonly used curing treatments are acridine mutagenes, UV, and ionizing radiation, thymine starvation, antibiotics and growth above optimal temperatures. Plasmids may be classified in terms of their mode of existence, spread and function. We have R-plasmids, col plasmids, virulence plasmids and metabolic plasmids. R-plasmid confers antibiotics resistance to the cell that contains them; they have gene that code for enzyme capable of destroying or modifying antibiotics. R-plasmid is of major concern to public health because they spread rapidly through out a population of cells. This is possible for several reasons; one of the reasons is that many R-factors are also conjugate plasmid. However a non conjugative R factor can be spread to other cells if it is present in a cell that also contains a conjugative plasmid. In such a cell, the R factor can sometimes be transferred when the conjugative plasmid is transferred. Even more troubling is that R-factor is readily transferred among species. Col plasmid contains genes for the synthesis of bacteriocins known as colicins which are directed against *E.coli.* Virulence plasmid encodes factors that make their hosts more pathogenic. Metabolic plasmids carry gene for enzymes that degrade substances such as aromatic compounds and sugars (Prescott *et al*., 2008).

2.22.1 Plasmid and bacterial resistance

Genes for drug resistance may be present on bacteria chromosomes, plasmids, transposons and integrons. Because they are often found on mobile genetic elements, they can freely exchange between bacteria. These genes can undergo spontaneous mutations in bacterial chromosomes, although they do not occur often, but can cause bacteria drug resistant. However, a bacterial pathogen is drug resistant because it has a plasmid bearing one or more resistance genes; such plasmids are called R-plasmids, plasmid resistance gene often code for enzymes that destroy or modify drugs, plasmid associated genes have been implicated in resistance to aminoglycosides, chloramphenicol, penicillin, cephalosporin, erythromycins, sulphonamides and others (Prescott *et al*., 2008). Once a bacterial cell posse R-plasmid, the plasmid may be transferred to other cells quite rapidly through normal gene exchange processes such as transduction, conjugation and transformation. Because a single plasmid may carry gene for resistance to several drugs, a pathogen population can become resistant to several antibiotics even though the infected patient is treated with one drug. Bacteria can resist the action of antibiotics by preventing the access to the target of the antibiotics, degrading the antibiotics or rapid extrusion of the antibiotics. The mechanism is shown in figures below.

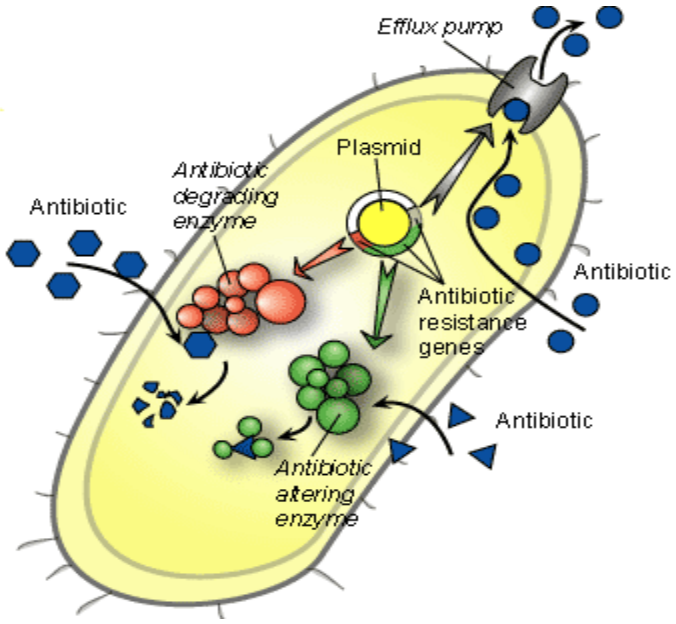

Figure 2.1: Antibiotics resistance mechanism (Prescott *et al*., 2008).

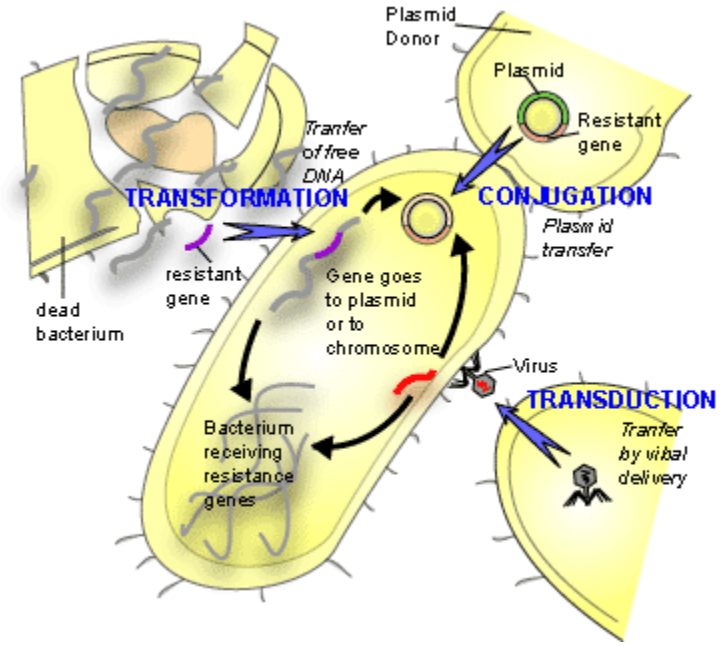

Figure 2.2: The horizontal exchange of genetic material like antibiotics resistance genes through plasmid by bacteria (Prescott *et al*., 2008).

2.23 Mode of transfer of resistance

Antibiotic resistant bacteria owe their drug insensitivity and ingenuity in developing resistance against our therapeutic regimens to resistance genes which they harbor or possess. It is these genes that resistant bacteria transfer to non resistant susceptible strains, thus compounding the problem of antibiotic resistance. Below are some of the major ways through which bacteria pass on their antibiotic resistance genes to susceptible non resistant bacteria:

2.23.1 Conjugation

Conjugation is the form of gene transfer and recombination in bacteria through which genetic materials are transferred from one bacterium to another through a direct cell - to - cell contact. It is the most important genetic transfer mechanism by which bacteria transfer their antibiotic resistance genes to susceptible bacteria. Conjugation is mediated by a particular kind of circular DNA called a plasmid, which replicates independently of the chromosome. Many plasmids carry genes that confer resistance to antibiotics. When two bacteria cells are in close proximity to each other a hollow bridge like structure known as the "pilus" forms between the two cells. This allows a copy of the plasmid as it is duplicated to be transferred from one bacterium to another. This process called conjugation enables a susceptible bacterium to acquire resistance genes to a particular antibiotic (Prescott *et al*., 2008).

2.23.2 Transduction

Transduction is the transfer of genetic material between bacteria by bacteriophages (bacterial viruses). Here, antibiotic resistance genes are incorporated into a phage capsule which is later injected into another bacterium (Prescott *et al*., 2008). In the process of transduction, bacterial DNA is transferred from one bacterium to another inside a virus that infects bacteria. These viruses are called bacteriophages or phage. When a phage infects a bacterium, it essentially takes

over the genetic process of the bacteria to produce more phage. During this process, bacterial DNA may inadvertently be incorporated into the new phage DNA. Upon bacterial death and lyses or breaking apart, these new phage goes on to infect other bacteria. This brings along genes from previously infected bacterium into the recipient bacterium. These genes might contain advantageous genes such as antibiotic resistance genes, which will leave the recipient bacterium resistant to a particular antimicrobial agent (e.g. antibiotics) (Prescott *et al*., 2008).

2.23.3 Transformation

Transformation is a mode of genetic transfer in bacteria in which a piece of free DNA (genetic materials) is taken up by a bacterium and integrated into the recipient genome. During this process, genes are transferred from one bacterium to another as "naked" DNA. When bacterial cells die and break apart, DNA can be released into the surrounding environment. Other bacteria in close proximity can scavenge this free floating DNA and incorporate them into their own DNA. This incorporated DNA can contain advantageous genes such as antibiotic resistance genes and benefit recipient bacterial cells (Prescott *et al*., 2008).

2.24 Control of antibiotic resistance

Antibiotic resistance can be controlled by one of the following methods: Hand washing as a measure of infection control. Review of antibiotic use in hospitals and updating clinicians, nurses, pharmacists, and patients on the rationale use of antibiotics. Good personal hygiene in both the hospital and in the community. Restriction of the use of human medicine in livestock and animal feeds formulation. Patronage of over - the - counter (OTC) drugs by patients for self medication without doctor's prescription should be discouraged. Patients should always endeavour to take full course of their drugs when under any medication (Prescott *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Population

This study was carried out at Abakaliki. Abakaliki is the capital city of Ebonyi State in Southeastern Nigeria. Its geographical coordinates are longitude 8^0 06'E and Latitude 6^0 20'N. The tropical climate is broadly of two seasons which are the rainy season (April to October) and dry season (November to March). Temperature throughout the year ranges between 21 ^{0}C to 29 ^{0}C and humidity is relatively high. The annual rainfall is about 1, 150mm. Its vegetation is of the sub-savannah rain forest. The inhabitants are primarily members of the Igbo tribe. Abakaliki is made up of four clans namely Ezza Ezekuna, Ngbo, Izzi and Ikwo. The estimated population of the state is 2.2 million according to the 2006 census. Nigerians from other communities also reside mainly within the state capital city. Inhabitants are predominantly farmers, civil servants and traders (National Bureau of Statistics, 2012).

This research project was limited to a tertiary hospital in Ebonyi State, owing to the fact that the hospital in question prescribes and administers antibiotics of different classes of beta lactam, and it also has a high influx of patients (in- and out- patients) with different ailments which resulted in a high turnout of bacterial isolates that was used for this research work under the study period.

3.2 Ethical Clearance

Ethical approval for the collection of clinical specimens and accessing of patients bio-data were obtained from the Management of Federal University Teaching Hospital Abakaliki, (FETHA). This research was carried out based on a thorough knowledge of the scientific literatures, satisfactory laboratory protocols and other relevant sources of information guiding this area of

research. Every fundamental study was done in line with the World Medical Association (WMA) declaration of Helsinki on the principles for medical research involving human subjects and identifiable human material or data (WMA Declaration of Helsinki, 2004). All bacterial isolates and identifiable human data or material used in undertaking this research project were treated and handled with utmost confidentiality. Appropriate caution was also observed in the conduct of this research work so as to avoid every negative impact of the study on the environment and other human subjects within the vicinity of the research work including the researchers. Results (both positive and negative) of the research work were also taken and recorded accurately based on existing guidelines in relevant scientific literatures, and the results were preserved without any alteration whatsoever.

3.3 Samples size

Sample size was determined using Cochran's formula; (Cochran, 1977).

$$N = \frac{\{Z^2 (pq)\}}{e^2}$$

N = Desired sample size

Z = Normal standard deviation at 95% confidence = 1.96

p = Prevalence = 0.252 as reported by Iroha *et al*., 2008a

q = Alternate proportion (1 – P = 0.748)

e^2 = Degree of precision/significance = 0.05

$$N = \frac{\{1.96^2 (0.252 \times 0.748)\}}{0.05^2}$$

$$N = \frac{\{3.842 (0.189)\}}{}$$

0.0025

$$N = \frac{0.726}{0.0025}$$

N= 290

A total of 1000 clinical specimens, high vaginal swab (n= 200; females - 200), wound swab (n= 350; males - 200, females - 150) and urine specimens (n= 450; males - 250, females - 200) were collected from patients attending Federal Teaching Hospital, Abakaliki, South Eastern Nigeria, between March, 2016 to Feburary, 2017. The demographic data (Age, Sex, Occupation, and Level of Education) of the patients were collected from the laboratory request forms in line with hospital record (Appendix 1). The target organisms were bacterial isolates of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* from clinical specimens of both in- and out-patients that attended the tertiary hospital under study.

3.4 Samples collection

Specimens were collected from male and female patients within the age range of 1 year and above 76 years and mean age of 38 years. Mid-stream urine specimens were collected from patients suspected of urinary tract infections (UTIs) using sterile universal container, each patient was given a sterile container and adviced on how to collect their urine specimen. Sterile swab sticks were used to collect high vaginal and wound swab from patients suspected of high vaginal and wound infections. The high vaginal specimens were collected by using sterile speculum to opon the mouth of the vagina before the swab stick was carefully inserted. The swab sticks were carefully removed and returned to their respective containers and labeled accordingly. The samples were transported to the microbiology laboratory of Ebonyi State University, Abakaliki for bacteriological analysis.

3.5 Preparation of Culture Media

The culture media used were Cystein Lactose Electrolyte Deficient (CLED) agar, Nutrient agar, Nutrient broth, Peptone water, MacConkey agar, Mannitol salt agar, Mueller Hinton agar, Luria-Bertani (LB) agar and Luria-Bertani (LB) broth. They were obtained in hydrated powdered formula and prepared according to the manufacturers instructions. A measured weight of the powder was dispensed into a conical flask and a definite volume of distilled water was added to it according to the manufacturer's recommendation. The flask was warmed gently over Bunsen flame using triple stand to dissolve the media properly. It was stirred during heating under flame. The flask was then properly stoppered and sterilized by autoclaving at 121 ^{0}C and 15 psi for 15 minutes. The flask was allowed to cool to about 45 ^{0}C to 50 ^{0}C before dispensing its contents into sterile petri dishes. Broth and agar slants were prepared by measuring a specific quantity of the dissolved media into Bijoux bottles and screw capped properly. The Bijoux bottles containing the media were autoclaved to achieve sterility. The Petri dishes and Bijoux bottles were placed on top of clean working bench till the media solidified. The agar and broths in the Bijoux bottles were allowed to cool before inoculating with specimens of choice.

3.6 Bacterial Isolation and Identification

The wound, high vaginal and urine specimens collected were cultured within 2 hours of collection on nutrient broth, before plating out the organisms on nutrient agar, MacConkey's agar, mannitol salt agar, CLED and incubated for 18 to 24 hours at 37 ^{0}C. The media were examined and colonies were sub-cultured to obtain discrete colonies. The discrete colonies were examined for their morphological appearances. All the isolates were Gram stained and subjected to biochemical characterization.

3.6.1 Morphological identification of the bacterial isolates

Pink or opaque yellow colonies on MacConkey or CLED agar respectively were assumed to be potential *E. coli* isolates. Pink or yellowish mucoid colonies on MacConkey or CLED agar were considered potential *K. pneumoniae* isolates. Golden yellow colonies on Mannitol salt agar were considered potential *S. aureus* (Cheesbrough, 2010).

3.6.2 Gram Staining

A smear from fresh pure cultures of the test bacterial isolates were made on grease-free glass slides labeled appropriately and heat fixed by passing it over the bunsen burner flame thrice. The slides were placed on a level surface and flooded with crystal violet and allowed to stand for one minute. The stain was washed off under running water. Lugol's iodine solution (mordant) was flooded on the smear and allowed to stand for 30 seconds. The Lugol's iodine was washed off under running water. The slide was placed in a slanting position and ethyl alcohol (decolourizer) was applied in drops for 30 seconds and washed off under running water. Finally, safranin (counter stain) was poured on the slide, allowed to stand for 30 seconds and washed off quickly with running water. The slide was placed on the rack for the smear to air dry and then observed under microscope using oil immersion objective lens. The colour, cell arrangement and shape of the bacteria were noted. Purple colour indicates Gram positive organisms while pink or red colour indicates Gram negative (Cheesbrough, 2010).

3.6.3 Biochemical Tests

The conventional biochemical tests done were indole test, citrate test, oxidase test, catalase test, coagulase test, methylred test, voges-proskauer test, sugar fermentation test (Cheesbrough, 2010).

3.6.3.1 Indole test

A loopful of the test isolate was cultured in peptone water for 48 hours at 37 ^{0}C. Three drops of kovac's reagent were added to the peptone water culture and observed for the appearance of a red ring above the peptone water.

3.6.3.2 Citrate Test

A slant was first prepared. A light suspension of the isolate was made in saline. The isolate was inoculated in citrate agar by stabbing, incubated 18 – 24hrs and thereafter observed for the development of deep blue colour which indicates citrate positive, while green colour meant negative.

3.6.3.3 Methyl Red Test

Freashly prepared peptone water was inoculated with a loopful of the test isolate and incubated at 35 ^{0}C for 48 hours. Then 5 drops of the indicator (methyl red solution) were added to the culture and then observed for a change in colour to red which indicates methyl red positive.

3.6.3.4 Voges Proskauer Test

The test isolate was cultured in peptone water for 48 hours at 37 ^{0}C. Then 1 ml of 10 % of KOH was added and allowed to stand for one hour. It was observed for the development of a pink colour which indicates Voges Proskauer positive and absence of pink colour indicates negative.

3.6.3.5 Motility Test

A semi solid medium containing 0.3 % nutrient agar dissolved in distilled water was used. Using a straight wire, the semi solid medium was stab inoculated with the test isolate and incubated at 37 ^{0}C for 24 to 48 hours. Zigzag and diffuse growth into the agar away from the stab line indicated motility.

3.6.3.6 Coagulase Test

A drop of plasma was mixed with a drop of the isolate from a broth culture. This was mixed well and kept for 5 to 10 minutes. The presence of coagulase was indicated by the production of agglutination.

3.6.3.7 Oxidase Test

A piece of filer paper was put in a clean and sterile petri dish. Then 3 drops of freshly prepared oxidase reagent (N, N-dimethyl-p-phenylenediamine hydrochloride) were added. Using a piece of sterile glass rod, a colony of the test isolate was smeared on the filter paper. Appearance of blue-purple colour within 10 seconds indicates oxidase positive.

3.6.3.8 Sugar Fermentation Test

This test was carried out to determine the ability of the isolates to metabolize sugar with the production of acid/gas or gas. Glucose, lactose and mannitol were prepared using peptone water. A pinch of bromocresol purple was added as indicator and 5 ml aliquots dispensed into Bijoux bottles containing Durham tubes and autoclaved at 115 ^{0}C for 10 minutes. It was allowed to cool and then inoculated with the test organism using sterile wire loop and incubated at 37 ^{0}C for 48 hours. A change in colour from purple to yellow indicated positive result while gas production was shown by the downward displacement of liquids in the Durham tubes (Cheesbrough, 2010).

3.6.3.9 Catalase test

A 2 ml of hydrogen peroxide solution was poured into a test tube and a glass rod was used to remove some colonies of the test isolate from overnight culture and immersed into hydrogen peroxide solution. Production of oxygen gas bubbles within seconds indicated catalase positive whereas absence of bubbles meant negative result (Cheesbrough, 2010).

3.7 Preparation of 0.5 McFarland turbidity standards

Turbidity standard equivalent to 0.5 McFarland was prepared by adding 1 ml of concentrated tetraoxosulphate (VI) acid (H_2SO_4) to 99 ml of distilled water, and dissolving 0.5 g of dehydrate barium chloride ($BaCL_2.2H_20$) in 50 ml of distilled water in separate reaction flasks respectively. Barium chloride solution (0.6 ml) was added to 99.4 ml of the tetraoxosulphate (VI) acid solution in a separate test tube, and the reaction mixture mixed well to form 0.5 McFarland turbidity standard. Small portion of the turbid solution was transferred to a capped test tube similar to the tube used for preparing the test microorganisms and stored at room temperature (28 ^{0}C). This small volume of capped turbid solution was always shaken properly before use, and it was used to adjust and compare the turbidity of the test bacteria to get a confluent growth on a growth or culture plate (Cheesbrough, 2010) when performing antibiotic susceptibility testing.

