OCCURRENCE OF CARBAPENEMASES AMONG ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE ISOLATED FROM URINE OF PATIENTS ATTENDING SELECTED HOSPITALS IN ZARIA, NIGERIA

BY

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DECEMBER, 2021

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADUBELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DOCTOR OF PHILOSOPHY DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCES,
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ZARIA

DECEMBER, 2021

DECLARATION

I declare that the work in this dissertation entitled "OCCURRENCE OF CARBAPENEMASES AMONG ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE ISOLATED FROM URINE OF PATIENTS ATTENDING SELECTED HOSPITALS IN ZARIA, NIGERIA" has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any other Institution.

_____ _____

Ibrahim Mohammed HUSSAINI Date

(P17LSMC9004)

CERTIFICATION

This dissertation entitled "OCCURRENCE OF CARBAPENEMASES AMONG ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE ISOLATED FROM URINE OF PATIENTS ATTENDING SELECTED HOSPITALS IN ZARIA, NIGERIA" by Ibrahim Mohammed HUSSAINI meets the requirement for the award of Doctor of Philosophy degreein Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

This study was carried out to determine the occurrence of carbapenemasesamong *Escherichia coli* and *Klebsiella pneumoniae*isolated from urine of patients attending selected hospitals in Zaria. A total of 302 mid-stream urine samples were collected, inoculated onto MacConkey agar by streaking and the plates were incubated for 24hours at 37ºC. Isolates with characteristic colonial morphology of *E. coli*and *K. pneumoniae* on MacConkey agar were characterized microscopically and biochemically. They were thenscreened for carbapenem resistance. Isolates that were resistant to carbapenem were screened for carbapenemase production phenotypically using the Modified Hodge Test (MHT), Carba NP test and modified Carbapenem Inactivation Method (mCIM)as described in the manual of Clinical Laboratory Standard Institute. All the carbapenem resistant isolates were screened for carbapenemase genes (bla_{KPC}, bla_{OXA} and bla_{NDM}) by PCR. The sensitivity, specificity and accuracy of the phenotypic tests at 95% CIs were calculated using the result of PCR as gold standard. Amplicons of the PCR positive samples were sequenced and the sequences were analyzed for sequence similarity by nucleotide BLAST. Multiple sequence alignment of the carbapenemase genesequences and reference sequences from the GenBank was done by ClustalW using BioEdit. Antibiotic susceptibility patterns of the carbapenem resistant isolates were determined by Kirby Bauer disc diffusion method. A total of 123 isolates consisting of 70 *E. coli* and 53 *K. pneumoniae* were isolated giving an occurrence rate of 23.18% and 17.55% respectively. Out of the 123 isolates screened for carbapenem resistance, 6 (4.88%) comprising of 2 isolates of *E. coli*(2.86%) and 4 isolates of *K. pneumoniae*(7.55%) were carbapenem resistant isolates. Phenotypically, the occurrence rate of carbapenemase producing *E. coli* was found to be 1.43%, 2.86% and 2.86% by MHT, mCIM and Carba NP test respectively. Using these phenotypic tests, the occurrence rate of carbapenemase producing *K. pneumoniae* was found to be 5.66%, 7.55% and 5.55% respectively.

Carbapenemase genes were detected in five out of the six carbapenem resistant isolates screened. The most frequently detected carbapenemase gene was *bla*$_{OXA}$ gene (57.14%) followed by *bla*$_{NDM}$ gene (42.86). *bla*$_{KPC}$ gene was not detected (0.0%).CarbaNP test had the highest sensitivity (100.0%) and specificity (100.0%). The detection rates of OXA and NDM carbapenemases were found be to 100.0% by Carba NP test and mCIM while the rates of OXA and NDM carbapenemases by MHT were found to be 75.0% and 33.33% respectively. Sequence similarity analysis revealed that the carbapenemase genes detected were similar to carbapenemase genes in the NCBI GenBank showing 98 – 100% identity. Nucleotide substitutionswith correspondingamino acid substitution were observed in the *bla*$_{OXA}$ gene sequences at various positions. All the isolates (100.0%) were susceptible to tigecycline and fosfomycin. However,the isolates were resistant to ceftriaxone (100.00%), ampicillin (100.00%), trimethoprim-sulphamethoxazole (100.00%), doxycycline (83.33%), nalidixic acid (66.67%) and chloramphenicol (66.67%). Most of the isolates (66.67%) were susceptible to amikacin and susceptibility to colistin was recorded in 16.67% of the isolates. All the isolates were resistant to multiple antibiotic with MAR indices ranging from 0.46 to 0.82. In conclusion, carbapenem resistance observed in the isolates was mediated by OXA and NDM carbapenemases. Carba NP was estimated to be the most sensitive, specific and accurate phenotypic test in the detection of carbapenemases. The carbapenem resistant isolates were susceptible to tigecycline, fosfomycin and amikacin.

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ABBREVIATIONS AND SYMBOLS

>	Greater than
≤	Less than or equal to
µL	Microliter
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CP	Carbapenemase producer
CRE	Carbapenem Resistant *Enterobacteriaceae*
CRKP	Carbapenem Resistant *Klebsiella pneumoniae*
df	Degree of freedom
ESBL	Extended Spectrum Beta-lactamases
GI	Gastrointestinal
HV	Hyper Virulent
ICU	Intensive Care Unit
KPC	*Klebsiella pneumoniae* Carbapenemase
LPS	Lipopolysaccharide
MAR I	Multiple Antibiotic Resistance Index
MDR	Multi Drug Resistant
mg/L	Milligram per Liter
MHT	Modified Hodge Test.
MIC	Minimum Inhibitory Concentration
mm	Millimeter
mmol/L	Millimoles per Liter

NCBI	National Center for Biotechnology Information
NDM	New Delhi metallo- β-lactamase
^{o}C	Degree Celsius
OMP	Outer Membrane Protein
OMPs	Outer membrane proteins
OXA	Oxacillinase
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
UTI	Urinary Tract Infection
WHO	World Health Organization
α	Alpha
β	Beta
μg	Microgram
χ^2	Chi square

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Klebsiella pneumoniae and *Escherichia coli* are important pathogens, causing various infections including pneumonia, bacteremia, septicemia, purulent infections,urinary tract infection, and liver abscess, all of which may occur in either a community or hospital setting (Yao *et al.,* 2015; Chiu *et al.,* 2018). One of the most serious health threats of the 21st century is Antimicrobial Resistance (AMR). Antimicrobial resistance challenges effective treatment of infectious diseases (WHO, 2015).

β-lactam antibiotics are often deemed the primary therapeutic options for infections caused by Gram negative bacteria. Among the β-lactams,carbapenems are considered the antibiotics of last resort. Once *K. pneumoniae* and *E. coli* isolates become non-susceptible tocarbapenems, they are often resistant to all currently available β-lactams and frequently resistant to non-β-lactam antibiotics. In the clinical context, the emergence of carbapenem non-susceptible isolates posesa serious threat to patient survival because infections caused by carbapenem non-susceptibleisolates have limited treatment options and are associatedwith high mortality (Chiu *et al.,* 2018).

Carbapenems have a penicillin-like five-membered ring, but the sulfur at C-1 in the five-membered ring is replacedwith a carbon atom and a double bond between C-2 and C-3 is introduced (Jeon*et al.,* 2015). They havethe broadest spectra of antimicrobial activity among all β-lactams and are primarily used to treatinfections by aerobic Gram-negative bacteria. The emergence and spread of acquired carbapenemresistance due to carbapenemases are a major concern of the public health and is considered a global sentinel event (Jeon*et al.,* 2015).

The broad spectrum of activity and stability to hydrolysis by most beta-lactamases of the carbapenem have made them the drugs of choice for the treatment of infections caused by cephalosporin-resistant Gramnegative bacilli, especially Extended Spectrum Beta-lactamases(ESBL) producing Gram-negative infections (Srinivasan *et al*., 2015).

Carbapenems are commonly used to treat infections causedby multidrug-resistant *Enterobacteriaceae*. During the lastdecade, carbapenem resistance has emerged among clinicalisolates of the *Enterobacteriaceae* family, and this isincreasingly attributed to the production of carbapenemases (Gupta *et al.,* 2013). Introduction of carbapenem into clinical practice for the treatment of serious bacterial infections caused by beta-lactam-resistant bacteria represented a great advancement (Srinivasan *et al*., 2015).

Carbapenems are widely used in the treatment of infection caused by ESBL producing Gram negative bacteria. However, as carbapenems are more frequently utilized, an increasing number ofCarbapenem Resistant *Enterobacteriaceae*(CRE) has been observed worldwide. The emergence of CRE isalarming, as antimicrobial treatment options are limited (Leavitt*et al.,* 2009).Carbapenem-resistant organisms (CROs) are of great significance to the medical community and are associated with highermortality rates than carbapenem-susceptible organisms (Esterly*et al.,* 2012). The Centers for Disease Control and Prevention (CDC) campaign to:Detect and Protect Against Antibiotic Resistance Initiative (known as the AR Initiative), specifically cites detection and tracking ofcarbapenem-resistant *Enterobacteriaceae*as highest priority (US CDC, 2016).

As such, accurate, rapid, diagnostic modalities to detect carbapenemase- producing *Enterobacteriaceae* (CPE) are needed to meetthese goals. Detecting these organisms is confounded due to the many ways organisms may become resistant to this class of

antimicrobials. These include mutations in porins that limit access of drugs to their site of action, alteration of penicillin-bindingprotein sites, up regulation of efflux pumps, and production of specific carbapenemases (Kosta*et al.,* 2017).

Carbapenemases are the most versatile family of β-lactamases and are able to hydrolyze carbapenems and other β-lactams (Jeon*et al.,* 2015).The carbapenemasesfall into three classes according to their aminoacid sequence: (1) Ambler Class A serine carbapenemases(serine beta-lactamases, inhibited by clavulanic acid), (2)Class B metallo-carbapenemases (metallo-beta-lactamases[MBLs], inhibited by metal chelators), and (3) Class Doxacillinase-type carbapenemases (expanded-spectrumoxacillinases) (Queenan and Bush, 2007). Among the many mechanisms conferring resistance to carbapenems, carbapenemases can efficiently hydrolyzecarbapenems and have become an important cause of antimicrobial resistance (Chiu *et al.,* 2018).

The Class A serine carbapenemases include:*Klebsiella pneumoniae* Carbapenemase(KPC), *Serratia marcescens* enzyme(SME), and Guiana Extended Spectrum β-lactamase(GES) enzymes; Class B metallo-β-lactamases (MBL) include:Imipenem Hydrolyzing β-lactamase(IMI), IMP,Verona integron–encoded metallo-β-lactamase(VIM) and New Delhi Metallo- β-lactamase(NDM) enzymes while Class D Oxacillinase (OXA) enzymes include OXA-48, OXA-181 and OXA-like enzymes (Bush and Jacoby, 2010). Most of the genes for these enzymeshave been identified on mobile genetic elements including plasmids and integrons; thus, transmission of carbapenemase-mediatedresistance has been detected among the *Enterobacteriaceae* as well as other Gram-negative rods, in particular *Pseudomonas* and*Acinetobacter* species (Bush and Jacoby, 2010).

This horizontal spread contributes to a reservoir of organisms both in clinical and environmental locations.Resistance can be mediated by a single mechanism, or combinations of the above, and when present with other extended-spectrum β-lactamases (e.g., ESBLs and AmpC), confers widespread resistance to multiple antimicrobials (Lutring and Limbago, 2016).

Of the Class A carbapenemases, the KPC family has thegreatest potential for spread due to its location on plasmids,especially since it is most frequently found in *K. pneumoniae*, an organism notorious for its ability toaccumulate and transfer resistance determinants. Inaddition, the clonal spread seen in several epidemicspoints to difficulties with infection control for thisorganism (Thomson, 2010).

In general, carbapenem resistance may be mediatedby three major mechanisms: (1) the hyperproduction of a β-lactamase with weak carbapenem-hydrolyzing activity(such as AmpC-type cephalosporinase or an Extended Spectrum β-Lactamase [ESBL]) combined with decreaseddrug permeability through the outer membrane (i.e., outermembrane porin loss or hyperproduction of efflux pumps),(2) a decreased affinity of the penicillin binding proteinsthat constitute target proteins for carbapenems, and (3) carbapenem-hydrolyzing β-lactamase production (Queenan and Bush, 2007; Gupta *et al.*, 2013).

In recent years, the emergenceof carbapenem-resistant *Enterobacteriaceae*, includingcarbapenem nonsusceptible *K. pneumoniae*, has made the treatment of infected patients particularly challenging (Perez and Van Duin, 2013; Huang*et al.,* 2014). Sincethe first report of carbapenem-resistant *K. pneumoniae*(CRKP) in 1996, many studies have been conducted toevaluate the clinical impact of CRKP. The CRKP infectionrelatedmortality rate is higher than those of ESBL producing strains andwild-type susceptible *K. pneumoniae*(Liu*et al.,* 2012). Moreover, infectionwith carbapenem-resistant strains seems to be one ofthe risk factors for

infection-related mortality (Gasink *et al.,* 2009; Ben-David*et al.,* 2012). Compared with other mechanismsof resistance, infections caused by carbapenemase-producing strainsresult in increased mortality (Mouloudi*et al.,* 2010).

1.2 Statement of Research Problem

Carbapenem utilization has increased following the emergence of resistance to third-generation cephalosporins. However, frequent use of carbapenems in the treatment of infections caused by MDR pathogens has led to the emergence of carbapenem-resistant enterobacterial isolates (Wang *et al.,* 2015a). Carbapenem resistance among*Enterobacteriaceae* is principally due to the production of carbapenemases. The less frequent mechanisms are the overproduction of AmpC-mediated β-lactamases or ESBLs in organisms with porinmutations (Nordmann*et al.,* 2011a; Nordmann*et al.,* 2012a; Demir*et al.,* 2015). KPC, NDM-1 and OXA-48 are the predominant mechanisms of carbapenem resistance in *Enterobacteriaceae* (Nordmann*et al.,* 2012a).

The global emergence of carbapenemase-producing *Enterobacteriaceae*(CPE) poses a threat to the achievements of modern medicine. The Centers for Diseases Control and Prevention and the World Health Organization have classified CPE as one of the most urgent antimicrobial-resistance threat. CPE rarely arise de novo; colonization and infection occur as a result of transmission of organisms, plasmids or transposons from person to person, with such transmission occurring predominantly in healthcare institutions (CDC, 2013b; WHO, 2018).

The rapid emergence and dissemination of carbapenemases poses a considerable threat to clinical patientcare and public health. These enzymes confer resistance tovirtually all β-lactam agents, including penicillins, cephalosporins,monobactams, and carbapenems.Most worrisome, treatment of infections caused by theseorganisms is extremely difficult because of their

multidrugresistance, which results in high mortality rates (Gupta *et al.,* 2013).The emergence of CRE,has proven to be a great challenge for physiciansnot only because of its multidrug resistance but alsobecause of the higher mortality and morbidity rates ofinfected patients (Gasink *et al.,* 2009; Ben-David*et al.,* 2012; Huang*et al.,* 2014).

1.3 Justification for the Study

Resistance to carbapenems is a significant therapeutic threat. The increasing frequency of carbapenemase enzymes among Gram-negative bacilli makes their early detectionand differentiation urgent.Early detection of producers of carbapenemases has nowbecome mandatory, it is crucial for controllingthe spread of carbapenemase-producing bacteria(Gupta *et al.,* 2013).

The carbapenemases KPCand New Delhi Metallo - β-lactamase (NDM) have been reported to have beenspreading around the world since late 2000s (Poirel*et al.,* 2010; Nordmann*et al.,* 2011a). OXA-48-producing *Enterobacteriaceae*have been reported in Europe, East-CentralAsia, and Africa (Carrër*et al.,* 2010; Nordmann*et al.,* 2011a).

The spreadof carbapenemaseproducing strains across the world have made it necessary for us to understandthe prevalence of these strains in hospitals. Detectionof CRI would allow physicians to formulatea policy of empirical therapy in high-risk units. Until recently, carbapenem was the only remaining optionfor treating serious multidrug resistant(MDR) enterobacterial infections. However, frequent utilization of carbapenems has led to the emergenceof carbapenem-resistant enterobacterial isolates (Wang *et al.,* 2015b), necessitating alteration to colistin and tigecycline as last-resort antibiotics (Osei Sekyere*et al.,* 2016).

Carbapenem resistant *Enterobacteriaceae* are both public health and therapeutic challenges. Detecting, preventing, and controlling these organisms requires a strategicand sustained effort. As the organisms are constantly altering their resistance mechanisms, it is critical to identify these organisms asrapidly and efficiently as possible (Kosta*et al.*, 2017).

There is need for a uniformand standardized phenotypic test for the detection of carbapenemases (Pasteran*et al.*, 2009). Despite these troublingtrends and the importance of this issue from both clinicaland public health perspectives, there are few studies on carbapenemase producing pathogens in this part of world. Hence,thisstudy was carried out to determine the occurrence of carbapenemasesamong *K. pneumonia*e and *E. coli* isolated from clinical samples. In addition, we evaluated the susceptibility pattern of the CRI to selected antibiotics which are the current treatment option for carbapenem resistant bacteria.

1.4 Aim and Objectives

1.4.1 Aim

The aim of the study was to determine the occurrence of carbapenemasesamong *Escherichia coli* and *Klebsiella pneumoniae*isolated from urine of patients attending selected hospitals in Zaria.

1.4.2 Objectives

The objectives of the study were to:

1. isolate and characterize *Escherichia coli*and *Klebsiella pneumoniae*from urine of patients attending selected hospitals in Zaria.
2. screen for carbapenem resistant*Escherichia coli*and *Klebsiella pneumoniae.*
3. phenotypically screen for carbapenemase producing *E. coli*and *K. pneumoniae* by Modified Hodge Test (MHT), Carba NP test and Modified Carbapenem Inactivation Methods (mCIM).
4. detect carbapenemase genes among the carbapenem resistant isolates by PCR.
5. compare the effectiveness of Modified Hodge Test, Carba NP test and Modified Carbapenem Inactivation Methods in the detection of carbapenemases using PCR as gold standard.
6. conduct sequence analysis on the carbapenemase genes detected.
7. determine the antibiotic susceptibility pattern of the carbapenem resistant isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Escherichia coli*

*Escherichia coli*is acommon commensal inhabitant of the gastrointestinal tract and urinary tract of human. It is one of the most important human pathogens hence the most-studied microorganism. *E. coli* is the mostfrequent aetiologic agent of urinary tract infections (UTIs) and bloodstream infection (Vila *et al.,* 2016).

Pathogenic strains of *E. coli* possess specialized virulence factors such as adhesins,toxins, iron-acquisition systems, polysaccharide coatsand invasins that are absent in commensal strains (Vila *et al.,* 2016).

2.1.1 Taxonomic classification of *Escherichia coli*

Domain = Bacteria

Phylum = *Proteobacteria*

Class = *Gammaproteobacteria*

Order = *Enterobacteriales*

Family = *Enterobacteriaceae*

Genus = *Escherichia*

Species = *Escherichia coli*(CABI, 2019)

2.1.2 Antibiotic Resistance Among*Escherichia coli*

Antibiotics play a vital rolein improving the health and wellbeing of people worldwide. Even though antibiotics have successfullyreduced infectious diseases, their exponentially increased use haveled to the emergence and spread of antibiotic resistance. Gram Negative Bacilli (GNB), including *E. coli,* have emerged as major players inresistance, with multidrug resistance nowbeing relatively common (Nordmann *et al.,* 2011a; Dortet *et al.,* 2014).

Antimicrobial resistance among*E. coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine, such as ampicillin. However, in the past two decades, increase have been observed in the emergence and spread of multidrug-

resistant bacteria, including strains resistant to newer antibiotics such as fluoroquinolones and extended-spectrum cephalosporins (Vila *et al.*, 2016).

Antibiotic resistance results in reduced efficacy of antibacterial agents,making the treatment of patients costly and difficult, oreven impossible (Tzouvelekis *et al*., 2014). In some cases, resistanceextends to the entire repertoire of the therapeutic agentsavailable (the so-called pan-drug resistant phenotypes), posinga formidable challenge to the antimicrobial therapy and turningback the clock to the pre-antibiotic era (Nordmann *et al.*, 2011a; WHO, 2014). This is particularly worrisome in view ofthe current dearth of new compounds active against Multi dru Resistant Gram Negative Bacilli (MDR-GNB) (Theuretzbacher, 2012; Tzouvelekis *et al*., 2014).

World Health Organization (WHO) has included*E. coli* in a list of the top nine microorganisms of international concerncausing the most common infections in different settings:in the community, in hospitals or transmitted throughthe food chain (WHO, 2014). Antimicrobial resistance global reporton surveillance by WHO in 2014 highlighted thirdgenerationcephalosporin and/or fluoroquinolone resistance inurinary tract and blood stream infections that limit empirictreatments (Vila *et al.*, 2016).

2.1.3 Carbapenem resistant *Escherichia coli*

The safety, bactericidal properties and clinical efficacy ofβ-lactam antibiotics place them among those most frequentlyprescribed for the treatment of bacterial infections. Carbapenems have the broadest spectrum of activity of all the β-lactams and are considered the drugs of choice to treat serious infections caused by ESBL producing *Enterobacteriaceae*(Vila *et al.*, 2016).

However, the use of carbapenems is being threatened by the emergence and spread of carbapenem-resistant *Enterobacteriaceae*(CRE)worldwide. The major mechanisms of

carbapenem resistance are: the presence of ESBLs or AmpC enzymesin combination with porin mutations and the productionof carbapenemases (Cuzon *et al*. 2010; Nordmann *et al.,* 2011a). Clinically relevant carbapenemases encounteredin *E. coli* belong to Ambler class A enzymes such as KPC and GES, class D enzymes such as OXA-48 or to metallo-β-lactamases (MBLs) such as IMP, VIM or NDM (Nordmann *et al.,* 2011a). The dissemination of these enzymesamong *E. coli* is a matter of great clinical concern given the major role of this pathogen as a cause of nosocomial aswell as community-acquired infections (Tzouvelekis *et al*., 2014). Carbapenemase-producing *E. coli*isolates are often resistant to most classes of antibiotics, leavingphysicians with very limited antibiotic choices, if any, for treating infected patients (Falagas *et al*., 2014; Tzouvelekis *et al*., 2014). In general, infections due to thisisolates were previouslylimited to immunocompromised/immunosuppressed individuals in hospital settings, however, CP-*E. coli* isolates are now spreading in the community and represent a rising threat to the general population (Nordmann *et al.,* 2011a; Canton *et al*., 2012; Falagas *et al*. 2014).

2.1.4 Virulence Factors of *Escherichia coli*

Escherichia coli possess diverse virulence factors, some of them related to pathogenicity islands, regions of DNA that are acquired by horizontal gene transfer (Vila *et al.,* 2016). These virulence factors are as follows:

(1) Adhesins: fimbriae or pili as type 1 fimbriae, P fimbriae (related with renal cells adherence), curli fimbriae, F1c/s fimbriae, F9 and type 3 fimbriae; and non-fimbrial adhesins (Afa/Dr adhesins, related with diarrheagic diseases) and autotransporter proteins (Ag43 adhesin and Upa uropathogenic autotransporter protein). These adhesins are responsible for adhesion to both urinary tract epithelial cells and urinary catheters, and promote biofilm formation.

(2) Toxins: endotoxin lipopolysaccharide (LPS), α-haemolysin (HlyA), CNF1 (cytotoxic necrotizing factor 1) and SPATEs (serine protease autotransporters of the *Enterobacteriaceae*) as Sat (Secreted Autotransported Toxin), Pic (Protease Involved in Colonization) or Vat (Vacuolating Autotrasported Protein). These toxins are related to dissemination in tissues, inflammatory response, cytotoxicity and resistance to neutrophils.

(3) Iron acquisition mechanisms: Haem receptors (iron Haem uptake regulated by ChuA and Hma) and siderophores (iron chelating molecules as enterobactin, aerobactin, salmochelin and yersiniabactin). Both mechanisms promote the availability of iron in the urinary tract and contribute to survival and persistence in the urinary tract. Zinc acquisition mechanisms are also important.

(4) Immune evasion mechanisms: suppression of induction of cytokines and chemokines (due to O antigens of LPS), serum resistance and protection against phagocytes (due to O antigens of LPS and K antigens of capsular polysaccharides) and motility (due to flagella with F antigens).

(5) Formation of biofilm: biofilm is an extracellular matrix that provides protection against antimicrobial treatment and host defense mechanisms and adherence to both epithelial cells and urinary catheters, and is responsible of persistence and recurrence of UTI. Biofilm formation is considered both a major virulence factor of chronic infections due to the inability of the immune system to eradicate the microorganisms and a clinical problem due to the failure of antibiotics to successfully eliminate them. In addition, biofilm formation can also play a role in certain stages of acute infections and is important for transmission of pathogens (Vila *et al.*, 2016).

2.1.5 Pathogenesis of *Escherichia coli*

Pathogenesis of *Escherichia coli* is due to their ability to invade tissues and produce toxins. The mechanisms of colonization include: adhesion, initial proliferation, extracellular production of invasins, followed by the ability to evade host defense mechanisms. Adhesion of bacteria to eukaryotic cell or a tissue surface requires a receptor and a ligand. Receptors are usually carbohydrate or peptide chain on the surface of eukaryotic cell. The bacterial ligand called adhesin is a macromolecular component of bacterial cell surface that specifically interacts with the receptor. The bacterial cell surface has properties determined by the molecular structure of the cell membrane and the cell envelope which includes the capsule or glycocalyx, Somatic layers, peptidoglycans, lipopolysaccharides, flagella, pili and fimbriae. The bacterial surface serves as a permeability barrier, contains: adhesins involved in adhesion, enzymes that catalyze important reactions for survival and protective structures against phagocytosis; antigens involved in bypassing activation of host defense mechanisms and endotoxins that cause inflammatory reactions in host (Manu *et al.,* 2011).

