CHAPTER ONE

INTRODUCTION

1.0 Introduction

Antibiotic resistance genes are crucial in the niche colonization of microorganisms since microbes need to combat antimicrobial compounds produced by other microorganisms and higher organisms in their immediate environment (Fernandez *et al*., 2012; Bush *et al*., 1995). Food-producing animals including poultry birds and cattle habouring multidrug resistance genes together with genes that mediate the production of some high-profile antibiotic hydrolyzing enzymes such as metallo-beta-lactamases (MBLs), extended spectrum beta-lactamases (ESBLs) and AmpC enzymes possess health risks to the human population particularly due to their potential contribution to the spread of drug resistant microorganisms in the community. More worrisome is the plethora of reports in many quarters about the prevalence of metallo-beta-lactamase (MBL) and AmpC beta-lactamase-producing bacteria in the community, and their possible link to food-producing animals (Toleman *et al*., 2005; Tian *et al*., 2012; Grundmann *et al*., 2010; Ben-Slama *et al*., 2010; Blanc *et al*., 2006; Bertrand *et al*., 2006; Bergenholtz *et al*., 2009). One of the biggest current challenges facing the health sector across the globe especially in the area of infection control and prevention is in the adequate containment of multidrug resistant Gram-negative organisms (MDRGNOs) such as *P. aeruginosa*, *Escherichia coli* and *Klebsiella* species. It is now more worrisome that MDRGNOs are not restricted to the hospital environment alone but bacterial pathogens including *Klebsiella* species, *P. aeruginosa* and pathogenic *E. coli* that produce enzymes that degrade some of the world's potent and effective

drugs (e.g. carbapenems and cephalosporins) are now found in the community. Measuring the prevalence of these organisms in both the hospital and community settings coupled with proper use of available drugs and proper susceptibility testing of these organisms before the prescription or use of some expanded spectrum drugs (especially in hospital environments) is critical to reducing the possible risks associated with infections due to MDRGNOs (Iroha *et al*., 2016; Javeed *et al*., 2011; Ejikeugwu *et al*., 2016). Both carbapenem-resistant *Enterobacteriaceae* and AmpC-producing Gram-negative bacteria inclusive of *E. coli, Klebsiella* species, *P. aeruginosa* and *Enterococcus* species have emerged in hospital environments and community settings as important health issue that are of global health concern (Akinduti *et al*., 2012; Akujobi *et al*., 2012; Leung *et al*., 2013; Vanwynsberghe *et al*., 2009; Aibinu *et al*., 2007). The AmpC β-lactamases are clinically important beta-lactamases because they confer antimicrobial resistance to the narrow-spectrum, expanded-spectrum and the broad-spectrum cephalosporins including cefotaxime, ceftazidime, ceftriaxone, aztreonam and the penicillins; and their resistance is also expressed towards the β- lactamase inhibitors such as amoxycillin-clavulanic acid (Shevade and Agrawal, 2013; Bush *et al*., 1995). The genes encoding AmpC β-lactamases are much more frequently chromosomal than plasmid-mediated. However, the genes for AmpC-β-lactamases can be transferred to organisms that do not harbour them (such as *K. pneumoniae*) through plasmids and other genetic transfer elements such as transposons. *Klebsiella* species especially *K. pneumoniae* is one of the few Gram-negative bacteria which do not possess a chromosomal AmpC β-lactamase, but the organism can acquire this resistant gene through the transfer of AmpC-containing plasmids from other *Enterobacteriaceae* harbouring the enzyme (Vanwynsberghe *et al*., 2009; Bush *et al*., 1995). The chromosomally mediated β-lactamase production of AmpC enzymes by Gram-negative bacteria takes place through the expression of

the AmpC gene which is either constitutive or inducible (Shevade and Agrawal, 2013). AmpC enzymes confer resistance to all classes of beta-lactams, except the carbapenems including ertapenem, meropenem and imipenem; and they are also not inhibited by clavulanic acid and other beta-lactamase inhibitors (Walsh *et al*., 2005; Bush *et al*., 1995). The economic cost of antimicrobial resistance goes beyond the morbidity and mortality associated with the medical condition caused by the organisms harbouring multidrug resistant genes; it also includes the loss of efficacy or ineffectiveness of some available antimicrobial drugs (Bush and Jacoby, 2010). In most parts of the world especially in the developing countries inclusive of Nigeria, the detection of resistant genes and/or multidrug resistant enzymes responsible for the negative response of pathogenic bacteria to potent antimicrobial onslaught is still ill-detected in our hospitals because routine antimicrobial susceptibility studies (as is obtainable in most of our hospitals) is almost ineffective in detecting these multidrug resistant organisms. The menace posed by multidrug resistant bacteria inclusive of environmental and hospital microbiota is well documented (Aibinu *et al*., 2007; Rossolini *et al*., 2001; Iroha *et al*., 2008; Hemalatha *et al*., 2007; Bush *et al*., 1995). Antimicrobial resistance is a natural biological phenomenon but it can be contained and averted through proper drug prescription, timely and appropriate detection of their emergence in a hospital setting and even amongst environmental microbiota. Selective pressure which can also be called evolutionary pressure is the natural force that allows a particular organism to evolve in a certain direction in an environment especially in the area of developing antibiotic resistant genes due to the undue exposure of such organisms to antimicrobial agents. This pressure causes the organism to develop resistance to the antibiotics they were exposed to in the environment. Selective pressure (particularly natural selection) is primarily responsible for the development of resistance to some available antimicrobial agents by pathogenic microorganisms (Madigan *et al*.,

2009; Prescott *et al*., 2005; ASM Report, 2015; Dawson *et al*., 2009). Most notably, the widespread use of antibiotics especially irrationally has resulted in the emergence of some drug-resistant disease-causing pathogenic bacteria that evolve into strains that are resistant to many classes of antibiotics (Bush and Jacoby, 2010; ASM Report, 2015). The use of drugs intended for human medicine in veterinary practices; livestock production, animal husbandry as well as the irrational use of antimicrobials by humans and without proper susceptibility studies and/or doctors recommendation are some of the factors that can stimulate selective or natural pressure as aforementioned amongst hospital and environmental microorganisms (ASM Report, 2015). Antibiotic treatment or usage can impact non-target microorganisms either in the community or hospital setting, and this enriches the pool of resistance genes available to pathogens in the environment (Tian *et al*., 2012). This impact of antibiotics on non-target microbes influences other beneficial microorganisms present in the healthy host; and the selective force imposed by an antibiotic can cause the accumulation of resistance determinants, which are often encoded on mobile genetic elements such as plasmids and transposons that are readily transmissible amongst other bacterial species in the environment (Tian *et al*., 2012). Metallo-beta-lactamases (MBLs) are beta-lactamase enzymes produced by pathogenic bacteria, and which hydrolyzes the carbapenems (e.g. imipenem, meropenem, and ertapenem) and render the antibiotics ineffective for treatment (Walsh *et al*., 2005; Rossolini *et al*., 2001). They are encoded by genes that have been procured by pathogenic bacteria either by mutation or by horizontal gene transfer from other resistant microbes. MBLs have high affinity for zinc ions (Zn^{2+}); and thus the enzyme is largely inhibited by chelating agents (e.g. EDTA and dipicolinic acid) *in vitro*. The susceptibility of MBL-producing bacteria to chelating agents is the basis upon which the enzyme can be detected phenotypically in pathogenic microorganisms (Walsh *et al*., 2005; Ejikeugwu *et al*.,

2014). Genes responsible for the expression of MBLs can also be chromosomally or plasmid-mediated as is also applicable with ESBLs and other resistance genes harboured by pathogenic microorganisms (Walsh *et al*., 2005; Aibinu *et al*., 2007). The carbapenems are often the last line of treatment option for a variety of infectious diseases including those caused by multidrug resistant organisms (e.g. ESBL positive bacteria). The carbapenems are very potent antimicrobial agents used for the treatment of serious Gram-negative bacterial infections, and because of the broad spectrum activity and stability of the carbapenems to most beta-lactamase enzymes, they have been widely used under restricted conditions in most hospitals worldwide as the first-line treatment for severe Gram-negative infections (Aibinu *et al*., 2007). MBLs are mainly expressed by non-lactose fermenters such as the *Pseudomonas* species and *Acinetobacter* species. The *Enterobacteriaceae* including *Escherichia coli* and *Klebsiella* species have also been reported to express MBLs (Walsh *et al*., 2005; Ejikeugwu *et al*., 2014). The presence or occurrence of MBL-producing bacteria in a hospital setting poses not only a therapeutic problem but also serious concern for infection control management in the health system (Toleman *et al*., 2005; Wadekar *et al*., 2013). MBLs may be disseminated in hospital environment through genetic transfer elements such as transposons, plasmids and integrons amongst clinically important bacteria; and the extensive use of the carbapenems especially irrationally has given impetus to the spread of organisms that produce the enzymes (Toleman *et al*., 2005; Walsh *et al*., 2005). Both Gram-positive and Gram-negative bacteria have these powerful weapons (i.e. multidrug resistance genes and/or enzymes) of antibiotic resistant genes at their disposal, but the production of these enzymes is mostly common in Gram-negative organisms – whose cell walls are exceptionally different and more resistant to antimicrobial agents than those of their Gram-positive counterparts (Madigan *et al*., 2009; Prescott *et al*., 2005). The presence of MBLs and AmpC

enzymes in a single pathogenic bacterium reduces the effectiveness of the beta-lactam and beta-lactamase inhibitor such as clavulanic acid, sulbactam and tazobactam. The problem of antibiotic resistance is real and this public health phenomenon is gradually spreading across the globe through different mechanisms (ASM Report, 2015). An estimated half of antibiotic prescriptions worldwide are usually inappropriate due to poor detection of resistant strains of bacteria and inadequate antimicrobial susceptibility testing (AST) in some hospitals (ASM Report, 2015). Multidrug resistant bacteria including those that produce metallo-β-lactamases (MBLs), AmpC β-lactamases and extended spectrum β-lactamases (ESBLs) in Gram-negative bacteria that comprises *E. coli, Klebsiella* species and *P. aeruginosa* in the community especially amongst animals and poultry birds is a constant source of the emergence and spread of antibiotic resistant bacteria in human population (Van den Bogaard *et al*., 2001; Usha *et al*., 2010; ASM Report, 2015; Tian *et al*., 2012; Bush and Jacoby, 2010). And these non-hospital sources of these pathogens are of public health importance due to the possibility of transmission of resistant bacteria from these animals to humans through the consumption of the meat and other products that come from them. The possible contamination of food and/or food-producing animals with multidrug resistant bacteria is now considered a potential source for the wide dissemination of AmpC β-lactamase and MBLs in the community; and this portend public health risks for the populace – since these organisms can be transmitted through the food chain in human population. Thus, this study investigated environmental isolates of *E. coli*, *P. aeruginosa* and *Klebsiella* species from poultry birds, anal swab of cows and abattoirs for the presence of MBL genes and AmpC genes, and as potential non-hospital sources of metallo-β-lactamases and AmpC β- lactamases in the community.

1.1 Aim and Objectives

1.1.1 Aim

The aim of this study was to determine by phenotypic and molecular techniques the occurrence of metallo-beta-lactamase (MBL) genes and AmpC enzyme genes amongst environmental isolates of *E. coli, P. aeruginosa* and *Klebsiella* species from abattoir and poultry sources.

1.1.2 Objectives

The specific objectives of this study are to:

1. Isolate and determine the prevalence of *E. coli, P. aeruginosa* and *Klebsiella* species from abattoir and the cloacae of poultry birds.
2. Determine the presence or not of β-lactamase, metallo-beta-lactamase (MBL) enzymes and AmpC enzymes in isolates of *E. coli, P. aeruginosa* and *Klebsiella* species by phenotypic techniques.
3. Determine the antimicrobial susceptibility profile and multiple antibiotic resistance index (MARI) of the isolated *E. coli, P. aeruginosa* and *Klebsiella* species.
4. Investigate the location (plasmid-borne or chromosomal) of the resistance determinants in the MBL and AmpC positive bacteria by plasmid curing experiment.
5. Determine the presence of MBL genes of ^{bla}IMP-1, ^{bla}IMP-2, ^{bla}VIM-1, and ^{bla}VIM-2 genes; and the AmpC genes of ^{bla}CMY-1, ACC, FOX-1 and DHA-1 genes in the MBL- and AmpC phenotypes of *E. coli, P. aeruginosa* and *Klebsiella* species of poultry and abattoir origin using multiplex PCR.

1.1.3 Statement of problem

The production of beta-lactamases such as metallo-beta-lactamases (MBLs) and AmpC enzymes by Gram-negative bacteria including *E. coli*, *Klebsiella* species and *P. aeruginosa* is one of the most potent weapons with which pathogenic bacteria use to evade the antimicrobial onslaught of some available antimicrobial agents in this era of bacterial resistance to antibiotics. The menace posed by multidrug resistant bacteria necessitates the need to detect by phenotypic and molecular characterization techniques the presence of MBLs and AmpC β – lactamases from environmental isolates and/or samples – owing to the fact that antibiotic resistance is an increasing problem in our health sector of today worldwide. And the ever increasing use of antimicrobial agents (especially those used for human medicine) in the rearing and/or breeding of livestock and poultry birds calls for a concerted effort to restrict and disallow such practices since they enable drug resistant strains of pathogenic bacteria to emerge and spread in the community.

Of particular concern is the fact that these resistant pathogens are responsible for some community-acquired infections that may lead to the hospitalization of the infected individuals. The hospitalization of such individuals can put them at risk of acquiring nosocomial infections. Many reports have elucidated the prevalence of multidrug resistant bacteria from both clinical and environmental samples in various states of Nigeria, but very few have established the genetic factors responsible for the prevailing antibiotic resistant cases occurring in such locations especially as is the case for MBL and AmpC positive bacteria from abattoir and poultry origin. The environmental reservoirs of antibiotic resistant bacteria and their genetic elements in this region are poorly understood. And the emergence and spread of resistant bacteria in the

environment especially amongst animals and poultry birds is very important to human health especially now that we experience an upsurge of zoonotic infections in our world.

In addition, there have been several reports on the reservation and occurrence of resistant bacteria in animals and poultry birds in other parts of the world. This portends danger to human health that consumes these animals and handles them as well. Down here in Nigeria, especially in Abakaliki, there is no extensive report or data showing the occurrence of MBL and AmpC-producing bacteria from the poultry and abattoir sources. When antibiotic resistant bacteria from environmental samples are not reported, human health may be at risk. This is because these drug resistant organisms could easily spread from these animals to humans through contact or consumption of animals and poultry products. This may cause an outbreak of community-acquired infections. Bacterial strains producing MBLs and AmpC enzymes in Gram-negative bacteria from food-producing animals portend serious public health risk. They could serve as route via which multidrug resistant bacteria could be transmitted through food chain or through food production environments.

More so, we have previously conducted series of studies on *E. coli, P. aeruginosa* and *Klebsiella* species from hospitals. In these studies, we only phenotypically screened for the presence of ESBLs, MBLs and AmpC enzymes in Enugu and Anambra states. These previous studies necessitated and informed the decision to do a study in Abakaliki metropolis of Ebonyi State, Nigeria in order to give credence to the growing occurrence of these multidrug resistant bacteria in our environment. It is in this view that this study sought to characterize and establish the genetic factors responsible for the occurrence, spread and infections caused by MBL- and AmpC-producing *E. coli, K. pneumoniae* and *P. aeruginosa* from environmental samples of broiler cloacal swab, swabs from anal regions of cow and swabs from abattoir tables.

1.1.4 JUSTIFICATION

The menace posed by multidrug resistant bacteria such as *Escherichia coli*, *Klebsiella* species and *Pseudomonas aeruginosa* necessitates the need to detect by phenotypic and molecular techniques the prevalence of these organisms in the community. Metallo-β-lactamases (MBLs) and AmpC enzymes are usually produced by Gram-negative bacteria; and these antibiotic-degrading enzymes give these organisms the exceptional ability to overcome the antimicrobial onslaughts of the carbapenems (for MBL-positive bacteria) and cephamycins such as cefoxitin and cefotetan (for AmpC positive bacteria). The presence of MBLs and AmpC β-lactamases from clinical isolates and non-clinical samples is important owing to the fact that antibiotic resistance is an increasing problem in our health sector worldwide. Infections caused by antibiotic resistant bacteria are usually associated with high morbidity and mortality; and they could also lead to long hospitalization and increased healthcare cost. Also, the long hospitalization of people infected with multidrug resistance bacteria can be predisposed to the acquisition of nosocomial infection. Gram-negative organisms that produce MBLs and AmpC enzymes are resistant to a wide variety of antimicrobial agents. It is therefore important to always be on the lookout for these organisms in both the hospital and non-hospital environment so that their emergence and spread can be forestalled. There is paucity of information regarding MBL-producing bacteria and AmpC positive bacteria in Abakaliki, Ebonyi State, Nigeria. This project helped to detect and determine by phenotypic and molecular detection methods the presence of MBLs and AmpC enzymes in *E. coli*, *Klebsiella* species and *P. aeruginosa* isolates from abattoir and poultry samples in Abakaliki, Ebonyi State Nigeria.

1.1.5 HYPOTHESIS

The hypothesis of this research work is as follows:

- Metallo-β-lactamase (MBL) and AmpC enzymes are produced by Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* species and *Pseudomonas aeruginosa.*

- Both MBL and AmpC enzymes are responsible for the antimicrobial resistance nature of some Gram-negative bacteria to the antimicrobial activity of the carbapenems and cephamycins respectively.

- MBL and AmpC-producing bacteria occur in abattoir and poultry samples.

- The genes that are responsible for MBL and AmpC enzyme production in Gram-negative bacteria are either chromosomally-mediated or plasmid-mediated.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of *Escherichia coli* infections

Escherichia coli is a Gram-negative, aerobic and rod-like bacterium found in the family *Enterobacteriaceae*, and it is a most frequent human pathogen that inhabits the intestinal tract of animals including humans as either pathogenic bacterium or normal microflora (Madigan *et al*., 2009; Brooks *et al*., 2004). *E. coli* is a normal flora of the intestinal tract of humans and animals; and *E. coli* can become pathogenic when they leave their usual sites or mutate to become pathogenic bacteria. Biochemically, *E. coli* is indole positive because of its ability to breakdown the amino acid, tryptophan to produce indole; and this test is used to identify and differentiate the bacterium from other enteric bacteria in the laboratory (Cheesbrough, 2000). Most of the diseases or infections caused by pathogenic *E. coli* only manifest when the host's immune system is weakened or compromised. *E. coli* frequently cause some nosocomial and community-acquired infections including urinary tract infections (UTIs), wound infections, respiratory tract infections, bacteraemia and sepsis (Zhang *et al*., 2010; Javeed *et al*., 2011; Madigan *et al*., 2009). Pathogenic *E. coli* is frequently resistant to antibiotics, and most of the infections caused by the bacterium are increasingly resistant to some commonly used antibiotics including those in the class of fluoroquinolones, quinolones, aminoglycosides and the beta-lactams (Zhang *et al*., 2010; Javeed *et al*., 2011). *E. coli* is a lactose positive bacterium, and thus the bacterium ferments lactose in culture media such as MacConkey agar to produce pinkish colouration or colonies on the growth media (Cheesbrough, 2000; Madigan *et al*., 2009). *E. coli* exist in different pathovars – through which the various clinical disease caused by the pathogenic strain of the bacterium in

humans are exhibited. The main pathovars of pathogenic *E. coli* include: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC). **Enteropathogenic *E. coli* (EPEC)** causes the classic infant diarrhea, and it is majorly responsible for some *E. coli* outbreaks in hospitals and nursing homes and even in residential homes (Madigan *et al*., 2009)**.** EPEC is still a major contributor to infant morbidity and mortality in the developing world even though the disease has been significantly contained in developed economies. **Enterotoxigenic *E. coli* (ETEC)** is responsible for causing cholera-like diarrhea (Madigan *et al*., 2009)**.** ETEC is the cause of traveler's diarrhea, and the pathogen produces a heat-labile and heat-stable enterotoxin that increases the pathogenicity and/or virulence of the organism *in vivo*. Clinically, ETEC is characterized by massive loss of fluid from the body of affected individuals – as is applicable in diarrheal diseases. ETEC affects people of all ages including adults and children. **Enteroinvasive *E. coli* (EIEC)** causes a dysentery-like diarrhea (Madigan *et al*., 2009)**.** EIEC produces verocytotoxins and invasins that promote its pathogenicity *in vivo*. The bacterium invades the mucosa of the colon (large intestine), and this causes inflammatory ulcers in the affected region. **Enterohaemorrhagic *E. coli* (EHEC)** is responsible for causing haemorrhagic colitis in humans (Madigan *et al*., 2009; Brooks *et al*., 2004)**.** Most individuals infected with EHEC develop haemolytic-uremic syndrome (HUS). EHEC infections are usually followed with significant anaemia, thrombocytopenia, and acute renal failure especially in HUS patients (Madigan *et al*., 2009; Brooks *et al*., 2004). **Enteroaggregative *E. coli* (EAEC)** causes watery diarrhea especially in infants and small children (Madigan *et al*., 2009). It can also cause haemorrhagic diarrhea in children and infants. EAEC adheres to the mucosal surfaces of the

small intestines, and their adherence mediates the production of toxins which has some similarity to the enterotoxins produced by ETEC.

2.2 Overview of *Pseudomonas aeruginosa* infections

Pseudomonas aeruginosa is a Gram-negative, oxidase-positive, aerobic and rod-like bacterium found in the family *Pseudomonadaceae*, and they are widespread in nature (Madigan *et al*., 2009). Unlike *E. coli*, *P. aeruginosa* is not an enteric organism. It is a free-living, non-enteric bacterium, commonly found in soil and water, and it can also be found on the surfaces of plants and occasionally on the surfaces of animals (Madigan *et al*., 2009). *P. aeruginosa* has biofilm-forming capabilities, and they are commonly found in moist or wet environments such as bath tubs, sinks and as common contaminants in hospitals. Recently, *P. aeruginosa* has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. Several strains of the organism are innately resistant to some commonly used antibiotics. *P. aeruginosa* is primarily a nosocomial pathogenic bacterium that is responsible for causing several bacterial-related infections especially cystic fibrosis (CF) and wound burn infections (Madigan *et al*., 2009). It is an opportunistic pathogen, and it causes infection in individuals with weakened or compromised immune system including cancer patients and HIV/AIDS patients. *P. aeruginosa* hardly causes infections in people with intact immune system (Madigan *et al*., 2009). Some of the infections in which *P. aeruginosa* is implicated as a causative factor include urinary tract infections (UTIs), otitis media, otitis externa, respiratory system infections, dermatitis, soft tissue infections, bacteremia, sepsis, bone and joint infections, gastrointestinal infections and wound/burn infections (Brooks *et al*., 2004). *P. aeruginosa* infection is a serious problem in

patients hospitalized with cancer, cystic fibrosis, and burns. The pathogen is a common contaminant of various hospital sites including doors, hands, hospital equipment, hospital beds, sinks and hospital floors from where possible hospital-acquired infections can emanate and spread. *P. aeruginosa* is notorious for its resistance to antibiotics, and the bacterium is naturally resistant to many antibiotics due to the permeability barrier associated with the outer membrane (OM) of Gram-negative bacterium (Madigan *et al*., 2009).

2.3 Overview of *Klebsiella* species infections

Klebsiella species including *K. pneumoniae* is a Gram-negative, aerobic and rod-like bacterium found in the *Enterobacteriaceae* family (Madigan *et al*., 2009). The main representative species of the genus *Klebsiella* includes *K. pneumoniae* and *K. oxytoca*; and these species have been identified as important cause of respiratory tract infections in humans (Brooks *et al*., 2004). *Klebsiella* species is a lactose-positive and non-motile bacterial organism. They also form capsules as a protective mechanism in harsh environments. Biochemically, *Klebsiella* species is urease positive and citrate positive because of the ability of the organism to break down urea to ammonia and carbon dioxide, and also to utilize citrate as its sole carbon source respectively (Cheesbrough, 2006). Urease test and citrate test is often used in the microbiology laboratory to identify and differentiate *Klebsiella* species from other members of the family *Enterobacteriaceae*. On culture media, *Klebsiella* species produce mucoid colonies and they are generally non-motile but positive for voges proskauer test (Cheesbrough, 2006). They are sometimes regarded as coliforms as is applicable to *E. coli* and other enteric bacteria (Brooks *et al*., 2004). *Klebsiella* species is the main causative agent of bacterial pneumonia in humans.

Klebsiella species also causes pneumonia in people who are predisposed to the pathogen especially in hospital settings. *Klebsiella* species is also an implicating causative factor in bacteremia and sepsis as well as in other blood stream infections. Most infections caused by *Klebsiella* species are fast becoming resistant to some commonly used antibiotics. Some strains and/or species of the organism inclusive of pathogenic *E. coli* and *P. aeruginosa* produce hydrolyzing enzymes such as metallo-beta-lactamases (MBLs), extended spectrum beta-lactamases (ESBLs) and AmpC enzymes which contribute to their multidrug resistant nature (Zhang *et al*., 2010; Javeed *et al*., 2011; Walsh *et al*., 2005; Jacoby and Munoz-Price, 2005).

2.4 Overview of Carbapenem-Resistant Gram-negative Bacteria

Carbapenems are broad-spectrum beta-lactam drugs that include imipenem, meropenem, ertapenem and doripenem. They are the most powerful class of antibiotics used for the treatment of bacterial infections caused by multidrug resistant bacteria including those that produce extended spectrum beta-lactamases (Walsh *et al*., 2005). Carbapenems have a pyrrolidine moiety as a side chain, and they possess broader spectrum of antimicrobial activity than the penicillins and cephalosporins (Madigan *et al*., 2009; Brooks *et al*., 2004; Walsh *et al*., 2005). They enter Gram-negative bacteria through outer membrane proteins (porins) and reach the periplasmic space of the target bacteria where they permanently acylate or cleave to the penicillin-binding proteins (PBPs) of the target organism. Carbapenems have the ability to bind specifically to multiple different PBPs, and thus inhibit the peptide cross-linking of N-acetylmuramic acid (NAM) and N-acetyl glucosamine (NAG) which are important reactions for the development of bacterial cell wall (Madigan *et al*., 2009; Brooks *et al*., 2004; Walsh *et al*., 2005). However, the

extensive and irrational use of this potent group of drugs (i.e. the carbapenems) has resulted in the emergence of carbapenem-resistant Gram-negative bacteria that render them inefficacious (Overturf, 2010; Walsh *et al* 2005; Toleman *et al*., 2005; Tortola *et al*., 2005). Metallo-β-lactamase (MBL) is one of such enzymes produced by Gram-negative bacteria, and which allow these organisms to be resistant to the carbapenems. The weakening of bacterial cell wall and autolysis (which are both mediated by the antimicrobial activity of carbapenems) result in the death of the target pathogenic bacteria *in vivo*. The carbapenems including imipenem, meropenem, ertapenem and doripenem have the broadest antimicrobial spectrum than the penicillins, cephalosporins or beta-lactamase inhibitor combinations such as amoxicillin-clavulanic acid. They are effectively used to treat bacterial diseases caused by Gram-positive and Gram-negative bacteria especially those that are multiply drug-resistant in nature. Carbapenems can also be co-administered along with other antimicrobial agents for effective treatment outcome. But the excessive use of the carbapenems alters the intestinal microflora and thus selects for carbapenem-resistant bacteria that produces carbapenemases (Walsh *et al*., 2005). Carbapenemases are carbapenem hydrolyzing enzymes, and they are broadly divided into two types based on the reactive site of the enzymes (Overturf, 2010). Serine carbapenemases (Class A carbapenemases) and metallo-β-lactamases (Class B carbapenemases) are the two major types of carbapenemases that hydrolyze the carbapenems (Overturf, 2010; Walsh *et al*., 2005). *Klebsiella pneumoniae* carbapenemases (KPC) is another group of the carbapenemases which mediate antibiotic resistance in *Klebsiella* species particularly *K. pneumoniae* (Overturf, 2010). The emergence of carbapenem resistance in Gram-negative bacteria including *E. coli, K. pneumoniae* and *P. aeruginosa* significantly limits possible treatment options for treating life-threatening infections caused by these pathogenic bacteria. Pathogenic bacteria that harbour

genes for the production of carbapenemases portend serious public health concern globally since the carbapenems are often the last line of drugs for the management of multidrug resistant infections. The fact that no newer broad-spectrum drugs are being developed against Gram-negative bacilli infections calls for the need to preserve these antibiotics through proper use. It is of utmost importance to detect these organisms from both hospital and environmental sources so that appropriate infection control practices can be implemented to control their spread and emergence in either the hospital environment or community. Serine carbapenemases are Ambler Class A enzymes and they are chromosomally- and plasmid-mediated enzymes i.e. they are carried by the bacterial chromosomes and can also be transmitted via plasmids found in the same organism respectively. The Class A serine carbapenemases include SME enzymes (first discovered in *Serratia marcescens*), IMI enzymes (for: imipenem hydrolyzing β-lactamase), KPC (for: *Klebsiella pneumoniae* carbapenemase), NMC (for: not metalloenzyme carbapenemase) and GES (for: Guiana extended spectrum). The serine Class A carbapenemases are commonly found in *Enterobacteriaceae* including *Escherichia coli* and *Klebsiella* species. They are rarely found in *Pseudomonas aeruginosa* isolates.

2.5 Metallo-beta-lactamase (MBL) enzymes

Metallo-beta-lactamases (MBLs) are carbapenem-hydrolyzing beta-lactamases which belong to molecular Class B of Ambler beta-lactamase classification, and which have the ability to hydrolyze and confer resistance to carbapenems (imipenem, meropenem, ertapenem) and other beta-lactam antibiotics (Tortola *et al*., 2005 and Thompson, 2010). Class B carbapenemases (i.e. the metallo-β-lactamases) are found in *Enterobacteriaceae*, *Acinetobacter* species and

Pseudomonas aeruginosa isolates. MBLs, which are a type of carbapenemases, are an emerging public health problem among clinically important Gram-negative organisms including *P. aeruginosa*, *A. baumannii* and the *Enterobacteriaceae* (Thompson, 2010). The carbapenems are very potent antimicrobial agents used for the treatment of serious Gram-negative bacterial infections including those that are ESBL-mediated; and because of the broad spectrum activity and stability of the carbapenems to most beta-lactamase enzymes, the carbapenems have been widely used under restricted conditions in most hospitals worldwide as the first-line treatment for severe Gram-negative infections (Franco *et al*., 2010). The MBLs are known to confer variable range of high resistance to all beta-lactam antibiotics except the monobactams and their presence in clinically important Gram-negative bacteria have put the use of the carbapenems under threat (Tortola *et al*., 2005 and Thompson, 2010).

The MBLs belong to a group of beta-lactamases which requires divalent cations (e.g. zinc ions) as cofactors for their enzyme activity, and they share four main characteristics as follows (Toleman *et al*., 2005 and Varaiya *et al*., 2008):

1. Activity against carbapenem antibiotics.
2. No clear hydrolysis of monobactams.
3. Inhibition by chelating agents such as ethylene diamine tetraacetic acid (EDTA) and dipicolinic acid.
4. Requirement of zinc ions (Zn^{2+}) for enzyme activity.

Genetically, the MBLs are either plasmid-mediated or chromosomally-mediated, and those that are plasmid-mediated (or encoded by transferable genes or elements such as integrons and transposons) are found in more resistant bacteria such as *P. aeruginosa*, *A. baumannii*, and the

Enterobacteriaceae while those that are chromosomally-mediated are found in bacterial strains such as *Bacillus cereus* and *Stenotrophomonas maltophilia* and in obscure non-clinical bacteria such as *Aeromonas* species (Walsh *et al*., 2005; Thompson, 2010 and Toleman *et al*., 2005). MBL genes are important resistance determinants considering the fact that most of these genes are carried as mobile gene cassettes (which can easily be integrated into the chromosomes of other susceptible organisms) on class one integrons with the potential to spread to other clinically important bacteria. And because the MBL genes are mainly plasmid-borne, thier spread to the population of pathogenic organisms is of great concern and a menace to our ability to fight and treat a wide variety of Gram-negative infections (Toleman *et al*., 2005 and Carfi *et al*., 1995). Opportunistic organisms from the environment are known to ubiquitously express MBLs chromosomally, and the reason for this is still arcane (Walsh *et al*., 2005 and Tortola *et al*., 2005). These bacteria including *Bacillus* species, *Chryseobacterium* species, *Flavobacterium* species, *Serratia* species, and *Stenotrophomonas* species seldom cause serious infections but yet are known to express these carbapenem-hydrolyzing enzymes in varying amounts from one bacterium to another (Walsh *et al*., 2005).

MBLs are disseminated through genetic elements such as transposons, plasmids and integrons amongst clinically important bacteria and the extensive use of the carbapenems are known to be responsible for the worldwide spread of these enzymes. The extra-chromosomally-mediated (transposable) MBLs include: the imipenemase (IMP) MBLs, Verona-imipenemase (VIM) MBLs, Sao Paulo MBL (SPM-1), and the German-imipenemase (GIM) MBLs (Walsh *et al*., 2005). *Pseudomonas aeruginosa* infections are treated with carbapenems such as meropenem and imipenem, and the increasing usage of these antibiotics together with other expanded spectrum drugs has resulted in the development of imipenem and meropenem-resistant *P.*

aeruginosa and other MBLs in *Enterobacteriaceae* (Tortola *et al*., 2005 and Overturf *et al*., 2010). Metallo-beta-lactamases are Ambler Class B beta-lactamases (or carbapenemases) and they constitute mainly of the enzyme types: IMP and VIM MBLs which are found in organisms such as *P. aeruginosa*, *A. baumannii* and the *Enterobacteriaceae* (Overturf, 2010). Other carbapenemases include: the *Klebsiella pneumoniae* carbapenemases (KPC) which is an Ambler Class A enzyme, four serine carbapenemases (SME, NMC-A, IMI, and rare GES) – which are all Ambler Class A enzymes, and a last group of MBLs known as the OXA carbapenemases (Walsh *et al*., 2005). The OXA-carbapenemases are weakly active against carbapenems and are inhibited by clavulanate poorly. They are largely restricted to *Pseudomonas* species and *Acinetobacter* species, and are rarely found in *Enterobacteriaceae* (Thompson, 2010 and Overturf, 2010). The SME carbapenemases derive their name from *Serratia marcescens* and other *Serratia* species, and they are chromosomally encoded while the imipenem-2 (IMI) carbapenemases and Guyana extended spectrum (GES) variants together with KPC are plasmid mediated carbapenemases (Tortola *et al*., 2005 and Thompson, 2010). The non-metallo-carbapenemase (NMC) is also plasmid-mediated and they can be inhibited by clavulanic acid together with GES, SME, NMC and KPC carbapenemases. Of much concern is another group of novel MBLs known as the New Delhi Metallo-beta-lactamase (NDM-1) producing bacteria which carry the NDM gene and confer resistance to carbapenems (Deshpande *et al*., 2010). NDM-1 is usually found in members of the *Enterobacteriaceae* and they differ from other beta-lactamase enzymes and are so named because they are known to originate from India and Pakistan (Deshpande *et al*., 2010). The NDM-1 enzyme has been identified in people who returned to the UK, USA and other parts of the world after undergoing surgery/medication in India or Pakistan. The incidence of this type of MBL is known to have emerged in 2007 (Mahajan and Tandon, 2010).

2.5.1 Classification of metallo-beta-lactamases

Metallo-β-lactamases was first classified in 1980 as serine carbapenemases by the Ambler classification scheme for beta-lactamase enzymes (Walsh *et al*., 2005). The classification of metallo-β-lactamases (MBLs) is based on several properties that include the substrate profile and/or affinity of the enzymes (e.g. its ability to hydrolyze any of the carbapenems such as imipenem), their sensitivity to a chelating agent (e.g. ethylene diamine tetra-acetic acid, EDTA), and their lack of inhibition by serine β-lactamases (Walsh *et al*., 2005; Tortola *et al*., 2005 and Thompson, 2010). Aside the Ambler classification of beta-lactamases, the Bush's classification of beta-lactamase enzymes further classified MBLs into a separate group of beta-lactamases mainly based on their functional properties such as their amino acid configuration and active site of the enzymes. It is noteworthy that all metallo-beta-lactamases hydrolyze imipenem (Walsh *et al*., 2005). However, the ability of the MBLs or carbapenemases to hydrolyze or breakdown imipenem varies considerably amongst the different types of MBLs available. According to Walsh *et al*. (2005), the classification of MBLs at a molecular level and the standardization of their molecular structure are almost impossible because the MBLs are a disparate group of proteins. Generally, MBLs can either be plasmid-mediated or chromosomally-borne (Walsh *et al*., 2005). Those MBLs that are plasmid mediated harbour transferable MBL genes that can easily be transmissible to non-MBL-producing bacteria in a given environment; and such a phenomenon spur the spread of carbapenem resistant bacteria in a given habitat. The transferable MBLs (i.e. the plasmid mediated MBLs) include IMI or IMP, SME, KPC, GES, VIM (for: Verona integrons encoded metallo-β-lactamases), GIM (for: German imipenemase), SPM (for: Sao Paulo metallo-β-lactamases) and New Delhi metallo-β-lactamases (NDM). They are in the

molecular Class B1 of Ambler classification of enzymes and functional group 3 of Bush's classification of beta-lactamases.

2.5.2 Chromosomal metallo beta-lactamases

Chromosomally-borne MBL-producing bacteria have their resistance traits in the chromosome of the organism (Walsh *et al*., 2005). The genes that mediate carbapenem resistance in these organisms are rarely transferable to susceptible bacteria in the environment. Chromosomally-borne MBLs were initially detected in environmental and opportunistic pathogenic bacteria that had no hospital origin (Walsh *et al*., 2005). The first metallo-β-lactamases were detected in *Bacillus cereus* (BCI, BCII), *Chryseobacterium meningosepticum* (BlaB or GOB-1), *Chryseobacterium indologenes* (IND-1), *Aeromonas* spp (CphA) and *Stenotrophomonas maltophilia* (L1) as chromosomally encoded MBLs (Walsh *et al*., 2005). The chromosomally mediated enzymes are also often co-regulated with serine β-lactamases. For example, two species of a bacterium expressing MBLs can be found to produce two to three different antibiotic-hydrolyzing enzymes. In particular, both *Aeromonas hydrophila* and *Aeromonas veronii* bv. sobia produce three different beta-lactamases including penicillinase (which hydrolyze the penicillins), a cephalosporinase (which hydrolyze the cephalosporins), and an MBL (which hydrolyze the carbapenems) (Walsh *et al*., 2005). These enzymes are usually co-expressed and over-expressed in the bacteria when high-level beta-lactam resistant mutant strains are selected in the environment. SME, IMI and NMC are some commonly encountered chromosomally encoded metallo-β-lactamases (MBLs).

2.5.2.1 SME type MBLs

SME is the acronym for "*Serratia marcescens* metallo-β-lactamase"; and it denotes the MBL isolated or naturally found in the organism, *Serratia marcescens*. These enzymes were first discovered in *S. marcescens* isolates from England in 1982. There are currently three (3) SME types viz: SME-1, SME-2, and SME-3 (Walsh *et al*., 2005). These enzymes have been sporadically observed in *S. marcescens* isolates throughout the United States of America and in some other parts of the world. However, no clonal spread among these isolates has been observed.

2.5.2.2 IMI type MBLs

IMI is the acronym for "imipenem hydrolyzing beta-lactamases". IMI enzymes was first discovered in an *Enterobacter cloacae* isolate in the United States of America in 1984; and the enzymes has since been rarely discovered in clinical isolates of *E. cloacae* in the U.S.A, France and Argentina (Walsh *et al*., 2005). Several types of IMI enzymes including IMI-1 and IMI-2 exist in clinical pathogens. IMI-2 was reported in China, and the enzyme was plasmid encoded.

2.2.3.3 NMC type MBLs

NMC is the acronym for "not metalloenzyme carbapenemase". NMC-A enzyme was isolated from *Enterobacter cloacae* isolates in France during 1990 and the enzyme was subsequently reported from the United States of America and Argentina (Walsh *et al*., 2005). It is worthy of note that NMC-A and IMI have 97 % amino acid homology and are related to SME-1 with 70 % amino acid homology (Walsh *et al*., 2005). Though these enzymes can hydrolyze extended spectrum cephalosporins, their rate of hydrolysis is comparatively less. Hydrolysis of cefoxitin

by these enzymes is also inefficient; and cefoxitin and imipenem can also induce the production of these enzymes in a given pathogenic bacterium (Walsh *et al*., 2005; Thompson, 2010).

2.6 Transferable metallo beta-lactamases

Transferable MBLs are plasmid-borne (encoded) carbapenemases that are transmissible amongst bacterial species (Walsh *et al*., 2005). Pathogenic bacteria expressing these enzymes possess transferable MBL genes that can be passed on to susceptible bacteria in a given environment. Transferable MBLs can also be called mobile MBLs because of the ease with which they could be transferred from one organism to another through mobile genetic transfer elements including plasmids and transposons (Walsh *et al*., 2005). Transferrable MBLs was first discovered in Japan in a *Pseudomonas aeruginosa* isolate which possessed an IMP gene (Walsh *et al*., 2005). Transferable MBLs include KPC, GES, IMP, VIM, SPM, GIM, SIM (Seoul imipenemase) and NDM (Walsh *et al*., 2005; Thompson, 2010). Transferable or mobile MBLs have also been reported in Brazil, England, Hong Kong, Australia, Portugal, Malaysia, Canada, Italy, USA, Singapore and Taiwan (Walsh *et al*., 2005). Apart from *P. aeruginosa* isolates from which the first transferable MBLs was discovered, these multidrug antibiotic degrading or hydrolyzing enzymes can also be harboured in *Acinetobacter* species, *Klebsiella* species, *Enterobacter* species, *Escherichia coli, Proteus* species, *Providencia* species, *Shigella flexneri, Serratia marcescens* and *Alcaligenes xylosoxidans* (Walsh *et al*., 2005). Plasmid-borne MBLs have now attained public health significance because of their global spread; and the majority of these mobile MBLs are found as gene cassettes in these organisms.