3.8 Susceptibility test using beta-lactam antibiotic discs

Mueller-Hinton agar plates were prepared following the manufacturer's instruction. A 0.5 McFarland's equivalent standards of the test organisms were inoculated on the surface of 90 mm diameter agar plates and spread using sterile swab stick for each organism. Antibiotic disc of ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), ceftriaxone (CRO; 30 µg), cefpodoxime (CPO; 10 µg), aztreonam (ATM; 30 µg), and cefepime (FEP; 30 µg) were placed 30 mm away

from each on the surface of the agar plates. The antibiotics were allowed to diffuse for about 10minutes and the plates were incubated at 37 ^{0}C for 18 to 24 hours. The zones of inhibition were determined and interpreted according to CLSI (Appendix 1). The control strains used for this study were *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *K. pneumoniae* ATCC 700603 (oxoid, UK). Isolates resistant to any of these cephalosporins (ceftazidime and cefotaxime) were subjected to ESBL phenotypic testing (CLSI, 2012).

3.9 Detection of beta-lactamase production using nitrocefin stick

E. coli, Klebsiella pneumoniae and Staphylococcus aureus strains isolated in this study were screened for beta-lactamase production using nitrocefin sticks (oxoid, UK). Nitrocefin is a chromogenic cephalosporin (incorporated in the nitrocefin test sticks) that changes colour from yellow to red on hydrolysis when the stick comes in contact with the test isolate. The nitrocefin sticks were removed from refrigerator, allowed to cool to room temperature (28 ^{0}C plus or minus 2^{0}C), and the colour coded end was used to touch the colonies and the stick was rotated to pick mass of the cells. Two drops of distilled water were used to moisten the tip of the stick and then allowed for 5-15 minutes and further observed for pink-red colour development (CLSI, 2012; Livermore and Brown, 2001).

3.10 Phenotypic determination of ESBL using double disc synergy test (DDST)

The colonies of *E. coli, K. pneumoniae* and *S. aureus* clinical isolates that were suspected to be potential ESBL producers were inoculated in peptone water and incubated at 37 ^{0}C for 2 - 6 hours. The turbidity was adjusted to 0.5 McFarland standards and spread plate was made on the surface of Mueller-Hinton agar plates using sterile swab stick for each organism (CLSI, 2012). Antibiotics disc of ceftazidime (CAZ), cefotaxime (CTX) (30 µg each) were placed at a distance

of 15 mm centre to centre from the centre disc containing amoxicillin plus clavulanic acid (AMC) (20 µg and 10 µg, respectively). The plates were incubated for 18 to 24 hours at 37 ^{0}C. An increase of ≥5 mm in the inhibition zone diameter for either of the cephalosporins (ceftazidime and cefotaxime) tested in combination with amoxycillin-clavulanic acid versus its zone when tested alone confirms ESBL production phenotypically (Pituot *et al.,* 2004).

3.11 ESBL test using brilliant ESBL agar

Brilliant ESBL agar (pale, off white, semi-opaque gel medium) is a chromogenic screening plate for detection of ESBL- producing organisms. The medium provides presumptive identification of ESBL- producing *E. coli* and *Klebsiella* spp direct from clinical specimens or cultured bacterial isolates in 24 hours. The medium was removed from refrigerator and allowed to cool at room temperature ((28 ^{0}C plus or minus 2^{0}C) before inoculation. Brilliance ESBL agar was inoculated direct with isolated pure colonies of *E. coli* and *Klebsiella pneumoniae* that were prepared to 0.5 McFarland turbidity equivalents. The inoculated plates were incubated for 18 to 24 hours at 37 ^{0}C. Negative plates were re-incubated for additional 24 hours. Blue or Pink and Green colonies were recorded as positive ESBL- producing *E. coli* and *Klebsiella pneumoniae* respectively (Thermo Scientific Oxoid Microbiology, 2013).

3.12 Genomic DNA Extraction

DNA was extracted from pure colonies of an overnight growth (18 to 24 hours) of bacteria isolates on Luria-Bertani (LB) agar (Merck, Germany). Genomic DNA extractions were performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's procedure (Appendix 3). The concentration and purity of the extracted DNA was determined using NanoDrop-Spectrophotometer at absorbance of A260/280 (Thermo scientific,

USA). Quality controls of the bacterial DNA extraction were carried out by testing all extracted isolates for 16S rRNA region (Azam *et al.,* 2016).

3.13 Plasmid DNA extraction

Overnight (18 to 24 hours) grown bacterial cells in 5 ml of LB broth (Merck, Germany) were harvested by centrifuging the 5 ml of each culture in 15 ml centrifuge tubes for 4 min at 4000 rpm (revolutions per minute) and the supernatant was discarded. A total of 600 µl of bacterial cells were measured into a clean 1.5 ml of micro-centrifuge tube. The plasmids DNA were extracted using Zyppy Plasmid Miniprep Kit (Zymo Research, Epigenetics, USA) according to the manufacturer's instruction (Appendix 3). The resulting plasmid preparations were stored at -20 ^{0}C for further use (Sharma *et al*., 2010).

3.14 PCR analysis of *bla*TEM, *bla*SHV and *bla*CTX-M genes

The isolated genomic DNA and plasmid DNA of ESBL positive isolates were screened by PCR method, using specific oligonucleotide primers to determine *bla*TEM, *bla*SHV and *bla*CTX-M group's genes (Appendix 4). The oligonucleotide primers were reconstituted with TE buffer according to the manufacturer's specification. A stock solution (10 µM) was prepared by dissolving 10 µL of the primers into 90 µL of Sigma water (Sigma-Aldrich, St. Louis, MO). Primer sequences and their size were used for the detection of *bla*TEM, *bla*SHV and *bla*CTX-M group's genes. The PCR reactions for detection of *bla*TEM, *bla*SHV and *bla*CTX-M groups genes were done in a total volume of 25 µl aliquot by mixing 12.5 µl of Master Mix Red, 10 µl of Sigma water (Nuclease free water), 0.25 µl of forward primer, 0.25 µl of reverse primer and 2 µl of the isolated genomic DNA/plasmid DNA in a PCR tube (Gudjónsdóttir, 2015). The PCR was done with a C1000 Touch^{TM} Thermo Cycler (Bio-Red) in Microbiology and Biotechnology Research Laboratory at North West University, South Africa.

3.15 Gel Electrophoresis

A mixture of 1.0 g of agarose (SeaKem, Lonza, USA) and 100 ml of 1 X Tris-acetate-ethylenediaminetetraacetate (TAE; pH 8.0) buffer (Bio-Rad) was used to make up a medium size of 1 % (w/v) agarose gel. The mixture was heated up for 3 minutes in a microwave oven for the agarose to completely dissolve, then it was cooled to about 50°C and ethidium bromide (1 µl/ml) was added to stain the gel. The molten gel was casted into a gel casting tray containing combs and allowed to solidify. After about 30 minutes the gel combs were carefully removed and the gel casting tray containing the gel was placed into a gel electrophoresis chamber or tank filled with TAE buffer (40 mM Tris, 20 mM acetic acid, and 100 mM EDTA pH 8.0). For each run, 5 µl of Extend Quick-Load DNA Ladder (1kb; New England, Bio Labs) and Lambda DNA/HindIII Marker 3 (2.5kb; Thermo Fisher Scientific) was added to one of the wells to estimate the band sizes and 5 µl of negative control, comprised of Sigma water (Nuclease free water), was added to another well. The plasmid DNA and genomic DNA/PCR products, 5 µl of each, were carefully loaded into the remaining wells. An electric current of 80 V; 400 mA (mini Ampere) was run through the gel for 60 minutes and gel was removed. Gels were visualized and photographed using a gel documentation system (Gel Doc 2000; Bio-Rad) (Gudjónsdóttir, 2015).

3.16 Sequencing and Blasting

After screening for the amplification of ESBL genes on both chromosomal and plasmid DNA, sequencing of PCR products was carried out using 16S rRNA gene to identify the isolates to species level. Direct sequencing of each PCR product was carried out using the Sanger dideoxynucleotide chain termination method with the ABI Prism BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Inc., Foster City, CA, USA) on an ABI Prism 3130 Automated Sequencer (Gudjónsdóttir, 2015). Sequencing was done at Inqaba Biotech in

Pretoria, South Africa. The nucleotide base sequences were cleaned up (base calling) for the presence of sense and antisense (chimeric sequences) using Chromas 2.6.4 version software to analyze the chromatograms, resulting from sequencing reaction for good quality sequence assurance. The resulting chromatograms (nucleotide sequences) obtained from various isolates were edited using the software program BioEdit (version 7.2, CA, USA), and aligned to create consensus sequence within BioEdit using Clustal W. Translated amino acid sequences were matched with similar sequences in the National Center for Biotechnology Information (NCBI) Web site (www.ncbi.nih.gov) using the Basic Local Alignment Search Tool (BLAST) and fasta files (consensus sequence) were deposited in GenBank. The GenBank nucleotide sequence accession numbers for the sequences studied in this work are detailed in Table 16 and Figure 1.

3.17 Phylogenetic Analysis

The phylogenetic analyses based on the 16S rRNA region for bacteria were used to characterize the *Escherichia coli* and *Klebsiella pneumoniae* in order to establish relationship among them. The partial 16S rRNA gene sequences obtained for the *Escherichia coli* and *Klebsiella pneumoniae* were utilized in the search of reference nucleotide sequence available in NCBI GenBank database using BlastN algorithm (Altschul *et al*., 1997). Multiple Alignment using Fasta Fourier Transform (MAFFT version 7.0) was employed in the multiple alignment of nucleotide sequences (Katoh and Standley, 2013) to align the identified and acquired seguences from NCBI Genbank, while trees were drawn based on two major techniques using Molecular Evolutionary Genetic Analysis (MEGA version 7.0). These techniques include the p-distance based (Neigbour-Joining (NJ) with cluster-based algorithm) used in calculating pairwise distance between sequences and group sequences that are most similar (Kumar *et al*., 2016).

3.18 Statistical Analysis

Statistical analysis was performed using SPSS 16.0 version software package. The frequency of isolated bacteria with patient demographic data and antibiotics resistance were compared and calculated using one-way ANOVA and Tukey post hoc test. Results were considered statistically significant where p value is less than 0.05 ($p < 0.05$).

CHAPTER FOUR

4.0 RESULTS

Morphological and Biochemical characterization of bacterial isolates

Morphological and biochemical characters of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were used to identify them phenotypically as presented in table 1.

Prevalence of bacterial isolates in clinical specimens

One thousand (1000) clinical specimens were collected from three different sources which include wound (350), high vaginal (200) and urine (450). Four hundred and fifty four (45.4 %) clinical isolates were identified from the 1000 clinical specimens. One hundred and seventy eight (39.2 %) out of the 454 clinical isolates were *S. aureus*; 84 (18.5 %) were *K. pneumoniae* while 192 (42.3 %) were *E. coli*. Among the specimens collected from wound (350), a total of 53 isolates were obtained. Out of these 53 isolates, 23 (43.4 %) were *S. aureus*, 11 (20.8 %) were *K. pneumoniae* while 19 (35.8 %) were *E. coli*. Out of the 99 isolates obtained from high vaginal specimens, 37 (37.4 %) were *S. aureus*, 21 (21.2 %) were *K. pneumoniae* while 41 (41.4 %) were *E. coli*. Three hundred and two isolates were obtained from 450 urine specimen. Out of these 302 isolates, 118 (39.1 %) were *S. aureus*, 52 (17.2 %) were *K. pneumoniae* while 132 (43.7 %) were *E. coli* (Table 2). Statistical analysis showed significant difference in the bacterial prevalence (*S. aureus, K. pneumoniae, E. coli*) among the different clinical specimens as determined by one-way ANOVA ($p = 0.016$ ($p < 0.05$)). The Tukey post hoc test conducted revealed that bacterial prevalence from wound specimens does not differ significantly from the bacterial prevalence from high vaginal specimens at ($p > 0.05$)) but the bacterial prevalence from urine specimen was significantly different from bacterial prevalence in wound specimens ($p = 0.017$ ($p < 0.05$)). Bacterial prevalence in the high vaginal specimen was also significantly different from the bacterial prevalence in the urine specimens ($p = 0.041$ ($p < 0.05$)) (Appendix 5).

Table 1: Morphological and Biochemical characterization of bacterial isolates

Gram Shape Appearance	Gram Staining Test	Catalase Test	Indole Test	Oxidase Test	Coagulase Test	Motility Test	Citrate Test	Voges Proskauer Test	Methyl Red Test	Lactose Sugar Test	Glucose Sugar Test	Mannitol Sugar Test	Suspected Organisms
Rod in shape	-ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	*Escherichia coli*
Rod in shape	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	*Klebsiella pneumoniae*
Cocci in shape	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	*Staphylococcus aureus*

-ve = Negative and +ve = Positive

Table 2: Prevalence of bacterial isolates in clinical specimens

Sample source	Sample size (Total No. of isolates)	*S. aureus,* No. (%)	*K. pneumoniae,* No. (%)	*E. coli,* No. (%)
Wound swab	350 (53)	23 (43.4)	11 (20.8)	19 (35.8)
High vaginal swab (HVS)	200 (99)	37 (37.4)	21 (21.2)	41 (41.4)
Urine	450 (302)	118 (39.1)	52 (17.2)	132 (43.7)
Total	**1000 (454)**	**178 (17.8)**	**84 (8.4)**	**192 (19.2)**

Distribution of bacterial isolates according to sex of the patients

Out of the 53 isolates obtained from wound swabs, 14 (26.4 %) and 9 (17.0 %) *S. aureus* were obtained from males and females respectively. Eight (15.1 %) and 3 (5.7 %) *K. pneumoniae* were obtained from males and females respectively while 7 (13.2 %) and 12 (22.6 %) *E. coli* were obtained from males and female respectively. Out of the ninety nine bacterial isolates obtained from high vaginal swabs, 37 (37.4 %) *S. aureus*, 21 (21.2 %) *K. pneumoniae*, and 41 (41.4 %) *E. coli* were obtained from female. However, out of the 302 isolates obtained from urine specimens, 43 (14.2 %) and 75 (24.8 %) *S. aureus* were obtained from males and females respectively; 33 (10.2 %) and 19 (6.3 %) *K. pneumoniae* were identified from males and females respectively, while 41 (13.6 %) and 91 (30.1 %) *E. coli* were obtained from males and females respectively (Table 3).

There was no statistically significant difference in the bacterial prevalence (*S. aureus*, *K. pneumoniae*, *E. coli*) between males and females from urine and wound swab specimens at ($p > 0.05$) (Appendix 5).

Table 3: Distribution of bacterial isolates according to sex of the patients

Sample source	Sample size (Total No. of isolates)	Isolates	Total No. of isolates (%)	No. positive for males (%)	No. positive for females (%)
Wound swab	350 (53)	*S. aureus*	23 (43.4)	14 (26.4)	9 (17.0)
		K. pneumoniae	11 (20.8)	8 (15.1)	3 (5.7)
		E. coli	19 (35.8)	7 (13.2)	12 (22.6)
High vaginal swab	200 (99)	*S. aureus*	37 (37.4)	0 (0.0)	37 (37.4)
		K. pneumoniae	21 (21.2)	0 (0.0)	21 (21.2)
		E. coli	41 (41.4)	0 (0.0)	41 (41.4)
Urine	450 (302)	*S. aureus*	118 (39.1)	43 (14.2)	75 (24.8)
		K. pneumoniae	52 (17.2)	33 (10.9)	19 (6.3)
		E. coli	132 (43.7)	41 (13.6)	91 (30.1)

Distribution of bacterial isolates according to age of the patients

The distribution of bacterial isolates according to age of the patients shows that *S. aureus*, *K. pneumoniae* and *E. coli* occurs more at the age bracket of 16-30, 31-45; and moderate at age bracket of 1-15, 46-60, and less at the age bracket of 61-75, 76 and above (Table 4). Age played significant role ($p < 0.05$) in the bacterial prevalence from high vaginal and urine specimens and in contrast, there was no statistically significant difference in the bacterial prevalence from wound swab specimens at ($p > 0.05$) (Appendix 5).

Table 4: Distribution of bacterial isolates according to age of the patients

Sample source	Isolates	1 - 15	16 - 30	31 - 45	46 - 60	61 - 75	76-Above
Wound swab	*S. aureus*	2	8	8	3	2	0
	K. pneumoniae	3	1	3	2	1	1
	E. coli	4	3	3	4	3	2
High vaginal swab	*S. aureus*	6	15	9	3	3	1
	K. pneumoniae	2	7	6	2	2	2
	E. coli	3	18	12	6	1	1
Urine	*S. aureus*	15	54	28	13	5	3
	K. pneumoniae	12	8	14	10	4	4
	E. coli	21	61	30	15	3	2

Distribution of bacterial isolates according to occupations of the patients

Students, farmers, traders and drivers harboured more of *S. aureus*, *K. pneumoniae* and *E. coli* isolates compare to house wives, civil servents, public servents and pupils. High prevalence of the isolates was observed in wound specimens among the farmers when compared to others. There was no bacterial isolates from wound specimens of public servants. Very low bacterial prevalence was observed among civil servants, public servants and house wives except for urine specimens where public servants showed significant numbers of these isolates (Table 5). Statistical analysis showed a significant occupational influence in the bacterial prevalence ($p < 0.05$) from all the three sources (Appendix 5).

Table 5: Distribution of bacterial isolates according to occupations of the patients

Sample source	Isolates	Pupils	Students	Farmers	Traders	Drivers	House wives	Civil servants	Public servants
Wound swab	*S. aureus*	3	4	7	3	4	2	0	0
	K. pneumoniae	2	1	4	1	1	1	1	0
	E. coli	2	2	8	1	3	1	2	0
High vaginal swab	*S. aureus*	0	12	10	6	0	4	3	2
	K. pneumoniae	0	8	6	4	0	0	1	2
	E. coli	0	21	5	8	0	2	4	1
Urine	*S. aureus*	4	32	16	18	22	6	8	12
	K. pneumoniae	6	8	8	8	10	2	6	4
	E. coli	3	31	15	19	41	4	4	15

Distribution of bacterial isolates according to educational levels of the patients

High prevalence rate of *S. aureus, K. pneumoniae* and *E. coli* isolates among secondary and undergraduate students were observed as recorded in the Table 6, except in wound swabs where we observed that primary, secondary and graduates harboured more of the isolates. Moderate prevalence was recorded in high vaginal and urine specimens obtained among graduates, whereas low prevalence was observed among nursery and primary pupils (Table 6). Level of education played significant role ($p < 0.05$) in the bacterial prevalence from high vaginal swab and urine specimens. However, there was no statistically significant difference in the bacterial prevalence from wound swab specimens at ($p > 0.05$) (Appendix 5).

