EPIDEMIOLOGY OF EXTENDED SPECTRUM BETA LACTAMASE (ESBL) PRODUCING ENTEROBACTERIACEAE ISOLATED FROM SELECTED HOSPITALS WITHIN OWERRI METROPOLIS IN IMO STATE.

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CERTIFICATION

This is to certify that this research study on "Epidemiology of Extended Spectrum Beta Lactamase (ESBL) Producing Enterobacteriaceae Isolated from Selected Hospitals Within Owerri Metropolis, Imo State" is the original work of MOSES, ABASIODIONG with registration number 20184137578 in partial fulfilment for the award of M.Sc. degree in Biotechnology under the supervision of DR. C. A. Nsofor and DR. R. N. Okechi of the Department of BIOTECHNOLOGY, School of Biological Sciences (SOBS), Federal University Of Technology, Owerri (FUTO).

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DEDICATION

To the true creator of all things, God! And to me, whom without, the work would be an illusion.

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Abstract

Over the past few decades, resistance to antibiotics has been of great concern to the general public, hence the several researches and measures to control it. Extended spectrum β-lactamases (ESBLs) are enzymes produced by plasmids possessed by enterobacteriaceae, capable of hydrolyzing broad spectrum cephalosporins and monobactam but inactive against cephamycins and imipenem compounds. Hence, the aim of this study was to determine the epidemiology of ESBL producing enterobacteriaceae from clinical samples within selected hospitals in Owerri metropolis by determining the resistant bacterial urine and stool, the antibiotic susceptibility pattern and phenotypically determining the ESBL producing isolates using the Double Disc Synergy Test (DDST) method as well as confirming the genes responsible for the resistance using the Polymerase Chain Reaction (PCR). Out of 300 analyzed urine and stool, 165 (55%) yielded bacterial growth; of which 107 (64.8%) was identified as *Escherichia coli (E. coli)* and 58 (35.2%) was identified as *Klebsiella pneumoniae (K. pneumoniae).* The overall resistance rate of the isolates to antibiotics include: Imipenem (50) 30.3%, Ceftazidime (128) 77.6%, Cefotaxime (118) 71.5%, Augmentin (72) 43.6%, Cefepime (133) 80.6%, Trimethoprim (63) 63%, Chloramphenecol (55) 55%, Septrin (65) 65%, Ciprofloxacin (54) 54%, Amoxicilin (65) 65%, Augmentin 2 (42) 42%, Gentamicin (44) 44%, Pefloxacin (49) 49%, Ofloxacin (60) 60% and Streptomicin (56) 56%. A total of 72 bacterial isolates (43.6%) were identified as ESBL producing, comprising 47 (65.3%) *E. coli* and 25 (34.7%) *K. pneumoniae*. Three (3) ESBL genes TEM, SHV and CTX-M were detected on seventeen (17) random isolates, with a prevalence rate of 5 (29.4%) for TEM/SHV, 9 (52.9%) for TEM/CTX, 4 (23.5%) for SHV/CTX, 4 (23.5%) for the three (3) genes and 2 (11.8%) did not possess any of the genes. Several factors have been traced to be a contributing factor to antibiotic resistance such as drug abuse, poor diagnosis, poor treatment of ailments, poverty, intake of fake and expired drugs, among others hence the need for need for public awareness on the dangers associated with abuse and intake of fake or expired of antibiotics. Most importantly the governments' provision of primary health care centers where patients can have easier access to health care workers is encouraged. The study underlined the high prevalence of antibiotic resistance among humans, hence the need to adopt quicker and more effective measures to combat them.

Keyword: Antibiotics, Enterobacteriaceae, resistance, Extended spectrum β-lactamases (ESBLs)

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Over the years, increase in antibiotics resistance has caused laboratory and global concern as it is responsible for high rate of life-threatening infections and mortality (Zaman, Hussain, Nye, Mehta, Mamun, & Hossain, 2017). These resistant bacteria have been found to be very difficult to combat hence, increasing treatment cost, reducing quality of life, leading to longer hospital stay, increasing family burden of infected individuals and eventually death of affected individuals if not properly managed. Antibiotics wrongly prescribed for viral infections by physicians have also contributed to antibiotic resistance as antibiotics are incapable of tackling viruses (Nsofor, Nwokenkwo, & Ohale, 2016).

Extended Spectrum Beta-Lactamase (ESBL) are plasmid mediated enzymes produced by Gram-negative bacteria which poses resistance to antibiotics such as Penicillin, Aztreonam, Aminoglycosides and susceptible to cephamycin such as cefoxitin and carbapenems comprising meropenem, imipenem, ertapenem, and doripenem (Poulou, Grivakou, Vrioni, Koumaki, Pittaras, Pournaras, & Tsakris, 2014; Ali, Ali, Khan, Han, & Gao, 2018). The prevalence of ESBL in hospitals has been reported to range between 6 and 88% in various hospitals (Sathya, Anuradha & Priyadharshini, 2013). ESBLs production among Enterobacteriaceae such as *Klebsiella pneumoniae* and *Escherichia coli* is considered the most common mechanism of resistance to

third-generation cephalosporins (Olowe, Adewumi, Odewale, Ojurongbe, & Adefioye, 2015). These enzymes (ESBLs) are diverse, complex and they evolve rapidly causing vital therapeutic challenges in the treatment of patients (Kajeguka, Nambunga, Kabissi, Kamugisha, Kassam, Nyombi, & Chilongola, 2015).

The major antibiotic usually chosen for treatment of infections included beta lactam antibiotics agents such as penicillins, cephalosporins, monobactams and carbapenems but reports of resistance followed thereafter (Sreekrishna, Babu, Ashokkumar & Sivakumar, 2012). Plasmid-mediated ESBL are encoded on the large plasmids conveying genes which transfer resistance to other antimicrobial agents, hence manifesting resistance to various classes of antibiotics (Ugah & Udeani, 2020).

As antibiotics resistant bacteria and ESBL producing isolates increase, it is necessary to recognize the dominant species that this enzyme as well as the most efficacious treatment options is necessary for each community (Gharavi, M., Zarei, J., Roshani- Asl, P., Yazdanyar, Z., Sharif, M. & Rashidi, N. 2021).

1.2 Problem Statement

The emergence and rapid dissemination of Extended-Spectrum Beta-Lactamase (ESBL)-producing Enterobacteriaceae pose a significant threat to public health, necessitating a comprehensive understanding of the epidemiological dynamics in specific geographic areas. Owerri metropolis, as a densely populated urban

center, faces unique challenges in managing infectious diseases. Despite the global concern regarding antibiotic resistance, there is a paucity of local data on the prevalence, distribution, and risk factors associated with ESBL-producing Enterobacteriaceae in clinical samples within Owerri metropolis. This study will address this gap by investigating the epidemiology of ESBL-producing Enterobacteriaceae obtained from clinical samples in Owerri metropolis. Understanding the epidemiology of ESBL-producing Enterobacteriaceae in Owerri metropolis is crucial for guiding effective antimicrobial stewardship programs, informing infection control measures, and ultimately safeguarding public health in the region.

1.3 Objectives

The aim of the study is to determine the epidemiology of ESBL producing enterobacteriaceae obtained from clinical samples within Owerri metropolis.

Objectives

1. To determine the prevalence of enterobacteriaceae among the different genders and age groups.
2. To characterize the isolates by determining their antibiotic susceptibility pattern.
3. To Phenotypically identify the ESBL- producing isolates
4. To identify ESBL genes

1.4 Justification of the Study

Knowing local ESBL carriage prevalence will help guide the medical practitioners on better clinical care and treatment procedures to patients.

It will guide the government into providing special care and financial support for further research to tackle the issue.

1.5 Scope of Research Work

This research work is set out to study, assess and identify ESBL producing enterobactericeae isolated from clinical samples in Owerri metropolis. This would add to the already existing information on molecular detection and characterization of ESBL producing enterobactericeae isolated from clinical samples.

CHAPTER TWO

LITERATURE REVIEW

2.1 Antibiotics and Resistance

Antibiotics are antibacterial drugs, which refer to natural metabolites of microorganisms such as fungi, actinomycetes, and bacteria and are used for killing or restricting growth of target bacteria

Generally, it is perceived that antibiotic production is triggered to compete for available space and nutrients and is likely vital for the survival and persistence of the host organisms. Enzymes that inactivate other antibiotics and develop resistance against them are secreted by the host bacterium as other secretory products of bacterial cells such as TpsA proteins, which are also thought to secrete and kill other competing similar bacteria (Piet, Ulsen, & Rahman, 2016; Rahman, 2018; Ulsen, 2014). Sir Alexander Fleming in 1928 observed that an opportunistic fungus, *Penicillium notatum*, was able to deny the growth of *Staphylococcus aureus* on an agar plate and in fact that novel discovery led to the road of application of those antimicrobial compounds against the microorganisms. The secreted inhibitory substance "penicillin" was thus soon showcased in the market that saved many lives during 1941 in England (Wright, 2019).

The discovery of penicillin and its potential use for restricting many infections inspired many scientists to look for other natural compounds and the quest of that golden antibiotic discovery era from 1945 to 1980 led to the

introduction of many successful antibiotics currently used in clinics. Remarkably, during this era, not only that a large number of new categories of antimicrobials were discovered, but they were also made available in the market for use against infections. From 1980s onward until recently 2000s, alongside discovering new antibiotics, emphasis was also made on improvements of already available antimicrobial drugs. Although discovery of the new antibiotics remained low during the last few years, yet, mining for more efficient and safe drugs is still an ongoing and unending expedition a hot issue in the last decades in the field of medicine. Recently a lot has been invested in formulating and designing new synthetic drugs that could replace antibiotics. Currently, in the shelf of antibiotics, there are drugs that are either semisynthetic, a modified natural product, or synthetic that is chemically designed in the laboratories, such as sulphonamides and quinolones (Normark & Normark, 2012).

Based on the diversity of the origin of these compounds, there are generally three different bases of antibacterial classification:

(i) Chemical structure,

(ii) Target site of the drugs, and

(iii) Impact of final outcome on the target such as bactericidal or bacteriostatic nature.

Categorization based on the end effect (bactericidal or bacteriostatic) can be ambiguous, because some drugs have bactericidal effect against one bacterium and bacteriostatic effect against another. Categorization based on the chemical

structure is inadequate because of the diversity among agents. Finally, grouping on the basis of target site is more appropriate because it helps in understanding the molecular basis and mode of action of antibacterial action.