2.6.1 KPC type MBLs

KPC is the acronym for "*Klebsiella pneumoniae* carbapenemase". KPC was first isolated in a *K. pneumoniae* isolate from the United States of America in 1996 (Tortola *et al*., 2005; Walsh *et al*., 2005). The resistance gene was associated with a large plasmid. There are currently over 12 known KPC enzymes. Though predominantly located or found in *K. pneumoniae* isolates, KPC enzymes have also been observed in *K. oxytoca, Salmonella enterica, Escherichia coli, Pseudomonas aeruginosa* and *Enterobacter cloacae* (Walsh *et al*., 2005; Thompson, 2010). KPC enzymes confer resistance to all penicillins, cephalosporins, aztreonam and imipenem. However, bacteria harbouring KPC enzymes still remain susceptible to inhibition by beta-lactamase inhibitors such as clavulanic acid (Walsh *et al*., 2005).

2.6.2 GES type MBLs

GES is the acronym for "Guyana extended spectrum". GES type MBLs was first observed in a *K. pneumoniae* isolate from French Guiana in 2000; and the enzymes of the GES family differ markedly from each other by 1-4 amino acid substitutions (Walsh *et al*., 2005). The genes encoding GES family of enzymes are located in integrons on the bacterial plasmids (Thompson, 2010; Tortola *et al*., 2005). GES type MBLs were initially taught to be ESBLs because of their exceptional ability in hydrolyzing extended spectrum cephalosporins. Currently there are over 22 known GES type MBLs; and these enzymes have also been observed in other bacteria aside *K. pneumoniae* including *P. aeruginosa* and *E. coli* isolates. GES type MBLs have also been reported in Greece, Portugal, South Africa, Japan, Korea and Argentina (Walsh *et al*., 2005).

2.6.3 IMP type MBLs

IMP is the acronym for "imipenem". IMP type MBLs are those class of MBLs that have action on imipenem (Walsh *et al*., 2005). They are transferable carbapenem resistance enzymes that were first detected in *P. aeruginosa* isolates from Japan in 1990 (Walsh *et al*., 2005; Thompson, 2010; Tortola *et al*., 2005). IMP type MBLs have also been reported in *Acinetobacter baumannii* and *Serratia marcescens* isolates aside *P. aeruginosa* from which they were first detected (Walsh *et al*., 2005). Over 37 different known IMP type MBLs occur around the world; and these enzymes are common among members of the bacterial family *Enterobacteriaceae* and *P. aeruginosa* isolates (Walsh *et al*., 2005).

2.6.4 VIM type MBLs

VIM is the acronym for "Verona integrons encoded metallo-β-lactamases". It is a class 1 integron associated MBLs that was first observed in a *P. aeruginosa* isolate from Italy in 1997 (Walsh *et al*., 2005; Thompson, 2010; Tortola *et al*., 2005). VIM type MBLs is closely related to BCII with only 39 % amino acid homology. VIM type MBLs is the second dominant group of acquired MBLs and it is known to be resistant to a range of beta-lactams including piperacillin, aztreonam, imipenem and ceftazidime (Walsh *et al*., 2005). Currently there are 34 known VIM type MBLs; and VIM-2 which was reported in a clinical isolate of *P. aeruginosa* from France is the most dominant MBL across Europe (Walsh *et al*., 2005; Tortola *et al*., 2005).

2.6.5 SPM-1 type MBLs

SPM is the acronym for "Sao Paulo metallo-β-lactamases". SPM-1 was first identified in a *Pseudomonas aeruginosa* isolate from Sao Paulo in Brazil (Walsh *et al*., 2005; Thompson,

2010). Genetic analysis has revealed that it is not a part of any integron but is associated with a new type of transposable structure (Walsh *et al*., 2005). *bla*SPM-1 is a part of mobile pathogenicity island located on a plasmid. SPM-1 type MBLs also has the ability to hydrolyze several beta-lactams including piperacillin, carbenicillin, penicillin, ampicillin, and cephalothin (Walsh *et al*., 2005). However, SPM-1 type MBLs is susceptible to aztreonam and clavulanic acid, a beta-lactamase inhibitor.

2.6.6 GIM-1 type MBLs

GIM is the acronym for "German imipenemase". GIM-type MBLs was first isolated from Germany in 2002. GIM type MBLs was isolated from *P. aeruginosa* clinical isolates in Germany; and they have no clear preference for any substrate and did not hydrolyze azlocillin, aztreonam, and the serine β-lactamase inhibitors (Walsh *et al*., 2005).

2.6.7 SIM-1 type MBLs

SIM is the acronym for "Seoul imipenemase". SIM type MBLs was first isolated from *Pseudomonas aeruginosa* isolates and *Acinetobacter baumannii* isolates during a large scale screening of imipenem resistant isolates in Seoul (Walsh *et al*., 2005; Tortola *et al*., 2005).

2.6.8 NDM-1 type MBLs

NDM is the acronym for "New Delhi metallo-β-lactamase". NDM-1 was first reported in 2009 from a *K. pneumoniae* isolate obtained from a Swedish patient of Indian origin, and who had received medical treatment in India (Walsh *et al*., 2005). NDM-1 is located on a 180 kb plasmid; and it expresses high-level resistance to all penicillins, cephalosporins, aztreonam, cefoxitin,

carbapenems and ciprofloxacin (Walsh *et al*., 2005; Overturf, 2010). NDM type metallo-β-lactamase enzyme is only susceptible to colistin. NDM-1 enzyme shares very little identity with other MBLs, with the most similar MBLs being VIM-1/VIM-2, with which it has only 32.4 % homology. Compared to VIM-2, NDM-1 displays tighter binding to most cephalosporins in particular, cefuroxime, cefotaxime, and cephalothin, and also to the penicillins (Walsh *et al*., 2005; Thompson, 2010). However, NDM-1 does not bind to the carbapenems as tightly as IMP-1 or VIM-2. NDM-1 not only is a new subclass of the B1 group of MBLs but also it possess novel amino acids near the active site, thus suggesting that it has a novel molecular structure from the other MBL types (Walsh *et al*., 2005; Overturf, 2010).

2.7 Epidemiology and treatment of infections caused by MBL-producing bacteria

The epidemiology of MBLs still remains largely unknown, but the enzymes have been detected in clinical isolates from some countries including Nigeria (Overturf, 2010; Walsh *et al*., 2005; Aibinu *et al*., 2007). MBLs started causing havoc in the world of bacterial resistance and became a major public health issue around the world (including USA, Greece, Italy, France, Germany, Israel, Poland, Spain, Hungary, India, UK) and more recently in Nigeria shortly after the introduction of carbapenems (drugs with exceptional intrinsic antibacterial activity and stability to most beta lactamases including ESBLs) into clinical medicine (Aibinu *et al*., 2007; Grundmann *et al*., 2010; Overturf, 2010; Walsh *et al*., 2005). MBL genes are first propagated in the genus *Pseudomonad's* (usually *P. aeruginosa*) before appearing in the *Enterobacteriaceae* including *K. pneumoniae* and *E. coli* (Walsh *et al*., 2005). In Nigeria, MBL have been detected and reported in clinical isolates of *P. aeruginosa* from Lagos, the Southwest Nigeria and in the southeastern part of Nigeria (Aibinu *et al*., 2007; Ejikeugwu *et al*., 2014). Strains of *K.*

pneumoniae and *E. coli* were confirmed to produce the VIM-1 type MBL in Spain, marking the first detection of MBL in that part of the world (Tortola *et al*., 2005). MBLs are very rare in the USA, but a recent study reported the presence of VIM-2 MBL-producing *P. aeruginosa* in a local hospital in Chicago (Lolans *et al*., 2005). In Iran, MBL-producing *P. aeruginosa* strains have also been detected in a recent study with about 50 % of *P. aeruginosa* strains showing MBL production phenotypically (Saderi *et al*., 2008). Elsewhere, it was also discovered that infections in intensive care units (ICUs) of a local hospital in India was caused by MBL-producing Gram-negative bacteria (Chakraborty *et al*., 2010). The discovery of a new type of metallo-beta-lactamase enzyme called the 'New Delhi Metallo-beta-lactamase-1' (NDM-1) in New Delhi, India in 2007 gave impetus to the devastating effect of this new form of resistance to the carbapenems (Mahajan and Tandon, 2010). The name 'New Delhi' was because the NDM-1 was first detected in a *K. pneumoniae* isolate of a 59 year old male patient who was from India but lives in Sweden and who often returned to India for medical treatment. But there is still controversy as to where NDM-1 actually originated from, and this has sparked a bone of contention between the Indian (because of the name 'New Delhi' that this type of carbapenemase was ascribed), British and Swedish government as to the originality or source of this type of carbapenemase enzyme (Mahajan and Tandon, 2010). *Enterobacteriaceae* (including *E. coli* and *K. pneumoniae*) are less clinically important in terms of MBL production than *Pseudomonas aeruginosa* and *Acinetobacter baumannii* whose genes are easily transferable; and MBL-producing bacteria have a broad spectrum resistance profile showing resistance to not only the expanded spectrum cephalosporins but also to other drug classes such as aminoglycosides and fluoroquinolones (Walsh *et al*., 2005). Nevertheless, MBL-producing bacteria still remains susceptible to polymyxins and tigecycline (HPA, 2008). No optimal line of treatment has yet

been ascertained for MBL-mediated infections as they have not been any extended *in vivo* studies to this regard.

2.8 Overview of AmpC enzymes

According to Akujobi *et al*. (2012), AmpC beta-lactamase enzymes began to gain importance in the health sector in the early 1970s as one of the mediators of antimicrobial resistance in Gram-negative bacteria including *Escherichia coli* and *Pseudomonas aeruginosa* (Akujobi *et al*., 2012). Chromosomal AmpC enzymes (which can also be called inducible AmpC enzymes) and plasmid-borne AmpC enzymes are the two main types of AmpC beta-lactamases that exist amongst bacteria especially in Gram-negative organisms in which these multidrug resistant enzymes are produced (Walsh *et al*., 2005; Akujobi *et al*., 2012). Inducible AmpC beta-lactamases are usually seen in *Citrobacter* species, *Morganella* species, *Enterobacter* species and *Serratia marcescens* while the plasmid-mediated AmpC enzymes are seen in other enteric and non-enteric bacteria including *Escherichia coli, Klebsiella* species and *Pseudomonas aeruginosa* (Akujobi *et al*., 2012). AmpC enzymes are broad-spectrum beta-lactamase enzymes that are usually encoded on bacterial chromosome, and which are active on cephamycins (e.g. cefoxitin and cefotetan) and oxyimino-β-lactam agents (Jacoby and Munoz-Price, 2005). They can also be plasmid encoded; and AmpC enzymes like other extended or expanded beta-lactamases such as ESBLs and MBLs confer on pathogenic Gram-negative bacteria the exceptional ability to be resistant to a wide array of beta-lactam drugs and non-beta-lactams (Jacoby and Munoz-Price, 2005). AmpC beta-lactamases are bacterial enzymes that hydrolyze third-generation extended spectrum cephalosporins and cephamycins (e.g. cefoxitin), thus

engendering antimicrobial resistance to these categories of antibiotics. AmpC beta-lactamases are differentiated from extended spectrum beta-lactamases (ESBLs) by the ability of the former (i.e. AmpC enzymes) to hydrolyze cephamycins (e.g. cefoxitin) and their lack of inhibition by clavulanic acid (Rudresh and Nagarathnamma, 2011). The expression of AmpC enzyme is typically inducible in several *Enterobacteriaceae* and other Gram-negative bacteria including but not limited to *Escherichia coli*, *Klebsiella* species, *Enterobacter* species and *Pseudomonas aeruginosa;* and the production of this enzyme facilitates the emergence under antibiotic pressure of highly resistant but stable depressed mutants of the organisms (Lafi *et al*., 2012). And these highly resistant but stably depressed mutants of the organisms have the ability to hydrolyze extended spectrum cephalosporins and other beta-lactam agents even though they may still remain susceptible to the carbapenems (e.g. imipenem and meropenem). The genes that codes for the production of AmpC enzymes in bacteria are normally chromosomally-mediated. Plasmid-mediated AmpC enzyme production in bacteria is also possible amongst bacterial organisms through genetic transfer mechanisms such as conjugation and transduction (Walsh *et al*., 2005; Jacoby and Munoz-Price, 2005). Thus, the genes for AmpC enzyme production can be transferred to other susceptible bacteria from organisms harbouring them through plasmids. *Klebsiella pneumoniae* is one of the few Gram-negative bacteria which do not possess a chromosomal AmpC beta-lactamase gene, but the organism can still acquire the gene for the enzyme production via the transfer of AmpC-containing plasmids from another AmpC-beta-lactamase positive bacteria. AmpC enzymes are intrinsic cephalosporinases found on the chromosomal DNA of many Gram-negative bacteria and opportunistic bacteria; and they confer resistance to a wide array of antibiotics including penicillins, 2^{nd} and 3^{rd} generation cephalosporins, cefoxitin and beta-lactamase inhibitors such as amoxicillin-clavulanic acid.

However, AmpC enzyme-producing bacteria are still susceptible to the carbapenems and fourth generation cephalosporins (e.g. cefepime). According to the European Food Safety Authority (EFSA), the concern today around the world that is of public health importance is not that AmpC positive bacteria exist but that a growing number of the AmpC enzymes have escaped on to plasmids and can be transferred to other bacteria within the environment or hospital setting (EFSA, 2011). The failure to detect AmpC beta-lactamase enzymes has no doubt contributed to the uncontrolled spread of AmpC positive bacteria; and in most of the cases these have also contributed to the treatment failures experienced in patients infected with such organisms (Jacoby and Munoz-Price, 2005). Of particular concern are the limited treatment options for infections caused by Gram-negative resistant bacteria leading to antibiotic selection pressure and consequent risk of the emergence of antibiotic resistant pathogens. Studies have shown that the onslaught of AmpC resistance represents a major challenge for physicians as these high-profile beta-lactamase hydrolyzing enzymes renders third-generation cephalosporins and the cephamycins increasingly ineffective in the treatment of bacterial related infections caused by AmpC-producing bacteria (Livermore and Brown, 2001; Lafi *et al*., 2012; Hemalatha *et al*., 2007; Shevade and Agrawal, 2013). The detection of AmpC beta-lactamases in bacterial isolates still remains problematic especially in those organisms that produce or have extended spectrum beta-lactamases (ESBLs); and this is due in part to the fact that ESBL-producing bacteria that also harbour genes for AmpC enzyme production mask the production of AmpC enzymes (Jacoby and Munoz-Price, 2005). This is why the Clinical Laboratory Standard Institute (CLSI) and other researchers recommend the use of several antimicrobial agents that also incorporates chelating agents such as ethylene diamine tetraacetic acid (EDTA) and boronic acid for the detection of AmpC enzymes from both environmental and hospital isolates (CLSI, 2011;

Thompson, 2010; Walsh *et al*., 2005; Livermore and Brown, 2001; Lafi *et al*., 2012; Hemalatha *et al*., 2007; Shevade and Agrawal, 2013). It is even more worrisome when clinical microbiology laboratories in some hospitals fail to detect these pathogens from clinically important specimens and/or other environmental samples. Bacterial resistance to the cephalosporins (especially 3rd-generation cephalosporins) and the cephamycins should raise a suspicion for possible production of AmpC beta-lactamases that warrants phenotypic confirmation (Jacoby and Munoz-Price, 2005). The confirmation of AmpC production in clinical and/or environmental pathogens is important for the patient's welfare because it will support the susceptibility test result by permitting the reservation of broad spectrum antibiotics (e.g. carbapenems) for more serious and complicated bacterial disease. And such a confirmatory test coupled with the antimicrobial susceptibility test results will help to select targeted narrow spectrum antibiotics for treatment rather than using drugs with broad spectrum activity, thereby minimizing the risk of selecting for, or promoting the development and spread of antimicrobial resistant bacteria pathogens in either the community or hospital environment.

2.8.1 Chromosomal AmpC enzymes

Chromosomal AmpC enzymes are AmpC beta-lactamases that are mediated by *AmpC* genes – which is usually located on the chromosomal DNA of the host bacteria (Walsh *et al*., 2005). The *ampC* genes are intrinsically located in the DNA of bacteria producing these enzymes; and they mediate the hyper-production of AmpC beta-lactamases in pathogenic bacteria. As aforementioned, bacterial organisms that harbour the *AmpC* genes include *Enterobacter* species, *Serratia marcescens, Citrobacter freundii, Morganella morganii, Hafnia* species and

Providencia species (Walsh *et al*., 2005; Jacoby and Munoz-Price, 2005). *Escherichia coli* and *Shigella sonnei* contain a chromosomal *AmpC* gene but due to a lack of the regulatory gene *AmpR,* the *AmpC* gene in *E. coli* and *S. sonnei* is not fully expressed in amounts large enough to confer resistance as is applicable in the case of the other organisms that hyper-produce AmpC beta-lactamases (*C. freundii*, *S. marcescens* and *Enterobacter* species) (Bush *et al.*, 1995; Bush and Jacoby, 2010; Walsh *et al*., 2005; Hemalatha *et al*., 2007). Though AmpC beta-lactamases are produced at low levels and may not cause resistance, these enzymes can still be produced at very high levels in other bacteria and cause resistance. Chromosomal AmpC beta-lactamases can be produced inducibly or constitutively. The inducible expression of *AmpC* gene occurs when the AmpC beta-lactamase is produced at a high level; and this inducibility usually occurs when the bacterial organism is exposed to inducing agents such as cephamycins and other broad-spectrum beta-lactam agents that drive its production (Walsh *et al*., 2005; Jacoby and Munoz-Price, 2005). However, this induction is usually temporary and the production of AmpC enzymes in such scenarios may be reversed when the antimicrobial agent inducing its production in the pathogens environment is removed. However, when mutation occurs in bacterial organisms producing AmpC beta-lactamases, the AmpC gene becomes permanently expressed at very high levels; and such pathogens are called permanently depressed mutants (Walsh *et al*., 2005; Jacoby and Munoz-Price, 2005; Bush *et al.*, 1995; Bush and Jacoby, 2010). These permanently depressed mutants of AmpC producers harbour plasmids that encode the genes responsible for the enzyme production; and these resistant traits can be passed on to susceptible bacteria in the environment through genetic transfer mechanisms such as conjugation or transformation.

2.8.2 Plasmid-mediated AmpC enzymes

Plasmid-mediated AmpC beta-lactamases are beta-lactamases produced by bacterial pathogens devoid of chromosomal *AmpC* genes but that may have acquired resistance plasmids of the resistance traits via antibiotic selection pressure, mutation or through gene transfer mechanisms (Walsh *et al*., 2005; Jacoby and Munoz-Price, 2005; Bush *et al.*, 1995; Bush and Jacoby, 2010). Plasmid-mediated AmpC beta-lactamases and/or genes have been known since 1989; and they have also been discovered around the world from both clinical and non-clinical bacterial isolates (Jacoby, 2009). The plasmid-mediated AmpC beta-lactamases unlike the chromosomal AmpC enzymes confer resistance to a broad spectrum of beta-lactams including ampicillin, piperacillin, temocillin, cephalothin, cefotaxime, ceftazidime, cefoxitin, cefotetan, cefmetazole, moxalactam, aztreonam, cefepime, cefpirome, imipenem, and meropenem (Jacoby, 2009). They are usually found in bacterial organisms that do not naturally carry the chromosomal *AmpC* gene. Plasmid-mediated AmpC beta-lactamases have also been detected in *Klebsiella* species, *Escherichia coli, Proteus* species and *Salmonella* species (Thompson, 2010; Hemalatha *et al*., 2007; Shevade and Agrawal, 2013). Plasmid-mediated AmpC beta-lactamases were first recognized in the early 1980s, and since then, these phenotypic traits of antimicrobial resistance have spread among members of the family *Enterobacteriaceae* and even to non-enteric bacteria such as *P. aeruginosa* (Rudresh and Nagarathnamma, 2011). Epidemiological studies have shown that AmpC enzyme producing bacteria are recovered from hospitalized patients after several days of admission to the hospital; and affected patients have often had prolonged stays in intensive care units (ICUs) and other high dependency units of hospitals (Manchanda *et al*., 2006; Lafi *et al*., 2012; Shevade and Agrawal, 2013; Hemalatha *et al*., 2007). Genes encoding the production of

AmpC beta-lactamases in bacteria are found on transferable plasmids, and these genetic elements can be transferred to other sensitive bacterial isolates in an environment. Thus, the determination of AmpC enzyme production from both clinical and environmental isolates is important because such a practice will help to reduce the incidence of the emergence of more resistant strains of bacteria, reduce the spread of resistant pathogens in the community and/or environmental settings, and thus help to guide physicians on the best choice of antimicrobial therapy to manage bacterial infections or diseases caused by these organisms and other resistant bacterial infections.

2.8.3 Treatment of infections caused by AmpC-producing bacteria

There is no consensus on the actual therapeutic measure for infections caused by AmpC-producing bacteria since AmpC-positive bacteria are multidrug resistant and thus are resistant to most first-line and second-line drugs. Bacterial strains with *AmpC* genes are often resistant to multiple antibiotics; and this makes the selection of an effective antibiotic difficult in the face of an infection by AmpC-producing bacteria (Jacoby, 2009). As a general rule and due to the multidrug resistant nature of AmpC-producing bacteria, beta-lactam/beta-lactamase inhibitor combinations and most cephalosporins and penicillins should be totally avoided because of the *in vitro* resistance, and the potential for AmpC induction or selection of high-enzyme-level mutants, and recorded poor clinical outcomes with these drugs (Jacoby, 2009). However, cefepime can be used for the treatment of bacterial infections caused by AmpC-producing bacteria because cefepime is a poor inducer of AmpC beta-lactamase production in bacteria; and the drug rapidly penetrates through the outer cell membrane of the target organism (Jacoby, 2009). Cefepime is also poorly hydrolyzed by AmpC beta-lactamase. Temocillin, a 6-α-methoxy derivative of ticarcillin is active *in vitro* against many *Enterobacteriaceae* irrespective of whether the AmpC

gene (*AmpC*) is chromosomally-borne or plasmid-mediated (Jacoby, 2009). The carbapenems can also be used for the treatment of infections caused by AmpC-producing bacteria. But the use of the carbapenems is usually followed by the emergence of carbapenem-resistant bacteria especially carbapenem-resistant *K. pneumoniae* (Jacoby, 2009). Fluoroquinolones can also be used to treat infections caused by AmpC-producing bacteria if the isolate shows *in vitro* susceptibility to the test agent; and fluoroquinolones is usually used for non-life-threatening diseases such as urinary tract infections (Jacoby, 2009). Tigecycline has also been reported to show good activity *in vitro* against AmpC-producing bacteria and thus could be used to treat infections caused by AmpC-producing bacteria (Jacoby, 2009).

2.9 Reasons for detecting multidrug resistant organisms in the microbiology laboratory

Multidrug resistant organisms inclusive of those that express metallo-beta-lactamase and AmpC enzymes are of immense clinical significance because they degrade a wide variety of available antimicrobial agents (particularly antibiotics used for treating bacterial related infections). Some of the reasons why such pathogenic organisms should be promptly detected from clinically relevant specimens of sick patients and/or environmental isolates are as follows:

- Detection of drug resistant organisms limits the unnecessary use of antibiotics.
- It guides physicians on the course or choice of antibiotic therapy for patients harbouring multidrug resistant organisms.
- It helps to manage high-risk patients effectively.
- It reduces the hospital stay or time of sick patients. Prolonged hospitalization predisposes sick people especially those in the intensive care units (ICUs) and other

high-dependency units (HDUs) of hospitals to a wide variety of nosocomial infections. This increases the suffering of the sick patient as well as cost of treatment.

- Detection of multidrug resistant bacteria prevents the emergence and spread of infectious disease and drug-resistant microbes in both the hospital environment and community.

When a sick patient visits the hospital and meets the doctors or nurses to find out what the problem is; it is the scientists in the laboratory (particularly the microbiologists) that are usually the first to know the infecting microorganism(s), and the results of the antimicrobial susceptibility test (AST) performed on the infecting pathogen(s) guides physicians on the choice of effective drugs to administer to the sick patient(s). However, it is unfortunate that most hospitals in Nigeria do not go beyond their routine AST technique to type down or detect the existence of multidrug resistant bacteria from clinically important specimens especially amongst very sick patients that have defied several antibiotic therapies. There is possibility that pathogenic bacteria expressing metallo-beta-lactamase and AmpC enzymes may be responsible for some infectious diseases that cause morbidity and mortality amongst sick people in this part of the world. But the inability of our hospital laboratories to be on the lookout for such multidrug resistant organisms may have allowed these microbes to emerge and spread within the hospital environment and community undetected. Antibiotic usage especially irrationally allows pathogenic bacteria and even susceptible bacteria to develop resistance to antimicrobial therapy; and such organisms continue to thrive even in the face of potent antimicrobial onslaught (WHO, 2001). Such a development portends danger for the health sector especially in this part of the world. It is therefore critical to put in place antibiotic stewardship in our hospitals; and our

laboratories should be well equipped to detect multidrug resistant bacteria from patient's samples in order to ensure the proper wellbeing of sick people. Antibiotic usage in livestock production and fish farming should also be discouraged since this practice allows bacterial pathogens to develop resistance through selective pressure and undue exposure of microbes to antimicrobial agents (WHO, 2001). When antibiotic usage is decreased, the resistance of microorganisms to these agents will also be on the decline since the irrational usage of drugs allows microbes to develop resistance via selective pressure.

2.10 Veterinary antimicrobials and the use of antibiotics in animal production

Veterinary antimicrobial agents are those drugs that are solely used for the treatment of animals, and have no application in human medicine. Nevertheless, some antimicrobial agents strictly reserved for human treatment have found their way into veterinary practice where animal and poultry keepers utilize these antimicrobials to encourage the growth and proper development of their animals. Such scenarios have encouraged the emergence and spread of drug resistant bacteria in the community; and these pathogens are responsible for a handful of community-acquired bacterial infections (Prescott *et al*., 2000; WHO, 2001, Walton, 1983). Veterinary antimicrobial agents have in so many ways contributed to the development of resistant strains of bacteria in human population, and this phenomenon is largely attributable to the incessant and irrational use of antimicrobials in poultry production, fish farming and animal/livestock production. Though antimicrobial agents meant for veterinary practice are used to manage infectious diseases in these animals, the antimicrobial residues in the animals being treated could still encourage the development of resistant bacteria that thrived in the face of the antimicrobial onslaught (Prescott *et al*., 2000; Usha *et al*., 2010). This factor coupled to the use of antibiotics

as growth promoting agents in animal/livestock production poses greater health risks in human populations who consume these animals as food (Usha *et al*., 2010). Resistant bacteria can spread from animals to humans through direct body contact with the animals as wells as consumption of meat from such animals. As aforementioned, antimicrobial agents are used in veterinary practice to treat and prevent microbial infection as well as to improve the efficiency and growth of food-producing animals including cattle, poultry birds and other livestock. But these antimicrobial agents are used in most cases without a prior antimicrobial susceptibility test on the isolated pathogen (as is applicable in human medicine before the administration of antibiotics); and this allows the farmer to use these agents irrationally and extensively. These scenarios encourages the development of drug resistant microorganisms in the animals; and humans become infected with these pathogens through several means that includes the consumption of animal milk, meat and other animal products (Usha *et al*., 2010). In order to slow the development of drug resistant bacteria in food-producing animals and in the non-hospital environment such as in abattoirs and poultry farms, it is critical to develop other sustainable measures that will rule out the use of antibiotics as growth promoting agents in the rearing of these animals. Vaccination, improved sanitation and the control of infectious diseases amongst the animals should be seen as alternative methods of improving animal yield and growth instead of using antibiotics for such purposes. The use of antibiotics especially those meant for human medicine should be restricted from veterinary practices. The need for using antibiotics for animal/livestock production can be reduced when these measures are instituted in the veterinary practice; and this will go a long way in containing the development and spread of drug-resistant bacteria in the community.

2.11 Factors that encourage the development and spread of resistance in the community and hospital environment

Several human, animal, microbial and environmental factors mediate the emergence and transmission of drug resistant pathogenic microorganisms in both the community and hospital environments. In addition to these factors, the indiscriminate and/or misuse of antimicrobial agents (especially those meant for human medicine) in the upbringing and rearing of livestock's and poultry birds greatly contribute to the emergence and spread of drug resistant bacteria. Naturally, some microbes are resistant to antimicrobial agents because of the genetic makeup of their structure. The human factors that contribute to the emergence and spread of antibiotic resistant bacteria include but not limited to the misuse of antibiotics, self prescription of drugs and the use of these agents as growth promoting factors in animal husbandry and other veterinary services (Usha *et al*., 2010). The dearth of knowledge regarding multidrug resistant bacteria including those that produce metallo beta lactamases and AmpC beta lactamases in our region coupled with the inability of most of our hospitals in detecting and reporting these resistant traits makes the status quo to prevail. According to Usha *et al*. (2010), patient-related factors are the major driving force of inappropriate antimicrobial use – since many patients believe that new and expensive drugs are more efficacious in combating microbial infections than older drugs. And this makes such patients to prefer expensive drugs with broader spectrum of activity over cheaper ones with narrow spectrum of activity. The indiscriminate use of these newer antimicrobial agents especially without doctor's prescription backed by a proper antimicrobial susceptibility test result helps novel antibiotic resistant genes and/or traits to emerge and spread. Similarly, most patients especially in developing nations resort to self medication due to the high

cost of medical care amongst other factors. The problem with self medication is that self medicated antimicrobials may be unnecessary, inadequately dosed and may not contain adequate amount of the active drug required for the killing or inhibition of the growth of the infecting pathogen *in vivo* (Usha *et al*., 2010). In some scenarios, patients under antimicrobial therapy fail to comply with the recommended and prescribed medication for their sickness, and some even stop to take their medication once they observe a kind of relief from their disease or infection. All these factors create the environment necessary for pathogens to develop resistance to these antimicrobial agents.

2.12 Prevention and control of antimicrobial drug resistance

It is true that antimicrobial resistance is usually a natural biological phenomenon associated with the growth of microorganisms. An inevitable untoward effect associated with the use of antimicrobial agents especially antibiotics is the emergence and spread of resistant pathogenic bacteria; and this development as aforementioned is seen as a natural biological phenomenon that is prevalent amongst microbial evolution. Microbes particularly bacteria acquire resistant genes from their environment through different genetic transfer mechanisms especially from resistant bacteria, and the underuse and/or overuse of these antimicrobial agents contributes significantly to the development and spread of drug resistant bacteria since bacteria acquire resistance through selective pressure. On the other hand, the incessant and undue use of antimicrobial agents in animal/livestock production especially as growth promoting agents (GPA) is not helping matters since antibiotic residues in these animals (following the usage of antibiotics in their rearing) could serve as possible route through which these drug resistant

microbes could develop and become transferred to humans who eat or come in contact with the animals (Usha *et al*., 2010, EFSA, 2011, WHO, 2001). The proper monitoring of the emergence and spread of antimicrobial resistance amongst animals and poultry birds through proper screening and detection of multidrug resistant bacteria from environmental samples and/or isolates is critical to controlling the emergence and spread of drug resistant bacteria in the community, and this will also help to assuage or stop the occurrence of community-acquired infections due to these organisms.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacteriological media and reagents: The culture media used in this research project include: Cetrimide selective media for the selective isolation of *Pseudomonas aeruginosa*, Mueller Hinton (MH) agar (Oxoid, UK), nutrient agar (Oxoid, UK), nutrient broth (Oxoid, UK), eosin methylene blue (EMB) agar (Oxoid, UK), MacConkey agar (Oxoid, UK), urea/urease agar (Oxoid, UK), and Simmon's citrate agar (Oxoid, UK). Other reagents include nitrocefin test sticks (Oxoid, UK), peptone water base and 0.5 McFarland Equivalence Turbidity Standard.

3.1.2 Instrument and Equipment: The equipment and instrument used in this research work include: 2720 thermal cycler (Applied Biosystems, Life technologies, USA), gel electrophoresis machine (Genlab, UK), hot air oven (Genlab, UK), incubator (Merck, Germany), UV illuminator (Genlab, UK), refrigerator, -20^{o}C freezer (Merck, Germany), microscope (Olympus, Germany), water bath (Fisher Scientific, USA), microcentrifuge machine (Fisher Scientific, USA), vortexing machine (Merck, Germany), and autoclave (Medica Instrument MFG. Co, Equitron, India). Other instrument include pipetting tips, micro-pipetting devices (Fisher Scientific, USA), bijou bottles, staining rack, analytical balance (Fisher Scientific, USA), test tube racks, aluminium foil, laboratory forceps, measuring rulers, indelible markers, paper tapes, Petri dishes, swab sticks, salvage papers, inoculating loop, conical flasks, measuring cylinder, glass slides, beakers, cotton wool, McCartney bottles, 10 ml and 20 ml syringes.

3.1.3 Reagents and solvents: The reagents and solvents used for this research project include: lugols iodine, crystal violet stain, safranin stain, acetone, immersion oil, ethylene diamine tetraacetic acid (EDTA), glycerol, distilled water, methyl-red reagent, voges-proskauer (VP) reagent, oxidase reagent, indole reagent, and peptone water.

3.1.4 Antibiotics: The antibiotic disks used in this study include: imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), ertapenem (ETP, 10 µg), cefoxitin (FOX, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CRO, 30 µg), cloxacillin (OB, 500 µg), ciprofloxacin (CIP, 10 µg), aztreonam (ATM, 30 µg), ampicillin (AMP, 10 µg), nitrofurantoin (F, 10 µg), oxacillin (OX, 10 µg), ofloxacin (OFX, 10 µg), amikacin (AK, 10 µg) and gentamicin (CN, 10 µg). All antibiotic disks were in the single disk format; and they were all procured from Oxoid Limited (Oxoid, UK).

3.1.5 Quality Control Strains: The quality control strains used in this research project include: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 10145. All quality control strains were procured from Oxoid Limited (Oxoid, UK).

3.1.6 PCR reagents and materials: The PCR reagents and materials for molecular studies used for the gene amplification process include: nuclease-free water, magnesium chloride ($MgCl_2$), agarose powder (Inqaba biotec, South Africa), electrophoresis machine (Fisher Scientific, USA), gel casting combs, gel casting tray, microcentrifuge tubes/Eppendorf (PCR) tubes, 70 % ethanol, micropipettes, pipetting tips, vortexing machine, ethidium bromide, gel electrophoresis tank, biosafety laminar flow cabinet (Fisher Scientific, USA), salvage paper, ice

cubes/packs, coloured loading dye/buffer, distilled water, Taq polymerase (Inqaba biotec, South Africa), Tris boric acid (TBE) buffer, 100 base pair (bp) DNA (molecular) ladder (Inqaba Biotechnical Industries, South Africa), PCR buffer (Inqaba Biotechnical Industries, South Africa), deoxynucleotide triphosphates (dNTPs), and Zymo Plasmid miniprep kit (Epigenetics Company, USA) for isolation of plasmid DNA (Inqaba Biotechnical Industries, South Africa). All PCR reagents and materials were procured from Inqaba Biotechnical Industries (Inqaba biotec, South Africa). [**The primer quotation and gene sequence of the oligonucleotide primers used for the amplification of AmpC genes and MBL genes is shown in Appendix IV**].

3.2 METHODS

3.2.1 Determination of Sample Size: Sample size determination for this study was determined by the Cochran's formular. And this was based on previously reported prevalence rates of Gram-negative bacteria producing multidrug resistant enzymes including metallo beta-lactamase (MBL) and AmpC enzymes in some parts of Nigeria (Abd El-Baky *et al*., 2013; Aibinu *et al*., 2007; Ejikeugwu *et al*., 2014; Akinduti *et al*., 2012). These studies reported prevalence's of between 10-50 % of MBL and AmpC enzyme production phenotypically amongst Gram-negative bacteria from both clinical and environmental isolates. In order to get sample size for this study, we pegged the prevalence rate of the production of AmpC enzyme and MBLs at 60 %. Sample size was done at 95 % confidence interval (CI) at a 0.05 precision; and the following formular (Cochran's formular) was used to evaluate this:

$$\mathbf{n = \frac{Z^2pq}{e^2}}$$

Where:

n = sample size

Z = standard normal deviation at 95 % confidence interval (which was 1.96)

p = proportion of target population (prevalence estimated at 60 %; this implies 60/100 = 0.6)

q = alternate proportion (1-p), which was calculated as: 1 – 0.6 = 0.4

e = desired level of precision (degree of precision/significance). This was taken as 0.05

Thus, using the sample size determination formular: $\mathbf{n = \frac{Z^2pq}{e^2}}$

$$\mathbf{n} = (1.96 \times 1.96) \times 0.6 \times 0.4 \,/\, (0.05 \times 0.05) = \mathbf{368.7936}$$

Thus, about 370 environmental samples (including cloacal swabs of poultry birds, anal swabs of cow and swab samples from abattoir tables) were needed for this research work.

3.2.2 Sampling: A total of three hundred and seventy (370) environmental samples were randomly collected from various abattoir tables (n=130), anal region of cow (n=120) and the cloacae of poultry birds particularly broilers (n=120) in Abakaliki metropolis over a one year period (July, 2015 – June, 2016) for this study. Overall, 120 rectal/cloacal swab samples was each collected aseptically from the cloacae of poultry birds and anal region of cows respectively while 130 swab samples were collected from the slaughter tables of abattoirs feacally contaminated with feacal samples of slaughtered animals. Ethical approval and/or clearance for obtaining the samples were not required since the animals were killed by the animal sellers for business. However, oral consent was obtained from the owners of the animals after properly explaining to them the reason for the sample collection; and oral approval was given to collect samples from the cloacae of poultry birds and anal swab of cows respectively. To get the swab samples, sterile swab stick(s) soaked in physiological (normal) saline was used to swab the cloacal and rectal region of the broilers and cows respectively; and the swabs containing the samples were each returned to their respective containers and properly labeled for identification. The slaughter benches of abattoirs were also aseptically swabbed with sterile swab stick(s); and each of the swabs containing the samples was aseptically returned to their respective containers and labeled properly. All the swab samples were transported not later than one hour after collection to the Applied Microbiology Laboratory Unit of Ebonyi State University, Abakaliki (Presco Campus) in their respective containers after collection; and they were each processed

within 24 hours after collection. Each of the samples were aseptically inoculated in single strength nutrient broth (Oxoid, UK) in labeled test tubes and incubated at 30^{o}C for 18-24 hrs prior to bacterial isolation (Cheesbrough, 2006). Bacterial growth in the inoculated test tubes was identified by the presence of turbidity after incubation. Tubes showing turbidity were each subcultured onto solid culture media plates for the isolation of the test bacteria used in this study.

3.2.3 Isolation of *Pseudomonas aeruginosa*: The environmental swab samples which included cloacal swab samples, samples from abattoir tables and rectal swabs of cow were each cultured in 5 ml single strength of nutrient broth (Oxoid, UK) and incubated overnight at 30^{o}C. And a loopful of the specimen or turbid solution was plated aseptically onto cetrimide selective agar (Oxoid, UK) plate(s) for the selective isolation of *Pseudomonas aeruginosa*. The culture plates were incubated at 30^{o}C for 18-24 hours. Suspected colonies of *P. aeruginosa* were subcultured onto freshly prepared cetrimide agar plate(s) for the isolation of discrete colonies of *P. aeruginosa*; and the presence of *P. aeruginosa* on the culture plates was determined qualitatively based on the colonial morphology or characteristics of the colonies on cetrimide plates. *P. aeruginosa* isolates produces greenish pigmentation on cetrimide selective agar due to their production of pyocyanin and pyoverdin pigments (Cheesbrough, 2006). Single colony of the suspected *P. aeruginosa* isolates from the cetrimide selective agar plate(s) was purified on freshly prepared nutrient agar (Oxoid, UK) plate(s); and the positive culture was selected for biochemical testing and microscopical examination using oxidase test, methyl red test, voges proskauer test and Gram staining technique (Cheesbrough, 2006). The *P. aeruginosa* isolates were stored in nutrient agar slants in McCartney bottles and kept in the refrigerator at ambient

temperature until needed. Isolated *P. aeruginosa* isolates were subsequently subcultured onto freshly prepared nutrient agar slants every two months for proper preservation of the isolates.

3.2.4 Isolation of *Escherichia coli*: To isolate *Escherichia coli* from the environmental swab samples (i.e. cloacal swabs of poultry birds, swabs of feacally-contaminated abattoir benches/tables and swabs of anal region of cow), a loopful of the turbid solution from the overnight nutrient broth culture were aseptically plated onto eosin methylene blue (EMB) agar and MacConkey agar (Oxoid, UK) plates. The culture plates were incubated at 30^{o}C for 18-24 hours. After incubation, suspected colonies of *E. coli* were aseptically subcultured onto freshly prepared EMB and MacConkey agar plate(s); and the *E. coli* colonies were presumptively identified based on their colonial morphology and appearance on culture media such as lactose-fermentation - resulting in the formation of pinkish colonies on the agar plate(s). *E. coli* also produces colonies with metallic green sheen on EMB agar and lactose-fermenting colonies on MacConkey agar plates (Cheesbrough, 2006). Single colony of the suspected *E. coli* from the MacConkey agar plates was purified on nutrient agar (Oxoid, UK) plate(s); and the positive cultures were microscopically identified using Gram staining technique. The cultures were also biochemically identified as *E. coli* using indole test, methyl red test, and voges proskauer test (Cheesbrough, 2006). The *E. coli* isolates was stored in nutrient agar slants in McCartney bottles and kept in the refrigerator at ambient temperature until further studies. Isolated *E. coli* isolates was subsequently subcultured onto freshly prepared nutrient agar slants every two months for proper preservation of the isolates.