Table 6: Distribution of bacterial isolates according to educational levels of the patients

Sample source	Isolates	Nursery	Primary	Secondary	Undergraduate	Graduate	Total
Wound swab	*S. aureus*	1	6	8	3	5	**23**
	K. pneumoniae	0	3	4	1	3	**11**
	E. coli	2	7	5	3	2	**19**
High vaginal swab	*S. aureus*	0	5	11	15	6	**37**
	K. pneumoniae	0	2	7	6	6	**21**
	E. coli	0	3	14	14	10	**41**
Urine	*S. aureus*	5	15	60	30	8	**118**
	K. pneumoniae	3	9	12	19	9	**52**
	E. coli	6	11	55	43	17	**132**

Antibiotic resistance profiles of the bacterial isolates obtained from wound specimens

Of the 53 isolates from wound specimens tested for antibiotics profile, 23 (43.4 %), 11 (20.8 %) and 19 (35.8 %) were *S. aureus, K. pneumoniae* and *E. coli* respectively. The resistance profiles of the 53 isolates are as follows: CAZ (*S. aureus* 52.2 %, *K. pneumoniae* 36.4 %, *E. coli* 36.8 %), CRO (*S. aureus* 43.5 %, *K. pneumoniae* 45.4 %, *E. coli* 57.9 %), CPO (*S. aureus* 82.6 %, *K. pneumoniae* 54.5 %, *E. coli* 73.7 %), ATM (*S. aureus* 69.6 %, *K. pneumoniae* 36.4 %, *E. coli* 52.6 %) and CTX (*S. aureus* 73.9 %, *K. pneumoniae* 72.7 %, *E. coli* 63.2 %). However, the isolates from wounds showed a higher degree of susceptibility to 4th-generation cephalosporins (cefepime) with susceptibility frequency of 60.9 % for *S. aureus*, 72.7 % for *K. pneumoniae* and 79.0 % for *E. coli* (Table 7).

Statistical analysis showed significant difference in the antibiotic resistance frequencies of *S. aureus*, *K. pneumoniae*, and *E. coli* from wound specimens ($p = 0.01$ ($p < 0.05$)). The Tukey post hoc test conducted revealed significant difference in the antibiotic resistance frequencies between *S. aureus* and *K. pneumoniae* ($p = 0.000$; $p < 0.05$), whereas there was no significant difference in the antibiotic resistance frequencies between *S. aureus* and *E. coli* ($p = 0.084$; at $p > 0.05$). Tukey post hoc test also showed no significant difference in the antibiotic resistance frequencies between *K. pneumoniae* and *E. coli* ($p = 0.052$; at $p > 0.05$) (Appendix 5).

Table 7: Antibiotic resistance profiles of the bacterial isolates obtained from wound specimens

Antibiotics (concentrations in µg)	***Staphylococcus aureus*** **No. (%)**		***Klebsiella pneumoniae*** **No. (%)**		***Escherichia coli*** **No. (%)**	
	S	**R**	**S**	**R**	**S**	**R**
Cefepime (30)	14 (60.9)	9 (39.1)	8 (72.7)	3 (27.3)	15 (79.0)	4 (21.0)
Ceftazidime (30)	11 (47.8)	12 (52.2)	7 (63.6)	4 (36.4)	12 (63.2)	7 (36.8)
Ceftriaxone (30)	13 (56.5)	10 (43.5)	6 (54.6)	5 (45.4)	8 (42.1)	11 (57.9)
Cefpodoxime (10)	4 (17.4)	19 (82.6)	5 (45.4)	6 (54.5)	5 (26.3)	14 (73.7)
Azatronam (30)	7 (30.4)	16 (69.6)	7 (63.6)	4 (36.4)	9 (47.4)	10 (52.6)
Cefotaxime (30)	6 (26.1)	17 (73.9)	3 (27.3)	8 (72.7)	7 (36.8)	12 (63.2)
Amoxycillin/Clavulanic acid (20/10)	13 (56.5)	10 (43.5)	5 (45.4)	6 (54.5)	11 (57.9)	8 (42.1)

S- Susceptible; R- Resistant

Antibiotic resistance profiles of bacterial isolates from high vaginal swab (HVS) specimens

Antibiotic sensitivity profiles of *S. aureus* (37), *K. pneumoniae* (21) and *E. coli* (41) isolated from high vaginal swab (HVS) specimens of patients is presented in table 8. The resistance profiles recorded are as follows: CAZ (*S. aureus* 32.4 %, *K. pneumoniae* 23.8 %, *E. coli* 39.0 %), CRO (*S. aureus* 27.0 %, *K. pneumoniae* 42.9 %, *E. coli* 53.7 %), CPO (*S. aureus* 43.2 %, *K. pneumoniae* 52.4 %, *E. coli* 46.3 %), ATM (*S. aureus* 35.1 %, *K. pneumoniae* 52.4 %, *E. coli* 41.5 %), CTX (*S. aureus* 45.9 %, *K. pneumoniae* 61.9 %, *E. coli* 63.4 %) and FEP (*S. aureus* 18.9 %, *K. pneumoniae* 14.3 %, *E. coli* 14.6 %). Notably, cefepime and ceftazidime had the strongest activity against the bacteria isolates from HVS; showing FEP (*S. aureus* 81.1 %, *K. pneumoniae* 85.7 %, *E. coli* 85.4 %) and CAZ (*S. aureus* 67.6 %, *K. pneumoniae* 76.2 %, *E. coli* 61.0 %) susceptibility (Table 8).

There was a statistically significant difference in the antibiotic resistance frequencies of *S. aureus*, *K. pneumoniae*, and *E. coli* in the high vaginal specimens ($p < 0.05$). The Tukey post hoc test conducted revealed that there was a statistically significant difference in the antibiotic resistance frequencies between *K. pneumoniae* and *E. coli* ($p = 0.005$; $p < 0.05$), whereas there was no statistically significant difference in the antibiotic resistance frequencies between *S. aureus* and *E. coli* ($p = 0.098$; at $p > 0.05$). Tukey post hoc test also showed that there was no statistically significant difference in the antibiotic resistance frequencies between *S. aureus* and *K. pneumoniae* ($p = 0.346$; at $p > 0.05$) (Appendix 5).

Table 8: Antibiotic resistance profiles of bacterial isolates from high vaginal swab (HVS) specimens

Antibiotics (concentrations in µg)	***Staphylococcus aureus*** **No. (%)**		***Klebsiella pneumoniae*** **No. (%)**		***Escherichia coli*** **No. (%)**	
	S	**R**	**S**	**R**	**S**	**R**
Cefepime (30)	30 (81.1)	7 (18.9)	18 (85.7)	3 (14.3)	35 (85.4)	6 (14.6)
Ceftazidime (30)	25 (67.6)	12 (32.4)	16 (76.2)	5 (23.8)	25 (61.0)	16 (39.0)
Ceftriaxone (30)	27 (73.0)	10 (27.0)	12 (57.1)	9 (42.9)	19 (46.3)	22 (53.7)
Cefpodoxime (10)	21 (56.8)	16 (43.2)	10 (47.6)	11 (52.4)	22 (53.7)	19 (46.3)
Azatronam (30)	24 (64.9)	13 (35.1)	10 (47.6)	11 (52.4)	24 (58.5)	17 (41.5)
Cefotaxime (30)	20 (54.1)	17 (45.9)	8 (38.1)	13 (61.9)	15 (36.6)	26 (63.4)
Amoxycillin/Clavulanic acid (20/10)	22 (59.5)	15 (40.5)	9 (42.9)	12 (57.1)	17 (41.5)	24 (58.5)

S- Susceptible; R- Resistant

Antibiotic resistance profiles of bacterial isolates from urine specimens of patients

Three hundred and two (302) isolates (118 (39.1 %), 52 (17.2 %) and 132 (43.7 %) were *S. aureus, K. pneumoniae* and *E. coli* respectively) from urine specimens were subjected to antibiotic sensitivity test. The antibiotic resistance profiles of these isolates were recorded as follows; FEP (*S. aureus* 21.2 %, *K. pneumoniae* 21.2 %, *E. coli* 28.0 %), CAZ (*S. aureus* 44.9 %, *K. pneumoniae* 51.9 %, *E. coli* 47.0 %), CRO (*S. aureus* 31.4 %, *K. pneumoniae* 59.6 %, *E. coli* 30.3 %), CPO (*S. aureus* 55.9 %, *K. pneumoniae* 46.2 %, *E. coli* 47.7 %), ATM (*S. aureus* 57.6 %, *K. pneumoniae* 67.3 %, *E. coli* 39.4 %) and CTX (*S. aureus* 36.4 %, *K. pneumoniae* 71.2 %, *E. coli* 62.1 %). High levels of resistance were observed among *K. pneumoniae* and *E. coli* isolates to the 3rd-generation cepholosporins, cefotaxime and ceftazidime (Table 9).

Statistical analysis showed significant difference in the antibiotic resistance frequencies of *S. aureus*, *K. pneumoniae*, and *E. coli* in the urine specimens ($p = 0.001$ ($p < 0.05$)). The Tukey post hoc test conducted revealed that there was a statistically significant difference in the antibiotic resistance frequencies between *S. aureus* and *K. pneumoniae* ($p = 0.011$; $p < 0.05$), whereas there was no statistically significant difference in the antibiotic resistance frequencies between *S. aureus* and *E. coli* ($p = 0.672$; at $p > 0.05$). Tukey post hoc test also showed significant difference in the antibiotic resistance frequencies between *K. pneumoniae* and *E. coli* ($p = 0.002$; $p < 0.05$) (Appendix 5).

Table 9: Antibiotic resistance profiles of bacterial isolates from urine specimens of patients

Antibiotics (concentrations in µg)	***Staphylococcus aureus*** **No. (%)**		***Klebsiella pneumoniae*** **No. (%)**		***Escherichia coli*** **No. (%)**	
	S	**R**	**S**	**R**	**S**	**R**
Cefepime (30)	93 (78.8)	25 (21.2)	41 (78.8)	11 (21.2)	95 (72.0)	37 (28.0)
Ceftazidime (30)	65 (55.1)	53 (44.9)	25 (48.1)	27 (51.9)	70 (53.0)	62 (47.0)
Ceftriaxone (30)	81 (68.6)	37 (31.4)	21 (40.4)	31 (59.6)	92 (69.7)	40 (30.3)
Cefpodoxime (10)	52 (44.1)	66 (55.9)	28 (53.8)	24 (46.2)	69 (52.3)	63 (47.7)
Azatronam (30)	50 (42.4)	68 (57.6)	17 (32.7)	35 (67.3)	80 (60.6)	52 (39.4)
Cefotaxime (30)	75 (63.6)	43 (36.4)	15 (28.8)	37 (71.2)	50 (37.9)	82 (62.1)
Amoxycillin/Clavulanic acid (20/10)	60 (50.8)	58 (49.2)	39 (75.0)	13 (25.0)	73 (55.3)	59 (44.7)

S- Susceptible; R- Resistant

Phenotypic confirmation of beta-lactamases and ESBLs producers

A total of 130 clinical isolates (41(31.5 %), 26(20.0 %), 63(48.5 %) *S. aureus, K. pneumoniae* and *E. coli* respectively) were positive for beta-lactamase production as represented in table 10. The isolates that produced beta-lactamase were subjected to ESBL detections using Double Disc Synergy Test and Brilliant ESBL Chromogenic Agar in line with CLSI. Out of the 130 beta-lactamase producing isolates screened in this study, 23 isolates were phenotypically confirmed ESBLs positive (1 (4.4 %) *S. aureus,* 7 (30.4 %) *K. pneumoniae* and 15 (65.2 %) *E. coli*). However, 7 (31.2 %) *K. pneumoniae* and 15 (68.2 %) *E. coli* were detected using Brilliant ESBL Chromogenic Agar and 1 (6.3 %) *S. aureus,* 5 (31.2 %) *K. pneumoniae* and 10 (62.5 %) *E. coli* were detected using Double Disc Synergy Test (Table 10).

Table 10: Phenotypic confirmation of beta-lactamases and ESBLs producers

Isolates	No. of isolates	No. resistant to beta-lactam antibiotics, No. (%)	Beta-lactamase positive by Nitrocefin stick, No. (%)	Confirmed ESBL producers by Brilliance agar, No. (%)	Confirmed ESBL producers by DDST method, No. (%)	Phenotypic confirmed ESBLs positive, No. (%)
S. aureus	178	92 (34.8)	41 (31.5)	0 (0.0)	1 (6.3)	1 (4.4)
K. pneumoniae	84	53 (20.1)	26 (20.0)	7 (31.8)	5 (31.2)	7 (30.4)
E. coli	192	119 (45.1)	63 (48.5)	15 (68.2)	10 (62.5)	15 (65.2)
Total	**454**	**264 (100)**	**130 (100)**	**22 (100)**	**16 (100)**	**23 (100)**

Double Disc Synergy Test (DDST)

Plate 1 shows a keyhole effect produced by *E. coli, S.aureus* and *K. pneumoniae* isolates expressing ESBL using DDST method. This appearance is the characteristic for ESBL-producing bacteria due to the synergistic effect produced between amoxicillin-clavulanic acid (AMC) (a beta-lactamase inhibitor) and the third generation cephalosporins (ceftazidime (CAZ) and cefotaxime (CTX)).

Plate 1: Picture of Double Disc Synergy Test, ESBL positive isolates.

Plate 2: Picture of ESBL- producing *K. pneumoniae* (green colour) and *E. coli* (blue and pink) on Brilliant ESBL Agar.

Source distributions of phenotypic detected ESBLs- producing isolates

A total of 23 ESBLs producing isolates (2 (8.7 %), 6 (26.1 %) and 15 (65.2 %)) were identified from wound, high vaginal and urine specimens respectively. Notably, a total of one (4.4 %) *S. aureus* was observed in wound specimens and none was obtainable in urine and high vaginal specimens. Seven (30.4 %) ESBL producing isolates were detected to be *K. pneumoniae*; two (8.7 %) and 5 (21.7 %) *K. pneumoniae* isolates were isolated from high vaginal and urine specimens respectively. None was detected from wound specimens. However, *E. coli* isolates had the highest number of ESBL producer with a total number of 15 (65.2 %) isolates. Wound specimens recorded 1 (4.4 %) *E. coli* isolate; high vaginal specimens had 4 (17.4 %) *E. coli* isolates while urine specimens had 10 (43.5 %) *E. coli* isolates (Table 11).

Table 11: Source distributions of phenotypic detected ESBLs- producing isolates.

Isolates	Wound, No. (%)	HVS, No. (%)	Urine, No. (%)	Total, No. (%)
Staphylococcus aureus	1 (4.4)	0 (0.0)	0 (0.0)	1 (4.4)
Klebsiella pneumoniae	0 (0.0)	2 (8.7)	5 (21.7)	7 (30.4)
Escherichia coli	1 (4.4)	4 (17.4)	10 (43.5)	15 (65.2)
Total	**2** (8.7)	**6** (26.1)	**15** (65.2)	**23 (100.0)**

Antibiotic resistance index of 23 ESBL- producing isolates

The results of susceptibility tests performed on the 23 ESBL- producing isolates from this study showed high resistance percentage index on the beta lactam antibiotics tested. *Staphylococcus aureus* isolate tested was 100 % resistant to all the antibiotics. However, all the 7 *Klebsiella pneumoniae* isolates tested were highly resistant to the antibiotics tested, with resistance percentage index ranging from 71.4 % to 100 %. The *Escherichia coli* isolates evaluated in this study showed a very high resistance index, which ranged from 80 % to 100 % (Table 12).

Table 12: Antibiotic resistance index of 23 ESBL- producing isolates

Antibiotics (concentration in µg)	***Staphylococcus aureus,* No. of resistant/ No. of tested**	**(%)**	***Klebsiella pneumoniae,* No. of resistant/ No. of tested**	**(%)**	***Escherichia coli,* No. of resistant/ No. of tested**	**(%)**
Cefepime (30)	1/1	(100)	5/7	(71.4)	12/15	(80.0)
Ceftazidime (30)	1/1	(100)	7/7	(100)	15/15	(100)
Ceftriaxone (30)	1/1	(100)	7/7	(100)	14/15	(93.3)
Cefpodoxime (10)	1/1	(100)	7/7	(100)	15/15	(100)
Azatronam (30)	1/1	(100)	6/7	(85.7)	13/15	(86.7)
Cefotaxime (30)	1/1	(100)	7/7	(100)	15/15	(100)
Amoxycillin/Clavulanic acid (20/10)	1/1	(100)	6/7	(85.7)	13/15	(86.7)

Source distributions of PCR detected ESBL- producing isolates

Molecular test using PCR identified the presence of *bla* genes in 20 isolates out of the 23 isolates characterized for the presence of ESBL genes. None of the *Staphylococcus aureus* was found to carry any of the ESBL genes using PCR methods. Five (25.0 %) *Klebsiella pneumoniae* were found to harbor the ESBL genes, one (5.0 %) of the isolates is isolated from high virginal swab and 4 (20.0 %) from urine specimens. Worthy to note, *Escherichia coli* had the highest number of ESBL genes, 15 (75.0 %) isolates were found to carry the *bla* genes, one (5.0 %) *E. coli* was identified from wound specimens, 4 (20.0 %) *E. coli* were isolated from high virginal swab and 10 (50.0 %) of *E. coli* from urine specimens. However, a total of one (5.0 %) isolate was identified in wound specimens, 5 (25.0 %) isolates from high virginal swab and 14 (70.0 %) isolates from urine specimens using PCR methods to harbor ESBL genes (Table 13).

Table 13: Source distributions of PCR detected ESBL- producing isolates.

Isolates	Wound, No. (%)	HVS, No. (%)	Urine, No. (%)	Total, No. (%)
Staphylococcus aureus	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Klebsiella pneumoniae	0 (0.0)	1 (5.0)	4 (20.0)	5 (25.0)
Escherichia coli	1 (5.0)	4 (20.0)	10 (50.0)	15 (75.0)
Total	**1** (5.0)	**5** (25.0)	**14** (70.0)	**20 (100.0)**

Distributions of ESBL genes among ESBL- producing isolates

The results of PCR products on gel electrophoresis showed that 11 (55.0 %) had *bla*TEM, 7 (35.0 %) had *bla*SHV and 9 (45.0 %) had *bla*CTX-M among the isolates characterized. Seven (7; 35.0 %) out of the 20 isolates harbored more than single ESBL genes, six (6) *E. coli* and one (1) *K. pneumoniae* and the remaining 13 (65.0 %) isolates had one of the ESBL genes each as enlisted in table 14 . The *E. coli* isolates harbored *bla*TEM/*bla*SHV, *bla*TEM/*bla*CTX-M-15 and *bla*SHV/*bla*CTX-M-15 and *K. pneumoniae* harbored *bla*SHV/*bla*TEM ESBL genes. Only *E. coli* isolate was observed to carry *bla*CTX-M-25 (n=1) and *bla*CTX-M-14 (n=2) ESBL gene (Table 14).