Antibiotics usually exert their effects through one of the five mechanisms:

(i) Cell wall synthesis inhibition (penicillins, monobactams, carbapenems, and bacitracin),

(ii) Cytoplasmic membrane inhibition (polymyxins),

(iii) Bacterial protein synthesis inhibition (chloramphenicol, lincosamides, macrolides, aminoglycosides, tetracycline, etc.),

(iv) Nucleic acid synthesis blocker (quinolones, nitroimidazoles, and rifampicin), and

(v) Folic acid synthesis blocker (sulphonamides, trimethoprim, etc.) (Samaha-Kfoury & Araj, 2013).

The β-lactam drugs are the most important and frequently used groups of antimicrobials that inhibit cell wall synthesis resulting in lysis of bacterial cells and thus are bactericidal. They have been categorized, on the basis of the chemical structure with the β-lactam ring, into six major groups: penicillins, cephalosporins, cephamycins, carbapenems, monobactams and β-lactamase inhibitors (Finch, 2018).

With the introduction of penicillin for the first time, people were optimistic to end the dominance of bugs and overcome the evils of pathogens; however, very soon, penicillin was seen to be ineffective against some infections due to emergence of

resistance against penicillin and the optimism was slashed (Abraham & Chain, 2019). Nevertheless, this phenomenon of emergence of resistance had accelerated the quest of human to overcome the resistance phenomenon by discovering next-generation antibiotics such as cephalosporin. The quest for discovering new antibiotics has begun in order to discover drugs that can kill all existing pathogens which led to the discovery of second generation (cefoxitin, cefotetan, cefmetazole, cefaclor, cefpodoxime and cefuroxime), third generation (cefixime,cefodizime, cefotaxime, cefteram and ceftizoxime), and fourth generation (cefepime, cefluprenam, cefoselis, cefozopran, cefpirome and cefquinome) cephalosporin drugs; however, at the same time bugs are fighting back for dominance and looking for new traits to adopt.

Meanwhile, the advancement of technology to unravel genetic composition in parallel with improvements in transcriptomic analysis helped biotech industry to engineer genetically modified strain with better production of improved antibiotics, such as engineering of *Streptomyces hygroscopicus* 5008 through metabolic engineering for enhanced production of validamycin (Tan *et. al,* 2015; Zhou & Zhong, 2015). Such an improvement towards production of new natural secreted antibiotics in response to enzymes that inactivate beta-lactams and carbapenems would certainly help overcoming shortage of new antibiotics. No doubt, there would have been a pool of resistance elements against all those naturally effective drugs and smart bugs were able to finally acquire them from nature and display them for their own defence translating emergence of horrible

broad resistance against those otherwise highly effective recently discovered drugs.

Antibiotics of β-lactam group are used globally and approximately 50% of all prescribed antimicrobials belong to this group. Unfortunately, however, resistance to this important safe and efficient class of antibiotics is increasing worldwide (Ciˇzman, 2018; Pitout *et. al* 2019). Emergence and increasing occurrence of resistance is putting a lot of pressure and presents challenge to healthcare experts. Resistance to β-lactam compounds is mainly due to the production of beta-lactamases (BLs) that hydrolyze and thereby inactivate beta-lactam antibiotics (Jacoby & Munoz-Price, 2015). The complexity and heterogeneity of BLs can be estimated from the constantly increased discoveries, and today more than 900 types, produced by many different species of bacteria, are recognized. Due to the broad spectrum activity against the latest cephalosporin (the extended spectrum cephalosporins), the extended spectrum beta-lactamases (ESBLs) are of specific concern. Blind and persistent use of antibiotics is thought to be one of the important triggers to provoke and spread antibiotic resistance. The objectives of the current manuscript is to accumulate concise updates regarding resistance offered by ESBL producing *Enterobacteriaceae* with special emphasis on structural and functional diversity, clinical significance and global epidemiology.

2.2 The β-Lactam Antibiotics

The β-lactams are a group of antibacterials comprising four major groups: penicillins, cephalosporins, monobactams and carbapenems (Samaha-Kfoury & Araj, 2013). See figure 2.1

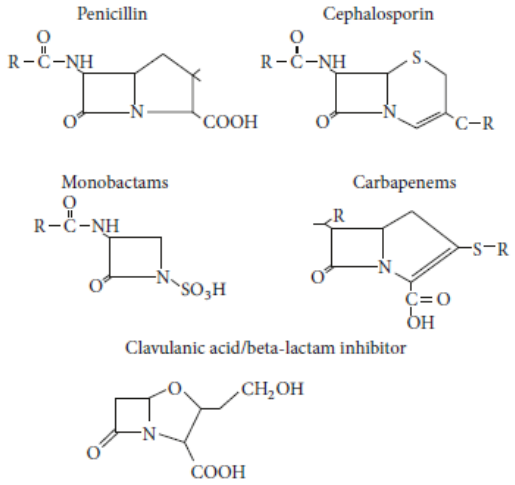


Figure 2.1 Structure of beta-Lactam compounds (Ali *et al.* 2018)

Structurally, they consist of a β- lactam ring, which consists of three carbon atoms and one nitrogen atom and is linked to a thiazolidine ring. In cephalosporins, the β-lactam ring and dihydrothiazine ring are merged; however, in the carbapenems, the β-lactam ring is joined with a hydroxyethyl side chain, deficient of an oxygen or sulphur atom in the bicyclic nucleus, while in monobactam there is no additional ring (Samaha-Kfoury & Araj 2013).

2.3 Mechanism of Action of β-Lactams.

There has been enormous improvement in understanding physiological principles of drug action, its metabolites, and fate by using the latest state-of-the-art genomic and functional techniques. No doubt, on-hand current improved technology helped us to elucidate many unknown principles and mechanism of actions and emergence of resistance of many antibacterial drugs. The β-lactams execute their antibacterial activity by inhibiting bacterial cell wall, peptidoglycan, and synthesis by preventing precise functioning of Penicillin Binding Protein (PBO), which is also known as transpeptidases (Parija, 2014). Peptidoglycan is a crucial structural constituent of the bacterial cell and periplasmic part. Apart from rigidity, it provides protection from the high internal osmotic pressure and gives an overall defined shape to a bacterial cell. PBP catalyzes cross-linking between amino acids of adjacent chains of amino acids that build into a mesh in the periplasmic space between the inner and outer membrane. Interestingly, β-lactam ring is similar to that of D-alanine-D-alanine of the N-acetylmuramic acid (NAM) pentapeptide; and thus PBPs "mistakenly" (due to very close shape resemblances) pick these up (β-lactam in fact) to use them as building blocks during cell wall synthesis. Bacterial cell pays for this mistake that leads to acylation of the PBP and thus eventually renders the enzyme (transpeptidases) inactive with inhibition of the transpeptidation reactions resulting in accumulation of cell wall precursor units that trigger activation of the cell wall autocatalytic system, leading to cell lysis (Zapun, 2018). By simultaneously blocking transpeptidases and activating

autolysin, β-lactams lead to disruption of the synthesis of cell wall and initiates its active destruction finally resulting into cell lysis and elimination of the bug.

2.4 Mechanism of Bacterial Resistance to β-Lactam Antibiotics.

Antibiotic or antimicrobial resistance arises when the otherwise effective antibiotics or antimicrobials are no more effective in controlling the pathogen. Resistance is noticed, or any bacterium is called resistant if it survives at higher dose of permissible drugs. When the antibiotic is not able to kill the pathogen then the term is equivalent to drug tolerance or drug failure. When the organism becomes resistant to more than one type of antibiotic then the organism is referred to as multidrug resistant. Bacteria acquire these features with time to resist a previously effective drug. Bacteria evolve a number of mechanisms of resistance. Particularly, for β-lactams, thus far four major ways have been known that are used by host bacteria to avoid the bactericidal effects of β-lactams.

(I) β-lactamase production that breaks the β-lactam ring and makes the antibiotic inactive before it reaches the PBP target. This mode of mechanism is very common in *Enterobacteriaceae* such as *Escherichia coli* and *Klebsiella pneumoniae (*Massova & Mobashery, 2019).

Subsequent sections of this work will focus on ESBL resistance

(II) Expressing altered and mutated PBPs, this mechanism is responsible for resistance to penicillin in pneumococci and methicillin resistance in Staphylococci *(*Fedarovich, 2018).

(III) Absence or reduced expression of outer membrane proteins (OMPs) in Gram-negative bacteria (Delcour, 2019).

(IV) Overexpression of efflux pumps, a system that ejects the antibiotics out of the cell with energy expenditure, thereby decreasing intracellular concentrations of the antibiotic.

Essentially, this active efflux system is comprised of a complex of specialized proteins that form a bridge between the cytoplasmic membrane and the outer membrane. A carrier protein in the cytoplasmic membrane, capable of capturing molecules located in the membrane or the cytoplasm, is linked to an "accessory protein," connected in turn with an outer membrane protein channel. Although the efflux of antimicrobials is the most common mode of acquired resistance to tetracyclines, it has been involved in developing resistance to other drugs including β-lactams (Li & Nikaido, 2019).

2.5. The β-Lactamases

β-Lactamases enzymes are produced by microorganisms and are able to break β-lactam molecules rendering them inactive and thus singularly or in part enable β-lactam resistance. So far, more than 500 β-lactamases have been reported produced by diverse bacteria. Beta-lactamases are thought to be the most common resistance mechanism that contributes to widespread resistance among Gram-negative bacteria (Bush & Jacoby, 2020). In Gram-negative microbes, the β-lactamase mediated resistance is either plasmid mediated or expressed

chromosomally. Nevertheless, the spread of β -lactamases is frequently linked to plasmid- mediated ESBLs, specifically the CTX-M family (Pitout, 2020). In Gram-negative organisms, chromosomally located inducible expression of β -lactamases is also common, while plasmid mediated enzymes are generally expressed constitutively. Generally, two classification systems for these enzymes are currently in use. The first is Ambler molecular classification or molecular classification, which is based on the conserved motifs and protein sequence that further categorize these enzymes into four such as classes A, B, C, and D enzymes. These enzymes utilize serine for β-lactam hydrolysis and class B metallo-enzymes that require divalent zinc ions (metal ion) for substrate hydrolysis. The second categorization is named as Bush, Jacoby and Medeiros functional classification, which group different β -lactamases according to their substrate and inhibitor profiles. This method correlates the beta-lactamases with phenotypes in clinical isolates. The latest updated functional classification is comprised of three groups: group 1 (class C), cephalosporinases; group 2 (classes A and D), broad spectrum, inhibitor resistant, extended spectrum β-lactamases, and serine carbapenemases; and group 3 (class B), metallo- β-lactamases (Ambler, 2019). Group 1 enzymes are usually encoded in the chromosome and are usually resistant to inhibition by clavulanic acid. AmpC expression is low, but when accumulated in large amount in the host cells it can offer resistance against carbapenems, mainly against ertapenem (Bradford, Urban, Mariano, Projan, Rahal, & Bush 2017). The traditional plasmid mediated CMY (blaCMY-1-136),

ACT, DHA, FOX, and so on are now outnumbered by the common plasmid-mediated subgroup 2be ESBLs. Subgroup 2a penicillinases, although a small group and with limited spectrum, are predominant β-lactamases produced by Gram-positive cocci such as staphylococci. Notably, subgroup 2b containing TEM-1, TEM-2, and SHV-1 enzymes efficiently hydrolyzes penicillins and early cephalosporins (cephaloridine) and is strongly inhibited by clavulanic acid and tazobactams. Furthermore, group 2be enzyme exhibits extended spectrum activity that hydrolyzes one or more oxyimino- β-lactamases (cefotaxime, ceftazidime, and aztreonam), derived from amino acid substitution in TEM-1, TEM-2, and SHV-1 (Queenan, Foleno, Gownley, Wira, & Bush, 2014).

