

**EXTENDED SPECTRUM BETA-LACTAMASE-  
PRODUCING BACTERIA: MOLECULAR STUDIES  
AND EFFECTS OF MEDICINAL PLANTS**

**BY**

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## CERTIFICATION PAGE

This is to certify that this work, "Extended Spectrum Beta-Lactamase-Producing Bacteria: Molecular Studies and Effects of Medicinal Plants", was carried out by Ohalete, Chinyere Ngozi, (20114773638) in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy (PhD) in Medical Microbiology in the Department of Microbiology, Federal University of Technology Owerri.

  
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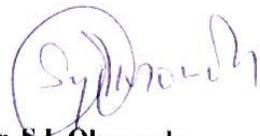
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## **DEDICATION**

To God Almighty, my parents, the entire Ezinna Cyriacus Ohalete's family and to all lovers of God, peace and seekers of knowledge.

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## ABSTRACT

Molecular studies of ES $\beta$ L-producing bacteria isolated from clinical samples was determined as well as the effects of medicinal plants on the isolates. The study was conducted from September 2013 to September 2015. A total of 480 bacterial isolates (250 from urine, 110 from wound swab & 120 from HVS & US) were used. The knowledge, attitude and practices (KAP) of health workers in Imo State towards multiple antibiotic resistant bacterial isolates and extended spectrum beta-lactamase producing bacteria was initially ascertained using structured questionnaire. Antimicrobial resistance profile was determined by the Kirby-Bauer technique. Phenotypic expression of  $\beta$ -lactamases production was performed by the double disk diffusion method. Genomic DNA extraction was by alkaline lysis method and hybridization effected with primers of the three  $\beta$ -lactamases genes, TEM, SHV and CTX-M. The isolated DNA and plasmids were analysed by the agarose gel electrophoresis. Plasmid curing was effected with acridine-orange. Extraction of the active components from plant material was conducted. Effects of the plant extracts on ES $\beta$ L producing bacteria and the minimum inhibitory concentration were also determined. Analysis of Variance (ANOVA) was used for the analysis of the data via SPSS software package. The result showed that the respondents had good knowledge of multiple antibiotic resistant bacterial infections and their causes. The results of screening the isolates with 12 antimicrobials showed that the isolates expressed high resistance rates. Examination of clinical samples showed higher prevalence of multiple antibiotic resistant (MAR) *Escherichia coli* (50.3%) than *P. aeruginosa* (43.3%) and *Klebsiella* species (36.6%). The prevalence of ES $\beta$ L-producing isolates was highest (67.6%) amongst *E. coli* than *Klebsiella* species (64.7%) and *P. aeruginosa* (57.7%). Gel electrophoresis of the amplified (PCR) genomic products showed that 36.7% were positive for TEM, 66.7% for SHV, and 23.3% for CTX-M genes. Phenotypic screening of isolates for extended spectrum beta-lactamase production before curing showed high resistance to the  $\beta$ -lactam antimicrobials and the  $\beta$ -lactamases inhibitors of amoxicillin/clavulanic acid combination. However, analysis of post curing showed a great reduction in rates of ES $\beta$ L positive isolates examined, *E. coli* 4(40%), *Klebsiella* species 3 (30%) and *P. aeruginosa* 4(40%) was found to harbour ES $\beta$ L genes as against, *E. coli* 8(80%), *Klebsiella* species 6 (60%) and *P. aeruginosa* 9(90%) before curing. Post curing results showed that half of the gene markers were borne on the plasmids while the other half were borne on the chromosomes. The plasmids and the beta-lactamase genes probably played important roles in the resistances identified. Studies on antibacterial effects of the medicinal plants showed that *Ocimum gratissimum* and *Xylopiya aethiopica* exhibited higher growth inhibitory effects on the bacterial isolates than *Allium sativum*, *Vernonia amygdalina* and *Garcinia kola*. The highest growth inhibitory effect was exhibited on *E. coli* and *Klebsiella* species by, *Ocimum gratissimum* and on *Escherichia coli* by *Xylopiya aethiopica*. The minimal inhibitory concentrations of the selected medicinal plant extracts on the test bacterial isolates were higher for ES $\beta$ L-bacteria than for non resistant isolates. Despite the high knowledge of MAR ES $\beta$ L discovered in this study by healthcare practitioners, it appears that much attention is not given to MAR and ES $\beta$ L-bacteria infections in the State. Extracts of commonly used medicinal plants in Imo State, Nigeria, such as those used in the present study are capable of inhibiting growth of MAR and ES $\beta$ L-producing bacteria. Therefore there is need for further investigations in terms of toxicological studies and purification of active components with a view to exploiting the plants in novel drug development.

**Keywords:** Beta-lactamase, clinical isolate, ES $\beta$ L-producing bacteria, medicinal plants,

Molecular

## CHAPTER ONE

### INTRODUCTION

#### 1.1 Background of the study

Extended Spectrum beta lactamases (ES $\beta$ L) were first described in the 1980s and they have been detected in *Klebsiella* species, and later in *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia marcescens* and other gram-negative bacilli (Kiratisin *et al.*, 2008). Extended-Spectrum Beta-lactamases (ES $\beta$ L) are  $\beta$ -lactamases capable of conferring bacterial resistance to penicillin and third generation cephalosporins, and aztreonam, but not the cephamycins or carbapenems (Paterson & Bonomo, 2005) and are usually encoded on plasmids which frequently carry genes encoding resistance to other classes of antibiotics. A good number of enteric gram-negative bacteria have been shown to possess naturally occurring chromosomally mediated genes that confer resistance on them to  $\beta$ -lactam antibiotics (Brown *et al.*, 2000). ES $\beta$ L strains are inhibited by  $\beta$ -lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Bradford, 2001).

ES $\beta$ LS constitute increasingly important causes of transferable multiple antibiotic resistances in gram-negative bacteria throughout the world. These bacteria have spread rapidly and have become a serious threat to human health worldwide (Poole, 2004; Gupta, 2007). The gram-negative bacilli especially *Pseudomonas* species and members of the family enterobacteriaceae are common causes of infections of many parts of the body. They account for more than 50% of all isolates in nosocomial infections (Talaro & Talaro, 1996).

The increasing prevalence of antibiotic resistance is a major health problem worldwide. The World Health Organization (WHO) and the European Commission (EC) recognized the importance of studying the emergence and determinants of resistance and the need for strategies

for its control (WHO, 2006). Previous studies have reported increasing prevalence of uropathogens resistant to commonly prescribed antibiotics. In some countries, resistance to fluoroquinolones has emerged. In developing countries, frequent irrational use of antibiotics predicts a worrisome increase in the prevalence of antimicrobial resistance (WHO, 2006).

Among the most prevalent bacterial pathogens capable of showing resistance to common antibiotics is *Escherichia coli* which is one of the most common causes of urinary tract infections (UTI) and other opportunistic infections such as wound abscess which can have serious clinical implications (Iroha *et al.*, 2009). Originally, ES $\beta$ L enzymes were derived from the wide spread TEM and SHV  $\beta$ -lactamase families. Today, over 110 derivatives of TEM ( $\beta$ -lactamase) and more than 63 derivatives of SHV  $\beta$ -lactamases are known (Weiner *et al.*, 1999). ES $\beta$ Ls undergo continuous mutations, causing the development of new enzymes showing expanded substrate profile. Presently, there are more than 300 different ES $\beta$ L variants. These have been clustered into 9 different structural and evolutionary families based on their amino acid sequences. TEM (Temoniera) and Sulphydryl variable (SHV) were the major types. However, CTX-M is more common in some countries (Paterson *et al.*, 2000).

The ES $\beta$ L enzymes are most commonly produced by *Klebsiella* species and *Escherichia coli*, but may also occur in other gram-negative bacteria including *Salmonella*, *Proteus*, *Citrobacter*, *Morganella*, *Serratia*, and *Shigella* species (Goussard & Courvalin, 1999). That ES $\beta$ Ls are encoded on plasmids and are, therefore, easily transmissible from one organism to another. This is a therapeutic challenge for physicians as resistant genes for other antimicrobials such as aminoglycosides, tetracycline, and trimethoprim/sulfamethoxazole are often present on the same plasmids (Weiner *et al.*, 1999), thereby contributing further to the narrowing of choices of antibiotics. Many clinical laboratories are not fully aware of the importance of ES $\beta$ Ls and a serious challenge facing clinical laboratories is that clinically relevant ES $\beta$ L-mediated

resistance is not always detected in routine susceptibility tests (Thomson, 2001; Catagay *et al.*, 2003). The National Committee for Clinical Laboratory Standards (NCCLS) recommends that microbiology laboratories should report ES $\beta$ L isolates of *E. coli* and *Klebsiella* species as resistant to all penicillins and cephalosporins including cefepime and aztreonam irrespective of their individual in vitro test results (NCCLS, 2000). Determination of TEM, SHV, and CTX-M genes by molecular techniques in ES $\beta$ L producing bacteria and their pattern of antimicrobial resistance can supply useful data about the epidemiology and risk factors associated with these infections (Jain & Mondal, 2008).

## **1.2 Statement of the problem**

In recent years, there has been increasing cases of multiple antibiotic resistant bacteria strains isolated from clinical patients in Nigerian hospitals. The socio-economic consequences of multiple antibiotic resistant bacteria infections are many; they cause treatment failures, as well as waste of resources to patients and health care providers. While some tertiary health institutions may be interested in further studies to know the cause of these and possible measures to combat the challenges, secondary and primary health-care centers appear to pay little or no attention to the problems and challenges of antibiotic resistant bacterial infections. Consequently, the lack of knowledge of multiple antibiotic resistant ES $\beta$ L has affected the attitude and practice of the medical practitioners towards the treatment of bacterial infections. The lack of facilities for ES $\beta$ L detection leads to incorrect identification of antibiotic resistance which may lead to inappropriate antibiotic prescription, which in turn may lead bacteria to produce new resistance genes by selective pressure.

In Imo State and Nigeria generally, people appear to be reluctant to go to hospitals for treatment and health care until they are critically ill. Many resort to road side chemist outfits, mobile medicine hawkers and traditional healthcare providers, resulting in drug abuse which leads to

bacterial mutation leading to increased drug resistance. A few others who seem to go to hospitals attend primary healthcare centers, without adequate knowledge of antibiotic resistant bacterial infections. A greater population of Nigerian patients stands the risk of facing the challenges of antibiotic resistant bacterial infections without proper remedy.

Much work has not been done on the prevalence, effects and management of multiple antibiotic resistant and extended spectrum beta lactamase producing bacterial pathogens causing diseases and infections in Nigeria. There is paucity of information on ES $\beta$ L and multiple antibiotic resistant bacteria (Iroha *et al.*, 2009; Nwaosu *et al.*, 2014; Azekhueme *et al.*, 2015). Previous studies concluded on phenotypic studies without advancing to molecular studies and possible treatment. The handicap and challenges posed by multiple antibiotics resistant and  $\beta$ -lactamase producing bacteria to patients, clinicians, students and researchers constitute the research problems of the present study.

### **1.3 Aim and objectives**

The aim of this study is to carry out molecular studies on extended spectrum beta lactamase (ES $\beta$ L) producing bacteria isolated from clinical samples and determine the effects of medicinal plants on them.

The specific objectives are to:

- I. examine the knowledge, attitude and practices of health workers in Imo State towards multiple antibiotic resistant bacterial isolates and extended spectrum beta lactamase producing bacterial isolates.
- II. determine the prevalence of ES $\beta$ L producing isolates of *Escherichia coli*, *Klebsiella*

species and *Pseudomonas aeruginosa* in clinical specimens (specifically urine, wound swab and HVS) in Imo State.

III.

ascertain the presence of ES $\beta$ L encoding genes -TEM, SHV and CTX-M types of ES $\beta$ L enzymes, carried by the isolates.

IV.

ascertain the possible role of plasmids in the ES $\beta$ L positive isolates by conducting plasmid curing and profiling protocols.

V.

determine the antibacterial susceptibility patterns of selected medicinal plants on identified ES $\beta$ L producing isolates.

#### **1.4 Justification for the study**

The need to ascertain the prevalence of multiple antibiotic resistant bacterial infections, the causes and possible remedies has made this present study absolutely necessary. Thus, this study will both provide the rudimentary data on the existence and prevalence of multiple antibiotic resistant ES $\beta$ L bacteria in Imo State to enhance cure and treatment as well as stir up interest on related studies among researchers in Nigerian Institutions. This study will also be of immense advantage to clinicians, and policy makers in health management to direct their priorities better.

This study will also create awareness on the importance of ES $\beta$ Ls and the serious challenge facing clinical laboratories since clinically relevant ES $\beta$ L-mediated resistance is not always detected in routine susceptibility test.

It will in addition educate the public on the consequences of frequent irrational use of antibiotics and the possible effects of the medicinal plants on multiple antibiotic resistant ES $\beta$ L producing bacteria.

The outcome of this study will help to enhance the knowledge of health practitioners on the detection of multiple antibiotic resistances due to ESβLs, which will affect the attitude and practice of medical practitioners towards the treatment of bacterial infections (infected patients).

### **1.5 Scope of study**

This study was carried out on the molecular biology of ESβL producing bacteria isolated from clinical samples and effects of selected medicinal plants on the bacterial isolates. The study was limited to bacterial isolates from health institutions within Imo State during the period of the study. The study does not include fungi isolates.

Molecular studies of ESβL producing isolates are limited to detection of TEM, SHV and CTX-M genes. Genomic DNA extraction, plasmid curing, profiling and PCR amplification.

Antibacterial effects of medicinal plants is limited to 5 selected plants: fresh leaves of *Vernonia amygdalina* Delile (bitter leaf) and *Ocimum gratissimum* L. (nchuanwu) and seeds of *Xylopia aethiopica* Dunal (Udah), *Garcinia kola* Heckel (bitter kola), and *Allium sativum* L. (garlic). The study of the antibacterial effects is limited to growth inhibition and the minimal inhibitory concentration of the test plants.

### **1.6 Limitations of the study**

This study was limited by some factors:

- I. the negative attitudes of respondents in the study area. Some health workers were non-chalant and reluctant to complete and return the study questionnaire. Out of 300 health workers selected for the study, 36 declined and could not return their completed

questionnaire, thereby limiting the study population to 264 medical laboratory scientist and medical doctors.

- II. the number of isolates used for this study was limited by improper identification by the clinical laboratories. Out of 480 isolates obtained from 602 clinical samples, 180 isolates were wrongly identified, thereby limiting the number of isolates used to 300. One hundred and twenty two samples had no bacterial isolates.
- III. the unwillingness of professional herbalists in Imo State to reveal the names of plants they use for treatment of bacterial infections, limited the selection to the ones commonly used by natives and non-professional herbalists. Most of the plants used are commonly used not only as herbal remedies but also for cooking and food preparations.

## CHAPTER TWO

### LITERATURE REVIEW

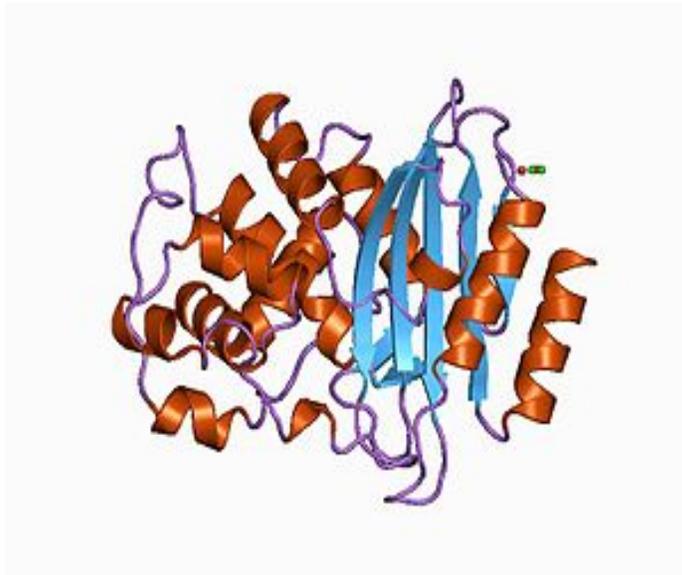
#### 2.1 Beta-lactamase

Beta-lactamases are enzymes produced by some bacteria. They are responsible for their resistance to beta-lactam antibiotics like penicillins, cephamycins, and carbapenems (ertapenem). Carbapenems are relatively resistant to beta-lactamase. These antibiotics have a common element in their molecular structure: a four-atom ring known as beta-lactam. The lactamase enzyme breaks the  $\beta$ -lactam ring open, deactivating the molecule's antibacterial properties. Beta-lactam antibiotics are typically used to treat a broad spectrum of Gram-positive and Gram-negative bacteria. Beta-lactamases are produced and secreted by Gram-negative bacteria, especially when antibiotics are present in the environment (Neu, 1969).

#### 2.2 Penicillinase

Penicillinase is a specific type of  $\beta$ -lactamase, showing specificity for penicillins, again by hydrolysing the beta-lactam ring. Molecular weights of the various penicillinases tend to cluster near 50 kilo Daltons. Penicillinase was the first  $\beta$ -lactamase to be identified. It was first isolated by Abraham and Chain (1940) from Gram-negative *E. coli* even before penicillin entered clinical use, but penicillinase production quickly spread to bacteria that previously did not

produce it or produced it only rarely. Penicillinase-resistant beta-lactams such as methicillin were developed, but there is now widespread resistance to even these.



**Figure 2.1: Structure of  $\beta$ -lactamase**

**Source:** Abraham and Chain (1940).

## Penicillin Resistance

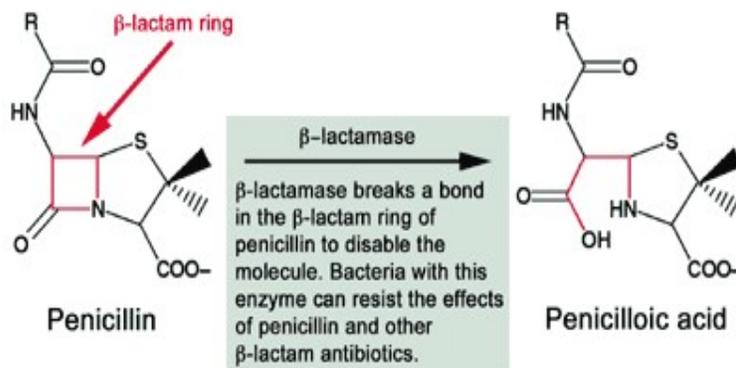


Figure 2.2: Structure of Penicillin Resistance

Source: Abraham and Chain (1940)

### 2.2.1 Classification of $\beta$ -lactamases

$\beta$ -lactamases are most classified according to two general schemes: the Ambler molecular classification scheme and the Bush-Jacoby-Mederos functional classification system (Ambler *et al.*, 1991; Rasmussen *et al.*, 1993; Bush *et al.*, 1995). The Ambler scheme divides  $\beta$ -lactamases into four major classes (A to D). The basis of this classification scheme rests upon protein homology (amino acid similarity) and not phenotypic characteristics. In the Ambler classification scheme,  $\beta$ -lactamases of class A, C and D are serine  $\beta$ -lactamases. In contrast the class B enzymes are metallo-  $\beta$ -lactamases. The Bush-Jacoby-Mederos classification scheme groups  $\beta$ -lactamases according to functional similarities (substrate and inhibitor profile). There are four main groups and multiple subgroups in this system. This classification scheme is of much more immediate relevance to the physician or microbiologist in a diagnostic laboratory because it considers  $\beta$ -lactamase inhibitors and  $\beta$ -lactam substrates that are clinically relevant. In this classification, ES $\beta$ Ls belong to group 2be or group 2d (OXA-type), the latter sharing most of the fundamental properties of group 2be enzymes though differing in being inhibitor resistant (Bush *et al.*, 1995). The 2be designation shows that these enzymes are derived from group 2b  $\beta$ -lactamases (for example, TEM-1, TEM-2 and SHV-1); the 'e' of 2be denotes that the  $\beta$ -lactamases have an extended spectrum. The ES $\beta$ Ls derived from TEM-1, TEM-2 or SHV-1 differs from their progenitors by as few as one amino acid. This result is a profound change in

the enzymatic activity of the ES $\beta$ Ls, so that they can now hydrolyze the third-generation cephalosporins or aztreonam, hence, there is extension of spectrum compared to the parent enzyme). Inhibition by  $\beta$ -lactamase inhibitors such as clavulanic acid and inability to hydrolyze cephamycins differentiates the ES $\beta$ Ls from the AmpC-type  $\beta$ -lactamases (group 1), which have third-generation cephalosporins as their substrates but which are not inhibited by clavulanic acid. Selection of stably de-repressed mutants which hyperproduce the AmpC-type  $\beta$ -lactamases has been associated with clinical failure when third-generation cephalosporins are used to treat serious infections with organisms producing these enzymes (Karim *et al.*, 2001).

In general, the fourth-generation cephalosporin, cefepime, is clinically useful against organisms producing AmpC-type  $\beta$ -lactamases (Sanders *et al.*, 1996) but may be less useful in treating ES $\beta$ L-producing organisms (Yuan *et al.*, 1998). Additionally, the metalloenzymes (group 3) produced by organisms such as *Stenotrophomonas maltophilia* can hydrolyze third-generation cephalosporins (and carbapenems) but are inhibited by ethylenediaminetetraacetic acid (EDTA), a heavy-metal chelator but not clavulanic acid (Wachino *et al.*, 2004).

### **2.2.2 Functional classification of $\beta$ -lactamases**

The following functional classification of beta lactamases has been proposed (Bush *et al.*, 1995).

#### **Group 1**

**Cephalosporinase:** Molecular Class C (not inhibited by clavulanic acid)

Group 1: Are cephalosporinases not inhibited by clavulanic acid, belonging to the molecular class C.

#### **Group 2**

Group 2 are penicillinases, cephalosporinases, or both inhibited by clavulanic acid, corresponding to the molecular classes A and D reflecting the original TEM and SHV genes. However, because of the increasing number of TEM- and SHV-derived  $\beta$ -lactamases, they were divided into two subclasses, 2a and 2b.

### **Group 2a**

**Penicillinase:** Molecular Class A

The 2a subgroup contains just penicillinases.

### **Group 2b**

**Broad-spectrum,** Molecular Class A

2b Opposite to 2a, 2b are broad-spectrum  $\beta$ -lactamases, meaning that they are capable of inactivating penicillins and cephalosporins at the same rate. Furthermore, new subgroups were segregated from subgroup 2b:

### **Group 2be**

**Extended-spectrum,** Molecular Class A

Subgroup 2be, with the letter "e" for extended spectrum of activity, represents the ES $\beta$ Ls, which are capable of inactivating third-generation cephalosporins (ceftazidime, cefotaxime, and cefpodoxime) as well as monobactams (aztreonam).

### **Group 2br**

**Inhibitor-resistant:** Molecular Class A (diminished inhibition by clavulanic acid).

The 2br enzymes, with the letter "r" denoting reduced binding to clavulanic acid and sulbactam, are also called inhibitor-resistant TEM-derivative enzymes; nevertheless, they are commonly still susceptible to tazobactam, except where an amino acid replacement exists at position met69.

### **Group 2c**

**Carbenicillinase,** Molecular Class A

Later subgroup 2c was segregated from group 2 because these enzymes inactivate carbenicillin more than benzylpenicillin, with some effect on cloxacillin.

### **Group 2d**

**Cloxacilanasase:** Molecular Class D or A

Subgroup 2d enzymes inactivate cloxacillin more than benzylpenicillin, with some activity against carbenicillin. These enzymes are poorly inhibited by clavulanic acid, and some of them are ESβLs. The correct term is "OXACILLINASE". These enzymes are able to inactivate the oxazolympenicillins like oxacillin, cloxacillin, and dicloxacillin. The enzymes belong to the molecular class D not molecular class A.

### **Group 2e**

**Cephalosporinase:** Molecular Class A

Subgroup 2e enzymes are cephalosporinases that can also hydrolyse monobactams, and they are inhibited by clavulanic acid.

## **Group 2f**

**Carbapenamase:** Molecular Class A

Subgroup 2f was added because these are serine-based carbapenemases, in contrast to the zinc-based carbapenemases included in group 3.

## **Group 3**

**Metalloenzyme:** Molecular Class B (not inhibited by clavulanic acid)

Group 3 are the zinc-based or metallo  $\beta$ -lactamases, corresponding to the molecular class B, which are the only enzymes acting by the metal ion zinc, as discussed above. Metallo B-lactamases is able to hydrolyse penicillins, cephalosporins, and carbapenems. Thus, carbapenems are inhibited by both group 2f (serine-based mechanism) and group 3 (zinc-based mechanism).

## **Group 4**

**Penicillinase:** No Molecular Class (not inhibited by clavulanic acid)

Group 4 are penicillinases that are not inhibited by clavulanic acid, and they do not yet have a corresponding molecular class.

**Table 2.1 Classification of beta-lactamases**

<b>Classification</b>	<b>β- Lactamases</b>	<b>Amino Acid</b>	<b>Examples</b>	<b>Inhibitor</b>
<b>Ambler class</b>				
A	Penicillinases	Serine	TEM-1, SHV, KPC CTX-M, SME-1	Clavulanate
B	Metallo-β- Lactamases	Zinc	IMP-1, VIM-1	EDTA
C	Cephalosporinases	Serine	AmpC	-
D	Oxacillinases	Serine	OXA-1	Sodium chloride
<b>Bush-Jacoby- Mederiros group<sup>12</sup></b>				
1	Cephalosporinases		AmpC	
2a	Penicillinases		PC1	
2b	Broad-spectrum Penicillinases		TEM-1, SHV-1	
2be	Extended-spectrum β- Lactamases		TEM-10, SHV-2, CTX-M-type	
2br	Inhibitor resistant		TEMs, 1RTs, TEM- 30,31	
2c	Carbenicillin hydrolyzing		PSE-1	
2d	Oxacillin hydrolyzing		OXA-1 to 11, PSE- 2	
2e	Cephalosporinases		FEC-1	
2f	Carbapenemases		KPC-1, KPC-2, SME-1	
3	Metallo- β- Lactamases		IMP-1, VIM-1, SPM-1	
4	Miscellaneous			
EDTA = ethylenediaminetetraacetic acid				

**Source:** Joseph and Kuti, (2008).

### **2.2.3 Molecular classification of $\beta$ -lactamases**

The molecular classification of  $\beta$ -lactamases is based on the nucleotide and amino acid sequences in these enzymes. To date, four classes are recognised (A-D), correlating with the functional classification. Classes A, C, and D act by a serine-based mechanism, whereas class B or metallo- $\beta$ -lactamases need zinc for their action (Ambler, 1980). "Penicillinase" was discovered in 1940 and renamed Beta-lactamase when the structure of the Beta-lactam ring was finally elucidated.

### **2.2.4 Resistance in gram-negative bacteria**

Among Gram-negative bacteria, the emergence of resistance to expanded-spectrum cephalosporins has been a major concern. It appeared initially in a limited number of bacterial species (*E. cloacae*, *C. freundii*, *S. marcescens*, and *P. aeruginosa*) that could mutate to hyperproduce their chromosomal class C  $\beta$ -lactamase. A few years later, resistance appeared in bacterial species not naturally producing AmpC enzymes (*K. pneumoniae*, *Salmonella sp.*, *P. mirabilis*) due to the production of TEM- or SHV-type ES $\beta$ Ls. Characteristically, such resistance has included oxyimino- (for example, cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam aztreonam), but not 7-alpha-methoxy-cephalosporins (cephamycins); in other words, (cefoxitin and cefotetan) have been blocked by inhibitors such as clavulanate, sulbactam, or tazobactam, and did not involve carbapenems. Chromosomal-

mediated AmpC  $\beta$ -lactamases represent a new threat, since they confer resistance to 7- $\alpha$ -methoxy-cephalosporins (cephamycins) such as cefoxitin or cefotetan are not affected by commercially available  $\beta$ -lactamase inhibitors, and can, in strains with loss of outer membrane porins, provide resistance to carbapenems (Philippon, *et al.*, 2002).

### **2.3 Extended-spectrum beta-lactamase (ES $\beta$ L)**

Members of the family Enterobacteriaceae commonly express plasmid-encoded  $\beta$ -lactamases (e.g., TEM-1, TEM-2, and SHV-1). Which confer resistance to penicillins but not to expanded-spectrum cephalosporins. In the mid-1980s, a new group of enzymes, the extended-spectrum beta-lactamases (ES $\beta$ Ls), was detected (first detected in Germany in 1983) (Knothe *et al.*, 1983). ES $\beta$ Ls are beta-lactamases that hydrolyze extended-spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam aztreonam. Thus, ES $\beta$ Ls confer resistance to these antibiotics and related oxyimino-beta lactams. In typical circumstances, they derive from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid configuration around the active site of these  $\beta$ -lactamases. This extends the spectrum of  $\beta$ -lactam antibiotics susceptible to hydrolysis by these enzymes. An increasing number of ES $\beta$ Ls not of TEM or SHV lineage has recently been described (Emery & Weymouth, 1997). The ES $\beta$ Ls are frequently plasmid encoded. Plasmids responsible for ES $\beta$ L production frequently carry genes encoding resistance to other drug classes, for example, aminoglycosides. Therefore, antibiotic options in the treatment of ES $\beta$ L-producing organisms are extremely limited. Carbapenems are the treatment of choice for serious infections due to ES $\beta$ L-producing organisms, yet carbapenem-resistant isolates have recently been reported. ES $\beta$ L-producing organisms may appear susceptible to

some extended-spectrum cephalosporins. However, treatment with such antibiotics has been associated with high failure rates.

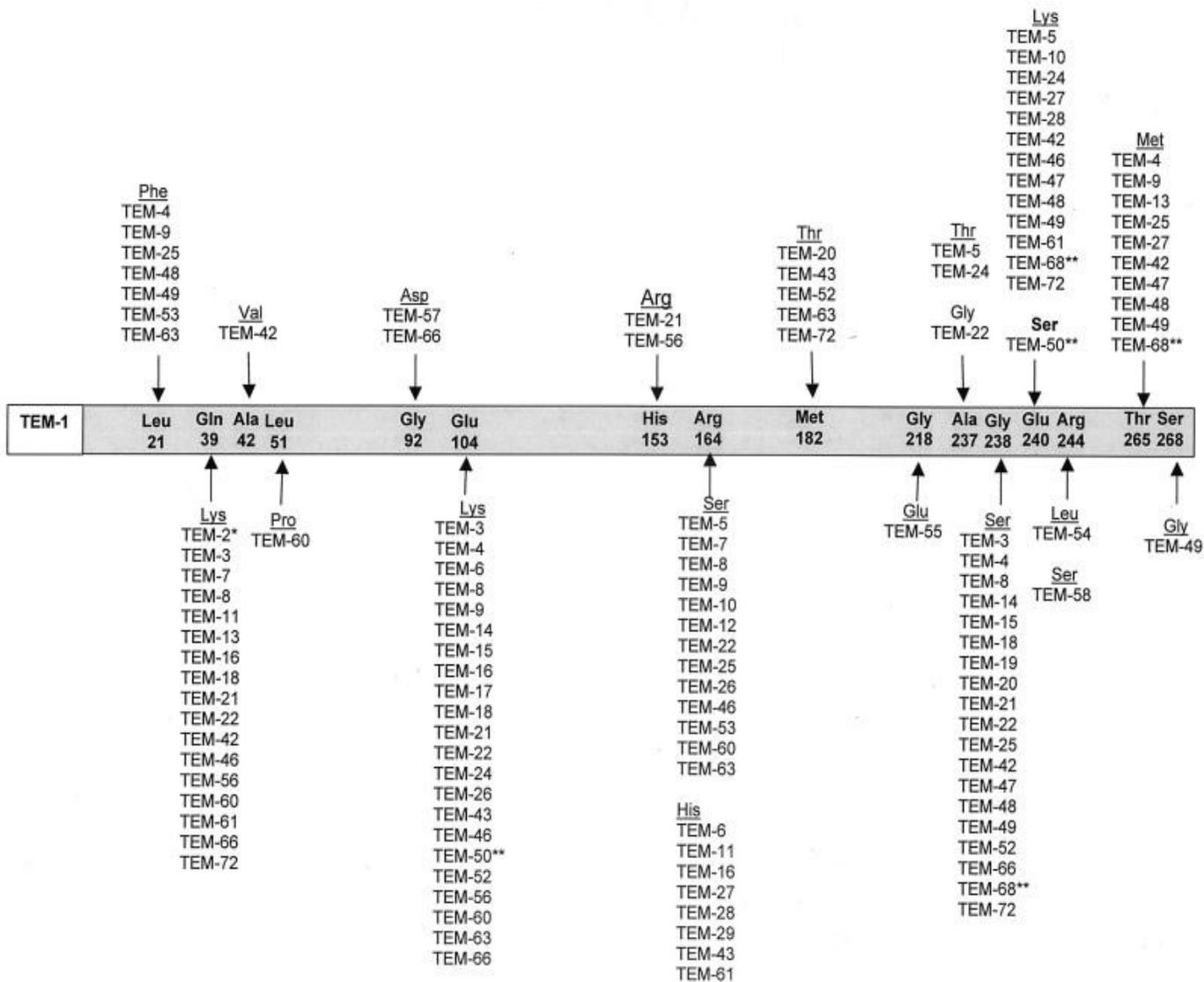
### **2.3.1 Diversity of ES $\beta$ L Types**

#### **2.3.1.1 TEM beta-lactamases (Class A)**

TEM-1 is the most commonly encountered beta-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Cooksey *et al.*, 1990). Also responsible for the ampicillin and penicillin resistance that is seen in *H. influenzae* and *N. gonorrhoeae* in increasing numbers. Although TEM-type beta-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found in other species of Gram-negative bacteria with increasing frequency. TEM-type ES $\beta$ Ls have been reported in genera of *Enterobacteriaceae* such as *Enterobacter aerogenes*, *Morganella morganii*, *Proteus mirabilis*, *Proteus rettgeri*, and *Salmonella* spp. (Bonnet *et al.*, 2000). Furthermore, TEM-type ES $\beta$ Ls have been found in non-*Enterobacteriaceae* gram-negative bacteria. The TEM-42  $\beta$ -lactamase was found in a strain of *P. aeruginosa*. Additionally, a recent report found the TEM-17  $\beta$ -lactamase being expressed from a plasmid in a blood culture isolate of *Capnocytophaga ochracea* (Rosenau *et al.*, 2000). The amino acid substitutions responsible for the ES $\beta$ L phenotype cluster around the active site of the enzyme and change its configuration, allowing access to oxyimino-beta-lactam substrates. Opening the active site to beta-lactam substrates also typically enhances the susceptibility of the enzyme to  $\beta$ -lactamase inhibitors, such as clavulanic

acid. Single amino acid substitutions at positions 104, 164, 238, and 240 produce the ES $\beta$ L phenotype, but ES $\beta$ Ls with the broadest spectrum usually have more than a single amino acid substitution. A number of amino acid residues are especially important for producing the ES $\beta$ L phenotype when substitutions occur at that position. They include glutamate to lysine at position 104, arginine to either serine or histidine at position 164, glycine to serine at position 238, and glutamate to lysine at position 240 (Fig.3).

In addition to  $\beta$ -lactamases TEM-1 through TEM-92 shown in Fig.2.3 and there has been a report of a naturally occurring TEM-like enzyme, TEM-AQ, that contained a number of amino acid substitutions and one amino acid deletion that have not been noted in other TEM enzymes (Perilli *et al.*,1997). Based upon different combinations of changes, currently 140 TEM-type enzymes have been described. TEM-10, TEM-12, and TEM-26 are among the most common in the United States (Bradford, 2001).



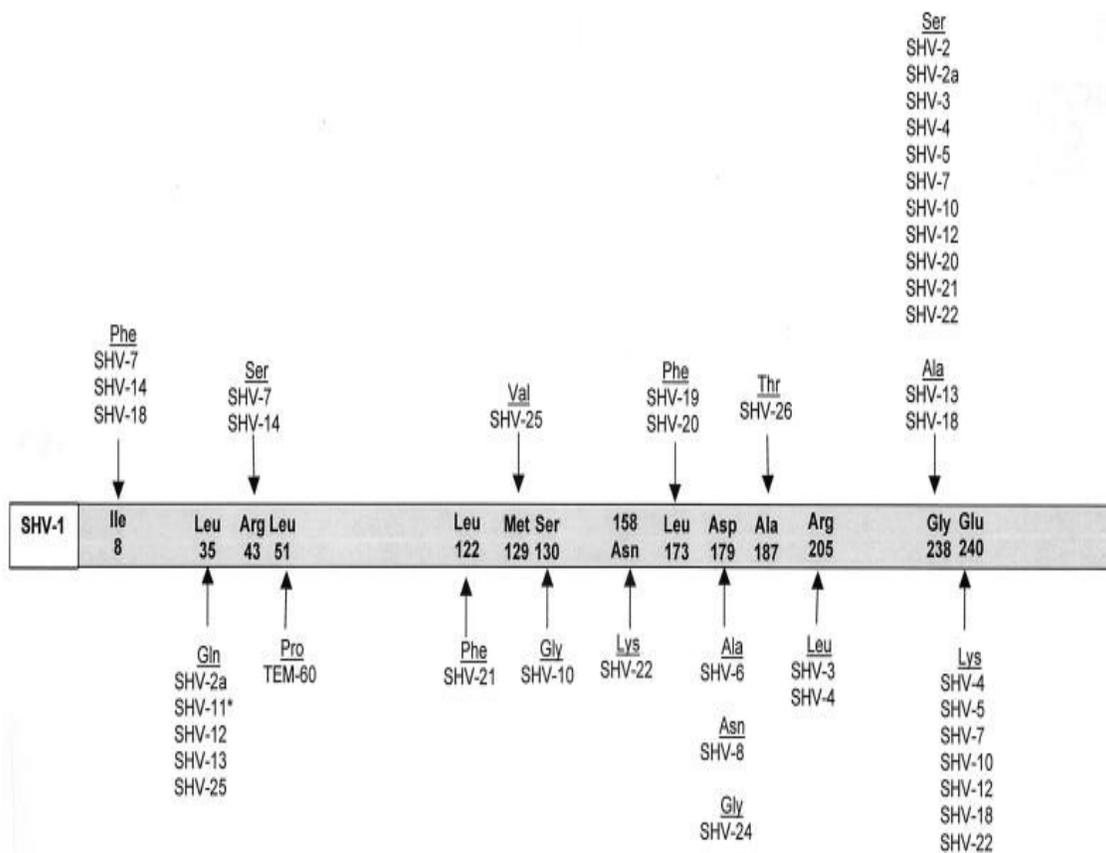
**Figure 2.3: Amino acid substitutions in TEM ESBL derivatives**

**Source:** Ambler *et al.* (1991).

The amino acids listed within the grey bar are those found in the structural gene of the TEM-1  $\beta$ -lactamase. The amino acid numbering is according to the scheme of Ambler *et al.* (1991).

### 2.3.1.2 SHV beta-lactamases (Class A)

SHV-1 shares 68 percent of its amino acids with TEM-1 and has a similar overall structure. The SHV-1 beta-lactamase is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. ESβLs in this family also have amino acid changes around the active site, most commonly at positions 238 or 238 and 240. Unlike the TEM-type β-lactamases, there are relatively few derivatives of SHV-1. Furthermore, the changes that have been observed in bla<sub>SHV</sub> to give rise to the SHV variants occur in fewer positions within the structural gene (Fig 2.4.). The majority of SHV variants possessing an ESβL phenotype are characterized by the substitution of a serine for glycine at position 238. A number of variants related to SHV-5 also have a substitution of lysine for glutamate at position 240. It is interesting that both the Gly238Ser and Glu240Lys amino acid substitutions mirror those seen in TEM-type ESβLs. The serine residue at position 238 is critical for the efficient hydrolysis of ceftazidime, and the lysine residue is critical for the efficient hydrolysis of cefotaxime (Huletsky *et al.*, 1993). More than 60 SHV varieties are known. They are the predominant ESβL type in Europe and the United States and are found worldwide. SHV-5 and SHV-12 are among the most common.



**Figure 2.4:** Amino acid substitutions in SHV ESBL derivatives

**Source:** Bradford (2001).

The amino acids listed within the grey bar are those found in the structural gene of the SHV-1  $\beta$ -lactamase (Bradford, 2001). The amino acid numbering is according to the scheme of Ambler *et al.* (1991).

### **2.3.1.3 CTX-M beta-lactamases (Class A)**

These enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates (e.g., ceftazidime, ceftriaxone, or cefepime). Rather than arising by mutation, they represent examples of plasmid acquisition of beta-lactamase genes normally found on the chromosome of *Kluyvera* species, a group of rarely pathogenic commensal organisms. These enzymes are not very closely related to TEM or SHV beta-lactamases in that they show only approximately 40% identity with these two commonly isolated beta-lactamases. More than 80 CTX-M enzymes are currently known. Despite their name, a few are more active on ceftazidime than cefotaxime. They have mainly been found in strains of *Salmonella enterica* serovar *Typhimurium* and *E. coli*, but have also been described in other species of Enterobacteriaceae and are the predominant ESBL type in parts of South America. They are also seen in Eastern Europe) CTX-M-14, CTX-M-3, and CTX-M-2 are the most widespread. CTX-M-15 is currently the most widespread type in *E. coli* in the UK and is widely prevalent in the community (Woodford *et al.*, 2006).

### **2.3.1.4 OXA Beta-lactamases (Class D)**

OXA beta-lactamases were long recognized as a less common but also plasmid mediated beta-lactamase variety that could hydrolyze oxacillin and related anti-staphylococcal penicillins. These beta-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d. The OXA-type beta-lactamases confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid. Amino acid substitutions in OXA enzymes can also give the ESBL phenotype. While most ESBLs have been found in *E. coli*, *K. pneumoniae*, and other Enterobacteriaceae, the OXA-type ESBLs have been found mainly in *P. aeruginosa*. OXA-type ESBLs have been found mainly in

*Pseudomonas aeruginosa* isolates from Turkey and France. The OXA beta-lactamase family was originally created as a phenotypic rather than a genotypic group for a few beta-lactamases that had a specific hydrolysis profile. Therefore, there is as little as 20% sequence homology among some of the members of this family. However, recent additions to this family show some degree of homology to one or more of the existing members of the OXA beta-lactamase family. Some confer resistance predominantly to ceftazidime, but OXA-17 confers greater resistance to cefotaxime and cefepime than it does resistance to ceftazidime.

## **2.4 Other beta-lactamases**

Other plasmid-mediated ES $\beta$ Ls, such as PER, VEB, GES, and IBC beta-lactamases, have been described but are uncommon and have been found mainly in *P. aeruginosa* and at a limited number of geographic sites. PER-1 is isolates in Turkey, France, and Italy; VEB-1 and VEB-2 is strains from Southeast Asia; and GES-1, GES-2, and IBC-2 is isolates from South Africa, France, and Greece. PER-1 is also common in multi-resistant acinetobacter species in Korea and Turkey. Some of these enzymes are found in Enterobacteriaceae as well, whereas other uncommon ES $\beta$ Ls (such as BES-1, IBC-1, SFO-1, and TLA-1) have been found only in Enterobacteriaceae.

### **2.4.1 Inhibitor-resistant $\beta$ -lactamases**

Although the inhibitor-resistant  $\beta$ -lactamases are not ES $\beta$ Ls, they are often discussed with ES $\beta$ Ls because they are also derivatives of the classical TEM- or SHV-type enzymes. These enzymes were at first given the designation IRT for inhibitor-resistant TEM  $\beta$ -lactamase; however, all have subsequently been renamed with numerical TEM designations. There are at least 19 distinct inhibitor-resistant TEM  $\beta$ -lactamases. Inhibitor-resistant TEM  $\beta$ -lactamases have been found mainly in clinical isolates of *E. coli*, but also some strains of *K. pneumoniae*,

*Klebsiella oxytoca*, *P. mirabilis*, and *Citrobacter freundii*. Although the inhibitor-resistant TEM variants are resistant to inhibition by clavulanic acid and sulbactam, thereby showing clinical resistance to the beta-lactam-lactamase inhibitor combinations of amoxicillin-clavulanate (co-amoxiclav), ticarcillin-clavulanate (co-ticarclav), and ampicillin/sulbactam, they normally remain susceptible to inhibition by tazobactam and subsequently the combination of piperacillin/tazobactam, although resistance has been described. This is no longer a primarily European epidemiology, it is found in northern parts of America often and should be tested for with complex UTI's (Bradford, 2001).

#### **2.4.2 AmpC-type $\beta$ -lactamases (Class C)**

AmpC type  $\beta$ -lactamases are commonly isolated from extended-spectrum cephalosporin resistant Gram-negative bacteria. AmpC  $\beta$ -lactamases (also termed class C or group 1) are typically encoded on the chromosome of many Gram-negative bacteria including *Citrobacter*, *Serratia* and *Enterobacter* species where its expression is usually inducible; it may also occur on *Escherichia coli* but is not usually inducible, although it can be hyperexpressed. AmpC type  $\beta$ -lactamases may also be carried on plasmids (Philippon *et al.*, 2002). AmpC  $\beta$ -lactamases, in contrast to ES $\beta$ Ls, hydrolyse broad and extended-spectrum cephalosporins (cephamycins as well as to oxyimino- $\beta$ -lactams) but are not inhibited by  $\beta$ -lactamase inhibitors such as clavulanic acid.

#### **2.4.3 Carbapenemases**

Carbapenems are famously stable to AmpC  $\beta$ -lactamases and extended-spectrum- $\beta$ -lactamases. Carbapenemases are a diverse group of  $\beta$ -lactamases that are active not only against the oxyimino-cephalosporins and cephamycins but also against the carbapenems. Aztreonam is stable to the metallo- $\beta$ -lactamases, but many IMP and VIM producers are resistant, owing to

other mechanisms. Carbapenemases were formerly believed to derive only from classes A, B, and D, but a class C carbapenemase has been described.