3.2.5 Isolation of *Klebsiella* species: To isolate *Klebsiella* species from the environmental samples (i.e. cloacal swabs of poultry birds, swabs of feacally-contaminated abattoir benches/tables and swabs of anal region of cow), a loopful of the turbid culture in the test tubes containing 5 ml double strength of nutrient broth (Oxoid, UK) was aseptically plated onto freshly prepared MacConkey agar (Oxoid, UK) plate(s) and eosin methylene blue (EMB) agar plates. The culture plates were incubated for 18-24 hours at 30^{o}C. Suspected colonies were subcultured onto freshly prepared culture media plates of MacConkey agar and eosin methylene blue (EMB) agar; and the culture plates were then incubated at 30^{o}C for 18-24 hours. *Klebsiella* species produce small, circular, elevated and mucoid colony on MacConkey agar; and on EMB agar, *Klebsiella* species colonies do not express metallic sheen colouration but they are also mucoid on EMB agar (Cheesbrough, 2006). Single colonies of the suspected *Klebsiella* species isolates were purified on nutrient agar (Oxoid, UK) plates; and the positive culture was tested microscopically and biochemically using urease test, methyl red test, voges proskauer test, citrate test and Gram staining technique (Cheesbrough, 2006). The *Klebsiella* species isolates was stored in nutrient agar slants in McCartney bottles and kept in the refrigerator at ambient temperature until further studies. Isolated *Klebsiella* species isolates was subsequently subcultured onto freshly prepared nutrient agar slants every two months for proper preservation of the isolates.

3.2.6 Confirmatory test: Quality control to determine and confirm the isolated organisms as *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella* species was done with the reference strains which include: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145 and *Klebsiella* species ATCC 700603 (Oxoid, UK); and this was based on the microscopical and

morphological and/or colonial characteristics of these control strains on culture media plates of MacConkey agar, cetrimide selective agar and EMB agar. The morphological appearances of the quality control organisms under the microscope were also used to evaluate the organisms isolated. These quality control strains were also used as standards for performing antimicrobial susceptibility studies.

3.2.7 Biochemical screening of bacterial isolates: All the isolated test bacteria including *E. coli*, *Klebsiella* species and *P. aeruginosa* isolates were characterized and identified microscopically and biochemically using microbiological identification techniques (Cheesbrough, 2006). The organisms were presumptively confirmed as *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella* species based on their reactions to Gram staining technique, colonial morphology on culture media, oxidase test, indole test, urease test, methyl red (MR) test, voges proskauer (VP) test and citrate test as it pertains to the identification of the individual organisms in the microbiology laboratory (Cheesbrough, 2006). According to Cheesbrough (2006), *Klebsiella* species is urease positive, VP positive and citrate positive, and it produces mucoid colonies on MacConkey agar and non-metallic sheen colonies that are mucoid on EMB agar; *Escherichia coli* is indole positive, and methyl red positive and it produces non-mucoid, pinkish colonies on MacConkey agar and colonies with metallic sheen on EMB agar; *Pseudomonas aeruginosa* produces greenish pigmentation and bluish pigmentation on Cetrimide selective agar and it is oxidase positive. *E. coli*, *Klebsiella* species and *P. aeruginosa* are all Gram-negative bacteria; and they appear pink-red under the microscope – since they all take up the colour of the counterstain (safranin) at the end of the Gram staining reaction. **[Details of protocol for carrying out these biochemical tests and Gram staining technique is explained in APPENDIX I].**

3.2.8 Antimicrobial susceptibility studies: Antimicrobial susceptibility studies was carried out on all the recovered test bacterial isolates based on the guideline of the Clinical Laboratory Standard Institute, CLSI (formerly known as the National Committee for Clinical Laboratory Standard, NCCLS) using sixteen (16) different single antibiotic disks (CLSI, 2011). The susceptibility of the test isolates to single disks of: imipenem (IPM), meropenem (MEM), ertapenem (ETP), cefoxitin (FOX), ceftazidime (CAZ), amikacin (AK), gentamicin (CN), cefotaxime (CTX), ceftriaxone (CRO), ciprofloxacin (CIP), ofloxacin (OFX), oxacillin (OX), ampicillin (AMP), aztreonam (ATM), nitrofurantoin (F) and cloxacillin (OB) was evaluated based on the modified Kirby-Bauer disk diffusion method on Mueller-Hinton (MH) agar plates (Oxoid, UK) as was previously described (Javeed *et al.*, 2011; Iroha *et al*., 2008; CLSI, 2011). Briefly, the test antibiotic disks were each aseptically placed at a distance of 15 mm apart on MH agar plates already swabbed or inoculated with the test isolates (adjusted to 0.5 McFarland turbidity standards). All culture plates were incubated at 30^{o}C for 18-24 hours and the zones of inhibition was recorded to the nearest millimeter using a meter rule and interpreted based on the CLSI criteria. The control strains were also run alongside the test isolates, and antimicrobial susceptibility test results were recorded based on their individual inhibition zone diameters (IZD) as susceptible (S), intermediate (I) and resistant (R). **The CLSI recommended breakpoints of the antibiotics used for susceptibility studies is shown in APPENDIX II. [Protocol for preparation of 0.5 McFarland turbidity standards is shown in APPENDIX I].**

3.2.9 Multiple antibiotic resistance index (MARI). Multiple antibiotic resistance indexes were determined for those bacterial isolates that showed multiple antibiotic resistances to the different classes of antibiotics used in this study. The multiple antibiotic resistance index (MARI) of multidrug resistant isolates in this study was determined by the method of Akinjogunla and Enabulele (2010). MARI was evaluated using the following formular:

$$\boldsymbol{MARI = \frac{A}{B}},$$

A = number of antibiotics to which the resistant bacteria was resistant to.

B = total number of antibiotics to which the resistant bacteria has been evaluated for.

3.3 PHENOTYPIC TESTS FOR METALLO BETA LACTAMASES (MBLs) AND AmpC ENZYMES

3.3.1 Nitrocefin test for beta-lactamase production: The production of beta-lactamase enzymes by Gram-negative bacteria including *E. coli*, *P. aeruginosa* and *Klebsiella* species is an important mechanism by which the organisms evade both *in vivo* and *in vitro* antimicrobial onslaught. Beta-lactamase production by resistant test isolates was evaluated using the Nitrocefin test sticks (Oxoid, UK) as described by the method of Akinduti *et al*. (2012). The nitrocefin stick is impregnated with a solution of nitrocefin, phosphate buffer and dimethysulphoxide. One end of the stick is coloured black (for handling) while the other end of the stick (coloured red) serves as the test point. Test isolates for beta-lactamase detection were brought to room temperature prior to carrying out the test; and a well separated representative colony of the test isolates was used for the test. The test isolates used for nitrocefin test for beta-lactamase detection were only cultures on nutrient agar plates. The nitrocefin stick was rotated several times around the representative colonies of the test isolate in order to pick up the cells; and because the reaction requires moisture to occur, one or two drops of distilled water was added to the test culture before bringing the nitrocefin stick in contact with the test isolate. The impregnated end of the nitrocefin stick (ladened with the test isolate) was examined for 5-15 minutes for a change in colour production. A positive reaction was shown by the development of a pink-red colour while a negative reaction was indicated as no colour change. Absence of a colour change showed that the test organism did not produce beta-lactamase enzyme while the presence of a pink-red colour indicated that the test organism produced beta-lactamase enzyme phenotypically. The colour of

the nitrocefin stick covered with the test isolate was compared with an unused nitrocefin stick in order to ensure correct reading and interpretation of the test result.

3.3.2 Screening for metallo-beta-lactamase (MBL) enzyme production: To phenotypically screen for the production of metallo-beta-lactamase (MBL) enzyme in the test isolates, all the test organisms were initially subjected to the three carbapenems which are: imipenem (IPM), meropenem (MEM), and ertapenem (ETP) in order to detect the level or pattern of their susceptibility to these antibiotics as per the CLSI criteria (CLSI, 2011; Ejikeugwu *et al*., 2014). The screening of the bacterial isolates for the production of MBL enzymes was conducted by the Kirby-Bauer disk diffusion technique. Each of the single antibiotic disks was placed at a distance of 25 mm apart and the plates were incubated at 30^{o}C for 18-24 hrs. MBL enzyme-producing isolates was suspected when the test organism(s) was resistant to any of the carbapenems used in the screening test. As per the CLSI criteria, isolates showing inhibition zone diameter (IZD) of ≤ 23 mm were considered and suspected to produce MBL enzyme; and these isolates were further tested using a phenotypic confirmation test (Ejikeugwu *et al*., 2014; Javeed *et al.*, 2011; Varaiya *et al*., 2008; CLSI, 2011; Aibinu *et al*., 2007; Walsh *et al*., 2005). **[The CLSI breakpoints for bacterial isolates screened for MBL enzyme production is shown in APPENDIX I].**

3.3.3 Screening for AmpC beta-lactamase production: Bacterial strains that produce AmpC beta-lactamase enzymes are resistant to the cephamycins (e.g. cefoxitin and cefotetan) but susceptible to the fourth generation cephalosporin, cefepime (Ejikeugwu *et al*., 2016; Manchanda *et al*., 2006; Vanwynsberghe *et al*., 2009; Shevade and Agrawal, 2013). The susceptibility of the test isolates to cefoxitin disk (30 µg) was used as the primary screening test to screen all the

environmental isolates for possible production of AmpC enzymes in line with the CLSI criteria (Lafi *et al*., 2012; CLSI, 2011). All the test isolates were each subjected to the antimicrobial activity of cefoxitin disk (30 µg) on an aseptically streaked Mueller-Hinton agar plate(s); and these were incubated at 30^{o}C for 18-24 hrs. AmpC enzyme production was suspected in those test isolates that showed reduced susceptibility to cefoxitin disk as per the breakpoints recommended by the CLSI (CLSI, 2011; Ejikeugwu *et al*., 2016). Test isolates showing inhibition zone diameter (IZD) ≤ 18 mm were selected and tested and/or confirmed phenotypically for the production of AmpC beta-lactamase enzyme. **[The CLSI breakpoints for bacterial isolates screened for AmpC enzyme production is shown in APPENDIX I].**

3.3.4 Inhibition based assay for detection of metallo-β-lactamase: Metallo-β-lactamase and other carbapenemases are inhibited by ethylenediamine tetraacetic acid (EDTA), boronic acid and dipicolinic acid (all chelating agents). Thus, the production of these enzymes by bacteria is detected phenotypically in the laboratory by subjecting the test pathogens to an inhibition based assay that employs any of the chelating agents. Test isolates found to be resistant to imipenem or meropenem (as described by the CLSI breakpoints for MBL screening) was evaluated phenotypically for metallo beta lactamase (MBL) production, and was carried out based on previously used methodologies (CLSI, 2011; Aibinu *et al*., 2007; Ejikeugwu *et al*., 2014; Saderi *et al*., 2008; Varaiya *et al*., 2008). Briefly, test bacteria (adjusted to 0.5 McFarland turbidity standards) were aseptically swabbed on Mueller-Hinton (MH) agar plates. Imipenem (10 µg) and meropenem (10 µg) disks impregnated with EDTA (1 µl) was aseptically placed on the MH agar plates. Supplementary imipenem (10 µg) and meropenem (10 µg) without EDTA was also placed alongside the carbapenem disks encumbered with the chelating agent (EDTA). The

chelating agent (EDTA) was tested on the test bacteria prior to the inhibition based assay to ensure they had no inhibitory effect on the test organisms. All plates were incubated at 30^{o}C for 18-24 hrs, and the zones of inhibition recorded using a meter rule. A difference of ≥ 7 mm between the zones of inhibition of any of the carbapenem disks with and without the chelating agents infers metallo-β-lactamase production phenotypically (Aibinu *et al*., 2007; Ejikeugwu *et al*., 2014; Saderi *et al*., 2008; Varaiya *et al*., 2008).

3.3.5 Hodges (Cloverleaf) Test for detection of metallo-β-lactamase: The Hodges or Cloverleaf test was performed by aseptically swabbing Mueller-Hinton (MH) agar plates with *Escherichia coli* ATCC 25922 strain. The inoculated MH agar plates were allowed for about 5 min; and imipenem (10 µg) single disks were aseptically placed at the center of the MH agar plates. The test bacteria (that showed reduced susceptibility to any of the carbapenems (as adjusted to 0.5 McFarland turbidity standards) were heavily streaked in three different directions from the edge of the imipenem (10 µg) disk to the center of the MH agar plates. Susceptibility plates were incubated for 18-24 hrs at 30^{o}C. The plates were macroscopically observed for indentation, and the growth of the test bacteria towards the imipenem (10 µg) susceptibility disk. Growth of test bacteria towards the carbapenem disk is indicative of metallo-β-lactamase production phenotypically (Aibinu *et al*., 2007; Ejikeugwu *et al*., 2014; Saderi *et al*., 2008; Varaiya *et al*., 2008).

3.3.6 Test for inducibility of chromosomal β-lactamase (AmpC enzymes): AmpC enzymes are chromosomally mediated enzymes, and they are produced in bacterial pathogens under induction by some cephalosporins (e.g. ceftazidime and cefotaxime) which induce their production (Livermore and Brown, 2001). These chromosomally-mediated enzymes (i.e. AmpC β-lactamases) ultimately destroy the antibiotics that stimulate their production by the pathogen. AmpC enzyme production was detected on bacterial isolates resistant to any of the third-generation cephalosporins (ceftazidime and cefotaxime) according to a previously described methodology (Coudron, 2005; Hemalatha *et al*., 2007). Single antibiotic disk containing 30 µg of cefoxitin and another cefoxitin (30 µg) disk impregnated with 5 µl of EDTA (a chelating agent) was aseptically placed on MH agar plate(s) already swabbed with the test bacteria. Susceptibility disks was placed at a distance of about 25 mm on the MH agar plate, and incubated at 30^{o}C for 18-24 hrs. A difference of ≥ 5 mm inhibition zone diameter around the cefoxitin disk (30 µg) alone and cefoxitin (30 µg) disk impregnated with EDTA confirms AmpC enzyme production phenotypically. AmpC enzymes are resistant to cefoxitin, amoxicillin-clavulanic acid and third-generation cephalosporins but susceptible to cefepime, a fourth generation cephalosporin and imipenem, a carbapenem. AmpC enzyme production was also confirmed phenotypically by evaluating the susceptibility of test pathogens to cefepime.

3.3.7 Ceftazidime-imipenem antagonism test (CIAT): AmpC enzyme production in the test organisms was also evaluated using the ceftazidime-imipenem antagonism test (CIAT) for the detection and confirmation of the presence of inducible AmpC beta-lactamases in the test bacteria. CIAT was performed using ceftazidime (30 µg) disk and imipenem disk (10 µg) as was previously described by Cantarelli *et al*. (2007). Ceftazidime disc and imipenem disk were

placed at a distance of 20 mm apart on Mueller-Hinton agar plate previously inoculated with a suspension of the test bacteria (adjusted to 0.5 McFarland turbidity standards) that showed reduced susceptibility to cefoxitin. A cefoxitin disk (30 µg) was also placed at a distance of 20 mm from the ceftazidime disk for comparison. All the susceptibility test plates were incubated at 30^{o}C for 18-24 hrs. Antagonism indicated by a visible reduction in the inhibition zone around the ceftazidime disk adjacent to the imipenem or cefoxitin disk was inferred as a positive inducible AmpC beta-lactamase production (Cantarelli *et al*., 2007).

3.3.8 Cefoxitin-cloxacillin double-disk synergy test (CC-DDST): CC-DDST was performed by the method of Polsfuss *et al*. (2011). CC-DDST test is a phenotypic test for AmpC enzyme detection; and the test is based on the inhibitory effect of cloxacillin on AmpC enzyme. Single disks containing 30 µg of cefoxitin was placed at a distance of 20 mm away from a disk containing 200 µg of cloxacillin on Mueller-Hinton agar plates already inoculated with the test bacteria (equivalent to 0.5 McFarland turbidity standards). The plates were incubated at 30^{o}C for 18-24 hours; and the zones of inhibition were recorded as per the CLSI criteria. A difference of ≥ 4 mm in the cefoxitin-cloxacillin inhibition zones minus the cefoxitin disk used alone was indicative of AmpC enzyme production phenotypically.

3.4 MOLECULAR CHARACTERIZATION OF MBL AND AmpC GENES

3.4.1 Preparation of bacterial stock cultures for gene amplification: Bacterial stock cultures for gene amplification were prepared by standard microbiological cultural technique (Helmy and Wasfi, 2014; Cheesbrough, 2006). The test bacterial cultures were transferred from stored cultures in the refrigerator, inoculated on double strength nutrient broth (Oxoid, UK) and incubated overnight at 30^{o}C for resuscitation of the organisms. And a loopful of the turbid suspension after incubation was inoculated on nutrient agar plates for purification. The plates were incubated at 30^{o}C for 18-24 hrs; and about 8-10 colonies from the overnight cultures were inoculated in 5 ml peptone water (Oxoid, UK) for further molecular studies. This stock culture served as source of bacterial cells and template DNA for further molecular studies.

3.4.2 Isolation and preparation of template DNA from bacterial cells: The isolation and preparation of template DNA for the amplification of AmpC genes and MBL genes from the bacterial isolates of *Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella* species was carried out by the method of Franco *et al*. (2010) and Perez-Perez and Hanson (2002) with little modification (Franco *et al*., 2010; Perez-Perez and Hanson, 2002). Single colony of each of the test isolates was inoculated from nutrient agar plates (Oxoid, UK) into 5 ml of peptone water (Oxoid, UK). The inoculated broth(s) was incubated at 30^{o}C for 18-24 hrs with mild shaking. Bacterial cells from the overnight broth culture were aseptically harvested by centrifugation technique at 5000 rpm for 5 minutes. After centrifugation in the centrifugal machine, the supernatant was decanted and the pellets collected at the bottom of the tube was then re-suspended in 100 µl of Tris-EDTA (TE) buffer. The cells in the TE buffer tubes were lysed by

heating at 100^{o}C for 10 minutes in a water bath; and the solution was centrifuged at 5000 rpm for 5 minutes (to remove cellular debris). An aliquot of the supernatant (2 µl) from the spinned or centrifuged solution was aseptically transferred to sterile Eppendorf tube(s) placed on ice. This aliquot was used as the source of the template DNA for gene amplification; and the solution was stored at -20^{o}C until use. The isolated DNA was washed and purified using the Zymo Plasmid miniprep kit (Epigenetics Company, USA). **[Step to step procedure for bacterial plasmid DNA isolation is shown in Appendix V].**

3.4.3 Multiplex PCR amplification of MBL genes: The presence of MBL genes including *bla*IMP-1, *bla*IMP-2, *bla*VIM-1, and *bla*VIM-2 in the test MBL bacterial phenotypes were determined according to the method of Shibata *et al*. (2003) and Franco *et al*. (2010). The multiplex PCR conditions for the gene amplification of MBL genes are shown in Table 3.1. Multiplex PCR amplification of MBL genes in the test isolates was performed in a thermal cycler (Lumex instrument, Canada) with a final volume of 26.5 µl in Eppendorf tubes using primers synthesized and supplied by Inqaba Biotechnical Industries Ltd (Inqaba Biotechnical Industries Ltd, South Africa). The 26.5 µl of the master mix contained 0.2 µl of Taq polymerase enzyme U/µl, 2.5 µl of 10X PCR buffer along with 2.5 µl $MgCl_2$, 1 µl of 10 pM from each of the forward and reverse primers (as shown in Table 3.1), 2.5 µl of dNTPs MIX (2 Mm), 3 µl of DNA template (from the test isolates), 14.8 µl of nuclease-free water. The master mix (as contained in the Eppendorf tubes) were properly vortexed in a vortexer prior to the gene amplification process in the thermal cycler. The Eppendorf tube(s) were each placed in the thermal cycler machine and the PCR program with the right PCR conditions pre-installed and started for the gene amplification process using the primers (Table 3.1). Overall, the initial

denaturation temperature was at 95^{o}C for 2 mins, and this was followed by 25 cycles of DNA denaturation at 95^{o}C for 30 sec. The primer annealing was carried out at 48^{o}C for 30 sec, and primer extension was carried out at 72^{o}C for 30 sec. After the last cycle, a final extension step was carried out at 72^{o}C for 2 mins. A 100 bp DNA molecular marker was used as the positive control (marker) during the gel electrophoresis process while the negative control was a PCR mastermix containing all other PCR reagents/materials without DNA. Gel electrophoresis of the multiplex PCR products was carried out in 1.5 % agarose gel (Inqaba Biotechnical Industries Ltd, South Africa) for 2 h at 80 V. **[The synthesis report of the primer production for MBL genes is shown in APPENDIX IX while the mastermix of the PCR setup is shown in APPENDIX X].**

Table 3.1: Multiplex PCR conditions for amplification of MBL genes

Gene target(s)	Primer sequence (5' to 3', as synthesized)	Expected amplicon size (bp)
*bla*IMP-1	F1 (5'-ACC GCA GCA GAG TCT TTG CC-3') R1 (5'-ACA ACC AGT TTT GCC TTA CC-3')	587
*bla*IMP-2	F2 (5'-GTT TTA TGT GTA TGC TTC C-3') R2 (5'-AGC CTG TTC CCA TGT AC-3')	678
*bla*VIM-1	F3 (5'-AGT GGT GAG TAT CCG ACA G-3') R3 (5'-ATG AAA GTG CGT GGA GAC-3')	261
*bla*VIM-2	F4 (5'-ATG TTC AAA CTT TTG AGT AAG-3') R4 (5'-CTA CTC AAC GAC TGA GCG-3')	801

F-forward primer**, R-**Reverse primer
Shibata *et al*., 2003.

3.4.4 Agarose gel electrophoresis of MBL genes: Agarose gel electrophoresis was carried out by the methods of Safari *et al*. (2015) and Shibata *et al*. (2003) using 1.5 % agarose gel in order to separate the amplified DNA molecules according to their individual sizes when an electric field is applied across the gel. The 1.5 % agarose gel was prepared by dissolving 3 g of the agarose powder in 250 ml of 0.5 X Tris boric acid EDTA (TBE) buffer. The agarose solution was then boiled until all the agarose was dissolved in a microwave oven. The molten agarose gel was cooled for five (5) minutes to about 50^{o}C, and 1.0 µl 10 mg/ml of ethidium bromide (EtBr) dye was added to the molten agarose gel. A comb was then placed in a sealed mould, and the molten agarose gel was poured into the sealed mould. The gel was allowed to cool for 20 mins before the seal and the comb was removed. Thereafter, the formed or solidified gel was placed in the gel electrophoresis vessel or chamber (Lumex instrument, Canada) that was filled with 0.5 X TBE buffer. And the PCR products (i.e. the amplified MBL genes in the Eppendorf tubes) was mixed with 1 µl 6 X coloured loading buffer per 5 µl of the PCR product and then aseptically pipetted into each of the well in the gel. A 5 µl of the DNA marker or ladder was initially loaded to the first well on the gel while the last well in the gel was filled with other PCR reagents without the DNA, and this served as the negative control. The gel was run at 80 V for 2 h; and then visualized with UV transilluminator (Scientico, India) at 260 nm. **[Protocol for preparation of 1.5 % of agarose gel and the running of the gel electrophoresis process in elucidated in APPENDIX VII and VIII].**

3.4.5 Multiplex PCR amplification of AmpC genes: Multiplex PCR protocol for the amplification of AmpC genes in the test bacterial isolates was carried out by previously described methodologies (Mohamudha *et al*., 2012; Helmy and Wasfi, 2014; Perez-Perez and

Hanson, 2002; El-Hady and Adel, 2015). All the bacterial isolates that were phenotypically positive for AmpC enzyme production were tested by multiplex PCR technique for the identification of specific AmpC genes carried on the bacterial plasmid DNA including FOX gene, ACC gene, DHA gene and CMY gene. The multiplex PCR amplification of AmpC genes from the test isolates was carried out in a thermal cycler (Lumex instrument, Canada) with a final volume of 26.5 µl in Eppendorf tubes using primers synthesized and supplied by Inqaba Biotechnical Industries Ltd (Inqaba Biotechnical Industries Ltd, South Africa). The primers and the PCR conditions used for the amplification of AmpC genes in the test organisms are shown in Table 3.2. The 26.5 µl of the master mix contained 0.2 µl of Taq polymerase enzyme U/µl, 2.5 µl of 10X PCR buffer along with 2.5 µl $MgCl_2$, 1 µl of 10 pM from each of the forward and reverse primers (as shown in Table 3.2), 2.5 µl of dNTPs MIX (2 Mm), 3 µl of DNA template (from the test isolates), 14.8 µl of nuclease-free water. The mastermix or master mixture was made on separate workbench to avoid contamination; and the mastermix was thawed on ice before use. The Eppendorf tube(s) were each placed on the thermal cycler machine and the multiplex PCR program with the right PCR conditions pre-installed and started for the gene amplification process using the primers (Table 3.2). Overall, the initial denaturation temperature was at 94^{o}C for 3 mins, and this was followed by 25 cycles of DNA denaturation at 94^{o}C for 30 secs. The primer annealing was carried out at 64^{o}C for 30 secs, and primer extension was carried out at 72^{o}C for 1 min. After the last cycle, a final extension step was carried out at 72^{o}C for 7 mins. A 100 bp DNA molecular marker was used as the positive control (marker) during the gel electrophoresis while the negative control was a PCR mastermix containing nuclease-free water in the place of the template DNA molecule. Gel electrophoresis of the multiplex PCR products was carried out using 1.5 % agarose gel (Inqaba Biotechnical Industries Ltd, South Africa) for 2

h at 80 V. **[The synthesis report of the primer production for AmpC genes is shown in APPENDIX IX while the mastermix of the PCR setup is shown in APPENDIX X].**

Table 3.2: Multiplex PCR conditions for amplification of AmpC genes

Gene target(s)	Primer	Primer sequence (5' to 3', as synthesized)	Expected amplicon size (bp)
CMY-1	MOXM-F	GCT GCT CAA GGA GCA CAG GAT	520
	MOXM-R	CAC ATT GAC ATA GGT GTG GTG C	
DHA-1	DHAM-F	AAC TTT CAC AGG TGT GCT GGG T	405
	DHAM-R	CCG TAC GCA TAC TGG CTT TGC	
ACC	ACCM-F	AAC AGC CTC AGC AGC CGG TTA	346
	ACCM-R	TTC GCC GCA ATC ATCC CT AGC	
FOX-1	FOXM-F	AAC ATG GGG TAT CAG GGA GAT G	190
	FOXM-R	CAA AGC GCG TAA CCG GAT TGG	

F-forward primer**, R-**Reverse primer
Perez-Perez and Hanson (2002)

3.4.6 Agarose gel electrophoresis of AmpC genes: Agarose gel electrophoresis was carried out by the method of Helmy and Wasfi (2014) and Perez-Perez and Hanson (2002) using 1.5 % agarose gel in order to separate the amplified DNA products according to their individual sizes when an electric field is applied across the gel (Perez-Perez and Hanson, 2002). The 1.5 % agarose gel was prepared by dissolving 3 g of the agarose powder in 0.5 X TBE buffer solution. The agarose solution was then heated until all the agarose was dissolved in a microwave oven. The molten agarose gel was cooled for five (5) minutes to about 50^{o}C before pouring, and 1.0 µl of 10 mg/ml of ethidium bromide (EtBr) dye was added to the agarose gel. A comb was then placed in a sealed mould, and the molten agarose gel was poured into the sealed mould. The gel was allowed to cool for 20 mins before the seal and the comb was removed. Thereafter, the formed or solidified gel was placed in the gel electrophoresis vessel or chamber that was filled with 0.5 X TBE buffer solution. And the PCR products (i.e. the amplified genes in the Eppendorf tubes) was mixed with 1 µl 6 X coloured loading buffer per 5 µl of the PCR product and then aseptically pipetted into the wells in the gel. A 5 µl of the DNA marker or ladder was initially loaded to the first well on the gel while the PCR reagent without DNA was loaded in the last well as a negative control. The gel was run at 80 V for 2 h; and then visualized with UV transilluminator at 260 nm. **[Protocol for preparation of 1.5 % of agarose gel and the running of the gel electrophoresis process in elucidated in APPENDIX VII and VIII].**

3.5 Plasmid curing: Plasmid curing experiment was undertaken to determine the location (plasmid or chromosomal) of the drug resistance determinants in the MBL and AmpC-enzyme positive bacteria including *E. coli, Klebsiella* species and *P. aeruginosa* phenotypes according to a previously described method (Iroha *et al*., 2010). Two colonies of individual MBL and AmpC-

positive bacteria each were grown in 5 ml nutrient broth (Oxoid, UK) that was also supplemented with 0.1 mg/ml of acridine orange. Positive and negative controls were also run alongside the curing experiments. The positive control tubes contained only the bacterial cells and no acridine orange while negative control tubes contained only acridine orange and no bacterial cells. All tubes were properly shaken and incubated for 18-24 hrs at 30^{o}C. After incubation, the tubes containing both bacterial cells and the acridine orange curing agent were selected, and a loopful of the test organism in the tube(s) was streaked aseptically on MacConkey agar (Oxoid, UK) plate(s) and cetrimide selective agar plates which served as the recovery medium (Iroha *et al*., 2010). The plates were incubated for 18-24 hrs at 30^{o}C, and the resultant colonies were sub-cultured onto freshly prepared MacConkey agar plates and cetrimide selective agar plates to get pure strains of the test bacteria. After sub-culturing and incubation, bacterial colonies that emanated from the recovery plates were checked for the loss of antibiotic resistance determinants (genes) by subjecting them to antibiotic susceptibility studies using drugs they showed reduced susceptibility to. Phenotypic tests for detection of MBL and AmpC enzymes were also carried out on the organisms to determine the loss or retention of their resistance trait. Isolates showing susceptibility to the antibiotics used and that were negative to the phenotypic tests were considered to have lost their plasmids following subjection to acridine orange (a mutagen).

3.6 Statistical analysis: Statistical analysis was carried out with the Statistical Package for Social Sciences (SPSS) version 23.0. The significance of AmpC positive isolates and MBL positive isolates was determined using Chi square tests at p-value < 0.05 and at a confidence

interval of 95 %. A p-value < 0.05 was considered statistically significant. **[Statistical analysis raw data is shown in APPENDIX XI].**

CHAPTER FOUR

RESULTS

4.1 RESULTS OF PRELIMINARY STUDIES

A total of three hundred and seventy (370) environmental samples that comprises of cloacal swabs of poultry birds (n = 120), anal swab samples from cow (n = 120) and swabs of abattoir tables (n = 130) were aseptically collected from a slaughter house (abattoir) and a poultry farm in Abakaliki metropolis respectively [Table 4.1]. Overall, *E. coli* isolates was isolated from 168 environmental swab samples out of the 370 environmental swab samples analyzed in this study. *E. coli* was isolated from 69 swab samples out of 130 swab samples from slaughter/abattoir tables, 51 swab samples out of 120 cloacal swab samples of poultry birds, and from 48 swab samples out of 120 swab samples from the anal region of cows (Table 4.2). The recovery rate of *E. coli* from the various environmental swab samples analyzed in this study was 41.1 %, 30.4 % and 28.6 % for swab samples from abattoir/slaughter tables, swab samples from the cloacal region of poultry birds and swabs from the anal region of cows (Table 4.2). *E. coli* fermented lactose, and thus produced pinkish colonies on MacConkey agar (Figure 4.1). But on eosin methylene blue (EMB) agar, *E. coli* produced colonies with metallic sheen (Figure 4.2).

Table 4.1: Distribution of samples collected and their sources

Sample	Source	Number
Cloacal swabs of poultry birds	Poultry	120
Anal swabs of cow	Abattoir	120
Swab samples from abattoir tables	Abattoir	130
Total		**370**

Table 4.2: Isolation rate of Gram-negative bacteria from the samples tested

Organism	Swabs from slaughter/abattoir benches (n = 130) n (%)	Cloacal swabs of poultry birds (n = 120) n (%)	Anal/rectal swabs of cow (n = 120) n (%)	Total
Escherichia coli	69 (41.1)	51 (30.4)	48 (28.6)	168
Pseudomonas aeruginosa	56 (38.1)	48 (32.7)	43 (29.3)	147
Klebsiella species	40 (28.4)	49 (34.8)	52 (36.9)	141

Keys:

n = number of isolates

% = percentage

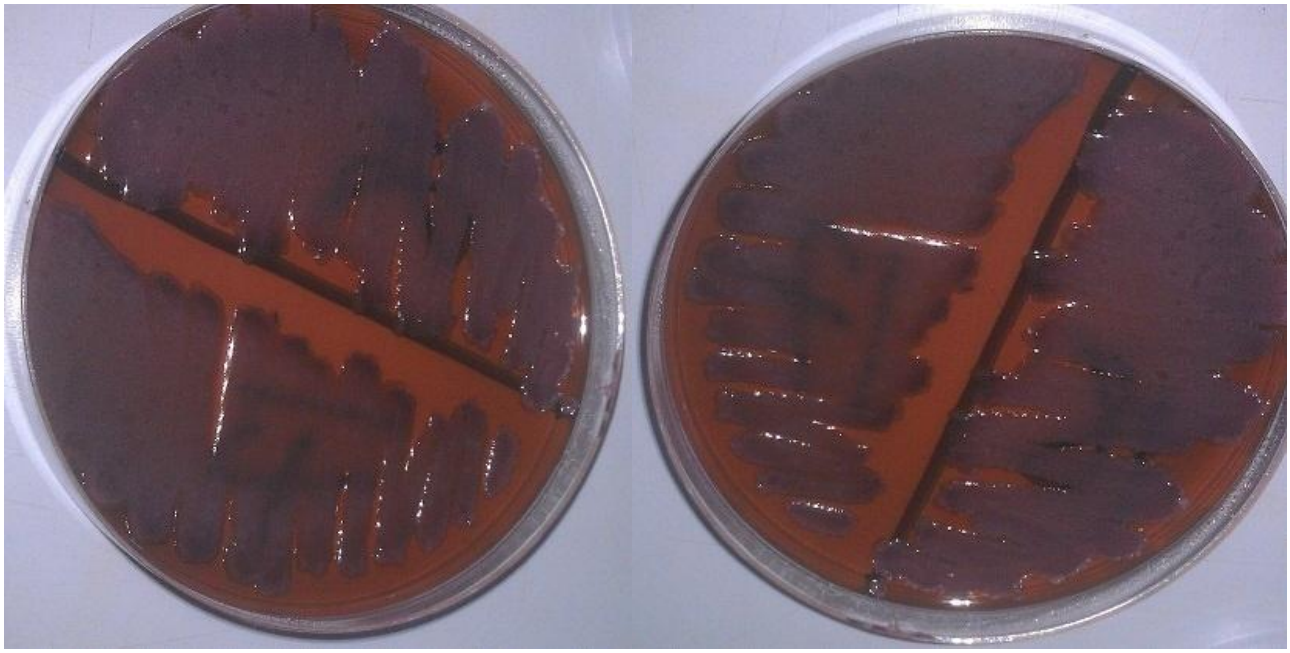

Figure 4.1 : *Escherichia coli* growing on MacConkey agar plates. *E. coli* ferments lactose, and thus produces pinkish colonies on MacConkey agar

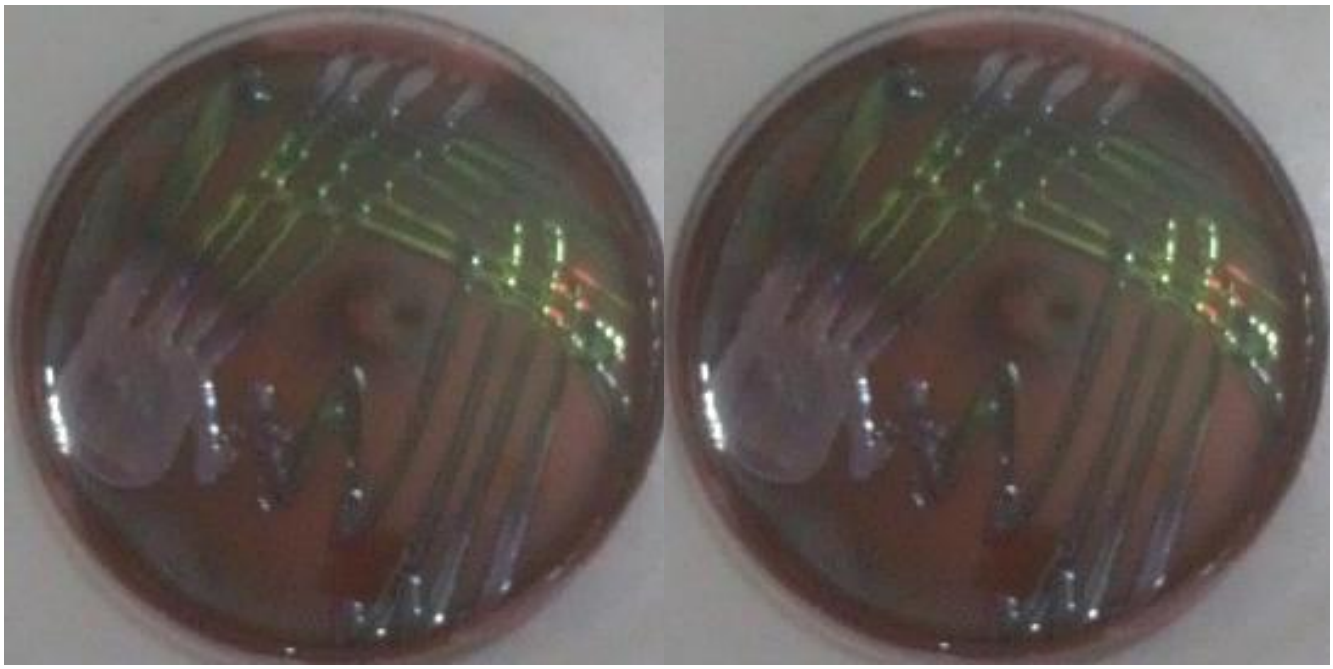

Figure 4.2: *Escherichia coli* growing on eosin methylene blue (EMB) agar plates. *E. coli* produces metallic sheen colonies (as shown in the figure) when grown on EMB

The recovery rate of *Klebsiella* species isolates from the environmental swab samples including swab samples from abattoir tables, cloacal swab samples from poultry birds and anal swab samples from cows was 28.4 %, 34.8 %, and 36.9 % respectively (Table 4.2). *Klebsiella* species produced large and mucoid colonies on MacConkey agar and on EMB agar (Figure 4.3). Isolates of *Klebsiella* species do not produce colonies with metallic sheen on EMB agar as is applicable with *E. coli* isolates. *P. aeruginosa* was isolated from 56 swab samples out of 130 swab samples from abattoir/slaughter tables, 48 swab samples out of 120 cloacal swab samples, and from 43 swab samples out of 120 anal swab samples from cows (Table 4.2). Isolates of *P. aeruginosa* produced greenish colonies on cetrimide selective agar due to the production of pyoverdin and pyocyanin (Figure 4.4). *E. coli* was the most prevalent isolated Gram-negative bacteria; and this was followed by *P. aeruginosa* and *Klebsiella* species in that order. All the isolated Gram-negative bacteria including *E. coli, Klebsiella* species and *P. aeruginosa* were biochemically identified using standard microbiological identification techniques including oxidase test, methyl red test, voges proskauer test, indole test, citrate test, urease test, and Gram staining technique (Table 4.3). Methyl red (MR) test is used to determine the fermentation pathway used by bacteria to ferment or utilize glucose; and bacteria ferment glucose to produce different acids including lactic acid and acetic acid. *E. coli* is methyl red positive (MR+) and voges proskauer negative (VP-) while *Klebsiella* species are methyl red negative (MR-) and voges proskauer positive (VP+). *P. aeruginosa* is a glucose non-fermenter unlike *E. coli* that is a lactose fermentor; and thus *P. aeruginosa* is methyl red negative (MR-) and voges proskauer negative (VP-).