2.6. Extended Spectrum β-Lactamases (ESBLs)

Among the β-lactamases, ESBLs are worthy of the attention of the scientific community over the last decades. Generally, ESBLs are plasmid-borne and are known for their ability to hydrolyze oxyimino-cephalosporin (3rd- and 4th-generation cephalosporins) and monobactams but not cephamycin such as cefoxitin and carbapenems comprising meropenem, imipenem, ertapenem, and doripenem. Furthermore, these are generally susceptible to β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. Classically, ESBLs are defined as enzymes originally derived or evolved from a narrow spectrum parent ESBLenzyme and thus gained the ability to inactivate the broad spectrum cephalosporins, penicillins, and aztreonam, but not the cephamycins (cefoxitin)

or carbapenems by hydrolytic activity and are inhibited by β-lactamase inhibitors, that is, clavulanic acid. The older and classical definition of ESBL includes derivatives from TEM-1, TEM-2, or SHV. Most recent definition divides ESBL into three main groups.

(i) ESBLA (class A ESBLs) comprises the most frequently found ESBL and the CTX-M, as well as SHV and TEM enzymes. These enzymes are mainly horizontally transferable and can be inactivated or inhibited by clavulanic acid.

(ii) ESBLM (miscellaneous ESBLs) are sectioned into ESBLM-C (class C, plasmid mediated AmpC) and ESBLM-D (class D). Acquired AmpC are the most frequently found ESBL in this class.

(iii) ESBLCARBA (ESBLs which degrade carbapenems) are divided into ESBLCARBA-A, ESBLCARBA-B, and ESBLCARBA-D (Zhao & Hu, 2018).

ESBLs are often found carried on large plasmids in addition to other resistance genes that confer resistance to antimicrobials such as aminoglycosides and sulphonamides (Storberg, 2016).

2.7. Types of ESBLs

Among the many ESBLs described in a variety of pathogens, CTX-M, TEM, and SHV types proved to be the most successful in terms of promiscuity

and dissemination across various epidemiological niches. It is thought that two main evolution strategies have been adopted by various Gram-negative bacteria such as *E. coli* and *K. pneumoniae*:

(i) The assortment of mutants with extended substrate specificity from the already prevalent TEM and SHV types of β-lactams; and

(ii) Uptake and capturing of novel broad spectrum β- lactamases genes from the naturally existing metagenome, coding for enzymes naturally endowed with ESBL activity.

Various reviews have been published on the types of ESBL β-lactamases (Zhao & Hu, 2018; (Cantón, Novais, Valverde, Machado, Peixe, Baquero, & Coque, 2017); however, they are evolving so rapidly that regular review of the accumulated knowledge on the structure and functional diversity of ESBL is necessary to update the readers.

2.7.1. CTX-M.

Although a bit recently discovered, CTX-M enzymes are the most increasingly reported types of enzymes associated with resistance. CTX-M enzymes are plasmid based encoded cefotaximases that constitute the fast growing family of ESBLs (Zhao & Hu, 2018). CTX-Ms are named after their extended activity against cefotaxime compared to ceftazidime and the origin of its first isolation (Munich, Germany) (Birbrair & Frenette, 2016). Among other ESBLs, CTX-M

enzymes have been proven to be the most efficacious in terms of promiscuity and its predominance abundance in diverse epidemiological settings, where they have largely replaced and outnumbered other ESBL types such as TEM9 (D'Andrea, Arena, Pallecchi, & Rossolini, 2019). Thus far, 172 CTXM variants have been reported to date with distinct amino acid sequence and functional characteristics along with expression of other resistance elements critically reducing response to treatment. Unlike the TEM and SHV ESBLs (see below), CTX-M type enzymes did not arise as a result of alterations of existing enzymes; they were acquired de novo by lateral gene transfer from *Kluyvera* sp. (D'Andrea, Arena, Pallecchi, & Rossolini, 2019). A phylogenetic tree can be drawn based on the amino acid sequence to determine the relatedness among the members of CTX-M β-lactamases. CTX-M has been divided into six sublineages or groups (CTX-M-1, CTX-M-2, CTXM-8, CTX-M-9, CTX-M-25, and KLUC, entitled after the first member of the group that was described). Members within a group have >94% amino acid relatedness and ≤90% relatedness across the groups. Additionally, there are about four CTX-M variants that exhibit a hybrid structure, namely, CTX-M-45 (formerly Toho-2), which is a hybrid of CTXM- 14 with a protein of unknown origin, and CTX-M-64, CTX-M-123, and CTX-M-132 that are hybrids of CTX-M- 15 with different segment CTX-M-14 (Zhao & Hu, 2018). While the main variants of CTX-Ms are biologically different, CTX-M-15 and CTX-M-14 are the most common variants detected globally in important microbes, followed by CTX-M-2, CTX-M-3, and CTX-M-1 (Levy, 2020). In the early 1990s, reports

from distant countries suggested the potential of spread of these enzymes and its ability to disseminate. During this time, diversification was also noticed, illustrated well by the CTX-M-3, closely related to CTX-M-1 differing in four amino acid positions (V77, D114, S140A, and N288D). In this context, CTX-M-10 was reported in the Mediterranean areas (Oliver, Pérez-Díaz, Coque, Baquero, & Cantón, 2019) and CTX-M- 15 in New Delhi (Karim, Poirel, Nagarajan, & Nordmann, 2017). The CTX-M-10 differs in two amino acids (at positions A27A and R38Q) from CTX-M-3, while CTX-M-15 differs in a single amino acid at position (D240G); presumably, all these three might have been produced from a common ancestor.

2.7.2. TEM ESBLs.

TEMs are mostly encoded by Gram-negative bacteria. Almost 90% of the resistance against ampicillin in Gram-negative bacteria is due to TEM encoded genes (Rice, Willey, Papanicolaou, Medeiros, Eliopoulos, Moellering Jr, & Jacoby, 2019). The TEM-type ESBLs are often plasmid mediated derived from mutations in the classic TEM (TEM-1 and TEM-2) genes by single or multiple amino acid substitution around the active site. *E. coli*, isolated from a patient named Temoneira (hence, named TEM) in Athens, Greece, harboring resistance encoded by *TEM-1* was the first ever report in 1965 (Steward, Wallace, Hubert, Lawton, Fridkin, Gaynes, & Tenover, 2020). TEM-1 is able to hydrolyze penicillin and 1st-generation cephalosporin such as cephaloridine. TEM-2 derived from the original TEM-1

enzymes as a result of single or multiple amino acid sequence mutations. All of them have a similar hydrolytic profile but each one has different isoelectric point, and hence not considered as ESBL (Karim, Poirel, Nagarajan, & Nordmann, 2017). In 1987 *Klebsiella pneumoniae* isolates spotted in France as early as 1984 were found to harbor a new plasmid mediated β-lactamase coined CTX-1 because of its greater activity against cefotaxime. The enzyme, now called TEM-3, differed from TEM-2 by double amino acid replacements (Alsterlund, Carlsson, Gezelius, Haeggman, & Olsson-Liljequist, 2019). Later on, TEM-5 and TEM-4 were discovered that were found 3 and 4 amino acid different from the parent TEM-1 (Alsterlund, Carlsson, Gezelius, Haeggman, & Olsson-Liljequist, 2019). TEM-12 was the 1st TEM-type ESBL detected in *Klebsiella oxytoca,* isolated in Liverpool, England, in 1982 (Vázquez, Bellido, García, & García-Rodríguez, 2016).

2.7.3. SHV ESBLs.

SHV types of enzymes are mostly found in *Klebsiella* species (especially *K. pneumoniae*) most often housed by a plasmid. However, a number of species have been shown to carry SHV-1 gene within the chromosome (Zhao & Hu, 2018). SHV denotes sulfhydryl variable as it was believed that the inhibition of SHV activity by chloromercuribenzoate was substrate-related and was found inconstant according to the substrate used for the assay (Randall, Clouting, Horton, Coldham, Wu, Clifton-Hadley, & Teale, 2017). SHV-2 was the 1st SHV-ESBL type detected in *Klebsiella ozaenae* isolated from Germany, in 1983.This

enzyme originated from point mutation in SHV-1 which resulted in substitution of glycine by serine at the 238 positions and extension of its hydrolytic substrate profile to include cefotaxime and to a minor degree ceftazidime (Perilli, Segatore, Mugnaioli, Celenza, Rossolini, Stefani, & Amicosante, 2018). Unlike TEM and CTX-M, SHV has few variants. Moreover, the substation of amino acid and position of substation is restricted to a limited and narrow region unlike the broad area of CTX-M.

2.7.4 OXA ESBLs.

The OXA type is remarkably increasing family of ESBLs. These β-lactamases differ from the TEM and SHV enzymes in that they fit into molecular class D and functional group 2d exhibiting oxacillin-hydrolyzing capabilities (Lederberg & Harrison, 2019). They mainly have been reported in *Pseudomonas aeruginosa* unlike TEM and SHV which are prevalent in Enterobacteriaceae. Most of these enzymes are resistance to ampicillin and cephalothin with high hydrolytic activity against oxacillin and cloxacillin, but poorly inhibited by clavulanic acid and cannot degrade the newer cephalosporins so they are not viewed as ESBLs. However, OXA-10 destroys (weakly) cefotaxime, ceftriaxone, and aztreonam, giving most microbes reduced susceptibility to these antimicrobials. Other OXA ESBLS include OXA-11, -14, -16, -17, -19, -15, -18, -28, -31, -32, -35, and -45 (Levy, 2020). Altogether, OXA type βlactamases is explosively increasing based

on the amino acid sequence variations and so far 498 variants have been reported and arranged in the database.