#### **2.4.4 IMP-type Carbapenemases (Metallo- $\beta$ -lactamases) (Class B)**

Plasmid-mediated IMP-type carbapenemases, 17 varieties of which are currently known, became established in Japan in the 1990s both in enteric Gram-negative organisms and in *Pseudomonas* and *Acinetobacter* species. IMP enzymes spread slowly to other countries in the Far East, were reported from Europe in 1997, and have been found in Canada and Brazil.

#### **2.4.5 VIM (Verona integron-encoded metallo- $\beta$ -lactamase) (Class B)**

A second growing family of carbapenemases, the VIM family, was reported from Italy in 1999 and now includes 10 members, which have a wide geographic distribution in Europe, South America, and the Far East and have been found in the United States. VIM-1 was discovered in *P. aeruginosa* in Italy in 1996; since then, VIM-2 - now the predominant variant - was found repeatedly in Europe and the Far East; VIM-3 and -4 are minor variants of VIM-2 and -1, respectively. VIM enzymes occur mostly in *P. aeruginosa*, also *P. putida* and very rarely, Enterobacteriaceae.

Amino acid sequence diversity is up to 10% in the VIM family, 15% in the IMP family, and 70% between VIM and IMP. Enzymes of both the families, nevertheless, are similar. Both are integron-associated, sometimes within plasmids. Both hydrolyse all  $\beta$ -lactams except monobactams, and evade all  $\beta$ -lactam inhibitors.

#### **2.4.6 OXA (Oxacillinase) group of $\beta$ -lactamases) (Class D)**

The OXA group of  $\beta$ -lactamases occur mainly in *Acinetobacter* species and are divided into two clusters. OXA carbapenemases hydrolyse carbapenems very slowly in vitro, and the high MICs

seen for some *Acinetobacter* hosts (>64 mg/L) may reflect secondary mechanisms. They are sometimes augmented in clinical isolates by additional resistance mechanisms, such as impermeability or efflux. OXA carbapenemases also tend to have a reduced hydrolytic efficiency towards penicillins and cephalosporins (Santillana *et al.*, 2007).

#### **2.4.7 KPC (*K. Pneumoniae* Carbapenemase) (Class A)**

A few class A enzymes, most noted the plasmid-mediated KPC enzymes, are effective carbapenemases as well. Ten variants, KPC-2 through KPC-11 are known, and they are distinguished by one or two amino-acid substitutions (KPC-1 was re-sequenced in 2008 and found to be 100% homologous to published sequences of KPC-2). KPC-1 was found in North Carolina, KPC-2 in Baltimore and KPC-3 in New York. They have only 45% homology with SME and NMC/IMI enzymes and, unlike them, can be encoded by self-transmissible plasmids. The class A *Klebsiella pneumoniae* carbapenemase (KPC) is currently the most common carbapenemase, which was first detected in North Carolina, US, in 1996 and has since spread worldwide (Nordmann *et al.*, 2009). A later publication indicated that Enterobacteriaceae that produce KPC were becoming common in the United States (Cuzon *et al.*, 2010).

#### **2.4.8 CMY (Class C)**

The first class C carbapenemase was described in 2006 and was isolated from a virulent strain of *Enterobacter aerogenes* (Kim *et al.*, 2006). It is carried on a plasmid, pYMG-1, and is, therefore, transmissible to other bacterial strains (Lee *et al.*, 2004).

#### **2.4.9 SME, IMI, NMC and CcrA**

In general, these are of little clinical significance.

CcrA (CfiA): Its gene occurs in c. 1-3% of *B. fragilis* isolates, but fewer produce the enzyme since expression demands appropriate migration of an insertion sequence. CcrA was known before imipenem was introduced, and producers have shown little subsequent increase.

#### **2.4.10 NDM-1 (New Delhi metallo- $\beta$ -lactamase) (Class B)**

New Delhi metallo-beta-lactamase

Originally described from New Delhi in 2009, this gene is now widespread in *Escherichia coli* and *Klebsiella pneumoniae* from India and Pakistan. As of mid-2010, NDM-1 carrying bacteria have been introduced to other countries (including the United States and UK), most probably due to the large number of tourists travelling the globe, who may have picked up the strain from the environment, as strains containing the NDM-1 gene have been found in environmental samples in India (Walsh *et al.*, 2011).

#### **2.5 Methods for ES $\beta$ L detection**

ES $\beta$ L testing involves two important steps. The first is a screening test with an indicator cephalosporin which looks for resistance or diminished susceptibility, thus identifying isolates likely to be harbouring ES $\beta$ Ls. The second one tests for synergy between an oxyimino cephalosporin and clavulanate, distinguishing isolates with ES $\beta$ Ls from those that are resistant for other reasons.

## **2.5.1 Screening for ESBL producers**

### **2.5.1.1 Disc-Diffusion methods**

The Clinical and Laboratory Standards Institute (CLSI) has proposed disk-diffusion methods for screening for ESBL production by *Klebsiellae pneumoniae*, *K. oxytoca*, *Escherichia coli* and *Proteus mirabilis*. Laboratories using disk-diffusion methods for antibiotic susceptibility testing can screen for ESBL production by noting specific zone diameters which indicate a high level of suspicion for ESBL production. Cefpodoxime, ceftazidime, aztreonam, cefotaxime or ceftriaxone disks are used. Since the affinity of ESBLs for different substrates is variable, the use of more than one of these agents for screening improves the sensitivity of detection (Wayne, 2009). However, it is adequate to use cefotaxime, which is consistently susceptible to CTX-M; and ceftazidime, which is a consistently good substrate for TEM and SHV variants. If only one drug can be used, then the single best indicator has been found to be cefpodoxime (Jarlier *et al.*, 1988; Steward *et al.*, 2000; Livermoore & Paterson, 2006). However, it has been seen that susceptibility testing with cefpodoxime can lead to a high number of false-positive results which can be due to mechanisms other than ESBL production (Livermoore & Paterson, 2006).

If isolates show resistance or diminished susceptibility to any of these five agents, it indicates suspicion for ESBL production, and phenotypic confirmatory tests should be used to ascertain the diagnosis.

### **2.5.1.2 Screening by dilution antimicrobial susceptibility tests**

The CLSI has proposed dilution methods for screening for ESBL production by *Klebsiellae pneumoniae* and *K. oxytoca*, *Escherichia coli* and *Proteus mirabilis*. Ceftazidime, aztreonam, cefotaxime or ceftriaxone can be used at a screening concentration of 1µg/ml or cefpodoxime at a concentration of 1µg/ml for *Proteus mirabilis*; or 4µg/ml, for the others. Growth at or above

this screening antibiotic concentration is suspicious of ES $\beta$ L production and is an indication for the organism to be tested by a phenotypic confirmatory test (Wayne, 2009).

### **2.5.1.3 Phenotypic confirmatory tests for ES $\beta$ L production**

Cephalosporin/clavulanate combination discs

The CLSI advocates use of cefotaxime (30 $\mu$ g) or ceftazidime (30 $\mu$ g) disks with or without clavulanate (10 $\mu$ g) for phenotypic confirmation of the presence of ES $\beta$ Ls in *Klebsiellae* and *Escherichia coli*, *P. mirabilis* and *Salmonella* species. The CLSI recommends that the disk tests be performed with confluent growth on Mueller-Hinton agar. A difference of  $\geq 5$  mm between the zone diameters of either of the cephalosporin discs and their respective cephalosporin/clavulanate disks is taken to be phenotypic confirmation of ES $\beta$ L production (Wayne, 2009).

For *Enterobacter* sp., *C. freundii*, *Morganella*, *Providentia* and *Serratia* spp., it is better to use cefepime or ceftiprome in the confirmatory tests as they are less prone to attack by the chromosomal AmpC beta lactamases, which may be induced by clavulanate in these species (Livermoore & Paterson, 2006).

### **2.5.1.4 Broth micro-dilution**

Phenotypic confirmatory testing can also be performed by broth microdilution assays using ceftazidime (0.25-128 $\mu$ g/mL), ceftazidime plus clavulanic acid (0.25/4-128/4 $\mu$ g/mL), cefotaxime (0.25-64 $\mu$ g/mL), or cefotaxime plus clavulanic acid (0.25/4 - 64/4 $\mu$ g/mL). Broth microdilution is performed using standard methods. Phenotypic confirmation is considered as  $\geq 3$  two fold serial-dilution decreases in minimum inhibitory concentration (MIC) of either cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone.

Steward *et al.* (2000) suggested using cefoxitin susceptibility in isolates with positive screening tests but negative confirmatory tests as a means of deducing the mechanism of resistance. ES $\beta$ L-producing isolates appear susceptible, while those with plasmid AmpC enzymes are resistant. However, resistance to cefoxitin seems to be increasing in ES $\beta$ L-producing isolates due to efflux or permeability changes or coexistence of ES $\beta$ Ls with AmpC enzymes. The usefulness of this screen test may thus be diminishing.

#### **2.5.1.5 Quality control when performing screening and phenotypic confirmatory tests**

Quality control recommendations are that simultaneous testing with a non-ES $\beta$ L-producing organism (*Escherichia coli* ATCC 25922) and an ES $\beta$ L-producing organism (*Klebsiella pneumoniae* ATCC 700603) also be performed (Wayne, 2009).

#### **2.5.1.6 Implications of positive phenotypic confirmatory tests**

According to CLSI guidelines, isolates which have a positive phenotypic confirmatory test should be reported as resistant to all cephalosporins (except the cephamycins, cefoxitin and cefotetan) and aztreonam, regardless of the MIC of that particular cephalosporin. Penicillins (for example, piperacillin or ticarcillin) are reported as resistant regardless of MIC, but  $\beta$ -lactam/  $\beta$ -lactamase inhibitor combinations (for example, ticarcillin-clavulanate or piperacillin-tazobactam) are reported as susceptible if MICs or zone diameters are within the appropriate range.

### **2.6 Other methods available for ES $\beta$ L detection**

Several other tests have been developed to confirm the presence of ES $\beta$ L.

### 2.6.1 Double-disc synergy test

In this, test disks of third-generation cephalosporins and augmentin are kept 30mm apart, centre to centre, on inoculated Mueller-Hinton agar (MHA) (Jarlier *et al.*, 1988). A clear extension of the edge of the inhibition zone of cephalosporin towards augmentin disk is interpreted as positive for ES $\beta$ L production. Evaluations of the double-disk diffusion test have revealed sensitivities of the method ranging from 79% to 97% and specificities ranging from 94% to 100% (Randegger & Hachler, 2001). While the double-disk diffusion test is technically simple, the interpretation of the test is quite subjective. Sensitivity may be reduced when ESBL activity is very low, leading to wide zones of inhibition around the cephalosporin and aztreonam disks, especially for *Proteus mirabilis* (Revathi & Singh, 1999). False-negative results have been observed with isolates harbouring SHV-2 (Thomson & Sanders, 1992; Randegger & Hachler, 2001). In isolates which are suspicious for harbouring ESBLs but are negative using the standard distance of 30 mm between disks, the test should be repeated using closer (for example, 20 mm) or more distant (for example, 40mm) spacing (Thomson & Sanders, 1992). A falsely positive test occurs for organisms such as *Stenotrophomonas maltophilia* because aztreonam is not a substrate for the metalloenzymes, and clavulanic acid inhibits other  $\beta$ -lactamases produced by this organism (Munoz & Garcia, 1998).

The double-disc diffusion ES $\beta$ L detection test as suggested by Jarlier *et al.* (1998). A disc containing amoxicillin-clavulanate (AMC) is placed in proximity to a disc containing ceftazidime (CAZ) or another oxyimino-cephalosporin. The clavulanate in the amoxicillin-clavulanate disc diffuses through the agar and inhibits the  $\beta$ -lactamase surrounding the ceftazidime disc. Enhancement of the zone of the ceftazidime disc on the side facing the amoxicillin-clavulanate disc is interpreted as a positive test.

### **2.6.2 Three-dimensional test**

The three-dimensional test gives phenotypic evidence of ES $\beta$ L-induced inactivation of extended-spectrum cephalosporins or aztreonam without relying on demonstration of inactivation of the  $\beta$ -lactamases by a  $\beta$ -lactamase inhibitor (Thomson & Sanders, 1992). In this test, the surface of the susceptibility plate is inoculated by standard methods for disk-diffusion testing, but additionally a circular slit is cut in the agar concentric with the margin of the plate. A heavy inoculum of the test organism ( $10^9$  to  $10^{10}$  CFU of cells) is pipette into the slit.  $\beta$ -lactam-impregnated disks are then placed on the surface of the agar 3mm outside of the inoculated circular slit.  $\beta$ -lactamase induced inactivation of each test antibiotic is detected by inspection of the margin of the zone of inhibition in the vicinity of its intersection with the circular three-dimensional inoculation. The presence of  $\beta$ -lactamase-induced drug inactivation is visualized as a distortion or discontinuity in the usually circular inhibition zone or as the production of discrete colonies in the vicinity of the inoculated slit.

### **2.6.3 Inhibitor-potentiated disk-diffusion test**

Antibiotic discs containing ceftazidime (30 $\mu$ g), cefotaxime (30 $\mu$ g), ceftriaxone (30 $\mu$ g) and aztreonam (30 $\mu$ g) are placed on the clavulanate-containing agar plates and regular clavulanate-free Mueller-Hinton agar plates. A difference in  $\beta$ -lactam zone width of  $\geq 10$  mm in the two media was considered positive for ES $\beta$ L production. A major drawback of the method is the need to freshly prepare clavulanate-containing plates. The potency of clavulanic acid begins to decrease after 72 hours. Cephalosporin/clavulanate zone size to cephalosporin zone size of 1.5 or greater was taken to signify the presence of ES $\beta$ L activity. Using this method, the sensitivity of the test for detecting ES $\beta$ Ls was 93% using both ceftazidime and cefotaxime. The test did not detect ES $\beta$ L production by strains producing SHV-6 (M'Zali *et al.*, 2000).

#### **2.6.4 Disc approximation test**

Cefoxitin (inducer) disc is placed at a distance of 2.5cm from cephalosporin disc (Revathi & Singh, 1999). Production of inducible  $\beta$ -lactamase is indicated by flattening of the zone of inhibition of the cephalosporin disc towards inducer disc by  $>1$  mm.

### **2.7 Commercially available methods for ES $\beta$ L detection**

#### **2.7.1 Vitek ES $\beta$ L Test**

A specific card which includes tests for ES $\beta$ L production has now been Food and Drug Administration (FDA) approved. The Vitek ES $\beta$ L test (bio Merieux Vitek, Hazelton, Missouri) utilizes cefotaxime and ceftazidime, alone (at 0.5 $\mu$ g/mL) and in combination with clavulanic acid (4 $\mu$ g/mL). Inoculation of the cards is identical to that performed for regular Vitek cards. Analysis of all wells is performed automatically once the growth control well has reached a set threshold (4-15 hours of incubation). A predetermined reduction in the growth of the cefotaxime or ceftazidime wells containing clavulanic acid, compared with the level of growth in the well with the cephalosporin alone, indicates presence of ES $\beta$ L. Sensitivity and specificity of the method exceed 90% (Sanders *et al.*, 1996).

#### **2.7.2 E - test**

The E-test ES $\beta$ L strip (AB Biodisk, Solna, Sweden) carries two gradients: on the one end, ceftazidime; and on the opposite end, ceftazidime plus clavulanic acid MIC is interpreted as the point of intersection of the inhibition ellipse with the E test strip edge. A ratio of ceftazidime MIC to ceftazidime-clavulanic acid MIC equal to or greater than 8 indicates the presence of ES $\beta$ L. The reported sensitivity of the method as a phenotypic confirmatory test for ES $\beta$ Ls is 87% to 100%, (Cormican *et al.*, 1996) and the specificity is 95% to 100%. The availability of

cefotaxime strips, as well as ceftazidime strips, improves the ability to detect ES $\beta$ L types, which preferentially hydrolyze cefotaxime, such as CTX-M type enzymes (Paterson & Bonomo, 2005).

### **2.7.3 MicroScan panels**

MicroScan panels (Dade Behring MicroScan, Sacramento, and CA.) comprise dehydrated panels for microdilution antibiotic susceptibility. Those used for ES $\beta$ L detection which contain combinations of ceftazidime or cefotaxime plus  $\beta$ -lactamase inhibitors have received Food and Drug Administration approval; and in studies of large numbers of ES $\beta$ L-producing isolates, they have appeared to be highly reliable (Komatsu *et al.*, 2003).

### **Becton Dickinson (BD) Phoenix Automated Microbiology System**

Becton Dickinson Biosciences (Sparks, Md) have introduced a short-incubation system for bacterial identification and susceptibility testing known as BD Phoenix (Leverstein-van *et al.*, 2002; Sturenburg *et al.*, 2003) The Phoenix ES $\beta$ L test uses growth response to cefpodoxime, ceftazidime, ceftriaxone and cefotaxime, with or without clavulanic acid, to detect the production of ES $\beta$ Ls. The test algorithm has been delineated by Sanguinetti *et al.* (2003). Results are usually available within 6 hours. The BD Phoenix ES $\beta$ L detection method detected ES $\beta$ L production in greater than 90% of strains genotypically confirmed to produce ESBLs. The method correctly detected ES $\beta$ L production by *Enterobacter*, *Proteus* and *Citrobacter* spp., in addition to *Klebsiellae* and *Escherichia coli* (Sanguinetti *et al.*, 2003).

## **2.8 Problems in Detection**

Identifying ES $\beta$ L-producing organisms is a major challenge for the clinical microbiology laboratory. Multiple factors contribute to this, including production of multiple different  $\beta$ -

lactamase types by a single bacterial isolate and the production of ES $\beta$ Ls by organisms that consecutively produce AmpC  $\beta$ -lactamases, varying substrate affinities and inoculum effect. The phenotypic confirmatory tests are highly sensitive and specific compared to genotypic confirmatory tests. However, there are a number of instances whereby the phenotypic confirmatory tests may be falsely positive or negative.

*Klebsiella pneumoniae* or *Escherichia coli* isolates which lack ES $\beta$ Ls but which hyperproduce SHV-1 may give false-positive confirmatory test results. Such isolates can have ceftazidime MICs as high as 32 $\mu$ g/mL (Rice *et al.*, 2000).

There are now numerous reports in which *Klebsiella pneumoniae* isolates have been found to harbor plasmid-mediated AmpC-type  $\beta$ -lactamases. Some of these organisms have been found to harbor both AmpC-type  $\beta$ -lactamases and ES $\beta$ Ls (Tzouvelekis *et al.*, 1999) The coexistence of both enzyme types in the same strain not only results in elevated cephalosporin MICs but may also give false-negative test results for the detection of ES $\beta$ Ls. The likely explanation is that AmpC-type  $\beta$ -lactamases resist inhibition by clavulanate and hence obscure the synergistic effect of clavulanate and cephalosporins against ES $\beta$ Ls.

For ES $\beta$ L-producing bacteria, there is a dramatic rise of MIC for extended-spectrum cephalosporins as the inoculum is increased beyond that used in routine susceptibility tests. Same isolates test susceptible at the standard inoculum and resistant at a higher inoculum. Therefore, false-negative results can occur with both screening and confirmatory tests when lower inocula are used (Queenan *et al.*, 2004).

Some ES $\beta$ L isolates may appear susceptible to a third-generation cephalosporin in vitro, particularly if relatively high breakpoints are used. However, treatment of infections due to an ES $\beta$ L-producing organism with third-generation cephalosporins may result in clinical failure even when the MIC is below the breakpoint and the ability of these enzymes to confer

resistance to weak-substrate cephalosporins is clear when MIC determinations are performed with heavy inoculum. This may be due to the variable affinity of these enzymes for different substrates and inoculum effect (Nathisuwan *et al.*, 2001).

Many ES $\beta$ L producers are resistant to combinations despite appearing sensitive *in vitro*. This could be due to hyper production making the inhibitor overwhelmed, relative impermeability of the host or co-production of inhibitor-resistant penicillanases (e.g., OXA-1). Since ES $\beta$ L production is usually plasmid mediated, it is possible for one specimen to contain both ES $\beta$ L-producing and non-ES $\beta$ L-producing cells of the same species. This suggests that for optimal detection, several colonies must be tested from a primary culture plate (Coudron *et al.*, 1997).

ESBL enzymes can be induced by certain antibiotics, amino acids or body fluids. Organism possessing genes for inducible  $\beta$ -lactamases show false susceptibility, if tested in the uninduced state (Revathi & Singh, 1999). All these factors make detection of ES $\beta$ Ls a complicated and complex task, and improvements in the ability of clinical laboratories to detect ES $\beta$ L are needed. Two opposing viewpoints have arisen in recognition of the poor outcome when patients with an infection due to an ES $\beta$ L-producing organism are treated with a cephalosporin to which it appears susceptible *in vitro*. Some investigators believe that alteration of cephalosporin breakpoints for Enterobacteriaceae by organizations such as the Clinical and Laboratory Standards Institute is a more appropriate endeavor than expanding efforts to detect ESBLs, which is too complex a task for a clinical microbiology laboratory. An advantage of such a change would be that organisms such as *Enterobacter* spp., which are not currently considered in CLSI guidelines for ES $\beta$ L detection, would be covered (Paterson & Bonomo, 2005).

Another viewpoint is that the inoculum effect is important for ES $\beta$ L-producing organisms. *In vitro*, the MICs of cephalosporins rise as the inoculum of ES $\beta$ L-producing organisms increases (Thauvin-Eliopoulos *et al.*, 1997). Thus, in the presence of high-inoculum infections (for

example, intra-abdominal abscess, some cases of pneumonia) or infections at sites in which drug penetration may be poor (for example, meningitis, endocarditis or osteomyelitis), physicians should avoid cephalosporins if an ES $\beta$ L-producing organism is present. Also severity of illness could have been greater in patients infected with organisms with higher MICs.

A point favouring efforts aimed at ES $\beta$ L detection is the infection control significance of detecting plasmid-mediated multi-drug resistance. There are epidemiologic implications for the detection of ES $\beta$ L-producing organisms, as the significance of this resistance may not be as apparent if organisms are simply reported as intermediate or resistant to individual cephalosporins. Outbreaks of ES $\beta$ L-producing organisms can be abruptly halted using appropriate infection-control interventions. Endemic transmission of ES $\beta$ L producers can also be curtailed, using infection-control measures and antibiotic management interventions. Detection of ES $\beta$ L production in organisms from samples such as urine may be important because this represents an epidemiologic marker of colonization and therefore has the potential to transfer such organisms to other patient.

## **2.9 Risk factors**

Patients at high risk for developing colonization or infection with ES $\beta$ L -producing organisms are often seriously ill with prolonged hospital stays and in whom invasive medical devices are present (urinary catheters, endotracheal tubes, central venous lines) for a prolonged duration (Paterson & Bonomo, 2005). In addition, other risk factors have been found in individual studies, including the presence of nasogastric tubes (Asensio *et al.*, 2000) gastrostomy or jejunostomy tubes (Weldhagen & Prinsloo, 2004) or arterial lines; (Pena *et al.*, 1997): administration of total parenteral nutrition, recent surgery, haemodialysis, (Crowley, 2001) decubitus ulcers and poor nutritional status (Weldhagen & Prinsloo, 2004).

Heavy antibiotic use is also a risk factor for acquisition of ES $\beta$ L-producing organism (Lautenbach *et al.*, 2001). Several studies have found a relationship between third-generation cephalosporin use and acquisition of an ES $\beta$ L-producing strain (Pessoa-Silva *et al.*, 2003). However, the greatest risk factor for nosocomial acquisition of an ES $\beta$ L-producing organism is accommodation in a ward or room with other patients with ES $\beta$ L-producing organisms (Livermoore & Paterson, 2006).

When less than the required dosage is taken or not taken within the prescribed time, antibiotic concentration in the tissues decreases to suboptimal levels increasing the frequency of antibiotic resistant organisms to develop and multiply. Factors within the intensive care unit setting such as mechanical ventilation and multiple underlying diseases also appear to contribute to bacterial resistance (Thomas, 1989).

Risk factors for colonization or infection with ES $\beta$ L-producing organisms, especially the CTX-M producers, include the history of a recent hospitalization; treatment with cephalosporins, penicillins and quinolones, age 65 years or higher, dementia and diabetes (Paterson & Bonomo, 2005). Although there is no conclusive evidence, one potential source of colonization with the ES $\beta$ L producers in the community may be the use of veterinary oxyimino cephalosporins like ceftiofur in livestock (Livermoore & Paterson, 2006).

In researches occasional animal-to-human spread of drug-resistant organisms has been demonstrated. Resistant bacteria can be transmitted from animals to humans in three ways: by consuming animal products (milk, meat, eggs, etc.), from close or direct contact with animals or other humans, or through the environment (Schneider & Garrett, 2009). In the first pathway, food preservation methods can help eliminate, decrease, or prevent the growth of bacteria in some food classes. Evidence for the transfer of antibiotic-resistant microorganisms from animals to humans has been scanty, and most evidence show that pathogens of concern in

human populations originated in humans and are maintained there, with rare cases of transference to humans (Hurd *et al.*, 2004).

### **2.9.1 Problems associated with ESβLs**

#### **Resistance**

As previously described, ESβL enzymes are plasmid mediate; meaning that genes encoding these enzymes are located on plasmids and are easily transferable to different bacteria. Most of these plasmids not only contain DNA encoding ESβL enzymes but also carry genes conferring resistance to several non-β-Lactam antibiotics (Jacoby & Sutton, 1991; Jacoby, 1994). Consequently, most ESβL isolates are resistant to many classes of antibiotics. The most frequent resistances found in ESβL-producing organisms are aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and sulfamethoxazole-trimethoprim (Jacoby & Sutton, 1991; Jacoby, 1994; Paterson *et al.*, 2000). Treatment of these multidrug-resistant organisms is a therapeutic challenge.

#### **Detection**

Identifying organisms that are ESβLs is a major challenge for the clinical microbiology laboratory. Due to the variable affinity of these enzymes for different substrates and the inoculum effect, some ESβL isolates may appear susceptible to a third-generation cephalosporin *in vitro*. However, treatment of infections due to an ESβL-producing organism with third-generation cephalosporins may result in clinical failure if the infection is outside the urinary tract (Jacoby, 1997). For example, a *K. pneumoniae* strain that harbors the TEM 26 enzyme may appear resistant to ceftazidime (MIC > 256 µg/ml) but sensitive to cefotaxime (MIC 0.5 µg/ml) *in vitro* (Rice *et al.*, 1991). If cefotaxime is used to treat that infection, treatment failure is likely. This is due to the fact that TEM 26 is highly effective in inactivating ceftazidime, even at low colony counts. However, with the higher colony count frequently found at infection sites,

the enzyme can inactivate cefotaxime and render the drug ineffective, resulting in a several-fold increase in MIC. Despite in vitro susceptibilities, reports of failures in both animal models and clinical settings are well documented when third-generation cephalosporins are used to treat ES $\beta$ L infections, unless the infection is confined to the urinary tract (Jorgensen & Ferraro, 2000; Jacoby *et al.*, 2003).

Two recent studies evaluated the ability of clinical laboratories to detect and report the presence of ES $\beta$ Ls in either *Klebsiella* isolates or *E. coli*. A survey in Connecticut found that 21% of laboratories failed to detect ES $\beta$ L-producing isolates (Tenover *et al.*, 1999). A proficiency testing project for clinical laboratories participating in the National Nosocomial Infections Surveillance System indicated that as many as 58% of laboratories failed to detect and report ES $\beta$ L isolates correctly (Steward *et al.*, 2000). These data suggest that improvements in the ability of clinical laboratories to detect ESBLs are needed. In addition, clinical pharmacists should be aware of the capability of the clinical laboratory in their hospital with regard to ES $\beta$ L testing.

Testing for the presence of ES $\beta$ Ls using techniques recommended by the NCCLS guidelines is not easily performed in clinical practice. Fortunately, automated testing systems designed to detect ES $\beta$ Ls recently were approved by the Food and Drug Administration and are now commercially available (Vitek and Microscan). Another product that is effective in ES $\beta$ L detection is ES $\beta$ L Etest (AB Biodisk). All of these testing methods have been shown to have greater than 90% sensitivity in ES $\beta$ L detection. A three-phase trial compared disk diffusion methods recommended by NCCLS guidelines with an automated growth-monitoring system (Vitek ES $\beta$ L). Results indicate that the two testing methods were comparable in both sensitivity (98.6% for disk diffusion vs 99.7% for Vitek ES $\beta$ L) and specificity (99.4% for disk diffusion and 100% for Vitek ES $\beta$ L) (Sanders *et al.*, 1996). The efficacy of ES $\beta$ L E-test also was

compared with the disk diffusion method. Results demonstrated that E-test was more sensitive than the disk diffusion method (100% vs 87%), whereas both methods were comparable in specificity (Cormican *et al.*, 1996). These advances are of great benefit to clinicians in overcoming the difficulties of ES $\beta$ L detection (Sanders *et al.*, 1996).

## **Outbreaks**

A number of nosocomial outbreaks (unit to unit and hospital to hospital) caused by ES $\beta$ L-producing organisms have been reported in the U.S (Rice *et al.*, 1990; Naumovski *et al.*, 1992). Although most outbreaks were limited to high-risk patient-care areas (i.e., ICUs, oncology units), the first report of an outbreak in nursing homes recently appeared in the literature (Wiener *et al.*, 1999). Therefore, the threat of these resistant organisms is not limited to institutions.

## **Morbidity and Mortality**

There has been no prospective controlled trial of antimicrobial therapy in ES $\beta$ L infections. However, several retrospective studies suggest that infections caused by ES $\beta$ L isolates are associated with increased morbidity and mortality compared with infections caused by non-ES $\beta$ L-producing organisms (Naumovski *et al.*, 1992; Medeiros, 1997). These data consistently indicate high clinical failure rates when third-generation cephalosporins were used to treat infections caused by ES $\beta$ L-producing organisms. Exceptions are certain minor infections, such as those of the urinary tract. Therefore, appropriate therapy for ES $\beta$ L infections is crucial.

## **2.10 Epidemiology of ES $\beta$ L-producing organisms**

**Europe:** ES $\beta$ L-producing organisms were first detected in Europe. Although the initial reports were from Germany (Knothe *et al.*, 1983) and England (Du Bois, 1995), the vast majority of

reports in the first decade after the discovery of ESβLs were from France (Philippon *et al.*, 2002; Sirot *et al.*, 1987). The first large outbreak in France to be reported occurred in 1986; 54 patients in three intensive care units were infected and spread of the infection to four other wards then occurred. The proliferation of ESβLs in France was quite dramatic. By the early 1990s, 25 to 35% of nosocomially acquired *Klebsiella pneumoniae* isolates in France were ESβL producing (Marty & Jarlier, 1998). However, in recent years, augmentation of infection control interventions has been accompanied by a decrease in incidence of ESβL-producing *Klebsiella pneumoniae* (Lucet *et al.*, 1999). In northern France, the proportion of *Klebsiella pneumoniae* isolates which were ESβL-producing fell from 19.7% in 1996 to 7.9% in 2000 (Albertini *et al.*, 2002). It is noteworthy; however, that 30.2% of *Enterobacter aerogenes* isolates in 2000 were ESBL producers (Albertini *et al.*, 2002). It is also important to note that while the proportion of *Klebsiella pneumoniae* isolates which are ESβL-producing may be decreasing in some parts of Western Europe, a significant increase may be occurring in Eastern Europe (Munoz & Garcia, 1998). Outbreaks of infection with ESβL-producing organisms have now been reported from virtually every European country.

There is considerable geographical difference in the occurrence of ESβLs in European countries. Within countries, hospital-to-hospital variability in occurrence may also be marked (Babini & Livermore, 2000). In a 1997-1998 survey of 433 isolates from 24 intensive care units in western and southern Europe, 25% of *klebsiellae* possessed ESβLs (Babini & Livermore, 2000). A similar survey was performed by the same group in 1994; the overall proportion of *klebsiellae* which possessed ESBLs did not differ significantly between the two time periods, but the percentage of intensive care units which recorded ESβL-producing *klebsiellae* rose significantly from 74% to >90% (Babini & Livermore, 2000). Another large study from more than 100 European intensive care units found that the prevalence of ESβLs in *klebsiellae* ranged from as low as 3% in Sweden to as high as 34% in Portugal (Hanberger *et al.*, 1999). A third

study, which included both intensive care unit and non-intensive care unit isolates from 25 European hospitals, found that 21% of *Klebsiella pneumoniae* isolates had reduced susceptibility to ceftazidime (usually indicative of ES $\beta$ L production, although it is acknowledged that other mechanisms of resistance may be responsible) (Fluit *et al.*, 2000). In Turkey, a survey of *Klebsiella* spp. from intensive care units from eight hospitals showed that 58% of 193 isolate harboured ES $\beta$ Ls (Gunseren *et al.*, 1999).

**North America:** First reports of ES $\beta$ L-producing organisms in the United States occurred in 1988 (Jacoby *et al.*, 1997). In 1989, significant infections with TEM-10-producing *Klebsiella pneumoniae* were noted in Chicago by Quinn and colleagues (Quinn *et al.*, 1989). Other early reports of outbreaks mainly described infections with TEM-type ES $\beta$ Ls, particularly TEM-10, TEM-12, and TEM-26 (Rice *et al.*, 1990; Naumovski *et al.*, 1992; Rasmussen *et al.*, 1993; Rice *et al.*, 1993). However, outbreaks with SHV-type ES $\beta$ Ls have also been described (Jacoby, 1997). CTX-M-type ES $\beta$ Ls have recently been described in the United States and Canada (Pitout *et al.*, 2007).

Assessment of the prevalence of ES $\beta$ L-producing organisms in the United States has been hampered by reliance of statistics examining resistance of organisms to third-generation cephalosporins, where resistance is defined as an MIC of  $\geq 32\mu\text{g/ml}$  (ceftazidime) or  $\geq 64\mu\text{g/ml}$  (cefotaxime/ceftriaxone). Since many ES $\beta$ L-producing organisms have MICs for third-generation cephalosporins between 2 and  $16\mu\text{g/ml}$ , the prevalence of ES $\beta$ L-producing organisms in the United States may have been underestimated in the past. Moland and colleagues have shown that ES $\beta$ L-producing isolates were found in 75% of 24 medical centers in the United States. In a survey of nearly 36,000 isolates from intensive care units in North America, nonsusceptibility of *Klebsiella pneumoniae* to third-generation cephalosporins averaged 13% (Neuwirth *et al.*, 2001). The percentage of isolates which were susceptible fell by

3% over the years 1994 to 2000 (Neuwirth *et al.*, 2001). National Nosocomial Infection Surveillance (NNIS) figures for the period January 1998 to June 2002 reveal that 6.1% of 6,101 *Klebsiella pneumoniae* isolates from 110 intensive care units were resistant to third-generation cephalosporins (NNIS, 2002). In at least 10% of intensive care units, resistance rates exceeded 25%. In non-intensive care unit inpatient areas, 5.7% of 10,733 *Klebsiella pneumoniae* isolates were ceftazidime resistant (NNIS, 2002). In outpatient areas, just 1.8% of 12,059 *Klebsiella pneumoniae* isolates and 0.4% of 71,448 *Escherichia coli* isolates were ceftazidime resistant (NNIS, 2002).

**South and Central America:** In 1988 and 1989 isolates of *Klebsiella pneumoniae* from Chile and Argentina were reported as harbouring SHV-2 and SHV-5 (Casellas and Goldberg, 1989). In retrospect, however a *Klebsiella pneumoniae* isolate kept lyophilized since 1982, during a preclinical study of cefotaxime in Buenos Aires, also proved to be a producer of an SHV-5 ES $\beta$ L. In 1989 an outbreak of multi-resistant *Salmonella enterica* serovar Typhimurium infections occurred in 12 of 14 Argentinian provinces. From these isolates a new non-SHV, non-TEM ES $\beta$ L named CTX-M-2 was identified (Bauernfeind, 1996; Radice *et al.*, 2002). Organisms with CTX-M-2 have spread throughout many parts of South America (Radice *et al.*, 2002). Other CTX-M enzymes (CTX-M-8, -9, and -16) have been discovered in Brazil (Bonnet *et al.*, 2000; Bonnet *et al.*, 2001). Curiously, TEM-type ES $\beta$ Ls have been very rarely reported from South America. As noted above, two novel non-TEM, non-SHV ES $\beta$ Ls have been recently reported from South America: GES-1, isolated from an infant previously hospitalized in French Guiana (Poirel *et al.*, 2000), and BES-1, from an ES $\beta$ L-producing *Serratia marcescens* isolate from a hospital in Rio de Janeiro (Bonnet *et al.*, 2000).

ES $\beta$ Ls have been found in 30 to 60% of *klebsiellae* from intensive care units in Brazil, Colombia, and Venezuela (Sader *et al.*, 1998; Mendes *et al.*, 2000; Sader *et al.*, 2000). Reports

of ES $\beta$ L-producing organisms also exist from Central America and the Caribbean Islands (Gonzalez-Vertiz *et al.*, 2001; Silva *et al.*, 2001).

**Africa and the Middle East:** Several outbreaks of infections with ES $\beta$ L-producing *Klebsiella* have been reported from South Africa (Shipton *et al.*, 2001), but no national surveillance figures have been published. However, it has been reported that 36.1% of *Klebsiella pneumoniae* isolates collected in a single South African hospital in 1998 and 1999 were ES $\beta$ L producers (Bell *et al.*, 2002). ES $\beta$ Ls have also been documented in Israel, Saudi Arabia, and a variety of North African countries (Borer *et al.*, 2002; Neuhauser *et al.*, 2003). Outbreaks of *Klebsiella* infections with strains resistant to third-generation cephalosporins have been reported in Nigeria and Kenya without documentation of ES $\beta$ L production (Akindele & Rotilu, 1997; Musoke & Revathi, 2000). A novel CTX-M enzyme (CTX-M-12) has been found in Kenya (Kariuki *et al.*, 2001). Characterization of ES $\beta$ Ls from South Africa has revealed TEM and SHV types (especially SHV-2 and SHV-5) (Hanson *et al.*, 2001; Pitout *et al.*, 2004). A nosocomial outbreak of infections with *Pseudomonas aeruginosa*, expressing GES-2 has been described in South Africa (Poirel *et al.*, 2002).

**Australia:** The first ES $\beta$ Ls to be detected in Australia were isolated from a collection of gentamicin-resistant *Klebsiella* spp. collected between 1986 and 1988 from Perth (Mulgrave, 1990). These were characterized as being of SHV derivation (Mulgrave & Attwood, 1993). In the last decade, ES $\beta$ L-producing organisms have been detected in every state of Australia and in the Northern Territory (Bell *et al.*, 2002; Howard *et al.*, 2002). Outbreaks of infection have occurred in both adult and pediatric patients. Overall, it appears that the proportion of *Klebsiella pneumoniae* isolates which are ES $\beta$ L producers in Australian hospitals is about 5% (Bell *et al.*, 2002).

**Asia:** In 1988, isolates of *Klebsiella pneumoniae* from China which contained SHV-2 were reported. In reports comprising limited numbers of isolates collected in 1998 and 1999, 30.7% of *Klebsiella pneumoniae* isolates and 24.5% of *Escherichia coli* isolates were ES $\beta$ L producers (Bell *et al.*, 2002). In a major teaching hospital in Beijing, 27% of *Escherichia coli* and *Klebsiella pneumoniae* blood culture isolates collected from through 1999 were ES $\beta$ L producers (Du Bois *et al.*, 1995). Of isolates collected from Zhejiang Province, 34% of *Escherichia coli* isolates and 38.3% of *Klebsiella pneumoniae* isolates were ES $\beta$ L producing (Yu *et al.*, 2002).

National surveys have indicated the presence of ES $\beta$ Ls in 5 to 8% of *Escherichia coli* isolates from Korea, Japan, Malaysia, and Singapore but 12 to 24% in Thailand, Taiwan, the Philippines, and Indonesia. Rates of ES $\beta$ L production by *Klebsiella pneumoniae* have been as low as 5% in Japan (Yagi *et al.*, 1997; Lewis *et al.*, 1999) and 20 to 50% elsewhere in Asia. However, there are clearly differences from hospital to hospital: it has been reported that a quarter of all *Klebsiella pneumoniae* isolates from a hospital in Japan in 1998 and 1999 were ES $\beta$ L producers (Bell *et al.*, 2002).

ES $\beta$ Ls of the SHV-2, SHV-5, and SHV-12 lineage initially dominated in those studies in which genotypic characterization has been carried out (Lee *et al.*, 2004). Newly described SHV-type ES $\beta$ Ls have recently been reported from Taiwan and Japan (Kurokawa *et al.*, 2000). However, the appearance of CTX-M ES $\beta$ Ls in India (Karim *et al.*, 2001; Poirel *et al.*, 2002) and China (Chanawong *et al.*, 2000; Wu *et al.*, 2001; Walsh *et al.*, 2005), and more frequent reports of outbreaks of infection with CTX-M-type ES $\beta$ Ls in Japan (Komatsu *et al.*, 2001), Korea (Pai *et al.*, 2001), and Taiwan (Yu *et al.*, 2002), raise suspicions that these may indeed be the dominant ES $\beta$ L types in Asia. Plasmid-mediated non-TEM, non-SHV ES $\beta$ Ls, showing homology to the chromosomal  $\beta$ -lactamases of *Klebsiella oxytoca* (Toho-1 and Toho-2), have been detected in

Japan (Ishii *et al.*, 1995). A new non-TEM, non-SHV ES $\beta$ L (VEB-1) has been reported from Thailand and Vietnam (Poirel *et al.*, 1999; Girlich *et al.*, 2002).

### **Molecular Epidemiology of Nosocomial Infections with ESBL-Producing Organisms**

More than 50 studies (describing in total more than 3,000 patients) have been published in peer-reviewed medical literature utilizing molecular typing methods in the study of the epidemiology of nosocomial infections with ES $\beta$ L-producing organisms (Paterson & Yu, 1999). More than 75% of the studies have addressed ES $\beta$ L-producing infections with *Klebsiella pneumoniae*. The predilection of ES $\beta$ Ls for *Klebsiella pneumoniae* has never been clearly explained. It should be noted that the parent enzyme of TEM-type ES $\beta$ Ls, TEM-1, is widespread in many other species. More relevant, given the frequent finding of SHV-type ES $\beta$ Ls in *Klebsiella pneumoniae*, may be the increased frequency of SHV-1 in *Klebsiella pneumoniae* versus other species. Almost all non-ES $\beta$ L-producing *Klebsiella pneumoniae* isolates have chromosomally mediated SHV-1  $\beta$ -lactamases (Babini & Livermore, 2000). In contrast, fewer than 10% of ampicillin-resistant *Escherichia coli* isolates harbour SHV-1.

Many ES $\beta$ L genes are on large plasmids; even prior to the advent of ES $\beta$ Ls, large multi-resistance plasmids were more common in *Klebsiellae* than *Escherichia coli*. Of importance may be the well-noted adaptation of *Klebsiellae* to the hospital environment. *Klebsiellae* survive longer than other enteric bacteria on hands and environmental surfaces, facilitating cross-infection within hospitals (Casewell & Phillip, 1981).

In 100% of the more than 50 studies previously mentioned, at least two patients were colonized or infected with genotypically similar strains, implying patient-to-patient transmission of the strain. A number of outbreaks have been described with dissemination of a single clone of genotypically identical organism (Gniadkowski *et al.*, 1998; Neuwirth *et al.*, 2001). Clones have been found to persist for more than 3 years.

However, in many hospitals a more complex molecular epidemiologic picture has emerged (Babini & Livermore, 2000). Recent reports have described the clonal dissemination of at least five different ES $\beta$ L-producing *Klebsiella* strains in the same unit at the same time (Fielt *et al.*, 2000). Additionally, members of a single epidemic strain may carry different plasmids (carrying different ES $\beta$ L genes) (Fielt *et al.*, 2000). Furthermore, genotypically non related strains may produce the same ES $\beta$ L due to plasmid transfer from species to species (Fielt *et al.*, 2000). Finally, although the same ES $\beta$ L may be prevalent in a particular unit of a hospital, they may be mediated by different plasmids. This may imply independent evolution via the effects of antibiotic pressure, or plasmid transfer from organism to organism.

Intensive care units are often the epicentres of ES $\beta$ L production in hospitals-in one large outbreak, more than 40% of all the hospital's ES $\beta$ L-producing organisms were from patients in intensive care units (Gori *et al.*, 1996). As was noted in the pre-ES $\beta$ L era, neonatal intensive care units can also be a focus of infections with multiply resistant *klebsiellae* (Linkin *et al.*, 2004). Intensive care units in tertiary referral hospitals may acquire patients already colonized with ES $\beta$ L-producing organisms, thereby triggering an outbreak of infection (Gori *et al.*, 1996; Shannon *et al.*, 1998).

Transfer of genotypically related ES $\beta$ Ls from hospital to hospital within a single city (Sader *et al.*, 1994; Yu *et al.*, 2002), from city to city (Yu *et al.*, 2002), and from country to country (Gori *et al.*, 1996; Fielt *et al.*, 2000; Shipton *et al.*, 2001; Yu *et al.*, 2002) has been documented. A notable clone has been an SHV-4-producing, serotype K-25 isolate of *Klebsiella pneumoniae* which has spread to multiple hospitals in France and Belgium (Yu *et al.*, 2002). Another notable dissemination has been of a TEM-24-producing *Enterobacter aerogenes* clone in France, Spain, and Belgium (De Gheldre *et al.*, 2001; Canton *et al.*, 2002). Intercontinental transfer has also been described (Shannon *et al.*, 1990).