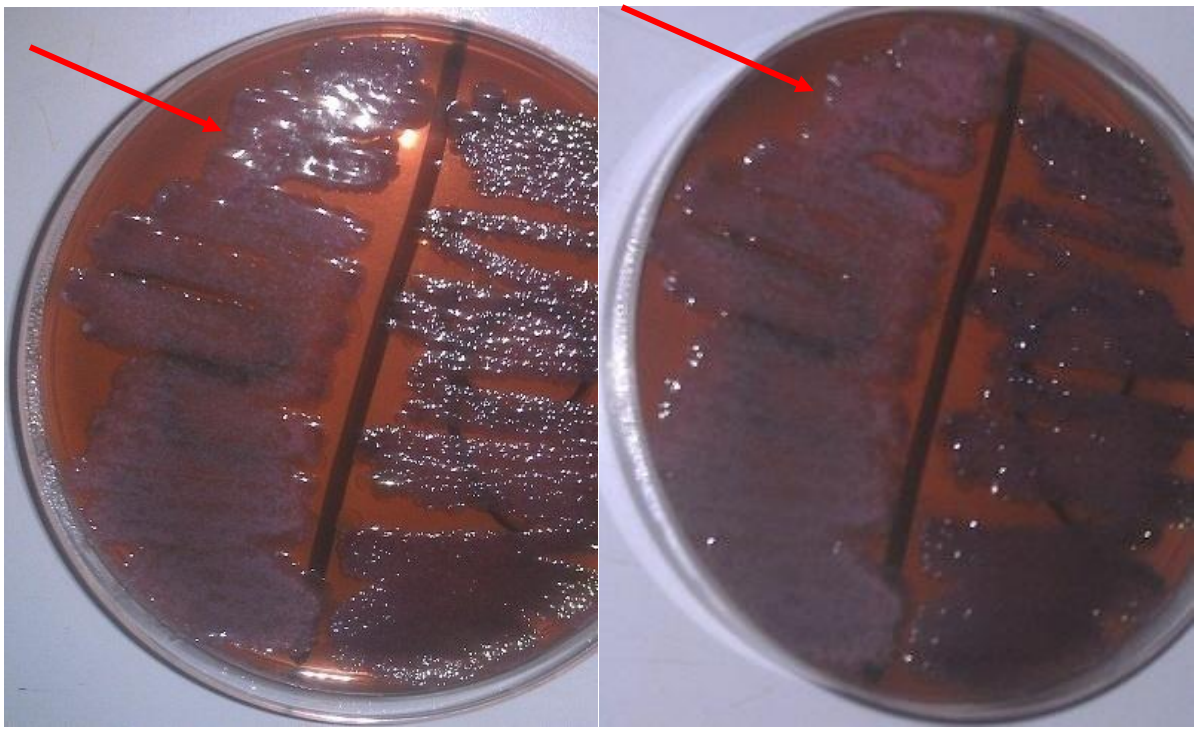

Figure 4.3: *Klebsiella* species growing on MacConkey agar plates (**arrows**). *Klebsiella* produces large and mucoid colonies with pinkish colouration on MacConkey agar

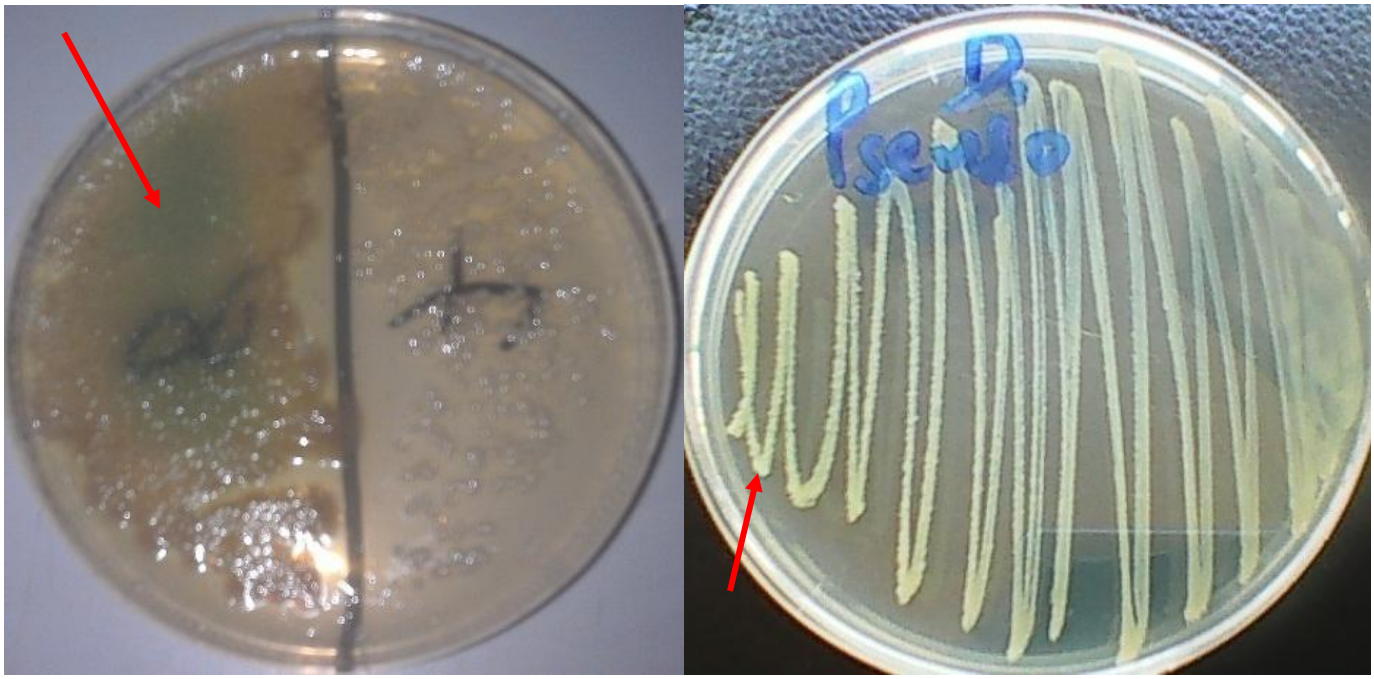

Figure 4.4: *Pseudomonas aeruginosa* growing on Cetrimide selective agar plate. *P. aeruginosa* produces greenish/bluish colouration on Cetrimide selective agar (**arrows**)

Table 4.3: Characterization and biochemical identification of the organisms

Bacteria	Family	Gram staining	Indole test	Oxidase test	Citrate test	Urease test	Methyl red test	Voges proskauer
Escherichia coli	*Enterobacteriaceae*	-ve	+ve	-ve	-ve	-ve	+ve	-ve
Klebsiella species	*Enterobacteriaceae*	-ve	-ve	-ve	+ve	+ve	-ve	+ve
Pseudomonas aeruginosa	*Pseudomonadaceae*	-ve	-ve	+ve	-ve	-ve	-ve	-ve

Key:

-ve = negative

+ve = positive

4.2 BETA LACTAMASE PRODUCTION

The result of the beta-lactamase production by *E. coli, Klebsiella* species and *P. aeruginosa* is shown in Figure 4.5. Beta-lactamase enzymes were phenotypically detected in the Gram-negative bacteria at varying rates. *E. coli* produced beta-lactamase enzyme at the rate of 38 % while *P. aeruginosa* produced the enzyme at the rate of 33 %. The rate of beta-lactamase production in *Klebsiella* species isolates was 29 %. This study employed 16 different single antibiotic disks to determine the antimicrobial susceptibility profile (antibiogram) of the isolated bacteria.

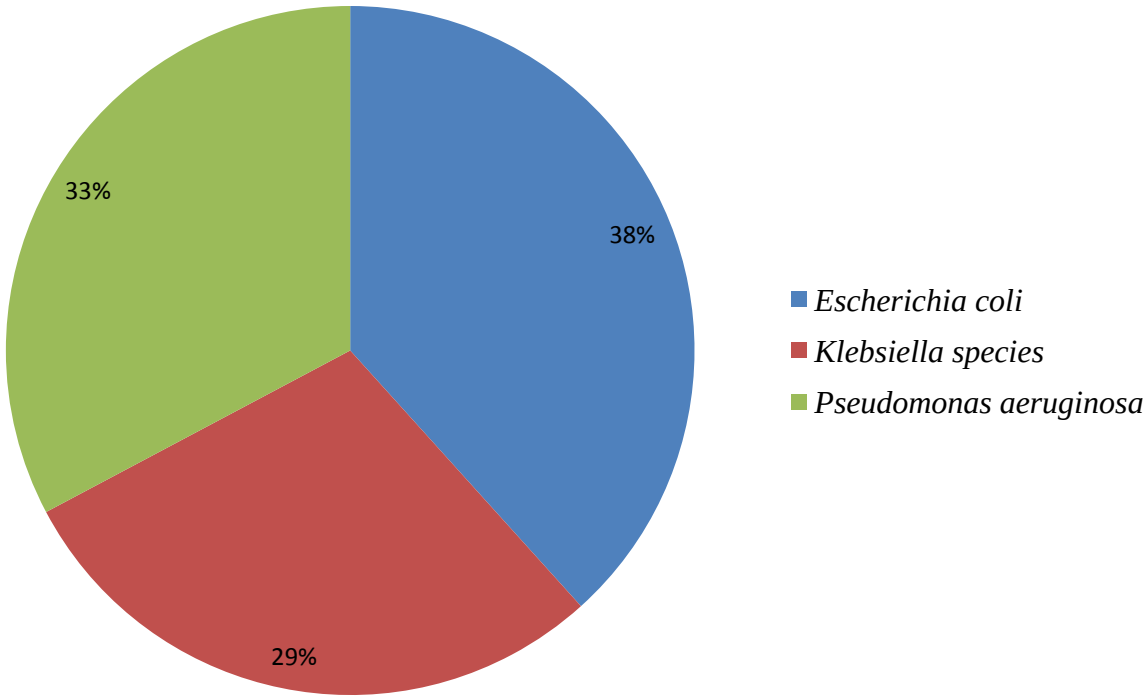

Figure 4.5: Beta-lactamase production by the test Gram-negative bacteria

4.3 SUSCEPTIBILITY RESULTS OF *ESCHERICHIA COLI*

The antibiotic susceptibility testing (antibiogram) results as shown in Table 4.4; reveal a very high rate of resistance among the test bacteria in the *Enterobacteriaceae* family including *E. coli* and *Klebsiella* species. High levels of resistance were noted in the *E. coli* isolates (Table 4.4). The *E. coli* isolates were highly resistant to the carbapenems, penicillins, aminoglycosides, fluoroquinolones and cephalosporins used in this study (Figure 4.6). Out of the 168 isolates of *E. coli* recovered from the environmental samples in this study, 160 (95.2 %) *E. coli* isolates were resistant to ceftriaxone. It was also found that 162 (96.4 %) and 165 (98.2 %) isolates of the *E. coli* isolates and 165 (98.2 %) *E. coli* isolates were resistant to ceftazidime and cefotaxime respectively – which are both third generation cephalosporins and broad spectrum antimicrobial agents. Very low levels of susceptibility of the *E. coli* isolates was also observed to cefoxitin, oxacillin, ofloxacin, amikacin, ciprofloxacin, and aztreonam with the resistance found to be at a rate of 74.4 %, 81.5 %, 70.8 %, 64.9 %, 81.5 % and 93.5 % respectively (Table 4.4). There was no statistical difference in the percentage susceptibility of the *E. coli* isolates when compared to those of *Klebsiella* species isolates to the tested antibiotics used in this study (p value > 0.05).

Table 4.4: Susceptibility of *Escherichia coli* and *Klebsiella* species to the tested antibiotics

Antibiotics (µg)	*Escherichia coli*		*Klebsiella* species	
	S n (%)	**R n (%)**	**S n (%)**	**R n (%)**
CRO (30)	8 (4.8)	160 (95.2)	15 (10.6)	126 (89.4)
FOX (30)	43 (25.6)	125 (74.4)	35 (24.8)	106 (75.2)
IPM (10)	81 (48.2)	87 (51.8)	83 (58.9)	58 (41.1)
CAZ (30)	6 (3.6)	162 (96.4)	25 (17.7)	116 (82.3)
ETP (30)	22 (13.1)	146 (86.9)	22 (15.6)	119 (84.4)
OX (5)	31 (18.5)	137 (81.5)	18 (12.8)	123 (87.2)
OFX (10)	49 (29.2)	119 (70.8)	49 (34.8)	92 (65.2)
CN (10)	73 (43.5)	95 (56.5)	55 (39.0)	86 (61.0)
AK (10)	59 (35.1)	109 (64.9)	67 (47.5)	74 (52.5)
CIP (10)	31 (18.5)	137 (81.5)	19 (13.5)	122 (86.5)
CTX (30)	3 (1.8)	165 (98.2)	5 (3.5)	136 (96.5)
MEM (10)	75 (44.6)	93 (55.4)	80 (56.7)	61 (43.3)
AMP 10)	50 (29.8)	118 (70.2)	43 (30.5)	98 (69.5)
ATM (30)	11 (6.5)	157 (93.5)	5 (3.5)	136 (96.5)
F (10)	39 (23.2)	129 (76.8)	36 (25.5)	105 (74.5)
OB (500)	65 (38.7)	103 (61.3)	38 (27.0)	103 (73.0)

(p value > 0.05)

Key: S = Susceptible, R = Resistant, IPM = imipenem, MEM = meropenem, ETP = ertapenem, FOX = cefoxitin, CAZ = ceftazidime, AK = amikacin, CN = gentamicin, CTX = cefotaxime, CRO = ceftriaxone, CIP = ciprofloxacin, OFX = ofloxacin, OX = oxacillin, AMP = ampicillin, ATM = aztreonam, F = nitrofurantoin, OB = cloxacillin

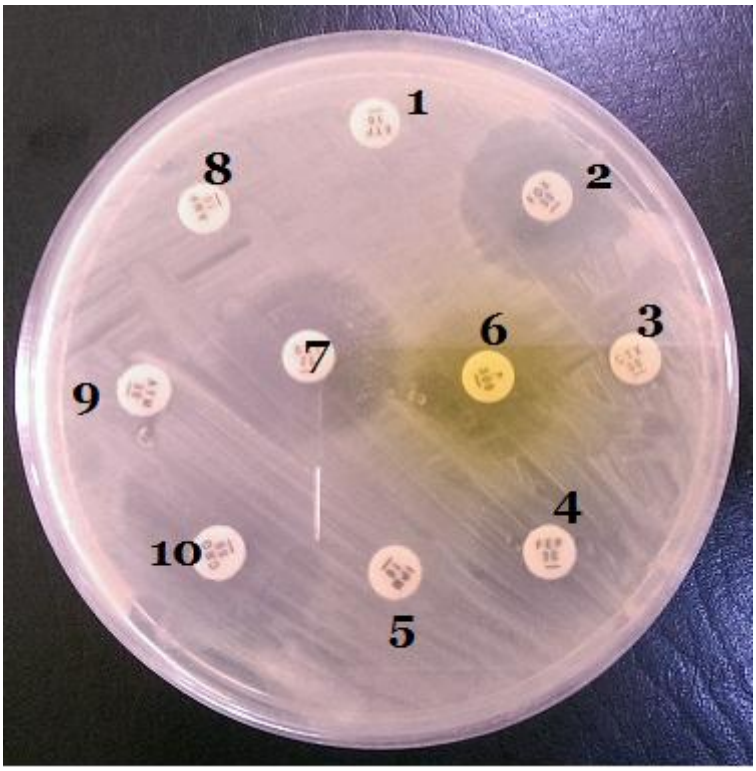

Figure 4.6: Susceptibility test plate of an *Escherichia coli* isolate (from cloacal swab of broiler) cultured on Mueller-Hinton agar plate.

Key:

1= Ertapenem (10 µg)
2= Cefoxitin (30 µg)
3 = Cefotaxime (30 µg)
4 = Ciprofloxacin (10 µg)
5 = Imipenem (10 µg)
6 = Nitrofurantoin (10 µg)
7 = Meropenem (10 µg)
8 = Ampicillin (10 µg)
9 = Aztreonam (30 µg)
10 = Ceftriaxone (30 µg)

4.4 SUSCEPTIBILITY OF *KLEBSIELLA* SPECIES AND *PSEUDOMONAS AERUGINOSA*

The antimicrobial susceptibility results of the *Klebsiella* species isolates recovered from the environmental samples is shown in Table 4.4. *Klebsiella* species was resistant to the various antibiotics used in this study (Figure 4.7). The resistance pattern most commonly observed amongst the *Klebsiella* species isolates was resistance to cefotaxime (96.5 %), aztreonam (96.5 %), ceftriaxone (89.4 %), oxacillin (87.2 %), ciprofloxacin (86.5 %), ceftazidime (82.3 %) and cloxacillin (73.0 %). The next most frequent resistance phenotypes of the *Klebsiella* species isolates was resistance to the carbapenems including imipenem (41.1 %), meropenem (43.3 %) and ertapenem (84.4 %) [Table 4.4]. The *Klebsiella* species isolates were resistant to ampicillin (69.5 %), nitrofurantoin (74.5 %), amikacin (52.5 %), and gentamicin (61.0 %). The cephamycin, cefoxitin had little inhibitory effect on the test *Klebsiella* species isolates as the organisms were highly resistant to this antibacterial agent (75.2 %). The antimicrobial susceptibility profile of *Pseudomonas aeruginosa* isolates is shown in Table 4.5. The *P. aeruginosa* isolates recovered from the environmental samples including cloacal swabs of poultry birds, swabs from anal region of cows and swabs from abattoir tables showed varying rates of resistance and susceptibility to the tested antibiotics used in this study. The antibiotic resistance pattern of the organism confirmed that more than 50 % of the *P. aeruginosa* isolates showed resistance to the carbapenems including imipenem (66.7 %), ertapenem (61.2 %) and meropenem (60.5 %). Reduced susceptibility of the *P. aeruginosa* isolates was also observed with resistance in cefoxitin (80.3 %), cefotaxime (79.6 %), ceftriaxone (64.6 %) and ceftazidime (54.4 %). High level resistance of the *P. aeruginosa* isolates to the tested antibiotics was also observed in this study especially to ertapenem, cefoxitin, nitrofurantoin, cefotaxime and ampicillin (Figure 4.8).

Table 4.5: Susceptibility test results of *Pseudomonas aeruginosa*

Antibiotics (µg)	*Pseudomonas aeruginosa*	
	S	**R**
	n (%)	**n (%)**
CRO (30)	52 (35.4)	95 (64.6)
FOX (30)	29 (19.7)	118 (80.3)
IPM (10)	49 (33.3)	98 (66.7)
CAZ (30)	67 (45.6)	80 (54.4)
ETP (30)	57 (38.8)	90 (61.2)
OX (5)	41 (27.9)	106 (72.1)
OFX (10)	54 (36.7)	93 (63.3)
CN (10)	65 (44.2)	82 (55.8)
AK (10)	53 (36.1)	94 (63.9)
CIP (10)	28 (19.0)	119 (81.0)
CTX (30)	30 (20.4)	117 (79.6)
MEM (10)	58 (39.5)	89 (60.5)
AMP 10)	28 (19.0)	119 (81.0)
ATM (30)	61 (41.5)	86 (58.5)
F (10)	44 (29.9)	103 (70.1)
OB (500)	58 (39.5)	89 (60.5)

(p value > 0.05)

Key: S = Susceptible, R = Resistant, IPM = imipenem, MEM = meropenem, ETP = ertapenem, FOX = cefoxitin, CAZ = ceftazidime, AK = amikacin, CN = gentamicin, CTX = cefotaxime, CRO = ceftriaxone, CIP = ciprofloxacin, OFX = ofloxacin, OX = oxacillin, AMP = ampicillin, ATM = aztreonam, F = nitrofurantoin, OB = cloxacillin

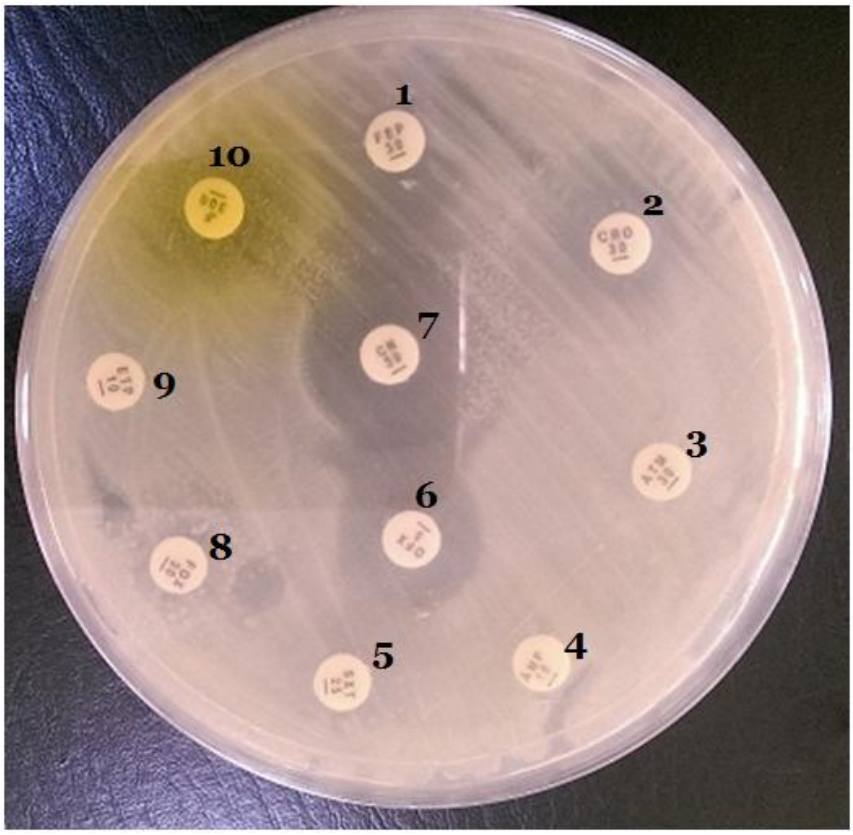

Figure 4.7: Susceptibility test plate of a *Klebsiella* species isolate (from the anal swab of cow) growing on Mueller-Hinton agar plate.

Key:

1= Meropenem (10 µg)
2= Ceftriaxone (30 µg)
3 = Aztreonam (30 µg)
4 = Ampicillin (10 µg)
5 = Imipenem (10 µg)
6 = Nitrofurantoin (10 µg)
7 = Gentamicin (10 µg)
8 = Cefoxitin (10 µg)
9 = Ertapenem (10 µg)
10 = Nitrofurantoin (10 µg)

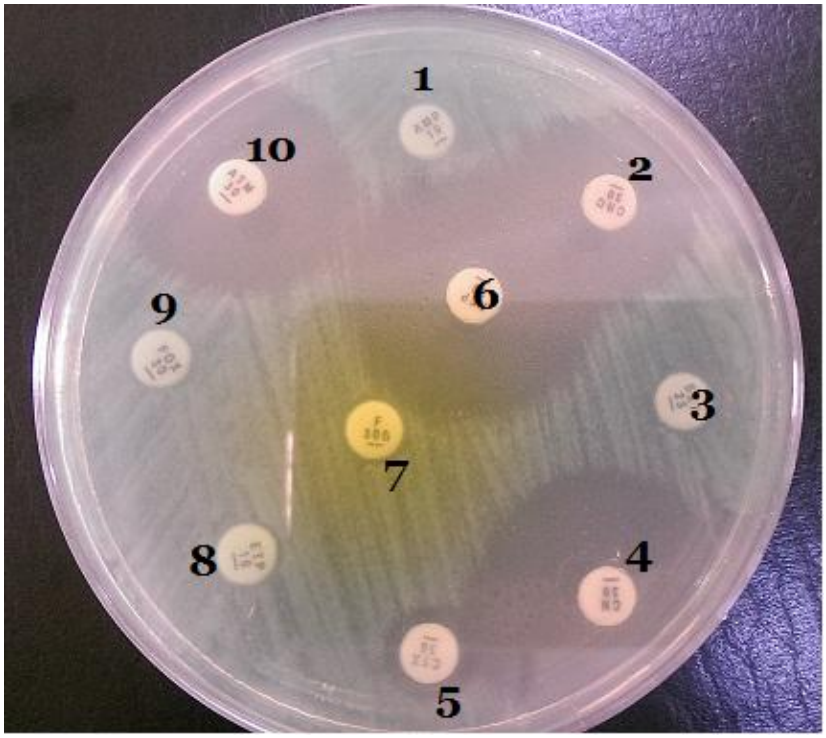

Figure 4.8: Susceptibility test plate of a *Pseudomonas aeruginosa* isolate (from abattoir sample) to a range of single antibiotic disks.

Key:

1= Ampicillin (10 µg)
2= Ceftriaxone (30 µg)
3 = Ciprofloxacin (30 µg)
4 = Gentamicin (10 µg)
5 = Cefotaxime (10 µg)
6 = Ofloxacin (10 µg)
7 = Nitrofurantoin (10 µg)
8 = Ertapenem (10 µg)
9 = Cefoxitin (10 µg)
10 = Aztreonam (10 µg)

4.5 SCREENING AND PHENOTYPIC CONFIRMATION TEST RESULTS FOR MBL AND AmpC ENZYME DETECTION

Phenotypic confirmation tests were carried out on suspected bacteria that produced metallo beta-lactamase (MBL) enzymes and AmpC enzymes based on the recommended screening test for MBL detection. All the isolated *E. coli* isolates (n=168), *Klebsiella* species isolates (n=141) and *P. aeruginosa* isolates (n=147) were tested for their susceptibility to any of the carbapenems, imipenem (10 µg) and meropenem (10 µg); and bacterial isolates found to be resistant to any of these carbapenems as per the CLSI criteria (isolates showing inhibition zone diameter of ≤ 23 mm) were considered and suspected to produce MBL enzymes phenotypically. Imipenem and meropenem have been recommended by the Clinical laboratory Standard Institute (CLSI) and other researchers for the screening of bacterial organisms for the presence of metallo beta-lactamase (MBL) enzymes. Figure 4.9 shows the result of the screening of all the bacterial isolates for their susceptibility to imipenem and meropenem. Out of 168 *E. coli* isolates, only 87 isolates (51.8 %) were resistant to imipenem while 93 *E. coli* isolates (55.4 %) were resistant to meropenem (Figure 4.9). Out of the 141 isolates of *Klebsiella* species recovered from the various environmental samples, 58 isolates (41.1 %) were resistant to imipenem while 61 *Klebsiella* species isolates (43.3 %) were resistant to meropenem (Figure 4.9). The *P. aeruginosa* isolates were also resistant to imipenem (n=98, 66.7 %) and meropenem (n=89, 60.5 %) as shown in Figure 4.9. Notably, the *P. aeruginosa* isolates were more resistant to the carbapenems (imipenem and meropenem) and cefoxitin than the *Klebsiella* species isolates and *E. coli* isolates (Figure 4.9).

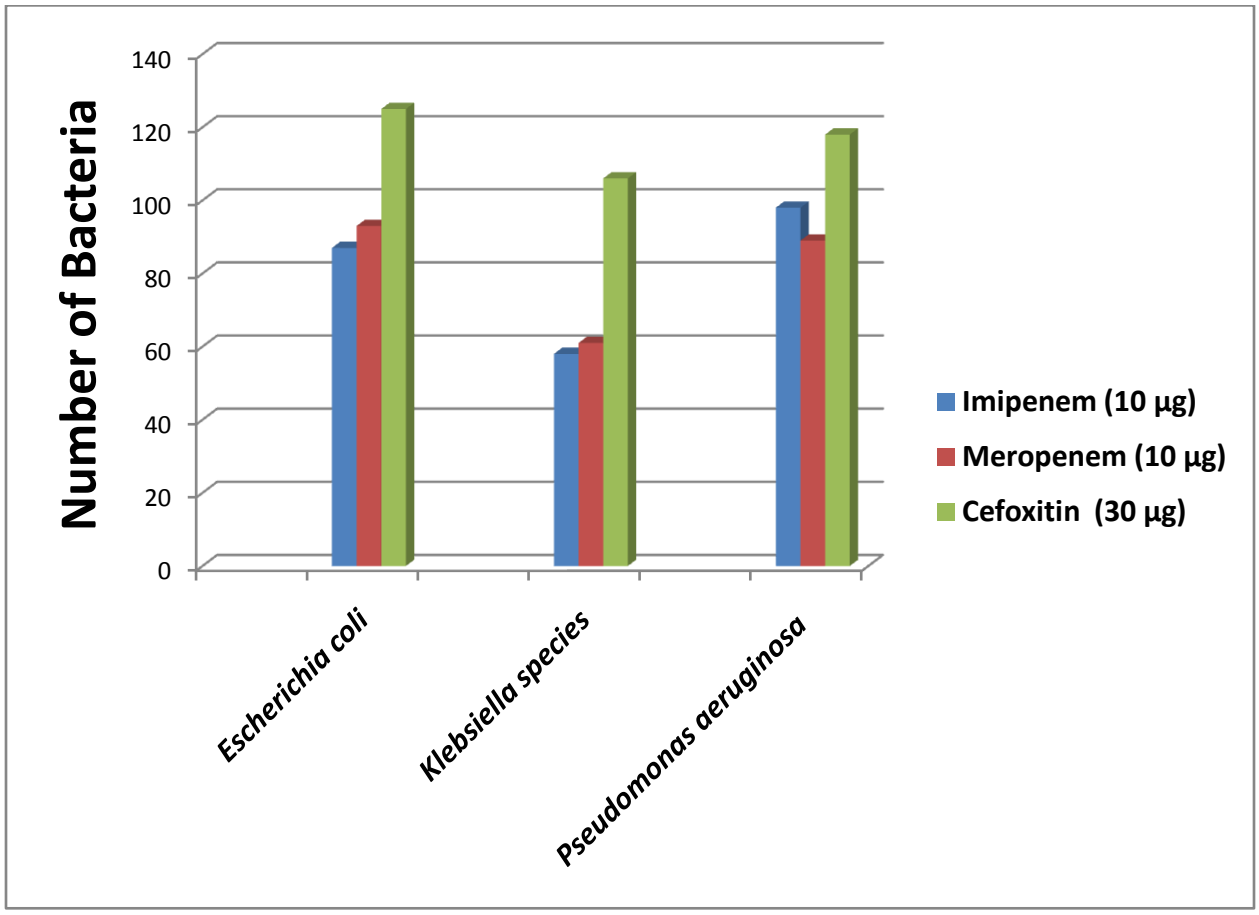

Figure 4.9: Number of Gram-negative bacteria resistant to imipenem (10 µg), meropenem (10 µg) and cefoxitin (30 µg)

The production of MBL enzymes was phenotypically detected using two methods: modified Hodges test (MHT) method and the inhibition based assay in which EDTA was used as a chelating agent. All the bacterial isolates found to be resistant to imipenem and meropenem in the screening test were further tested using the MHT method (Figure 4.10) and the inhibition based assay using EDTA. The result of the phenotypic confirmation of MBL production in the test bacteria is shown in Table 4.6. In this study, MBL producing bacteria was phenotypically detected in a total of 22 *E. coli* isolates out of the 168 isolates of *E. coli* recovered from the various environmental samples. Eight (8) *E. coli* isolates (11.6 %) from abattoir tables were phenotypically confirmed as MBL producers while seven (7) isolates of *E. coli* were respectively characterized as MBL producers from cloacae swab samples and samples from the anal region of cow (Table 4.6). Bacterial organisms expressing metallo beta-lactamase (MBL) enzymes phenotypically, produces zone enhancement around a carbapenem disk impregnated with a chelating agent (e.g. EDTA) since this compound inhibit the action of zinc ion in the active site of the enzyme (Figure 4.11). However, organisms that do not express MBL enzymes were completely susceptible to the carbapenems including imipenem and meropenem as shown in Figure 4.12. The prevalence of MBL-producing *Klebsiella* species and *P. aeruginosa* isolates positive for MBL production is shown in Table 4.6. Of the 141 *Klebsiella* species isolates isolated from the environmental samples, 9 isolates were identified as MBL-positive bacteria using the inhibition based assay and 9 isolates were also positive by the modified Hodges test (MHT) method (Table 4.6). There was no statistical difference amongst the different test isolates and sample source of *E. coli, Klebsiella* species and *P. aeruginosa* for the production of MBL in this study (p value > 0.05).

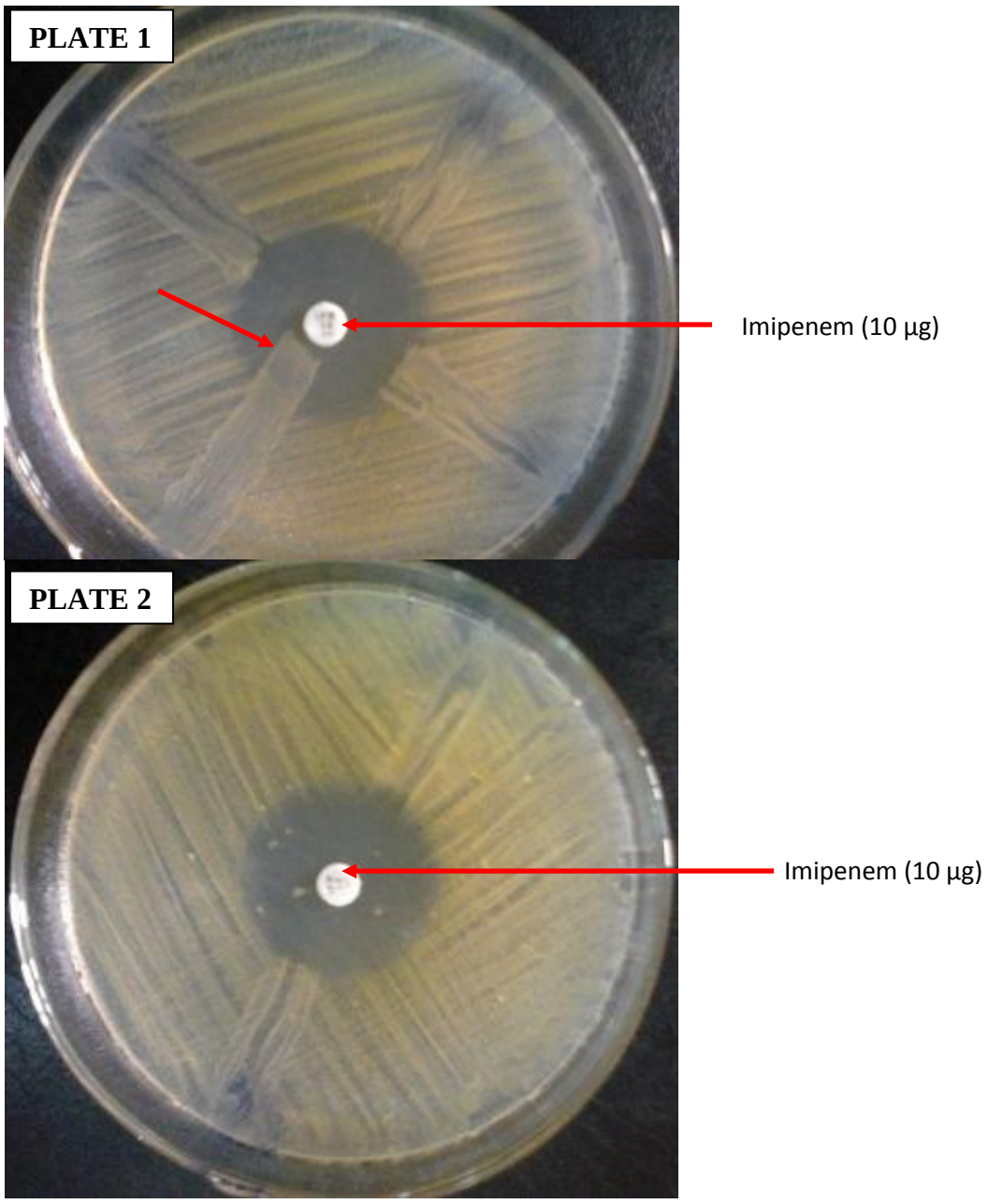

Figure 4.10: *Escherichia coli* isolate tested for the production of metallo beta-lactamase enzyme using the Modified Hodges Test (MHT) method showing a positive result (PLATE 1) and a negative result (PLATE 2). Bacteria expressing MBL enzymes are resistant to the carbapenems, and they tend to remain undaunted by the antimicrobial action of these drugs (arrow)

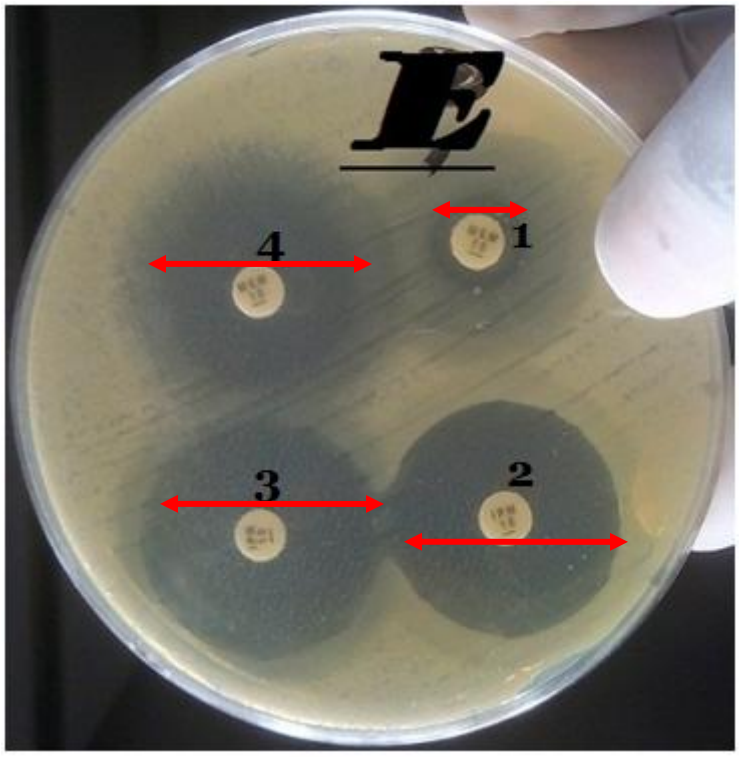

Figure 4.11: A strain of *Escherichia coli* tested using the disk test (inhibition based assay using EDTA) showing a positive result for the production of metallo-β-lactamase enzyme phenotypically. The difference in zone size between imipenem and meropenem disks used alone and imipenem and meropenem impregnated with EDTA differ by ≥7 mm.

Key:
1 = Meropenem (10 µg)
2 = Imipenem (µg)
3 = Imipenem (µg)
4 = Meropenem (10 µg)

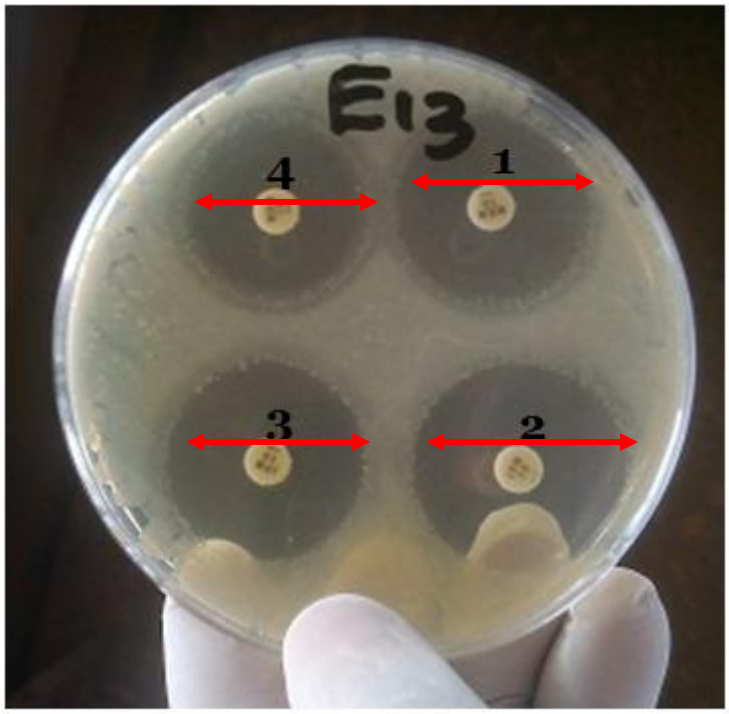

Figure 4.12: *Escherichia coli* isolate tested for the production of metallo-β-lactamase (MBL) enzyme by disk method, showing a negative result. Organisms that do not express metallo beta-lactamase enzymes are completely susceptible to imipenem and meropenem as shown in this picture.

Key:
1 = Meropenem (10 µg)
2 = Imipenem (µg)
3 = Imipenem (µg)
4 = Meropenem (10 µg)

A total of 6 *Klebsiella* species isolates from abattoir tables were MBL-positive while 7 isolates of *Klebsiella* species from the cloacal swabs of poultry birds were identified as MBL producers. Only 5 isolates of *Klebsiella* species were identified as *Klebsiella* species from the anal swab samples of cow (Table 4.6). *Klebsiella* species isolates that do not produce MBL enzyme phenotypically are completely susceptible to imipenem and meropenem (Figure 4.13). The occurrence rate of MBL-positive *P. aeruginosa* isolates in this study was 12.5 % (samples from abattoir tables), 14.6 % (cloacal swab samples) and 18.6 % (samples from anal swab of cow) [Table 4.6]. Of the 147 *P. aeruginosa* isolates tested, 98 isolates (66.7 %) appeared to produce MBL enzymes. *P. aeruginosa* isolates found to produce MBL enzymes are completely resistant to imipenem while those *P. aeruginosa* isolates that do not express MBL enzymes are completely susceptible to the drug (Figure 4.13). In this study, *P. aeruginosa* isolates that produce MBL enzymes were phenotypically detected in abattoir samples (15.0 %), cloacal swab samples (14.3 %), and from anal swab samples of cow (9.6 %) as shown in Table 4.6. A total of 106 *Klebsiella* species was found to be resistant to cefoxitin while 118 isolates of *P. aeruginosa* showed reduced susceptibility to cefoxitin. Nine (9) isolates of *P. aeruginosa* (16.1 %) from abattoir tables were confirmed as AmpC enzyme producers while 7 *P. aeruginosa* isolates (14.6 %) were confirmed as AmpC enzyme producers by the disk methods used in this study. It was also found in this study that 8 isolates of *P. aeruginosa* (18.6 %) from the anal swabs of cow was phenotypically confirmed AmpC enzyme producers as shown in Table 4.7. This study shows that AmpC enzyme production amongst the *Klebsiella* species isolates was 15.0 % (for isolates from abattoir tables), 12. 2 % (for isolates from poultry birds) and 11.5 % (for isolates from the anal swabs of cow) as shown in Table 4.7. Bacterial isolates producing AmpC enzymes were susceptible to cloxacillin but resistant to cefoxitin (Figure 4.14).