Toxigenesis is the ability to produce toxins (exotoxins and/or endotoxins). Exotoxins are secreted by the bacterial cells, acting on the host tissue. Endotoxins are constituent molecules of the bacterial cell, often the term refers to the lipopolysaccharides which are constituents of the outer membrane of Gram-negative bacteria. Endotoxins can be released only when the bacterial cell is affected by defense mechanisms of host cells or the activity of antibiotics (penicillins or cephalosporins). Both types of bacterial toxins- soluble and cell associated may produce cytotoxic effects on some host tissue sites distant from the original point of invasion and multiplication. Some toxins have an important role even in colonization and invasion (Manu *et al.,* 2011).

2.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae was first described by Carl Friedlander in 1882 as a bacterium isolated from the lungs of patients who had died from pneumonia (Martin and Bachman, 2018) and was initially known as Friedlander's bacterium. It's a Gram-negative straight rod (between 0.3 and 1.8 µm in size), non-motile, lactose-fermenting, facultative anaerobic bacterium (De Jesus *et al.*, 2015) that resides in the environment, including in soil and surface waters, on medical devices (Paczosa and Mecsas, 2016), plants, animals and humans (Martin and Bachman, 2018).

Itreadily colonizes mucosal surfaces, including the gastrointestinal (GI) tract and oropharynx, where the effects of its colonization appear benign (Dao *et al.,* 2014; Rock *et al.,* 2014; Paczosa and Mecsas, 2016). From these sites it can gain entry to other tissues and cause severe infections in humans. *K. pneumoniae* is an extremelyresilient bacterium whose success as a pathogen seems tofollow the model of "the best defense for a pathogen is a gooddefense" rather than "the best defense for a pathogen is a goodoffense." This is exemplified by the ability of these bacteria toevade and survive, rather than actively suppress many componentsof the immune system and grow at many sites in hosts (Paczosa and Mecsas, 2016).

*Klebsiella pneumoniae*is a pathogenic bacterium and has a mucoid phenotype on agar medium that is conferredby the polysaccharide capsule attached to the bacterial outer membrane and ferments lactose (Magill*et al*., 2014).

Ithas recently gained notoriety as an infectious agent due to a rise in the number of severe infections andthe increasing scarcity of effective treatments. These concerningcircumstances have arisen due to the emergence of *K. pneumoniae*strains that have acquired additional genetic traits and becomeeither hypervirulent (HV) or antibiotic resistant. *Klebsiella pneumoniae*causes a wide range of infections, includingpneumonias, urinary tract infections, bacteremia, andliver

abscesses.*K. pneumoniae* strains have become increasingly resistantto antibiotics, rendering infection by these strains very challengingto treat (Paczosa and Mecsas, 2016).

Klebsiella pneumoniae has a large accessory genomeof plasmids and chromosomal gene loci. This accessory genome divides *K. pneumoniae*strains into opportunistic, hypervirulent, and multidrug-resistant groups(Martin and Bachman, 2018).

2.2.1 Taxonomic classification of *Klebsiella pneumoniae*

Domain = Bacteria

Phylum = *Proteobacteria*

Class = *Gammaproteobacteria*

Order = *Enterobacteriales*

Family = *Enterobacteriaceae*

Genus = *Klebsiella*

Species = *K. pneumoniae*(De Jesus *et al.,* 2015).

2.2.2 Clinical manifestations of *Klebsiella pneumoniae* infections

Klebsiella pneumoniae is associated with a myriad of infections, rangingfrom blood, respiratory, urinary to intra-abdominal infections, especially in incapacitatedpatients (Struve*et al.,* 2008; Brisse*et al.,* 2009; Schroll*et al.,* 2010; Nordmann*et al.,* 2011a). Clinical manifestation of infections (both community-associated and healthcare-associated) caused by *K. pneumoniae* is dependent on the quantity and type of virulence factors expressed(Yu*et al.,* 2007; Schroll*et al.,* 2010).

Patients on admission in the Intensive Care Unit (ICU) are at higher risk of *Klebsiella pneumoniae*infection (Gasink*et al.,* 2009). In the clinical setting, *K. pneumoniae* is second only to *E. coli* in causingcatheter-associated urinary tract infections and is an important blood stream pathogen (Schroll*et al.,* 2010; Bamford*et al.,* 2011).

K. pneumoniae is also implicated in community-associatedpneumonia, pyogenic liver abscess, rhinoscleroma, atrophic rhinitis and less frequentlymeningitis, necrotising fasciitis and prostatic abscess (Brisse*et al.,* 2009; Schroll*et al.,* 2010). Rhinoscleroma andatrophic rhinitis are specifically caused by *K. pneumoniae* subsp. *rhinoscleromatis* and *K.pneumoniae* subsp. *ozaenae*, respectively (Brisse*et al.,* 2009).

*Klebsiella pneumoniae*is largely thought of as an opportunistic pathogen, but the emergence ofhypervirulent strains over the past decade have demonstrated the capacity to infect otherwisehealthy individuals (Yu *et al.,* 2007; Bamford*et al.,* 2011).

The virulence factors expressed could contribute to the range of clinical manifestations ofinfections, but the geographical restriction of certain manifestations could alternately bedependent on host factors typical to that region (Yu *et al.,* 2007; Bamford*et al.,* 2011). Host factors could include thefrequency of diabetes mellitus, genetic predilections, underlying prevalent diseases, alcoholism,socioeconomic determinants and the availability of quality healthcare (Yu *et al.,* 2007; Bamford*et al.,* 2011).

2.2.3 Antibiotic resistance in *Klebsiella pneumoniae*

Over the last few decades, there has been a concerning rise in theacquisition of resistance to a wide range of antibiotics by *K. pneumoniae*strains. UTIs have become recalcitrant to treatment as a result of the emergence and spread of antibiotic resistance. This has also resulted in

infections such as pneumonia and bacteremiabecoming increasingly life-threatening (Boucher *et al.,* 2009; Kuehn, 2013).

Klebsiella species are known to serve as reservoir for antibiotic resistance genes and they can spread these genes to other Gram-negativebacteria. In fact, many of the antibiotic-resistant genes nowcommonly found in multidrug-resistant organisms were firstlydescribed in *Klebsiella*(Bengoechea and Sa Pessoa, 2019). With the emergence of carbapenem resistant isolates, limited therapeutic options are left forpatients infected with multidrug-resistant *K. pneumoniae.* Alarmingly, recent studieshave recognized that several *K. pneumoniae* virulent andmultidrug-resistant clones have access to a mobile pool of virulenceand antimicrobial resistance genes (Holt *et al.* 2015; Lam*et al.* 2018). This has made it possible for the emergenceof a multidrug resistant, hypervirulent *K. pneumoniae* clone capableof causing untreatable infections in healthy individuals (Bengoechea and Sa Pessoa, 2019).

Two major types ofantibiotic resistance have been commonly observed in *K. pneumoniae*.One mechanism involves the expression of ESBLs, which render bacteria resistantto cephalosporins and monobactams. The other mechanism ofresistance, which is even more troubling, is the expression of carbapenemasesby *K. pneumoniae*, which renders bacteria resistantto almost all available β-lactams, including the carbapenems (CDC, 2015).

The first case of *K. pneumoniae* expressing a carbapenemasewas identified in North Carolina in 1996 and thus, this type of carbapenemase is called *K. pneumoniae* carbapenemase - KPC (Paczosa and Mecsas, 2016). Additional carbapenemases,such as MBL, NDM-1, IMP, and VIM, have since been found in *K.pneumoniae* strains (Pitout*et al.,* 2015).

Due to a lack of availableeffective treatments, *K. pneumoniae* infections caused byESBL-producing and carbapenem-resistant bacteria have significantlyhigher rates of morbidity and mortality than infections withnonresistant bacteria (CDC, 2013a).

Three modes of antibiotic resistance exist in *K. pneumoniae* viz: drug modification orenzymatic inactivation, antibiotic target modification or decreased concentrations of antimicrobialdrugs within cells (possible by reduced permeability) and increased efflux activity (Nordmann and Poirel, 2008;Page *et al.,* 2010; Fernández *et al.,* 2011; Kumar *et al.,* 2011). These modes of action are encoded either intrinsically or acquired throughmutation and resistance gene acquisition (Poole, 2004; Fernández *et al.,* 2011).

Genetic elements conferring potentialresistance genes are easily transferred horizontally both intra- and interspecies among *Enterobacteriaceae*due to the closegenetic resemblance between bacteria of the *Enterobacteriaceae*family (Fernández *et al.,* 2011; Kumar *et al.,* 2011).

Changes in membrane permeability and drug flux can be influenced by variable expression andregulation of the efflux pumps (Kumar *et al.,* 2011). Modification or loss of the OmpK35 and OmpK36 porin proteins can affect resistance in various ways either leading to elevated minimum inhibitory concentrations (MICs) or resistancetowards carbapenems and expanded-spectrum cephalosporins, reduced fluoroquinolonesusceptibility, or it may occasionally confer additional cross-resistance to quinolones, aminoglycosidesand co-trimoxazole within broad-spectrum β-lactamase- or ESBL-producers (García-Sureda*et al.,* 2011; Tsai*et al.,* 2011). An additional modification to the outer membrane aiding in resistance, other than porinloss, is the upregulation of capsule polysaccharide (CPS) production in *K. pneumoniae* (Kocsis and Szabó, 2013).

Biofilm confers survival advantages in the form of improvedresistance to host immune defences, resistance to biocides, increased resistance to antimicrobialcompounds and higher plasmid transfer rates within that environment, which couldinclude antibiotic resistance genes (Schroll *et al.,* 2010; Fernández *et al.,* 2011; Hennequin*et al.,* 2012; Soto, 2013).

The reducedantimicrobial drug effect against bacterial populations within a biofilm is largely unclear butcould be as a result of several mechanisms acting in conjunction, such as: (i) poor compounddiffusion, (ii) the slower growth and uptake of antibiotics by the bacteria in mature biofilm(>24 hours old), (iii) the production of antimicrobial inactivating enzymes, (iv) general stressresponses, (v) the expression of efflux pumps and (vi) the presence of persister cells(Fernández *et al.,* 2011; Hennequin*et al.,* 2012; Bernier*et al.,* 2013; Soto, 2013). Biofilm formation in *K. pneumoniae* is influenced by cell densitydependentquorum sensing signaling *via* the non-specific bacterial type-2 QS regulatorymolecules, AI-2 autoinducers (De Araujo*et al.,* 2010). The mannose-resistant *Klebsiella*-like (MR/K) haemagglutininsor "Mrk proteins" are encoded by the genes *mrk*ABCDF within an operon and form partof type 3 fimbriae, which is important in mediating biofilm formation in *K. pneumoniae* (Wilksch*et al.,* 2011).Antimicrobial drug resistance can increase up to 1000-fold for bacterial cells existing withinthe biofilm (De La Fuente-Núñez*et al.,* 2013; Soto, 2013).

Resistance againstβ-lactam antibiotics are mainly mediated by β-lactamase enzymeproduction, which is capable of hydrolysing third-generation cephalosporins and monobactams (Elhani*et al.,* 2010; Page *et al.,* 2010).

2.2.4 Virulence factorsof *Klebsiella pneumoniae*

K. pneumoniae employs many virulence factorsin surviving and circumventing the host immune response. Capsule, lipopolysaccharide (LPS), siderophores andfimbriae are currently the well characterized virulence factors of *K. pneumoniae*(Figure 2.1). Other virulence factors of *K. pneumoniae*have beenidentified however they are notyet thoroughly characterized. These virulence factors include outer membrane proteins (OMPs), porins, effluxpumps, iron transport systems, and genes involved in allantoinmetabolism (Paczosa and Mecsas, 2016).

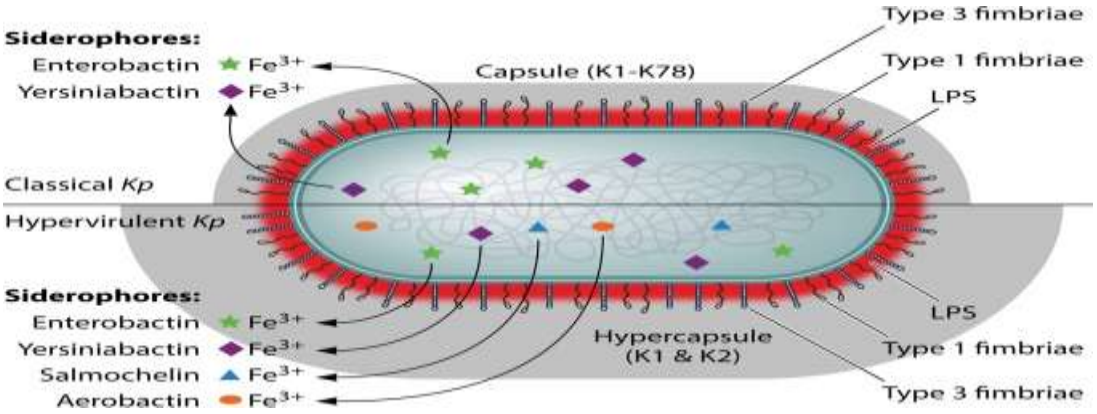

Figure 2. 1: Annotated Diagram of *K. pneumoniae* showing the Four Most Characterized Virulence Factors Source: Paczosa and Mecsas (2016)

i. Capsule as a virulence factor in *K. pneumoniae*

Capsule is a polysaccharide matrix that coats the bacterial cell. Itplays a vital role in *K. pneumoniae* virulence and is arguably the most thoroughlystudied virulence factor of *K. pneumoniae* (Figure 2.2). Strains of *K. pneumoniae* that lack capsule are dramatically less virulent compared to the encapsulated strains in mouse models, based on decreasedbacterial loads in the lungs, lower rates of mouse mortality,and an inability of the bacteria to spread systemically (Paczosa and Mecsas, 2016).

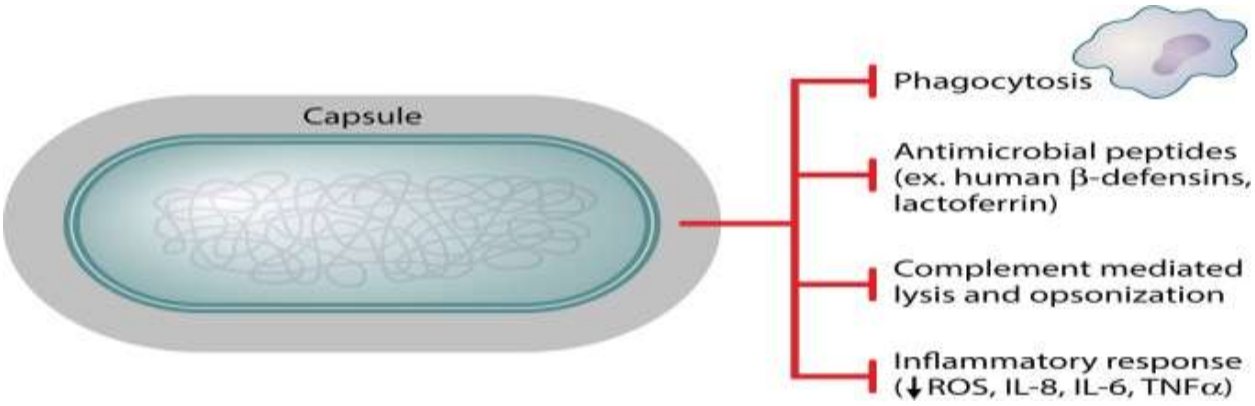

Figure 2. 2: Role of capsule in *K. pneumoniae* virulenceSource: Paczosa and Mecsas (2016).

Furthermore, hypervirulent *K. pneumoniae* strains produce ahypercapsule, also known as being hypermucoviscous, which consistsof a mucoviscous exopolysaccharide bacterial coating that ismore robust than that of the typical capsule. This hypercapsulemay contribute significantly to the pathogenicity of hypervirulent *K. pneumoniae*(Yeh*et al.,* 2007).Both classical capsule and hypervirulent hypercapsule are made up ofstrain-specific capsular polysaccharides termed K antigens (i.e., K1 and K2, up through K78) (Pan*et al.,* 2008).

ii. Lipopolysaccharide (LPS) as a virulence factor in *K. pneumoniae*

Lipopolysaccharide, also known as endotoxin, is a major and necessary componentof the outer layerof all Gram-negative bacteria cell membrane. Although there is considerable variation in LPSstructures among bacterial species, it is typically comprised of anO antigen, a core oligosaccharide, and lipid A (Figure 2.3) (Raetz *et al.,* 2009; De Majumdar *et al.,* 2015).

Nine different O-antigen types have been identified in *K. pneumoniae* isolates with O1 being the most common. Lipopolysaccharide is an important virulence factor that protectsagainst humoral defenses however, it's also a strong immune activator. The lipid portion of bacterial LPS, lipid

A, is well known for being a potent ligand of TLR4, a pattern recognition receptor.TLR4 stimulation leads to the production of cytokines andchemokines that help recruit and activate cellular responses, including neutrophils and macrophages, which clear *K. pneumoniae*infection and control spread to other tissues (Paczosa and Mecsas, 2016).

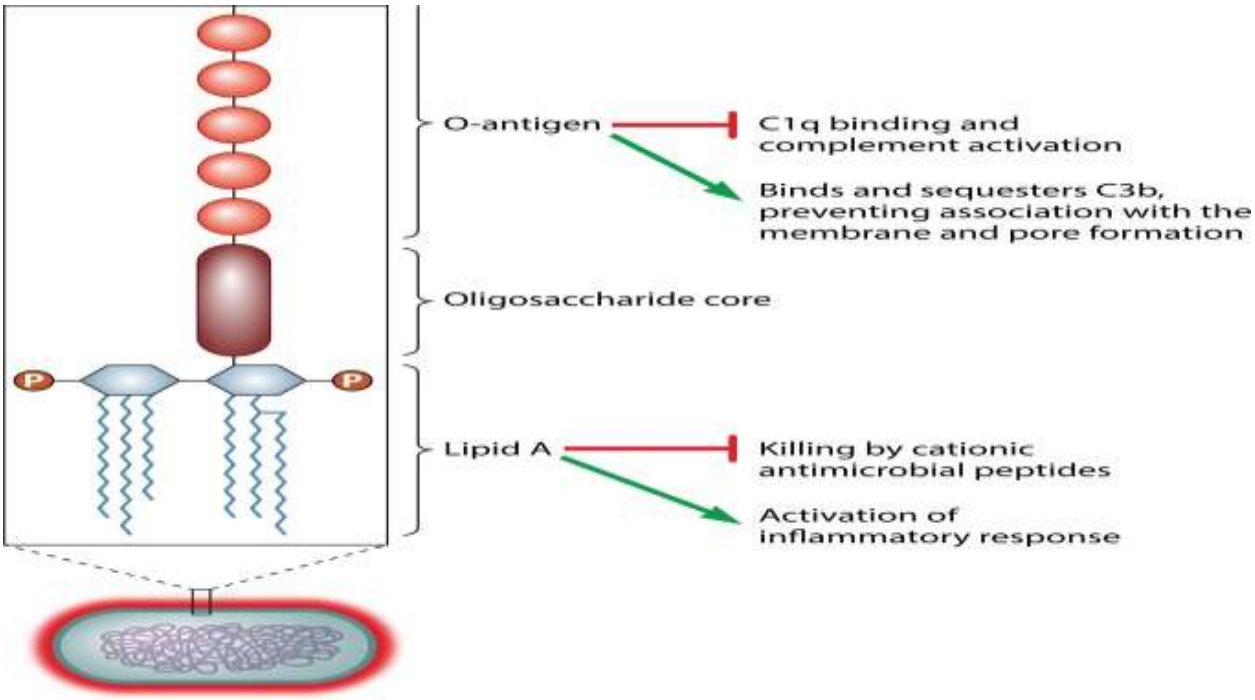

Figure 2. 3: Role of Lipopolysaccharide in *K. pneumoniae* virulenceSource: Paczosa and Mecsas (2016)

iii. Type 1 and 3 Fimbriae as a virulence factor in *K. pneumoniae*

Fimbriae represent another class of *K. pneumoniae* virulence factorsand are important mediators of *K. pneumoniae* adhesion. In *K. pneumoniae*, type 1 and 3 fimbriae are the majoradhesive structures that have been characterized as pathogenicity factors (Figure 2.4) (Paczosa and Mecsas, 2016).

Type 1 fimbriae are thin, thread-like protrusions on the bacterialcell surface and are expressed in 90% of both clinical andenvironmental *K. pneumoniae* isolates as well as almost all membersof the *Enterobacteriaceae*(Klemm and Schembri, 2000; Stahlhut *et al.*, 2009).

K. pneumoniae type 1 fimbriaebind D-mannosylated glycoproteins, and therefore, binding bytype 1 fimbriae is frequently termed “mannose-sensitive” binding.Type 3 fimbriae are helix-like filaments. In a mannersimilar to that of type 1 fimbriae, the type 3 fimbria-encodingoperon is found in and expressed by almost all *K. pneumoniae*isolates. In contrast to type 1 fimbriae, type 3 fimbriae are “mannoseinsensitive” and therefore do not bind mannose. While aspecific cell surface receptor has not yet been identified for type 3fimbriae, they have been shown to bind extracellular matrix proteinssuch as type IV and V collagens (Paczosa and Mecsas, 2016).

K. pneumoniae utilizes environmental cues to regulate the expressionof its type 1 fimbriae. For example, type 1 fimbria genesare expressed in the urinary tract but not in the GI tract or lungs. This observation is in line with the fact that *K. pneumoniae*type 1 fimbriae contribute to UTIs. Like their type 1 counterparts, type 3 fimbriae are not neededfor GI tract colonization or for virulence in the lung. Type 3 fimbriaecan bind to bladder epithelial cells grown in culture, but inmouse model systems, they do not seem to contribute to UTIs. Moreover, in these organs, types 1 and 3 are not functionallyredundant; i.e., a strain with a double knockout of theclusters encoding both type 1 and type 3 fimbriae had virulenceequal to that of a WT strain in the lungs (Struve*et al.*, 2009).

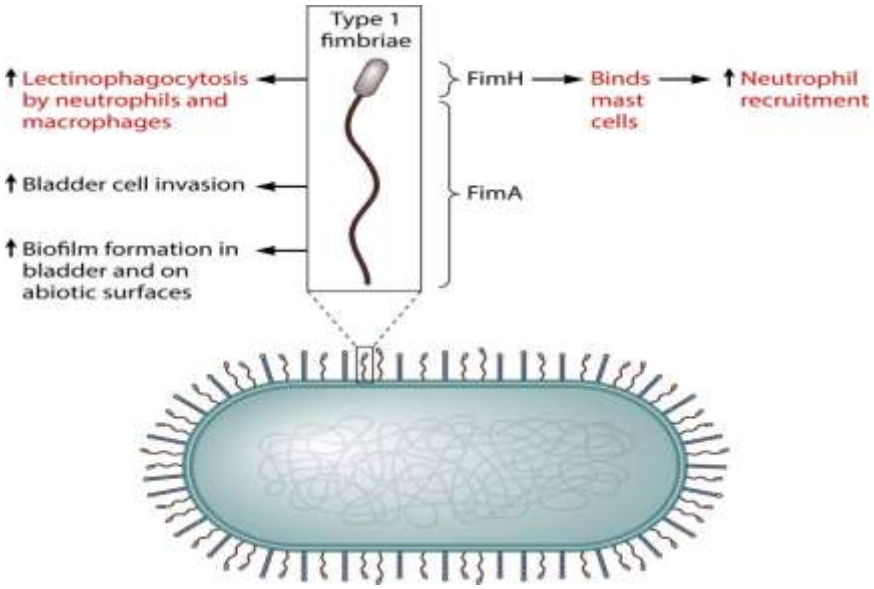

Figure 2. 4: Role of fimbriae in *K. pneumoniae* infection and biofilm formation

Source: Paczosa and Mecsas, 2016

iv. Siderophores as a virulence factor in *K. pneumoniae*

Siderophoresare molecules withhigh affinity for iron which is a limited resource required by *K. pneumoniae* andmust be acquired from the environment during infection. Iron is not readily available in thehost during infection, primarily because, as part of the nonspecificimmune response, the host sequesters it to restrict the growth of a number of possible pathogens (Paczosa and Mecsas, 2016).

K.pneumoniae acquire iron predominantly through the secretion of siderophores that steal iron fromhost iron-chelating proteins or scavenge it from the environment (Figure 2.5). *K. pneumoniae* strains encode several siderophores, and theexpression and contribution of each siderophore to virulence vary. The production of more than one siderophore by *K. pneumoniae*may be a means of optimizing successful colonization ofdifferent tissues and/or avoiding neutralization of one siderophoreby the host (Bachman*et al.,* 2012).

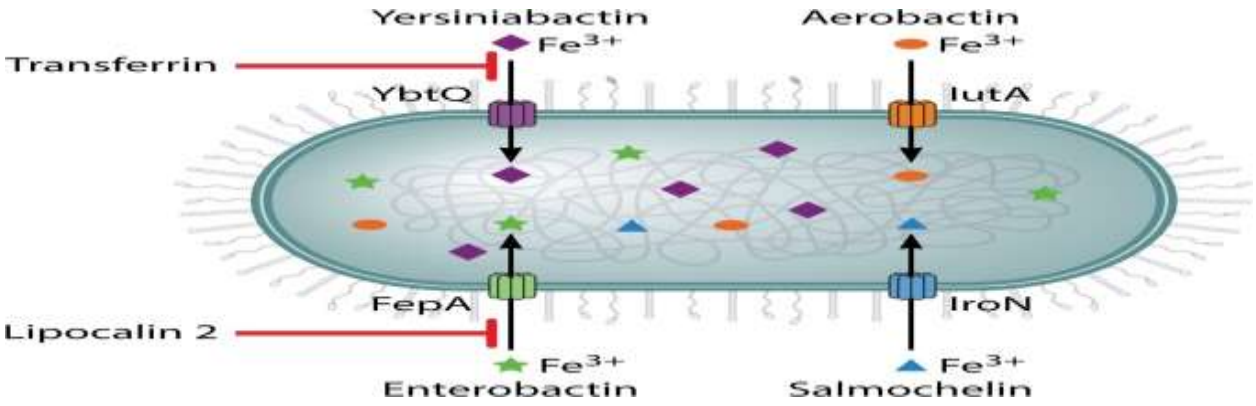

Figure 2. 5: Acquisition of iron from the environment by *K. pneumoniae* using siderophores
Source: Paczosa and Mecsas(2016).