2.7.5 Minor Extended Spectrum β-Lactamases.

During the last ten years, class A β-lactamases have been designated, including SFO, BES, BEL, TLA, GES, PER, and VEB types. Some of these minor ESBL are infrequently identified or are very restricted; others are becoming locally prevalent or are progressively isolated globally.

2.8 Genetic Factors Contributed to Successful Dissemination of ESBLs

Factors associated with genetics contributing to current highly penetrating global dissemination of ESBL are poorly understood. However, molecular epidemiology of ESBL indicated a strong association of ESBL spread with conjugative plasmids and successful clones (Naseer & Sundsfjord, 2017). Mobile genetic elements with the ability to jump between different loci of the same chromosome or from chromosome to plasmid or vice versa have been shown implicated in the spread of resistance elements. Therefore, it is crucial to understand the molecular features of the plasmids and associated genetic elements that help resistance elements to transfer between species. In addition to circular plasmid with high efficiency of transformation, transposons are DNA fragments able to move from one place to another in the bacterial nucleic acids through transposition and can be inserted nonspecifically at any place in the

bacterial nucleic acids. There are numerous different kinds of transposons, but normally they contain a transposase promoting transposition, overturned repeats in the ends, and short direct repeats of target nucleic acids bracketing the transposons. Conjugative transposons are one more type holding genes for conjugative transfer from one cell to another. Though it rarely happens and is highly regulated, transposition is one of the genetic factors that contribute to spread of resistance elements. The most important element that has been shown highly associated with successful transmission of resistance elements is integrons (Ali, Ur Rahman, Zhang, Shahid, Zhang, Liu, & Han, 2016). These are genetic elements incorporated in transposons found on sets of plasmids and in the bacterial chromosome. These gene capturing systems are progressed from site specific recombination mechanisms, and a general integrin encodes a DNA integrase gene *(int)* and a neighboring recombination site *(att1) (*Mazel, 2016). Generally, within the variable region, multiple resistance associated genes could be integrated in the form of gene cassettes that are basically incorporated in the attachment site (attI) of the integron which can have many cassettes at once. Different integrons classes have been described, and classes 1, 2, and 3 have been associated with antibiotic resistance. Most often, they encode for multiple resistances and thus associated with coresistance. Importantly, integrons by itself are not able to jump, but their gene cassettes could be mobilized as they are often found as part of the transposons or plasmid and thus could be integrated into secondary sites, thereby conferring new resistance phenotype. Therefore, plasmid replicon typing is quite

useful technique to analyse the strength of the ESBL to be associated with other coresistance and types of integrons that have potential of promiscuity and no specific integration and transmission. It has been suggested that the association of IncFII plasmid encoding ESBL type CTX-M-15 in the well-adapted strain of *E. coli* ST131-O25:H11 is linked to its successful widespread global dissemination (Naseer & Sundsfjord, 2017; Peirano, van der Bij, Gregson, & Pitout, 2019). In addition to those described elements, other genetic elements such as toxin-antitoxin systems have been pointed that bacteria most probably would use this system to maintain the resistance elements. A very recent phenomenon of multiple addiction systems was reported in plasmids bearing bla CTX-M types dependent on the multiple addiction system for plasmid maintenance and probably transmission (Tamang, Nam, Gurung, Jang, Kim, Jung, & Lim, 2018). An addiction system or a toxin-antitoxin system helps sustain plasmids in bacteria while replicating in the host by eliminating/killing plasmid-free cells resulting from segregation or replication defects. In short, this is vital to understand the genetic structural and functional elements that contribute to promote maintain and spread of resistance elements either directly or indirectly. The insertion sequences (IS) on the one hand can cut and paste ESBL encoding genes between the plasmids and, on the other hand, can enhance the expression of these enzymes.

2.9 Factors Affecting the Spread and Considerations

To simplify the resistance phenomenon, we can keep working and concentrate on two main factors, the antibiotics themselves that provide selective pressure to dominate which have the second important element/factor: the genes. If, for example, either the antibiotic or the resistance genes did not exist, we would not have the phenomenon of resistance after all. In fact, referring to the clinical cases, finding of resistance to new drugs by a pathogen is not unexpected, as antibiotics and other organic molecules resembling antibiotics are always found in the environment. Bacteria, after their many millennia of existence, might have confronted them at some point and that would have affected their growth; to survive, bacteria would have acquired resistance to these molecules. However, the emergence of these traits in the clinical isolates or in the hospital setting is what actually warns the clinicians about the use of certain drugs. In this respect, discovery of the new resistance elements in bacteria, for example, in the commensal, foretells future complications in the pathogens in clinical isolates of that hospital, community. In the United States alone, an estimated 9.45×10^6 kg of antibiotics is used annually; half of this is provided to people for use during sickness and the rest for agricultural use (Lederberg & Harrison, 2019).

These drugs in hospitals are commonly delivered parenterally, while in the community they are delivered through oral prescription. About 7×10^6 kg of antibiotics, mainly penicillin and tetracycline, are routinely used annually in animals as growth promoters (Lederberg & Harrison, 2019). On top of that an

estimated 4.5 $\times 10^4$ kg of antibiotics, chiefly tetracycline and streptomycin, are sprayed over the fruits and crops annually. Most of these would certainly reside in environment as residues for some time and bacteria would confront them at a point. A pool of genes resistant to these antibiotics would certainly arise in the environment in response to selective pressure provided by the existing residues of these antibiotics. Above is the amount used in a country where antibiotics are narrowly and strictly prescribed. But based on the prevailing practices about how antibiotics are provided in US, it is nevertheless hard to estimate that they are currently using higher amount of antibiotics in clinical, agriculture and food animals. This blind use of antibiotics is thus encouraging new elements of resistance to emerge against antibiotics. In this way, antibiotics are excreted in the environment, water, crops, and so on, where the antibiotics keep exerting selective pressure resulting in "posttherapy" environmental selection phase of the antibiotic (Levy, 2020). During such phase, the antibiotic concentration would be less than the therapeutic concentration, which is ideal for selecting resistance. Thus, considering this scenario, it may not be the use of persistent high concentration of antibiotics in clinics for treatment (treatment period), rather the slow and persistent release of low amount of antibiotics in the environment (post-therapy period) that provide ample amount of time for bacteria to develop resistance. Altogether, we should really change our mentality and course of action against the use of antibiotics. First, we should emphasize implementing the shorter course of antibiotics. Secondly, recycling of the antibiotics should be

considered if new antibiotics are available in the market. Thirdly, we should alongside invest in synthesizing or discovering new antibiotics such as improvement of the current tetracycline drugs; understanding the mechanism of resistance to currently available tetracycline drugs would help improve the efficacy. A chemical free, nonclassical approach to retreat the resistance problem would be the recovery of the vulnerable strain. We need to encourage the growth of the susceptible strain to take over the resistant one. One of the approaches would be to reintroduce the susceptible flora in the form of probiotic. As we understand more and more the chain of producing resistance elements that provide impetus to the rise of antibiotic resistance in human, animal, food, and environment, we need to block those chains and encourage the forward lane for improving the susceptible strains. Also, instead of attacking and jumping from one factor to another, sticking to one of the responsible factors and eliminating it eventually would help us to eliminate resistant bacteria. We need to bring "peace" instead of attacking and conquering the bacteria. Commensal bacteria are our allies and we need to encourage them and to take over the resistant ones. Thus, a time will arrive that once we have been sick of bacteria, they will be susceptible to antibiotics and would be easy to eliminate (Ali, *et al.*, *2*018).

2.10 CARBAPENEMASES

Carbapenemases are diverse enzymes that vary in their ability to hydrolyze carbapenems and other beta- lactams. Detection of carbapenemase is a crucial

infection control issue because they are often associated with extensive antibiotic resistance, treatment failures and infection- associated mortality. Among the beta- lactamases, the carbapenemases, especially transferrable metallo- beta- lactamases (MBLs) are the most dreaded because of their ability to hydrolyze virtually all drugs in that class, including the carbapenems. The major concern is with transmissible carbapenemases. The transmissible enzymes can be acquired unpredictably by important nosocomial pathogens such as *Pseudomonas aeruginosa, Acinetobacter baumannii* and members of the family Enterobacteriaceae. The chromosomal enzymes occur predictably in less common pathogens such as *Stenotrophomonas maltophilia, Aeromonas spp., Chryseobacterium spp.,* and others (Tamang, 2018). In addition to their resistance to all beta- lactams, the MBL producing strains are frequently resistant to aminoglycosides and fluoroquinolones (Peirano, 2019). However, they usually remain susceptible to polymyxins. Unlike carbapenem resistance due to several other mechanisms, the resistance due to MBL and other carbapenemase production has a potential for rapid dissemination, as it is often plasmid- mediated (Oliver, 2019). Consequently, the rapid detection of carbapenemase production is necessary to initiate effective infection control measures to prevent their dissemination

CHAPTER THREE

MATERIALS AND METHODS

3.1 STUDY POPULATION

The study was conducted within Owerri metropolis in Imo state, Nigeria. Clinical samples (stool and urine samples) were collected from inpatients both male and female of age groups in the selected hospitals.

3.2 SAMPLE COLLECTION

A total of 300 (146 urine and 154 stool) non replicated clinical samples of in-patients were collected from selected hospitals. These samples were collected using sterile bottles after which, was properly packed and transported to the laboratory immediately for bacterial isolation and incubation.

The following measures were taken before and after sample collection;

(i) Sterile collection bottles were distributed to various laboratory attendants in different hospitals used for this research.

(ii) After collection, the surface of the collection bottles were properly tightened to avoid spillage and rinsed with ethanol to avoid contamination and then was carefully placed in a polythene bags and transported to the laboratory.

3.3 BACTERIA ISOLATION

The media were prepared according to manufacturers' instructions.

MaConkey Agar: 12.8g of MacConkey agar was weighed into a conical flask containing 250 mL of distilled water and 1g of Cefoxitin antibiotics. The solution in the conical flask was properly sealed with cotton and aluminum foil, autoclaved at 121° C for 15 minutes, allowed to cool for 45° C and aseptically poured into sterile petri dishes and allowed to gel. The samples were cultured by dipping a sterile swab stick into the samples and evenly robbing the swab stick against the medium in the petri dish. The petri dishes were then put into the incubator and left for 48 hours at 37^{O}C. Bacteria isolates sensitive to Cefoxitin will not survive while positive growth will be found on plates containing bacteria isolates resistant to the antibiotics, Cefoxitin.

Nutrient Agar: 7 g of Nutrient agar was weighed and poured into a conical flask containing 250 mL of distilled water and swirled to obtain a homogenous mixture. The solution in the conical flask was properly sealed with cotton and aluminum foil, autoclaved at 121℃ for 15 minutes, allowed to cool to 45℃ and aseptically poured into petri dishes and allowed to gel. The bacteria isolates were sub-cultured on nutrient agar in order to provide additional nutrients for their growth.