Table 14: Distributions of ESBL genes among ESBL- producing isolates

ESBL genes	*E. coli*	*K. pneumoniae*	*S. aureus*	**Total No. of isolate positive**
TEM	3	2	0	5
TEM/SHV	2	1	0	3
TEM/CTX-M-15	3	0	0	3
Total Number	**8**	**3**	**0**	**11 (55.0 %)**
SHV	2	1	0	3
SHV/CTX-M-15	1	0	0	1
SHV/TEM	2	1	0	3
Total Number	**5**	**2**	**0**	**7 (35.0 %)**
CTX-M-15	1	1	0	2
CTX-M-14	2	0	0	2
CTX-M-25	1	0	0	1
CTX-M-15/TEM	3	0	0	3
CTX-M-15/SHV	1	0	0	1
Total Number	**8**	**1**	**0**	**9 (45.0 %)**

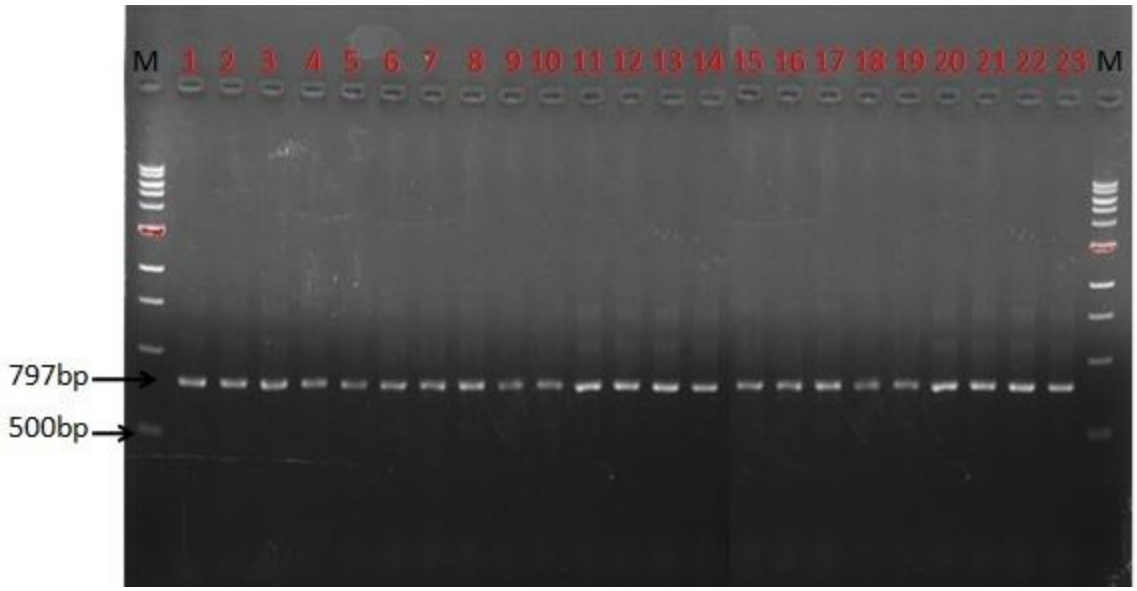

Plate 3: Agarose gel electrophoresis of PCR amplification of 16S rRNA among the bacteria isolates. Lane M = 1kb Molecular Marker, Lane 1-2, 6-12, 14-19 = *E. coli*, Lane 13 = *S. aureus*, Lane 3-5, 20-23 = *K. pneumoniae*.

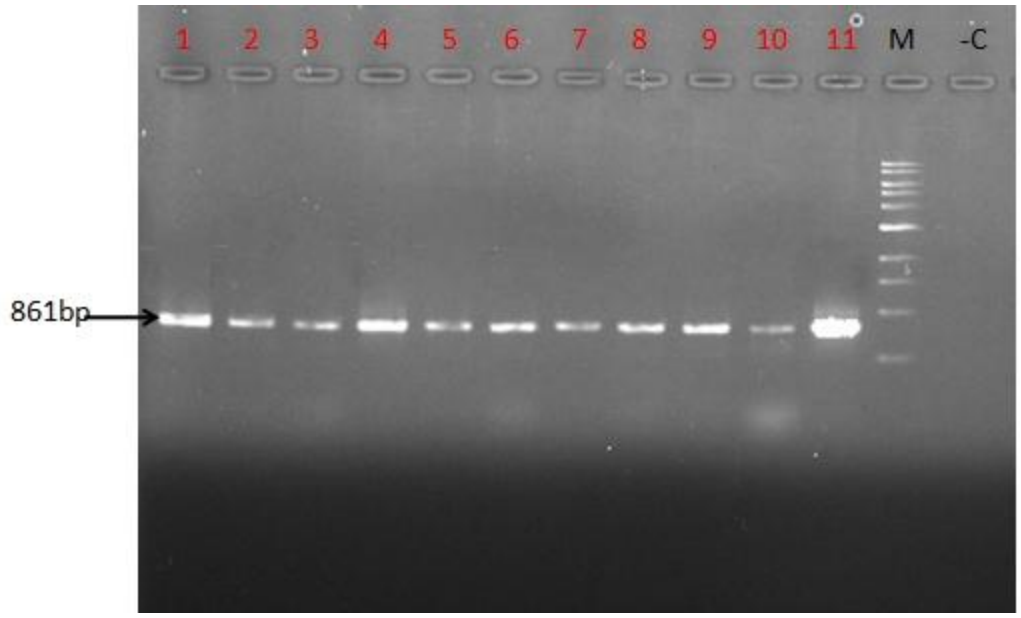

Plate 4: Agarose gel showing PCR products of amplified *bla*TEM gene among the bacteria isolates. Lane M = 1kb Molecular Marker, -C = Negative control, Lane 1-8 = *E. coli*, Lane 9-11 = *K. pneumoniae*.

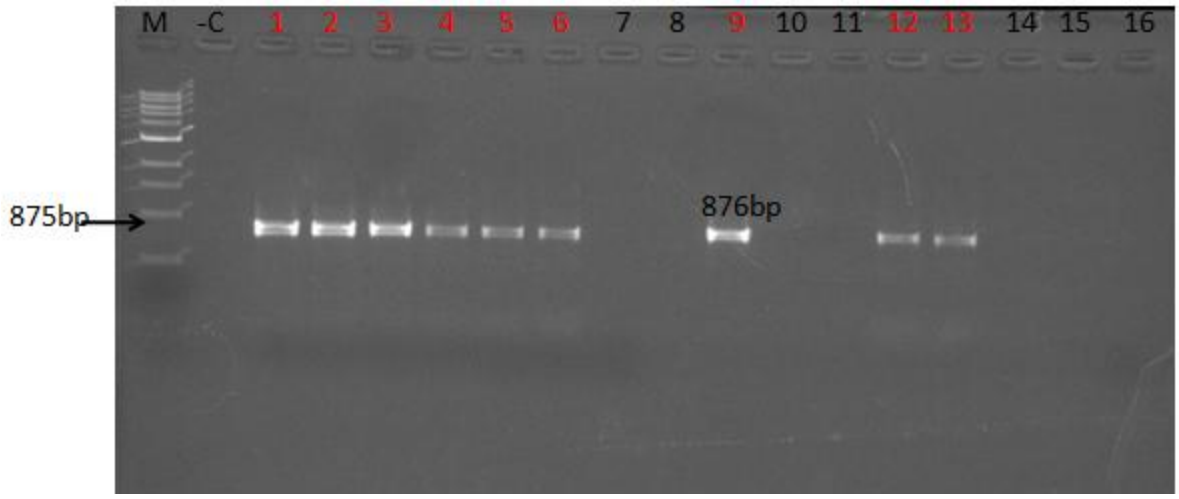

Plate 5: Agarose gel showing PCR products of amplified *bla*CTX-M group's gene among the bacteria isolates. Lane M = 1kb Molecular Marker, -C = Negative control, Lane 1-6 = *bla*CTX-M-15, Lane 9 = *bla*CTX-M-25, Lane 12-13 = *bla*CTX-M-14.

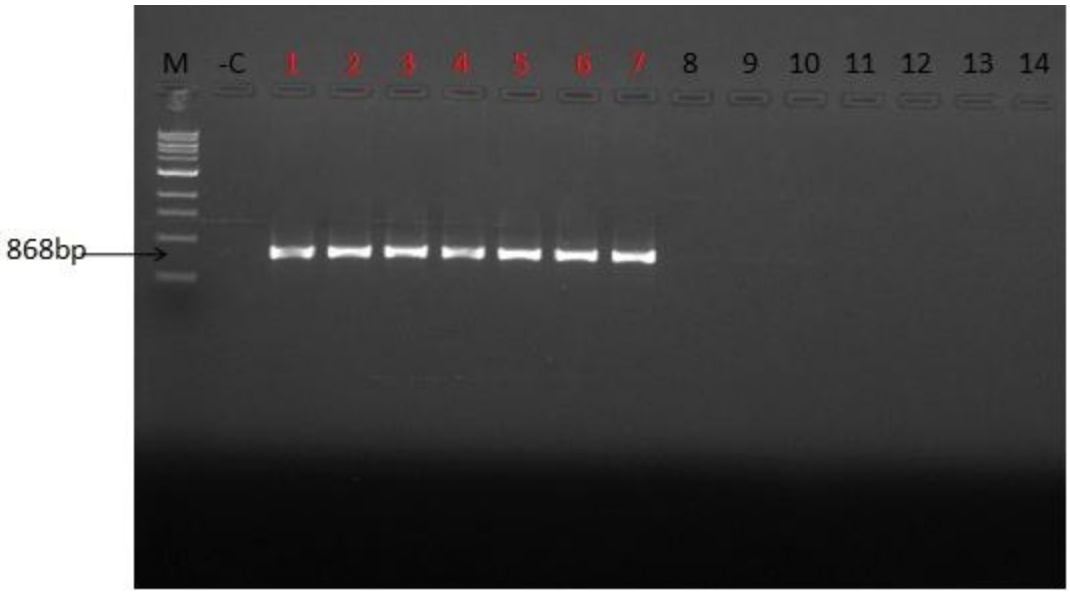

Plate 6: Agarose gel showing PCR products of amplified *bla*SHV gene among the bacteria isolates. Lane M = 1kb Molecular Marker, -C = Negative control, Lane 1-5 = *E. coli*, Lane 6-7 = *K. pneumoniae*.

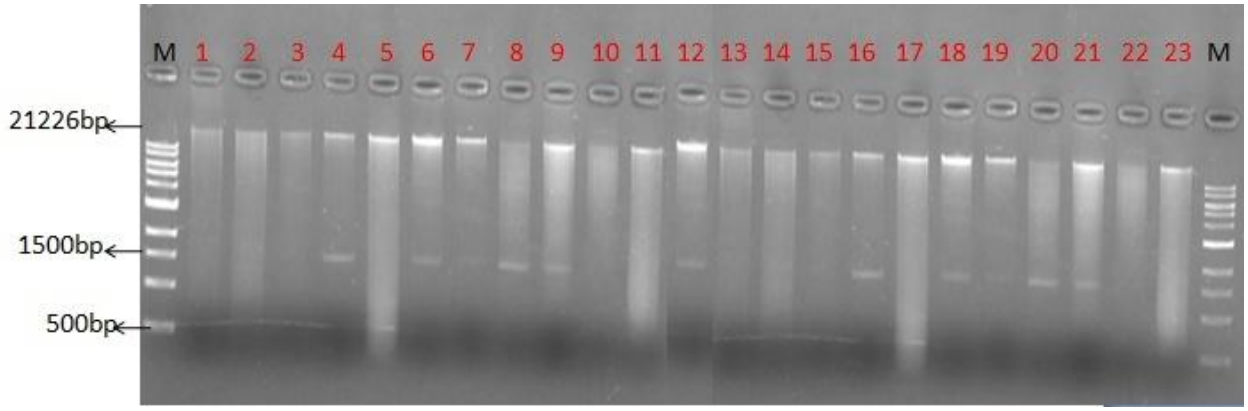

Plate 7: Gel electrophoretic separation profile of plasmid DNAs isolated from bacterial isolates.

Lane M = 1kb Molecular Marker, Lane 1-2, 6-12, 14-19 = *E. coli*, Lane 13 = *S. aureus*, Lane 3-5, 20-23 = *K. pneumoniae* plasmid amplicons.

ESBL genes locations among ESBL- producing isolates

Plasmid DNA was tested for the presence of ESBL genes using PCR and primers specific for ESBL genes. Out of the 11 *bla*TEM ESBL- producing isolates, 6 *E. coli* and two (2) *Klebsiella pneumoniae* were found to harbor the *bla*TEM in their plasmid. Thus, two (2) *E. coli* and one (1) *K. pneumoniae* harbored *bla*TEM in their chromosome. Four (4) *E. coli* and one (1) *K. pneumoniae* were found to harbor *bla*SHV in their plasmid out of the seven *bla*SHV ESBL positive isolates. Of the 9 *bla*CTX-M ESBL- producing isolates, six (6) *E. coli* and one (1) *K. pneumoniae* harbor the *bla*CTX-M in their plasmid DNA. Notably, the isolates caring more than one ESBL genes harbor the genes in their plasmid DNA. Out of the 20 ESBL producing isolates, 13 (65.0 %) isolates *E. coli* (10, 50.0 %) and *Klebsiella pneumoniae* (3, 15.0 %) were found to harbor the genes in their plasmid DNA/genomic DNA (Table 15).

Table 15: ESBL genes locations among ESBL- producing isolates

ESBL genes	**Plasmid/Chromosomal DNA (n = 13; 65.0 %)**		**Chromosomal DNA only (n = 7; 35.0 %)**	
	E.coli (10; 50%)	*K. pneumoniae* (3; 15%)	*E.coli* (5; 25%)	*K. pneumoniae* (2; 10%)
*bla*TEM	6	2	2	1
*bla*SHV	4	1	1	1
*bla*CTX-M	6	1	2	0

16S rRNA identity of bacterial isolates

The 16S rRNA nucleotide sequences from the isolates harboring ESBL genes in their chromosomal DNA and plasmid DNA were matched with similar sequences in the NCBI Web site using BLAST. The BLAST query revealed that the strains were closely related to pathogenic *Escherichia coli* and *Klebsiella pneumoniae*. The identities of all bacterial isolates and their GenBank accession numbers are presented in the table 16.

Table 16: 16S rRNA identity of bacterial isolates

Isolate code	GenBank accession no.	Predicted organism	Matched strain ID	GenBank accession no.	Similarity (%)	*bla* genes present	Gene Location
EUGBO2	MG566066	*Escherichia coli*	EPEC	MF919608.1	100	TEM/ SHV	Plasmid/ Chromosome
EUGBO6	MG566067	*Escherichia coli*	B. pat37	KF951534.1	100	TEM/ CTXM15	Plasmid/ Chromosome
EUGBO10	MG566068	*Escherichia coli*	HPCAQ7CR13	JQ512961.1	100	TEM/ CTXM15	Plasmid/ Chromosome
EUGBO15	MG566069	*Escherichia coli*	NBRC102203	KY655088.1	100	TEM/ SHV	Plasmid/ Chromosome
EUGBO16	MG566070	*Escherichia coli*	D3	KT984389.1	100	TEM/ CTXM15	Plasmid/ Chromosome
EUGBO19	MG566071	*Escherichia coli*	RE10	FJ789678.1	100	SHV/ CTXM15	Plasmid/ Chromosome
EUGBO21	MG566072	*Klebsiella pneumoniae*	B11C	KJ725232.1	100	SHV/ TEM	Plasmid/ Chromosome

Phylogenetic tree reconstruction for the bacteria isolates

Neighbor-Joining method of phylogenetic tree based on partial 16S rRNA gene sequence, showing the phylogenetic relationships between *Escherichia coli* and *Klebsiella pneumoniae* and the most closely related strains from the GenBank. Numbers at the nodes indicate the levels of bootstrap support based on 1000 replicates data sets. Only values greater than 50% are shown. The scale bar indicates 0.05 base substitutions per site. Sequences obtained in this study are denoted with a green diamond shape. The 1000 replicates of bootstrap test revealed the percentage of replicate trees in which the associated taxa clustered together next to the branches using the Neighbor-Joining method to be 100 percent similarity match to the compared isolates in NCBI Genbank. The percentage similarity matches are presented in figure 1.

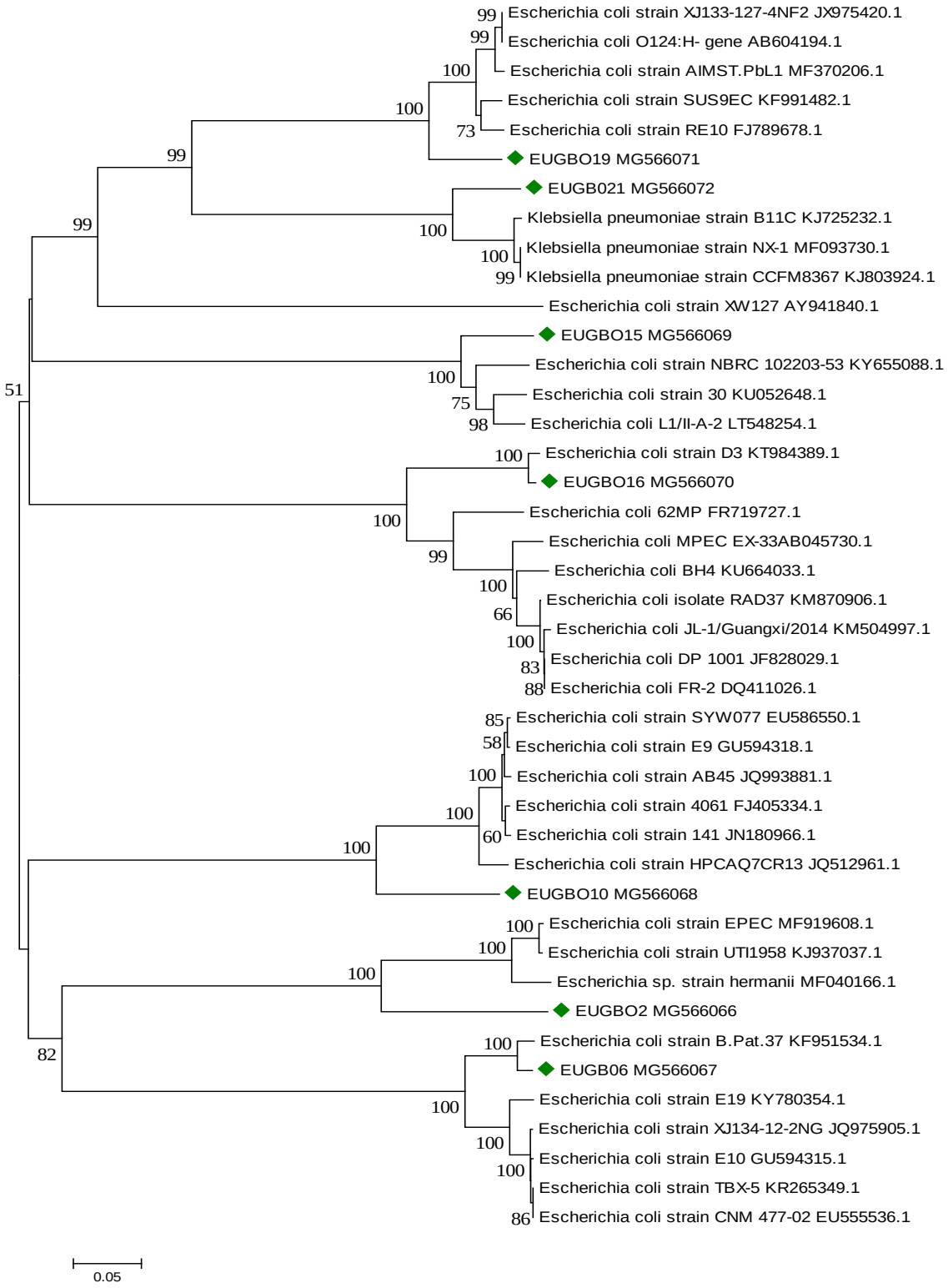

Figure 1: Phylogenetic tree showing the evolutionary relationships among the bacteria isolates.