Several siderophores are expressed in*K. pneumoniae*, including enterobactin, yersiniabactin, salmochelin, and aerobactin. The affinity of these siderophores for iron ranges from aerobactinwith the lowest to enterobactin with the highest.While the expression of the other siderophores is less conserved,enterobactin expression is almost ubiquitous among both classicaland HV *K. pneumoniae* strains and is therefore considered to bethe primary iron uptake system utilized by *K. pneumoniae* (El Fertas-Aissani*et al.*, 2013).

Yersiniabactin was originally discovered in the Gram-negativebacterial pathogen *Yersinia* as part of a *Yersinia* high-pathogenicityisland, but this siderophore has since been identified in otherbacteria, including *K. pneumoniae* (Paczosa and Mecsas, 2016).

Salmochelin is a c-glucosylated form of enterobactin.This modification is carried out by genes found on either the chromosomeor a plasmid within the *iroA* gene cluster, *iroBCDE* (Hsieh*et al.*, 2008).

Aerobactin is a citrate-hydroxamate siderophore. It is rarelyexpressed by classical nosocomial *K. pneumoniae* clinical isolates,as it is found in only about 6% of classical strains, yet is present

in93 to 100% ofHV*K. pneumoniae* isolates. Thepresence of aerobactin is always associated with a hypercapsule,although not all hypercapsulated strains possess this siderophore (Paczosa and Mecsas, 2016).

v. Outer Membrane Proteins (OMPs) as a virulence factor in *K. pneumoniae*

Several OMPs have been noted to be important for *K. pneumoniae*virulence, including outer membrane protein A (OmpA),peptidoglycan-associated lipoprotein (Pal), and murein lipoprotein(LppA), which are encoded by genes of the same names.OmpA aids in *K. pneumoniae* virulence, at least in part, throughprotection against the innate immune response (Hsieh*et al.,* 2013).

vi. Porins as a virulence factor in *K. pneumoniae*

Down regulation of OmpK35 and OmpK36 porins appears to play a role in antibiotic resistance as these porins are oftenpoorly or not expressed in antibiotic-resistant strains of *K. pneumoniae* (Chen*et al.,* 2010; Shin*et al.,* 2012). Antibiotic resistance of carbapenem resistant *K. pneumoniae*strains significantly decreasesfollowing restoration of the expressionof *ompK35* or *ompK36.*So also deletion of *ompK36* from a K2 HV *K. pneumoniae* strain results in an increasedresistance to certain antibiotics *in vitro*. Furthermore,while the deletion of *ompK35* did not change the susceptibility of*K. pneumoniae* to certain antibiotics, the concurrent deletion ofboth *ompK35* and *ompK36* led to antibiotic resistance that washigher than that with even the *ompK36* single-deletion mutant (Tsai*et al.,* 2011).

vii. Pumps and Transporters as a virulence factor in *K. pneumoniae*

Efflux pumpssuch as AcrB have been implicated in both virulence and antibiotics resistance of *K. pneumoniae*(Padilla *et al.,* 2010; Bialek-Davenet *et al.,* 2015). Thiscontribution to virulence

was demonstrated in a mouse model ofpneumonic infection, where infection with an *acrB* deletion mutantin a K2-expressing *K. pneumoniae* strain resulted in a decreasedbacterial load in the lungs compared to that with the WTstrain, demonstrating that AcrB enhances bacterial fitness in thelungs (Padilla *et al.*, 2010).

viii. Allantoin Metabolism as a virulence factor in *K. pneumoniae*

Bacteria can obtaincarbon and nitrogen from their environmentthrough metabolism of allantoin. An operon comprising of genes involved in allantoin metabolism was identifiedin *K. pneumoniae.* Transcription of this operon was upregulatedin HV *K. pneumoniae* strains compared to classical strains (Paczosa and Mecsas, 2016).

2.2.5 Carbapenem-Resistant *Klebsiella pneumoniae* (CR-Kp)

The use of carbapenems in the treatment of infections caused by ESBL producing Gram negative bacteria might has resulted in the emergence of carbapenem resistance in *Enterobacteriaceae*especially *K. pneumoniae.* In 2013 the CDC declared CRE anurgent threat to public health in the United States (CDC, 2014).

Klebsiella species account for about 80% of the approximately 9,000 infections due to CRE (CDC, 2014).Carbapenem resistance is primarily driven by the accessorygenome, sometimes in combination with mutations in thecore genome. Carbapenem resistance in *K. pneumoniae* can bemediated in part through up-regulation of efflux pumps (Filgona*et al*., 2015) and alteration of outer membrane porins in thecore genome (Martin and Bachman, 2018), and hyperproduction ofESBL enzymes or AmpC β-lactamases in the accessory genome(Bush and Jacoby, 2010). For instance, hyperproduction of anESBL or AmpC enzyme combined with a porinmutation can leadto a resistance phenotype, particularly to ertapenem (García-Fernández *et al*., 2010).

However the most worrisomemechanism of carbapenem resistance is through plasmidmediated carbapenemases (Samuelsen *et al.,* 2009; Breurec *et al*., 2013).

2.3 Carbapenems

Carbapenems are bactericidal β-lactam antimicrobials (Figure 2.6) with proven efficacy in the treatment of severe infections caused by ESBL producing Gram negative bacteria (Hawkey and Livermore, 2012). Carbapenems such as imipenem, meropenem, doripenem, ertapenem, panipenem and biapenem, are in use globally due the rising cephalosporin resistance in *Enterobacteriaceae*(Codjoe and Donkor, 2018).

Theyare broad spectrum antibiotics with a unique structure defined by a carbapenem coupled to a β-lactam ring which confers protection against hydrolysis by most β-lactamases such as metallo-β-lactamase (MBL) as well as ESBLs (Codjoe and Donkor, 2018).

Carbapenems are considered one of the most reliable drugs for treating bacterialinfections hence, the emergence and spread of resistance to these antibiotics constitute a major publichealth concern (Datta and Wattal, 2010; Livermore, 2012; Meletis, 2016).Recent emerging mechanisms of resistance accumulate through the spread of carbapenem-hydrolysing β-lactamases leaving narrow therapeutic options (Patel and Bonomo, 2013).

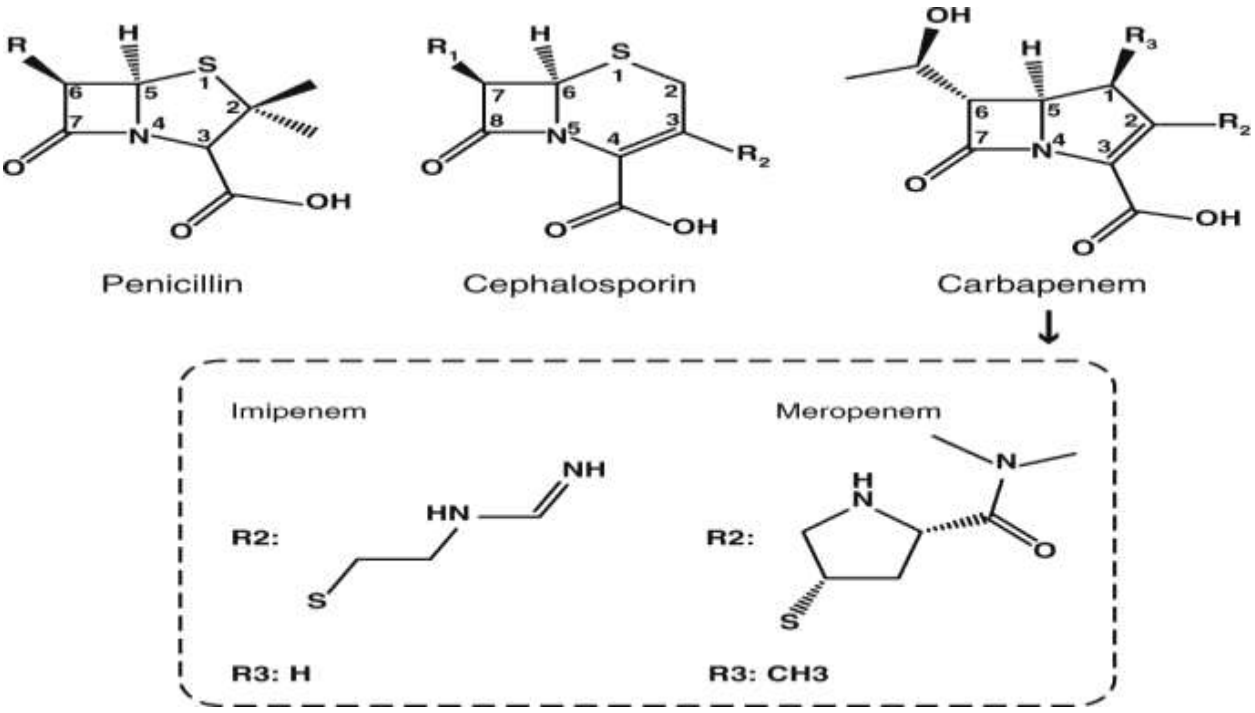

Figure 2. 6: Classical Molecular Examples of Carbapenems Source: Feng *et al.* (2017)

2.3.1 Discovery and developments of carbapenems

In the late 1960s, as bacterial β-lactamases emerged andthreatened the use of penicillin, the search for β-lactamaseinhibitors began in earnest. By 1976, the first β-lactamaseinhibitors were discovered; these olivanic acids were natural products produced by theGram-positive bacterium *Streptomyces clavuligerus*. Olivanicacids possess a "carbapenem backbone" (a carbon at the 1position, substituents at C-2, a C-6 ethoxy, and *sp2*-hybridizedC-3) and act as broad-spectrum β-lactams. Due tochemical instability and poor penetration into the bacterialcell, the olivanic acids were not further pursued. Shortlythereafter, two superior β-lactamase inhibitors were discovered namely:

(i) clavulanic acid from *S. clavuligerus*, which is the first clinically available β-lactamase inhibitor, and

(ii) thienamycin from *Streptomyces cattleya* which was the first "carbapenem" and eventually serve as the parent or model compound for all carbapenems.

A series of other carbapenems were also identified; however, the discovery of thienamycin was paramount (Papp-Wallace *et al.,* 2011).

The term "carbapenem" is defined as the 4:5 fused ringlactam of penicillins with a double bond between C-2 and C-3but with the substitution of carbon for sulfur at C-1 (Figure 2.7). Thehydroxyethyl side chain of thienamycin is a radical departurefrom the structure of conventional penicillins and cephalosporins,all of which have an acylamino substituent on the β-lactam ring; the stereochemistry of this hydroxyethyl sidechain is a key attribute of carbapenems and is important for activity. Remarkably, thienamycin demonstrated potentbroad-spectrum antibacterial and β-lactamase inhibitory activity (Papp-Wallace*et al.,* 2011).

Althoughthienamycin is a "natural product" and the biosynthetic pathway was determined, yields from thepurification process were low. With time, the synthetic preparationof thienamycin assumed greater importance, especially as a key derivative, imipenem, was discovered. Thienamycin was found to be unstable inaqueous solution, sensitive to mild base hydrolysis (above pH8.0), and highly reactive to nucleophiles, such as hydroxylamine,cysteine, and even thienamycin's own primary amine. The chemical instability of thienamycin stimulated thesearch for analogous derivatives with increased stability. Dueto the continued evolution of cephalosporin-resistant Gramnegativeand Gram-positive pathogens, compounds derivedfrom thienamycin were anticipated to have even greater value with time (Papp-Wallace*et al.,* 2011).

The first developed carbapenem was the *N*-formimidoyl derivative, imipenem. Imipenem and a closely related carbapenem, panipenem, identified later, were more-stablederivatives of

thienamycin and less sensitive to base hydrolysis in solution. In 1985, imipenem became the first carbapenem available for the treatmentof complex microbial infections. Imipenem, like its parent,thienamycin, demonstrated high affinity for PBPs and stabilityagainst β-lactamases. However, both imipenem andpanipenem were susceptible to deactivation by dehydropeptidaseI (DHP-I), found in the human renal brush border. Therefore, coadministration with an inhibitor, cilastatin or betamipron, was necessary (Papp-Wallace*et al.,* 2011).

Along the journey to the discovery of more-stable carbapenemswith a broader spectrum, the other currently availablecompounds, meropenem, biapenem, ertapenem, and doripenem, were developed, andseveral novel carbapenems were also identified. A major advance in this"synthetic journey" was the addition of a methyl group to the 1-β position. This modification was found to beprotective against DHP-I hydrolysis. Several carbapenemswere identified with this modification in the subsequent 2 decades;many were similar to the currently available carbapenems,having a 1-β-methyl and a pyrrolidine ring at C-2. These novel carbapenems included antipseudomonal carbapenems,anti-methicillin-resistant *S. aureus* (MRSA) carbapenems (i.e cationic and dithiocarbamate carbapenems), orallyavailable carbapenems, trinem carbapenems, a dual quinolonyl-carbapenem, and others (Papp-Wallace*et al.,* 2011).

2.3.2 Chemistry of carbapenems

The carbon atom at positionC-1 of carbapenems plays a vital role in the potency and broad spectrum of carbapenems as well as their stability against β-lactamases. The hydroxyethyl R_2side chain is also responsible for their resistance to hydrolysis by β-lactamases. In addition, carbapenems with an *R* configuration at C-8 are also very potent. The *trans* configuration ofthe β-lactam ring at C-5 and C-6 results in stability against β-lactamases (Figure 2.7). Carbapenems

with a pyrrolidine moiety (panipenem,meropenem, ertapenem, and doripenem) amongvarious cyclic amines as a side chain have a broader antimicrobial spectrum (Papp-Wallace*et al.*, 2011).

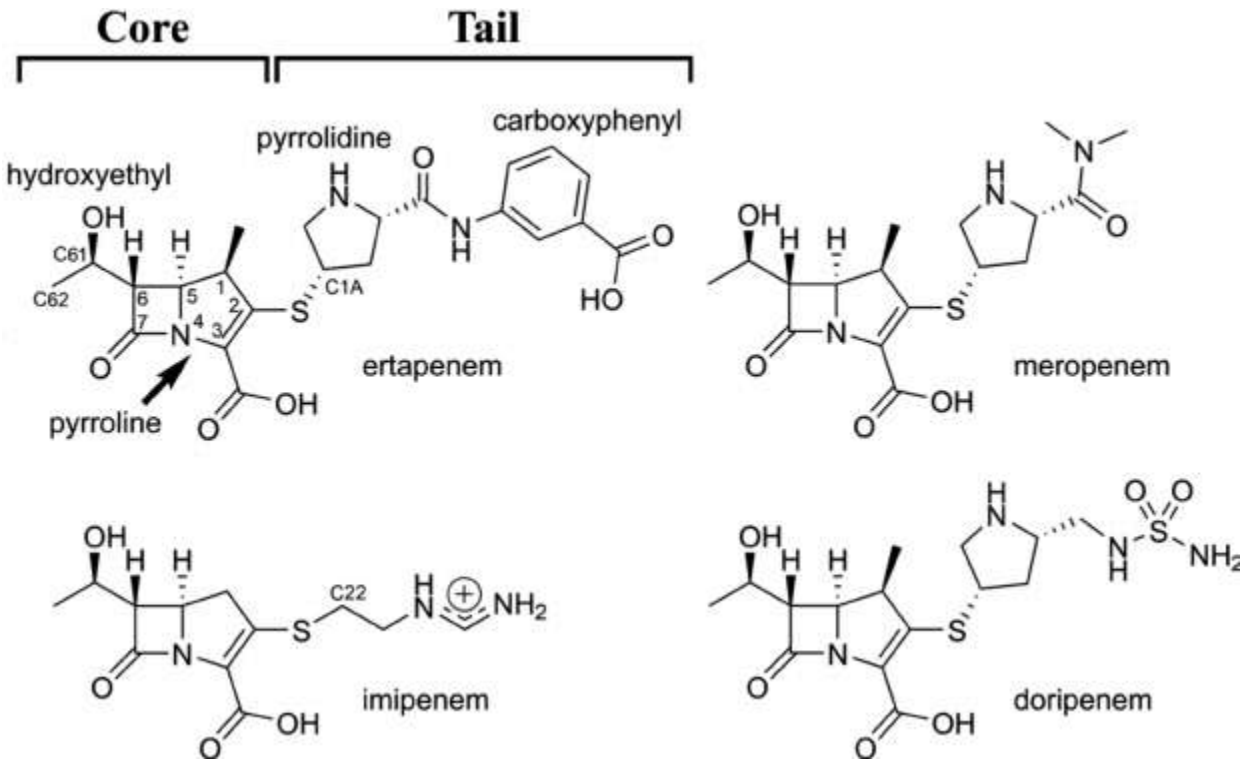

Figure 2. 7: General Structure and Chemistry of Carbapenems Source: Stewart*et al.* (2015)

2.3.3 Synthesis of Carbapenems

Production of carbapenems through fermentation is not efficient; hence chemical approaches have been developed for the synthesis of carbapenems. Natural products such as L-Cysteine, L-Valine, L-α-amino adipic acid and *S*-adenosyl-Methionineare often used asstarting material for the production of carbapenems.The syntheticapproach was largely influenced by the desired stereochemistry of the final compound. Carbapenems are unique comparedto other β-lactams, which tend differ in both R_1 and R_2side chains, most modifications of carbapenems are at the R_1 side chain (at position C-2)(Papp-Wallace*et al.*, 2011).

2.3.4 Mechanism of action of Carbapenems

Mode of action of carbapenems is first initiated by penetration of the bacterial cellwall and binding to penicillin-binding proteins (PBPs). The inactivation of an inhibitor ofautolytic enzymes within the cell wall results in the killing of the bacteria. Inhibition of PBPs 2 and 3 generally occurs in Gram-negative bacillus-shaped bacteria toform spherical cells and filamentous organisms, respectively (Codjoe and Donkor, 2018).

The main target of carbapenems is the inhibition of transpeptidase during bacterial cell wall synthesis (Figure 2.8). The lethal effects are thought toresult in cell death by autolytic action within the bacterial cell (Van Dam*et al.*, 2009). Repression of the PBPs affects the vitalityof the cell wall which weakens the glycan backbone due to autolysis and eventually the cell is destroyed by osmotic pressure in Gram-negative bacteria (Papp-Wallace*et al.*, 2011; Meletis, 2016).

Carbapenems are generally preferred over antibiotics in the treatment of invasive or life-threatening infections because of their concentration-independent killing effect on the infecting bacteria (Abbott*et al.*, 2013; Watkins and Bonomo, 2013). They are broad-spectrum and act against Gram-positive, Gram-negative bacteria and including anaerobes. Cyclic aminecarbapenems with pyrrolidine derivatives such as meropenem, doripenem, panipenem andertapenem possess a wider spectrum of activity.Comparative clinical trials have established the efficacy of Imipenem/cilastatin and meropenem in the treatment of a varietyof infections including complicated intra-abdominal infections, skin and skin structure infections,community-acquired pneumonia, nosocomial pneumonia, complicated urinary tract infections,meningitis (meropenem only) and febrile neutropenia (Codjoe and Donkor, 2018).

Doripenem ishighly stable against hydrolysis by most β-lactamases and have lower Minimum InhibitoryConcentrations (MICs) comparative to meropenem and imipenem against*Acinetobacter baumannii* and *Pseudomonas aeruginosa*. It is less susceptible to carbapenemase hydrolysis and hydrolysed more slowly (from 2 to 150 fold) compared to imipenem. Ertapenem has a relatively limited effectiveness against *Pseudomonas aeruginosa* compared to meropenem or imipenem (Codjoe and Donkor, 2018).

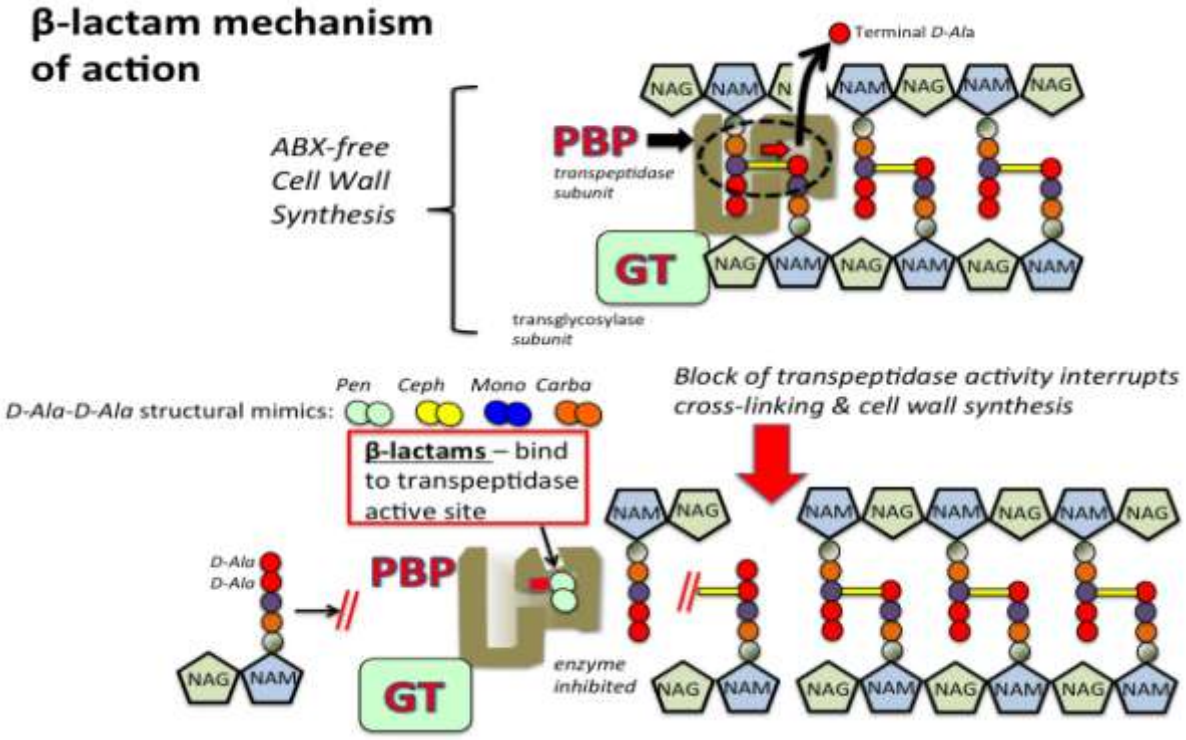

Figure 2. 8: Mechanism of action of carbapenems Source: Stewart *et al.* (2015).

2.3.5 Carbapenem Usage and Side Effects

Effective use of carbapenems in combination therapy in order to enhance successes in patient outcomeshas been reviewed because of resistance due to carbapenemases (Lakshmi*et al.*, 2014). Bacteria produce β-lactamase enzymes that are capable of degradingβ-lactam antibiotics,

however,these enzymes are unable to effectively degrade carbapenem when combined with β-lactamase inhibitors for *in vivo* use. When combined with imipenem, cilastatin inhibits the renal metabolism of imipenem and prolongs its half-life (Codjoe and Donkor, 2018).

Imipenem, meropenem and doripenem have *in vivo* half-lives of approximately 1 h, while ertapenemhas a half-life of approximately 4 h making it suitable for once-daily administration. Imipenem isnoted for its dose-dependent gastrointestinal side effects as compared with the other carbapenems (Zhanel*et al.*, 2007).

Ertapenem has the lowest activity against *Pseudomonas* species and othernon-fermentative Gram-negative bacteria. Different kinds of carbapenems are used in clinicalpractice as antipseudomonal agents; doripenem, imipenem and meropenem. The agents; ertapenem,imipenem and meropenem are poorly absorbed orally and require parenteral administration to beeffective. Recently approved doripenem is of value among the available carbapenems fortreating serious infections (Watkins and Bonomo, 2013).

Carbapenems have trivial hepatic metabolism effects leading to hepatotoxicity with jaundice, although this is an uncommon medical condition for these agents (Zhanel*et al.*, 2007). Most carbapenems are subject to degradation by the enzyme dehydropeptidase-1 (DHP-1) located in renal tubules and require co-administration with a DHP-1 inhibitor such as cilastatin. The later types of carbapenem agentsincluding doripenem and ertapenem require no β-lactamase inhibitor as they are made stable intheir mode of activity against Gram-negative bacterial infections. These compounds vary in theirbinding to PBP, thereby giving unique differences of activity towards different types of organisms(Codjoe and Donkor, 2018).

2.3.6 Carbapenem resistance

Infections caused by Carbapenem-Resistant *Enterobacteriaceae* (CRE) is considered an importantchallenge in health-care settings and a growing concern worldwide (Schwaber and Carmeli, 2008; Nordmann*et al.*, 2011a). Carbapenems arevery effective antimicrobials and administered intravenously with no reported cause of allergic reactions inhospitals (Cunha*et al.*, 2008). The important role of carbapenems in the treatment of infections caused by Gram-negative pathogens cannot be overemphasized. Carbapenem resistance might arise as result of intrinsic or acquired resistance mechanisms or both.

2.3.6.1 Intrinsic resistance of Gram-Negative Bacilli

Several bacterial (commensals and pathogens) are naturally resistant to certain classes of antibiotics, this type of resistance called intrinsic resistance. Theoccurrence of this type of resistance limits and complicates drug selections for treatment. It can also increase the risk of developing acquired resistance. For example, Gram-negative bacteria reduce the uptake of β-lactam drugs, by selectively altering their cell membrane porin channels. Reduction of outermembrane permeability in this manner prevents the β-lactams from reaching their targets (Codjoe and Donkor, 2018).