Although ES $\beta$ L-producing organisms can be introduced into intensive care units, epidemics of infection from intensive care units to other parts of the hospital have been well documented to occur (Jarlier *et al.*, 1988; Shannon *et al.*, 1998). Likewise, ES $\beta$ Ls may spontaneously evolve outside of the intensive care unit. Units noted to have been affected by outbreaks include neurosurgical, burns (Revathi & Singh, 1999), renal, obstetrics and gynaecology (Gaillot *et al.*, 1998), haematology and oncology (Naumovski *et al.*, 1992), and geriatric units (Gouby *et al.*, 1994; NNIS, 2002). Nursing homes and chronic care facilities may also be a focus of infections with ES $\beta$ L-producing organisms. In these settings, clonal spread has also been documented (Rice *et al.*, 1990; Weldhagen & Prinsloo, 2004).

**Risk Factors for Colonization and Infection with ES $\beta$ L producers:** Numerous studies have used a case-control design with which to assess risk factors for colonization and infection with ES $\beta$ L-producing organisms (Weldhagen & Prinsloo, 2004). Analysis of the results of these studies yields a plethora of conflicting results, likely due to the differences in study populations, selection of cases, selection of controls, and sample size. Nevertheless, some generalizations can be made. Patients at high risk for developing colonization or infection with ES $\beta$ L-producing organisms are often seriously ill patients with prolonged hospital stays and in whom invasive medical devices are present (urinary catheters, endotracheal tubes, central venous lines) for a prolonged duration. The median length of hospital stay prior to isolation of an ES $\beta$ L producer has ranged from 11 to 67 days, depending on the study of Weldhagen and Prinsloo, (2004). In addition to those already mentioned, a myriad of other risk factors have been found in individual studies, including the presence of nasogastric tubes, gastrostomy or jejunostomy tubes (Weldhagen & Prinsloo, 2004) and arterial lines (Lucet *et al.*, 1999), administration of total parenteral nutrition, recent surgery, haemodialysis, decubitus ulcers (Weldhagen & Prinsloo, 2004), and poor nutritional status.

Heavy antibiotic use is also a risk factor for acquisition of an ES $\beta$ L-producing organism. Several studies have found a relationship between third-generation cephalosporin use and acquisition of an ES $\beta$ L-producing strain (Lautenbach *et al.*, 2001; Eveillard *et al.*, 2002; Pessoa-Silva *et al.*, 2002; Lee *et al.*, 2004; Kim *et al.*, 2006). Other studies, which were underpowered to show statistical significance, showed trends towards such an association (the P values in all three studies were between 0.05 and 0.10) (Weldhagen & Prinsloo, 2004). Furthermore, a tight correlation has existed between ceftazidime use in individual wards within a hospital and prevalence of ceftazidime-resistant strains in those wards (Rice *et al.*, 1996). In a survey of 15 different hospitals, an association existed between cephalosporin and aztreonam usage at each hospital and the isolation rate of ES $\beta$ L-producing organisms at each hospital (Saurina *et al.*, 2000).

Use of a variety of other antibiotic classes has been found to be associated with subsequent infections due to ES $\beta$ L-producing organisms. These include quinolones (Lautenbach *et al.*, 2001; Weldhagen & Prinsloo, 2004), trimethoprim-sulfamethoxazole (Lautenbach *et al.*, 2001; Weldhagen & Prinsloo, 2004), aminoglycosides (Asensio *et al.*, 2000; Lautenbach *et al.*, 2001), and metronidazole (Lautenbach *et al.*, 2001). Conversely, prior use of  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations, penicillins, or carbapenems seems not to be associated with frequent infections with ES $\beta$ L-producing organisms.

**Nursing Homes and ES $\beta$ L Producers:** There is some evidence that nursing homes may serve as a portal of entry for ES $\beta$ L-producing organisms into acute-care hospitals. Conversely, patients with hospital-acquired colonization or infection may return to their nursing home with ES $\beta$ L carriage (Bradford *et al.*, 2001).

In a point prevalence study in the skilled care floor of a Chicago nursing home, 46% of residents were colonized with ES $\beta$ L-producing organisms (all *Escherichia coli*) (Weldhagen &

Prinsloo, 2004). These patients had been in the nursing home, without inter-current hospitalization, for a mean of more than 6 months. Patients from this nursing home, as well as seven other nursing homes, served as a reservoir for introduction of ES $\beta$ L-producing organisms into an acute-care hospital (Weldhagen & Prinsloo, 2004).

Within nursing homes, antibiotic use is a risk factor for colonization with ES $\beta$ L-producing organisms. Antibiotic use is frequent in nursing homes; in one recent study, 38% of nursing home residents had taken a systemic antibiotic in the previous month (Smith *et al.*, 2000). Use of third-generation cephalosporins has been identified as a predisposing event in some (Rice *et al.*, 1990), but not all studies (Weldhagen & Prinsloo, 2004). In contrast to the situation in acute-care hospitals, use of orally administered antibiotics (ciprofloxacin and/or trimethoprim-sulfamethoxazole) may also be a risk for colonization with an ES $\beta$ L-producing strain (Weldhagen & Prinsloo, 2004). Nursing home residents would appear to have several additional risk factors for infection with ES $\beta$ L-producing organisms. They are prone to exposure to the microbial flora of other residents, especially if they are incontinent and require frequent contact with health care providers. It has been well documented that hand washing rates are low among nursing home personnel (Denman & Burton, 1992). Urinary catheterization and decubitus ulcers are frequent (Smith *et al.*, 2000), and have been associated with colonization of non-ES $\beta$ L-producing, antibiotic-resistant gram-negative bacilli (Muder *et al.*, 1997).

**Community-Acquired Infections:** A survey of more than 2,500 isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* isolated from non-hospitalized patients in France in 1993 revealed no truly community-acquired infections. Five out of 107 strains of *Klebsiella pneumoniae* produced ES $\beta$ Ls, but all were isolated from patients staying in a nursing home (Goldstein *et al.*, 1995). However, in recent years there has been a wide variety of reports of true community-acquired infections with ESBL-producing organisms.

Several community-acquired pathogens that commonly cause diarrhoea have been found to be ES $\beta$ L producers. ES $\beta$ L-producing *Salmonella* infections remain a concern in many parts of the world (Baraniak *et al.*, 2000; Kruger *et al.*, 2004; Pessoa-Silva *et al.*, 2002). Additionally, a *Shigella flexneri* isolate from the stool of an Algerian child admitted to hospital with dysentery and harboring SHV-2 has been detected (Fortineau *et al.*, 2001). SHV-11 has been detected in *Shigella dysenteriae* (Ahamed & Kundu, 1999) as have a variety of TEM- and CTX-M-type ES $\beta$ Ls in *Shigella sonnei* (Radice *et al.*, 2002; Kim *et al.*, 2006). Finally, *Vibrio cholerae* isolates and Shiga toxin-producing *Escherichia coli* isolates which are ES $\beta$ L producers have been found (Ishii *et al.*, 1995; Petroni *et al.*, 2002).

In the previous years, there have been several reports of true community-acquired infection or colonization with ES $\beta$ L-producing *Escherichia coli* (Pitout *et al.*, 2004; Rodriguez-Bano *et al.*, 2004; Woodford *et al.*, 2006). These reports have come from Spain, Israel, the United Kingdom, Canada, and Tanzania. Typically, patients have developed urinary tract infection with CTX-M-producing *Escherichia coli*. Some urinary tract infections have been associated with bacteraemia. The majority of isolates have been resistant to commonly used first-line agents for urinary tract infection such as trimethoprim-sulfamethoxazole, ciprofloxacin, gentamicin, and ceftriaxone. Rodriguez-Bano, in Seville, Spain, performed a case-control study examining risk factors for ES $\beta$ L-producing *Escherichia coli* infections in non-hospitalized patients and found that *Diabetes mellitus*, prior quinolone use, recurrent urinary tract infections, prior hospital admissions, and older age were independent risk factors (Rodriguez-Bano *et al.*, 2004). Pitout, in Calgary, Canada, showed that 22 cases of ES $\beta$ L-producing *Escherichia coli* infection occurred per year per 100,000 populations greater than 65 years of age (Pitout *et al.*, 2004). The cause of this sudden upsurge in community-acquired infections with ES $\beta$ L-producing organisms is not yet clear, but associations with foodstuffs, animal consumption of antibiotics, and frequent patient contact with health care facilities need to be explored.

## **2.11 Mechanisms of transfer of resistance genes**

Over several millennia, bacteria have become adept at dealing with innumerable substances that threaten their survival. For them, antibiotics are just another group of poisonous compounds, the lethal effects of which have to be neutralized in some way. The effectiveness of the various strategies employed is according to Bennett (2008), attested by the impressive speed with which resistant versions of human pathogens have emerged to every antibiotic that has been introduced into clinical practice throughout the last 6 or 7 decades. The author further stated that none of the changes that confer antimicrobial resistance is acquired by design, rather changes to the genetic blueprints of all bacteria are made at random.

Changes can be made to a bacterium's genetic inheritance in two ways: i) by mutations that alter the pre-existing DNA of the cell - these alterations, base changes and DNA deletions and inversions (Avison & Bennett, 2005), change genes already possessed but do not add new genes to the cell genome-and ii) by acquisition of new genetic material that is capture of new genes into the cell, which expands the genome. The second method is largely, although not exclusively, responsible for the development of antibiotic-resistant variant strains of bacteria which cause infections of man and animals.

The phenomenon of gene acquisition implies gene transfer from some outside source, usually other bacteria. Bacteria have three methods by which DNA may be transferred from one cell to another, transformation, transduction and conjugation. Conjugation is promoted by genetic elements in bacteria called plasmids.

### **2.11.1 Mobile bacterial genetic elements**

Mobile genetic elements fall into two gene types: elements that can move from one bacterial cell to another which in terms of antibiotic resistance includes resistance plasmids and

conjugative resistance transposons, and elements that can move from one genetic location to another in the same cell. The latter include resistance transposons, gene cassettes and ISCR-Promoted gene mobilization.

### **2.11.2 Bacterial plasmids and antimicrobial resistance**

The elements that move many bacterial genes from one bacterial cell to another, the so-called horizontal gene transfer, are bacterial plasmids, specifically conjugative plasmids, that is, those able to promote their own transfer and the transfer of other plasmids from one bacterial cell to another. Plasmids are small auxiliary, dispensable chromosomes. In general, they exist separately from and are replicated independently of the main bacterial chromosome, although the majority of replication functions are provided by the host cell. Most plasmids investigated so far are circular, double-stranded DNA molecules that range in size from those with just 2-3 genes (2-3 kb) to elements that are equivalent to 10% or more of the host cell chromosome, that is, accommodates 400 genes or more. A resistance plasmid is any plasmid that carries one or more antibiotic resistance genes (it may also be for example, a metabolic plasmid, because it encodes a metabolic function or a virulence plasmid because it possesses one or more virulence genes. Carriage of one type of gene does not preclude carriage of other types that do not contribute towards maintenance and spread of the plasmid).

Plasmid encoded antibiotic resistance encompasses most, if not all classes of antibiotics currently in clinical use and includes resistance to many that are at the forefront of antibiotic therapy. Notable among these are commonly used cephalosporins, fluoroquinolones and aminoglycosides. Many resistance plasmids are conjugative, that is, they encode the functions necessary to promote cell-to-cell DNA transfer, particularly their own transfer. Others are mobilizable when helped by a conjugative plasmid co-resident in the cell.

In general, mobilizable plasmids lack the genes that encode the functions that enable cells to couple prior to DNA transfer (which are provided by the conjugative plasmid) but do encode the functions needed specifically for transfer of their own DNA. Accordingly, mobilizable resistance plasmids tend to be relatively small, often less than 10 kb in size, encoding only a handful of genes including resistance gene(s), whereas conjugative plasmids tend to be somewhat larger, 30 kb or more (resistance plasmids of 100 kb or more are not unusual), reflecting the sizable amount of DNA (20-30 kb) needed to encode the conjugation functions that permit cell-to-cell coupling, particularly between Gram-negative bacteria (Bennett, 2008).

Such coupling is mediated by an external filamentous appendage called a sex pilus, which essentially acts like a grappling hook to join donor and recipient cells and which is then retracted into the donor to effect envelope-to-envelope contact, when a DNA transfer pore forms to bridge the cytoplasmic compartment of the conjoined cells (Wilkins, 1995). Conjugative plasmids in Gram-positive bacteria tend to be smaller than those in Gram-negative bacteria, reflecting a somewhat different mechanism of cell-to-cell coupling (Bennett, 2008), which requires less genetic information. Conjugation is a replicative process that leaves both donor and recipient cells with a copy of the plasmid (Wilkins, 1995).

Conjugative plasmids can exhibit broad and narrow host range. For the latter, transfer is restricted generally to and between a small number of similar bacterial species. Broad host denotes an element able to transfer between widely different bacterial species and, some broad host range plasmids from Gram-negative bacteria appear to have no host limitation within the division and, using genetic constructs assembled in the test tube, have shown to be able to transfer to, but not to survive in both Gram-positive and unicellular eukaryotic microbes (Bennett, 2008).

One broad host range plasmid is the resistance plasmid RPI (also known as RP4 and RK4), first identified in a clinical strain of *Pseudomonas aeruginosa*. This plasmid appears to be transferred productively to most, if not all Gram-negative bacteria (Bennett, 2008). Many other, unrelated resistance plasmids are also known to have broad host ranges (Thomas, 1989).

What determines host range could, according to Bennett (2008), be the possibility that it reflects the nature of the surface receptor on the potential recipient cell needed by the particular conjugation machinery of the plasmid. If the potential recipient cell lacks this structure, then plasmid transfer to it will not occur. If the distribution of the receptor is limited, then the plasmid would exhibit a narrow host range. Another possibility, the author maintains, is that although transfer of the plasmid is successful, the recipient cell is unable to support its replication. Both broad host range and narrow host range plasmids are common.

Indeed, plasmids are common in most bacterial species, identifying a large pool of mobile genetic information. Furthermore, carriage of multiple plasmids is not at all uncommon (Bennett, 2008). Multi-resistant plasmids found in bacterial pathogens must have arisen in the past few decades (Séveno *et al.*, 2002). The use of antibiotics possibly has not increased the spread of bacterial clones carrying such plasmids but encouraged the dissemination of antibiotic resistance genes (Hughes & Data, 1983). Conjugation is thought to be the primary route of broad host range DNA transfer between different genera of bacteria (horizontal transfer). In principle, it requires only the addition of a short DNA sequence—the origin of transfer (*ori T*) to a replicative element to render it mobilizable by conjugation, by trans-action of the transfer functions of another plasmid (Davies, 1994).

Many different bacterial species have been shown to participate in sex factor-directed mating', hence conjugation can be viewed as a non-species-specific-process' (Davies, 1994; Mazocher & Davies, 1991). There are now numerous examples of horizontal gene transfers between bacterial

species, genera or families and even between bacteria and eukaryotes (Amabile-Cuevas & Chicurel, 1992).

Bacteria also possess other mobile elements called transposable elements, which can transfer within and/or between bacterial cells. Transposable elements can associate with oily elements such as chromosomes, plasmids or bacteriophages and may allow the establishment of antibiotic resistance in bacterial hosts in which a plasmid can not replicate (Top *et al.*, 2000). Thus, transposable elements can be transferred horizontally to other bacterial cells (intercellular mobility), either by transduction, in which case the vehicle is a phage, or a conjugative transposon (Merlin *et al.*, 2000). Phage-mediated transfer of genetic elements in natural environment is thought to occur at low frequency although it might be comparable with respect to its frequency, to conjugation and / or transformation encountered in soil (Marsh & Wellington, 1994). The stability and time abundance of the phage suggest that they can mediate horizontal gene transfer in ecosystems such as the marine environments (Jiang & Patti, 1998).

### **2.11.3 Transformation**

Transformation is another method by which genetic materials can be transferred between bacterial cells. Natural transformation in soil has been underestimated because of the belief that released DNA is rapidly degraded. Contrary to this, DNA bound to mineral surfaces has been shown to retain its transforming ability (Romanowski *et al.*, 1993; Nielsen *et al.*, 1997).

Bacterial transformation is also likely to be significant for the resistance gene transfer process in nature, as most bacterial genera are competent for transformation under some conditions (Lorenz & Wackernagel, 1994). Even *E. coli* is transformable under natural conditions. Webb & Davies (1994) speculated that antibiotic resistance genes were disseminated from DNA of the antibiotic-producing micro organisms which contaminated the commercial preparations of antibiotics (Webb & Davies 1993, 1994). Antibiotic gene transfer could occur through natural

transformation of bacterial cells in the human or animal intestine. Orpin *et al.* (1986) reported the natural transformation of a ruminant bacterium, *Selenomonas ruminantium*.

#### **2.11.4 Mechanisms of Bacterial Resistance to Antimicrobials**

There are four main mechanisms by which microorganisms exhibit resistance to antimicrobials and these include:

- a) Alteration of target site: An example is the alteration of penicillin binding proteins PBPs, the binding target site of penicillin's. These alterations have been noticed in methicillin resistant *Staphylococcus aureus* (MRSA) and other penicillin-resistant bacteria.
- b) Alteration of metabolic pathway: As an example, some sulphonamide-resistant bacteria would no longer require para-amino benzoic acid (PABA). PABA is an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulphonamides. Instead, like mammalian cells, they would now be able to utilize preformed folic acid.
- c) Drug inactivation or modification: An example is the enzymatic modification of penicillin G in some penicillin-resistant bacteria through the production of  $\beta$ -lactamases.  $\beta$ -Lactamases have also been known to be produced generally against  $\beta$ -lactam antibiotics. These enzymes are usually plasmid-borne.
- d) Reduced drug accumulation: This is achieved by decreasing drug permeability and/or increasing active efflux (pumping out) of the drugs across the cell surface.

### **2.11.5 Drug inactivation or modification**

One method by which drug inactivation is achieved is by the production of enzymes called  $\beta$ -lactamases and extended spectrum  $\beta$ -lactamases. These act generally against the  $\beta$ -lactam antimicrobials agents which are the most common treatment for bacterial infections. The  $\beta$ -lactam consist of four major groups: penicillin's, cephalosporins, monobactams and carbapenems (Table 2.3).

### **2.11.6 The $\beta$ -lactam antibiotics and $\beta$ -lactamases**

All  $\beta$ -lactam antibiotics have a  $\beta$ -lactam ring, which can be hydrolysed by  $\beta$ -lactamases. The groups differ from each other by additional rings (thiazolidine ring for penicillin's, cephem nucleus for cephalosporins, none for monobactams, double ring structure for carbapenems). The various antibiotics in each group differ by the nature of one or two side chains (Samaha & Araj, 2003).

The  $\beta$ -lactam antibiotics act on bacteria through two mechanisms targeting the inhibition of cell wall synthesis (Dbaibo, 2000). Firstly, they are incorporated in the bacterial cell wall and inhibit the action of the transpeptidase enzyme responsible for completion of the cell wall.

Secondly, they can attach to the penicillin-binding-proteins (PBPs) that normally suppress cell wall hydrolases, thus freeing these hydrolases which in turn act to lyse the bacterial cell wall. To bypass these antimicrobial mechanisms of action, bacteria resist by producing  $\beta$ -lactam inactivating enzymes ( $\beta$ -lactamases) or mutated types of PBPs.

**Table 2.2: Antimicrobial Agents, their Modes of Action and the Corresponding Mechanisms of Bacterial Resistance**

<b>Antimicrobial</b>	<b>Mode of action</b>	<b>Resistance mechanism</b>
$\beta$ -lactam	Inhibit cell wall synthesis cell division	$\beta$ -lactamases, altered PBPs
Glycopeptideds (azoles, cycloserine)	Inhibit Cell wall division	Blocking of drug access to pentapeptide
Aminoglycopeptides (spectinomycin)	Inhibit protein synthesis (bind to 30s ribosome)	Enzymatic inactivation, altered target, impermeability
Macrolids	Inhibit protein synthesis (bind to 50s ribosomes)	Altered target, enzymatic inactivation
Tetracycline	Inhibit protein synthesis (affect rRNA binding to 30s)	Efflux, altered target impermeability, enzymatic inactivation.
Chloramphenico (lincosamides, streptogramin)	Inhibit protein synthesis (bind to 50s ribosomes)	Enzymatic inactivation, altered target, impermeability
Quinolones	Replication: inhibits DNA gyrase	Altered target, enzymatic impermeability
Rifampin	Transcription: inhibits DNA dependent RNA polymerase	Altered target, enzymatic impermeability
Sulfonamides	Folic acid synthesis	Altered target
Trimethoprim	Folic acid synthesis	Altered target, impermeability
Polyenes(nystatin, amphotericin B)	Cell membrane permeability	Ergosterability deficient mutants

**Sources:** Cheesbrough (2010).

The synthesis of  $\beta$ -lactamases is either chromosomal (constitutive), like in *Pseudomonas aeruginosa*, or plasmid-mediated (inducible), like in *Aeromonas hydrophila* 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. In the Gram positive bacteria,  $\beta$ -lactamases are 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 (Stratton, 2000). Beta-lactamase enzymes destroy the  $\beta$ -lactam ring by major mechanisms of action:

- a) Most common  $\beta$ -lactamases have a serine based mechanism of action. They are divided into three major classes (A, C and D) on the basis of the amino acid sequences. They contain an active site consisting of a narrow longitudinal groove, with a cavity on its floor (the oxyanion pocket) which is loosely constructed in order to have conformational flexibility in terms of substrate binding (Stratton, 2000). Close to this lies the serine residue that irreversibly reacts with the carbonyl carbon of the  $\beta$ -lactam ring, resulting in an open ring (inactive  $\beta$ -lactam) and regenerating the  $\beta$ -lactamase. These enzymes are active against many penicillins, cephalosporins and monobactams.
- b) A less commonly encountered group of  $\beta$ -lactamases is the metallo  $\beta$ -lactamases, or class B  $\beta$ -lactamases. These use a divalent transition metal ion, most often zinc, linked to a histidine or cysteine residue or both to react with the carbonyl group of the amide bond of most penicillins, cephalosporins and carbapenems, but not monobactams (Dbaibo, 2000).

## 2.12 Reduced drug accumulation by efflux

Efflux pumps are transport proteins localized in the cytoplasmic membrane involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment (Webber & Piddock, 2003). These proteins found in both Gram positive and negative bacteria as well as in eukaryotic organisms (Bambeke *et al.*, 2000). They are active transporters, meaning that they require a source of chemical energy to perform their function.

Some are primary active transporters utilizing adenosine tri-phosphate hydrolysis as a source of energy, while others are secondary active transporters (uniporters, symporters and antiporters), in which transport is coupled to an electrochemical potential difference created by pumping out hydrogen and sodium ions outside the cell.

In the prokaryotic kingdom, there are five major families of efflux transporters. According to Lomovskaya *et al.* (2001), these are the major facilitator (MF), multidrug and toxic efflux (MATE), resistance-nodulation-division (RND), small multidrug resistance (SMR) and ATP binding cassette (ABC). All these utilize the proton motive force as an energy source (Paulsen *et al.*, 1998), apart from the ABC family which utilizes ATP hydrolysis to drive the export of substrates. While MFS dominates in Gram positive bacteria, the RND family is unique to the Gram negatives. Transporters that efflux multiple substrates including antibiotics, have not evolved in response to the stresses of the antibiotic era. All bacterial genomes studied contain several different efflux pumps; thus indicating their ancestral origins. It has been estimated that approximately 5-10% of all bacterial genes are involved in transport and a large proportion of these encode efflux pumps (Lomovskaya *et al.*, 2001).

Although antibiotics are the most clinically important substrates of efflux systems, it is probable that most efflux pumps have other natural physiological functions as both antibiotic susceptible

and resistant bacteria carry and express these genes. In many cases, efflux pump genes are part of an operon with a regulatory gene controlling expression. Increased expression is associated with resistance to the substrates, e.g. resistance to bile salts (Thanassi *et al.*, 1997).

Genes encoding efflux pumps can be found on plasmids, but their carriage on the chromosome gives the bacterium an intrinsic mechanism that allows survival in a hostile environment (e.g. the presence of antibiotics), and so mutant bacteria that over-express efflux pumps genes can be selected without the acquisition of new genetic material. It is possible that these pumps arose so that noxious substances could be transported out of the bacterium, allowing survival. According to Li *et al.* (1994), it is now widely accepted that the 'intrinsic resistance' of gram negative bacteria relative to gram positive bacteria is a result of the activity of efflux systems.

The broad substrate range of efflux systems is of concern, as often over expression of a pump will result in resistance to antibiotics of more than one class as well as some dyes, detergents and disinfectants (including some commonly used biocides). Cross resistance is also a Problem: exposure to any one agent that belongs to the substrate profile of a pump would favour over expression of that pump and consequent cross resistance to all other substrates of the pump. These may include clinically relevant antibiotics. An example of this is seen with the mexAB system of *Pseudomonas aeruginosa*; mutants that over produce mexAB are less: susceptible if not to a wide range of antibiotics (flouoroqumnolones,  $\beta$ -lactams, chloramphenicol and trimethoprim), but also triclosan, a commonly used household biocide (Chuachuen *et al.*, 2001).

Over expression of a multidrug resistance efflux pump alone often does not confer high level, clinically significant resistance to antibiotics. It has been demonstrated that expression of the mex systems of *Pseudomonas aeruginosa* and the acrAB efflux system of *E. coil* is greatest when the bacteria are stressed, e.g. growth in a nutrient-poor medium, growth to stationary

phase or osmotic shock; these inhospitable conditions may be relevant to the situation within an infection (Rand *et al.*, 2002).

Unregulated over expression of efflux pumps is potentially disadvantageous to the bacterium as not only will toxic substrates be exported, but also nutrients and metabolic intermediates may be lost. Work with *Pseudomonas aeruginosa* has suggested that mutants over expressing Mex pump are less able to withstand environmental stress and are less virulent than their wild type counterparts (Sanchez *et al.*, 2001).

As a result, the expression of pumps is tightly controlled. However, mutants and clinical isolates that over express efflux pumps are stable and commonly isolated; it may be that such mutants accumulate compensatory mutations allowing them to grow as well as wild-type.

**Table 2.3: Groups and examples of  $\beta$ -lactam antimicrobial agents**

<b>Beta-lactam group</b>	<b>Examples of antimicrobials</b>
Penicillins	Penicillins G, Penicillins  Penicillanase resistant Penicillins: methicillin, Nafcillin, oxacillin, cloxacillin.  Aminopenicillins: ampicillin, amoxicillin  Ureidopenicillins: mezlicillin, piperacillin  Carboxypenicillins: carbenicillin, ticarcillin
Cephalosporins	1. First generation: cefazolin, cephalothin, cephalexin  2. Second generation: cefuroxime, cefaclor, cefamandole, cefamycins (cefotetan, cefoxitin)  3. Third generation: cefotaxims, ceftriazone, cefpodoxime, ceftizoxime, celeperazone, ceftazidime  4. Fourth generation: cefepime, ceftazidime
Carbapenems	Imipenem, meropenem, ertapenem
Monobactams	Aztrenam

**Source:** Samaha and Araj, (2003).

### **2.13 Impact of efflux pumps on antimicrobial resistance**

The impact of efflux mechanisms is large and usually attributed to the following:

- a) The genetic elements encoding efflux pumps may be encoded on chromosomes and/or plasmids, thus, contributing to both intrinsic (natural) and acquired resistance respectively. As an intrinsic mechanism of resistance, efflux pump genes can survive a hostile environment (for example in the presence of antibiotics), which allows for the selection of mutants that over- express these genes. Being localized on transposable genetic elements as plasmids or transposons is also advantageous for the micro organisms as it allows for the easy spread of efflux genes between distant species.
- b) Antibiotics can act as inducers and regulators of the expression of some efflux pumps
- c) Expression of several efflux pumps in a given bacterial species may lead to a broad spectrum of resistance when considering the shared substrates of some multi drug efflux pumps, where one efflux pump may confer resistance to a wide range of antimicrobials.

### **2.14 Treatment options**

The factors which determine the choice of antibiotics and other management options include a) site of infection; b) severity of infection; c) presence of a prosthetic device or implant; d) metabolic parameters — liver and renal function; e) patient-related factors such as age, pregnancy, lactation. The therapeutic options for ESBL-producing organisms are very limited. ESBLs confer on them the ability to be resistant to most  $\beta$ -lactam antibiotics except cephamycins and carbapenems. In addition, the plasmids bearing genes-encoding ESBLs frequently also carry genes encoding resistance to other antimicrobial agents, such as

aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (Livermoore and Paterson, 2006).

There have also been increasing reports of plasmid-encoded decrease in susceptibility to quinolones, frequently in association with plasmid-mediated cephalosporin resistance. (Jacoby *et al.*, 2003) There appears to be a strong association between quinolone resistance and ESBL production, (Paterson and Bonomo, 2005) even in the absence of plasmid-encoded decrease in quinolone susceptibility, although the reason for this association is not well understood. Fluoroquinolones may be used for the treatment of uncomplicated urinary tract infections (UTIs) when found to be susceptible, although increasing *in vitro* resistance of ESBL producers to quinolones will limit the role of these antibiotics in the future. Studies have found carbapenems to be superior to quinolones for treatment of serious infections caused by ESBL-producing organisms. (Paterson *et al.*, 2000)

Some infections due to organisms testing resistant to ceftazidime but susceptible to cefotaxime or ceftriaxone have responded to treatment with these alternate cephalosporins. However, MICs of these agents rise dramatically as the inoculum is increased.

Thus isolates giving a positive synergy test are inferred to have ESBLs, and all cephalosporins should be avoided as therapy, irrespective of susceptibility results. Cefamycins, such as cefoxitin and cefotetan, although active *in vitro*, are not recommended for treating such infections, because of the relative ease with which these strains decrease the expression of outer membrane proteins, rendering them resistant.

Although ESBL activity is inhibited by clavulanic acid,  $\beta$ -lactam/  $\beta$ -lactamase inhibitor combinations are not considered optimal therapy for serious infections due to ESBL producers as their clinical effectiveness against serious infections due to ESBL-producing organisms is

controversial (Paterson and Bonomo, 2005). The majority of ESBL-producing organisms produce more than one  $\beta$ -lactamase, often in different amounts. Additionally, it is well known that ESBL-producing organisms may continue to harbour parent enzymes (for example, SHV-1 or TEM-1). Hyper production of these non-ESBL-producing  $\beta$ -lactamases or the combination of  $\beta$ -lactamase production and porin loss can also lead to a reduction in activity of  $\beta$ -lactamase inhibitors.

Also  $\beta$ -lactam/  $\beta$ -lactamase inhibitor combinations are subject to rising MICs as inoculum rises. As a result, infections with high organism burden (intra-abdominal collections, sepsis) may be associated with sufficient  $\beta$ -lactamase production to overcome the effects of the  $\beta$ -lactamase inhibitor. However, they may be useful for less serious infections, such as uncomplicated non-bacteremic lower urinary tract infection, because the infection is localized and the antibiotic is excreted in large amounts through the urine. They have also been found to be a good option for the treatment of uncomplicated community-acquired infections due to ESBL producers, especially since they have the advantage of oral administration. The advantages of using  $\beta$ -lactamase inhibitors are that by inhibiting ESBLs they appear to impair the emergence and spread of *Klebsiella*-carrying resistance plasmids. Furthermore, administration of inhibitors may exert in vitro pressure on ESBLs, thereby facilitating their reverse mutation to less harmful enzymes.

There is also concern that misuse of carbapenems in uncomplicated cases will result in carbapenem resistance. Thus the therapeutic options are limited to carbapenems, colistin, polymyxin, temocillin, tigecycline for serious infections. However uncomplicated infections like non-bacteremic urinary tract infections can be managed with a variety of antibiotics, depending on their susceptibility. These include oral antibiotics like trimethoprim, nitrofurantoin, fosfomicin, co-amoxiclav, mecillinam; or intravenous agents like

aminoglycoside (gentamicin, amikacin) and inhibitor combinations (Bhattacharya, 2006). Among these carbapenems are the drugs of choice for serious infections with ESBL producers. Imipenem and meropenem are preferred in nosocomial infections, while etrapenam is preferred in community-acquired infections (Shah & Isaacs, 2003).

Although *in vitro* studies have demonstrated no synergy, additivity or antagonism in combination therapy (carbapenem + aminoglycoside), the bactericidal activity of imipenem in combination with amikacin was found to be greater than that of imipenem alone. This was due to the faster killing rates of amikacin. Thus carbapenems may be combined with a second agent (amikacin) for the first few days in the treatment of life-threatening infections like septicemia, hospital-acquired pneumonia, intra-visceral abscesses. Tigecycline, temocillin, colistin and polymyxin are reserved for patients resistant to all of the other antibiotics, including the carbapenems.

### **2.15 Prevention and control**

Proper infection-control practices and barriers are essential to prevent spreading and outbreaks of ES $\beta$ L-producing bacteria. The reservoir for these bacteria seems to be the gastrointestinal tract of patients (Samaha & Araj, 2003). Alternative reservoirs could be the oropharynx, colonized wounds and urine. The contaminated hands and stethoscopes of healthcare providers are important factors in spreading infection between patients (Samaha & Araj, 2003). Essential infection-control practices should include avoiding unnecessary use of invasive devices such as indwelling urinary catheters or IV lines, hand washing by hospital personnel, increased barrier precautions, and isolation of patients colonized or infected with ES $\beta$ L producers.

At an institutional level, practices that can minimize the spread of such organisms include clinical and bacteriological surveillance of patients admitted to intensive care units and

antibiotic cycling; as well as policies of restriction, especially on the empirical use of broad-spectrum antimicrobial agents such as the third-and fourth-generation cephalosporins and quinolones (Samaha & Araj, 2003; Paterson & Bonomo, 2005).

Some authors have suggested that use of  $\beta$ -lactam/  $\beta$ -lactamase inhibitor combinations, rather than cephalosporins, as workhorse empirical therapy for infections suspected as being due to gram-negative bacilli, may facilitate control of ES $\beta$ L producers (Paterson *et al.*, 2000; Rice *et al.*, 1996). However, many organisms now produce multiple  $\beta$ -lactamases, which may reduce the effectiveness of  $\beta$ -lactam/  $\beta$ -lactamase inhibitor combinations (Baraniak *et al.*, 2000; Shen *et al.*, 2001).

## **2.16 Organisms of interest**

### **2.16.1 *Escherichia coli***

*Escherichia coli* (*E. coli*) are a Gram-negative, facultatively anaerobic, rod-shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms) (Singleton, 1999). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (Vogt & Dippold, 2005). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and preventing colonization of the intestine with pathogenic bacteria as reported by Hudault *et al.* (2001).

Eckburg *et al.* (2005) postulated that *E. coli* and other facultative anaerobes constitute about 0.1% of gut flora, and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination (Feng *et al.*, 2002). A growing body of research, though, has examined

environmentally persistent *E. coli* which can survive for extended periods outside of a host. Most *E. coli* strains do not cause disease, but virulent strains can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible for hemolytic-uremic syndrome, peritonitis, mastitis, septicemia, and Gram-negative pneumonia (Todar, 2007).

There is one strain, *E. coli* 0157:H7, that produces a toxin called the Shiga Toxin (classified as a bioterrorist agent). This toxin causes premature destruction of the red blood cells which then clog the body's filtering system, the kidneys causing hemolytic-uremic syndrome (HUS). This in turn causes strokes due to small clots of blood which lodge in capillaries in the brain. This causes the body parts controlled by this region of the brain to not work properly. In addition, this strain causes the buildup of fluid (since the kidneys do not work) leading to edema around the lungs and legs and arms. This increase in fluid build up especially around the lungs impedes the functioning of the heart, causing an increase in blood pressure (Feng *et al.*, 2002).

Uropathogenic *E. coli* (UPEC) is one of the main causes of urinary tract infections. It is part of the normal flora in the gut and can be introduced in many ways. In particular for females, the direction of wiping after defecation (wiping back to front) can lead to fecal contamination of the urogenital orifices. Anal intercourse can also introduce this bacterium into the male urethra, and in switching from anal to vaginal intercourse, the male can also introduce UPEC to the female urogenital system (Feng *et al.*, 2002).

#### **2.16.1.1 Diseases caused by *E. coli***

Most *E. coli* strains do not cause disease, but virulent strains can cause gastroenteritis, urinary tract infections, and neonatal meningitis (Todar, 2007). It can also be characterized by severe

abdominal cramps, diarrhea that typically turns bloody within 24 hours, and sometimes fever. In rarer cases, virulent strains are also responsible for bowel necrosis (tissue death) and perforation without progressing to hemolytic-uremic syndrome, peritonitis, mastitis, septicemia, and Gram-negative pneumonia.

### **Intestinal Diseases Caused by *E. coli***

As a pathogen, *E. coli* is best known for its ability to cause intestinal diseases. Five classes (virotypes) of *E. coli* that cause diarrheal diseases are now recognized: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC).

### **Enterotoxigenic *E. coli* (ETEC)**

ETEC is an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. In the U.S., it has been implicated in sporadic waterborne outbreaks, as well as due to the consumption of soft cheeses, Mexican-style foods and raw vegetables. The diseases vary from minor discomfort to a severe cholera-like syndrome. ETEC are acquired by ingestion of contaminated food and water, and adults in endemic areas evidently develop immunity. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid-encoded.

ETEC may produce a heat-labile enterotoxin (LT) that is similar in molecular size, sequence, antigenicity, and function to the cholera toxin (CTX). ETEC may also produce a heat stable toxin (ST) that is of low molecular size and resistant to boiling for 30 minutes. The infective dose of ETEC for adults has been estimated to be at least  $10^8$  cells; but the young, the elderly and the infirm may be susceptible to lower numbers.

Symptoms ETEC infections include diarrhea without fever

### **Enteroinvasive *E. coli* (EIEC)**

EIEC closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. EIEC penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes a dysentery-like diarrhea with fever. EIEC apparently lack fimbrial adhesins but do possess a specific adhesin that, as in *Shigella*, is thought to be an outer membrane protein. Also, like *Shigella*, EIEC are invasive organisms. They do not produce LT or ST toxin.

### **Enteropathogenic *E. coli* (EPEC)**

EPEC induce a profuse watery, sometimes bloody, diarrhea. They are a leading cause of infantile diarrhea in developing countries. Outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Pathogenesis of EPEC involves a plasmid-encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells and a non fimbrial adhesin designated intimin, which is an outer membrane protein that mediates the final stages of adherence. They do not produce ST or LT toxins.

Through volunteer feeding studies the infectious dose of EPEC in healthy adults has been estimated to be  $10^6$  organisms.

Some types of EPEC are referred to as diffusely adherent *E. coli* (DAEC), based on specific patterns of adherence. They are an important cause of traveler's diarrhea in Mexico and in North Africa.

### **Enteroaggregative *E. coli* (EAEC)**

The distinguishing feature of EAEC strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhea without invading or causing inflammation. This suggests that the organisms produce an enterotoxin of some sort. Recently, a distinctive heat-labile plasmid-encoded toxin has been isolated from these strains, called the EAST (EnterAggregative ST) toxin. They also produce a hemolysin related to the hemolysin produced by *E. coli* strains involved in urinary tract infections. The role of the toxin and the hemolysin in virulence has not been proven. The significance of EAEC strains in human disease is controversial.

### **Enterohemorrhagic *E. coli* (EHEC)**

EHEC are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome (HUS). EHEC are characterized by the production of verotoxin or Shiga toxins (Stx). Although Stx1 and Stx2 are most often implicated in human illness, several variants of Stx2 exist. There are many serotypes of Stx-producing *E. coli*, but only those that have been clinically associated with HC are designated as EHEC. Of these, O157:H7 is the prototypic EHEC and most often implicated in illness worldwide. The infectious dose for O157:H7 is estimated to be 10 - 100 cells; but no information is available for other EHEC serotypes. EHEC infections are mostly food or water borne and have implicated undercooked ground beef, raw milk, cold sandwiches, water, unpasteurized apple juice and vegetables.

EHEC are considered to be "moderately invasive". Nothing is known about the colonization antigens of EHEC but fimbriae are presumed to be involved. The bacteria do not invade mucosal cells as readily as *Shigella*, but EHEC strains produce a toxin that is virtually identical to the Shiga toxin. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of EHEC strains to cause HUS. The toxin is phage encoded and its production is enhanced by iron deficiency.

### **2.16.1.2 Antimicrobial resistance**

Infection with *Escherichia coli* and *Salmonella* can result from the consumption of contaminated food and water. Both of these bacteria are well known for causing nosocomial (hospital-linked) infections, and often, these strains found in hospitals are antibiotic resistant due to adaptations to wide spread antibiotic use (Davies & Davies, 2010). When both bacteria are spread, serious health conditions arise. Many people are hospitalized each year after becoming infected, with some dying as a result. Since 1993, some strains of *E. coli* have become resistant to multiple types of fluoroquinolone antibiotics.

Although mutation alone plays a huge role in the development of antibiotic resistance, there was a study done recently that found that high survival rates after exposure to antibiotics could not be accounted for by mutation alone (Adam *et al.*, 2008). This study focused specifically on *Escherichia coli*'s development of resistance to three antibiotic drugs: ampicillin, tetracycline, and nalidixic acid. At the conclusion of the study, these researchers found that some antibiotic resistance in *E. coli* developed due to epigenetic inheritance rather than by direct inheritance of a mutated gene. This was further supported by their data showing that reversion back to antibiotic sensitivity was relatively common as well. According to Adam *et al.* (2008) this could only be explained by epigenetics. Epigenetics is a type of inheritance where gene expression is altered rather than the genetic code itself. There are many modes by which this alteration of gene expression can occur. This includes methylation of DNA and histone modification;

however, the important idea is that both inheritance of random mutations and epigenetic markers can result in the expression of antibiotic resistance genes.

### **2.16.2 *Klebsiella pneumoniae***

*Klebsiella pneumoniae* is a Gram-negative, nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. Although found in the normal flora of the mouth, skin, and intestines, it can cause destructive changes to human lungs if aspirated, specifically to the alveoli resulting in bloody sputum. In the clinical setting, it is the most significant member of the *Klebsiella* genus of *Enterobacteriaceae*. *K. oxytoca* and *K. rhinoscleromatis* have also been demonstrated in human clinical specimens. In recent years, *Klebsiellae* have become important pathogens in nosocomial infections as reported by Ryan and Ryan (2004).

It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions. As a free-living diazotroph, its nitrogen fixation system has been much-studied, and is of agricultural interest, as *K. pneumoniae* has been demonstrated to increase crop yields in agricultural conditions (Riggs *et al.*, 2001).

Members of the *Klebsiella* genus typically express two types of antigens on their cell surfaces. The first, O antigen is a component of the lipopolysaccharide (LPS), of which 9 varieties exist. The second is K antigen, a capsular polysaccharide with more than 80 varieties. Both contribute to pathogenicity and form the basis for serogrouping (Podschun & Ullmann, 1998).

#### **2.16.2.1 Diseases caused by *Klebsiella***

The most common condition caused by *Klebsiella* bacteria outside the hospital is pneumonia, typically in the form of bronchopneumonia and also bronchitis. These patients have an increased tendency to develop lung abscess, cavitation, empyema, and pleural adhesions. It has

a death rate of about 50%, even with antimicrobial therapy. The mortality rate can be nearly 100% for people with alcoholism and bacteremia.

In addition to pneumonia, *Klebsiella* can also cause infections in the urinary tract, lower biliary tract, and surgical wound sites. The range of clinical diseases includes pneumonia, thrombophlebitis, urinary tract infection, cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis, meningitis, and bacteremia and septicemia. For patients with an invasive device in their bodies, contamination of the device becomes a risk; for example, neonatal ward devices, respiratory support equipment, and urinary catheters put patients at increased risk. Also, the use of antibiotics can be a factor that increases the risk of nosocomial infection with *Klebsiella* bacteria. Sepsis and septic shock can follow entry of the bacteria into the blood.

Two unusual infections of note from *Klebsiella* are rhinoscleroma and ozena. Rhinoscleroma is a chronic inflammatory process involving the nasopharynx. Ozena is a chronic atrophic rhinitis that produces necrosis of nasal mucosa and mucopurulent nasal discharge.

#### **2.16.2.2 Antimicrobial Resistance**

*Klebsiella* organisms are often resistant to multiple antibiotics. Current evidence implicates plasmids as the primary source of the resistance genes. *Klebsiella* with the ability to produce extended-spectrum beta-lactamases (ESBL) are resistant to many classes of antibiotics. The most frequent resistances include resistance to aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and trimethoprim/sulfamethoxazole (Rashid & Ebringer, 2007).

Infection with carbapenem-resistant Enterobacteriaceae (CRE) or carbapenemase-producing Enterobacteriaceae is emerging as an important challenge in health-care settings. One of many

CREs is carbapenem-resistant *Klebsiella pneumoniae* (CRKP) (Limbago *et al.*, 2011). Over the past 10 years, a progressive increase in CRKP has been seen worldwide; however, this new emerging nosocomial pathogen is probably best known for an outbreak in Israel that began around 2006 within the healthcare system there. In the USA, it was first described in North Carolina in 1996; since then CRKP has been identified in 41 states; and is recovered routinely in certain hospitals in New York and New Jersey. It is now the most common CRE species encountered within the United States (Yigit *et al.*, 2001).

CRKP is resistant to almost all available antimicrobial agents, and infections with CRKP have caused high rates of morbidity and mortality, in particular among persons with prolonged hospitalization and those critically ill and exposed to invasive devices (e.g., ventilators or central venous catheters). The concern is that carbapenem is often used as a drug of last resort when battling resistant bacterial strains. New slight mutations could result in infections for which there is very little, if anything, healthcare professionals can do to treat patients with resistant organisms (Limbago *et al.*, 2011).