CHAPTER FIVE

5.0 DISCUSSIONS

5.1 Discussions

The increasing spread of resistance to extended spectrum cephalosporin among bacteria isolates is becoming a great concern worldwide and thereby complicates antibiotic therapy and limits therapeutic options. Highest number of bacterial isolates was obtained from urine, followed by high vaginal swab and wound specimens had the least number of bacteria isolates. The high occurrence of isolates from urine and high vaginal specimens is an indication of a possible poor personal hygiene on the part of the patient and poor hospital hygienic practices which may give room for the spread of clinically important organisms. This finding is in line with the works of Behroozi *et al.* (2010) and Ramazanzadah (2010) who both reported high prevalence of antibiotic resistance bacteria in urine specimens.

The higher prevalence of *S. aureus* and *E. coli* observed could be attributed to poor personal hygiene since these organisms are normal flora of the gastrointestinal tract and colon. This research discovered that females harboured more of these bacteria than males. This is attributed to the physiology of female anatomy. It calls for proper advocacy for the general public to imbibe good personal hygiene as a way of curtailing antibiotic resistant organisms. The isolates of *S. aureus, K. pneumoniae* and *E. coli* were present in the entire age bracket under review. However, high prevalence of *S. aureus*, and *E. coli* were observed within the age bracket of 16-30, 31-45 years. This could be attributed to youth restiveness, adolescence and other bad practices exhibited by people within this age bracket. Low prevalence of *S. aureus, K. pneumoniae* and *E. coli* was observed within the age bracket of 61-75 and above76 years. Age played significant role ($p < 0.05$) in the bacterial prevalence from high vaginal swab and urine

specimen and there was no statistically significant difference in the bacterial prevalence from wound swab specimen ($p > 0.05$) (Appendix 5). This study shows that students and drivers harboured more *S. aureus, K. pneumoniae* and *E. coli* isolates. The high prevalence of this isolates among students is attributed to youth restiveness and adolescence practices. Drivers are known to be sex hunkers; they harbour multiple sexual partners. This practice could help to transmit these organisms.

High prevalence of all the isolates was observed in wound specimens among the farmers when compared to others. This could be as a result of the exposure of their body to soil and poor personal hygienic practices. Civil servants, public servants and house wives recorded very low prevalence of the isolates; except from urine specimens (Table 5). Statistical analysis showed a significant occupational influence in the bacterial prevalence ($p < 0.05$) from all the three sources (Appendix 5). This research observed high prevalence of the isolates among secondary and undergraduate students. Moderate prevalence was recorded among graduates whereas low prevalence was obtained among nursery and primary pupils. This is because these children have not been exposed to bad practices of life which could encourage the contraction and dissemination of these pathogens. Level of education played significant role ($p < 0.05$) in the bacterial prevalence from high vaginal swab and urine specimen, except in wound samples (Appendix 5).

Statistical analysis showed significant difference in the antibiotic resistance frequencies of *S. aureus, K. pneumoniae,* and *E. coli* in the wound specimens ($p < 0.05$). High level of resistance was observed among the isolates. This is in line with the work of Okesola and Adeniji, (2010) where they isolated multi drug resistance organisms in Abeokwuta, Nigeria. It is possible that there is a high abuse of antibiotics, which warranted the high level of resistance that was noted

among the isolates used in this research. However, the isolates from wound showed a higher degree of susceptibility to 4th-generation cephalosporins (cefepime) with susceptibility profiles of *S. aureus* 60.9 %, *K. pneumoniae* 72.7 %, and *E. coli* 79.0 %. This is akin to previous researches in which cefepime was reported as drug of choice for treatment of bacterial infections caused by ESBL-producing organisms (Walsh *et al.*, 2005 and Paterson, *et al.*, 2005). Isolates obtained from high vaginal recorded high resistances to 3rd-generation cepholosporins which includes cefotaxime, ceftriaxone, and cefpodoxime. The same high levels of resistance potential ESBL-producing clinical isolates obtainable in this research have also been previously reported (Bradford, 2001). The findings of this research were in agreement with a similar study done in Nigeria, 2008 and Tehran in 2006 where they observed high rates of resistance among *E. coli*, and *K. pneumoniae* obtained from clinical origin (Mehrgan *et al*., 2010; Iroha *et al.*, 2008b and Dzierzanowska-Fangrat *et al.*, 2005). Statistical analysis showed significant difference in the antibiotic resistance frequencies of *S. aureus*, *K. pneumoniae*, and *E. coli* in the high vaginal swab specimen ($p < 0.05$).

High levels of resistance were observed among *K. pneumoniae* and *E. coli* isolates from urine to the 3rd-generation cepholosporins (cefotaxime and ceftazidime). These could be as a result of these isolates harbouring some enzymes (such as ESBL) that hydrolysis these drugs as reported in work done in South India (Padmini *et al.*, 2008). The natural consequence or phenomenon which allows pathogens to easily adapt to antibiotics (selective pressure imposed on organisms by drugs) is one of the most single reasons why these microorganisms develop antimicrobial resistance (Bush and Jacoby, 2010). Statistical analysis showed significant difference in the antibiotic resistance frequencies of *S. aureus*, *K. pneumoniae*, and *E. coli* in the urine specimens ($p< 0.05$). Higher degree of susceptibility was obtainable among all the clinical isolates from

urine specimens to 4th-generation cephalosporins (cefepime). This finding is in line with the work of Lye *et al.* (2008), who reported that 4th-generation cephalosporins (cefepime) are effective against bacterial isolates that were resistance to 3rd-generation cephalosporins. However, *K. pneumoniae* and *E. coli* has been reported, that they acquire a transmissible form of antibiotic resistance genes via genetic transfer routes such as conjugation (STRAMA, 2007). According to a study done in China, the resistance of bacteria (especially the *Enterobacteriaceae*) to available antibiotics has dramatically risen and amongst other form of resistance in bacteria such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE) have contributed to this phenomenon in recent times, and reducing the efficacy of these commonly used antimicrobial agents (Zhang *et al.,* 2010). These resistances posed by organisms on some groups of powerful antibiotics including penicillins and cephalosporins to mention but a few, which have been used over the years to treat infections like urinary tract infections (UTI's), wound infection, high vaginal infections, post-operative infections and blood infections (bacteremia and septicemia) are no longer effective to a large extent as they used to be during their first introduction into clinical medicine some decades ago (Ejikeugwu *et al*., 2013).

Among the 454 isolates identified, a total of 130 (28.6 %) bacteria were detected to produce beta-lactamase and 23 (17.7 %) were confirmed extended spectrum beta-lactamase producer phenotypically and further investigated molecularly for presence of various ESBL genes (Table 10). The presence of beta-lactamase and ESBLs among the isolates as observed in this study is line with work of Livermore, (2012); Pitout and Laupland, (2008) where they stated that persistent exposure of bacterial strains to beta-lactam antibiotics has induced continuous dynamic production and mutation of beta-lactamase in these bacteria thus, expanding their

activity even against the newly developed beta-lactam antibiotics. However, 23 (17.7 %) isolates (1 (4.4 %) *S. aureus,* 7 (30.4 %) *K. pneumoniae* and 15 (65.2 %) *E. coli*) were phenotypically confirmed as ESBLs positive. Out of the 23 (17.7 %) isolates confirmed ESBL producers, 16 isolates were identified using DDST whereas 22 isolates were confirmed using brilliant ESBL agar (Table 10.) Notably, some of the isolates that were ESBL negative when tested using DDST, were ESBL positive when confirmed using Brilliant ESBL agar. The presence of ESBL among the isolates as identified in this study is in accordance with a study done in Enugu, Nigeria, where ESBL – producing bacteria have been detected phenotypically in clinical isolates of patients that attended a tertiary hospital in the South-Eastern part of Nigeria; and 25.2 % of *E. coli* isolates out of one hundred and twenty three *E. coli* isolates that was screened were found to be positive for ESBL production (Iroha *et al.,* 2008a). Elsewhere in the Northern part of Nigeria, a high prevalence of ESBL production (41.2 %) from Enterobacteriaceae isolated from patients that attended a tertiary hospital in the region has also been reported (Yusha'u *et al*., 2010a). The findings of this work also agrees with the research done by Aibinu *et al.* (2003), Chau and Obegbunam (2007) and Iroha *et al.* (2010), where they found ESBL-producing *E. coli* and *K. pneumoniae* among patients diagnosed of wound infections, high vaginal infections and Urinary Tract Infections in Nigeria.

The prevalence of ESBL production by 22 bacteria isolates among the tested Enterobacteriaceae in this study (31.2 % *K. pneumoniae* and 62.5 % *E. coli*) is substantial and consistent with data from similar works done in Singapore (44.0 % *Klebsiella,* in contrast to 16.1 % *E. coli*), Pakistan (58.7 % *K. pneumoniae*) and Kano, Nigeria (66.7 % ESBL production in Enterobacteriaceae) (Yusha'u *et al*., 2010b; Chlebicki *et al*., 2004 and Ullah *et al*., 2009). This is still similar to the work done in University of Maryland Medical Centre, where out of 117 clinical isolates of

Escherichia coli and *Klebsiella* spp analyzed for ESBL-production, 29 (25.0 %) were ESBL-positive (Anthony *et al*., 2007). However, this results of ESBL production of 62.5 % *E. coli* and 31.2 % *K. pneumoniae* is in contrast to a work done in Saudi Arabia where ESBL production was 4.4 % and 95.6 % for *K. pneumoniae* and *E. coli* respectively (Kader *et al*., 2009) and in Egypt where ESBL production was 19.0 % for *E. coli* and 14.0 % in *K. pneumoniae* (Nevine *et al*., 2010). The finding of this study is also in agreement with similar works done in Eastern Nigeria, where the ESBL production was 56.6 % *E. coli* and in contrast to 59.4 % *K. pneumoniae* (Iroha *et al*., 2010). The false negative results in ESBL detection phenotypically as observed using DDST is as result of co-production of other related enzymes such as metallo beta-lactamases (Walsh *et al.,* 2005), plasmid-mediated and chromosomal inducible AmpC beta-lactamase in the test bacteria other than ESBLs (Jacoby and Munoz-Price, 2005) and (Paterson and Bonomo, 2005). However, the brilliant result observed using Brilliant ESBL agar is as result of formulation with antibiotic and chromogenic mixture which inhibits every other enzymes present and allow only the organisms producing ESBL enzyme to grow and produce colour pigments.

The high level of ESBL-producing *E. coli* from urine specimens as obtainable in this research is in line with the work of Niki *et al*., (2011) who reported extended-spectrum beta-lactamase-producing *Escherichia coli* strains as one of the major course of urinary tract infections among patients. Also in agreement with the research done by Chau and Obegbunam (2007) and Iroha *et al.* (2010) who differently reported ESBL-producing *E. coli* and *K. pneumoniae* among patients diagnosed of wound infections, high vaginal infections and urinary tract infections in Nigeria. All the phenotypic identified ESBL producing isolates presented high resistance percentage index on the beta lactam antibiotics tested as shown in table 12. Thus, this isolates were further

investigated genotypically for the presence of ESBL genes, as a results of their high reduced susceptibility to ceftazidime, which is the best indicator for TEM and SHV derived ESBLs, cefotaxime which is the best indicator for CTX-M types of ESBLs and cefpodoxime which is a good indicator for all ESBLs types (Bush and Jacoby, 2010). Thus, *E. coli* and *K. pneumoniae* remain the major ESBL-producing organisms isolated worldwide (Jacoby and Munoz-Price, 2005).

Quality control of the Genomic DNA extract of the phenotyped ESBL producing isolates was carried out by testing all the isolates for 16S rRNA hyper variable region and all the isolates successfully amplified (Plate 3). Molecular test using PCR identified the presence of *bla* genes in 20 (86.9 %) isolates out of the 23 isolates characterized for the presence of ESBL genes. The absence of ESBL genes in the remaining three isolates could be as a result of them encoding different ESBL gene such as OXA other than the ESBL genes investigated in this research. None of the *Staphylococcus aureus* was found to carry any of the ESBL genes using PCR methods. Five (25.0 %) *Klebsiella pneumoniae* were found to harbor the ESBL genes. Worthy to note, *Escherichia coli* had the highest number of ESBL genes, 15 (75.0 %) isolates were found to carry the *bla* genes (Table 13). This agreed with work done in Egypt, where the prevalence of ESBL among *E. coli* isolates was 61.1 % and *Klebsiella* species was 38.9 % (Saedii *et al*., 2017). The presence of ESBL genes as observed in this study is in line with the observations of Bush and Jacoby, (2010) where they find out that ESBL genes is more common in *E. coli* and *K. pneumoniae* from clinical origin. These result is similar to those of past researchers where majority of ESBL isolates were identified mostly to be *E. coli* and *K. pneumoniae* (Azam *et al.,* 2016; Shehani and Sui, 2013; Iroha *et al.,* 2010). Jacoby and Munoz-Price, (2005) identified *E. coli* and *K. pneumoniae* as the most notorious ESBL producing organisms. This research

observed that urine isolates (14, 70.0 %) harbored majority of the ESBL genes and *Escherichia coli* (10, 50.0 %) had the highest prevalence. This finding agrees with the work done in Burkina Faso where urine samples constituted the major source of ESBL producers. The highest prevalence of ESBL was recorded in *Escherichia coli* (50.0 %) with gene prevalence of *bla*-CTX-M (49.0 %) and *bla*-TEM (73.0 %) (Zongol *et al.,* 2015). Niki *et al*., 2011 reported that *Escherichia coli* are the most predominant ESBL producing organism in urine of patient diagnosed of urinary tract infections.

This research reported the presence of ESBL genes in Enterobacteriaceae using PCR and has shown that 11 (55.0 %) isolates harbored *bla*TEM (Plate 4), *E. coli* (n=8) and *K. pneumoniae* (n=3), 7 (35.0 %) isolates had *bla*SHV (Plate 6), *E. coli* (n=5) and *K. pneumoniae* (n=2) and 9 (45.0 %) isolates harbored *bla*CTX-M (Plate 5), *E. coli* (n=8) and *K. pneumoniae* (n=1). However, 7 (35.0 %) out of the 20 isolates harbored more than single ESBL genes, six (6) *E. coli* and one (1) *K. pneumoniae* and the remaining 13 (65.0 %) isolates had one of the ESBL genes each as enlisted in table 14. The presence of *bla*SHV, *bla*TEM and *bla*CTX-M in this work agreed with most other reports of researchers (Mojtaba *et al*., 2012; Laupland *et al*., 2008; Mehrgan *et al*., 2010). The *E. coli* isolates harbored *bla*TEM/*bla*SHV (n=2), *bla*TEM/*bla*CTX-M-15 (n=3) and *bla*SHV/*bla*CTX-M-15 (n=1) and *K. pneumoniae* harbored *bla*SHV/*bla*TEM (n=1) ESBL genes. The genotype *bla*TEM/*bla*CTX-M had the highest frequency among the multiple ESBL genes produced by the isolates and is in line with the work of Reem *et al*., (2016) where TEM/CTX-M had the highest occurrences among the multiple ESBL genotypes. The PCR-products in gel electrophoresis showed genomic band patterns with molecular weight of 16S rRNA (797 bp, Plate 3), *bla*TEM (861 bp, Plate 4), *bla*SHV (868 bp, Plate 6), *bla*CTX-M-15 (875 bp, Plate 5), *bla*CTX-M-14 (875 bp, Plate 5) and *bla*CTX-M-25 (876 bp, Plate 5) genes.

The observation of more than single ESBL genes in some of the isolates in this study is in accordance with the research work of Varun and Parijath, (2014) where they observed the presence of multiple ESBL genes (*bla*SHV and *bla*TEM) in *E. coli* and *K. pneumoniae* isolates. This research is also in agreement with the work of Justin *et al.,* (2011) were they identified most of the *E. coli* and *K. pneumoniae* isolates to encoded CTX-M-1/CTX-M-9, CTX-M-1/SHV, CTX-M-9/SHV ESBL genes. Only *E. coli* isolate was observed to carry *bla*CTX-M-25 (n=1) and *bla*CTX-M-14 (n=2) ESBL gene. None of the CTX-M group 2 and group 8 ESBL genes was identified among the isolates evaluated in this research. The *bla*TEM genes were observed to have the highest occurrence, followed by *bla*CTX-M genes and *bla*SHV genes had the lowest occurrence. The high occurrence of *bla*TEM and *bla*CTX-M genes in this study agreed with the work of Geenen *et al.,* (2010) where they observed that *bla*TEM and *bla*CTX-M genes is the most widespread ESBL genes among the human population, while the harboring strains of *bla*SHV species are also known to cause infections in some cases.

The findings of this also akin with the research of Mojtaba and Behnaz, (2012) where they observed the prevalence of *bla*TEM and *bla*SHV to be 65.5 % and 15 % respectively and 14 isolates (19.0 %) to carry both *bla*TEM and *bla*SHV genes among ESBL-producing Enterobacteriaceae. Omar *et al*., (2013) in the research on prevalence of TEM, SHV and CTXM genes in *Escherichia coli* and *Klebsiella* spp from urinary isolates discovered that the communist prevalence ESBL gene was CTX-M gene followed by TEM while the least one was SHV gene. The presence of CTX-M, SHV and TEM gene were confirmed in 52.3 % of the isolates and is in accordance with the findings of this research work. Koshesh *et al.,* (2017) in their research identified the presence of *bla*TEM (42.5 %) and *bla*SHV (24.0 %) genes in *Escherichia coli* recovered from patients with urinary tract infections. Justin *et al*., (2011) in their study also

identified multiple ESBL genes in bacteria isolates. CTX-M enzymes appear worldwide, this includes CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-15, CTX-M-9, and CTX-M-25 (Paterson *et al*., 2005) and have been reported in enteric bacteria especially in *E. coli* from France, Italy, Portugal, Spain, USA, Germany, and in some parts of Africa too with varying prevalence (Jacoby and Munoz-Price, 2010; Iroha *et al*., 2010). Some studies have shown prevalence of ESBL-producing *Enterobacteriaceae* in hospitals in African countries including Algeria, Morocco, South Africa and Nigeria (Iabadene *et al.*, 2008; Sekhsokh *et al.*, 2008; Pitout *et al.*, 2008; Aibinu *et al.*, 2003 and Iroha *et al*., 2008) and are similar to the findings of this presence study.

Plasmid DNA was extracted from each of the bacterial isolates and the presence of plasmid were confirmed in all the isolates. The isolates harbored plasmid and the molecular weight of the plasmid ranged from 1500 bp to 21226 bp and some of the isolates harboured double plasmid (Plate 7). Plasmid DNA was tested for the presence of ESBL genes using PCR and primers specific for ESBL genes. Out of the 11 *bla*TEM ESBL- producing isolates, six (6) *E. coli* and two (2) *Klebsiella pneumoniae* were found to harbor the *bla*TEM in their plasmid. Four (4) *E. coli* and one (1) *K. pneumoniae* were found to harbor *bla*SHV in their plasmid out of the seven *bla*SHV ESBL positive isolates. Of the 9 *bla*CTX-M ESBL- producing isolates, six (6) *E. coli* and one (1) *K. pneumoniae* harbor the *bla*CTX-M in their plasmid DNA. Notably, the isolates caring more than one ESBL genes harbour the genes in their plasmid DNA. Out of the 20 ESBL producing isolates, 13 (65.0 %) isolates *E. coli* (10, 50.0 %) and *Klebsiella pneumoniae* (3, 15.0 %) were found to harbor the genes in their plasmid DNA/genomic DNA.