Soil bacteria have been reported to transmit different antimicrobial resistance genes to clinical pathogens. Carriage of such genes was laterally transferred to clinicalpathogens and multiple mobilization sequences including non-coding regions were identified inshort-read sequence data from many soil bacteria (Forsberg *et al*., 2012).

2.3.6.2 Acquired resistance of Gram-Negative Bacilli

Multiple resistance mechanisms such as enzymatic inactivation, target site mutation and efflux pumps have been acquired by bacteria. The development and emergence of inactivating enzymes were established early following the discovery and clinical introduction of the β-lactam class of antibiotics. Over the years, the β-lactamases have extended their spectra of antibiotic hydrolysis: from penicillinasesto cephalosporinases, then to ESBLs and recently to carbapenemases (Garcia, 2013).

Many of the acquired carbapenemases found in *Enterobacteriaceae* are plasmid-mediated and there are several ways by which they can be spread amongst bacterial isolates. In addition, there areother important mechanisms conferring carbapenem-resistance that have been observed in recent times. In the presence of plasmid AmpCs in combination with ESBL enzymes, Gram-negative bacteria can become unsusceptible to carbapenem agents (Bedeni´c*et al.*, 2014).

The most common carbapenem resistance mechanisms in *Pseudomonas aeruginosa*are overexpression of efflux pumps and lossof porin. Other β-lactams may be affected by this mechanism. *Pseudomonas aeruginosa*efflux-pump overexpression occurs more regularly when meropenem is used when compared withimipenem. Both commensals and pathogenic bacteria have different mechanisms of using their effluxpumps to remove amphipathic or lipophilic substances in and out of the cells. These mechanismshave been recognized in other organisms such as *Enterobacteraerogenes* and *Klebsiella* species against imipenem agent (Codjoe and Donkor, 2018).

Generally, Gram-negative bacteria are more resistant to a large number of antimicrobials and other chemotherapeutic agents than Gram-positive bacteria due to difference in cell wall, decreased membrane permeability, efflux pumps and possession of various broad-spectrum-β-

lactamases such as ESBL and/or AmpC cephalosporinase. The resistance may be attributed to the presence ofbroad-specificity drug-efflux pumps (Armand-Lefèvre*et al.,* 2013).

2.3.7 Risk Factors for Acquisition of CRE Infection

There are certain riskfactors that predisposea person to infection by CRE and other MDR Gram-negatives bacteria such as ESBL producers. Exposure to these resistant organisms can cause serious infections in patients with the following reported risk factors: immune-suppression, admission to intensive care unit (ICU), mechanical ventilation, previous exposure to antimicrobials, organ or stem-cell transplantation and prolonged hospital stay (Gasink*et al.,* 2009; Arnold*et al.,* 2011). With respect to antibiotic usage, the risk of CRE acquisition appears to be much higher in the developing world particularly in sub-Saharan Africa where there is a predominance of irrational use of such drugs (Donkor*et al.,* 2011; Donkor*et al.,* 2012).

Nosocomial infections caused by CRE, mainly *Klebsiella pneumoniae*, have been encountered most commonly in ventilator-associated pneumonia, bacteremia, urinary tract and surgical site infections. Most carbapenemase genes are carried on transposon or plasmid which increasesthe risk of spreading in many hospital settings around the world. In clinical situations,where KPC-producing bacteria are a major concern, early intervention has to be taken to preventdeath by administering effective empiric antimicrobials when the patient is immunocompromised,undergoing organ transplants or during cancer treatment (Codjoe and Donkor, 2018).

2.4 Carbapenemases

Carbapenemases are specific β-lactamases with the ability tohydrolyze carbapenems and other β-lactam antibiotics. Production of carbapenemases appearsto be the most widespread cause of

carbapenem resistance,since the documentation of their distribution in different bacterialspecies is extensive (Walsh, 2010).

During the lastdecade, carbapenem resistance has emerged among clinicalisolates of the *Enterobacteriaceae* family, and this isincreasingly attributed to the production of carbapenemases.The rapid emergence and dissemination of theseenzymes poses a considerable threat to clinical patientcare and public health. These enzymes confer resistance tovirtually all β-lactam agents, including penicillins, cephalosporins,monobactams, and carbapenems (Gupta *et al.*, 2013).

The types of carbapenemases found in *Enterobacteriaceae* are mostly KPC, VIM, IMP, NDM and OXA-48, each of them has their unique properties. KPC enzymes hydrolyse all β-lactams (although they hydrolyse cephamycins at a low level) and their activity is only inhibited partially *in vitro* by clavulanic acid, tazobactam and boronic acid. The metallo-β-lactamases (IMP, VIM and NDM) hydrolyse all β-lactams except aztreonam and their activity is not affected by any of the inhibitors that are in clinical use, but they can be inhibited *invitro* with compounds such as zinc chelators (e.g. EDTA). The OXA-48-type enzymes hydrolyse aminopenicillins, ureidopenicillins and carbapenems at low levels, but do not significantly hydrolyse broad-spectrum cephalosporins. Their activity is not affected by the inhibitors in clinical use, but they are inhibited by NaCl *in vitro* (Nordmann and Poirel, 2013).

2.4.1 Classes of Carbapenemases

All the carbapenemases identified in *Enterobacteriaceae*belong to 3 classesof β-lactamases: the Ambler classes A, B and D β-lactamases.

2.4.1.1 Class A Carbapenemases

Class A Carbapenemases are all known to effectively hydrolyze carbapenemsand are partially inhibited by clavulanic acid. Some are chromosomally encoded (non-metallo carbapenemase A [NmcA], *Serratia marcescens* enzyme [Sme], Imipenem Hydrolyzing β lactamase-1 [IMI-1], *Serratia fonticola* carbapenemase-1 [SFC-1]), and others are plasmid encoded (*K. pneumoniae* carbapenemase [KPC], Imipenem Hydrolyzing β lactamase-2 [IMI-2], and Guiana Extended Spectrum β lactamase [GES]). KPCsare the most clinically common enzymes in this group.The first KPC producer (KPC-2 in *K. pneumoniae*) wasidentified in 1996 in the eastern United States (Codjoe and Donkor, 2018). Of these, the KPCs are the most prevalent and after a few yearsof its discovery, had spread worldwide and caused outbreaks in many Asian, North American andEuropean countries as well as in Africa (Codjoe and Donkor, 2018). KPC producers have been reported, mostly from nosocomial *K. pneumoniae* isolates and to a much lesser extent from *E. coli* (especially in Israel) and from other enterobacterial species (Nordmann *et al*., 2009). The level of resistance to carbapenems of KPC producers may vary markedly; ertapenem is the carbapenem that has the lowest activity (Nordmann *et al.*, 2009; Navon-Venezia *et al*., 2009). KPC producers are usually multidrug resistant (especially to all β-lactams), and therapeutic options for treating KPC related infections remain limited (Nordmann *et al*., 2009). Death rates attributed to infections with KPC producers are high (>50%) (Patel *et al.*, 2008; Schwaber *et al*., 2008; Borer *et al*., 2009).

2.4.1.2 Class B carbapenemases

Class B metallo-β-lactamases (MBLs) are mostly of the Verona integron–encoded metallo-β-lactamase (VIM) and IMP types and, more recently, of the New Delhi metallo- β-lactamase-1 (NDM-1) type. These enzymes hydrolyze all β-lactams except aztreonam.Their activity is

inhibited by EDTA but not by clavulanic acid (Walsh *et al.*, 2005). Class B carbapenemase genes in *Enterobacteriaceae* are mostly encoded on transferable plasmids. These genes can also be encoded on the chromosome (Diene and Rolain, 2014). Most MBL producers are hospital acquired and multidrug-resistant *K.pneumoniae* (Codjoe and Donkor, 2018). Resistance levels to carbapenems of MBL producers may vary. Death rates associated with MBL producers range from 18% to 67% (Daikos *et al*., 2009).

In contrast to several other carbapenemase genes, the $bla_{\text{NDM-1}}$ gene is not associated with a single clone but rather with nonclonally related isolates and species. It has been identified mostly in *E. coli* and *K.pneumoniae* and to a lesser extent in other enterobacterial species (Kumarasamy *et al.,* 2010; Nordmann *et al.,* 2011b).

2.4.1.3 Class D Carbapenemases

The class D carbapenemases (also called OXA carbapenemases) were among the earliest detected β lactamases. Initially their substrate hydrolyzing profile was limited to penicillins, however they have evolved to confer resistance to cephalosporins and carbapenems. They are now considered a major hindrance in the clinical efficacy of carbapenems (Evans and Amyes, 2014). These enzymes are poorly inhibited by EDTA or clavulanic acid and have a low hydrolyzing activity against carbapenems with higher activity against imipenem than meropenem (Codjoe and Donkor, 2018). These class of carbapenemase genes are also located on both plasmid and chromosome (Diene and Rolain, 2014).

The first identified OXA-48 carbapenemase producer was a MDR *K. pneumoniae* (which was also resistant to carbapenem)isolated from a patient in Istanbul, Turkey in 2001 (Evans and Amyes, 2014). Since then, OXA-48 producers have been extensively reported from Turkey as a source of nosocomial outbreaks. Their worldwide distribution now includes countries in Europe,

in the southern and eastern part of the Mediterranean Sea, and Africa (Benouda *et al.,* 2010; Carrër *et al.,* 2010; Cuzon *et al.,* 2011; Moquet *et al.,* 2011; Poirel *et al.,* 2011).

2.4.2 Detection of carbapenemase - producing *Enterobacteriaceae* (CPE)

Carbapenemase production can be detected through phenotypic techniques or molecular techniques.

2.4.2.1 Phenotypic detection of carbapenemase - producing *Enterobacteriaceae* (CPE)

The baseline detection test for carbapenemase production is screeningfor carbapenem resistance by disc diffusion test using carbapenem (imipenem, ertapenem or meropenem)and third generation cephalosporins (cefoperazone, cefotaxime, ceftazidime, ceftizoxime or ceftriaxone). Isolates that test intermediate or resistant to one or more carbapenems and resistant to one or more third generation cephalosporins are potential carbapenemase producer (CLSI, 2015).

i. Modified Hodge Test

The Modified Hodge Test (MHT) is a generic phenotypic test that is useful in the demonstration of carbapenemase enzymes production. Multiple isolates (up to eight) can be tested on a single Mueller-Hinton agar plate.

This test is carried out by preparing a 0.5 McFarland standard suspension (using either direct colony suspension or growth method) of *E. coli* ATCC® 25922 (the indicator organism) in broth or saline, and then 1:10 dilution will be prepared in saline or broth. Then the diluted inocula will be inoculated on Mueller Hinton agar plate as for the routine disk diffusion procedure. Appropriate number ofertapenem or meropenem disks will then be placed on the plate. Using a swab, 3 to 5 colonies of test or type culture (QC) organism grown overnight on a blood agar plate will then be picked and inoculated in a straight line out from the edge of the disk. The

streak should be at least 20-25 mm in length. Following incubation at 37^{o}C, the plates should be examined for enhanced growth around the test or QC organism streak at the intersection of the streak and the zone of inhibition. “Enhanced growth” means positive for carbapenemase production while “No enhanced growth” means negative for carbapenemase production (CLSI, 2015).

In low income countries this test may be the only available tool for detecting Carbapenemase producing *Enterobacteriaceae* and should be considered as an initial step in the absence of more sophisticated methods. It is simple to perform and require no special reagents or media. However, false-positive results can occur in isolates that produce ESBL or AmpC enzymes coupled with porin loss. False-negative results are occasionally noted (e.g. some isolates producing NDM carbapenemase) and it only applies to *Enterobacteriaceae*(CLSI, 2015).

ii. Carbapenemase Nordmann-Poirel test

The Carba NP (Carbapenemase Nordmann-Poirel) test was developed by Nordmann*et al.* (2012a). This test is performed as follows: overnight culture of the test organism from Mueller Hinton agar is re-suspended in a Tris-HCl 20 mmol/L lysis buffer and vortex mixed for 5 secs. This lysate is then mixed with 100 µL of an aqueous indicator solution consisting of 0.05% phenol red with 0.1 mmol/L $ZnSO_4$, previously adjusted to pH 7.8 and 12 mg/mL imipenem-cilastatin or 6 mg/mL of imipenem standard powder (reaction tube). The control tube is then prepared as above but without antibiotic. The tubes are then incubated at 35 °C and monitored for 2 hrs for color change from red to orange/yellow in the antibiotic-containing tube, which was interpreted as a positive result (CLSI, 2018).

The test's specificity and sensitivity were 100% when results were compared with those from molecular-based methods, the reference standard for identifying carbapenemase genes. The test could also be used to quickly identify carbapenem- resistant isolates from fecal specimens screened for multidrug-resistant bacteria. This capability would be valuable in preventing outbreaks. Its use as a home-made test and may contribute to the global surveillance network. The Carba NP test perfectly differentiates carbapenemase producers from strains that are carbapenems resistant due to non–carbapenemase-mediated mechanisms, such as combined mechanisms of resistance (outer-membrane permeability defect associated with overproduction of cephalosporinase and/or extended-spectrum β-lactamases) or from strains that are carbapenem susceptible but express a broad-spectrum β-lactamase without carbapenemase activity (extended-spectrum β-lactamase, plasmid and chromosome-encoded cephalosporinases) (Nordmann*et al.,* 2012a).

iii. Modified Carbapenem Inactivation Method

A new phenotypic method for the detection of carbapenemase production, the carbapenem inactivation method (CIM), was first described by van der Zwaluw*et al*. (2015). A modification of this method (modified carbapenem inactivation method) in order to improve the performance of the method was described by CLSI (2018).This test is based on the principle that when a 10 μg meropenem (MEM) disk is incubated for 4 hrs in trypticase soy broth (TSB) inoculated with a carbapenemase producing microorganism, the carbapenem in the disk is degraded by the carbapenemase; in contrast, if the test microorganism does not produce carbapenemase, MEM retains its antimicrobial activity after incubation in the bacterial suspension. The disk is removed from the suspension and placed onto a Mueller Hinton agar (MHA) plate seeded with a suspension of a carbapenem-susceptible indicator organism; following overnight incubation, the

zone of inhibition is measured to determine whether the MEM had been hydrolyzed (growth of the indicator organism close to the disk), or is still active (a large zone of inhibition around the disk).

The initial description of the CIM reported very promising results, including high sensitivity for the detection of a variety of carbapenemases and excellent specificity. The test is straightforward to perform and interpret, and involves low-cost materials readily available in clinical laboratories (van der Zwaluw*et al.*, 2015).

iv. Boronic Acid based screening test

A fourth phenotypic method of detecting carbapenemases (KPCs specifically) involves the use of boronic acid (BA)-based compounds. BA was originally described in the 1980s as a reversible inhibitor of class C β-lactamases and has been used in combination disc tests for the identification of AmpC-producing isolates (Hirsch and Tam, 2010). Several disc tests combining BA compounds such as phenylboronic acid and 3-aminophenyl boronic acid (APB), have proved to be highly sensitive and specific for the detection of KPC production. Tsakris *et al*. (2009) tested discs containing 400 mg of phenylboronic acid as an inhibitor and several β-lactams as the antibiotic substrates against 57 KPC-producing isolates. Their result shows a significantly increased (≥5 mm) inhibition zone diameters when used in combination with cefepime and all carbapenems (imipenem, meropenem and ertapenem) compared with zones produced by the β-lactam discs alone (Tsakris *et al*., 2009; Hirsch and Tam, 2010). Meropenem, imipenem and cefepime were the most sensitive and specific (100% for all), while meropenem demonstrated the largest difference in inhibition zone diameters.

Additionally, Doi *et al*. (2008) found that the addition of APB to ertapenem or meropenem (but not imipenem) discs resulted in an increased zone diameter ≥5 mm for 10 KPC-producing isolates when compared with the carbapenem disc alone. Optimal sensitivity and specificity was found using 300 mg of 3-aminophenyl boronic acid with a cut-off of a 5 mm difference in zone diameter. A third group investigated the utility of APB for detection of other class A carbapenemases (Pasteran *et al*., 2009). They found BA-based MIC tests utilizing imipenem–APB to have 100% sensitivity and specificity to differentiate class A carbapenemase-producing bacteria from non-carbapenemase-producing bacteria when using a cut-off of ≥3-fold reduction in MIC compared with imipenem alone. In summary, these BA-based methods have shown promising results and appear practical for use in a clinical laboratory setting as a similar methodology/algorithm was recommended for the phenotypic confirmation of ESBLs (Hirsch and Tam, 2010).

v. Use of screening media

Another phenotypic screening method is the media screening method. The currently available screening media however cannot detect all types of carbapenemase producers with high sensitivity and high specificity.

Three screening media are currently known, however they are mostly for carbapenem-resistance detection rather than carbapenemase-production detection (Nordmann and Poirel, 2013). The first marketed screening medium was the CHROMagar KPC medium, which contains a carbapenem (CHROMagar, Paris, France) (Moran-Gilad *et al.*, 2011). It detects carbapenem-resistant bacteria only if they exhibit high-level resistance to carbapenems. This chromogenic medium has been shown to have a sensitivity of 100% and specificity of 98.4% relative to polymerase chain reaction (PCR) (Samra *et al.,* 2008). However, this selective agar is unable to

detect OXA-48-like carbapenemase producers because of the low MICs for imipenem. Its main disadvantage therefore remains its lack of sensitivity since it does not detect carbapenemase producers exhibiting a low level of carbapenems resistance, as observed for several MBL or OXA-48 producers (Nordmann *et al.*, 2011b).

The second screening medium also contains a carbapenems (CRE Brilliance, Thermo Fisher Scientific, UK). It detects KPC and MBL producers well, and most but not all OXA-48 producers (Withey and Scopes, 2011; Girlich *et al.*, 2012).

Finally, the third screening mediadeveloped (SuperCarba) contains cloxacillin, zinc and ertapenem. It shows excellent sensitivity and specificity for detection of any kind of carbapenemase producer (not only high-level carbapenem-resistant isolates) (Nordmann *et al.*, 2012a). Compared with the two other media, it also shows improved sensitivity and specificity for detecting all types of carbapenemase producers (including the OXA-48 producers) when present in low amounts in stools. Once carbapenem-resistant isolates are selected on SuperCarba medium, it is recommended that Carba NP test should be used for detecting carbapenemase activity. Then, if needed, molecular identification of the carbapenemase genes may be performed (Nordmann and Poirel, 2013).

2.4.2.1 Genotypic Technique

i. Polymerase Chain Reaction(PCR) for carbapenemase genes

The gold standard for identifying carbapenemase-producing *Enterobacteriaceae* remains the use of molecular techniques (Nordmann *et al.*, 2012a). This involves the detection of genes encoding for carbapenemases. Most of these techniques are based on PCR and may be followed by a sequencing step if a precise identification of the carbapenemase gene is needed (e.g. VIM type,

KPC type, NDM type or OXA-48 type) (Avlami *et al.,* 2010; Poirel *et al.,* 2011). They are either single or multiplex PCR techniques. A PCR technique performed directly on colonies can give results within 4–6 hrs (or less when using real-time PCR technology) with excellent sensitivity and specificity. Similarly, other molecular techniques are useful for this purpose (Cuzon *et al.,* 2012). The main disadvantages of the molecular-based technologies are their cost, the requirement for trained microbiologists and inability to detect any novel carbapenemase gene. So also, these methods are beyond the scope of less well financed laboratory systems. Sequencing of the genes is interesting mostly for research and epidemiological purposes. Precise identification of the type of carbapenemase is not actually needed for treating patients or for preventing outbreaks. These molecular techniques may be mostly used in reference laboratories (Nordmann *et al.,* 2012b).

ii. Commercial DNA microarray for detection of carbapenemases

Commercial DNA microarray such as the Check-MDR CT102 DNA microarray can be used for the detection of the most prevalent carbapenemases (NDM, VIM, KPC, OXA-48 and IMP) and ESBL gene families (SHV, TEM and CTX-M). The system combines ligation-mediated amplification with detection of amplified products on a microarray to detect the various carbapenemase genes ($bla_{\text{OXA-48}}$, bla_{NDM}, bla_{IMP}, bla_{VIM} and bla_{KPC}), $bla_{\text{CTX-M}}$ groups ($bla_{\text{CTX-M}}$ groups 1, 2 and 9, or combined 8/25), and the most prevalent ESBL-associated single nucleotide polymorphisms (SNPs) in bla_{TEM} and bla_{SHV} variants. The microarray assays are performed according to the manufacturer's instructions using software version 20110215T170816R29 and involves the use of two separate rooms (one room for DNA isolation and ligation, and one for amplification, hybridization and detection). The time to result of the microarray system is usually

8 hours (3 hours for DNA isolation and 5 hours for ligation, amplification and detection) (Cohen *et al*., 2012).

2.5 Treatment Options for Infections caused by Carbapenem Resistant *Enterobacteriaceae*(CRE)

Carbapenems are considered as last-resort antibiotics for the treatment of infections caused by multidrug-resistant Gram-negative bacteria. With the increasing use of carbapenems in clinical practice, the emergence of carbapenem-resistant pathogens now poses a great threat to human health. Currently, antibiotic options for the treatment of carbapenem-resistant *Enterobacteriaceae* (CRE) are very limited, with polymyxins, tigecycline, fosfomycin, and aminoglycosides as the mainstays of therapy. The need for new and effective anti-CRE therapies is urgent (Sheu *et al.,* 2019).

Antimicrobial treatment of CRE infections has been challenged by the emergence of morecomplex resistance phenotypes as well as economic and regulatory pressures. Agents such aspolymyxins and tigecycline have recently seen resurgence in their clinical usage (particularly colistin)in the management of multidrug-resistant Gram-negative infections, particularly CRE includingcarbapenem-resistant *Acinetobacter baumannii*in most hospitals (Hagihara*et al.,* 2014).

2.5.1 Polymyxins as a treatment option for infections caused by CRE

The initial target of polymyxins is LPS of outer membrane; they can selectively bind to LPS, coincident with its narrow spectrum of antibacterial activity against Gram-negative bacteria. The bactericidal activity of polymyxin is through membrane lysis of the bacterial cell (Velkov*et al.,* 2010). The polymyxins are active agents and attain sufficient serum levels in the treatment of seriousbloodstream and CRE infections. The agents produce additive or synergistic effects on humansagainst multidrug-resistant organisms including *Acinetobacter baumannii* isolates when

combined withanother antimicrobial agents such as tigecycline. In a study conducted by Lee *et al*. (2009), 16 patientswith recurrent infections caused by KPC-producing *Klebsiella pneumoniae* evaluated that three out oftwelve managed with polymyxin monotherapy experienced polymyxin resistance in their treatment. Increased MIC of polymyxin B (range = 1.5 – 1,024 µg/mL) for CRKP isolates from patients treated with polymyxin B was reported by Lee*et al.* (2009).

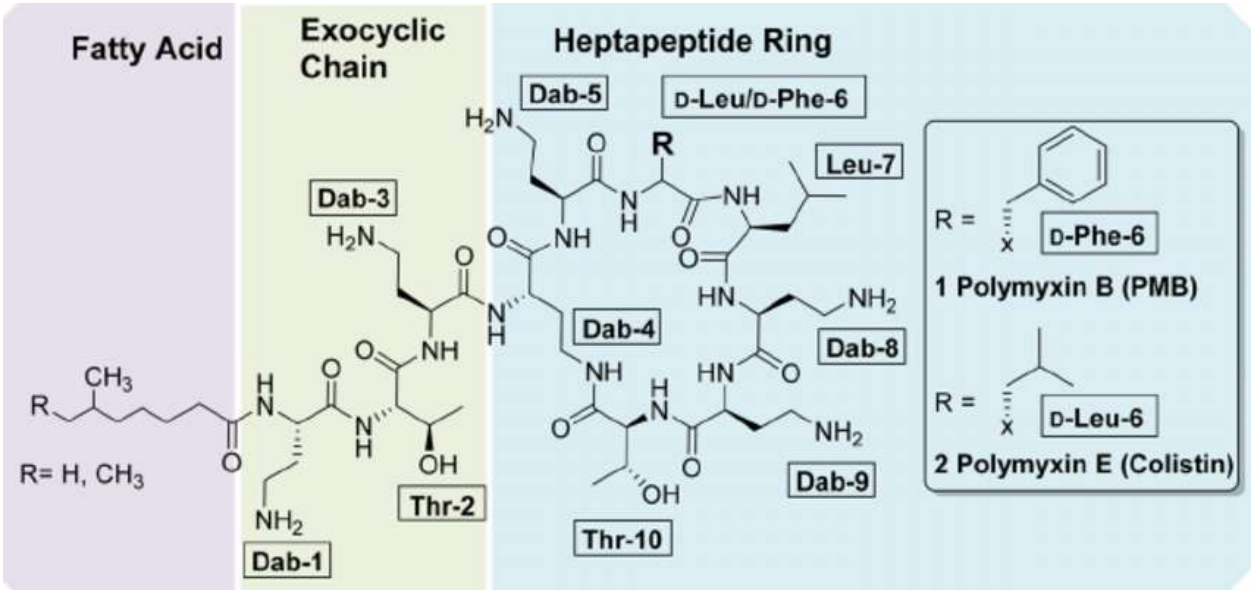

Figure 2. 9: Molecular Structure of Polymyxins Source: Gallardo-Godoy*et al.* (2016).

2.5.2 Tigecyclineas a treatment option for infections caused by CRE

Tigecycline, a glycylcycline, is active *in vitro* against most carbapenem-resistant *Escherichia coli*.Tigecyclinebinds to the bacterial 30S ribosome and blocks the entry of transfer RNA, this prevents protein synthesis by halting the incorporation of amino acids into the peptide chain and thus limit bacterial growth (Greer, 2006). The drug is licensed for most complicated intra-abdominal, skin and soft tissue infections.Interestingly, a study reports success in various infections caused by carbapenemase producers (Arnold*et al.,* 2011). In a study conducted by

Kontopidou*et al.* (2014), the MIC of tigecycline for most patients with bacteremia or ventilator-associated pneumonias caused by CRKP was estimated to be 2 µg/mL.