### **2.16.3 *Pseudomonas* species**

*Pseudomonas* is a Gram - negative, ubiquitous, aerobes that are present in water, soil and plants. They are very common in nature and can be isolated from a large variety of natural sources, occurring in animal faeces, feed, drinking water and foodstuffs. The most prominent specie, *P. aeruginosa* is also frequently isolated from tap water in patient rooms (Valles *et al.*, 2004), a number of strain are notorious for their nutritional versatility towards organic low molecular weight compounds in media totally devoid of organic growth factors. *Pseudomonas* is able to multiply on a wide range of substrates and may proliferate by utilizing nutrient derived from

unsuitable materials used in the construction of water distribution systems and domestic plumbing installation (Place *et al.*, 1996).

According to Boyd *et al.* (1991), *Pseudomonas* sp, compared with other Gram negative bacteria appear to possess the ability to be viable and culturally for unusually long period of exposure to adverse conditions. In recent years particular attention has been paid to this microorganism because of its increasing significance as a human pathogen and its high rate of resistance to most antibiotics.

*Pseudomonas aeruginosa* is an opportunistic human pathogen characterized by an innate resistance to multiple antimicrobial agents (Poole, 2001). *P. aeruginosa* accounts for a significant proportion of nosocomial infection and like all nosocomial pathogens have the tendency to acquire new antibiotic resistant traits (Livrelli *et al.*, 1996).

#### **2.16.3.1 Diseases caused by *Pseudomonas aeruginosa***

**Endocarditis.** *Pseudomonas aeruginosa* infects heart valves of IV drug users and prosthetic heart valves. The organism establishes itself on the endocardium by direct invasion from the blood stream.

#### **Respiratory infections**

Respiratory infections caused by *Pseudomonas aeruginosa* occur almost exclusively in individuals with a compromised lower respiratory tract or a compromised systemic defense mechanism. Primary pneumonia occurs in patients with chronic lung disease and congestive heart failure. Bacteremic pneumonia commonly occurs in neutropenic cancer patients undergoing chemotherapy. Lower respiratory tract colonization of cystic fibrosis patients by mucoid strains of *Pseudomonas aeruginosa* is common and difficult, if not impossible, to

eradicate.

### **Bacteremia and septicemia**

*Pseudomonas aeruginosa* causes bacteremia primarily in immunocompromised patients. Predisposing conditions include hematologic malignancies, immunodeficiency relating to AIDS, neutropenia, diabetes mellitus, and severe burns. Most *Pseudomonas* bacteremia is acquired in hospitals and nursing homes. *Pseudomonas* accounts for about 25 percent of all hospital acquired Gram-negative bacteremias.

### **Central nervous system infections**

*Pseudomonas aeruginosa* causes meningitis and brain abscesses. The organism invades the CNS from a contiguous structure such as the inner ear or paranasal sinus, or is inoculated directly by means of head trauma, surgery or invasive diagnostic procedures, or spreads from a distant site of infection such as the urinary tract.

### **Ear infections including external otitis**

*Pseudomonas aeruginosa* is the predominant bacterial pathogen in some cases of external otitis, including "swimmer's ear". The bacterium is infrequently found in the normal ear, but often inhabits the external auditory canal in association with injury, maceration, inflammation, or simply wet and humid conditions.

### **Eye infections**

*Pseudomonas aeruginosa* can cause devastating infections in the human eye. It is one of the most common causes of bacterial keratitis, and has been isolated as the etiologic agent of

neonatal ophthalmia. *Pseudomonas* can colonize the ocular epithelium by means of a fimbrial attachment to sialic acid receptors. If the defenses of the environment are compromised in any way, the bacterium can proliferate rapidly through the production of enzymes such as elastase, alkaline protease and exotoxin A, and cause a rapidly destructive infection that can lead to loss of the entire eye.

### **Bone and joint infections**

*Pseudomonas* infections of bones and joints result from direct inoculation of the bacteria or the hematogenous spread of the bacteria from other primary sites of infection. Blood-borne infections are most often seen in IV drug users and in conjunction with urinary tract or pelvic infections. *Pseudomonas aeruginosa* has a particular tropism for fibrocartilagenous joints of the axial skeleton. *Pseudomonas aeruginosa* causes chronic contiguous osteomyelitis, usually resulting from direct inoculation of bone and is the most common pathogen implicated in osteochondritis after puncture wounds of the foot.

### **Urinary tract infections**

Urinary tract infections (UTI) caused by *Pseudomonas aeruginosa* are usually hospital-acquired and related to urinary tract catheterization, instrumentation or surgery. *Pseudomonas aeruginosa* is the third leading cause of hospital-acquired UTIs, accounting for about 12 percent of all infections of this type. The bacterium appears to be among the most adherent of common urinary pathogens to the bladder uroepithelium. As in the case of *E. coli*, urinary tract infection can occur via an ascending or descending route. In addition, *Pseudomonas* can invade the bloodstream from the urinary tract, and this is the source of nearly 40 percent of *Pseudomonas* bacteremias.

### **Gastrointestinal infections**

*Pseudomonas aeruginosa* can produce disease in any part of the gastrointestinal tract from the oropharynx to the rectum. As in other forms of *Pseudomonas* disease, those involving the GI

tract occur primarily in immunocompromised individuals. The organism has been implicated in perirectal infections, pediatric diarrhea, typical gastroenteritis, and necrotizing enterocolitis. The GI tract is also an important portal of entry in *Pseudomonas* septicemia and bacteremia.

#### **Skin and soft tissue infections, including wound infections, pyoderma and dermatitis**

*Pseudomonas aeruginosa* can cause a variety of skin infections, both localized and diffuse. The common predisposing factors are breakdown of the integument which may result from burns, trauma or dermatitis; high moisture conditions such as those found in the ear of swimmers and the toe webs of athletes, hikers and combat troops, in the perineal region and under diapers of infants, and on the skin of whirlpool and hot tub users. Individuals with AIDS are easily infected. *Pseudomonas* has also been implicated in folliculitis and unmanageable forms of acne vulgaris.

#### **2.16.3.2 Antimicrobial Resistance**

Multiple drug resistance (MDR) strains of *P.aeruginosa* are often isolated among patients suffering from nosocomial infections, particularly those in the intensive care unit. Thus infections caused by *P.aeruginosa* are particularly problematic because the organism is inherently resistant to many drug classes and is able to acquire resistance to all effective antimicrobial drugs (Gales *et al.*, 2001).

As an opportunistic infectious pathogen *P. aeruginosa* can often lead to life threatening diseases. For example, *P. aeruginosa* is the main cause of mortality in cases of microbial bacteremia and the second most common bacteria causing sepsis in the ICU (Marra *et al.*, 2006; Vincent *et al.*, 2006). *Pseudomonas aeruginosa* has been implicated in urinary tract infections, burn wounds, ventilator- associated pneumonia and multi organ system failure (Rello *et al.*, 2006).

Antimicrobial resistance has also been reported in environmental isolates of *P. aeruginosa*. Jensen *et al.* (2001) reported resistance to carbadox, chloramphenicol, nalidixic acid, nitrofurantoin, streptomycin and tetracycline in *P. aeruginosa* isolated from Danish agricultural soil. In a related study, Akinbowale *et al.* (2006), reported isolates of *P. aeruginosa* from agricultural and environments resistant to ampicillin, amoxicillin, cephalexin, cephalothin. Other antibiotics included cefoperazone, chloramphenicol, florfenicol, trimethoprim-sulfamethoxazole, nalidixic acid, erythromycin, oxolinic acid, tetracycline and oxytetracycline. Similarly, Gad *et al.* (2007) reported higher antibiotic resistance in his environmental isolates of *P. aeruginosa* in Egypt. Mechanisms of resistance used by isolates were  $\beta$ -lactamase production and multiple drug resistance efflux pumps. Their result showed that 95% of the environmental *P. aeruginosa* isolates were  $\beta$ -lactamase producers.

## **2.17 Medicinal plants**

Botany and medicine have been closely linked throughout history. Prior to this century, medical practitioners whether allopath (medical doctors), homeopaths, naturopaths, herbalist or shamans had to know the plants in the area and how to use them since many of their drugs were derived from plants (Akujobi *et al.*, 2004). Around 1900, 80% of the drugs were derived from plants. However, in the decades that followed, the development of synthetic drugs from petroleum products caused a sharp decline in the pre-eminence of drugs from live plant sources (Akujobi *et al.*, 2004).

Sofowora (1993) defined medicinal plants as plants in which one or more of the organs contains substances that can be used for therapeutic purposes or which is precursors for the manufacturing of drugs and are useful for disease therapy. The use of medicinal plants predates the introduction of antibiotics and other modern drugs into the African continent. Since

medicinal plants do not merely save people from feeling pain but also permit them to emerge unscathed, then they deserve investigation. The active components in these medicinal plants are expected to be detrimental to the growth of at least some microorganisms especially the disease causing ones e.g. *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* etc.

Plants show enormous versatility in synthesizing complex materials which have no immediate obvious growth or metabolic functions. These complex materials are referred to as secondary metabolites. Plants secondary metabolites have recently been referred to as phytochemicals. Phytochemicals are naturally occurring and biologically active plant compounds that have potential disease inhibiting capabilities. It is believed that phytochemicals may be effective in combating or preventing disease due to their antioxidant effect (Halliwell & Gutteridge, 1992). Antioxidants protect other molecules (*in vivo*) from oxidation when they are exposed to free radicals and reactive oxygen species which have been implicated in the aetiology of many diseases and in food deterioration and spoilage (Halliwell & Gutteridge, 1992).

All plants produce chemical compounds as part of their normal metabolic activities. These phytochemicals are divided into (1) primary metabolites such as sugars and fats, which are found in all plants; and (2) secondary metabolites-compounds which are found in a smaller range of plants, serving a more specific function (Meskin, 2002). For example, some secondary metabolites are toxins used to deter predation and others are pheromones used to attract insects for pollination. It is these secondary metabolites and pigments that can have therapeutic actions in humans and which can be refined to produce drugs-examples are inulin from the roots of dahlias, quinine from the cinchona, morphine and codeine from the poppy, and digoxin from the foxglove (Meskin, 2002). Toxic plants even have use in pharmaceutical development.

Plants synthesize a bewildering variety of phytochemicals, but most are derivatives of a few biochemical motifs (Springbob & Kutchan, 2009).

Alkaloids are a class of chemical compounds containing a nitrogen ring. Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, and animals, and are part of the group of natural products (also called secondary metabolites). Many alkaloids can be purified from crude extracts by acid-base extraction. Many alkaloids are toxic to other organisms. They often have pharmacological effects and are used as medications, as recreational drugs, or in entheogenic rituals. Examples are the local anesthetic and stimulant cocaine; the stimulant caffeine; nicotine; the analgesic morphine; the antibacterial berberine; the anticancer compound vincristine; the antihypertension agent reserpine; the cholinomimetic galatamine; the spasmolysis agent atropine; the vasodilator vincamine; the anti-arhythmia compound quinidine; the anti-asthma therapeutic ephedrine; and the antimalarial drug quinine. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste.

Polyphenols (also known as phenolics) are compounds contain phenol rings. The anthocyanins that give grapes their purple color, the isoflavones, the phytoestrogens from soy and the tannins that give tea its astringency are phenolics.

Glycosides are molecules in which a sugar is bound to a non-carbohydrate moiety, usually a small organic molecule. Glycosides play numerous important roles in living organisms. Many plants store chemicals in the form of inactive glycosides. These can be activated by enzyme hydrolysis, which causes the sugar part to be broken off, making the chemical available for use. Many such plant glycosides are used as medications. In animals and humans, poisons are often bound to sugar molecules as part of their elimination from the body. An example is the cyanoglycosides in cherry pits that release toxins only when bitten by a herbivore.

Terpenes are a large and diverse class of organic compounds, produced by a variety of plants, particularly conifers, which are often strong smelling and thus may have had a protective function. They are the major components of resin, and of turpentine produced from resin. (The name "terpene" is derived from the word "turpentine"). Terpenes are major biosynthetic building blocks within nearly every living creature. Steroids, for example, are derivatives of the triterpene squalene. When terpenes are modified chemically, such as by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids. Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. Essential oils are used widely as natural flavor additives for food, as fragrances in perfumery, and in traditional and alternative medicines such as aromatherapy. Synthetic variations and derivatives of natural terpenes and terpenoids also greatly expand the variety of aromas used in perfumery and flavors used in food additives. Vitamin A is an example of a terpene. The fragrance of rose and lavender is due to monoterpenes. The carotenoids produce the reds, yellows and oranges of pumpkin, corn and tomatoes.

Medicinal plants have been used for centuries before the advent of orthodox medicine. Leaves, flowers, stems, roots, seeds, fruit, and bark can all be constituents of herbal medicines. The medicinal values of these plants lie in their component phytochemicals, which produce the definite physiological actions on the human body. The most important of these phytochemicals are alkaloids, tannins, flavonoids and phenolic compounds (Hill, 1952).

Infectious diseases with increasing trends of drug resistant microorganisms have been common global problem posing enormous public health concerns (Iwu *et al.*, 1999). The global emergence of antimicrobial resistant bacterial strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections (Hancock & Harrison 2000). According to WHO (2002), the available antimicrobial drugs are costly and beyond the reach of the common man in many poor countries. It was estimated by Anon (1987) that more

than two thirds of the world's population relied on plant derived drugs. Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world (Ashish *et al.*, 2011). These plant-based systems will continue to play an essential role in healthcare, especially in rural areas around the world.

### **2.17.1 *Vernonia amygdalina***

*Vernonia amygdalina* is a tropical plant belonging to the family Compositae and is used widely as vegetable and medicinal plant. It has the common name bitter leaf (Ibrahim *et al.*, 2000). It is a shrub of about 2m - 5m with a petiolate leaf of about 6 mm in diameter and elliptic shape. The leaves are green with a characteristic odour and bitter taste. It does not produce seeds and has to be distributed or propagated through cutting. It grows under a range of ecological zones in Africa and produces a larger mass of forage and it is drought tolerant.

It is majorly used for human consumption. *Vernonia* has to be washed to remove the bitter taste (Iwu, 1993). Furthermore, the root provides one of the commonly used chew sticks in Nigeria due to alleged beneficial effect on dental caries (Aregheore *et al.*, 1998). The leaves and bark in Ethiopian local medicine are used as purgative against menstrual pain and wound dressing (Uhegbu & Ogbuchi, 2004).

### **2.17.2 *Allium sativum***

*Allium sativum* L., commonly called garlic is a widely cultivated herbal plant, belonging to the family Alliaceae. Garlic is a plant with long, flat grass like leaves and a papery hood around the flowers (Milner, 2005). The greenish white or pink flowers are grouped together at the end of a long stalk. The stalk rises directly from the flower bulb, which is the part of the plant used as food and medicine (Awonubi, 1988). The most active components of fresh garlic are alliin and an enzyme called allinase. When garlic is chewed, chopped, bruised or cut, these compounds

mix to form allicin, which is responsible for garlic's strong smell. Its medicinal claims have included cures for toothaches, cold, coughs and other viral infections, open wounds and evil demons (Fluck, 1973).

### **2.17.3 *Ocimum gratissimum***

*Ocimum gratissimum* L. (Lamiaceae) is an herbaceous shrub notably found in tropical countries including Nigeria, where it is commonly called Clove basil, Sweet basil, teabush, Scent leaf or fever plant; but it is also popularly known with different local names in Nigeria (Nupe: Tanmotsungi-wawagi; Epira: Ireru; Hausa: Dai doya ta gida; Yoruba: Efinrin ajase; Ibo: Nchuanwu-meaning mosquito repellent (Gill, 1992). Amongst the Igbos of south eastern Nigeria, the fresh leaves are beaten or spread around the walls of bedrooms in the evenings to repel mosquitoes which bite and transmit diseases (Obiajuru, 1995).

Many species of the genus *Ocimum* namely: *Ocimum americanum*, *Ocimum basilicum*, *Ocimum canum*, *Ocimum gratissimum*, *Ocimum sanctum* and *Ocimum suave* have been reputed for various medicinal uses (Sofowora, 1993). Several ethnobotanical surveys show that *Ocimum gratissimum* was among the plants reported in Nigerian communities to be used traditionally to treat bacterial infections such as enteric diseases viz: diarrhoea, dysentery and other gastrointestinal infections; upper respiratory tract infections associated with coughing, pneumonia, asthma and bronchitis; urogenital infections including sexually transmitted diseases, skin infections (dermatitis, eczema, scabies), wounds and ulcers; headache, insect bites, nasal bleeding, stroke, measles, paludism; and bacterial fevers such as typhoid fever and diabetes and veterinary problems (Chukwuka, 2011). It is also used in the treatment of epilepsy, shigellosis, trypanosomiasis, convulsion, pile and anaemia in Nigeria (Idika, 2008), and implicated in the oral hygiene in Nigeria (Matekaire & Bwakur, 2004).

Comprehensive biological activities of *O. gratissimum* has been reviewed (Prabhu *et al.*, 2009) and it is associated with antibacterial, antifungal, hypoglycaemic, antipyretic, antioxidant, anti-inflammatory, chemo- preventive, anti-carcinogenic, free radical scavenging, radio protective, antidermatophytic activities, and numerous other pharmacological use (Janssen *et al.*, 1989). Earlier reports have also shown the smooth muscle contracting and antimutagenic activity (Janssen *et al.*, 1986) as well as its anti-diarrhoeal effects in experimental animals (Onajobi, 1989), high antiviral indices against HIV-1 and HIV-2 (Ayisi & Nyadedzor, 2003); shigelloidal properties (Iwalokun *et al.*, 2001), anti- trypanosomal effects (Adamu *et al.*, 2009), immunobiological activity (Atal *et al.*, 1986), gastro- protective properties (Akah *et al.*, 2007), controlling agent for food spoilage and mycotoxin producing fungi (Nguefack *et al.*, 2004), disintegrant properties of its seed mucilage (Ravikumar *et al.*, 2007), and as a relaxant on isolated ileum from guinea pig (Madeira *et al.*, 2002). Its essential oil has mosquito repellent, insecticidal properties (Mwangi *et al.*, 1995). The essential oil of *O. gratissimum* and its main component eugenol were reported to be efficient in inhibiting *Haemonchus contortus* (Pessoa *et al.*, 2002). Currently, basil is mainly used as a culinary herb as well as perfumes and cosmetics (Ross, 2003).

#### **2.17.4 *Garcinia kola***

*Garcinia kola* seed belongs to the Clusiaceae family. The seed of *Garcinia kola* is chewed as masticatory, stimulant and for its bitter taste in traditional hospitality, cultural and social ceremonies (Olaleye *et al.*, 2009). *Garcinia kola* plant is referred to as a wonder plant as every part of it has been found to be of medicinal importance. Bitter cola is a highly valued ingredient in African ethnomedicine because of its varied and numerous uses which are social and medicinal thus making the plant an essential ingredient in folk medicine. It has a bitter taste followed by slight sweetness. Despite its bitter taste, *Garcinia kola* nuts are commonly eaten as a snack and used for their stimulant effects, due to high caffeine content. Bitter cola has been

used as an antidote for cases of poison or suspected poisons as it is chewed to prevent the development of any infection or poison when food is suspected to be contaminated by bacteria. The medicinal relevance of *Garcinia kola* cannot be over emphasized as several researches have been reported on its medicinal uses. Adesuyi *et al.* (2012) reported that the medicinal properties of *Garcinia kola* seed can be classified under purgative antiparasitic and antimicrobial. Adesuyi *et al.* (2012) also reported that the antimicrobial nature of *Garcinia kola* seed has been attributed to the benzophenone and flavonones present in the plant while the anti-inflammatory effect is believed to result from the inhibition of the cyclooxygenase enzyme. The medicinal relevance of *Garcinia kola* seed is based mainly on the phytochemical components of the plant which is known to contain several phytochemicals noted for their medicinal importance. These phytochemicals are natural bioactive compounds found in plants. They are non-nutritive plant chemicals that have been isolated from bitter kola seed such as oleoresin, tannin, saponins, alkaloids and cardiac glycosides, bioflavonoid such as kolaflavonone and 2-hydroxyflavonoids, chromanols, (Terashima *et al.*, 2002). Eisner, (1990) reported that medicinal plants such as *Garcinia kola* are believed to be an important source of new chemical substances with potential therapeutic benefits. Phytochemical screening showed presence of steroids, flavonoids, cardiac glycosides, tannins, saponins.

### **2.17.5 *Xylopi aethiopica* (Udah)**

*Xylopi aethiopica* commonly known as “African guinea pepper” or “Ethiopian pepper” is wide spread in tropical Africa, Zambia, Mozambique and Angola (Puri & Talata, 1978). In Nigeria, it is found all over the low land rain forest and most fringe forest in the Savanna zones of Nigeria. Negro pepper as it is also known as been used as a pepper substitute in Europe and India. It is common in ethno-medicine in West Africa. This is due to its preservative effect; the fruit extract has been shown to be active as antimicrobial agent against gram positive and gram negative

bacteria. *Xylopi aethiopica* has anti-spirochoetal properties so that it works also as a preventive measure.

*Xylopi aethiopica* has been important in the treatment of primary, secondary and tertiary stages of syphilis. It has been used for treating rheumatism and arthritis as well as other inflammatory conditions. Numerous research studies have confirmed the spice's anti-inflammatory and antipyretic (fever reducing) properties. Indian researchers reported antiarthritic and anti-inflammatory actions of one of the compounds of *X. callednimbidin*.

The seeds are mainly used by traditional medicine healers and can also serve as an alternative to pepper (Cai, 1997). It has also been reported to be used as a flavour in palm wine. The bark of the tree when steeped in palmwine is given for attacks of asthma and rheumatism.

A fruit extract or decoction of the bark as well as of the fruit is useful in the treatment of bronchitis and dysenteric conditions. In Congo, it is used for the attacks of asthma, stomach aches and rheumatism (Burkill, 1995). *Xylopi aethiopica* has been reported to be recommended to women who have newly given birth as a tonic in the Ivory Coast and Nigeria as a woman remedy, it is taken also to encourage fertility and for ease of childbirth (Burkill, 1995). The sauce is usually given to women after delivery to relieve pains, promote healing and lactation. Sometimes, a combination of *X. aethiopica* with other plant types or a combination of different parts of *X. aethiopica* is used to achieve the desired effects (Fall *et al.*, 2003). Among the conditions treated with *X. aethiopica* in traditional medicine are cough (fruits and roots of the plant) bronchitis, dysentery and biliousness (fruits and stem bark) and boils and sores (leaves and bark) (Mshana *et al.*, 2000).

## CHAPTER THREE

### MATERIALS AND METHOD

#### 3.1 Materials

##### 3.1.1 Study area

This study was carried out in selected public and private health institutions (hospitals and diagnostic laboratories) in Imo State, South-East Nigeria. Imo State is located in the South- East of Nigeria between the latitudes  $5^{\circ}29'N$ ,  $7^{\circ}2'E$  longitude. Imo State comprises of three Senatorial zones, namely, Owerri, Orlu, Okigwe. The citizens are greatly diversified professionals including traders, farmers, artisans, public/civil servants, politicians, students (Ohalete *et al.*, 2011). The study area covered all the three senatorial zones of the State. (See figure 5). Imo State has an average annual rainfall of 1800 - 2500mm, an average annual relative humidity of 80%, an average annual temperature of  $28^{\circ}C$  and an altitude of about 100m above sea level (Imo ADP, 1990). The State belongs to the tropical rain forest zone of Nigeria, which encourages the habitation for diverse forest and livestock products. It experiences two major seasons: rainy and dry seasons. The rainy season occurs between April to October and give a relieve in August refered to as “august break” while the dry season occurs between November to March.

Imo State has a population of about 3,934,899 million men and women. It has five major tertiary institutions of higher learning: two universities, two polytechnics and one college of education. Generally, the cost of living is average (Nigerian Muse, 2006).

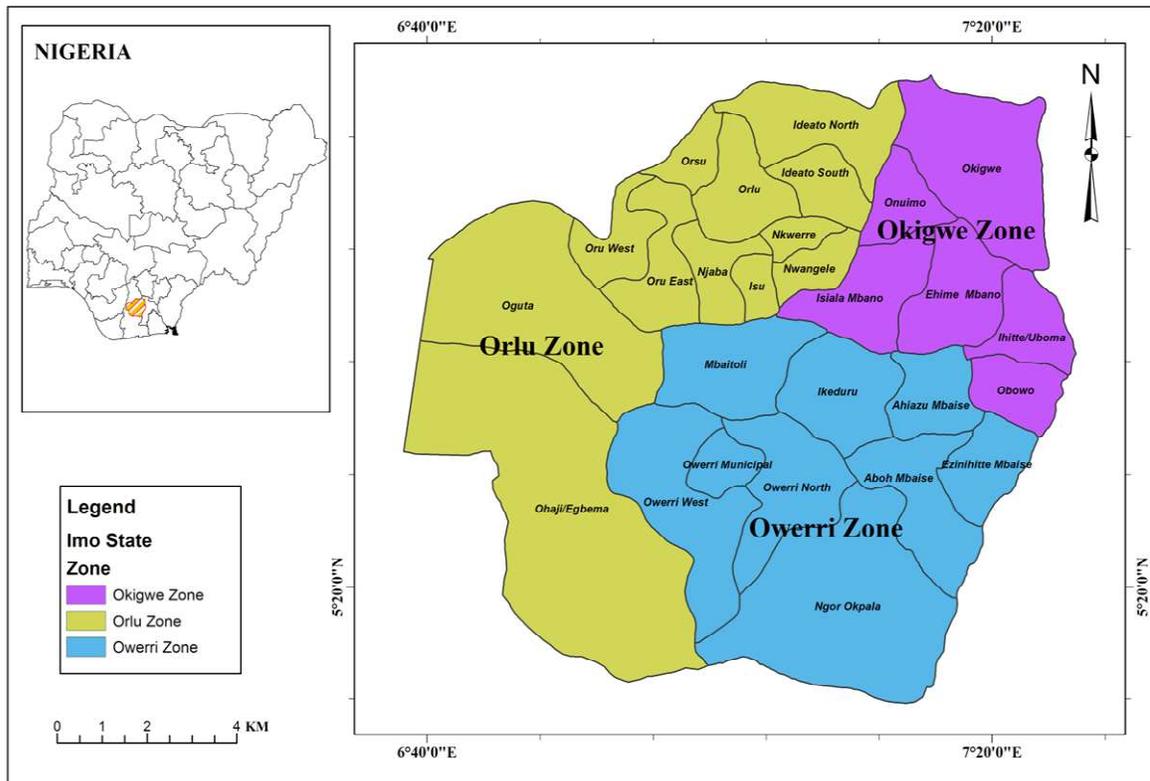


Figure 3.1: Imo State showing the Senatorial zones.

Source: Pat-Mbano and Chikwendu (2015)

### **3.1.2 Ethical clearance**

Ethical permit for this study (Appendix 1) was obtained from Imo State Ministry of health, Owerri.

### **3.1.3 Selection of respondents**

Three public health institutions (one from each senatorial zone) were selected and 20 private health institutions (10 from Owerri and 5 from each of Okigwe and Orlu) were selected. The selection was based on health facilities with higher number of patients and professional health workers in each zone. Hospitals having less than 20 patients per week were excluded. Also diagnostic laboratories having less than 20 cultures per week also excluded.

### **3.1.4 Test samples**

The samples used for this study were clinical samples: uro-genital samples (urine, high vaginal swabs and urethral swabs), and wound swabs collected from different health centers in Imo State.

### **3.1.5 Antimicrobial agents and plant extracts**

The antimicrobial agents used for this study were commercially prepared antibiotic discs (oxoid) including: ceftazidime (30 $\mu$ g), cefotaxime (30 $\mu$ g), ceftriazone (30 $\mu$ g), cefepime (30 $\mu$ g), gentamicin (30 $\mu$ g), ticarcillin (75 $\mu$ g), carbenecillin (100 $\mu$ g), piperacillin (100 $\mu$ g), ciprofloxacin (5 $\mu$ g), ampicillin (30 $\mu$ g), aztreonam (30 $\mu$ g), imipenem (10 $\mu$ g) and amoxicillin+clavulanic acid (30 $\mu$ g). Also selected plant materials commonly used in Imo State and other parts of Nigeria for treatment of bacterial infections, including: *Vernonia amygdalina*

(bitter leaf), *Xylopia aethiopica* (Udah), *Garcinia kola* (bitter kola), *Ocimum gratissimum* (nchuanwu) and *Allium sativum* (garlic), were employed in the study.

### 3.1.6 Test media

The media used for this study were oxoid brands of Mueller-Hinton agar, cystein lactose electrolyte deficient (CLED) medium, MacConkey agar (without salt) chocolate agar and Mueller-Hinton broth.

### 3.1.7 Standard organisms used

The following standard organisms obtained from the Nigerian Institute of Medical Research Yaba Lagos, were used as controls during the course of the project:

<b>Name of organism</b>	<b>Number</b>
<i>Escherichia coli</i>	ATCC 25922
<i>Klebsiella pneumoniae</i>	ATCC 700603
<i>Pseudomonas aeruginosa</i>	ATCC 27853

(Wayne, 2009).

### 3.1.8 Research questionnaire

The instrument used for collection of data in this study was a structured questionnaire, comprising of 4 sections: Section A: socio-demographic data comprised of 7 questions, Section B: Knowledge of multi-drug resistant/extended spectrum beta lactamase producing bacteria, comprised of 8 questions, Section C: attitude towards antibiotic resistant bacteria, comprised of 9 questions and Section D: Practice towards Extended spectrum beta lactamase producing bacteria, comprised of 6 questions (Appendix 2).

## **3.2 Methods**

### **3.2.1 Administration of questionnaires**

The knowledge, attitude and practices of health workers in Imo State towards extended spectrum beta lactamase (ES $\beta$ L) producing bacteria were determined using structured questionnaire (Appendix 2). The questionnaire was administered to health workers including medical doctors and medical laboratory scientists working at various hospitals and diagnostic laboratories in the 3 senatorial zones of Imo State. Healthworkers who gave consent were given the questionnaire, and requested to indicate their consent by signing the consent column on the first page. As much as possible, they were requested to complete the questionnaires and return them on the spot. A few who were not disposed to do so were allowed to complete and return the questionnaire within 24 hours.

### **3.2.2 Collection of samples**

The selected hospitals and diagnostic laboratories in Imo State were given freshly prepared agar slants on weekly basis. They were instructed to isolate cultures of *Escherichia coli*, *Klebsiella* species and *Pseudomonas aeruginosa* isolated from urine, high vaginal swab (HVS), urethral smear (U/S) wound swabs (wound infections such as burns, surgical wounds, and orthopedic wounds) onto the agar slants. A total of 602 clinical samples were collected by the selected clinical laboratories. A total of 480 clinical isolates: 250 from urine, 120 from HVS and U/S and 110 from wound were obtained from these samples. All the isolates were collected in Mueller-Hinton agar slants in sterile screw capped bijou bottles and transported to the laboratory. In the laboratory, the isolates were subcultured to obtain pure cultures and for identification test. Out

of the 480 isolates obtained, 300 were confirmed with bacteriological and biochemical identification test to be the required organisms. One hundred and eighty isolates were wrongly identified by the clinical laboratories and were not used for further tests. Stock cultures of the 300 confirmed isolates were prepared using Mueller-Hinton agar slants in bijou bottles. They were stored in the refrigerator at 4°C for further studies.

### **3.2.3 Sterilization of materials**

The materials used for this study were sterilized, using standard techniques. Glass wares were sterilized in the hot air oven at 160 °C for 1 hour. Culture media were sterilized by autoclaving at 121°C and 15psi pressure unit for 15 minutes. Inoculation wire loops were sterilized by flaming to red hot over a bunsen flame. “Hockey stick” was sterilized by dipping in absolute alcohol and bringing it over a burning flame to burn off. Bench top and working areas were disinfected with purit® antiseptic and swabbing with 75% ethanol. Sterile disposable hand gloves and face masks were worn and changed after each procedure to ensure aseptic conditions.

### **3.2.4 Preparation of media**

The media used for this study were prepared according to the manufacturers’ instructions.

#### **Agar slants**

Thirty eight grams (38g) of Mueller- Hinton agar powder was dissolved in 1 litre of deionized water and allowed to stand for 10 minutes for complete dissolution. It was swirled to homogenize and distributed 10 ml into bijou bottles. The bottles were covered with their screw caps and sterilized by autoclaving at 121°C and 15 psi pressure unit for 15 minutes. They were placed in slanting position in a fume cupboard and allowed to cool, solidify and stored in the refrigerator until required for use.

### **CLED (Cystine lactose electrolyte deficient)**

Thirty six grams (36g) of the medium was dissolved in 1 litre of deionized water and was swirled to homogenize. It was sterilized at 121°C for 15 minutes and 20 mls of prepared media were poured into sterile Petri dishes. They were allowed to solidify and stored in the refrigerator until required for use.

### **MacConkey agar**

Fifty grams (50g) of MacConkey agar (without salt) powder was dissolved in 1 litre of deionized water and allowed to stand for 10 minutes for complete dissolution. It was swirled to homogenize and sterilized by autoclaving at 121°C and 15 psi pressure unit for 15 minutes. It was allowed to cool to about 40 °C and was distributed 20 ml into each sterile Petri dish. They were allowed to cool, solidify and stored in the refrigerator until required for use.

### **Mueller-Hinton agar**

Thirty eight grams (38g) of Mueller Hinton agar powder was dissolved in 1 litre of deionized water and allowed to stand for 10 minutes for complete dissolution. It was swirled to homogenize and was autoclaved at 121°C for 15 minutes. Allowed to cool to about 40 °C and 20 mls were poured into sterile Petri dishes. They were allowed to cool and solidify at room temperature. They were stored in the refrigerator until required for use.

### **Chocolate agar**

Thirty-six grams (36g) of nutrient agar was dissolved in 1 litre of deionized water and allowed to stand for 10 minutes for complete dissolution. It was swirled to homogenize and sterilized by

autoclaving at 121°C for 15 minutes. It was allowed to cool to about 50 °C, then 50 mls of defibrinated sheep blood was added and swirled to homogenize. It was allowed to cool to about 40 °C and 20 mls of the media poured into sterile Petri dishes. They were allowed to cool and solidify at room temperature. They were stored in the refrigerator until required for use.

### **3.2.5 Processing of test samples**

The agar slants containing the test isolates were properly labeled and stored in the refrigerator for subsequent studies. The test isolates were sub-cultured from the agar slant onto Chocolate agar, MacConkey agar and CLED medium and incubated at 37 °C for 24 hours for morphological characteristics and identification and to obtain pure isolates before carrying out the various tests in the study.

### **3.2.6 Identification and confirmation of the test isolates**

The bacterial isolates *Klebsiella* species, *Escherichia coli*, *Pseudomonas aeruginosa* from different hospitals and diagnostic laboratories were identified using their growth morphological characteristics on different media used, bacteriological identification tests (Gram staining and motility test) and biochemical identification tests (catalase, oxidase, fermentation test and IMVIC test-indole production, methyl red, Voges Proskeur, citrate utilization) (Obiajuru & Ozumba, 2009; Cheesbrough, 2010).

### **3.2.7 Bacteriological examinations of the test isolates**

The purified samples were subjected to the following bacteriological examinations;

#### **1. Gram Staining**

The isolates were sub-cultured from the agar slant onto CLED agar medium and incubated at 35°C for 18 hours to obtain a pure culture. A discrete colony of each isolate from the 18 hour

pure culture was emulsified in a drop of physiological saline on grease-free slide and smeared to produce thick film. It was heat-fixed over a bunsen flame and stained with crystal violet for 30 seconds. The primary stain was drained and the slide covered with Lugol's iodine for 60 seconds, and subsequently drained, and washed with water. This was followed by a few drops of acetone for few seconds and washed off immediately with clean water. Counter stained with neutral red for 2 minutes. The slide was blot-dried and examined microscopically with oil immersion objective (Cheesbrough, 2010).

## **2. Motility Test (Wet preparation)**

A drop of sterile distilled water was placed on slides and using a sterile wire loop a colony of each isolate was picked and emulsified in the water. The slides were covered with their slips and examined microscopically for motility using 10x and 40x objectives (Obiajuru & Ozumba, 2009).

## **Biochemical identification tests**

### **1. Catalase test**

Two drops of 3% hydrogen peroxide was placed on clean grease-free, slide using clean Pasteur pipette. A discrete colony of 18 hour pure culture of the isolate was emulsified in the hydrogen peroxide using clean glass rod. Production of effervescence or gas bubbles immediately indicates positive reaction, (Cheesbrough, 2010).

### **2. Oxidase test**

A piece of metal-free filter paper was placed in a petri dish and 2 drops of freshly prepared oxidase reagent was placed on the filter paper. A colony of the 18 hour pure culture isolate was smeared on the filter paper using a glass rod. Observation of blue-purple change in colour within few seconds indicates positive result (Cheesbrough, 2010).

### **3. Citrate utilization**

Three to four milliliters sterile Simmon's citrate medium was inoculated with 18 hour pure culture of test isolate, using sterile straight wireloop. The inoculated broth was incubated at 35°C for 4 days and observed daily for growth. A colour change from green to blue of the medium indicates positive result (Cheesbrough, 2010).

### **4. Indole production test**

Three milliliters of peptone water containing tryptophan was dispensed into different bijou bottles. The tubes were inoculated with the test organisms and incubated at 35°C for 24 hours. 0.5ml of Kovac's reagent (Isoamyl alcohol, P-dimethyl amino benzaldehyde and conc. Hydrochloric acid) was added to each tube and shaken gently. Formation of deep red colour in the inoculated tubes within 10 minutes indicates positive result (Cheesbrough, 2010).

### **5. Methyl Red (MR) test**

The test isolates was inoculated in 5ml sterile glucose phosphate broth and incubated at 35°C for 48 hours. Four drops of methyl red solution was added later to it. Presence or appearance of bright red colour indicates positive result (Cheesbrough, 2010).

### **6. Voges Proskauer (VP) test**

Two milliliters of sterile glucose phosphate peptone water was inoculated with the test organism and incubated at 35°C for 48 hours. A little amount of creatine was added and mixed. Three milliliters of sodium hydroxide was added and mixed properly. The bottle cap was removed and left at room temperature for 1 hour. Colour change of the medium to pink red coloration indicates a positive result (Cheesbrough, 2010).

## **7. Sugar Fermentation tests**

Two percent carbohydrate (maltose, mannitol or lactose) peptone water sugar medium was prepared in tubes. A Durham tube was placed into each tube containing the medium. A suspension of the test isolate was inoculated and the tube plugged with non-absorbent cotton wool. It was incubated at 35°C for 7 days and examined daily for acid and / or gas production (Obiajuru & Ozumba, 2009).

### **3.3 Antibiotic susceptibility tests**

Antibiotic susceptibility test was carried out on the isolates using conventional antibiotics routinely used in hospitals and Diagnostic laboratories within Imo State, to select multiple antibiotic resistant isolates. The selected antibiotics are single discs of: (ceftazidime (CAZ) 30 µg, cefotaxime (CTX)30 µg, ceftriazone (CRO)30 µg, cefepime(CP)30 µg, gentamicin (CN)30 µg, ticarcillin (TIC)75 µg, carbenecillin (CAR)100 µg, piperacillin (PRL) 100 µg, ciprofloxacin (CIP)5 µg, ampicillin(AMP) 30 µg, aztreonam (ATM)30 µg, imipenem (IPM)10 µg, amoxicillin+clavulanic acid (AMC) 30 µg (Oxoid,UK.). Isolates which gave positive results for multiple antibiotic resistances were subjected to phenotypic testing for extended spectrum Beta-Lactamase production. Those which were positive for ESBL by phenotypic tests were used for molecular testing for TEM, SHV and CTX-M genes, plasmid curing using acridine orange and Plasmid profiling by Gel Electrophoresis.

#### **3.3.1 Standardization of inocula**

The cultures were standardized, using the method of Cheesbrough (2010). Pure isolates were transferred into sterile prepared normal saline and the cultures were then adjusted to 0.5

McFarland turbidity standards by checking the turbidity level in comparison with the standard (See appendix 3)

### **3.3.2 Antimicrobial susceptibility testing**

Antimicrobial resistance testing of isolates to determine antimicrobial resistance was done using disc diffusion method (Bauer *et al.*, 1966). Isolates from pure culture were grown in Mueller-Hinton broth at 37°C for 4 hours. Cultures were standardized to 0.5 McFarland standards depending on the turbidity of the broth. A swab stick full of broth culture was spread on the surface of already prepared Mueller-Hinton agar plate. The plates were left for 30 minutes to dry before the antimicrobial discs were applied on to their surface using sterile forceps. These were then kept on the bench for another 30 minutes to dry before inverting and incubating at 35°C for 24 hours. All the isolates were inoculated on triplicate plates. After incubation, the sizes of inhibition zones were measured with a meter rule and recorded in millimeter to the nearest whole number. Zones of inhibition were interpreted as resistant or sensitive, using the interpretative chart of the zone sizes of the Kirby-Bauer sensitivity test method (Cheesbrough, 2010), according to the recommendation of CLSI (2012).

These antimicrobials, their classes, standard disc concentrations and different zone sizes of inhibition are presented in Table 3.1 They were all of Oxoid, UK. Multiple drug resistant (MDR) in this work is defined as resistant to three or more antimicrobial classes according to Felagas *et al.* (2006).

**Table 3.1: Antimicrobials tested, disc concentrations and their zones of inhibition**

S/NO	Antimicrobial class	Antimicrobial/concentration ( $\mu\text{g}$ )	Zone sizes of inhibition(mm) (resistant)
1	Penicillins	Ampicillin(10)	<13
		Carbenicillin(100)	<14
		Mezlocillin(75)	<14
		Penicillin G(10)	<28
2	Cephalosporins	Cefotaxime(30)	<14
		Ceftazidime(30)	<14
		Cefuroxime(30)	<14
		Aztreonam(30)	<27
		Imipenem(10)	<27
		Cefepime(30)	<14
		Cefpodoxime(30)	<14
3	Tetracyclines	Tetracycline(30)	<14
		Doxycycline(30)	<14
4	Aminoglycosides	Gentamicin(30)	<12
		Kanamycin(30)	<12
		Streptomycin(10)	<12
5	Macrolides	Erythromycin(15)	<13
6	Quinolones	Ciprofloxacin(5)	<15
		Norfloxacin(10)	<15
		Ofloxacin(10)	<15
		Enrofloxacin(5)	<15
7	Sulphonamides/trimethoprm	Cotrimoxazole(25)	<10
8	Chloramphenicol	Chloramphenicol(30)	<12
9	Glycopeptides	Vancomycin(30)	<15

**Source:** Cheesbrough, (2010) and CLSI, (2012).

### **3.4 Phenotypic testing of extended spectrum beta-lactamase (ES $\beta$ L) producing bacteria.**

In accordance with CLSI (2012) antibiotic disks of ceftazidime, aztreonam, cefotaxime, and ceftriaxone were used for screening of ES $\beta$ L production by phenotypic method. More than one of these agents was used for screening to improve the sensitivity of ES $\beta$ L detection. The test was performed by the double disk diffusion test (DDST) as described by Kumar, *et al.* (2014) and Chikwendu *et al.* (2011).

Inoculum with turbidity equivalent to 0.5 McFarland standards was prepared from colonies on agar plates. Mueller-Hinton agar plates were inoculated by lawn culture method using a sterile cotton swab. With a sterile forceps ceftazidime, cefotaxime, ceftriaxone, and aztreonam disc were placed on the Mueller-Hinton agar plate and the plate was incubated at 35°C for 24 hours.

#### **Interpretation of results**

Zones given below, against respective antibiotic disc indicate potential ES $\beta$ L producer. If any strain was suspected as ES $\beta$ L producer then phenotypic confirmatory tests were done.

- Ceftazidime  $\leq$  22 mm or
- Aztreonam  $\leq$  27 mm or
- Ceftriaxone  $\leq$  25 mm or
- Cefotaxime  $\leq$  27 mm

#### **(b) Phenotypic confirmatory methods**

Isolates found to be resistant to ceftazidime and cefotaxime were picked for further testing for ES $\beta$ L gene production. Standardized isolates were streaked on Mueller-Hinton agar plates using a sterile cotton swab. With sterile forcep, discs containing the standard concentration of

ceftazidime and cefotaxime were placed 15mm centre to centre away from amoxicillin/clavulanic acid disc (20:10µg) (Oxoid, UK). Inoculated media were incubated for 24hrs at 35°C.

Results were interpreted according to the criteria established by the CLSI (2012). A  $\geq 5$  mm increase in zone of inhibition of growth for the single discs as compared with the zone around the combination disc was confirmatory for the result of ES $\beta$ L producing isolates.

### **3.5 Molecular detection of TEM, SHV and CTX-M genes**

Eighty seven ES $\beta$ L positive isolates were obtained. Ten of each test organism comprising at least 3 ES $\beta$ L positive isolates from each test sample were selected for molecular study. A total of 30 ES $\beta$ L positive isolates from the 87, comprising of *E. coli* (10), *Klebsiella species* (10) and *Pseudomonas aeruginosa* (10), were selected and tested for the presence of TEM, SHV, and CTX-M genes.