The amplification of *bla* genes in the chromosomal DNA and plasmid DNA suggested that ESBLs expression among these organisms are controlled by both chromosomal and plasmid DNA. The presence of ESBL genes in the plasmid DNA as observed in this research is in accordance with the work done by Sharma *et al*., (2010) were they used PCR to identify ESBL genes in plasmid DNA, 30 per cent ESBL positive isolates using TEM primer and 38 per cent using SHV primer, whereas PCR for both plasmid and chromosomal DNA had 56 per cent positivity for TEM. CTX-M enzymes, particularly CTX-M-15, have disseminated throughout all continents as a result of plasmids (Ruppé, 2010). CTX-M-producing *E. coli* and *K. pneumoniae* are becoming increasingly involved in urinary tract infections (Arpin *et al.,* 2009). Barlow *et al.,* (2008) found that *bla*CTX-M genes are mobilized to plasmids almost ten times more frequently than other class A β-lactamases. The genes encoding ESBLs are usually located on plasmids that are highly mobile and can harbor resistance genes to several other unrelated classes of antimicrobials (Pitout *et al*., 2005). A shift in the distribution of different ESBLs plasmid mediated, TEM and SHV derived enzyme has occurred in Africa and Europe, with a dramatic increase of CTX-M enzyme over TEM and SHV variants (Baudry *et al.,* 2009). The presence of *bla*CTX-M, *bla*SHV, and *bla*TEM ESBL genes in plasmid as observed in this research agrees with most other reports viewed (Sharma *et al*., 2010).

Some bacterial cannot be fully identified to species level from phenotypic characteristics, thus sequencing the 16S rRNA hyper variable region is the best method of characterizing them. The amplification of the 16S rRNA region was performed successfully using a set of universal primers (27F and 805R) by targeting 16S V3-V4 hyper variable region (appendix 5 and Plate 3). The PCR products of the seven bacteria isolates that harbored the *bla* genes in both chromosomal DNA and plasmid DNA were sequenced. The Sanger sequenced 16S rRNA nucleotide

sequences (fasta files) from all bacterial strains were matched with similar sequences in the NCBI Web site using BLAST. The BLAST query covers, exponential value, identity similarities, total and maximum score revealed that the strains were closely related to multiply antibiotic resistant pathogenic *Escherichia coli* and *Klebsiella pneumoniae.* The identities of all bacteria isolates, the *bla* gene present, locations of *bla* gene, closest matched strain identity, percentage similarty and their GenBank accession numbers are presented in table 16. The amino acid nucleotide sequences of this isolates can be found in appendix 5.

Phylogenetic tree of the 16S rRNA sequences of the seven bacterial isolates that harbored the *bla* genes in both chromosomal DNA and plasmid DNA was reconstructed using Molecular Evolutionary Genetic Analysis (MEGA, version 7.0) computational analysis (Kumar *et al*., 2016). The 16S rRNA sequences of the isolates were aligned with 42 reference nucleotide sequences of the 16S rRNA of closely related taxa retrieved from the NCBI GenBank data library (Figure 1). The relationships were based on evolutionary distances using the p- distance method (Nei and Kumar, 2000). The p-distance based method inferred the evolutionary relationship using Neighbor-Joining (NJ) clustered-based algorithm statistical method. The concatenated Neighbor-Joining (NJ) revealed the optimal tree of 3.58246628 branch length with 596 positions in the final dataset (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in 1000 replicates of bootstrap test are shown next to the branches (Felsenstein, 1985). Based on the cluster algorithm, Neighbor-Joining tree revealed the percentage of evolutionary relationship with the *Escherichia coli* and *Klebsiella pneumoniae* based on the degree of differences between the sequences. The concatenated Neighbor-Joining showed that the multiple antibiotic resistant ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* in this study have a very high homologous similarity of 100% with the matched

isolates within NCBI GenBank based on their nucleotide sequences. The high similarities observed among the isolates in this research compare with the isolates in NCBI GenBank shows that there are not a novel isolates.

5.2 Conclusions and Recommendations

In conclusions this study has provided updated knowledge on extended spectrum cephalosporin resistance levels in *Escherichia coli, K. pneumoniae* and *S. aureus* isolates from Federal Teaching Hospital Abakaliki. The high prevalence resistance rate of beta-lactam antibiotics as observed in this research is due to over prescription, self-medicate by most patients, obtaining prescriptions for non-adapted drugs from unqualified personals and refusal to follow drug prescription and completion of drug regime, thus restricting sales and usage of cephalosporin is recommended. This research observed that resistances were mediated by ESBLs and revealed the presence of multiple ESBL genes in genomic DNA and plasmid DNA of clinical isolates and this may encourage high dissemination of ESBL genes. Detection of *bla*TEM, *bla*SHV and *bla*CTX-M groups both on plasmid and chromosome gave better understanding of ESBL production. Both *K. pneumoniae* and *Escherichia coli* were found to harbor the ESBL gene using phenotypic and molecular (PCR) methods, while *S. aureus* isolate was found to be ESBL producer using phenotypic method according to CLSI. Among the isolates the most frequent occurring ESBL gene was *bla*TEM (55.0 %) gene followed by *bla*CTX-M (45.0 %) while the least one was *bla*SHV (35.0 %) gene. This research has confirmed that CTX-M-15 is the most predominant ESBL subtype genotype that is harbored in plasmid and also identified the first CTX-M-14 and CTX-M-25 producing *E. coli* in clinical isolates from FETHA. Sequencing revealed that the strains were closely related to multiply antibiotic resistant pathogenic *Escherichia coli* and *Klebsiella pneumoniae.* Phylogenetic tree of the 16S rRNA sequences of the seven bacteria isolates and nucleotide sequences of the matched isolates in NCBI Genbank reconstructed using Molecular Evolutionary Genetic Analysis (MEGA) revealed 100 percent similarities. We therefore recommend multilocus sequence typing (MLST) to know if the fast spreading

international clone ST131 has reached Nigeria. The isolates in this study could harbor either R-plasmid or virulence plasmids, since R-plasmid contains gene that code for enzyme capable of destroying or modifying antibiotics, we therefore suggest further characterization of plasmid encoding ESBL genes to know the type of plasmid that encode ESBL genes and other antibiotics resistance genes. Determination of *bla*TEM, *bla*SHV and *bla*CTX-M genes by molecular techniques in ESBL producing bacteria may give a valuable clinical data about their epidemiology and risk factors associated with these infections. Therefore, ESBL producing organisms should be promptly identified for appropriate antibiotic prescription and proper implementation of infection control measures.

There is also need for sensitive diagnostic methods for ESBL detection in order to guide therapy, monitor the development of resistance in both the community and hospital settings and to implement any interventional measures as a way of curtailing the economic loss and negative clinical effect of ESBL - producing bacteria. A good number of Microbiology laboratories across the hospitals in Nigeria and other parts of Africa are not fully aware of the clinical importance of ESBLs, how to detect and report them in their routine laboratory practices. This may be due to lack of basic awareness and resources to undertake such studies in areas where there is a proven knowledge about these pathogens. ESBL detection can be very expensive to perform and time consuming as it is different from routine antimicrobial susceptibility studies performed in the microbiology laboratories.

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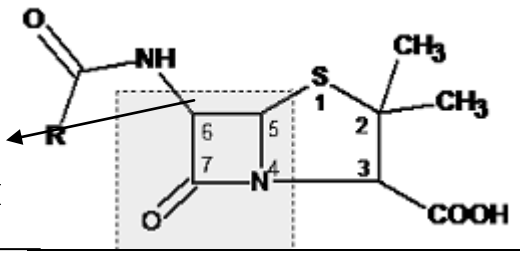
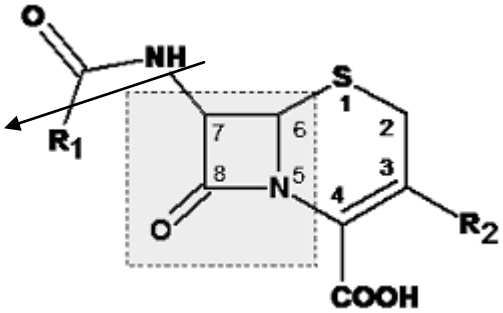
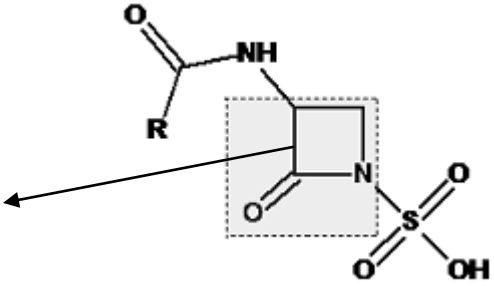
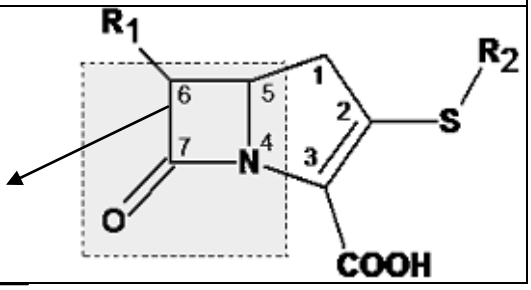
Appendix 1

(A) Structure of the main groups of beta - lactam antibiotics

PENICILLINS	 **BETA – LACTAM RING**
CEPHALOSPORINS	 **BETA – LACTAM RING**
MONOBACTAMS	 **BETA – LACTAM RING**
CARBAPENEMS	 **BETA – LACTAM RING**

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(B) ESBL screening breakpoints zone diameter (ZD) interpretive standards for Enterobacteriaceae in millimeter (mm).

Disc	Content (µg)	S (BP, mm)	I (BP, mm)	R (BP, mm)
Cefpodoxime	10	≥21	15-20	≤14
Ceftazidime	30	≥18	15-17	≤14
Cefotaxime	30	≥23	15-22	≤14
Ceftriaxone	30	≥23	15-22	≤14
Aztreonam	30	≥22	16-21	≤15
Cefepime	30	≥18	15-17	≤14

BP = Breakpoint; **R** = Resistant; **S** = Susceptible; **I** = Intermediate (CLSI, 2012).

(C) ESBL screening breakpoints zone diameter (ZD) interpretive standards for *Staphylococcus aureus* in millimeter (mm).

Disc	Content (µg)	S (BP, mm)	I (BP, mm)	R (BP, mm)
Cefpodoxime	10	≥21	18-20	≤17
Ceftazidime	30	≥18	15-17	≤14
Cefotaxime	30	≥23	15-22	≤14
Ceftriaxone	30	≥23	14-22	≤13
Cefepime	30	≥18	15-17	≤14
Aztreonam	30	≥22	16-21	≤15

BP = Breakpoint; **R** = Resistant; **S** = Susceptible; **I** = Intermediate (CLSI, 2012).

(D) ESBL confirmatory test guide

Discs	Content (µg)	Interpretation
Ceftazidime	30	A 5 mm increase in zone diameter for either antibiotic tested in combination with clavulanic acid versus its zone when tested alone confirms ESBL production phenotypically.
Ceftazidime + clavulanic acid AND	30/10	
Cefotaxime	30	
Cefotaxime + clavulanic acid	30/10	
Amoxicillin-clavulanic acid	20/10	

Scottish Center for Infection and Environmental Health (2004)

(E) Demographic distribution of patient specimens based on the hospital records

Age	1 - 15	16 - 30	31 - 45	46 - 60	61 - 75	76 - Above		
Wound	56	59	59	58	58	58		
HVS	15	37	37	37	37	37		
Urine	75	75	75	75	75	75		
Occupation	**Pupils**	**Students**	**Farmers**	**Traders**	**Drivers**	**House wives**	**Civil servants**	**Public servants**
Wound	40	45	45	45	45	45	45	40
HVS	0	35	35	35	0	35	30	30
Urine	50	56	56	57	58	56	56	56
Education	**Nursery**	**Primary**	**Secondary**	**Undergraduate**	**Graduate**			
Wound	40	80	80	75	75			
HVS	0	50	50	50	50			
Urine	50	100	100	100	100			

Appendix 2

Equipment and Media

Equipment

ABI Prism 3130 Automated Sequencer

Aluminium foil

Antibiotic disc

Autoclave (Apex group, SA-300VL)

Bijou bottles

Bunsen burner

Centrifuge

Conical flask

Cotton wool

Electric weighing balance

Face mask

Filta Matrix Laminar flow

Gel documentation system (Gel Doc)

Gel Electrophoresis machine

Hand glove

Ice pack

Incubator

Masking tape

Measuring cylinder

Microwave oven

NanoDrop-Spectrophotometer

Oven (Scientific, Series 2000)

Petri dishes

Pipette

Refrigerator

Thermo Cycler (PCR)

Vortex Geniez

Media

CLED agar

Eosin methylene blue (EMB) agar

Luria-Bertani (LB) agar

Luria-Bertani (LB) broth

Nutrient agar

Nutrient broth

MacConkey agar

Mannitol salt agar

Mueller Hinton agar

Peptone water

Appendix 3

(A) QIAamp DNA Mini Kit Protocol (Qiagen, Hilden, Germany)

Pure colony of an overnight growth of bacteria isolates on Luria-Bertani (LB) agar (Merck, Germany) were harvested by removeing bacteria from culture plate with an inoculation loop and suspended in 180 µl of buffer ATL (supplied in the QIAamp DNA Mini Kit) by vigorous stirring into the 1.5 ml micro-centrifuge tube. Addition of 20 µl proteinase K (lysing enzyme) were added, mixed by vortexing, and incubated in a shaking water bath at 56°C until the cells were completely lysed. The tubes were briefly centrifuge to remove drops from the inside of the lid. Addition of 200 µl buffer AL (lysing buffer) to the sample was made, mixed by pulse-vortexing for 15 s, and incubated at 70°C for 10 min and briefly centrifuged again. Addition of 200 µl ethanol (96 -100%) was added to the samples and mixed by pulse-vortexing for 15 s and briefly centrifuged at 6000 x *g* (8000 rpm). The supernatant (including the precipitate) was transferred to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed, centrifuged at 6000 x *g* (8000 rpm) for 1 min and the tube containing the filtrate was discarded. The QIAamp Mini spin column was returned to clean 2 ml collection tube. Addition of 500 µl buffer AW1 (washing buffer) was addad without wetting the rim, the cap was closed, centrifuged at 6000 x *g* (8000 rpm) for 1 min and the tube containing the filtrate was discarded. The QIAamp Mini spin column was returned to clean new 2 ml collection tube. Addition of 500 µl buffer AW2 was added without wetting the rim, the cap was closed, centrifuged at full speed of 20000 x *g* (14000 rpm) for 3 min and the tube containing the filtrate was discarded. The QIAamp Mini spin column was placed in a clean 1.5 ml micro-centrifuge tube, addition of 200 µl buffer AE (elution buffer) was added, the cap was closed and incubated at a room temperature

for 5 min, before centrifuged at 6000 x *g* (8000 rpm) for 1 min. The eluted genomic DNA was stored at -80^{0}C for further use.

(B) Zyppy Plasmid Miniprep Kit, Quick Protocol (Zymo Research, USA)

Overnight grown bacterial cells in 5 ml of LB broth (Merck, Germany) were harvested by centrifuging the 5 ml of each culture in 15 ml centrifuge tubes for 4 min at 4000 rpm (revolutions per min), 600 µl of bacterial culture was added to 1.5 ml micro-centrifuge tube. A total of 100 µl of 7X Lysis buffer (blue) was added, mixed by inverting the tube 4 - 6 times and incubated for 1 - 2 minutes. Addition of 350 µl of cold Neutralization buffer (yellow) was made and mixed thoroughly for complete neutralization to occur. Bacterial isolates were centrifuged for 2 - 4 minutes at 11,000 x g. The supernatant was transferred to Zymo-spin^{TM} IIN column in a collection tube and centrifuged for 15 seconds at 11,000 x g. The flow-through was discarded and the Zymo-spin^{TM} IIN column was returned back to the same tube. A total of 200 µl of Endo-Wash buffer was added to the column and centrifuged for 30 seconds at 11,000 x g. Another 400 µl of Zyppy^{TM} Wash buffer was added to the column and centrifuged for 1 minute at 11,000 x g. The column was transferred into a clean 1.5 ml micro-centrifuge tube, 30 µl of Zyppy^{TM} Elution buffer was added directly to the column matrix and incubated for one minute at room temperature. The Zymo-spin^{TM} IIN column was centrifuged for 30 seconds at 11,000 x g to elute the plasmid DNA. The eluted plasmid DNA was stored at -80^{0}C for further use.

Appendix 4

List of primers used

Primers name	Target Gene	Primer sequence (5'-3')[1]	Amplicon product size (bp)	
27	16S rRNA	F-AGT TTG ATC MTG GCT CAG	797	Gudjónsdóttir, 2015
805		R-GGA CTA CHA GGG TAT CTA AT		
TEM	*bla*TEM	F-ATGAGTATTCAACATTTCCGTGT	861	Azam *et al.,* 2016
		R-TTACCAATGCTTAATCAGTGAGG		
CTX-M	*bla*CTX-M	F-SCS ATG TGC AGY ACC AGT AA	585	Gudjónsdóttir, 2015
		R-ACC AGA AYV AGC GGB GC		
CTX-M-1gp	*bla*CTX-M-15	F-ATGGTTAAAAAATCACTGCGYCAGTTCACGC	875	Azam *et al.,* 2016
		R-TTACAAACCGTYGGTGACGATTTTAGCCG		
CTX-M-2gp	*bla*CTX-M-2	F-ATGATGACTCAGAGCATTCGCC	742	
		R-TCGTTGGTGGTGCCATAATCTCC		
CTX-M-8gp	*bla*CTX-M-8	F-AACGCACAGACGCTCTACC	517	
		R-GGGTAGCCCAGCCTGAAT		
CTX-M-9gp	*bla*CTX-M-14	F-ATGGTGACAAAGAGAGTGCAACGG	875	
		R-TTACAGCCCTTCGGCGATGATTC		
		F-ATGATGAGAAAAAGCGTAAGGCGGG		
CTX-M-25gp	*bla*CTX-M-25	R-TTAATAACCGTCGGTGACAATTCTGGC	876	
SHV	*bla*SHV	F-TCGTTATGCGTTATATTCGCC	868	Reem *et al.,* 2016
		R-GGTTAGCGTTGCCAGTGCT		

(Manufactured by Whitehead Scientific Ltd, Cape Town, South Africa)

PCR criteria for the amplification of 16S rRNA and *bla* genes

16S rRNA
Step 1 Denaturing of DNA 94^0C for 7min

Step 2 Denaturing of DNA 94^0C for 30sec

Step 3 Hybridization of primers to bacteria DNA 48^0C for 40sec

Step 4 Elongation of primers 72^0C for 2min

Steps 2-4 repeated for 25 times

Step 5 Finial elongation of primers 72^0C for 7min

Step 6 Samples is cooled down to 4^0C

*bla*TEM gene

Step 1 Denaturing of DNA 94^0C for 5min

Step 2 Denaturing of DNA 94^0C for 1min

Step 3 Hybridization of primers to bacteria DNA 50^0C for 30sec

Step 4 Elongation of primers 72^0C for 1min

Steps 2-4 repeated for 30 times

Step 5 Finial elongation of primers 72^0C for 10min

Step 6 Samples is cooled down to 4^0C

*bla*CTX-M groups gene (*bla*CTX-M-15, blaCTX-M-2, *bla*CTX-M-8, *bla*CTX-M-14, *bla*CTX-M-25)
Step 1 Denaturing of DNA 94^0C for 5min

Step 2 Denaturing of DNA 94^0C for 1min

Step 3 Hybridization of primers to bacteria DNA 58^0C (59^0C, 55^0C, 55^0C, 57^0C, 56^0C) for 30sec