Muller-Hinton Agar:

27 g of Muller-Hinton Agar was weighed and poured into a conical flask containing 250 mL of distilled water and swirled to obtain a homogenous mixture. The solution in the conical flask was properly sealed with cotton and aluminum foil, autoclaved at 121℃ for 15 minutes, allowed to cool to 45℃ and aseptically poured into petri dishes and allowed to gel.

3.4 GRAM STAINING

A suspension of each isolate was prepared and smeared on a clean grease free slide and air dried for a couple of minutes. Crystal Violet was poured on the slide and kept for about 30 seconds to 1 minute and rinsed with water. Iodine was poured on the dry slide and immediately washed away with clean water. Safranin was added for about 1 minute and washed with water. After proper air drying, it was observed under the microscope with x100 magnification.

3.5 ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing was carried out on all the isolates using the Kirby-Bauer disc diffusion method as recommended by the CLSI (2010). Sterile petri-dishes of Mueller Hinton agar were prepared according to manufacturer's specification. Colonies of an overnight culture were suspended in sterile water. A sterile cotton wool swab was inserted into each test tube containing the standardized inoculums suspension, rotated with firm pressure on the side wall of

the test tube to remove excess fluid and used to inoculate the entire surface of the Mueller Hinton agar plate.

The antibiotics used in the testing include Ceftazidime 30 µg, Cefotaxime 30 µg, Cefpime 30 µg, Imipenem 30 µg, Gentamicin 30 µg, Ofloxacin 5 µg, chloramphenicol 20 µg, Streptomicin 20 µg, Ofloxacin 5 µg, Amoxicilin Clavulanic Acid 30 µg (Rapidlabs, UK). All plates were incubated at 37^0C for 24 hours and the diameter of zones of inhibition was measured to the nearest millimetre using a transparent ruler.

3.6 PHENOTYPIC DETECTION OF ESBL BY DOUBLE-DISK SYNERGY TEST (DDST) METHOD

Phenotypic detection of ESBL was carried out using Double-Disk Synergy Test (DDST) method according to Drieux (2008). Several plates of Mueller Hinton ager were prepared and 30 µg disc of Ceftazime, Cefotaxime, and Cefpime, were placed 50 mm centre to centre from the amoxicillin clavolanic acid disc (10 µg). The Standardized inoculums were inoculated into Mueller Hinton agar plate and incubated at 37^0C overnight. Enhanced zones of inhibition between any of the betalactam discs and the centre disc were recorded as ESBL producers according to the (CLSI, 2010) criteria.

3.7 DNA EXTRACTION

3.7.1 Materials:

Quick-DNATM Miniprep Plus Kit (Zymo Research), Centrifuge (EPPENDORF, GERMANY), Vortex Mixer, Block Heater (WEALTEC CORP, TAIWAN), Microwave Oven (SCANFROST, CHINA), Pipettes, Digital Scale, Microcentrifuge Tubes, Gel Tank, Gel comb, Scientific Power Pack (CLEAVER SCIENTIFIC, TAIWAN), Gel Documentation System (VILBER, GERMANY). Genomic DNA was extracted using Quick-DNATM Miniprep Plus Kit (Catalog Nos. D3024 & D3025), according to recommended protocol.

3.7.2 Protocol

4 µl of Genomic Lysis Buffer was added to 1 µl of liquid sample (4:1), mixed briefly by vortexing, and stood at room temperature for 8 minutes. The mixture was centrifuged at 10,000 x g for 8 minutes. The mixture was transferred to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuged at 10,000 x g for one minute. The flow through was discarded with the Collection Tube and the Zymo-Spin™ IICR Column transferred to a new Collection Tube. 100 µl of DNA Pre-Wash Buffer was added to the spin column and centrifuged at 10,000 x g for one minute. 250 µl of g-DNA Wash Buffer was then added to the spin column and centrifuged at 10,000 x g for one minute. The spin column was transferred to a clean microcentrifuge tube and 65 µl of DNA Elution Buffer was added to the spin column and incubated for 3 minutes at room temperature and then

centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was stored at -20ºC for future use.

3.8 PCR PROTOCOL

12.5 µl of One Taq Quick-Load 2X Master Mix with Standard Buffer (New England Biolabs Inc.); 0.5 µl each of forward and reverse primers; 8.5 µl of Nuclease free water and 3 µl of DNA template was used to prepare 25 µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to an Eppendorf nexus gradient Mastercycler (Germany).

The primers TEM, SHV, CTX, (See Table below for primer sequences) were used with amplification conditions as shown on the Table below

3.9 GEL ELECTRPHORESIS

This was done using the method of Lee *et al*. (2012). A 1% agarose was dissolved in 100ml 0f SBE buffer and microwaved at 650 wavelengths for 3 minutes until a clear crystal solution was seen. The gel was then cooled down under running tap water. Ethidium bromide of 14µl was added into the gel and mixed gently to allow visibility. The gel was cast on the already prepared tray containing the comb and base. The gel was left to solidify for 30 minutes. The comb was gently removed inside the electrophoretic chamber containing SBE buffer solution to ensure that the wells were not damaged. DNA of 6µl from each sample were gently loaded into the wells. After loading, the electrophoretic chamber was then

closed and connected to power supply. The gel was run at 100V for 45 minutes and the DNA bands was photographed under aplegen UV light transilluminator.

Table3.1. Primer sequences and their cycling parameters

Primer name	**Primer Sequence (5'- 3')**	**Cycling Parameters**
Bla TEM_seq Bla TEM_rev	TCAACATTTCCGTGTCG CTGACAGTTACCAATGCTTA	**Initial denaturation**: 94ºC, 5 minutes **Denaturation**: 94ºC, 30 seconds **Annealing**: 55ºC, 1 minute **Extension**: 72ºC, 2 minutes **Final extension**: 72ºC, 5 minutes
Bla CTX-M_seq Bla CTX-M_rev	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	**Initial denaturation**: 94ºC, 5 minutes **Denaturation**: 94ºC, 30 seconds **Annealing**: 55ºC, 1 minute **Extension**: 72ºC, 2 minutes **Final extension**: 72ºC, 5 minutes
Bla SHV (forward) Bla SHV (reverse)	CGCCTGTGTATTATCTCCCT CGAGTAGTCCACCAGATCCT	**Initial denaturation**: 94ºC, 5 minutes **Denaturation**: 94ºC, 30 seconds **Annealing**: 54ºC, 30 seconds minute **Extension**: 72oC, 1 minutes **Final extension**: 72oC, 10 minutes

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Distribution of bacterial isolates based on gender and age group

A total of 300 non replicated stool and urine were obtained from five (5) hospitals within Owerri metropolis. A total of 165 (55%) of the collected stools and urins econtained bacterial isolates positive against Cefotaxime (Table 4.1). A total of 131 clinical samples were obtained from male subjects (55.6% of stool samples and 44.4% urine samples) and 169 stools and urines were obtained from female subjects (59.9% stool samples and 40.1% urine samples). 50.4% (66) of the isolates from male subjects were found to be resistant to the antibiotics while 58.7% (99) of the isolates from the female subjects were resistant to the antibiotics, Cefotaxime as seen in Figure 4.1.

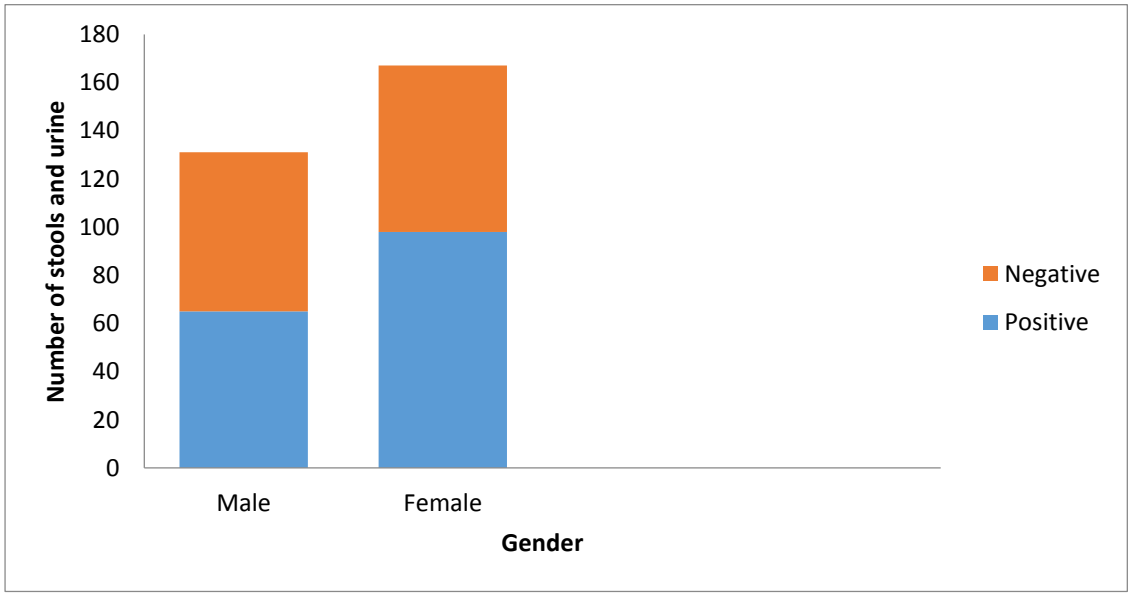


Figure 4.1: Distribution of samples based on gender

Based on age group, 15% (47) were obtained from subjects within the ages of 1-20, 24% (72) from subjects within the ages of 21-40, 23% (69) from subjects within the ages of 41-60, 22% (65) from subjects within the ages of 61-80 and 16% (47) from subjects above 80years as described in Figure 4.2.