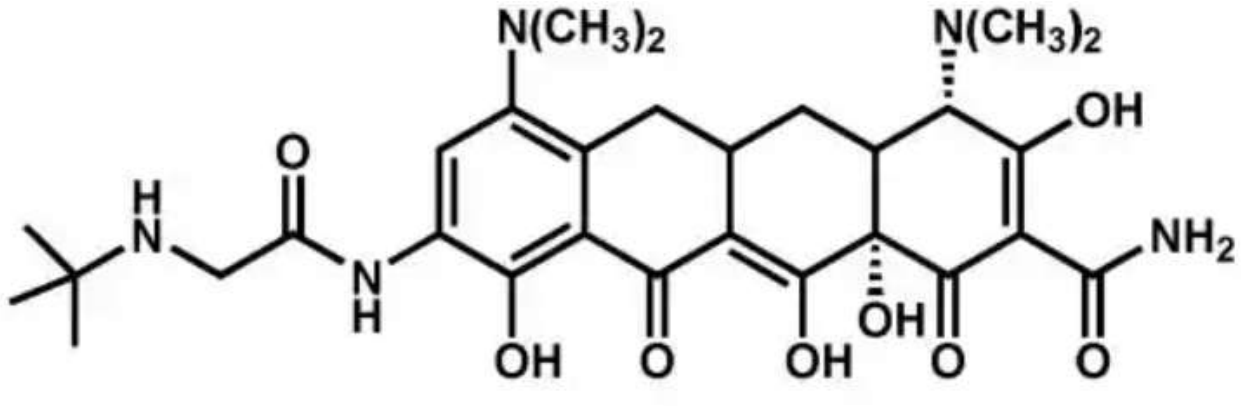

Figure 2. 10: Molecular Structure of Tigecycline Source: Olson *et al.* (2006).

2.5.3 Aminoglycosidesas a treatment option for infections caused by CRE

Aminoglycosides inhibit protein synthesis by binding to the A- site of the 16S ribosomal RNA with high affinity and alter the conformation of the A-site. This interaction promotes mistranslation by inducing codon misreading on delivery of the aminoacyl transfer RNA (Krause*et al.,* 2016). Aminoglycosides, notably gentamicin, amikacin and tobramycin have different *in vitro* activities. Treatments of CRE infections may depend on the susceptible organism as studies have showngentamicin activity against gentamicin-susceptible strains in urinary tract infections have positiveoutcomes. Of the aminoglycosides, amikacin appears to be the more active against CREs whencompared with gentamicin or tobramycin (Abbott *et al.,* 2013). While in another study, remarkably low activitywas observed to amikacin and tobramycin for infections caused by MDR Gram-negative bacteria. This maybe due to gentamicin modifying enzymes which have been carried by these MDR organisms. The useof aminoglycosides as monotherapy against carbapenemase-producing *Klebsiella pneumoniae* infectionsare considered

ineffective, and therefore not recommended for clinical management of patients (Satlin*et al.*, 2011; Hara *et al.*, 2013).

Different MICs of aminoglycosides were reported for CRKP by Galani *et al.* (2019). They reported MIC_{90} of amikacin (128 mg/L), gentamicin (64 mg/L) and tobramycin (256 mg/L) for KPC producers; MIC_{90} of amikacin (64 mg/L), gentamicin (> 256 mg/L) and tobramycin (128 mg/L) for NDM producers; MIC_{90} of amikacin (> 256 mg/L), gentamicin (256 mg/L) and tobramycin (64 mg/L) for VIM producersand $MIC_{90}$of amikacin (32 mg/L), gentamicin (> 256 mg/L) and tobramycin (> 256 mg/L) for OXA-48 producers.

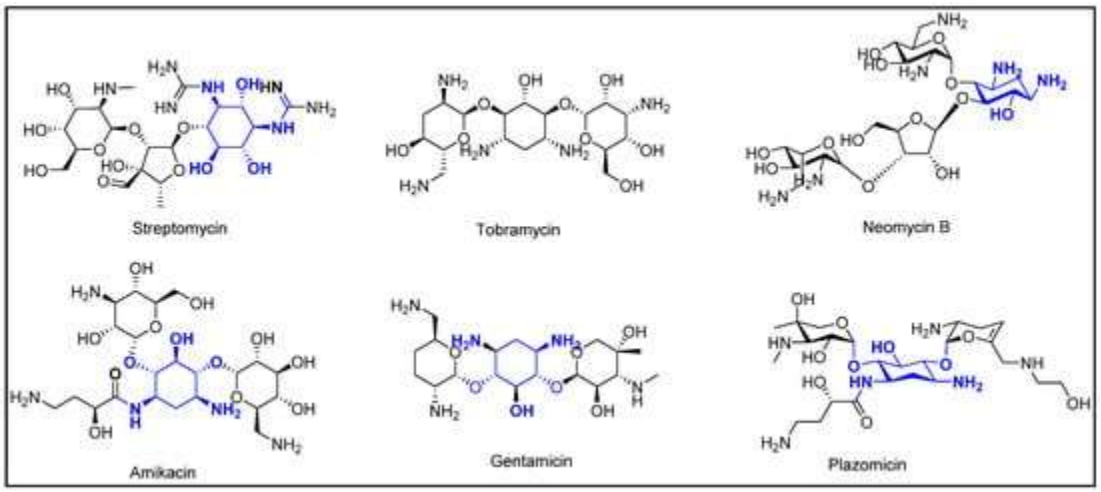

Figure 2. 11: Molecular Structure of Aminoglycosides Source: Serio *et al.* (2018)

2.5.4 Fosfomycinas a treatment option for infections caused by CRE

Fosfomycin, a bactericidal antibiotic that inhibits bacterial cell wall biogenesis has seenits use renewed globally in response to the recent threat of antimicrobial resistance includingcarbapenem-resistant *Klebsiella pneumoniae*isolates (Falagas*et al.*, 2008; Neuner*et al.*, 2012). The drug is effectively used to treaturinary tract infections and has low rates of resistance. However, poor outcomes may occur whentreating complicated *Pseudomonas aeruginosa* as a

urinary pathogen. Many patients that developedtreatment failure were immunosuppressed or had urethral stents due to the use of fosfomycin asmonotherapy in kidney transplant cases (Codjoe and Donkor, 2018). Fosfomycin MIC of ≤ 32 mg/L was reported by Kaase *et al.* (2014) for most of the CRE isolates.

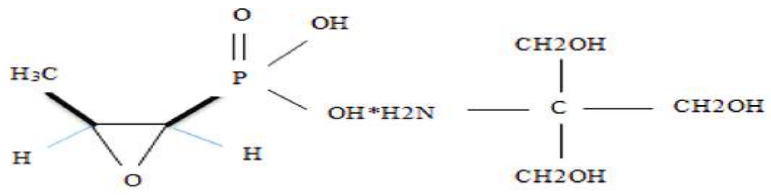

Figure 2. 12: Molecular Structure of Fosfomycin-trometamol

Source: Aghamali *et al.* (2018).

2.5.5 Combination therapiesas a treatment option for infections caused by CRE

Combination therapies have shown remarkable outcomes in dealing with MDR and CREinfections. The most commonly used combinations are colistin, polymyxin B or tigecycline combinedwith a carbapenem. In a retrospective study by Qureshi*et al*. (2012) on patients with bacteremia,the monotherapy, either tigecycline or colistin-polymyxin B alone had 58% mortality rate ascompared with 13% for the tigecycline or colistin-polymyxin B combined with a carbapenem ona 28-day assessment, and this was observed in infections caused by KPC-producing *Klebsiella pneumoniae* isolates (Qureshi*et al*., 2012)

In future, treatments for infections caused by carbapenemase producers may involve new β-lactamaseinhibitors such as methylidene penems; avibactam, MK-7655; the maleic acid derivative ME1071;(‘neoglycoside’) plazomicin, a novel aminoglycoside; the polymyxin derivatives NAB739 and NAB7061;and the siderophore monosulfactam, BAL30072 combined with cephalosporins and novel antimicrobialagents that are effective against these pathogens

such as solithromycin and omadacycline (Patel and Bonomo, 2011; Fernandes and Martens, 2017).

2.5.6 High-Dose and Prolonged-Infusion of Carbapenems

The fact that wide disparities of carbapenem MICs exist, evenamong CPE isolates, complicates the discourse for the roleof carbapenems in the treatment of CRE or CPE. Severalstudies have investigated the efficacy of carbapenems againstCPKP in animal models and suggested that with a higher doseof carbapenems it is possible to attain reliable reductions inbacterial density in isolates with lower carbapenem MICs (Bulik and Nicolau, 2010; Bulik *et al*., 2010;Souli *et al*., 2011). Daikos and Markogiannakis (2011) proposed,based on several animal infection model studies, that highdose,prolonged-infusion carbapenems can achieve bactericidaleffects in immunocompetent animals infected by KPC-producing*K. pneumoniae* isolates with MICs up to 8 mg/L.

In addition to carbapenem-containing combinations, thestrategy of high-dose (2 g every 8 h) carbapenem withprolonged infusion (over 3 h) was also found to be associatedwith better outcomes in CPKP infections (Tumbarello *et al*., 2015; Daikos *et al*., 2014).

2.5.7 Double-Carbapenem Therapy (DCT)as a treatment option for infections caused by CRE

The most well-investigatedDCT is the combination of ertapenem(with a standard infusion time of 30–60 min) prior to a prolongedinfusion of meropenem or doripenem over 3–4 h, with high-dosemeropenem of 2 g every 8 h being most commonly applied. Thisregimen originated from the revolutionary approach proposed byBulik and Nicolau (2011), as a salvage option for CPKP. The studyvalidated enhanced activities for both ertapenem and doripenemin combination, using an in vitro chemostat and *in vivo* murinethigh infection model (Bulik and Nicolau, 2011). The

rationalefor this combination came from the hypothesis that ertapenemmight play a sacrificial role, being preferentially hydrolyzed dueto its greater affinity to KPC (Sheu*et al.,* 2019), permittingthe concomitant administration of carbapenem to sustain a highconcentration. Some in vitro studies show a beneficial effect oflower MICs of meropenem (MIC ≤ 128 mg/L) (Oliva *et al*., 2017) or doripenem (MIC ≤ 16 mg/L) (Wiskirchen *et al*., 2013)with regard to ertapenem-based DCT.

2.5.8 New Antibiotics

i. Ceftazidime/Avibactamas a treatment option for infections caused by CRE

Ceftazidime/avibactam (CAZ/AVI, AvyCazR , Allergan Inc.,Jersey City, NJ, United States) is a new β-lactam/β-lactamaseinhibitor combination recently approved for the treatment of infections in the United States in February 2015 (Kayeand Pogue, 2015), and for the treatment of Hospitalacquiredpneumonia (HAP) and ventilator-associated pneumonia inJanuary 2018. Unlike most β-lactamase inhibitors, avibactam isnot a β-lactam. Avibactam is a novel synthetic non-β-lactam(diazabicyclooctane)/β-lactamase inhibitor that inhibits a widerange of β-lactamases, including Ambler Class A (GEM, SHV,CTX-M, and KPC), Class C (AmpC), and some Class D (OXA-48) b-lactamases (de Jonge *et al*., 2016). It does not inhibit Class BMBLs (IMP, VIM, VEB, and NDM) (Syue *et al*., 2016; Wong andvan Duin, 2017). The addition of avibactam restores ceftazidimeactivity against various *Enterobacteriaceae* and *P. aeruginosa*,therefore expanding the activity spectrum of ceftazidime to MDRGram-negative bacteria. Vaborbactam is a novel boron-containing serine-β-lactamaseinhibitor which confers activity against certain meropenemresistantbacteria by inhibiting Ambler Class A and C serinecarbapenemases, such as KPC (Castanheira *et al*., 2017).

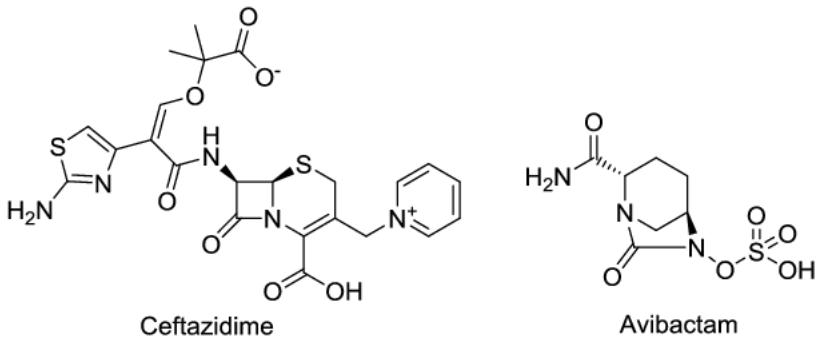

Figure 2. 13: Molecular Structure of Ceftazidime/Avibactam Source: Qin *et al.* (2014).

ii. Plazomicinas a treatment option for infections caused by CRE

Plazomicin is a next-generation aminoglycoside syntheticallyderived from sisomicin, which retains activity against bacteriacontaining aminoglycoside-modifying enzymes (Castanheira *et al*., 2018). Plazomicin (ZemdriTM,Achaogen, Inc., San Francisco, CA, United States) was approvedin June 2018 by the FDA for the treatment of adults with complicated urinary tract infectionsincluding pyelonephritis who have limited or no alternativetreatment options, with a recommended dose of 15 mg/kgevery 24 h for normal renal function (Sheu*et al.*, 2019).

Studies have shown that plazomicin is morepotent than other aminoglycosides against KPC-producing*Enterobacteriaceae*(Sheu*et al.*, 2019).

Figure 2. 14: Molecular Structure of Plazomicin Source Nainu *et al.* (2021)

iii. Eravacyclineas a treatment option for infections caused by CRE

Eravacycline, a synthetic fluorocycline antibacterial agent ofthe tetracycline class, has broad-spectrum antimicrobial activityagainst Gram-positive, Gram-negative, and anaerobic bacteria,exception for *P. aeruginosa* (Zhanel *et al*., 2016). It disrupts bacterial protein synthesis by binding to the 30S ribosomal subunit, preventing the incorporation of amino acid residues into the elongating peptide chains. Eravacycline(XeravaTM, Tetraphase Pharmaceuticals, Inc., Watertown, MA,United States) was approved by the FDA in August 2018 forthe treatment of complicated intra-abdominal infections (Sheu*et al.*, 2019). MICs of eravacycline between 0.5 to 1 µg/mL were reported by Livermore *et al.* (2016).

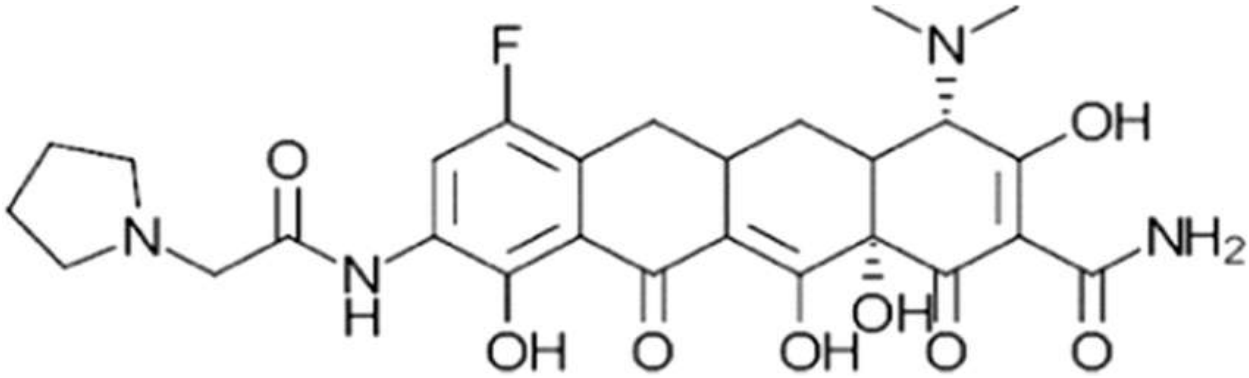

Figure 2. 15: Molecular Structure of Eravacycline Source: Lee *et al.* (2019).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Population

The study was conducted in Zaria. It is a major city in Kaduna State in northern Nigeria, as well as a Local Government Area. It is also known as Zazzau and also one of the original seven Hausa city-states. Zaria lies within the coordinates latitude 11^{o} 7', 11^{o} 12'N and longitude 07^{o} 41' E (Chigor *et al.*, 2010).

The selected hospitals were Hajiya Gambo Sawaba General Hospital, Kofar Gayan, Zaria city; Ahmadu Bello University Medical Center, Samaru, Zaria and Major Ibrahim Bello Abdullahi Memorial Hospital, Sabon-Gari, Zaria.

3.2 Sample Size Determination

The sample size of the study was determined using the prevalence of carbapenemases among *Enterobacteriaceae* (10.2%) report Mohammed *et al*. (2015) and the Kish Leisle formula.

$N=Z^2 P (1-P)/d^2$

Where N = sample size

P = prevalence of carbapenemases among *Enterobacteriaceae* (10.2%) (Mohammed *et al*.,2015)

Z = confidence interval (1.96)

D = allowable error (5%)

$N=Z^2 P (1-P)/d^2$

$N = 1.96^2 x 0.102 x (1-0.102)/ (0.05)^2$

N= 141 samples.

A total of 302 clinical samples were collected to increase statistical precision and minimize error.

3.3.1 Inclusion criteria

Patients referred to the Microbiology laboratory by the clinician for suspected cases of Urinary Tract Infection who consented.

3.3.2 Exclusion criteria

Patients referred to the Microbiology laboratory by the clinicianfor suspected cases other than Urinary Tract Infection and those who did not consent.

3.4 Ethical approval

Ethical approval (Appendix I) was obtained from the ethical committee of Kaduna State Ministry of Health (MOH/ADM/744/VOL.1/763).

3.5 Collection of Samples

A total of 302urine samples were collected from patients sent to Microbiology laboratory of the selected hospitals in Zaria using convenience sampling technique. The patients were given sterile, dry, wide-necked, leak-proof container and requested to provide 10–20 mL of midstream urine. The containers were also labeled appropriately.

3.6 Isolation and Characterization of *Escherichia coli* and *Klebsiella pneumoniae*

3.6.1 Isolationof *Escherichia coli* and *Klebsiella pneumoniae*

The samples were inoculated on MacConkey agar and incubated for 24 hrs aerobically at 37ºC. The isolates were identified by their morphological characteristics on MacConkey agar. Isolates that appeared as pink mucoid colonies on MacConkey agar after incubation at 37^{o}C for 24 hrs were considered presumptive *Klebsiella pneumoniae.* While pink small non mucoid colonies

were considered presumptive *Escherichia coli.* These isolates were Gram stained (Akter *et al.*, 2014).

3.6.2 Gram Staining

A smear was prepared from the 24 hrs culture of the colonies, a well separated colony (pure culture) was placed directly into a drop of normal saline on a clean, dried, grease-free slide, smeared and then allowed to air-dry. The smear was heat-fixed by passing the slide over a Bunsen flame for three quick successions.The slide was flooded with crystal violet solution and allowed to stand for one minute, then washed with slow-running water and then flooded with Gram's iodine (mordant) and allowed to act for also 1 min. The slide was washed with water and decolourized with 95% alcohol for 15 secs. The slide was rinsed with water and then counter-stained with safranin for another 30 secs. The slide was finally rinsed with slow-running water, allowed to air-dry and then examined microscopically under oil immersion objective lens after adding a drop of oil immersion (Cowan and Steel, 2003). Isolates that appeared as Gram negative rods were subcultured on nutrient agar slant and then stored in a refrigerator before they were further characterized biochemically.

3.6.3 Biochemical Characterization of the isolates

The isolates were characterized using the following biochemical tests:

3.6.3.1 Indole test

Indole test was carried out by inoculating the suspected colonies into 1% peptone water and then the inoculated peptone water was incubated at 37^{o}C for 24 hrs. After 48 hrs incubation, 0.5 mL of Kovacs reagent was added and shaken. A positive reaction was indicated by the development

of a red colour in the reagent layer above the broth. Negative reaction was indicated by a yellow colour (Cowan and Steel, 2003).

3.6.3.2 Methyl Red - Voges-Proskauer test:

This test was carried out by inoculating 5 mL of MR-VP broth with the suspected coloniesand then the inoculated broth was incubated at 37^{o}C for 48 hrs. After 48 hrs of incubation, 1ml of the cultured broth was transferred to a test tube to which 2 drops of Methyl Red solution was added and then shaken. Formation of red colour on addition of the indicator signifies a positive methyl red test and an orange or yellow colour signifies a negative test.

To the rest of the broth, 6 drops of 5% α-Naphthol solution was added followed by 2 drops of 40% potassium hydroxide. The tube was shaken and placed in a slope. Development of a red colour starting from the liquid – air interface within 15 mins to 1 hr indicates a VP positive test. No colour change indicates VP negative test (Cowan and Steel, 2003).

3.6.3.3 Citrate utilisation test

This test was carried out by inoculating the suspected colonies on Simmons' citrate agar slant and the inoculated slant was then incubated at 37^{o}C for 72 hrs. Development of a deep blue colour indicates a positive reaction while if the original green colour is maintained it means citrate was not utilized (Cowan and Steel, 2003).

3.6.3.4 Urease test

Urease test was carried out by inoculating Christensen's urea agar slant with the suspected coloniesand then the inoculated Christensen's urea agar slant was incubated at 35^{o}C for 48 hrs.

The development of bright pink or red colour indicates a positive reaction (Cowan and Steel, 2003).

3.6.3.5 Sugar Fermentation Test

Tubes of Triple Sugar Iron Agar (TSI Agar) was inoculated by stabbing the butt and streaking the slope. The tubes were then incubated at 37^{o}C for 24 hrs. After incubation, the tubes were observed for colour change (from red to yellow), gas production and blackening of the butt. Red slant (Alkaline) indicates lactose or sucrose not fermented, yellow slant (acidic) indicates lactose or sucrose fermented, red butt indicates that glucose was not fermented and yellow butt indicates glucose fermentation. While bubbles or cracks in the medium indicate gas production, blackening of the butt indicates H_2S production (Cowan and Steel, 2003).

3.6.3.6 Motility test

This test was carried out by stab-inoculating the tubes of motility medium with the suspected colonies. A fine stab with a needle was made to a depth of about one third the total volume of the medium. The medium was then incubated at 37^{o}C for 24hrs. If the medium turns cloudy (turbid) after incubation, it means the organism is motile but if growth is restricted to the line of inoculation and the rest of the medium remains clear, then the organism is non motile (Cowan and Steel, 2003).

3.7 Screening for Carbapenem Resistant *Klebsiella pneumoniae* and *Escherichia coli*

Screening for carbapenemresistant isolates of *Klebsiella pneumoniae*and *Escherichia coli*was carried as follows. Biochemically identified isolates of *Klebsiella pneumoniae*and *Escherichia coli*were standardized by comparing their turbidity with that of 0.5 McFarland (1.5 x 10^8 CFU/mL) standard and subjected to antibiotics susceptibility test on Mueller Hinton agar by

modified Kirby-Bauer disc diffusion technique using imipenem (10 µg) and ceftriaxone (30µg) antibiotic discs.

Briefly, a sterile swab was dipped into the standardized inoculum tube and then excess fluid was removed by rotating the swab against the side of the tube. The Mueller Hinton agar was then inoculated by swabbing the swab stick three times over the surface of the agar, rotating the plate at approximately 60^{o} each time to ensure even distribution of the inocula. The plates were kept at room temperature for 5 mins for the surface of the agar to dry (Acharya, 2013).

Using a sterile forcep, the discs were placed one at a time on the plates and pressed gently to ensure complete contact with the agar surface. The plates were kept at room temperature for 5minutes before incubation at 37^{o}C for 24 hrs. The sizes of the zones of inhibition were measured with the aid of a ruler to the nearest millimetre(Acharya, 2013). Using the published CLSI guidelines, the susceptibility or resistance of the isolates to each of the antibiotic tested was determined (CLSI, 2019).

Isolates that were non-susceptible to imipenem and ceftriaxone were further screened for carbapenemase production by the Modified Hodge Test, Carba NP test and Modified Carbapenem Inactivation Methods as recommended by the Clinical Laboratory Standards Institute (CLSI, 2018).

3.8 Phenotypic Screening for Carbapenemase Production

3.8.1 Modified Hodge Test (MHT)

A 0.5 McFarland (1.5 X 10^{8}CFU/mL) standardized suspension of the indicator organism (*Escherichia coli* ATCC 25922) was prepared in normal saline and then a 1:10 dilution of it in normal saline was inoculated on Mueller Hinton Agar plate as a lawn. The plate was allowed to

dry for 10 mins. Meropenem disc was then placed at the middle of the inoculated Mueller Hinton Agar plate. Using a sterilizedwire loop 5 colonies of test isolates grown overnight was picked and inoculated in a straight line out from the edge of the disc. Following incubation at 37^{o}C for 20 hrs, the MHA plate was examined for enhanced growth of the indicator organism around the test isolates at the intersection of the streak and the zone of inhibition. Enhanced growth of the indicator organism (*Escherichia coli* ATCC 25922) means the test isolate is positive for carbapenemase production while no enhanced growth of the indicator organism means the isolate is negative for carbapenemase production (CLSI, 2015).

3.8.2 Carbapenemase Nordmann-Poirel (Carba NP) Test

The Carba NP test is based on the principle of acidimetry. In the acidimetric method, hydrolysis of beta-lactam ring results in a drop in pH, causing a colour change of phenol red indicator from red to yellow.

The Carba NP test was performed following the protocol described in CLSI manual (2018). Briefly, the isolates were grownovernight on Mueller-Hinton agar (MHA). The bacterial colony was scraped off with a sterilized wireloop and suspended in a 1.5 mL Eppendorf tube containing 100 µL of 20mMTris-HCl lysisbuffer and mixed using a vortex device for 5 secs. This lysate was mixed with 100 µL of an aqueous indicator solution consisting of0.05% phenol red with 0.1 mmol/L $ZnSO_4$, previously adjustedto pH 7.8 and 12 mg/mL imipenem-cilastatin injectable form (equivalent to 6 mg/mL of imipenemstandard powder) (reaction tube). The control tube was prepared as above but without imipenem. The tubes were then incubated at 35°C and monitored throughout 2 hrs forcolor change from red to orange/yellow in the antibiotic-containing tube, which was interpreted as a positive result.