#### **3.5.1 Genomic DNA extraction**

GeneJET Genomic DNA Purification Kit (Thermo Scientific) as described by Hassan *et al.* (2014) was used for the genomic DNA extraction. Cells were grown in Mueller Hinton broth overnight. The over night grown cells were then harvested and suspended in 150µl of digestion buffer in a 1.5ml microcentrifuge tube and centrifuged for 10minutes. The supernatant was discarded. The pellet was resuspended in 180 µl of digestion solution. Proteinase k solution of 20 µl was added and mixed thoroughly by vortexing. Sample was incubated at 56°C for 30 minutes, 20 µl of RNase. A solution was added and mixed by vortexing and incubated at room temperature for 10minutes. To the sample was added 200µl of lysis solution, mixed thoroughly by vortexing for about 15 seconds until a homogenous mixture is obtained. To the mixture 400µl of 50% ethanol was added and mixed properly by vortexing. The prepared lysate was

transferred to a GeneJET Genomic DNA Purification Column inserted in a collection tube and centrifuged for 1minute. The flow through tube in the collection tube was discarded and inserted into a new 2ml collection tube and 500µl of Wash Buffer II added and then centrifuged for 3minutes. The collection tube containing the flow through solution was discarded and the GeneJET Genomic DNA Purification Column transferred into a sterile 1.5ml microcentrifuge tube and 200µl of Elution Buffer added to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA and incubated at room temperature for 2minutes and centrifuged. The purification column was discarded and the purified DNA stored immediately in the Biosafety cabinet at -20°C.

#### **Protocol for DNA amplification using the genomic guided sequence method**

PCR master mix (2x) was gently vortexed after thawing and briefly centrifuged. The thin walled PCR tube was placed on ice and the following added for each 50µl reaction.

Quick load one Taq one step PCR master mix (2x)	25µl
Forward primer(10 µm)	(1 µl)
Reverse primer(10 µm)	(1µl)
Template DNA	1-10 µl
Nuclease free water	Make up to 50 µl
Total Volume	50 µl

The sample was gently vortexed and spinned down. The PCR was performed using the recommended thermal cycling conditions. Five to ten microlitre of the PCR product was analyzed on a 1.5g agarose gel electrophoresis stained with ethidium bromide.

### **3.5.2 Plasmid curing with acridine orange**

Plasmid curing is a term used to describe the removal of plasmids from micro organisms that possess them. This is usually done in order to study some characters of the organism that may have been conferred upon them by the presence of these plasmids. This helps to determine whether such characters are chromosomal or plasmid borne. It can be effected with the use of some dyes like acridine orange. The method of plasmid curing used in this study is that of Salisbury *et al.* (1972).

#### **Method:**

Overnight cultures of the isolates to be tested were standardized by completely diluting them 100 fold. 200µl of this diluted culture was taken with a micro-pipette and put into 20mls of warmed nutrient broth, after which 2mls of freshly prepared solution of acridine orange (1ml/ml) was added. The mixture was incubated for 72hours at 37°C in a water bath with intermittent vigorous shaking. At the end of 72hours, 100 µl of the mixture was diluted 10 fold by adding 900 µl of nutrient broth. The broth was then plated out on Mueller-Hinton agar plates and after the surface had dried appropriately, the antimicrobial discs were placed on the agar surface. Plates were incubated again at 35°C for 18-24 hours and antimicrobial sensitivity or resistance determined by measuring the zone sizes of inhibition and further interpreting the results.

### **3.5.3 Plasmid profiling by gel electrophoresis**

The plasmid extraction according to Hassen *et al.* (2014) was carried out by the protocol described by the manufacturer of the extraction kit (Thermo Scientific, UK). Over night culture of 0.5-5ml of bacteria culture was centrifuged in a clear 1.5ml tube at full speed for 20 seconds in a microcentrifuge. The supernatant was discarded. Red coloured 200µl of P1 Buffer was added to the eppendorf tube and resuspended into the pellet completely, by vortexing. Green coloured 200µl of P2 Buffer was added to the eppendorf tube and mixed by inverting the tube 4 times. Cells are completely lysed when the solution appears clear, purple and viscous. After 2 minutes 400µl of yellow coloured P3 buffer was added and mixed gently by thoroughly mixing not vortexing. The sample turned yellow colour when the neutralization was completed. The lysate was allowed to incubate at room temperature for 2 minutes. The sample was centrifuged for 2 minutes, and the supernatant was transferred into the Zymo -Spin™ IIN column. The Zymo -Spin™ IIN with the collection tube was centrifuge for 30 seconds. The flow-through in the collection tube was discarded. The Zymo -Spin™ IIN column was returned to the collection tube, 200µl of endo-wash buffer was added and centrifuged for 30 seconds. To the column 400µl of plasmid wash buffer was added and centrifuged for 1minutes. The column was transferred into a clean 1.5ml eppendorf tube and 30µl of DNA elution buffer was then added to the column and centrifuged for 30 seconds to elute the plasmid DNA.

Electrophoresis of the plasmid DNA was carried out on 0.8% agarose gel in a 0.5% concentration of Tris-Borate-EDTA (TBE) buffer. Agarose gel was prepared by boiling 0.8g of agarose powder in 100mls of 0.5X TBE buffer. After boiling, the solution was allowed to cool and 10µl of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes and the comb removed. 20 µl of the plasmid DNA sample were then loaded into the wells after mixing with 2 µl of bromophenol blue. A DNA molecular weight marker (HIND III

digest of  $\lambda$  DNA) was also loaded into one of the wells. The gel was thereafter electrophoresed in a horizontal tank at a constant voltage of 60V for about 1 hour 30minutes.

After electrophoresis, plasmid DNA bands were identified by fluorescence of bound ethidium bromide, using a short wave ultraviolet light transilluminator. Photographs were taken, using a digital camera.

### **3.5.4 Polymerase chain reaction (PCR) amplification of extended spectrum beta lactamase gene (ES $\beta$ L), using primers for SHV, TEM and CTX-M genes.**

#### **Preamble**

The polymerase chain reaction (PCR) is a technique for making copies or amplifying a specific sequence of DNA in a short period of time. Here, the target DNA to be amplified is added to a thin-walled tube and mixed with deoxyribnucleotides (Datp, dCTP, dGTP and dTTP), buffer and DNA polymerase. A pair set of primers is added to the mixture. Primer is short oligonucleotides, usually around 20 to 30 nucleotides long. These primers are complementary to nucleotides flanking opposite ends of the target DNA to be amplified. The reaction tube is then placed in an instrument called a thermal cycler. This is a sophisticated heating block that is capable of rapidly changing temperature over very short time intervals. The thermal cycler takes the stages, denaturation, hybridization (or annealing) and extension or elongation.

In denaturation, the reaction tube is heated to approximately 94-96 °C, causing separation of target DNA to single strands. In hybridization the tube is cooled slightly to around 50 °C to 65 °C which allows the primers to hydrogen bond to complementary bases at opposite ends of the target sequence. During extension, the last stage of a PCR cycle, the temperature is usually raised to about 70 to 75 °C and DNA polymerase copies the target DNA by binding to the 3' ends of each primer and using the primers as templates. DNA polymerase adds nucleotides to

the 3' end of each primer to synthesize a complementary strand. At the end of one cycle, the amount of target DNA would have been doubled.

The thermal cycler repeats these three stages again according to the total number of cycles determined by the researcher, usually 20 or 30 cycles. The polymerase chain reaction has wide applications in research and medicine such as making DNA probes, studying gene expression, amplification minute amounts of DNA to detect viral pathogens and bacterial infections, detecting trace amounts of DNA from tissues at a crime scene and even amplifying ancient DNA from fossilized dinosaur tissue (Thieman & Palladino, 2009).

### **PCR protocol using chromosomal DNA of isolates**

PCR reactions were conducted, using bacterial genomic DNA as substrate and specific primers used (Erlich *et al.*, 1991). Amplification reaction was performed in a 25 $\mu$ l volume containing 5 $\mu$ l of DNA template preparation, 1 $\mu$ l of forward and reverse primers each, PCR master mix 10 $\mu$ l (consisting of deoxynucleoside triphosphates (dNTPs), MgCl<sub>2</sub>, PCR buffer, Taq polymerase) and 8 $\mu$ l sterile distilled water. Thermal cycling was conducted in an Eppendorf Master Cycler Gradient with the following temperature profile: initial denaturation (94°C for 2mins); denaturation at (94°C for 45s for TEM and SHV and 30s for CTX-M ); primer annealing at ( 32°C for 45s for TEM and SHV and 58°C for 30s for CTX-M ); primer extension (72°C for 1 min) and final extension step (72°C for 7mins for TEM and SHV and 5mins for CTX-M ). The amplification products were analysed by gel electrophoresis on a 1.5% agarose gel. Assayed products were electrophoresed for 1 hour at 100v in TBE buffer and visualized by ethidium bromide staining. PCR amplicon size was calculated by comparison to molecular weight size marker (100-1517bp DNA ladder).

Gene	Sequence	References
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**Table 3.2:** The primers used for the detection of *E.coli*, *Pseudomonas* sp & *Klebsiella* sp

<i>E.coli</i> F	5'TTCTTCGGTATCCTATTCCC3'	Olsvik & Strockbine(1993)
<i>E.coli</i> R	5'ATGCATCTCTGGTCATTGTA3'	Olsvik & Strockbine(1993)
<i>Pseudomonas</i> species F	5'GACGGGTGAGTAATGCCTA3'	Aghamiri <i>et al.</i> (2014)
<i>Pseudomonas</i> species R	5'CACTGGTGTTCCTTCCTATA3'	Aghamiri <i>et al.</i> (2014)
<i>Pseudomonas aeruginosa</i> F	5'ATGGAAATGCTGAAATTCGGC3'	Aghamiri <i>et al.</i> (2014)
<i>Pseudomonas aeruginosa</i> R	5'CTTCTTCAGCTCGACGCCACG3'	Aghamiri <i>et al.</i> (2014)
<i>Klebsiella</i> species F	5'TCCCCTGGTGTGGCTAATATTGC3'	Temprano <i>et al.</i> (2001)
<i>Klebsiella</i> species R	5'GCGCTAGTCACTACTGGCTATCCT3'	Temprano <i>et al.</i> (2001)
<i>Klebsiella pneumonia</i> F	5'GAGCGCTACGACAGTCCCAGATAT3'	Temprano <i>et al.</i> (2001)
<i>Klebsiella pneumonia</i> R	5'CATACCTTTAACGCTCAGTTCGAC3'	Temprano <i>et al.</i> (2001)

**Key: F – Forward primer**  
**R – Reverse primer**

Gene	Sequence	References
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**Table 3.3: The primers used to screen for ESBL genes**

TEM	F	5'ATTTTCTTCTGATGACG	Sharma <i>et al.</i> (2010)
TEM	R	5'TTACCAATGCTTAATCA3'	Sharma <i>et al.</i> (2010)
SHV	F	5'TTAACTCCCTGTTAGCCA3'	Sharma <i>et al.</i> (2010)
SHV	R	5'GATTTGCTGATTTGCCCC3'	Sharma <i>et al.</i> (2010)
CTX-M	F	5'TTAATGATGACTCAGAGCATTC 3'	Erlich <i>et al.</i> (1991)
CTX-M	R	5' GATACCTCGCTCCATTTATTG 3'	Erlich <i>et al.</i> (1991)

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**Key: F – Forward primer**  
**R – Reverse primer**

### **3.6 Collection and identification of the plant materials**

The five (5) medicinal plants, *Garcinia kola* Heckel (bitter kola), *Vernonia amygdalina* Delile (bitter leaf), *Allium sativum* L. (garlic), *Xylopi aethiopica* Dunel (Udah), *Ocimum gratissimum* L. (Nchanwu) were identified and authenticated at the National Root Crops Research Institute, Umudike, Umuahia, Abia State by a taxonomist.

#### **3.6.1 Preparation of plant materials**

Fresh leaves of *Vernonia amygdalina* (bitter leaf) and *Ocimum gratissimum* (nchuanwu) were collected from unpolluted environment. Seeds of *Xylopi aethiopica* (Udah), *Garcinia kola* (bitter kola), and *Allium sativum* (garlic) was purchased from Ekeukwu Owerri market. All were respectively washed and rinsed with distilled water. The plant materials were dried, initially at room temperature, and finally in a thermostatically controlled hot air oven at 40°C, until it maintained constant weight (Obiajuru & Ozumba, 2009).

The dried leaves and seeds were ground into fine powder in an electric blender. The powdered materials were stored in screw capped bottles and kept in the refrigerator at 4°C until required for use (Ameh, 2010).

#### **3.6.2 Extraction of active components from plant materials**

The active principles of the various medicinal plants were extracted, using Soxhlets extractor and rotary evaporator, in the chemistry laboratory of Imo State University Owerri. Twenty five grammes of each powdered materials, was extracted in 250ml of 95% methanol to extract the active principles, using soxhlets apparatus as described by Harborne (1998) and Mbata *et al.* (2009).

The solvent, 250ml of methanol was added to a round bottom flask, which is attached to a soxhlets extractor and condenser. The round bottom flask was placed on an isomantle. The

crushed plant material was loaded into the thimble, which was placed inside the soxhlet extractor. The solvent was heated using the isomantle, the solvent boiled and vapourized into the condenser. The condensed extract dripped into the reservoir. Once the level of solvent reached the siphon, the solvent flowed back into the flask and refluxed, the cycle begins again. The process continued until extraction of the active principles was determined as the refluxing solvent turned clear. The crude extract was allowed to evaporate to dryness, using rotary evaporator (Harborne, 1998).

### **3.6.3 Drying of the extracts**

Rotary evaporation is the process of removing the volume of a solvent by evaporation, by distributing the solvent in a thin film across the interior of a vessel at low temperature and reduced pressure. This promotes the rapid removal of excess solvent from less volatile samples. Rotary evaporator has four major components: water bath, rotor, condenser and solvent trap. Additionally, a vacuum pump needs to be attached as well as a bump trap and round bottom flask containing the sample to be concentrated.

#### **Procedure**

The extracted plant material was put half full in the round bottom flask. The water pump was turned on and the condenser gets cold. The vacuum was turned on. The round bottom flask was clamped to the bump trap. The vent was opened. The rotary evaporator was lowered into the water bath, and then the temperature and the rotor turned on. Rotation allows the liquid in the flask to splash up onto the sides of the round bottom flask, allowing more surface area to be exposed and speed evaporation. Rotation also helps to prevent bumping. The sample was allowed to spin under vacuum. The sample was allowed to boil as long as the bubbles do not reach the neck. The system was repressurized by fully opening the stop cock to cease boiling. After evaporation, the vacuum is turned off, the vent is released, the round bottom flask is

removed from the water. The spinner and temperature is turned off. The round bottom flask is removed from the bump trap and the extract was recovered.

### **3.7 Phytochemical screening of the plant materials**

#### **3.7.1 Qualitative analysis**

Phytochemical screening for alkaloids, saponins, flavonoids, tannins and HCN were carried out according to the methods of Odebiyi and Sofowora (1978) and Harborne (1998).

##### **Test for tannins**

Extract of the test plant (1.0g) was weighed into a beaker and 10 ml of distilled water added. The mixture was boiled for 5 minutes. Two drops of 5%  $\text{FeCl}_3$  were then added. Production of greenish precipitate indicated the presence of tannins.

##### **Test for saponins**

The powdered extract (0.5g) was introduced into a tube containing 5.0 ml of distilled water, the mixture was vigorously shaken for 2 minutes, formation of froth indicated the presence of saponins.

##### **Test for flavonoids**

The powdered extract (0.5g) was introduced into a tube containing 3 ml of distilled water, a volume 1 ml of 10% sodium hydroxide was added. A yellow coloration indicated the presence of flavonoids.

### **Test for alkaloids**

The extract of the plant sample (0.5g) was stirred with 5 ml of 1% HCL in a steam bath. The solution obtained was cooled and filtered and few drops of Mayer's reagents/ picric acid was added to the filtrate. A cream precipitate indicated the presence of alkaloid.

### **Test for glycosides**

The powdered extract (0.5g) was introduced into a tube containing 3 ml of distilled water. To a volume of 3 ml, 2 ml of chloroform was added. Tetraoxosulphate VI acid was carefully added to form a layer. A reddish brown color at interface indicated the presence of a steroidal ring.

## **3.7.2 Quantitative determination of the phytochemicals**

### **3.7.2.1 Alkaloids**

The alkaloids were extracted using a slightly modified method of Maxwell, *et al.* (1995). The dried powdered sample was blended and alkaloids were extracted from 20g of the sample using 100mls of 10% acetic acid and left to stand for 4 hours. The extract was filtered to remove cellular debris and then concentrated to about one quarter to the original volume by evaporation over a steam bath.

One percent  $\text{NH}_4\text{OH}$  was added drop wise until no precipitate occurred. The alkaloid was dried to constant weight in an oven and the percentage alkaloid calculated as:

$$\% \text{ Alkaloid} = \frac{\text{weight of residue}}{\text{weight of sample}} \times \frac{100}{1}$$

### **3.7.2.2 Saponins**

The dried powdered samples of the plants were ground and 10g of the samples were put into conical flasks and 100ml of 20% aqueous ethanol added. The samples in conical flasks were

put into a water bath and stirred continuously under a constant temperature of 55°C for 12 hours. After this, the sample solution was filtered with whatman number 1 filter paper and residue was extracted with another 200mls of 2% ethanol. The extracts were combined reduced to about 40ml by evaporation and then transferred to a separating funnel, and equal volume (40ml) of diethyl ether was added to it. After mixing well, there was partition; the other layer was discarded while the aqueous layer was reserved. The aqueous layer was re-extracted with the ether after which its pH was brought to 4.5 with drop-wise addition of dilute NaOH solution.

Saponins in the extract were taken up in successive extraction with 60ml and 30ml portion of butanol. The combined extract (precipitate) was washed with 5% NaCl solution, and evaporated to dryness in a previously weighed evaporating dish. The saponin was then dried in an oven at 60°C (to remove any residual solvent) and cooled in a desiccator, then re-weighed; the saponin extracted was taken and expressed as a percentage.

$$\% \text{ Saponins} = \frac{\text{weight of residue}}{\text{weight of sample}} \times \frac{100}{1}$$

### 3.7.2.3 Flavonoids

The method used for the determination of flavonoid was that of Bohn and Kocipai (1994). Five grams (5g) of the blended sample were extracted repeatedly and separated with 50mls of 40% aqueous methanol at room temperature. The whole solution was filtered with whatman's filter paper into beaker. The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and then dried in an electric oven to a constant weight. The flavonoid content was expressed in percentage as % flavonoid.

$$\% \text{ flavonoid} = \frac{\text{weight of residue}}{\text{weight of sample}} \times \frac{100}{1}$$

### 3.7.2.4 Tannin

The Follinns-Dennis spectrophotometer method by Pearson (1976) was used. One gram (1g) of the dry test sample was dispensed in 50mls of distilled water and shaken for 3mins in a shaker.

The mixture was filtered and the filtrate used for the analysis. 5mls of the extract was measured into 50mls volumetric flask and diluted with 35mls of distilled water. Similarly, 5mls of standard tannic acid solution and 5mls of distilled water were measured, diluted with 35mls of distilled water and put in different flasks to serve as standard and blank respectively.

One milliliter (1ml) of Follins- Dennis reagent was added to each of the flasks followed by 2.5mls of saturated sodium carbonate solution. The content of each flask was made up with distilled water and incubated for 90mins at room temperature. The absorbance of the developed colour was measured at 760nm wavelength with the reagent blank at zero. The experiment was repeated two more times to get an average.

The tannin content was calculated as shown below:

$$\%Tannin = \frac{100}{w} \times \frac{AU}{AS} \times \frac{C}{1000} \times \frac{Vf}{Va} \times D$$

Where,

W = Weight of sample analyzed.

Au = Absorbance of the test sample

AS = Absorbance of standard tannin solution

C = Concentration of standard in mg/ml

Vf = Volume of filtrate analyzed

D = Dilution factor, where applicable.

Va = Volume of extract used

### 3.7.2.5 Determination of cyanogenic glycoside (HCN)

The alkaline picrate colourimeter method described by Balagopalan *et al.* (1988) was used. The alkaline picrate solution was prepared by dissolving 1g of picrate and 5g of sodium carbonate in a small volume of minimally warm water and the volume made up to 200ml with distilled water. The picrate paper was prepared by dipping rectangular pieces of filter paper in picric acid solution and dried. Each test sample (1g) was dispersed in 200ml of distilled water in a 250ml conical flask. An alkaline picrate paper was suspended inside the flask and held in place with the stopper used to cork the flask. Care was taken to ensure that the picrate paper did not touch the surface of the mixture in the flask. These were incubated at room temperature for 18hrs (overnight) and then each picrate paper was carefully removed and eluted in 60ml of distilled water. A standard cyanide solution was prepared (0.05M). The absorbance of the standard solution prepared was read first before each of the samples replicates was read. The absorbance of the sample solutions as well as those of the standard were measured spectrophotometrically at 540nm using the reagent blank to set the instrument at zero. The cyanide content was determined by calculation as shown below:

$$\text{HCN mg/kg} = \frac{100}{W} \times \frac{A_u}{A_s} \times C$$

Where:

- W = Weight of sample
- A<sub>u</sub> = Absorbance of sample
- A<sub>s</sub> = Absorbance of standard solution
- C = Concentration of standard solution

### **3.8 Effects of plant extracts on extended spectrum $\beta$ -lactamase producing bacteria**

Extended Spectrum beta lactamases (ES $\beta$ L) positive bacterial isolates were subjected to susceptibility tests using plant extracts commonly used in Imo State, South-Eastern Nigeria to determine the antimicrobial effects of the plant extracts on these isolates. The well-in-agar technique as described by Ogueke *et al.* (2007) and Amadi *et al* (2013) was determined.

#### **3.8.1 Dilution of plant materials**

Serial dilutions of the crude extracts were prepared using the method of Akujobi *et al.*, (2004). Crude extracts of the different plant materials were evaporated to dryness. 1 mg, 2 mg, 4 mg, 6 mg, 8 mg and 10 mg of each dried extract were dissolved in 50% dimethyl sulphoxide (DMSO) to obtain the different concentrations of 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1 mg/ml. These were stored at 15°C until required for use.

#### **3.8.2 Test isolates**

Antibacterial susceptibility test was carried out on 10 isolates each of suspected ES $\beta$ L-positive and 10 isolates of non- ES $\beta$ L producing bacteria each of *Klebsiella* species, *Escherichia coli* and *Pseudomonas aeruginosa* using the crude extracts of the selected plant materials. The plant extracts were tested on non-ES $\beta$ L producing bacteria to ascertain the antibacterial efficacy of the plant extracts on non- ES $\beta$ L producing bacteria.

#### **3.8.3 Antibacterial susceptibility testing of the plant extracts**

This was carried out, using the well in agar method as described by Ogueke *et al.* (2007) and Amadi *et al* (2013). In this method, 0.1ml of 3 hour Mueller-Hinton broth culture of test organisms corresponding to 0.5 McFarland standard culture was inoculated on solid Mueller-

Hinton agar plates, using spread-plate method. Sterile bent glass rod spreader (Hockey stick) was used to spread the inoculum evenly on the surface of the medium. Seven wells (5.0mm diameter) were then made in the plates using a sterile cork borer. To the six wells 0.02ml of each of the 6 dilutions of the different crude extracts was delivered into each well, using a sterile automatic pipette. To the seventh well, 0.02 ml of the diluents was delivered as control and labeled accordingly. All the extracts were inoculated on triplicate plates. The plates were allowed on the bench for 40 minutes for pre-diffusion of the extract. The plates were incubated at 37 °C for 24 hours and examined for bacterial growth inhibition. The diameter zone of growth inhibition on the different plates was measured in millimeters, using a transparent metric rule. Those with out any sign of inhibition is resistant.

### **3.9 Minimum inhibitory concentration**

Five isolates each of the ES $\beta$ L producing and 5 isolates each of the non- ES $\beta$ L producing test bacterial isolates were used to determine the minimal inhibitory concentration of the different medicinal plant extracts. Thirty eight grammes of Mueller-Hinton agar powder was dissolved in 1 litre of deionized water and swirled to homogenize. It was dispensed 10 ml into different MacCarteny bottles. They were sterilized by autoclaving and allowed to cool to about 40 °C. To the different bottles containing the sterile medium, 0.2 ml of the different dilution of the crude extract plant extract was dispensed. Each was swirled to homogenize and dispensed into sterile petri dish. They were allowed to cool and solidify. To the surface of the different medium containing different concentrations of the crude plant extracts, 0.1 ml of the test isolates were inoculated using the spread plate technique (Nweze *et al.*, 2004). They were incubated at 37 °C for 24 hours and examined for bacterial growth. The lowest concentration of crude extract, showing bacterial growth inhibition (no growth) was reported as the minimal inhibitory concentration (MIC) (Cheesbrough, 2010).

### **3.10 Statistical analysis**

Two way analysis of variance (ANOVA) at 95% level of significance, via SPSS software package and Chi square were used to test for significant differences in the variables considered in this study.

## **CHAPTER FOUR**

### **RESULTS AND DISCUSSION**

#### **4.1 Results**

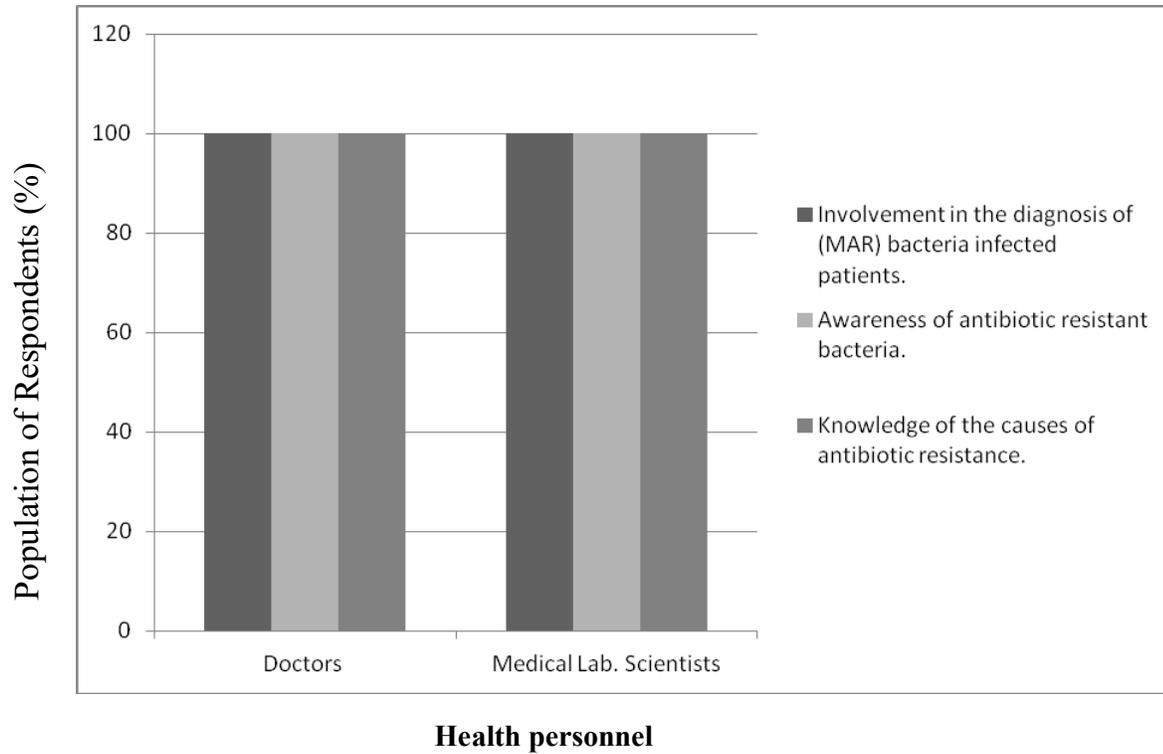
##### **4.1.1 Knowledge, attitude and practice towards beta-lactamase- producing bacteria**

###### **4.1.1a Involvement of multiple antibiotic resistant (MAR) bacteria**

Two hundred and sixty four health workers (152 medical doctors and 112 medical laboratory scientists) were interviewed. In this study out of the 300 copies of the structured questionnaire distributed, 264 were filled and returned. All the health workers interviewed were involved in the diagnosis and/or treatment of bacteria infected patients in their hospitals or diagnostic laboratories. See fig. 4.1.1 below.

###### **4.1.1b Awareness of antibiotic resistant bacteria**

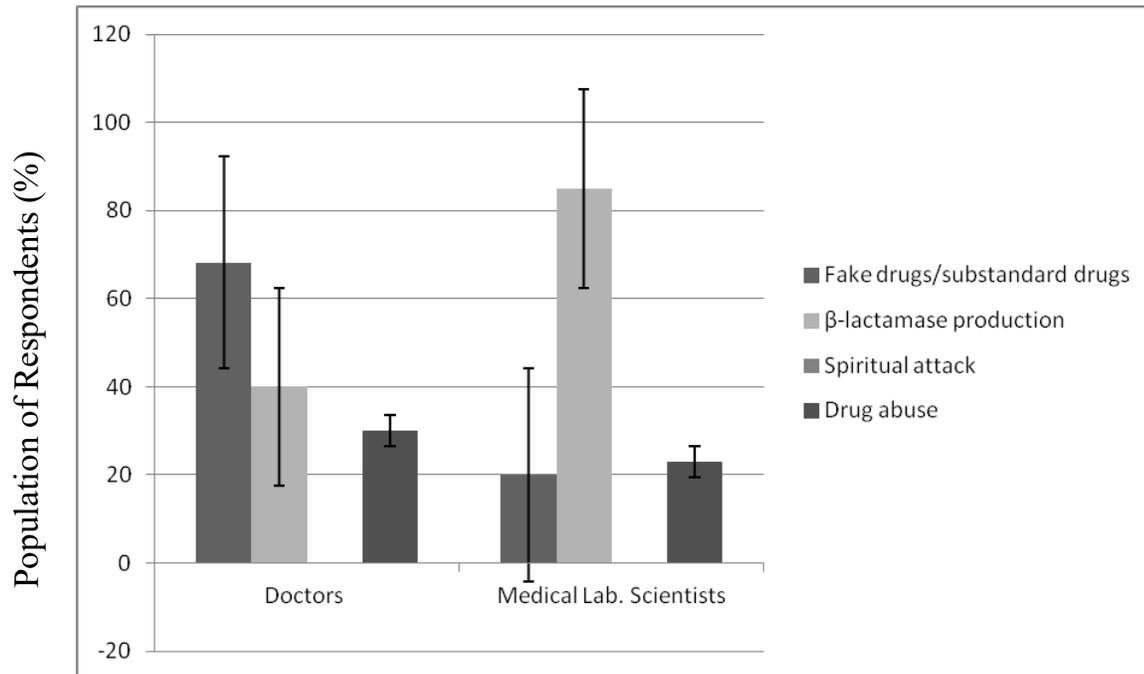
The findings of this study showed that all the respondents are aware of antibiotic resistant bacteria. All the respondents had good knowledge of the causes of antibiotic resistance. See fig. 4.1.1 below.



**Figure 4.1.1: Health workers, involvement, awareness and knowledge of antibiotic resistant bacteria.**

#### **4.1.1c Causes of antibiotic resistance**

Sixty eight percent of medical doctors and 22% of medical laboratory scientists said fake drugs is one of the causes of antibiotic resistance, while 41% of medical doctors and 87% of medical laboratory scientists said  $\beta$ -lactamase production. Thirty percent of medical doctors and 22% of medical laboratory scientist said drug abuse while non of the health practioners ticked spiritual attack. This finding is as illustrated in Fig. 4.1.2 below.



**Health personnel**

**Figure 4.1.2: Causes of antibiotic resistant**

#### **4.1.1d Encountered drug resistant bacteria in the hospital**

Out of 264 respondents interviewed, 256 (97%) have encountered cases of antibiotic resistant bacterial infected patients in their hospitals or diagnostic laboratories. Table 4.1.1 below summarizes the distribution of respondents who have encountered antibiotic resistant bacterial – infected patients in their health institutions

#### **4.1.1e Frequency with which the respondents encounter cases of antibiotic resistant bacteria in their hospitals or diagnostic centres**

One hundred and thirty two (86.8%) doctors and 60 (53.6%) medical laboratory scientists encounter antibiotic resistant bacteria infected patients at least once in a week, 16 (10.5%) doctors and 24 (21.4%) medical laboratory scientists encounter antibiotic resistant bacteria infected patients at least once in a month, 8 (5.3%) doctors and 8 (7.1%) medical laboratory scientists encounter antibiotic resistant bacteria infected patients at least 1 in every 10 days and 4 (2.6%) doctors and 12 (10.7%) medical laboratory scientists encounter antibiotic resistant bacteria infected patients at least once daily (See table 4.1.2 below). Analysis of the data using ANOVA at 95% confidence level showed strong positive correlation between medical doctors and medical laboratory scientists in the frequency at which they encounter cases of antibiotic resistance.

**Table 4.1.1: Frequency and percentage distribution of health workers based on the cases of drug resistant bacteria encountered.**

<b>Professional Group</b>	<b>Number Examined</b>	<b>Number that have Encountered Antibiotic Resistant Bacteria (%)</b>	
Medical Doctors	152	152	(100)
Med. Lab. Scientists	112	104	(92.9)
<b>Total</b>	<b>264</b>	<b>256</b>	<b>(97.0)</b>

**Table 4.1.2: Frequency and percentage distribution of health workers according to frequency of cases of drug resistant bacteria.**

<b>RESPONDENTS</b>	<b>No Examined</b>	<b>FREQUENCY OF ENCOUNTER (%)</b>			
		<b>1 per Week</b>	<b>1 per Month</b>	<b>1 in 10 Days</b>	<b>1 Daily</b>
Medical Doctors	152	132 (86.8)	16 (10.5)	8 (5.3)	4 (2.6)
Med. Lab Scientists	112	60 (53.6)	24 (21.4)	8 (7.1)	12 (10.7)
		<b>192</b>	<b>40</b>	<b>16</b>	<b>16</b>
<b>Total</b>	<b>264</b>	<b>(72.7)</b>	<b>(15.2)</b>	<b>(6.1)</b>	<b>(6.1)</b>

#### **4.1.1f Bacteria most commonly involved in MAR**

The study showed that 88 (57.9%) and 88 (78.6%) medical laboratory scientists reported that Gram-positive bacteria were the group most commonly encountered in antibiotic resistant bacterial infected patients while 56 (36.8%) doctors and 16 (14.3%) medical laboratory scientists reported that Gram-negative bacteria were more frequently encountered. See table 4.1.3a below.

#### **Species of organisms involved in MAR**

Fifty two (34.2%) doctors and 52 (46.4%) medical laboratory scientists encounter *Escherichia coli*, 24 (15.8%) doctors and 28 (25%) medical laboratory scientists encounter *Pseudomonas aeruginosa*, 40 (26.3%) doctors and 16 (14.3%) medical laboratory scientists encounter *Staphylococcus aureus* while 36 (23.7%) doctors and 48 (42.9%) medical laboratory scientists encounter *Klebsiella* species . This result is as presented on table 4.1.3b below. Analysis of the data using ANOVA showed no significant difference ( $P>0.05$ ) in the bacteria and species of organisms involved in MAR between different groups and within different groups of respondents.

**Table 4.1.3a: Bacteria most commonly involved in MAR**

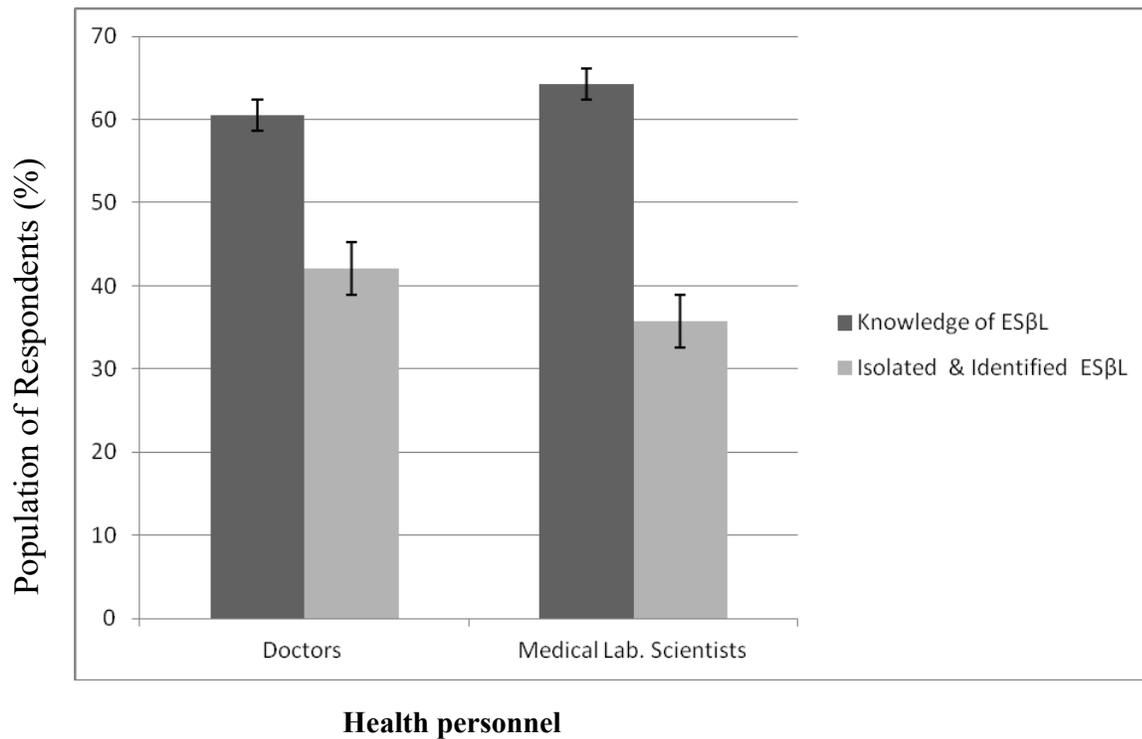
<b>RESPONDENTS</b>	<b>Gram +ve bacteria</b>	<b>Gram -ve bacteria</b>
Medical Doctors (n = 152)	88(57.9%)	56 (36.8%)
Med. Lab. Scientists (n = 112)	88(78.6%)	16 (14.3%)
<b>Total</b>	<b>176(66.7%)</b>	<b>72 (27.3%)</b>

**Table 4.1.3b: Frequency and percentage distribution of health workers according to the species of bacteria identified.**

RESPONDENTS	No Examined	BACTERIA SPECIES ENCOUNTERED (%)							
		<i>S. aureus</i>		<i>E. coli</i>		<i>Pseudomonas aeruginosa</i>		<i>Klebsiella</i> Species	
<b>Medical Doctors</b>	<b>152</b>	40	(26.3)	52	(34.2)	24	(15.8)	36	(23.7)
<b>Med. Lab Scientists</b>	<b>112</b>	16	(14.3)	52	(46.4)	28	(25.0)	48	(42.9)
		<b>56</b>		<b>104</b>		<b>52</b>		<b>84</b>	
<b>Total</b>	<b>264</b>	<b>(21.2)</b>		<b>(39.4)</b>		<b>(19.7)</b>		<b>(31.8)</b>	

#### **4.1.1g Knowledge of ES $\beta$ L**

On knowledge of extended spectrum beta lactamase producing bacteria, 92 (60.5%) doctors and 72 (64.3%) medical laboratory scientists have heard of and are aware of extended spectrum beta lactamase producing bacteria. Fig 4.1.3 below summarizes the awareness and distribution of respondents who have isolated and identified ES $\beta$ L in their health institutions. As shown, 64 (42.1%) doctors and 40 (35.7%) medical laboratory scientists have identified ES $\beta$ L producing bacteria in their hospitals and / or diagnostic laboratory.



**Figure 4.1.3: Distribution of Respondents based on ESBL identification.**

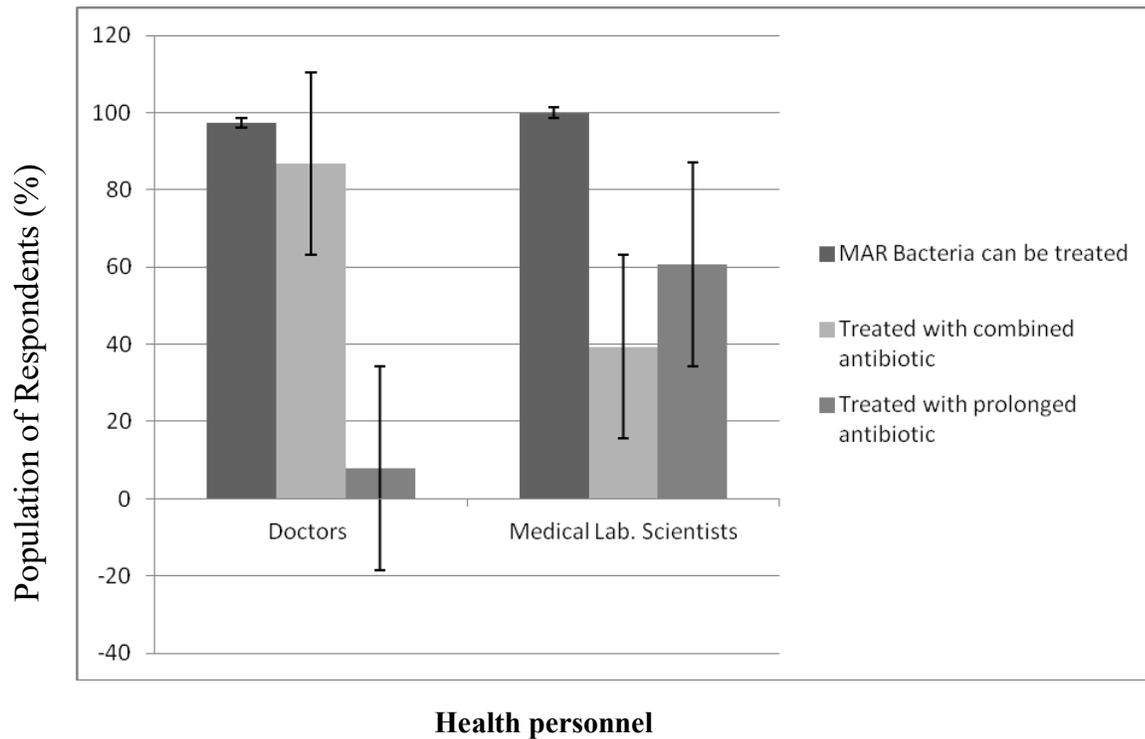
## **4.1.2 Attitude of health workers towards antibiotic resistant bacteria**

### **4.1.2a Treatment of MAR bacteria**

One hundred and forty six (97.4%) doctors and 112 (100%) medical laboratory scientists believed that multiple antibiotic resistant bacteria can be treated. See figure 4.1.4 below.

### **4.1.2b Treatment approach**

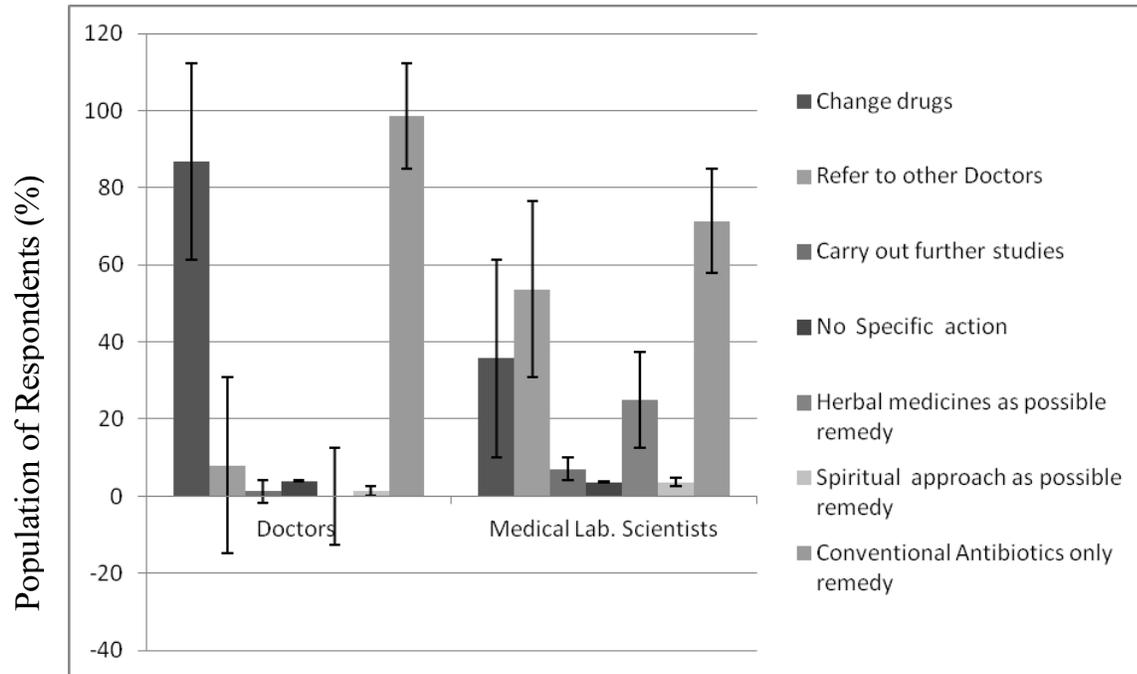
Of the 152 doctors who participated in the study, 132 (86.8%) said multi-antibiotic resistant bacterial infections can be treated with combined antibiotic therapy, 12 (7.9%) can be treated by prolonged antibiotic therapy. Similarly, 44 (39.3%) out of 112 medical laboratory scientists believed multi-antibiotic resistant bacterial infections can be treated, using combined antibiotic therapy while 68 (60.7%) believed they can be treated with prolonged antibiotic therapy. Analysis of the data using Chi square showed significant difference ( $p < 0.05$ ) in attitude towards multi-antibiotic resistant bacteria infection between medical doctors and medical laboratory scientists. All medical laboratory scientists believe that MAR- bacterial infections can be treated but only 97.4% of medical doctors believed that MAR-bacterial infections can be treated. While a greater majority of medical doctors (86.8%) believed that MAR-bacterial infections can be treated using combined antibiotic therapy, a greater majority of medical laboratory scientists (60.7%) believed they can be treated, using prolonged antibiotic therapy. Attitude of Medical doctors and medical laboratory scientists in Imo State towards management of MAR bacteria is summarized in Fig. 4.1.4 below.