Step 4 Elongation of primers 72^0C for 1min

Steps 2-4 repeated for 30 times

Step 5 Finial elongation of primers 72^0C for 10min

Step 6 Samples is cooled down to 4^0C

*bla*SHV gene
Step 1 Denaturing of DNA 95^0C for 2min

Step 2 Denaturing of DNA 94^0C for 1min

Step 3 Hybridization of primers to bacteria DNA 50^0C for 30sec

Step 4 Elongation of primers 72^0C for 1min

Steps 2-4 repeated for 30 times

Step 5 Finial elongation of primers 72^0C for 5min

Step 6 Samples is cooled down to 4^0C

Appendix 5

(A) Statistical Analysis

```
ONEWAY Specimen_size BY Specimen_type
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

ANOVA

Specimen_size

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11702.889	2	5851.444	8.890	.016
Within Groups	3949.333	6	658.222		
Total	15652.222	8			

Post Hoc Tests

Multiple Comparisons

Specimen_size

Tukey HSD

(I) Specimen_type	(J) Specimen_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Wound_swab	High_vaginal_swab	-15.33333	20.94791	.755	-79.6073	48.9406
	Urine	-83.00000*	20.94791	.017	-147.2739	-18.7261
High_vaginal_swab	Wound_swab	15.33333	20.94791	.755	-48.9406	79.6073

	Urine	-67.66667*	20.94791	.041	-131.9406	-3.3927
Urine	Wound_swab	83.00000*	20.94791	.017	18.7261	147.2739
	High_vaginal_swab	67.66667*	20.94791	.041	3.3927	131.9406

*. The mean difference is significant at the 0.05 level.

ONEWAY High_vaginal_swab BY Specimen size between male and female

/STATISTICS DESCRIPTIVES

/MISSING ANALYSIS.

ANOVA

High_vaginal_swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1633.500	1	1633.500	29.170	.006
Within Groups	224.000	4	56.000		
Total	1857.500	5			

ONEWAY Urine BY Specimen_type

/STATISTICS DESCRIPTIVES

/MISSING ANALYSIS.

ANOVA

Urine					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	770.667	1	770.667	1.058	.362

Within Groups	2914.667	4	728.667		
Total	3685.333	5			

ONEWAY Wound_swab BY Specimen_type

/STATISTICS DESCRIPTIVES

/MISSING ANALYSIS.

ANOVA

Wound_swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.167	1	4.167	.236	.653
Within Groups	70.667	4	17.667		
Total	74.833	5			

ONEWAY High_vaginal_swab BY Age_distribution

/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS

/POSTHOC=TUKEY ALPHA(0.05).

ANOVA

High_vaginal_swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	329.833	5	65.967	7.710	.002
Within Groups	102.667	12	8.556		
Total	432.500	17			

```
ONEWAY Wound_swab BY Age_distribution
 /STATISTICS DESCRIPTIVES HOMOGENEITY
 /MISSING ANALYSIS
 /POSTHOC=TUKEY ALPHA(0.05).
```

ANOVA

Wound_swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26.278	5	5.256	1.245	.348
Within Groups	50.667	12	4.222		
Total	76.944	17			

Post Hoc Tests

Multiple Comparisons

Wound_swab

Tukey HSD

(I) Age_distribution	(J) Age_distribution	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1_15	16_30	-1.00000	1.67774	.989	-6.6354	4.6354
	31_45	-1.66667	1.67774	.911	-7.3021	3.9687
	46_60	.00000	1.67774	1.000	-5.6354	5.6354
	61_75	1.00000	1.67774	.989	-4.6354	6.6354
	76_above	2.00000	1.67774	.833	-3.6354	7.6354
16_30	1_15	1.00000	1.67774	.989	-4.6354	6.6354

	31_45	-.66667	1.67774	.998	-6.3021	4.9687
	46_60	1.00000	1.67774	.989	-4.6354	6.6354
	61_75	2.00000	1.67774	.833	-3.6354	7.6354
	76_above	3.00000	1.67774	.507	-2.6354	8.6354
31_45	1_15	1.66667	1.67774	.911	-3.9687	7.3021
	16_30	.66667	1.67774	.998	-4.9687	6.3021
	46_60	1.66667	1.67774	.911	-3.9687	7.3021
	61_75	2.66667	1.67774	.620	-2.9687	8.3021
	76_above	3.66667	1.67774	.311	-1.9687	9.3021
46_60	1_15	.00000	1.67774	1.000	-5.6354	5.6354
	16_30	-1.00000	1.67774	.989	-6.6354	4.6354
	31_45	-1.66667	1.67774	.911	-7.3021	3.9687
	61_75	1.00000	1.67774	.989	-4.6354	6.6354
	76_above	2.00000	1.67774	.833	-3.6354	7.6354
61_75	1_15	-1.00000	1.67774	.989	-6.6354	4.6354
	16_30	-2.00000	1.67774	.833	-7.6354	3.6354
	31_45	-2.66667	1.67774	.620	-8.3021	2.9687
	46_60	-1.00000	1.67774	.989	-6.6354	4.6354
	76_above	1.00000	1.67774	.989	-4.6354	6.6354
76_above	1_15	-2.00000	1.67774	.833	-7.6354	3.6354
	16_30	-3.00000	1.67774	.507	-8.6354	2.6354
	31_45	-3.66667	1.67774	.311	-9.3021	1.9687
	46_60	-2.00000	1.67774	.833	-7.6354	3.6354
	61_75	-1.00000	1.67774	.989	-6.6354	4.6354

ONEWAY Urine BY Age_distribution

/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS

/POSTHOC=TUKEY ALPHA(0.05).

ANOVA

Urine					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3028.444	5	605.689	3.890	.025
Within Groups	1868.667	12	155.722		
Total	4897.111	17			

Post Hoc Tests

Multiple Comparisons

Urine

Tukey HSD

(I) Age_distribution	(J) Age_distribution	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1_15	16_30	-25.00000	10.18896	.213	-59.2239	9.2239
	31_45	-8.00000	10.18896	.965	-42.2239	26.2239
	46_60	3.33333	10.18896	.999	-30.8906	37.5572
	61_75	12.00000	10.18896	.839	-22.2239	46.2239
	76_above	13.00000	10.18896	.792	-21.2239	47.2239
16_30	1_15	25.00000	10.18896	.213	-9.2239	59.2239
	31_45	17.00000	10.18896	.574	-17.2239	51.2239

	46_60	28.33333	10.18896	.129	-5.8906	62.5572
	61_75	37.00000*	10.18896	.032	2.7761	71.2239
	76_above	38.00000*	10.18896	.027	3.7761	72.2239
31_45	1_15	8.00000	10.18896	.965	-26.2239	42.2239
	16_30	-17.00000	10.18896	.574	-51.2239	17.2239
	46_60	11.33333	10.18896	.867	-22.8906	45.5572
	61_75	20.00000	10.18896	.414	-14.2239	54.2239
	76_above	21.00000	10.18896	.366	-13.2239	55.2239
46_60	1_15	-3.33333	10.18896	.999	-37.5572	30.8906
	16_30	-28.33333	10.18896	.129	-62.5572	5.8906
	31_45	-11.33333	10.18896	.867	-45.5572	22.8906
	61_75	8.66667	10.18896	.951	-25.5572	42.8906
	76_above	9.66667	10.18896	.926	-24.5572	43.8906
61_75	1_15	-12.00000	10.18896	.839	-46.2239	22.2239
	16_30	-37.00000*	10.18896	.032	-71.2239	-2.7761
	31_45	-20.00000	10.18896	.414	-54.2239	14.2239
	46_60	-8.66667	10.18896	.951	-42.8906	25.5572
	76_above	1.00000	10.18896	1.000	-33.2239	35.2239
76_above	1_15	-13.00000	10.18896	.792	-47.2239	21.2239
	16_30	-38.00000*	10.18896	.027	-72.2239	-3.7761
	31_45	-21.00000	10.18896	.366	-55.2239	13.2239
	46_60	-9.66667	10.18896	.926	-43.8906	24.5572
	61_75	-1.00000	10.18896	1.000	-35.2239	33.2239

*. The mean difference is significant at the 0.05 level.

ONEWAY Urine BY Occupation

/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS

/POSTHOC=TUKEY ALPHA(0.05).

ANOVA

Urine					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1371.167	7	195.881	2.972	.034
Within Groups	1054.667	16	65.917		
Total	2425.833	23			

Post Hoc Tests

Multiple Comparisons

Urine

Tukey HSD

(I) Occupation	(J) Occupation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Pupils	Students	-19.33333	6.62906	.134	-42.2841	3.6175
	Farmers	-8.66667	6.62906	.883	-31.6175	14.2841
	Traders	-10.66667	6.62906	.739	-33.6175	12.2841
	Drivers	-20.00000	6.62906	.112	-42.9508	2.9508
	House_wives	.33333	6.62906	1.000	-22.6175	23.2841

	Civil_servants	-1.66667	6.62906	1.000	-24.6175	21.2841
	Public_servants	-6.00000	6.62906	.981	-28.9508	16.9508
Students	Pupils	19.33333	6.62906	.134	-3.6175	42.2841
	Farmers	10.66667	6.62906	.739	-12.2841	33.6175
	Traders	8.66667	6.62906	.883	-14.2841	31.6175
	Drivers	-.66667	6.62906	1.000	-23.6175	22.2841
	House_wives	19.66667	6.62906	.123	-3.2841	42.6175
	Civil_servants	17.66667	6.62906	.203	-5.2841	40.6175
	Public_servants	13.33333	6.62906	.504	-9.6175	36.2841
Farmers	Pupils	8.66667	6.62906	.883	-14.2841	31.6175
	Students	-10.66667	6.62906	.739	-33.6175	12.2841
	Traders	-2.00000	6.62906	1.000	-24.9508	20.9508
	Drivers	-11.33333	6.62906	.682	-34.2841	11.6175
	House_wives	9.00000	6.62906	.863	-13.9508	31.9508
	Civil_servants	7.00000	6.62906	.957	-15.9508	29.9508
	Public_servants	2.66667	6.62906	1.000	-20.2841	25.6175
Traders	Pupils	10.66667	6.62906	.739	-12.2841	33.6175
	Students	-8.66667	6.62906	.883	-31.6175	14.2841
	Farmers	2.00000	6.62906	1.000	-20.9508	24.9508
	Drivers	-9.33333	6.62906	.841	-32.2841	13.6175
	House_wives	11.00000	6.62906	.711	-11.9508	33.9508

	Civil_servants	9.00000	6.62906	.863	-13.9508	31.9508
	Public_servants	4.66667	6.62906	.996	-18.2841	27.6175
Drivers	Pupils	20.00000	6.62906	.112	-2.9508	42.9508
	Students	.66667	6.62906	1.000	-22.2841	23.6175
	Farmers	11.33333	6.62906	.682	-11.6175	34.2841
	Traders	9.33333	6.62906	.841	-13.6175	32.2841
	House_wives	20.33333	6.62906	.103	-2.6175	43.2841
	Civil_servants	18.33333	6.62906	.172	-4.6175	41.2841
	Public_servants	14.00000	6.62906	.447	-8.9508	36.9508
House_wives	Pupils	-.33333	6.62906	1.000	-23.2841	22.6175
	Students	-19.66667	6.62906	.123	-42.6175	3.2841
	Farmers	-9.00000	6.62906	.863	-31.9508	13.9508
	Traders	-11.00000	6.62906	.711	-33.9508	11.9508
	Drivers	-20.33333	6.62906	.103	-43.2841	2.6175
	Civil_servants	-2.00000	6.62906	1.000	-24.9508	20.9508
	Public_servants	-6.33333	6.62906	.975	-29.2841	16.6175
Civil_servants	Pupils	1.66667	6.62906	1.000	-21.2841	24.6175
	Students	-17.66667	6.62906	.203	-40.6175	5.2841
	Farmers	-7.00000	6.62906	.957	-29.9508	15.9508
	Traders	-9.00000	6.62906	.863	-31.9508	13.9508
	Drivers	-18.33333	6.62906	.172	-41.2841	4.6175

	House_wives	2.00000	6.62906	1.000	-20.9508	24.9508
	Public_servants	-4.33333	6.62906	.997	-27.2841	18.6175
Public_servants	Pupils	6.00000	6.62906	.981	-16.9508	28.9508
	Students	-13.33333	6.62906	.504	-36.2841	9.6175
	Farmers	-2.66667	6.62906	1.000	-25.6175	20.2841
	Traders	-4.66667	6.62906	.996	-27.6175	18.2841
	Drivers	-14.00000	6.62906	.447	-36.9508	8.9508
	House_wives	6.33333	6.62906	.975	-16.6175	29.2841
	Civil_servants	4.33333	6.62906	.997	-18.6175	27.2841

```
ONEWAY High_vaginal_swab BY Occupation
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

ANOVA

High_vaginal_swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	448.625	7	64.089	8.270	.000
Within Groups	124.000	16	7.750		
Total	572.625	23			

Post Hoc Tests

Multiple Comparisons

High_vaginal_swab

Tukey HSD

(I) Occupation	(J) Occupation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Pupils	Students	-13.66667*	2.27303	.000	-21.5362	-5.7971
	Farmers	-7.00000	2.27303	.101	-14.8696	.8696
	Traders	-6.00000	2.27303	.211	-13.8696	1.8696
	Drivers	.00000	2.27303	1.000	-7.8696	7.8696
	House_wives	-2.00000	2.27303	.984	-9.8696	5.8696
	Civil_servants	-2.66667	2.27303	.929	-10.5362	5.2029
	Public_servants	-1.66667	2.27303	.994	-9.5362	6.2029
Students	Pupils	13.66667*	2.27303	.000	5.7971	21.5362
	Farmers	6.66667	2.27303	.130	-1.2029	14.5362
	Traders	7.66667	2.27303	.059	-.2029	15.5362
	Drivers	13.66667*	2.27303	.000	5.7971	21.5362
	House_wives	11.66667*	2.27303	.002	3.7971	19.5362
	Civil_servants	11.00000*	2.27303	.003	3.1304	18.8696
	Public_servants	12.00000*	2.27303	.001	4.1304	19.8696
Farmers	Pupils	7.00000	2.27303	.101	-.8696	14.8696

	Students	-6.66667	2.27303	.130	-14.5362	1.2029
	Traders	1.00000	2.27303	1.000	-6.8696	8.8696
	Drivers	7.00000	2.27303	.101	-.8696	14.8696
	House_wives	5.00000	2.27303	.400	-2.8696	12.8696
	Civil_servants	4.33333	2.27303	.566	-3.5362	12.2029
	Public_servants	5.33333	2.27303	.328	-2.5362	13.2029
Traders	Pupils	6.00000	2.27303	.211	-1.8696	13.8696
	Students	-7.66667	2.27303	.059	-15.5362	.2029
	Farmers	-1.00000	2.27303	1.000	-8.8696	6.8696
	Drivers	6.00000	2.27303	.211	-1.8696	13.8696
	House_wives	4.00000	2.27303	.653	-3.8696	11.8696
	Civil_servants	3.33333	2.27303	.814	-4.5362	11.2029
	Public_servants	4.33333	2.27303	.566	-3.5362	12.2029
Drivers	Pupils	.00000	2.27303	1.000	-7.8696	7.8696
	Students	-13.66667*	2.27303	.000	-21.5362	-5.7971
	Farmers	-7.00000	2.27303	.101	-14.8696	.8696
	Traders	-6.00000	2.27303	.211	-13.8696	1.8696
	House_wives	-2.00000	2.27303	.984	-9.8696	5.8696
	Civil_servants	-2.66667	2.27303	.929	-10.5362	5.2029
	Public_servants	-1.66667	2.27303	.994	-9.5362	6.2029
House_wives	Pupils	2.00000	2.27303	.984	-5.8696	9.8696

	Students	-11.66667*	2.27303	.002	-19.5362	-3.7971
	Farmers	-5.00000	2.27303	.400	-12.8696	2.8696
	Traders	-4.00000	2.27303	.653	-11.8696	3.8696
	Drivers	2.00000	2.27303	.984	-5.8696	9.8696
	Civil_servants	-.66667	2.27303	1.000	-8.5362	7.2029
	Public_servants	.33333	2.27303	1.000	-7.5362	8.2029
Civil_servants	Pupils	2.66667	2.27303	.929	-5.2029	10.5362
	Students	-11.00000*	2.27303	.003	-18.8696	-3.1304
	Farmers	-4.33333	2.27303	.566	-12.2029	3.5362
	Traders	-3.33333	2.27303	.814	-11.2029	4.5362
	Drivers	2.66667	2.27303	.929	-5.2029	10.5362
	House_wives	.66667	2.27303	1.000	-7.2029	8.5362
	Public_servants	1.00000	2.27303	1.000	-6.8696	8.8696
Public_servants	Pupils	1.66667	2.27303	.994	-6.2029	9.5362
	Students	-12.00000*	2.27303	.001	-19.8696	-4.1304
	Farmers	-5.33333	2.27303	.328	-13.2029	2.5362
	Traders	-4.33333	2.27303	.566	-12.2029	3.5362
	Drivers	1.66667	2.27303	.994	-6.2029	9.5362
	House_wives	-.33333	2.27303	1.000	-8.2029	7.5362
	Civil_servants	-1.00000	2.27303	1.000	-8.8696	6.8696

*. The mean difference is significant at the 0.05 level.

ONEWAY Wound_swab BY Occupation

/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS

/POSTHOC=TUKEY ALPHA(0.05).