3.8.3 Modified Carbapenem Inactivation Method (mCIM)

The Modified Carbapenem Inactivation Methodwas carried out as described in the CLSI manual (2018). For each isolate to be tested, an overnight culture of the isolate on blood agar was emulsified in 2 mLtrypticase soy broth (TSB) and then vortexed for 15 secs. Meropenem disc (10μg)was then added to each tube using sterile forcep or a single disc dispenser. The entire disc was immersed in the suspension and then incubated at 35°C ± 2°C in ambient air for 4 hrs ± 15 mins. Just before or immediately following completion of the TSB-meropenem disc suspension incubation, a0.5 McFarland suspension of *E. coli* ATCC 25922 in normal saline was prepared. The standardized inoculum of *E. coli* ATCC 25922 was inoculated on MHA plate as for the routine disc diffusion procedure making sure the inoculum suspension preparation and MHA plate inoculation steps are each completed within 15 minutes. The plates were allowed to dry for 10 mins before adding the meropenem discs. The meropenem disc was removed from each TSB-meropenem disc suspension using a sterilized wire loop by placing the flat side of the loop against the flat edge of the disc and using surface tension,the disc was pulled out of the liquid. The disc was carefully dragged and pressed along the inside edge of the tube to expel excess liquid from the disc. After removing the disc from the tube it was placed on the MHA plate previously inoculated with the meropenem-susceptible *E. coli* ATCC 25922 indicator strain. The MHA plates were inverted and incubated at 35°C ± 2°C in ambient air for 18–24 hrs. Following incubation, the zones of inhibition was measured as for the routine disc diffusion method using CLSI, 2019 manual.

Isolates with zone diameter of 6-15 mm or presence of pinpoint colonies within a 16-18 mm zone were considered carbapenemase positive isolates. If the test isolate produces a

carbapenemase, the meropenem in the disc was hydrolyzed and there was no inhibition or limited growth inhibition of the meropenem-susceptible *E. coli* ATCC 25922.

Isolates with zone diameter of ≥ 19 mm (clear zone) were considered carbapenemase negative isolates. If the test isolate does not produce carbapenemase, the meropenem in the disc was not hydrolyzed and growth of the meropenem-susceptible *E. coli* ATCC 25922 was inhibited.

Isolates with zone diameter of 16–18 mm, zone diameter of ≥ 19 mm and the presence of pinpoint colonies within were considered carbapenemase indeterminate isolates.

3.9 Molecular Detection of Carbapenemase Genes by PCR

3.9.1 DNA extraction

Crude genomic DNA for PCR was extracted from the isolates using the heat lysis method. Briefly, colonies from overnight culture of the isolates were transferred into Eppendorf tubes containing 1 mL of nuclease-free water and boiled at 100 ^{o}C for 5 minutes in a heating block and subsequently frozen at -4 ^{o}C for 7 mins. This was followed by centrifugation for 10 minutes at 16,000 rpm (Espinosa *et al*.,2013). One microliters (1 µL) of the supernatant was used as the DNA template for PCR. All isolates were screened for the resistance genes encoding KPC, NDM and OXA by PCR assay using previously described primers (Table 3.1).

3.9.2 Detection of carbapenemase genes by PCR

PCR was performed in accordance with Inqaba Biotec's in-house protocol using10 µL of NEB OneTaq 2X master mix with standard buffer (Catalogue No. M0482S), 1 µL of each primer (10 µM), 7 µL Nuclease free water (Catalogue No. E476)and 1 µL of DNA template. The PCR conditions were as follows: initial denaturation at 94 °C for 5 mins, followed by 35 cycles of

denaturation at 94 °C for 30 secs, annealing at 50°C for 30 s, and extension at 68 °C for 1 min. Final extension was at 68 °C for 10 mins.

3.9.3 Agarose Gel Eletrophoresis

The PCR amplicons were visualized after running at 100 V for 90 mins on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye (Inqaba Biotec's in-house protocol).

3.9.4Carbapenemase Gene Sequencing

PCR products were cleaned using ExoSAP Protocol as follows:Exo/SAP master mix was first prepared by adding 50 µL of Exonuclease I (Catalogue No. NEB M0293L) 20 U/µLand 200 µL Shrimp Alkaline Phosphatase (Catalogue No. NEB M0371) 1 U/µL in a 0.6 mL micro-centrifuge tube. The reaction mixture was then prepared with 10 µL of Amplified PCR Product and 2.5 µL of ExoSAP Mix. The mixture was mixed properly and then incubated at 37°C for 15 mins after which the reaction was stopped by heating the mixture at 80°C for 15 mins. Fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions. The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053). The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50cm array, using POP7. Sequence chromatogram analysis was performed using FinchTV analysis software.

The carbapenemase gene sequences obtained were compared with those in NCBI database. A minimum sequence percent identity of ≥ 98.00% and 100.00% coverage was used to confirm the genes. Sequences of the carbapenemase genes wereedited, aligned with reference sequences from the GenBank and translated into amino acid sequences using BioEdit version 7.2.5.

The evolutionary history of the carbapenemase genes was inferred by the Maximum Likelihood methodusing MEGA 7. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed.

3.10 Determination of Susceptibility Pattern of Carbapenem Resistant Isolates

All the carbapenemase producing isolates were screened for their susceptibility to colistin (10 mg), ceftriaxone (30 µg), nalidixix acid (30 µg), doxycycline (30 µg), amikacin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), trimethoprim-sulphamethoxazole (23.75/1.25 µg),gentamicin (10µg), tigecycline (15 µg) and fosfomycin (200 µg) using the Modified Kirby-Bauer disc diffusion method as described above. Interpretation was done as per CLSI (2019) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2019) breakpoints (Appendix I).

3.11Determination of the MAR Index of theCarbapenem Resistant Isolates

The MAR index of the carbapenem resistant isolates was calculated using the formula below as described by Olonitola *et al*. (2007).

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which the isolate is resistant}}{\text{Total number of antibiotics used}}$$

3.12 Data Analysis

Chi square (χ^2) was used to analyze the relationship between the occurrence *K. pneumoniae* and *E. coli* among the various categories of data while the effectiveness of the methods (Modified Hodge Test, Carba NP and Modified Carbapenem Inactivation Methods) was determined by calculating their accuracy, sensitivity and specificity.

Table 3. 1: Primer Sequences Used for the Detection of Carbapenemase Genes.

Gene	Primer	Sequences (5' – 3')	Expected amplicon size (bp)	References
***bla*$_{KPC}$**	KPC-F	ATGTCACTGTATCGCCGTCT	893	Huang *et al.*,2014
	KPC-R	TTTTTCAGAGCCTTACTGCCC		
***bla*$_{NDM}$**	NDM-F	GGTTTGGCGATCTGGTTTTC	550	Mohammed *et al.*, 2015
	NDM-R	CGGAATGGCTCATCACGATC		
***bla*$_{OXA}$**	OXA-F	AACGGGCGAACCAAGCATTTT	597	Mlynarcik*et al.*,2016
	OXA-R	GAGCACTTCTTTTGTGATGGCT		

CHAPTER FOUR

4.0 RESULTS

4.1 Overall Occurrence of *Escherichia coli* and *Klebsiella pneumoniae*Among Patients Attending Selected Hospitals in Zaria

Table 4.1 shows the overall occurrence of *Escherichia coli*and *Klebsiella pneumoniae*in urine of patients attending selected hospitals in Zaria. From the 302 urine samples collected in this study, 70 isolates of *Escherichia coli*and 53 isolates of *Klebsiella pneumoniae*were gotten giving an occurrence of 23.18% and 17.55% respectively. The overall occurrence in this study was 40.73% (123/302). The difference observed in the occurrence of *E. coli* and *K. pneumoniae* was not statistically significant ($p \geq 0.05$).

4.2 Occurrence of *Escherichia coli and Klebsiella pneumoniae* based on Hospital

Occurrence of *Escherichia coli and Klebsiella pneumoniae*based on hospital is presented in Table 4.2. Out of the 100 urine samples collected from Major Ibrahim Bello Abdullahi Memorial Hospital (MIBAMH), 18 *E. coli* and 21 *K. pneumoniae* were isolated giving an occurrence of 18.00% and 21.00% respectively. The occurrence of *Escherichia coli*and *Klebsiella pneumoniae* in Ahmadu Bello University Medical Center (ABUMC) was found to be 20.00% and 10.00% respectively while the occurrence of *Escherichia coli*and *Klebsiella pneumoniae*in Hajiya Gambo Sawaba General Hospital (HGSGH) was found to be 31.37% and 21.57% respectively. The overall occurrence was found to be higher in HGSGH (52.94%), followed by MIBAMH (39.00%). The least occurrence was found in ABUMC (30.00%). The difference observed in theoccurrence of *E. coli* and *K. pneumoniae* in the selected hospital was statistically significant ($p \leq 0.05$).

Table 4. 1: Overall Occurrence of *Escherichia coli* and *Klebsiella pneumoniae* in Urine of Patients Attending Selected Hospitals in Zaria

Isolate	No. of samples examined	No. of samples positive	Occurrence (%)
Escherichia coli	302	70	23.18
Klebsiella pneumoniae	302	53	17.55
Overall occurrence	**302**	**123**	**40.73**

$\chi^2 = 2.614$, p = 0.1060 df =1

***** The difference observed in the occurrence of *E. coli* and *K. pneumoniae* was not statistically significant (p = 0.1060)

Table 4. 2: Occurrence of *Escherichia coli* and *Klebsiella pneumoniae* based on Hospital

Hospital	No. of samples examined	*Escherichia coli*		*Klebsiella pneumoniae*	
		No. positive	Occurrence (%)	No. positive	Occurrence (%)
MIBAMH	100	18	18.00	21	21.00
ABUMC	100	20	20.00	10	10.00
HGSGH	102	32	31.37	22	21.57
Total	**302**	**7023.18**		**5317.55**	

E. coli: $\chi^2 = 6.410$, p = 0.0406 df =2

K. pneumoniae: $\chi^2 = 6.096$, p = 0.0475 df =2

Key: MIBA = Major Ibrahim Bello Abdullahi Memorial Hospital; ABUMC = Ahmadu Bello University Medical Center; HGS = Hajiya Gambo Sawaba General Hospital

***** The difference observed in theoccurrence of *E. coli* and *K. pneumoniae* in the selected hospital was statistically significant ($p \leq 0.05$).

4.3 Occurrence of *Escherichia coli and Klebsiella pneumoniae* based on Gender

A total of 113 males and 189 females were examined in this study. The occurrence of *E. coli* was found to be 18.58% and 25.93% in males and females respectively, while the occurrence of *K. pneumoniae* was found to be 15.04% and 19.05% in males and females respectively (Table 4.3). There was no statistically significant difference in the occurrence of *E. coli* and *K. pneumoniae* based on gender ($p \geq 0.05$).

4.4 Occurrence of *Escherichia coli and Klebsiella pneumoniae* based on Age Group

Occurrence of *E. coli and K. pneumoniae* based on age group is presented in Table 4.4. Age group 11-20 years had the highest occurrence of both *E. coli* (42.86%) and *K. pneumoniae* (35.71%) followed by age group ≤ 10 years with occurrence of 36.36% and 30.30% for *E. coli* and *K. pneumoniae* respectively. Age group ≥ 51 years had the least occurrence of both *E. coli* (7.69%) and *K. pneumoniae* (7.69%). The differences observed in the occurrences of *E. coli* and *K. pneumoniae* based on age group were statistically significant ($p \leq 0.05$).

4.5 Overall Occurrence of Carbapenem Resistant Isolates

Figure 4.1 shows the overall occurrence of carbapenem resistant isolates. Out of the isolates screened for carbapenem resistance, 6 (4.88%) were found to be carbapenem resistant isolates while the remaining 117 (95.12%) isolates were carbapenem susceptible isolates.

4.6 Occurrence of Carbapenem Resistant *Escherichia coli* and *Klebsiella pneumoniae*

Out of the 70 *E. coli* isolates screened for carbapenem resistance, 2 were found to be carbapenem resistant *E. coli* giving an occurrence of 2.86%. So also, 4 isolates of *K. pneumoniae* were found to be carbapenem resistant *K. pneumoniae* out of the 53 *K. pneumoniae* isolates screened giving an occurrence of 7.55% (Table 4.5). The difference in the occurrence of carbapenem resistant *E. coli* and *K. pneumoniae* was not statistically significant ($p \geq 0.05$).

Table 4. 3: Occurrence of *Escherichia coli* and *Klebsiella pneumoniae* based on Gender

Gender	No. of samples examined	*Escherichia coli*		*Klebsiella pneumoniae*	
		No. positive	Occurrence (%)	No. positive	Occurrence (%)
Male	113	21	18.58	17	15.04
Female	189	49	25.93	36	19.05
Total	302	70	23.18	53	17.55

E. coli:$\chi^2 = 2.141$, p = 0.1434 df =1

K. pneumoniae:$\chi^2 = 0.783$, p = 0.3761 df =1

***** There was no statistically significant difference in the occurrence of *E. coli* and *K. pneumoniae* based on gender ($p \geq 0.05$).

Table 4. 4: Occurrence of *Escherichia coli* and *Klebsiella pneumoniae* based on Age Group

Age group (years)	No. of samples examined	*Escherichia coli*		*Klebsiella pneumoniae*	
		No. positive	Occurrence (%)	No. positive	Occurrence (%)
≤ 10	33	12	36.36	10	30.30
11 - 20	42	18	42.86	15	35.71
21 – 30	97	21	21.65	18	18.56
31 – 40	98	16	16.33	8	8.16
41 – 50	19	2	10.53	1	5.26
≥ 51	13	1	7.69	1	7.69
Total	302	70	23.18	53	17.55

E. coli: $\chi^2 = 18.526$, p = 0.0024 df =5

K. pneumoniae: $\chi^2 = 22.177$, p = 0.0005 df =5

***** The differences observed in the occurrences of *E. coli* and *K. pneumoniae* based on age group were statistically significant ($p \leq 0.05$).

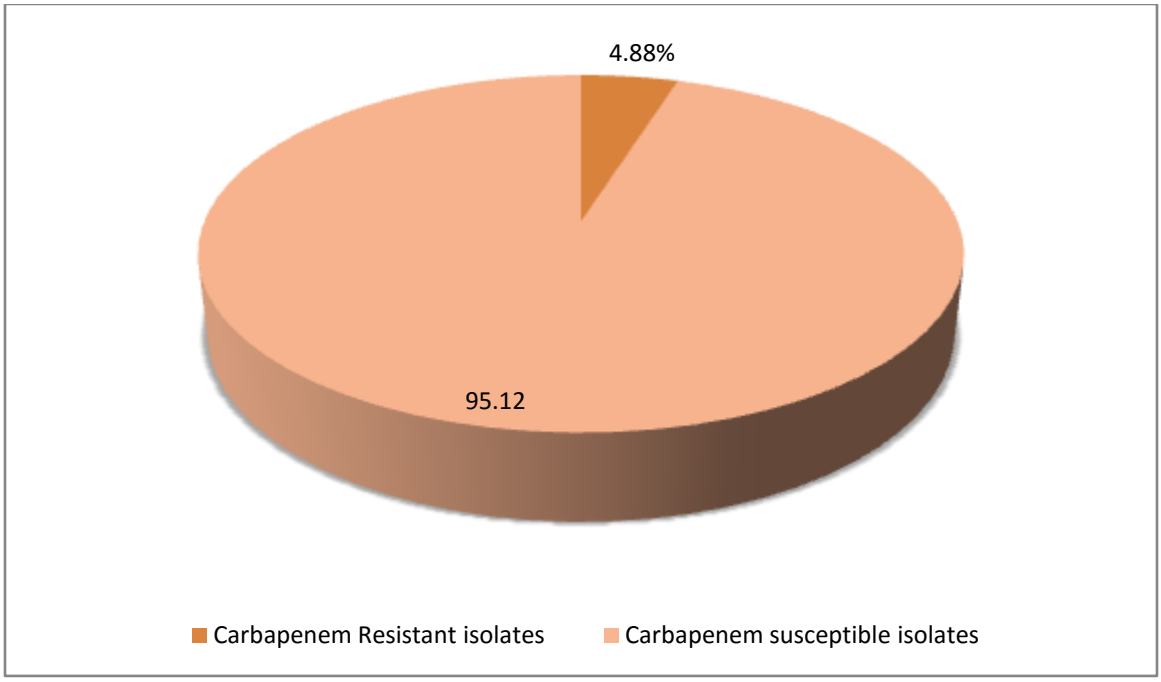

Figure 4. 1: Overall Occurrence of Carbapenem Resistant Isolates among *E. coli* and *K. pneumoniae* Isolated From Urine of Patients Attending Selected Hospitals in Zaria

Table 4. 5: Occurrence of Carbapenem Resistant *E. coli* and *K. pneumoniae*

Isolate	No. of isolates screened	No. of Carbapenem resistant isolates	Occurrence of Carbapenem resistant isolates (%)
E. coli	70	2	2.86
K. pneumoniae	53	4	7.55
Total	123	6	4.88

$\chi^2 = 1.430$, $p = 0.2318$ $df = 1$

*****The difference in the occurrence of carbapenem resistant *E. coli* and *K. pneumoniae* was not statistically significant ($p \geq 0.05$).

4.7 Phenotypic Detection of Carbapenemase Production by mCIM, MHT and Carba NP Test

All the 6 carbapenem resistant isolates (100.00%) were positive for carbapenemase production by Modified Carbapenem Inactivation Method (mCIM). Four of the six carbapenem resistant isolates (66.67%) were positive for carbapenemase production by Modified Hodge Test (MHT) while five of the carbapenem resistant isolates (83.33%) were positive for carbapenemase production by the Carba NP test (Table 4.6).

4.8 Occurrence of Carbapenemase Producing *Escherichia coli* and *Klebsiella pneumoniae* based on the Different Detection Methods

The occurrence of carbapenemase producing *Escherichia coli*was found to be 1.43%, 2.86% and 2.86% by MHT, mCIM and Carba NP respectively while the occurrence of carbapenemase producing *Klebsiella pneumoniae*was found to be 5.66%, 7.55% and 5.66% by MHT, mCIM and Carba NP test respectively. The overall occurrence of carbapenemase producing isolates was 3.25%, 4.88% and 4.07% by MHT, mCIM and Carba NP test respectively (Table 4.7).

4.9 Detection of Carbapenemase Genes by PCR

Plate I represents the agarose gel electrophoresis result of PCR amplicons for carbapenemase genes. bla_{KPC} gene was not detected in any of the carbapenem resistant isolates screened. While bla_{OXA} gene (amplicon size of 597 bp)and bla_{NDM} gene (amplicon size of 550 bp) were detected in 4 and 3 carbapenem resistant isolates respectively.

4.10 Percentage Distribution of Carbapenemase Genes

The most frequently detected carbapenemase gene was bla_{OXA} gene (57.14%) followed by bla_{NDM} gene (42.86). bla_{KPC} gene was not detected (0.0%) in this study (Figure 4.2).

Table 4. 6: Screening for Carbapenemase Production Using Different Detection Techniques

Isolate code	Isolate identity	Carbapenemase production/Percentage positive (%)		
		MHT	**mCIM**	**Carba NP**
GUM015	*Klebsiella pneumoniae*	-	+	+
MUF002	*Escherichia coli*	+	+	+
MUF012	*Escherichia coli*	-	+	+
AUM023	*Klebsiella pneumoniae*	+	+	+
GUF078	*Klebsiella pneumoniae*	+	+	-
GUF084	*Klebsiella pneumoniae*	+	+	+
		4/6 (66.66)	6/6 (100.00)	5/6 (83.33)

Key: MHT = Modified Hodge Test; mCIM = Modified Carbapenem Inactivation Method;Carba NP = Carba NP test; + = carbapenemase producer; - = non carbapenemase producer

Table 4. 7: Occurrence of Carbapenemase Producing *Escherichia coli* and *Klebsiella pneumoniae* based on the Different Detection Methods

Methods	*Escherichia coli* (n = 70)		*Klebsiella pneumoniae* (n = 53)		Overall (n = 123)	
	No. of CP	Occurrence (%) of CP	No. of CP	Occurrence (%) of CP	No. of CP	Occurrence (%) of CP
MHT	1	1.43	3	5.66	4	3.25
mCIM	2	2.86	4	7.55	6	4.88
Carba NP	2	2.86	3	5.66	5	4.07

Key: CP = Carbapenemase producers; n = number of isolates; MHT = Modified Hodge Test; mCIM = Modified Carbapenem Inactivation Method; Carba NP = Carba NP test

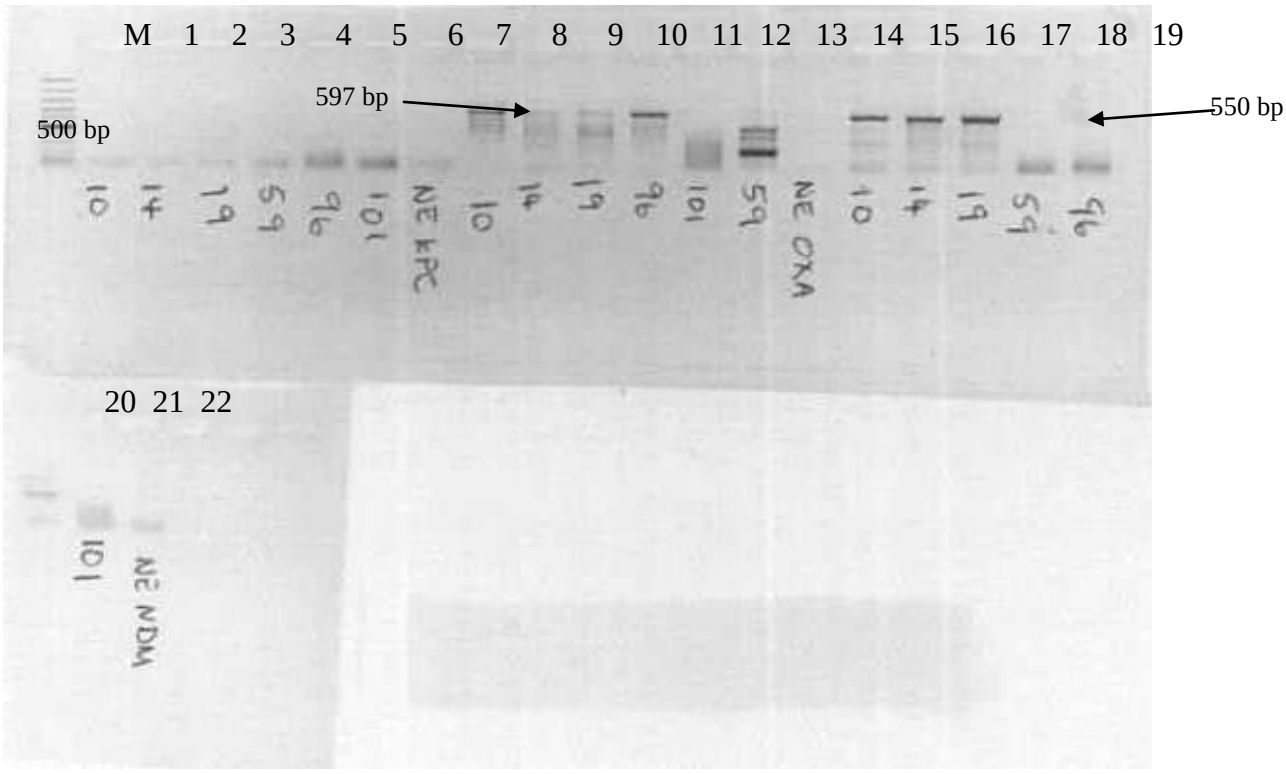

Plate I: Agarose Gel Eletrophoresis of Amplicons of Carbapenemase Genes.

Lane M Is 100 bp Molecular Ladder, Lanes 8, 9, 10 and 11 had bands corresponding to 597 bp (*bla*$_{OXA}$) while lanes 15, 16 and 17 had bands corresponding to 550 bp (*bla*$_{NDM}$). Lanes 7, 14 and 22 were negative controls.