**Figure 4.1.4 Respondents' Attitude towards treatment of MAR bacteria infection**

#### **4.1.2c Management / treatment approach towards multiple antibiotic resistant bacteria**

Findings from the study showed that 132 (86.8%) doctors manage and treat multiple antibiotic resistant bacteria infections by changing drugs after some time, 12 (7.9%) refer the patients to other specialist doctors, 2 (1.3%) carry out further studies on the patients and 6 (3.9%) have no specific action. Similarly, 60 (53.6%) medical laboratory scientists refer multiple antibiotic resistant bacterial infected patients to medical doctors, 40 (35.7%) recommend change of drugs, 8 (7.1%) carry out further studies on the patient and 4 (3.6%) have no specific action. On the use of alternative approach to treatment, 150 (98.7%) medical doctors insisted that conventional antibiotics is the only remedy for treatment and 2 (1.3%) believed that spiritual approach such as prayers could offer treatment. Eighty (71.4%) medical laboratory scientists said conventional antibiotics are the only remedy, 28 (25.0%) said herbal medicines could provide effective treatment and 4 (3.6%) believed spiritual approach such as prayers can provide treatment. Statistical analysis of the data using Analysis of variance (ANOVA) showed strong positive correlation ( $p < 0.05$ ) in the attitude and treatment/management approach between medical doctors and medical laboratory scientists. While a greater majority of medical doctors (98.7%) insisted on their professional approach (use of conventional antibiotics), a good number of medical laboratory scientists (25.0%) gave hope that research and trials on alternative approach such as the use of medicinal plants might have positive result leading to effective treatment (See figure 4.1.5 below).



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**Figure 4.1.5: Management / treatment approach towards multiple antibiotic resistant bacteria**

#### **4.1.2d Facilities for ES $\beta$ L detection**

Of the 152 medical doctors who participated in this study, 32 (21.0%) have facilities for ES $\beta$ L detection in their hospitals, others are not sure their hospitals have facilities for this. Sixty eight (60.7%) medical laboratory scientists have facilities for detection of ES $\beta$ L producing bacteria. Twenty eight (18.4%) doctors and 60 (53.6%) medical laboratory scientists said they use double disc method for detection of ES $\beta$ L in their hospitals or diagnostic laboratories while 4 (2.6%) medical doctors and 8 (7.1%) medical laboratory Scientists use other methods such as ES $\beta$ L E-tests or combination discs 3 methods for detection of ES $\beta$ L-producing bacteria. Table 4.1.4 below summarizes the availability of ES $\beta$ L-detection facilities and methods adopted by different hospitals and diagnostic laboratories for detection of ES $\beta$ L-producing bacteria in Imo State. Statistical analysis of the data using ANOVA showed strong positive correlation between availability of facilities and diversification of methods of detection amongst medical doctors and medical laboratory scientists. Whereas only few medical doctors (21.0%) think they have facilities to detect ES $\beta$ L-producing bacteria in their hospitals, a greater majority (60.7%) of medical laboratory scientists are sure they have facilities for ES $\beta$ L detection and they are sure of the methods they use.

**Table 4.1.4: Availability of facilities and knowledge of method for ESβL detection**

Respondent	Number Examine	Number that have Facilities		Method for Detection of EsβL			
				Double Disc		E – test or Combination disc 3.	
Medical Doctors	152	32	(21.0)	28	(18.4)	4	(2.6)
Med. Lab Scientists	112	68	(60.7)	60	(53.6)	8	(7.1)
			<b>100</b>		<b>88</b>		<b>12</b>
<b>Total</b>	<b>264</b>		<b>(37.9%)</b>		<b>(33.3%)</b>		<b>(4.5%)</b>

#### **4.1.2e Involvement in research and dissemination of research findings**

Out of 152 medical doctors that participated in the study, 44 (28.9%) are involved in research and dissemination of research information in their hospitals. Out of 112 medical laboratory scientists, 44 (39.3%) are involved in research and dissemination of research information in their hospital or diagnostic centre. See figure 4.1.6 below.

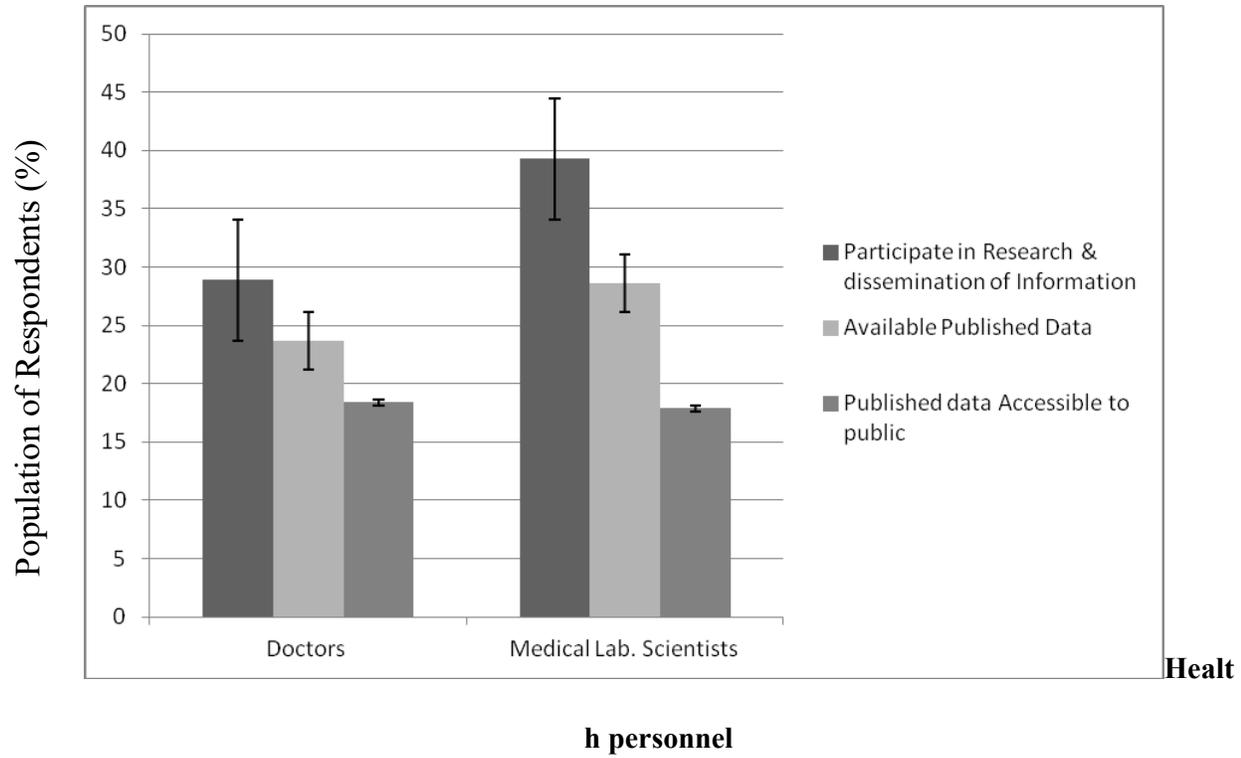
#### **4.1.2f Available published data to the public**

Only 36 (23.7%) doctors and 32 (28.6%) medical laboratory scientists have published data or evidence of their research finding. See figure 4.1.6 below.

#### **4.1.2g Published data accessible to the public**

Twenty eight (18.4%) doctors and 20 (17.9%) medical laboratory scientists believe their published research findings are accessible to the public. See figure 4.1.6 below.

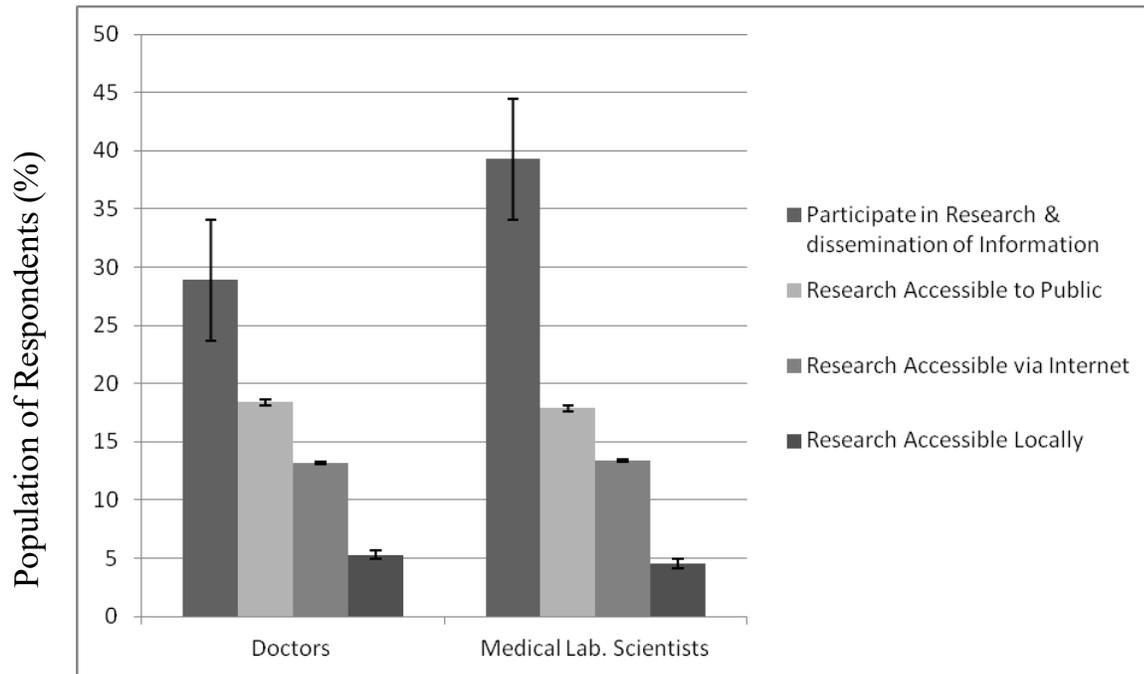
Fig. 4.1.6 below summarizes distribution of medical doctors and medical laboratory scientists who are involved in research and dissemination of research information in their places of work as well as the availability of such information to the public. Analysis of the data using ANOVA showed strong positive correlation ( $p < 0.05$ ) in participation and availability of research information between doctors and medical laboratory scientists in Imo State. More medical laboratory scientists (60.7%) than doctors (21.0%) claimed availability of facilities to detect ESβL bacteria in their places of work (Figure 4.1.6) and more medical laboratory scientists (39.3%) than doctors (28.9%) are involved in research and dissemination of research information. However, more doctors (18.4%) than medical laboratory scientists (17.9%) think their research information are accessible to the public.



**Figure 4.1.6: Participation in Research and Availability of Research information**

#### **4.1.2h Means of assessment of data / research information**

The findings of this study showed that out of 264 doctors and medical laboratory scientist who participated in the study, 28.9% of doctors and 39.3% of medical laboratory scientists are involved in research and dissemination of research information. Of this number, 48 (18.2%) comprising of 28 (18.4%) doctors and 20 (17.9%) medical laboratory scientists believe their research publications are accessible to the public. Thirty five (13.3%), comprising 20 (13.2%) doctors and 15 (13.4%) medical laboratory scientists said their research information are accessible on the internet; 13 (4.9%) comprising of 8 (5.3%) doctors and 5 (4.5%) medical laboratory scientists said their research publications are available locally and on request. Fig. 4.1.7 below shows data on the number of doctors and laboratory scientists in Imo State involved in research and dissemination of research information and means of accessing of their research publications. Analysis of the data, using ANOVA, shows no significant difference ( $p > 0.05$ ) in the population and distribution of researchers and research publications between doctors and medical laboratory scientists in Imo State. Also, there was no correlation in the availability of research facilities and means of dissemination of research information between doctors and medical laboratory scientists in Imo State. Whereas more medical laboratory scientists (60.7%) than doctors (21.0%) have facilities for research, there is no significant difference in the number of doctors and medical laboratory scientists who disseminate information via internet or local means.



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**Figure 4.1.7: Distribution of doctors and medical laboratory scientists involved in research and means of dissemination of their research information.**

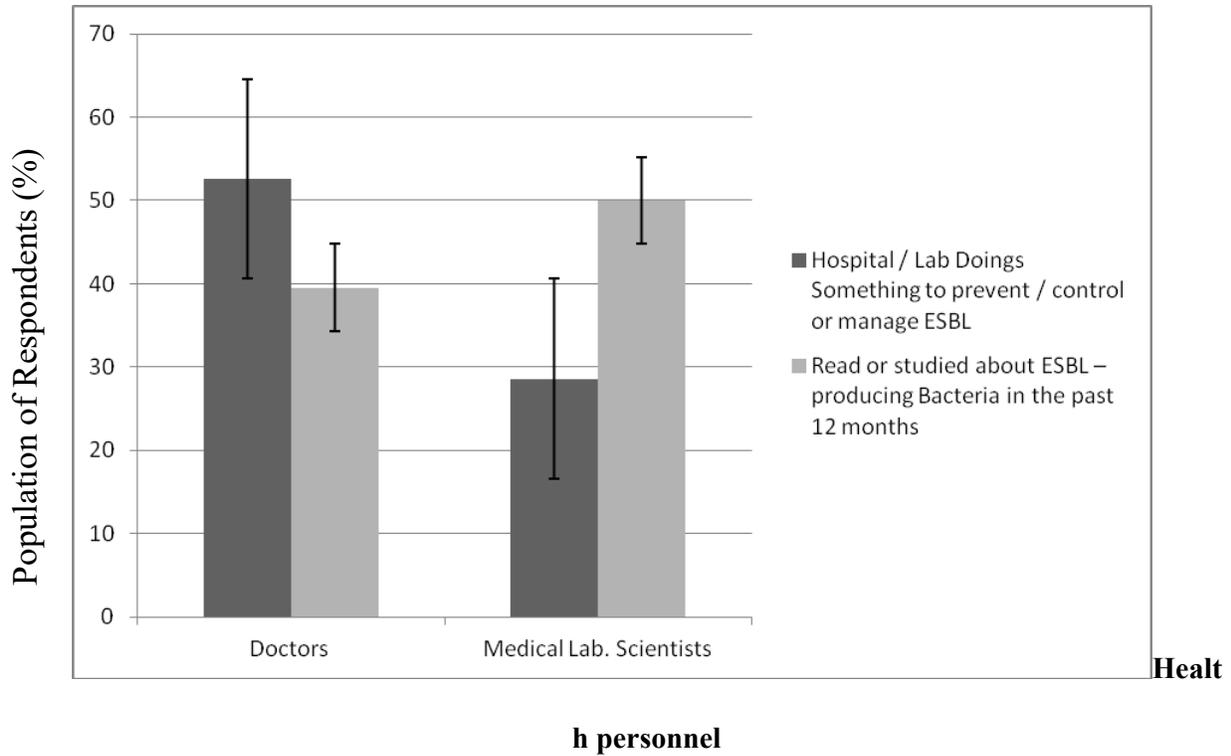
### **4.1.3 Practices towards ESβL**

#### **4.1.3a Reading or studying about ESβL-producing bacteria**

In the past 12 months, 116 (43%) respondents comprising of 60 (39.5%) doctors and 56 (50%) medical laboratory scientists have read or studied about ESβL-producing bacteria. See figure 4.1.8 below.

#### **4.1.3b Doing something towards prevention, control and management of ESβL**

One hundred and twelve (42.4%) respondents comprising of 80 (52.6%) doctors and 32 (28.6%) medical laboratory scientists said their hospitals and diagnostic centres are presently doing something towards prevention, control and management of ESβL-producing bacterial infections. Fig. 4.1.8 below summarizes data on the distribution of respondents who have read or studied about ESβL-producing bacteria in the past 12 months and those whose health institutions are presently doing something towards prevention, control and management of ESβL-producing bacteria. Statistical analysis of the data using ANOVA showed negative correlation ( $p > 0.05$ ) between involvement of health institutions in prevention, control or management of ESβL producing bacteria and attitude of health workers (doctors and medical laboratory scientists) towards reading and studying about ESβL-producing bacteria. Whereas 52.6% of the doctors reported that their hospital are presently doing something in prevention, control and management of ESβL-producing bacteria, only 39.5% of doctors have read or studied about ESβL-producing bacteria in the last 12 months. Conversely, 28.6% medical laboratory scientists said their hospital and / or diagnostic laboratories are presently doing something on prevention, control and management of ESβL-producing bacteria but 50% of medical laboratory scientists have read or studied about ESβL-producing bacteria in the last 12 months.



**Figure 4.1.8: Distribution of respondents who read or studied about ESβL-producing bacteria in the past 12 months and those whose health institutions are presently doing something towards prevention, control and management of ESβL-producing bacteria**

### **4.1.3c Actions taken by respondents' hospitals towards prevention, control and management of ESβL-producing bacteria**

#### **i Continuous education, enlightenment of staff**

Fourty four (28.9%) doctors and 44 (39.3%) medical laboratory scientists indicated that their hospitals engaged in continuous education, enlightenment of staff as a means towards prevention, control and management of ESβL-producing bacteria. See figure 4.1.9 below.

#### **ii Proper disposal of hospital wastes**

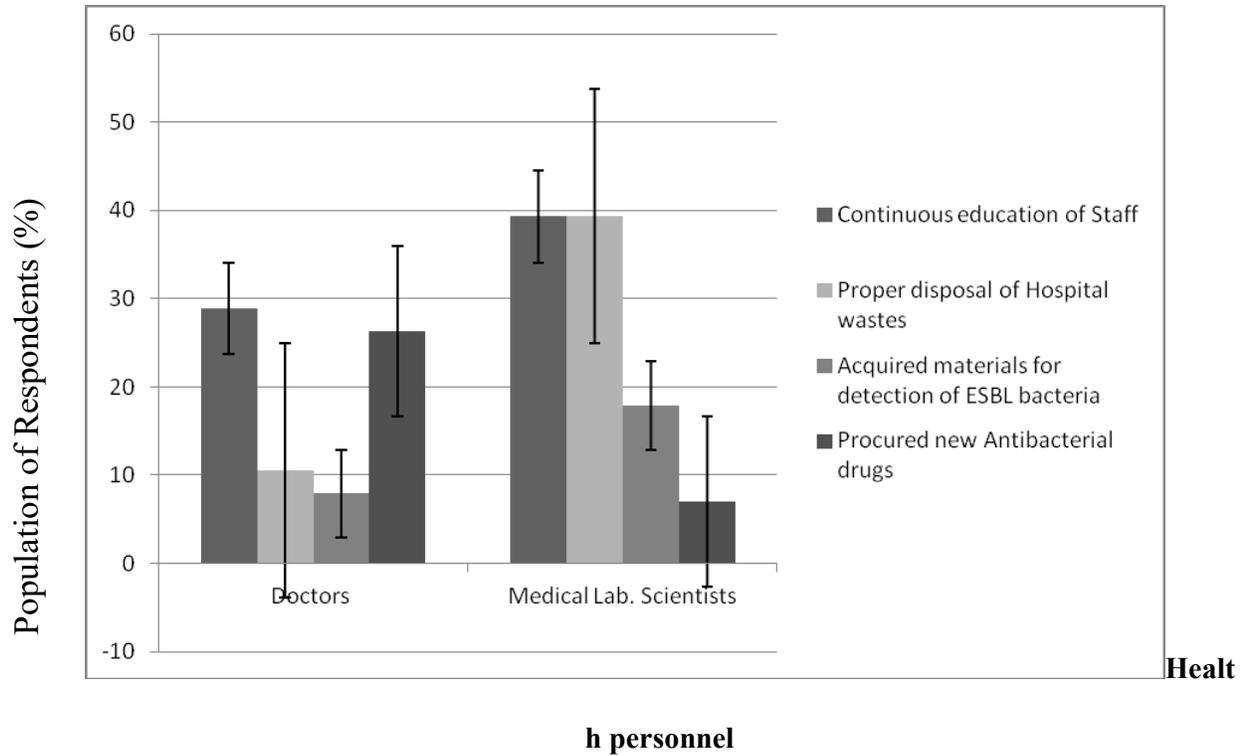
Sixteen (10.5%) doctors and 44 (39.3%) medical laboratory scientists said their hospitals adopted proper disposal of hospital wastes as a measure towards prevention, control and management of ESβL-producing bacteria. See figure 4.1.9 below.

#### **iii Acquired materials for detection of ESβL-producing bacteria**

Twelve (7.9%) doctors and 20 (17.9%) medical laboratory scientists said their hospitals acquired materials for detection of ESβL-producing bacteria. See figure 4.1.9 below.

#### **iv Procurement of new antibacterial drugs**

Fourty (26.3%) doctors and 8 (7.1%) medical laboratory scientists said their hospitals embarked on procurement of new antibacterial drugs to deal with ESβL-producing bacteria measure towards prevention, control and management of ESβL. Fig 4.1.9 below summarizes the actions taken by Respondents' Hospitals towards prevention, control and management of ESβL-producing bacteria.



**Figure 4.1.9: Measures taken by respondents' Hospitals towards prevention, control and management of ESBL-producing bacteria.**

#### **4.1.3d Data on the status of ES $\beta$ L-producing bacteria infection in respondents' hospitals in the last 3 months of this study**

##### **i Encountered ES $\beta$ L within the last 3months**

As shown, 8 (5.3%) doctors and 20 (17.9%) medical laboratory scientists said they have encountered ES $\beta$ L-producing bacteria infected patients in their hospitals within the last 3months. See figure 4.1.10 below.

##### **ii Treated the patients with different antibiotics**

Seven (4.6%) doctors said they treated the patients with different conventional antibiotics. See figure 4.1.10 below.

##### **iii Referred patients to specialist**

One (0.7%) doctors referred the patients to specialist colleagues, while 8 (7.1%) medical laboratory scientists referred the patients to specialist doctors for management. See figure 4.1.10 below.

##### **iv Embarked on further studies of ES $\beta$ L**

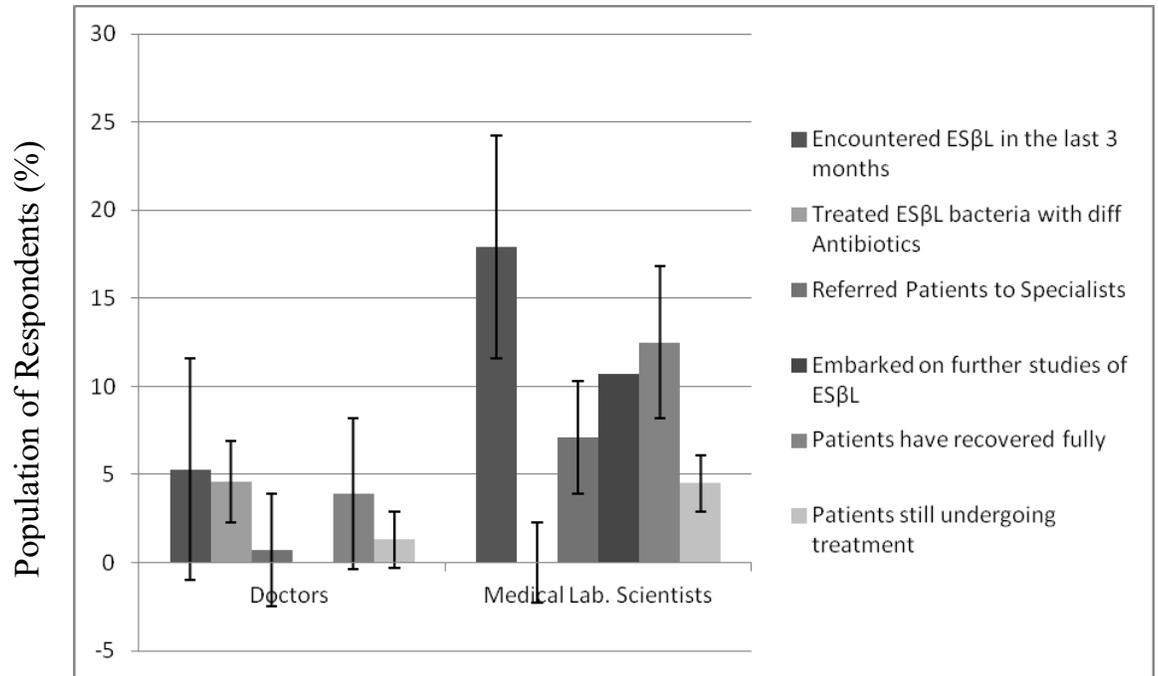
Twelve (10.7%) medical Laboratory Scientists embarked on further studies of the patients and the ES $\beta$ L-producing bacterial isolates. See figure 4.1.10 below.

##### **v Patients have fully recovered**

Six (3.9%) doctors reported that the patients have fully recovered while 14 (12.5%) medical laboratory scientists said the patients have recovered. See figure 4.1.10 below.

#### **vi Patients are still undergoing treatment**

Two (1.3%) medical doctors said the patients are still undergoing treatment 5 (4.5%) medical laboratory scientists said the patients are still undergoing treatment. While 1 (0.9%) could not account for the state of the patients at the time of this study. Fig 4.1.10 below summarizes the data on the status of ESBL-producing bacteria infection in respondents' hospitals in the last 3 months of this study.



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**Figure 4.1.10: Status of ESBL-producing bacteria infection in respondents' hospitals in the last 3 months**

#### 4.1.4 Laboratory investigation of clinical bacterial isolates for antibiotic resistance

##### 4.1.4.1 Isolation, characterization and identification of bacterial isolates

A total of 110 wound swabs and 370 uro-genital samples comprising 250 urine samples, 50 Urethral smear and 70 High vaginal swabs were collected and cultured for isolation of bacteria used for the study. Out of 250 urine samples, 98 (39.2%) had *Escherichia coli*, 45 (18%) had *Klebsiella* species and 19 (7.6%) had *Pseudomonas aeruginosa*. Out of 120 HVS and U/S examined, 25 (20.8%) had *Escherichia coli*, 23 (19.2%) had *Klebsiella* species and 13 (10.8%) had *Pseudomonas aeruginosa*. Out of 110 wound swabs examined, 24 (21.8%) had *Escherichia coli*, 25 (22.7%) had *Klebsiella* species and 28 (25.5%) had *Pseudomonas aeruginosa*. All together, 147 *Escherichia coli*, 93 *Klebsiella* species and 60 *Pseudomonas aeruginosa* giving a total of 300 bacterial pathogens isolated.

Summarize the prevalence of test isolates in clinical samples examined bacterial isolates. As shown, the most prevalent clinical isolate was *Escherichia coli* (30.6%), followed by *Klebsiella* species (19.1%) and *Pseudomonas aeruginosa* (12.5%) was the least. Specifically, the most prevalent bacteria in urogenital samples was *Escherichia coli* (39.2% urine and 20.8% U/S and HVS), while the most prevalent bacteria in wound swabs was *Pseudomonas aeruginosa* (25.5%). Statistical analysis of the data, using Chi square showed no significant difference ( $p > 0.05$ ) in the prevalence of bacterial pathogens between the different clinical samples examined.

**Table 4.1.5: Prevalence of Test Bacterial Isolates in Clinical Samples**

Sample	Prevalence (%)			Total
	<i>Escherichia coli</i>	<i>Klebsiella species</i>	<i>Pseudomonas aeruginosa</i>	
Urine (n = 250)	98 (39.2)	45 (18.0)	19 (7.6)	162 (64.8)
HVS/US (n = 120)	25 (20.8)	23 (19.2)	13 (10.8)	61 (50.8)
Wound Swab (n = 110)	24 (21.9)	25 (22.7)	28 (25.5)	77 (70.0)
<b>Total</b>	<b>147 (30.6)</b>	<b>93 (19.1)</b>	<b>60 (12.5)</b>	<b>300 (62.5)</b>

P &gt; 0.05

Key: HVS = High Vaginal Swap

US = Urethral Swap



#### 4.1.4.2 Antibacterial resistance of test organisms

Antibiotic resistance of the test organisms is summarized on Table 4.1.7. As shown, 73 (49.7%) *Escherichia coli* were susceptible to 3 or more antibiotics, 74 (50.3%) were resistant to 3 or more antibiotics. Out of 93 *Klebsiella* isolates, 59 (63.4%) were susceptible to 3 or more antibiotics and 34 (36.6%) were resistant to 3 or more antibiotics. Out of 60 *Pseudomonas aeruginosa* isolates, 34 (56.7%) were susceptible to 3 or more antibiotics and 26 (43.3%) were resistant to 3 or more antibiotics. Statistical analysis of the data using Chi square showed significant difference ( $p < 0.05$ ) in the resistant pattern between the test clinical bacterial isolates. *Pseudomonas aeruginosa* exhibited higher resistant (43.3%) than *Klebsiella* species (36.6%). Both *Klebsiella* species and *Pseudomonas aeruginosa* were more susceptible to the selected antibiotics than *Escherichia coli*. Thus, the prevalence of multiple antibiotic resistant (MAR) bacteria was higher amongst *Escherichia coli* (50.3%) than *Pseudomonas aeruginosa* (43.3%) and *Klebsiella* species (36.6%).

**Table 4.1.7: Antibiotic resistant Profile of the test organisms**

Antibiotic	ANTIBIOTIC RESISTANCE (%)		
	<i>Escherichia coli</i>	<i>Klebsiella species</i>	<i>Pseudomonas aeruginosa</i>
	(n = 147) resistance	(n = 93) resistance	(n = 60) resistance
Cefotaxime	66 (44.9)	45 (48.4)	14(23.3)
Ciprofloxacin	96 (65.3)	89 (95.7)	44(73.3)
Ceftazidime	118 (80.3)	30(32.3)	32(53.3)
Carbecillin	83 (56.5)	63(67.7)	41(68.3)
Ceftriazone	118 (80.3)	42(45.2)	12(20.0)
Ticarcillin	120(81.6)	30(32.3)	41(68.3)
Cefepime	99(67.4)	76(81.7)	47(78.3)
Azetreonam	66(44.9)	45(48.4)	37(61.7)
Piperacillin	65(44.2)	59(63.5)	40(66.7)
Gentamicin	81(55.1)	66(71.0)	33(55.0)
Imipenem	92(62.6)	86(92.5)	56(93.3)
Ampicillin	79(53.7)	61(65.6)	31(51.7)

#### **4.1.4.3 Prevalence of multiple antibiotic resistant (MAR) bacteria in Clinical samples**

The most prevalent MAR-bacteria in all the clinical samples was *Escherichia coli* (45.8% in wound swab 51.0% in urine, 52.0% in U/S and HVS). The least prevalent MAR-bacteria in urogenital samples was *Pseudomonas aeruginosa* (Urine = 42.1%, U/S and HVS = 46.2%), while the least prevalent MAR-bacteria in wound swab was *Klebsiella* species (24.0%). Statistical analysis of the data using Chi square showed no significant difference ( $p > 0.05$ ) in the prevalence of MAR-resistant between the groups of bacterial isolates used. Table 4.1.8 summarizes the prevalence of multi-antibiotic resistant (MAR) bacteria in Clinical samples examined.

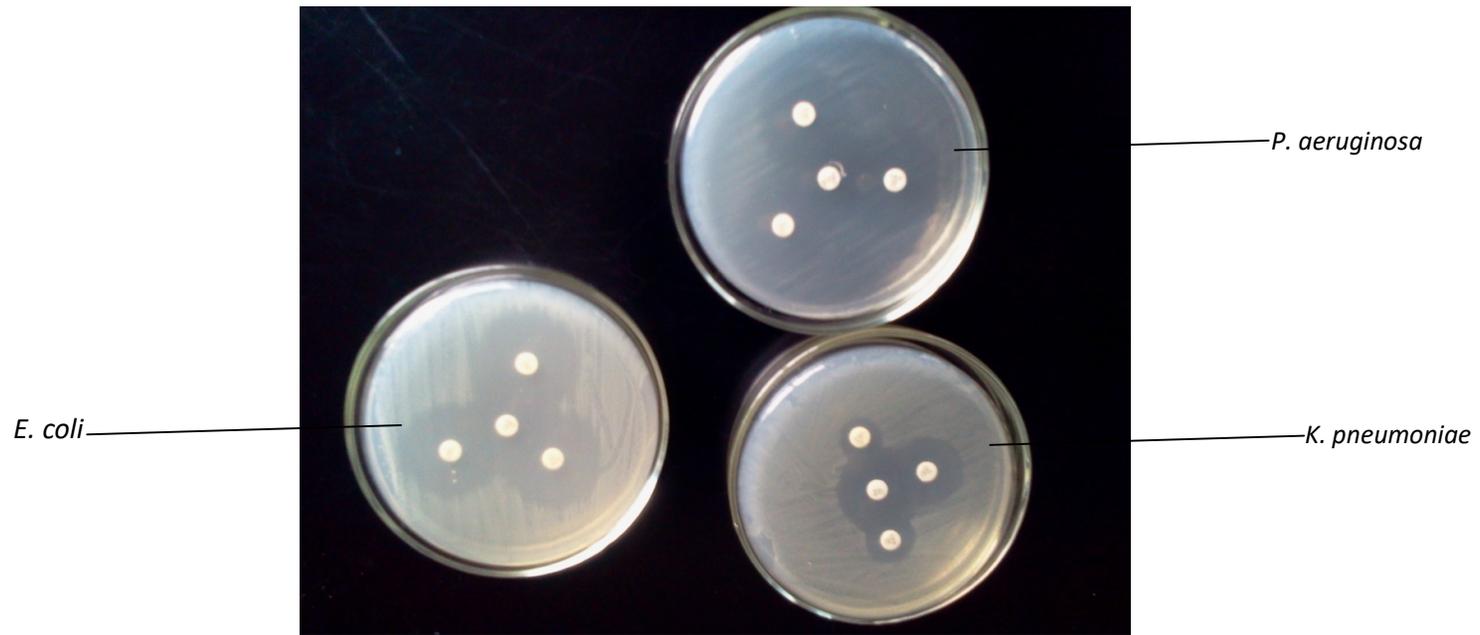
**Table 4.1.8: Prevalence of multiple antibiotic resistant (MAR) isolates in clinical samples**

Sample	Isolate	Antibiotic resistant Profile (%) Resistant to 3 or more antibiotics		
		Total		
Urine (n = 250)	<i>Escherichia coli</i> (n = 98)	50	(51.0)	75(30%)
	<i>Klebsiella species</i> (n = 45)	17	(44.4)	
	<i>Pseudomonas aeruginosa</i> (n = 19)	8	(42.1)	
US/HVS (n =120)	<i>Escherichia coli</i> (n = 25)	13	(52.0)	30(25%)
	<i>Klebsiella species</i> (n =23)	11	(47.8)	
	<i>Pseudomonas aeruginosa</i> (n = 13)	6	(46.2)	
Wound swab (n = 110)	<i>Escherichia coli</i> (n = 24)	11	(45.8)	29(26%)
	<i>Klebsiella species</i> (n = 25)	6	(24.0)	
	<i>Pseudomonas aeruginosa</i> (n = 28)	12	(42.9)	
<b>Total</b>	<b>300</b>	<b>134(44.7%)</b>		

Key: HVS = High Vaginal Swap  
US = Urethral Swap

#### 4.1.4.4 Phenotypic screening for ESβL-producing bacteria using double disc synergy (DDST)

The double disc synergy phenotypic method (Plate 4.1.1) was used to screen the multiple antibiotic resistant bacterial isolates for ESβL-production. The prevalence of ESβL-producing bacteria amongst MAR isolates is summarized on Table 4.1.9. As shown, 50 (67.6) out of 74 *Escherichia coli* were ESβL positive. This comprised of 38 (95.0%) out of 50 *Escherichia coli* from Urine samples and 12 (63.2%) out of 19 from HVS and U/S. Out of 34 MAR - *Klebsiella* species, 22 (64.7%) were ESβL-positive. This comprised of 11 (55.0%) out of 17 *Klebsiella* from urine samples, 10 (83.3%) out of 11 from HVS and U/S and 1 (50.0%) out of 6 from wound swabs. Also, 15 (57.7%) out of 26 MAR-*Pseudomonas aeruginosa* were positive for ESβL-production. This comprised 5 (83.3%) out of 8 from urine samples and 10 (62.5%) out of 12 from wound swabs. Statistical analysis of the data, using chi square shows significant difference ( $p < 0.05$ ) in the prevalence of ESβL-producing strains between the different species of bacteria exhibiting multiple antibiotic resistance. The prevalence of ESβL-producing strains was highest (67.6%) amongst *Escherichia coli* than *Klebsiella* species (64.7%) and *Pseudomonas aeruginosa* (57.7%).



**Plate 4.1.0: Double Disc Synergy Screening for ESβL – producing bacteria**

**Table 4.1.9: Prevalence of ESβL-producing bacteria amongst MAR isolates**

ALL SAMPLES (%)			
ISOLATE	SOURCE	ESβL POSITIVE ISOLATES (%)	TOTAL
<i>Escherichia coli</i> (n = 74)	URINE (n = 50)	38 (95.0)	50 (67.6)
	HVS/US (n = 19)	12 (63.2)	
	WOUND SWAB (n=11)	0	
<i>Klebsiella species</i> (n = 34)	URINE (n = 17)	11 (55.0)	22 (64.7)
	HVS/US (n = 11)	10 (83.3)	
	WOUND SWAB (n =6)	1 (50.0)	
<i>Pseudomonas aeruginosa</i> (n = 26)	URINE (n = 8)	5 (83.3)	15 (57.7)
	HVS/US (n = 6)	0	
	WOUND SWAB (n = 12)	10 (62.5)	
			87 (64.9)

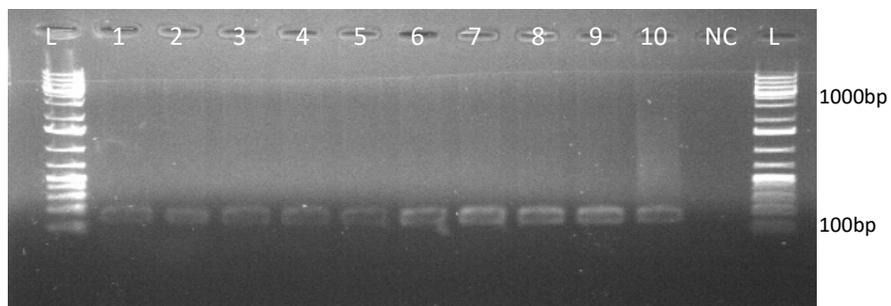
Key: HVS = High Vaginal Swap  
US = Urethral Swap

#### **4.1.5 Molecular examination of bacterial isolates**

##### **4.1.5.1: Molecular identification of bacterial isolates**

###### **4.1.5.1a Electrophoretic pattern for the molecular identification of *Escherichia coli***

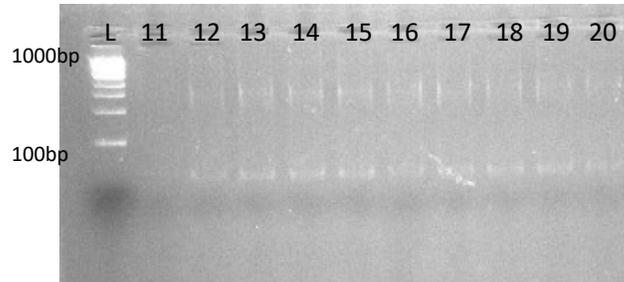
All of the ten representative isolates 1-10 were positive for *Escherichia coli*. Their sizes were 160bp respectively (See plate 4.1.1 below)



**Plate 4.1.1:** Polymerase chain reaction results for clinical bacterial isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Sample1-10 are positive for *Escherichia coli* with bands at 160bp. NC is a no DNA template control.

#### **4.1.5.1b Electrophoretic pattern for the molecular identification of *Klebsiella pneumoniae***

Nine out of the ten representatives isolates 12-20, were positive for *klebsiella* species. Their sizes were 850bp, 250bp and 50bp. One isolate number 11 was negative for *klebsiella* species. Nine of the *klebsiella* species were all negative for *Klebsiella pneumoniae*. (See plates 4.1.2 and 4.1.3 below).



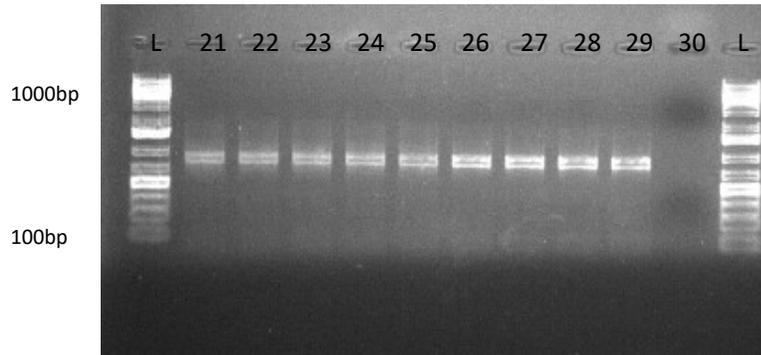
**Plate 4.1.2:** Polymerase chain reaction results for clinical bacterial isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 12-20 are positive for *Klebsiella* species with bands at 850bp, 250bp and 50bp while Sample 11 was negative for *Klebsiella* species.



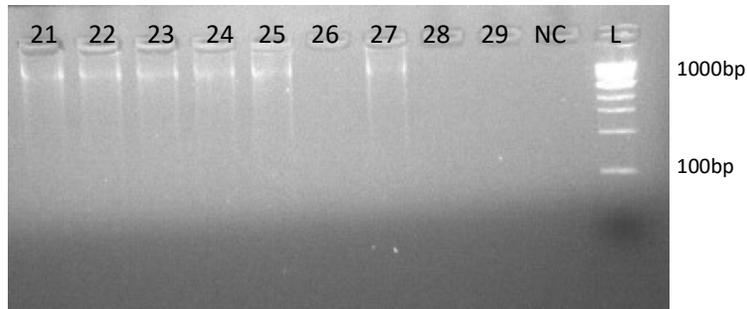
**Plate 4.1.3:** Polymerase chain reaction results for clinical *Klebsiella* species analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 12-20 are negative for *Klebsiella pneumoniae*. NC is a no DNA template control.

#### **4. 1.5.1c Electrophoretic pattern for the molecular identification of *Pseudomonas aeruginosa***

Nine out of the ten representative isolates 21-29 were positive for *Pseudomonas* species. Their sizes were 650bp respectively while a sample 30 was negative. Six out of the 9 isolates were positive for *Pseudomonas aeruginosa* while 3 isolates were negative (See plates 4.1.4 and 4.1.5 below).



**Plate 4.1.4:** Polymerase chain reaction results for clinical bacterial isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 21-29 are positive for *Pseudomonas* species with bands at 650bp while sample 30 is negative for *Pseudomonas* species.



**Pale 4.1.5:** Polymerase chain reaction results for clinical *Pseudomonas* species analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 21-25 and 27 are positive for *Pseudomonas aeruginosa* with bands at 1000bp while samples 26, 28 and 29 are negative for *Pseudomonas aeruginosa*. NC is a no DNA template control.

#### **4. 1.5.1d Summary of the result of electrophoretic pattern of the molecular identification of the bacterial isolates**

All the 10 isolates were positive for *Escherichia coli*. Out of the 10 isolates of *Klebsiella* species, 9 was positive for *Klebsiella* species but none was positive for *Klebsiella pneumoniae*. Out of the 10 isolates of *Pseudomonas* species, 9 was positive for *Pseudomonas* species but 6 was positive for *Pseudomonas aeruginosa* while 3 was negative (See table 4.1.10 below).

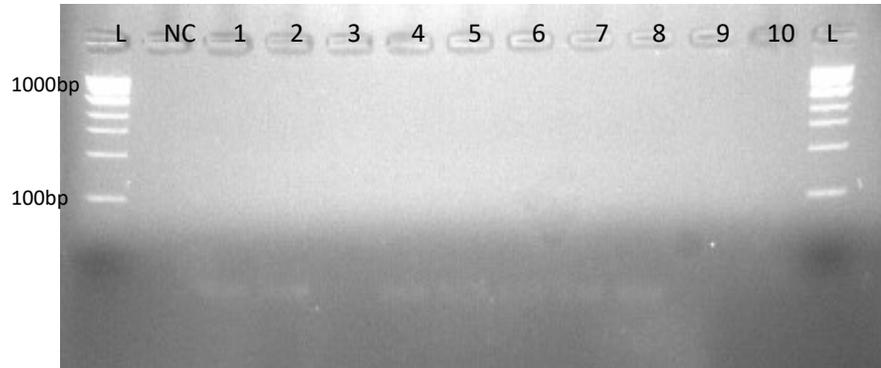
**Table 4.1.10: Molecular identification of bacterial isolates**

<b>Isolates</b>	<b>Number Examined</b>	<b>Number Confirmed</b>
<i>Escherichia coli</i>	10	10 with bands at 160bp
<i>Klebsiella</i> species	10	9 with bands 850bp, 250bp, 50bp
<i>Klebsiella pneumoniae</i>	9	None
<i>Pseudomonas</i> species	10	9 with bands at 650bp
<i>P. aeruginosa</i>	9	6 with bands at 1000bp

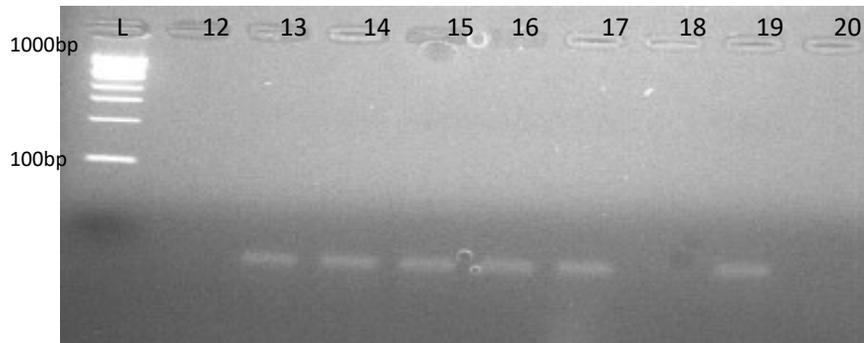
#### **4.1.5.2 Detection of ES $\beta$ L genes**

##### **4.1.5.2a Gel electrophoresis of the PCR amplified products for the detection of (SHV) $\beta$ -lactamase genes for the representative isolates.**

Isolates 1,2,4-8, 13-17,19 and 20 showed amplification of size 50bp for SHV  $\beta$ -lactamase gene while isolates 21-25 and 27 showed amplification of size 50bp and 90bp. However, there was no amplification for the SHV  $\beta$ -lactamase gene for isolates 3,9,10,12,18,20,26 and 29 ( See plates 4.1.6a - 4.1.8a below).