ANOVA

Wound_swab					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	73.958	7	10.565	7.044	.001
Within Groups	24.000	16	1.500		
Total	97.958	23			

Post Hoc Tests

Multiple Comparisons

Wound_swab

Tukey HSD

(I) Occupation	(J) Occupation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Pupils	Students	.00000	1.00000	1.000	-3.4622	3.4622
	Farmers	-4.00000*	1.00000	.018	-7.4622	-.5378
	Traders	.66667	1.00000	.997	-2.7955	4.1288
	Drivers	-.33333	1.00000	1.000	-3.7955	3.1288
	House_wives	1.00000	1.00000	.968	-2.4622	4.4622

	Civil_servants	1.33333	1.00000	.873	-2.1288	4.7955
	Public_servants	2.33333	1.00000	.334	-1.1288	5.7955
Students	Pupils	.00000	1.00000	1.000	-3.4622	3.4622
	Farmers	-4.00000*	1.00000	.018	-7.4622	-.5378
	Traders	.66667	1.00000	.997	-2.7955	4.1288
	Drivers	-.33333	1.00000	1.000	-3.7955	3.1288
	House_wives	1.00000	1.00000	.968	-2.4622	4.4622
	Civil_servants	1.33333	1.00000	.873	-2.1288	4.7955
	Public_servants	2.33333	1.00000	.334	-1.1288	5.7955
Farmers	Pupils	4.00000*	1.00000	.018	.5378	7.4622
	Students	4.00000*	1.00000	.018	.5378	7.4622
	Traders	4.66667*	1.00000	.005	1.2045	8.1288
	Drivers	3.66667*	1.00000	.034	.2045	7.1288
	House_wives	5.00000*	1.00000	.003	1.5378	8.4622
	Civil_servants	5.33333*	1.00000	.001	1.8712	8.7955
	Public_servants	6.33333*	1.00000	.000	2.8712	9.7955
Traders	Pupils	-.66667	1.00000	.997	-4.1288	2.7955
	Students	-.66667	1.00000	.997	-4.1288	2.7955
	Farmers	-4.66667*	1.00000	.005	-8.1288	-1.2045
	Drivers	-1.00000	1.00000	.968	-4.4622	2.4622
	House_wives	.33333	1.00000	1.000	-3.1288	3.7955

	Civil_servants	.66667	1.00000	.997	-2.7955	4.1288
	Public_servants	1.66667	1.00000	.707	-1.7955	5.1288
Drivers	Pupils	.33333	1.00000	1.000	-3.1288	3.7955
	Students	.33333	1.00000	1.000	-3.1288	3.7955
	Farmers	-3.66667*	1.00000	.034	-7.1288	-.2045
	Traders	1.00000	1.00000	.968	-2.4622	4.4622
	House_wives	1.33333	1.00000	.873	-2.1288	4.7955
	Civil_servants	1.66667	1.00000	.707	-1.7955	5.1288
	Public_servants	2.66667	1.00000	.202	-.7955	6.1288
House_wives	Pupils	-1.00000	1.00000	.968	-4.4622	2.4622
	Students	-1.00000	1.00000	.968	-4.4622	2.4622
	Farmers	-5.00000*	1.00000	.003	-8.4622	-1.5378
	Traders	-.33333	1.00000	1.000	-3.7955	3.1288
	Drivers	-1.33333	1.00000	.873	-4.7955	2.1288
	Civil_servants	.33333	1.00000	1.000	-3.1288	3.7955
	Public_servants	1.33333	1.00000	.873	-2.1288	4.7955
Civil_servants	Pupils	-1.33333	1.00000	.873	-4.7955	2.1288
	Students	-1.33333	1.00000	.873	-4.7955	2.1288
	Farmers	-5.33333*	1.00000	.001	-8.7955	-1.8712
	Traders	-.66667	1.00000	.997	-4.1288	2.7955
	Drivers	-1.66667	1.00000	.707	-5.1288	1.7955

	House_wives	-.33333	1.00000	1.000	-3.7955	3.1288
	Public_servants	1.00000	1.00000	.968	-2.4622	4.4622
Public_servants	Pupils	-2.33333	1.00000	.334	-5.7955	1.1288
	Students	-2.33333	1.00000	.334	-5.7955	1.1288
	Farmers	-6.33333*	1.00000	.000	-9.7955	-2.8712
	Traders	-1.66667	1.00000	.707	-5.1288	1.7955
	Drivers	-2.66667	1.00000	.202	-6.1288	.7955
	House_wives	-1.33333	1.00000	.873	-4.7955	2.1288
	Civil_servants	-1.00000	1.00000	.968	-4.4622	2.4622

*. The mean difference is significant at the 0.05 level.

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ONEWAY High_vaginal_swab BY Educational_levels
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
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ANOVA

High_vaginal_swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	284.933	4	71.233	8.219	.003
Within Groups	86.667	10	8.667		
Total	371.600	14			

Post Hoc Tests

Multiple Comparisons

High_vaginal_swab

Tukey HSD

(I) Educational_levels	(J) Educational_levels	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nursery	Primary	-2.66667	2.40370	.798	-10.5774	5.2441
	Secondary	-10.33333*	2.40370	.011	-18.2441	-2.4226
	Undergraduate	-11.33333*	2.40370	.006	-19.2441	-3.4226
	Graduate	-7.00000	2.40370	.090	-14.9108	.9108
Primary	Nursery	2.66667	2.40370	.798	-5.2441	10.5774
	Secondary	-7.66667	2.40370	.059	-15.5774	.2441
	Undergraduate	-8.66667*	2.40370	.031	-16.5774	-.7559
	Graduate	-4.33333	2.40370	.422	-12.2441	3.5774
Secondary	Nursery	10.33333*	2.40370	.011	2.4226	18.2441
	Primary	7.66667	2.40370	.059	-.2441	15.5774
	Undergraduate	-1.00000	2.40370	.993	-8.9108	6.9108
	Graduate	3.33333	2.40370	.649	-4.5774	11.2441
Undergraduate	Nursery	11.33333*	2.40370	.006	3.4226	19.2441
	Primary	8.66667*	2.40370	.031	.7559	16.5774
	Secondary	1.00000	2.40370	.993	-6.9108	8.9108

	Graduate	4.33333	2.40370	.422	-3.5774	12.2441
Graduate	Nursery	7.00000	2.40370	.090	-.9108	14.9108
	Primary	4.33333	2.40370	.422	-3.5774	12.2441
	Secondary	-3.33333	2.40370	.649	-11.2441	4.5774
	Undergraduate	-4.33333	2.40370	.422	-12.2441	3.5774

*. The mean difference is significant at the 0.05 level.

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ONEWAY Urine BY Educational_levels
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
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ANOVA

Urine					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2833.733	4	708.433	4.010	.034
Within Groups	1766.667	10	176.667		
Total	4600.400	14			

Post Hoc Tests

Multiple Comparisons

Urine

Tukey HSD

(I) Educational_levels	(J) Educational_levels	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nursery	Primary	-10.33333	10.85255	.870	-46.0500	25.3833
	Secondary	-37.66667*	10.85255	.038	-73.3833	-1.9500
	Undergraduate	-26.00000	10.85255	.193	-61.7166	9.7166
	Graduate	-6.66667	10.85255	.969	-42.3833	29.0500
Primary	Nursery	10.33333	10.85255	.870	-25.3833	46.0500
	Secondary	-27.33333	10.85255	.162	-63.0500	8.3833
	Undergraduate	-15.66667	10.85255	.616	-51.3833	20.0500
	Graduate	3.66667	10.85255	.997	-32.0500	39.3833
Secondary	Nursery	37.66667*	10.85255	.038	1.9500	73.3833
	Primary	27.33333	10.85255	.162	-8.3833	63.0500
	Undergraduate	11.66667	10.85255	.815	-24.0500	47.3833
	Graduate	31.00000	10.85255	.098	-4.7166	66.7166
Undergraduate	Nursery	26.00000	10.85255	.193	-9.7166	61.7166
	Primary	15.66667	10.85255	.616	-20.0500	51.3833
	Secondary	-11.66667	10.85255	.815	-47.3833	24.0500

	Graduate	19.33333	10.85255	.433	-16.3833	55.0500
Graduate	Nursery	6.66667	10.85255	.969	-29.0500	42.3833
	Primary	-3.66667	10.85255	.997	-39.3833	32.0500
	Secondary	-31.00000	10.85255	.098	-66.7166	4.7166
	Undergraduate	-19.33333	10.85255	.433	-55.0500	16.3833

*. The mean difference is significant at the 0.05 level.

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ONEWAY Wound_swab BY Educational_levels
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
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ANOVA

Wound_swab					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	437.067	4	109.267	1.167	.382
Within Groups	936.667	10	93.667		
Total	1373.733	14			

Post Hoc Tests

Multiple Comparisons

Wound_swab

Tukey HSD

(I) Educational_levels	(J) Educational_levels	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nursery	Primary	-4.33333	7.90218	.980	-30.3401	21.6734
	Secondary	-4.66667	7.90218	.973	-30.6734	21.3401
	Undergraduate	-1.33333	7.90218	1.000	-27.3401	24.6734
	Graduate	-15.33333	7.90218	.358	-41.3401	10.6734
Primary	Nursery	4.33333	7.90218	.980	-21.6734	30.3401
	Secondary	-.33333	7.90218	1.000	-26.3401	25.6734
	Undergraduate	3.00000	7.90218	.995	-23.0067	29.0067
	Graduate	-11.00000	7.90218	.646	-37.0067	15.0067
Secondary	Nursery	4.66667	7.90218	.973	-21.3401	30.6734
	Primary	.33333	7.90218	1.000	-25.6734	26.3401
	Undergraduate	3.33333	7.90218	.992	-22.6734	29.3401
	Graduate	-10.66667	7.90218	.669	-36.6734	15.3401
Undergraduate	Nursery	1.33333	7.90218	1.000	-24.6734	27.3401
	Primary	-3.00000	7.90218	.995	-29.0067	23.0067
	Secondary	-3.33333	7.90218	.992	-29.3401	22.6734

	Graduate	-14.00000	7.90218	.438	-40.0067	12.0067
Graduate	Nursery	15.33333	7.90218	.358	-10.6734	41.3401
	Primary	11.00000	7.90218	.646	-15.0067	37.0067
	Secondary	10.66667	7.90218	.669	-15.3401	36.6734
	Undergraduate	14.00000	7.90218	.438	-12.0067	40.0067

ONEWAY Resistance_Wound_Swab BY Bacterial_isolates

/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS

/POSTHOC=TUKEY ALPHA(0.05).

ANOVA

Resistance_Wound_Swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	232.286	2	116.143	11.614	.001
Within Groups	180.000	18	10.000		
Total	412.286	20			

Post Hoc Tests

Multiple Comparisons

Resistance_Wound_Swab

Tukey HSD

(I) Bacterial_isolates	(J) Bacterial_isolates	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S_aureus	K_pneumoniae	8.14286*	1.69031	.000	3.8289	12.4568
	E_coli	3.85714	1.69031	.084	-.4568	8.1711
K_pneumoniae	S_aureus	-8.14286*	1.69031	.000	-12.4568	-3.8289
	E_coli	-4.28571	1.69031	.052	-8.5997	.0282
E_coli	S_aureus	-3.85714	1.69031	.084	-8.1711	.4568
	K_pneumoniae	4.28571	1.69031	.052	-.0282	8.5997

*. The mean difference is significant at the 0.05 level.

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ONEWAY Susceptible_wound_swab BY Bacterial_isolates
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
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ANOVA

Susceptible_wound_swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	66.952	2	33.476	3.348	.058

Within Groups	180.000	18	10.000		
Total	246.952	20			

Post Hoc Tests

Multiple Comparisons

Susceptible_wound_swab

Tukey HSD

(I) Bacterial_isolates	(J) Bacterial_isolates	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S_aureus	K_pneumoniae	3.85714	1.69031	.084	-.4568	8.1711
	E_coli	.14286	1.69031	.996	-4.1711	4.4568
K_pneumoniae	S_aureus	-3.85714	1.69031	.084	-8.1711	.4568
	E_coli	-3.71429	1.69031	.099	-8.0282	.5997
E_coli	S_aureus	-.14286	1.69031	.996	-4.4568	4.1711
	K_pneumoniae	3.71429	1.69031	.099	-.5997	8.0282

ONEWAY Resistance_High_vaginal_swab BY Bacterial_isolates

/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS

/POSTHOC=TUKEY ALPHA(0.05).

ANOVA

Resistance_High_vaginal_swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	315.810	2	157.905	6.713	.007
Within Groups	423.429	18	23.524		
Total	739.238	20			

Post Hoc Tests

Multiple Comparisons

Resistance_High_vaginal_swab

Tukey HSD

(I) Bacterial_isolates	(J) Bacterial_isolates	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S_aureus	K_pneumoniae	3.71429	2.59251	.346	-2.9022	10.3308
	E_coli	-5.71429	2.59251	.098	-12.3308	.9022
K_pneumoniae	S_aureus	-3.71429	2.59251	.346	-10.3308	2.9022
	E_coli	-9.42857*	2.59251	.005	-16.0451	-2.8121
E_coli	S_aureus	5.71429	2.59251	.098	-.9022	12.3308

	K_pneumoniae	9.42857*	2.59251	.005	2.8121	16.0451

*. The mean difference is significant at the 0.05 level.

ONEWAY Susceptible_High_vaginal_swab BY Bacterial_isolates

/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS

/POSTHOC=TUKEY ALPHA(0.05).

ANOVA

Susceptible_High_vaginal_swab

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	619.810	2	309.905	13.174	.000
Within Groups	423.429	18	23.524		
Total	1043.238	20			

Post Hoc Tests

Multiple Comparisons

Susceptible_High_vaginal_swab

Tukey HSD

(I) Bacterial_isolates	(J) Bacterial_isolates	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S_aureus	K_pneumoniae	12.28571*	2.59251	.000	5.6692	18.9022
	E_coli	1.71429	2.59251	.788	-4.9022	8.3308

K_pneumoniae	S_aureus	-12.28571*	2.59251	.000	-18.9022	-5.6692
	E_coli	-10.57143*	2.59251	.002	-17.1879	-3.9549
E_coli	S_aureus	-1.71429	2.59251	.788	-8.3308	4.9022
	K_pneumoniae	10.57143*	2.59251	.002	3.9549	17.1879

*. The mean difference is significant at the 0.05 level.

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ONEWAY Resistance_Urine BY Bacterial_isolates
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
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ANOVA

Resistance_Urine

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3747.524	2	1873.762	9.578	.001
Within Groups	3521.429	18	195.635		
Total	7268.952	20			

Post Hoc Tests

Multiple Comparisons

Resistance_Urine

Tukey HSD

(I) Bacterial_isolates	(J) Bacterial_isolates	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S_aureus	K_pneumoniae	24.57143*	7.47634	.011	5.4906	43.6523
	E_coli	-6.42857	7.47634	.672	-25.5094	12.6523
K_pneumoniae	S_aureus	-24.57143*	7.47634	.011	-43.6523	-5.4906
	E_coli	-31.00000*	7.47634	.002	-50.0808	-11.9192
E_coli	S_aureus	6.42857	7.47634	.672	-12.6523	25.5094
	K_pneumoniae	31.00000*	7.47634	.002	11.9192	50.0808

*. The mean difference is significant at the 0.05 level.

```
ONEWAY Susceptible_Urine BY Bacterial_isolates
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

ANOVA

Susceptible_Urine

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9740.857	2	4870.429	24.895	.000

Within Groups	3521.429	18	195.635		
Total	13262.286	20			

Post Hoc Tests

Multiple Comparisons

Susceptible_Urine

Tukey HSD

(I) Bacterial_isolates	(J) Bacterial_isolates	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S_aureus	K_pneumoniae	41.42857*	7.47634	.000	22.3477	60.5094
	E_coli	-7.57143	7.47634	.579	-26.6523	11.5094
K_pneumoniae	S_aureus	-41.42857*	7.47634	.000	-60.5094	-22.3477
	E_coli	-49.00000*	7.47634	.000	-68.0808	-29.9192
E_coli	S_aureus	7.57143	7.47634	.579	-11.5094	26.6523
	K_pneumoniae	49.00000*	7.47634	.000	29.9192	68.0808

*. The mean difference is significant at the 0.05 level.

(B) Amino acid sequences of ESBL- producing isolates harboring the *bla* gene in both chromosomal DNA and plasmid DNA.

>Seq2 (EUGBO2)

GAGAGAGCTTGCTCTCTTGGAGGAGAGGGGGGCGCGTGAGAATGTATGGGGATACG
CGCCCTAGAGGGGGGTATATATACTCAAAAGAGTGTAATACCCCGTATACTCCCTAC
AGGGGAAAGGGGGAACTTCCAAGATCTCTCTAATTATGAACCCCTATGGGATAATA
TATAAGTGGAGGGAAAAAGTTCACCGGGCMCGATCCGTAGCTGGTGTGAGAGGACG
ACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGGGCAAGCCTGATCCAGCCATTCCGCGTGTATGAAGAAGG
CCCTTGGGTTGTAAAGTACTTTCAGCTGGGGAAGAAGAGGTATCTCCTAATAACGCA
AACGATTGACGGTACCGTCAGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCG
GTAATACGCAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGCAGG
CGGTTTAGTAAGTCAGATGTGAAATCCCAGGGCTTAACCTTGGAATTGCATTTAAGA
CTGGTCAGCTAGAGTCGTCAGAGGGGGGTAGATTCCGGAAGTGGTAAGGCCGTGAA
ATGCGTAGAACCCGGGGAAAGGCGCCCTTGGAAACTTGACTCAAGACGAAAAGGGG
AAAAAA

>Seq6 (EUGBO6)

GCTTCTCGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAG
AGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAATCTCTTAGGAGCAAAG
CAGGGGAACTTCGGTCCTTGCGCTATCGGATGAACCCATATGGGATTAGCTAGTAGG
TGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAG
GGTTGTAAAGTACTTTCAGTCGGGAGGAAGGCGTTGATGCTAATATCATCAACGATT
GACGTTACCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGA
TTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAGACTGGTCA
GCTAGAGTATTGCAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAG
ATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTG

>Seq10 (EUGBO10)

CATGGCTCAGATTGAACGCTAGCGGGATGCTTTACACATGCAAGTCGAACGGCAGC
GCGAGAGAGCTTGCTCTCTTGGCGGCGAGTGGCGGACGGGTGAGTAATATATCGGA
ACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCC
CTACGGGGGAAAGGGGGGGATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCG
GATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTG
AGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTA
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CAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
TGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTG
CATTTTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGTGTAGCA
GTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATA
ATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACAGGATTAGATACC

>Seq15 (EUGBO15)

GAAACTTGGTTCTCTTTGACGAGTGGCGGACGGGTGAGTAATGTATGGGGATCTGCC
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GCAAAGCAGGGGATCTTCGGTCCTCTCTCTATATGGATGAACCCATATGGGATTAGC
TAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTGTGAGAGGAT
GATCAGCACACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATCCCGCGTGTATGAAGAA
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GCAACGATTGACGCTACCTGCAGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA
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GACTGGTCAGCTAGAGTCTGTGTAGAGGGGGGTAGAATTCCATGTGTAGCAGTGAA
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CAA

>Seq16 (EUGBO16)

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GAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTAC
TCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGG
GTGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGAATGAGCCGATGTCGGATTAG
CTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGA
TGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAA
GGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAATACCCG
GTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGC

GGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAG
GTGGTTATTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGAT
ACTGGATGACTAGAATGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAAT
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CACTGAGGCACGAAAGCGTGGGGAGCAAACAGGA

>Seq19 (EUGBO19)

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CGCATAATCTCTCAGGAGCAAAGCGGGGGAACTTCGGTCCTCTCGCTATCGGATGAA
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CTGGTTTGAGAGGAGGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGCAACCCTGATGCAGCCATCCC
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G

>Seq21 (EUGBO21)

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AGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
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GAATATTGCACAATGGGGGCAAGCCTGATGCAGCCATTCCGCGTGTATGAAGAAGG
CCTTAGGGTTGTAAAGTACTTTCAGCAGGGAGGAAGGGGTTGAGGCTAATATCATC
AACGATTGACGTTACCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCG
GTAATACGCAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGCAGG
CGGTCAGTAAAGTTAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTATGA
CTGGCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCAGTGAAATG
CGTAGAAATGTGGAGGAATACCG

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Chief Medical Director

Our Ref: FETHA/AD/ECMC/VOL.1/15

Date: 4th February, 2015

Mr. Emmanuel N. Ugbo
Department of Applied Microbiology
Ebonyi State University, Abakaliki

ETHICAL CLEARANCE

Following your application and subsequent interview, you are hereby informed that Ethical Clearance Management Committee of Federal Teaching Hospital Abakaliki has approved Ph.D Project Research Thesis "Detection of Extracted spectrum Beta lactamase in clinical isolates of *Klebsiella* spp, *Escherichia coli* and *Staphylococcus aureus* from soft tissue infections in Abakaliki, South Eastern Nigeria" by Mr. Ugbo Emmanuel Nnabuike with the Registration Number: 2012487014F. Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, to be carried out in out establishment.

The management believed that you are going to carryout this research according to rules and regulation guiding Hospital Ethical.

Yours faithfully

Chief C. C. Ogbu
Director, Administration