Table 4.6: Prevalence of metallo-β-lactamase (MBL) producing bacteria as determined by inhibition-based assay and MHT methods

Organism (n)	Source	Total n (%)
Escherichia coli (69)	Abattoir	8 (11.6)
Escherichia coli (51)	Poultry	7 (13.7)
Escherichia coli (48)	Anal swabs of cow	7 (14.6)
Pseudomonas aeruginosa (56)	Abattoir	7 (12.5)
Pseudomonas aeruginosa (48)	Poultry	7 (14.6)
Pseudomonas aeruginosa (43)	Anal swabs of cow	8 (18.6)
Klebsiella species (40)	Abattoir	6 (15.0)
Klebsiella species (49)	Poultry	7 (14.3)
Klebsiella species (52)	Anal swab of cow	5 (9.6)

p value > 0.05

Key:

MBL = metallo-β-lactamase

n = number of isolates

MHT = Modified Hodges test

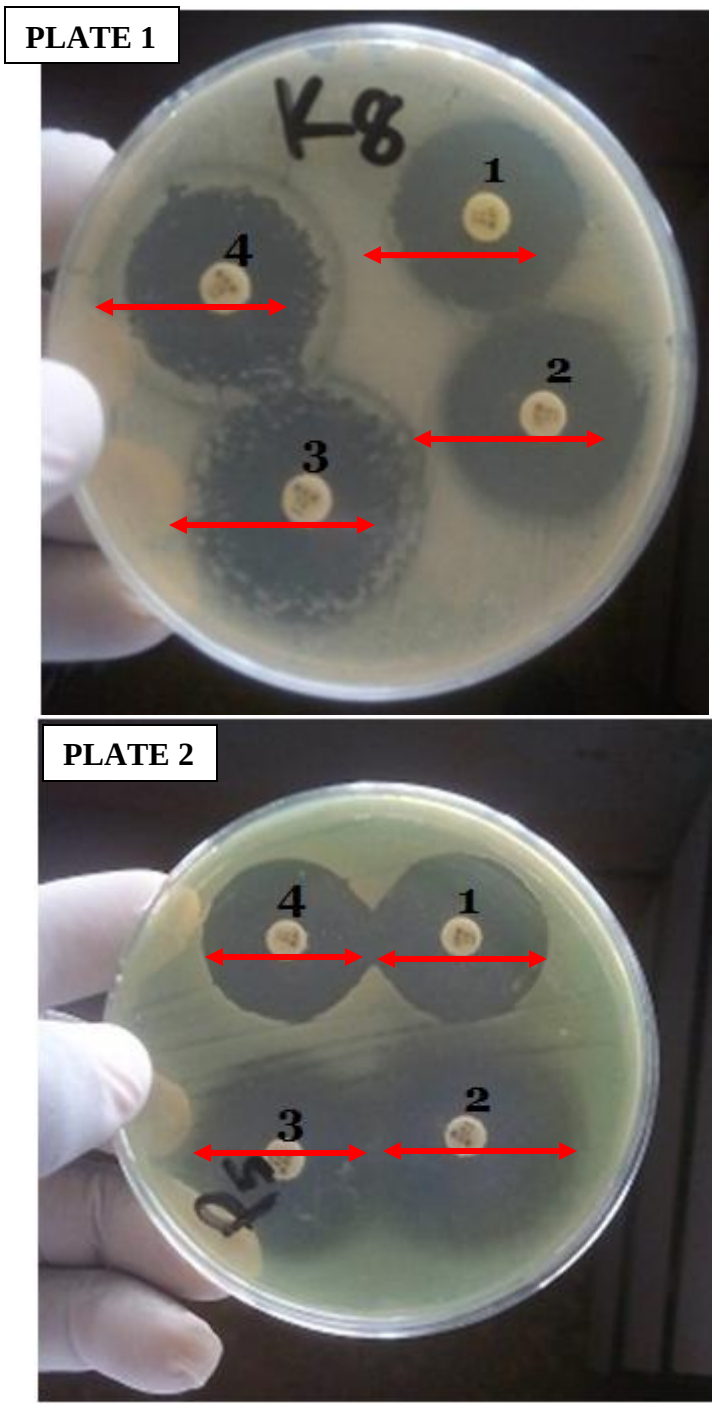

Figure 4.13: Strains of *Klebsiella* species (**PLATE 1**) and *Pseudomonas aeruginosa* (**PLATE 2**) tested for the production of metallo-β-lactamase (MBL) enzyme phenotypically using the disk method.

Key:

1 = Meropenem (10 µg)
2 = Imipenem (µg)
3 = Imipenem (µg)
4 = Meropenem (10 µg)

Table 4.7 shows the result of the AmpC enzyme production amongst the environmental isolates of *E. coli, Klebsiella* species and *P. aeruginosa* from cloacal swabs of poultry birds, anal swabs of cow and swab samples of abattoir tables. AmpC enzyme production was suspected when any of the test isolates showed reduced susceptibility to cefoxitin. Among the 168 isolates of *E. coli* tested in this study, 125 *E. coli* isolates were found to be resistant to cefoxitin; and these isolates were phenotypically tested for AmpC enzyme production. A total of 6 *E. coli* isolates (8.7 %) from abattoir tables were phenotypically confirmed to produce AmpC enzymes by these three methods while 5 isolates of *E. coli* (9.8 %) from cloacal swab samples were phenotypically detected to produce AmpC enzymes (Table 4.7). AmpC enzyme production was also detected in 6 isolates of *E. coli* (12.5 %) from the anal swabs of cow. The result of this study demonstrate that AmpC enzyme production was high in anal swab samples, followed by cloacal swab samples and then samples from abattoir tables. The statistical analysis conducted on the occurrence of AmpC enzyme positive *E. coli, Klebsiella* species and *P. aeruginosa* from the various sample sources showed that it was not statistically significant (p value > 0.05).

Table 4.7: Prevalence of AmpC-enzyme producing bacteria as determined by inducibility test, CIAT and CC-DDST methods

Organism (n)	Source	Total n (%)
Escherichia coli (69)	Abattoir	6 (8.7)
Escherichia coli (51)	Poultry	5 (9.8)
Escherichia coli (48)	Anal swabs of cow	6 (12.5)
Pseudomonas aeruginosa (56)	Abattoir	9 (16.1)
Pseudomonas aeruginosa (48)	Poultry	7 (14.6)
Pseudomonas aeruginosa (43)	Anal swabs of cow	8 (18.6)
Klebsiella species (40)	Abattoir	6 (15.0)
Klebsiella species (49)	Poultry	6 (12.2)
Klebsiella species (52)	Anal swab of cow	6 (11.5)

p value > 0.05

Key:

n = number of isolates

CIAT = ceftazidime-imipenem antagonism test

CC-DDST = Cefoxitin-cloxacillin double disc synergy test

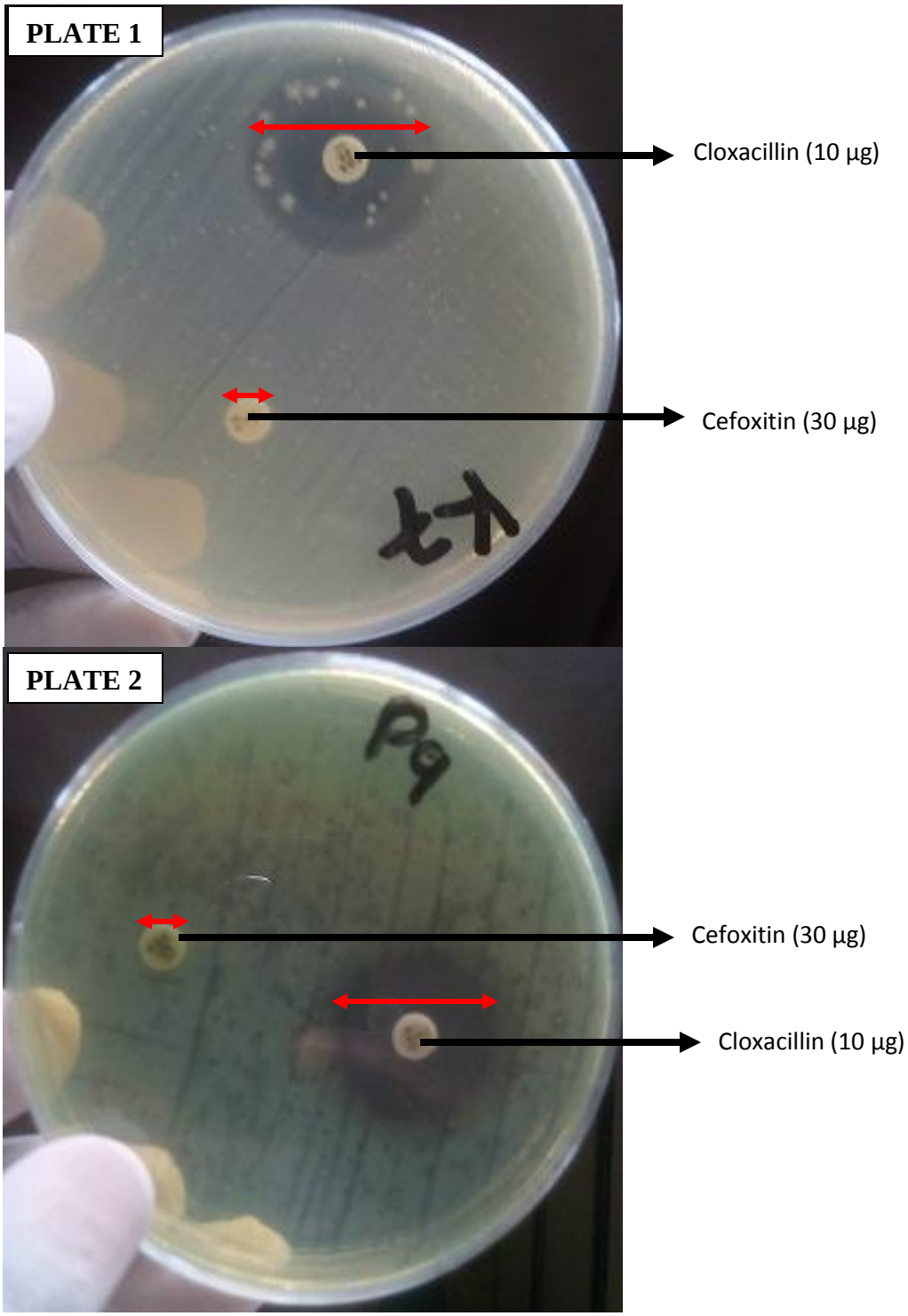

Figure 4.14: Strains of *Klebsiella* species (**PLATE 1**) and *Pseudomonas aeruginosa* (**PLATE 2**) tested for the production of AmpC enzymes using the disk method of cefoxitin-cloxacillin double disk synergy test (CC-DDST). Both organisms express the AmpC enzymes phenotypically, as they were susceptible to cloxacillin. However, the organisms showed complete resistance to cefoxitin, indicating that they produce AmpC enzymes

The result of the multiple antibiotic resistance indexes of MBL- and AmpC-positive bacteria is shown in Table 4.8. The MBL- and AmpC-positive *Klebsiella* species have MARI of 0.4 to 0.5 while the MBL- and AmpC positive *E. coli* isolates have MARI of 0.4 to 0.6. The *P. aeruginosa* isolates that were positive for MBL- and AmpC enzyme production had MARI of 0.7 to 0.8. The MARI result showed that the MBL- and AmpC-positive *Klebsiella* species and *E. coli* isolates were multiply resistant to more than four antibiotic while the *P. aeruginosa* isolates that were positive for MBL- and AmpC enzyme production were multiply resistant to more than seven antibiotics.

Table 4.8: Multiple antibiotic resistance index (MARI) of MBL- and AmpC positive bacteria

Isolates	MARI
K23	0.6
K10	0.7
K39	0.5
K22	0.6
K29	0.4
K18	0.8
K31	0.5
K25	0.6
K21	0.7
K42	0.8
K47	0.4
K11	0.6
K99	0.5
E21	0.6
E78	0.4
E14	0.5
E12	0.4
E26	0.4
E56	0.5
P88	0.7
P55	0.8
P61	0.8
P93	0.7
P44	0.7

K = *Klebsiella* species; E = *Escherichia coli*; P = *Pseudomonas aeruginosa*

4.6 RESULTS OF GENOTYPIC ANALYSIS USING MULTIPLEX PCR

The result of gene amplification of MBL phenotypes in the test isolates for the detection of MBL genes is shown in Table 4.9. It was observed that only the IMP-type MBL gene family (particularly the IMP-1 genes and IMP-2 genes) were detected by multiplex PCR in this study. It was observed from our study that the prevalence of MBL-producing strains detected with multiplex PCR from the test bacterial isolates that were phenotypically detected as MBL-positive isolates were 54.6 % in the *E. coli* isolates, 83.3 % in the *Klebsiella* species isolates and 72.7 % in the *P. aeruginosa* isolates (Table 4.9). Only the IMP MBL gene family was detected by PCR in this study. The VIM-1 and VIM-2 MBL genes were not detected in the MBL phenotypes. Overall, the prevalence of IMP-1 gene in the MBL phenotypes was 36.4 %, 50 % and 54.5 % for *E. coli, Klebsiella* species and *P. aeruginosa* respectively (Table 4.9). However, it was observed in this study that the occurrence rate of the IMP-2 genes in this study was less than the IMP-1 gene – which was detected more among the MBL phenotypes (Table 4.9). IMP-2 genes were detected at the rate of 18.2 %, 33.3 % and 18.2 % for *E. coli, Klebsiella* species and *P. aeruginosa* isolates respectively (Table 4.9). Figure 4.15 and Figure 4.16 showed the PCR amplification of gel products of IMP-1 genes and IMP-2 genes respectively, in MBL phenotypes of *E. coli* and *Klebsiella* species. As shown, IMP-1 MBL gene has a base-pair (bp) band of 587 bp (Figure 4.15) while the IMP-2 gene has a base-pair of 678 bp (Figure 4.16). There was no statistical difference (p value > 0.05) in the detection of MBL genes of ^{bla}IMP-1 and ^{bla}IMP-2 genes amongst the *E. coli, Klebsiella* species and *P. aeruginosa* isolates evaluated in this study.

Table 4.9: Detection of metallo-β-lactamase (MBL) gene variants in *E. coli*, *Klebsiella* species and *P. aeruginosa* MBL phenotypes by multiplex PCR

Gene (s)	*Escherichia coli* (n=22) No. (%) of isolates	*Klebsiella* species (n=18) No. (%) of isolates	*Pseudomonas aeruginosa* (n=22) No. (%) of isolates
*bla*IMP-1	8 (36.4)	9 (50)	12 (54.5)
*bla*IMP-2	4 (18.2)	6 (33.3)	4 (18.2)
Total	12 (54.6)	15 (83.3)	16 (72.7)

p value > 0.05

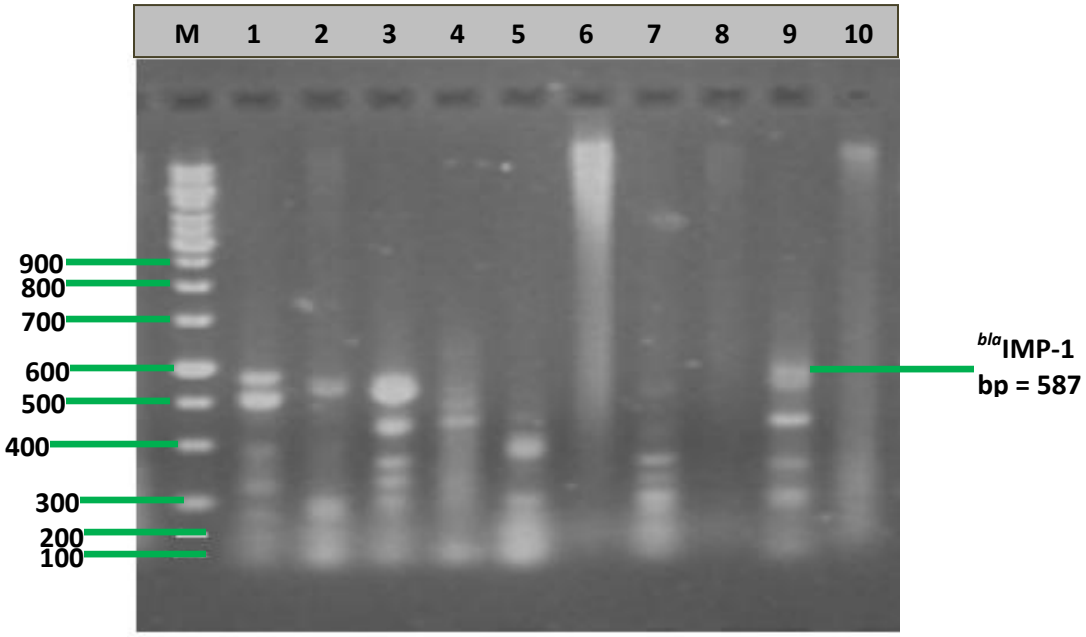

Figure 4.15: Amplification of MBL genes from *Escherichia coli*.

Key:

Lane M = DNA markers/ladder (100 bp)

Lanes 1 - 9 = Different DNA samples

Lane 10 = Negative control which contains all PCR reagents excluding DNA.

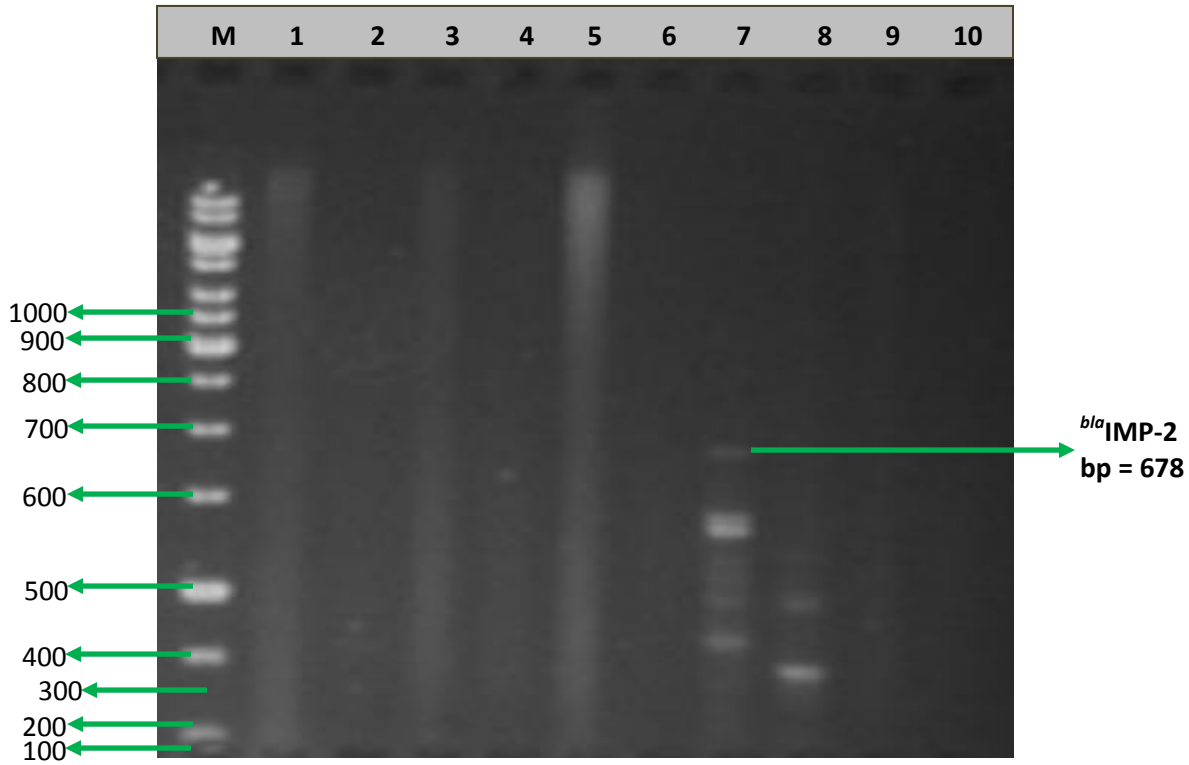

Figure 4.16: Amplification of MBL genes from *Klebsiella* species and *P. aeruginosa*. phenotypes.

Key:

Lane M = DNA markers/ladder (100 bp)

Lanes 1 - 9 = Different DNA samples

Lane 10 = Negative control which contains all PCR reagents excluding DNA.

Table 4.10 shows the result of gene amplification of AmpC genes in the *E. coli, Klebsiella* species and *P. aeruginosa* isolates. It was discovered that only the CMY-1 genes and FOX-1 genes were detected in the test bacterial isolates used in this study. In total, 17 isolates of *E. coli,* 18 isolates of *Klebsiella* species and 24 isolates of *P. aeruginosa* were examined genotypically using multiplex PCR for AmpC encoding genes. Out of the series of AmpC genes investigated in this study, only the CMY-1 genes and the FOX-1 genes were harboured by the test bacterial isolates. It was observed also, that out of the 17 isolates of *E. coli,* only 3 and 2 *E. coli* isolates harboured the CMY-1 genes (17.6 %) and the FOX-1 genes (11.8 %) respectively. Four (4) out of the 18 *Klebsiella* species harboured the CMY-1 genes while only one (1) isolate of *Klebsiella* species harboured the FOX-1 genes. Also in this study, 7 isolates of the tested *P. aeruginosa* isolates were detected by multiplex PCR to harbour the CMY-1 genes while only 3 *P. aeruginosa* isolates harboured the FOX-1 genes. The CMY homologues particularly the CMY-1 genes were the most predominant AmpC genes detected in this study; and this was followed by the FOX-1 genes which were least detected in the test populations of *E. coli, Klebsiella* species and *P. aeruginosa* phenotypes. Electrophoretic analysis of the test isolates after gene amplification using PCR technique revealed bands in the electrophoretogram; and this was positive for CMY-1 genes with a base pair of 520 bp (Figure 4.17) and FOX-1 genes with a base pair of 190 bp (Figure 4.18). There was no statistical difference (p value > 0.05) in the detection of AmpC genes of CMY-1 and FOX-1 genes amongst the *E. coli, Klebsiella* species and *P. aeruginosa* isolates evaluated in this study. The distribution of MBL and AmpC genes from the bacterial isolates according to their sources is shown in Table 4.11. MBL and AmpC genes were detected more from isolates emanating from abattoir samples than from poultry samples. The distribution of the MBL and AmpC genes in the study isolates is shown in Table 4.11.

Table 4.10: Detection of AmpC enzyme gene variants in *E. coli*, *Klebsiella* species and *P. aeruginosa* AmpC phenotypes by multiplex PCR

Gene (s)	*Escherichia coli* (n=17) No. (%) of isolates	*Klebsiella* species (n=18) No. (%) of isolates	*Pseudomonas aeruginosa* (n=24) No. (%) of isolates
CMY-1	3 (17.6)	4 (22.2)	7 (29.2)
FOX-1	2 (11.8)	1 (5.6)	3 (12.5)
Total	5 (29.4)	5 (27.8)	10 (41.7)

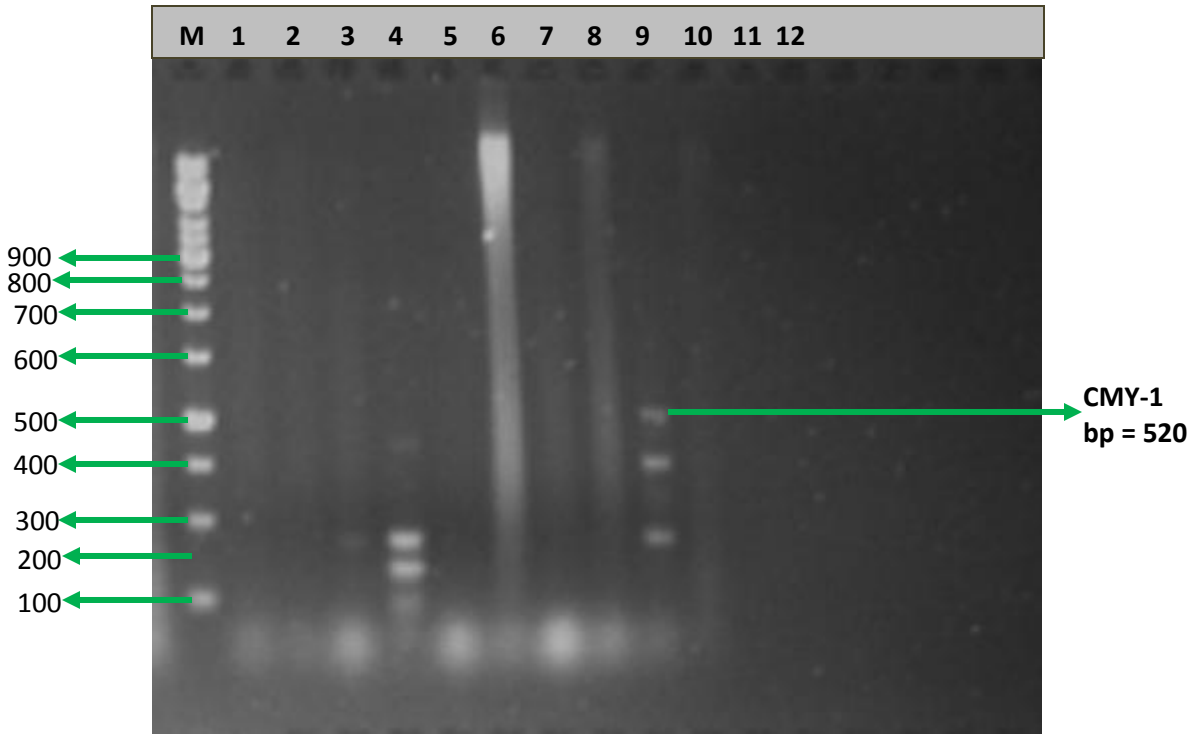

Figure 4.17: Amplification of AmpC genes from *Escherichia coli*

Key:

Lane M = DNA markers/ladder (100 bp)

Lanes 1 - 9 = Different DNA samples

Lane 10 = Negative control which contains all PCR reagents excluding DNA.

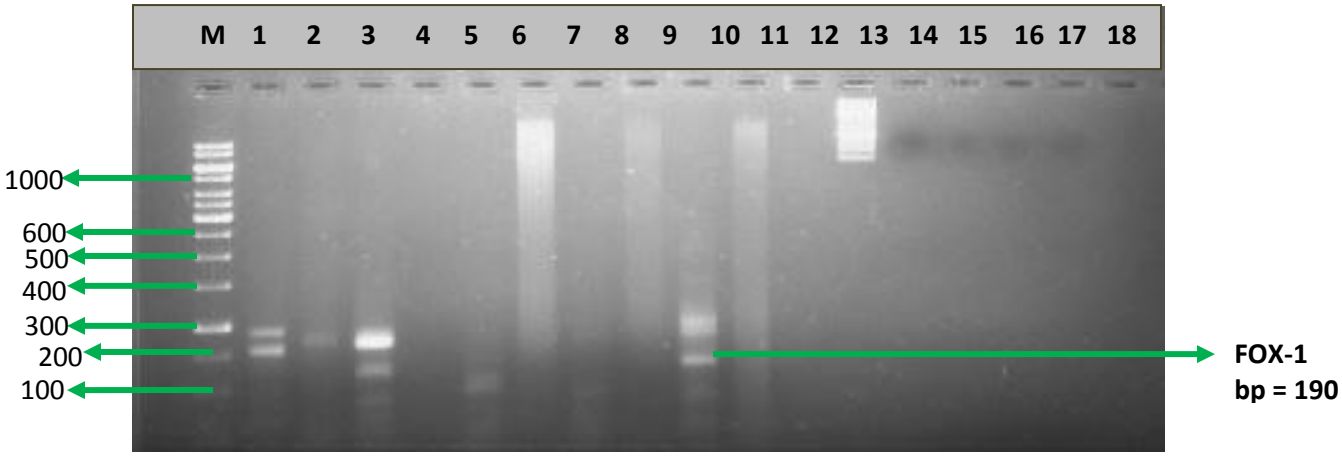

Figure 4.18: Amplification of AmpC genes from *Klebsiella* species and *Pseudomonas aeruginosa*

Key:

Lane M = DNA markers/ladder (100 bp)

Lanes 1 - 9 = Different DNA samples

Lane 10 = Negative control which contains all PCR reagents excluding DNA.

Table 4.11: Distribution of MBL genes and AmpC genes within study isolates based on their sources

Isolate code	Organism	Source	Phenotype	Gene detected	Inference
E21	*E. coli*	Abattoir	MBL +ve	IMP-1	MBL gene
E78	*E. coli*	Abattoir	MBL +ve	IMP-1	MBL gene
E14	*E. coli*	Abattoir	MBL +ve	IMP-1	MBL gene
E12	*E. coli*	Abattoir	MBL +ve	IMP-1	MBL gene
E26	*E. coli*	Abattoir	MBL +ve	IMP-1	MBL gene
E56	*E. coli*	Abattoir	MBL +ve	IMP-1	MBL gene
E10	*E. coli*	Poultry	MBL +ve	IMP-1	MBL gene
E55	*E. coli*	Poultry	MBL +ve	IMP-1	MBL gene
E20	*E. coli*	Abattoir	MBL +ve	IMP-2	MBL gene
E31	*E. coli*	Abattoir	MBL +ve	IMP-2	MBL gene
E42	*E. coli*	Abattoir	MBL +ve	IMP-2	MBL gene
E46	*E. coli*	Poultry	MBL +ve	IMP-2	MBL gene
E60	*E. coli*	Abattoir	AmpC +ve	CMY-1	AmpC gene
E78	*E. coli*	Abattoir	AmpC +ve	CMY-1	AmpC gene
E101	*E. coli*	Poultry	AmpC +ve	CMY-1	AmpC gene
E53	*E. coli*	Poultry	AmpC +ve	FOX-1	AmpC gene
E12	*E. coli*	Poultry	AmpC +ve	FOX-1	AmpC gene
K23	*Klebsiella* species	Poultry	MBL +ve	IMP-1	MBL gene
K10	*Klebsiella* species	Poultry	MBL +ve	IMP-1	MBL gene
K39	*Klebsiella* species	Poultry	MBL +ve	IMP-1	MBL gene
K22	*Klebsiella* species	Abattoir	MBL +ve	IMP-1	MBL gene
K29	*Klebsiella* species	Abattoir	MBL +ve	IMP-1	MBL gene
K18	*Klebsiella* species	Abattoir	MBL +ve	IMP-1	MBL gene
K31	*Klebsiella* species	Poultry	MBL +ve	IMP-1	MBL gene
K25	*Klebsiella* species	Poultry	MBL +ve	IMP-1	MBL gene
K21	*Klebsiella* species	Abattoir	MBL +ve	IMP-1	MBL gene
K42	*Klebsiella* species	Abattoir	MBL +ve	IMP-2	MBL gene
K47	*Klebsiella* species	Abattoir	MBL +ve	IMP-2	MBL gene
K11	*Klebsiella* species	Poultry	MBL +ve	IMP-2	MBL gene
K99	*Klebsiella* species	Abattoir	MBL +ve	IMP-2	MBL gene
K20	*Klebsiella* species	Abattoir	MBL +ve	IMP-2	MBL gene
K49	*Klebsiella* species	Abattoir	MBL +ve	IMP-2	MBL gene
K76	*Klebsiella* species	Abattoir	AmpC +ve	CMY-1	AmpC gene
K71	*Klebsiella* species	Abattoir	AmpC +ve	CMY-1	AmpC gene
K80	*Klebsiella* species	Abattoir	AmpC +ve	CMY-1	AmpC gene
K99	*Klebsiella* species	Abattoir	AmpC +ve	CMY-1	AmpC gene

Table 4.11 continues

K19	*Klebsiella* species	Poultry	AmpC +ve	FOX-1	AmpC gene
P88	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P55	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P61	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P93	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P44	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P10	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P20	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P45	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P23	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P11	*P. aeruginosa*	Poultry	MBL +ve	IMP-1	MBL gene
P65	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P45	*P. aeruginosa*	Abattoir	MBL +ve	IMP-1	MBL gene
P32	*P. aeruginosa*	Poultry	MBL +ve	IMP-2	MBL gene
P21	*P. aeruginosa*	Abattoir	MBL +ve	IMP-2	MBL gene
P54	*P. aeruginosa*	Poultry	MBL +ve	IMP-2	MBL gene
P87	*P. aeruginosa*	Poultry	MBL +ve	IMP-2	MBL gene
P14	*P. aeruginosa*	Abattoir	AmpC +ve	CMY-1	AmpC gene
P99	*P. aeruginosa*	Abattoir	AmpC +ve	CMY-1	AmpC gene
P80	*P. aeruginosa*	Poultry	AmpC +ve	CMY-1	AmpC gene
P8	*P. aeruginosa*	Poultry	AmpC +ve	CMY-1	AmpC gene
P2	*P. aeruginosa*	Abattoir	AmpC +ve	CMY-1	AmpC gene
P13	*P. aeruginosa*	Abattoir	AmpC +ve	CMY-1	AmpC gene
P94	*P. aeruginosa*	Poultry	AmpC +ve	CMY-1	AmpC gene
P82	*P. aeruginosa*	Abattoir	AmpC +ve	FOX-1	AmpC gene
P49	*P. aeruginosa*	Poultry	AmpC +ve	FOX-1	AmpC gene
P75	*P. aeruginosa*	Poultry	AmpC +ve	FOX-1	AmpC gene

4.5 PLASMID CURING ANALYSIS RESULT

The result of the plasmid curing analysis of the MBL-positive and AmpC-positive *E. coli* isolates, *Klebsiella* species and *P. aeruginosa* using acridine orange (0.1 mg/ml) as a mutagen is shown in Table 4.12. The antimicrobial activity of the cured bacterial isolates were determined using broad-spectrum antibiotics including ceftriaxone, cefoxitin, imipenem, ceftazidime, ertapenem, cefotaxime, and meropenem. The plasmid curing experiment carried out in this study revealed that most of the antibiotic resistant *E. coli* isolates, *Klebsiella* species and *P. aeruginosa* isolates (which were positive for MBL production and AmpC enzyme production) were plasmid mediated since most of the organisms showed susceptibility (i.e. cured) to the tested antibiotics to which they were previously resistant to (APPENDIX III). However, some of the *E. coli* isolates, *Klebsiella* species and *P. aeruginosa* isolates still remained resistant to the same tested antibiotics after treatment with acridine orange dye (Tables 4.12). Out of the 12 isolates of *Klebsiella* species that were treated with acridine orange dye (0.1 mg/ml), 10 *Klebsiella* isolates showed no enzyme activity after curing while 4 isolates showed enzyme activity. This result shows that the 10 (71.4 %) *Klebsiella* species isolates harboured their resistant traits in the plasmid while the resistant genes of the other four isolates were chromosomally-borne since they still exhibited less susceptibility to the tested antibiotics after curing. Only 4 (80 %) isolates of *E. coli* and 3 (60 %) isolates of *P. aeruginosa* harboured their resistant traits in their plasmids (Table 4.12). The *E. coli* isolates, *Klebsiella* species and *P. aeruginosa* isolates that showed susceptibility to the tested antibiotics after treatment with acridine orange have their resistant traits on a plasmid; and thus the antibiotic resistance phenotypes/genotype of these organisms are plasmid-mediated.

Table 4.12: Result of plasmid curing analysis using 0.1 mg/ml of acridine orange

Organisms	Pre-curing (No. of isolates)	Cured isolates		Post-curing (Resistant)	
		n	%	n	%
Klebsiella species	14	10	71.4	4	28.6
Escherichia coli	5	4	80	1	20
Pseudomonas aeruginosa	5	3	60.0	2	40

Table 4.13 shows the co-production of MBL and AmpC enzymes in the *E. coli*, *Klebsiella* species and *P. aeruginosa* isolates according to their sources and the antibiotic resistance genes that each of the isolates co-produced.

Only one *Klebsiella* species isolate and one *P. aeruginosa* isolate was from cloacal swab samples. The rest of the *E. coli* isolates, *Klebsiella* species and *P. aeruginosa* isolates that co-produced MBL enzymes and AmpC enzymes were from abattoir samples (Table 4.13). The genes co-harboured by these MBL phenotypes and AmpC phenotypes were the IMP-1 genes and CMY-1 genes that is responsible for the co-production of MBL and AmpC enzymes in the test bacterial isolates respectively (Table 4.13). The multiple antibiotic resistance index (MARI) of these bacterial isolates that co-produced MBL and AmpC enzymes shows that the isolates had MARI of between 0.6 and 0.8. This shows that the *E. coli, Klebsiella* species and *P. aeruginosa* isolates that co-produced MBL and AmpC enzymes in this study were multiply resistant to more than 6 different antibiotics from different classes as used in this study.

Table 4.13: Co-production of MBL enzymes and AmpC enzymes

Isolates	Source	MBL + AmpC	Gene detected	MARI
K23	Abattoir	+	IMP-1, CMY-1	0.6
K22	Poultry	+	IMP-1, CMY-1	0.6
K11	Abattoir	+	IMP-2, CMY-1	0.6
E21	Abattoir	+	IMP-1, CMY-1	0.6
E56	Abattoir	+	IMP-1, CMY-1	0.5
P61	Poultry	+	IMP-1, CMY-1	0.8
P93	Abattoir	+	IMP-1, CMY-1	0.7
P44	Abattoir	+	IMP-1, CMY-1	0.7

Key:
K = *Klebsiella*
E = *Escherichia*
P = *Pseudomonas*
+ = Positive for co-production of MBL enzymes and AmpC enzymes
MARI = multiple antibiotic resistance index

CHAPTER FIVE

DISCUSSION, CONCLUSION, RECOMMENDATIONS AND CONTRIBUTION TO KNOWLEDGE

5.1 Discussion

In this study, environmental samples including cloacal swabs of poultry birds, anal swabs of cow and swab samples from abattoir benches/tables were bacteriologically analyzed for the isolation of *Escherichia coli, Klebsiella* species and *Pseudomonas aeruginosa*; and these organisms were phenotypically and genotypically tested for the production of multidrug resistant genes including genes responsible for the production of metallo-beta-lactamase (MBL) and AmpC enzymes. These environmental samples were chosen for this study to isolate *Klebsiella* species, *P. aeruginosa* and *E. coli* for the identification of MBL- and AmpC-producing isolates because of their close association with humans. The increasing reports of the development and spread of multidrug resistance genes (including genes for MBL and AmpC enzyme production) amongst these organisms in both the community and hospital environments also necessitated the choice for these samples and class of Gram-negative bacteria (Aibinu *et al*., 2007; Akujobi *et al*., 2012; Ben-Slama *et al*., 2010; Bergenholtz *et al*., 2009; Usha *et al*., 2010). Moreover, these organisms portend public health risk due in part to their antibiotic resistant nature and their ability to cause several bacterial infections in human populace. The source of the samples used in this study has serious connotation or association with humans because man depends on these animals for their source of food (meat) and protein. Abattoirs/slaughter houses and poultry farms are good grounds for the breeding, development and spread of antibiotic resistant bacteria including *E.*

coli, Klebsiella species and *P. aeruginosa* because antibiotics are used irrationally in these environments for the breeding and/or production and rearing of these food-producing animals. This has made such environments like abattoirs and poultry farms to be good areas for the selection of antibiotic resistant traits and also for the dissemination of resistant traits or phenotypes to other susceptible bacteria in the same environment.

E. coli isolates was isolated from 168 environmental swab samples out of the 370 environmental swab samples analyzed in this study. *E. coli* was isolated from 69 swab samples out of 130 swab samples from slaughter/abattoir tables, 51 swab samples out of 120 cloacal swab samples of poultry birds, and from 48 swab samples out of 120 swab samples from the anal region of cows. And recovery rate of *E. coli* from the various environmental swab samples analyzed in this study was 41.1 %, 30.4 % and 28.6 % for swab samples from abattoir/slaughter tables, swab samples from the cloacal region of poultry birds and swabs from the anal region of cows.

E. coli, Klebsiella species and *P. aeruginosa* have been previously noted as producers of metallo-beta-lactamase enzymes and AmpC enzymes; and such phenotypes confers on the bacteria the exceptional ability to be resistant to a wide variety of antibiotics (Bashir *et al*., 2011; Chakraborty *et al*., 2010; Shevade and Agrawal, 2013). The irrational use of antibiotics in the rearing and production of food-producing animals such as cow and poultry birds is very strategic for the development and dissemination of antibiotic resistant bacteria in any community – as this study has shown the occurrence of such bacteria in abattoir samples, cloacal swab samples and samples from the anal region of cows. The recovery rate of *Klebsiella* species isolates from the environmental swab samples including swab samples from abattoir tables, cloacal swab samples from poultry birds and anal swab samples from cows was 28.4 %, 34.8 %, and 36.9 % respectively.

The occurrence of Gram-negative bacteria including *E. coli, Klebsiella* species and *P. aeruginosa* in environmental samples (as obtainable in this study) have been previously reported both within Nigeria and in other parts of the world. (Akinduti *et al*., 2012; Ben-Slama *et al*., 2010; Bertrand *et al*., 2006; Bergenholtz *et al*., 2009; Leung *et al*., 2013; Ejikeugwu *et al*., 2016). Akinduti *et al*. (2012) also reported in their study that *E. coli, Klebsiella* species and *P. aeruginosa* were the most prevalent organisms isolated from environmental samples including samples from poultry farms. These results also showed that *E. coli*, followed by *P. aeruginosa* were the most prevalent Gram-negative bacteria isolated from environmental samples including samples from poultry birds (Bergenholtz *et al*., 2009; Leung *et al*., 2013; Ben-Slama *et al*., 2010). All the isolated Gram-negative bacteria were tested for the production of beta-lactamase enzymes using nitrocefin sticks.

Beta-lactamase enzymes were phenotypically detected in the Gram-negative bacteria at varying rates. *E. coli* produced beta-lactamase enzyme at the rate of 38 % while *P. aeruginosa* produced the enzyme at the rate of 33 %. The rate of beta-lactamase production in *Klebsiella* species isolates was 29 %. A previous study has shown that the presence of beta-lactamase enzyme in bacteria provides opportunity for the horizontal transmission of these enzymes from one organism to another (Bush and Jacoby, 2010). The recurring mutation in the genetic makeup of these beta-lactamase genes has been attributed to the development and dissemination of multidrug resistant bacteria with mutated forms of beta-lactamases in the community including *E. coli, Klebsiella* species and *P. aeruginosa* that produce MBL and AmpC enzymes (Jacoby and Munoz-Price, 2005; Bush and Jacoby, 2010). Beta-lactamase enzyme is responsible for the inactivation of the antimicrobial potency of most antimicrobial agents especially the beta-lactam agents such as antibiotics in the group: cephalosporins, penicillins and carbapenems.