**Plate 4.1.6A:** Polymerase chain reaction results for clinical *Escherichia coli* isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 1, 2, 4, 5, 6, 7 and 8 are positive for ES $\beta$ L (SHV) resistance gene with bands at 50bp while Samples 3, 9 and 10 are negative for ES $\beta$ L (SHV) resistance genes. NC is a no DNA template control.



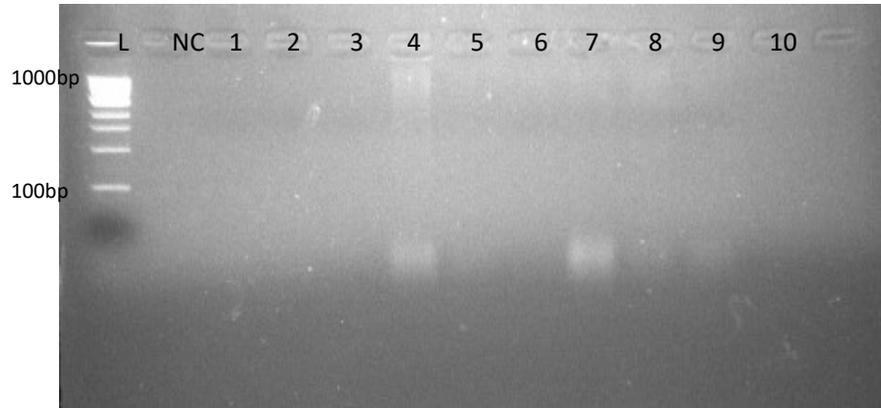
**Plate 4.1.7A:** Polymerase chain reaction results for clinical *Klebsiella* species isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 13, 14, 15, 16, 17 and 19 are positive for ESβL (SHV) resistance gene with bands at 50bp while Samples 12, 18 and 20 are negative for ESβL (SHV) resistance genes.



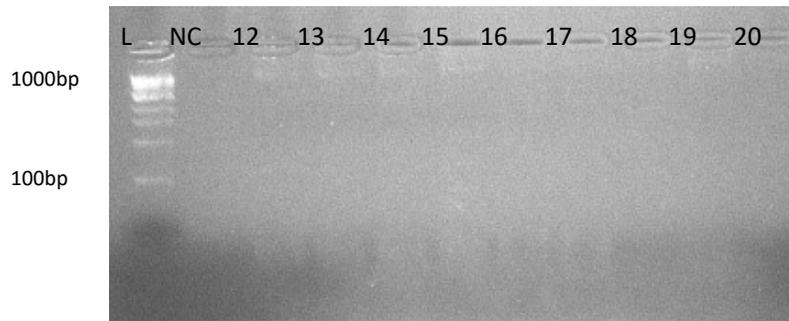
**Plate 4.1.8A:** Polymerase chain reaction results for clinical *Pseudomonas* species isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 21, 22, 23, 24, 25 and 27 are positive for ES $\beta$ L (SHV) resistance gene with bands at 50 and 90bp, sample 28 is positive for ES $\beta$ L (SHV) resistance gene with bands at 50 while Samples 26 and 29 are negative for ES $\beta$ L (SHV) resistance gene. NC is a no DNA template control.

**4.1.5.2b Gel electrophoresis of the PCR amplified products for the detection of (TEM)  $\beta$ -lactamase genes for the representative isolates.**

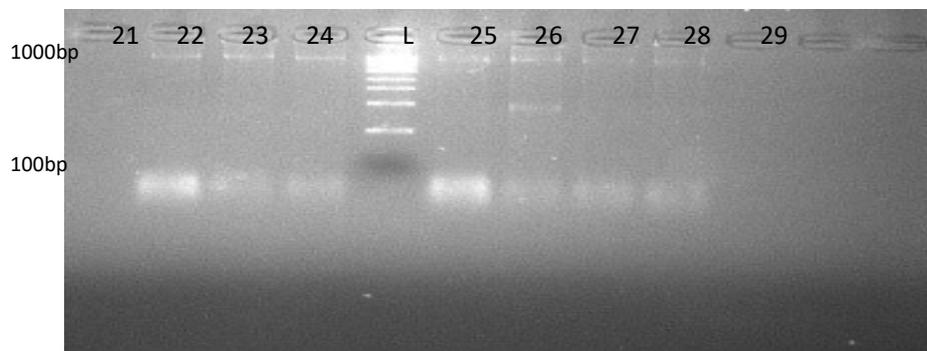
Isolates 4, 7-9,22-28 showed amplification of size 50bp for TEM  $\beta$ -lactamase gene out of 1-30 isolates. However, there was no amplification for TEM  $\beta$ -lactamase gene for the rest of the isolates. (See plates 4.1.6b - 4.1.8b below)



**Plate 4.1.6B:** Polymerase chain reaction results for clinical *Escherichia coli* analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 4, 7, 8 and 9 are positive for ES $\beta$ L (TEM) resistance gene with bands at 50bp while Samples 1, 2, 3, 5, 6 and 10 are negative for ES $\beta$ L (TEM) resistance gene. NC is a no DNA template control.



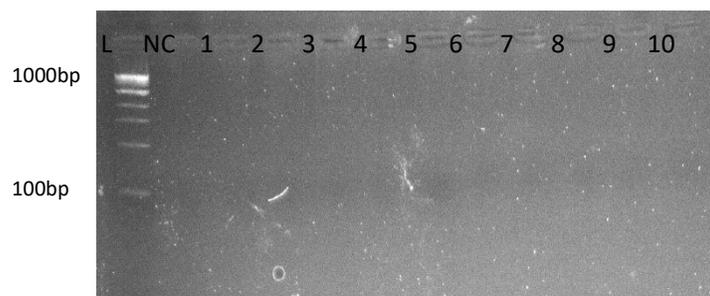
**Plate 4.1.7B:** Polymerase chain reaction results for clinical *Klebsiella* species analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 12 - 20 are negative for ES $\beta$ L (TEM) resistance genes.



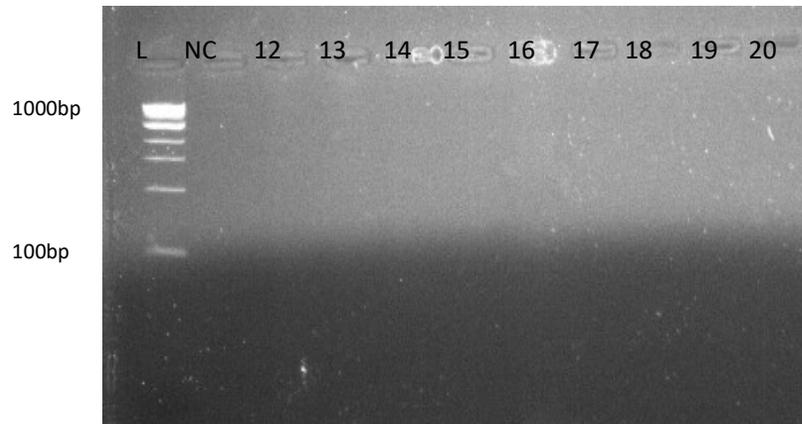
**Plate 4.1.8B:** Polymerase chain reaction results for clinical *Pseudomonas* species isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 22, 23, 24, 25, 26, 27 and 28 are positive for ES $\beta$ L (TEM) resistance gene with bands at 50bp while Samples 21 and 29 are negative for ES $\beta$ L (TEM) resistance gene.

**4.1.5.2c Gel electrophoresis of the PCR amplified products for the detection of (CTX-M)  $\beta$ -lactamase genes for the representative isolates.**

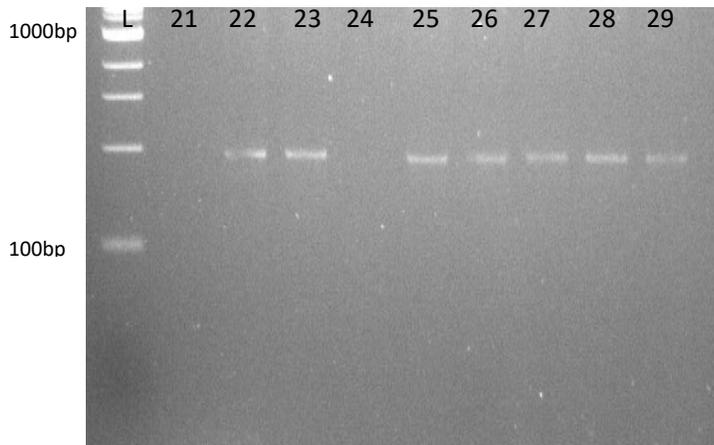
Isolates 22,23,25-29 showed amplification of size 200bp for CTX-M  $\beta$ -lactamase gene out of 1-30 isolates. However there was no amplification for CTX-M  $\beta$ -lactamase gene for the rest of the isolates (See plates 4.1.6c - 4.1.8c below).



**Plate 4.1.6C:** Polymerase chain reaction results for clinical *Escherichia coli* isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 1 - 10 are negative for ESβL (CTX-M) resistance genes. NC is a no DNA template control.



**Plate 4.1.7C:** Polymerase chain reaction results for clinical *Klebsiella* species isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 12 - 20 are negative for ESβL (CTX-M) resistance genes.



**Plate 4.1.8C:** Polymerase chain reaction results for clinical *Pseudomonas* species isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 22, 23, 25, 26, 27, 28 and 29 are positive for ESβL (CTX-M) resistance gene with bands at 200bp while Samples 21 and 24 are negative for ESβL (CTX-M) resistance gene.

#### **4.1.5.2d Summary of the results of gel electrophoresis of the PCR amplified products**

At least isolates of *Escherichia coli*, *Klebsiella* species. and *Pseudomonas aeruginosa* showed amplification for one type of  $\beta$ -lactamase gene or the other. Out of the 10 isolates of *Escherichia coli* examined, 4 (40.0%) had TEM-genes and 7 (70.0) had SHV genes. Of the 10 isolates of *Pseudomonas aeruginosa* examined, 7 (70.0%) had TEM genes, 6 (60%) had SHV, and 7 (70%) had CTX-M. Six (60.0%) out of 10 *Klebsiella* species examined had SHV. The most commonly gene detected was SHV, followed by TEM and CTX-M. The findings on detection of ES $\beta$ L are summarized in Table 4.1.11. As shown, out of the 30 ES $\beta$ L positive isolates examined, 7(23.3%) were positive for CTX-M genes, 11 (36.7%) were positive for TEM genes and 19 (63.3%) were positive for SHV genes.

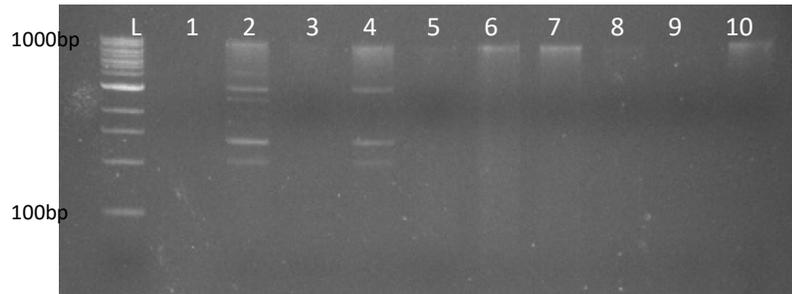
**Table 4.1.11: Beta-lactamase-genes detected in ESβL-positive isolates**

<b>ISOLATES/% OCCURRENCE</b>				
<b>ESβL GENE</b>	<b><i>E.coli</i> (n = 10)</b>	<b><i>Klebsiella spp</i> (n = 10)</b>	<b><i>P. aeruginosa</i> (n = 10)</b>	<b>Total (n = 30)</b>
<b>TEM</b>	4 (40)	0 (0)	7 (70)	11 (36.71)
<b>SHV</b>	7 (70)	6 (60)	6 (60)	19 (63.3)
<b>CTX-M</b>	0 (0)	0 (0)	7 (70)	7 (23.3)
	8(80)	6(60)	9(90)	23(76.7%)

### **4.1.5.3 Plasmid profiling of the representative isolates**

#### **4.1.5.3a Plasmid profiles of the representative isolates 1-10 from MAR *Escherichia coli***

Out of the 10 isolates, 5 plasmids were isolated from isolates 2, 4, 6, 7 & 10, the rest had no plasmids. Among the 5 isolates, 2 isolates, 2 & 4 are positive for plasmid genes with multiple bands at 200, 260, 480, 500 & 1000bp respectively. Isolates 6, 7 & 10 are positive for plasmid genes with bands at 1000bp (plate 4.1.9A).



**Plate 4.1.9A:** Plasmid profile of multiple drug resistance *Escherichia coli* isolates analyzed with 0.8% agarose gel electrophoresis. L is 100bp-1000bp ladder (molecular marker). Samples 2 and 4 are positive for plasmid genes with multiple bands at 200, 260, 480, 500 and 1000bp respectively, samples 6, 7 and 10 are positive for plasmid genes with bands at 1000bp while samples 1, 3, 5, 8 and 9 are negative for plasmid genes. NC is a no template control.

#### **4.1.5.3b Plasmid profiles of the representative isolates 11-20 from MAR *Klebsiella* species**

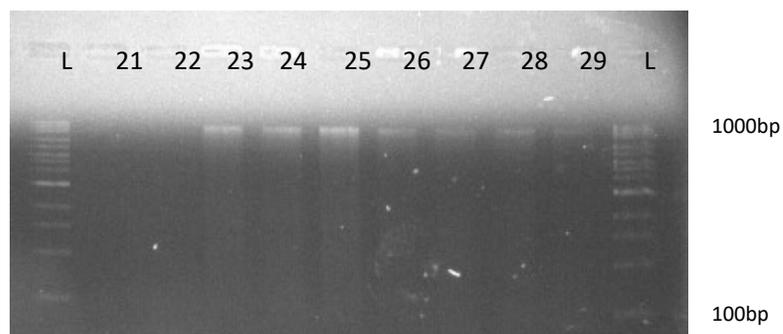
Four plasmids were isolated from isolates 16, 17, 19 & 20 while no plasmids were isolated from isolates 12-15 & 18. The isolated plasmids were of the size 1000bp, 900bp, 850bp, 800bp as shown in plate 4.1.9B.



**Plate 4.1.9B:** Plasmid profile of multiple drug resistance *Klebsiella* species isolates analyzed with 0.8% agarose gel electrophoresis. L is 100bp-1000bp ladder (molecular marker). Samples 16, 17, 19 and 20 are positive for plasmid genes with bands at 1000bp, 900bp, 850bp, 800bp while samples 12, 13, 14, 15 and 18 are negative for plasmid genes.

#### **4.1.5.3c Plasmid profiles of the representative isolates 21-30 from MAR *Pseudomonas* species**

Seven plasmids were isolated from isolates 23-29, while no plasmids were isolated from isolates 21 & 22. The isolated plasmids were of the size 1000bp as shown in plate 4.3.9C.



**Plate 4.1.9C:** Plasmid profile of multiple drug resistance *Pseudomonas* species isolates analyzed with 0.8% agarose gel electrophoresis. L is 100bp-1000bp ladder (molecular marker). Samples 23-29 are positive for plasmid genes with bands at 1000bp while samples 21 and 22 are negative for plasmid genes.

#### **4.1.5.3d Summary of the results of plasmid profiling of the representative isolates**

Sixteen (53.3%) of the representative isolates were found to harbor plasmids. Two isolates (6.7) had plasmids with multiple bands, while the rest had single bands. Five (50.0%) *Escherichia coli*, 4 (40.0%) *Klebsiella* species and 7 (70.0%) *Pseudomonas aeruginosa* were positive for plasmid profiling. Two (20.0%) *Escherichia coli* were positive for plasmids with multiple bands while 7 (70.0%) *Pseudomonas aeruginosa*, 4 (40.0%) *Klebsiella* species and 3 (30.0%) *Escherichia coli* were positive for plasmid with single band. The Plasmid Profile of multiple drug resistant bacterial Isolates are summarized on table 4.1.12.

**Table 4.1.12: Plasmid profile of multiple drug resistant bacterial isolates**

<b>Isolates</b>	<b>Number positive for plasmid profiling.</b>	<b>Number positive for plasmid with multiple band.</b>	<b>Number positive for plasmid with single band.</b>
<i>Escherichia coli</i> (n = 10)	5 (50.0)	2 (20.0)	3 (30.0)
<i>Klebsiella</i> species (n = 10)	4 (40.0)	0	4 (40.0)
<i>P.aeruginosa</i> (n = 10)	7 (70.0)	0	7 (70.0)
<b>Total</b>	<b>16 (53.3)</b>	<b>2 (6.7)</b>	<b>14 (46.7)</b>

#### **4.1.5.4 Post-curing antimicrobial resistance profile of representative isolates.**

The findings on the post-curing detection of ES $\beta$ L are summarized on table 4.1.13. As shown, out of 30 ES $\beta$ L positive isolates examined, *Escherichia coli* 4(40%), *Klebsiella* species 3 (30%) and *Pseudomonas aeruginosa* 4(40%) was found to harbour ES $\beta$ L genes after plasmid curing.

**Table 4.1.13 Post-curing antimicrobial resistance profile of representative isolates**

<b>Isolates</b>	<b>Number Examined</b>	<b>Number producing ESBL (%)</b>
<i>E.coli</i>	10	4(40)
<i>Klebsiella spp</i>	10	3(30)
<i>P.aeruginosa</i>	10	4(40)
<b>Total</b>	30	11(36.7)

#### **4.1.6. Antibacterial Effects of Selected Medicinal Plants on MAR Bacteria**

##### **4.1.6.1 Phytochemical Analysis of Medicinal Plants**

Phytochemical composition of the selected medicinal plants: leaves of *Ocimum gratissimum*, *Vernonia amygdalina* and seeds of *Allium sativum*, *Garcinia kola* and *Xylopia aethiopica*. The leaves of *Ocimum gratissimum*, *Vernonia amygdalina* and seeds of *Allium sativum*, *Garcinia kola* and *Xylopia aethiopica* contain Saponins, Tannins, Flavonoids, Alkaloids and HCN (See table 4.1.14 below).

**Table 4.1.14: Active Principles in the Selected Medicinal Plants**

Sample	Tannins	Saponins	Flavonoids	Alkaloids	HCN
<i>Ocimum gratissimum</i>	++	+	++	++	+++
<i>Garcinia kola</i>	+	++	+	+++	+
<i>Vernonia amygdalina</i>	+	+	++	+	+
<i>Allium sativum</i>	+	+	+++	+	+
<i>Xylopi aethiopica</i>	+	+	+	++	+

**Key:** + = Slightly present  
 ++ = Present  
 +++ = Deeply present  
 - = Absent

#### 4.1.6.2 Quantitative phytochemical analysis

The results of the quantitative phytochemical analysis of five medicinal plants namely: *Ocimum gratissimum*, *Garcinia kola*, *Vernonia amygdalina*, *Allium sativum* and *Xylopi aethiopica* are present on table 4.1.15 below. *Ocimum gratissimum* contains Tannins  $0.752 \pm 0.002$ , Saponins  $0.293 \pm 0.023$ , Flavonoids  $0.373 \pm 0.023$ , Alkaloids  $0.307 \pm 0.023$  and HCN  $1.577 \pm 0.029$ .

*Garcinia kola* showed Tannins  $0.337 \pm 0.001$ , Saponins  $1.510 \pm 0.967$ , Flavonoids  $2.00 \pm 0.052$ , Alkaloid  $1.950 \pm 0.098$  and HCN  $0.690 \pm 0.030$ . *Vernonia amygdalina* revealed Tannins  $0.360 \pm 0.005$ , Saponins  $2.330 \pm 0.110$ , Flavonoid  $9.140 \pm 0.051$ , Alkaloids  $0.710 \pm 0.030$  and HCN  $2.140 \pm 0.236$ . *Allium sativum* exhibited Tannins  $0.310 \pm 0.028$ , Saponins  $0.290 \pm 0.011$ , Flavonoids  $6.820 \pm 0.103$ , Alkaloids  $0.410 \pm 0.230$  and HCN  $0.510 \pm 0.023$ . *Xylopi aethiopica* recorded Tannins  $0.780 \pm 0.002$ , Saponins  $0.440 \pm 0.020$ , Flavonoid No Result, Alkaloids  $0.193 \pm 0.230$  and HCN  $0.370 \pm 0.023$ .

**Table 4.1.15: Quantitative phytochemical Composition**

Sample	Tannins	Saponins	Flavonoids	Alkaloids	HCN
<i>Ocimum gratissimum</i>	0.752±0.002	0.293±0.023	0.373±0.023	0.307±0.23	1.577±0.029
<i>Garcinia kola</i>	0.337±0.001	1.510±0.967	2.000±0.052	1.950±0.098	0.690±0.030
<i>Vernonia amygdalina</i>	0.360±0.005	2.330±0.110	9.140±0.051	0.710±0.030	2.140±0.236
<i>Allium sativum</i>	0.310±0.028	0.290±0.011	6.820±0.103	0.410±0.230	0.510±0.023
<i>Xylopia aethiopica</i>	0.780±0.002	0.440±0.020	No Result	0.193±0.230	0.370±0.023

The results are percentage mean ± standard deviation (SD)

#### 4.1.6.3 Antibacterial Effects of Selected Medicinal Plants on ES $\beta$ L-Bacteria

The antibacterial effects of the selected medicinal plants (*Ocimum gratissimum*, *Garcinia kola*, *Vernonia amygdalina*, *Allium sativum* and *Xylopiya aethiopica*) on test bacterial isolate (*Escherichia coli*, *Klebsiella species* and *Pseudomonas aeruginosa*) showed that *Ocimum gratissimum*, inhibited growth of 10 (100%) out of 10 *Escherichia coli*, 10 (100%) *Klebsiella species* and 9 (90.0%) *Pseudomonas aeruginosa* with mean zones of growth inhibition of 19.0mm, 18.0mm and 16.0mm respectively. It also inhibited growth of 7 (70.0%) out of 10 ES $\beta$ L *Escherichia coli*, 6 (60.0%) ES $\beta$ L *Klebsiella species* and 5 (50.0%) ES $\beta$ L *Pseudomonas aeruginosa* with mean zones of growth inhibition 12.0mm, 11.0mm and 13.0mm respectively (See table 4.1.16 below).

*Garcinia kola*, inhibited growth of 5 (50.0%) out of 10 *Escherichia coli*, 3 (30.0%) *Klebsiella species* and 4 (40.0%) *Pseudomonas aeruginosa* with mean zones of growth inhibition 12.0mm, 13.0mm and 11.0mm respectively. Similarly, it inhibited growth of 2 (20.0%) out of 10 ES $\beta$ L *Escherichia coli*, 1 (10.0%) ES $\beta$ L *Klebsiella species* and 2 (20.0%) ES $\beta$ L *Pseudomonas aeruginosa* with mean zones of growth inhibition 8.0mm, 6.0mm and 10.0mm respectively (See table 4.1.17 below).

**Table 4.1.16: Antibacterial Effects of *Ocimum gratissimum*, on test bacterial isolates**

<b>Isolates</b>	<b>Suceptibility rate (%)</b>	<b>Mean zone of growth inhibition (mm)</b>
<i>Escherichia coli</i> (n = 10)	10 (100.0)	19.0
ES $\beta$ L <i>Escherichia coli</i> (n = 10)	7 (70.0)	12.0
<i>Klebsiella</i> species (n = 10)	10 (100.0)	18.0
ES $\beta$ L <i>Klebsiella</i> species (n = 10)	6 (60.0)	11.0
<i>Pseudomonas aeruginosa</i> (n = 10)	9 (90.0)	16.0
ES $\beta$ L <i>Pseudomonas aeruginosa</i> (n = 10)	5 (50.0)	13.0

**Table 4.1.17: Antibacterial Effects of *Garcinia kola*, on test bacterial isolates**

<b>Isolates</b>	<b>Suceptibility rate (%)</b>	<b>Mean zone of growth inhibition (mm)</b>
<i>Escherichia coli</i> (n = 10)	5 (50.0)	12.0
ES $\beta$ L <i>Escherichia coli</i> (n = 10)	2 (20.0)	8.0
<i>Klebsiella</i> species (n = 10)	3 (30.0)	13.0
ES $\beta$ L <i>Klebsiella</i> species (n = 10)	1 (10.0)	6.0
<i>Pseudomonas aeruginosa</i> (n = 10)	4 (60.0)	11.0
ES $\beta$ L <i>Pseudomonas aeruginosa</i> (n = 10)	2 (20.0)	10.0

*Vernonia amygdalina*, inhibited growth of 6 (60.0%) out of 10 *Escherichia coli* with mean zone of growth inhibition 12.0mm. It also inhibited growth of 6 (60.0%) *Klebsiella* species and 5 (50.0%) *Pseudomonas aeruginosa* with mean zones of growth inhibition 14.0mm respectively. It inhibited growth of 2 (20.0%) out of 10 ES $\beta$ L *Klebsiella* species and 2 (20.0%) out of 10 ES $\beta$ L *Pseudomonas aeruginosa* with mean zones of growth inhibition 9.0mm and 10.0mm respectively (See table 4.1.18 below).

*Allium sativum* inhibited growth of 10 (100.0%) out of 10 *Escherichia coli* with mean zone of growth inhibition 14.0mm. It also inhibited growth of 7 (70.0%) *Klebsiella* species and 8 (80.0%) *Pseudomonas aeruginosa* with mean zones of growth inhibition 15.0mm and 18.0mm respectively. It inhibited growth of 6 (60.0%) out of 10 ES $\beta$ L *Escherichia coli*, 3 (30.0%) out of 10 ES $\beta$ L *Klebsiella* species and 2 (20.0%) out of 10 ES $\beta$ L *Pseudomonas aeruginosa* with mean zones of growth inhibition 10.0mm, 10.0mm and 14.0mm respectively (See table 4.1.19 below).

**Table 4.1.18: Antibacterial Effects of *Vernonia amygdalina*, on test bacterial isolates**

<b>Isolates</b>	<b>Suceptibility rate (%)</b>	<b>Mean zone of growth inhibition (mm)</b>
<i>Escherichia coli</i> (n = 10)	6 (60.0)	12.0
ES $\beta$ L <i>Escherichia coli</i> (n = 10)	0 (0.0)	0.0
<i>Klebsiella</i> species (n = 10)	6 (60.0)	14.0
ES $\beta$ L <i>Klebsiella</i> species (n = 10)	2 (20.0)	9.0
<i>Pseudomonas aeruginosa</i> (n = 10)	5 (50.0)	14.0
ES $\beta$ L <i>Pseudomonas aeruginosa</i> (n = 10)	2 (20.0)	10.0

**Table 4.1.19: Antibacterial Effects of *Allium sativum* on test bacterial isolates**

<b>Isolates</b>	<b>Suceptibility rate (%)</b>	<b>Mean zone of growth inhibition (mm)</b>
<i>Escherichia coli</i> (n = 10)	10 (100.0)	14.0
ES $\beta$ L <i>Escherichia coli</i> (n = 10)	6 (60.0)	10.0
<i>Klebsiella</i> species (n = 10)	7 (70.0)	15.0
ES $\beta$ L <i>Klebsiella</i> species (n = 10)	3 (30.0)	8.0
<i>Pseudomonas aeruginosa</i> (n = 10)	8 (80.0)	18.0
ES $\beta$ L <i>Pseudomonas aeruginosa</i> (n = 10)	2 (20.0)	14.0

*Xylopiya aethiopica* inhibited growth of 10 (100.0%) out of 10 *Escherichia coli* with mean zone of growth inhibition 16.0mm. It also inhibited growth of 9 (90.0%) *Klebsiella* species and 6 (60.0%) *Pseudomonas aeruginosa* with mean zones of growth inhibition 18.0mm and 14.0mm respectively. It inhibited growth of 8 (80.0%) out of 10 ES $\beta$ L *Escherichia coli*, 3 (30.0%) out of 10 ES $\beta$ L *Klebsiella* species and 2 (20.0%) out of 10 ES $\beta$ L *Pseudomonas aeruginosa* with mean zones of growth inhibition 10.0mm, 11.0mm and 12.0mm respectively (See table 4.1.20 below).

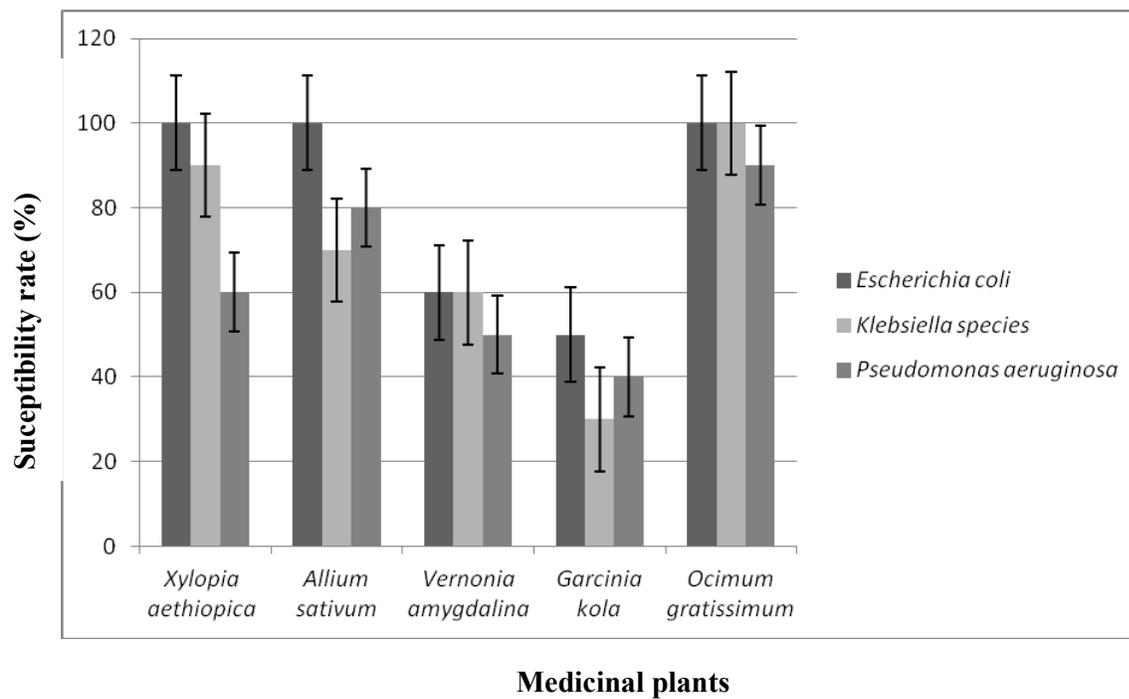
Statistical analysis of the data using chi square showed significant difference ( $p < 0.05$ ) in the susceptibility pattern between bacteria and ES $\beta$ L bacterial isolates towards crude extracts of the selected medicinal plants, the number of bacterial isolates susceptible to the medicinal plant extracts was remarkably higher than the number of ES $\beta$ L isolates susceptible to the medicinal plant extracts.

**Table 4.1.20: Antibacterial Effects of *Xylopi*a *aethi*opica**

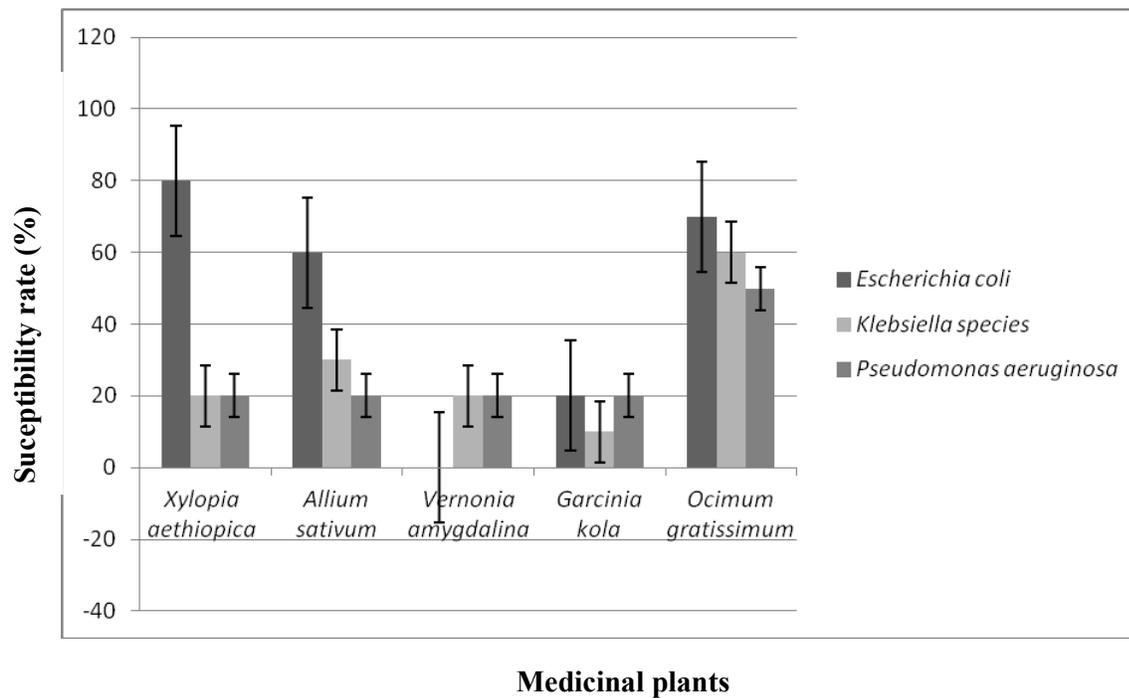
Isolates	Suceptibility rate (%)	Mean zone of growth inhibition (mm)
<i>Escherichia coli</i> (n = 10)	10 (100.0)	16.0
ES $\beta$ L <i>Escherichia coli</i> (n = 10)	8 (80.0)	10.0
<i>Klebsiella</i> species (n = 10)	9 (90.0)	18.0
ES $\beta$ L <i>Klebsiella</i> species (n = 10)	3 (30.0)	11.0
<i>Pseudomonas aeruginosa</i> (n = 10)	6 (60.0)	14.0
ES $\beta$ L <i>Pseudomonas aeruginosa</i> (n = 10)	2 (20.0)	12.0

#### 4.1.6.4 Comparative analysis of the susceptibility of test isolates to plant extracts

*Ocimum gratissimum* and *Xylopia aethiopica* exhibited higher growth inhibitory effects on the bacterial isolates than *Allium sativum*, *Vernonia amygdalina* and *Garcinia kola*. Comparatively, all the selected medicinal plants exhibited higher growth inhibitory effects on non-ES $\beta$ L bacterial isolates than their respective ES $\beta$ L isolates. *Vernonia amygdalina* exhibited no growth inhibitory effect on ES $\beta$ L *Escherichia coli*. Statistical analysis of the data, using chi square, showed significant difference ( $p < 0.05$ ) in the growth inhibitory effects of the medicinal plants between the non- ES $\beta$ L bacterial isolates and their respective ES $\beta$ L isolates. The highest growth inhibitory effect (100%) was exhibited on *Escherichia coli* and *Klebsiella* species by, *Ocimum gratissimum* and on *Escherichia coli* by *Xylopia aethiopica*. Figure 4.1.11 and 4.1.12 below show comparative analysis of the susceptibility pattern between the different test isolates.



**Figure 4.1.11 Comparative analysis of the susceptibility pattern between the different test isolates**



**Figure 4.1.12: Comparative analysis of the susceptibility pattern between different ESBL isolates**

#### 4.1.6.5 Minimum inhibitory concentration (MIC)

*Xylopiya aethiopica* exhibited inhibitory growth on 4 out of 5 *Escherichia coli* and 4 out of 5 ES $\beta$ L-*Escherichia coli* with minimal inhibitory concentrations of 0.4 mg/ml and 0.8 mg/ml respectively. The MIC on *Klebsiella* species and ES $\beta$ L-*Klebsiella* species are 0.6 mg/ml and 0.8 mg/ml respectively while the MIC on *Pseudomonas aeruginosa* and ES $\beta$ L-*Pseudomonas aeruginosa* are 0.4 mg/ml and 0.8 mg/ml respectively. See table 4.1.22 below.

**Table 4.1.21: Minimal inhibitory concentration of *Xylopi*a *aethi*o*p*i*c*a extracts on bacterial isolates**

Isolates	Number Inhibited	Growth inhibitory effects					
		1 mg/ml	0.8mg/ml	0.6mg/ml	0.4mg/ml	0.2mg/ml	0.1mg/ml
<i>Escherichia coli</i> (n = 5)	4	N	N	N	N	G	G
ES $\beta$ L - <i>Escherichia coli</i> (n = 5)	4	N	N	G	G	G	G
<i>Klebsiella</i> species (n = 5)	5	N	N	N	G	G	G
ES $\beta$ L - <i>Klebsiella</i> species (n = 5)	3	N	N	G	G	G	G
<i>Pseudomonas aeruginosa</i> (n = 5)	4	N	N	N	N	G	G
ES $\beta$ L - <i>Pseudomonas aeruginosa</i> (n = 5)	3	N	N	G	G	G	G

KEY: G = Bacterial Growth recorded      N = No Bacterial Growth recorded       MIC

*Allium sativum* exhibited growth inhibitory effect on 5 out of 5 *Escherichia coli* and 4 out of 5 ES $\beta$ L-*Escherichia coli* with minimal inhibitory concentrations of 0.6 mg/ml and 0.8 mg/ml respectively. The MIC on *Klebsiella* species and ES $\beta$ L-*Klebsiella* species are 0.8 mg/ml and 0.8 mg/ml respectively while the MIC on *Pseudomonas aeruginosa* and ES $\beta$ L-*Pseudomonas aeruginosa* are 0.6 mg/ml and 0.8 mg/ml respectively. See table 4.1.23 below.

**Table 4.1.22: Minimal inhibitory concentration of *Allium sativum* extracts on bacterial isolates**

Isolates	Number Inhibited	Growth inhibitory effects					
		1 mg/ml	0.8mg/ml	0.6mg/ml	0.4mg/ml	0.2mg/ml	0.1mg/ml
<i>Escherichia coli</i> (n = 5)	5	N	N	N	G	G	G
ES $\beta$ L - <i>Escherichia coli</i> (n = 5)	4	N	N	G	G	G	G
<i>Klebsiella</i> species (n = 5)	4	N	N	G	G	G	G
ES $\beta$ L - <i>Klebsiella</i> species (n = 5)	3	N	N	G	G	G	G
<i>Pseudomonas aeruginosa</i> (n = 5)	4	N	N	N	G	G	G
ES $\beta$ L - <i>Pseudomonas aeruginosa</i> (n = 5)	2	N	N	G	G	G	G

**KEY:** G = Bacterial Growth recorded      N = No Bacterial Growth recorded       = MIC

*Garcinia kola* exhibited growth inhibitory effect on 5 out of 5 *Escherichia coli* and 3 out of 5 ES $\beta$ L-*Escherichia coli* with minimal inhibitory concentrations of 0.4 mg/ml and 0.6 mg/ml respectively. The MIC on *Klebsiella* species and ES $\beta$ L-*Klebsiella* species are 0.6 mg/ml and 0.8 mg/ml respectively while the MIC on *Pseudomonas aeruginosa* and ES $\beta$ L-*Pseudomonas aeruginosa* are 0.6 mg/ml and 0.8 mg/ml respectively. See table 4.1.24 below.

**Table 4.1.23: Minimal inhibitory concentration of *Garcinia kola* extracts on bacterial isolates**

Isolates	Number Inhibited	Growth inhibitory effects					
		1 mg/ml	0.8mg/ml	0.6mg/ml	0.4mg/ml	0.2mg/ml	0.1mg/ml
<i>Escherichia coli</i> (n = 5)	5	N	N	N	N	G	G
ES $\beta$ L - <i>Escherichia coli</i> (n = 5)	4	N	N	N	G	G	G
<i>Klebsiella</i> species (n = 5)	3	N	N	N	G	G	G
ES $\beta$ L - <i>Klebsiella</i> species (n = 5)	5	N	N	G	G	G	G
<i>Pseudomonas aeruginosa</i> (n = 5)	4	N	N	N	G	G	G
ES $\beta$ L - <i>Pseudomonas aeruginosa</i> (n = 5)	3	N	N	G	G	G	G

KEY: G= Bacterial Growth recorded      N = No Bacterial Growth recorded       MIC

*Vernonia amygdalina* exhibited growth inhibitory effect on 3 out of 5 *Escherichia coli* and none out of 5 ES $\beta$ L-*Escherichia coli* with minimal inhibitory concentrations of 0.4 mg/ml. It inhibited growth of 5 out of 5 *Klebsiella* species and 4 out of 5 ES $\beta$ L-*Klebsiella* species with MIC of 0.6 mg/ml and 0.8 mg/ml respectively while the MIC on *Pseudomonas aeruginosa* and ES $\beta$ L-*Pseudomonas aeruginosa* are 0.6 mg/ml and 0.8 mg/ml respectively. See table 4.1.25 below.

**Table 4.1.24: Minimal inhibitory concentration of *Vernonia amygdalina* extracts on bacterial isolates**

Isolates	Number Inhibited	Growth inhibitory effects					
		1 mg/ml	0.8mg/ml	0.6mg/ml	0.4mg/ml	0.2mg/ml	0.1mg/ml
<i>Escherichia coli</i> (n = 5)	5	N	N	N	N	G	G
ES $\beta$ L - <i>Escherichia coli</i> (n = 5)	0	G	G	G	G	G	G
<i>Klebsiella</i> species (n = 5)	5	N	N	N	G	G	G
ES $\beta$ L - <i>Klebsiella</i> species (n = 5)	4	N	N	G	G	G	G
<i>Pseudomonas aeruginosa</i> (n = 5)	4	N	N	N	G	G	G
ES $\beta$ L - <i>Pseudomonas aeruginosa</i> (n = 5)	3	N	N	G	G	G	G

KEY: G = Bacterial Growth recorded      N = No Bacterial Growth recorded       = MIC

*Ocimum gratissimum* exhibited growth inhibitory effect on 5 out of 5 *Escherichia coli* and 4 out of 5 ES $\beta$ L-*Escherichia coli* with minimal inhibitory concentrations of 0.2 mg/ml and 0.4 mg/ml respectively. It inhibited growth of 4 out of 5 *Klebsiella* species and 3 out of 5 ES $\beta$ L-*Klebsiella* species with MIC of 0.6 mg/ml and 0.8 mg/ml respectively while it inhibited growth of 5 out of 5 *Pseudomonas aeruginosa* and 3 out of 5 ES $\beta$ L-*Pseudomonas aeruginosa* with MIC of 0.6 mg/ml and 0.8 mg/ml respectively. See table 4.1.26 below.

**Table 4.1.25: Minimal inhibitory concentration of *Ocimum gratissimum* extracts on bacterial isolates**

Isolates	Number Inhibited	Growth inhibitory effects					
		1 mg/ml	0.8mg/ml	0.6mg/ml	0.4mg/ml	0.2mg/ml	0.1mg/ml
<i>Escherichia coli</i> (n = 5)	5	N	N	N	N	N	G
ES $\beta$ L - <i>Escherichia coli</i> (n = 5)	4	N	N	N	N	G	G
<i>Klebsiella</i> species (n = 5)	4	N	N	N	G	G	G
ES $\beta$ L - <i>Klebsiella</i> species (n = 5)	3	N	N	G	G	G	G
<i>Pseudomonas aeruginosa</i> (n = 5)	5	N	N	N	G	G	G
ES $\beta$ L - <i>Pseudomonas aeruginosa</i> (n = 5)	3	N	N	G	G	G	G

**KEY:** G = Bacterial Growth recorded      N = No Bacterial Growth recorded       = MIC

## **4.2 Discussion of Findings**

Multiple antibiotic resistant bacterial infection is a major challenge to healthcare in Nigeria and Imo State in particular. A number of factors are responsible for the development of antibiotic resistance by bacterial species. According to Paterson and Bonomo (2005), Extended-Spectrum Beta-lactamases (ES $\beta$ L) are capable of conferring bacterial resistance to penicillin and third generation cephalosporins, and aztreonam, but not the cephamycins or carbapenems. The findings of the present study revealed a high prevalence of multiple antibiotic resistant (MAR) and ES $\beta$ L-producing bacteria from private and public health facilities in Imo State.