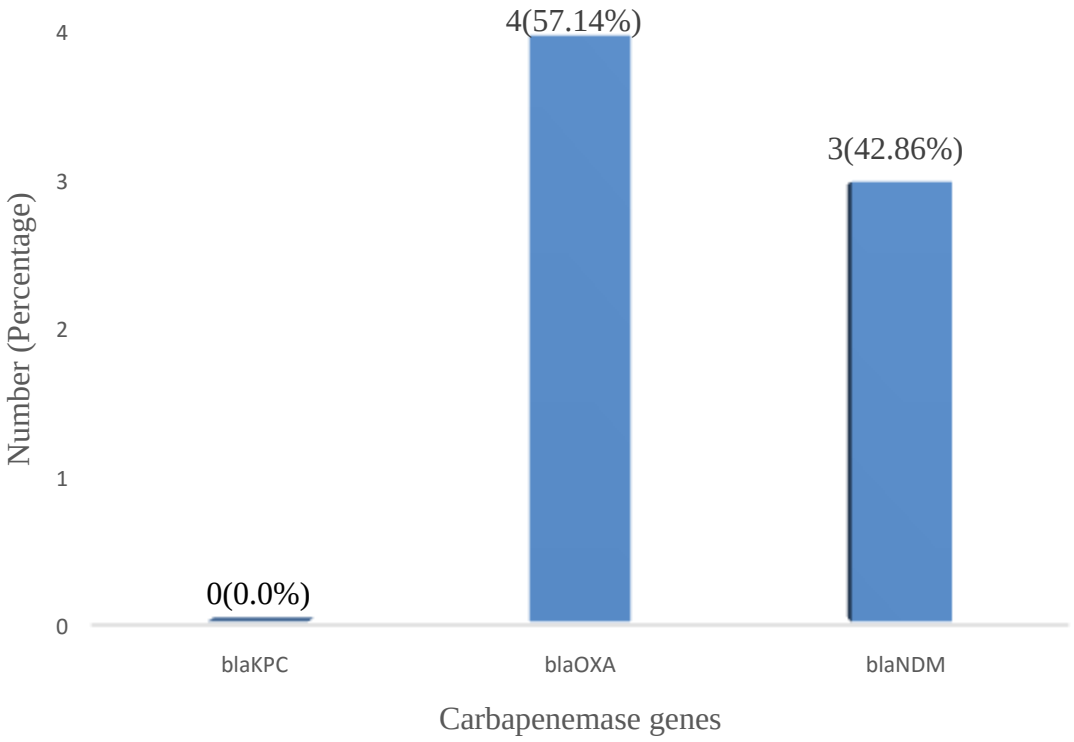

Figure 4. 2 : Percentage Distribution of Carbapenemase Genes among Bacterial Isolates from Urine of Patients Attendin Selected Hospitals in Zaria

4.11 Distribution of Carbapenemase Genes among the Carbapenem Resistant *Klebsiella pneumoniae* and *Escherichia coli*

Distribution of carbapenemase genes among carbapenem resistant *Klebsiella pneumoniae*and *Escherichia coli* is illustrated in Table 4.8. bla_{NDM} and bla_{OXA} genes were co-detected in two isolates namely GUM015 and AUM023. bla_{OXA} was detected alone in isolates MUF002 and GUF084 while bla_{NDM} alone was detected in isolate MUF012.

4.12 Occurrence of Carbapenemase Producing Isolates based on PCR

The occurrence of carbapenemase production *Escherichia coli* was found to be 2.86% while that of carbapenemase producing *Klebsiella pneumoniae*was found to be 5.66% by PCR. The overall occurrence of carbapenemase producing isolates in this study was 4.07% (Table 4.9). There was no statistically significant difference in the occurrence of carbapenemase producing *E. coli* and *K. pneumoniae* ($p \geq 0.05$).

4.13 Efficacy of Phenotypic Tests in the Detection of Carbapenemases

Table 4.10 shows the performance of the MHT, mCIM and Carba NP test in the detection of carbapenemases. Carba NP test had the highest sensitivity (100.0%) followed by mCIM (100.0%) while MHT had the least sensitivity (60.0%). The most specific phenotypic test was Carba NP test (100.0) while mCIM and MHT were less specific (0.0%). Likewise, Carba NP test had the highest accuracy (100.0%).

4.14 Detection Rates of OXA and NDM Carbapenemases by the Phenotypic Tests

The detection rates of OXA and NDM carbapenemases were found be to 100.0% by Carba NP test and mCIM while the rates of OXA and NDM carbapenemases by MHT were found to be 75.0% and 33.33% respectively (Table 4.11).

Table 4. 8 : Distribution of Carbapenemase Genes among Carbapenem Resistant *Klebsiella pneumoniae* and *Escherichia coli*

Isolate code	Isolate identity	Carbapenemase gene(s) detected
GUM015	*Klebsiella pneumoniae*	bla_{NDM}, bla_{OXA}
MUF002	*Escherichia coli*	bla_{OXA}
MUF012	*Escherichia coli*	bla_{NDM}
AUM023	*Klebsiella pneumoniae*	bla_{NDM}, bla_{OXA}
GUF084	*Klebsiella pneumoniae*	bla_{OXA}
GUF078	*Klebsiella pneumoniae*	None detected

Table 4. 9: Occurrence of Carbapenemase Producing Isolates based on PCR

Isolate	No. of CPs	Occurrence of CPs (%)
***Escherichia coli*(n = 70)**	2	2.86
***Klebsiella pneumoniae*(n = 53)**	3	5.66
Overall (n = 123)	5	4.07

$\chi^2 = 0.608$, $p = 0.4356$ $df = 1$

* There was no statistically significant difference in the occurrence of carbapenemase producing *E. coli* and *K. pneumoniae* based on PCR ($p \geq 0.05$).

Table 4. 10: Efficacy of Phenotypic Tests in the Detection of Carbapenemases

Tests	MHT	mCIM	Carba NP
Sensitivity (%)	60.00	100.00	100.00
95% CI	14.66 - 94.73	47.82 - 100.00	47.82 - 100.00
Specificity (%)	0.00	0.00	100.00
95% CI	0.00 - 97.50	0.00 - 97.50	35.88 - 99.58
Accuracy (%)	50.00	83.33	100.00
95% CI	11.81 - 88.19	35.88 - 99.58	54.07 - 100.00

MHT = Modified Hodge Test, mCIM = Modified Carbapenem Inactivation Method, CI = Confidence Interval

Table 4. 11: Detection Rates of OXA and NDM by the Phenotypic Tests

	No. (%) detected		
Carbapenemases	**MHT**	**mCIM**	**Carba NP**
OXA (4)	3 (75.00)	4 (100.00)	4 (100.00)
NDM (3)	1 (33.33)	3(100.00)	3 (100.00)

4.15 Sequence Similarity of Carbapenemase Genes Detected With Genes in the Genbank

Sequence similarity analysis revealed that the carbapenemase genes were similar to carbapenemase genes in the NCBI GenBank showing 98 – 100% identity. The *bla*$_{NDM}$ genes detected in isolates GUM015 and MUF012 were 100% similar to *bla*$_{NDM}$detected in *E.coli* F070 from Myanmar (AP023238.1), *E.coli* YJ6 from Myanmar (AP0233236.1) and *K.pneumoniae* KJ10 from India (MT462582.1). However, the *bla*$_{NDM}$ gene detected in isolateAUM023 was 99.46% similar to these strains in the NCBI GenBank. The *bla*$_{OXA}$ genes detected in isolates GUM015 and MUF002 were 99.54% similar to *bla*$_{OXA}$ detected in *K. pneumoniae* N83 from Egypt (MK341123.1), 98.47% similar to *K. pneumoniae* KPTR1-18from Russia (MK867763.1) and 98.47% similar to *E. coli* LAU-OXAfrom Lebanon (CP045282.1) (Table 4.12).The evolutionary history of the carbapenemase genes as inferred by the Maximum Likelihood method is presented in Figure 4.3.

4.16: Nucleotide and Amino Acid Substitutions in Sequences

Positions, a nucleotide and amino acid substitution observed in the sequences is presented in Table 4.13. Nucleotide substitutionswere not observed in the *bla*$_{NDM}$ gene sequences, however nucleotide substitutionswere observed in the *bla*$_{OXA}$ gene sequences. At position 389 of *bla*$_{OXA}$ gene detected in isolates GUM015, cytosine (C) was substitutedwith Guanine (G) with a corresponding amino acid substitutionfrom Threonine was change Arginine. Likewise, at position 194 of *bla*$_{OXA}$ gene detected in isolates AUG023, Thymine (T) was substituted with Guanine (G) with a corresponding amino acid substitutionfrom Serine to Isoleucine. While a change at position 518 of *bla*$_{OXA}$ gene detected in isolates AUG023, cytosine (C) substitution with Guanine (G) resulted in a stop codon. All the substation types were transversion (a point mutation that result in substitution of purine forpyrimidine or pyrimidine for purine).

Table 4. 12: Sequence Similarity of Carbapenemase Genes Detected with Genes in the NCBI Genbank

Identified bacteria	Gene detected	Hits (Country)	E-value	Percent Identity (%)	Accession number
K.pneumoniae GUM015	*bla*$_{NDM}$	*E.coli* F070 (Myanmar)	0.0	100.00	AP023238.1
		E.coli YJ6 (Myanmar)	0.0	100.00	AP023236.1
		K.pneumoniae KJ10 (India)	0.0	100.00	MT462582.1
	bla$_{OXA}$	*K. pneumoniae* N83 (Egypt)	0.0	99.54	MK341123.1
		K. pneumoniae KPTR1-18(Russia)	0.0	98.47	MK867763.1
		E. coli LAU-OXA(Lebanon)	0.0	98.47	CP045282.1
K.pneumoniae AUM023	*bla*$_{OXA}$	*K. pneumoniae* AAKS3(India)	0.0	98.58	LC583817.1
		K. pneumoniae AAKS4(India)	0.0	98.21	LC583818.1
		K. pneumoniae AAKS2 (India)	0.0	98.06	LC583816.1
	bla$_{NDM}$	*E.coli* F070 (Myanmar)	0.0	99.46	AP023238.1
		E.coli YJ6 (Myanmar)	0.0	99.46	AP023236.1
		K.pneumoniae KJ10 (India)	0.0	99.46	MT462582.1
E.coli MUF002	*bla*$_{OXA}$	*K. pneumoniae* N83 (Egypt)	0.0	99.54	MK341123.1
		K. pneumoniae KPTR1-18(Russia)	0.0	98.47	MK867763.1
		E. coli LAU-OXA(Lebanon)	0.0	98.47	CP045282.1
K.pneumoniae GUF084	*bla*$_{OXA}$	*K. pneumoniae* AAKS3(India)	0.0	99.55	LC583817.1
		K. pneumoniae AAKS4(India)	0.0	99.25	LC583818.1
		K. pneumoniae AAKS2 (India)	0.0	99.10	LC583816.1
E.coli MUF012	*bla*$_{NDM}$	*E.coli* F070 (Myanmar)	0.0	100.00	AP023238.1
		E.coli YJ6 (Myanmar)	0.0	100.00	AP023236.1
		K.pneumoniae KJ10 (India)	0.0	100.00	MT462582.1

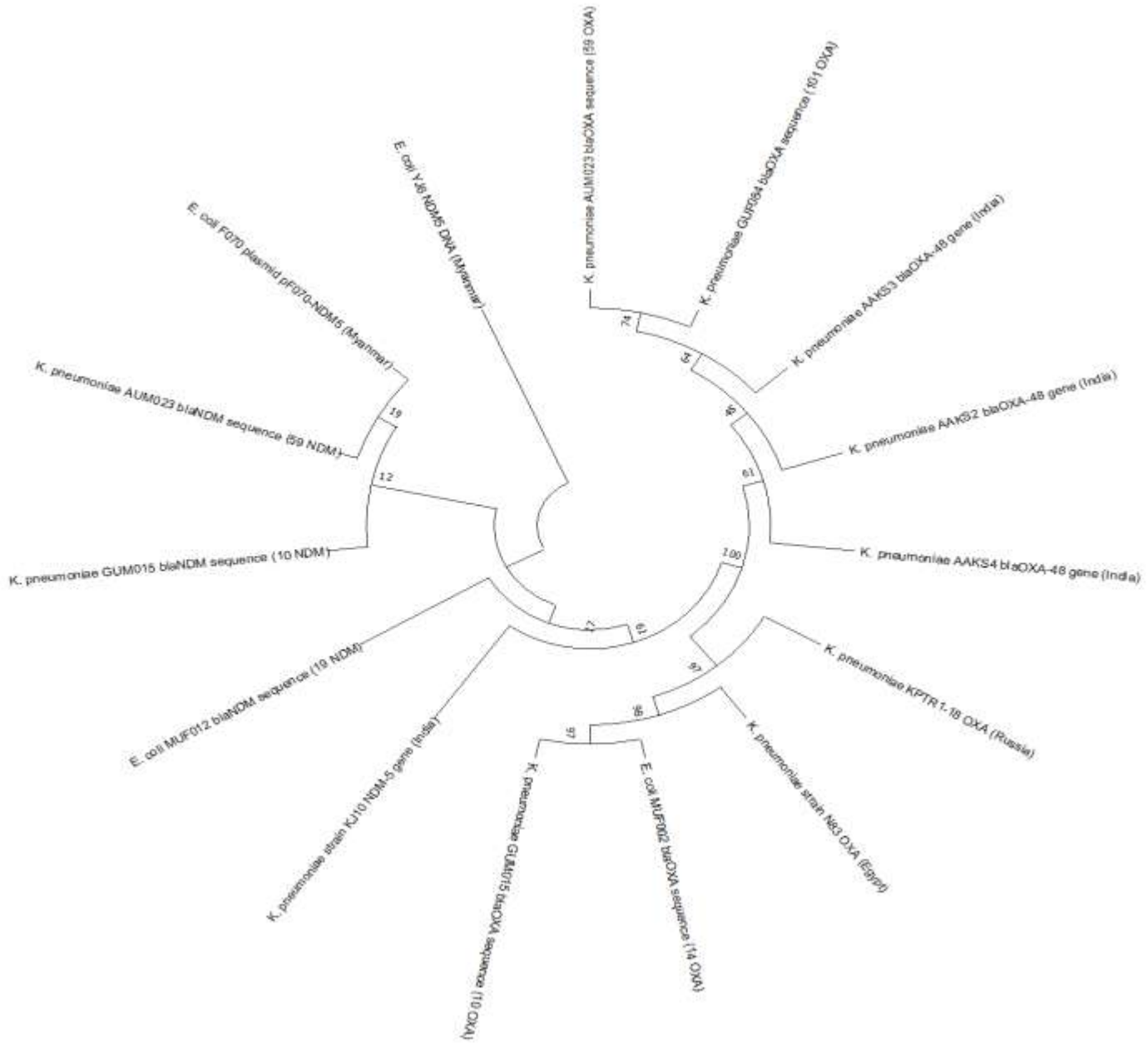

Figure 4. 3: Evolutionary Analysis of the Carbapenemase Genes Detected and Carbapenemase Genes from the NCBI Genbank by Maximum Likelihood Method

Table 4. 13: Positions, Nucleotide and Amino Acid Substitutions in the Sequences

Gene	Hits	Identified bacteria	Mutation	Type of substitution	Mutation result	*Significance of Mutation
***bla*$_{NDM}$**	*E.coli* F070 (AP023238.1)	*K. pneumoniae* GUM015	ND	NA	ND	-
	E.coli F070 (AP023238.1)	*K. pneumoniae* AUM023	ND	NA	ND	-
	E.coli F070 (AP023238.1)	*E.coli* MUF012	ND	NA	ND	-
***bla*$_{OXA}$**	*K. pneumoniae* N83 (MK341123.1)	*K. pneumoniae* GUM015	389 A<u>C</u>G→A<u>G</u>G	Transversion	Thr→Arg	Missense
			428 T<u>T</u>T→T<u>A</u>T	Transversion	Phe→Tyr	Missense
			626 A<u>T</u>A→A<u>A</u>A	Transversion	Ile→Lys	Missense
	K. pneumoniae N83 (MK341123.1)	*E.coli* MUF002	389 A<u>C</u>G→A<u>G</u>G	Transversion	Thr→Arg	Missense
			428 T<u>T</u>T→T<u>A</u>T	Transversion	Phe→Tyr	Missense
			626 A<u>T</u>A→A<u>A</u>A	Transversion	Ile→Lys	Missense
	K. pneumoniae AAKS3 (LC583817.1)	*K. pneumoniae* AUM023	194 AG<u>T</u>→AG<u>G</u>	Transversion	Ser→Ile	Missense
			388 <u>C</u>GA→<u>G</u>GA	Transversion	Arg→Gly	Missense
			391 <u>C</u>GG→<u>G</u>GG	Transversion	Arg→Gly	Missense
			430 T<u>T</u>T→<u>A</u>TT	Transversion	Phe→Ile	Missense
			518 T<u>C</u>A→T<u>G</u>A	Transversion	Val→Stop	Nonsense
			587 <u>G</u>GT→<u>C</u>GT	Transversion	Gly→Arg	Missense
	K. pneumoniae AAKS3 (LC583817.1)	*K. pneumoniae* GUF084	194 AG<u>T</u>→AG<u>G</u>	Transversion	Ser→Ile	Missense
			407 T<u>T</u>C→T<u>G</u>C	Transversion	Phe→Cys	Missense
			589 <u>G</u>GT→<u>C</u>GT	Transversion	Gly→Arg	Missense

* Missense mutation: the mutation resulted in a different amino acid encoded however there was no apparent change in function. Nonsense mutation: the mutation resulted in a stop codon. **ND** : Not Detected, **NA** : Not Applicable.

4.17 Antibiotic Susceptibility Pattern of the Carbapenem Resistant Isolates

Table 4.14a and Table 4.14b show the diameter of the zones of inhibition and antibiotic susceptibility pattern of the carbapenem resistant isolates respectively. The isolates were resistant to ceftriaxone (100.00%), ampicillin (100.00%), trimethoprim-sulphamethoxazole (100.00%) and doxycycline (83.33%). So also the isolates were moderately resistant to nalidixic acid (66.67%) and chloramphenicol (66.67%). Low resistance rate were recorded against gentamicin (33.33%), colistin (16.67%) and amikacin (0.00%). Most of the isolates (66.67%) were susceptible to amikacin, while 33.33% of the isolates were susceptible to gentamicin and nalidixic acid. Susceptibility to chloramphenicol and colistin was recorded in 16.67% of the isolates. All the isolates (100.0%) were susceptible to tigecycline and fosfomycin.

4.18 Percentage Susceptibility of Carbapenem Resistant *Escherichia coli* and *Klebsiella pneumoniae* to the Test Antibiotics

Half of the *Escherichia coli*isolates (50.00%) were susceptible to nalidixic acid, amikacin, chloramphenicol, colistin and gentamicin. However none of the *Escherichia coli*isolates (0.00%) were susceptible to ceftriaxone, doxycycline, ampicillin and trimethoprim-sulphamethoxazole**.** Most of the *Klebsiella pneumoniae*isolates (75.00%) were susceptible to amikacin, while 25.00% of the *Klebsiella pneumoniae*isolates were susceptible to nalidixic acid and gentamicin. However, none of the *Klebsiella pneumoniae*isolates were (0.00%) susceptible to ceftriaxone, doxycline, ampicillin, trimethoprim-sulphamethoxazole, chloramphenicol and colistin (Table 4.15). All the isolates of both *K. pneumoniae* and *E. coli* were 100.0%susceptible to tigecycline and fosfomycin.

Table 4.14 a: Diameters of the zones of inhibition of the antibiotics against the isolates

Antibiotics (disc content)	Zones of inhibition (mm)					
Isolate codes	GUM015	MUF002	MUF012	AUM023	GUF078	GUF084
Ceftriaxone (30µg)	6	17	13	18	6	6
Nalidixic acid (30µg)	6	6	22	6	20	9
Doxycycline (30µg)	9	9	9	9	12	6
Amikacin (30µg)	15	21	15	23	27	20
Ampicillin (10µg)	6	6	6	6	6	6
Trimethoprim-sulphamethoxazole (23.75/1.25µg)	6	6	6	6	6	6
Chloramphenicol (30µg)	9	6	19	6	14	12
Colistin (10µg)	12	12	14	9	13	12
Gentamicin (10µg)	6	9	17	9	18	13
Tigecycline (15µg)	20	22	23	20	22	24
Fosfomycin (200µg)	16	17	18	16	17	17

Table 4. 14b: Antibiotic Susceptibility Pattern of the Carbapenem Resistant Isolates

Antibiotic (disc content)	Number (%) of isolates		
n = 6	**Susceptible**	**Intermediate**	**Resistant**
Ceftriaxone (30µg)	0 (0.00)	0 (0.00)	6 (100.00)
Nalidixic acid (30µg)	2 (33.33)	0 (0.00)	4 (66.67)
Doxycycline (30µg)	0 (0.00)	1 (16.67)	5 (83.33)
Amikacin (30µg)	4 (66.67)	2 (33.33)	0 (0.00)
Ampicillin (10µg)	0 (0.00)	0 (0.00)	6 (100.00)
Trimethoprim-sulphamethoxazole (23.75/1.25µg)	0 (0.00)	0 (0.00)	6 (100.00)
Chloramphenicol (30µg)	1 (16.67)	1 (16.67)	4 (66.67)
Colistin* (10µg)	1 (16.67)	4 (66.67)	1 (16.67)
Gentamicin (10µg)	2 (33.33)	1 (16.67)	3 (50.00)
Tigecycline* (15µg)	6 (100.00)	0 (0.00)	0 (0.00)
Fosfomycin (200µg)	6 (100.00)	0 (0.00)	0 (0.00)

* EUCAST breakpoints were because there are no CLSI breakpoints for colistin and tigecycline for *Enterobacteriaceae.*

Table 4. 15: Percentage Susceptibility of Carbapenem Resistant *Escherichia coli* and *Klebsiella pneumoniae* to the Test Antibiotics

Antibiotic (disc content)	Number (%) of isolates susceptible	
	Escherichia coli (n = 2)	*Klebsiella pneumoniae* (n = 4)
Ceftriaxone (30µg)	0 (0.00)	0 (0.00)
Nalidixic acid (30µg)	1 (50.00)	1 (25.00)
Doxycycline (30µg)	0 (0.00)	0 (0.00)
Amikacin (30µg)	1 (50.00)	3 (75.00)
Ampicillin (10µg)	0 (0.00)	0 (0.00)
Trimethoprim-sulphamethoxazole (23.75/1.25µg)	0 (0.00)	0 (0.00)
Chloramphenicol (30µg)	1 (50.00)	0 (0.00)
Colistin* (10µg)	1 (50.00)	0 (0.00)
Gentamicin (10µg)	1 (50.00)	1 (25.00)
Tigecycline* (15µg)	2 (100.00)	4 (100.00)
Fosfomycin (200µg)	2 (100.00)	4 (100.00)

* EUCAST breakpoints were because there are no CLSI breakpoints for colistin and tigecycline for *Enterobacteriaceae.*

4.19 Resistance Patterns and Multiple Antibiotic Resistance (MAR) Indices of the Carbapenem Resistant Isolates

The resistance patterns and MAR indices of the carbapenem resistant isolates is presented in Table 4.16. Isolate GUM015 was non-susceptible to 9 antibiotics (resistance pattern = CRO,NA,DO,AK,AMP,SXT,C,CT,CN; MAR index = 0.82). Three isolates: GUF084, AUM023 and MUF002 (resistance pattern = CRO,NA,DO,AMP,SXT,C,CT,CN; MAR index = 0.73) were non-susceptible to 8 antibiotics, while isolates GUF078 (resistance pattern = CRO,DO,AMP,SXT,C,CT; MAR index = 0.55) and MUF012 (resistance pattern = CRO,DO,AK,AMP,SXT; MAR index = 0.46) were non-susceptible to 6 and 5 antibiotics respectively.

Table 4. 16: Resistance Pattern and MAR index of the Carbapenem Resistant Isolates

Isolate code	Identified bacteria	Carbapenemase gene(s) detected	Resistance pattern	MAR index
GUM015	*K. pneumoniae*	bla_{NDM}, bla_{OXA}	CRO,NA,DO,AK,AMP,SXT,C,CT,CN	0.82
MUF002	*E. coli*	bla_{OXA}	CRO,NA,DO,AMP,SXT,C,CT,CN	0.73
MUF012	*E. coli*	bla_{NDM}	CRO,DO,AK,AMP,SXT	0.46
AUM023	*K. pneumoniae*	bla_{NDM}, bla_{OXA}	CRO,NA,DO,AMP,SXT,C,CT,CN	0.73
GUF078	*K. pneumoniae*	None detected	CRO,DO,AMP,SXT,C,CT	0.55
GUF084	*K. pneumoniae*	bla_{OXA}	CRO, NA,DO,AMP,SXT,C,CT,CN	0.73

Key: CRO = Ceftriaxone,NA = Nalidixic acid, DO = Doxycycline, AK = Amikacin, AMP = Ampicillin, SXT = Trimethoprim-sulphamethoxazole, C = Chloramphenicol, CT = Colistin, CN = Gentamicin

CHAPTER FIVE

5.0 DISCUSSION

In this study, the overall occurrence of *Escherichia coli*and *Klebsiella pneumoniae*in urine of patients attending selected hospitals in Zaria was 23.18% and 17.55% respectively. Poor personal hygiene, study population and overcrowded nature of some hospital wards could account for these high occurrences observed in this study.Higher occurrence rates of *E. coli* (56.0%) and *K. pneumoniae* (20.0%) were reported by Giwa *et al.* (2018) in Zaria. So also, higher occurrence rate of *K. pneumoniae* (55.0%) was reported by Chikwendu*et al.* (2010).

The fact that *E. coli* is ranked first as a urinary tract pathogen and that it constitutes a large proportion of the urinary tract flora argues in favour of the higher occurrence of *E. coli* (23.18%) compared to *K. pneumoniae* (17.55%) observed in this study. This implies that *E. coli* uses it well characterized virulence factor to colonize urinary tract better than *K. pneumoniae.*This is in agreement with the result of Giwa *et al.* (2018) in Zaria who also reported higher occurrence of *E. coli* (56.0%) compared to *K. pneumoniae* (20.0%).

The higher occurrences of *E. coli* (31.37%) and *K. pneumoniae* (21.57%) observed among patients attending Hajiya Gambo Sawaba General Hospital might likely be due to the poor personal hygienic practices among the patients attending this hospital.

The higher occurrences of *E. coli*and *K. pneumoniae* observed in females (25.93% and 19.05% respectively) compared to males (18.58% and 15.93% respectively) might be due to theanatomical differences in females (short urethra as well as the small distance between anal and vaginal opening) which may ease invasion and colonization by these bacteria. Our finding of

higher occurrence in females is in agreement with the finding of Ndzime *et al.* (2021) who also reported higher occurrence among females in Franceville, Gabon.

The higher occurrences of *E. coli* and *K. pneumoniae* observed among age groups of ≤ 10 years (36.36% and 30.30% respectively) and 11-20 years (42.86% and 35.71% respectively)may be because of the higher hospitalization rate of patients within this age groups.

Emergence and spread of CRE worldwideis of great public health concern as there are limited antibiotics that can be used against these strains. The increasing number ofhospital acquired and community acquired infections caused by CRE especially carbapenem resistant*K. pneumoniae* and *E. coli* isolates is increasingthe burden on the health care system (Ssekatawa *et al.,* 2018).