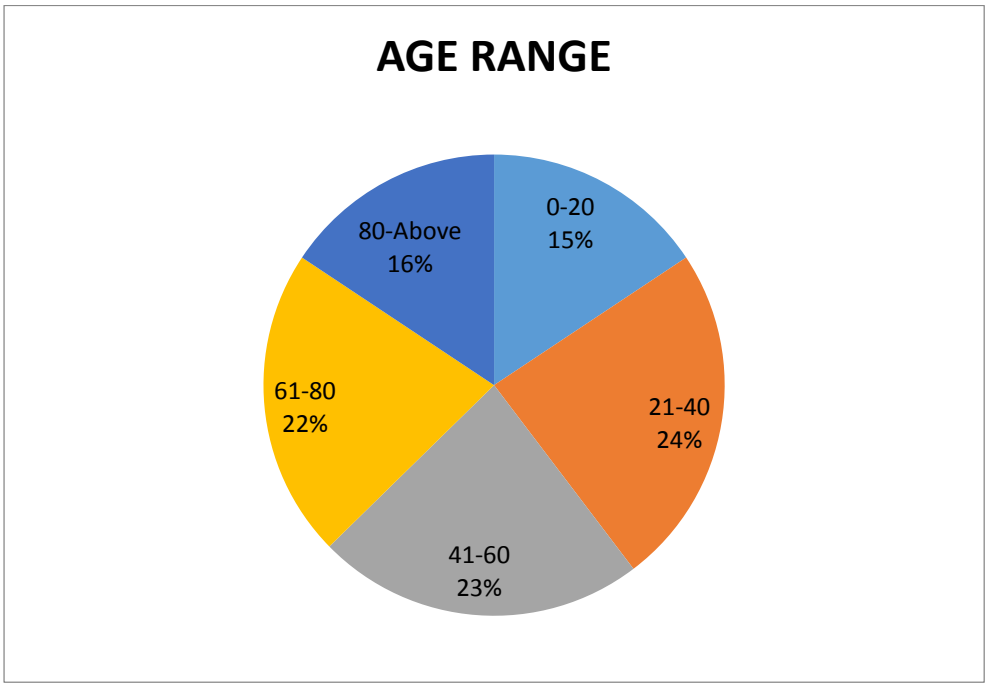


Figure 4.2: Distribution based on age range.

From the 165 ESBL positive isolates obtained, 64.8% (107) bacterial isolates were discovered to be *Escherichia coli* and 35.2% (58) bacterial isolates were discovered to be *Klebsiella pneumoniae*. Table 4.2 highlights the distribution of these enterobacteriaceae based on the different hospitals in which they were collected and Figure 4.3 shows a positive bacterial growth.

Table 4.1 Distribution of *Escherichia coli* and *Klebsiella pneumoniae* and its frequency within the positive samples.

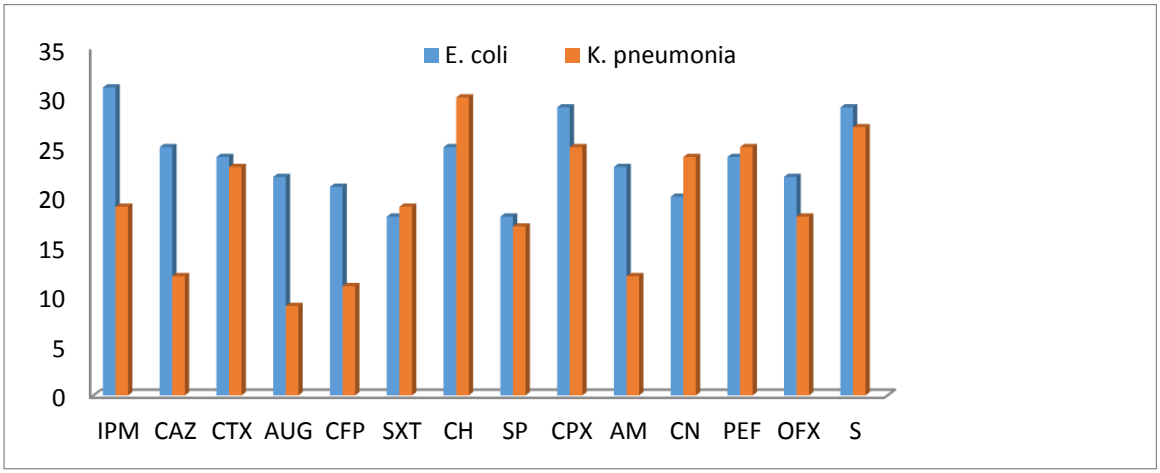
S/N	*SAMPLE SOURCE*	*TOTAL*	*E. c*	*% E. c*	*K. p*	*% K. p*
1	FUTO Health Centre	37	27	73	10	18.5
2	Good Health Medical Center Ihiagwa	51	33	64.7	18	31.7
3	Imo State Specialist Hospital Umuguma	26	15	57.7	11	37.5
4	Edge Medical Center	28	18	64.3	10	27.8
5	Our Lady Of Mercy Hospital & Maternity	23	14	60.9	9	30.8
	Total	**165**	**107**	**64.8**	**58**	**35.2**

Legend: *Escherichia coli* (E.c), frequency of *Escherichia coli* (%E. c), *Klebsiella pneumoniae* (K.p), Frequency of *Klebsiella pneumoniea* (%K.p).

Figure 4.3: A plate of MacConkay Agar showing growth of enterobacteriaceae after 24 hours of incubation.

4.1.2 ANTIBIOTICS SUSCEPTIBILITY

Each of the 165 positive resistant bacterial isolates was subjected to 15 different antibiotics and their susceptibility was measured and recorded.



Legend: Imipenem (IPM), Ceftazidime (CAZ), Cefotaxime (CTX), Augmentin (AUG), Cefepime (CFP), Trimethoprim (SXT), Chloramphenecol (CH), Septrin (SP), Ciprofloxacin (CPX), Augmentin (Local disc) (AM), Gentamicin (CN), Pefloxacin (PEF), Ofloxacin (OFX) and Streptomicin (S)

Figure 4.4: Antibiotics susceptibility pattern of *Escherichia coli* and *Klebsiella pneumoniae*

Figure 4.4 showed the resistivity profile of the two enterobacterioceae (*Escherichia coli* and *Klebsiella pneumoniae*) focused in this study. 79.6% of the resistant isolates were found to be *Escherichia coli* as compared to *Klebsiella pneumoniae.*

TABLE 4.2: Antibiotic resistance profile of the isolates to the antibiotics.

ANTIMICROBIAL AGENTS	NUMBER OF ANTIBIOTICS	NUMBER OF ISOLATES
CFP, CAZ, CTX, AUG	4	32
IPM, CFP, CAZ, CTX, AUG	5	27
SXT, IPM, CFP, CAZ, CTX, AUG	6	23
CH, SXT, IPM, CFP, CTX, AUG, CAZ	7	19
SP, CH, SXT, IPM, CFP, CTX, AUG, CAZ	8	18
CPX, SP, CH, SXT, IPM, CFP, CTX, AUG, CAZ	9	18
AM, CPX, SP, CH, SXT, IPM, CFP, CTX, AUG, CAZ	10	15
AU, AM, CPX, SP, CH, SXT, IPM, CFP, CTX, AUG, CAZ	11	14
CN, AU, AM, CPX, SP, CH, SXT, IPM, CFP, CTX, AUG, CAZ	12	11
PEF, CN, AU, AM, CPX, SP, CH, SXT, IPM, CFP, CTX, AUG, CAZ	13	9
OFX, PEF, CN, AU, AM, CPX, SP, CH, SXT, IPM, CFP, CTX, AUG, CAZ	14	6
S, PEF, CN, AU, AM, CPX, SP, CH, SXT, IPM, CFP, CTX, AUG, CAZ	15	5

Legend: Imipenem (IPM), Ceftazidime (CAZ), Cefotaxime (CTX), Augmentin (AUG), Cefepime (CFP), Trimethoprim (SXT), Chloramphenecol (CH), Septrin (SP), Ciprofloxacin (CPX), Augmentin (Local disc) (AM), Gentamicin (CN), Pefloxacin (PEF), Ofloxacin (OFX) and Streptomicin (S)

4.1.3 PHENOTYPIC DETECTION OF ESBL PRODUCING ISOLATES

Phenotypic detection of ESBL using the double disc synergy test (DDST) method according to Drieux (2008) was carried out on all the 165 bacterial isolates and 43.6% (72 isolates) were found to be positive. *Escherichia coli* consisted of 65% (47) of the positive ESBL producing isolates while *Klebsiella pneumoniae* consisted of the remaining (25) 35%. Figures 4.5 and 4.6 depict positive results for ESBL producing isolates.

Figure 4.5

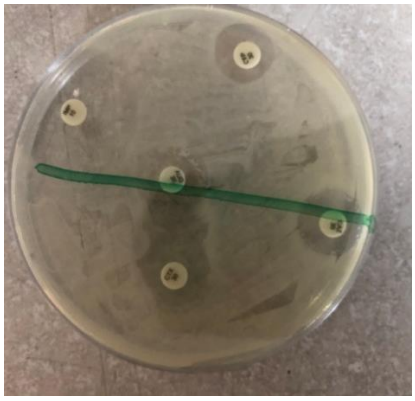


Figure 4.6

Figures 4.5&4.6*:* Phenotypic detection of ESBL production based on Double Disc Synergy Test (DDST) method.

From the figure, a positive ESBL result is indicated when the zone of inhibition around any of the cephalosporin disc is augmented in he direction of the Amoxicilin Clavulanic Acid disc placed in the center plate

4.41. PCR DETECTION OF ESBL GENES

About (17) Random phenotypically confirmed ESBL producing isolates were chosen for molecular detection of ESBL gene, SHV, CTX and TEM and at least one of the gene was confirmed present in all the isolates as shown in Table 4.3

Table 4.3: Antibiotic resistant profile for the 17 isolates for PCR

S/N	ISOLATE	CTX	SHV	TEM	TOTAL
1	ABU170	+	-	-	1
2	ABF60	+	-	+	2
3	ABU58	+	-	+	2
4	ABU183	+	+	+	3
5	ABU134	+	-	+	2
6	ABU112	+	+	+	3
7	ABU138	+	-	+	2
8	ABU139	+	-	-	1
9	ABU59	+	-	+	2
10	ABU150	-	+	-	1
11	ABU126	+	+	+	3
12	ABU135	+	-	-	1
13	ABU136	+	-	-	1
14	ABU142	-	+	+	2
15	ABU126	-	-	+	1
16	ABU189	+	-	-	1
17	ABU40	+	+	+	3
		14	6	11	

Legend: ABU-urine sample, ABF- stool sample

Out of the 17 bacteria isolates, 70.1% (14) of the isolates possessed the CTX gene, 64.7% (6) possessed the TEM gene and (11) 35.3% possessed the SHV gene. 29.4% (5) possessed both TEM and SHV gene, 60% (10) possessed TEM and CTX gene, 23.5% (4) possessed SHV and CTX gene while (2) 11.7% of the

isolate didn’t possess any of the gene, and 23.5% (4) possessed all the three (3) genes.

The distribution of Extended Spectrum Beta-Lactamase genes with relationship to isolate source, isolate name, antimicrobial resistance profile as well as the resistant gene(s) are showed in Table 4.4.