### **4.2.1 Knowledge, attitude and practices of healthcare workers towards beta-lactamase-producing bacteria**

The findings of this study revealed that many health workers in Imo State, South-Eastern Nigeria involved in the diagnosis and/or treatment of bacteria infected patients are aware of bacteria including ES $\beta$ L producers. Sixty eight percent ( 68%) of the doctors and 20% of the medical laboratory scientists (MLS) attributed the cause of antibiotic resistance in the country to fake drugs, while 41% and 87% of the doctors and MLS respectively, attributed this problem to growing cases of antibiotic resistance to  $\beta$ -lactamase production by bacteria pathogens. Thirty percent of doctors and 22% of MLS believed that this is caused by abuse of antibiotics. This finding is supported by the work of Smith *et al.* (2000), who reported that antibiotic use is a risk factor for colonization with ES $\beta$ L-producing organisms. Similarly, Rice *et al.* (1990), Pessoa-Silva *et al.* (2003) and Weldhagen and Prinsloo, (2004) reported that the use of third-generation cephalosporins is a predisposing factor in some cases of ES $\beta$ L-production.

#### 4.2.2 Facilities for ES $\beta$ L detection

The present study showed that many hospitals and diagnostic centres in Imo State have facilities to detect ES $\beta$ L producing bacteria. However, only few health workers in the state show interest on detection of ES $\beta$ L-producing bacteria in their hospitals. This result supports the work of Thomson, (2001) and Catagay *et al.* (2003) which revealed that many clinical laboratories in Istanbul are not fully aware of the importance of ES $\beta$ Ls. Consequently, this poses a serious challenge to clinical laboratories, so much so that clinically relevant ES $\beta$ L-mediated resistance is not always detected in routine susceptibility tests. Iroha *et al.* (2009) supports this in his work where he said, unfortunately, information on infections caused by ES $\beta$ L producing organism (*E.coli*) are limited particularly in our environment and many clinicians are yet to fully appreciate the immense significance of detecting ES $\beta$ Ls. These can lead to the out break of multiple drug resistant gram negative pathogens that could bring about expensive control effects and therapeutic failures in patients who receive inappropriate antibiotics.

Also, the present work revealed that very few health workers in the State, 28.9% and 39.3% of doctors and MLS respectively are involved in research and dissemination of research findings in their hospitals. Out of these few who are involved in research and dissemination of research findings, only 23.7% of doctors and 28.6% of MLS have published data or evidence of their research findings. Furthermore, amongst the very few who have published data on their research, majority said such publications are accessible or available locally or on request (See figure 4.1.7). Many hospitals and diagnostic centres in Imo State are said to be doing something towards prevention, control and management of ES $\beta$ L-producing bacterial infections, yet it appears that they do not carry the staff along or the health workers in their hospitals are nonchalant about it. Furthermore, the present study showed that majority of the health workers are aware that their hospitals are engaged in continuing education, training

and enlightenment of their staff as a means towards prevention, control and management of ES $\beta$ L-producing bacterial infections. Consequently, in the past 12 months, 60.5% of doctors and 50% of MLS have read/attended training about ES $\beta$ L-producing bacteria.

#### **4.2.3 MAR and ES $\beta$ L-producing bacteria not given adequate attention**

Majority of the health practitioners who participated in this study have encountered cases of antibiotic resistant bacteria infected patients in their hospitals and diagnostic laboratories frequently. Doctors (52.6%) and MLS (28.6%) said their health facilities are presently doing something towards prevention, control and management of MAR and ES $\beta$ L-producing bacterial infections. However, 39.5% and 50% of doctors and MLS respectively in the study have not read or studied about ES $\beta$ L-producing bacteria in the past 12 months from the time of this study, yet they are in active practice and have been encountering MAR and ES $\beta$ L producing bacterial infections.

Few hospitals adopted proper disposal of hospital wastes as a measure towards prevention, control and management of ES $\beta$ L-producing bacteria. 26.3% and 7.1% of doctors and MLS respectively said their hospitals acquired materials for detection of ES $\beta$ L-producing bacteria and embarked on procurement of new antibacterial drugs to deal with ES $\beta$ L-producing bacteria. If this is implemented, it will help to solve the problem envisaged by previous researchers, Tenover *et al.* (1999) and Steward *et al.* (2000); who believed that the inability of diagnostic laboratories to detect ES $\beta$ L-producing bacteria failed the management of patients. A survey in Connecticut found that 21% of laboratories failed to detect ES $\beta$ L-producing isolates (Tenover *et al.*, 1999). A proficiency testing project for clinical laboratories participating in the National Nosocomial Infections Surveillance System indicated that as many as 58% of laboratories failed to detect and report ES $\beta$ L isolates

correctly (Steward *et al.*, 2000). These data suggest that improvements in the ability of clinical laboratories to detect ESβLs are needed.

#### **4.2.4 Treatment and management of MAR-ESβL-producing bacterial infections**

The attitude of health workers in Imo State towards management of MAR and ESβL-producing bacterial infections according to the present study varied widely. While many of the practitioners believe that MAR-bacterial infections can be treated, a few believed that MAR-bacterial infections cannot be treated. Specifically, many stated that they treat MAR-bacterial infections using combined antibiotic therapy which agrees with Paterson *et al.* (2000) and some adopted prolonged antibiotic therapy, changing drugs after some time. This is similar with the report of Bhattacharya, (2006) who says that uncomplicated infections like non-bacteremic urinary tract infections can be managed with a variety of antibiotics, depending on their susceptibility.

About (86.8%) of doctors and some MLS (35.7%) claimed they have managed and treated multiple antibiotic resistant bacteria infections by changing from one antibiotic to another, only 7.9% of doctors and 53.6% of MLS refer the patients to other specialist doctors. Similarly, 1.3% and 7.1% of doctors and MLS respectively, carried out further studies on the patients. It is worthy of note that a few health workers in the State; 1.3% and 3.6% of doctors and MLS respectively believed that spiritual approach such as prayers could offer treatment to multiple antibiotic resistant bacteria infected patients, while an overwhelming majority, 98.7% and 71.4% of doctors and MLS respectively insisted that conventional antibiotics are the only remedy. 25% of MLS believed that herbal medicines could provide effective treatment.

#### 4.2.5 Test bacterial isolates and antibiotic resistance

The knowledge of local antimicrobial resistance trends among clinical isolates is important in guiding clinician's to prescribe appropriate antibiotics (Blomberg *et al.*, 2005). In the present study, the prevalence of multiple antibiotic resistant (MAR) bacteria was higher among *E. coli* (50.3%) than *Pseudomonas aeruginosa* (43.3%) and *Klebsiella* Species (36.6%). This finding is in line with the work of Nwosu *et al.* (2014), who reported 54.5% and 45.5% prevalence rate in *E. coli* and *Klebsiella* species respectively in Aba, Abia State. Similarly, Wong-Beringer, (2001) reported a high percentage of ES $\beta$ L (83%) where *K. pneumoniae* and *E. coli* were most frequently associated with ES $\beta$ L production in the United States. The high rate of MAR observed in this study is worrisome and would undoubtedly be responsible for cases of treatment failures in the state.

In this study, the overall prevalence of ES $\beta$ L producing isolates from clinical sample was 64.9%. This finding is supported by the previous study of Iroha *et al.* (2008) who reported 52.4% in South-Eastern Nigeria from 2003-2007. Also Okesola and Adeniji (2010) reported 58.6% prevalence in Enugu, Nigeria.

Global reports show that in Sudan Ahmed *et al.* (2013) recorded 59.6% of ES $\beta$ L producing bacteria while in India Rao *et al.* (2014) recorded 57.5% prevalence. On the other hand, the result of the present study is however higher than the 7.5% of ES $\beta$ L producers recorded in Ogun State by Olowe and Aboderin (2010) and 16% ES $\beta$ L producers reported in South-Eastern Nigeria (Akujobi *et al.*, 2010). The variation in ES $\beta$ L prevalence rates reported between geographical areas, and countries may be attributed to the complex epidemiology of ES $\beta$ L, specific type of bacteria involved and methods used for ES $\beta$ L detection among other factors. The presence of ES $\beta$ L enzymes in these organisms is because expanded spectrum

beta lactamase are commonly included in the empirical antibiotic regimens for treatment of gram negative organisms.

In this study, *E. coli* was identified as the major ES $\beta$ L producer (67.6%) followed by *Klebsiella* spp. (64.7%) and *P. aeruginosa* (57.7%). This is similar to the work carried out in Uyo-Nigeria by Azekhueme *et al.* (2015) where *E. coli* was identified as the major ES $\beta$ L producer (52.5%) followed by *K. pneumoniae* 47.5%.

Similar result was obtained by Nwosu *et al.* (2014) in Aba, Abia State of Nigeria where ES $\beta$ L producers, made up of 50.8%. *E. coli* and 50.4% *Klebsiella* spp. were isolated. The high prevalence of ES $\beta$ L producing bacteria reported in this study may be indication of the uncontrolled cephalosporin's used and thus could culminate to serious treatment challenges in the study area.

#### **4.2.6 Molecular studies on bacterial isolates**

The most common gene responsible for ES $\beta$ L production in this study was SHV, followed by TEM and CTX-M. Majority of the ES $\beta$ L-positive *Escherichia coli* and *Klebsiella* species had SHV genes, but amongst ES $\beta$ L-positive *Pseudomonas aeruginosa* TEM genes, SHV, and CTX-M gene appears to be evenly distributed. Using specific primers for TEM, SHV, and CTX-M 23(76.7%) of the 30 ES $\beta$ L positive isolates could be type for one or more genes. The negative amplification in the remaining isolates may be due to the presence of other ES $\beta$ L gene which was not explored further. In the present study, 36.7%, 63.3% and 23.3% of 30 ES $\beta$ L isolates harboured TEM, SHV and CTX-M genes respectively. This finding is consistent with the report of Babini and Livermore, (2000) and Jemima and Vergese, (2008) in which TEM and SHV were molecularly found in *E.coli*.

Similarly, 50%, 14.89% and 11.70% ES $\beta$ L rates for TEM, SHV and CTX-M type beta-lactamases were recorded in a study carried out in a Turkish hospital (Elif *et al.*, 2010).

However, the prevalence rate of 11(36.7%) TEM type ES $\beta$ L producing recorded in this study is lower than the 50% prevalence rate recorded in a Turkish hospital (Elif *et al.*, 2010). Also, the prevalence rate of 7(23.3%) CTX-M type ES $\beta$ L reported in the study is lower than the 19(83%) recorded in another study conducted in India (Padmini, 2008). This decrease could be differences in environmental factors.

Most isolates in this study were positive for plasmids profiling. Specifically 70% *Pseudomonas aeruginosa*, 40% *Klebsiella* species and 30% *Escherichia coli* were positive for plasmid with single band while 20% *Escherichia coli* were positive for plasmid with multiple bands. Elsewhere Messi *et al.* (2005) detected plasmids in 46% of *Pseudomonas aeruginosa* isolates. In another study, Stanisich (1988) reported that plasmids do not possess any of the set of genes needed by the cell for basic growth and multiplication; rather they carry genes that may be useful periodically to enable the cell to exploit particular environmental situations. Thus, plasmids carry a considerable variety of genes, including those that confer antibiotic resistance and resistance to a number of toxic heavy metals, such as mercury, cadmium and silver, as well as those that provide enzymes that expand the nutritional ability of the cell, virulence determinants that permit invasion of and survival in animal systems and functions that enhance the capacity to repair DNA damage. These factors suggest reasons why multiple antibiotic resistant bacteria are rapidly increasing in the environment and patients in Imo State. Previous work by Iroha *et al.* (2009) reported increasing prevalence of antibiotic resistant bacteria infections in Nigeria. The present study has shown high prevalence of antibiotic resistant bacteria infections amongst patients in Imo State, South Eastern Nigeria. The antibiotic resistance which is a major health challenge in the State is mostly caused by the plasmid carrying bacteria especially *Escherichia coli* ,

*Pseudomonas aeruginosa* and *Klebsiella* species as indicative in the present work. Consequently, health care providers and health policy makers in the State should take step up actions to combat these infections.

Curing of the isolates using acridine orange was carried out in the present study and the results showed that 4(40%) *Escherichia coli*, 3(30%) *Klebsiella* species and 4(40%) *Pseudomonas aeruginosa* carry ES $\beta$ L-genes as against 8(80%) *Escherichia coli*, 6(60%) *Klebsiella* species and 9(90%) *Pseudomonas aeruginosa* which harbour ES $\beta$ L –genes prior to curing. This finding shows that half of the gene-markers that confer resistance are carried by plasmids while the other half is carried by the chromosomes. Thus the plasmids and the beta-lactamase genes are probably responsible for antibiotic resistance observed in this study. Chikwendu *et al.* (2011) supported this observation in their report which stated that half of these gene markers are borne on the plasmids while the other half are borne on the chromosomes.

#### **4.2.7 Antibacterial effects of selected medicinal plants on ES $\beta$ L bacteria**

Analysis of the selected medicinal plants showed that *Ocimum gratissimum*, *Vernonia amygdalina*, *Allium sativum*, *Xylopia aethiopica* and *Garcinia kola* contain saponins, tannins, flavonoids, alkaloids and HCN. These findings corroborates the reports of previous workers (Akerele, 1990, Sim, 1992, Obiajuru, 1995), who identified alkaloids, flavonoids, tannins, glycosides and saponins in *Ocimum gratissimum*, *Adenia cissampeloides*, *Garcinia kola* and *Vernonia amygdalina*. The presence of these active principles in medicinal plants have been reported to exhibit antimicrobial effects. Nweze *et al.* (2004) reported that the bioactive principles of *Allium sativum* are believed to be responsible for the observed antimicrobial effect of the plant extract. Iwu (1993) reported that the bitter taste of *Vernonia amygdalina* is due to antinutritional factors such as alkaloids, saponins, tannins and glycosides. According to Iwu (1993), this stimulates the digestive system as well as reduces fever. In different

countries, these plants are used for various medicinal purposes and as food condiments. They are widely consumed amongst the Igbos of south eastern Nigeria. *Ocimum gratissimum* and *Xylopia aethiopica* are used to season and add flavor to different types of food especially for nursing mothers. They are believed to prevent intestinal infections. The findings of this study show *Ocimum gratissimum*, *Allium sativum*, *Xylopia aethiopica* exhibited reasonable antibacterial effects on ES $\beta$ L-positive bacterial isolates. The result of the present work appears to justify the local use of these plants in the treatment of some ailments.

*Vernonia amygdalina* exhibited growth inhibitory effects on 20% of ES $\beta$ L positive *Klebsiella* species and 10% ES $\beta$ L-positive *Pseudomonas aeruginosa*. However, it exhibited inhibitory effects on 60% of non- ES $\beta$ L-positive *Escherichia coli*, 60% of non- ES $\beta$ L positive *Klebsiella* species and 50% non- ES $\beta$ L-positive *Pseudomonas aeruginosa*. However, *Vernonia amygdalina* exhibited growth inhibitory effects on ES $\beta$ L positive *Escherichia coli*. The zones of growth inhibition exhibited by *Vernonia amygdalina* on ES $\beta$ L-positive bacteria ranged from 9mm to 10mm while on non- ES $\beta$ L-positive bacteria the zone of growth inhibition ranged from 12 -14mm. These zones of growth inhibition were slightly higher than that reported by Okigbo and Mmekka (2008) who observed mean zone of growth inhibition of 10-15mm on *Staphylococcus aureus* and *E. coli*. The difference in these two studies may be due to the difference in test organisms used and method of extraction. Whereas the present study used soxhlet extraction method, Okigbo and Mmekka (2008) used ethanol infusion technique. The soxhlet method probably extracted more active ingredients than ethanol infusion and therefore exhibited higher degree of growth inhibition.

Interestingly, the study showed that while *Vernonia amygdalina* had no visible activity for ES $\beta$ L-positive *Escherichia coli*, it inhibited 60% of non- ES $\beta$ L-positive *Escherichia coli*.

The reason for this is not very certain, but the influence of the resistant genes may be culpable.

Previous researchers Hamowia and Saffaf (1994) reported that *Vernonia amygdalina* contains complex active components that are pharmacologically useful. The roots and the leaves are used in ethno-medicine to treat fever, hiccups, kidney problems and stomach discomfort. The stem and root divested of the bark are used as chewing sticks in many West African countries like Cameroon, Ghana and Nigeria. Akujobi *et al.* (2006) evaluated the antibacterial properties and reported that it contains cardiac glycosides, saponins, tannins and alkaloids. According to Akujobi *et al.* (2006), the extracts of the leaves of *Vernonia amygdalina* are used among the traditional palm wine tapers in Eastern Nigeria to control the microbial load and foaming in palm wine. Palm wine is an alcoholic beverage produced from a variety of palms such as oil palm.

*Xylopiya aethiopica* seed exhibited growth inhibitory effects on 80% ES $\beta$ L - positive *E. coli*, 30% ES $\beta$ L- positive *klebsiella* species and 20% ES $\beta$ L-positive *Pseudomonas aeruginosa* as well as 100% non-ES $\beta$ L-positive *E. coli*, 90% non-ES $\beta$ L-positive *klebsiella* species and 60% non-ES $\beta$ L-positive, *Pseudomonas aeruginosa*. The zone of growth inhibition exhibited by *Xylopiya aethiopica* on ES $\beta$ L positive bacteria ranged from 10mm –12mm while that on non ES $\beta$ L positive bacteria ranged from 18-20mm. This is in line with the report of Ezeifeke *et al.* (2004) that the seed extracts of *Xylopiya acthiopica* has inhibitory effects on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli* with zone of growth inhibition ranging from 6.0 to 10.2mm.

*Ocimum gratissimum* inhibited the growth of 70% ES $\beta$ L -positive *E. coli*, 60% ES $\beta$ L – positive *Klebsiella spp.* and 50% ES $\beta$ L - positive *Pseudomonas aeruginosa* as well as 100%

non- ES $\beta$ L-positive *E. coli*, and *Klebsiella spp.* and 90% non-ES $\beta$ L- positive *Pseudomonas aeruginosa*. The inhibition zone diameter recorded ranged from 16 -19mm. This is consistent with the works of Mbajiuka *et al.* (2014), who reported that ethanol extract of *Ocimum gratissimum* exhibited growth inhibitory effects on *Staphylococcus aureus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *E. coli* with mean zones of growth inhibition ranging from 12mm to 15mm. The difference in the result in these studies may be due to difference in types of isolates used. Whereas Mbajiuka *et al.* (2014) worked on gram positive cocci (*S. aureus* and *S. faecalis*) and gram negative rods (*E.coli* and *Pseudomonas aeruginosa*), the present study was only on gram-negative rods (*E. coli*, *Klebsiella spp.* and *Pseudomonas aeruginosa*). Furthermore, Mbajiuka *et al.* (2014), used filter paper discs dipped in crude extract while the present study used measured volume and concentration of crude extract in standard well in agar. The filter paper discs used by Mbajiuka *et al.* (2014) may not have collected enough extract to exhibit reasonable antibacterial effects on the isolates. Previous researchers (Akerlele, 1990, Jedlickova, 1992 & Obiajuru, 1995) reported that *Ocimum gratissimum* has broad spectrum antibacterial effects.

Worthy of note also is the effectiveness of antimicrobial agent varies with the nature of organisms being treated. Also the presence of the active principles is influenced by several factors such as age of the plants, method of extraction and extracting solvent. It is possible that the leaves of *Ocimum gratissimum* contain high concentration of the antimicrobial compounds or different antimicrobial agents based on the values of minimum inhibitory concentration and the zone of inhibitions obtained.

*Allium sativum* extract exhibited growth inhibitory effects on ES $\beta$ L-positive bacteria with mean zones of growth inhibition ranging from 8mm on ES $\beta$ L-*Klebsiella spp.* to 14mm on ES $\beta$ L-*Pseudomonas aeruginosa*. On the non- ES $\beta$ L-positive bacteria, the zones of growth

inhibition ranged from 14mm on *E. coli* to 18mm on *Pseudomonas aeruginosa*. Other researchers, Akintobi *et al.* (2013) reported that *Allium sativum* extract inhibited growth of *Staphylococcus aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Proteus mirabilis*, with mean zones of growth inhibition ranging from 6mm to 19mm. The slight difference between the result of these researchers and the present study may be explained by the difference in test organisms, and methods of extraction of active principles of *Allium sativum*.

*Garcinia kola* exhibited growth inhibitory effects on the ES $\beta$ L-positive bacteria, *Garcinia kola* inhibited growth of 20% *E. coli* and *Pseudomonas aeruginosa* respectively as well as 10% ES $\beta$ L *Klebsiella spp*, However, it exhibited growth inhibitory effects on 60% non-ES $\beta$ L positive pseudomonas 50% non-ES $\beta$ L *E. coli* and 30% non-ES $\beta$ L - positive *Klebsiella spp*. with mean zones of growth inhibition ranging from 11mm on *Pseudomonas aeruginosa* to 13mm on *Klebsiella* species. In a related study, Okigbo and Mmekka (2008) reported that *Garcinia kola* extracts inhibited growth of 3 pathogens: *Staphylococcus aureus*, *E. coli* and *candida albicans*, with mean zones of growth inhibition ranging from 20mm for *E. coli* to 26mm for *Staplycoccus aureus*. The mean zones of growth inhibition reported by Okigbo and Mmekka (2008) were similar to the findings of the present study. The similarity of the two results may be explained by the similarities in environmental and social conditions of the study areas. Okigbo and Mmekka (2008) carried out their study in Awka, Anambra state which shares common boundary with Imo State, the study area of the present study. Both Anambra and Imo indigenes produce and consume similar species of *Garcinia kola*. Also the socio-cultural useage of *Garcinia kola* in Anambra State and Imo State are similar. It is likely that the strains of bacteria infecting patients in Awka are related to the strains infecting patients in Imo State. Nwokocha *et al.* (2010) reported antihepatoxic effect of *Garcinia kola* supplemented diet on mercury toxicity. Ajobeson and Aine (2004) reported that *Garcinia kola* seed has been found useful in the treatment of stomach ache and gastritis.

The result of this study which underpinned the encouraging inhibitory effect of *Garcinia kola* against challenging pathogens is immensely significant particularly at a time when multiple drug resistance is posing serious treats to health care developing system in developing nations. These plant materials apparently hold a great promise in the pharmaceutical industry in the quest to annihilate multiple drug resistant pathogens.

## CHAPTER FIVE

### CONCLUSION AND RECOMMENDATIONS

#### 5.1 Major findings of this study

This study has established that

- I. ES $\beta$ L-producing bacterial infections are less recognized and not given adequate attention in Nigeria.
- II. Many health workers in Nigeria give little or no attention to continuous study, research and dissemination of information on current health challenges.
- III. Many hospitals involved in research and facility development to combat current health challenges in the country do not carry their staff along or most staff are nonchalant about this and thus not carried along.
- IV. Multiple antibiotic resistant bacterial infections are major challenges in health care services in Nigeria and Imo State in particular.
- V. Gram-negative ES $\beta$ L-producing bacteria, including, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella* species, are the probable causes of antibiotic -resistant bacterial infections in Nigeria and Imo State in particular.
- VI. The most common genes associated with ES $\beta$ L production in Imo State include SHV, followed by TEM and CTX-M.
- VII. Some Nigerian medicinal plants are likely to provide remedy for multiple antibiotic resistant ES $\beta$ L bacterial infections.

## 5.2 Conclusion

The study established that antibiotic resistant bacteria infections caused by ES $\beta$ L-producing bacteria are common in Imo State. Health workers in the State are aware of this health challenge and had good knowledge of the causes. The different clinical approach adopted by health workers in the State to treat and manage antibiotic resistant bacteria infections are inadequate.

About 25-30% of clinical isolates from different hospitals and laboratories in Imo State are multiple antibiotic resistant strains. The multiple antibiotic bacteria strains are more prevalent in uro-genital samples than wound samples.

Amongst the MAR-bacteria strains in Imo State, 58-68% was ES $\beta$ L-positive. The prevalence of ES $\beta$ L-positive bacteria in Imo State was higher amongst *Escherichia coli* and *Klebsiella* species than *Pseudomonas aeruginosa*.

About 76.7% of ES $\beta$ L-positive harboured beta-lactamase genes. *Escherichia coli* harboured the TEM and the SHV genes, *Klebsiella* species harboured only one type of gene SHV while *Pseudomonas aeruginosa* harboured three types of gene, TEM, SHV and CTX-M.

Medicinal plants used in this study exhibited antibacterial activity against ES $\beta$ L-producing bacteria which were resistant to conventional antibiotics commonly used. This observation suggests the possibility of developing novel antibacterial agents from these medicinal plants, which will be of immense advantages in the treatment and management of ES $\beta$ L-bacteria infections.

### 5.3 Contribution to knowledge

This study has contributed to the following to scholarship.

- I. There has been paucity of published work on ES $\beta$ L producing bacteria and possible effects of medicinal plants on ES $\beta$ L-producing bacteria in Imo State. The present study has provided rudimentary data on molecular studies and effects of medicinal plants on ES $\beta$ L producing bacteria. Researchers, students and health-policy makers in Nigeria and Imo State will benefit immensely from this knowledge.
- II. This study has established that ES $\beta$ L producing bacteria is a major health challenge in health care delivery in Imo State and Nigeria in general.
- III. This study revealed that many health institutions in the State are not giving adequate attention to ES $\beta$ L-producing bacterial infections and some of the approaches of health workers to these infections are not yielding desirable results.
- IV. This study has shown that ES $\beta$ L producing bacteria infections are major causes of delays and treatment failures in Imo State, leading to waste of drugs and scarce resources.
- V. This study has shown that most ES $\beta$ L producing bacteria in Imo State possess plasmid genes with multiple bands.
- VI. This study has shown that some medicinal plants used in Imo State and other parts of Nigeria have antibacterial effects on clinical bacteria isolates. The study further established that some medicinal plants may be effective for treatment of multiple antibiotic resistant/ ES $\beta$ L bacterial infections.

#### **5.4 Recommendations**

This study has x-rayed one of the current health challenges in the country. It has not by any means exhausted the study and solutions to the problem. Thus the following recommendations are made:

- I. Further studies should be carried out on the purification and characterization of the active principles of these medicinal plants with a view to developing possible new drugs that will effectively deal with ES $\beta$ L-producing bacteria.
- II. Government and health policy makers in the country should educate health workers adequately on current health challenges such as the growing prevalence of ES $\beta$ L-producing bacterial infections, through sponsored seminars, workshops and short term training.

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## APPENDICES

### Appendix 1: Ethical Permit for the Study

Ohalete Chinyere.N  
Federal University of  
Technology,  
Owerri.  
Imo State.  
27/07/2015

The Permenent Secretary,  
Ministry Of Health,  
Imo State.

Sir,

#### **REQUEST FOR RESEARCH ETHICAL PERMIT**

I am a research student of the above institution, working on the prevalence of extended beta lactamase producing bacteria in health facilities within Imo State. My studies involve collection of antibiotic resistant bacteria from public and private health institution within Imo State, as well as administration of questionnaires to health workers for collection of data on knowledge, attitude and practice towards beta-lactamase producing bacteria.

I hereby apply for ethical permit to enable me proceed with the studies. Licensed and qualified health professionals working in public hospitals in the State will be recruited as field assistants for the collection and handling of clinical samples/isolates. The finding will be held in strict confidence and used purely for academic purposes.

Thanks for your usual co-operations.

Yours Faithfully,

**Ohalete Chinyere.N.**  
**Research student**

# GOVERNMENT OF IMO STATE OF NIGERIA

Telegrams:

Telephone:

Your Ref:.....

SON/MH/PLD/84/1/167.....

*(All replies to be addressed to the Hon. Commissioner)*



MINISTRY OF HEALTH

..... DEPT

OWERRI

19<sup>th</sup> Nov. 2015

.....

Ohalete Chinyere  
Dept. of Microbiology  
Federal University of Technology  
Owerri

**Re: Ethical Permit**

Ethical Permit is hereby granted to you to carry out your research on your topic "Studies on the prevalence of extended beta lactamase producing bacteria on clinical isolates in Imo State".

You should follow / adhere strictly to the proposal as approved by your department.

**Anozie L.O.**  
Director Planning, Research & Statistics  
Ministry of Health

## Appendix 2: Research Questionnaire

Department of Microbiology,  
Federal University of Technology,  
Owerri Imo State, Nigeria.

4<sup>th</sup> September, 2013.

Sir/Madam,

### **RESEARCH QUESTIONNAIRE**

I am a post graduate student in the Department of Microbiology, Federal University of Technology, Owerri (FUTO). I am carrying out studies on extended spectrum beta- lactamase (ES $\beta$ L) producing bacteria from clinical isolates in Imo State. I am soliciting for your assistance in the collection of data for knowledge, attitude and practice (KAP) towards ES $\beta$ L bacteria among health workers in Imo State. Kindly complete the attached questionnaire for me.

Your opinion will be held in strict confidence and used purely for academic purposes. It has nothing to do with your personal identification or that of your institution.

Thanks for your assistance.

**Ohalete Chinyere**

Research student.

Do you agree to participate in the study?

YES

NO

## QUESTIONNAIRE

### SECTION A: Socio - Demographic Data

- 1) Index No \_\_\_\_\_
- 2) Age: 20-30  31-40  41-50  51& above
- 3) Sex: Male  Female
- 4) Occupation: Medical Doctor   
Lab. Scientist
- 5) Location of health institution: Owerri   
Orlu   
Okigwe
- 6) Do you work in a Government owned Hospital.  
Yes  No
- 7) How long have you worked in this institution:  
1-5yrs   
6-10yrs   
11-15yrs   
16 & above

### SECTION B: Knowledge of Multi Drug Resistance (MDR)/Extended Spectrum Beta Lactamase (ES $\beta$ L)

- 1) Are you involved in diagnosis/ management / treatment of bacteria infected patient?  
Yes  No
- 2) If yes, have you heard of resistant or antibiotic resistance bacteria? Yes  No
- 3) If yes, in your own opinion what do you think causes antibiotic resistant.  
Fake drug/ substandard drugs   
 $\beta$ -lactamase production

- Spiritual attack
- Drug abuse
- 4) Have you encountered drug resistant bacteria in your hospital? Yes  No
- 5) If yes how commonly do you isolate/encounter Antibiotic resistant bacteria?
- Once in a week
- Once in a month
- One in every ten
- Once in a day
- 6) Which groups of bacteria are commonly involved?
- Gram -ve bacteria
- Gram +ve bacteria
- 7) Which species of Organisms did you identify?
- Mostly *Escherichia coli*
- Mostly *Stahpylococcus aureus*
- Mostly *Klebsiella pneumoniae*
- Mostly *Pseudomonas aeruginosa*
- Others (specify) \_\_\_\_\_
- 8) Have you heard of ESβL? YES  NO

**SECTION C: Attitude towards antibiotic resistant bacteria.**

- 1) What do you usually do when you encounter multi antibiotic resistant bacteria?
- Refer the patient to another hospital
- Change to different antibiotics
- Carry out further studies on the nature and cause of resistance
- Subject the isolated organism for antibiotic resistance screening
- No specific action

2) Do you think multiple antibiotic resistant bacteria can be treated? Yes  No

3) If yes, what is the treatment approach?

Combined antibiotic therapy

Prolonged antibiotic therapy

Herbal/traditional remedy

Spiritual approach

Others (specify) \_\_\_\_\_

4) Do you have any facilities for ES $\beta$ L detection? YES  NO

5) If yes, what method?

ES $\beta$ L Etest

Double disk synergy (DDS) methods on Mueller Hinton agar.

Combination disks.

Molecular detection

6) Is your hospital involved in research and dissemination of research information?

Yes  No

7) If yes, are there published data / documented evidence of  $\beta$ -lactamase producing bacteria / multi antibiotic resistance bacteria isolates from your hospital? Yes

No

8) If yes, is such information available / accessible to the public? Yes  No

9) If yes, how accessible are they?

Local library

Internet

On request

No idea

**SECTION D: Practices towards Extended Spectrum Beta Lactamases (ESBL).**

- 1) In the past 12 months, have you read or studied about ESBL producing bacteria? Yes  No
- 2) Is your hospital doing anything presently towards prevention /control and management of these types of bacteria? Yes  No
- 3) If yes, what is your hospital doing presently?
- Continues education and enlightenment of staffs
  - Importation of new drugs
  - Proper disposal / management of hospital waste
  - Acquiring/acquired material for ESBL detection
  - No specific action
- 4) In the last 3 months, have you encountered patients with ESBL bacteria? Yes  NO
- 5) If yes, what did you do to the patient?
- Referred to another place
  - Improved /concerted antibiotic management
  - Spiritual assistance
  - Use of local/herbal remedies
  - Refer the patients for ESBL screening
- 6) What is the present state of such patients?
- Gradually improving
  - Fully recovered
  - Not much improvement
  - Still hoping to recover

### Appendix 3: Composition of media and reagents used

#### (i) Formulae for Media and Reagents

##### (a) CLED AGAR (Cystine lactose electrolyte deficient)

Approximate formula in g/l:

Beef Extract	3.0
Lactose	10.0
Bromthymol Blue	0.02
Gelatin Peptone	4.0
L-Cystine	4.0
Agar	0.128
	15.0

Final pH  $7.3 \pm 0.2$

##### (b) MACCONKEY AGAR

Peptone	2.0g
Lactose	10.0g
Bile salt	5.0g
Sodium chloride	5.0g
Neutral red	0.075g
Agar	12.0g
Distilled water	1litre

##### (c) MUELLER-HINTON AGAR

Beef extract	2g
Acid Hydrolysate of casein	17.5g
Starch	1.5g
Agar	17g

Final PH  $7.3 \pm 0.1$  at 25°C

**(d) PHYSIOLOGICAL SALINE**

FORMULA AND PREPARATION

NaCl - 8.5g (0.85% W/V)

Distilled water - 1000.0cm<sup>3</sup>

Dissolve 8.5g in 1000.0cm<sup>3</sup> distilled water. Mix thoroughly, dispense into bottles and sterilize at 121<sup>0</sup>C.

**(e) PEPTONE WATER BROTH**

FORMULA AND PREPARATION

Peptone - 10.0g

NaCl - 5.0g

Distilled water - 1000.0cm<sup>3</sup>

Adjust ph to 7.0 – 7.4. Dispense into bottles and sterilize at 121<sup>0</sup>C.

**(f) METHYL-RED INDICATOR**

FORMULA AND PREPARATION

Methyl red - 0.10g

Ethanol (95%) - 30.0ml

Dissolve methyl red in 95% ethanol. Add 200mls distilled water to make up for 500ml.

**(g) OXIDASE REAGENT**

FORMULA AND PREPARATION

Para-amino dimethylphenylenediamine

Monohydrochloride - 0.01g

Distilled water - 100.0ml

Reagents should be prepared and used fresh. Store in dark prior to use.

(h) **KOVAC'S (INDOLE) REAGENT**

FORMULA AND PREPARATION

P-Dimethylaminobenzaldehyde	- 5.0g
Concentrated hydrochloric acid	- 25.0g
Tertamyl-alcohol	- 75.0ml

Dissolve (i) in (ii) and (iii) and mix thoroughly by shaking.

(i) **VOGES-PROSKAUER (V-P) REAGENT**

FORMULA AND PREPARATION

Solution A: Alpha Naphthol	- 5.0g
Absolute ethanol	- 100.0ml
Solution B: KOH	- 40.0g
Distilled water	- 100.0ml

Mix A and B and shake thoroughly to homogenize.

(j) **PREPARATION OF TURBIDITY STANDARD** (equivalent to McFarland 0.5)

1. Prepare a 1%v/v solution of sulphuric acid by adding 1ml of concentrated sulphuric acid to 99ml of water. Mix well.

Caution: Concentrated sulphuric acid is hygroscopic and highly corrosive, therefore do not mouth pipette, and never add water to acid.

2. Prepare a 1% w/v solution of barium chloride by dissolving 0.5g of dehydrate barium chloride ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in 50ml of distilled water.
3. Add 0.6ml of the barium chloride solution to 99.4ml of the sulphuric acid solution, and mix.

Transfer a small volume of the turbid solution to a capped tube or screw-cap bottle of the same type as used for preparing the test and control inocula.

When stored in a well-sealed container in the dark at room temperature (20-28<sup>0</sup>c), the standard can be kept for up to 6 months

#### Appendix 4: Characterization and Identification of Bacterial Isolates

Isolates	No. Isolated	Growth Morphology on Mueller Hinton Agar	Bacteriological Tests					Biochemical Tests						Possible Organism	
			Oxygen Req.	Cell Morph.	Motility	Gram Stain	Catalase	Oxidase	Lactose	Glucose	Indole production	Methyl Red	Voges production		Citrate Utilization
1.	147	Cream white round raised colony, smooth edges and pinkish on MacConkey.	Facultative anaerobe	Straight rods in singles	+	-	+	-	+	+	+	+	-	-	<i>Escherichia coli</i>
2.	60	Larger opaque, Blueish-green pigment.	Anaerobic / facultative anaerobe	Slightly curved rods	+	-	+	+	-	-	-	-	-	+	<i>Pseudomonas aeruginosa</i>
3.	93	Mucoid grey white and mucoid pinkish on MacConkey.	Facultative anaerobe	Straight rod shaped.	-	+	+	-	+	+	-	-	+	+	<i>Klebsiella</i> species

KEY: + = Positive reaction  
 - = Negative reaction

**Appendix 5: Statistical analysis of the quantitative analysis result.**

**Quantitative Analysis of Tanin**

SAMPLE	ABS	AU/AS	%TANIN	MEAN%TANIN	REMARKS
<i>Ocimum gratissimum</i>	2.560				5.0g was used
A	0.770	0.3008	0.752		
B	0.768	0.3000	0.750	0.752	C=5mg/ml
C	0.771	0.3012	0.753		
<i>Garcinia kola</i>					
A	0.346	0.1354	0.338		
B	0.344	0.1344	0.336		
C	0.346	0.1352	0.338	0.337	
<i>Vernonia amygdalina</i>					
A	0.346	0.1490	0.37		
B	0.344	0.1444	0.36		
C	0.346	0.1460	0.36	0.36	
<i>Allium sativum</i>					
A	0.187	0.1242	0.311		
B	0.187	0.1242	0.311		
C	0.190	0.1262	0.316	0.31	
<i>Xylopi aethiopica</i>					
A	0.796	0.3109	0.777		
B	0.800	0.3125	0.781		
C	0.801	0.3129	0.782	0.780	

$$\% \text{ Tannin} = 100/5 \times \text{au/as} \times 5/1000 \times 50/2$$

$$= 2.5(\text{au/as})$$

## QUANTITATIVE ANALYSIS OF SAPONIN

SAMPLE	W1	W2		% SAPONIN	MEAN% Saponin	REMARKS
<i>Ocimum gratissimum</i>						5.0g was used
A	8.662	8.676	0.014	0.014		
B	9.677	9.693	0.016	0.016		
C	11.620	11.634	0.014	0.014	0.07	
<i>Garcinia kola</i>						
A	11.146	11.167	0.021	0.42		
B	11.024	11.120	0.096	1.92		
C	12.030	12.140	0.11	2.2	1.51	
<i>Vernonia amygdalina</i>						
A	8.030	8.149	0.119	2.38		
B	11.031	11.142	0.12	2.4		
C	12.030	12.140	0.11	2.2	2.33	
<i>Allium sativum</i>						
A	8.664	8.682	0.018	0.36		
B	8.674	9.691	0.017	0.34		
C	9.442	9.46	0.018	0.36	0.29	
<i>Xylopi aethiopica</i>						
A	13.146	13.167	0.021	0.42		
B	11.628	11.651	0.023	0.46		
C	16.512	16.534	0.022	0.44	0.44	

$$\% \text{ Saponin} = \frac{w_2 - w_1}{5} \times \frac{100}{1} = 20 (w_2 - w_1)$$

## QUANTITATIVE ANALYSIS OF HCN

Sample	Abs	Au/as	HCN mg/kg	Mean % HCN	Remarks
STD	1.025				
<i>Ocimum gratissimum</i>					
A	0.033	0.0322	1.61		1.0g sample was used
B	0.032	0.0312	1.56		
C	0.032	0.0312	1.56	1.58	
<i>Garcinia kola</i>					
A	0.001	0.04	2.00		
B	0.001	0.04	2.00		
C	0.001	0.0404	2.02	2.0	
<i>Vernonia amygdalina</i>					
A	0.188	0.1834	9.17		
B	0.188	0.1834	9.17		
C	0.186	0.1815	9.08	9.14	
<i>Allium sativum</i>					
A	0.139	0.1356	6.7		
B	0.141	0.1376	6.88		
C	0.141	0.1376	6.88	6.82	
<i>Xylopiya aethiopica</i>	Not Detected				

$$\text{HCNmg/kg} \frac{100}{1.0} \times \frac{au}{as} \times 0.05 = 50 (au/as)$$

### QUANTITATIVE ANALYSIS OF FLAVONOID

SAMPLE	W <sub>1</sub>	W <sub>2</sub>	W <sub>2</sub> - W <sub>1</sub>	% FLAVONOID	MEAN % FLAVONOID	REMARKS
<i>Ocimum gratissimum</i>						
A	1.012	1.030	0.018	0.36		5.0g sample
B	1.014	1.034	0.020	0.40		was used
C	1.017	1.035	0.018	0.36	0.373	
<i>Garcinia kola</i>						
A	1.008	1.100	0.092	1.84		
B	1.001	1.101	0.01	2.0		
C	1.001	1.102	0.101	2.02	1.95	
<i>Vernonia amygdalina</i>						
A	1.022	1.058	0.036	0.72		
B	1.024	1.058	0.034	0.68		
C	1.028	1.065	0.037	0.74	0.41	
<i>Allium sativum</i>	1.026	1.047	0.021	0.42		
	1.031	1.05	0.019	0.38		
	1.028	1.049	0.021	0.42		
<i>Xylopi aethiopica</i>						
	1.016	1.025	0.009	0.18		
	1.018	1.027	0.009	0.18		
	1.021	1.032	0.011	0.22	0.193	

$$\% \text{ Flavonoid} = \frac{(W_2 - W_1) \times 100}{5 \quad 1}$$

## QUANTITATIVE ANALYSIS OF ALKALOID

Sample	W <sub>1</sub>	W <sub>2</sub>	W <sub>2</sub> -W <sub>1</sub>	% alkaloid	Mean % Alkaloid	Remarks
<i>Ocimum gratissimum</i>						5.0g sample was used
A	1.016	1.032	0.016	0.32		
B	1.014	1.028	0.014	0.28		
C	1.012	1.028	0.016	0.32	0.31	
<i>Garcinia kola</i>						
A	1.024	1.058	0.034	0.68		
B	1.024	1.060	0.036	0.72		
C	1.021	1.054	0.033	0.66	0.69	
<i>Vernonia amygdalina</i>						
A	1.421	1.542	0.121	2.42		
B	1.400	1.500	0.100	2.00		
C	1.410	1.511	0.101	2.02	2.14	
<i>Allium sativum</i>						
A	1.024	1.048	0.024	0.48		
B	1.022	1.048	0.026	0.52		
C	1.024	1.05	0.026	0.52	0.51	
<i>Xylopi aethiopica</i>						
A	1.022	1.040	0.018	0.36		
B	1.024	1.042	0.018	0.36		
C	1.020	1.040	0.020	0.40	0.37	

$$\% \text{ Alkaloid} = W_2 - W_1 / 5 \times 100/1$$

$$= 20 (W_2 - W_1) s$$

### Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Tanin						
<i>O. gratissimum</i>	3	.75	.75	.7517	.00088	.00153
<i>G. kola</i>	3	.34	.34	.3373	.00067	.00115
<i>V. amygdalina</i>	3	.36	.37	.3633	.00333	.00577
<i>A. sativum</i>	3	.31	.32	.3127	.00167	.00289
<i>X. aethiopica</i>	3	.78	.78	.7800	.00153	.00265
Valid N (listwise)	3					

### Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Saponin						
<i>O. gratissimum</i>	3	.01	.02	.0147	.00067	.00115
<i>G. kola</i>	3	.42	2.20	1.5133	.55261	.95715
<i>V. amygdalina</i>	3	2.20	2.40	2.3267	.06360	.11015
<i>A. sativum</i>	3	.34	.36	.3533	.00667	.01155
<i>X. aethiopica</i>	3	.42	.46	.4400	.01155	.02000
Valid N (listwise)	3					

### Descriptive Statistics

HCN	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
<i>O. gratissimum</i>	3	1.56	1.61	1.5767	.01667	.02887
<i>G. kola</i>	3	.01	.10	.0703	.03017	.05225
<i>V. amygdalina</i>	3	9.08	9.17	9.1400	.03000	.05196
<i>A. sativum</i>	3	6.70	6.88	6.8200	.06000	.10392
<i>X. aethiopica</i>	0					
Valid N (listwise)	0					

### Descriptive Statistics

Flavonoid	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
<i>O. gratissimum</i>	3	.36	.40	.3733	.01333	.02309
<i>G. kola</i>	3	1.84	2.02	1.9533	.05696	.09866
<i>V. amygdalina</i>	3	.68	.74	.7133	.01764	.03055
<i>A. sativum</i>	3	.38	.42	.4067	.01333	.02309
<i>X. aethiopica</i>	3	.18	.22	.1933	.01333	.02309
Valid N (listwise)	3					

## Descriptive Statistics

Alkaloid	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
<i>O. gratissimum</i>	3	.28	.32	.3067	.01333	.02309
<i>G. kola</i>	3	.66	.72	.6867	.01764	.03055
<i>V. amygdalina</i>	3	2.00	2.42	2.1467	.13679	.23692
<i>A. sativum</i>	3	.48	.52	.5067	.01333	.02309
<i>X. aethiopica</i>	3	.36	.40	.3733	.01333	.02309
Valid N (listwise)	3					



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