Beta-lactam antimicrobial agents have beta-lactam ring in their molecular/chemical structure. Thus, microbes that express beta-lactamase enzymes have the innate ability to ward-off any antimicrobial action from these drugs. Beta-lactamase enzymes cleaves the beta-lactam ring of these antibiotics, thus breaking open the beta-lactam ring (which is responsible for the antimicrobial action or potency of the drug) of the antibiotics, and by this means the drug is rendered inefficacious *in vivo*. Beta-lactamase enzymes are responsible for various resistance mechanisms experienced or expressed by Gram-negative bacteria including *E. coli, Klebsiella* species and *P. aeruginosa*. These organisms have also acquired some multidrug resistant genes such as those that mediate the expression of AmpC enzymes and metallo-beta-lactamase (MBL) enzymes – which have now allowed these bacteria to be multiply resistant to many antibiotics (Bush and Jacoby, 2010; Jacoby and Munoz-Price, 2005). According to Jacoby and Munoz-Price (2005), mutation in the active sites of beta-lactamase enzymes have resulted in the development and spread of broad-spectrum beta-lactamase enzymes including extended spectrum beta-lactamase (ESBL) enzymes, metallo beta-lactamases (MBLs) and AmpC enzymes. Antibiotic resistance including those mediated by ESBLs, MBLs and AmpC enzymes flourishes wherever antibiotics are abused, misused and dispensed or administered at antimicrobial levels that are lower than the required treatment guidelines or usage. Pathogenic bacteria including *E. coli, Klebsiella* species and *P. aeruginosa* that produces these enzymes (MBL and AmpC enzymes in particular) have over the years acquired resistant genes – which have given them the exceptional ability to be multiply resistant to several classes of antibiotics. To test bacterial isolates for the presence of multidrug resistant enzymes such as MBLs and AmpC enzymes, it is most desirable to test all the isolates with a combination of several antibiotics that will allow the detection of the prevalent resistant mechanisms in the test organisms.

The *E. coli* isolates were completely resistant to the carbapenems, penicillins, aminoglycosides, fluoroquinolones and cephalosporins used in this study. Out of the 168 isolates of *E. coli* recovered from the environmental samples in this study, 160 (95.2 %) *E. coli* isolates were resistant to ceftriaxone. It was also found that 162 (96.4 %) isolates of the *E. coli* isolates and 165 (98.2 %) *E. coli* isolates were resistant to ceftazidime and cefotaxime – which are both third generation cephalosporins and broad spectrum antimicrobial agents. Very low levels of susceptibility of the *E. coli* isolates was also observed with cefoxitin, oxacillin, ofloxacin, amikacin, ciprofloxacin, and aztreonam with resistance rate of 74.4 %, 81.5 %, 70.8 %, 64.9 %, 81.5 % and 93.5 % respectively. The antibiogram results also showed that 103 (61.3 %) of the *E. coli* isolates and 129 (76.8 %) *E. coli* isolates were highly resistant to cloxacillin and nitrofurantoin. The *E. coli* isolates were also highly resistant to the carbapenems including imipenem (51.8 %), ertapenem (86.9 %) and meropenem (55.4 %). The high levels of resistance of *E. coli* isolates from environmental samples to some conventional antibiotics in this study have been reported in the Netherlands, Nigeria, and Uganda (van den Bogaard *et al*., 2001; Majalija *et al*., 2010; Ogunleye *et al*., 2008). Bergenholtz *et al*. (2009) also reported high resistance of *E. coli* isolates from environmental samples to some conventional antibiotics as reported in this study. In Uganda, it was also reported that 168 *E. coli* isolates out of 182 *E. coli* isolates were resistant to several antibiotic classes including penicillins, fluoroquinolones, aminoglycosides and tetracyclines (Majalija *et al*., 2010). The high profile level of resistance observed in *E. coli* isolates has been attributable to the production of beta-lactamase enzymes including AmpC enzymes and metallo-beta-lactamase – which gives these organisms the ability to be multiply resistant to antimicrobial agents. And the use of antibiotics as growth promoting

agents in the production and rearing of animals contributes a great deal to the development and spread of antimicrobial resistance in the community (EFSA, 2011).

Klebsiella species was multiply resistant to the various antibiotics used in this study. The resistance pattern most commonly observed amongst the *Klebsiella* species isolates was resistance to cefotaxime (96.5 %), aztreonam (96.5 %), ceftriaxone (89.4 %), oxacillin (87.2 %), ciprofloxacin (86.5 %), ceftazidime (82.3 %) and cloxacillin (73.0 %). The next most frequent resistance phenotypes of the *Klebsiella* species isolates was resistance to the carbapenems including imipenem (41.1 %), meropenem (43.3 %) and ertapenem (84.4 %). The *Klebsiella* species isolates were resistant to ampicillin (69.5 %), nitrofurantoin (74.5 %), amikacin (52.5 %), and gentamicin (61.0 %). The cephamycin, cefoxitin had little inhibitory effect on the test *Klebsiella* species isolates as the organisms were highly resistant to this antibacterial agent (75.2 %). The frequency of antimicrobial resistance of *Klebsiella* species isolates to the tested antibiotics has been previously reported in Australia (Leung *et al*., 2013). In Enugu State, higher levels of resistance were recorded amongst *Klebsiella* species isolates recovered from sewage samples (Eze, 2012). This study which is similar to ours also recorded higher levels of resistance to other Gram-negative bacteria including *P. aeruginosa* and *E. coli*.

The *P. aeruginosa* isolates recovered in this study also showed high level resistance to the carbapenems including imipenem (66.7 %), ertapenem (61.2 %) and meropenem (60.5 %). Reduced susceptibility of the *P. aeruginosa* isolates was also observed in cefoxitin (80.3 %), cefotaxime (79.6 %), ceftriaxone (64.6 %) and ceftazidime (54.4 %). This study revealed that none of the tested antibiotics showed complete activity against the *P. aeruginosa* isolates, thus revealing the multidrug resistant nature of the test organism. Several reports around the world revealed that *P. aeruginosa* is notorious for its resistance to antibiotics due to the permeability

barrier associated with the outer membrane (OM) layer of the organism (Fernandez *et al*., 2012; Walsh *et al*., 2005: Wegener, 2003*;* Madigan *et al*., 2009). In Brazil, high-level resistance of *P. aeruginosa* isolates to the carbapenems has also been reported (Franco *et al*., 2010). All the *P. aeruginosa* isolates also showed reduced susceptibility to oxacillin, ofloxacin, ciprofloxacin, ampicillin, aztreonam, nitrofurantoin and cloxacillin; and this result is similar to results obtained by Aibinu *et al*. (2007) and Olutayo and Abimbola (2016) who both reported similar levels of reduced susceptibility of *P. aeruginosa* isolates to antibiotics in southwest Nigeria. Bashir *et al*. (2011) and Akinduti *et al*. (2012) also reported in their study carried out in Kashmir and Abeokuta respectively that *P. aeruginosa* is a multidrug resistant organism that is notoriously resistant to several antibiotic classes. The multidrug resistant nature of *P. aeruginosa* isolates has been confirmed by Fernandez *et al*. (2012) and Lolans *et al*. (2005) in the Americas and United States of America respectively.

The resistance rate of *E. coli* to the carbapenems (imipenem and meropenem) in this study is similar to the work of Olutayo and Abimbola (2016) who recently reported in Southwest Nigeria that *E. coli* isolates from abattoirs are highly resistant to imipenem and meropenem. They also showed in their study that 100 *E. coli* isolates recovered from abattoir effluents were resistant to imipenem and meropenem (Olutayo and Abimbola, 2016). This result also concur with the work of Rossolini *et al*. (2001) who reported that *Enterobacteriaceae* from environmental samples were resistant to the carbapenems, imipenem and meropenem. In Southwest Nigeria, Ogunleye *et al*. (2008) reported that *E. coli* isolates recovered from poultry were resistant to imipenem and meropenem; and in Southeast Nigeria, the resistance of *E. coli* to the carbapenems (as obtainable in this study) have also been reported (Ejikeugwu *et al*., 2014). Out of the 141 isolates of *Klebsiella* species recovered from the various environmental samples, 58 isolates (41.1 %) were

resistant to imipenem while 61 *Klebsiella* species isolates (43.3 %) were resistant to meropenem. The level of resistance recorded for the *P. aeruginosa* isolates recovered in this study is different from the work of Olutayo and Abimbola (2016) – who reported in a similar study that none of the 60 *P. aeruginosa* isolates recovered from abattoir effluents showed resistance to imipenem and meropenem. In a recent study in southeast Nigeria, similar level of resistance amongst *P. aeruginosa* isolates from abattoir has also been reported (Iroha *et al*., 2016). In Switzerland, the resistance of *Klebsiella* species to the carbapenems has also been reported as obtained in this study (Zurfluh *et al*., (2013). The frequency of *Klebsiella* species resistance to the carbapenems (imipenem and meropenem) in this study is also similar to previous studies both in Nigeria and outside of Nigeria where higher resistance rates was recorded amongst *Klebsiella* species to the carbapenems (Lafi *et al*., 2012; Javeed *et al*., 2011; Akinduti *et al*., 2012).

Antibiotics are usually added to the feed and water of food-producing animals to promote growth and limit bacterial infection in the animals. But such supplementation of antibiotics in the water and feed of food-producing animals poses health risk to the human population due to the possible development and dissemination of antibiotic resistance in the food chain (Shea, 2004). And once antibiotic resistance-conferring genes have appeared by mutation in the bacteria population, they can be easily mobilized between different strains and species – and thus continue to spread in the region. Antimicrobial resistant genes can be transferred to humans directly (through contact with these animals) or indirectly (through consumption of food). According to Shea (2004), antimicrobial agents are widely used in food animal production either for therapeutic measures or for prophylactic measures, and as growth promoting agents. And several researchers have also reported in their study that the use of sub-therapeutic doses of antibiotics in healthy animals for prophylaxis and growth promotion, coupled with the imprecise dosages of drugs given to sick

animals and healthy ones helps to spur the development and spread of resistance in the community through selective pressure (Hammer, 2002; Shea, 2004; Li *et al*., 2014; Rosengren *et al*., 2008; Wegener, 2003). Li *et al*. (2014) and Shea (2004) reported that the use of one antimicrobial agent may give rise to the development of multidrug resistant bacteria (MDRB) – since several antibiotic resistance genes may be linked and transferred together on mobile genetic elements such as transposons and plasmids harboured by these organisms. Human beings can also be exposed to antibiotic resistant bacteria through the consumption of food colonized by resistant organisms, through infection from antimicrobial resistant microbes in the environment and through contamination of food during processing.

Phenotypic confirmation tests were carried out on suspected bacteria that produced metallo beta-lactamase (MBL) enzymes and AmpC enzymes based on the recommended screening test for MBL detection (CLSI, 2011; Chakraborty *et al*., 2010; Walsh *et al*., 2005). All the isolated *E. coli* isolates (n=168), *Klebsiella* species isolates (n=141) and *P. aeruginosa* isolates (n=147) were tested for their susceptibility to any of the carbapenems, imipenem (10 µg) and meropenem (10 µg) as was previously described (Ejikeugwu *et al*., 2014; Javeed *et al.*, 2011; Varaiya *et al*., 2008); and bacterial isolates found to be resistant to any of these carbapenems as per the Clinical and Laboratory Standard Institute (CLSI) criteria (isolates showing inhibition zone diameter of ≤ 23 mm) were considered and suspected to produce MBL enzymes phenotypically. Imipenem and meropenem have been recommended by the Clinical Laboratory Standard Institute (CLSI) and other researchers for the screening of bacterial organisms for the presence of metallo beta-lactamase (MBL) enzymes (CLSI, 2011; Walsh *et al*., 2005; Chakraborty *et al*., 2010; Thompson, 2010; Toleman *et al*., 2005; Tortola *et al*., 2005). In this study, MBL producing bacteria was phenotypically detected in a total of 22 *E. coli* isolates out of the 168 isolates of *E.*

coli recovered from the various environmental samples. Eight (8) *E. coli* isolates (11.6 %) from abattoir tables where phenotypically confirmed as MBL producers while seven (7) isolates of *E. coli* were respectively characterized as MBL producers from cloacae swab samples and samples from the anal region of cow. The occurrence of MBL-producing *E. coli* isolates in this study is similar to the work of Leung *et al*. (2013) in Australia – who reported the occurrence of MBL-producing *E. coli* from environmental samples. Chakraborty *et al*. (2010) also reported similar prevalence of *E. coli* isolates positive for metallo beta-lactamase enzyme production in India. This result is also similar to the work of Bashir *et al*. (2011) – who recorded higher prevalence of MBL-producing *E. coli* isolates in their study carried out in Kashmir. The prevalence of MBL-producing *E. coli* isolates in this study also agreed with the work of Chouchani *et al*. (2011) – who reported the occurrence of MBL-producing *E. coli* isolates (13 %) in their study. There was no statistical difference (p value > 0.05) in the phenotypic detection of MBLs in the *E. coli*, *Klebsiella* species and *P. aeruginosa* isolates evaluated in this study.

A total of 6 *Klebsiella* species isolates from abattoir tables were MBL-positive while 7 isolates of *Klebsiella* species from the cloacal swabs of poultry birds were identified as MBL producers. Only 5 isolates of *Klebsiella* species were identified as MBL producers from the anal swab samples of cow. The occurrence of *Klebsiella* species producing metallo-beta-lactamase enzyme have been reported in Nigeria, Asia, Europe and other parts of Africa (Akinduti *et al*., 2012; Ejikeugwu *et al*., 2014; Chakraborty *et al*., 2010; Deshpande *et al*., 2010). These report on the occurrence of MBL-producing *Klebsiella* species from both clinical and environmental isolates are similar to our results. However, a similar work done in Australia showed that none of the *Klebsiella* species isolates recovered from environmental samples produced metallo beta-lactamase enzyme (Leung *et al*., 2013). This result is different from ours in which MBL-positive

Klebsiella species isolates were recovered from environmental samples of cloacal swabs of poultry birds, swab samples of the anal region of cow and swab samples of abattoir tables. In India, Wadekar *et al*. (2013) reported a higher prevalence of MBL-positive *Klebsiella* species, a result which is higher than ours. A recent study carried out in Japan showed that MBL-producing *Klebsiella* species isolate has also been recovered from non-hospital environment, and this study gives impetus to the occurrence of MBL-producing bacteria in the community as shown in our study (Okazaki *et al*., 2016).

Of the 147 *P. aeruginosa* isolates tested and screened for the phenotypic production of metallo beta-lactamase (MBL) enzyme, 98 isolates (66.7 %) appeared to produce MBL enzymes, as determined by the CLSI recommended screening test using imipenem (10 µg) antibiotic disk. *P. aeruginosa* isolates found to produce MBL enzymes are completely resistant to the antimicrobial action of imipenem while those *P. aeruginosa* isolates that do not express MBL enzymes are completely susceptible to the drug. In this study, *P. aeruginosa* isolates that produce MBL enzymes were phenotypically detected in abattoir samples (15.0 %), cloacal swab samples (14.3 %), and from anal swab samples of cow (9.6 %). In all, a total of 18 *P. aeruginosa* isolates was phenotypically detected to produce MBL enzymes by the inhibition based assay and the modified Hodges test (MHT) method as was previously described (Lee *et al*., 2003; Aibinu *et al*., 2007; Ejikeugwu *et al*., 2014). This result is not in agreement with those reported by Abd El-Baky *et al*. (2013) – in which 31 isolates of *P. aeruginosa* were phenotypically detected to produce MBL enzymes in Asia. Akinduti *et al*. (2012) also reported a lower rate of MBL-positive *P. aeruginosa* isolates (3.3 %) in their study carried out in Southwest Nigeria. Shibata *et al*. (2003) also reported a higher occurrence rate of MBL-producing-*P. aeruginosa* isolates in their study in which 116 *P. aeruginosa* isolates were discovered phenotypically to produce MBL

enzymes in Japan. However, the result of MBL enzyme production amongst the *P. aeruginosa* isolates screened in this study is in agreement with the report of Saderi *et al*. (2008) who reported similar prevalence of MBL-producing *P. aeruginosa* isolates in their study carried out in Iran. *P. aeruginosa* isolates are intrinsically resistant to antimicrobial agents; and their ability to express resistance phenotypes such as MBL enzymes adds to their multidrug resistant nature. The production of AmpC enzymes by the test bacterial isolates was phenotypically detected using three techniques including the inducibility test, ceftazidime-imipenem antagonism test (CIAT), and the cefoxitin-cloxacillin double disk synergy test (CC-DDST) as was previously described (Shevade and Agrawal, 2013; Thompson, 2010).

Among the 168 isolates of *E. coli* phenotypically tested in this study for AmpC enzyme production, 125 *E. coli* isolates were found to be resistant to cefoxitin; and these isolates were phenotypically tested for AmpC enzyme production. A total of 6 *E. coli* isolates (8.7 %) from abattoir tables were phenotypically confirmed to produce AmpC enzymes while 5 isolates of *E. coli* (9.8 %) from cloacal swab samples were phenotypically detected to produce AmpC enzymes. AmpC enzyme production was also detected in 6 isolates of *E. coli* (12.5 %) from the anal swabs of cow. The result of this study demonstrate that AmpC enzyme production was high in anal swab samples, followed by cloacal swab samples and then samples from abattoir tables. In India, the prevalence of AmpC-producing *E. coli* isolates was reported to be 12.5 % (Manoharan *et al*., 2012), and this result is similar to ours. However, the prevalence of AmpC enzyme-producing *E. coli* isolates in Iran was reported to be 37.2 % in a similar report (Mansouri *et al*., 2014), and this result is higher than the occurrence rate of AmpC enzyme-producing *E. coli* isolates in this study. In Czech Republic, 2 *E. coli* isolates from cloacal swabs of poultry birds was reported as AmpC-positive bacteria (Kolar *et al*., 2010). The prevalence of AmpC

enzyme-producing Gram-negative bacteria in Nigeria especially from environmental isolates is not known probably due to limited surveillance studies on such multidrug resistance organisms in this region. And it could also be that research works on AmpC enzyme producing Gram-negative bacteria are not published. There was no statistical difference (p value > 0.05) in the phenotypic detection of AmpC enzymes amongst the *E. coli, Klebsiella* species and *P. aeruginosa* isolates evaluated in this study.

However, the difficulty experienced in the accurate detection of AmpC enzyme-producing Gram-negative bacteria has contributed to the limited data on the occurrence of these organisms in this region. A total of 106 *Klebsiella* species was found to be resistant to cefoxitin while 118 isolates of *P. aeruginosa* showed reduced susceptibility to cefoxitin. All these isolates were phenotypically tested for the production of AmpC enzymes as described previously (Polsfuss *et al*., 2011). Nine (9) isolates of *P. aeruginosa* (16.1 %) from abattoir tables were confirmed as AmpC enzyme producers while 7 *P. aeruginosa* isolates (14.6 %) were confirmed as AmpC enzyme producers by the disk methods used in this study. It was also found in this study that 8 isolates of *P. aeruginosa* (18.6 %) from the anal swabs of cow was phenotypically confirmed AmpC enzyme producers. The prevalence of AmpC enzyme production in this study is similar to the work of Akinduti *et al*. (2012) who reported similar rates of AmpC enzyme producers in southwest Nigeria. The prevalence of AmpC-positive *P. aeruginosa* isolates in this study is lower than the report of Abd El-Baky *et al*., (2013) who reported higher prevalence of AmpC-positive *P. aeruginosa* (72.4 %) in their study. Our result is dissimilar to the result of Mansouri *et al*. (2014) who reported 37.2 % prevalence rate of AmpC-positive *K. pneumoniae* in their study carried out in Iran. Mohamudha *et al*. (2012) also showed in their study carried out in India that AmpC positive bacteria abound in the community. When AmpC enzymes are produced at

low levels, it is not associated with resistance. But resistance is possible at high level production of AmpC enzymes in organisms that are AmpC positive (Jacoby, 2009).

At high level production of AmpC enzymes, AmpC positive bacteria including *E. coli, P. aeruginosa* and *Klebsiella* species can become resistant to a wide array of beta-lactam antibiotics including the cephalosporins (with exception to cefepime, a fourth generation cephalosporin), beta-lactamase inhibitors and the cephamycins including cefoxitin and cefotetan (Jacoby, 2009). However, studies have shown that AmpC-positive bacteria still remain susceptible to the carbapenems including imipenem, meropenem and ertapenem (Thompson, 2010; Jacoby, 2009). Multiple antibiotic resistances in bacterial population in the community is gradually becoming a threat to some available antibiotics because organisms with genes for multiple antibiotic resistance have the ability to be multiply resistant to a wide variety of antimicrobial agents including the beta-lactams and fluoroquinolones.

In this study, the multiple antibiotic resistance index (MARI) of AmpC- and MBL-producing bacteria were investigated according to the method of Akinjogunla and Enabulele (2010). A bacterium is regarded as multidrug resistant if it is resistant to at least three different antimicrobial agents. The MBL- and AmpC-positive *Klebsiella* species have MARI of 0.4 to 0.5 while the MBL- and AmpC positive *E. coli* isolates have MARI of 0.4 to 0.6. The *P. aeruginosa* isolates that were positive for MBL- and AmpC enzyme production had MARI of 0.7 to 0.8. The MARI result showed that the MBL- and AmpC-positive *Klebsiella* species and *E. coli* isolates were multiply resistant to more than four antibiotics while the *P. aeruginosa* isolates that were positive for MBL- and AmpC enzyme production were multiply resistant to more than seven antibiotics. Bacterial organisms can become resistant to multiple antibiotics if they harbour genes that confer antimicrobial resistance to different antibiotic classes; and the MARI results are

similar to previous studies (Ogunleye *et al.*, 2008; Majalija *et al*., 2010; Van den Bogaard *et al*., 2001).

Only the *bla*IMP-1 and *bla*IMP-2 MBL genes were detected by multiplex PCR in this study. The *bla*VIM-1 and *bla*VIM-2 MBL genes were not detected in the MBL phenotypes. Overall, the prevalence of *bla*IMP-1 gene in the MBL phenotypes was 36.4 %, 50 % and 54.5 % for *E. coli*, *Klebsiella* species and *P. aeruginosa* respectively. *bla*IMP-2 genes were detected at the rate of 18.2 %, 33.3 % and 18.2 % for *E. coli*, *Klebsiella* species and *P. aeruginosa* isolates respectively. Previous studies have shown that the *bla*IMP-1 and *bla*IMP-2 genes occur worldwide; and these genes have been reported in members of the *Enterobacteriaceae* and some non-enteric bacterial organisms including *P. aeruginosa* and *A. baumannii* (Walsh *et al*., 2005; Thompson, 2010; Tortola *et al*., 2005). The rate of prevalence of MBL genes in the *E. coli* phenotypes in our study is much lower than the result of Adwan *et al*. (2016) who reported 87.4 % carriage rate of *bla*IMP-1 genes in *E. coli* isolates in their own study. Similar rates of *bla*IMP-1 and *bla*IMP-2 MBL gene prevalence as obtainable in our study have also been reported by Chouchani *et al*. (2011) in Tunisia. However, Enwuru *et al*. (2011) in Lagos, Nigeria reported that the prevalence of MBL positive bacteria harbouring the *bla*IMP-1 and *bla*IMP-2 genes in their study was between 40-50 % and Mansouri *et al*. (2014) in Iran also reported lesser prevalence of MBL genes in their study.

The prevalence of *bla*IMP-1 MBL genes and *bla*IMP-2 MBL genes in the tested *Klebsiella* species and *P. aeruginosa* isolates was 50 % and 54.5 % respectively. This is in agreement to the result of Aibinu *et al*. (2007) and Franco *et al*. (2010) who both reported higher prevalence rates of MBL genes amongst *P. aeruginosa* isolates in Lagos, Nigeria and Brazil respectively. Mansouri *et al*. (2014) reported similar prevalence of MBL genes amongst *Klebsiella* species in their study.

However, in a similar study conducted in Iraq, Anoar *et al*. (2014) reported that the prevalence of IMP-1 gene amongst *Klebsiella* species and *P. aeruginosa* isolates was 3.95 %. In Japan, Shibata *et al*. (2003) reported that out of a total of 357 isolates of Gram-negative bacteria carrying ^{bla}IMP-1 MBL genes, 116 isolates were *P. aeruginosa*, 23 isolates were *Klebsiella* species while 17 isolates were *E. coli*.

These result reported by Shibata *et al*. (2003) is not in agreement with ours where the number of bacterial isolates that were genotypically confirmed by multiplex PCR to harbour ^{bla}IMP-1 MBL gene was 8 *E. coli* isolates, 9 *Klebsiella* species isolates and 12 *P. aeruginosa* isolates. The presence of ^{bla}IMP-1 MBL genes and ^{bla}IMP-2 MBL genes among the *E. coli, Klebsiella* species and *P. aeruginosa* isolates analyzed in this study may be due to several risk factors associated with the acquisition, development and spread of antibiotic resistance genes in the community – of which the undue exposure and use of antibiotics in animal husbandry and in livestock production have been implicated as a key factor driving the development and spread of drug resistant bacteria in the community (Usha *et al*., 2010; Shea, 2004). ^{bla}IMP-1 MBL genes and ^{bla}IMP-2 MBL genes has been shown to be the most prevalent MBL genes harboured by Gram-negative bacteria including *E. coli, Klebsiella* species and *P. aeruginosa*; and these MBL genes occur worldwide (Mansouri *et al*., 2014; Walsh *et al*., 2005; Rossolini *et al*., 2001).

It was discovered that only the CMY-1 AmpC genes and FOX-1 AmpC genes were detected in the test bacterial isolates used in this study. The DHA-1 AmpC genes and ACC AmpC genes was not detected by multiplex PCR in the *E. coli, Klebsiella* species and *P. aeruginosa* isolates recovered in this study. Totally, 17 isolates of *E. coli*, 18 isolates of *Klebsiella* species and 24 isolates of *P. aeruginosa* were examined genotypically using multiplex PCR for AmpC encoding genes. Out of the series of AmpC genes investigated in this study, only the CMY-1 genes and the

FOX-1 genes were harboured by the test bacterial isolates. It was observed also, that out of the 17 isolates of *E. coli*, only 3 *E. coli* isolates and 2 *E. coli* isolates harboured the CMY-1 genes (17.6 %) and the FOX-1 genes (11.8 %). Four (4) out of the 18 *Klebsiella* species harboured the CMY-1 genes while only one (1) isolate of *Klebsiella* species harboured the FOX-1 genes. Also in this study, 7 isolates of the tested *P. aeruginosa* population were detected by multiplex PCR to contain the CMY-1 genes while only 3 *P. aeruginosa* isolates harboured the FOX-1 genes.

The CMY homologues particularly the CMY-1 AmpC genes were the most predominant AmpC genes detected in this study; and this was followed by the FOX-1 genes which were least detected in the test populations of *E. coli*, *Klebsiella* species and *P. aeruginosa* phenotypes. The CMY gene homologues and the FOX gene homologues are usually the most prevalent AmpC genes found in Gram-negative bacteria, and which mediate their resistance to the cephamycins (Walsh *et al*., 2005; Thompson, 2010). The prevalence of *E. coli* phenotypes and *Klebsiella* species phenotypes harbouring the CMY-1 genes in this study is not in agreement with the report of El-Hady and Adel (2015) who reported that CMY-1 genes was detected in 68.2 % of *Klebsiella* species and 80 % of *E. coli*. The same report also showed that FOX-1 genes were detected in 40.9 % *Klebsiella* species and 33.3 % *E. coli* isolates (El-Hady and Adel, 2015). This result reported by El-Hady and Adel (2015) in Egypt is higher than ours in which FOX-1 genes was detected in only 11.8 % *E. coli* phenotypes and 5.6 % *Klebsiella* species. Also in Egypt, Helmy and Wasfi (2014) reported that AmpC-producing strains were detected by multiplex PCR in 88.46 % isolates amongst cefoxitin resistant *E. coli* and *Klebsiella* species; and this same study also reported that the CMY homologues were the most prevalent genes detected while the FOX family of AmpC genes was detected in low percentage.

The low percentage of FOX family of AmpC genes detected by Helmy and Wasfi (2014) is in accordance with ours in which the FOX family of AmpC genes was the least detected AmpC genes in our study. In Iran, Mansouri *et al*. (2014) reported that the prevalence of AmpC-producing strains of *E. coli* and *Klebsiella* species was 37.2 %, a result that is similar to ours (Table 4.10). In China, Zhu *et al*. (2013) reported that the CMY homologues were the only AmpC genes detected in their study. This result is similar to the result obtained in our study in which the CMY gene homologues were the most prevalent AmpC genes detected. In another related study carried out in Pakistan, it was also reported that the CMY genes and FOX genes were the most prevalent AmpC genes detected by PCR in *E. coli* isolates (Hussain *et al*., 2011).

Out of the 12 isolates of *Klebsiella* species that was treated with acridine orange dye (0.1 mg/ml), 10 isolates of *Klebsiella* species showed no enzyme activity after curing while 4 isolates showed enzyme activity. This result shows that the 10 (71.4 %) *Klebsiella* species isolates harboured their resistance traits in their plasmid while the resistance genes of the other four isolates were chromosomally-borne since they still exhibited less susceptibility to the tested antibiotics after curing. Only 4 (80 %) isolates of *E. coli* and 3 (60 %) isolates of *P. aeruginosa* harboured their resistance traits in their plasmids. The *E. coli* isolates, *Klebsiella* species and *P. aeruginosa* isolates that showed susceptibility to the tested antibiotics after treatment with acridine orange has their resistance traits on a plasmid; and thus the antibiotic resistance phenotypes/genotype of these organisms are plasmid-mediated.

Bacterial isolates that showed resistance to the tested antibiotics after treatment with acridine orange dye harbour their resistance phenotypes/genotypes in their chromosomes; and thus their resistance genes are said to be chromosomally-borne. The plasmid curing analysis carried out on the resistant *E. coli* isolates, *Klebsiella* species and *P. aeruginosa* isolates using acridine orange

reveal that the antibiotic resistance markers or traits of these multidrug resistant organisms (MDROs) were stably lost (for those that were plasmid-mediated) while others (i.e. those that were chromosomally-borne) were not lost after curing. This result is in line with the work of Akinjogunla and Enabulele (2010), Akortha and Filgona (2009) and Ojo *et al*. (2014) who reported that the genetic elements responsible for antimicrobial resistance in Gram-negative bacteria including *E. coli, Klebsiella* species and *P. aeruginosa* could be plasmid-borne or chromosomally-borne.

The level of co-production of MBL enzymes and AmpC enzymes was detected amongst the *E. coli, Klebsiella* species and *P. aeruginosa* isolates that were positive for the production of MBLs and AmpC enzymes. The frequency of co-production of MBL enzymes and AmpC enzymes by a bacterium has been previously reported by Abd El-Baky *et al*. (2013) in Asia and Akinduti *et al*. (2012) in Southwest Nigeria. In this study, MBL and AmpC enzyme co-production was detected in 3 *Klebsiella* species isolates; 2 *E. coli* isolates and 3 isolates of *P. aeruginosa*. These genes have been previously reported to be the most prevalent MBL genes and AmpC genes amongst bacteria in the family *Enterobacteriaceae* and even in non-enteric organisms such as *P. aeruginosa* (Walsh *et al*., 2005; Thompson, 2010; Mansouri *et al*., 2014; Walsh *et al*., 2005; Rossolini *et al*., 2001). This result is in line with the report of Abd El-Baky *et al*. (2013) and Akinduti *et al*. (2012) who showed that both enteric and non-enteric bacteria can co-produce MBL and AmpC enzymes. The ability of a bacterium to co-produce more than one resistance trait gives the organism the ability to be multidrug resistant in nature. And this makes such bacterial pathogens to remain viable even in the face of potent antimicrobial agents required to kill or inhibit their growth in any environment.

5.2 Conclusion

Drug resistance is fast becoming such a significant public health problem that there are bacteria for which most antibiotics no longer work. The effective monitoring of the development and spread of antimicrobial resistance in zoonotic pathogens especially those that produce high profile antibiotic degrading enzymes like MBLs and AmpC beta-lactamases is critical to the containment of any disease outbreak due to these microbes. And the colonization of slaughtered farm animals and poultry birds with bacteria producing MBLs and AmpC beta-lactamases portend serious public health implications for the general public (who consume these food-producing animals' habouring multidrug resistant pathogens).

Human consumption of such contaminated foods could serve as routes via which multidrug resistant pathogenic bacteria reach humans to cause infection. This study evaluated by phenotypic and molecular techniques the prevalence, antibiogram and molecular typing of environmental isolates of *E. coli, Klebsiella* species and *P. aeruginosa* harbouring MBL genes and AmpC genes. The most prevalent bacteria isolated was *E. coli* (n=168), and this was followed by *P. aeruginosa* (n=147) and *Klebsiella* species (n=141). Beta-lactamase production was highest in *E. coli* isolates (38 %). However, *P. aeruginosa* isolates and *Klebsiella* species expressed beta-lactamase enzyme at the rate of 33 % and 29 % respectively.

The antimicrobial susceptibility test results show that the *E. coli, Klebsiella* species and *P. aeruginosa* isolates were multiply resistant to the tested antibiotics. The *E. coli* isolates were found to be highly resistant to gentamicin (56.5 %), ceftriaxone (95.2 %), cefoxitin (74.4 %), ceftazidime (96.4 %), ertapenem (86.9 %), nitrofurantoin (76.8 %), ciprofloxacin (81.5 %), oxacillin (81.5 %) and cefotaxime (98.2 %) while the *Klebsiella* species was found to be resistant

to ceftriaxone (89.4 %), cefotaxime (96.5 %), aztreonam (96.5 %), ciprofloxacin (86.5 %), oxacillin (87.2 %), ertapenem (84.4 %), ceftazidime (82.3) and cefoxitin (75.2 %). *P. aeruginosa* isolates were most resistant to ampicillin (81.0 %), ciprofloxacin (81.0 %), cefoxitin (80.3 %), cefotaxime (79.6 %), oxacillin (72.1 %) and nitrofurantoin (70.1 %). MBL was phenotypically detected in a total of 22 *E. coli* isolates and 22 *P. aeruginosa* isolates while a total of 18 *Klebsiella* species isolates produced MBL phenotypically. AmpC enzymes were phenotypically detected in 17 isolates of *E. coli*, 24 isolates of *P. aeruginosa* and 18 isolates of *Klebsiella* species.

MBL genes were predominantly detected in *P. aeruginosa* isolates (72.7 %), and this was followed by *Klebsiella* species (83.3 %) and *E. coli* isolates (54.6 %). ^{bla}IMP-1 MBL genes and ^{bla}IMP-2 MBL genes were the MBL genes detected in the bacterial isolates by multiplex PCR technique. The CMY AmpC genes was the most prevalent AmpC genes detected in the *E. coli* isolates (CMY-1, 17.6 %), *Klebsiella* species isolates (CMY-1, 22.2 %) and *P. aeruginosa* isolates (CMY-1, 29.2 %). However, FOX genes was also detected by multiplex PCR in the bacteria isolates but at a lower percentage in *E. coli* (FOX-1, 11.8 %), *Klebsiella* species (FOX-1, 5.6 %) and *P. aeruginosa* (FOX-1, 12.5 %). Plasmid curing analysis showed that 10 (71.4 %) *Klebsiella* species isolates harboured their resistance traits in their plasmid, while 4 (80 %) isolates of *E. coli* and 3 (60 %) isolates of *P. aeruginosa* harboured their resistance traits in their plasmids.

MBL and AmpC enzyme co-production was detected in only 3 *Klebsiella* species isolates, 2 *E. coli* isolates and 3 isolates of *P. aeruginosa*. The most prevalent genes detected amongst the MBL and AmpC phenotypes that co-produced MBL and AmpC enzymes were ^{bla}IMP-1 MBL genes and CMY-1 AmpC genes. The ^{bla}IMP-2 MBL gene was only detected in one *Klebsiella*

species isolate that also harbour gene for AmpC enzyme production. The findings from this research study are a major concern as scientific evidence from around the world continues to suggest that the overuse and inappropriate use of antibiotics in animal husbandry and poultry production contribute significantly to the emergence and spread of antibiotic resistant bacteria in the community. And the untoward aspect of this is that these organisms are capable of being transmitted to humans through the food chain.

5.3 Recommendations

The following recommendations were deduced from this research work:

- Strict and reliable surveillance measures should be put in place by the government to monitor the emergence and spread of drug resistant bacteria in the community especially from abattoirs and poultry sources.
- Antibiotics meant for human use should not be used for the rearing and propagation of farm animals and poultry birds - since these activities allow drug resistant bacteria to emerge and spread in the community.
- Antimicrobial resistance surveillance and monitoring units should be set up across the nation in order to keep the emergence and spread of drug resistant pathogens at bay.
- Public awareness on the judicious use of antibiotics should be carried out regularly in both the community and hospital environment to assuage the spread of resistant microbes.

5.4 Contributions to knowledge

- First, this is the first report of ^{bla}IMP-1 MBL genes amongst *E. coli, P. aeruginosa* and *Klebsiella* species isolated from abattoir and poultry samples in Abakaliki, Nigeria.
- Secondly, this is also the first report of ^{bla}IMP-2 MBL genes amongst *E. coli, P. aeruginosa* and *Klebsiella* species isolated from abattoir and poultry samples in Abakaliki, Nigeria.
- Thirdly, this is the first report of CMY-1 genes that mediate AmpC enzyme production amongst *E. coli, P. aeruginosa* and *Klebsiella* species isolated from abattoir and poultry samples in Abakaliki, Nigeria.
- Fourthly, this is also the first report of FOX-1 genes that mediate AmpC enzyme production amongst *E. coli, P. aeruginosa* and *Klebsiella* species isolated from abattoir and poultry samples in Abakaliki, Nigeria.
- Fifthly, the phenotypic techniques used for the detection of MBL and AmpC phenotypes in this study can be applied by hospital personnel and healthcare workers in the detection of these enzymes from both the hospital and community in order to timely dectect them so that their spread can be contained, and therapy administered appropriately.
- This study described the molecular characterization of MBL genes (^{bla}IMP-1 and ^{bla}IMP-2) and AmpC genes (CMY-1 and FOX-1) from environmental isolates of *Escherichia coli, Klebsiella* species and *Pseudomonas aeruginosa* that emanated from abattoir and poultry samples. These genes are responsible for the

multidrug resistant nature of bacteria harbouring them, and thus their timely detection will help to guide therapy and control the spread of drug resistant bacteria in the environment.

- This study also reports that abattoir and poultry sources are likely breeding grounds for the emergence and spread of drug resistant bacteria, and this is due in part to the fact that antibiotics are usually used as growth promoting agents for the rearing and breeding of these animals.
- This work can serve as basis for the development and establishment of antibiotic resistant surveillance unit's across the nation – since it has shown that environmental isolates (particularly those of abattoir and poultry origin) with link to humans harbour drug resistant bacteria.

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APPENDIX I

1.0 PURIFICATION, IDENTIFICATION AND MAINTENANCE OF BACTERIAL ISOLATES

All the bacterial isolates (*E. coli*, *Klebsiella* species and *P. aeruginosa*) were purified on nutrient agar plates, and these were re-confirmed and identified by their characteristic appearances on growth media, Gram staining, and conventional biochemical testing techniques including indole test, citrate test, oxidase test, voges proskauer (VP) test, methyl red (MR) test and urease test. Before maintenance, the purity of the bacterial isolates was checked by Gram staining and was maintained on nutrient agar slants (Oxoid, UK), stored in the refrigerator and sub-cultured to new nutrient agar slants on a monthly basis during the course of this research work (Cheesbrough, 2006).

2.0 PREPARATION OF MUELLER-HINTON AGAR PLATES

Mueller-Hinton (MH) agar plates were prepared by dissolving 3.8 g of MH agar powder in 100 ml of distilled water (to make 100 ml of MH agar) according to the manufacturer's instructions. This was heated over a Bunsen burner flame to dissolve the ingredients of the agar powder. Twenty milliliters each of the reaction mixture was then dispensed aseptically into McCartney bottles using syringes, and this was then sterilized by autoclaving at 121^{o}C for 15 mins at 15 psi. After sterilization, the molten agar medium was allowed to cool to about 40-50^{o}C before pouring plate. Poured culture plates were allowed on the bench for the solidification of the culture medium (Cheesbrough, 2006).

3.0 PREPARATION OF EOSIN METHYLENE BLUE (EMB) AGAR

Eosin methylene blue (EMB) agar plates were prepared by dissolving 3.6 g of eosin methylene blue (EMB) agar powder in 100 ml of distilled water (to make 100 ml of EMB agar) according to the manufacturer's instructions. This was heated over a Bunsen burner flame to dissolve the ingredients of the EMB agar powder. Twenty milliliters each of the reaction mixture was then dispensed aseptically into McCartney bottles using syringes, and this was then sterilized by autoclaving at 121^{o}C for 15 mins at 15 psi. After sterilization, the molten agar medium was allowed to cool to about 40-50^{o}C before pouring plate. Poured culture plates were allowed on the bench for the solidification of the culture medium (Cheesbrough, 2006).

4.0 PREPARATION OF MAcCONKEY AGAR

MacConkey agar plates were prepared by dissolving 5.2 g of MacConkey agar powder in 100 ml of distilled water (to make 100 ml of MacConkey agar) according to the manufacturer's instructions. This was heated over a Bunsen burner flame to dissolve the ingredients of the nutrient agar powder. Twenty milliliters each of the reaction mixture was then dispensed aseptically into McCartney bottles using syringes, and this was then sterilized by autoclaving at 121^{o}C for 15 mins at 15 psi. After sterilization, the molten agar medium was allowed to cool to about 40-50^{o}C before pouring plate. Poured culture plates were allowed on the bench for the solidification of the culture medium (Cheesbrough, 2006).