The overall occurrence of carbapenem resistant isolates in Zaria was 4.88%. This occurrence raises concern since carbapenems are not commonly prescribed and used in the selected hospitals in Zaria, they are usually reserved as last drug of resort for the treatment of infections caused by multidrug resistant GNB. The occurrence of CRE observed in Zaria and other regions where carbapenems are not prescribed or less commonly prescribed may be as a result of international travel of patients into this regionsfromcountries where CRE is endemic (Olowo-okere *et al.,* 2019).

However, the overall occurrence of carbapenem resistant isolates in this study is lower than 36.8% reported by Enwuru *et al.* (2011) in Southwest Nigeria, 9.7% (meropenem resistant isolates) and 12.4% (ertapenem resistant isolates) reported by Mohammed *et al.* (2015) in Maiduguri, 15.2% reported by Oduyebo *et al.*(2015) in Lagos, 7.7% reported by Anibijuwon*et al.* (2018) in Ogbomoso and Osogbo, Southwest Nigeria, 7.61% reported by Alaka*et al.* (2019) in Ile Ife and 28.2% reported by Olowo-okere *et al.* (2019) in Sokoto. The higher

occurrenceobserved in these studies might be due to differences in targeted bacteria, study population, study design and type of screening technique used.

The occurrences of carbapenem resistant *E. coli* and carbapenem resistant *K. pneumoniae* in this study were 2.86% and 7.55% respectively. The most prevalent carbapenem resistant bacteria in this study was *K. pneumoniae*. This might be linked to its ability to acquire and accumulate genes coding for antibiotic resistance as reported by WHO (2017). In line with this finding, *K. pneumoniae* was indicated as one of the MDR bacteria that constitute an immediate threat to human health (WHO, 2017; Fasciana *et al.,* 2019). Higher occurrence of carbapenem resistant *K. pneumoniae* observed in this study is in agreement with the report of Ssekatawa*et al.* (2018) in East Africa, global data about carbapenem resistance in USA (CDC, 2013b) and India (Ne Gelband*et al.,* 2015). So also, Oduyebo, *et al*. (2015) reported higher occurrence of carbapenem resistant *K. pneumoniae* (14.5%) compared to the occurrence of carbapenem resistant *E. coli* (7.8%) in Lagos.

Higher occurrence of carbapenem resistant *K. pneumoniae* of 19.05% was previously reported by Mukail *et al*. (2019) in Zaria. The difference observed in the occurrence might be because their isolates were from various clinical samples (urine, blood, HVS, sputum and wound swab).

Four (66.67) of the carbapenem resistant isolates (CRIs) were positive for carbapenemase production by MHT. This finding is contrary to the result of a study conducted by Mohammed *et al.* (2015), where 82.1% of carbapenem resistant *Enterobacteriaceae*were positive for carbapenemase production by MHT. The differences observed in the detection rates might be due to difference in principle, sensitivity, specificity and accuracy of the methods in the detection of carbapenemases.

The overall occurrence of carbapenemase producing isolates in this study was 4.88% (as detected by mCIM) 4.07% (as detected by Carba NP test) and 3.25% (as detected by MHT). Occurrence of carbapenemase producing isolates in hospital setting has negative health implicationsas it can easily spread among patients and health workers. So also the carbapenemase gene can spread to other pathogens because they are located on highly mobile genetic elements (Potter *et al.,* 2016). This could also result to prolonged hospital stay and increased cost of treatment due to treatment failure.

The occurrence of carbapenemase producing isolate observed in this study is lower than 9.3% reported by Motayo*et al.* (2013) in Abeokuta, 11.9% reported by Yusuf *et al.* (2014) in Kano, 10.2% reported by Mohammed *et al.* (2015) in Maiduguri, 12.4% reported by Oduyebo, *et al*. (2015) in Lagos, 8.71% reported by Alaka *et al.* (2019) in Ile Ife. These differences observed in the occurrence carbapenemase producing isolates might be due to difference in type of samples, method of detection or study population.

The occurrence of carbapenemase producing *E. coli* was 1.43% (as detected by MHT) and 2.86 (as detected by Carba NP test and mCIM). While the occurrence of carbapenemase producing *K. pneumoniae* was 7.55% (as detected by mCIM) and 5.66% (as detected by MHT and Carba NP test). The emergence of carbapenemase producing *E. coli* and *K. pneumoniae* is of great clinical concern because these bacteria are known as the major cause of nosocomial infection (Khan *et al.,* 2015).

This result is in contrast with the occurrence of carbapenemase producing *E. coli* (11.5%) and *K. pneumoniae* (13.3%) reported by Yusuf *et al.,* (2014) in Kano. Higher occurrence of carbapenemase producing *Klebsiella* species (23.9%) was reported by Mukail *et al*. (2019) in Zaria. However, this occurrence is higher than 0% occurrence of carbapenemase producing *K.*

pneumoniae reported by Hussaini *et al.* (2017) in Zaria and 0.15% reported by Jones *et al.* (2005) in Israel.

The higher occurrence of carbapenemase producing *K. pneumoniae* compared to carbapenemase producing *Escherichia coli*observed in this study is in line with the report of Landman *et al.* (2007), Yusuf *et al.* (2012), Yusuf *et al.* (2014) and Mohammed *et al.* (2015).

Carbapenem resistance traits such asdecreased outer membrane permeability, overexpression of β-lactamases, production ofcephalosporinase and porin loss are not transferable like the carbapenemasegenes. This explains why carbapenem resistant bacteria that are notcarbapenemase producers are considered to be of much less importance from apublic health perspective compared to carbapenemase producing carbapenem resistant bacteria. The spread of carbapenemaseproducers is an importantclinical issue in the control of antibiotic resistantGNB (Nordmann *et al.*, 2012a).

Carbapenemase genes were detected in five out of the six CRIs screened. This indicated that the carbapenem resistance in these five isolates was mediated by carbapenemases while carbapenem resistance in the remaining one isolate might to due to either production of other carbapenemase genes that were not targeted in this study, over production of other β-lactamases, porin loss or reduced permeability. This finding is in consonance with those of Nordmann*et al.* (2011a);Nordmann*et al.* (2012a)and Demir*et al.* (2015) who reported that carbapenem resistance among*Enterobacteriaceae* is principally due to the production of carbapenemases while the less frequent mechanisms are the overproduction ofAmpC-mediated β-lactamases or ESBLs in organisms with porin mutations.

The carbapenem resistance determinants in this study were bla_{OXA} (57.14%) and bla_{NDM} (42.86%), bla_{KPC} was not detected in any of the CRIs. Similar phenomenon where OXA and NDM were the dominant carbapenemases in *E. coli* and *K. pneumoniae* were reported by Nordmann*et al.* (2012a); Zowawi*et al.* (2014) in countries of the GulfCooperation Counciland Al-Agamy*et al.* (2018) in Riyadh, KSA.

The most frequently detected carbapenemase gene was the *bla*OXA (57.14%) which codes for OXA carbapenemase. Detection of OXA carbapenemases in *Enterobacteriaceae*is of major public health concern due to their ability to mutate rapidly thereby resulting in expanded spectrum of activity (Mathers *et al*., 2013;Codjoe and Donkor, 2018). This finding is supported by the report that OXA gene is the predominant mechanism and major contributor tocarbapenem resistance in *Enterobacteriaceae* by Evans and Amyes (2014).

Two isolates co-harboured bla_{OXA} and bla_{NDM} genes, this indicates carriage of multiple carbapenemase genes on a plasmid which can serve as source of multidrug resistance and may represent an emerging threat. Similar finding was also reported by Kumarasamy*et al.* (2010) in India, Pakistan, and the UK, Nordmann *et al.* (2011c), Zowawi*et al.* (2014) in countries of the GulfCooperation Council, Protonotariou *et al.* (2019) and van der Zwaluw *et al*. (2020) in the Netherlands.

The overall occurrence of carbapenemase genes was 4.07% while the occurrence of carbapenemase genes in *E. coli* and *K. pneumoniae* was 2.86% and 5.66% respectively. Higher occurrence of carbapenemase genes in *K. pneumoniae* might be due its permeability to mobile genetic elements hence the high frequency and diversity of resistance genes observed in it. Higher occurrence of carbapenemase genes in *K. pneumoniae* compared to *E. coli* was also reported by van der Zwaluw *et al*. (2020) in the Netherlands.

Carba NP test was found to be the most sensitive, specific and accurate phenotypic test for the detection of carbapenemase producers. No false-negative or false-positive results were observed using Carba NP test. This is in line with result of Nordmann*et al.* (2012b), however it is in contrast to the results of Manohar*et al.* (2018) and Zhou *et al.* (2018), who observed false-negative result for OXA-48 and KPC-2 producers respectively using Carba NP test.

MHT was the least sensitive, specific and accurate phenotypic test, it give false-negative results for isolate MUF012 harbouring bla_{NDM} gene and isolate GUM015 harbouring bla_{OXA} and bla_{NDM} genes. It also gave a false-positive result for isolate GUF078 which was not harbouring any of the carbapenemase genes targeted. The false-positive result is likely due to the inability of the test to differentiate between carbapenemase and other β-lactamases such as AmpC and ESBL. This finding is similar to the report of Manohar*et al.* (2018) where MHT gave false-positive results for ESBLsproducing isolates andfalse-negative results for NDM-1 and OXA-48-like carbapenemase producing isolates. Similarly, false negative results were reported by Zhou *et al.* (2018) for NDM-1 producer and false positive results for ESBL and AmpC producers.

Carba NP test and mCIM had higher detection rates for both OXA and NDM carbapenemases compared to MHT. The low detection rate of NDM by MHT compared to Carba NP test and mCIM observed in this study is similar to the finding of Zhou *et al.*(2018) who also reported low detection rate of NDM by MHT.

Analysis of the carbapenemase gene sequences revealed some level of polymorphism in the bla_{OXA} genes however, this was not observed in the bla_{NDM} genes. In line with this finding, Diene and Rolain (2014) reported the OXA carbapenemases are the most variable carbapenemase. Furthermore, analysis of bla_{OXA}genesequences revealed that these variations resulted from

preferential alteration due to antibiotic selective pressure. Despite the polymorphism observed in the *bla*$_{OXA}$ sequences, the active site regions are relatively conserved (Evans and Amyes, 2014).

The fast rate at which *bla*$_{OXA}$ genes are evolving coupled with their diversity suggest that a number of these*bla*OXA genes may rapidly evolve to be resistant to new carbapenemase inhibitors (Evans and Amyes, 2014).

The 100.0 % resistance rate exhibited by the CRIs against ceftriaxone and ampicillin is not surprising because carbapenemases are known to confer resistance against all β-lactam antibiotics. This may also be attributed to co-production of both ESBL and carbapenemases, resulting in hydrolysis of almost all β-lactam antibiotics. This is in agreement with the report of Olowo-okere *et al.* (2019) and Pawar*et al.* (2020) who reported high resistance rates of carbapenem resistant isolates to β-lactam antibiotics.

The high trimethoprim-sulphamethoxazoleresistance rate (100.00%) observed might be as a result of selective pressure due to its extensive use in the treatment of uncomplicated UTI and as prophylaxis. Similarly, low cost and low toxicity of the chloramphenicol and tetracyclines has resulted in their frequent use in human and veterinary medicine. This may account for the high resistance rates observed to chloramphenicol and doxycycline.

So also, the resistance observed against nalidixic acid (66.67%) may be linked to the excessive use of quinolonesas an empiric treatment for UTI. This is in agreement with the result of Giwa *et al.* (2018) who reported high resistance rate to nalidixic acid. High level of resistance of CRIs against trimethoprim-sulphamethoxazole (73.5%) and nalidixic acid (91.7%) was also reported by Pawar*et al.* (2020).

The CRIs were however susceptible to amikacin (66.67%) and gentamicin (33.33%). This is likely due to the fact that amikacin and gentamicin are administered intravenously, very expensive and less frequently prescribed. So also this may be due to the refractory property of amikacin against most aminoglycosidemodifying enzymes. This report corroborate with that of Giwa *et al.* (2018) and Almugadam *et al.* (2018). This report however disagrees with the finding of Pawar*et al.* (2020) where 82.9% of CRIs were gentamicin resistant.

This finding is similar to the report of Olonitola *et al.* (2007), where they reported that amikacin was the most effective antibiotic against ESBL producing *Escherichia coli*and *Klebsiella pneumoniae.* Lower susceptibility rate of CRIs to amikacin (45.3%) was also reported by Pawar*et al.* (2020).

Only 16.67% of the CRIs were susceptible to colistin, which is one of the treatment options for infection caused CRIs. The low colistin susceptibility rate observed in this study might be due the emergence and spread of plasmid encoded transferable *mcr-1* gene that confer resistance to colistin among *Enterobacteriaceae*(Gharaibeh and Shatnawi, 2019). This finding is in contrast with the results of Huang *et al*. (2014) and Pawar*et al.* (2020), who reported 92.6% and 84.7% colistin susceptibility rates of CRIs respectively.

High tigecycline and fosfomycin susceptibility levels (100.0%) were observed in this study, these antibiotics are part of the treatment options for infections caused by CRE. Expanded spectrum activity of tigecycline against ESBL and carbapenemase producers have been reported to be linked to the presence of 9-t-butyl-glycylamido side chain at C-9 of minocycline central skeleton (Roy *et al.,* 2013). Resistance to fosfomycin is mostly acquired through chromosomal mutations which are not transferable easily, this might account for the high susceptibility rate observed to fosfomycin (Schito, 2003). The 100.0% tigecycline susceptibility rate of CRIs

observed is similar to 100.0% tigecycline susceptibility rate reported by Mulla *et al.* (2016) but higher than tigecycline susceptibility rates of 88.3% reported by Hu *et al.* (2012)and 65.9% reported by Pawar*et al.* (2020). Almugadam *et al.* (2018) also reported that most CRE isolates were susceptible to tigecycline and fosfomycin. This finding however disagrees with the 8.0% and 36.0% tigecycline and fosfomycin resistance rates of CRIs respectively reported by Fasciana *et al*. (2019).Care should be taken when using tigecycline due to its side effect profiles, moreover the US Food and Drug Administration discourages routine use of tigecycline because of increased risk of death (US FDA, 2014; Logan *et al.*, 2015).

The most effective antibiotics against the CRIs in this study were amikacin (66.67%), tegicycline (100.00%) and fosfomycin (100.00%). This is in consonance with the findings of Abid *et al.* (2021) in Qatar who also reported that most of the CRIs were susceptible to tigecycline, fosfomycin and amikacin.

All the CRIs were resistant to more than three antibiotics tested, this implies that the isolates are MDR isolates. The fact that plasmids bearing carbapenemase genesalso carry determinants of resistance to multiple classes of antibiotics lend credence to the high level of multidrug resistance observed in CRIs. A single resistance plasmid may carry multiple genes coding for resistance to multiple antibiotics thereby making an isolate simultaneously resistant to several antibiotics. This is in line with previous reports by Munoz-Price and Quinn(2009), Bush and Fisher (2011), Logan (2012) and Logan *et al.* (2015).

The MAR indices of the CRIs ranged from 0.46 to 0.82. This implies that the isolates originated from high risk sources where antibiotics are often used since their MAR indices are greater than 0.2.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, *E. coli* and *K. pneumoniae* were isolated from urine of patients attending selected hospitals in Zaria at the rate of 23.18% and 17.55% respectively. The occurrence of *E. coli* and *K. pneumoniae* were higher in females and patients within the age group of 11-20 years.

Carbapenem resistance among clinical isolates of *E. coli* and *K. pneumoniae* in Zaria occurs at the rate of 2.86% and 7.55%, while the overall occurrence rate of CRIs in Zaria was 4.88%.

Phenotypically, the occurrence rate of carbapenemase producing *E. coli* was found to be 1.43%, 2.86% and 2.86% by MHT, mCIM and Carba NP respectively while the occurrence rate of carbapenemase producing*K. pneumoniae* by MHT, mCIM and Carba NP was 5.66%, 7.55% and 5.66% respectively.

Carbapenemase genes were detected in five out of the six CRIs screened by PCR. bla_{OXA} and bla_{NDM} carbapenemase genes were the resistance determinants in the CRIs, with bla_{OXA} been the most common carbapenemase gene. Carbapenemase genes occurred at the rate of 2.86% and 5.66% in *E. coli* and *K. pneumoniae* respectively.

Carba NP test was the most sensitive (100.0%), specific (100.0%) and accurate (100.0%) phenotypic test for the detection of carbapenemase producing isolates.

The sequences of the carbapenemase genes detected were 98-100% similar to other carbapenemase genes in the GenBank.

The CRIs were resistant against ceftriaxone (100.0%), ampicillin (100.0%), trimethoprim-sulphamethoxazole (100.0%), doxycycline (83.33%), chloramphenicol (66.67%) and nalidixic

acid (66.67%). However, the isolates were susceptible to tigecycline (100.0%), fosfomycin (100.0%) and amikacin (66.67%).

6.2 Recommendations

1. The use of carbapenems should be controlled and reserved for treatment of life threateninginfections caused by Gram negative bacteria. Uncontrolled sales of antibiotics in the streets, markets and pharmacy shops should be stopped.
2. All CRIs should be screened for carbapenemase production to prevent the spread of carbapenemase producing organisms.
3. Carba NP test should be recommended for use in laboratories for phenotypic detection of carbapenemases.
4. The committee responsible for the use of antibiotics should consider tigecycline, fosfomycin and amikacin among the probable treatment options for infection caused by CRIs, however the use should be monitored to avoid excessive toxicity and emergence of resistance.
5. Actions necessary for the prevention of antimicrobial drug resistance for all patient populations and implementation of infection control practices should be initiated to limit the spread of carbapenemases producers.

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APPENDICES

MINISTRY OF HEALTH AND HUMAN SERVICES
KADUNA STATE, NIGERIA

NHREC/17/03/2018

MOH/ADM/744/VOL.1/763

26TH JULY, 2019

NOTICE OF EXPEDITED REVIEW AND APPROVAL

OCCURRENCE OF CARBAPENEMASES AMONG ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE ISOLATED FROM URINE OF PATIENTS ATTENDING SELECTED HOSPITALS IN ZARIA, NIGERIA

Name of Principal Investigator : HUSSAINI IBRAHIM MOHAMMED

Address of Ethical Approval : DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA.

Date of receipt of Application : 24TH JULY, 2019

Date of Ethical Approval : 26TH JULY 2019

Expiry Date of Approval : 26TH JULY, 2020

This is to inform you that the Research described in the submitted Protocol, the Consent Forms, advertisements and other participant information materials have been reviewed and given Expedited approval by the the Health Research Ethics committee (HREC).

If there is delay in starting the research or any change, inform the HREC so that the dates of approval can be adjusted accordingly.

However, Researcher is kindly requested to submit a copy of his/her findings to the State Ministry of Health, please

Dr. JOSEPH SUNDAY
For: Chairman

SMOH Resource Centre Kaduna

Independence Way, PM.B 2014, Kaduna, Kaduna State - Nigeria.
Tel: +234 (0) 818 407 8693 | Website: www.kdsg.gov.ng | Email: health@kdsg.gov.ng

Appendix I: Ethical Approval from Ministry of Health and Human Services, Kaduna State

INFORMED CONSENT FORM (ICF)

This Informed Consent Form is for patients with suspected causes of urinary tract infection, respiratory tract infection or wound infection attending some selected hospitals in Zaria, Kaduna State. We are inviting you to participate in this research work titled **"Occurrence of Carbapenemases among *Escherichiacoli* and *Klebsiellapneumoniae* Isolated From Urine of Patients Attending Selected Hospitals in Zaria"**

Principal Investigator: Hussaini, Ibrahim Mohammed

Collaborating Investigators: Dr A.B. Suleiman

Prof. O.S. Olonitola

Prof. R.A. Oyi

Name of Organization: Department of Microbiology

Ahmadu Bello University, Zaria - Nigeria

Name of Sponsor: Self

Name of Proposal: Ph.D Research Proposal

This Informed Consent Form has two parts:

- Information Sheet (to share information about the research with you)
- Certificate of Consent (for signatures if you agree to take part)
  You will be given a copy of the full Informed Consent Form

PART I: INFORMATION SHEET

Introduction

I am Hussaini, Ibrahim Mohammed, a postgraduate student of the Department of Microbiology, Ahmadu Bello University, Zaria, carrying out a research work under the supervision of Dr. A.B. Suleiman, Prof. O.S. Olonitola and Prof. R.A. Oyi

We are conducting a research work on **"Occurrence of Carbapenemases among *Escherichiacoli* and *Klebsiellapneumoniae* Isolated From Urine of Patients Attending Selected Hospitals in Zaria"**

I will give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask me to pause as we go through the information and I will take time to explain. If you have questions, you can ask me, the study doctor or the staff.

Purpose of the research

The purpose of this research is to determine Occurrence of Carbapenemases among *Escherichiacoli* and *Klebsiellapneumoniae* Isolated From Urine of Patients Attending Selected Hospitals in Zaria.

Participant selection

We are inviting all patients with suspected causes of urinary tract infection, respiratory tract infection or wound infection attending some selected hospitals in Zaria to participate in research.

- *Do you know why we are asking you to take part in this study? YES.....NO……..*
- *Do you know what the study is about? YES..... NO. .....*

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive at this clinic will continue and nothing will change. If you choose not to participate in this research project, you will be offered the treatment that is routinely offered in this clinic/hospital. You may change your mind later and stop participating even if you agreed earlier.

- *If you decide not to take part in this research study, do you know what your options are?YES…… NO……*
- *Do you know that you do not have to take part in this research study, if you do not wish to? YES…… NO …….*
- *Do you have any questions? YES……. NO. ……*

Procedures and Protocol

When you have agreed to participate in this research, your urine, sputum or wound swab will be collected and transported to the laboratory for processing. At the end of the research leftover samples will be discarded.

Risks

There is a risk that you may share some personal or confidential information by chance, or that you may feel uncomfortable talking about some of the topics. However, we do not wish for this to happen. You do not have to answer any question or take part in the research if you feel the question(s) are too personal or if talking about them makes you uncomfortable.

Benefits

If you participate in this research, you will have the following benefits:

- ✓ Your will be screen for *Klebsiella pneumoniae* and *Escherichia coli*..
- ✓ *Antibiotic susceptibility testing will be carried out to determine the drug to be used.*
- ✓ *Isolates resistant to carbapenem will be screen for carpanemase enzyme.*

Confidentiality

With this research, it is possible that if others in the community are aware that you are participating, they may ask you questions. We will not be sharing the identity of those participating in the research.

The information that we collect from this research project will be kept confidential. Information about you that will be collected during the research will be put away and no-one but the researchers will be able to see it. Any information about you will have a study number on it in place of your name. Only the principal investigator will know what your number is and we will lock that information up with a lock and key. It will not be shared with or given toanyone except the principal investigator.

- *Did you understand the procedures that we will be using to make sure that any information that we as researchers collect about you will remain confidential?.....*
- *Do you have any questions about them?.....*

Sharing the Results

The knowledge that we get from doing this research will be shared with you through clinic meetings with your health care provider before it is made widely available to the public. Confidential information will not be shared. There will be small meetings in the community and these will be announced. After these meetings, we will publish the results in order that other interested people may learn from our research.

Right to Refuse or Withdraw

You do not have to take part in this research if you do not wish to do so, refusing to participate will not affect your treatment at this clinic in any way. You will still have all the benefits that you would otherwise have at this clinic. You may stop participating in the research at any time that you wish without losing any of your rights as a patient here. Your treatment at this clinic will not be affected in any way.

Who to Contact

If you have any questions you may ask your health care provider now or later even after the study has begun. If you wish to ask questions later, you may contact any of the following:

Hussaini, Ibrahim Mohammed 08142446864, 08076420648.

- *Do you know that you do not have to take part in this study if you do not wish to? ……*
- *Do you know that you can ask me questions later, if you wish to? ……*
- *Do you know that I have given the contact details of the person who can give you more information about the study?.....*
- *You can ask me any more questions about any part of the research study, if you wish to. Do you have any questions?.....*

PART II: CERTIFICATE OF CONSENT

I have read the foregoing information, or it has been read and translated to me in a language that I understand. I have also talked it over with my health care provider to my satisfaction. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risks and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have received a copy of this consent form and additional sheet to keep for myself. I therefore consent voluntarily to participate as a participant in this research.

Name of Participant: .....

Signature of Participant: .....

Date: .....

Day/month/year

Statement by Witness

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness: ..... AND Thumb print of participant

Signature of witness: _____

Date _____

Day/month/year

Statement by the Researcher/Person Taking Consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. Urine, sputum or wound swab sample will be taken

I confirm that sufficient information, including about risks and benefits, to make an informed decision have been fully explained to the participant. The participant was given an opportunity to ask questions about the study, and all the questions asked by participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Name of Researcher/person taking the consent: .....

Signature of Researcher /person taking the consent: _____

Date _____

Day/month/year

Appendix II: Informed consent

Antibiotics	Disc content (µg)	Interpretive Categories and Zone Diameter Breakpoints (mm)		
		Susceptible	Intermediate	Resistant
Imipenem	10	≥ 23	20 – 22	≤ 17
Ceftriaxone	30	≥ 23	20 – 22	≤ 17
Nalidixic acid	30	≥ 19	14 – 18	≤ 13
Doxycycline	30	≥ 14	11 – 13	≤ 10
Amikacin	30	≥ 17	15 – 16	≤ 14
Ampicillin	30	≥ 17	14 – 16	≤ 13
Trimethoprim-sulphamethoxazole	23.75/1.25	≥ 16	11 – 15	≤ 10
Chloramphenicol	30	≥ 18	13 – 17	≤ 12
Gentamicin	10	≥ 15	13 – 14	≤ 12
Fosfomycin	200	≥ 16	13 – 15	≤ 12
Colistin*	10	≥ 14	12 – 13	≤ 11
Tigecycline*	15	≥ 18	-	< 18

* EUCAST breakpoints were because there are no CLSI breakpoints for colistin and Tigecycline for *Enterobacteriaceae.*

Appendix III: Zone Diameter Breakpoints for *Enterobacteriaceae*