Gel Electrophoresis Photos
CTX-M GENE

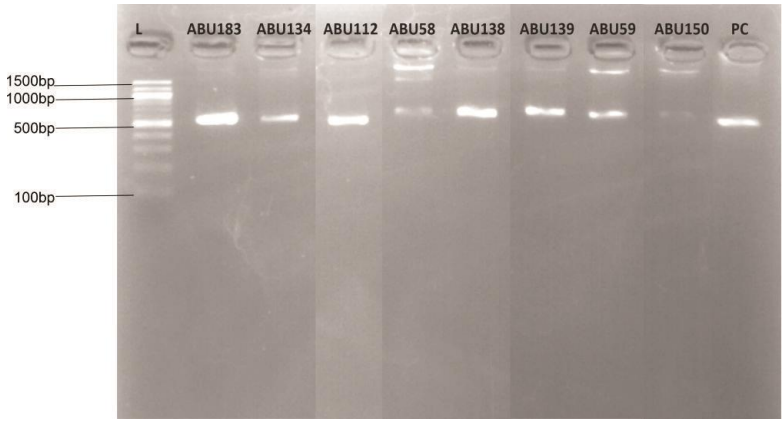


Figure 4.7: Agarose gel showing PCR amplified products of blaCTX-M gene. Lane L=1kb DNA ladder, strain ABU150 is blaCTX-M negative while all the other strains are blaCTX-M positive

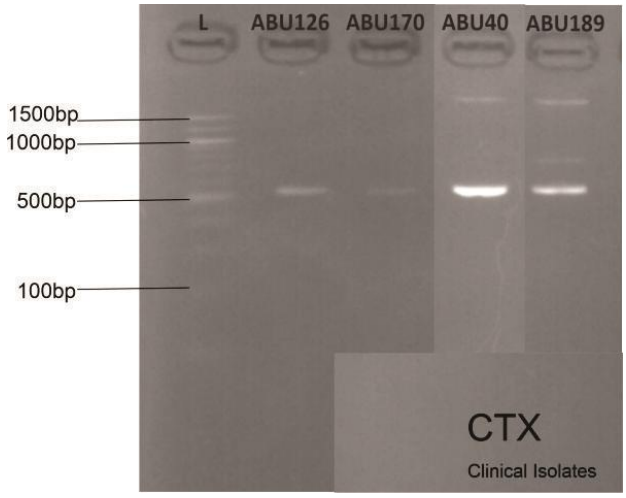


Figure 4.8: Agarose gel showing PCR amplified product of blaCTX-M gene. Lane L=1kb DNA ladder, all the strains are blaCTX-M positive

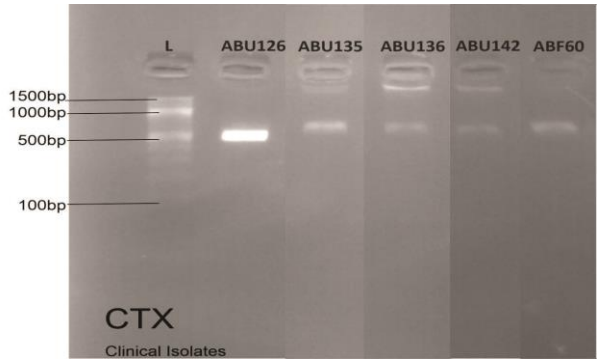


Figure 4.9: Agarose gel showing PCR amplified product of blaCTX-M gene. Lane L=1kb DNA ladder, all the strains are blaCTX-M positive

SHV GENES

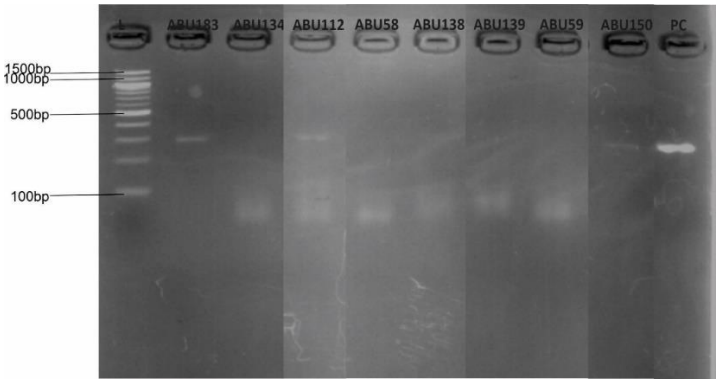


Figure 4.10: Agarose gel showing PCR amplified product of blaSHV gene. Lane L=1kb DNA ladder, strain ABU134, ABU58, ABU138, ABU139 and ABU59, are blaSHV negative while all the other strains are blaSHV positive

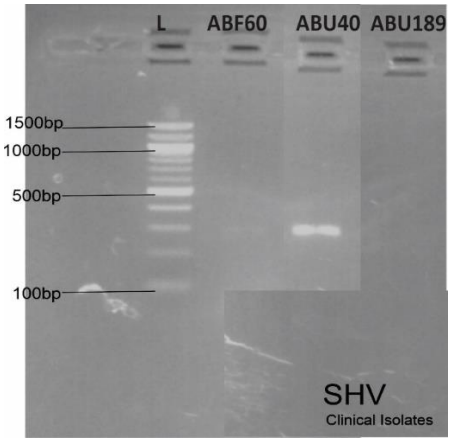


Figure 4.11: Agarose gel showing PCR amplified product of blaSHV gene. Lane L=1kb DNA ladder, strain ABU60, ABU138, ABU58, are blaSHV negative while all the other strains are blaSHV positive

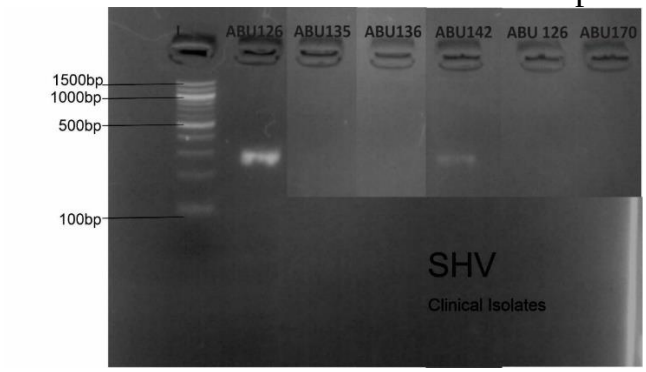


Figure 4.12: Agarose gel showing PCR amplified product of blaSHV gene. Lane L=1kb DNA ladder, strain ABU135, ABU136, ABU126 and ABU170, are blaSHV negative while all the other strains are blaSHV positive

TEM GENES

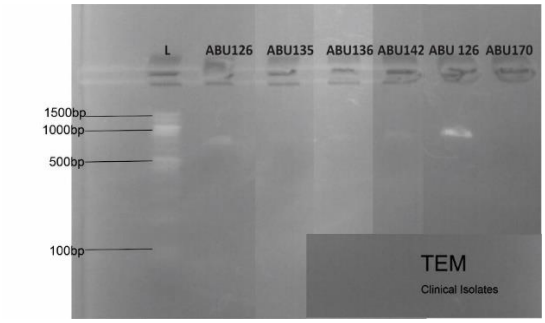


Figure 4.13: Agarose gel showing PCR amplified product of blaTEM gene. Lane L=1kb DNA ladder, strain ABU135, ABU136, and ABU170, are blaTEM negative while all the other strains are blaTEM positive

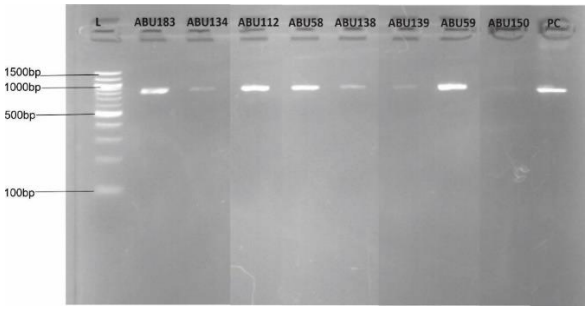


Figure 4.14: Agarose gel showing PCR amplified product of blaTEM gene. Lane L=1kb DNA ladder, strain ABU150 is blaTEM negative while all the other strains are blaTEM positive

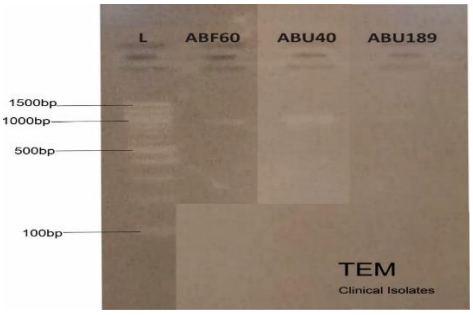


Figure 4.15: Agarose gel showing PCR amplified product of blaTEM gene. Lane L=1kb DNA ladder, strain ABU189, is negative while all the other strains are blaTEM positive

Table 4.4 Distribution of Extended–spectrum β– lactamase (ESBL) gene among the isolates

ISOLATE ID	ISOLATE SOURCE	ISOLATE NAME	ANTIMICROBIAL RESISTANCE PROFILE	ESBL GENE	TOTAL NUMBER OF ESBL GENE
ABU170	Urine	*Escherichia coli*	CEF, AUG, CTX, IMP	blaCTX-M	1
ABF60	Stool	*Escherichia coli*	CEF, CAZ, AUG, CTX, IMP,	blaCTX-M & blaTEM	2
ABU58	Urine	*Escherichia coli*	CEF, CAZ, AUG, CTX, SP	blaCTX-M & blaTEM	2
ABU183	Urine	*Klebsiella pneumoniae.*	CEF, CAZ, AUG, CTX,	blaCTX-M, blaSHV & blaTEM	3
ABU134	Urine	*Escherichia coli*	CEF, CAZ, AUG, CTX, IMP	blaCTX-M & blaTEM	2
ABU112	Urine	*Escherichia coli*	CEF, CAZ, AUG, CTX, IMP	blaCTX-M, blaSHV & blaTEM	3
ABU138	Urine	*Klebsiella pneumoniae.*	CEF, CEF, AUG, CTX, IMP	blaCTX-M & blaTEM	2
ABU139	Urine	*Escherichia coli*	CEF, AUG, CTX, IMP	blaCTX-M	1
ABU59	Urine	*Klebsiella pneumoniae.*	CAZ, AUG, CTX, IMP	blaCTX-M & blaTEM	2
ABU150	Urine	*Klebsiella pneumoniae.*	CEF, CAZ, AUG, IMP	blaSHV	1
ABU126	Urine	*Escherichia coli*	CEF, CAZ, AUG, CTX, IMP	blaCTX-M, blaSHV & blaTEM	3
ABU135	Urine	*Escherichia coli*	CEF, CAZ, AUG, CTX	blaCTX-M	1
ABU136	Urine	*Klebsiella pneumoniae.*	CEF, CAZ, AUG, IMP	blaCTX-M	1
ABU142	Urine	*Klebsiella pneumoniae.*	CEF, CAZ, AUG, CTX, IMP	blaSHV & blaTEM	2
ABU126	Urine	*Escherichia coli*	CEF, CAZ, AUG, CTX, IMP	blaTEM	1
ABU189	Urine	*Escherichia coli*	CEF, AUG, CTX, IMP	blaCTX-M	1
ABU40	Urine	*Klebsiella pneumoniae.*	CEF, CAZ, AUG, IMP, CEF	blaCTX-M, blaSHV & blaTEM	3