5.0 PREPARATION OF CETRIMIDE SELECTIVE AGAR FOR SELECTIVE ISOLATION OF *PSEUDOMONAS AERUGINOSA*

Cetrimide selective agar (CSA) plates were prepared by dissolving 45.3 g of cetrimide selective agar powder in 900 ml of distilled water (to make 1000 ml of CSA) according to the manufacturer's instructions. This was heated over a Bunsen burner flame to dissolve the ingredients of the nutrient agar powder. After which, 10 ml of glycerol was added to the boiled medium prior to sterilization, and this was properly shaken for even mixture. Twenty milliliters each of the reaction mixture was then dispensed aseptically into McCartney bottles using syringes, and this was then sterilized by autoclaving at 121^{o}C for 15 mins at 15 psi. After sterilization, the molten agar medium was allowed to cool to about 40-50^{o}C before pouring plate. Poured culture plates were allowed on the bench for the solidification of the culture medium (Cheesbrough, 2006).

6.0 PREPARATION OF NUTRIENT AGAR PLATES

Nutrient agar plates were prepared by dissolving 2.8 g of nutrient agar powder in 100 ml of distilled water (to make 100 ml of nutrient agar) according to the manufacturer's instructions. This was heated over a Bunsen burner flame to dissolve the ingredients of the nutrient agar powder. Twenty milliliters each of the reaction mixture was then dispensed aseptically into McCartney bottles using syringes, and this was then sterilized by autoclaving at 121^{o}C for 15 mins at 15 psi. After sterilization, the agar medium was allowed to cool to about 40-50^{o}C before pouring plate. Poured culture plates were allowed on the bench for the solidification of the culture medium (Cheesbrough, 2006).

7.0 PREPARATION OF NUTRIENT BROTH

Nutrient broth was prepared by dissolving 1.3 g of nutrient broth powder in 100 ml of distilled water according to the manufacturer's instructions. The reaction mixture was heated to 100^{o}C over a Bunsen burner flame in order to dissolve the ingredients in the nutrient broth powder. After which, 5 ml each of the nutrient broth was dispensed aseptically into capped test tubes which was then sterilized by autoclaving at 121^{o}C for 15 mins at 15 psi. The nutrient broth was allowed to cool to 50^{o}C after autoclaving, and this was stored in the refrigerator until use (Cheesbrough, 2006).

8.0 PREPARATION OF PEPTONE WATER

Peptone water tubes were prepared by dissolving 1.5 g of peptone water base (powder) in 100 ml of distilled water (to make 100 ml of peptone water medium) according to the manufacturer's instructions. This was heated over a Bunsen burner flame to dissolve the ingredients of the peptone water base. Five (5) milliliters each of the reaction mixture was then dispensed aseptically into sterile test tubes using 5 ml syringes, and this was then sterilized by autoclaving at 121^{o}C for 15 mins at 15 psi. After sterilization, the peptone water medium was allowed to cool to about 40-50^{o}C before use (Cheesbrough, 2006).

9.0 CITRATE TEST

AIM: To differentiate between strains of *Enterobacteriaceae* based on their ability to utilize citrate as their sole carbon source.

PRINCIPLE: The principle is based on the ability of an organism to utilize citrate as its only carbon source. If the test organism utilizes citrate as its sole carbon source, the enzyme citrase (produced by the test bacteria) hydrolyzes citrate into oxaoloacetic acid and acetic acid. The oxaoloacetic acid is then hydrolyzed into pyruvate and carbondioxide (CO_2). CO_2 reacts with the components of the citrate agar medium to produce sodium trioxocarbonate IV (Na_2CO_3). This results to alkalinization of the medium; and thus the medium changes colour from a greenish colour to a bluish colouration in the medium. The change in the colour of the medium is because the alkaline pH of the citrate medium turns the pH indicator in the medium (i.e. bromothymol blue) from green to blue. And this indicates a positive test result (Cheesbrough, 2006).

PROCEDURE

- Streak a speck of the test bacterium on the citrate agar slant.
- Incubate tube at 30^oC for 18-24 hrs.
- Look out for a colour change after incubation.
- The presence of a blue colouration is positive while the absence of it shows a negative test result.
- *Klebsiella pneumoniae* is citrate positive.

10.0 UREASE TEST

AIM: To detect the production of urease enzymes from strains of *Enterobacteriaceae*.

PRINCIPLE: The principle is based on the breakdown of urea to ammonia (NH_3) and CO_2 by urease (enzyme produced by urease positive organisms), which turns the medium alkaline. This is seen as a change in colour of the indicator (phenol red) in the medium to pink-red (Cheesbrough, 2006).

PROCEDURE:

- Streak a speck of the test organism on the urea agar slant.
- Incubate tube at 30^{o}C for 18-24 hrs.
- Observe the tube for a colour change. Colour change of the medium to pink-red is positive for *Klebsiella pneumoniae*. Absence of it shows a negative test result.

11.0 INDOLE TEST

AIM: To identify strains of *Enterobacteriaceae* that breakdown tryptophan with the release of indole.

PRINCIPLE: The principle is based on the reaction between indole produced from the breakdown of tryptophan, with the 4(P)-dimethylamino benzaldehyde contained in the Kovac's reagent. The final reaction results in the production of a pink-red colouration which identifies the organism (Cheesbrough, 2006).

PROCEDURE:

- Collect a speck of the test isolate
- Emulsify it in the peptone water medium and incubate at 30^{o}C for 18-24 hrs.
- Add 0.5 ml of Kovac's reagent after incubation.

- Allow tube to stand. Look out for the formation of a pink or red coloration ring at the top of the medium within 10 minutes.
- Presence of this coloration is positive for the presence of *Escherichia coli* which is indole positive. Absence of it shows a negative result.

12.0 OXIDASE TEST

AIM: To detect the presence of cytochrome-c-oxidase in bacteria, as an aid in differentiating *Pseudomonas aeruginosa* which is oxidase positive from other Gram-negative rods which are oxidase negative (Cheesbrough, 2006).

PRINCIPLE: The principle is based on the oxidation of phenylenediamine in the oxidase reagent by cytochrome-c-oxidase produced by the oxidase positive bacteria. This results to the production of a deep purple (bluish) color (Cheesbrough, 2006).

PROCEDURE:

- Add few drops of 1 % aqueous solution of the oxidase reagent on a filter paper placed on a clean glass slide.
- Collect a speck of the test bacterium using a clean glass slide.
- Smear the inoculums on the area of the filter paper containing the oxidase reagent.
- Look out for a purple coloration within 5-10 seconds. When present, it gives a positive test result for *P. aeruginosa*.

13.0 METHYL RED (MR) AND VOGES PROSKAUER (VP) TEST

Aim: To detect the fermentation pathway used by bacteria to metabolize or ferment glucose.

Principle: Some organisms ferment sugars first to lactic acid then to other acids such as formic and acetic acid. Methyl red is used to detect the presence of these acids (with low pH, < 5.0) in the methyl red (MR) test. In a MR test, the tube turns red in a positive test result, while a yellow tube is indicative of a negative result. However, other bacteria convert lactic acid or glucose into alcohols such as butanediol in VP test. In such scenario, the precursors or butanediol (acetyl carbinol or acetoin) is readily detected using the Barritt's reagents. A red colour is indicative of a positive test result while the absence of a red colour production is indicative of a negative test result and this is shown by the appearance of a brownish-green to yellow colour (Cheesbrough, 2006).

PROCEDURE:

- Inoculate both tubes of MR-VP broth with the test organism; and label one tube MR and the other tube VP. Incubate both tubes at 37^{o}C.
- After two days take out the one labeled VP, remove 1.0 ml of culture from tube and add this volume to a sterile tube.
- Add 18 drops of Barritt's A (alpha-naphthol) to the tube containing the 1.0 mL of test culture.
- Add 18 drops of Barritt's B (KOH) to the tube containing the 1.0 mL test culture.
- Shake the tube vigorously every 15 seconds for 5-10 minutes. The reaction is dependent upon the presence of ample oxygen. Allow the tube to sit for 5 more minutes. A red, usually blood red or brick red shows positive VP test result. After 7 days remove the tube labeled MR and add 8 drops of methyl red. Mix the tube. Any hint of red (orange,

peach, pink) is a positive result. *E. coli* is MR positive and VP negative. *Klebsiella* species is MR negative and VP positive. *P. aeruginosa* is MR negative and VP negative.

14.0 GRAM STAINING

AIM: To identify and differentiate Gram-positive bacteria from Gram-negative bacteria.

PRINCIPLE: The principle is based on the physical nature of the bacterial cell wall and the composition of their peptidoglycan layer, which leafs Gram-positive organism(s) undecolorized and Gram-negative organism(s) decolorized by acetone after staining (Cheesbrough, 2006).

PROCEDURE:

- Make a smear of the test bacteria on a clean glass slide.
- Fix the smear by heat fixing.
- Cover the slide with crystal violet stain and wash off with water after 30 seconds.
- Cover with Lugol's iodine and wash off with water after 30 seconds.
- Cover with acetone/ethanol and wash off with water immediately.
- Cover with safranin and wash off with water after 30 seconds.
- Allow slides to dry. View under the microscope using 100x objective lens oil immersion objective.
- Gram-positive organisms appear purple-blue while Gram-negative organisms appear pink-red under the microscope.

15.0 STANDARDIZATION OF TEST BACTERIA

All test bacteria were standardized individually before use by inoculating a 5 ml normal saline in sterile test tubes with loopful(s) of an overnight culture of the purified organism

from a nutrient agar slant. Afterwards, dilutions using loopful of the test bacterium and sterile water were carried out in order to get microbial population of 10^5 CFU/ml by comparing it with 0.5 McFarland turbidity standards (Cheesbrough, 2006).

16.0 PREPARATION OF 0.5 M ETHYLENE-DIAMINE TETRA-ACETIC ACID (EDTA)

EDTA solution (0.5 M) was prepared by dissolving disodium EDTA salt (186.1 g) in 1000 ml of deionized water. The pH of the EDTA solution was taken to 8.0 by adding sodium hydroxide (NaOH) pellets. The EDTA solution was then sterilized by autoclaving at 121^{o}C for 15 mins at 15 psi, and the 0.5 M EDTA solution was capped in an air tight tube and stored at room temperature until use (Cheesbrough, 2006).

17.0 PREPARATION OF 0.5 MCFARLAND TURBIDITY STANDARD

Turbidity standard equivalent to 0.5 McFarland was prepared by adding 1 ml of concentrated tetraoxosulphate (VI) acid to 99 ml of distilled water, and dissolving 0.5 g of dehydrate barium chloride ($BaCl_2.2H_20$) salt in 50 ml of distilled water in separate reaction flasks respectively. Barium chloride solution (0.6 ml) was added to 99.4 ml of the tetraoxosulphate (VI) acid solution in a separate test tube, and the reaction mixture mixed well to form 0.5 McFarland turbidity standard. Small portion of the turbid solution was transferred to a capped test tube similar to the tube used for preparing the test microorganisms and stored at room temperature (28^{o}C). This small volume of capped turbid solution was always shaken properly before use, and it was used to adjust and compare the turbidity of the test bacteria to get a confluent growth on a growth or culture plate when performing antibiotic susceptibility testing (Cheesbrough, 2006).

18.0 SUSCEPTIBILITY BREAKPOINTS RECOMMENDED BY CLSI FOR METALLO-BETA-LACTAMASE (MBL) PHENOTYPIC DETECTION (CLSI, 2011)

Carbapenems	**Disc diffusion breakpoints (zone diameters in mm)**			**MIC breakpoints (µg/ml)**		
	R	**I**	**S**	**R**	**I**	**S**
Imipenem 10 µg	≤ 23	20-22	≥ 19	≤ 1	2	≥ 4
Meropenem 10 µg	≤ 23	20-22	≥ 19	≤ 1	2	≥ 4
Ertapenem 10 µg	≤ 23	20-22	≥ 19	≤ 0.25	0.5	≥ 1
Doripenem 10 µg	≤ 23	20-22	≥ 19	≤ 1	2	≥ 4

R= Resistance, **S=**Susceptible, **I=**Intermediate, **MIC=**Minimum inhibitory concentration

19.0 SUSCEPTIBILITY BREAKPOINTS RECOMMENDED BY CLSI FOR AmpC ENZYME PHENOTYPIC DETECTION (CLSI, 2011)

Cephamycins	**Disc diffusion breakpoints (zone diameters in mm)**			**MIC breakpoints (µg/ml)**		
	R	**I**	**S**	**R**	**I**	**S**
Cefotetan 10 µg	≤ 12	13-15	≥ 16	≤ 1	2	≥ 3
Cefoxitin 10 µg	≤ 14	15-17	≥ 18	≤ 1	2	≥ 3
Cefmetazole 10 µg	≤ 15	15-16	≥ 21	≤ 1	.5	≥ 1

APPENDIX II

1.0 PERFORMANCE STANDARDS FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING AS GIVEN BY THE CLINICAL LABORATORY STANDARD INSTITUTE (CLSI)

Drug	Disc code	Potency	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Ertapenem	ETP	10 µg	≤ 15	16-18	≥ 19
Cefoxitin	FOX	30 µg	≤ 14	15-17	≥ 18
Amikacin	AK	10 µg	≤ 14	15-16	≥ 17
Ampicillin	AMP	10 µg	≤ 13	14-16	≥ 17
Aztreonam	ATM	30 µg	≤ 17	18-20	≥ 21
Nitrofurantoin	F	10 µg	≤ 14	15-16	≥ 17
Ceftriaxone	CRO	30 µg	≤ 19	20-22	≥ 23
Cloxacillin	OB	500 µg	≤ 12	13-14	≥ 15
Oxacillin	OX	10 µg	≤ 12	13-14	≥ 15
Cefotaxime	CTX	30 µg	≤ 22	23-25	≥ 26
Ceftazidime	CAZ	30 µg	≤ 17	18-20	≥ 21
Ciprofloxacin	CIP	5 µg	≤ 15	16-20	≥ 21
Gentamicin	CN	10 µg	≤ 12	13-14	≥ 15
Imipenem	IPM	10 µg	≤ 13	14-15	≥ 16
Meropenem	MEM	10 µg	≤ 13	14-15	≥ 16
Ofloxacin	OFX	5 µg	≤ 12	13-15	≥ 16

2.0 Inhibition zone diameter (IZD) of *Escherichia coli* isolates (n=168)

	ANTIBIOTICS															
Isolate No.	**CRO**	**FOX**	**IPM**	**CAZ**	**ETP**	**OX**	**OFX**	**CN**	**AK**	**CIP**	**CTX**	**MEM**	**AMP**	**ATM**	**F**	**OB**
	INHIBITION ZONE DIAMETER, IZD (mm)															
E1	17	14	12	12	0	0	16	15	15	14	17	9	12	17	13	10
E2	14	6	23	0	0	0	18	12	18	16	16	18	10	8	15	0
E3	15	15	19	16	22	0	18	15	14	19	15	20	10	11	16	0
E4	14	15	16	18	15	0	0	15	18	0	23	9	20	0	0	12
E5	18	0	22	11	15	0	13	15	16	17	22	22	10	0	0	12
E6	0	0	22	0	0	0	15	15	4	16	0	16	0	12	20	0
E7	19	0	14	16	4	0	26	16	22	10	16	16	20	10	11	14
E8	18	6	26	16	18	0	14	22	10	22	26	24	10	0	20	18
E9	0	4	24	12	0	0	14	0	5	0	5	24	16	20	18	0
E10	18	0	16	0	0	0	22	22	18	24	30	16	18	16	0	10
E11	10	18	25	18	26	0	15	24	10	20	14	24	23	11	16	10
E12	15	0	19	17	0	0	6	22	18	10	15	18	20	0	0	15
E13	23	18	22	6	0	22	22	22	22	24	15	24	18	0	0	0
E14	17	13	14	14	0	0	15	16	19	15	14	12	20	10	11	17
E15	14	21	20	5	22	0	19	0	0	20	13	20	23	0	0	0
E16	10	0	18	15	0	0	24	12	22	18	26	22	6	9	10	0
E17	12	13	34	0	26	0	14	20	16	20	14	19	5	9	10	14
E18	20	13	16	12	0	0	21	22	18	26	18	18	7	12	10	20
E19	20	28	26	14	30	0	16	18	20	18	16	24	16	10	5	8
E20	14	2	24	15	16	0	19	24	15	23	13	17	20	0	0	18
E21	12	0	0	0	0	0	22	18	18	24	0	8	14	16	18	0
E22	22	0	26	18	18	0	13	19	17	13	0	26	8	17	0	0
E23	19	9	29	15	0	0	19	19	21	26	0	16	15	14	10	12
E24	4	12	26	6	0	0	0	8	17	2	0	15	16	12	14	20
E25	13	0	23	0	0	0	10	20	14	18	0	9	0	12	20	15
E26	0	0	24	0	0	0	22	28	26	28	0	17	20	0	0	0
E27	15	14	26	17	0	0	22	20	14	22	17	6	16	23	20	19
E28	0	0	0	0	0	0	16	12	20	19	7	8	23	0	0	18
E29	0	0	15	1	0	0	15	0	0	17	0	0	20	18	12	9
E30	24	0	20	19	13	0	16	22	18	22	0	18	9	12	10	17
E31	6	0	24	0	0	0	0	24	22	23	0	17	18	20	0	0
E32	9	0	24	0	0	0	0	24	22	23	0	17	18	20	9	10
E33	12	8	9	10	20	22	23	18	9	10	0	0	0	12	8	10
E34	20	12	23	18	17	16	10	9	17	0	20	12	18	20	13	15
E35	12	19	18	12	10	10	11	12	8	9	20	0	0	0	0	0
E35	20	12	0	0	0	0	12	18	16	10	12	18	16	13	15	19
E36	0	0	0	0	0	12	17	18	12	23	19	20	10	16	18	18

E37	20	19	18	16	17	2	8	9	16	7	8	12	17	20	18	10
E38	21	22	28	12	10	17	18	18	16	14	0	0	0	16	18	12
E39	20	12	10	18	12	11	10	17	18	16	17	12	12	10	11	11
E40	20	23	22	12	16	17	18	19	0	0	0	0	12	19	10	11
E41	12	16	18	16	0	0	0	0	20	23	18	15	17	16	18	20
E42	20	18	16	16	18	15	14	10	11	20	15	13	11	10	20	0
E43	12	18	16	18	11	10	23	18	12	10	0	0	0	0	0	0
E44	18	16	12	18	19	10	11	20	12	8	9	10	0	20	11	12
E45	20	0	0	0	0	20	18	12	20	16	18	12	10	11	10	13
E46	12	10	16	12	20	18	11	10	14	0	0	0	0	12	13	10
E47	10	20	18	19	13	14	16	18	20	23	19	18	20	0	0	10
E48	14	18	18	14	2	0	0	6	18	6	24	14	0	20	18	12
E49	6	14	0	14	0	0	0	5	0	10	6	23	23	10	18	12
E50	18	0	18	20	3	0	0	8	0	0	16	12	20	12	19	18
E51	8	15	14	1	0	0	0	2	3	11	9	15	12	18	16	15
E52	17	6	14	14	14	0	0	4	0	16	14	0	12	10	20	18
E53	10	10	12	13	16	0	9	2	14	0	9	16	0	20	11	17
E54	0	13	6	15	0	0	0	6	8	9	18	6	23	20	12	18
E55	8	11	21	14	4	0	6	4	15	9	14	21	21	12	18	20
E56	12	18	20	12	10	9	8	15	22	20	1	0	0	12	19	17
E57	10	18	11	18	12	11	10	9	20	22	17	11	15	18	20	22
E58	12	10	18	20	3	5	8	19	17	0	0	0	0	0	0	0
E59	20	12	18	20	10	11	18	18	12	10	11	9	8	2	1	11
E60	13	10	18	20	16	18	12	11	9	8	12	10	11	18	12	13
E61	8	11	21	14	4	0	6	4	15	9	14	21	10	11	18	19
E62	13	17	19	3	13	0	0	9	0	5	0	15	10	12	13	10
E63	15	11	20	13	0	0	2	5	0	0	13	24	10	20	12	13
E64	14	0	0	14	3	0	0	6	15	4	14	13	10	18	11	20
E65	17	10	0	9	4	0	5	3	14	19	16	16	10	20	10	3
E66	15	0	10	4	6	0	0	5	0	17	16	16	18	12	19	10
E67	21	0	15	13	3	0	0	6	17	21	11	20	12	18	12	13
E68	6	0	19	13	0	0	4	3	21	6	20	18	20	18	8	19
E69	13	0	12	12	0	0	5	11	12	5	18	12	20	12	18	11
E70	20	23	12	18	10	11	9	0	0	0	12	18	12	19	0	20
E71	12	10	8	18	16	17	12	14	10	11	10	18	20	16	18	16
E72	12	3	9	18	20	22	16	15	11	10	19	18	19	20	11	16
E73	20	12	10	19	7	8	16	18	12	10	11	20	16	13	14	20
E74	12	18	17	19	20	21	23	16	18	20	12	17	18	19	20	11
E75	20	12	13	18	18	16	12	16	17	18	19	12	20	11	10	12
E76	9	10	8	7	1	3	7	16	18	20	19	18	12	11	10	20
E77	12	12	20	19	18	3	9	8	12	18	19	20	12	17	20	10
E78	20	12	18	7	9	0	12	13	0	0	0	0	20	12	10	11
E79	12	23	19	20	10	11	18	20	18	15	16	11	16	10	9	8
E80	20	12	21	11	10	18	17	12	17	9	3	1	12	10	11	20
E81	15	11	20	13	0	0	2	5	0	0	13	24	20	12	12	10

E82	12	20	10	18	11	0	0	0	12	18	16	9	7	10	11	20
E83	20	11	10	8	18	12	14	19	9	16	17	11	10	20	22	21
E84	14	0	0	14	3	0	0	6	15	14	14	13	20	11	2	9
E85	12	10	19	20	22	12	8	10	19	18	12	11	13	9	0	12
E86	20	12	10	11	18	16	8	9	0	12	10	0	20	21	18	16
E87	12	18	19	10	11	12	6	8	12	20	2	21	20	12	18	9
E88	17	10	0	9	4	0	5	3	14	16	18	18	19	20	1	0
E89	20	12	19	18	10	11	21	20	0	20	17	12	11	10	8	9
E90	17	10	0	9	5	6	12	13	10	11	20	23	11	9	10	20
E91	16	16	17	19	0	4	9	0	5	8	3	19	14	16	18	20
E92	15	0	10	4	6	0	0	5	0	17	0	16	16	20	11	10
E93	20	10	11	12	19	20	10	9	8	11	10	20	17	12	14	16
E94	11	10	9	8	11	10	20	23	10	9	8	12	13	11	10	5
E95	12	19	10	20	21	23	19	12	10	18	0	20	12	18	12	12
E96	21	0	15	13	3	0	0	6	17	0	21	11	12	20	19	16
E97	6	0	19	13	0	0	4	3	21	6	20	18	20	11	10	15
E98	13	0	12	12	0	0	5	11	12	5	18	12	11	2	8	19
E99	4	11	20	14	0	0	7	0	7	16	15	16	0	15	20	10
E100	7	21	14	0	0	0	10	9	18	15	4	23	20	11	12	18
E101	17	17	17	8	0	7	10	17	17	20	0	24	12	18	10	18
E102	15	20	24	13	0	0	6	11	15	0	17	18	20	10	11	2
E103	9	21	19	14	14	0	16	10	17	12	0	22	20	11	10	9
E104	12	10	18	20	10	9	18	16	15	2	8	9	11	10	20	21
E105	6	13	17	11	0	0	6	10	22	21	4	17	11	10	18	20
E106	7	0	17	7	0	0	15	6	13	13	0	23	20	12	11	10
E107	14	0	25	0	0	0	18	19	20	23	16	23	2	9	12	18
E108	9	0	17	0	14	0	12	10	18	19	5	24	0	21	12	20
E109	4	0	13	17	0	0	8	14	14	19	4	16	12	10	9	20
E110	10	0	26	14	0	0	7	4	14	0	31	20	12	11	2	10
E111	6	5	17	0	0	0	16	13	13	17	0	14	10	18	16	14
E112	14	11	18	21	20	18	17	15	16	17	10	11	0	10	8	9
E113	20	12	18	17	21	18	9	10	12	17	0	0	0	20	12	10
E114	0	0	0	0	0	20	12	17	18	19	10	11	20	21	13	10
E115	12	21	18	9	10	11	12	3	8	17	6	8	12	20	-	9
E116	19	18	10	21	20	9	18	17	18	12	10	10	11	12	17	19
E117	20	12	11	0	0	0	0	13	12	17	18	17	16	20	12	13
E118	12	11	10	17	18	17	12	12	21	20	9	10	0	0	12	13
E119	12	18	2	7	18	14	13	7	0	19	18	13	17	6	3	5
E120	20	19	18	12	15	17	13	12	18	0	12	10	9	8	12	20
E121	12	19	10	12	17	0	0	0	20	21	8	9	10	12	10	12
E122	20	12	17	18	9	10	7	8	12	17	11	10	15	16	12	20
E123	12	10	19	18	17	12	9	8	3	2	1	12	21	19	18	10
E124	20	12	18	17	15	14	3	9	10	21	11	18	17	16	12	18
E125	12	18	17	10	9	8	12	10	12	1	9	10	20	23	12	10
E126	19	0	10	10	0	0	10	20	12	11	21	0	0	8	0	0

E127	8	15	0	18	10	0	15	9	13	12	8	0	0	0	0	19
E128	8	0	0	0	0	0	14	17	15	10	0	20	0	18	0	18
E129	0	0	0	0	0	0	0	0	9	0	0	0	0	10	0	12
E130	14	7	11	0	0	0	10	0	7	13	18	18	0	0	0	9
E131	19	16	14	12	18	0	17	20	8	0	20	15	0	0	0	3
E132	14	0	0	0	8	0	15	28	10	0	0	20	0	11	0	10
E133	15	0	11	20	0	10	15	0	0	20	15	0	0	0	14	12
E134	0	0	0	0	0	12	0	0	0	0	0	0	0	18	0	11
E135	24	0	0	0	0	11	26	0	0	0	0	0	0	8	0	15
E136	23	0	0	0	0	19	28	0	20	0	0	0	0	0	0	0
E137	0	0	18	0	0	13	15	26	21	12	10	20	0	0	0	0
E138	21	0	12	17	10	14	20	0	2	0	0	11	0	0	0	0
E139	20	0	0	18	0	12	0	19	1	0	0	0	0	0	0	20
E140	19	0	10	0	0	20	17	29	8	20	9	13	0	10	18	21
E141	22	19	12	0	7	11	29	25	10	12	10	10	0	22	0	18
E142	0	9	0	12	7	0	10	11	11	10	12	0	0	0	0	12
E143	15	0	10	13	0	0	13	23	19	0	0	11	0	28	0	16
E144	26	0	0	18	0	0	20	29	18	0	0	12	0	17	0	13
E145	0	21	10	8	12	0	13	21	12	12	11	18	0	27	0	12
E146	0	9	0	9	0	0	9	25	11	13	18	20	0	28	0	14
E147	19	0	0	0	0	0	0	20	13	12	20	12	0	0	0	12
E148	16	22	10	0	6	12	17	26	14	10	0	13	0	0	0	8
E149	14	11	8	12	0	13	16	0	15	0	12	0	0	10	0	9
E150	10	0	10	13	17	14	8	13	17	20	0	20	0	12	0	10
E151	0	0	14	10	17	16	0	0	18	17	0	12	0	20	0	18
E152	0	21	10	0	22	18	10	18	20	18	0	14	0	26	0	12
E153	0	11	21	20	0	20	17	19	11	10	20	0	0	0	0	10
E154	28	14	0	11	19	18	0	9	10	11	12	0	0	0	0	0
E155	10	14	0	21	29	19	12	0	12	20	18	14	0	0	9	10
E156	10	12	22	16	12	20	0	12	19	10	0	10	0	0	16	11
E157	26	12	12	15	19	12	12	18	12	9	20	8	14	10	0	12
E158	0	0	0	10	20	10	10	19	9	12	2	18	0	12	0	13
E159	0	22	10	13	12	12	9	20	18	13	12	20	0	20	12	10
E160	19	0	20	14	13	20	8	0	20	14	20	11	0	26	10	18
E161	16	15	21	16	14	9	7	23	2	20	12	12	0	13	20	0
E162	14	12	10	17	18	8	12	12	5	18	10	10	0	16	16	0
E163	10	10	19	18	0	7	14	15	0	19	11	10	10	10	11	20
E164	0	20	12	20	20	10	20	3	0	8	9	8	0	11	12	24
E165	0	21-	18	0	12	11	0	9	0	0	8	0	0	12	8	0
E166	0	12	15	12	11	12	0	12	11	12	3	0	0	18	19	22
E167	28	18	10	12	10	13	0	10	12	13	4	18	0	9	18	24
E168	10	8	0	13	12	8	12	11	14	18	9	20	0	10	20	20

KEY: CRO = ceftriaxone, FOX = cefepime, IPM = imipenem, CAZ = ceftazidime, ETP = ertapenem, OX = oxacillin, OFX = ofloxacin, CN = gentamicin, AK = amikacin, CIP = ciprofloxacin, CTX = cefotaxime, MEM = meropenem, AMP = ampicillin, ATM = aztreonam, F = nitrofurantoin, OB = cloxacillin; 0 = No zone of inhibition

3.0 Inhibition zone diameter (IZD) of *Klebsiella pneumoniae* isolates (n=141)

	ANTIBIOTICS															
Isolate No.	**CRO**	**FOX**	**IPM**	**CAZ**	**ETP**	**OX**	**OFX**	**CN**	**AK**	**CIP**	**CTX**	**MEM**	**AMP**	**ATM**	**F**	**OB**
	INHIBITION ZONE DIAMETER, IZD (mm)															
K1	14	0	20	17	0	0	24	14	19	14	14	16	0	10	21	20
K2	16	14	24	19	14	0	18	24	16	17	24	14	0	12	20	1
K3	14	18	22	24	2	0	14	22	18	8	24	14	0	20	12	0
K4	14	0	20	16	0	0	0	14	19	0	18	20	0	12	11	2
K5	14	14	24	14	4	0	22	16	18	26	24	26	0	12	10	10
K6	14	0	24	24	10	0	0	19	18	19	21	24	0	14	9	3
K7	16	20	15	19	0	0	20	21	13	17	17	19	0	18	5	12
K8	14	0	19	19	0	0	16	15	13	16	15	16	0	10	3	18
K9	10	7	20	13	0	0	14	15	14	15	11	18	0	20	12	20
K10	20	4	20	12	12	0	24	22	16	24	18	24	0	0	13	10
K11	24	24	18	17	0	0	22	20	14	24	22	24	0	0	18	11
K12	14	0	17	24	10	0	22	20	16	22	23	20	0	12	12	12
K13	12	19	20	0	12	11	10	12	9	8	2	8	10	11	20	11
K14	0	0	0	20	12	18	18	17	15	12	20	18	12	9	11	13
K16	20	11	17	16	13	14	15	19	10	12	13	17	0	10	12	11
K17	21	23	24	12	10	11	12	17	16	15	8	9	10	0	0	0
K18	11	12	10	20	2	8	9	16	17	20	11	0	0	20	11	12
K19	20	11	9	8	6	4	5	6	8	12	20	18	0	12	10	18
K20	2	23	11	9	10	11	17	18	12	10	11	12	13	8	7	20
K21	0	0	0	0	0	0	20	12	18	17	16	18	15	16	2	9
K22	20	12	12	12	18	17	18	18	9	10	2	11	10	0	12	16
K23	9	8	0	0	12	13	15	16	7	1	20	21	3	19	10	11
K24	11	9	18	17	16	12	13	14	16	17	16	19	20	19	21	10
K25	2	8	19	17	18	12	11	10	2	8	19	10	20	21	17	18
K26	28	20	22	30	4	0	22	24	20	30	20	24	12	20	10	11
K27	18	14	22	22	24	0	20	18	21	21	20	24	1	8	19	10
K28	22	0	18	14	0	0	20	22	18	26	26	24	20	12	10	11
K29	23	0	23	22	0	0	22	22	0	0	22	26	7	8	18	12
K30	20	22	22	24	28	0	16	22	20	26	24	22	10	12	13	9
K31	24	20	22	4	2	0	18	22	18	22	24	24	20	1	9	18
K32	12	8	0	16	0	0	22	24	20	28	-	24	12	11	8	9
K33	0	0	21	20	0	0	14	19	16	18	26	22	10	12	9	10
K34	9	0	22	22	4	0	18	24	24	0	21	28	20	21	19	0
K35	10	0	24	12	6	0	24	26	21	26	28	24	12	13	10	9
K36	16	0	0	18	4	0	15	19	16	2	0	20	2	12	12	10
K37	7	0	17	0	2	0	16	20	19	23	0	15	20	11	12	18
K38	22	0	21	14	0	0	23	22	24	0	0	24	19	10	1	0

K39	24	0	23	16	0	0	22	11	23	28	0	25	17	8	12	23
K40	9	0	26	13	0	0	1	20	19	1	0	25	0	0	0	0
K41	22	18	0	24	0	0	24	0	17	27	0	0	12	18	16	10
K42	6	0	0	14	0	0	2	15	22	0	0	20	0	0	11	10
K43	0	0	0	16	0	0	13	18	17	15	0	0	12	16	17	20
K44	20	12	21	0	12	0	20	-	10	20	11	12	13	14	8	9
K45	12	10	18	0	10	0	0	12	13	0	10	17	10	20	12	17
K46	13	18	11	18	11	10	18	9	12	11	12	18	21	20	18	17
K47	12	19	20	11	10	9	8	10	20	23	12	12	18	17	10	11
K48	20	11	10	19	18	9	10	16	11	12	15	16	17	18	20	11
K49	12	11	9	8	10	15	14	3	8	7	12	19	18	17	10	0
K50	0	0	0	0	0	0	20	11	10	9	0	0	12	13	4	6
K51	10	9	8	16	7	12	17	18	9	19	18	10	0	10	2	11
K52	12	13	20	11	0	0	9	8	18	16	2	1	17	18	20	12
K53	20	11	10	8	9	1	12	16	17	18	9	19	18	12	11	8
K54	18	11	10	12	16	17	18	12	14	16	20	19	1	9	1	10
K55	20	19	10	11	18	16	17	14	15	10	10	0	0	0	20	12
K56	20	11	12	1	9	18	18	16	7	18	0	0	20	1	2	19
K57	2	8	12	11	10	11	12	13	18	16	11	10	12	20	12	11
K58	10	11	12	13	12	12	19	18	13	10	11	12	18	9	8	1
K59	12	13	16	17	18	20	21	12	18	8	9	1	8	7	17	18
K60-	0	0	0	0	0	20	12	18	19	9	10	11	0	0	20	12
K61	12	20	0	0	2	9	8	10	11	0	12	18	9	18	16	14
K62	20	0	21	12	9	8	12	17	16	15	13	8	9	10	11	18
K63	11	10	0	2	9	18	16	17	18	20	23	12	11	10	9	10
K64	12	18	19	10	20	12	23	19	18	2	9	13	6	18	9	11
K65	0	0	0	20	21	12	18	17	16	9	8	1	9	12	10	0
K66	10	18	0	12	13	19	8	10	0	0	0	20	21	12	18	16
K67	21	18	10	11	0	0	12	3	9	19	12	21	0	0	11	21
K68	2	9	18	16	11	12	13	0	20	21	3	9	18	10	11	19
K69	10	12	11	19	18	17	0	0	12	0	20	12	11	10	8	9
K70	20	12	18	19	9	8	11	1	9	8	13	16	7	12	18	10
K71	11	19	18	14	15	6	18	5	0	0	0	12	13	14	7	8
K72	18	20	11	21	11	0	9	6	8	6	9	12	13	14	8	12
K73	21	11	18	12	13	18	19	2	9	12	10	11	21	4	10	13
K74	11	2	9	18	17	12	13	8	0	0	20	12	18	13	9	18
K75	21	9	8	9	12	12	11	10	12	20	8	19	14	17	8	18
K76	11	10	18	16	11	2	7	8	9	11	10	23	12	0	20	1
K77	20	12	12	11	19	18	17	16	11	2	9	0	20	0	0	12
K78	20	8	3	1	17	16	18	0	0	0	0	0	0	0	0	0
K79	12	11	9	8	12	13	18	19	10	11	23	12	11	18	17	2
K80	0	0	0	12	3	18	19	10	11	12	18	16	0	20	21	13
K81	12	3	9	18	17	16	11	10	12	0	0	20	12	18	16	2
K82	20	18	12	11	17	16	15	13	14	0	20	19	18	20	11	20
K83	0	0	20	2	9	12	18	16	7	8	12	12	11	10	20	0

K84	20	12	10	9	8	12	0	0	20	21	3	8	19	18	16	20
K85	12	13	8	9	12	18	19	16	15	11	10	-	20	12	13	18
K86	22	0	20	16	4	0	0	8	18	0	20	14	11	16	16	12
K87	22	18	24	24	22	0	0	6	17	0	28	25	12	12	24	20
K88	22	16	20	18	24	0	0	0	0	4	22	22	13	2	18	5
K89	22	3	20	22	24	0	0	0	14	2	24	28	10	8	22	4
K90	8	15	19	19	1	0	0	4	17	0	9	0	0	13	19	8
K91	18	18	22	24	19	0	0	5	20	0	24	19	0	12	24	19
K92	14	15	15	19	23	0	0	7	17	4	24	24	0	19	19	19
K93	24	20	23	24	3	0	0	2	19	0	24	24	0	18	26	14
K94	24	16	24	19	24	0	0	8	18	16	24	28	0	23	22	15
K95	24	14	24	26	26	0	0	9	28	14	0	24	0	21	20	12
K96	24	20	24	22	14	0	14	5	20	16	24	24	0	10	22	20
K97	0	0	20	20	21	0	0	11	15	0	24	20	0	19	19	13
K98	22	11	22	24	3	0	22	5	16	0	26	0	20	1	14	20
K99	28	22	24	19	0	0	4	13	18	4	19	22	2	3	20	17
K100	9	0	24	14	0	0	14	6	20	4	20	23	22	2	0	12
K101	4	4	20	24	0	0	6	19	18	6	0	24	20	8	0	0
K102	28	6	26	0	0	0	5	12	20	11	0	23	11	7	21	0
K103	8	14	22	0	0	0	4	14	20	24	5	28	2	9	0	0
K104	24	0	0	24	0	0	4	13	18	4	0	22	10	19	0	2
K105	9	0	24	0	22	0	6	9	18	4	24	26	12	12	0	12
K106	5	22	20	0	12	0	0	22	0	4	0	20	20	12	0	13
K107	20	0	26	0	0	0	0	0	18	6	4	24	12	3	19	2
K108	18	14	22	19	0	0	0	24	12	2	24	14	9	10	0	1
K109	3	0	24	0	0	0	0	12	20	9	5	20	8	11	0	7
K110	11	12	24	0	7	0	0	16	12	10	0	11	11	18	0	9
K111	12	10	24	0	8	0	0	12	10	12	12	18	12	16	0	18
K112	13	12	24	12	10	0	0	10	3	13	10	12	16	17	0	13
K113	14	13	25	10	18	0	0	11	8	10	9	16	17	12	0	21
K114	15	8	22	8	12	0	0	8	9	11	2	13	18	21	20	20
K115	18	9	21	9	18	0	0	9	12	20	8	9	12	20	21	12
K116	9	0	12	2	20	0	0	10	12	9	18	0	2	10	12	10
K117	6	0	18	3	12	0	0	23	18	2	12	0	9	8	17	12
K118	0	20	12	7	10	0	0	7	17	8	18	12	18	3	18	9
K119	0	21	10	8	11	0	0	5	16	12	12	17	11	12	1	8
K120	20	22	20	12	20	0	12	0	0	17	20	20	10	9	9	6
K121	22	18	0	17	3	0	0	0	13	16	9	0	12	1	3	0
K122	21	16	0	18	9	0	21	3	16	0	11	0	0	13	8	0
K123	22	4	0	22	8	0	5	4	18	0	12	0	0	2	9	4
K124	22	14	0	20	17	0	13	6	16	3	19	0	0	10	12	5
K125	22	17	0	16	18	0	5	1	18	4	22	0	0	12	11	7
K126	8	15	11	12	2	0	3	7	17	0	20	0	0	15	0	2
K127	12	17	10	18	12	0	3	5	7	0	21	12	0	16	0	8
K128	18	13	9	15	10	0	2	10	19	3	8	2	11	5	0	9

K129	12	18	8	16	13	0	2	4	14	16	23	8	19	9	12	5
K130	14	0	12	18	16	0	12	12	15	15	20	18	16	8	20	11
K131	23	11	18	15	3	0	10	5	18	15	21	16	14	10	17	5
K132	22	20	23	23	21	0	11	18	18	0	20	18	13	12	12	13
K133	21	0	19	18	23	0	17	11	19	0	0	19	10	0	12	16
K134	20	5	18	21	22	0	18	13	18	4	22	10	2	0	7	19
K135	0	7	21	18	21	0	12	8	19	3	22	11	9	0	13	12
K136	21	14	14	23	2	0	13	21	17	5	21	24	12	0	20	14
K137	26	0	22	18	18	0	11	0	19	10	20	22	20	0	13	13
K138	9	0	23	25	22	0	0	12	20	23	18	27	21	0	10	9
K139	8	21	23	21	4	0	4	18	20	4	19	21	22	0	19	22
K140	23	0	23	18	22	0	6	20	17	4	18	27	20	0	12	0
K141	9	14	19	20	4	0	12	8	17	6	0	18	21	0	20	24

KEY: CRO = ceftriaxone, FOX = cefepime, IPM = imipenem, CAZ = ceftazidime, ETP = ertapenem, OX = oxacillin, OFX = ofloxacin, CN = gentamicin, AK = amikacin, CIP = ciprofloxacin, CTX = cefotaxime, MEM = meropenem, AMP = ampicillin, ATM = aztreonam, F = nitrofurantoin, OB = cloxacillin; 0 = No zone of inhibition

4.0 Inhibition zone diameter (IZD) of *Pseudomonas aeruginosa* isolates (n=147)

	ANTIBIOTICS															
Isolate No.	**CRO**	**FOX**	**IPM**	**CAZ**	**ETP**	**OX**	**OFX**	**CN**	**AK**	**CIP**	**CTX**	**MEM**	**AMP**	**ATM**	**F**	**OB**
	INHIBITION ZONE DIAMETER, IZD (mm)															
P1	23	0	17	14	0	0	13	15	16	15	0	14	0	10	4	0
P2	0	0	17	0	14	0	0	14	15	22	0	24	0	3	5	0
P3	12	0	26	22	0	0	14	18	12	26	0	0	0	12	12	0
P4	12	0	22	16	0	0	14	18	12	28	7	16	0	16	10	0
P5	0	0	18	14	0	0	18	16	12	28	12	24	0	0	0	0
P6	14	0	28	18	0	0	16	18	12	14	30	0	2	0	0	0
P7	12	0	28	24	0	0	16	18	16	26	0	4	5	5	0	0
P8	18	0	11	20	0	12	23	10	20	12	0	20	0	0	0	0
P9	18	0	12	0	0	2	19	23	12	20	0	23	0	23	0	0
P10	21	10	0	0	6	12	10	22	22	21	0	18	0	6	0	0
P11	25	12	13	11	0	8	22	20	12	0	0	14	0	28	0	0
P12	22	12	18	10	0	10	16	21	16	11	0	0	0	25	0	0
P13	23	20	21	12	0	11	19	10	18	13	0	0	0	28	0	0
P14	18	0	20	22	0	2	11	11	0	16	0	0	0	21	0	0
P15	0	0	23	21	0	10	18	0	0	18	0	0	0	0	0	0
P16	19	0	11	21	9	2	20	17	0	0	0	12	0	17	0	2
P17	22	12	0	23	9	8	0	19	0	0	23	0	0	10	0	22
P18	12	9	0	22	7	9	0	10	0	0	20	0	0	27	0	20
P19	20	8	0	23	0	10	23	0	20	0	12	14	0	20	0	12
P20	10	10	0	12	0	13	20	0	23	23	28	13	0	20	0	10
P21	8	12	0	20	0	17	0	10	21	24	29	17	0	20	0	17
P22	15	11	0	12	0	11	22	0	12	20	10	18	0	24	0	18
P23	13	10	0	26	0	12	20	0	10	0	23	19	0	0	0	11
P24	14	3	0	26	0	12	17	21	17	0	28	0	0	24	20	10
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P26	9	0	0	21	20	11	12	10	12	11	0	0	0	23	19	18
P27	23	0	0	22	25	16	11	16	22	0	27	0	0	20	9	0
P28	25	0	0	16	22	14	17	18	10	23	14	0	0	10	18	0
P29	24	20	0	18	20	17	18	0	10	0	29	0	0	23	12	0
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P33	0	0	12	11	23	8	0	12	13	14	0	28	17	20	18	11
P34	0	0	20	10	25	9	0	20	19	0	29	21	12	23	17	20
P35	12	0	12	2	22	2	0	11	20	0	9	20	10	21	12	18
P36	10	0	18	9	20	1	0	18	2	20	0	23	0	28	14	15
P37	12	0	11	13	10	7	23	19	12	12	0	28	0	27	17	11

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P42	22	0	0	27	20	12	12	0	12	19	0	20	-	20	21	20
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P47	13	0	0	26	20	0	12	16	0	22	0	21	0	22	22	12
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P49	16	0	0	20	22	0	0	12	20	16	0	20	0	14	20	0
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P78	12	5	0	0	19	0	0	9	10	0	0	0	0	20	0	0
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P143	23	10	0	0	9	18	0	0	0	0	0	3	2	0	20	0
P144	20	0	0	0	12	12	20	0	12	0	0	28	8	12	0	0
P145	23	0	0	23	20	13	11	0	17	12	27	20	23	27	0	0
P146	23	12	12	22	0	2	12	12	18	18	26	0	20	12	0	11
P147	24	20	12	21	0	2	9	20	19	20	8	0	12	21	0	20

KEY: CRO = ceftriaxone, FOX = cefepime, IPM = imipenem, CAZ = ceftazidime, ETP = ertapenem, OX = oxacillin, OFX = ofloxacin, CN = gentamicin, AK = amikacin, CIP = ciprofloxacin, CTX = cefotaxime, MEM = meropenem, AMP = ampicillin, ATM = aztreonam, F = nitrofurantoin, OB = cloxacillin; 0 = No zone of inhibition

APPENDIX III

RESULTS OF PLASMID CURING ANALYSIS

Table 1: Plasmid curing analysis of MBL-positive and AmpC-positive *Klebsiella* species (n=14) with acridine orange (0.1 mg ml^{-1})

Antibiotics	Pre-curing (No. of resistant isolates)	Cured		Post-curing Resistant (%)	
		n	(%)	n	(%)
CRO	13	10	77	4	30.8
FOX	12	9	75	3	25.0
IPM	14	11	78.6	2	14.3
CAZ	10	8	80	3	30.0
ETP	11	9	81.8	3	27.3
CTX	13	10	76.9	4	30.8
MEM	13	9	69.2	2	15.4

Key:

CRO = Ceftriaxone
FOX = Cefoxitin,
IPM = Imipenem
CAZ = Ceftazidime
ETP = Ertapenem
CTX = Cefotaxime
MEM = Meropenem

Table 2: Plasmid curing analysis of MBL-positive and AmpC-positive *E. coli* (n=5) with acridine orange (0.1 mg ml-^{1})

Antibiotics	Pre-curing (No. of resistant isolates)	Cured		Post-curing Resistant (%)	
		n	(%)	n	(%)
CRO	4	3	75.0	1	25.0
FOX	5	3	60.0	2	40.0
IPM	5	4	80.0	1	20.0
CAZ	3	2	66.7	1	33.3
ETP	4	2	50.0	1	25.0
CTX	3	2	66.7	1	33.3
MEM	4	3	75.0	1	25.0

Key:

CRO = Ceftriaxone
FOX = Cefoxitin,
IPM = Imipenem
CAZ = Ceftazidime
ETP = Ertapenem
CTX = Cefotaxime
MEM = Meropenem

Table 3: Plasmid curing analysis of MBL-positive and AmpC-positive *P. aeruginosa* (n=5) with acridine orange (0.1 mg ml^{-1})

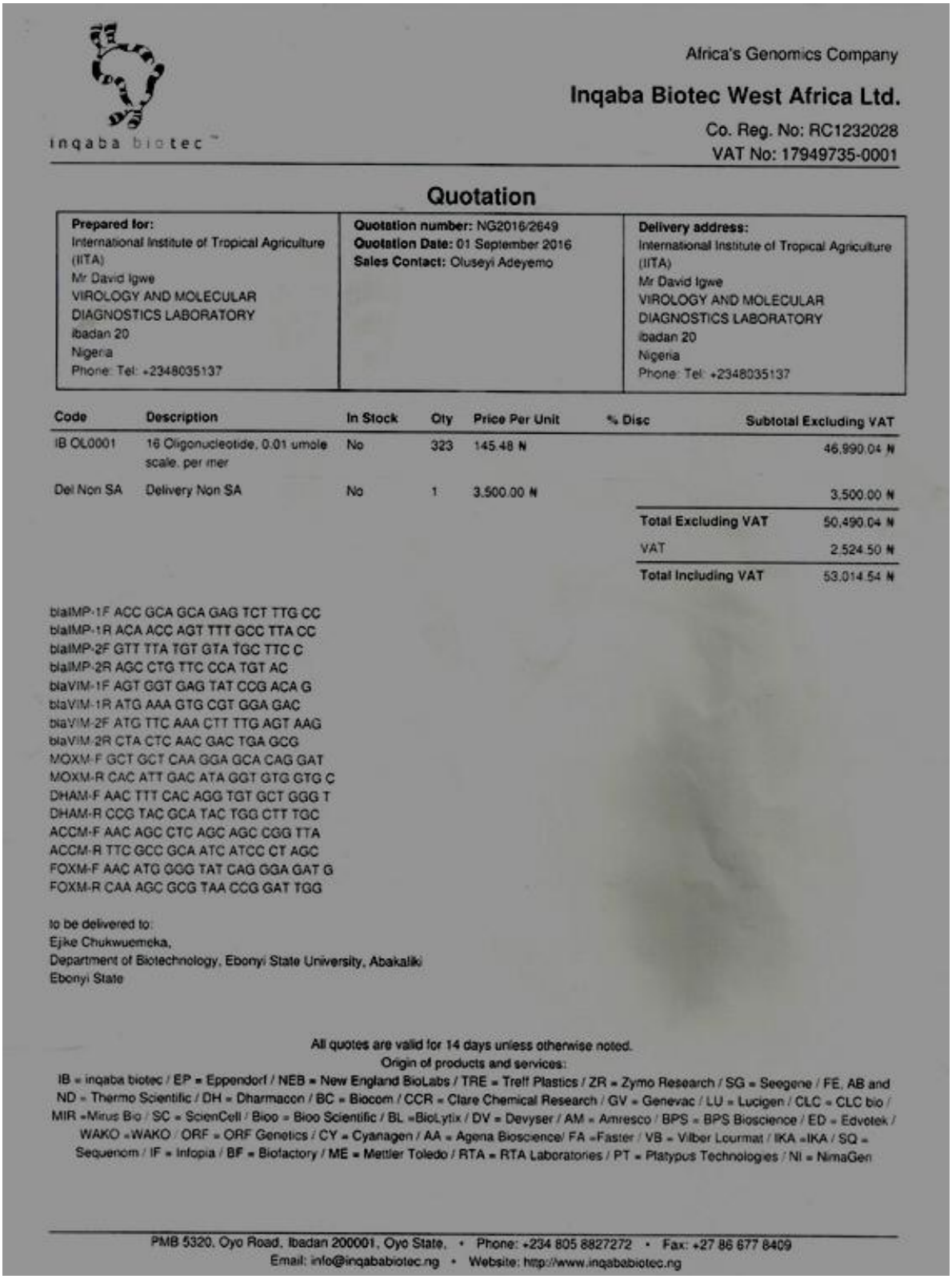
Antibiotics	Pre-curing (No. of resistant isolates)	Cured		Post-curing Resistant (%)	
		n	(%)	n	(%)
CRO	5	3	60.0	1	20
FOX	5	4	80.0	1	20
IPM	4	3	75.0	1	25
CAZ	4	3	75.0	1	25
ETP	5	4	80.0	2	40
CTX	4	3	75.0	1	25
MEM	5	3	60.0	2	40

Key:

CRO = Ceftriaxone
FOX = Cefoxitin,
IPM = Imipenem
CAZ = Ceftazidime
ETP = Ertapenem
CTX = Cefotaxime
MEM = Meropenem

APPENDIX IV

QUOTATION FOR THE OLIGONUCLEOTIDE PRIMERS USED FOR PCR



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Quotation

Prepared for:	Quotation number: NG2016/2649 Quotation Date: 01 September 2016 Sales Contact: Oluseyi Adeyemo	Delivery address:
International Institute of Tropical Agriculture (IITA) Mr David Igwe VIROLOGY AND MOLECULAR DIAGNOSTICS LABORATORY Ibadan 20 Nigeria Phone: Tel: +2348035137		International Institute of Tropical Agriculture (IITA) Mr David Igwe VIROLOGY AND MOLECULAR DIAGNOSTICS LABORATORY Ibadan 20 Nigeria Phone: Tel: +2348035137

Code	Description	In Stock	Qty	Price Per Unit	% Disc	Subtotal Excluding VAT
IB OL0001	16 Oligonucleotide, 0.01 umole scale, per mer	No	323	145.48 ₦		46,990.04 ₦
Del Non SA	Delivery Non SA	No	1	3,500.00 ₦		3,500.00 ₦
					Total Excluding VAT	50,490.04 ₦
					VAT	2,524.50 ₦
					Total Including VAT	53,014.54 ₦

blaIMP-1F ACC GCA GCA GAG TCT TTG CC
blaIMP-1R ACA ACC AGT TTT GCC TTA CC
blaIMP-2F GTT TTA TGT GTA TGC TTC C
blaIMP-2R AGC CTG TTC CCA TGT AC
blaVIM-1F AGT GGT GAG TAT CCG ACA G
blaVIM-1R ATG AAA GTG CGT GGA GAC
blaVIM-2F ATG TTC AAA CTT TTG AGT AAG
blaVIM-2R CTA CTC AAC GAC TGA GCG
MOXM-F GCT GCT CAA GGA GCA CAG GAT
MOXM-R CAC ATT GAC ATA GGT GTG GTG C
DHAM-F AAC TTT CAC AGG TGT GCT GGG T
DHAM-R CCG TAC GCA TAC TGG CTT TGC
ACCM-F AAC AGC CTC AGC AGC CGG TTA
ACCM-R TTC GCC GCA ATC ATCC CT AGC
FOXM-F AAC ATG GCG TAT CAG GGA GAT G
FOXM-R CAA AGC GCG TAA CCG GAT TGG

to be delivered to:
Ejike Chukwuemeka,
Department of Biotechnology, Ebonyi State University, Abakaliki
Ebonyi State

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Email: info@inqababiotec.ng • Website: http://www.inqababiotec.ng

APPENDIX V

PREPARATION OF TEST BACTERIAL ISOLATES FOR DNA EXTRACTION

1. The test bacterial isolates (growing and stored in a slant of nutrient agar) was subcultured onto nutrient agar plates and incubated at 30^{o}C for 18-24 hrs (Cheesbrough, 2006).
2. After incubation, sufficient amount of the test bacterial isolates was transferred from the nutrient agar plate onto 5 ml peptone water (broth) in sterile test tubes using sterile inoculating loop; and the tubes were incubated at 30^{o}C for 18-24 hrs. This overnight broth culture served as the source of bacterial culture for the isolation of bacterial DNA required for the gene amplification process using polymerase chain reaction (PCR) technique. Bacterial plasmid DNA isolation was carried out using the Zymo® Plasmid miniprep kit.
3. Microcentrifuge tubes were arranged on test tube rack and labeled according to the number of isolates to be analyzed.
4. An aliquot of 1.5 ml of the test organism from the overnight broth culture was pipetted into the labeled microcentrifuge tubes using sterile pipetting tips and micropipette. And the microcentrifuge tubes were corked properly to avoid any spillage.
5. The Eppendorf tubes containing the test bacterial cells were inserted into a microcentrifuge machine for spinning while ensuring that the tubes in the centrifuge machine are well balanced.
6. An aliquot of 8 ml lysis buffer was added to 25 ml of the bacterial suspension in a microcentrifuge tube, and the tubes were inverted several times to ensure even mixture.

7. The tubes were allowed to stand for about 3 minutes in order to give time for the lysis to take place. After this, 10 ml of neutralization buffer was added to the solution and the tubes were inverted several times to allow for even mixture of the solution.
8. The neutralized lysate was added into the ZymoPURE syringe filter and this was clarified in a conical tube.
9. An aliquot of binding buffer was added to the cleared lysate in the conical tube and this was mixed thoroughly.
10. After this, the entire mixture in the conical tube was added into the Zymo-Spin V-P Column Assembly, and the column was made tight in order to allow all the liquid to pass through the column completely.
11. The reservoir in the top of the Zymo-Spin V-P Column Assembly was removed and discarded. An aliquot of 5 ml ZymoPURE wash 1 was added to the conical reservoir and the vacuum was turned on until all the liquid has passed completely through the vacuum.
12. An aliquot of 5 ml ZymoPURE wash 2 was added to the conical reservoir, and the vacuum was turned on until all the liquid has passed completely through the vacuum.
13. The liquid in the conical reservoir was removed and discarded, and the Zymo-Spin V-P Column Assembly was placed in a collection tube.
14. The solution in the tube was centrifuged at 10,000 rpm for 1 minute in a microcentrifuge machine to remove any residual wash buffer. The column was transferred into a 1.5 ml tube and an aliquot of 200 µl of ZymoPURE elution buffer was added directly to the column matrix. The solution was incubated at room temperature for 2 minutes and then centrifuged at 10,000 rpm for 1 minute in a microcentrifuge.
15. The plasmid DNA was eluted and stored at -20^{o}C until use

APPENDIX VI

PROCEDURE FOR RE-SUSPENSION OF PRIMERS AND RE-CONSTITUTION OF PRIMERS WITH OTHER PCR REAGENTS AND TEMPLATE DNA SAMPLES FOR MULTIPLEX PCR TECHNIQUE

1. The microcentrifuge tubes containing the forward and reverse primers were gently spinned in a centrifuge machine in order to bring the contents of the tubes to the bottom of the tube prior to re-suspension (Perez-Perez and Hanson, 2002).
2. To re-suspend the synthesized DNA primers for PCR technique, a given aliquot of nuclease-free water was aseptically added to each of the microcentrifuge tubes containing the forward and reverse primers for MBL genes and AmpC genes respectively (as synthesized by Inqaba Biotechnical Industries Ltd, South Africa), and as follows:
    - **For *bla*IMP-1F:** An aliquot of 616 µl of nuclease-free water was added.
    - **For *bla*IMP-1R:** An aliquot of 592 µl of nuclease-free water was added.
    - **For *bla*IMP-2F:** An aliquot of 641 µl of nuclease-free water was added.
    - **For *bla*IMP-2R:** An aliquot of 559 µl of nuclease-free water was added.
    - **For *bla*VIM-1F:** An aliquot of 549 µl of nuclease-free water was added.
    - **For *bla*VIM-1R:** An aliquot of 522 µl of nuclease-free water was added.
    - **For *bla*VIM-2F:** An aliquot of 648 µl of nuclease-free water was added.
    - **For *bla*VIM-2R:** An aliquot of 579 µl of nuclease-free water was added.
    - **For MOXM-F:** An aliquot of 576 µl of nuclease-free water was added.
    - **For MOXM-R:** An aliquot of 583 µl of nuclease-free water was added.
    - **For DHAM-F:** An aliquot of 701 µl of nuclease-free water was added.

- **For DHAM-R:** An aliquot of 576 µl of nuclease-free water was added.
- **For ACCM-F:** An aliquot of 610 µl of nuclease-free water was added.
- **For ACCM-R:** An aliquot of 622 µl of nuclease-free water was added.
- **For FOXM-F:** An aliquot of 518 µl of nuclease-free water was added.
- **For FOXM-R:** An aliquot of 534 µl of nuclease-free water was added.

3. After the addition of the different aliquot of nuclease-free water to each of the microcentrifuge tube(s) containing the respective forward and reverse primers for the MBL genes and AmpC genes as elaborated above, the tubes were carefully corked and shaken vigorously to ensure even mixture of the primers and the nuclease-free water.

4. After this, the microcentrifuge tubes containing the primers were placed on the test tube rack to allow for the dissolution of the primers in the nuclease-free water. The tubes were allowed overnight at ambient temperature in the refrigerator until further use. This mix served as the source of the forward and reverse primers that was used for the multiplex PCR technique (Perez-Perez and Hanson, 2002).

5. To carry out the multiplex PCR amplification of MBL genes in the test isolates, a final volume of 26.5 µl reaction mixture comprising: 0.2 µl of Taq polymerase enzyme U/µl, 2.5 µl of 10X PCR buffer, 2.5 µl of $MgCl_2$, 1 µl of 10 pM from each of the forward and reverse primers, 2.5 µl of dNTPs, 3 µl of DNA template (from the test isolates), 14.8 µl of nuclease –free water. This was the master mix reaction used for the multiplex PCR amplification of MBL genes.

6. The master mix was properly vortexed in a vortexer prior to the gene amplification process in the thermal cycler. The Eppendorf tube(s) were placed in the heating (block)

chamber of the thermal cycler machine and the multiplex PCR program with the right PCR conditions were set and the process was allowed to run.

7. The initial denaturation temperature (for MBL gene amplification) was at 95^{o}C for 2 mins, and this was followed by 25 cycles of DNA denaturation at 95^{o}C for 30 sec. The primer annealing was carried out at 48^{o}C for 30 sec, and primer extension was carried out at 72^{o}C for 30 sec. After the last cycle, a final extension step was carried out at 72^{o}C for 2 mins.

8. To carry out the multiplex PCR amplification of AmpC genes in the test isolates, a final volume of 26.5 µl of the master mix comprising: 0.2 µl of Taq polymerase enzyme U/µl, 2.5 µl of 10X PCR buffer along with 2.5 µl $MgCl_2$, 1 µl of 10 pM from each of the forward and reverse primers, 2.5 µl of dNTPs MIX (2 Mm), 3 µl of DNA template (from the test isolates), and 14.8 µl of nuclease-free water was used. This was the master mix reaction used for the multiplex PCR amplification of AmpC genes.

9. The master mix was properly vortexed in a vortexer prior to the gene amplification process in the thermal cycler. The Eppendorf tube(s) were placed in the heating (block) chamber of the thermal cycler machine and the multiplex PCR program with the right PCR conditions were set and the process was allowed to run.

10. The initial denaturation temperature (for AmpC gene amplification) was at 94^{o}C for 3 mins, and this was followed by 25 cycles of DNA denaturation at 94^{o}C for 30 secs. The primer annealing was carried out at 64^{o}C for 30 secs, and primer extension was carried out at 72^{o}C for 1 min. After the last cycle, a final extension step was carried out at 72^{o}C for 7 mins.

11. All PCR products were analyzed in 1.5 % agarose gel electrophoresis technique.

APPENDIX VII

PROTOCOL FOR PREPARATION OF 1.5 % AGAROSE GEL

1. To prepare 1.5 % agarose gel, 3 g of agarose powder and 200 ml of Tris boric acid ethylene diamine tetraacetic acid (TBE) buffer solution is required (Perez-Perez and Hanson, 2002).
2. A volume of 200 ml of TBE buffer solution was measured using a clean measuring cylinder and this was dispensed into a clean/sterile beaker.
3. Three (3) gram (g) of the agarose powder was measured out using an analytical balance and a weighing paper.
4. The measured agarose powder was dispensed into a clean conical flask and the 200 ml of TBE buffer solution was added to it.
5. The conical flask containing the agarose and the TBE buffer solution was swirled vigorously to ensure even mixture of the agarose solution.
6. After swirling, the conical flask containing the solution was heated to boil for one minute in a microwave oven. The heating was allowed to continue until the solution became clear, and swirling of the solution was intermittently carried out during the heating process.
7. The heating was stopped once the agarose starts to boil to avoid over spilling in the microwave, and the conical flask was swirled until the mixture was clear. If the agarose was not dissolved completely, the finished gel will have regions of different concentrations; and this will make the nucleic acid samples not to separate correctly.

8. The heated agarose gel was allowed to cool to about 60^{o}C before pouring the molten gel into the gel casting chamber. Hot gel damages the gel casting box or tray. The heated or hot gel was placed in a water bath regulated at 60^{o}C.
9. The gel tray was placed into the gel casting chamber and appropriate casting comb(s) was inserted into the chamber. The casting combs creates cavities known as wells in the gel, and it is into these wells that the DNA samples was pipetted into.

APPENDIX VIII

PROTOCOL FOR GEL ELECTROPHORESIS TECHNIQUE

1. An aliquot of 6 µl of ethidium bromide dye was added to the molten gel prior to pouring. Ethidium bromide is a mutagen and a possible carcinogen that absorbs into the skin and could be breathed into the body via the respiratory tract. And thus, hand gloves should be used and changed once ethidium bromide dye is handled and nose mask should also be used to avoid contamination.
2. The conical flask containing the molten agarose gel and the ethidium bromide was swirled vigorously to ensure even mixture of the gel and the dye.
3. Molten agarose gel was poured into the gel casting chamber to a depth of about 5-7 mm with the casting comb in place. All bubbles formed during this process were gently removed using the ends of a pipetting tip. Bubble formation prevents free flow of electric current through the gel during the electrophoresis process.
4. The molten gel was allowed for about 20-30 minutes to solidify. The poured gel was allowed to cool in the gel chamber or tray and when its appearance was opaque, it was ready for the next stage. Usually, the colour of the gel changes from colourless to slightly opaque. While the gel is setting, it is important not to move the gel tray or disturb the gel itself – since this could create gel of non-uniform fitness.
5. After solidification or setting, the casting combs were removed carefully by pulling it upwards firmly and in a smooth and continuous motion. Some resistance is usually felt as the casting comb was removed from the solidified gel. Ignore it and gently remove the comb in an upward position as earlier stated (Perez-Perez and Hanson, 2002).

6. The set gel contained in a gel tray was transferred into a gel rig while ensuring that the wells on the cast gel was on the cathode (left) side of the gel rig.
7. The gel rig containing the gel was filled with 0.5 X TBE buffer solutions until the gel was entirely covered by it. Usually, the buffer solution should be above the surface of the wells in the gel at a distance of about 2 mm.
8. The gel chamber or rig was placed on the surface of a dark background in order to easily visualize or see the wells during loading of the DNA samples. The dark background helps you to see the wells when adding the DNA samples.
9. Before loading the DNA samples to be analyzed into the wells of the gel, a coloured loading buffer (5 X bromophenol blue with ficole) was added to each of the DNA samples in a microcentrifuge tube. The coloured loading buffer is made up of dye and a glycerol of ficole solution. The dye colour helps to visualize the loading of the samples into the wells and it also helps to keep track of the migrating DNA samples in the gel while the glycerol or ficole makes the DNA samples heavy so that they sink to the bottom of the wells.
10. The coloured loading buffer was mixed properly with the DNA samples in a microcentrifuge tube in order to get a uniform colour. This was done by using the micropipette by pipetting the solution in the tubes up and down in a take-and-release manner.
11. The labeled microcentrifuge tubes containing the DNA samples were arranged in a test tube rack in the same order that they will be loaded into the wells.
12. The samples were aseptically pipetted into the various wells on the gel starting from the left hand side to the right hand side while ensuring that the end of the pipetting tip was

held in a parallel manner to avoid stabbing the wells. The DNA marker was pipetted into the first well of the gel while the rest of the wells were left for the DNA samples analyzed. Samples were added to the wells at an angle in order to avoid putting a hole in the bottom of the well(s). All air bubbles in the tip of the pipetting tips were removed before loading the DNA samples; and care was taken not to touch the sides of the well or the bottom with the end of the pipetting tips. Damaged wells causes DNA samples to leak out and this could cause contamination of the process. Each of the DNA samples was loaded using a new pipetting tip.

13. Care was taken not to load the DNA samples too quickly. Loading the samples too quickly could cause the samples to flow out of the wells.

14. The gel tank was allowed in the same position after loading the DNA samples. Moving the gel chamber could knock out the samples out of the wells, and this could cause sample contamination.

15. The gel tank was covered with its lid while ensuring that the lids of the tank are properly attached in the correct way.

16. The voltage was set at 100 volts and the gel was allowed to run for 75 minutes. The presence or rising of bubbles at the negative electrode (cathode) shows that the gel electrophoresis process is working. This process creates an electric current through the buffer on the gel and this starts the process for the size separation of the DNA fragments.

17. After running the gel, the power source of the gel rig was turned off and the lid or cover of the gel tank was removed from the gel box using gloved hands.

18. The gel was gently removed from the gel slab or gel casting tray and transferred onto an ultraviolet (UV) light box in a UV illuminator fitted with a camera.

19. The UV illuminator was switched on and the gel image was displayed on a computer screen.

20. The image seen on the gel was photographed, stored as a digital file and printed out as the final result of the gel electrophoresis process. This image is called an electrophoretogram; and it shows the different bands (sizes) of the separated DNA sample in relation to the DNA ladder (marker) used (Perez-Perez and Hanson, 2002).

APPENDIX IX

SYNTHESIS REPORT OF PRIMER PRODUCTION

Name:	blaIMP-1F		**Barcode: S4B17**	**Length:** 20 bases	
Sequence:	ACCGCAGCAGAGTCTTTGCC				
OD	12.7091	**MW min \ max**	6077.4\6077.4	**5' Mod**	None
nmoles	61.58	**GC % min \ max**	60\60	**3' Mod**	None
Tm min \ max	64.5\64.5			**Purification**	Standard
For a 100 µM stock solution add 615.75 µl water or buffer					
Comments					

Name:	blaIMP-1R		**Barcode: S4B18**	**Length:** 20 bases	
Sequence:	ACAACCAGTTTTGCCTTACC				
OD	12.0973	**MW min \ max**	6011.6\6011.6	**5' Mod**	None
nmoles	59.21	**GC % min \ max**	45\45	**3' Mod**	None
Tm min \ max	64.5\64.5			**Purification**	Standard
For a 100 µM stock solution add 592.13 µl water or buffer					
Comments					

Name:	blaIMP-2F		**Barcode: S4B19**	**Length:** 19 bases	
Sequence:	GTTTTATGTGTATGCTTCC				
OD	12.0118	**MW min \ max**	5790.9\5790.9	**5' Mod**	None
nmoles	64.06	**GC % min \ max**	36.84\36.84	**3' Mod**	None
Tm min \ max	53.69\53.69			**Purification**	Standard
For a 100 µM stock solution add 640.63 µl water or buffer					
Comments					

Name:	blaIMP-2R		**Barcode: S4B1A**	**Length:** 17 bases	
Sequence:	AGCCTGTTCCCATGTAC				
OD	9.4506	**MW min \ max**	5121.2\5121.2	**5' Mod**	None
nmoles	61.58	**GC % min \ max**	52.94\52.94	**3' Mod**	None
Tm min \ max	57.19\57.19			**Purification**	Standard
For a 100 µM stock solution add 558.88 µl water or buffer					
Comments					

Name:	blaVIM-1F		**Barcode: S4B1B**	**Length:** 19 bases	
Sequence:	AGTGGTGAGTATCCGACAG				
OD	11.8503	**MW min \ max**	5892.3\5892.3	**5' Mod**	None
nmoles	54.86	**GC % min \ max**	52.63\52.63	**3' Mod**	None
Tm min \ max	60.16\60.16			**Purification**	Standard
For a 100 µM stock solution add 548.63 µl water or buffer					
Comments					

Name:	blaVIM-1R		**Barcode: S4B1D**	**Length:** 18 bases	
Sequence:	ATGAAAGTGCGTGGAGAC				
OD	11.2404	**MW min \ max**	5612.1\5612.1	**5' Mod**	None
nmoles	52.21	**GC % min \ max**	50\50	**3' Mod**	None
Tm min \ max	57.62\57.62			**Purification**	Standard
For a 100 µM stock solution add 522.08 µl water or buffer					
Comments					

Name:	blaVIM-2F		**Barcode: S4B17E**	**Length:** 21 bases	
Sequence:	ATGTTCAAACTTTGAGTAAG				
OD	15.5287	**MW min \ max**	6458.9\6458.9	**5' Mod**	None
nmoles	64.81	**GC % min \ max**	28.57\28.57	**3' Mod**	None
Tm min \ max	52.8\52.8			**Purification**	Standard
For a 100 µM stock solution add 648.11 µl water or buffer					
Comments					

Name:	blaVIM-2R		**Barcode: S4B1F**	**Length:** 18 bases	
Sequence:	CTACTCAACGACTGAGCG				
OD	11.2328	**MW min \ max**	5468.1\5468.1	**5' Mod**	None
nmoles	57.9	**GC % min \ max**	55.56\55.56	**3' Mod**	None
Tm min \ max	59.9\59.9			**Purification**	Standard
For a 100 µM stock solution add 579.01 µl water or buffer					
Comments					

Name:	MOXM-F		**Barcode: S4B21**	**Length:** 21 bases	
Sequence:	GCTGCTCAAGGAGCACAGGAT				
OD	13.6781	**MW min \ max**	6479.4\6479.4	**5' Mod**	None
nmoles	57.66	**GC % min \ max**	57.14\57.14	**3' Mod**	None
Tm min \ max	64.52\64.52			**Purification**	Standard
For a 100 µM stock solution add 576.65 µl water or buffer					
Comments					

Name:	MOXM-R		**Barcode: S4B22**	**Length:** 22 bases	
Sequence:	CACATTGACATAGGTGTGGTGC				
OD	14.0334	**MW min \ max**	6789.8\6789.8	**5' Mod**	None
nmoles	58.25	**GC % min \ max**	50\50	**3' Mod**	None
Tm min \ max	62.67\62.67			**Purification**	Standard
For a 100 µM stock solution add 582.54 µl water or buffer					
Comments					

Name:	DHAM-F		**Barcode: S4B23**	**Length:** 22 bases	
Sequence:	AACTTTCACAGGTGTGCTGGGT				
OD	16.4312	**MW min \ max**	6780.9\6780.9	**5' Mod**	None
nmoles	70.13	**GC % min \ max**	50\50	**3' Mod**	None
Tm min \ max	62.67\62.67			**Purification**	Standard
For a 100 µM stock solution add 701.29 µl water or buffer					
Comments					

Name:	DHAM-R		**Barcode: S4B24**	**Length:** 21 bases	
Sequence:	CCGTACGCATACTGGCTTTGC				
OD	12.0232	**MW min \ max**	6372.7\6372.7	**5' Mod**	None
nmoles	57.64	**GC % min \ max**	57.14\57.14	**3' Mod**	None
Tm min \ max	64.52\64.52			**Purification**	Standard
For a 100 µM stock solution add 576.38 µl water or buffer					
Comments					

Name:	ACCM-F		**Barcode: S4B25**	**Length:** 21 bases	
Sequence:	AACAGCCTCAGCAGCCGGTTA				
OD	13.9422	**MW min \ max**	6399.4\6399.4	**5' Mod**	None
nmoles	61.04	**GC % min \ max**	57.14\57.14	**3' Mod**	None
Tm min \ max	64.52\64.52			**Purification**	Standard
For a 100 µM stock solution add 610.43 µl water or buffer					
Comments					

Name:	ACCM-R		**Barcode: S4B26**	**Length:** 21 bases	
Sequence:	TTCGCCGCAATCATCCCTAGC				
OD	12.8307	**MW min \ max**	6301.6\6301.6	**5' Mod**	None
nmoles	62.16	**GC % min \ max**	57.14\57.14	**3' Mod**	None
Tm min \ max	64.52\64.52			**Purification**	Standard
For a 100 µM stock solution add 621.64 µl water or buffer					
Comments					

Name:	FOXM-F		**Barcode: S4B27**	**Length:** 22 bases	
Sequence:	AACATGGGGTATCAGGGAGATG				
OD	13.6097	**MW min \ max**	6887.6\6887.6	**5' Mod**	None
nmoles	51.77	**GC % min \ max**	50\50	**3' Mod**	None
Tm min \ max	62.67\62.67			**Purification**	Standard
For a 100 µM stock solution add 517.68 µl water or buffer					
Comments					

Name:	FOXM-R		**Barcode: S4B27**	**Length:** 21 bases	
Sequence:	CAAAGCCGTAACCGGATTGG				
OD	12.7091	**MW min \ max**	6077.4\6077.4	**5' Mod**	None
nmoles	50.58	**GC % min \ max**	50\50	**3' Mod**	None
Tm min \ max	62.52\62.52			**Purification**	Standard
For a 100 µM stock solution add 518.75 µl water or buffer					
Comments					

APPENDIX X

MASTERMIX OF THE PCR SETUP

REAGENTS	1X (µl)	X24 (µl)
10XB	2.5	60.0
$MgCl_2$	1.25	30.0
dNTPs	2.0	48.0
Primers	1.0	X8 primers
Template DNA	2.0	Each
DNA Taq	0.2	4.8
Nuclease-free water	9.05	217.2
Total	25.0	

B = PCR buffer

Taq = Taq polymerase

APPENDIX XI

Statistical Analysis Results

Proposed Hypothesis:

H_0 = There is no significant difference between the presence or number of gene variants (mbl, ampc) and the organisms that harbored these genes.

H_1 = There is significant difference between the presence or number of gene variants (mbl, ampc) and the organisms that harbored these genes.

```
DATASET ACTIVATE DataSet1.
NEW FILE.
DATASET NAME DataSet3 WINDOW=FRONT.
WEIGHT BY Frequency.
CROSSTABS
  /TABLES=mbl_variants BY Organisms
  /FORMAT=AVALUE TABLES
  /STATISTICS=CHISQ PHI
  /CELLS=COUNT EXPECTED ROW
  /COUNT ROUND CELL.
```

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
mbl_variants * Organisms	43	100.0%	0	0.0%	43	100.0%

mbl_variants * Organisms Crosstabulation

			Organisms			Total
			Escherichia coli	Klebsiella species	Pseudomonas aeruginosa	
mbl_variants	IMP-1	Count	8	9	12	29
		Expected Count	8.1	10.1	10.8	29.0
		% within mbl_variants	27.6%	31.0%	41.4%	100.0%
	IMP-2	Count	4	6	4	14
		Expected Count	3.9	4.9	5.2	14.0
		% within mbl_variants	28.6%	42.9%	28.6%	100.0%
Total		Count	12	15	16	43
		Expected Count	12.0	15.0	16.0	43.0
		% within mbl_variants	27.9%	34.9%	37.2%	100.0%

NB: The **"count"**represents the **"observed count".** The**expected count** is what we would expect when there is no relationship between the two variables

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.798[a]	2	.671
Likelihood Ratio	.805	2	.669
Linear-by-Linear Association	.273	1	.601
N of Valid Cases	43		

a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is 3.91.

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.136	.671
	Cramer's V	.136	.671
N of Valid Cases		43	

df = (R-)(C-1)

=(2-1)x(3-1) = 2

At α= 0.05 and df = 2; $\chi^2_{critical}$ = 0.798

Decision Rule:
Since χ^2_{cal} = 0.798< $\chi^2_{critical}$ = 5.99, then, we accept H_o and fail to accept H_1.

Phi and **Cramer's V** are both tests of the strength of association.

Phi is interpretable as a nonparametric correlation coefficient, and means just the same thing as the Pearson r in terms of the strength and direction of the relationship between these two variables (gene variants and organisms). In this case, phi = 0.136, which is a weak positive relationship between the two variables.
•The strength of association between the variables is moderate (0.136)

Effect size:
Cramer's V- applies to tables where at least one variable had only two categories; for example, 2 x 2 tables, 2 x 3 tables, etc
Small = .10 medium = .30 large = .50
Cramer's V = .20 – small effect size in this study

NB: the **expected count** is what we would expect when there is no relationship between the two variables

Recall that the significance level (alpha) sets a standard on how the extreme data must be before rejecting the null hypothesis. To reject the null, data must meet a significance level of 0.05. Sig. = 0.05 means that data would have occurred by chance at most 5% of that time.

- If p value (sig.) ≤ .05, then, reject the null. Test is Statistically significant
- If p value (sig.) > .05, then, fail to reject the null. Test is not-Statistically significant

NB: The result is always stated in relation to the null hypothesis, not the alternate.

```
CROSSTABS
  /TABLES=ampc_variants BY Organisms
  /FORMAT=AVALUE TABLES
  /STATISTICS=CHISQ PHI
  /CELLS=COUNT EXPECTED ROW
  /COUNT ROUND CELL
  /BARCHART.
```

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
ampc_variants * Organisms	20	100.0%	0	0.0%	20	100.0%

ampc_variants * Organisms Crosstabulation

			Organisms			Total
			Escherichia coli	Klebsiella species	Pseudomonas aeruginosa	
ampc_variants	CMY-1	Count	3	4	7	14
		Expected Count	3.5	3.5	7.0	14.0
		% within ampc_variants	21.4%	28.6%	50.0%	100.0%
	FOX-1	Count	2	1	3	6
		Expected Count	1.5	1.5	3.0	6.0
		% within ampc_variants	33.3%	16.7%	50.0%	100.0%
Total		Count	5	5	10	20
		Expected Count	5.0	5.0	10.0	20.0
		% within ampc_variants	25.0%	25.0%	50.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.476[a]	2	.788
Likelihood Ratio	.483	2	.785
Linear-by-Linear Association	.082	1	.774
N of Valid Cases	20		

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is 1.50.

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.154	.788
	Cramer's V	.154	.788
N of Valid Cases		20	

Plasmid curing analysis

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Pmid_curin * Organisms	48	100.0%	0	0.0%	48	100.0%

			Organisms			Total
			Escherichia coli	Klebsiella species	Pseudomonas aeruginosa	
Pmid_curin	pre-curing	Count	5	14	5	24
		Expected Count	5.0	14.0	5.0	24.0
		% within Pmid_curin	20.8%	58.3%	20.8%	100.0%
		% of Total	10.4%	29.2%	10.4%	50.0%
	cured isolates	Count	4	10	3	17
		Expected Count	3.5	9.9	3.5	17.0
		% within Pmid_curin	23.5%	58.8%	17.6%	100.0%
		% of Total	8.3%	20.8%	6.3%	35.4%
	post-curing	Count	1	4	2	7
		Expected Count	1.5	4.1	1.5	7.0
		% within Pmid_curin	14.3%	57.1%	28.6%	100.0%
		% of Total	2.1%	8.3%	4.2%	14.6%
Total		Count	10	28	10	48
		Expected Count	10.0	28.0	10.0	48.0

	% within Pmid_curin	20.8%	58.3%	20.8%	100.0%
	% of Total	20.8%	58.3%	20.8%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.490[a]	4	.974
Likelihood Ratio	.489	4	.975
Linear-by-Linear Association	.094	1	.759
N of Valid Cases	48		

a. 5 cells (55.6%) have expected count less than 5. The minimum expected count is 1.46.

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.101	.974
	Cramer's V	.071	.974
N of Valid Cases		48	