4.2 DISCUSSION

The presence of antibiotics resistance gene in bacteria has caused a very tremendous negative impact among humans in the past decades. The result of this work showed a high prevalence of ESBL production among the enterobateriaceae: *Escherichia coli* and *Klebsiella pneumoniae.*

Higher resistance prevalence was recorded among bacteria isolated from females (59.4%) compared to isolates from male (40.6%). Many scholars such as Shakya (2017) that recorded 73.9% resistance prevalence from isolates among female subjects and 21.1% among male subjects prevalence. Akinlabi *et. al* (2020) also confirmed a higher resistance of 58.7% among bacterial isolates from female subjects compared to 41.3% from male. One of the several factors that could contribute to the high prevalence of resistant bacteria among female subjects is the structure and physiology of the female reproductive system which makes them more prone to bacterial infections, mostly urinary tract infection (UTI). In this part of the world, women are more exposure to contaminated water, foods, domestic animals and household wares due to their frequent involvement with farming, petty trading of perishable goods and domestic chores.

Of the 300 non repetitive samples collected and observed, 55% were observed to be antibiotics resistant consisting of 64.8% *Escherichia coli* and 35.2% *Klebsiella pneumoniae.* Other publications are in accordance with this range of distribution. Ampaire *et. al* (2017) observed a high prevalence of *Escherichia coli* (55.9%) in her work as compared to *Klebsiella pneumonia*

(26.1%). Shakya *et. al* in her hospital case study of ESBL producing *Escherichia coli and Klebsiella pneumonia* in 2017, she had a higher rate of *Escherichia coli* (80.9%) isolates with a very low rate of *Klebsiella pneumonia* (3.8%). Egbule and Odih (2020) observed a higher frequency of 81% *Escherichia coli* and 9.5% *Klebsiella pneumonia* among the bacterial isolates investigated. This work assessed the prevalence of *Escherichia coli* in the gastrointestinal track of animals (humans). High number of resistance *Escherichia coli* in the body is dangerous and alarming as it depicts that most antibiotics taken will be ineffective against the bacteria

Plasmid production of Beta Lactamase is highly linked to multidrug resistance. Most of the bacterial samples were resistant to several antibiotics of the 2nd and 3rd cephalosporin generation which are considered very important by WHO (Egbule, Iweriebor & Odum 2021). Bacterial samples were highly resistant to Ceftazidime (77.6%), Cefotaxime (71.5%), Augmentin (81.2%), Cefepime (80.6%), Septrin (65%), Streptomicin (56%), Ofloxacin (60%), and Trimethoprim (63%), but mildly resistant to Gentamicin (44%), Chloramphenecol (55%), Pefloxacin (49%) and Ciprofloxacin (54%) and sensitive to Imipenem (30.3%).

This antimicrobial resistivity result obtained were in accordance with recent researches across the globe. Leski *et al.* (2016) observed a resistance frequency of Ceftazidime (100%), Imipenem (0%), Chloramphenecol (53.8%) and Gentamicin (7.7%) while Anitha and Anuraoha (2019) also observed similar

resistance range of Cefotaxime (85%), Ceftazidime (77%), Ciprofloxacin (44%), Augmentin (92%) and Imipenem (16%). Egbule, Iweriebor and Odum (2020) recorded a resistance of Augmentin (100%), Ceftazidime (100%), Ciprofloxacin (62.5%), Gentamicin (65.4%), Ofloxacin (55.8%) and Imipenem (9.6%) while Putra, Rustini & Marlina (2021) observed a resistance frequency of Augmentin (86%), Cefotaxime (60%), Ceftazidime (75.6%) and Imipenem (16.3%). Nsofor et. al (2016) also observed similar high antibiotics resistance in Cefotaxime (42.3%), Chloramphenecol (70.1%), Ciprofloxacin (38.5%) and Gentamicin (31.7%).

The high resistance rate could be associated to a couple of factors such as the abuse of these antibiotics, consumption of plants or animals treated with antibiotics having residue of these drugs on them, production of Beta-Lactamse by the host among others. Most of the bacterial samples were found to be susceptible to Imipenem (IPM) and it is presently highly recommended for treatment of bacterial infections.

Double Disc Synergy Test (DDST) method is the most randomly used method for phenotypic detection of ESBL using Ceftazidime, Cefotaxime, Cefepime and Amoxillin Clavulanic Acid (Augmentin) on Muller-Hinton agar. In this research, 43.6% of the bacterial isolates tested positive to the phenotypic detection test for ESBL. Olowe *et al.* (2015) observed a higher number of ESBL isolated after a DDST of 78.9%, Sreekrishna *et. al* (2012) observed 58.3% while Sales (2014) observed 53.9% of positive ESBL isolates. This result explains that

one-in-five enterobacteriaceae isolated from these subjects possessed antibiotics resistance gene which degrades any antimicrobial medication, thereby making the drug ineffective. 65% of the ESBL positive isolates were identified to be *Escherichia coli* and 35% *Klebsiella pneumonia.* Ugah & Udeani (2020) had a lower number of *Escherichia coli* (30.8%) and 19.3% *Klebsiella pneumonia.* Kajeguka *et al.* (2018) observed a closer result having 44.4% *Escherichia coli* and 40.7% *Klebsiella pneumonia.*

The three antibiotics resistance genes, CTX, TEM and SHV investigated indicated a high prevalence of CTX gene (70.1%) which is in accordance with prior researches from other Nigerian scholars. The gene has been found to be the highest prevalent antibiotics resistant gene in Nigeria. Ogefere (2015), *Ogbolu et al.* (2013), Aibinu *et al.* (2003), Omeregie (2013), Soge et al. (2006). 23.5% of the bacteria isolates possessed all three antibiotics resistant genes, this alarming result showed the effective resistance possessed by these isolates against any antibiotics. Individuals with these isolates in them would experience longer hospital stay, delay in healing and total ineffective treatment as most of the average antibiotics that would previously tackle the bacterial infection would be ineffective.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The incidence of infections caused by beta-lactam-resistant organisms due to the production of ESBL has increased in recent years. These resistant bacteria has been found to be very difficult to combat hence, increasing treatment cost, reducing quality of life, leading to longer hospital stay, increasing family burden of infected individual and eventually death of affected individuals if not properly managed. Several factors have contributed to high prevalence of these resistant bacteria across the globe and in Nigeria. Over the counter sales of antibiotics without prescription by a pharmacist or physician, dosage abuse, expired or fake antibiotics among others are ways resistance to antibiotics develop over time.

The Nigerian National Bureau of Statistics as at 2020 estimates about 40% or 83 million Nigerians live in poverty. This poverty rate leaves the individuals with no access to clean water, clean food or clean habitat free from contamination or bacterial attack. With no access to these amenities, they are infested with bacteria and in an attempt to get better self-medicate, sometimes causing more damage to them, leading to antimicrobial resistance in this case. Sales of expired or fake drugs by pharmacies and drug dispensing shops have also led to medical complications on the patient thereby leading to antimicrobial resistance. Treatment of bacterial infection with herbs by local traditional herbalist has also been a challenge and a known cause of bacterial resistance to antimicrobials.

These herbs with their various concentrations do alter the bacterial load on the host but sometimes leave bacterial strains behind with trace resistance ability thereby leading to ineffectiveness of drugs over time. Poor diagnosis and treatment of ailment is also a leading cause of antimicrobial resistance in this part of the world. Wrong administration of antibiotics for a non-bacterial ailment could lead to bacterial strains gaining resistance for the administered drug.

5.2 Recommendation

There is need for proper public enlightenment on the prevalence of infections caused by these resistant bacteria by public health workers. Hospitals and appropriate health workers should be trained on proper identification of these resistant bacteria before recommending antibiotics for such infections. There is need for proper awareness on the damages that can arise from the abuse of antibiotics, Poor diagnosis and treatment of ailment, treatment of ailment by traditional herbalist among others. Social media awareness could be used to target the younger generation which are the ones with higher rate of these resistant bacterial strains. The pharmaceutical association of Nigeria (PAN) should carry-out regular checks on pharmacies to ensure fake and expired drugs are not sold. Pharmacies should enforce the sales of antibiotics on prescription as it would reduce abuse. The government should provide proper basic amenities to a greater number of its people. Provision of good water supply in all the villages around the nation will increasingly decrease the rate of bacterial infection as most of the

daily regular activities require water. With provision of more easy-access and less expensive primary health care centers across the nation, there would be a reduced rate of patronage on herbalists and untrained health care providers. Some individuals stay away from secondary and tertiary health care facilities because of the stress and cost implications and sometimes it is distant away from their location. Provision of health care centers in every village or region will provide most of the individuals' access to proper medical attention by trained professionals. There is need for the government to fund more scientific researches to help investigate the national prevalence of these resistant bacteria to have sufficient workable data and researches on the manufacture of appropriate medication against these bacteria.

Contribution to knowledge

This study provides valuable data on the prevalence of ESBL-producing Enterobacteriaceae within Owerri Metropolis. Understanding the distribution of these antibiotic-resistant strains is crucial for local healthcare management and infection control. The study identifies specific Enterobacteriaceae species that are prevalent in this region and are capable of producing Extended-Spectrum Beta-Lactamases. This information is essential for targeted surveillance and intervention strategies. More light shed on the antibiotic resistance patterns of ESBL-producing strains which is crucial for guiding empirical antibiotic therapy and formulating antibiotic stewardship programs to combat the rising issue of

multidrug-resistant bacteria. The study contributes to the identification of specific ESBL genes (CTX, SHV & TEM) responsible for antibiotic resistance. This information is crucial for tracking the genetic basis of resistance and informing future research on targeted therapies. The findings can contribute to the development and refinement of healthcare policies and guidelines related to antibiotic use, infection prevention, and control measures within the studied hospitals and, more broadly, in the region, as well as contribute to raising awareness among healthcare professionals, policymakers, and the public about the challenges posed by ESBL-producing Enterobacteriaceae. Education initiatives based on the study's findings may promote responsible antibiotic use.

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